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Jacqueline Farinha Shimizu

Avaliação de compostos naturais e sintéticos como antivirais contra o vírus do Chikungunya e Enterovírus A-71.

São José do Rio Preto
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RESUMO

Nas últimas décadas, diversos vírus que tinham sua ocorrência limitada a pequenas regiões se espalharam pelo globo, causando epidemias e preocupação entre as autoridades de saúde. Apesar dos inúmeros avanços no tratamento das infecções virais, vários destes vírus ainda não possuem tratamento especifico e eficaz. Adicionalmente, a alta taxa de resistência e o surgimento de novas mutações, torna a busca por novos antivirais desafiadora e de extrema importância. Desta forma, o presente trabalho teve como objetivo investigar a atividade antiviral de compostos de origem natural ou sintética contra os vírus da Chikungunya (CHIKV) e Enterovirus A71 (EV-A71). Contra o CHIKV, 48.750 compostos sintéticos foram inicialmente avaliados *in silico* por *docking* molecular, dos quais 12 compostos demonstraram apresentar interação com a região de ligação a ADP-ribose do dominío macro da proteína viral não estrutural 3 (nsP3), e foram selecionados para ensaios *in vitro*. Ensaios de viabilidade celular foram realizados para determinar a máxima concentração não tóxica de cada composto, que foi utilizada nos ensaios anti-CHIKV em células de hepatocarcimona humano Huh-7, transfectadas com os replicons subgenômicos do CHIKV. Os resultados demonstraram que os compostos C5 e C13 na concentração de 20 µM inibiram 53 e 76% da replicação do CHIKV em células Huh-7, respectivamente. Contra o EV-A71, 6 proteínas isoladas da peçonha de serpentes foram testadas em concentrações não tóxicas em células Vero infectadas, sendo avaliadas a atividade virucida, protetiva e contra a replicação do EV-A71. Das toxinas testadas, a crotamina e MJTX-II apresentaram atividade para as três etapas avaliadas, inibindo até 100% da infectividade viral. A MJTX-I e a crotapotina apresentaram atividade virucida e protetiva, enquanto a crotoxina apresentou atividade virucida, e a PLA contra na replicação viral. Os resultados obtidos demonstraram que os compostos sintéticos testados contra o CHIKV apresentaram atividade moderada, porém significante, contra a replicação do vírus. Adicionalmente, as proteínas isoladas da peçonha de serpentes demostraram potente atividade antiviral contra o EV-A71. Mais estudos são necessários para avaliar o potencial dessas moléculas como modelos de antivirais, bem como para um melhor entendimento do mecanismo de ação antiviral.

Palavras–chave: antivirais; virus do Chikungunya; Enterovirus A71; peçonha de serpente; docking molecular.

ABSTRACT

In the last decades, several viruses that had their occurrence limited to small regions spread through the globe, causing epidemics and concern among health authorities. Despite the numerous advances in the treatment of viral infections, several of these viruses have no specific and effective treatment yet. In addition, the high rate of resistance and the emergence of new mutations, makes the search for new antivirals challenging and extremely important. The present work aimed to investigate the antiviral activity of compounds from natural or synthetic origin against Chikungunya virus (CHIKV) and Enterovirus A71 (EV-A71). Against CHIKV, 48,750 synthetic compounds were initially evaluated *in silico* by molecular docking, of which 12 compounds demonstrated to be interacting with the ADP-ribose binding region of viral non-structural protein 3 (nsP3) macro domain and were selected for *in vitro* assays. Cell viability assays were performed to determine the maximum non-toxic concentration of each compound and used in anti-CHIKV assays in human hepatocarcinoma cells (Huh-7) transiently transfected with the CHIKV subgenomics replicons. The results demonstrated that the C5 and C13 compounds at 20 µM inhibited 53 and 76% of CHIKV replication in Huh-7 cells, respectively. Against EV-A71, 6 proteins isolated from snake venom were tested at non-toxic concentrations in infected Vero cells, and the virucidal, protective and anti-EV-A71 replication activity was evaluated. From the tested toxins, crotamine and MJTX-II presented activity for the three steps evaluated, inhibiting up to 100% of viral infectivity. MJTX-I and crotapotin showed virucidal and protective activity, while crotoxin showed virucidal activity, and PLA against viral replication. The results obtained demonstrated that the synthetic compounds tested against CHIKV showed moderate yet significant activity aginst virus replication. In addition, proteins isolated from snake venom demonstrated potent antiviral activity against EV-A71. Further studies are needed to assess the potential of these molecules as antiviral models, as well for a better understanding of the mechanism of antiviral action.

Keywords: antivirals; Chikungunya virus; Enterovirus A71; snake venoms; molecular docking.

LISTA DE ABREVIATURAS E SIGLAS

ACBD3	*acyl- CoA* (acil-CoA)
ADP	Adenosina difosfato
C	Proteína do Capsídeo
CHIKV	Vírus do Chikungunya
DENV	Vírus do Dengue
DMEM	Dulbecco's Modified Eagle Medium (Meio básico modificado por Dulbecco)
DMSO	Dimetilsulfóxido
ECSA	*East / Central / South Africa* (Leste/Centro/Sul da África)
EV-A71	Enterovirus A71
HCV	*Hepatitis C virus (*Vírus da hepatite C)
HFMD	*Hand-Foot-and-Mouth disease* (doença Mão-Pé-e-Boca)
Huh-7	*Hepatocyte derived cellular carcinoma cell line* (Linhagem de carcinoma celular derivado de hepatócito)
hSCARB2	Human scavenger receptor B2
kB	*Kilobase* (Quilobase)
kDA	*Kilodalton* (Quilodalton)
MOI	*Multiplicity of infection* (Multiplicidade de infecção)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (Brometo de 3-(4',5'-dimetiltiazol-2'-ila)-2,5-difeniltetrazol)
nsP	*non-structural proteins* (Proteínas não estruturais)
nsP3	*non-structural protein 3* (Proteína não estrutural 3)
OPAS	Organização Pan Americana de Saúde
OMS	Organização Mundial da Saúde
ORFs	*Open Reading Frame* (Regiões de leitura aberta)
OSBP	*Oxysterol- binding protein* (Proteína ligante de oxiesterol)
PI4KB	*Phosphatidylinositol4-kinase-β* (Fosfatidilinositol 4-quinase-β)
RE	Retículo endoplasmático
RD	*Rhabdomyosarcoma cells* (Células de rabdomiossarcoma)
RNA	*Ribonucleic acid* (Ácido ribonucleico)
YFV	*Yellow fever virus* (Vírus da febre amarela)
WNV	*West Nile virus* (Virus do Nilo Ocidental)
WA	*West African* (Oeste Africano)
ZIKV	*Vírus do ZIKV*

µg	Micrograma

SUMÁRIO

CAPÍTULO I:

Fundamentação Teórica

1.1 INTRODUÇÃO

Há algumas décadas, várias epidemias vêm preocupando os órgãos de sáude pública mundial. O vírus do Chikungunya (CHIKV), anteriormente restrito a pequenas regiões da Ásia e África, hoje é responsável por diversos surtos em mais de 60 países, segundo a Organização mundial da Saúde (OMS - World Health Organization – WHO) (WHO, 2019).

O Enterovirus A71 (EV-A71) foi responsável na década de 1970 por pequenos surtos de meningite asséptica nos Estados Unidos, na Europa e na Austrália, e grandes surtos de doenças semelhantes à poliomielite na Bulgária e Hungria. No final da década de 90, o vírus se disseminou globalmente (PONS-SALORT; PARKER; GRASSLY, 2015).

Avanços tecnológicos como a descoberta de modelos biológicos *in vitro* e a engenharia molecular permitiram o isolamento e cultivo de vírus, assim como a possibilidade de avaliar o ciclo replicativo viral na busca por novos fármacos. Apesar do desenvolvimento de tais ferramentas, ainda existe uma escassez de tratamentos eficazes aprovados pelos órgãos de saúde contra infecções virais.

Diante deste contexto, o estudo de compostos que possam atuar como antivirais é de interesse econômico para melhorar o bem-estar de indivíduos acometidos por infecções virais, visando a melhoria dos tratamentos existentes e o desenvolvimento de novas terapias contra infecções que atualmente possuem apenas tratamentos paliativos.

1.1.2 Vírus do Chikungunya

Histórico, transmissão e manifestações clínicas

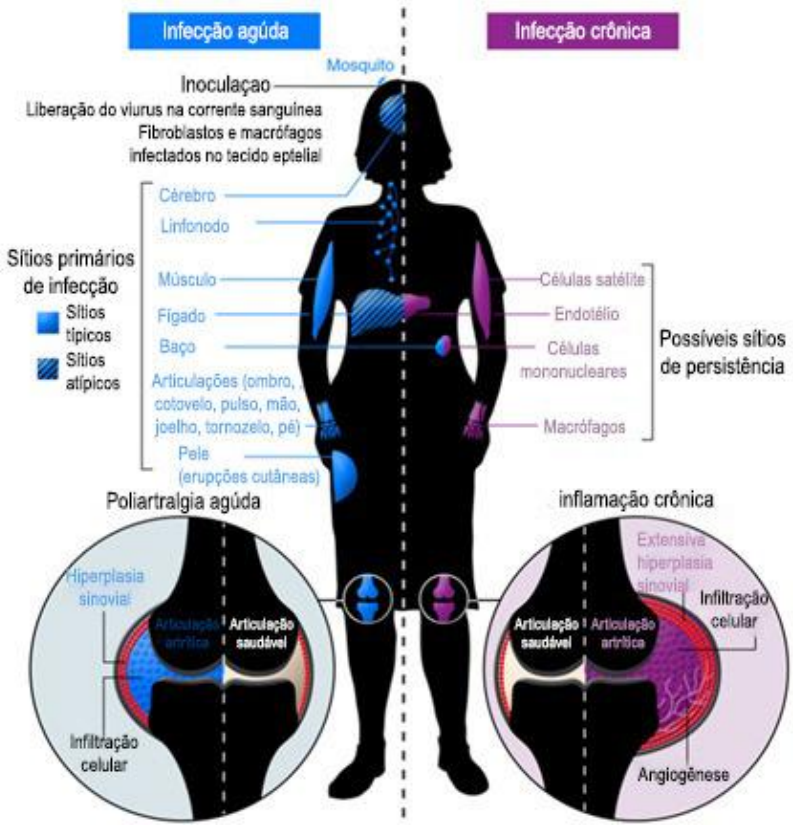
O CHIKV foi descrito pela primeira vez em 1950 na região da Tanzânia (LUMSDEN, 1955). Inicialmente, este vírus causava pequenos surtos em regiões da África e Ásia, e a partir de 2004 começaram a ocorrer surtos maiores da infecção (MCSWEEGAN et al., 2015). Desde então, a epidemia vem se espalhando e afetando milhares de pessoas em todo o mundo (STAPLES; BREIMAN; POWERS, 2009; WHO, 2019). O primeiro caso da infecção nas Américas foi registrado em dezembro de 2013 (WHO, 2017), e o primeiro caso autóctone da infecção no Brasil foi registrado em 13 de setembro de 2014 (MINISTÉRIO DA SAÚDE, 2014). Segundo dados do Ministério da Saúde, em 18 de outubro do mesmo ano já haviam sido confirmados 682 casos autóctones de CHIKV no Brasil (MINISTÉRIO DA SAÚDE, 2014). Em 2016, a Organização Pan Americana de Saúde (OPAS) registrou 349.936 casos suspeitos de febre Chikungunya e 146.914 foram confirmados no Caribe e nas Américas (P.A.H.O,

2017). O CHIKV ganhou destaque devido a emergência de surtos em todo o mundo, principalmente nas Américas, tendo sido identificado em mais de 100 países (CDC, 2019).

A febre do Chikungunya é uma arbovirose transmitida aos seres humanos pela picada de fêmeas de mosquitos infectados pelo vírus do Chikungunya (CHIKV), sendo os principais vetores os mosquitos *Aedes aegypti* e *Aedes albopictus* (CAREY, 1971; SILVA; DERMODY, 2017). Após entrar na corrente sanguínea, o vírus infecta macrófagos, fibroblastos e células endoteliais, e posteriormente apresenta uma intensa replicação no fígado, e principalmente em tecidos musculares, articulares e fibroblastos da pele (DUPUIS-MAGUIRAGA et al., 2012; SCHILTE et al., 2010).

A infecção pode ser assintomática, entretanto, entre 70 a 92 % dos casos são sintomáticos, apresentando uma fase aguda inicial de cerca de 3 a 7 dias (BRASIL., 2015; THIBERVILLE et al., 2013). A infecção aguda leva a uma resposta inflamatória dos tecidos infectados, caracterizada por uma extensa infiltração de macrófagos e monócitos, principalmente, podendo também ocorrer infiltração de neutrófilos, células *natural killers* e linfócitos. A alta taxa de replicação viral e a resposta imune do hospedeiro resultam em mialgia e poliartralgia nas articulações distais (KREJBICH-TROTOT et al., 2011) (Figura 1).

Figura 1. Infecção aguda e crônica pelo CHIKVIinfecção aguda e crônica pelo CHIKV. O esquema mostra os tecidos de replicação do CHIKV nas fases agudas e crônicas. Adaptado de (SILVA; DERMODY, 2017).



