



UNIVERSIDADE FEDERAL DE UBERLÂNDIA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA E PARASITOLOGIA
APLICADAS



**DERIVADOS DE BENZOTIAZOL COMO INIBIDORES DO CICLO REPLICATIVO
DO VÍRUS CHIKUNGUNYA**

SHIRAZ FEFERBAUM LEITE

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SHIRAZ FEFERBAUM LEITE

Dissertação apresentada ao
colegiado do Programa de Pós-
Graduação em Imunologia e
Parasitologia Aplicadas como
requisito parcial para obtenção do
título de mestre.

Orientadora: Profa. Dra. Ana
Carolina Gomes Jardim

Coorientador: Prof. Dr. Celso de
Oliveira Rezende Júnior

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Agradeço a todos os meus amigos, a presença de vocês na minha vida faz toda a diferença

"If I have seen further it is by standing on the shoulders of Giants."

Isaac Newton

LISTA DE ABREVIATURAS E SIGLAS

AUD	<i>Alphavirus Unique Domain</i> (AUD)
CHIKF	<i>Chikungunya fever</i> (Febre chikungunya)
CHIKV	<i>Chikungunya virus</i> (Vírus chikungunya)
DMEM	<i>Dulbecco's Modified Eagle Medium</i> (Meio Básico Modificado por Dulbecco)
DMSO	<i>Dimetilsulfoxide</i> (Dimetilsulfóxido)
IS	Índice de Seletividade
MOI	<i>Multiplicity of infection</i> (Multiplicidade de infecção)
MTT	<i>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</i> (Brometo de 3-(4',5'-dimetiltiazol-2'-ila)-2,5-difeniltetrazol)
MXRA8	<i>Matrix Remodeling Associated 8</i> (Remodelação de Matriz Associada 8)
nsP	<i>Non-structural proteins</i> (Proteínas não estruturais)
ORFs	<i>Open Reading Frame</i> (Regiões de leitura aberta)
TF	<i>Transframe</i> (Transquadro)
RE	Retículo Endoplasmático
RNA	<i>Ribonucleic Acid</i> (Ácido ribonucleico)
RPdR	RNA Polimerase dependente de RNA
SAR	<i>Structure Activity Relationship</i> (Relação entre Estrutura e Atividade)
ZIKV	<i>Zika virus</i> (Vírus da Zika)

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RESUMO

O vírus chikungunya (CHIKV) é o patógeno causador da febre chikungunya (CHIKF). Embora represente um importante problema de saúde, principalmente em países tropicais, permanece como uma doença negligenciada e reemergente. O tratamento é baseado em medidas paliativas para controlar sintomas como febre, dor de cabeça e exantemas. A doença pode se tornar crônica e resultar em poliartralgias, que podem levar à artrite. Como não há medicamentos licenciados para tratar CHIKF, a busca por terapias é essencial. Nesse contexto, nós avaliamos 20 sulfonamidas sintéticas contra CHIKV-*nanoluc in vitro*, com o objetivo principal de identificar novos inibidores antivirais. Dentre os compostos testados, 13 são derivados heteroaromáticos: 1-3 são derivados de tiazol e benzimidazol; 4-13 estão associados a derivados de benzotiazol (BTA); e 7 são sulfonamidas derivadas de ligações de ácido carboxílico. Os BTAs 6, 9, 11 e 13 exibiram a atividade inibitória mais potente com base em ensaios de dose-resposta. Esses compostos mostraram índice de seletividade (IS) de 13,8, 5,8, 4,4 e 11, respectivamente. Além disso, estes compostos demonstraram atividade pós-entrada, inibindo a replicação do CHIKV em até 98%. Curiosamente, o composto 9 apresentou efeito antiviral em diferentes estágios do ciclo replicativo viral, como virucida (55%), pré-tratamento (69%) e entrada dos vírus na célula hospedeira (98%). A análise de ancoragem molecular revelou as maiores pontuações de afinidade entre os derivados de BTA e as proteínas não estruturais do CHIKV. Além disso, dado que 9 também exibiu ação inibitória na entrada viral nas células hospedeiras, a ancoragem molecular foi conduzida com a glicoproteína do envelope do CHIKV da mesma forma, apresentando uma pontuação de -8,01 kcal/mol. A análise de espectroscopia de infravermelho corroborou o efeito de 9 nas partículas virais, indicando interação com o complexo de glicoproteína e lipídios. Os dados apresentados neste trabalho podem fornecer uma fonte de informações relevante para futuras pesquisas *in vivo* e ensaios clínicos para desenvolvimento de tratamentos direcionados à CHIKF.

Palavras-chave: antiviral, vírus chikungunya, derivados de benzotiazol.

ABSTRACT

Chikungunya virus (CHIKV) is the causative pathogen of Chikungunya Fever (CHIKF). It has become a major health problem, mostly in tropical countries, and remains as a neglected and reemergent disease. The treatment is based on palliative measures to control symptoms such as fever, headache, and exanthemas. The disease can become chronic and result in polyarthralgia, that may lead to arthritis. Since there are no licensed drugs to treat CHIKF, the search for therapies is essential. In this sense, here we evaluated 20 synthetic sulfonamides against CHIKV-*nanoluc in vitro* with the primary goal of identifying novel antiviral inhibitors. Among the tested compounds, 13 are heteroaromatic derivatives: 1-3 are thiazole and benzimidazole derivatives; 4-13 are associated with benzothiazole (BTA) derivatives; and 7 are sulfonamides derived from carboxylic acid bonds. BTA derivatives 6, 9, 11 and 13 exhibited the most potent inhibitory activity based on dose-response assays. These compounds displayed selective index (SI) of 13.8, 5.8, 4.4, and 11, respectively. Furthermore, these compounds demonstrated post-entry activity, inhibiting CHIKV replication in up to 98%. Interestingly, compound 9 displayed antiviral effect on different stages of viral replicative cycle, such as virucidal (55%), pre-treatment (69%), and virus entry to the host cells (98%). The molecular docking analysis revealed the highest affinity scores between BTA derivatives and the non-structural proteins of CHIKV. Additionally, given that 9 also exhibited inhibitory action on viral entry to the host cells, molecular docking was also conducted with the envelope glycoprotein of CHIKV, yielding a -8.01 kcal/mol score. Infrared spectroscopy analysis corroborated the effect of 9 on virus particles, by indicating interaction with the glycoprotein complex and lipids. The data presented in this work can provide a source for future *in vivo* and clinical research in the development of treatments for CHIKF.

Keywords: antiviral, chikungunya virus, benzothiazole derivatives.

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CAPÍTULO I

Fundamentação Teórica

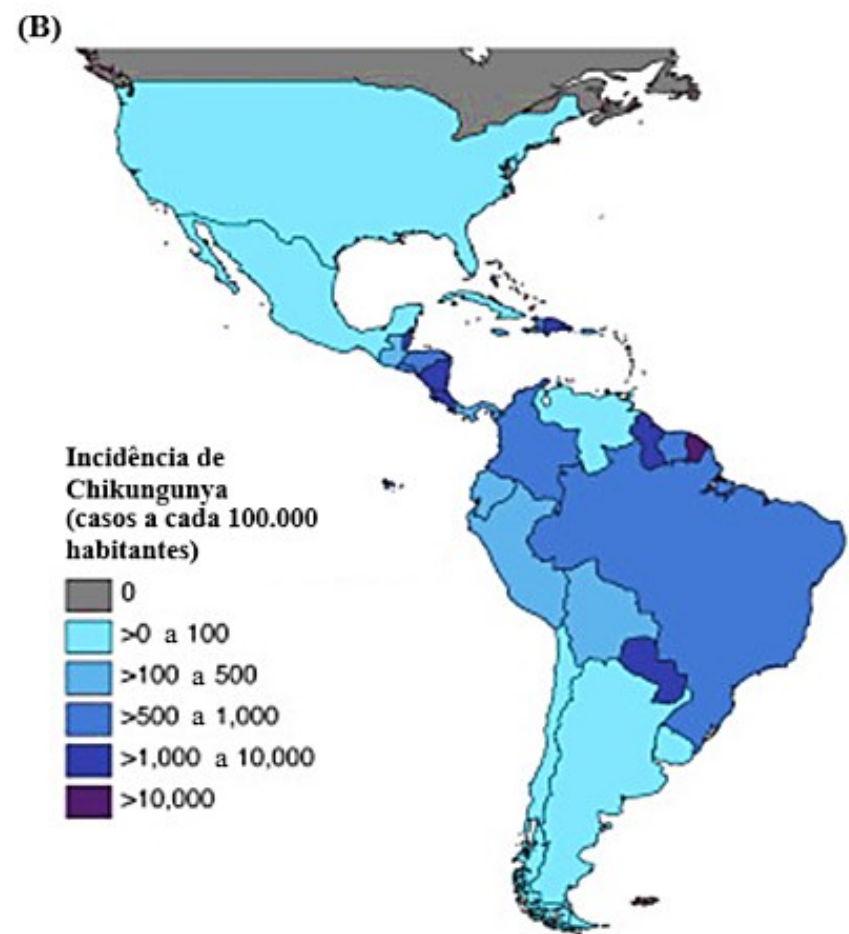
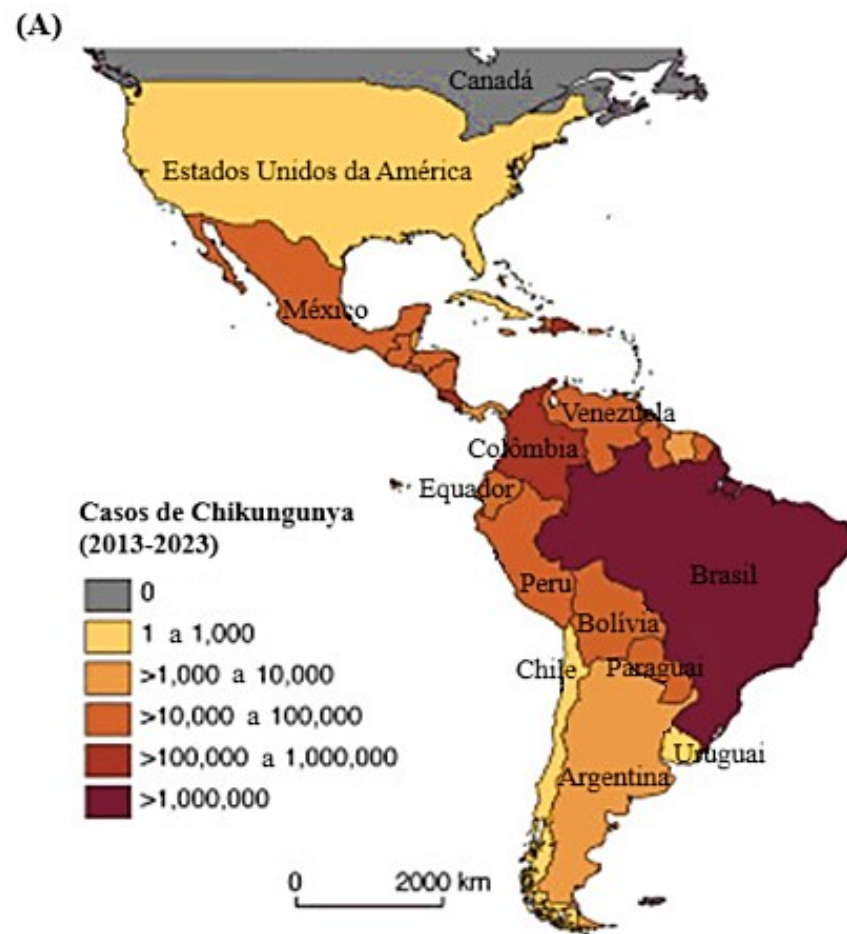
INTRODUÇÃO

História e Epidemiologia do Vírus Chikungunya

Entre 1952 e 1953 ocorreu uma epidemia viral na Tanzânia, caracterizando-a como o primeiro país do leste africano a reportar o vírus chikungunya (CHIKV), isolado do soro de um paciente febril (ROBINSON, 1955). A etimologia da palavra chikungunya se originou do dialeto Kimakonde, e significa “aquele que se curva”, fazendo alusão à postura dos indivíduos que desenvolviam artralgia (KUMAR et al., 2021). Nos anos seguintes, outros surtos foram relatados em diferentes países do centro e sul da África (WAHID et al., 2017).

Posteriormente, houve expansão das ocorrências epidêmicas para países asiáticos, como Tailândia, Índia, Sri Lanka e Filipinas (KHONGWICHIT et al., 2021). Em 2005, a ilha de *Reunion*, localizada no Oceano Índico, passou por um surto massivo associado a casos mais graves que progrediam para o envolvimento do sistema nervoso central (DE LIMA CAVALCANTI et al., 2022). Nas Américas, a primeira evidência de incidência do CHIKV foi na Ilha de *Saint Martin* em dezembro de 2013 (LEPARC-GOFFART et al., 2014). Entre dezembro de 2013 e junho de 2023 foram reportados 3.684.554 casos distribuídos por 50 países americanos (**Figura 1A e 1B**) (DE SOUZA et al., 2024).

Figura 1: Epidemiologia do chikungunya nas Américas. (A) Casos cumulativos de chikungunya reportados à Organização Pan Americana de Saúde (PAHO) referentes a dezembro de 2013 a junho de 2023. **(B)** Incidência cumulativa de casos de chikungunya a cada 100.000 habitantes.



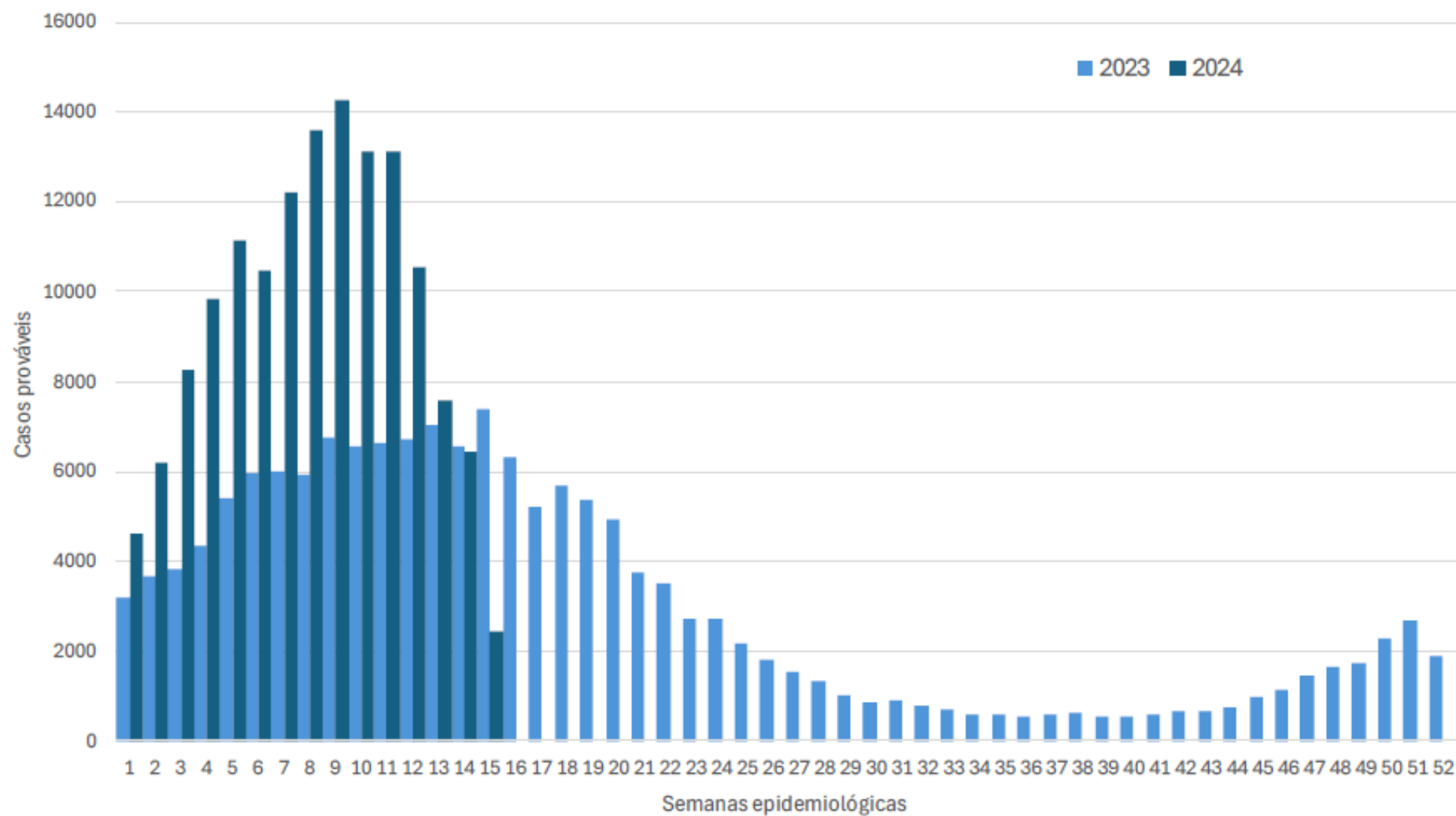
Adaptado de (DE SOUZA et al., 2024).

