



UNIVERSIDADE ESTADUAL PAULISTA  
“JÚLIO DE MESQUITA FILHO”  
Campus de São José do Rio Preto

Suely da Silva

**Atividade antiviral de compostos naturais e sintéticos na  
replicação do ZIKV *in vitro*.**

São José do Rio Preto

2018

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de São José do Rio Preto.  
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*“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota”.*

*Madre Teresa de Calcutá*

## Resumo

A febre Zika é uma arbovirose resultante da infecção pelo vírus Zika (ZIKV). Em 2016 o ZIKV foi considerado pela Organização Mundial da Saúde um grave problema da saúde pública, devido ao número de casos de má formação congênita e distúrbios neurológicos vinculados à infecção, como microcefalia e síndrome de Guillain-Barré. Não existe atualmente antiviral específico contra o ZIKV. Neste contexto, torna-se necessário a busca por um antiviral seguro e que trate a infecção. Compostos naturais e seus derivados sintéticos vêm contribuindo significativamente para desenvolvimento de novos fármacos e têm ganhado destaque como potenciais antivirais, tornando-se uma possibilidade para o tratamento de infecções causadas pelo ZIKV. Sendo assim, o presente trabalho teve como objetivo avaliar os efeitos de compostos naturais e análogos sintéticos obtidos com base em estruturas químicas naturais no ciclo replicativo do ZIKV *in vitro*. Um total de 166 compostos dentre eles, flavonoides, alcaloides (naturais e sintéticos) e derivados do ácido antranílico foram analisados quanto à atividade antiviral pela infecção e tratamento de células Vero. Os dados obtidos mostraram que o composto sintético FAM E3, da classe diarilaminas derivadas do ácido antranílico (FAMs), inibiu um mínimo de 99.92% a etapa de replicação viral. Ensaio adicionais mostraram que o potencial de inibição é dose-dependente e este composto não apresenta ação antiviral na etapa de entrada do ciclo viral. Para elucidar o mecanismo de ação, investigamos a capacidade de FAM E3 em intercalar em dsRNA e inibir a polimerase viral. Os resultados mostraram que o composto não atua nessas vias. Ensaio de docking molecular sugerem que FAM E3 apresenta alta afinidade com a helicase NS3 e o complexo NS2B3-NS3, sugerindo estas proteínas como possíveis alvos do composto. Outras análises são necessárias para estabelecer o mecanismo de ação do FAM E3, contudo este composto apresenta potencial terapêutico para o tratamento da febre Zika.

**Palavras-chave:** *Flavivírus*; antivirais; compostos naturais; derivados sintéticos; vírus Zika; Diarilaminas derivadas do ácido antranílico.

## ABSTRACT

Zika fever is an arboviruses resulting from infection cause by Zika virus (ZIKV). In 2016 the ZIKV was considered by the World Health Organization a serious public health problem due to the number of cases of congenital malformation and neurological disorders linked to infection, such as microcephaly and Guillain-Barré syndrome. There is no specific antiviral against infection ZIKV. In this context, it becomes necessary to search for a safe antiviral to treat this infection. Therefore molecules derived from natural sources and their synthetic analogues are a potential alternative for the investigation of active compounds to treat the infection. Natural compounds and their synthetic derivatives have contributed significantly to the development of new drugs and have been acquiring a high profile as potential antivirals, becoming a possibility for the treatment of infections caused by ZIKV. Thus, the present work had as objective to evaluate the effects of natural compounds and synthetic analogues obtained based on natural chemical structures in the replicative cycle of ZIKV in vitro. A total of 166 compounds among them, flavonoids, alkaloids (natural and synthetic) and anthranilic acid derivatives were analyzed for antiviral activity by infection and treatment of Vero cells. The data obtained showed that the synthetic compound FAM E3, from the class diarylamines derived from anthranilic acid (FAMs), inhibited a minimum of 99.92% of the viral infection. Additional assays have shown that the inhibition potential is dose-dependent and this compound does not present antiviral action at the entry stage of the viral cycle. To elucidate the mechanism of action, we investigated the ability of FAM E3 to intercalate into dsRNA and inhibit viral polymerase. The results showed that the compound does not act in these pathways. Molecular docking assay suggests that FAM E3 exhibits high affinity with the NS3 helicase and the NS2B3-NS3 complex, suggesting these proteins as possible targets of the compound. Other analyzes are needed to establish the mechanism of action of FAM E3, however this compound has therapeutic potential for the treatment of Zika fever.

**Keywords:** *Flavivirus*; antivirals; natural compounds; synthetic derivatives; diarylamines derived from anthranilic acid; Zika virus.

## SUMÁRIO

### ***CAPÍTULO I: FUNDAMENTAÇÃO TEÓRICA ..... 12***

|                                                      |           |
|------------------------------------------------------|-----------|
| <b>1. Introdução .....</b>                           | <b>15</b> |
| 1.1. Histórico e Epidemiologia .....                 | 15        |
| 1.2. Classificação.....                              | 17        |
| 1.3. Transmissão .....                               | 17        |
| 1.4. Patogênese e Implicações na Saúde Pública ..... | 18        |
| 1.5. Genoma viral .....                              | 20        |
| 1.6. Proteínas virais .....                          | 22        |
| 1.7. Ciclo reprodutivo do ZIKV .....                 | 23        |
| 1.8. Compostos naturais no tratamento do ZIKV .....  | 25        |
| <b>2. Objetivos.....</b>                             | <b>28</b> |
| 2.1. Objetivos específicos .....                     | 28        |
| <b>3. Referências Bibliográficas .....</b>           | <b>29</b> |

### ***CAPÍTULO II: A REVIEW OF THE ONGOING RESEARCH ON ANTI-ZIKA TREATMENTS ..... 41***

|                                                                |           |
|----------------------------------------------------------------|-----------|
| <b>1. Introduction.....</b>                                    | <b>44</b> |
| <b>2. The ZIKA virus .....</b>                                 | <b>45</b> |
| <b>3. Therapeutical potential of anti-ZIKV molecules .....</b> | <b>46</b> |
| <b>4. Concluding Remarks.....</b>                              | <b>50</b> |
| <b>5. References .....</b>                                     | <b>51</b> |

### ***CAPÍTULO III: DIARYLAMINE DERIVED FROM ANTHRANILIC ACID INHIBITS THE ZIKV REPLICATION..... 69***

|                                                        |           |
|--------------------------------------------------------|-----------|
| <b>1. Introduction .....</b>                           | <b>73</b> |
| <b>2. Material and Methods .....</b>                   | <b>74</b> |
| 2.1. Compounds.....                                    | 74        |
| 2.2. ZIKV construction.....                            | 75        |
| 2.3. Cell Culture .....                                | 76        |
| 2.4. Cell viability assay .....                        | 76        |
| 2.5. Virus assays.....                                 | 76        |
| 2.6. Antiviral assays .....                            | 77        |
| 2.7. Dose-dependent assay .....                        | 77        |
| 2.8. ZIKV entry e/or replication assay.....            | 78        |
| 2.9. Pre-treatment assay .....                         | 78        |
| 2.10. dsRNA Intercalation assay .....                  | 78        |
| 2.11. Inhibition of SP6 polymerase transcription ..... | 79        |
| 2.12. Immunofluorescence assay .....                   | 79        |
| 2.13. Molecular docking .....                          | 79        |

|                                                    |                                                                                                  |            |
|----------------------------------------------------|--------------------------------------------------------------------------------------------------|------------|
| 2.14.                                              | Statistical analysis.....                                                                        | 80         |
| <b>3.</b>                                          | <b>Results .....</b>                                                                             | <b>80</b>  |
| 3.1.                                               | Inhibitory effect of natural compounds and their synthetic analogues on ZIKV infection.....      | 80         |
| 3.2.                                               | The antiviral activity of FAM E3 is not related to the entry step of ZIKV in the host cell ..... | 81         |
| 3.3.                                               | FAM E3 inhibits the replication ZIKV step in Vero cells .....                                    | 82         |
| 3.4.                                               | FAM E3 antiviral mechanisms.....                                                                 | 82         |
| <b>4.</b>                                          | <b>Discussion .....</b>                                                                          | <b>83</b>  |
| <b>5.</b>                                          | <b>References .....</b>                                                                          | <b>87</b>  |
| <b><i>CAPÍTULO IV: ARTIGOS PUBLICADOS.....</i></b> |                                                                                                  | <b>120</b> |

## ***Capítulo I: Fundamentação Teórica***

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## 1.Introdução

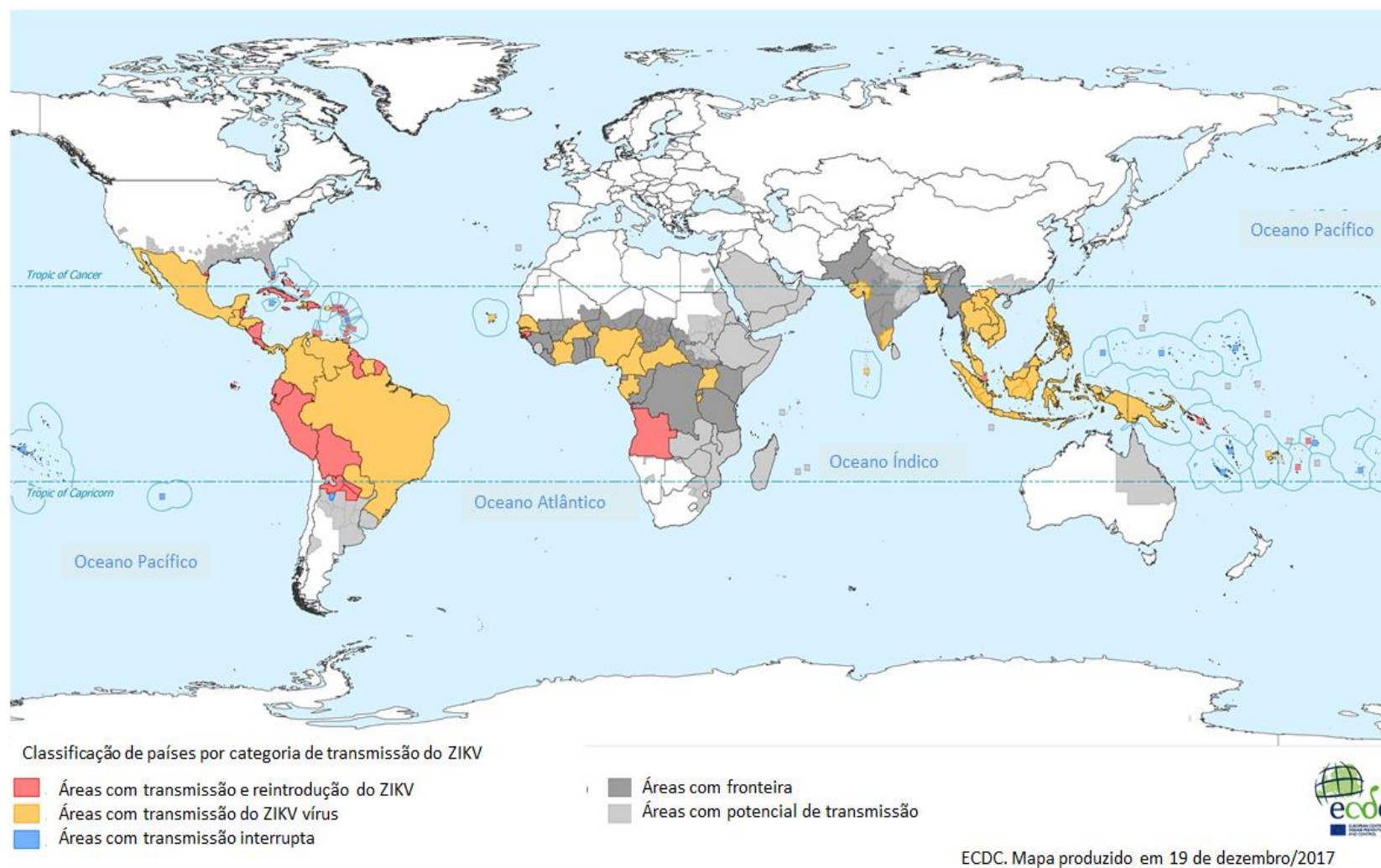
### 1.1. Histórico e Epidemiologia

A febre Zika é uma arbovirose transmitida aos seres humanos pela picada de mosquitos infectados com vírus Zika (ZIKV) (AUBRY et al., 2016). O ZIKV foi isolado pela primeira vez em 1947 na Floresta Zika na Uganda de um macaco *Rhesus* (DICK; KITCHEN; HADDOW, 1952; VINET; ZHEDANOV, 2010a). Posteriormente, o vírus foi relatado na mesma região em isolado de um *pool* mosquito *Aedes Africanus* (DICK; KITCHEN; HADDOW, 1952; HADDOW et al., 2012; MARANO et al., 2015).

Por décadas o vírus permaneceu endêmico apenas na África e Ásia. Os primeiros casos de infecção em humanos foram descritos em 1954, primeiro em Uganda e depois na Nigéria, com o isolamento do vírus de pacientes infectados. Posteriormente casos esporádicos da infecção por ZIKV foram registrados em Uganda (SIMPSON, 1964a), Nigéria (FAGBAMI, 1979) e Indonésia (VINET; ZHEDANOV, 2010b). Somente em 2007 que pequenos surtos foram documentados em outras regiões (PINTO JUNIOR et al., 2015).

O primeiro registro do ZIKV fora do continente Africano e Asiático ocorreu em YAP, uma ilha da Federação dos Estados da Micronésia (DUFFY et al., 2009; LANCIOTTI et al., 2008; SONG et al., 2017). Em 2007 o vírus alcançou o leste da Polinésia e outras regiões do pacífico entre 2013/2014, chegando ao continente Americano em fevereiro de 2015 (AUBRY et al., 2016). Em maio de 2015 a transmissão autóctone do vírus foi reportada no Brasil, principalmente na região Nordeste, a partir de casos suspeitos de infecção por vírus da dengue (DENV) (CAMPOS; BANDEIRA; SARDI, 2015; ZAMMARCHI et al., 2015; ZANLUCA et al., 2015). Nesta época no estado de Pernambuco houve um aumento abrupto do número de casos de recém-nascidos com microcefalia. Ao investigar o período gestacional das mães dessas crianças foi constatado que a maioria delas apresentou sintomas típicos de infecção causada pelo ZIKV no primeiro trimestre da gravidez. Posteriormente os casos de microcefalia na região e em outros estados brasileiros puderam ser relacionados à infecção causada por vírus Zika (TEIXEIRA et al., 2016). A Organização Mundial da Saúde declarou em fevereiro de 2016 a epidemia causada pelo ZIKV como um grave problema de saúde pública, devido às infecções com ZIKV estarem relacionadas aos casos de má formação congênita (W.H.O, 2016). Embora medidas de controle e prevenção venham sendo tomadas, a situação geral do risco global não alterou. O ZIKV continua a se espalhar geograficamente em regiões com grande predominância do inseto vetor (W.H.O, 2017)(**Figura 1**).

**Figura 1.** Países e territórios com casos de transmissão do ZIKV.



Fonte: Centro europeu de prevenção e controle de doenças, estado da transmissão de ZIKV no mundo em dezembro de 2017.

## 1.2. Classificação

O ZIKV pertence à família *Flaviviridae*, gênero *Flavivirus*, sendo filogeneticamente relacionado aos vírus da Dengue (DENV), vírus do Nilo Ocidental (WNV), vírus da Febre Amarela (YFV), vírus da Encefalite Japonesa (JEV) e vírus da Encefalite transmitida por carrapatos (TBEV) (SUMMERS et al., 2015).

Análises filogenéticas do genoma viral revelaram que o ZIKV se originou na África e desde então divergiu em duas linhagens principais, a Africana, representada pela estirpe viral MR 766 isolada na África Ocidental em 1947 (DICK; KITCHEN; HADDOW, 1952), e Asiática/ Americana, representada pela estirpe viral P6740 isolada da Malásia em 1966 (SMITH-BINDMAN, 2010). A irradiação do protótipo Asiático para o Ocidente fez com que este sofresse uma adaptação natural ao novo ambiente, possibilitando o aparecimento da linhagem Americana e, dentro desta, o surgimento de alguns subgrupos como a estirpe brasileira (MLAKAR et al., 2016), Porto Rico (THOMAS et al., 2016), Haiti, Guatemala e Suriname (ENFISSI et al., 2016).

## 1.3. Transmissão

Arbovírus como o vírus Zika geralmente apresentam dois ciclos de transmissão distintos, o ciclo silvestre e o urbano. No ciclo silvestre o vírus é mantido entre primatas não humanos e o vetor artrópode em florestas. O ciclo urbano envolve a transmissão viral entre os insetos vetores e humanos na cidade (WEAVER; BARRETT, 2004).

A transmissão do ZIKV se dá principalmente pela picada da fêmea de mosquitos do gênero *Aedes*, que incluem *Aedes aegypti*, *A. albopictus*, *A. africanus*, *A. luteocephalus*, *A. vitattus*, *A. furcifer*, *A. hensilii* e *A. apicoargenteus* (VINET; ZHEDANOV, 2010a). No ciclo urbano as espécies que mais se destacam são o *A. aegypti* e *A. albopictus*, ambas distribuídas amplamente em zonas tropicais e subtropicais, destacando a capacidade de adaptação do *A. albopictus* a zonas mais frias (KRAEMER et al., 2015). Além do gênero *Aedes*, o ZIKV foi isolado de outros mosquitos vetores como *Anopheles coustani*, *Culex perfuscus* e *Mansonia uniformis* (DIALLO et al., 2014; FAYE et al., 2013; VASILAKIS; WEAVER, 2017).

Os primatas selvagens são os principais reservatórios não humanos, no entanto anticorpos contra ZIKV foram detectados em morcegos (ANDRAL et al., 1968), cabras

(DARWISH et al., 1983), roedores (DARWISH et al., 1983) e ovelhas (DARWISH et al., 1983). Embora os anticorpos anti-ZIKV foram confirmados em outras espécies, não há nenhuma evidência conclusiva para a existência de um reservatório não primata (VINET; ZHEDANOV, 2010a). Além do mosquito, outros veículos de transmissão devem ser considerados como a transfusão sanguínea (MUSSO et al., 2014), transmissão sexual (MCCARTHY, 2016) e transmissão perinatal (VENTURA et al., 2016).

#### **1.4. Patogênese e Implicações na Saúde Pública**

Em 2016 a Organização Mundial da Saúde declarou o surto do ZIKV como emergência de saúde pública, principalmente por estar relacionado ao número de casos de malformações congênitas (microcefalia) vinculadas às gestantes infectadas pelo vírus (W.H.O, 2016). No Brasil, em 2016 foram registrados 215.319 casos suspeitos de infecção pelo ZIKV, sendo 60,7% destes confirmados para a doença. Além disso, 8 óbitos foram registrados distribuídos entre os estados do Rio de Janeiro, Espírito Santo, Maranhão e Paraíba (BRASIL, 2017a). Em 2017 embora o número de casos suspeitos tenha reduzido para 17.321, metade destes foram confirmados para a infecção sendo o Centro-Oeste e Norte do Brasil os estados mais afetados com registro de óbito em Rondônia e no estado de São Paulo (BRASIL, 2017b).

Por ser um vírus emergente e negligenciado, a patogênese do ZIKV ainda não está bem elucidada. (HAMEL et al., 2015). Sabe-se que as células da pele humana (epiderme e derme) são os alvos primários dados a capacidade de o mosquito transmitir o vírus por meio da picada. Desta forma foi mostrado por Hamlet et al., a capacidade que o ZIKV de infectar fibroblastos, queratinócitos e células dendríticas mediada pelos receptores de entrada TAM (AXL ou Tyro3), DC-SIGN, TIM-1, TIM-4, (HAMEL et al., 2015). O receptor AXL é altamente expressado em células da glia radiais sustentando a hipótese de que células neurais são mais permissíveis ao ZIKV o que propicia o desenvolvimento da microcefalia (NOWAKOWSKI et al., 2016).

O vírus Zika também tem tropismo pelas células progenitoras neurais humanas (CPNHs) infectando e replicando com grande eficiência, sendo os neurônios menos suscetíveis à infecção. A infecção por ZIKV em células neuronais desencadeia a apoptose celular, desregula o ciclo celular, bem como impede a diferenciação celular das CPNHs,

efeitos esses que também contribuem o desenvolvimento de microcefalia (LI et al., 2016; TANG, 2016).