Fonte: SILVA; DERMODY, 2017

Os primeiros sintomas se manifestam após um período de incubação de dois a quatro dias após infecção (FIGUEIREDO; FIGUEIREDO, 2014). Os principais sintomas da febre Chikungunya são dores articulares e musculares, com febre aguda, náuseas, exantema e fadiga. Os sintomas são muitos semelhantes aos da dengue, porém, a febre do Chikungunya tem como principal característica fortes dores nas articulações. Além disso, em casos raros são observadas encefalopatias, hepatites e miocardites, podendo também levar a óbito (DAS et al., 2010).

Quando os sintomas persistem, é caracterizada a fase crônica que pode permanecer por meses ou anos, Rodrígues-Morales e colaboradores, após uma revisão sistemática de trabalhos publicados entre 2007 e 2015, calcularam uma prevalência de aproximadamente 40% de cronificação em 5702 pacientes que foram acompanhados por mais de 18 meses (RODRÍGUEZ-MORALES et al., 2016). A replicação viral nesses casos continua além dos sítios primários, em células endoteliais, no fígado, células mononucleares no baço, macrófagos no interior do líquido sinovial e tecidos adjacentes, e células satélites dentro do músculo (HOARAU et al., 2010; SILVA; DERMODY, 2017) (Figura 3).

Alguns fatores como idade avançada e problemas articulares são descritos na literatura com fatores de risco para a cronificação. Na fase crônica há a persistência das dores nas mesmas articulações acometidas durante a fase aguda, e os indivíduos podem apresentar limitações de movimento (CHHABRA et al., 2008; LO PRESTI et al., 2014).

Atualmente, não há antivirais aprovados contra a infecção por CHIKV (MCSWEEGAN et al., 2015). As terapias são utilizadas nos casos sintomáticos e são paliativas, consistindo de administração de analgésicos e antitérmicos como paracetamol e dipirona, associados à hidratação e repouso. Nos casos de dor refratária, analgésicos opióides como tramadol e codeína podem ser associados ao paracetamol e dipirona (WHO, 2017).

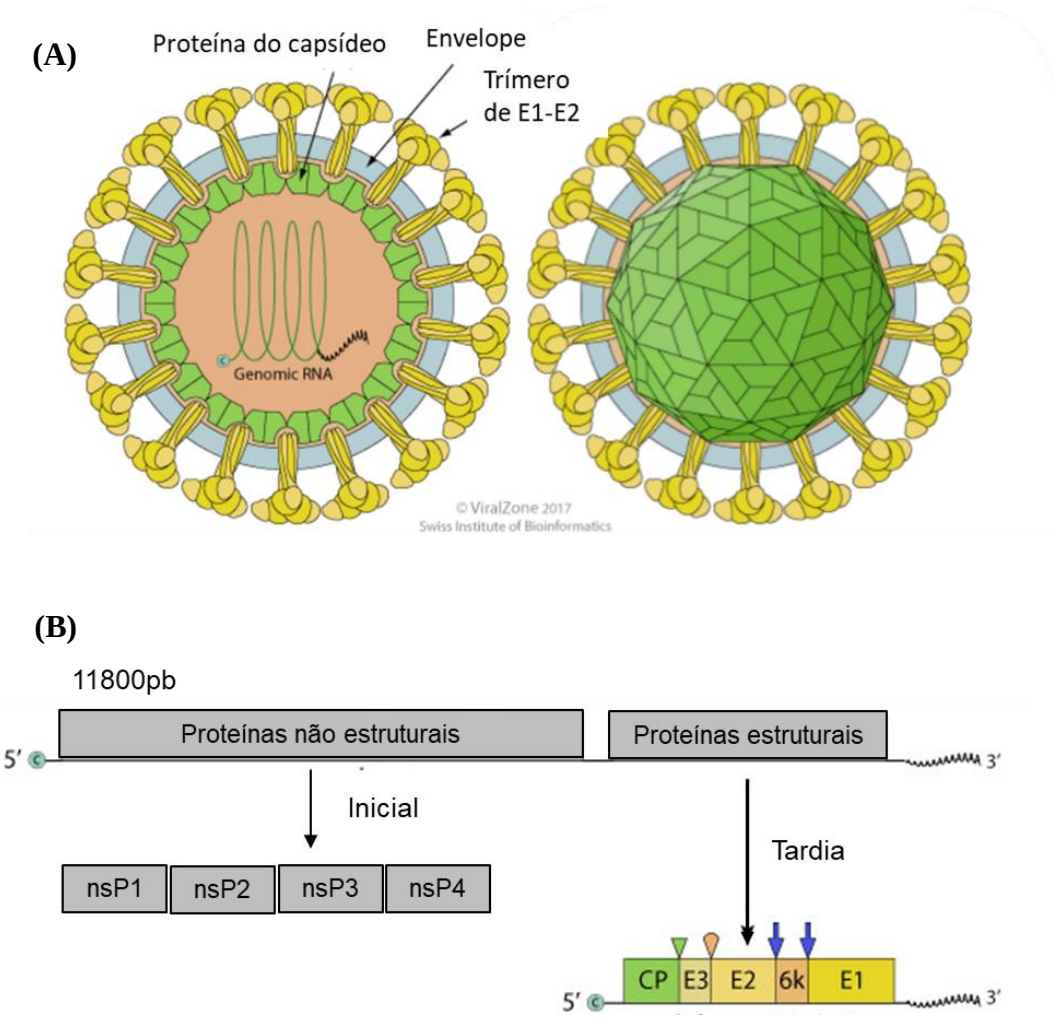
Estudos para o desenvolvimento de vacinas e antivirais específicos para CHIKV ainda estão em fases iniciais de desenvolvimento (MCSWEEGAN et al., 2015; ROSSI et al., 2019; ZHANG et al., 2019), demonstrando a importância da busca por compostos que possam exercer ação contra o vírus.

Classificação e ciclo replicativo

O CHIKV pertence à família *Togaviridae* e ao gênero *Alphavirus* (CHEN et al., 2018). Por ser uma arbovirose, apresenta ciclo replicativo em hospedeiros vertebrados e em artrópodes, tendo com principais vetores os mosquitos *Aedes aegypit* e *Aedes albopictus* (ARIAS-GOETA et al., 2014; LUMSDEN, 1955)..

A partícula viral mede aproximadamente 70 nm de diâmetro. É formada por um capsídeo icosaédrico, envolto por um envelope lipídico derivado da membrana plasmática de célula hospedeira, onde estão inseridas as glicoproteínas virais E1 e E2 (RASHAD; MAHALINGAM; KELLER, 2014) (Figura 2).

Figura 2.. Esquema representativo do Chikungunya. ***(A)*** *Estrutura da partícula viral do CHIKV.* ***(B)*** *Esquema do RNA do CHIKV. Reproduzido de ViralZone* (ALPHAVIRUS, [s.d.]).



Fonte: ViralZone, 2017

O genoma viral é constituído de uma fita simples de ácido ribonucleico (RNA - *Ribonucleic acid)* de polaridade positiva, com aproximadamente 11,8 quilobases (kB), apresentando um cap na região 5' e uma cauda poli-A na região 3'. Possui duas regiões de leitura aberta (ORFs – *Open Reading Frame*). A primeira ocupa os dois terços do terminal 5', que codifica uma poliproteína precursora, que é clivada em 4 proteínas não estruturais (nsP1-P4), as quais se associam para formar o complexo replicativo. A segunda codifica a poliproteína precursora que sera clivada nas 5 proteínas estruturais, as proteínas do capsídeo (C), E1, E2, E3 e 6K (LUM; NG, 2015; STRAUSS; STRAUSS, 1994).

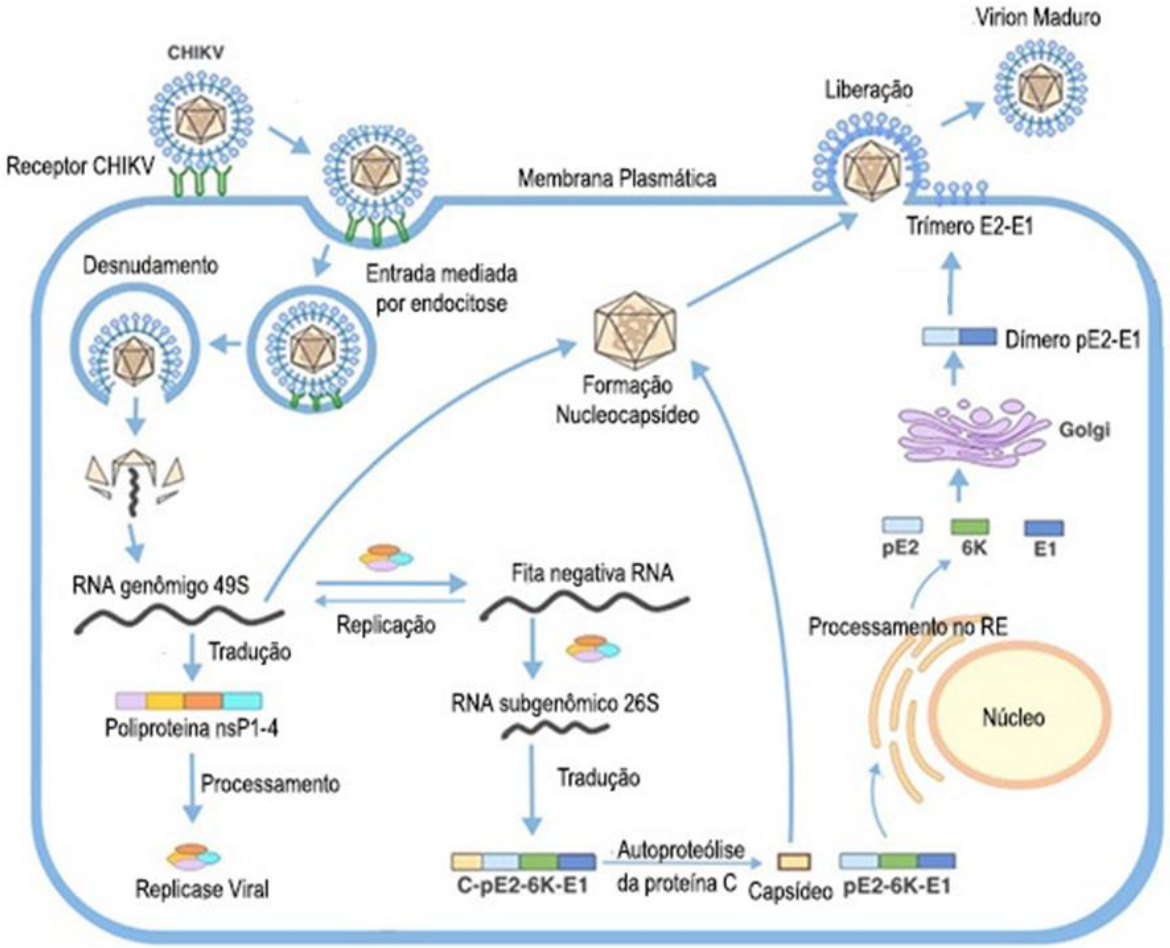
As glicoproteínas E1 e E2 estão associadas à superfície do vírion, e estão envolvidas com a adsorção e entrada do vírus nas células susceptíveis (VOSS et al., 2010). A proteína E3 parece estar envolvida com as etapas de montagem das novas partículas virais e no direcionamento das proteínas estruturais ao retículo endoplasmático (UCHIME; FIELDS;

KIELIAN, 2013). O papel da proteína 6K ainda não está claro, mas parece estar associado com a montagem do vírion (LEUNG; NG; CHU, 2011).

A nsP1 possui atividade de metil e guaniltransferase, ligando o complexo de replicação às membranas. A nsP2 tem função de helicase em sua porção N-terminal e de protease na porção C-terminal, enquanto a nsP4 atua como uma RNA polimerase viral RNA dependente. A nsP3 é dividida em 3 domínios. O macro domínio se encontra na porção N-terminal da proteína, o qual se liga a ADP-ribose. Após o domínio macro, se observa o domínio de ligação ao zinco, o qual ainda não possui um consenso sobre sua função no ciclo replicativo do CHIKV (SHIN et al., 2012). Na porção C-terminal se encontra o domínio hiper variável, o qual possui função de regulação de proteínas celulares (AHOLA; MERITS, 2016; MALET et al., 2009; RUPP et al., 2015)

A infecção das células inicia-se pela ligação da glicoproteína viral E2 a receptores de superfície celular, como a proibitina (WINTACHAI et al., 2012) e os glicosaminoglicanos (SILVA et al., 2014), seguida da internalização da partícula viral por meio de endocitose mediada por clatrinas (BERNARD et al., 2010). Posteriormente, ocorre a fusão de membranas virais e do endossomo devido a alterações de pH, liberando o nucleocapsídeo viral, o qual é desmontado. O RNA genômico viral 49S é então liberado no citoplasma (HOORNWEG et al., 2016) e traduzido em uma poli proteína viral que é processada e clivada pela protease viral nsP2, resultando nas proteínas não estruturais nsP1-4 (SOLIGNAT et al., 2009). As proteínas estruturais, que formam a partícula viral, são expressas posteriormente a partir de um RNA mensageiro subgenômico (26S), o qual é traduzido em uma poli proteína durante replicação viral (AHOLA; MERITS, 2016; SOLIGNAT et al., 2009). As proteínas E1 e E2 são inseridas no retículo endoplasmático (RE), onde sofrem inicialmente modificações pós-traducionais. Em seguida, são encaminhadas ao complexo de Golgi, onde são amadurecidas e depositadas na membrana plasmática (VOSS et al., 2010). As proteínas do capsídeo são agrupadas e juntamente com o RNA genômico 49S formam o nucleocapsídeo, o qual é direcionado para a região onde se encontram as proteínas E1 e E2 na membrana plasmática, resultando no brotamento dos vírions maduros (MENDES; KUHN, 2018) (Figura 3).