A partir de análises filogenéticas, foram determinadas quatro linhagens de genótipos relacionadas à origem geográfica das epidemias: ECSA (*East Central South African* – Leste Centro Sul Africano), WA (*West African* – Oeste Africano), *Asian* (Asiática) e IOL (*Indian Ocean Lineage* – Linhagem Oceano Índico) (POWERS et al., 2000). A IOL derivou da linhagem ECSA, devido os surtos na ilha de *Reunion* entre 2005 e 2006, e posteriormente se alastrou para Índia e Ásia, resultando também em transmissões autóctones na Europa Mediterrânea (ARANKALLE et al., 2007; LIUMBRUNO et al., 2008).

A circulação de CHIKV no Brasil vem sendo reportada desde 2014, com o início das ocorrências, em sua maioria, restritas à região Nordeste (NUNES et al., 2015). No entanto, o clima e a densidade populacional permitiram a ampliação dos casos para outros estados, sendo registrados 1.659.167 de casos desde 2016, e se tornando o epicentro da epidemia de CHIKV nas Américas (**Figura 1A**) (DE SOUZA et al., 2024).

Conforme o último boletim epidemiológico de 2024, entre as semanas epidemiológicas (SE) 1 e 15, foram notificados 188.836 casos suspeitos, sendo 143.831 prováveis (**Figura 2**) (Organização Pan-Americana da Saúde, 2024). Comparado à 2023 (n = 86.044), houve aumento de 67% no número de casos em relação ao mesmo período. A região Sudeste compreende 72% (n = 103.891) dos casos, dos quais 62,1% (n = 89.330) se concentram em Minas Gerais, onde há registro de 41 óbitos (Organização Pan-Americana da Saúde, 2024).

Figura 2: Casos prováveis de chikungunya por SE de 2023 e 2024, Brasil. Atualização epidemiológica dos casos de chikungunya na região das Américas.



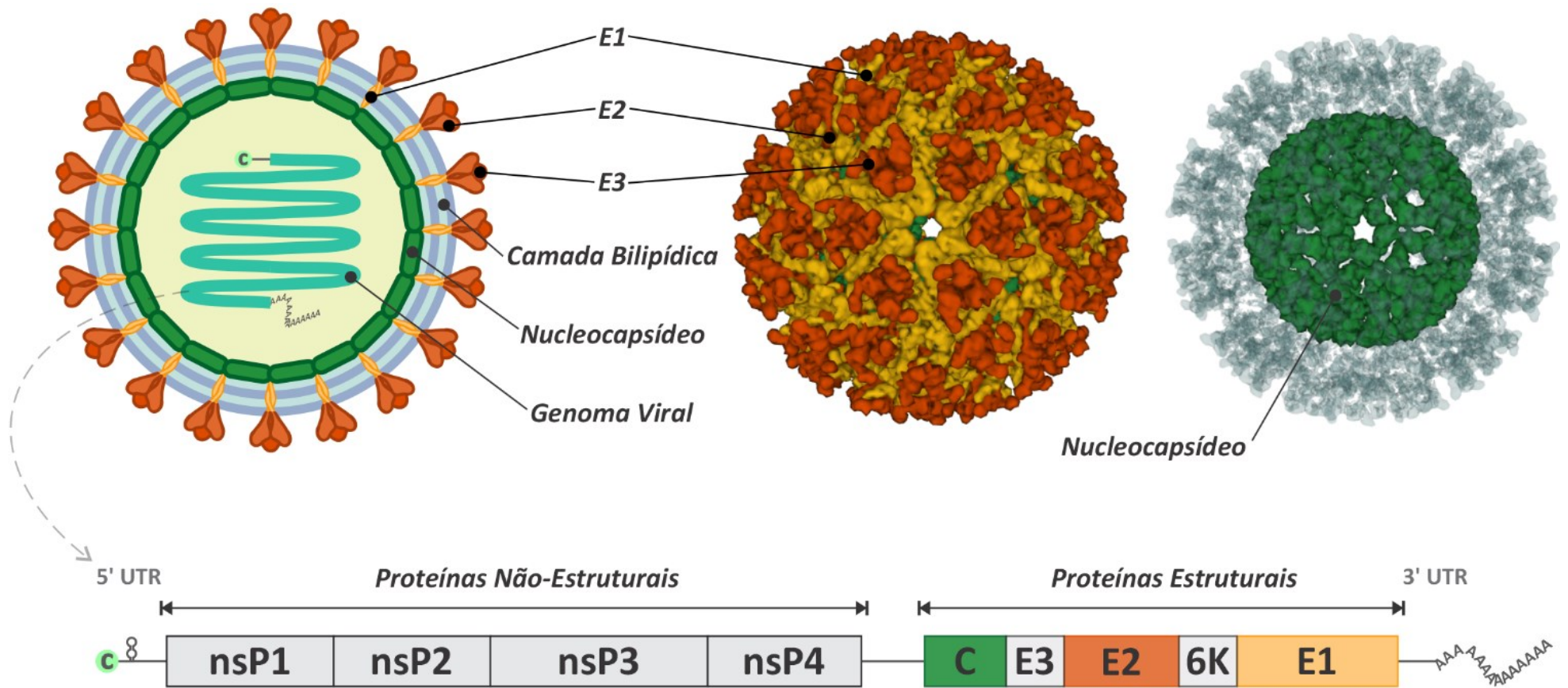
OPAS/OMS - 22 de abril de 2024.

Partícula Viral e Ciclo Replicativo

O CHIKV pertence ao gênero *Alphavirus* e família *Togaviridae*. A partícula envelopada possui diâmetro de aproximadamente 70 nanômetros (nm) e seu nucleocapsídeo icosaédrico abriga um genoma de RNA fita simples positiva com extensão aproximada de 18 kilobases (kb). O RNA genômico (RNAg) contém duas regiões de leitura aberta (*Open Reading Frame* - ORF), que codificam uma poliproteína não estrutural, de onde se originam as proteínas não estruturais (nsPs) 1, 2, 3 e 4; e uma poliproteína estrutural, posteriormente clivada, resultando nas glicoproteínas de envelope (E1, E2, E3), capsídeo (C) e proteínas 6K e TF (*transframe*). Adicionalmente, o RNAg possui a presença de “cap” na região 5’UTR (*Untranslated Region* – Região Não Traduzida) e cauda poliadenilada na parte 3’UTR, assemelhando-se ao RNA mensageiro (RNAm) celular e permitindo a tradução imediata das proteínas virais quando o genoma é liberado no citoplasma da célula hospedeira (

Figura 3) (KHONGWICHIT et al., 2021).

Figura 3: Representação esquemática da partícula viral e genoma do CHIKV.

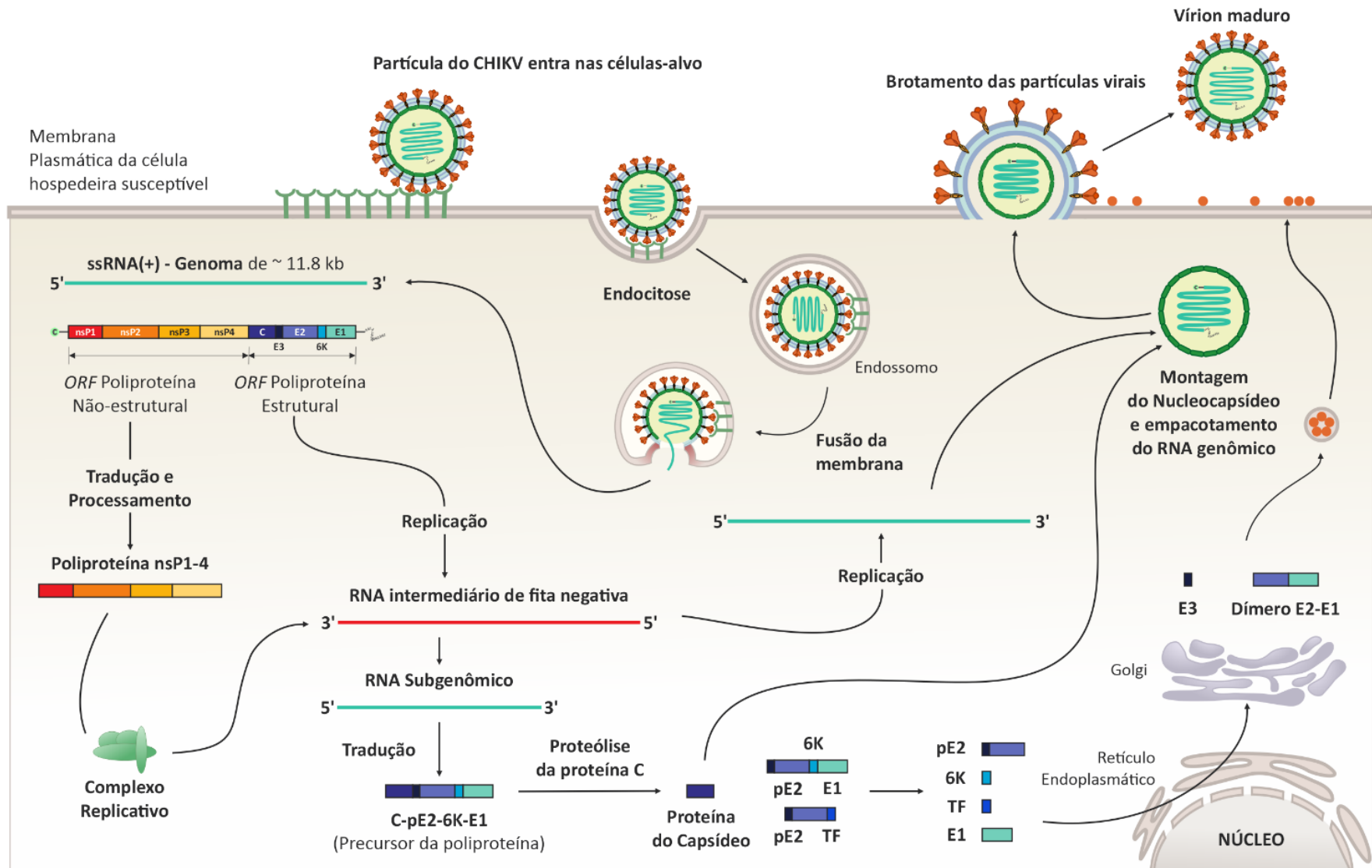


Adaptado de (RUIZ et al., 2023).

O CHIKV se replica em células de *Aedes aegypti* e *Aedes albopictus*, vetores artrópodes, e por isso é considerado um arbovírus (CIOTA, 2019). O ciclo replicativo viral se inicia com a ligação das glicoproteínas de envelope do CHIKV aos receptores da célula hospedeira. O receptor mais conhecido por essa interação com vírus artrítogênico é o MXRA8 (*Matrix Remodeling Associated 8* - Remodelação de Matriz Associada 8), amplamente expresso em fibroblastos dérmicos e sinoviais, osteoblastos, condrócitos e células de músculo esquelético (HOORNWEG et al., 2016; ZHANG et al., 2018). Em seguida, ocorre a penetração por endocitose mediada por clatrin, na qual o pH ácido do endossomo gera mudanças conformacionais que proporcionam a exposição da glicoproteína E1 do envelope viral, responsável por mediar a fusão do vírus com a membrana endossomal (KIELIAN; REY, 2006). Isso permite o desmonte do nucleocapsídeo e liberação do RNA genômico no citoplasma, logo traduzido pelos ribossomos celulares para gerar a poliproteína não estrutural (p1234). A p1234 é subsequentemente processada em nsP1, nsP2, nsP3 e nsP4, que formam o complexo replicativo responsável pela síntese da fita intermediária negativa de RNA (SCHWARTZ; ALBERT, 2010). Esta fita serve como molde para a produção de novas cópias do RNA genômico (g) e subgenômico (sg), que, por sua vez, contém os genes da poliproteína estrutural pC-pE2-6K/TF-E1 (SCHWARTZ; ALBERT, 2010). A proteína C é liberada devido à sua atividade autoproteolítica e direcionada para a montagem do nucleocapsídeo e empacotamento do material genético, enquanto as outras proteínas são clivadas por peptidases no Retículo Endoplasmático (RE) (AGGARWAL et al., 2015). No Complexo de Golgi, proteases do tipo furina clivam a pE2 em E2, que está envolvida na ligação ao receptor, e em E3, que facilita o dobramento da precursora pE2 e sua posterior associação com a proteína E1 (CONSTANT et al., 2021). Após a associação, o heterodímero E1-E2 é transportado até a membrana plasmática, onde se integra externamente ao nucleocapsídeo, formando uma partícula viral completa que será liberada da célula hospedeira por brotamento (PÉREZ-PÉREZ et al., 2019) (

Figura 4).

Figura 4: Representação esquemática do ciclo replicativo do CHIKV.



Adaptado de (PASCHOALINO et al., 2023).

Proteínas Estruturais e Não Estruturais do CHIKV

As proteínas estruturais e não estruturais do CHIKV são intrinsecamente importantes, uma vez que desempenham papéis cruciais em cada estágio do ciclo replicativo viral. Dentre as proteínas estruturais, a proteína C forma o nucleocapsídeo, responsável pela proteção e estabilidade do material genético (NORANATE et al., 2014). As proteínas de envelope E1 e E2 se associam em heterodímeros, ancorados na porção externa da partícula viral, e estão envolvidas no processo de adsorção e penetração. E1 é uma glicoproteína de fusão que media a interação com a membrana através de sua região hidrofóbica, enquanto E2 se liga nos receptores de superfície da célula. Além disso, quando o vírion está maduro, E2 está posicionada acima de E1, protegendo-a contra a exposição prematura do sítio hidrofóbico (MANGALA PRASAD et al., 2022). Por outro lado, acredita-se que a glicoproteína E3 esteja relacionada com a estabilização do heterodímero e manutenção de um pH equilibrado até que os vírions sejam liberados das células hospedeiras (UCHIME; FIELDS; KIELIAN, 2013). 6K é essencial para o brotamento viral devido sua atividade de canal iônico, ao mesmo tempo que TF demonstrou ter função semelhante, dado que compartilha a maior parte da sequência de aminoácidos com a 6K (SNYDER et al., 2013).