A microcefalia é uma desordem neural em que a criança nasce com a circunferência do crânio e cérebro muito reduzidos. Esta condição leva à incapacidade motora, sensorial e cognitiva do portador (TANG, 2016; WOODS; BOND; ENARD, 2005). Vários estudos tem relacionado o período da emergência do vírus no Brasil com o número de crianças que tem nascido com o distúrbio desde então (BRASIL et al., 2016; CAUCHEMEZ et al., 2016; MAGALHÃES-BARBOSA et al., 2016; SCHULER-FACCINI et al., 2016). A microcefalia causada pelo ZIKV ocorre pela transmissão vertical do vírus que pode acontecer até o primeiro trimestre de gestação, sendo os macrofágos transplacentários e citotrofoblastos alvos primários da infecção viral na placenta (QUICKE et al., 2016). A presença de anticorpos contra o ZIKV em amostras coletadas de neonatos com microcefalia e tecido de placenta de mulheres infectadas pelo vírus já foram confirmadas (BAYER et al., 2016; DE ARAÚJO et al., 2016). Recentemente foi mostrado que uma mutação no aminoácido de posição 139 da proteína estrutural prM aumentou o tropismo do ZIKV por células neurais (YUAN et al., 2017a).

O ZIKV também pode estar relacionado à síndrome de Guillain-Barré (GBS), distúrbio neurológico que afeta principalmente adultos acima de 30 anos. A GBS é uma doença autoimune caracterizada por desordem motora e, algumas vezes, perda sensorial dos músculos faciais e respiratórios (CRÉANGE, 2016; GOODFELLOW; WILLISON, 2016). É um distúrbio que também pode ser desencadeado por outros vírus como DENV (CAROD-ARTAL et al., 2013; RALAPANAWA; KULARATNE; JAYALATH, 2015) e CHIKV. (AGARWAL et al., 2017). A primeira relação entre GBS e ZIKV surgiu em 2013, durante o surto viral na Polinésia Francesa, quando exames sorológicos de 42 pacientes com GBS demonstraram infecção por ZIKV (MUSSO; NILLES; CAO-LORMEAU, 2014). Uma nova relação dos casos da síndrome pôde ser feita com o crescente número de casos de pacientes infectados com ZIKV, quando o vírus se espalhou no continente Americano de 2015 à 2016 (BROUTET et al., 2016; THOMAS et al., 2016). Outras investigações sugerem a associação do ZIKV com outras desordens neurológicas como meningo-encefalite (CARTEAUX et al., 2016) e mielite aguda (MÉCHARLES et al., 2016).

### 1.5.Genoma viral

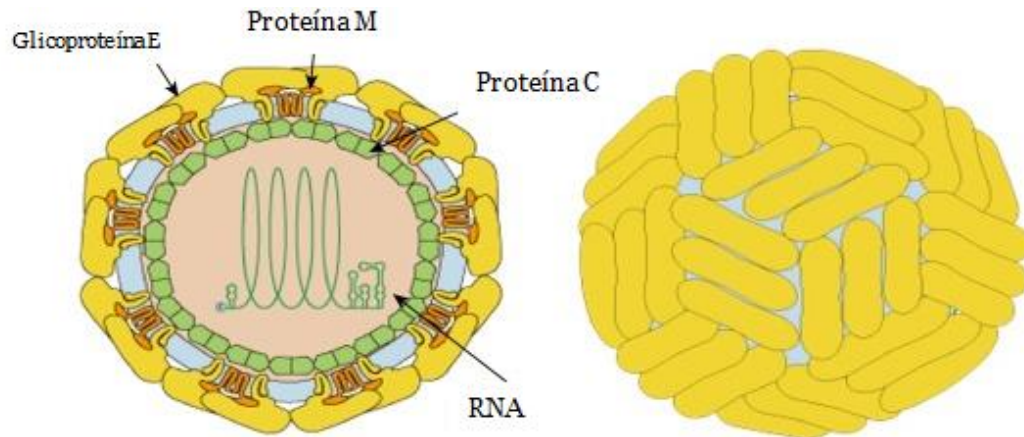
A partícula viral é formada por um nucleocapsídeo icosaédrico, uma bicamada lipídica (envelope) derivada de membranas do hospedeiro onde estão inseridas duas glicoproteínas virais (proteínas E e M). (SHANKAR; PATIL; SKARIYACHAN, 2017). No interior da partícula encontra-se o genoma viral constituído de RNA de fita simples de polaridade positiva de aproximadamente 11 Kb de comprimento (HAYES, 2009).

O genoma do ZIKV possui única região aberta de leitura ("*open reading frame*"-ORF) que é traduzida em uma poliproteína de aproximadamente 3200 aminoácidos (aa) na sequência C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. Após ser sintetizada esta poliproteína, é clivada por proteases virais e celulares em 7 proteínas não estruturais (NS), relacionadas à etapa de replicação viral (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5) e 3 proteínas estruturais capsídeo (C), pré-membrana (prM) e de envelope (E) que farão parte das novas partículas virais (HADDOW et al., 2012; FAYE et al., 2014) **(Figura 2A)**.

A ORF se encontra flanqueada por regiões não traduzidas (*Untranslated Region - UTR*), altamente conservadas: 5'-UTR (107 nts) e 3'-UTR (429 nts) de grande importância para a replicação viral. No início da região 5'UTR existe uma estrutura Cap 1 formada por uma guanosina conectada ao primeiro nucleotídeo do RNA por intermédio de uma ligação trifosfato, de grande importância para o processo de tradução do genoma viral. (GÖERTZ et al., 2017; ZHU et al., 2016). Assim como em outros *Flavivirus* as regiões 5' e 3' do genoma do ZIKV se dobram em estruturas secundárias de RNA (*Stem-loop*) que codificam regiões de grande importância para a fase inicial de replicação. Além de se ligarem as proteínas específicas das células hospedeiras estas estruturas atuam na síntese de RNA de polaridade negativa realizada pela polimerase viral NS5 e auxiliam a tradução dependente de Cap (ZHU et al., 2016). **(Figura 2B)**

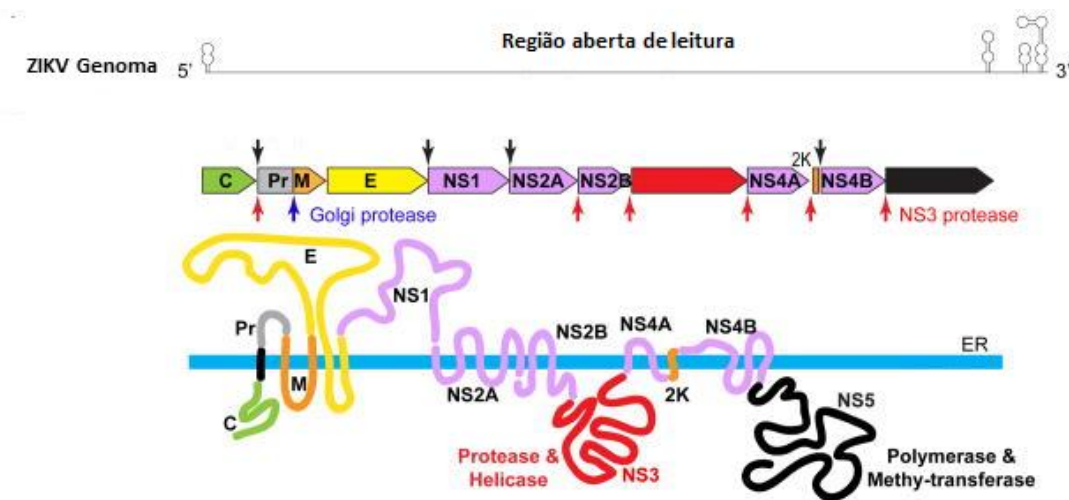
**Figura 2.** (A) Representação esquemática da partícula viral, demonstrando as proteínas do Core (C), do envelope (E), proteína de membrana (M) e RNA viral. (B). Estrutura geral da poliproteína viral codificada pelo ZIKV, demonstrando as proteínas estruturais e não estruturais.

A)



Fonte: Adaptado de Kaushik et al, 2016

B)



Fonte: Adaptado de Ming et al., 2016

## 1.6. Proteínas virais

A proteína do Core (C) é um dímero que se mantém associado ao genoma formando o nucleocapsídeo vira. A proteína E e proteína prM/M estão diretamente relacionadas com a etapa da entrada viral (SIROHI; KUHN, 2017). Além de mediar a interação vírus/célula hospedeira, a proteína E está envolvida em outras etapas do ciclo replicativo como processo de fusão, penetração e tropismo celular (CHÁVEZ et al., 2010) (**Figura 3**).

NS1 é a primeira glicoproteína não estrutural apresentando aproximadamente 46–55 kDa e 342aa NS1 pode ser encontrada em vários compartimentos celulares, sendo que a forma intracelular é essencial para a manutenção do complexo replicativo enquanto a NS1 extracelular está envolvida com a resposta imune da célula hospedeira (MACKENZIE; JONES; YOUNG, 1996; PRYOR; WRIGHT, 1994).

As proteínas NS2 são proteínas hidrofóbicas que estão relacionadas com o complexo de replicação viral. NS2A participa na síntese de RNA e montagem viral, além de estar envolvida em processos importantes como na evasão do vírus à resposta ao sistema imunológico, rearranjo da membrana e processamento da proteína NS1 (FAJARDO-SÁNCHEZ; GALIANO; VILLALÁIN, 2017; XIE et al., 2013). Já a proteína NS2B atua no processamento da poliproteína viral (MILLER; SPARACIO; BARTENSCHLAGER, 2006).

A protease de *Flavivirus* NS3 é uma preteínase responsável pelo processamento da poliproteína em vários sítios dentro da região não-estrutural do genoma (LEUNG et al., 2001). Em sua forma basal, a NS3 é inativa, e a sua forma ativa requer a fusão de sua região N-Terminal (~180aa) com a região C-terminal (~40aa) do cofator NS2b, formando o complexo NS2B/NS3 que dará início ao processo proteolítico (LEUNG et al., 2001; LI et al., 2015; ZHANG et al., 2016).

As proteínas NS4A e NS4B são codificadas pela região NS4. NS4A se destaca tanto na replicação e pós-tradução da poliproteína viral como na clivagem de NS4B e NS5A. Também funciona como um cofator para protease NS3 (BUTKIEWICZ et al., 1996; TANJI et al., 1995). NS4B está envolvida na formação dos constituintes de membrana que forma o complexo de replicação viral (BUCKLEY; GOULD, 1988).

A proteína NS5 é uma metil-transferase (MTase) N-terminal e uma RNA polimerase dependente de RNA C-terminal (RdRp), sendo fundamental para a replicação de novas partículas virais. A metil-transferase facilita a tradução do genoma introduzindo o Cap1 no mRNA viral da região 5' (DAFFIS et al., 2010; ISSUR et al., 2009; ZHAO et al., 2017;

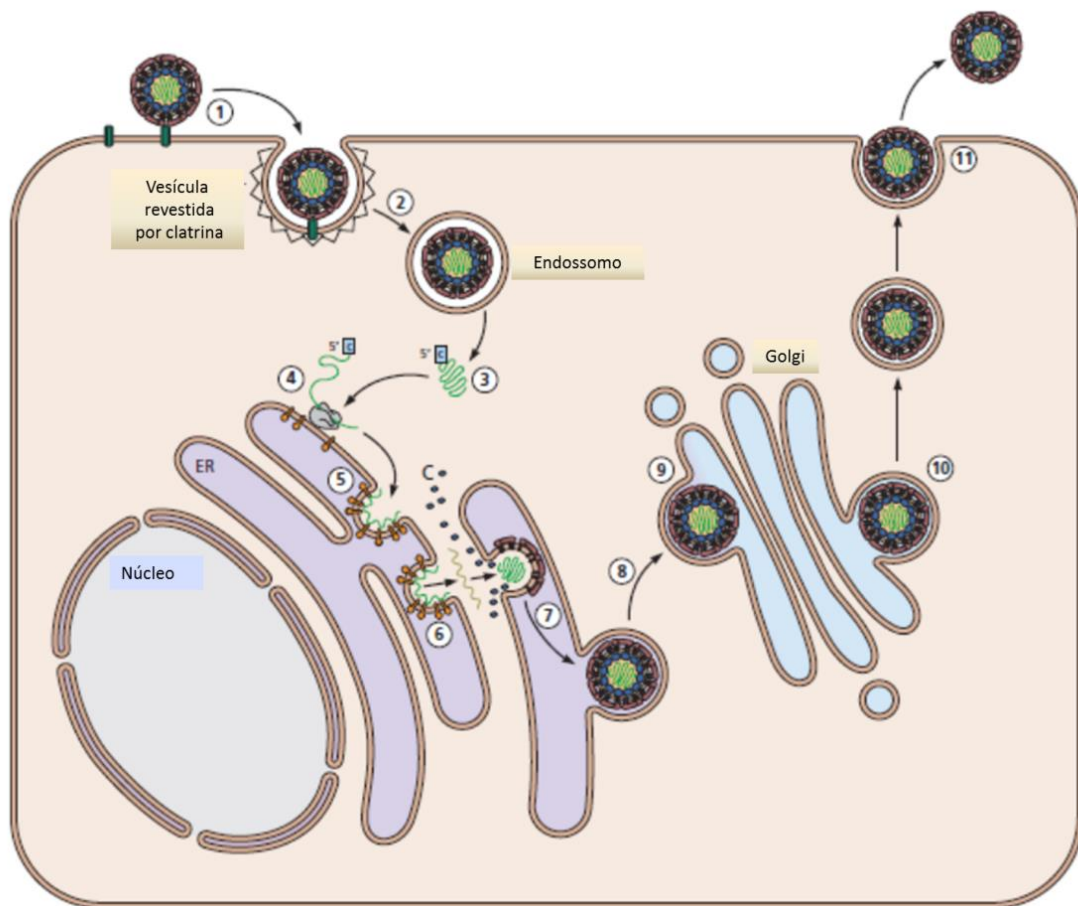
ZHENG et al., 2013), enquanto a RdRp é responsável por iniciar a síntese de novas fitas de RNA.

### 1.7. Ciclo reprodutivo do ZIKV

Diferentes tipos de células e diferentes espécies são suscetíveis à infecção por ZIKV como células progenitoras neurais, da glia radial e da epiderme (GUO, 2016; HAMEL et al., 2015; LI et al., 2012; MEERTENS et al., 2017; SAIZ; MARTÍN-ACEBES, 2017a; TANG et al., 2016). A primeira etapa para a infecção é a interação da glicoproteína E do envelope viral com receptores específicos do hospedeiro como AXL ( LIU et al., 2016; MEERTENS et al., 2017; NOWAKOWSKI et al., 2016; SAVIDIS et al., 2016) , DC-SIGN, TYOR3 e TIM 1 (HAMEL et al., 2015). A entrada do ZIKV na célula ocorre por fusão do envelope viral com o endossomo derivado da membrana da célula hospedeira, processo este mediado pela proteína clatrina(SAIZ et al., 2016)

A célula hospedeira oferece um ambiente com pH adequado para que, após a entrada da partícula viral, ocorra o desnudamento do genoma (SAIZ et al., 2016). Uma vez que ocorre a liberação do material genético no citoplasma, a ORF é traduzida em uma poliproteína viral que é processada dando origem às proteínas virais. O ZIKV assim como outros *Flavivirus* induz a formação de vesículas derivadas do retículo endoplasmático rugoso onde ocorre a replicação do genoma. As proteínas não-estruturais se organizam nessas vesículas para formar o complexo replicativo onde sintetizam RNAs de polaridade negativa, usando como molde o genoma viral que foi liberado após o desnudamento. Novos RNAs de polaridade positiva são produzidos a partir do molde de fita negativa e são utilizados para a tradução de proteínas e para compor novas partículas virais. Após a montagem e maturação das novas partículas virais, estas são transportadas por vesículas do complexo de *Golgi* e pela via secretora para fora da célula (BYLER; OGUNGBE; SETZER, 2016; SAIZ et al., 2016; SHANKAR; PATIL; SKARIYACHAN, 2017). **(Figura 3).**

**Figura 3.** Ciclo reprodutivo do ZIKV. (1) Ligação da partícula viral ao receptor de superfície celular. (2) Endocitose viral. (3) Fusão das membranas virais e celulares (4) Tradução da poliproteína viral (5) Sítio de replicação (6) Multiplicação do genoma viral (7) Sítio de montagem (8) Transporte das partículas virais imaturas pela via secretora. (9) Marturação da partícula viral (10) vírus maduro. (11) Liberação da partícula viral.



Fonte: Adaptado de Flint, 2015.

### 1.8. Compostos naturais no tratamento do ZIKV

Atualmente, não existe tratamento específico para a infecção causada por ZIKV, (W.H.O, 2016). Sendo assim a busca por moléculas ativas para o tratamento da infecção tem sido constante (ALAM et al., 2017; SAIZ; MARTÍN-ACEBES, 2017b). O uso de plantas para fins medicinais é uma prática antiga e um dos aspectos importantes da investigação de moléculas naturais com propriedades terapêuticas ativas é que estas servem de modelos para síntese de moléculas análogas, mantendo ou aprimorando as propriedades ativas, como já vêm sendo realizado ao longo dos anos pelas indústrias farmacêuticas (BALUNAS; KINGHORN, 2005). Tais conhecimentos vêm sendo utilizado na composição de vários fármacos importantes como a salicina, extraída da *Salix alba* e popularmente conhecido como aspirina, e a morfina e a codeína, extraídas do *Papaver somniferum* (NEWMAN; CRAGG; SNADER, 2000). Muitos compostos naturais são descritos com enorme potencial terapêutico, apresentando diversas atividades como anticâncer, antimaláricas (VAN AGTMAEL, 1999), antidepressivas (CÍCERO BEZERRA FELIPE et al., 2007) e antivirais (JARDIM et al., 2015; MULLER et al., 2014; SERKEDJIEVA; NIKOLOVA; KIRILOV, 2010). Com isso, compostos naturais se apresentam como uma alternativa em potencial (COSTA-LOTUFO et al., 2010) para o tratamento do ZIKV. Adicionalmente, compostos naturais e seus derivados são moléculas facilmente metabolizadas pelo corpo humano, o que pode vir a apresentar efeitos colaterais reduzidos aos pacientes em tratamento (KITAZATO; WANG; KOBAYASHI, 2007).

Dentre as moléculas naturais existe a classe dos compostos flavonoides, estes são metabólitos secundários amplamente distribuídos no reino vegetal. Atuam como agentes antioxidantes, protegendo o tecido vegetal e contribuindo para sua pigmentação (DI CARLO et al., 1999). São encontrados em uma variedade de alimentos e têm ganhado destaque por desempenhar atividades como anti-inflamatória, antialérgica, antioxidante, anticancerígena dentre outras (MIDDLETON; KANDASWAMI; THEOHARIDES, 2000). Alguns flavonoides naturais demonstraram atividade antiviral como o galato-3-epigallocatequina (EGCG) (CALLAND et al., 2012a; CIESEK et al., 2011; ZHAO et al., 2017), a quecertina (GONZALEZ et al., 2009), a narigerina (NAHMIAS et al., 2008), a curcumina ((KUTLUAY et al., 2008) e a luteolina/apigenina (CALLAND et al., 2012b; LIU et al., 2012). O flavonoide curcumina teve suas propriedades antivirais descritas para o ZIKV e CHIKV *in vitro* inibindo a etapa de replicação viral. Adicionalmente dois análogos sintéticos da curcumina: demethoxycurcumina e bisdemethoxycurcumina, apresentaram mecanismo de ação e

toxicidade semelhante à curcumina natural (MOUNCE et al., 2017). O EGCG é outro flavonoide natural, isolado do extrato do chá verde com propriedades antivirais já descritas na entrada do HCV na célula hospedeira (CALLAND et al., 2012a) e que também mostrou ser eficiente na infecção do ZIKV impedindo a entrada do vírus na célula (CARNEIRO et al., 2016).