Figura 3. Ciclo replicativo do CHIKV. Esquema mostra todas as etapas do ciclo replicativo do CHIKV, retirado de (ABDELNABI; NEYTS; DELANG, 2015)



Fonte: ABDELNABI; NEYTS; DELANG, 2015

Em relação à variabilidade do genoma viral o CHIKV pode ser dividido em 3 linhagens principais: Oeste Africano (WA - *West African*), Asiática e a do Leste/Centro/Sul da África (ECSA - *East / Central / South Africa*). Análises filogenéticas demonstraram que as linhagens de CHIKV relacionadas aos surtos recentes são a Asiática e a ECSA (TEO et al., 2015; WEAVER; FORRESTER, 2015). Nas Américas, os casos relacionados a infecção por CHIKV são principalmente associados às linhagens ECSA e a Asiática (WEAVER, 2014).

1.1.3 Enterovírus A-71

Histórico, transmissão e manifestações clínicas

O EV-A71 foi isolado pela primeira vez em 1969 (SCHMIDT; LENNETTE; HO, 1974), e foi responsável por grandes surtos da doença Mão-Pé-e-Boca (HFMD - *Hand-Foot-and-Mouth disease*) em todo o mundo (MESSACAR et al., 2018).

No Japão, na década de 70, foram observadas as primeiras associações entre EV-A71 e HFMD com quadros clínicos mais graves (ISHIMARU et al., 1980). Na mesma década, casos de EV-A71 e sintomatologia semelhante à poliomielite foram relatados, apresentando 20% de paralisia e 5% de letalidade na Europa, América do Norte e Austrália (SOLOMON et al., 2010). A frequência de casos mais graves associados ao EV-A71 permitiu um aumento na detecção do vírus (CRABOL et al., 2017; HO et al., 1999). Epidemias em larga escala ocorreram na região do sudeste asiático desde 1997, com surtos associados a casos letais relatados na Malásia, Taiwan, Cingapura, China, Hong Kong, Japão, Coréia, Vietnã e Camboja, (SHIMIZU et al., 2004).

Atualmente, o EV-A71 é considerado endêmico em muitos países do Sudeste Asiático, e um vírus emergente em outros, com sérias preocupações quanto ao potencial de disseminação em regiões extremamente populosas (WANG et al., 2015). Como exemplo, a China registrou em 2012 mais de 2 milhões de casos e 567 óbitos (WANG et al., 2015).

A transmissão do vírus ocorre por via fecal/oral, através do contato com objetos infectados (CHANG et al., 2002). O EV-A71 pode ser encontrado nas secreções respiratórias de uma pessoa infectada, como saliva, muco nasal ou expectoração, nas fezes, e nas bolhas presentes nas mãos, pés e boca (WONG et al., 2010). O vírus pode ser transmitido enquanto houver a presença de bolhas, e as fezes podem ser fonte de infecção por várias semanas após o desaparecimento das bolhas (WANG; LIU, 2009).

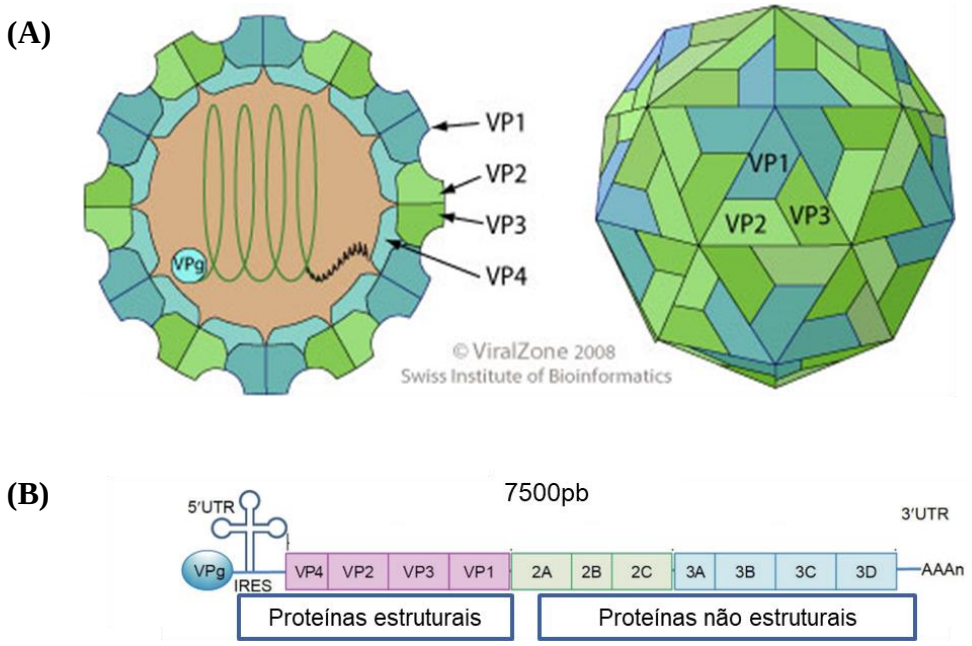
O EV-A71 é a principal causa da HFMD em crianças menores de 5 anos. Os primeiros sintomas aparecem após um período de incubação de 3 a 5 dias. A infecção caracteriza-se por apresentar febre e exantema infantil com bolhas nas mãos, pés, nádegas e boca (LEE, 2016). Entretanto, a infecção por EV-A71 também pode estar associada a casos mais graves de encefalite, meningite asséptica, edema pulmonar, paralisia flácida aguda e miocardite, podendo resultar em sequelas de longo prazo e alta taxa de mortalidade, especialmente em crianças e pessoas com imunodeficiência (DUONG et al., 2016). Portanto, o EV-A71 é considerado o enterovírus não-polio mais neurotrófico atualmente (BAGGEN et al., 2018; HUANG; SHIH, 2014).

Não existe atualmente antivirais específicos licenciados para o tratamento de pessoas com infecção por EV-A71. Os indivíduos em casos mais graves, como doenças neurológicas, podem ser hospitalizados para receber terapia de suporte (CDC, 2018).

Classificação e ciclo replicativo

O sorotipo EV-A71 pertence a espécie *Enterovirus A* ao gênero *Enterovirus* da família *Picornaviridae*, da ordem *Picornavirales*. É um vírus não envelopado, de RNA de fita simples e polaridade positiva (SCHMIDT; LENNETTE; HO, 1974). A partícula viral apresenta aproximadamente 30 nm e é composta por um capsídeo com simetria icosaédrica, o qual contém o RNA com cerca de 7,5 kb (PLEVKA et al., 2012) (Figura 4).

Figura 1. Esquema representativo do Enterovírus A-71. (A) Estrutura da partícula viral. Reproduzido de ViralZone (PICORNAVIRIDAE, [s.d.]). *(B) Esquema do RNA do Enterovírus A-71.*



Fonte: ViralZone, 2008

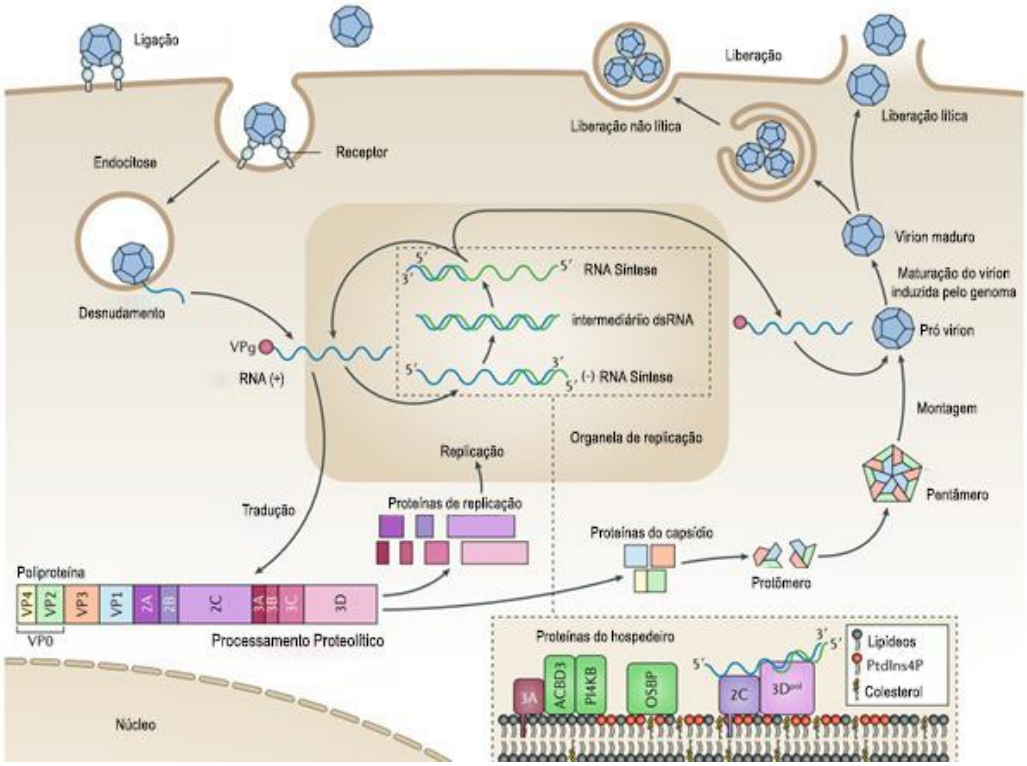
Após a ligação aos receptores celulares, como o ligante-1 da glicoproteína P-selectina, anexina II, sulfato de heparina, proteoglicanos, proibitina e hSCARB2 (KOBAYASHI; KOIKE, 2020), a partícula viral penetra na célula por endocitose. A liberação do RNA viral no citoplasma ocorre por meio da diminuição do pH no endossomo e interação com os receptores da membrana celular (HUSSAIN et al., 2011). O genoma viral está covalentemente ligado ao peptídeo viral 3B (VPg). Este peptídeo funciona como um primer para o ínicio da replicação do RNA viral, que é traduzido em uma poliproteína precursora de 250 quilodalton (kDA – *kilodalton)* (CHEN et al., 2013). A poliproteína é clivada nas 4 proteínas estruturais VP1, VP2,

VP3 e VP4. As proteínas VP2 e VP4 estão inicialmente unidas, formando a VP0 e são clivadas ao final do ciclo replicativo. Além das proteínas estruturais, o EV-A71 possui 7 proteínas não estruturais (2A, 2B, 2C, 3A, 3B, 3C and 3D) (HUANG et al., 2014; PLEVKA et al., 2012) (Figura 5).

A replicação do RNA viral se inicia pela síntese de um RNA de fita simples e polaridade negativa pela RNA polimerase dependente de RNA viral (3D), que serve como molde para a síntese de novas moléculas de RNA de polaridade positiva. A replicação ocorre nas chamadas organelas de replicação membranosa, onde as proteínas virais 2B, 2C e 3A auxiliam na formação de um ambiente lipídico, junto com proteínas hospedeiras que se ligam à acil-CoA (ACBD3), fosfatidilinositol 4-quinase-β (PI4KB) e proteína ligante de oxiesterol (OSBP) (STRATING; VAN KUPPEVELD, 2017). As novas moléculas de RNA positivas podem servir de molde para a síntese de novas proteínas virais, ou serem empacotadas e liberadas em novos vírions (Figura 5) (BAGGEN et al., 2018).

Para a formação do capsídeo, as proteínas estruturais se organizam inicialmente em protômeros e depois em pentâmeros. O RNA viral é então encapsulado, formando os pré vírions, e com o auxílio do mecanismo de replicação que cliva a proteína VP0 em VP2 e VP4 são convertidos em vírions maduros (CHOW et al., 1987). Os virions maduros saem da célula hospedeira através de lise celular, entretanto, estudos recentes demonstram que a liberação viral também pode ocorrer através da formação de vacúolos, sem a necessidade de lise celular (BAGGEN et al., 2018; MUTSAFI; ALTAN-BONNET, 2018; TOO et al., 2016) (Figura 5).

Figura 2. Ciclo replicativo dos Enterovirus. Esquema representativo do ciclo de replicação do gênero Enterovirus, retirado de (BAGGEN et al., 2018).



Fonte: (BAGGEN et al., 2018).