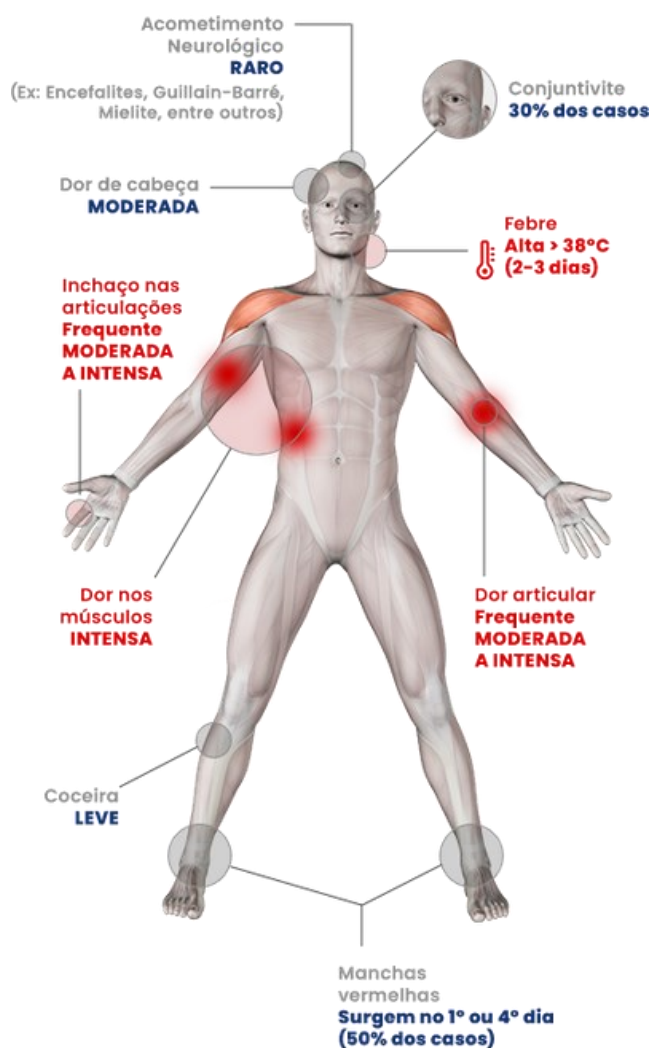
Alternativamente, as proteínas não estruturais são organizadas em complexos replicativos, sendo expressas em conjunto e dependentes das atividades umas das outras para assegurar a replicação do RNA viral (SPUUL et al., 2011). NsP1 e nsP2 apresentam propriedades *capping*, na qual a primeira possui ação de guanina metiltransferase (MTase) e guanililtransferase (GTase), enquanto a segunda contém um domínio RTPase que hidrolisa a ligação de fosfato na região final da 5', além de ação helicase (LAW et al., 2023). A proteína nsP3 possui três domínios distintos, conferindo-lhe multifuncionalidade: o macrodomínio exibe atividade hidrolase e é essencial para a iniciação da replicação viral; o domínio único de alphavirus (*Alphavirus Unique Domain* - AUD) está envolvido na montagem viral; por fim, a porção hipervariável permite sua participação no recrutamento de fatores da célula hospedeira, na formação de sítios replicativos e na modulação da resposta imune do hospedeiro (GAO et al., 2019; SRIVASTAVA et al., 2024). Finalmente, a nsP4 representa o fator essencial no processo de alongamento do genoma, conhecida como RNA Polimerase dependente de RNA (RPdR) (FREIRE et al., 2022).

Febre Chikungunya

A transmissão do CHIKV ocorre principalmente pela inoculação da saliva do mosquito fêmea (gênero *Aedes*) infectado nos humanos, embora haja relatos de transmissão vertical (GÉRARDIN et al., 2008). Após inoculado, o CHIKV se replica nas células fibroblásticas dérmicas, queratinócitos, melanócitos e células endoteliais, que permitem a disseminação do vírus através do sistema circulatório e linfático para todos os órgãos (KAM et al., 2009).

O período de incubação da infecção varia de 3 a 7 dias (CAI et al., 2023). Geralmente a CHIKF é caracterizada por sintomas autolimitantes na fase aguda, como febre alta, erupções cutâneas, inchaços e dores articulares e musculares (**Figura 5**) (BURT et al., 2017). Quando a infecção progride para uma condição crônica, os indivíduos podem enfrentar poliartralgias persistentes por meses ou até anos, que, em alguns casos, evoluem para artrite (SCHWARTZ; ALBERT, 2010).

Figura 5: Sintomas da febre chikungunya.



Ainda que o CHIKV não seja considerado um vírus neurotrópico, existem registros que o correlacionam com complicações neurológicas como encefalite, mielopatia e neuropatia periférica (MEHTA et al., 2018). Ademais, embora incomum, foram registradas infecções fatais por CHIKV que levaram os pacientes à óbito, sobretudo recém-nascidos e idosos com comorbidades (LEVI; VIGNUZZI, 2019).

Profilaxia e Tratamento da Febre Chikungunya

As profilaxias usuais contra o CHIKV se concentram no combate ao vetor através de químicos aduictidas e larvicidas, métodos mecânicos que evitam água parada, e técnicas de armadilha para captura e morte do mosquito (WAHID et al., 2017). Ainda, existem tecnologias de manipulação biológica para supressão ou modificação da população vetorial a fim de reduzir a transmissão do vírus, como infecção com parasita *Wolbachia* e mosquitos transgêneros que geram fêmeas estéreis (WANG et al., 2021). Embora sejam medidas profiláticas importantes, é necessária a associação com outras formas de controle do vetor e vacina.

Diversas vacinas foram desenvolvidas contra CHIKV, entretanto, a primeira delas a chegar na última fase clínica até o presente momento foi a VLA1553, baseada em vírus atenuado e de dose única (BARTHOLOMEEUSEN et al., 2023a). De acordo com estudos realizados nos Estados Unidos, a vacina desenvolvida pela companhia Valneva promoveu resposta sorológica robusta, independente de infecção prévia por CHIKV (SCHNEIDER et al., 2023). Em 2020, firmou-se uma parceria entre a Valneva e o Instituto Butantan para aplicação de testes clínicos da vacina no Brasil, o primeiro país endêmico a aprovar e produzir o imunizante (Instituto Butantan, 2023).

Até o momento, não há tratamento antiviral licenciado contra a CHIKF (BARTHOLOMEEUSEN et al., 2023b). O controle dos sintomas se baseia em medidas paliativas através do uso de analgésicos e antitérmicos para minimização de dores e febre, além de hidratação e descanso (VU; JUNGKIND; LABEAUD, 2017). Neste contexto, compostos sintéticos podem se apresentar como fonte promissora para o desenvolvimento de novos fármacos antivirais.

Compostos Sintéticos como Recurso para a Identificação de Candidatos a Fármacos

Historicamente, compostos naturais de diversas origens são utilizados como fonte de identificação de medicamentos, desde antimicrobianos a antineoplásicos (ATANASOV et al.,

2021; HARVEY; EDRADA-EBEL; QUINN, 2015). A produção de moléculas sintéticas a partir da estrutura química de um produto natural possui vantagens relevantes, como facilidade de produção em larga escala, possibilidade de patente e o aprimoramento da bioatividade, aumentando a especificidade para determinados alvos (SILVA et al., 2019).

Anteriormente aos testes *in vitro* e *in vivo*, existem técnicas computacionais capazes de prever o sítio de interação entre um composto e um patógeno, bem como as ligações envolvidas no processo. Um dos modos de acessar estes dados é através do *docking* molecular, ferramenta *in silico* amplamente utilizada para descobrir novas moléculas, seja para o reposicionamento de fármacos, antevisão de efeitos adversos, entre outros fins (PINZI; RASTELLI, 2019). Desta forma, os métodos *in silico* auxiliam na seleção de moléculas sintéticas a partir da estrutura química de um produto natural, bem como no aprimoramento dessas moléculas sintéticas afim de se obter uma melhor correlação entre estrutura-atividade (GUHA, 2013).

O uso de análogos sintéticos tem se mostrado como alternativa recorrente para múltiplos tratamentos, uma vez que já exibiram diversos efeitos biológicos (GRANCHI, 2022). Neste contexto, a sulfonamida é uma molécula orgânica sintética, presente em compostos biologicamente ativos, incluindo antibióticos, agentes anti-hipertensivos e medicamentos antitireoidianos (GAFFER et al., 2024). Além disso, esse grupo constitui alguns medicamentos comercializados, envolvidos na inibição enzimática, como o Amprenavir, um inibidor de protease do vírus da imunodeficiência humana (HIV), e o Sultiam, empregado como um anticonvulsivante que atua inibindo a enzima anidrase carbônica (KALAY, 2022). Na química medicinal, a fração sulfonamida é aplicada como bioisótero para o grupo carboxílico, uma vez que pode formar as mesmas ligações de hidrogênio, além dos átomos de oxigênio de ambos os grupos estarem posicionados em distâncias semelhantes (NGUYEN et al., 2021). Essa troca pode evitar algumas desvantagens do grupo carboxílico, como toxicidade, instabilidade metabólica e difusão passiva limitada em membranas biológicas (WAN et al., 2021).

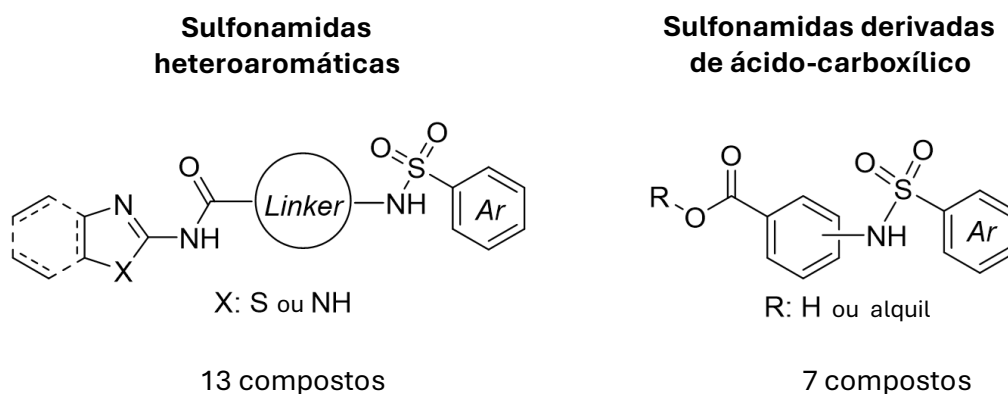
Alternativamente, o benzotiazol (BTA) é uma molécula heterocíclica, cujo núcleo se constitui da fusão entre os anéis de benzeno e tiazol (PRAJAPATI et al., 2014). Devido sua manipulação flexível em várias posições dos anéis, os derivados de benzotiazol têm ganhado atenção como potenciais fármacos (ASIRI et al., 2020). Estudos demonstraram um amplo espectro de atividades biológicas de derivados do BTA, como analgésica e anti-inflamatória (KUMAR; SINGH, 2021), fungicida (LIU et al., 2013), antidiabética (MELTZER-MATS et al., 2013), antimicrobiana (POONIA et al., 2022) e antitumoral (PHILOPPES; LAMIE, 2019). Ainda, derivados de BTA tem demonstrado efeitos antivirais contra diversos vírus, como o da

dengue (DENV) (WU et al., 2015), da hepatite C (HCV) (LI et al., 2012), da herpes simplex (HSV) (ABDEL-AZIZA; ABDEL-WAHAB; BADRIA, 2010) e da Influenza A (GALOCHKINA et al., 2019).

A associação entre sulfonamidas e compostos heterocíclicos, como os BTAs, foi recentemente descrita como híbridos moleculares, ou seja, a união de dois ou mais farmacóforos que formam uma estrutura-base (IVASIV et al., 2019). Levando em consideração a diversidade de atividades biológicas reportadas para ambos, essas moléculas têm o potencial de apresentar atividades farmacológicas sinérgicas e maior habilidade de se ligar a mais de um alvo (SANDHU et al., 2014).

Em um trabalho recente realizado pelo nosso grupo de pesquisa, uma série de derivados de BTA foi avaliada contra o ZIKV *in vitro* (DIAS et al., 2023). No presente trabalho, investigamos 20 sulfonamidas, 13 associadas à fragmentos heteroaromáticos e 7 derivadas de ácido-carboxílico (**Figura 6**).

Figura 6: Estrutura química geral dos compostos investigados neste trabalho.



Os 13 compostos baseados em fragmentos heterocromáticos são subdivididos em: 1-3 representam sulfonamidas com inserções de tiazol ou benzimidazol; 4-12 caracterizam sulfonamidas ligadas à derivados de BTA; 13 representa um derivado de BTA que não possui sulfonamida em sua estrutura (**Figura 7: Estrutura química representativa das sulfonamidas heteroaromáticas**). Alternativamente, os outros 7 compostos são sulfonamidas derivadas de ácido carboxílico, conforme a ilustra a **Figura 8: Estrutura química representativa das sulfonamidas derivadas de ácido carboxílico**..

Figura 7: Estrutura química representativa das sulfonamidas heteroarômicas.

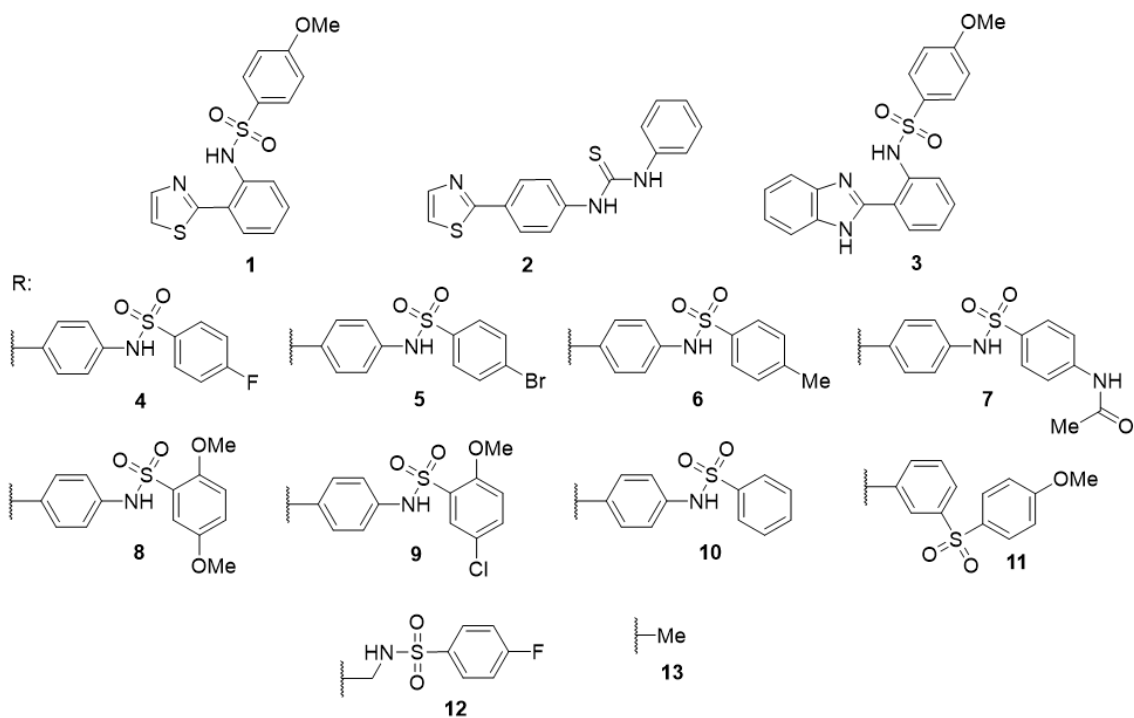
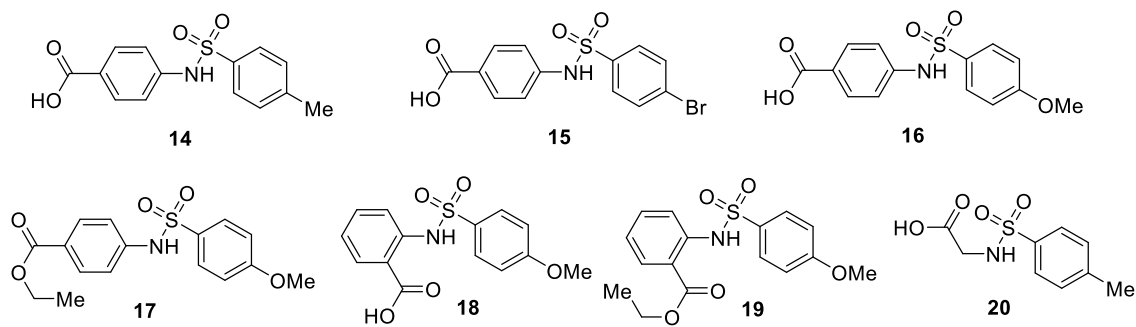


Figura 8: Estrutura química representativa das sulfonamidas derivadas de ácido carboxílico.