Os alcaloides são outra classe de moléculas naturais, obtidos também a partir do metabolismo secundário de planta, sendo moléculas heterocíclicas nitrogenadas, fisiologicamente ativas em mamíferos, incluindo humanos (CHOWAŃSKI et al., 2016). O alcaloide Piridoacridina e seus análogos têm mostrado múltiplas atividades biológicas, destacando ação anticancerígena antiparasitária e antimicrobiana (PLODEK; BRACHER, 2016). Em estudo realizado com os alcaloides Matrine e Oxymatrine presentes em raízes de plantas *Sophora*, a atividade antiviral destes compostos foi demonstrada no tratamento de pacientes portadores de HCV (AZZAM et al., 2007). O alcaloide Miriberina A, isolado de *Myrioneuron faberi*, também demonstrou inibir o ciclo replicativo do HCV (HUANG et al., 2007). As acridonas são uma subclasse de alcaloides que vêm ganhando destaque na literatura por atuar inibindo o ciclo replicativo de diferentes tipos de vírus como: Vírus da Herpes tipo 1 (HSV-1) (AKANITAPICHAT; LOWDEN; BASTOW, 2000), Vírus da imunodeficiência humana (HIV-1) (FUJIWARA et al., 1999), vírus da febre hemorrágica (HFV) (SEPÚLVEDA et al., 2013), Vírus da Dengue (MAZZUCCO et al., 2015) e o vírus da Hepatite C (CAMPOS et al., 2017; STANKIEWICZ-DROGON et al., 2008; STANKIEWICZ-DROGOŃ et al., 2010).

Além disso, a literatura tem mostrado a atuação de derivados de ácidos naturais e sintéticos como antivirais para diferentes tipos de vírus, por exemplo, o ácido rosmarínico que reduziu a mortalidade de cobaias infectadas com o vírus da Encefalite Japonesa (SWARUP et al., 2007). Os ácidos 1-metoxioxalil-3,5-dicaffeoilquinico e 3,5-dicofiroilquinico e seu análogo sintético L-chicórfico atuam como inibidores da integrase do HIV *in vitro* (ROBINSON et al., 1996). Merino-Ramos e colaboradores descreveram as propriedades antivirais do ácido Nordihydroguaiarético e seu derivado Tetra-O-Metil Nordihydroguaiarético no ciclo replicativo do WNV e ZIKV (MERINO-RAMOS et al., 2017). Em estudos anteriores, o ácido Nordihydroguaiarético também mostrou atividade na replicação viral do DENV e HCV (SOTO-ACOSTA et al., 2014; SYED; SIDDIQUI, 2011).

No atual contexto, o desenvolvimento de novos fármacos antivirais pela síntese de moléculas com base em estruturas naturais ativas se apresenta de forma vantajosa para a produção em escala comercial. As moléculas sintéticas podem ser manipuladas para

potencializar suas atividades terapêuticas de acordo com cada necessidade, o que as tornam inéditas e passíveis de patente. Além disso, tais moléculas têm apresentado várias vantagens químicas, baixa toxicidade, na maioria dos casos, o que são condições favoráveis para o desenvolvimento de uma terapêutica futura.

## 2. Objetivos

O presente trabalho teve como objetivo avaliar o potencial antiviral de compostos naturais e seus derivados sintéticos no ciclo replicativo do ZIKV *in vitro*.

### 2.1. Objetivos específicos

- Avaliar a citotoxicidade de compostos naturais e sintéticos na linhagem de células Vero, por meio de ensaios de viabilidade celular (MTT) e estabelecer as concentrações viáveis para tratamento das células;
- Produzir ZIKV *in vitro* para infecção de células Vero, na presença ou na ausência dos compostos em concentrações específicas, e avaliar a atividade antiviral por leitura de luminescência;
- Determinar a concentração efetiva de inibição em 50% ( $EC_{50}$ ), concentração citotóxica em 50% ( $CC_{50}$ ) e Índice de Seletividade ( $IS = CC_{50}/EC_{50}$ ) de cada composto ativo, avaliando assim os valores ótimos de concentração para o tratamento celular e o potencial antiviral de cada composto;
- Analisar as etapas do ciclo replicativo do ZIKV inibidas pelo tratamento com os compostos ativos.

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***Capítulo II: A Review of the Ongoing Research on Anti-  
ZIKA Treatments***

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## **A review of the ongoing research on anti-Zika treatments**

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## ABSTRACT

The Zika fever is an arboviral disease resulting from the infection with ZIKA virus (ZIKV). The virus is transmitted to humans by the bite of *Aedes* mosquitos, mainly *Aedes aegypti* and *Aedes albopictus*. ZIKV has been detected for decades in African and Asian regions and since 2007 has spread to other continents, among them, infections are most reported in Americas. This can be explained by the the presence of vectors in highly populated and tropical regions where people are susceptible to contamination. The ZIKV has been considered by the World Health Organization as a serious public health problem due to the increasing number of cases of congenital malformation and neurological disorders related to its infection, such as microcephaly, syndrome Guillain-Barré, meningo- encephalitis and myelitis. There is no vaccine or specific antiviral against ZIKV. Infection is best prevented by avoiding mosquito bite and treatment of infected patients is palliative. In this context, the search for efficient antivirals is necessary but remains challenging. Here, we aim to review the molecules that have been described to interfere the ZIKV life cycle and discuss their potential use in ZIKV therapy.

**Keywords:** Zika fever, ZIKV, treatment, antiviral, drug screening

## 1. Introduction

ZIKV (strain MR 766) was first isolated (DICK, 1952) from the serum sample of a Rhesus monkey during a research on Yellow fever virus (YFV) in the Zika forest, Uganda 1947. In 1948, the virus was isolated from a pool of *Aedes Africanus* (*Stegomyia*) mosquitoes in the same forest (DICK; KITCHEN; HADDOW, 1952; MARANO et al., 2015). The first cases of human infection were reported in 1950 in Africa and later in Asia, and remained restricted to these regions until 2007 (AUBRY et al., 2016; SIMPSON, 1964b). Since then, ZIKV has spread geographically with reports of infection in the North Pacific [6,8], French Polynesia and South Pacific (CAO-LORMEAU, 2014). The first records in the Americas dated from 2015, when ZIKV spread across the Pacific Ocean to invade Brazil, Suriname, and Columbia (CHAN et al., 2016). Since 2015, eighty-four countries reported new introduction or re-introduction of ZIKV and although prevention and control measures are being taken the risk of infection still a reality, mainly in regions with large mosquito vector presence (WHO, 2017)

ZIKV is mainly transmitted by the bite of female mosquito of *Aedes aegypti* and *Aedes albopictus*. The latter is prevalent in the Asia Southeast and due to its ability to adapt to different environments it has have been found in the Americas, Pacific Island, Australia and Africa. *Aedes albopictus* is a potential vector for more than 20 arboviruses including important members of the Flaviviridae and Togaviridae families such as Dengue, and Chikungunya, respectively (PAUPY et al., 2009). Both species can co-exist in the same regions although, previous researches demonstrated the superiority of *A. Albopictus*, this specie is considered the second main vector of DENV and CHIKV (PAUPY et al., 2009; WONG et al., 2013). Indeed the transmission by others species of the *Aedes* genus should be considered including *Aedes africanus*, *Aedes luteocephalus*, *Aedes vitattus*, *Aedes furcifer*, *Aedes hensilii* and *Aedes apicoargenteus* (CALVEZ et al., 2016). The successful virus transmission by these species is related to their high capacity of proliferation in tropical and subtropical regions [14,16]. Although wild primates are the main non humans reservoirs, anti-ZIKV antibodies have been also identified in rodents and domestic animals (DARWISH et al., 1983; VOROU, 2016). Additionally, others transmission routes have been confirmed as blood transfusion (MUSSO et al., 2014), sexual (MCCARTHY, 2016), perinatal and transplacental transmissions (CHAN et al., 2016; VENTURA et al., 2016).

Zika fever is considered a mild disease with symptoms as headache, fever, malaise and joint pain (PERREIRA et al., 2013). However, since reemerged, ZIKV has been associated

with the increasing number of microcephaly cases, Guillain-barré syndrome, meningo-encephalitis and myelitis in highly affected areas like the Brazilian North Eastern State (PETERSEN et al., 2016b; SAMARASEKERA; TRIUNFOL, 2016).

## **2. The ZIKA virus**

ZIKV belongs to the Flaviviridae family and genus Flavivirus, being phylogenetically related to dengue virus (DENV), West Nile virus (WNV) and Yellow fever virus (YFV) (DUFFY et al., 2009). Recently, the structural similarity among the virions of ZIKV and other Flavivirus was demonstrated by cryoelectron microscopy (cryo-EM) (SIROHI et al., 2016). The viral nucleocapsid of Flavivirus is involved by a bilayer lipid membrane ranging from 25 to 30 nm diameter derivate from host cells, in which viral glycoproteins are inserted.

As others flavivirus, the virus entry into the cell by endocytosis derived by the interaction of the envelope proteins and specific receptors on the host cell (CHAMBERS et al., 1990). The process of internalization is intermediated by clathrin protein in low pH environment. Viral genome is released into the host cytoplasm where will be translated. ZIKV genome is a positive-sense single-stranded RNA of approximately 11kb length that is uncoated immediately after virus entry into the host cells (BUATHONG et al., 2015). The viral genome has a single "open reading frame" (ORF) flanked by untranslatable regions 5' and 3' of 106 nucleotides and 429 nucleotides, respectively. The polyprotein encoded by this ORF will be proteolytically cleaved into three structural proteins (capsid protein, pre membrane/membrane protein and envelope glycoprotein) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (HADDOW et al., 2012; LINDENBACH; RICE, 2003; VAN HEMERT; BERKHOUT, 2016). The NS3 and NS5 proteins are essential for viral replication, processing high catalytic activity (BOLLATI et al., 2010). After the viral genome translation, the new viral particles are released from the cells restarting the infection process (LINDENBACH; RICE, 2003).

Phylogenetic analysis of the ZIKV genome indicates the existence of three lineages: West Africa, Asian/American (ADIGA, 2016; SHAPSHAK et al., 2015) and Brazilian ZIKV (CUGOLA et al., 2016). Further analyses of Asiatic and African ZIKV strains isolated from infected mosquitoes, monkeys and humans showed significant amino acids variations throughout the sequence of viral polyprotein (WANG et al., 2016). The same study associated the human strains isolated during the recent outbreaks with the viral strain P6-740 (from

Asian mosquitoes, 1966) and demonstrated that all strains have a common ancestor. However, all the recent strains presented a minimum of 400 amino acid mutations when compared with P6-740 which can interfere with the viral replicative efficiency, fitness and transmissibility (WANG et al., 2016).

### 3. Therapeutical potential of anti-ZIKV molecules

Currently, there is no approved vaccine or antiviral against ZIKV infection. The main strategy for controlling ZIKV is to prevent breeding of mosquitoes (WHO., 2016). Treatment is palliative and includes rest and fluids intake. Acetaminophen or paracetamol are used to alleviate headache, fever and myalgia. The use of the aspirin or others salicylates is not recommended in childhood to avoid the risk of Reye's syndrome. Others non-steroidal anti-inflammatory drugs (NSAIDS) should be avoided until the certainty of dengue no-infection (ATIF et al., 2016; CDC, 2016). Here we aim to review the molecules that have been described to interfere in the ZIKV replication life cycle (**figure 1; Table 1**).

In the search for effective antivirals, research centers have screened compounds which previously demonstrated to possess antiviral activity against other pathogens for their therapeutical potential against ZIKV infection (XU et al., 2016). As an example, nucleoside analogues showed to inhibit the viral replication of the herpes simplex virus 1 and 2 (HSV-1 and HSV-2) (DE CLERCQ, 2000), cytomegalovirus (CMV) (BEADLE et al., 2006), hepatitis B virus (HBV) (MURATA et al., 2016) human immunodeficiency virus (HIV) [42,45] and hepatitis C virus (CARROLL et al., 2003).

Eyer and coworkers investigated the anti-ZIKV efficacy of a panel of 29 nucleosides analogues by using an infected culture system. Vero cells were infected with ZIKV at a multiplicity of infection (MOI) of 0,1 in the presence or absence of compounds ranging 0-100µM. Five days post-infection (d.p.i.), the cell viability was determined by cytotoxicity assay and the viral titers were determined by plaque assay. The nucleoside analogues 7-desaza-2'-C -methyladenosine (7-desaza-2'-CMA), 2'-C-methyladenosine (2'-CMA), 2'-C -methylcytidine (2' CMC), 2'-C -methylguanosine (2'-CMG) and 2'-C -methyluridine (2'-CMU) reduced cytopathic effect caused by ZIKV infection, demonstrating the anti-ZIKV potential of these compounds (EYER et al., 2016).

Another research group screened 6.000 pharmacologically active compounds against ZIKV infection measuring the caspase-3 activity and cell viability of treated human neural

cells. Emricasan, also known as IDN-6556 or PF-03491390, is an inhibitor of the pan-caspase pathway that presented a protective effect against ZIKV-induced death of cells in glioblastoma SNB-19 cells. This effect was observed when cells were previously infected by ZIKV strains MR766 (1947 Ugandan strain), FSS13025 (2010 Cambodian strain), PRVAB659 (2015 Puerto Rican strain) and treated with 0.13-0.9 $\mu$ M (IC<sub>50</sub>) concentration of the compound. Also, emricasan reduced the number of active (cleaved) caspase-3-expressing forebrain-specific hNPCs infected by FSS13025 strain in both monolayer and 3D organoid cultures. Although cell proliferation was not affected by the emricasan treatment of ZIKV-exposed brain organoids, the data showed that ZIKV antigen persisted in both cultures. Therefore, the authors concluded that emricasan presented neuroprotective activity for hNPCs but did not inhibited ZIKV- replication.

The niclosamide, an anti helminthic approved by the Food Drug Administration (FDA), and the cyclin-dependent kinase inhibitor PHA-690509 demonstrated to significantly inhibited the ZIKV infection in vitro (XU et al., 2016). The SNB-19 cells were treated with these compounds at increasing concentrations one hour prior inoculation with FSS-13025, PRVABC59 or MR766 ZIKV strains (MOI=1). Twenty four hours post inoculation, cells were harvested and the protein NS1 expression evaluated by western blotting. Results demonstrated that these compounds significantly inhibited the viral infection of the three strains of ZIKV. Additionally, the effects of these compounds on early and post entry stages were investigated. The results showed that nicosamide and PHA-690509 inhibited the ZIKV infection post entry, probably blocking the viral replication. Additionally, the combined therapy with emricasan and PHA-690509 presented an additional inhibition effect on caspase activity, showing the synergic effect of these compounds (XU et al., 2016).

Similar analyses were performed by Barrows and coworkers who investigated the anti-ZIKV activity of a library of 774 drugs approved by the Food and Drug Administration (FDA). Huh-7.0 cells were pre-treated with compounds for 1 hour, inoculated with ZIKV MEX-I-7/15 (MOI=0.4), fixed twenty four h.p.i. and labeled with immunofluorescent antibodies against the viral envelope proteins. The majority of drugs showed no significant effect, however thirty compounds demonstrated to be effective decreasing virus infectivity. Eight compounds (ivermectin, daptomycin, mycophenolic acid (MPA), sertraline, pyrimethamine, cyclosporine A, azathioprine and mefloquine) were selected for tests in human cell lines derived from cervical cancer (HeLa), placental (JEG3), and neural tissues (hNSC). In Hela cells all the eight compounds reduced infectivity rates and four of these compounds presented anti-ZIKV activity in JEG3 cells. For flow cytometry analyses, hNSC

cells were maintained in the presence of drugs and ZIKV (MOI=3) for ninety six hours. The results showed that MPA, cyclosporine A and ivermectin significantly inhibited ZIKV MEX-I-7 infection. In human amnion epithelial cells (HAECs), ivermectin, daptomycin, sertraline, and mefloquine inhibited viral infection at the maximum concentration of 16 $\mu$ M. Ivermectin, sertraline, and mefloquine demonstrated higher antiviral effect, however, significant reduce of cell viability was observed. Ivermectin (EC<sub>50</sub>=1 $\mu$ M) and MPA (EC<sub>50</sub>=10 $\mu$ M) demonstrated the most potent action on ZIKV-replication (BARROWS et al., 2016).

Byler and co-workers generated structural models of ZIKV protein (NS3 helicases, NS5 methyltransferase, RNA-dependent RNA polymerases and NS2B-NS3 proteases) by homology modeling techniques to analyze in silico interactions between these viral proteins and molecules derived from plants. Two thousand and two hundred sixty three compounds isolated from herbs including alkaloids, polyphenolic and terpenoids were tested. For ZIKV NS2B-NS3, only the bis-indole alkaloids flinderole A and flinderole B were in agreement with the Lipinski's rules (experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings) (LIPINSKI, C.A.; LOMBARDO, F.; DOMINY, B.W.; FEENEY, 1997). For ZIKV NS3 helicase, the isoquinoline alkaloid cassiarin D, 3-Omethyl diplacone, exiguaflavanone A, sesqui terpenoid lactucopicrin, aurone kanzonol V, chalcones angusticornin B, balsacone B, and kanzonol Y, and the lignans hibalactone and kaerophyllin showed high binding activity with the ATP site. The polyphenolic compounds cimiphenol, cimiracemate B, and rose marinic acid presented strong docking energies with NS5 methyl transferase while Zika virus NS5 RNA-dependent RNA polymerase presented docking ligand properties with polyphenolic compound 4,7-digalloylcatechin, prenylated chalcone, 2,4,4 -trihydroxy-3,3 -diprenylchalcone, bis-indole alkaloid flinderole and lignan di-O-demethyl isoguaiacin. All tested polyphenolic compounds showed to be the greatest ligands, demonstrating high affinity to several protein sites due to the higher potential to target viral proteins. The lowest binding energy compounds were monoterpenoids and triterpenoids. Other phytochemicals as balsacone B, kanzonol V, cinnamoyl chinaxanthol, cimiphenol, and rosemarinic acid isolated from *Populus balsamifera*, *Glycyrrhiza glabra*, *Echinacea root*, *Cimicifuga racemosa* and *Rosmarinus officinalis*, respectively showed great docking properties concerning the viral proteins that highlights the potential antiviral of the molecules available at the nature (BYLER; OGUNGBE; SETZER, 2016).

The anti-ZIKV potential of the natural compound epigallocatechin gallate (EGCG) was demonstrated in a research conducted by Carneiro and coworkers. They investigated the

virucidal potential of the EGCG compound against ZIKV by using Vero cells infected with ZIKV BR or MR766 strains, they demonstrated that EGCG at 200 and 25 $\mu$ M reduced a minimum of 90% and 85% of foci numbers of ZIKV BR and MR766, respectively. Their data showed that EGCG acts strongly inhibiting the ZIKV entry in to Vero cells (CARNEIRO et al., 2016).

Chloroquine is another compound approved by the FDA to treat malaria and can be administrated to pregnant woman. The anti-ZIKV properties of chloroquine were tested in Vero cells, Human brain microvascular endothelial cells (hBMEC) and human neural stem cells (hNSCs) infected with ZIKV MR766 for 5 days and treated with the compound at concentrations ranging from 6.25 to 50 $\mu$ M. The viral envelope E protein was labeled with 4G2 antibody, specific to flavivirus, to measure the viral infectivity. The data obtained showed decreasing virus infection in a dose-dependent manner. Results were corroborated by immunofluorescence and flow cytometry analyses which demonstrated that chloroquine at 50 $\mu$ M reduced up to 65% and 50% ZIKV infection in Vero cells and hBMEC, respectively, with no effect on cell viability. In hNSCs, chloroquine at 50 $\mu$ M reduced 57% of the ZIKV infection after 4 days of treatment with 70% of cell viability. Chloroquine at 25 $\mu$ M was also tested in Vero cells infected with the ZIKV BR isolate that showed to decrease up to 16-folds of the viral RNA levels in the supernatant. Moreover, chloroquine was tested for distinct stages of ZIKV infection, demonstrating to block the early stages of the replication cycle (DELVECCHIO et al., 2016).