Os enterovírus pode ser classificado em quatro espécies (A, B, C e D), baseados na homologia da proteína VP1(NASRI et al., 2007). O EV-A71 é classificado como um sorotipo da espécie *Enterovirus A* e pode ser dividido em três genogrupos (A, B e C), esses são subdivididos em subgenogrupos (KOBAYASHI; KOIKE, 2020).

1.1.4 Compostos com potencial terapêutico

A utilização de tratamentos à base de compostos naturais é uma prática antiga, sendo que muitos compostos naturais e seus derivados vêm sendo utilizados como medicamentos (BALUNAS; KINGHORN, 2005). Estes compostos são descritos com enorme potencial terapêutico, apresentando diversas atividades biológicas como anticâncer (COSTA-LOTUFO et al., 2010), antimalárica (VAN AGTMAEL, 1999), antidepressiva (CÍCERO BEZERRA FELIPE et al., 2007) e antiviral (JARDIM et al., 2015; LOU et al., 2013; MULLER et al., 2014; SHIMIZU et al., 2017a).

Dentre os compostos naturais, os venenos animais de diferentes origens são descritos na literatura com inúmeras atividades biológicas, havendo registros da sua utilização pela humanidade desde a antiguidade (BHATTACHARJEE; BHATTACHARYYA, 2014). Seu

potencial terapêutico é vasto, apresentando, por exemplo, atividade anti-cancer (MAHADEVAPPA; MA; KWOK, 2017), contra doenças neurodegenerativas (DE SOUZA et al., 2018), anti-inflamatória (WANG; QIN, 2018) e antivirais (DA MATA et al., 2017). Dentre os venenos animais, a peçonha de serpentes tem sido uma fonte terapêutica inovadora, por ser uma mistura complexa de metalo-proteínas, fosfolipases e enzimas proteolíticas (BAILEY; WILCE, 2001; FABIAN VILLALTA-ROMERO, 2017).

O primeiro componente purificado e cristalografado de um veneno animal foi a crotoxina, uma proteína da peçonha de *Crotalus durissus terrificus* (Slotta K, 1938). Atualmente, a crotoxina já é descrita apresentar atividade imunomoduladora, anti-inflamatoria, anti-tumoral, anti-microbiana e atividade analgésica documentadas (Yan, 2006; Zhu, et al., 2008; Sampaio, 2006; Sampaio, 2010). Em 1971, foi isolado um peptídeo da peçonha de *Bothrops jararaca,* o qual deu origem a um dos principais agentes anti-hipertensivos utilizados como inibidor da enzima conversora da angiotensina I, o captopril (ONDETTI et al., 1971; RUPAMONI THAKUR; ASHIS K. MUKHERJEEA, 2017).

Dentre uma diversidade de aplicações para compostos extraídos de animais, componentes do veneno de *Crotalus durissus terrificus* demonstraram efeito antiviral contra o vírus Rocio (família *Flaviviridae*), vírus Oropouche (família *Bunyaviridae*), vírus Mayaro (família *Togaviridae*) (MULLER et al., 2014) e contra o vírus da Hepatite C (família *Flaviviridae*) (SHIMIZU et al., 2017b). Alguns compostos encontrados em venenos animais também apresentam atividade anti-HIV (UZAIR et al., 2018), HCV, SARS-CoV, vírus da influenza aviária (H5N1) e sarampo (LI et al., 2011; PETRICEVICH; MENDONÇA, 2003; XING et al., 2012; YAN et al., 2011). Com isso, a utilização de substâncias provenientes da peçonha de serpentes pode ser vista como alternativa para o desenvolvimento de novos antivirais com potencial terapêutico contra diferentes vírus.

Os compostos sintéticos podem se apresentar como uma alternativa aos compostos naturais. Moléculas naturais, apesar de amplamente estudados, podem apresentar limitações quanto a quantidade de princípio ativo isolado, o que dificulta sua aplicação como tratamento (BALUNAS; KINGHORN, 2005). Além disso, compostos naturais não são patenteáveis, sendo este um obstáculo para sua aplicação na comercialização como fármaco em grande escala. Deste modo, a utilização de compostos sintéticos baseados na estrutura de compostos naturais vem sendo cada vez mais estudada (JI et al., 2010; MADAN et al., 2014; NOWAKOWSKA, 2007), pois permitem a produção de grandes quantidades de compostos e a possibilidade de modificações químicas que possam melhorar a atividade antiviral da molécula.

Na literatura podemos observar a descrição de inúmeros compostos, de diferentes origens que apresentam atividade antiviral. E também diferentes metodologias empregadas na busca e seleção de compostos com potencial terapêutico. Os compostos podem ser selecionados baseando-se na atividade antiviral para outros vírus, por pertencerem a mesma classe de compostos com atividade antiviral descrita na literatura (BOLDESCU et al., 2017; VAZQUEZ-CALVO et al., 2017). A avaliação da atividade antiviral dos compostos pode ter início com testes *in vitro* (VARGHESE et al., 2016), assim como, uma pré-avaliação *in silico*, buscando quais compostos seriam mais ativos para um determinado alvo e assim restringir a quantidade de compostos a serem testados *in vitro* (GIMENO et al., 2019).

1.2 OBJETIVOS

Objetivo Geral

Selecionar por *virtual screening* e avaliar *in vitro* compostos sintéticos contra a proteína nsp3 do CHIKV.

Avaliar a atividade antiviral de compostos proteínas isoladas da peçonha de serpentes brasileiras contra o *Enterovirus A71*.

Objetivos capítulo I

- Selecionar *in silico* compostos sintéticos que apresentem afinidade pela região do sítio de ligação ADP-ribose, no dominío Macro da proteína nsP3 do CHIKV.
- Avaliar a viabilidade celular dos compostos selecionados em células BHK-21.
- Avaliar *in vitro* a atividade antiviral dos compostos selecionados na replicação do CHIKV

Objetivos capítulo II

- Investigar a atividade antiviral de proteínas isoladas da peçonha de serpentes brasileiras no ciclo replicativo do EV-A71 *in vitro*.
- Avaliar a viabilidade celular dos compostos selecionados em células Vero.
- Avaliar a atividade antiviral dos compostos selecionados *in vitro* nas etapas virucida, protetiva pós entrada.

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Capítulo II

Is the ADP ribose site of the Chikungunya virus NSP3 Macro domain a target for antiviral approaches?

Is the ADP ribose site of the Chikungunya virus NSP3 Macro domain a target for antiviral approaches?

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Highlights

- Virtual screening identifies molecules that binding to ADP-ribose site of CHIKV

- Two selected compounds inhibited CHIKV replication in vitro

- Compounds are probably interfering in the early CHIKV replication

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Abstract

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

Introduction

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

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

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

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

Previously, the macro domain of CHIKV nsP3 has been described as a potential target in the development of anti-alphavirus compounds (Nguyen et al., 2014; Subudhi et al., 2018; Vijayasri and Hopper, 2017). Despite the interest in the macro domain of nsP3, there is a lack of *in vitro* or *in vivo* studies on drugs that bind to this region of nsP3. Most are computational studies, with

no further *in vitro* or in *vivo* validation (Nguyen et al., 2014; Vijayasri and Hopper, 2017). Computational methods can be useful to refine the search for new antiviral drugs, reducing efforts and costs to approve antivirals, however, the validation of these analysis is essential.

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

Material and Methods

In silico screening

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

Compounds

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

Cell lines and replicons

The human hepatoma (Huh-7) and rhabdomyosarcoma (RD) cell lines were grown in DMEM, supplemented with 10% FBS, 0.5 mM non-essential amino acids and 100 units/mL penicillin-streptomycin. Murine myoblast cells (C2C12) were grown in DMEM supplemented with 20% FBS and 100 units/mL penicillin-streptomycin. All cells were maintained at 37 °C with 5% CO_2 in a humidified incubator.

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

Cell viability assay

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

RNA Transcription and electroporation

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

replaced with compound-containing media in different concentrations and incubated for 24 hours.

Replication assay

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

Statistical Analysis. Individual experiments were performed in triplicate and all assays were performed a minimum of three times in order to confirm the reproducibility of the results. Statistical analysis was performed using GraphPad Prism software. A Kruskal-Wallis test followed by post hoc analysis by Dunn's test was used for multiple comparisons. P values of less than 0.05 (indicated by asterisks) were considered statistically significant.

Results

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

To identify potential inhibitors against the macro domain of CHIKV nsP3, the structure of CHIKV nsP3 complexed with ADP-ribose (Protein Data Bank - 3GPO) was used. The ADP-ribose substrate was manually removed from the ADP-ribose binding site and a library containing 48,750 small synthetic molecules were docked to this region of the protein with Schrodinger's docking tool Glide in vHTS mode using default parameters. The compounds were ranked based on Glide docking scores, a prediction of binding affinity, and the top 1000 scoring compounds were re-docked using Glide XP mode, a higher precision scoring function.

After that, the top 50 compounds were visually inspected. And 12 compounds were selected for biological testing based on the docking score (**Table 1**); inspection of hydrogen bonds between ADP-ribose binding site of CHIKV nsP3 and the compounds; the conformation of each docking pose and the position of the compound inside the binding site relative to the ADP-ribose.

Activity of compounds against CHIKV replication

Initial in vitro activity of compounds selected by docking analysis

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

Compounds with anti-CHIKV activity and related analogues

In silico analysis showed predicted hydrogen bonding interactions of compounds C5 and C13 with residue Y114 and van der Waals interactions/hydrophobic effect with residue V113 (**Figure 2A**)**.** After initial *in vitro* screening, compounds C5 and C13 were selected for further assays and structural analogues were purchased from ChemDiv (**Figure 2B, Table 2**).

The EC_{50} and CC_{50} of C5, C13 and their analogues were determined by performing replication and viability assays in cells treated with a range of concentrations of each compound. Huh-7 cells were electroporated with CHIKV-Fluc SGR RNA, seeded in 48 well plates and treated with a two-fold dilution (ranging from 1.5-100 μM) of each compound at 4 h.p.e. Cell viability and CHIKV replication levels were measured 24 h post treatment (**Figure 3**). The selective

index (SI) was calculated by dividing the value of CC_{50} by EC_{50} (CC_{50}/EC_{50}). The EC_{50} and CC_{50} values and SI of C5, C13 and their analogues are shown in Table 3. Compound C5 showed EC_{50} of 12.7 μM and CC_{50} of 38.9 μM, and demonstrated lower EC_{50} value than its analogues 1C5, 2C5, 3C5 and 4C5. Compound C13 presented a slightly higher EC_{50} (11.9 μM) and CC_{50} (36.8 μM). While the C13 analogue 1C13 showed a decrease in EC_{50}, the value o6 CC_{50} decreased almost by a half.

Investigation of C5 and C13 compounds mechanisms

Compounds C5 and C13 presented the highest SI and their effects on CHIKV replication were further investigated. To this end, the CHIKV-D-Luc replicon containing two reporter genes, the renilla luciferase (Rluc) and firefly luciferase (Fluc) was used, the replicon is described in (Roberts et al., 2017). The reporter Rluc is present in the hypervariable region of nsP3 of CHIKV and its expression indicate both input translation and early replication levels. Alternatively, the reporter Fluc replaces the virus structural genes and it is only expressed from a subgenomic RNA, thus indicating that RNA replication has occurred (**Figure 4A**). In order to investigate a possible mechanism of C5 and C13 were acting during early or late replication, Huh-7 cells were electroporated with CHIKV-D-Luc and treated with C5 (**Figure 4B**) or C13 (**Figure 4C**) at non-toxic concentrations (concentrations above 80% of cell viability were considerated non-toxic). The data obtained demonstrated that the treatment with C5 (**Figure 4B**) and C13 (**Figure 4C**) compound significantly decreased Rluc levels, but not Fluc. Therefore, suggesting that these compounds are most likely interfering with early replication events.

Finally, the effects of C5 and C13 were also investigated in muscle derived cell lines. For this, human rhabdomyosarcoma (RD) cells and murine myoblast (C2C12) cells were used. However, the results demonstrated that C5 and C13 were only able to inhibit CHIKV replication at concentrations that also demonstrated some cell toxicity (**Figure 5**). At 100 μM, C5 inhibited

100% and 70% of CHIKV replication in RD and C2C12, respectively (cell viability 65% and 49%) **(Figure 5A and 5B)**. C13 inhibited CHIKV replication in 95% and 64% in RD and C2C12, respectively (cell viability 70% and 51%) **(Figure 5B and 5D)**.

Discussion

Virtual screening has the advantage of reducing costs/time to identify hit compounds when compared to screening large numbers of compounds in an HTS approach (Piccirillo and Amaral, 2018). In this study, the macro domain of CHIKV nsP3 was investigated since this protein has been reviewed and described to participate in several processes of the viral cycle. The macro domain of CHIKV nsP3 possesses ADP-ribose binding and ADP-ribose hydrolase activity, removing ADP-ribose from mono(ADP-ribosyl)ated proteins, this activity has been shown to be important for viral replication (McPherson et al., 2017). CHIKV mutants with reduced hydrolase activity showed slower virus replication and decrease of virulence, and mutants with no hydrolase activity were unable to replicate in mammalian or mosquito cells (McPherson et al., 2017). Therefore, a compound that can block CHIKV nsP3 hydrolase activity is proposed to be a target for antiviral therapy. Additionally, the macro domain of nsP3 is a well conserved region in the *Alphavirus* genus and other positive single strand RNA viruses, demonstrating the potential to be a broad spectrum antiviral (Eckei et al., 2017; Gregor et al., 2016).