OBJETIVOS

Objetivo Geral

O objetivo deste trabalho foi avaliar a atividade de 20 compostos sintéticos que incluem sulfonamidas associadas a moléculas heterocíclicas, derivado de BTA, ou derivadas de ligações com ácido carboxílico, no ciclo replicativo do CHIKV *in vitro*.

Objetivos Específicos

- Avaliar a citotoxicidade dos 20 compostos sintéticos, que compreendem sulfonamidas associadas a moléculas heterocíclicas ou derivadas de ligações com ácido carboxílico, em células BHK-21, por meio de ensaios de viabilidade celular;
- Avaliar a atividade antiviral desses compostos em células BHK-21 infectadas por CHIKV, por meio de ensaios antivirais;
- Determinar a concentração efetiva de inibição de 50% (EC₅₀), concentração citotóxica de 50% (CC₅₀) e índice de seletividade ($IS = CC_{50} / EC_{50}$) dos derivados de BTA que apresentaram as melhores taxas de atividade antiviral, de forma a avaliar o potencial antiviral destas moléculas;
- Analisar a atividade dos compostos selecionados em diferentes etapas do ciclo replicativo do CHIKV, para compreender os estágio do ciclo replicativo viral inibidos por esses compostos;
- Investigar as interações dos compostos com as proteínas do CHIKV por meio de *docking molecular*, para identificar potenciais alvos virais e sugerir possíveis mecanismos de ação;
- Observar as interações dos compostos ativos com o vírion do CHIKV, por meio da técnica de espectroscopia ATR-FTIR, a fim de obter um melhor entendimento das interações entre vírus e composto que afetam os estágios iniciais da infecção.

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CAPÍTULO II

Manuscript:

BENZOTHAZOLE DERIVATIVES AS INHIBITORS OF CHIKUNGUNYA VIRUS REPLICATIVE CYCLE

*Este capítulo está em formato de manuscrito com algumas alterações estruturais para melhor se adequar ao formato da dissertação. O artigo em questão será submetido à revista *Antiviral Research*.

BENZOTHAZOLE DERIVATIVES AS INHIBITORS OF CHIKUNGUNYA VIRUS REPLICATIVE CYCLE

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ABSTRACT

Chikungunya virus (CHIKV) is the causative pathogen of Chikungunya Fever (CHIKF). It has become a major health problem mostly in tropical countries and remains as a neglected and reemergent disease. The treatment is based on palliative measures to control symptoms such as fever, headache, and exanthemas, which further can become chronic and initiate polyarthralgia that may lead to arthritis. Since there are no licensed drugs to treat CHIKF, the search for therapies is essential. In this sense, we aimed to screen twenty synthetic sulfonamides derivatives against CHIKV-*nanoluc* *in vitro* with the primary goal of identifying novel inhibitors. Among these compounds, thirteen are heteroaromatic derivatives containing thiazole, benzimidazole, and benzothiazole (BTA) moieties, while seven are sulfonamides containing ester and carboxylic acid groups.. BTA derivatives **6**, **9**, **11** and **13** exhibited the most potent inhibitory activity based on dose-response assays with EC₅₀ values ranging from 14.9 to 63.1 μ M.. These compounds displayed selective index (SI) of 13.8, 5.8, 4.4, and 11.0, respectively. Furthermore, all of them demonstrated post-entry activity, inhibiting CHIKV replication in up to 98%. Interestingly, compound **9** displayed antiviral effect on different stages of viral replicative cycle, such as virucidal (55%), pre-treatment (69%), and entry (98%). The molecular docking analysis revealed the highest affinity scores between these BTA derivatives and the non-structural proteins of CHIKV. Additionally, given that **9** also exhibited inhibitory action on viral entry to the host cells, molecular docking was also conducted with the envelope glycoprotein of CHIKV, yielding a -8.01 kcal/mol score. Infrared spectroscopy analysis corroborated the effect of **9** on virus particles, by indicating interaction with the glycoprotein complex and lipids. The data presented here can provide a source for future *in vivo* and clinical research in the development of treatments for CHIKF.

1. INTRODUCTION

Chikungunya virus (CHIKV) is an enveloped arbovirus, belonging to the *Alphavirus* genus and *Togaviridae* family, transmitted by the female bite of *Aedes aegypti* or *Aedes albopictus* in urban cycles (Burt et al., 2012). CHIKV exhibits a single-stranded positive-sense RNA with an 11.8 kb genome consisting of two open reading frames (ORF), one that encodes four non-structural proteins (nsP1, nsP2, nsP3, and nsP4), and the second that encodes five structural proteins (C – capsid; 6K/TF – transframe; E1/E2/E3 – envelope glycoprotein) (Montalvo Zuribia-Flores et al., 2023).

Responsible for triggering Chikungunya fever (CHIKF), CHIKV has been a global health problem, mainly in the Americas, Africa, and some regions of Asia due to the tropical climate favorable to the development of mosquito reservoirs (de Souza et al., 2024). The illness may lead to chronic complications, such as severe arthritis, limiting the quality of life of infected individuals (Ahmed Imad et al., 2021).

Currently, there is no licensed drug to treat CHIKF, restricting medical management to medications that alleviate the symptoms. In this context, novel compounds can be synthesized based on organic scaffolds to enhance and target their pharmacological properties, leading to the development of effective and safe drugs for a wide range of treatments (Philip, 2015; Sáenz-Galindo et al., 2020). Sulfonamide exemplify a structure present in biologically active compounds, including antibiotics, antihypertensive agents, and antithyroid medications (Gaffer et al., 2024). Furthermore, this group encompass some commercialized drugs involved in enzyme inhibition like Amprenavir, which is a protease inhibitor of human immunodeficiency virus (HIV), and Sultiam, employed as an anticonvulsant that acts as carbonic anhydrase enzyme inhibitor (Kalay, 2022). In medicinal chemistry, sulfonamide moiety can be applied as a bioisostere for the carboxylic acid group, as it is capable of forming the same hydrogen bonds and its oxygen atoms are positioned at similar distances (Nguyen et al., 2021). This exchange can avoid some disadvantages of the carboxylic group, such as toxicity, metabolic instability and limited passive diffusion in biological membranes (Wan et al., 2021).

Alternatively, BTA possesses a chemical structure composed of oxygen, nitrogen, and sulfur, where its nucleus is formed by a fusion of thiazole and benzene rings (Irfan et al., 2020). It is rarely found in some natural products like the flavour compound produced by *Aspergillus clavatus*, cranberries aroma and fermentation culture extracts of

Micrococcus sp., a marine bacterium (Le Bozec and Moody, 2009). Due to versatile manipulation in various ring positions, their derivatives have gained attention as optimized drugs candidates (Asiri et al., 2020). A plethora of BTA derivatives have demonstrated broad-spectrum biological activity, such as antitumor (Cai et al., 2013), anti-diabetic (Meltzer-Mats et al., 2013), analgesic (None et al., 2021), antiparasitic (Lapierre et al., 2024) and antibacterial (Zhu et al., 2021). In addition, antiviral activities against herpes simplex virus (HSV) (Abdel-Aziza et al., 2010), influenza A virus (Galochkina et al., 2019), and dengue virus (DENV) (Wu et al., 2015) have also been described.

The association between sulfonamides and heterocycle compounds like BTAs has been recently described as molecular hybrids, which is the union of two or more pharmacophores in a scaffold (Ivasiv et al., 2019). Taking into account the amount of biological activities displayed by both, this may lead to synergical pharmacological activities and increased ability to bind to more than one target (Sandhu et al., 2014).

Here we investigated the antiviral effects of sulfonamides containing heteroaromatic and carboxylic-acid insertions in their chemical structure (**Figure 1**), which were previously described as inhibitors of ZIKV (Dias et al., 2023), against CHIKV *in vitro*. The results found here can underscore the significant promise of BTA derivatives (heteroaromatic insertions) as antiviral drug candidates that can effectively inhibit the replication of arboviruses.

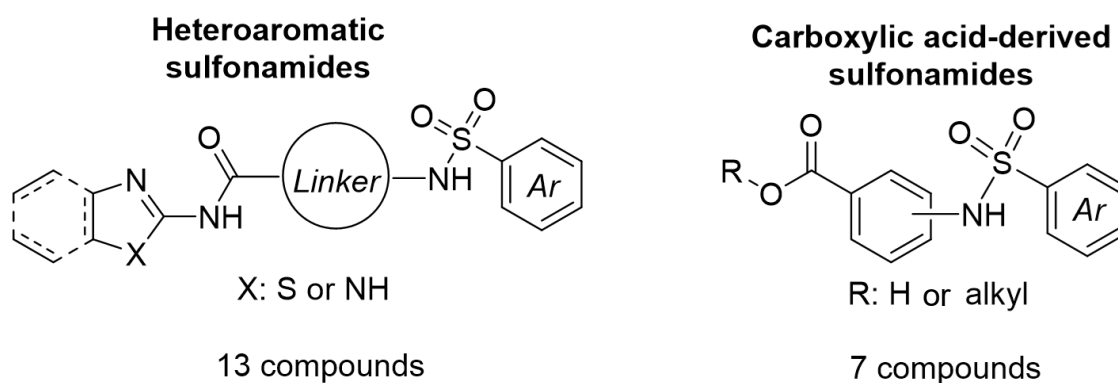


Figure 1. General chemical structure of the compounds investigated in this work..

2. MATERIALS AND METHODS

2.1. Compounds

All the sulfonamides were synthesized as previously reported (Dias et al., 2023; Lapierre et al., 2024). The twenty compounds evaluated in this work were divided into two classes. **Figure 2** shows the chemical structure of the thirteen representative heteroaromatic sulfonamide compounds, except for compound **13**, which is not a sulfonamide, but rather a simplified derivative of BTA with a *N*-acetyl amino fragment. These thirteen compounds are divided into three subclasses, where **1-2** are thiazole derivatives, **3** is a benzimidazole derivative, while compounds **4-13** are BTA derivatives. **Figure 3** shows the chemical structure of the seven representative carboxylic acid-derived sulfonamide compounds.

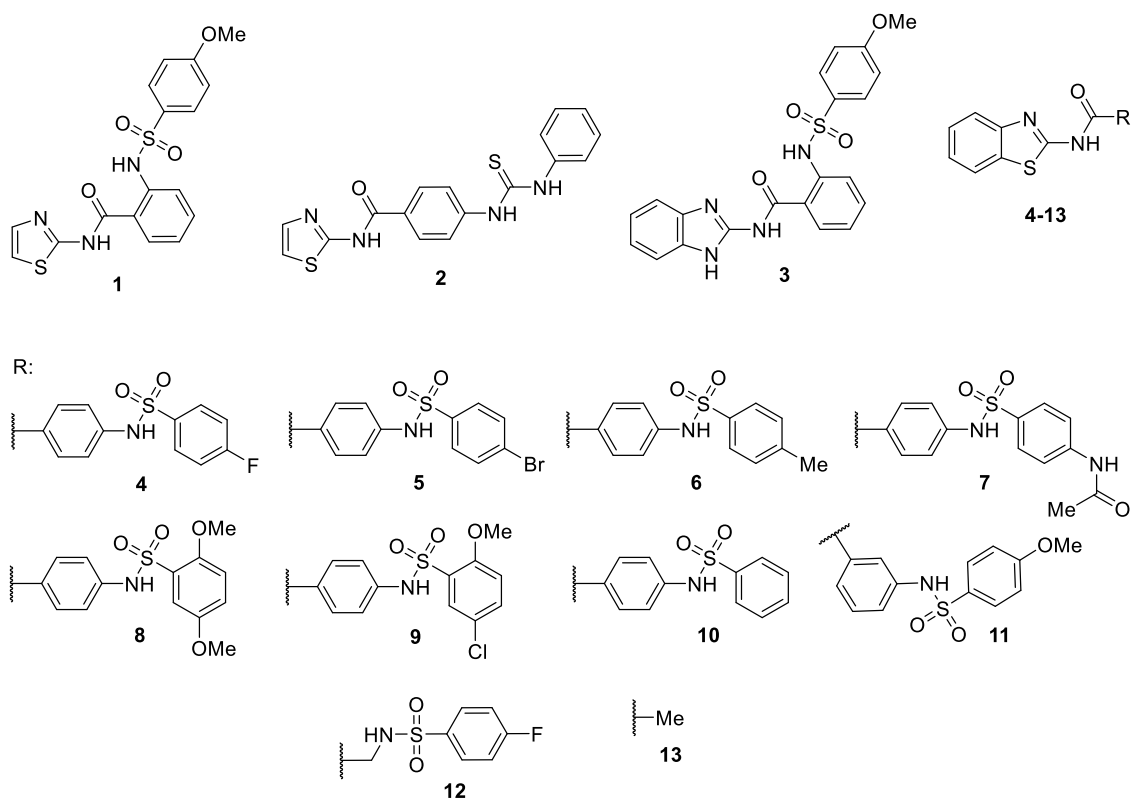


Figure 2. Chemical structures of the representative heteroaromatic sulfonamide compounds.

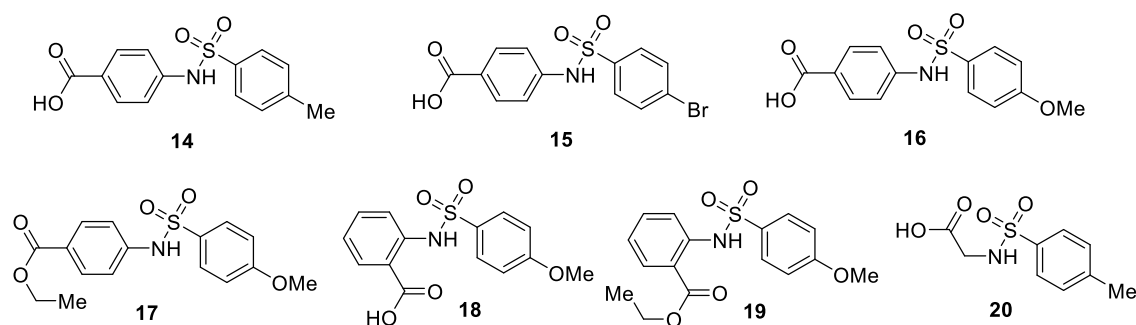


Figure 3. Chemical structure of the representative carboxylic acid-derived sulfonamide compounds.

Compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C in 50 mM stock solutions. Dilutions of compounds in cell culture media were performed immediately prior to the assays to a final concentration of DMSO of 0.1%.

2.2. Cell culture

BHK-21 cells (fibroblasts derived from Syrian golden hamster kidney; ATCC® CCL-10™) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 100U/mL of penicillin (Hyclone Laboratories), 100 mg/mL of streptomycin (Hyclone Laboratories), 1% dilution of non-essential amino acids (Hyclone Laboratories) and 5% of fetal bovine serum (FBS, Hyclonen Laboratories) in a 5% CO₂ incubator at 37 °C.

2.3. Virus sample

The CHIKV-expressing nanoluciferase reporter (CHIKV-*nanoluc*) used for the antiviral assays is based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype). This construct was produced, rescued, and titrated as previously described (Andrade Santos et al., 2021).

2.4. Cell viability and determination of the cytotoxic concentration of 50% (CC₅₀)

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich®) assay. For screening assays, BHK-21 cells were plated to 96 well plates at a density of 2×10^4 cells per well and incubated overnight at 37 °C. Medium containing each compound at 50 and 10 μM was added, and cells were

incubated for 24 h. DMSO 0.1% was used as an untreated control. After this, the medium was replaced with the MTT solution at 1 mg/mL, cells were incubated for 30 minutes, after which the MTT solution was removed and replaced with 100 μ L of DMSO (dimethyl sulfoxide) to solubilize the formazan crystals. The absorbance was measured at 560 nm on the Glomax microplate reader (Promega®). Cell viability was calculated according to the equation $(T/C) \times 100\%$, where T and C represent the mean optical density of the treated and untreated control groups, respectively.