Kuivanen and coworkers investigated the antiviral activity of the compounds SaliPhe, obatoclastax and gemcitabine in human telomerase reverse transcriptase-immortalized retinal pigment (RPE) cells infected with ZIKV FB-GWUH-2016 strain. They analyzed ZIKV-mediated cellular apoptosis, transcription, signaling and metabolism. Immunofluorescence assay showed that these compounds at non-cytotoxic concentrations inhibited the synthesis of viral protein 24 hours post infection. The reduction of RNA viral expression and production of infectious viral particles was also confirmed by RT-qPCR or plaque assay 48 h.p.i, respectively. Also, it was analyzed whether the compounds inhibited ZIKV-mediated cell death. Initially, short treatments were performed with each compound, starting at 0, 2, 4, 6 and 8 h.p.i. The infected cells were maintained with each compound for 2 hours and then replaced by media culture. A second experiment was performed by adding each compound at 0, 2, 4, 6, 8 and 10 h.p.i, but the media culture containing compound was not replaced. For both treatments, the cell viability was measured 48 h.p.i. The results demonstrated that the cellular viability was maintained when cells were treated up to 4 h.p.i., and gemcitabine

protected cells when added 10 h.p.i and media was not replaced (KUIVANEN et al., 2016). These compounds also inhibited the ZIKV-mediated activation of caspases 3, 7 and 8, responsible for cellular apoptosis. SaliPhe, obatoclax and gemcitabine were evaluated by their activity on two signaling phosphoprotein which are mainly affected by ZIKV infection, the transcription factor CREB and the cyclin-dependent kinase inhibitor 1B (p27). For a better understand of SaliPhe and obatoclax effects on the antiviral genes expressions, cells were treated with compounds at 1 $\mu$ M and infected with ZIKV at MOI=8,5. After 10 hours, the gene expression profiling was analyzed by RNA quantification. The antiviral genes expression was impaired by SaliPhe and obatoclax but not for gemcitabine. The research results showed that these compounds presented different mechanisms of action and therefore, combined therapy could be a great alternative against ZIKV infection (KUIVANEN et al., 2016).

#### **4. Concluding Remarks**

Since the ZIKV infection is currently a serious public health problem, many research centers have directed efforts in the search for a drug with potent anti-ZIKV activity. In this review we discussed some approaches that have been performed for this purpose, being the most drugs either pre-approved by FDA or mainly based on molecules previously documented to possess antiviral effect against other diseases, as Ebola, Giardíase, Malaria, Hepatitis C, Herpes, Hepatitis B and syndrome of the human immunodeficiency. Some molecules have been effective on the ZIKV infection virus in vitro by inhibiting the viral entry and/or replication, or by decreasing death induced by infection. However, further research is clearly needed to the development of effective and safe antiviral that could be used for the control of ZIKV infection with no significant side effects.

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**Table 1.** Compounds tested with potential anti-ZIKV known.

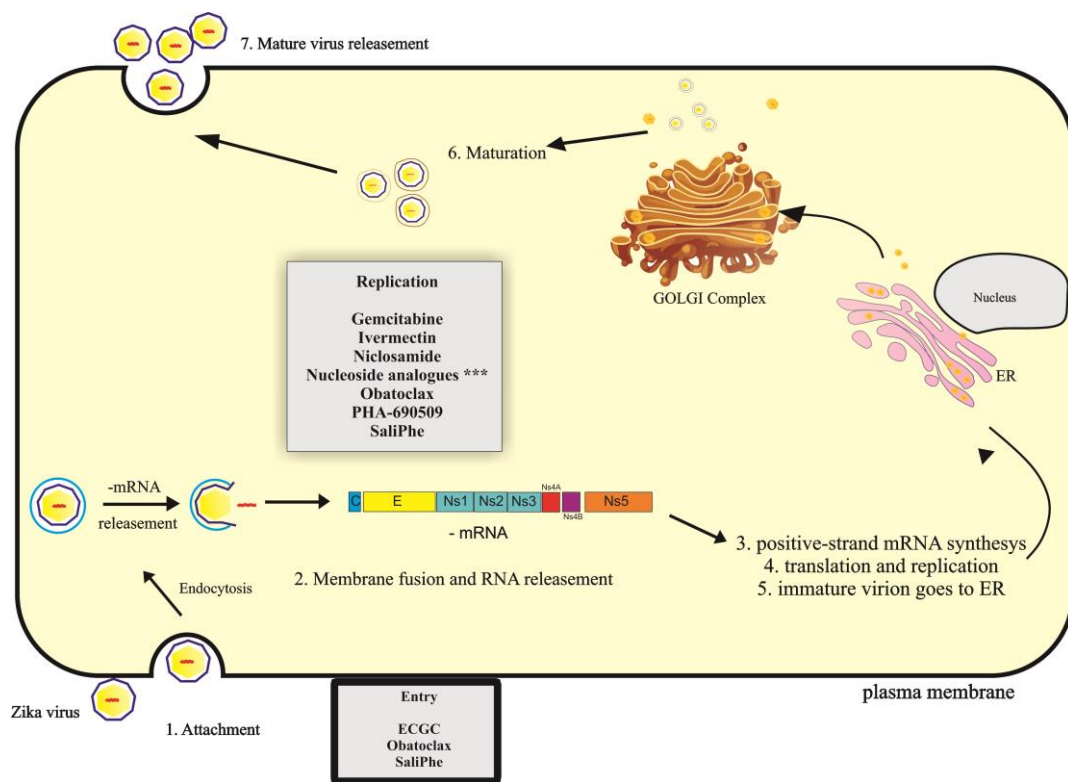
| <b>Compounds</b>                                                                                                            | <b>Validated Cells</b>                                 | <b>Mechanism of Action</b>                                         | <b>References</b>         |
|-----------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------|--------------------------------------------------------------------|---------------------------|
| <b>Emricasan</b>                                                                                                            | Glioblastoma SBN-19, hNPCS and human astrocytes cells. | Reduces cellular apoptosis by inhibition of caspase -3 activities. | (XU et al., 2016)         |
| <b>Nicosamide, PHA-690509</b>                                                                                               | Glioblastoma SBN-19, hNPCS and human astrocytes cells. | Inhibit viral replication                                          | (XU et al., 2016)         |
| <b>Chloroquine</b>                                                                                                          | Vero, hNSC and hBMEC cells                             | Inhibits early stages of replicative cycle                         | (DELVECCHIO et al., 2016) |
| <b>Ivermectin, daptomycin, Mycophenolic acid (MPA), Sertraline, Pyrimethamine, Cyclosporine A, Azathioprine, Mefloquine</b> | HuH 7 cells                                            | Inhibit the viral infection                                        | (BARROWS et al., 2016)    |

|                    |                                                                           |                                                                                                                                                                                                                 |                         |
|--------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|
| <b>SaliPhe</b>     | human telomerase reverse transcriptase-immortalized retinal pigment (RPE) | Inhibits endocytic uptake of ZIKV and protein viral synthesis. Prevents caspase 8, 3 and 7 activation.                                                                                                          | (KUIVANEN et al., 2016) |
| <b>Gemcitabine</b> | Human telomerase reverse transcriptase-immortalized retinal pigment (RPE) | Interferes with transcription of viral RNA. Inhibits viral protein synthesis. Prevents caspase 8, 3 and 7 activation, Changes the phosphorylation status of the CREB phosphoprotein affected by ZIKV infection. | (KUIVANEN et al., 2016) |
| <b>Obatoclax</b>   | Human telomerase reverse transcriptase-immortalized retinal pigment (RPE) | Inhibits endocytic uptake of ZIKV and viral protein synthesis Prevents caspase 8, 3 and 7 activation. Protects the phosphorylation status of p27 phosphoprotein .                                               | (KUIVANEN et al., 2016) |
| <b>Heparin</b>     | human neural progenitor cells (hNPCs)                                     | Inhibits caspase 3 activity mediated by ZIKV infection.                                                                                                                                                         | (GHEZZI et al., 2017)   |

|                                                                                                                                                                                                                                                                                                                                   |                                                                    |                                                                         |                         |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------|-------------------------------------------------------------------------|-------------------------|
| <b>Epigallocatequina galato</b>                                                                                                                                                                                                                                                                                                   | Vero cells                                                         | Inhibits the viral entry                                                | (CARNEIRO et al., 2016) |
| <b>Nucleoside analogues:</b><br><b>7-desaza-2'- C -</b><br><b>methyladenosine (7-desaza-</b><br><b>2'-CMA),</b><br><b>2'- C-methyladenosine (2'-</b><br><b>CMA),</b><br><b>2'- C -methylcytidine (2'</b><br><b>CMC),</b><br><b>2'- C -methylguanosine (2'-</b><br><b>CMG),</b><br><b>2'- C -methyluridine (2'-</b><br><b>CMU)</b> | Vero, Human neuroblastoma (UKF-NB-4 and porcine kidney (PS) cells. | Reduce cytopathic effect, cell death and inhibit the viral replication. | (EYER et al., 2016)     |

## Figure and Legend

**Figure 1.** Schematic representation of Zika virus replicative cycle. ZIKV replicative cycle and inhibitory drugs of viral entry and replication. Epigallocatequina galato; \*\*\*7-desaza-2'- C -methyladenosine (7-desaza-2'-CMA); 2'-C-methyladenosine (2'-CMA); 2'- C -methylcytidine (2' CMC); 2'-C-methylguanosine (2'-CMG) and 2'-C-methyluridine (2'-CMU).



***Capítulo III: Diarylamine Derived From Anthranilic acid  
Inhibits the ZIKV Replication***

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## Diarylamine derived from anthranilic acid inhibits the ZIKV replication

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## ABSTRACT

The *Flaviviridae* family phylogenetically groups arboviruses of great importance for the public health such as Zika virus (ZIKV), Dengue virus (DENV), Yellow Fever (YFV), West Nile virus (WNV) and Japanese Encephalitis virus (JEV). ZIKV is an emergent arbovirus in regions of the Pacific and America transmitted mainly by mosquitoes of the genus *Aedes*. Several researches have related the emerging outbreak of ZIKV infections with the increasing number of cases of neurological disorders such as microcephaly and Guillain-Barré syndrome. Although continuous efforts have been made to develop an effective therapy for ZIKV infection, there is no approved antiviral. Here we screened a library of 166 natural and synthetic compounds which include flavonoids, alkaloids and diarylamines derived from anthranilic acid (FAMs) to access their antiviral potential against ZIKV infection *in vitro*. First, we evaluated cell cytotoxicity of each compound and the maximum non-toxic concentration was used in antiviral assays. Vero cells were infected with ZIKV Brazilian strain which expresses the reporter gene Nanoluciferase (ZIKV-Nanoluc) in the presence or absence of the compounds, at different times of the viral cycle, and infectivity levels were measured 72 hours post infection. One synthetic diarylamine derived from anthranilic acid (FAM E3) demonstrated potential as an inhibitors of ZIKV replication step by reducing up to 86.82% of viral infectivity. No effect was observed in ZIKV viral entry. We analyzed the possible mechanisms of action of FAM E3 in the intercalation of dsRNA and in the interaction with viral SP6 viral polymerase. The results showed that FAM E3 does not act on these respective pathways. Molecular docking analysis suggests high affinity of FAM E3 with ZIKV NS3 helicase and NS2B-NS3 complex, suggesting these proteins as possible targets of the compound.

**Keywords:** *Flavivirus*, antivirals, natural compounds, synthetic derivatives diarylamines derived from anthranilic acid, Zika virus.

## 1. Introduction

Zika virus (ZIKV) is a mosquito - borne isolated for first time in 1947 from a *Rhesus* monkey in the Zika forest, Uganda (DICK; KITCHEN; HADDOW, 1952). ZIKV remained for long time endemic to the African and Asian regions until 2007, since then the virus has geographically spread to other continents (CAO-LORMEAU, 2014; CHAN et al., 2016; DUFFY et al., 2009; LANCIOTTI et al., 2008; SONG et al., 2017). In 2015, the ZIKV outbreak had a worldwide impact due to the large number of people infected and mainly related to the development of neurological disorders in newborns (microcephaly) and adults (Guillain Barre), being considered a serious public health problem (WHO, 2016).

ZIKV belongs to *Flaviviridae* family and genus *Flavivirus* (KUNO et al., 1998). As others *Flavivirus*, the viral genome is a positive single-stranded RNA with a single open reading region (ORF) which is translated in a polyprotein that will be cleaved by a combination of cellular and viral proteases into seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5) which are involved in maintaining the dynamic complex of replication and assembly of the viral particle. Three structural capsid (C), pre-membrane (prM) and the envelope (E) proteins are responsible for the formation of the viral particle (HOU et al., 2017; SIROHI et al., 2016; SIROHI; KUHN, 2017). NS3 and NS5 protein coordinate most of the catalytic activities necessary to maintain the machinery, among them the capping and the replication of the RNA. NS3 N-terminal region (RNA triphosphatase-NS3RTPase) coordinates cap-related activities while the NS3 C-terminal (RNA helicase-NS3Hel) is related to viral RNA synthesis, as processing of the viral polyprotein (BOLLATI et al., 2010). To activate the protease function, NS3 must be associated with the NS2B protein, which form the NS3-NS2B proteolytic complex (LEUNG et al., 2001). NS5 protein is mainly responsible for synthesizing the complementary strands of RNA from the existing mRNA. The NS5 domain is N-terminal methyl transferase (MTase) and C-terminal domain (RdRp) an RNA-dependent RNA polymerase (DAFFIS et al., 2010; ISSUR et al., 2009).

Similarly to other arboviruses as Dengue virus (DENV), Yellow Fever virus (YFV) and Chikungunya virus (CHIKV), ZIKV is mainly transmitted by *Aedes spp.* of mosquitoes (MUSSO et al., 2015; PAUPY et al., 2009; WONG et al., 2013). Nevertheless, other sources of infection acquisition have been reported as blood transfusion (MUSSO et al., 2015), sexual (MCCARTHY, 2016; NICASTRI et al., 2016), perinatal and transplacental transmissions (CHAN et al., 2016; VENTURA et al., 2016). There is no approved antiviral for ZIKV

infection. The treatment of infected individuals is palliative and consists of fluid intake and resting. Acetaminophen is prescribed for alleviate symptoms of fever and pain (ATIF et al., 2016).

Historically, the therapeutic properties of natural compounds have been described for the treatment of various diseases, including infections caused by viruses as Hepatitis C virus (HCV) (CALLAND et al., 2012b; JARDIM et al., 2015), Human Immunodeficiency Virus (HIV) (WAN; CHEN, 2014), CHIKV (DA SILVA-JÚNIOR et al., 2017), DENV and West Nile virus (WNV) (VÁZQUEZ-CALVO et al., 2017). Natural molecules have presented advantageous features as the high chemical diversity, low cost of production and efficient metabolization (KITAZATO; WANG; KOBAYASHI, 2007; LI; VEDERAS, 2009) However, compounds isolated from natural sources are not patentable and isolation process is time consuming (KITAZATO; WANG; KOBAYASHI, 2007; MARTINEZ et al., 2015). In this scenario, the chemical structure of the natural compounds has been used for the discovery and synthesis of new molecules that can be manipulated for better bioactivity and produced on a large scale (BUTLER, 2008; MARTINEZ et al., 2015; NEWMAN; CRAGG; SNADER, 2000).

In this work, we evaluated the antiviral activity of natural compounds isolated from Brazilian plants and theirs synthetic analogs on ZIKV infection *in vitro*. Our data showed that one synthetic diarylamines derived from anthranilic acid significantly inhibited the viral replication step of ZIKV *in vitro*.

## **2. Material and Methods**

### **2.1. Compounds**

A library of a hundred and sixty-six compounds is composed of natural compounds isolated from Brazilian plants and theirs synthesized analogues, and includes chalcones and derivatives, curcuminoids, acridones, and diarylamines derived from anthranilic acid were tested. The compounds were synthesized by the group of Dr Luis Octavio Regasini at the Institute of Bioscience, Language and Exact Science (IBILCE) of Sao Paulo State University (UNESP) in Sao Jose do Rio Preto, Brazil, using the protocol previously described (HERATH; MÜLLER; DIYABALANAGE, 2004) The compounds were dissolved in Dimethyl sulfoxide (DMSO) and stored at - 20°C. Dilutions of the compounds in complete

medium were made immediately prior to the experiments to reach a maximum final concentration of 0.1 % DMSO. For all the assays performed, control cells were treated with medium added with DMSO at the final concentration of 0.1 %. Obatoclax (OLX) at 0,125 $\mu$ M (AdooQ Bioscience) was used as a positive control for inhibition of ZIKV infectivity (SAIZ; MARTÍN-ACEBES, 2017b).

## 2.2. ZIKV construction

The ZIKV construction was kindly provided by collaborators of Institute of Technology, University of Tartu. The details of the design of the ZIKV-Nanoluciferase (Nanoluc) infectious clone (**Figure 1**) was described previously (MUTSO et al., 2017). Briefly, plasmids containing ZIKV-Nanoluc infectious cDNA were constructed from synthetic DNA fragments (Genscript, USA) that encode the polyprotein of the Brazilian ZIKV strain (BeH819015) and the sequences of 5' and 3' untranslated regions (UTRs) of ZIKV PE243/2015. To assemble the construct designated, pCCI-SP6-ZIKV full length cDNA was split into specific regions of the genome by recognition sites for Sall, SmaI, NaeI and BstBI. SP6 RNA polymerase promoter was inserted at the 5' region end of the ZIKV genome of A-SP6 fragment. A-SP6 was cloned into a high copy number pUC57Kan plasmid and transferred to a pCC1BAC vector designating the pCCI-ZIKV-SP6 construction (Epicenter, USA). For maintenance and propagation of the plasmid containing the pCCI-SP6-ZIKV, the *E. coli* Turbo strain (New England Biolabs) was used. The insertion of the reporter gene encoding Nanoluc was performed similarly to the protocol described previously for WNV (MCGEE et al., 2010).

Complete amplification of the viral genome of pCCI-SP6-ZIKV-Nanoluc was performed using a PCR reaction with Phusion High Fidelity (Thermo Fisher) enzyme and designed primers ZIKV-Forward (CG ATT AAG TTG GGT AAC GCC AGG GT) and ZIKV-Reverse (T AGA CCC ATG GAT TTC CCC ACA CC). PCR product with a complete viral genome sequence was purified with the DNA clean and concentration kit (Zymo Research) and *in vitro* transcription was performed using the RiboMAX™ Large-scale RNA Production Systems kit (Promega), as instructed by the manufacturers.

### 2.3. Cell Culture

Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 100U/mL penicillin (Gibco Life Technologies), 100mg/ml, streptomycin (Gibco Life Technologies), 1% non-essential amino acids (Gibco Life Technologies) and 10% fetal bovine serum (FBS; Hyclone) at 37 °C in a humidified 5% CO<sub>2</sub> incubator.

### 2.4. Cell viability assay

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 a density of  $1 \times 10^4$  per well and incubated overnight at 37°C in a humidified 5% CO<sub>2</sub> incubator. Drug-containing medium at different concentrations was added to the cell culture. After 72 h of incubation at 37 °C, the media was removed and a solution containing MTT at the final concentration of 1 mg/mL was added to each well, incubated for 30 min at 37 °C in a humidified 5% CO<sub>2</sub> incubator and replaced with 100µl of DMSO to solubilize the formazan crystals. Absorbance was measured by optical density (OD) of each well at 562 nm, using a spectrophotometer. Cell viability was calculated according to the equation  $(T/C) \times 100\%$ , where T and C represent the mean optical density of the treated group and control group, respectively. All experiments were performed in quadruplicates and repeated a minimum of three times. Compounds concentrations for further assays were selected considering 80% of cell viability. The cytotoxic concentration of 50% (CC<sub>50</sub>) was calculated using Prism (Graph Pad).

### 2.5. Virus assays

For virus assays  $8 \times 10^6$  Vero cells were electroporated with 10µg of RNA viral transcript in 4mm cuvettes, 450V, 600 µF, two pulses of 8 seconds. After electroporation, cells were suspended in culture media supplemented with 1% penicillin, 1% non-essential amino acids and 2% FBS and placed into 25cm<sup>2</sup> cell culture flask and monitored during 5 days. At the end of this time, the viral supernatant was collected and stored at -80 °C.

To determine the viral titer, Vero cells at the density of  $3 \times 10^5$  per well were seeded in a 6 wells plate for 24 h prior the infection, the cells were infected with ZIKV-Nanoluc at 10-fold serially diluted for 2 hour at 37°C. The inoculum was removed and the cells were washed with PBS to completely remove the virus and added of cell culture media supplemented with 1% penicillin, 1% non-essential amino acids, 2% FBS and 2% de carboxyl methyl cellulose (CMC). The infected cells were incubated for 5 days at a CO<sub>2</sub> incubator at 37°C. The media was removed and cells were fixed with 4% formaldehyde, stained with 0.5% violet crystal and the viral foci were counted.

## **2.6. Antiviral assays**

For antiviral assay, a density of  $1 \times 10^4$  Vero cells was seeded per well into 96-well plates 24 h prior the infection. The ZIKV-Nanoluc at a multiplicity of infection (MOI=0.1) and compounds were simultaneously added to cells. Samples were harvested in Renilla lysis buffer (Promega) at 72 hours post-infection (h.p.i.) and virus infectivity was quantified by measuring luciferase activity using the Renilla Luciferase Assay System (Promega). The effective concentration of 50% (EC<sub>50</sub>) was calculated using Prism (Graph Pad). The values of CC<sub>50</sub> and EC<sub>50</sub> were used to calculate the selectivity index (SI = CC<sub>50</sub>/EC<sub>50</sub>). DMSO and OLX were used as controls.