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

active than the original compounds. Moreover, C5 and C13 demonstrated interference in the early steps of CHIKV replication (for example formation of the replication complex) rather than after RNA replication has occurred.

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

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

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

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

Conclusion

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

Author contributions

Jacqueline Farinha Shimizu: Methodology, Investigation, Visualization and Writing-Original draft preparation. **Daniel Oliveira Silva Martins:** Writing-Original draft preparation. **Martin J. McPhillie:** Software, Conceptualization, Formal analysis. **Grace C. Roberts:** Methodology. **Carsten Zothner:** Methodology. **Andres Merits:** Methodology, Resources.

Mark Harris: Funding acquisition, Supervision, Resources and Conceptualization. **Ana Carolina Gomes Jardim:** Writing-Reviewing, Visualization, Editing and Supervision.

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

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

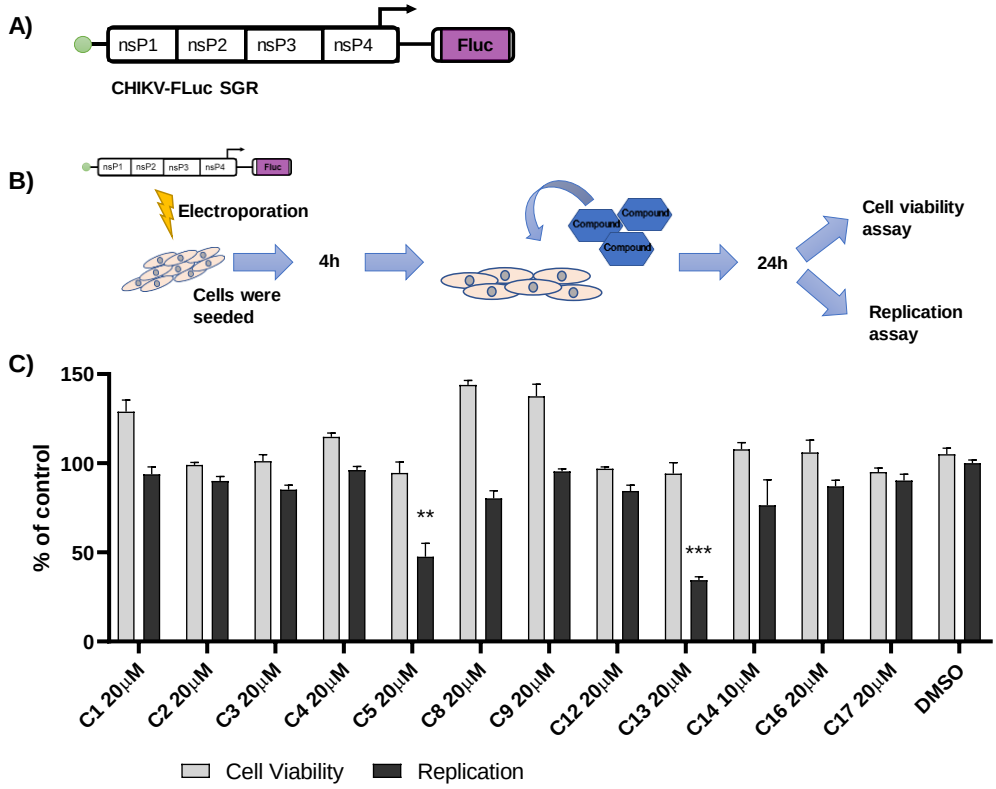

Figure 1*: In vitro* screening of 12 MCCB compounds selected from docking analyses. CHIKV-Fluc SGR replicon scheme **(A)**. Schematic representation of *in vitro* initial trials of MCCB compounds **(B)**. The results of the treatment with maximum non-toxic concentration of Huh-7 cells harbouring CHIKV-Fluc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

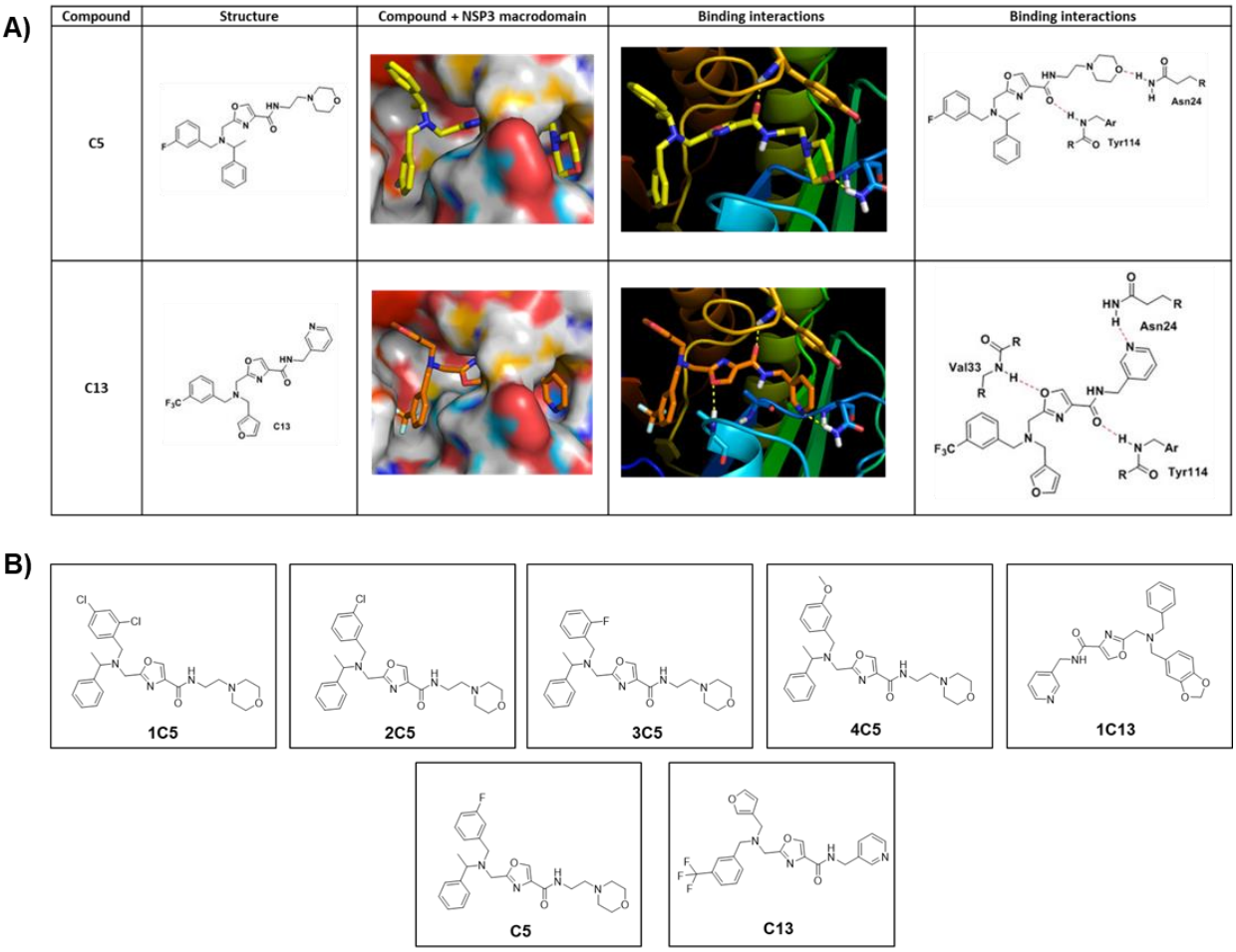

Figure 2. Structure of compounds C5, C13 and analogues. **(A)** Predicted binding conformations of C5 and C13 docked within to the ADP-ribose binding site of CHIKV nsP3 macro domain. Predicted hydrogen bonding interactions between 2D compound structures and CHIKV nsP3 amino acids are shown with a dotted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

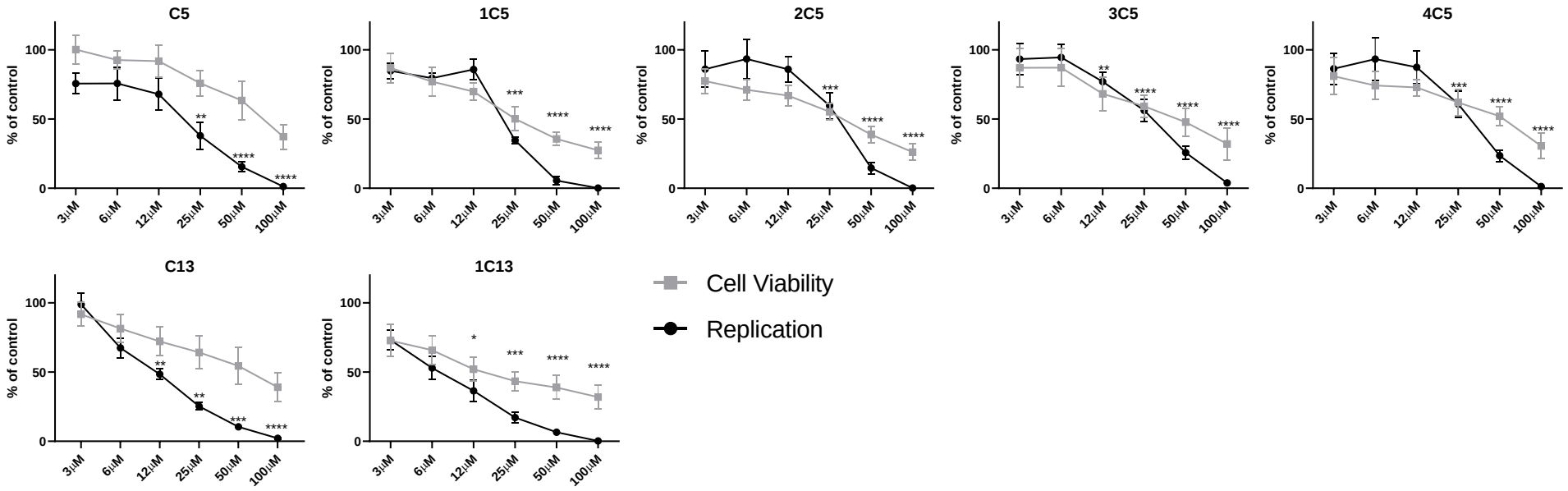

Figure 3: Activity of selected compounds and analogues against CHIKV replication. Huh-7 cells were electroporated with CHIKV-Fluc-SGR RNA, seed and treated 4 h later with compounds at concentrations ranging from 100µM to 3µM. Cell viability (■) was measured by MTT and replication (●) by luciferase assay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

Table 3. EC_{50} and CC_{50} of C5 and C13 analogues.

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

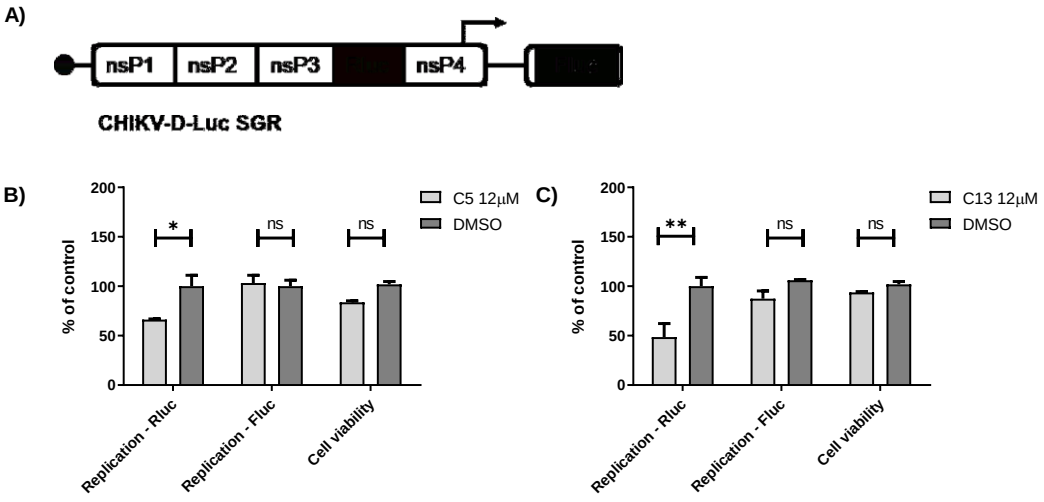

Figure 4: Effect of C13 and C5 at different stages in CHIKV replication. CHIKV-D-Luc wild type RNA **(A)** were electroporated in Huh-7 cells used to analyse the activity of C5 **(B)** and C13 **(C)**. DMSO was used as non-treated control. The asterisks indicate statistically significant differences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