For determination of the cytotoxic concentration of 50% (CC_{50}), BHK-21 cells were plated to 48 well plates at a density of 5×10^4 cells per well and incubated overnight at 37 °C. Medium containing two-folds serial dilutions at concentrations ranging from 1.6 to 200 μ M for **6**, **9**, **11**, and from 3.1 to 400 μ M for **13**, was added to cells and incubated for 16 h. Subsequently, the medium was replaced by MTT solution at 1 mg/mL and proceeded as described above. CC_{50} was calculated using non-linear regression at GraphPad Prism 8.0.

2.5. Antiviral activity and determination of the effective concentration of 50% (EC_{50})

To assess the antiviral activity of the compounds, BHK-21 cells were seeded at a density of 5×10^4 cells per well into 48 well plates 24 h before the assays. Cells were infected with CHIKV-*nanoluc* at a multiplicity of infection (MOI) of 0.1 for antiviral trials in the presence of the compounds at the highest non-cytotoxic concentration analyzed through the viability assay. DMSO 0.1% was used as an untreated control. Samples were harvested using Passive Lysis Buffer (Promega®) at 16 h post-infection (h.p.i.), and virus replication levels were quantified by measuring nanoluciferase activity using the *Renilla* luciferase Assay System (Promega®). Data were calculated according to the equation $(T/C) \times 100\%$, where T and C represent the mean of luminescence signals of the treated and untreated control groups, respectively.

Similarly to CC_{50} , EC_{50} assays were conducted through the treatment of cell with two-fold serial dilutions of the compounds, at concentrations ranging from 1.5 to 200 μ M for **6**, **9**, **11**, and from 3.1 to 400 μ M for **13**, in the presence of CHIKV-*nanoluc* at a MOI of 1. EC_{50} was calculated using non-linear regression at GraphPad Prism 8.0. The values of CC_{50} and EC_{50} were used to calculate the selectivity index ($SI = CC_{50} / EC_{50}$).

All the infection assays were performed at a BSL-2 laboratory under the authorization number CBQ: 163/02 and process SEI: 01.245.006267/2022–14 from the CTNBio - National Technical Commission for Biosecurity from Brazil.

2.6. Time-of-drug-addition assays

For all the time-of-drug-addition assays BHK-21 cells at 5×10^4 cells per well were seeded in 48 well plates 24 h before infection and treatment. Compounds **6**, **9**, and **11** were evaluated at 10 μ M, whereas **13** was at 50 μ M, determined at viability screening as the highest non-cytotoxic concentrations. All infections were performed at MOI of 1, except for the virucidal protocol (MOI= 5). The efficiency of virus replication was assessed by measurement of nanoluciferase activity at 16 h.p.i using the *Renilla*-luciferase assay system (Promega).

In the pretreatment assay, cells were treated for 1 h with compounds prior to the infection, extensively washed with phosphate-buffered saline (PBS) [1x], and added of CHIKV-*nanoluc* for 1 h. Then, cells were washed with PBS [1x] to remove unbound virus, and added to DMEM 2%. In the entry inhibition assay, cells were infected using media containing the compound and virus for 1 h, washed with PBS [1x], and incubated with fresh medium. The virucidal activity was assessed using the same setting, except that the inoculum containing compound and virus were incubated for 1 h before it was added to the cells. Finally, in the post-entry assay, cells were infected with CHIKV-*nanoluc* for 1 h, washed extensively with PBS [1x], and then incubated in a compound-containing medium for 16 h. DMSO 0.1% was used as untreated control for all protocols. Data were calculated according to the equation $(T/C) \times 100\%$, where T and C represent the mean of luminescence signals of the treated and untreated control groups, respectively.

2.7. Molecular docking

Structures of nsPs 1, 2, 3 and the envelope glycoprotein complex (E1E2E3) of CHIKV were obtained from Protein Data Bank (PDB) from the entries 7X01, 3TRK, 6W0T, and 3N42, respectively. NsP4 structure was obtained by *in silico* modeling using AlphaFold2 from ColabFold v1.5.5 (Mirdita et al., 2022). Potential binding sites were predicted by the SiteFinder MOE tool for all proteins. The compounds **6**, **9**, **11**, and **13** were docked to all nsPs using the MOE (2024) program. All proteins were submitted to MOE QuickPrep to correct structural problems. Molecular docking was performed using

the Triangle Matcher placement method (30 poses) and London dG as the scoring function. Refining was conducted using induced fit with GBVI/WSA dG as a scoring function. The best GBVI/WSA dG ranked was selected by each complex for the 3D and 2D analysis. 3D images were generated by MOE and ChimeraX (Meng et al., 2023) programs and 2D were generated by MOE.

2.8. Infrared Spectral data analysis

The infrared spectra of the samples (CHIKV, the compound **9**, and the combination of CHIKV+**9**) were recorded using Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy with the Agilent Cary 630 FTIR (Agilent Technologies, Santa Clara, CA, USA). For this analysis, the lipidic region (3050-2800 cm^{-1}) and the fingerprint region (1800-800 cm^{-1}) were examined. The samples were prepared separately with the compound **9** and/or CHIKV (Grosche et al., 2023; Ruiz et al., 2023). A volume of 3 μL from each sample was placed on the diamond cell, forming a thin layer on the ATR crystal. The spectra were recorded at a resolution of 2 cm^{-1} with 64 scans. The second derivative spectra were derived from the original data and plotted using Origin Pro 9.8.0.200 software (OriginLab, Northampton, MA, USA). Adjustments were made using the Savitzky-Golay algorithm with a polynomial order of 2 and a 20-point window (Cassani et al., 2023; Ruiz et al., 2023). The tentative assignments were derived from infrared spectra in biological tissues (Movasaghi et al., 2008).

2.9. Statistical analysis

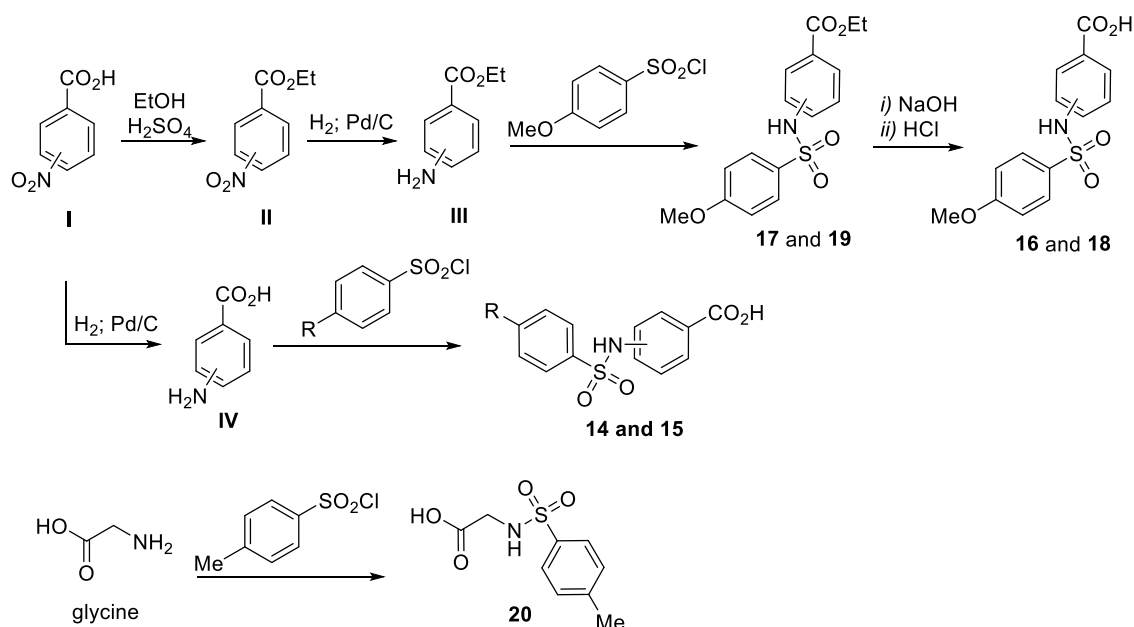
Individual experiments were performed in triplicates, and all assays were performed a minimum of three times to confirm the reproducibility of the results. GraphPad Prism 8.0 software was used to assess normal distribution and statistical differences of means. $p < 0.05$ values were considered statistically significant.

3. RESULTS

3.1. Synthesis

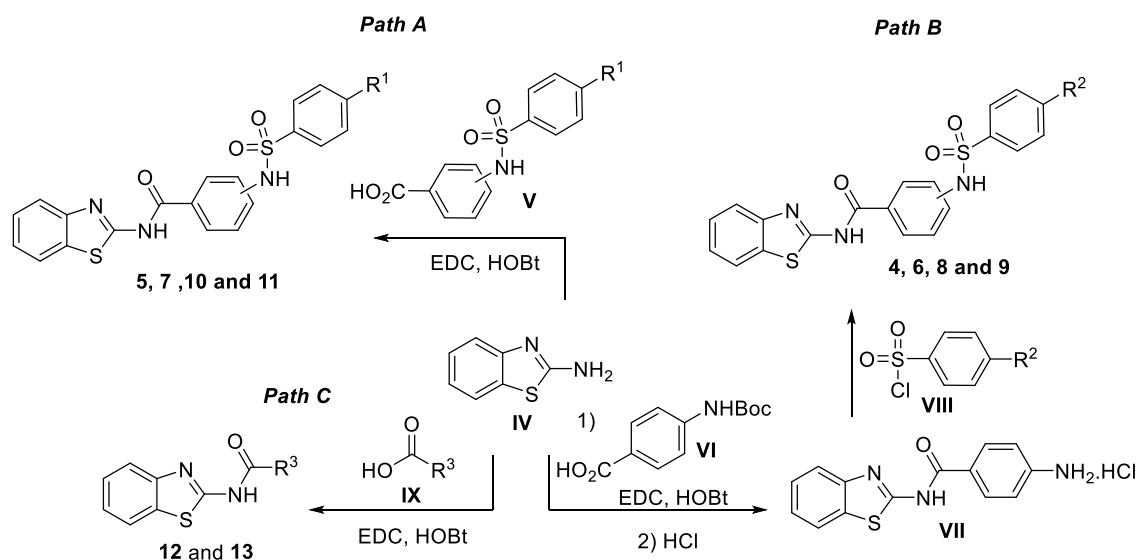
Carboxylic acid-derived sulfonamide compounds (**14-20**) was synthesized according to Scheme 1. Ethyl esters **17** and **19** were prepared by esterifying carboxylic acid **I** with ethanol in the presence of sulfuric acid, followed by nitro group reduction of **II** using hydrogen gas and palladium(0) catalyst, and subsequent sulfonylation of **III** with

4-methoxybenzenesulfonyl chloride. Carboxylic acids **14** and **15** were obtained through the reduction of the corresponding nitrobenzoic acids with hydrogen gas and palladium(0), followed by sulfonamide formation with various sulfonyl chlorides. Compound **20** was synthesized by sulfonylation of glycine with 4-methylsulfonyl chloride.



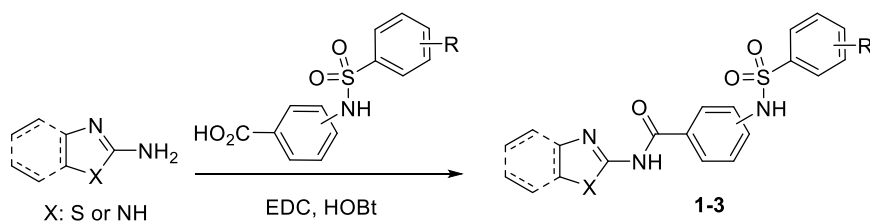
Scheme 1. Synthesis of carboxylic acid-derived sulfonamide compounds (**14-20**).

The heteroaromatic sulfonamides (**1-13**) were synthesized as outlined in Schemes 2 and 3. BTA derivatives were prepared via three alternative routes using 2-aminobenzothiazole **IV** as a starting material (Path 1-3, Scheme 2). Compounds **5**, **7**, **10-13** were synthesized through amidation of **IV** with various carboxylic acids using EDC and HOBt as coupling reagents (Path A and Path C), while compounds **4**, **6**, **8**, and **9** were prepared by amidation of **IV** with carboxylic acid **VI** followed by sulfonylation with different benzenesulfonyl chlorides (**VIII**) (Path B).



Scheme 2. Synthesis of heteroaromatic sulfonamides 4-13.

Compounds **1-3** were synthesized through amidation reactions between thiazole or benzimidazole and the corresponding carboxylic acids, using EDC and HOBT as coupling reagents (Scheme 3).



Scheme 3. Synthesis of heteroaromatic sulfonamides 1-3.

3.2 Antiviral activity of sulfonamide compounds against CHIKV

To assess the antiviral effect of the sulfonamide derivatives, the cytotoxicity of these compounds was first investigated. To this, BHK-21 cells were treated with each compound at concentrations of 50 and 10 μ M. Analyzing the effect on cell viability, we found that treatment with the heteroaromatic sulfonamide compounds **2**, **8** and **13** at 50 μ M presented no significant cytotoxicity ($p = 0,6865$, $p > 0,9999$ and $p = 0,9988$, respectively) (**Figure 4A; Supplementary Material 1**). At 10 μ M, all the compounds were viable to the cells, except for **4** (81%; $p = 0.0282$), **5** (56%; $p < 0.0001$), and **10** (69%; $p < 0.0001$) (**Figure 4A; Supplementary Material 1**).

For the carboxylic acid-derived sulfonamide group, only **14** (81%; $p = 0.0210$) and **20** (80%; $p = 0.0262$) were viable to the cells at 50 μM , while the others were viable at 10 μM (**Figure 4B; Supplementary Material 1**). With a exception, compounds **15** (50 μM - 74% - $p = 0.0002$; 10 μM - 80% - $p = 0.0099$) and **17** (50 μM - 79% - $p = 0.0132$; 10 μM - 74% - $p = 0.0011$) demonstrated to be cytotoxic at both concentrations (**Figure 4B; Supplementary Material 1**).