## **2.7. Dose-dependent assay**

To evaluate if antiviral effect was dose- dependent,  $1 \times 10^4$  Vero cells were previously distributed in 96 wells plates. 24 h after, a concentration ranging from 1µM to 10µM of the compound were added to the cells simultaneously with ZIKV-Nanoluc supernatant at MOI= 0.1. 72 h.p.i the media was completely removed. The cells was washed with PBS and harvested in Renilla lysis buffer and subjected to luminescence reading. Cell viability was analyzed concomitantly.

## **2.8. ZIKV entry e/or replication assay**

To access the effect of compounds on ZIKV entry to the host cells, supernatant containing ZIKV-Nanoluc (MOI=0.5) and compounds were simultaneously added to cells ( $1 \times 10^4$ ) and incubated for 2 h at 37°C. Cells were washed extensively with PBS to complete removal of virus and compounds, and were replaced with fresh media. Samples were harvested in Renilla lysis buffer (Promega) at 72 h post-infection (h.p.i.) and virus infectivity was quantified by measuring luciferase activity using the Renilla Luciferase Assay System (Promega).

Alternatively, cells were infected with viral supernatant containing ZIKV-Nanoluc (MOI=0.5) for 2 hours, the viral inoculum was completely removed by extensively washing to remove viral particles, and compounds were added. The inhibition of ZIKV replication was measured by quantifying Renilla luciferase activity 72 h.p.i as previously described. DMSO and OLX were used as controls.

## **2.9. Pre-treatment assay**

Vero cells at a density of  $1 \times 10^4$  cell per well were incubated with FAM E3 for 2 hour at 37 °C in a humidified 5% prior to infection. After incubation, cells were washed extensively and added of ZIKV-Nanoluc (MOI=0.5) for 2 h. Then, the inoculum was removed and the cells were washed to completely remove non-endocytosed virus and fresh media was added. After 72 hours the viral supernatant was removed and the cells were washed with PBS, lysed with Renilla lysis buffer and subjected to luminescence reading DMSO and OLX were used as controls.

## **2.10. dsRNA Intercalation assay**

To investigate whether the compound interacts with the dsRNA the protocol was performed as described by (KRAWCZYK et al., 2009). The dsRNA obtained was quantified and 15nM incubated with FAM E3 (3μM) at room temperature for 45 min and submitted to

agarose gel analysis. DMSO and Doxorubicin at 100 $\mu$ M (DOX) were used as negative and positive control, respectively.

### 2.11. Inhibition of SP6 polymerase transcription

Assuming that viral polymerases have similar topology and functions (GNIAZDOWSKI et al., 2005; STANKIEWICZ-DROGON et al., 2008), we evaluated the effect of the compound at *in vitro* transcription. To this end, complete amplification and purification of the viral genome of pCCI-SP6-ZIKV-Nanoluc was performed as described in **section 2.2**. SP6 RiboMAX <sup>TM</sup> Large-scale RNA Production Systems kit (Promega) reaction was set up as manufactures instruction using pCCI-SP6-ZIKV-Nanoluc as target with the addition of FAM E3 (3 $\mu$ M). After incubation the RNA was quantified, samples were resolved in a 1% agarose gel, and results were analyzed by densitometry. DMSO vehicle was used as control.

### 2.12. Immunofluorescence assay

For immunofluorescence assay, 2 x10<sup>5</sup> Vero cells were grown in 6-well plates 24 h prior infection. The ZIKV – Nanoluc at MOI=0.1 and compounds were simultaneously added to cells. 72 h.p.i the cells were fixed with 4% paraformaldehyde and washed with PBS and blocking buffer (BB) containing: 0.1% Triton X-100 (Vetec Labs, BR), 0.2% bovine albumin (BSA) and PBS for 30 min. Then, cells were incubated with primary polyclonal antibody rabbit anti-NS3 diluted in BB for 1 h. Anti-rabbit IgG, Alexa Fluor 594 conjugated, was used as secondary antibody. Cells were washed and labelled for nuclei and lipid droplets (LDs), with DAPI and BODYPI 493/503, respectively. Images were analyzed at EVOs cell imaging systems fluorescence microscopy (Thermo Fisher Scientific).

### 2.13. Molecular docking

The molecular docking between FAM E3 and ZIKV proteins was analyzed on Glide (FRIESNER et al., 2006; HALGREN et al., 2004) by extra precision (XP) mode. The

crystallographic structure NS2B-NS3 protease ; NS3 helicase; NS5 methyltransferase; NS5 polymerase and envelope protein was obtained from the protein database PDB ID: 5H4I; PDB ID: 5GJ; PDB ID: 5KQR; PDB ID: 5U04; PDB ID: 5LBV respectively and imported to Maestro interface (SCHRÖDINGER RELEASE, 2015a). The proteins were performed on Protein Preparation Wizard (MADHAVI SASTRY et al., 2013) and ligand on LigPrep tool (SCHRÖDINGER RELEASE, 2015b). The protonation state of viral protease and compound were calculated on software EPIK (GREENWOOD et al., 2010; SHELLEY et al., 2007) and the energy minimized using the OPLS-2005 force field (BANKS et al., 2005).

#### **2.14. Statistical analysis**

Individual experiments were performed in triplicate and all assays were performed a minimum of two times in order to confirm the reproducibility of the results. Differences between means of readings were compared using analysis of variance (one-way or two-way ANOVA) or Student *t* test using Graph Pad Prism 5.0 software (Graph Pad Software). *P* values of less than 0.001 (indicated by asterisks) were considered to be statistically significant.

### **3. Results**

#### **3.1. Inhibitory effect of natural compounds and their synthetic analogues on ZIKV infection**

To evaluate whether the panel of Brazilian natural compounds and their synthetic analogues could inhibit ZIKV infectivity, we performed a screening assay using a luciferase genomic replicon construct (ZIKV-Nanoluc). One hundred and sixty-six natural and synthetic compounds were evaluated, distributed by class: flavonoids, alkaloids and anthranilic acid derivatives. First, Vero cells were treated with 50, 10, 2 and 0.4µM of each compound and incubated for 72 h to access the cytotoxicity of the compounds. Then, Vero cells were added of viral supernatant containing ZIKV-Nanoluc at a MOI=0.1 in the presence or absence of each compound at non-toxic concentration. Infectivity levels were assessed 72 h.p.i. by

Renilla luciferase assay (**Figure 2**). Most compounds showed little or no effect on cell infectivity or cytotoxicity (**Supplementary material**). The chemical class of compounds which presented stronger antiviral activity was the synthetic diarylamines derived from anthranilic acid (FAMs). Of this class, eight compounds significantly inhibited ZIKV infection when compared to DMSO and OLX controls. The FAMs B3 10 $\mu$ M, B4 40 $\mu$ M, C1 50 $\mu$ M, D1 30 $\mu$ M, E3 10 $\mu$ M, E5 50  $\mu$ M and I5 50 $\mu$ M significantly inhibited 43.92%, 51.33%, 38.18%, 76.8%, 99.92%, 44.20% and 59.50%, respectively ( $p < 0.001$ ). However FAM B4, FAM D1, FAM E3 and FAM I5 compounds decreased cell viability at the established pre-concentrations (**Supplementary material**).

Of all compounds, FAM E3 showed better inhibition, so we investigated the highest non-toxic concentration this compound performing a dose dependent assay. We treated infected cell with ZIKV-Nanoluc supernatant at concentrations into scale of 1 $\mu$ M to 10 $\mu$ M and concomitantly we also evaluated the cell viability in these different concentrations. After 72 h.p.i the data of absorbance and luminescence levels was normalized and analyzed (**Figure 3A**). Our data showed that concentrations from 7 $\mu$ M to 10 $\mu$ M are able to inhibit a minimum of 99.67% of viral replication with viability up to 65%. For concentrations from 6 $\mu$ M to 3 $\mu$ M there was an inhibition up to 96% and a gradual increase in viability. At 2 $\mu$ M and 1 $\mu$ M FAM E3 presented inhibition of about 79.08% and 17.84%, respectively, with more than 92.29% and 100% of cell viability. For further analysis we established the concentration of 3 $\mu$ M that presented approximately 96.00% of inhibition and 88.00% of cell viability (**Figure 3B**), with  $EC_{50}$  of 2.59 $\mu$ M,  $CC_{50}$  of 8.0 $\mu$ M and SI of 3.08 (**Figure 4**).

### 3.2. The antiviral activity of FAM E3 is not related to the entry step of ZIKV in the host cell

To analyze in which stage of the ZIKV replicative cycle FAM E3 is interfering, virus and/or compounds were added to Vero cells at different times over the assay. To evaluate the activity of compound on virus entry, Vero cells were treated simultaneously with compounds at specific non-toxic concentrations and viral supernatant containing ZIKV-Nanoluc at a  $MOI=0.5$  for 2 hours at 37°C. Then, the inoculum was removed; the cells were washed with PBS extensively and added with fresh media for 72 h (**Figure 5A**). The results showed that there was no decrease in luminescence levels suggesting that FAM E3 does not affect ZIKV entry in to the host cell (**Figure 5B**).

We further investigated whether FAM E3 exerted some protective effect on the cells. Media contain FAM E3 at 3 $\mu$ M was added to the cells and incubated for two hours. Then the cells were washed and supernatant of ZIKV-Nanoluc at MOI=0.5 was added. After 72h cells were washed, lysed and the luminescence levels and viability was analyzed (**Figure 5C**). The data showed that the treatment of the cell with the compound inhibited 19.2% the ZIKV infection, non-significant value ( $P < 0.001$ ). OLX positive control inhibited 97.28% of viral infectivity (**Figure 5D**).

### 3.3. FAM E3 inhibits the replication ZIKV step in Vero cells

We also studied the effect of FAM E3 on replication step. For this, Vero cells were first incubated with ZIKV-Nanoluc at MOI=0.5 for 2 h, the inoculum was removed and washed extensively. Media containing compound was added and ZIKV infectivity was measured by luminescence levels 72 h.p.i (**Figure 5E**) and cell viability was analyzed concomitantly. The data showed that FAM E3 decreased viral activity up to 86.82% maintaining the cell viability at 89.12%. OLX control presented up to 97% of inhibition (**Figure 5F**). These data suggest that the antiviral potential of FAM E3 is related to its ability to inhibit some process within the viral replication stage.

### 3.4. FAM E3 antiviral mechanisms

To investigate the possible mechanisms of action of FAM E3, we first analyzed if this compound had the ability to intercalate in dsRNA. An *in vitro* synthesized dsRNA (15Nm) was incubated with FAM E3 compound (3 $\mu$ M), DMSO (0.1%) and DOX (100 $\mu$ M) negative and positive controls, respectively, for 45min and analyzed by agarose gel. Densitometry analysis showed that FAM E3 intercalated with dsRNA only 10.28% while the positive control DOX intercalated up to 93% (**Figure 6 A and B**).

In an attempt to elucidate whether FAM E3 interacted with the viral RdRp preventing the RNA synthesis, we performed an *in vitro* transcription assay based on SP6 RNA polymerase with FAM E3 being added as a component of the reaction. Results were analyzed in an agarose gel and the intensity of the bands was measured by densitometry. The data showed that FAM E3 does not exhibit high anti-enzymatic properties in SP6 RNA

polymerase, with about 7.9% of inhibition of RNA transcription. The difference was not significant when compared to DMSO control (**Figure 7A and B**). These data were corroborated by RNA quantification (**Figure 7C**).

Another possible mechanism of action investigated was whether the compound altered the cell lipid metabolism. To this end, we infected Vero cells with ZIKV supernatant and treated with FAM E3, DMSO and OLX. After the incubation period the cells were fixed and indirect immunofluorescence was performed labelling the nucleus, NS3 and lipid droplets, with specific dyes. Treatment with the compound decreased the lipid droplet levels when compared to DMSO control, and also reduced the NS3 viral protein expression. (**Figure 8**).

We also evaluated the molecular docking of FAM E3 with the viral proteins: NS2B-NS3 protease; NS3 helicase; NS5 methyltransferase; NS5 polymerase and envelope protein. For this analysis we used Glide program, in extra precision (XP) mode. At **Table 1** displays the docking scores of FAM E3 with each ZIKV protein available and respective binding sites. The best docking scores were observed for NS2B-NS3 protease (-5.66 kcal/mol) and NS3 helicase (-7.95, -7.29 kcal/mol) (**Table 1**). The prediction of interaction between FAM E3 and NS3 helicase occurred at binding sites related to RNA strand and ATP molecule (**Figure 9 A and B; Table 1**). The prediction of interaction with NS2B-NS3 protease occurred at the binding site described previously for fragment1H-benzo[d]imidazol-1-yl)methanol) (ZHANG et al., 2016) (**figure 10 A and B; Table 1**). FAM E3 docked with two NS5 methyltransferase domain: S-adenosylmethionine (SAM) (-3.86 kcal/mol), Guanosine-5'-triphosphate (GTP)/ cap binding site (-2.84 kcal/mol) and with the active site (-3.32 kcal/mol), a catalytic tetrad of Lys61–Asp146–Lys182–Glu218 (ZHAO et al., 2017). Concerning FAM E3 and NS5 polymerase there was interacting between active site (-3.35 kcal/mol) and RNA strand site (-3.59 kcal/mol). As E protein has a binding site located between the domain I and II, the interaction was computationally predicted, showed docking score of -4.44 kcal/mol.

#### 4. Discussion

ZIKV is an emerging arbovirus that has drawn attention since the current outbreak started in 2015. The World Health Organization (WHO) has declared that although prevention and control measures have been taken, the global risk for infection has not decreased (W.H.O,

2017). Despite the continuous efforts on the development of effective antiviral, there is no specific treatment for ZIKV infection.

In this study we evaluated the antiviral activity of natural compounds isolated from Brazilian plants and their synthetic analogs on ZIKV infection *in vitro*. Among the compounds screened, we selected a molecule, FAM E3, which demonstrated the highest inhibition of ZIKV infection. FAM E3 is an intermediary molecule obtained during the production of synthetic acridones. The chemical structure of FAMs is based on two aromatic rings and one hydrogen molecule linked to an amine group (LAYER. ROBERT W., 2000). We analyzed the antiviral potential of FAM E3 against ZIKV infection. FAM E3 at 3 $\mu$ M was able to inhibit approximately 96.38% of the viral infection while maintaining viability of 88.08 %. We further investigated the effects of FAM E3 on ZIKV replication and entry in the host cells. Our results demonstrated that this compound acts on the replication stage of ZIKV with 86.82% of inhibition. We also found that the antiviral activity of FAM E3 is not related to ZIKV entry into cells.

Others studies have shown that acid derivatives compounds have interfered in the replicative cycle of different virus families. Zanello and coworkers showed that N-sulfonylanthranilic acid derivatives inhibited the replication of DENV by inactivating the RNA-dependent RNA polymerase (RdRp), essential for the synthesis of the viral genome (ZANELLO et al., 2015). Also, Quinic acid derivatives inhibited the DENV-3 replication in a RepDV3-Huh 7.5 cells sub genomic system (MERINO-RAMOS et al., 2017). Additionally, anthranilic acid derivatives were reported to act as allosteric inhibitors binding to HCV NS5B polymerase inhibiting viral replication (NITTOLI et al., 2007; VRONTAKI et al., 2016).

In an attempt to elucidate the possible mechanisms of action of FAM E3 in the replicative cycle of ZIKV, we investigated the ability of this compound to interact with dsRNA. The ability of some compounds to inhibit the replication step by intercalating with dsRNA already has been described in the literature for another flaviviridae family member, the HCV (CAMPOS et al., 2017; STANKIEWICZ-DROGON et al., 2008), however according to our results FAM E3 intercalated minimally with dsRNA and values were not significant to relate to an effective antiviral mechanism.

Antiviral mechanisms of action already described for some compounds are associated with the ability of interaction with the structural and non-structural proteins of viruses (LEUNG et al., 2001; LIM et al., 2017; MACDONALD et al., 2003). In this context we tested whether FAM E3 prevented the replication step by acting on viral RdRp NS5. We analyzed the effect of FAM E3 in an *in vitro* transcription system based on SP6 RNA polymerase, since

the viral polymerases present structural and functional similarities (STANKIEWICZ-DROGON et al., 2008). No significant inhibition of viral transcription was observed by the addition of FAM E3, suggesting that the replication inhibition does not involve NS5 protein.

Some studies have shown that non-structural proteins of ZIKV have been targeted by viral inhibitors (ROY et al., 2017; SAHOO et al., 2016; STEPHEN; LIN, 2017; ZHANG et al., 2017). Here we performed a molecular docking analysis to verify how the compounds could interact with the viral proteins: NS2B-NS3 protease; NS3 helicase; NS5 methyltransferase; NS5 polymerase and envelope protein. We found that FAM E3 is predicted to have high affinity with NS2B-NS3 protease and NS3 helicase showed good docking scores. According to docking results FAM E3 could interact with the RNA strand site and ATP molecule site of NS3 helicase, with docking scores of -7.95 kcal/mol and -7.29 kcal/mol, respectively. The interaction with NS2B-NS3 complex occurs by fragment based sites (ZHANG et al., 2016) with docking score of -5.66 kcal/mol. Due to its importance in the replication stage the viral helicases have been the target of study for new antiviral drugs. *Flavivirus* helicases have an ATP site that provides the chemical energy required for unwinding viral RNA during replication process (TIAN et al., 2016). This is a potential mechanism by which FAM E3 inhibits viral replication. Our results are consistent with findings described by Chan et al (2017) that showed the antiviral activity exhibited by the Bromocriptine compound was also related to the ability of this compound to inhibit the complex ZIKV-NS2B-NS3 protein, by molecular docking and in vitro analysis (CHAN et al., 2017). Also, Novobiocin compound has anti-ZIKV activity reported to inhibit the replication step of ZIKV and authors suggest also by molecular docking that the inhibition is due to an interaction with NS2B-NS3 protease blocking the activation of this protein (YUAN et al., 2017b).

Knowing that host cell lipids provide a replication platform for various types of viruses, including *Flavivirus* several studies have shown that the antiviral potential of some compounds is related with the cellular lipid reduction and as consequence preventing viral morphogenesis. Under this perspective we decided to investigate whether FAM E3 alters the lipid metabolism of the cell inhibiting the viral replication step. We evaluated the effect of this compound by labeling the cellular LDs after infecting and treating the cell. The results showed that FAM E3 reduced the LDs in Vero cells and as expected decreased the NS3 protein due to its anti-ZIKV action. In a study conducted by Merino-Ramos et al. the hypolipidemic properties of the Nordihydroguaiaretic Acid (NDGA) and synthetic derivative (Tetra-O-Methyl Nordihydroguaiaretic Acid) against Zika Virus was shown. The mechanism

of action of NDGA was related to reduction of expression of two main molecules, both responsible for cell lipids synthesis. NDGA phosphorylates the energy regulatory molecules (AMPK) and increases their activity. At activated mode the AMPK inhibits two regulatory elements of sterol and protein synthesis (SREBP 1 and SREBP 2), both precursor proteins involved in lipid synthesis (MERINO-RAMOS et al., 2017). This same mechanism of action for NDGA has been shown for other *Flavivirus* such as HCV (SYED; SIDDIQUI, 2011) and DENV (SOTO-ACOSTA et al., 2014). This is a possible mechanism by which FAM E3 is decreasing lipid synthesis hindering the replication and morphogenesis of ZIKV. Curiously, Heaton et al showed that the NS3 DENV protein is responsible for recruitment of fatty acid synthase (FASN) (HEATON et al., 2010). Since our molecular docking assay suggests that FAM E3 has high affinity with NS3 helicase domain we can thus suppose that once the NS3 of ZIKV is inactivated, there is also a reduction of lipid recruitment which interferes in viral replication.

Also, the chemical scaffold of the FAMs has been observed in several anti-cancer drugs, demonstrating the medicinal properties of these molecules. The study conducted by Wang and coworkers has shown that altering the radicals of both aromatic rings A and B of FAMs can improve the therapeutic activity of these molecules against different types of cancers (WANG et al., 2012). In addition, etravirine and rilpivirine are two non-nucleoside analogues Inhibitors of HIV-1 approved by the Food and Drug Administration (FDA) that are also based on the structural nucleus of a diarylamine (DAS et al., 2004; MILLER et al., 2011; TIAN et al., 2013). Although diarylamine activity is not reported for viruses other than HIV, these data show that these molecules previously demonstrated to possess antiviral activity. It suggests that these molecules may interfere not only on ZIKV replication, but also against other viruses.