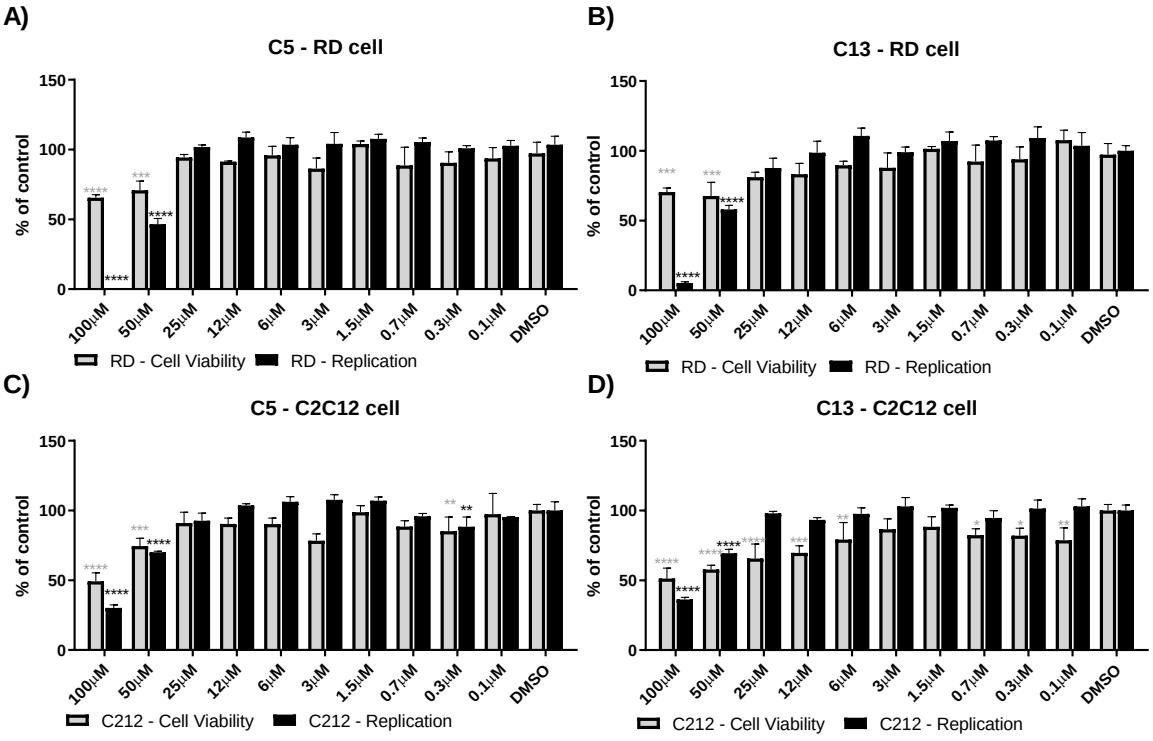

Figure 5. Activity of C5 and C13 in RD and C2C12 cell lines. Cells were electroporated with CHIKV-Fluc RNA, seeded and after 4h treated with C5 or C13. Cell viability and replication were measured in RD (**A** and **B**) and C2C12 cells (**C** and **D**). DMSO was used as non-treated control. The asterisks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

Capítulo III

Proteins isolated from Brazilian snakes venom possess antiviral activity against Enterovirus 71.

Proteins isolated from Brazilian snakes venom possess antiviral activity against Enterovirus 71

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Abstract

Enterovirus 71 belongs to *Enterovirus* genus from *Picornaviridae* family. The virus is mainly associated to the hand, foot and mouth disease (HFMD). In the recent years, the EV-A71 has caused several outbreaks around the world and there is no approved antiviral against this virus. Therefore, the search for novel anti-EV-A71 drugs is challenging and extremely important for healthy system. In this context, compounds isolated from snake venoms has shown to possess antiviral activity against a range of viruses. Here, six proteins isolated from the venom of Brazilian snakes were investigated by their antiviral activity against EV-A71 infection. Vero cells were infected with EV-A71 in the presence or absence of compounds at non-toxic concentrations, and infectivity levels were measured to analyse the virucidal, protective and on replication activities of each protein. The results demonstrated that crotoxin presented virucidal activity (56%) and PLA2-CB significantly inhibited virus replication in 89%. Crotapotin presented both virucidal and protective activities (38 and 60%, respectively). MJTX-I was able to significantly inhibited virus replication (99%) and presented virucidal activity (88%). More interestingly, crotamine and MJTX-II showed a broad-spectrum activity inhibiting the three stages evaluated in up to 99 and 100%, respectively. The obtained data demonstrate that the proteins isolated from venom of snakes are potent inhibitor of EV-A71 and could be used as templates to the development of future novel antiviral.

Introduction

The Enterovirus 71 (EV-A71) is one of the major causes of hand, foot and mouth disease (HFMD), characterized by fever and rash with blisters on the hands, feet, buttocks and mouth (K. Y. Lee 2016). The EV-A71 infection may also be associated with more severe cases of encephalitis, aseptic meningitis and myocarditis, which could result in long-term sequelae and a high mortality rate, especially in children (Duong et al. 2016; Huber et al. 1998; Messacar et al. 2018; Q. Zhang et al. 2014). EV-A71 starts to replicates in gastrointestinal and respiratory epithelium cells, however, the virus can infect neurons and cardiomyocytes (Muehlenbachs et al. 2015).The EV-A71 belongs to *Enterovirus* genus from *Picornaviridae* family (Brown and Pallansch 1995). It is a non-enveloped virus, with icosadrical capsid and a single positive RNA strain as genome (Schmidt et al. 1974). The RNA encodes a polyprotein that is cleaved in four structural proteins and seven non-structural proteins (Belsham and Sonenberg 1996; Wimmer et al. 1993)

This virus has been responsible for outbreaks associated to neurological complications in children (T. C. Lee et al. 2009; Messacar et al. 2018). In the recent years, epidemics in Southeast Asian countries generated concern about the potential of EV-A71 dissemination in extremely populous regions. In 2012, China registered more than 2 million cases and 567 deaths related to HFMD (Y. Wang et al. 2015).

Currently, there is no approved antiviral therapy against enterovirus infection. The search for therapeutics against EV-A71 possesses importance for several countries and could provide a substantial benefit to the global health systems. In this context, animal venoms has shown to be an interesting source of compounds with several biological activities as anti-inflammatory, anti-cancer and cardiovascular activity (Biswas et al. 2012; Koh and Kini 2012; P. Samy et al. 2012). Proteins isolated from animal venoms have also showed to possess antiviral activity (Vilas Boas

et al. 2019) against hepatitis C (Shimizu et al. 2017), dengue and yellow fever (V. D. Muller et al. 2014) and measles virus (Petricevich and Mendonça 2003).

In this study, the antiviral activity of proteins isolated from Brazilian venom snakes *Bothrops moojeni* and *Crotalus durissus terrificus* was investigated against EV-A71. The data obtained displayed that these compounds strongly inhibited virus replication and presented virucidal and protective effects against EV-A71 infection in Vero cells.

Material and methods

Venoms and isolated proteins

The crude venom of *Bothrops moojeni* was purchased from serpentarium "Serpentário Proteínas Bioativas Ltda" of Batatais/SP, registered at the Ministry of the Environment, nº 471301. For *Crotalus durissus terrificus*, the crude venom was purchased from serpentarium "Centro de Extração de Toxinas Animais", registered at the Ministry of the environment, nº 3002678. Isolation and purification of the proteins Crotamim, Crotapotin, Crotoxin and PLA2-CB were carried out at the Laboratory of Toxinology of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (IBAMA authorization: 1/35/1998/000846-1), under the supervision of Professor Suely Vilela Sampaio, as previously described in (V. D. M. Muller et al. 2012), using Itzhaki and Gill method (Itzhaki and Gill 1964). MjTX-I and MjTX-II were isolated by ion-exchange chromatography on CM-Sepharose (Pharmacia) as previously described in (Lomonte et al. 1990). Lyophilized proteins were dissolved in PBS (Phosphate-Buffered Saline), filtered, aliquoted and stored at -80˚C. Stocks were diluted in DMEM immediately prior to the experiments. PBS was used as untreated control. The source of each protein is shown on Table 1.

Cell culture

African green monkey kidney epithelial cells (Vero) were grown in minimal Eagle's medium (MEM; Sigma–Aldrich, USA) supplemented with 100 U/mL penicillin (Gibco Life Technologies, USA), 100 U/mL streptomycin (Gibco Life Technologies, USA), 1% HEPES (Gibco Life Technologies, USA) and 10% fetal bovine serum (FBS; Gibco Life Technologies, USA) at 37˚C in a humidified 5% CO2.

Virus stocks and Titration

The enterovirus EV-A71 B2 infectious clone was kindly provided by Professor Nicola Stonehouse. The full description of EV-A71 B2 infectious clone is in preparation for publication (Kingston et al, in preparation). Briefly, EV-A71 B2 was isolated and cloned under a T7 promoter to produce homogeneous infection, plasmids were linearised, transcribed using T7 RiboMAX Express system (Promega) and RNA were transfected in Vero cells using of Lipofectamine 2000 (Invitrogen). For virus titration, EV-A71 cell culture supernatants were 10-fold serial diluted in MEM medium and used to infect Vero cells at 37 °C and 5% CO2. Cells were fixed with 4% paraformaldehyde (PFA) 72 hours post-infection (hpi) and stained with crystal violet 0.5% for 30 min. Cytopathic effect was counting using the 50% tissue culture infectious dose (TCID50) method and calculated by the Spearman & Kärber algorithm as described in Killington and Hierholzer (Killington and Hierholzer 1996).

Antiviral assays

The activity of the proteins isolated from snake venoms was evaluated in different steps of EV-A71 replicative cycle.

To evaluate whether compounds presented a protective effect against EV-A71 infection, Vero cells were pre-treated with each compound for 1 hour at 37 °C in a humidified 5% CO2 incubator prior to the infection. After incubation, cells were washed extensively to remove compounds and were infected with EV-A71cc for 1 hour. Infectious supernatant was removed, additional washes were performed to virus removal and fresh media was added.

For virucidal assay, infectious supernatant was prior incubated with each protein for 1 h at 37 °C and then used to infect naive Vero cells. Virus and protein were incubated with cells for 1 h at 37 °C. The inoculum was removed; cells were washed three times with PBS to completely remove virus and proteins and replaced by fresh media.

To investigate the antiviral activity on EV-A71 replication, Vero cells were added of with infectious supernatant for 1 hour, washed with PBS to remove non-endocytosed virus particles and replaced by compound containing media.

For all antiviral assays, the supernatant was collected 72 hpi and used to infected Vero cells in 96 well plates. Cells were washed with PBS after 1 hour and incubated with fresh media for 72 hours. The titers were determined by TCID50 method as described above.

Cell Viability

Cell viability was measured by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Sigma–Aldrich) method. Vero cell were seeded in a 96-well plate at 5 x 10^3 cells per well and incubated overnight at 37ºC in a humidified 5% CO2 incubator. Compound-containing medium was added to the cell culture and incubated for 72 hours. Supernatant was removed and MEM containing MTT at 1 mg/mL was added to each well, incubated for 30 min at 37 ºC in a humidified 5% CO2 incubator and replaced with 100µL of DMSO to solubilize the formazan crystals. The absorbance was measured at 560 nm on Infinite F50 microplate reader (Tecan).

Western Blot

Cells were lysed in PLB buffer (Promega) added of protease inhibitors (Sigma-Aldrich). Protein concentration of samples was calculated using Pierce BCA Protein Assay Kit (Thermofisher). Ten micrograms of protein were resolved by SDS/PAGE and transferred to a PVDF membrane. Membranes were blocked in 10% (w/v) dried skimmed milk powder in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Membranes were probed with Mouse anti-enterovirus VP0 (anti-VP0) monoclonal antibody, secondary anti-mouse a peroxidase-conjugated antibody (Sigma) and ECL substrate (Thermo scientific).

Statistical

All assays were performed a minimum of three times in order to confirm the reproducibility of the results. Differences between means of readings were compared using the Kruskal-Wallis test followed by post hoc analysis by Dunn's test was used for multiple comparisons. P values of less than 0.05 (indicated by asterisks) were considered statistically significant.

Results

Proteins isolated from Brazilian snake venoms inhibit multiple stages of EV-A71 life cycle

To analyse the antiviral potential of proteins isolated from Brazilian snake venoms on EV-A71 infection, we first determined the non-toxic concentration of MJTX-I, MJTX-II, Crotapotin, Crotoxin, and PLA2-CB in the naive Vero cells. For this, cells were treated with 50, 25, 5 and 1 µg/mL of each protein for 72 hours to access cytotoxicity of the compounds. With exception of PLA2-CB, all tested proteins demonstrated a minimum of 87% of cell viability (Figure 1) at the highest concentrations tested. PLA2-CB showed a dose dependence effect on treated cells,

reaching non-toxic concentrations at 5 µg/mL or lower (Figure 1). From this data, the highest non-cytotoxicity concentrations of each protein were selected for the antiviral assays (Table 1). To access the antiviral activity of each venom proteins, their protective activity on the host cells was investigated. For this, Vero cells were pre-treated with the compounds for 1 hour, followed by washes with PBS to remove any trace of compounds, and infection with EV-A71cc virus for 1 hour. Supernatant was replaced by fresh media after further PBS washes, and cells were incubated for 72 hours (Figure 2A). The results demonstrated that MJTX-II, Crotamin e Crotapotin reduced virus titers to 76, 62 and 60% ($P<0.001$), respectively (Figure 2B and C), suggesting that these compounds could act in the host cells preventing virus infection. In contrast, MJTX-I and CX increased virus infectivity in 47 and 49%, respectively ($P<0.001$) (Figure 2B and C), suggesting that these proteins could interfer with host factor that are associated to the benefit of virus infectivity.