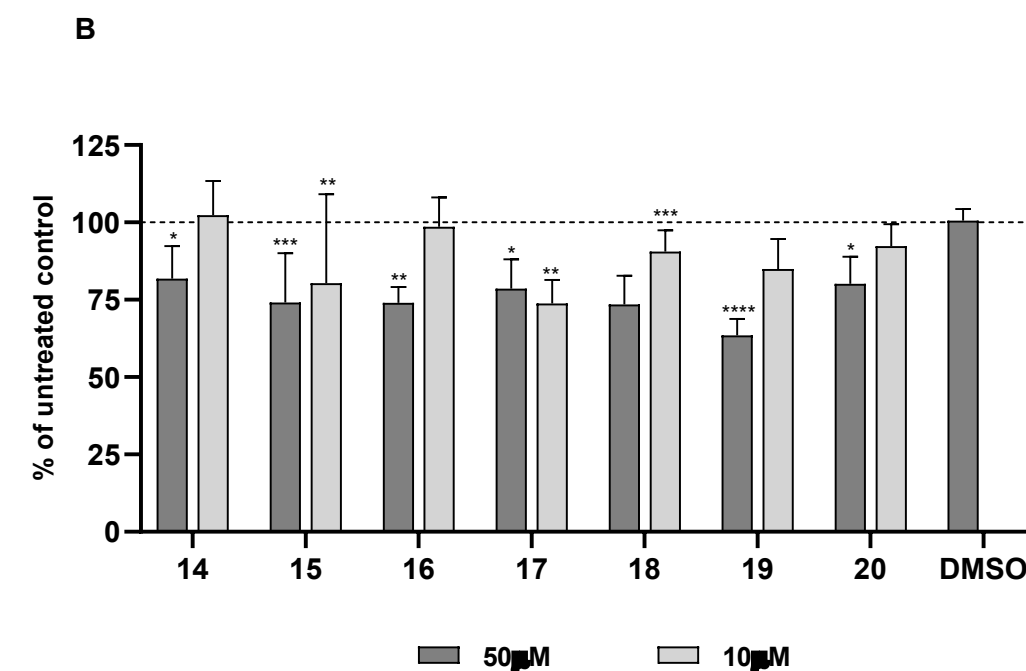
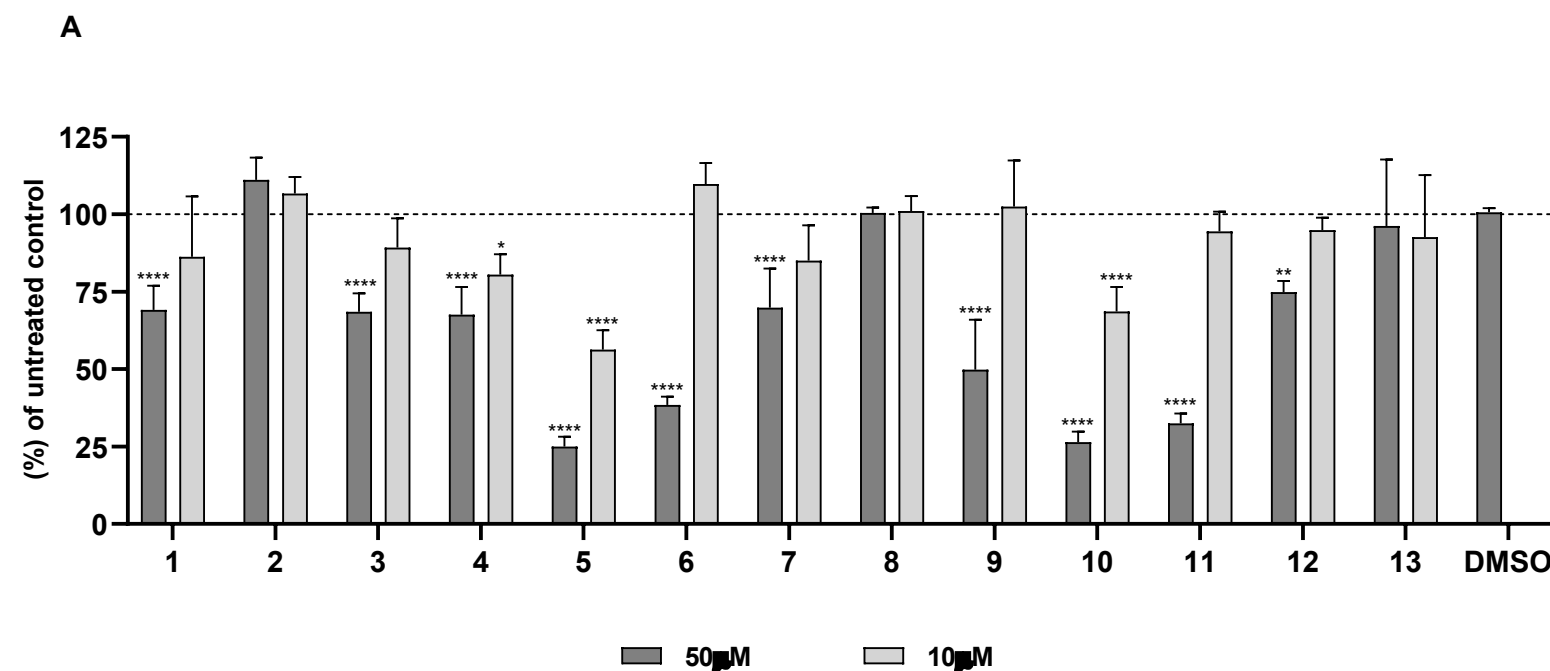


Figure 4. Effect of sulfonamide compounds on BHK-21 viability. Cell viability was accessed by treating BHK-21 cells with (A) heteroaromatic sulfonamide compounds, in which 1-3 are thiazole and benzimidazole derivatives and 4-13 are BTA derivatives; and (B) carboxylic acid-derived sulfonamide compounds, at 50 and 10 μ M. After treatment, the medium with compound was removed and replaced by fresh medium with MTT at 1 mg/mL and incubated for 30 min. After incubation, medium was removed, and the crystals solubilized with DMSO. Surviving cells were measured by absorbance (560 nm). Mean values of three independent experiments each measured in quadruplicate including the standard deviation are shown. DMSO 0.1% was used as an untreated control. P values < 0.05 were considered significant. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; and (****) $p < 0.0001$.

Then, BHK-21 cells were infected with CHIKV-*nanoluc* at MOI of 0.1 and treated with the compounds at the highest non-cytotoxic concentration to evaluate their antiviral activity. For compounds that showed cytotoxicity at 50 and 10 μ M, such as **4**, **5**, **10**, **15** and **17**, antiviral assay was performed at 2 μ M. Viability assay was performed in parallel for all compounds.

All the compounds from heteroaromatic sulfonamide group showed significant inhibition of CHIKV replication ($p < 0,0001$), where BTA derivatives **6** (90%), **9** (95%), **11** (98%) and **13** (94.5%) presented the strongest antiviral effects, highlighting their potential as promising scaffolds for the development of CHIKV antiviral drugs (**Figure 5A**). On the other hand, none of the carboxylic acid-derived sulfonamide compounds were capable of inhibiting CHIKV infection above 80%. Compounds **16**, **17** and **19** presented inhibition rates between 12 to 72% ($p < 0.01$) (**Figure 5B**).

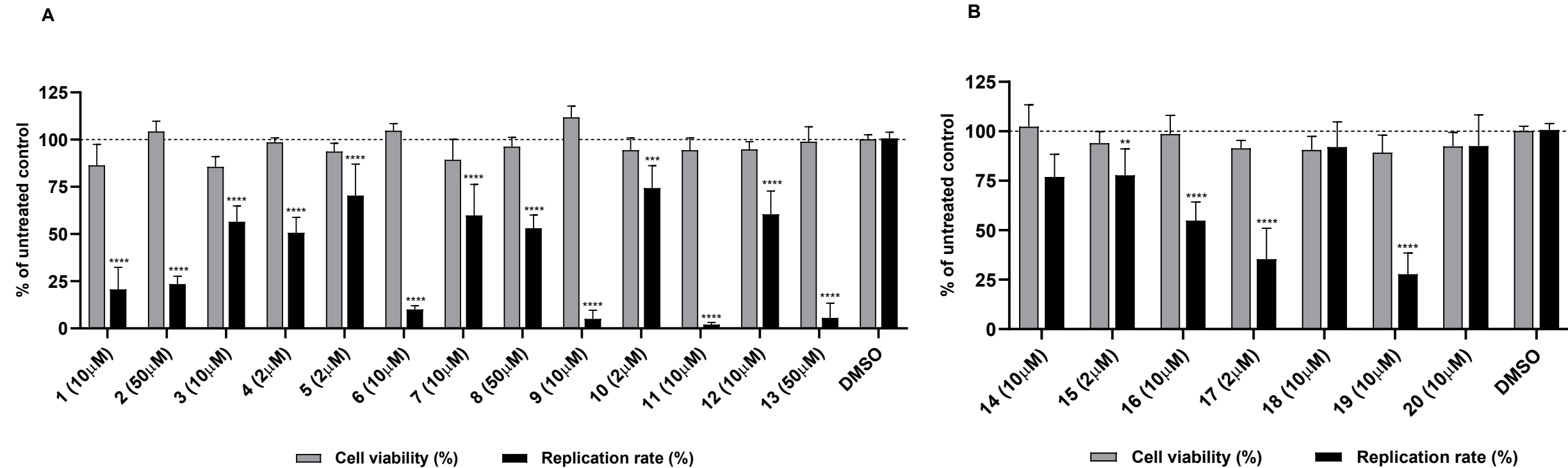


Figure 5. Effect of sulfonamides on BHK-21 viability and CHIKV infection. BHK-21 cells were infected with CHIKV-*nanoluc* at an MOI of 0.1 and simultaneously treated with (A) heteroaromatic sulfonamides and (B) carboxylic acid-derived sulfonamide group, at the highest non-cytotoxic concentration. After 16 h.p.i, cells were lysed, and a *Renilla*-luciferase assay was performed to assess CHIKV replication rates. Cell viability was obtained by treating BHK-21 cells with the compounds at specific concentrations. After treatment, the medium with the compound was removed, replaced with medium with MTT at 1 mg/mL and incubated for 30 min. After incubation, MTT was removed, and the crystals solubilized with DMSO. Surviving cells were measured by absorbance (560 nm). DMSO 0.1% was used as an untreated control. Mean values of two independent experiments each measured in triplicates, including the standard deviation, are shown. P values < 0.05 were considered significant. (**) $p < 0.01$; (***) $p < 0.001$; and (****) $p < 0.0001$.

3.3. BTA derivatives of sulfonamide groups possess robust response against CHIKV *in vitro*

From screening tests, the compounds with inhibition over 80% (**6**, **9**, **11**, and **13**) were selected for further evaluation. The effective concentration of 50% (EC_{50}), cytotoxic concentration of 50% (CC_{50}), and selective index ($SI = CC_{50}/EC_{50}$) were determined. Compounds **6** and **9** demonstrated, respectively, EC_{50} of 14.9 μ M and 17.0 μ M, CC_{50} of 205.2 μ M and 97.9 μ M, and a SI of 13.8 and 5.8 (**Figure 6A and 6B**). While **11** and **13** showed, in this order, EC_{50} of 63.1 μ M and 41.6 μ M, CC_{50} of 276.0 μ M and 459.3 μ M,

and SI of 4.4 and 11.0, respectively (Figure 6C and 6D).

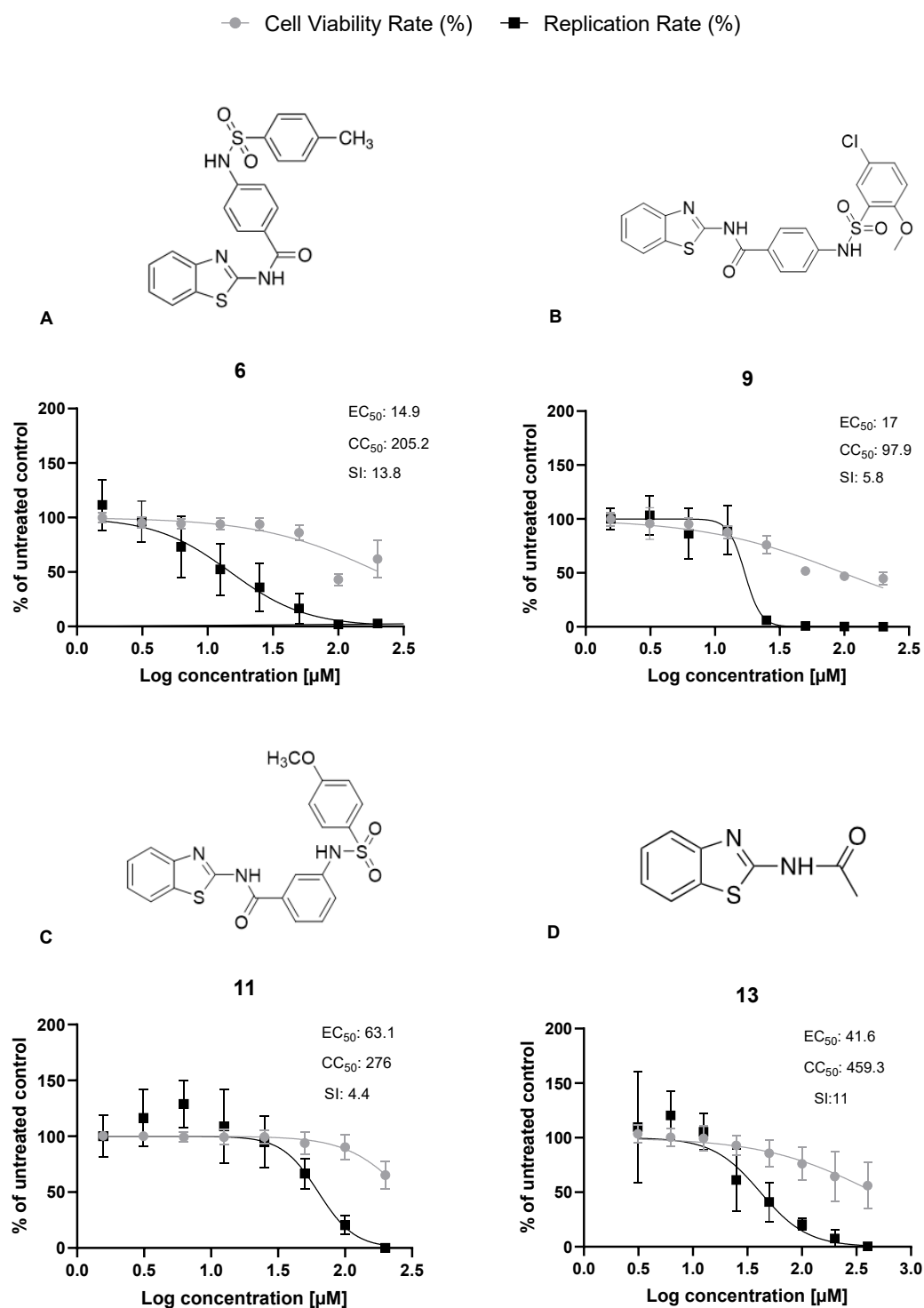


Figure 6. Dose-response curve of the compounds with highest rates of inhibition of CHIKV replication. BHK-21 cells were treated with compounds 6 (A), 9 (B), 11 (C), and 13 (D) at concentrations ranged from 1.6 to 200 μM, except for 13, which ranged from 31 to 400 μM. CHIKV replication was quantified by measuring nanoluciferase activity (black squares) and cell viability was measured using an MTT assay (grey dots). The mean values of three independent experiments each measured in triplicates,

including the standard deviation, are shown. All images were generated using GraphPad Prism 8.0 applying a non-linear regression.

3.4. BTA derivatives reveal strong post-entry activity, with a multiple effect of compound 9

As shown in **Figure 7**, time-of-drug-addition assays were carried out for **6**, **9**, **11**, and **13** to further investigate the effects of these compounds on different stage of CHIKV replicative cycle. For this, BHK-21 cells were infected with CHIKV-*nanoluc* at an MOI of 5 for the virucidal test and an MOI of 1 for the pretreatment, entry, and post-entry stages, and the compounds were added to the cells as described in methods. Interestingly, the results demonstrated that **9** inhibited all stages of replicative cycle investigated, showing 55% of inhibition at virucidal test, 69% at pretreatment, 79% on entry stage and 98% on post-entry (**Figure 7A-D**). Compounds **11** and **13** were capable to significantly inhibit entry ($p < 0.05$) (**Figure 7C**) and post-entry stages ($p < 0.0001$) (**Figure 7D**), presenting stronger effect after viral entry to the host cells. Alternatively, compound **6** reduced 54% of viral post-entry to the host cells ($p < 0.0001$) (**Figure 7D**).