In summary, we evaluated the antiviral properties of natural compounds isolated from Brazilian plants and their synthesized analogues on ZIKV infection. Here we describe FAM E3 as a potent inhibitor of ZIKV replication. This is a preliminary description of the antiviral properties of FAM E3 compound and additional analyses will be required to better understand how this compound inhibits the ZIKV replication.

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## Figures and Legends

Figure 1. ZIKV- Nanoluc. Schematic representation of ZIKV-Nanoluc that continuously expresses the Nanoluciferase luminescence enzyme Adapted from MUTSO et al., 2017.



Figure 2. Compound screening scheme.

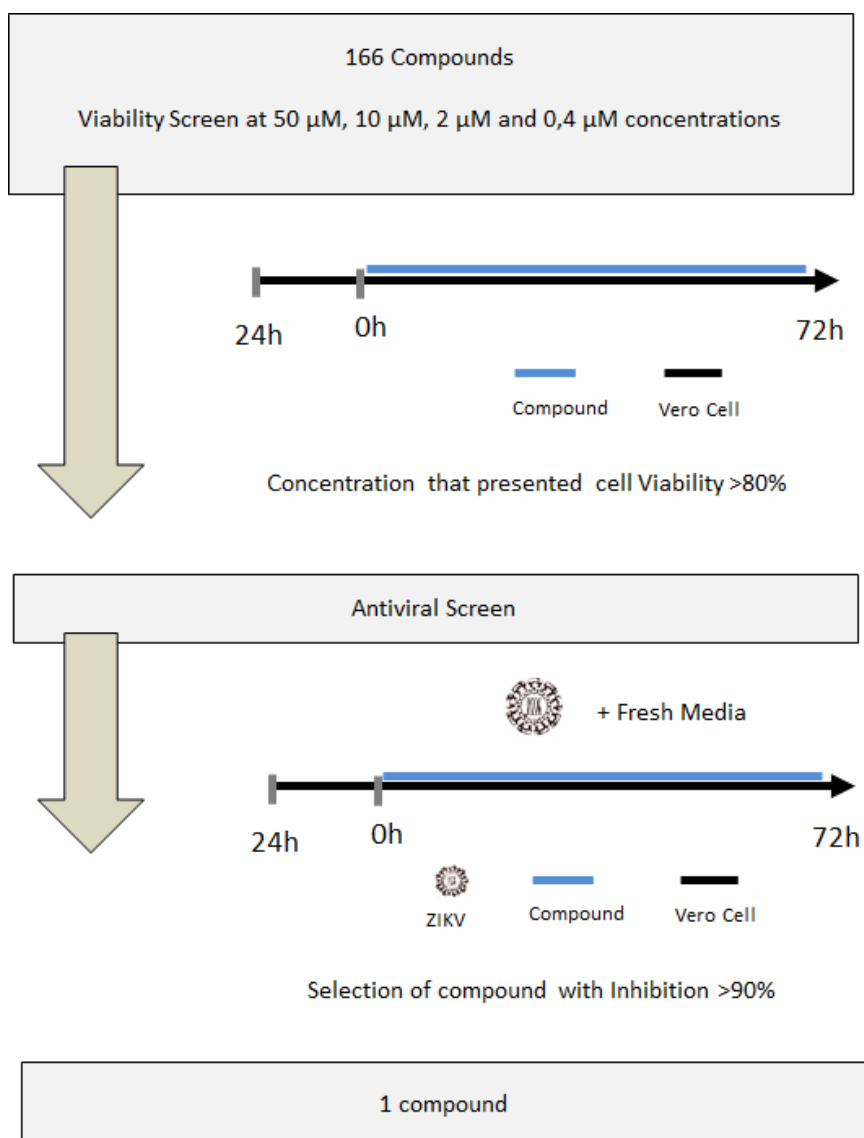


**Figure 3.** Dose dependent assay. Vero cell were infected with ZIKV Nanoluc at a MOI=0.1 and simultaneously treated with different concentration of FAM E3 (1µM to 10µM/ml) for 72 h (A). The cell viability and infectivity were evaluated by MTT and Renilla luciferase assays, respectively (B). Mean values of individual experiments were performed in quadruplicate and all assays were performed a minimum of three times in order to confirm the reproducibility of the results. P < 0.001 was considered significant.

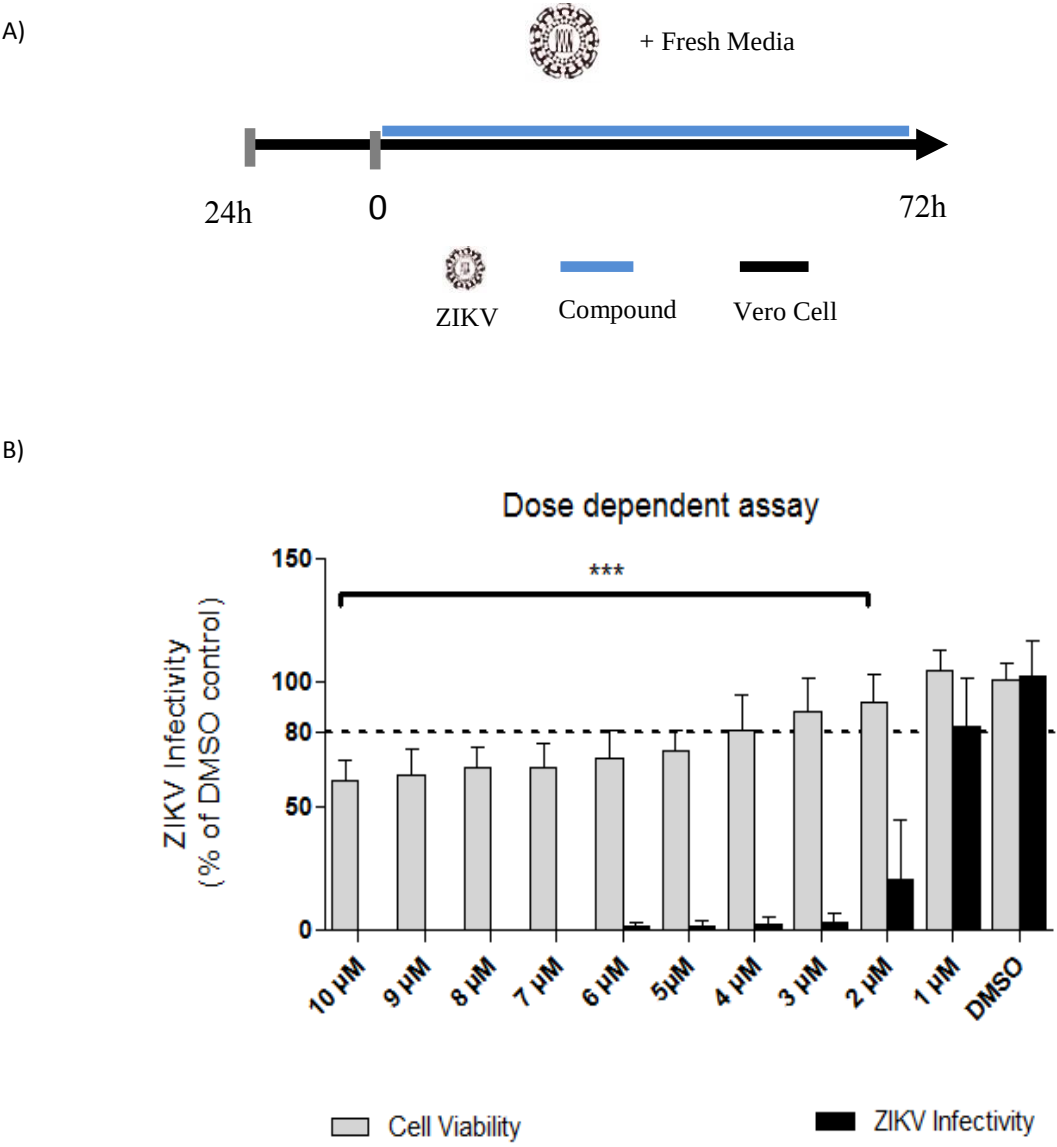


**Figure 4.** Effective and cytotoxic concentration of FAM E3. Vero cells were treated with increasing concentrations of FAM E3 from 200 at 0,10 $\mu$ M/ml. Replication efficiency was measured by luciferase assay (indicated by ■) and cellular viability measured using an MTT assay (indicated by ●). Mean values of two independent experiments each measured in quadruple including the standard deviation are shown.

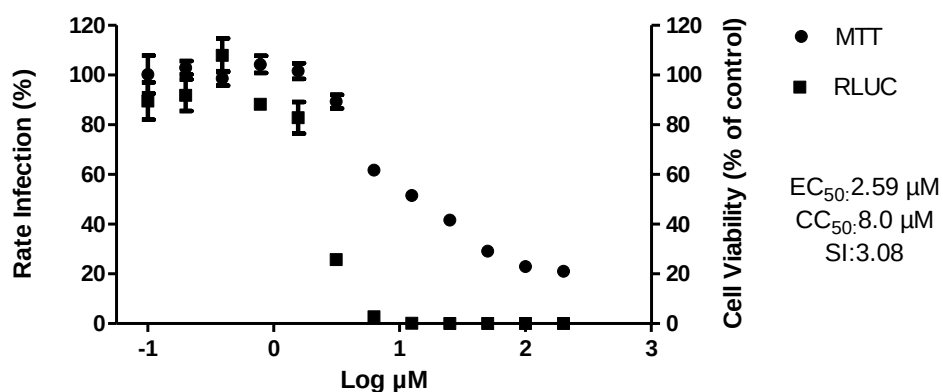


**Figure 5.** Effect of FAM E3 on ZIKV entry, pretreatment and replication. (A) Vero cells were treated with ZIKV-Nanoluc at a MOI=0.5 and compound simultaneously for 2 h; cells were washed to remove the virus and replaced with fresh media. (B) ZIKV infectivity was measured by Renilla Luciferase luminescence 72 h.p.i. (C) Vero cells were treated with compound 2h. Then, cells were washed extensively and infected with ZIKV-Nanoluc at a MOI=0.5 for 2h. The inoculum was removed and the cells washed extensively and replaced with fresh media. (D) ZIKV infectivity was measured by Renilla Luciferase luminescence 72 h.p.i. (E) Vero cells were infected with ZIKV-Nanoluc at a MOI=0.5 for 2 h. The virus was removed, cells were washed and added of fresh media containing compound. (F) ZIKV infectivity was measured by Renilla Luciferase luminescence 72 h.p.i. DMSO was used as negative control and Obatoclax as positive control for infectivity inhibition. Mean values of two independent experiments each measured in quadruple including the standard deviation are shown.  $P < 0.001$  was considered significant.

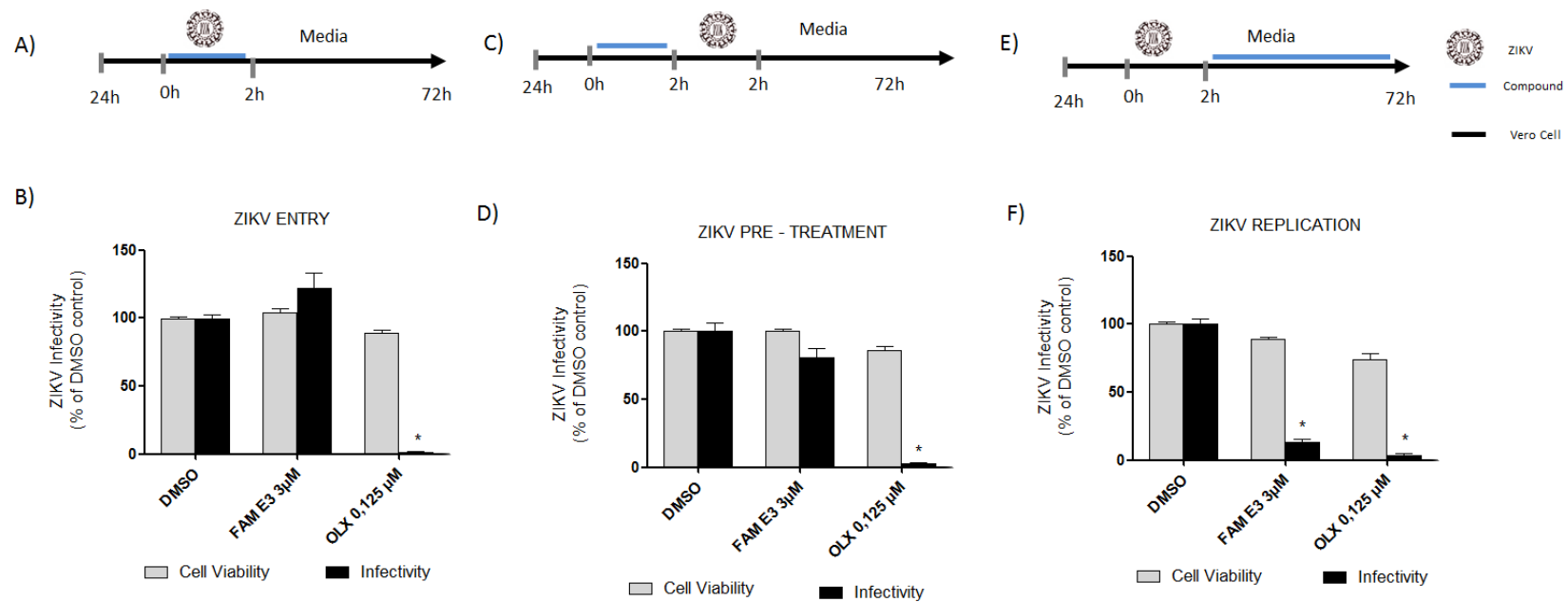


**Figure 6.** dsRNA intercalation. To elucidate the action mechanisms of FAM E3, 15nM of synthesized dsRNA was incubated with the compound and negative and intercalating positive controls, DMSO and Doxorubicin 100 $\mu$ M (DOX), respectively for 45 minutes at room temperature. The reaction was subjected to (A) electrophoresis 1% agarose gel and (B) densitometry analysis

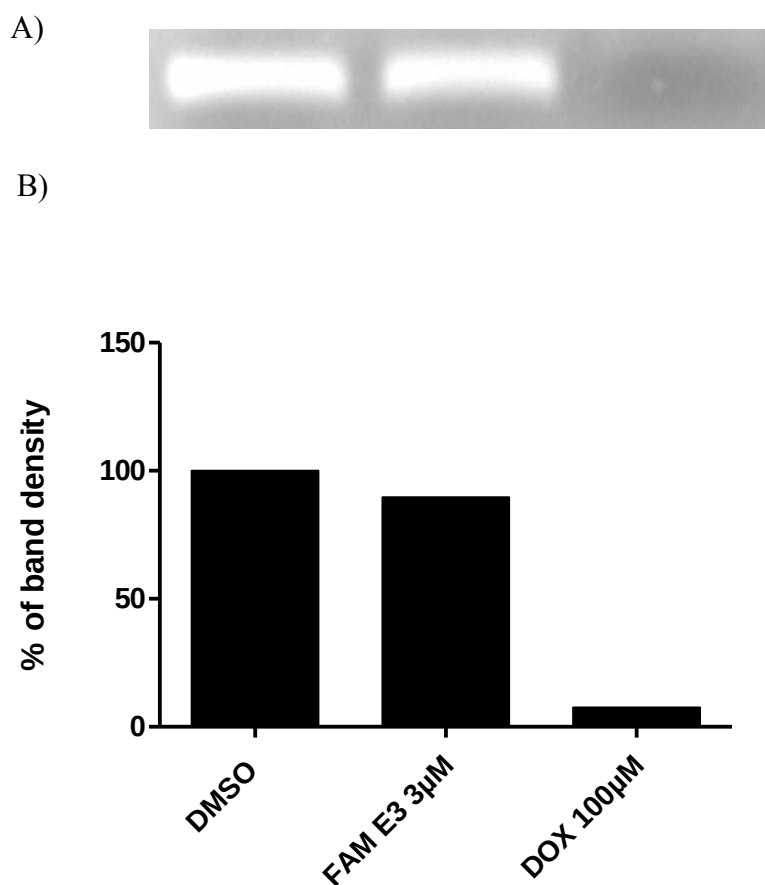


**Figure 7.** Effect of FAM E3 on in vitro transcription. FAME3 and 5µg pCCI-SP6-ZIKV amplicon purified was inserted at in vitro transcription reaction by according to SP6 RNA polymerase kit (Promega). Reaction was submitted to (A) agarose gel, (B) densitometry and (C) quantification by NanoDrop™ spectrophotometers analysis.

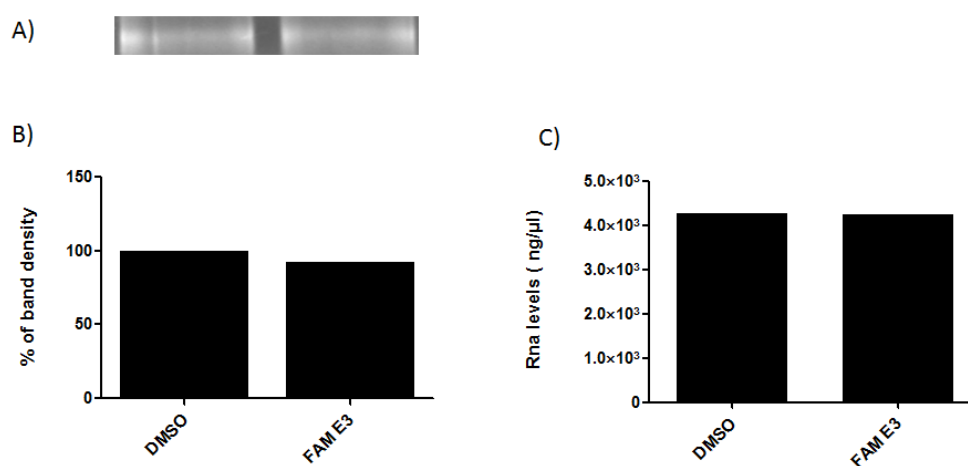


**Figure 8.** Vero cells were infected with ZIKV at MOI=0.5 and treated with FAM E3 (3 $\mu$ M), negative (DMSO) and positive (OLX) controls for 72h. Cells were fixed and nuclei, Lipid droplets (LDs) and viral protein ZIKV NS3 were labeled with DAPI (blue), ZIKV anti-NS3 primary antibody (red) and BODYPI 493/503 (green). Scale bar 100nm.