To evaluate the virucidal activity of proteins, infectious supernatant was prior incubated with each protein for 1 hour and then used to infect naive Vero cells for further 1 hours. Supernatant was replaced by fresh media after further PBS washes and incubated for 72 hours (Figure 2D). All proteins, except for PLA2-CB, showed significant virucidal inhibition (Figure 2E and F). Crotamin and MJTX-II knocked down the virus, and MJTX-I and Crotoxin significantly reduced virus infectivity to 88 and 56%, respectively (Figure 2E and F).

The effect of venom proteins was also investigated on EV-A71 pos entry steps. Vero cells were infected with EV-A71 for 1 hour, washed with PBS to remove non-endocytosed virus particles and replaced by compound containing media (Figure 3A). The proteins MJTX-I, MJTX-II knocked down virus replication, while Crotamin and PLA2-CB reduced 97% and 89% of EV-A71 pos entry, respectively (Figure 3B and C). The EV-A71 levels of replication were also evaluated by the expression of EV-A71 VP0 protein which was also significantly reduced in the presence of MJTX-I, MJTX-II, Crotamin and PLA2-CB (Figure 3D).

Discussion

Currently, there is no specific antiviral against EV-A71 infection, a virus associated to the hand, foot and mouth disease HFMD (CDC 2018). It is considered an endemic virus in Pacific-Asia, where there are concerns about the potential of dissemination in extremely populous area (Y. Wang et al. 2015). Thus, the search for active compounds against EV-A71 is required.

Proteins isolated from snake venoms demonstrated antiviral activity against virus from different family as *Flaviviridae, Paramyxoviridae, Retroviridae, Bunyaviridae* and *Togaviridae* (V. D. Muller et al. 2014; Petricevich and Mendonça 2003; Shimizu et al. 2017; Y. J. Zhang et al. 2003). According to our knowledge, this is the first description of proteins isolated from snake venoms possessing antiviral activity against *Picornaviridae* family.

The proteins crotoxin, crotapotin and PLA2-CB investigated in this study were isolated from *Crotalus durissus terrificus* and previously demonstrated antiviral activity against HCV, a RNA enveloped virus that belong to *Flaviviridae* family (Shimizu et al. 2017). Against HCV, PLA2-CB was able to protect host cells of virus infection, reduced virus replication and also presented virucidal activity. Crotoxin acted as a virucidal compound and inhibiting HCV release, and Crotapotin inhibited virus release.

Muller and coworkers showed a direct effect of PLA2-CB present on Dengue virus envelope by cleaving glycerophospholipid, that would cause disruption of the lipid bilayer and destabilization of the envelope proteins on the virus surface. They also investigated the virucidal effect of PLA2-CB on others enveloped virus (Rocio virus, Oropouche virus, and Mayaro virus) and non-enveloped virus (Coxsackie B5 virus, a non-enveloped virus from *Picornaviridae* family). PLA2-CB showed virucidal activity against all the enveloped vírus tested, however, PLA2-CB was not able to inhibit Coxsackie B5 virus.

Corroborating the Muller and coworkers results, our results showed that PLA2-CB was unable to inhibit EV-A71 (a non-enveloped virus) in the pre-treatment and virucidal steps. PLA2-CB is a phospholipase and the lack of virucidal and/or protective activity could be related to the absence of an envelope on EV-A71.

However, PLA2-CB demonstrated an inhibition of 89% in EV-A71 pos entry assays. The EV-A71 proteins 2B, 2C and 3A act in the formation of a lipid environment for EV-A71 replication, named membranous replication organelles (Strating and van Kuppeveld 2017), which is essencial to the virus replication. One possible mechanism of action of PLA2-CB on EV-A71 replication could be the disruption of membranous replication organelles, abrogating virus replication. However, this hypothesis needs to be further investigated.

Our results showed for the first time the antiviral activity of crotamin on all steps of virus replicative cycle tested in this study. Crotamin is a low molecular weight cationic polypeptide from *Crotalus durissus terrificus* (CHANG and TSENG 1978) that demonstrated to present cell penetrating property (Radis-Baptista and Kerkis 2011), and antimicrobial (Yount et al. 2009) and antifungal activity (Yamane et al. 2013). Rodrigues and collaborators showed that crotamin was able to interact with lipid bilayers and penetrate cells via different entry mechanisms (Rodrigues et al. 2012). Muller and colleagues investigated the anti-DENV and YFV activities, both eveloped viruses that present replication associated with lipid metabolism. However, crotamin did not show any effect against these viruses (V. D. M. Muller et al. 2012).

The proteins MJTX-I and MJTX-II are two phospholipases from *Bothrops moojeni* venom which showed inhibition of replication and virucidal activity against EV-A71 in this study. MJTX-I also showed a protective effect against EV-A71, being active against all stages of virus replication investigated in this study. In the literature, there are no reports of biological activities linked to these two proteins from *B. moojeni*.

Despite the lack of envelope on EV-A71, the virus presents depression in the capsid named canyons, that are important regions where cellular receptors bind to the virus particle (Rossmann et al. 2002). The canyons can harbours pocket factor that are hydrophobic pocket filled with a lipids, involved in regulating particle stability (X. Wang et al. 2012). In EV-A71, the pocket factor is partly exposed on the viral particle (Plevka et al. 2014) and could be accessed by antiviral. Wang and colleagues showed that a receptor binding that engage to VP1 causes a collapse on pocket factor (X. Wang et al. 2012). Thus, MJTX-I, MJTX-II and crotamin that demonstrated a significant virucidal activity, could be acting on this pocket factor and destabilizing virus particle. However, more studies are need for a better understand of how these two phospholipases are acting on EV-A71 entry

Altogether, the results demonstrated that all the evaluated venom proteins inhibited a minimum of one stage of EV-A71 replicative cycle. More interestingly, MJTX-II and Crotamin were able to significantly inhibit all stages of virus infection investigated.

This study is the first report of protein isolated from Brazilian snake venoms with antiviral activity against EV-A71.

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Tables

Table 1. Activity of proteins isolated from snake venoms.

Protein	*Venom Origim*	*Non-toxic concentration*	*Cell viability (%)*	*Protective activity (%)*	*Virucidal activity (%)*	*Inhibition of replication (%)*
MJTX-I	*B. moojeni*	*50µg/mL*	107	-	88	99
MJTX-II	*B. moojeni*	*50µg/mL*	104	76	98	100
Crotamin	*C. durissus terrificus*	*50µg/mL*	93	62	99	97
Crotapotin	*C. durissus terrificus*	*50µg/mL*	96	60	38	11
Crotoxin	*C. durissus terrificus*	*50µg/mL*	87	-	56	-
PLA2-CB	*C. durissus terrificus*	*5µg/mL*	97	-	-	89

Figures and legends

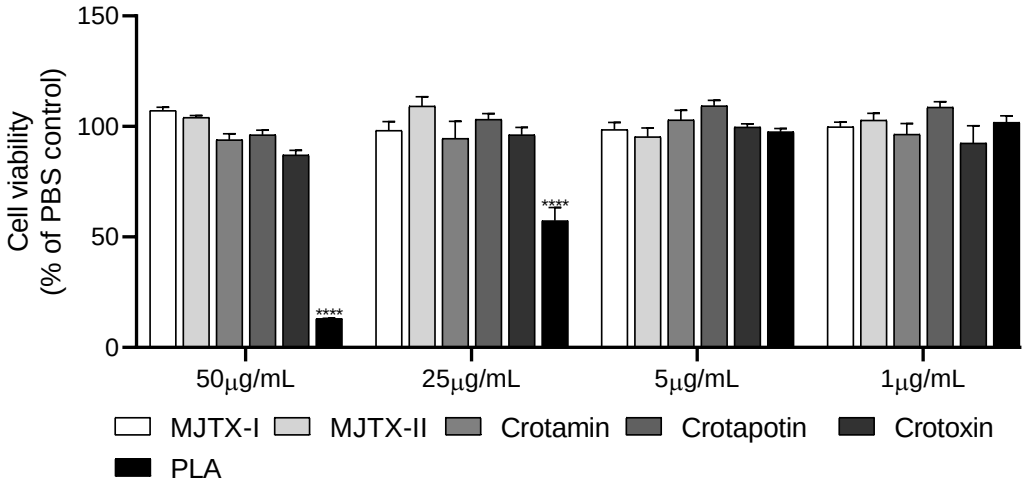

Figure 1: Cell viability of proteins isolated from Brazilian snakes venoms. No infected Vero cells were plated in a 96 well plates and treated with proteins for 72 hours. PBS was used as untreated control. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. The asterisks indicate statistically significant differences between each compound and PBS control. $P < 0.05$ was considered significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$).

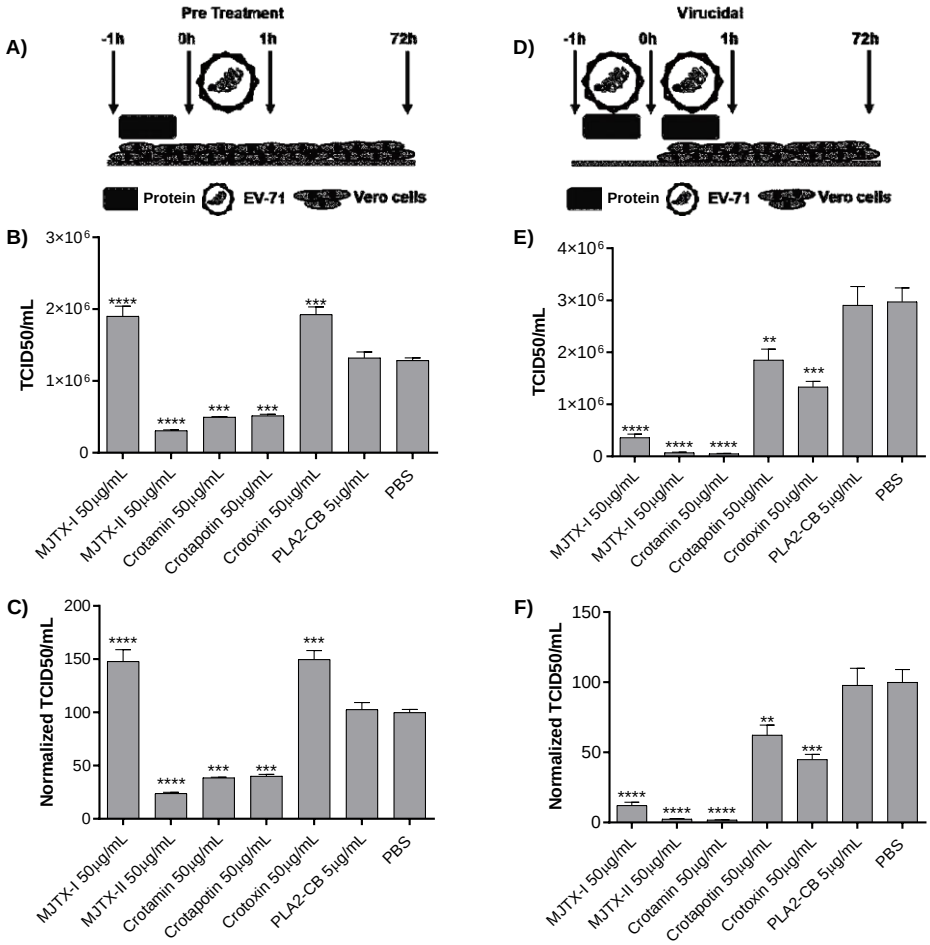

Figure 2: Protective and virucidal effects of proteins against enterovirus 71 infection. Virus supernatant and proteins were added in different times to the cells and virus was titrated by TCID50 method. Pre-treatment assay: Vero cells were treated with proteins for 1 h, washed with PBS and infected with EV-A71 for 1 h. Then, cells were washed and replaced with fresh media and incubated for 72 h (A), supernant were collected and titrated (B) and normalized with PBS control (C). For virucidal assay: EV-A71 were incubated with proteins for 1 h prior to the infection on Vero cells. Cells were added of the mixture for 1 h, washed and fresh media was added, cells were incubated for 72 h (D), supernant were collected and titrated (E) and normalized with PBS control (F). PBS was used as untreated infected control. The asterisks indicate statistically significant differences between each compound and PBS control. $P < 0.05$ was considered significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$).