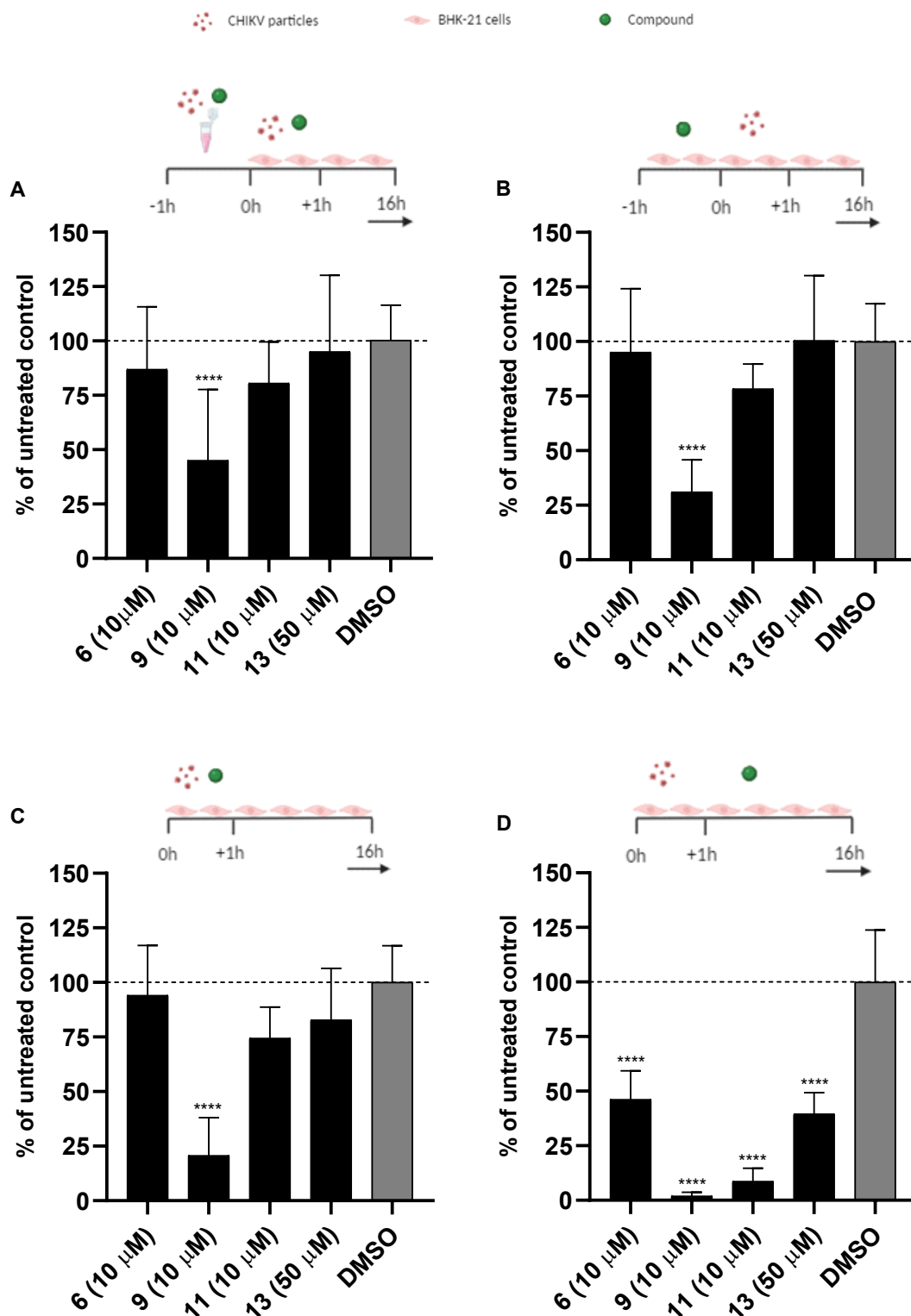


Figure 7. BTA derivatives activity on different stages of CHIKV-*nanoluc* replicative cycle. (A) Virucidal. BTA derivatives and CHIKV-*nanoluc* (MOI of 5) were incubated for 1 h. Then, the inoculum was added to the cells for an additional hour. Cells were washed and fresh medium was added. **(B) Pre-treatment.** BHK-21 cells were treated with the compounds for 1 h, cells were washed to remove compounds and were infected with CHIKV-*nanoluc* at MOI of 1 for 1 h. Then, the medium was removed, cells were

washed to remove unbound virus, and a fresh medium was added. **(C) Entry.** BHK-21 cells were infected with CHIKV-*nanoluc* (MOI of 1) and simultaneously treated with the compounds for 1 h. Subsequently, cells were washed, and a fresh medium was added. **(D) Post-entry.** BHK-21 cells were infected with CHIKV-*nanoluc* (MOI of 1) for 1 h, cells were washed with PBS to remove unbound virus, and treated with the respective derivatives. All infection assays were quantified 16 h.p.i through the measurement of luminescence levels. DMSO was used as an untreated control. Mean $\pm$ SD values of a minimum of three independent experiments, each measured in triplicate. P values < 0.05 were considered significant. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; and (****) $p < 0.0001$. A schematic representation of each time-based assay is indicated above in each graphic. Images were generated using GraphPad Prism 8.0 and BioRender.

3.5. Insights on the molecular interactions between BTA derivatives and CHIKV proteins

According to the time-of-drug-addition assays, the four tested compounds demonstrated post-entry activity. Therefore, molecular docking calculations were performed with all CHIKV non-structural proteins. 2D diagrams and 3D images were generated to illustrate the optimal interactions exhibited by **6**, **9**, **11**, and **13** with the CHIKV proteins (**Figure 8**).

The compound **6** exhibited the most favorable docking GBVI/WSA dG score (indicative of the lowest binding energy in kcal/mol) for nsP4 (-7.97) among the other docked proteins, resulting in the formation of four hydrogen bonds with Arg521, Arg556, Glu553, and Ser4 (**Table 1; Figure 8A**). Concerning **9**, the best score was obtained by the nsP3 (-8.28), with interactions involving three hydrogen bonds (Val113, Gly112, and Thr111) and two arene-arene bonds with Trp148 (**Table 1; Figure 8B**). The interactions between **11** and nsP1 were characterized by three hydrogen bonds with residues Cys82, Met84 and Arg92 (**Figure 8C**). Compound **13** showed its highest docking score with the nsP2 protein (-5.42) and two hydrogen bonds with Trp1084 (**Table 1; Figure 8D**).

Furthermore, as **9** also demonstrated virucidal and entry activity, docking analysis were performed between this compound and the envelope glycoprotein complex, to better understand the molecular interactions involved. Regarding the envelope complex, the compound **9** demonstrated a score of -8.01, where one hydrogen bond (with Tyr233 from E2) and one arene-H bond (with Ile167 from E1) were identified (**Table 1; Figure 8B**).

Table 1. Molecular docking scores between BTA derivatives and nonstructural and structural proteins of CHIKV.

Compounds	<i>GBVI/WSA dG (kcal/mol)</i>				
	nsP1	nsP2	nsP3	nsP4	Glycoproteins (E1-E2-E3)
6	-7.60	-7.35	-7.55	-7.97	*
9	-7.70	-7.63	-8.28	-7.38	-8.01
11	-8.06	-7.90	-7.80	-8.00	*
13	-5.35	-5.42	-5.33	-5.28	*

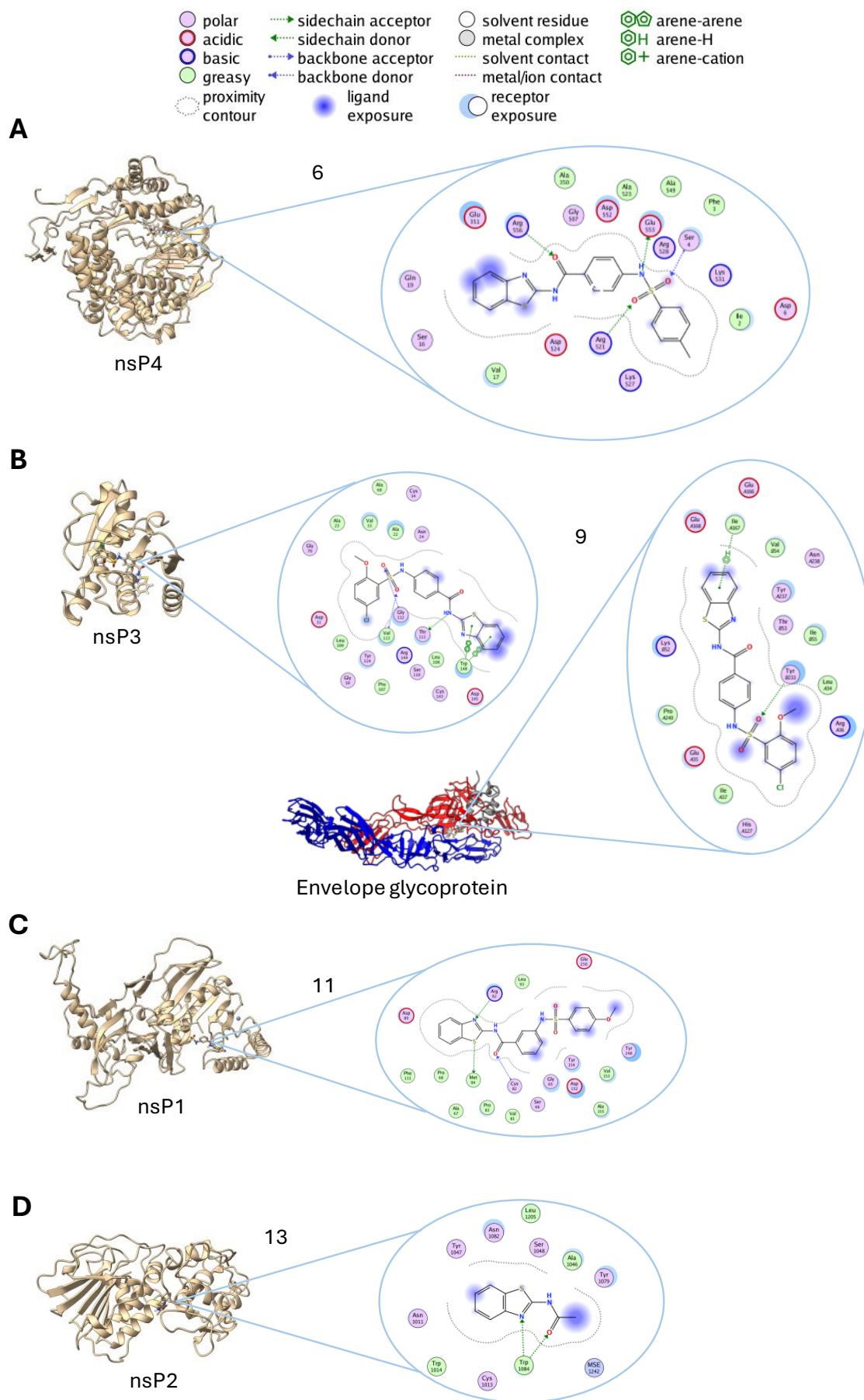


Figure 8. 2D and 3D interactions between BTA derivatives and CHIKV proteins are shown by molecular docking analysis. **A)** 6 and nsP4 interact mostly through four hydrogen bonds with Arg521, Arg556, Glu553, and Ser4. **B)** 9 exhibited three hydrogen bonds (Val113, Gly112, and Thr111) and two arene-arene bonds between Trp148 with nsP3; and one hydrogen bond (with Tyr233 from E2) and one arene-H bond (with Ile167 from E1) were identified with the glycoprotein complex. The blue chain represents E1; red represents E2; and grey represents E3. **C)** 11 showed strong molecular interactions with nsP1 by three hydrogen bonds with residues Cys82, Met84, and Arg92. NsP4 structure was obtained by *in silico* modeling using AlphaFold2. **D)** 13 revealed two hydrogen bonds with Trp1084 from nsP2. NsP1, 2, 3 and the envelope glycoprotein complex (E1E2E3) structures were obtained from Protein Data Bank (PDB) from the entries 7X01, 3TRK, 6W0T, and 3N42, respectively.

3.6. Infrared spectroscopy suggests interactions of compound 9 with CHIKV particles

The infrared spectra of the compound 9, CHIKV-*nanoluc*, and compound 9 plus CHIKV-*nanoluc* are represented in **Figure 9**. We found molecular vibrational modes in CHIKV-*nanoluc* related to lipids, proteins, carbohydrates, and nucleic acids. The spectra of 9 also expressed several vibrational modes in the fingerprint region at 1800–600 cm^{-1} . The second derivative spectra of the 9 plus CHIKV-*nanoluc* at 3040 cm^{-1} presented an additional vibrational mode that was not present in the compound 9 or CHIKV-*nanoluc* alone, suggesting interactions between the compound and virus related to stretching of C-H in lipids. Besides, the vibrational modes at 1418 cm^{-1} and 1040 cm^{-1} were present in the CHIKV-*nanoluc* and it was reduced in the compound 9 plus CHIKV-*nanoluc*, which suggests additional interaction in symmetric vibration of COO⁻ in polysaccharides and stretching C-O of riboses, respectively.

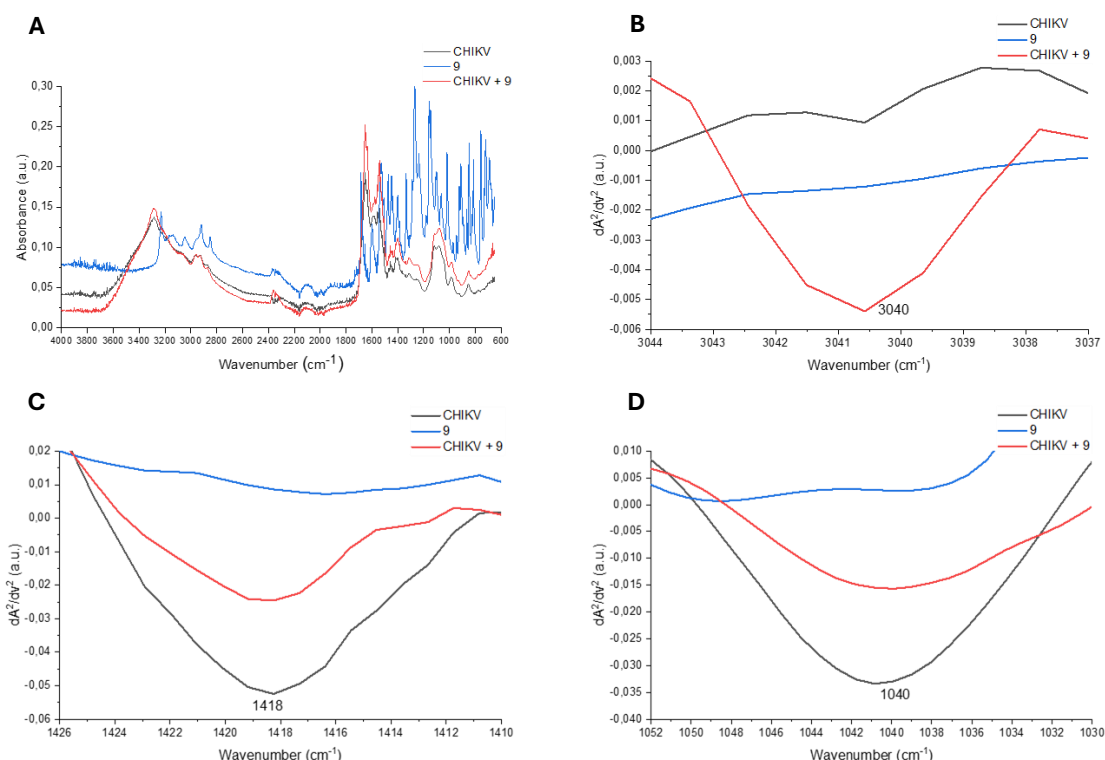


Figure 9. Infrared spectroscopy indicates molecular interactions between the compound **9**, CHIKV-*nanoluc*, and compound **9** plus CHIKV-*nanoluc*. Representative scheme of the ATR-FTIR technology with CHIKV-*nanoluc* (black line), the compound **9** (blue line), compound **9** plus CHIKV-*nanoluc* (red line). The original spectra are depicted in panel A. Panels B, C, and D depict the second-derivative spectra of vibrational modes at 3040, 1418 e 1040 cm^{-1} , respectively.

4. DISCUSSION

In this work, we evaluated the anti-CHIKV activity of twenty sulfonamide derivative compounds. These compounds have previously demonstrated potent and selective anti-ZIKV activity, highlighting their potential to inhibit other viruses (Dias et al., 2023). All compounds were evaluated through cell viability and antiviral activity employing BHK-21 cells and CHIKV expressing the *nanoluciferase* reporter.