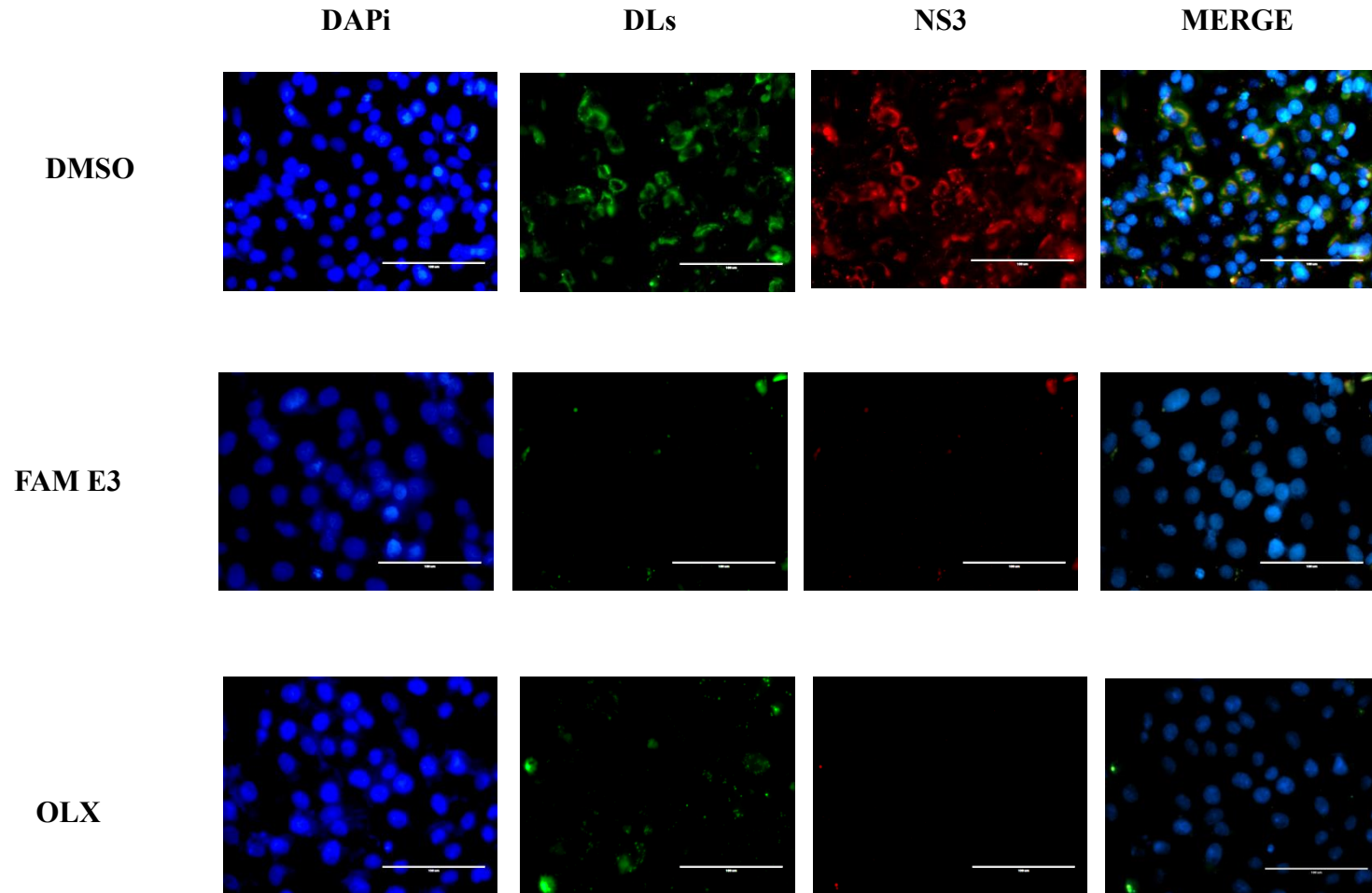


**Figure 9.** (A) Protein-ligand interactions of FAM E3 compound with NS2B-NS3 protease protein. (B) Predicted complex structure of NS2B-NS3 protease and FAM E3 compound.

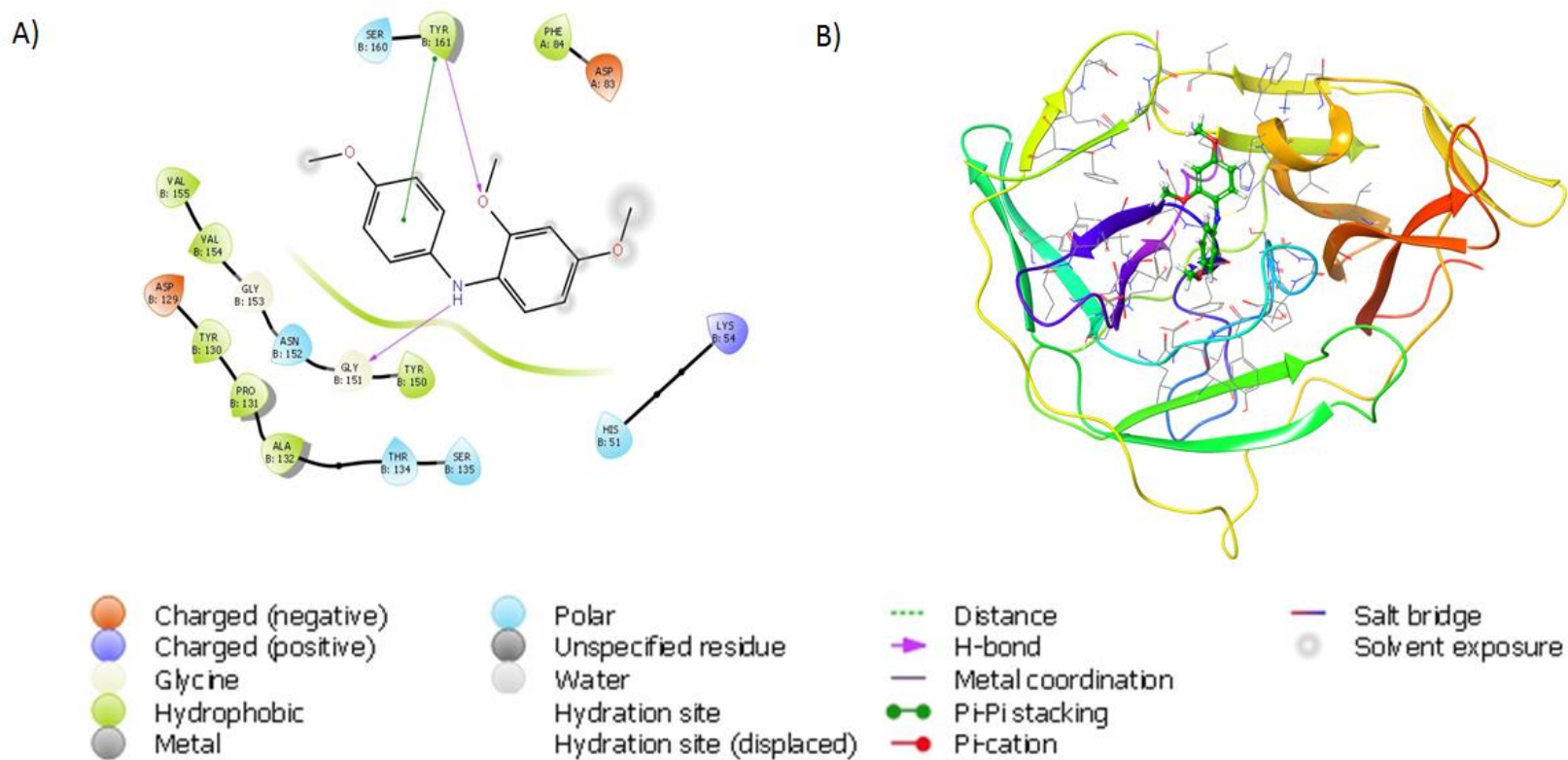
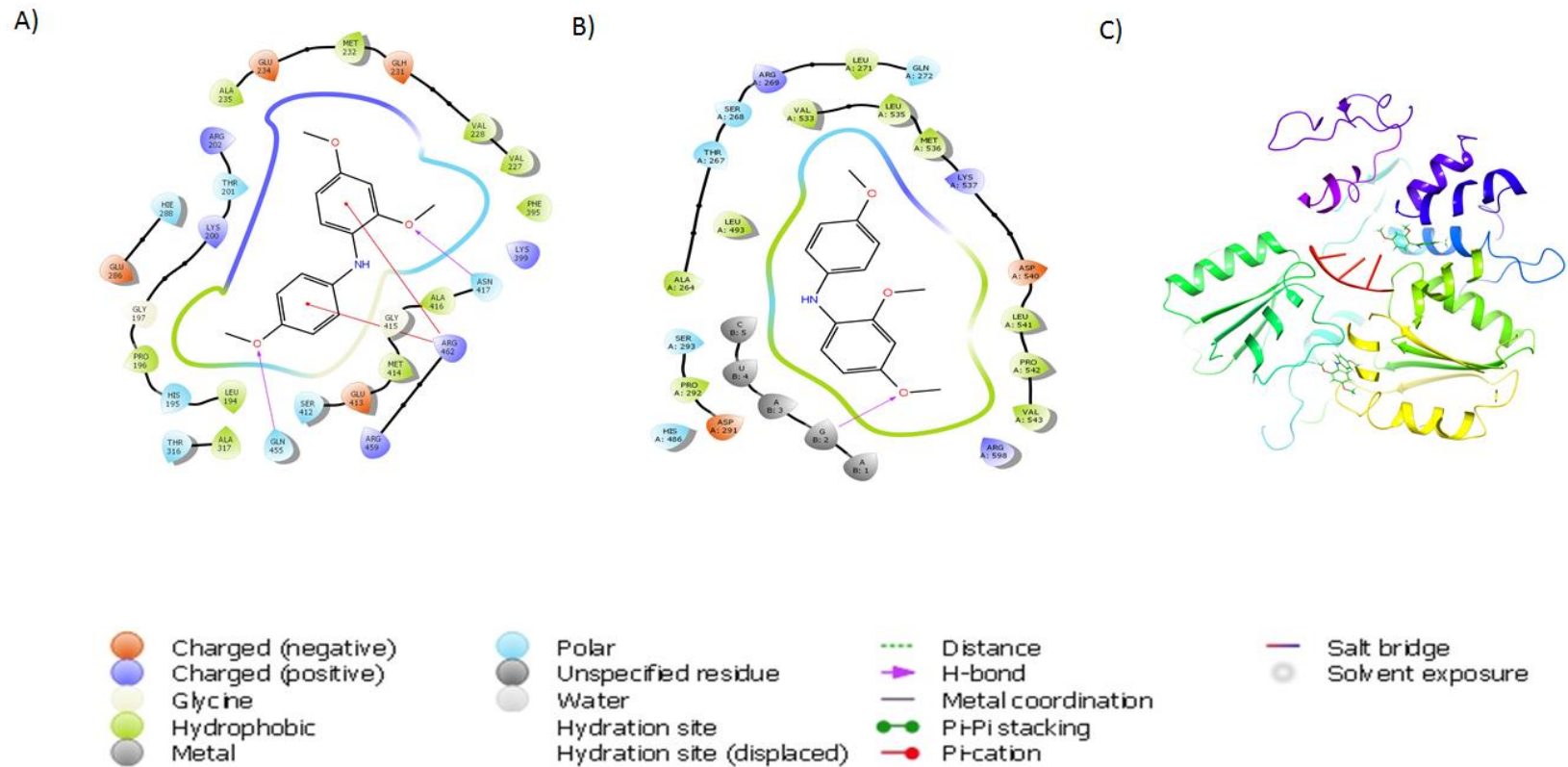


**Figure 10.** Protein-ligand interactions of FAM E3 compound with NS3 helicase protein, at (A) ATP and NS3 helicase protein, at (B) RNA binding site. (C) Predicted complex structure of NS3 helicase and FAM E3 compound, in ATP and RNA binding sites (docking calculations were performed independently).



## SUPPLEMENTARY MATERIAL

| Compound | Concentration $\mu$ M | Cell viability% | Infectivity % | Cytotoxicity % | Inhibition % | Effective inhibition % |
|----------|-----------------------|-----------------|---------------|----------------|--------------|------------------------|
| Naturais |                       |                 |               |                |              |                        |
| 3*20     | 2                     | 50.85           | 116.22        | 49.15          | -16.22       | -                      |
| 3*43     | 2                     | 65.51           | 102.49        | 34.49          | -2.49        | -                      |
| AC. B    | 2                     | 96.00           | 128.29        | 4.00           | -28.29       | -                      |
| AC. CLO  | 2                     | 95.17           | 101.75        | 4.83           | -1.75        | -                      |
| Ac. O    | 2                     | 91.52           | 134.28        | 8.48           | -34.28       | -                      |
| CATEQ    | 50                    | 74.47           | 124.34        | 25.53          | -24.34       | -                      |
| CITR     | 50                    | 116.55          | 96.29         | -16.55         | 3.71         | 20.26                  |
| CRI      | 10                    | 122.78          | 74.46         | -22.78         | 25.54        | 48.32                  |
| CUMA     | 50                    | 115.42          | 101.39        | -15.42         | -1.39        | 14.04                  |
| E27      | 50                    | 95.86           | 160.54        | 4.14           | -60.54       | -                      |
| E28      | 50                    | 94.70           | 138.23        | 5.30           | -38.23       | -                      |
| E29      | 50                    | 101.16          | 178.29        | -1.16          | -78.29       | -                      |
| E30      | 50                    | 96.67           | 121.52        | 3.33           | -21.52       | -                      |
| E31      | 50                    | 101.69          | 169.12        | -1.69          | -69.12       | -                      |
| E40      | 50                    | 101.58          | 149.69        | -1.58          | -49.69       | -                      |
| E42      | 50                    | 98.93           | 137.61        | 1.07           | -37.61       | -                      |
| E43      | 50                    | 96.65           | 99.13         | 3.35           | 0.87         | -                      |

|              |           |               |               |               |               |              |
|--------------|-----------|---------------|---------------|---------------|---------------|--------------|
| <b>E44</b>   | <b>50</b> | <b>99.07</b>  | <b>91.35</b>  | <b>0.93</b>   | <b>8.65</b>   | <b>7.71</b>  |
| <b>E45</b>   | <b>50</b> | <b>99.19</b>  | <b>94.56</b>  | <b>0.81</b>   | <b>5.44</b>   | <b>4.63</b>  |
| <b>E46</b>   | <b>50</b> | <b>99.94</b>  | <b>137.72</b> | <b>0.06</b>   | <b>-37.72</b> | <b>-</b>     |
| <b>E5</b>    | <b>50</b> | <b>102.03</b> | <b>126.60</b> | <b>-2.03</b>  | <b>-26.60</b> | <b>-</b>     |
| <b>E51</b>   | <b>50</b> | <b>102.19</b> | <b>115.90</b> | <b>-2.19</b>  | <b>-15.90</b> | <b>-</b>     |
| <b>E52</b>   | <b>50</b> | <b>93.59</b>  | <b>101.07</b> | <b>6.41</b>   | <b>-1.07</b>  | <b>-</b>     |
| <b>E6</b>    | <b>50</b> | <b>94.51</b>  | <b>103,85</b> | <b>5.49</b>   | <b>-3.85</b>  | <b>-</b>     |
| <b>E7</b>    | <b>50</b> | <b>92.16</b>  | <b>74,54</b>  | <b>7.84</b>   | <b>25.46</b>  | <b>17.62</b> |
| <b>F18</b>   | <b>10</b> | <b>68.98</b>  | <b>85,54</b>  | <b>31.02</b>  | <b>14.46</b>  | <b>-</b>     |
| <b>K1</b>    | <b>50</b> | <b>92.78</b>  | <b>116,69</b> | <b>7.22</b>   | <b>-16.69</b> | <b>-</b>     |
| <b>K3</b>    | <b>50</b> | <b>94.14</b>  | <b>126,15</b> | <b>5.86</b>   | <b>-26.15</b> | <b>-</b>     |
| <b>K4</b>    | <b>50</b> | <b>92.14</b>  | <b>125,59</b> | <b>7.86</b>   | <b>-25.59</b> | <b>-</b>     |
| <b>K5</b>    | <b>10</b> | <b>71.45</b>  | <b>120,41</b> | <b>28.55</b>  | <b>-20.41</b> | <b>-</b>     |
| <b>K6</b>    | <b>50</b> | <b>95.51</b>  | <b>159,67</b> | <b>4.49</b>   | <b>-59.67</b> | <b>-</b>     |
| <b>K7</b>    | <b>50</b> | <b>94.31</b>  | <b>129,15</b> | <b>5.69</b>   | <b>-29.15</b> | <b>-</b>     |
| <b>MORI</b>  | <b>50</b> | <b>119.10</b> | <b>131,74</b> | <b>-19.10</b> | <b>-31.74</b> | <b>-</b>     |
| <b>PIPER</b> | <b>50</b> | <b>96.04</b>  | <b>156,64</b> | <b>3.96</b>   | <b>-56.64</b> | <b>-</b>     |
| <b>PIPGA</b> | <b>10</b> | <b>117.23</b> | <b>131,91</b> | <b>-17.23</b> | <b>-31.91</b> | <b>-</b>     |
| <b>PN2</b>   | <b>10</b> | <b>81.58</b>  | <b>88,49</b>  | <b>18.42</b>  | <b>11.51</b>  | <b>-</b>     |
| <b>RUTI</b>  | <b>50</b> | <b>120.65</b> | <b>112,31</b> | <b>-20.65</b> | <b>-12.31</b> | <b>8.34</b>  |
| <b>S.1</b>   | <b>50</b> | <b>103.15</b> | <b>78,83</b>  | <b>-3.15</b>  | <b>21.17</b>  | <b>24.32</b> |
| <b>S.2</b>   | <b>50</b> | <b>97.45</b>  | <b>172,02</b> | <b>2.55</b>   | <b>-72.02</b> | <b>-</b>     |

|             |           |               |               |               |               |              |
|-------------|-----------|---------------|---------------|---------------|---------------|--------------|
| <b>S.3</b>  | <b>50</b> | <b>89.35</b>  | <b>99.68</b>  | <b>10.65</b>  | <b>0.32</b>   | <b>-</b>     |
| <b>S.4</b>  | <b>50</b> | <b>62.89</b>  | <b>49.33</b>  | <b>37.11</b>  | <b>50.67</b>  | <b>13.56</b> |
| <b>S.5</b>  | <b>50</b> | <b>93.10</b>  | <b>78.68</b>  | <b>6.90</b>   | <b>21.32</b>  | <b>14.42</b> |
| <b>S.6</b>  | <b>50</b> | <b>65.12</b>  | <b>77.10</b>  | <b>34.88</b>  | <b>22.90</b>  | <b>-</b>     |
| <b>S.7</b>  | <b>10</b> | <b>91.45</b>  | <b>74.16</b>  | <b>8.55</b>   | <b>25.84</b>  | <b>17.30</b> |
| <b>S.8</b>  | <b>10</b> | <b>88.35</b>  | <b>76.09</b>  | <b>11.65</b>  | <b>23.91</b>  | <b>12.26</b> |
| <b>UMBE</b> | <b>50</b> | <b>119.38</b> | <b>144.74</b> | <b>-19.38</b> | <b>-44.74</b> | <b>-</b>     |

### Chalcones

|            |           |               |               |              |                |          |
|------------|-----------|---------------|---------------|--------------|----------------|----------|
| <b>T35</b> | <b>50</b> | <b>96.58</b>  | <b>215.14</b> | <b>3.42</b>  | <b>-115.14</b> | <b>-</b> |
| <b>T36</b> | <b>50</b> | <b>109.34</b> | <b>153.66</b> | <b>-9.34</b> | <b>-53.66</b>  | <b>-</b> |