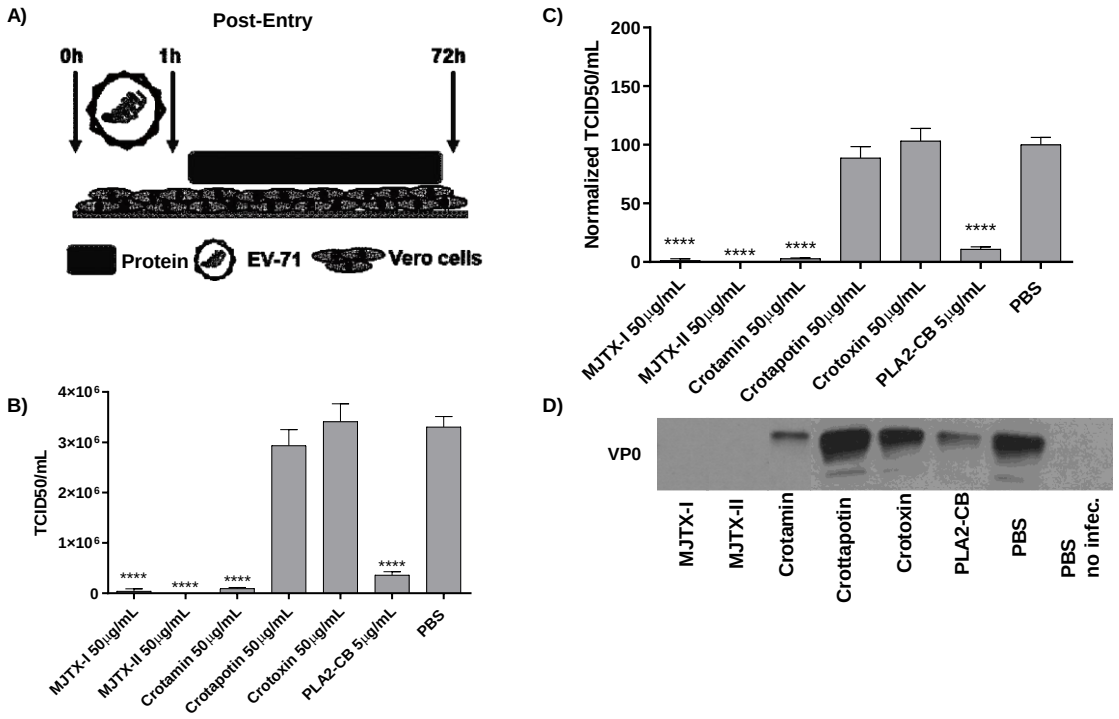

Figure 3. Effect of proteins on enterovirus 71 post-entry. Vero cells were infected with virus supernatant for 1h, washed to the removal of non-endocyted virus and compounds were added. Cells were incubated for 72h (A). Replication efficiency was measured by using TCID50 method assay (B and C) and western blotting assays (D). PBS was used as untreated infected control. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. The asterisks indicate statistically significant differences between each compound and PBS control. $P < 0.05$ was considered significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$).

Capítulo IV
Considerações Finais

Considerações Finais

- Os resultados deste trabalho demonstram que os compostos testados, poderão servir de base para novos estudos em busca de terapias antivirais.
- O sistema virtual é um facilitador no processo de busca por novos antivirais, no entanto, não elimina outras variáveis em um sistema vivo e precisa ser combinada com ensaios biológicos.
- As proteínas isoladas da peçonha de serpentes apresentaram atividade inibitória significante e que fornecerão informação para o potencial desenvolvimento de novas terapias antivirais.

Perspectivas para o futuro.

Mais estudos são necessários para avaliar mecanismos de ação antiviral dessas moléculas, além da investigação de testes *in vivo* e vias de entrega para esses compostos.

Apêndice A

Artigos Publicados

PLOS ONE

RESEARCH ARTICLE

Multiple effects of toxins isolated from *Crotalus durissus terrificus* on the hepatitis C virus life cycle

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Abstract

Hepatitis C virus (HCV) is one of the main causes of liver disease and transplantation worldwide. Current therapy is expensive, presents additional side effects and viral resistance has been described. Therefore, studies for developing more efficient antivirals against HCV are needed. Compounds isolated from animal venoms have shown antiviral activity against some viruses such as *Dengue virus*, *Yellow fever virus* and *Measles virus*. In this study, we evaluated the effect of the complex crotoxin (CX) and its subunits crotapotin (CP) and phospholipase A_2 (PLA_2-CB) isolated from the venom of *Crotalus durissus terrificus* on HCV life cycle. Huh 7.5 cells were infected with HCVcc JFH-1 strain in the presence or absence of these toxins and virus was titrated by focus formation units assay or by qPCR. Toxins were added to the cells at different time points depending on the stage of virus life cycle to be evaluated. The results showed that treatment with PLA_2-CB inhibited HCV entry and replication but no effect on HCV release was observed. CX reduced virus entry and release but not replication. By treating cells with CP, an antiviral effect was observed on HCV release, the only stage inhibited by this compound. Our data demonstrated the multiple antiviral effects of toxins from animal venoms on HCV life cycle.

Introduction

Hepatitis C is a disease caused by Hepatitis C virus (HCV) infection, essentially characterized by liver inflammation. Chronic infection may progress to cirrhosis or hepatocellular carcinoma and represents one of the major causes of liver diseases and transplants [1]. Approximately 130–150 million people are chronically infected worldwide [2].

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Flavonoids from *Pterogyne nitens* Inhibit Hepatitis C Virus Entry

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Hepatitis C virus (HCV) is one of the leading causes of liver diseases and transplantation worldwide. The current available therapy for HCV infection is based on interferon-α, ribavirin and the new direct-acting antivirals (DAAs), such as NS3 protease and NS5B polymerase inhibitors. However, the high costs of drug design, severe side effects and HCV resistance presented by the existing treatments demonstrate the need for developing more efficient anti-HCV agents. This study aimed to evaluate the antiviral effects of sorbifolin (1) and pedalitin (2), two flavonoids from *Pterogyne nitens* on the HCV replication cycle. These compounds were investigated for their anti-HCV activities using genotype 2a JFH-1 subgenomic replicons and infectious virus systems. Flavonoids 1 and 2 inhibited virus entry up to 45.0% and 78.7% respectively at non-cytotoxic concentrations. The mechanism of the flavonoid 2 block to virus entry was demonstrated to be by both the direct action on virus particles and the interference on the host cells. Alternatively, the flavonoid 1 activity was restricted to its virucidal effect. Additionally, no inhibitory effects on HCV replication and release were observed by treating cells with these flavonoids. These data are the first description of 1 and 2 possessing *in vitro* anti-HCV activity.

Hepatitis C virus (HCV) was identified in 1989 as the causative agent of hepatitis C[1]. It infects millions of people worldwide and is the major cause of liver disease and transplantation. According to World Health Organization (WHO), more than 350,000 people die currently from liver disease related to HCV infection[2].

HCV is an enveloped, single stranded positive-sense RNA virus, which belongs to the Flaviviridae family, genus *Hepacivirus*[3]. There is no effective vaccine for prevention of the HCV infection and, until recently, the only treatment for HCV infected patients was based on pegylated interferon and ribavirin association (PEG-IFN + RBV)[4]. The availability of new direct acting antivirals (DAAs) such as simeprevir, daclatasvir and sofosbuvir have increased rates of sustained virological response (SVR) with treatment efficacies as high as 90% for most common HCV genotypes[5–8]. However, the current treatments present several side effects, high costs[9] and resistant variants were described even for the recent therapies approved by Food and Drugs Administration (FDA)[10,11]. Therefore, despite the introduction of interferon-free regimens, the therapy regime in many countries is still based on PEG-IFN + RBV.

The high costs and potential for developing viral resistance presented by the existing treatments demonstrate the need for improving therapeutic options against HCV. In this context, natural sources have demonstrated to provide a wide source of compounds, which can be evaluated for their antiviral properties[12].

Flavonoids represent an important class of compounds, which are produced by plants as a response to microbial infections[13]. Several flavonoids have been described to possess antiviral activities. Epigallocatechin from green tea was demonstrated to inhibit replication of Enterovirus 71[14], Chikungunya virus[15] and HCV[16]. Naringenin from grapefruit showed antiviral effects against Herpes simplex virus type 1 (HSV-1)[17] and HCV[18]. Silibin and ladanein have also demonstrated anti-HCV activities by inhibiting the viral entry step[19,20]. In this context, the high structural similarity between ladanein and flavonoids from *Pterogyne nitens* Tul. (Leguminosae) and other active flavonoids encouraged us to search for their potential anti-HCV activity by inhibition of viral entry.

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Virology Journal

REVIEW Open Access

Plant-derived antivirals against hepatitis c virus infection

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Abstract

Hepatitis C virus (HCV) infection is a worldwide public health burden and it is estimated that 185 million people are or have previously been infected worldwide. There is no effective vaccine for prevention of HCV infection; however, a number of drugs are available for the treatment of infection. The availability of direct-acting antivirals (DAAs) has dramatically improved therapeutic options for HCV genotype 1. However, the high costs and potential for development of resistance presented by existing treatment demonstrate the need for the development of more efficient new antivirals, or combination of therapies that target different stages of the viral lifecycle. Over the past decades, there has been substantial study of compounds extracted from plants that have activity against a range of microorganisms that cause human diseases. An extensive variety of natural compounds has demonstrated antiviral action worldwide, including anti-HCV activity. In this context, plant-derived compounds can provide an alternative approach to new antivirals. In this review, we aim to summarize the most promising plant-derived compounds described to have antiviral activity against HCV.

Keywords: Hepatitis C, Natural compounds, Antivirals

Background

Hepatitis C virus (HCV) infection is a worldwide public health burden and it is estimated that 185 million people are or have previously been infected worldwide, representing almost 3% of the global population. Most infections persist and chronically infected individuals have a high risk to develop liver cirrhosis and hepatocellular carcinoma after 10 to 30 years of infection [1–3].

HCV transmission occurs by parenteral via. The main route of transmission was blood transfusion until in the 1990s when Food and Drug Administration (FDA) authorized medical centers to screen blood for HCV. Nowadays, the sharing of contaminated syringes by drug users and inadequate sterilization of medical equipment are the main transmission routes [4–6].

There is no effective vaccine for prevention of HCV infection, however, a number of drugs are available for the treatment of infection. Until recently, the standard therapy was based on pegylated interferon (IFN) plus ribavirin (RBV), resulting in a sustained virological response in approximately 50% of patients infected with HCV genotypes 1a/1b and 80% of those infected with genotypes 2 or 3 [7–9]. The availability of new, direct-acting antivirals (DAAs) targeting the NS3/4A protease, NS5B polymerase and NS5A protein have dramatically improved therapeutic options for HCV genotype 1 [10, 11]. However, the high costs and potential for development of resistance presented by existing treatment demonstrate the need for the development of more efficient new antivirals, or combination of therapies that target different stages of the viral lifecycle. The efforts to develop innovative anti-HCV drugs are challenged by the viral high mutation rate, which allows the rapid emergence of resistant strains to DAAs directed to the NS5A and NS3 regions. Further issues to overcome include the development of a drug which can impair the virus with limited side effects on the host cell and the affordable access to care in developing countries.

Over the past decades, there has been substantial study of compounds extracted from plants that have activity against a range of microorganisms that cause

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A diarylamine derived from anthranilic acid inhibits ZIKV replication

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Zika virus (ZIKV) is a mosquito-transmitted Flavivirus, originally identified in Uganda in 1947 and recently associated with a large outbreak in South America. Despite extensive efforts there are currently no approved antiviral compounds for treatment of ZIKV infection. Here we describe the antiviral activity of diarylamines derived from anthranilic acid (FAMs) against ZIKV. A synthetic FAM (E3) demonstrated anti-ZIKV potential by reducing viral replication up to 86%. We analyzed the possible mechanisms of action of FAM E3 by evaluating the intercalation of this compound into the viral dsRNA and its interaction with the RNA polymerase of bacteriophage SP6. However, FAM E3 did not act by these mechanisms. *In silico* results predicted that FAM E3 might bind to the ZIKV NS3 helicase suggesting that this protein could be one possible target of this compound. To test this, the thermal stability and the ATPase activity of the ZIKV NS3 helicase domain ($NS3^{Hel}$) were investigated *in vitro* and we demonstrated that FAM E3 could indeed bind to and stabilize $NS3^{Hel}$.

Zika virus (ZIKV) is a mosquito - transmitted virus first isolated in 1947 from a *Rhesus* monkey in the Zika forest, Uganda[1]. ZIKV remained endemic to the African and Asian regions until 2007, since then the virus has spread to other continents[2–6]. Notably, in 2015, the ZIKV outbreak had a worldwide impact and was considered a serious public health problem due to the large number of people infected and the development of neurological disorders in neonates (microcephaly) and adults (Guillain Barre syndrome)[7].

Similar to other arboviruses such as Dengue virus (DENV), Yellow Fever virus (YFV) and Chikungunya virus (CHIKV), ZIKV is mainly transmitted by *Aedes spp.* of mosquitoes[8–10]. Nevertheless, other sources of infection acquisition have been reported, including blood transfusion[9], sexual[11,12], perinatal and transplacental transmissions[5,13]. Recently, it has been suggested that ZIKV may also have a sylvatic transmission cycle which could increase the frequency of human reinfection[14].

ZIKV belongs to the *Flaviviridae* family and genus *Flavivirus*[15]. As other members of the genus, the viral genome is a positive single-stranded RNA with one open reading frame (ORF), translated in a polyprotein that is

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