From investigated compounds, seventeen exhibited statistically significant inhibition of CHIKV replication, although compounds **6**, **9**, **11**, and **13** presented the highest replication inhibition rates (more than 80% at 10 μM for **6**, **9** and **11**, and 50 μM for **13**), demonstrating a dose-response activity against CHIKV with EC_{50} of 14.9, 17.0, 63.1 and 41.6 μM and SIs of 13.8, 5.8, 4.4, and 11.0, respectively.

Carboxylic acid-derived sulfonamide compounds (**14-20**) exhibited the weakest inhibition of CHIKV replication (Figure 5B), suggesting that a heteroaromatic moiety is

crucial for antiviral efficacy. Furthermore, all four of the most potent compounds (**6**, **9**, **11**, and **13**) are BTA derivatives, highlighting the significance of this scaffold in antiviral activity.

Compounds **6** and **9** were identified as the most potent compounds, exhibiting EC₅₀ values of 14.9 and 17.0 μ M, respectively. The structural variation between these two compounds resides in the substituents attached to the benzenesulfonyl moiety. Specifically, compound **6** possesses a methyl substituent at the 4-position, whereas compound **9** bears a methoxy substituent at the 2-position and a chloro substituent at the 5-position.

In addition, it was shown through time-of-drug-addition assay that **6**, **9**, **11**, and **13** possess strong activity on post-entry stage. Furthermore, **9** demonstrated activity on all staged of CHIKV replicative cycle investigated here.

The production of synthetic compounds from the skeleton of organic molecules has demonstrated relevant advantages, such as ease of large-scale production, the possibility of patenting and the improvement of bioactivity, and increasing specificity for certain targets. Through the structure-activity relationship (SAR) commonly applied in medicinal chemistry, BTA derivatives have shown to possess strong antiviral properties, acting as allosteric inhibitors of NS2A/NS3 proteases of DENV (Wu et al., 2015) and ZIKV (Maus et al., 2021), NS3 and NS5B of HCV (Li et al., 2012; Manfroni et al., 2012), and the reverse transcriptase of HIV (Akbay et al., 2003). Similarly, our group demonstrated the action of **6**, **9**, and **11** against ZIKV *in vitro*, those with BTA insertions (Dias et al., 2023).

The strongest effects observed for all derivatives were on the post-entry stage of CHIKV replicative cycle, suggesting that the interference of CHIKV replication could be related to the replicative complex of CHIKV nsPs. In this context, the molecular docking calculations performed here corroborates with this hypothesis. Each of these nsPs plays many roles on virus replication, assembled into a viral replicative complex, fundamental for CHIKV genome replication and transcription (Bartholomeeusen et al., 2018). NsP1 possesses capping attributes, functioning as both a methyltransferase and guanylyltransferase; nsP2 operates as RNA triphosphatase, nucleoside triphosphatase (NTPase), and RNA helicase; nsP3 is a multifunctional protein involved in several process of recruiting host cell factors to improve viral replication, participates in the formation of replicative sites and modulates host immune response; and, ultimately, nsP4 is a key enzyme to the replicative process, known as RNA-dependent-RNA polymerase,

responsible mainly for catalyzing genome elongation (Freire et al., 2022; Schwartz and Albert, 2010; Subudhi et al., 2018).

In contrast, **9** was capable of interfering with all the stages of CHIKV replication cycle investigated. The results obtained here corroborate our previous published findings with ZIKV. However, in the experiments conducted with the BHK-ZIKV replicon, **9** did not show a significant inhibition, indicating that its mode of action might not be exclusively related to the nsPs (Dias et al., 2023). The molecular interactions between the compound **9** and CHIKV-*nanoluc* detected at 3040 cm^{-1} suggest interactions related to the stretching of C-H in the lipid membrane of the virus. These lipidic interactions could explain the virucidal effect of this compound, given that CHIKV is an enveloped virus surrounded by a bilipid layer derived from the host cell, as well as may justify the inhibition observed at the entry stage (Caglioti et al., 2013). Additionally, CHIKV replicates into lipidic membranes structures that could be also affected by this compound, disrupting the replication complex (Spuul et al., 2011).

Besides, the changes in the infrared spectra at 1418 cm^{-1} and 1040 cm^{-1} in the compound **9** plus CHIKV-*nanoluc* suggest superficial interaction in symmetric vibration of COO⁻ in polysaccharides, that may be related to the glycosidic bonds between the proteins and glucose molecules present in the envelope, and stretching C-O of riboses, respectively. These changes are in accordance with molecular docking based suggested in bioinformatics assays, which indicates interactions in hydrogen and arene-H bonds of the glycoprotein complex. Moreover, a study by Djuidje and collaborators demonstrated that BTA derivatives can exert antioxidant properties (Djuidje et al., 2022). This finding could support the observed protective effects of **9** on BHK-21 cells, preventing CHIKV infection, when administered at the pre-treatment stage, suggesting a possible mode of action.

The data presented here demonstrate that BTA derivatives exhibit anti-CHIKV activity by inhibiting some points of its replicative cycle *in vitro*. In this regard, these compounds represent templates to the development of potential new drug candidates against CHIKF, highlighting the importance of synthesizing molecules based on improving their pharmacological and pharmacokinetic properties. Nevertheless, further analysis are necessary to better understand the involved mechanisms of action and to assess the effects of these compounds *in vivo* systems for the development of new antiviral strategies.

Supplementary Material

 Ordinary one-way ANOVA Multiple comparisons							
1	Number of families	1					
2	Number of comparisons per family	26					
3	Alpha	0.05					
4							
5	Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	AA-?
6	DMSO 0.1% vs. 1 50 μ M	31.56	14.38 to 48.73	Yes	****	<0.0001	A 1 50 μ M
7	DMSO 0.1% vs. 10 μ M	14.34	-4.472 to 33.16	No	ns	0.2610	B 10 μ M
8	DMSO 0.1% vs. 2 50 μ M	-10.48	-29.29 to 8.334	No	ns	0.6865	C 2 50 μ M
9	DMSO 0.1% vs. 10 μ M	-6.050	-24.86 to 12.76	No	ns	0.9936	D 10 μ M
10	DMSO 0.1% vs. 3 50 μ M	32.10	13.29 to 50.92	Yes	****	<0.0001	E 3 50 μ M
11	DMSO 0.1% vs. 10 μ M	11.42	-7.397 to 30.23	No	ns	0.5679	F 10 μ M
12	DMSO 0.1% vs. 4 50 μ M	33.10	14.29 to 51.91	Yes	****	<0.0001	G 4 50 μ M
13	DMSO 0.1% vs. 10 μ M	20.10	1.290 to 38.92	Yes	*	0.0282	H 10 μ M
14	DMSO 0.1% vs. 5 50 μ M	75.68	56.86 to 94.49	Yes	****	<0.0001	I 5 50 μ M
15	DMSO 0.1% vs. 10 μ M	44.35	25.53 to 63.16	Yes	****	<0.0001	J 10 μ M
16	DMSO 0.1% vs. 6 50 μ M	62.17	43.35 to 80.98	Yes	****	<0.0001	K 6 50 μ M
17	DMSO 0.1% vs. 10 μ M	-9.113	-27.93 to 9.700	No	ns	0.8461	L 10 μ M
18	DMSO 0.1% vs. 7 50 μ M	30.83	13.66 to 48.01	Yes	****	<0.0001	M 7 50 μ M
19	DMSO 0.1% vs. 10 μ M	15.64	-1.539 to 32.81	No	ns	0.0993	N 10 μ M
20	DMSO 0.1% vs. 8 50 μ M	0.2400	-18.57 to 19.05	No	ns	>0.9999	O 8 50 μ M
21	DMSO 0.1% vs. 10 μ M	-0.3433	-19.16 to 18.47	No	ns	>0.9999	P 10 μ M
22	DMSO 0.1% vs. 9 50 μ M	50.80	33.62 to 67.97	Yes	****	<0.0001	Q 9 50 μ M
23	DMSO 0.1% vs. 10 μ M	-1.787	-18.96 to 15.39	No	ns	0.9996	R 10 μ M
24	DMSO 0.1% vs. 10 50 μ M	74.23	55.41 to 93.04	Yes	****	<0.0001	S 10 50 μ M
25	DMSO 0.1% vs. 10 μ M	32.04	13.22 to 50.85	Yes	****	<0.0001	T 10 μ M
26	DMSO 0.1% vs. 11 50 μ M	68.20	49.39 to 87.02	Yes	****	<0.0001	U 11 50 μ M
27	DMSO 0.1% vs. 10 μ M	6.223	-12.59 to 25.04	No	ns	0.9933	V 10 μ M
28	DMSO 0.1% vs. 12 50 μ M	25.77	6.958 to 44.59	Yes	**	0.0014	W 12 50 μ M
29	DMSO 0.1% vs. 10 μ M	5.783	-13.03 to 24.60	No	ns	0.9941	X 10 μ M
30	DMSO 0.1% vs. 13 50 μ M	4.458	-12.72 to 21.63	No	ns	0.9988	Y 13 50 μ M
31	DMSO 0.1% vs. 10 μ M	8.016	-9.159 to 25.19	No	ns	0.8783	Z 10 μ M

Supplementary Fig 1. Values acquired through the statistic analysis from viability assays. All treated cells were compared to DMSO 0.1% untreated control by One-way ANOVA and Dunnett's multiple comparisons test.

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CAPÍTULO III

Considerações finais

Considerações finais

Dos 20 compostos sintéticos avaliados, as sulfonamidas 6, 9, 11 e 13 apresentaram a atividade antiviral mais potente contra CHIKV sem citotoxicidade para as células BHK-21, demonstrando bons índices de seletividade nos ensaios de dose-resposta. Além disso, todos exibiram ação inibitória na pós-entrada, enquanto o composto 9 teve ação em diversas etapas do ciclo replicativo viral, como virucida, pré-tratamento e na entrada dos vírus nas células. Sendo assim, ensaios de ancoragem molecular foram conduzidos com as proteínas estruturais e não estruturais do CHIKV para avaliar potenciais interações entre as proteínas virais e os compostos 6, 9, 11 e 13. Adicionalmente, o ensaio de FTIR mostrou interações do composto 9 com lipídios, corroborando a ação destes compostos em membranas virais, como o envelope viral, e potencialmente em membranas celulares relacionadas à replicação viral. Os compostos destacados neste trabalho possuem em sua composição derivados de benzotiazol, indicando moléculas potenciais para serem adicionalmente investigadas no tratamento da CHIKF.



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Effect of proteins isolated from Brazilian snakes on enterovirus A71 replication cycle: An approach against hand, foot and mouth disease

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ABSTRACT

Enterovirus A71 (EVA71) belongs to the *Picornaviridae* family and is the main etiological agent of hand, foot, and mouth disease (HFMD). There is no approved antiviral against EVA71, and therefore the search for novel anti-EVA71 therapeutics is essential. In this context, the antiviral activity of proteins isolated from snake venoms has been reported against a range of viruses. Here, the proteins CM10 and CM14 isolated from *Bothrops moojeni*, and Crotamin and PLA2_{CB} isolated from *Crotalus durissus terrificus* were investigated for their antiviral activity against EVA71 infection. CM14 and Crotamin possessed a selective index (SI) of 170.8 and 120.4, respectively, while CM10 and PLA2_{CB} had an SI of 67.4 and 12.5, respectively. CM14 inhibited all steps of viral replication (protective effect: 76 %; virucidal: 99 %; and post-entry: 99 %). Similarly, Crotamin inhibited up to 99 % of three steps. In contrast, CM10 and PLA2_{CB} impaired one or two steps of EVA71 replication, respectively. Further dose-response assays using increasing titres of EVA71 were performed and CM14 and Crotamin retained functionality with high concentrations of EVA71 (up to 1000 TCID₅₀). These data demonstrate that proteins isolated from snake venom are potent inhibitors of EVA71 and could be used as scaffolds for future development of novel antivirals.

1. Introduction

Enterovirus A71 (EVA71) 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 [1]. EVA71 infection may also be associated with more severe cases of encephalitis, aseptic meningitis, and myocarditis, which could result in long-term sequelae and high mortality rates, especially in children [2–5]. EVA71 belongs to the *Enterovirus* genus from the *Picornaviridae* [6]. It is a non-enveloped virus, with an icosahedral capsid and a single-stranded positive RNA genome [7]. The viral RNA contains one open reading frame (ORF) that encodes a polyprotein cleaved into four structural proteins (VP1, VP2, VP3, and

VP4) and seven non-structural proteins (P2-2A, 2B, 2C, P3-3A, 3B, 3C, and 3D) [8–11].

Transmission occurs mainly via fecal-oral or oropharyngeal secretions, being highly contagious. In addition, it can also contaminate surfaces and promote transmission by fomites [12]. Interestingly, the incidence increases at the warmest times of the year [13]. EVA71 replicates in gastrointestinal and respiratory epithelial cells, however, the viremia can result in infection of neurons and cardiomyocytes [14]. An infected individual can be asymptomatic, and in most cases the infection is self-limiting, however, symptomatic cases can progress to HFMD. Thus, the infection may reach the central nervous system and cause dysregulation of the autonomic nervous system, resulting in

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Insights into enterovirus a-71 antiviral development: from natural sources to synthetic nanoparticles

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Abstract

Enteroviruses are pathogens responsible for several diseases, being enterovirus A71 (EVA71) the second leading cause of hand, foot, and mouth disease (HFMD), especially in Asia-Pacific countries. HFMD is mostly common in infants and children, with mild symptoms. However, the disease can result in severe nervous system disorders in children as well as in immunosuppressed adults. The virus is highly contagious, and its transmission occurs via fecal-oral, oropharyngeal secretions, and fomites. The EVA71 burdens the healthy systems and economies around the world, however, up to date, there is no antiviral approved to treat infected individuals and the existent vaccines are not available or approved to be used worldwide. In this context, an extensive literature research was conducted to describe and summarize the recent advances in natural and/or synthetic compounds with antiviral activity against EVA71. The summarized data presented here might simply encourage the future studies in EVA71 antiviral development, by encouraging further research encompassing these compounds or even the application of the techniques and technologies to improve or produce new antiviral molecules.

Keywords Enterovirus A-71 · HFMD · Antivirals · Natural and synthetic compounds

Introduction

Enterovirus A71 (EVA71) is a member of the *Picornaviridae* family and represents a persistent global health problem since it is responsible for several outbreaks, mainly in the Asia-Pacific regions (Huang and Shih 2014). EVA71 is the etiological agent of the hand, foot, and mouth disease (HFMD) and herpangina, being especially serious for children under five years old (Lin et al. 2019a). The severe form of the disease includes neurological complications such as aseptic meningitis, brainstem, and/or cerebellar encephalitis,

acute flaccid paralysis, besides myocarditis, rapid fatal pulmonary oedema, and haemorrhage (McMinn 2002).

EVA71 is a non-enveloped virus with an icosahedral shape and a single-stranded positive sense RNA genome of approximately 7.4 kb with one open reading frame (ORF) (McMinn 2002) (Fig. 1A and 1B). The ORF region encodes a polyprotein of approximately 2100 amino acids, which is hydrolysed to form three precursor proteins: P1, P2, and P3. P1 is cleaved into four structural proteins: VP1, VP2, VP3, exposed on the capsid surface, and VP4, located internally of the capsid (Fig. 2A–C). P2 and P3, on the other hand, are cleaved in seven non-structural proteins, known as P2–2A, 2B, 2C; P3–3A, 3B, 3C, and 3D (Solomon et al. 2010) (Fig. 1A and 1B).

The viral replicative cycle begins with the EVA71 binding to the cell receptors mediated mainly by the scavenger receptor B2 (SCARB2; clathrin-dependent endocytosis) (Kobayashi and Koike 2020). However, the receptors sialomucin, leukocyte membrane protein 1 (PSGL-1; caveolin dependent endocytosis), annexin family protein 2 (Anx2), sialylated glycans, and heparan sulphate, are also known for mediate viral entry into the host (Dang et al. 2014; Nishimura et al. 2009; Yamayoshi et al. 2009, 2014; Zhou et al. 2019).

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