### Chalcones derived from Apocinines

|            |           |               |               |              |                |          |
|------------|-----------|---------------|---------------|--------------|----------------|----------|
| <b>A14</b> | <b>2</b>  | <b>101.11</b> | <b>142.60</b> | <b>-1.11</b> | <b>-42.60</b>  | <b>-</b> |
| <b>A15</b> | <b>2</b>  | <b>97.13</b>  | <b>244.20</b> | <b>2.87</b>  | <b>-144.20</b> | <b>-</b> |
| <b>A16</b> | <b>50</b> | <b>90.69</b>  | <b>258.45</b> | <b>9.31</b>  | <b>-158.45</b> | <b>-</b> |
| <b>A17</b> | <b>10</b> | <b>96.04</b>  | <b>291.41</b> | <b>3.96</b>  | <b>-191.41</b> | <b>-</b> |
| <b>A20</b> | <b>10</b> | <b>31.81</b>  | <b>190.48</b> | <b>68.19</b> | <b>-90.48</b>  | <b>-</b> |
| <b>A3</b>  | <b>2</b>  | <b>94.46</b>  | <b>207.16</b> | <b>5.54</b>  | <b>-107.16</b> | <b>-</b> |

|                         |            |               |               |               |                |             |
|-------------------------|------------|---------------|---------------|---------------|----------------|-------------|
| <b>APO</b>              | <b>50</b>  | <b>120.51</b> | <b>148.41</b> | <b>-20.51</b> | <b>-48.41</b>  | <b>-</b>    |
| <b>B1</b>               | <b>2</b>   | <b>117.03</b> | <b>194.70</b> | <b>-17.03</b> | <b>-94.70</b>  | <b>-</b>    |
| <b>B3</b>               | <b>0,4</b> | <b>110.63</b> | <b>175.06</b> | <b>-10.63</b> | <b>-75.06</b>  | <b>-</b>    |
| <b>B4</b>               | <b>2</b>   | <b>95.97</b>  | <b>298.21</b> | <b>4.03</b>   | <b>-198.21</b> | <b>-</b>    |
| <b>B6</b>               | <b>0,4</b> | <b>96.68</b>  | <b>226.06</b> | <b>3.32</b>   | <b>-126.06</b> | <b>-</b>    |
| <b>C3</b>               | <b>0,4</b> | <b>94.25</b>  | <b>191.43</b> | <b>5.75</b>   | <b>-91.43</b>  | <b>-</b>    |
| <b>D2</b>               | <b>2</b>   | <b>97.99</b>  | <b>162.29</b> | <b>2.01</b>   | <b>-62.29</b>  | <b>-</b>    |
| <b>D3</b>               | <b>2</b>   | <b>97.13</b>  | <b>141.02</b> | <b>2.87</b>   | <b>-41.02</b>  | <b>-</b>    |
| <b>E1</b>               | <b>10</b>  | <b>115.99</b> | <b>201.28</b> | <b>-15.99</b> | <b>-101.28</b> | <b>-</b>    |
| <b>E13</b>              | <b>2</b>   | <b>100.33</b> | <b>153.62</b> | <b>-0.33</b>  | <b>-53.62</b>  | <b>-</b>    |
| <b>E2</b>               | <b>10</b>  | <b>108.65</b> | <b>175.71</b> | <b>-8.65</b>  | <b>-75.71</b>  | <b>-</b>    |
| <b>E20</b>              | <b>2</b>   | <b>100.53</b> | <b>142.17</b> | <b>-0.53</b>  | <b>-42.17</b>  | <b>-</b>    |
| <b>E21</b>              | <b>10</b>  | <b>88.58</b>  | <b>132.60</b> | <b>11.42</b>  | <b>-32.60</b>  | <b>-</b>    |
| <b>F1</b>               | <b>2</b>   | <b>105.21</b> | <b>154.17</b> | <b>-5.21</b>  | <b>-54.17</b>  | <b>-</b>    |
| <b>F2</b>               | <b>10</b>  | <b>108.33</b> | <b>122.76</b> | <b>-8.33</b>  | <b>-22.76</b>  | <b>-</b>    |
| <b>Hydrochalcones</b>   |            |               |               |               |                |             |
| <b>T 1</b>              | <b>10</b>  | <b>97.83</b>  | <b>107.55</b> | <b>2.17</b>   | <b>-7.55</b>   | <b>-</b>    |
| <b>T 10</b>             | <b>10</b>  | <b>93.29</b>  | <b>86.72</b>  | <b>6.71</b>   | <b>13.28</b>   | <b>6.58</b> |
| <b>T 10<sup>a</sup></b> | <b>10</b>  | <b>96.68</b>  | <b>114.17</b> | <b>3.32</b>   | <b>-14.17</b>  | <b>-</b>    |
| <b>T 10B</b>            | <b>2</b>   | <b>102.91</b> | <b>153.40</b> | <b>-2.91</b>  | <b>-53.40</b>  | <b>-</b>    |
| <b>T 11</b>             | <b>10</b>  | <b>99.25</b>  | <b>136.86</b> | <b>0.75</b>   | <b>-36.86</b>  | <b>-</b>    |

|                         |             |               |               |               |                |              |
|-------------------------|-------------|---------------|---------------|---------------|----------------|--------------|
| <b>T 11 A</b>           | <b>50</b>   | <b>110.83</b> | <b>115.94</b> | <b>-10.83</b> | <b>-15.94</b>  | <b>-</b>     |
| <b>T 11 B</b>           | <b>10</b>   | <b>101.78</b> | <b>110.00</b> | <b>-1.78</b>  | <b>-10.00</b>  | <b>-</b>     |
| <b>T 2</b>              | <b>2</b>    | <b>85.41</b>  | <b>83.24</b>  | <b>14.59</b>  | <b>16.76</b>   | <b>2.17</b>  |
| <b>T 26</b>             | <b>10</b>   | <b>84.92</b>  | <b>453.41</b> | <b>15.08</b>  | <b>-353.41</b> | <b>-</b>     |
| <b>T 27</b>             | <b>10</b>   | <b>102.34</b> | <b>131.62</b> | <b>-2.34</b>  | <b>-31.62</b>  | <b>-</b>     |
| <b>T 3</b>              | <b>10</b>   | <b>97.68</b>  | <b>96.24</b>  | <b>2.32</b>   | <b>3.76</b>    | <b>1.44</b>  |
| <b>T 31</b>             | <b>50</b>   | <b>94.77</b>  | <b>565.29</b> | <b>5.23</b>   | <b>-465.29</b> | <b>-</b>     |
| <b>T 32</b>             | <b>2</b>    | <b>97.11</b>  | <b>177.70</b> | <b>2.89</b>   | <b>-77.70</b>  | <b>-</b>     |
| <b>T 33</b>             | <b>10</b>   | <b>92.54</b>  | <b>373.29</b> | <b>7.46</b>   | <b>-273.29</b> | <b>-</b>     |
| <b>T 34</b>             | <b>10</b>   | <b>98.45</b>  | <b>77.80</b>  | <b>1.55</b>   | <b>22.20</b>   | <b>20.65</b> |
| <b>T 37 *</b>           | <b>0.08</b> | <b>97.15</b>  | <b>144.79</b> | <b>2,85</b>   | <b>-44.79</b>  | <b>-</b>     |
| <b>T 37<sup>a</sup></b> | <b>0.08</b> | <b>103.15</b> | <b>128.81</b> | <b>-3.15</b>  | <b>-28.81</b>  | <b>-</b>     |
| <b>T 4</b>              | <b>2</b>    | <b>92.54</b>  | <b>397.67</b> | <b>7.46</b>   | <b>-297.67</b> | <b>-</b>     |
| <b>T 6</b>              | <b>10</b>   | <b>96.75</b>  | <b>412.94</b> | <b>3.25</b>   | <b>-312.94</b> | <b>-</b>     |
| <b>T 8</b>              | <b>10</b>   | <b>92.99</b>  | <b>412.25</b> | <b>7.01</b>   | <b>-312.25</b> | <b>-</b>     |
| <b>T 9</b>              | <b>50</b>   | <b>102.07</b> | <b>123.63</b> | <b>-2.07</b>  | <b>-23.63</b>  | <b>-</b>     |
| <b>T11 C</b>            | <b>10</b>   | <b>99.90</b>  | <b>111.89</b> | <b>0.10</b>   | <b>-11.89</b>  | <b>-</b>     |
| <b>T11D</b>             | <b>10</b>   | <b>101.18</b> | <b>125.50</b> | <b>-1.18</b>  | <b>-25.50</b>  | <b>-</b>     |
| <b>Curcuminoids</b>     |             |               |               |               |                |              |
| <b>CH1</b>              | <b>0.4</b>  | <b>99.72</b>  | <b>120.47</b> | <b>0.28</b>   | <b>-20.47</b>  | <b>-</b>     |
| <b>CH2</b>              | <b>0.4</b>  | <b>102.11</b> | <b>129.74</b> | <b>-2.11</b>  | <b>-29.74</b>  | <b>-</b>     |

|                       |            |                |               |               |               |              |
|-----------------------|------------|----------------|---------------|---------------|---------------|--------------|
| <b>CH5</b>            | <b>0.4</b> | <b>95.62</b>   | <b>89.79</b>  | <b>4.38</b>   | <b>10.21</b>  | <b>5.83</b>  |
| <b>AC7</b>            | <b>4</b>   | <b>99.75</b>   | <b>95.04</b>  | <b>0.25</b>   | <b>4.96</b>   | <b>4.71</b>  |
| <b>AC10</b>           | <b>10</b>  | <b>104.63</b>  | <b>117.07</b> | <b>-4.63</b>  | <b>-17.07</b> | <b>-</b>     |
| <b>Nitrochalcones</b> |            |                |               |               |               |              |
| <b>E03</b>            | <b>2</b>   | <b>100.96</b>  | <b>75.06</b>  | <b>-0.96</b>  | <b>24.94</b>  | <b>25.90</b> |
| <b>E05</b>            | <b>2</b>   | <b>98.29</b>   | <b>89.66</b>  | <b>1.71</b>   | <b>10.34</b>  | <b>8.63</b>  |
| <b>E07</b>            | <b>10</b>  | <b>100.44</b>  | <b>86.19</b>  | <b>-0.44</b>  | <b>13.81</b>  | <b>14.25</b> |
| <b>E13</b>            | <b>2</b>   | <b>100.33</b>  | <b>153.62</b> | <b>-0.33</b>  | <b>-53.62</b> | <b>-</b>     |
| <b>E14</b>            | <b>10</b>  | <b>106.50</b>  | <b>89.21</b>  | <b>-6.50</b>  | <b>10.79</b>  | <b>17.29</b> |
| <b>E20</b>            | <b>2</b>   | <b>100.53</b>  | <b>142.17</b> | <b>-0.53</b>  | <b>-42.17</b> | <b>-</b>     |
| <b>E21</b>            | <b>10</b>  | <b>88.58</b>   | <b>132.60</b> | <b>11.42</b>  | <b>-32.60</b> | <b>-</b>     |
| <b>E23</b>            | <b>10</b>  | <b>104.23</b>  | <b>81.25</b>  | <b>-4.23</b>  | <b>18.75</b>  | <b>22.98</b> |
| <b>E26</b>            | <b>50</b>  | <b>88.09</b>   | <b>58.39</b>  | <b>11.91</b>  | <b>41.61</b>  | <b>29.70</b> |
| <b>E29</b>            | <b>50</b>  | <b>98.06</b>   | <b>85.32</b>  | <b>1.94</b>   | <b>14.68</b>  | <b>12.74</b> |
| <b>E30</b>            | <b>10</b>  | <b>99.53</b>   | <b>95.82</b>  | <b>0.47</b>   | <b>4.18</b>   | <b>3.71</b>  |
| <b>E37</b>            | <b>2</b>   | <b>95.72</b>   | <b>79.57</b>  | <b>4.28</b>   | <b>20.43</b>  | <b>16.15</b> |
| <b>E43</b>            | <b>10</b>  | <b>98.30</b>   | <b>87.88</b>  | <b>1.70</b>   | <b>12.12</b>  | <b>10.42</b> |
| <b>E46</b>            | <b>10</b>  | <b>102.73</b>  | <b>73.83</b>  | <b>-2.73</b>  | <b>26.17</b>  | <b>28.89</b> |
| <b>E47</b>            | <b>10</b>  | <b>115.66</b>  | <b>75.42</b>  | <b>-15.66</b> | <b>24.58</b>  | <b>40.24</b> |
| <b>T23</b>            | <b>10</b>  | <b>113.524</b> | <b>59.84</b>  | <b>-13.52</b> | <b>40.16</b>  | <b>53.68</b> |

|             |           |               |               |              |               |              |
|-------------|-----------|---------------|---------------|--------------|---------------|--------------|
| <b>T24</b>  | <b>10</b> | <b>104.20</b> | <b>60.00</b>  | <b>-4.20</b> | <b>40.00</b>  | <b>44.20</b> |
| <b>T25</b>  | <b>10</b> | <b>103.64</b> | <b>65.59</b>  | <b>-3.64</b> | <b>34.41</b>  | <b>38.06</b> |
| <b>T28</b>  | <b>10</b> | <b>96.34</b>  | <b>103.71</b> | <b>3.66</b>  | <b>-3.71</b>  | <b>-</b>     |
| <b>T29</b>  | <b>50</b> | <b>98.80</b>  | <b>94.87</b>  | <b>1.20</b>  | <b>5.13</b>   | <b>3.92</b>  |
| <b>T30</b>  | <b>10</b> | <b>97.91</b>  | <b>112.04</b> | <b>2.09</b>  | <b>-12.04</b> |              |
| <b>T35</b>  | <b>50</b> | <b>97.96</b>  | <b>101.77</b> | <b>2.04</b>  | <b>-1.77</b>  | <b>-</b>     |
| <b>T38</b>  | <b>2</b>  | <b>99.73</b>  | <b>90.05</b>  | <b>0.27</b>  | <b>9.95</b>   | <b>9.68</b>  |
| <b>T39</b>  | <b>10</b> | <b>85.58</b>  | <b>92.05</b>  | <b>14.42</b> | <b>7.95</b>   | <b>-</b>     |
| <b>T41</b>  | <b>10</b> | <b>92.36</b>  | <b>72.25</b>  | <b>7.64</b>  | <b>27.75</b>  | <b>20.10</b> |
| <b>T42</b>  | <b>50</b> | <b>101.16</b> | <b>132.75</b> | <b>-1.16</b> | <b>-32.75</b> | <b>-</b>     |
| <b>T45</b>  | <b>10</b> | <b>107.46</b> | <b>113.98</b> | <b>-7.46</b> | <b>-13.98</b> | <b>-</b>     |
| <b>T46A</b> | <b>50</b> | <b>103.12</b> | <b>85.97</b>  | <b>-3.12</b> | <b>14.03</b>  | <b>17.16</b> |
| <b>T46B</b> | <b>50</b> | <b>102.91</b> | <b>87.87</b>  | <b>-2.91</b> | <b>12.13</b>  | <b>15.04</b> |

### Acridones

|              |           |               |               |              |               |              |
|--------------|-----------|---------------|---------------|--------------|---------------|--------------|
| <b>FAC 1</b> | <b>10</b> | <b>89.75</b>  | <b>79.69</b>  | <b>10.25</b> | <b>20.31</b>  | <b>10.06</b> |
| <b>FAC 2</b> | <b>50</b> | <b>69.35</b>  | <b>116.59</b> | <b>30.65</b> | <b>-16.59</b> | <b>-</b>     |
| <b>FAC 3</b> | <b>10</b> | <b>99.33</b>  | <b>110.50</b> | <b>0.67</b>  | <b>-10.50</b> | <b>-</b>     |
| <b>FAC 4</b> | <b>10</b> | <b>102.02</b> | <b>126.72</b> | <b>-2.02</b> | <b>-26.72</b> | <b>-</b>     |
| <b>FAC 5</b> | <b>10</b> | <b>101.73</b> | <b>141.19</b> | <b>-1.73</b> | <b>-41.19</b> | <b>-</b>     |
| <b>FAC 6</b> | <b>10</b> | <b>103.54</b> | <b>110.45</b> | <b>-3.54</b> | <b>-10.45</b> | <b>-</b>     |
| <b>FAC 7</b> | <b>50</b> | <b>104.52</b> | <b>64.33</b>  | <b>-4.52</b> | <b>35.67</b>  | <b>40,19</b> |

|              |           |               |               |              |               |              |
|--------------|-----------|---------------|---------------|--------------|---------------|--------------|
| <b>FAC 8</b> | <b>10</b> | <b>102.85</b> | <b>98.89</b>  | <b>-2.85</b> | <b>1.11</b>   | <b>3.95</b>  |
| <b>FAC14</b> | <b>10</b> | <b>105.04</b> | <b>121.00</b> | <b>-5.04</b> | <b>-21.00</b> | <b>-</b>     |
| <b>FAC15</b> | <b>2</b>  | <b>99.96</b>  | <b>113.35</b> | <b>0.04</b>  | <b>-13.35</b> | <b>-</b>     |
| <b>FAC16</b> | <b>10</b> | <b>73.63</b>  | <b>60.18</b>  | <b>26.37</b> | <b>39.82</b>  | <b>13.45</b> |
| <b>FAC17</b> | <b>10</b> | <b>84.37</b>  | <b>111.04</b> | <b>15.63</b> | <b>-11.04</b> | <b>-</b>     |
| <b>FAC18</b> | <b>2</b>  | <b>96.01</b>  | <b>117.23</b> | <b>3.99</b>  | <b>-17.23</b> | <b>-</b>     |
| <b>FAC19</b> | <b>2</b>  | <b>97.34</b>  | <b>103.76</b> | <b>2.66</b>  | <b>-3.76</b>  | <b>-</b>     |
| <b>FAC20</b> | <b>50</b> | <b>86.85</b>  | <b>111.83</b> | <b>13.15</b> | <b>-11.83</b> | <b>-</b>     |
| <b>FAC21</b> | <b>10</b> | <b>101.03</b> | <b>136.72</b> | <b>-1.03</b> | <b>-36.72</b> | <b>-</b>     |
| <b>FAC22</b> | <b>2</b>  | <b>101.25</b> | <b>112.95</b> | <b>-1.25</b> | <b>-12.95</b> | <b>-</b>     |

**Diarylamines derived from anthranilic acid**

|                |           |               |               |               |               |              |
|----------------|-----------|---------------|---------------|---------------|---------------|--------------|
| <b>FAM-A3</b>  | <b>10</b> | <b>106.64</b> | <b>99.22</b>  | <b>-6.64</b>  | <b>0.78</b>   | <b>7.41</b>  |
| <b>FAM-A3'</b> | <b>2</b>  | <b>119.18</b> | <b>119.50</b> | <b>-19.18</b> | <b>-19.50</b> | <b>-</b>     |
| <b>FAM-A5</b>  | <b>10</b> | <b>115.54</b> | <b>110.77</b> | <b>-15.54</b> | <b>-10.77</b> | <b>4.77</b>  |
| <b>FAM B3</b>  | <b>10</b> | <b>107.72</b> | <b>56.08</b>  | <b>-7.72</b>  | <b>43.92</b>  | <b>51.64</b> |
| <b>FAM B4</b>  | <b>40</b> | <b>76.74</b>  | <b>48.67</b>  | <b>23.26</b>  | <b>51.33</b>  | <b>28.97</b> |
| <b>FAM C1</b>  | <b>50</b> | <b>100.26</b> | <b>60.66</b>  | <b>-0.26</b>  | <b>39.34</b>  | <b>39.59</b> |
| <b>FAM C3</b>  | <b>10</b> | <b>119.04</b> | <b>93.69</b>  | <b>-19.04</b> | <b>6.31</b>   | <b>25.35</b> |
| <b>FAM C5</b>  | <b>10</b> | <b>112.63</b> | <b>97.71</b>  | <b>-12.63</b> | <b>2.29</b>   | <b>14.92</b> |
| <b>FAM D1</b>  | <b>30</b> | <b>61.82</b>  | <b>20.48</b>  | <b>38.18</b>  | <b>79.52</b>  | <b>41.34</b> |

|               |            |               |               |               |               |              |
|---------------|------------|---------------|---------------|---------------|---------------|--------------|
| <b>FAM I3</b> | <b>10</b>  | <b>114.56</b> | <b>29.59</b>  | <b>-14.56</b> | <b>70.41</b>  | <b>84.98</b> |
| <b>FAM E1</b> | <b>10</b>  | <b>117.68</b> | <b>98.94</b>  | <b>-17.68</b> | <b>1.06</b>   | <b>18.74</b> |
| <b>FAM L3</b> | <b>10</b>  | <b>112.23</b> | <b>101.17</b> | <b>-12.23</b> | <b>-1.17</b>  | <b>11.06</b> |
| <b>FAM I5</b> | <b>50</b>  | <b>61.85</b>  | <b>40.06</b>  | <b>38.16</b>  | <b>59.50</b>  | <b>21.34</b> |
| <b>FAM G3</b> | <b>0,4</b> | <b>114.25</b> | <b>118.20</b> | <b>-14.25</b> | <b>-18.0</b>  | <b>-</b>     |
| <b>FAM F3</b> | <b>10</b>  | <b>114.37</b> | <b>116.88</b> | <b>-14.37</b> | <b>-16.88</b> | <b>-</b>     |
| <b>FAM E3</b> | <b>10</b>  | <b>51.30</b>  | <b>0.57</b>   | <b>38.48</b>  | <b>99.73</b>  | <b>60.95</b> |
| <b>FAM F5</b> | <b>50</b>  | <b>114.49</b> | <b>108.20</b> | <b>-14.49</b> | <b>-8.20</b>  | <b>6.29</b>  |
| <b>FAM D5</b> | <b>50</b>  | <b>102.42</b> | <b>93.56</b>  | <b>-2.42</b>  | <b>6.44</b>   | <b>8.85</b>  |
| <b>FAM A1</b> | <b>10</b>  | <b>110.71</b> | <b>94.46</b>  | <b>-10.71</b> | <b>5.54</b>   | <b>16.26</b> |
| <b>FAM B1</b> | <b>50</b>  | <b>101.41</b> | <b>122.05</b> | <b>-1.41</b>  | <b>-22.05</b> | <b>-</b>     |
| <b>FAM D3</b> | <b>2</b>   | <b>140.36</b> | <b>131.15</b> | <b>-40.36</b> | <b>-31.15</b> | <b>9.21</b>  |
| <b>FAM E5</b> | <b>50</b>  | <b>101.24</b> | <b>55.8</b>   | <b>-1.24</b>  | <b>44.20</b>  | <b>45.44</b> |

## ***Capítulo IV: Artigos Publicados***

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## RESEARCH ARTICLE

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

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**Data Availability Statement:** All relevant data are within the paper.

## 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<sub>2</sub> (PLA<sub>2</sub>-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<sub>2</sub>-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.