ÍTALO ESPOSTI POLY DA SILVA

EFEITO ANTIVIRAL DE XANTENODIONAS E UMA 1,3 DICETONA CONTRA O VÍRUS ZIKA

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de Magister Scientiae.

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APROVADA: 28 de fevereiro de 2018.

Róbson Ricardo Teixeira

Michelle Dias de Oliveira Teixeira

Sérgio Oliveira de Paula
(Orientador)

Aos meus pais, Jocimar e Lia, dedico.

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“No! Try not! Do ... or do not! There is no try.”
(Mestre Yoda- Star Wars Ep V)

RESUMO

SILVA, Ítalo Esposti Poly, M.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Efeito antiviral de xantenodionas e uma 1,3 dicetona contra o vírus Zika**. Orientador: Sérgio Oliveira de Paula.

O vírus Zika (ZIKV) é um arbovírus pertencente à família Flaviviridae, se tornando uma preocupação mundial depois das grandes epidemias na Polinésia Francesa em 2013 e principalmente nas Américas em 2015. Com sintomas muito parecidos com os de outros flavivirus como Dengue e Chikungunya, que incluem dores musculares e de cabeça, febre e exantema, o ZIKV também foi relacionado com várias complicações neurológicas como microcefalia em recém-nascidos e síndrome de Guillain-Barre em adultos. De acordo com o Ministério da Saúde, em 2016 foram registrados 216.207 casos prováveis de febre pelo ZIKV, sendo 8 óbitos confirmados laboratorialmente e 10.039 casos de microcefalia foram notificados. Considerando a alta prevalência de infecção por ZIKV em certas áreas, os riscos que o vírus possui para o cérebro em desenvolvimento, e o fato de até o presente momento não existirem vacinas ou profilaxias específicas licenciadas, um tratamento efetivo capaz de regredir a infecção seria de grande valia. Assim sendo, neste trabalho foi avaliado pela primeira vez a atividade antiviral de compostos inéditos derivados de xanthenodionas contra o ZIKV, onde o composto foi capaz de inibir completamente a infecção em células Vero bem como reduziu a carga viral no cérebro de camundongos Swiss recém-nascidos, por uma interação direta do composto com a partícula viral através da proteína E, bloqueando a adsorção do vírus.

ABSTRACT

SILVA, Ítalo Esposti Poly, M.Sc., Universidade Federal de Viçosa, February, 2018. A**ntiviral effect of xanthenediones and an 1,3 diketone against Zika virus.** Advisor: Sérgio Oliveira de Paula.

The Zika virus (ZIKV) is an arbovirus belonging to the Flaviviridae family, becoming a global concern after the great epidemics in French Polynesia in 2013 and especially in the Americas in 2015. With symptoms very similar to those of other flaviviruses such as Dengue and Chikungunya including head and muscle pain, fever and rash, ZIKV has also been related to various neurological complications such as microcephaly in newborns and Guillain-Barre syndrome in adults. According to the Ministry of Health, in 2016 216,207 probable cases of ZIKV fever were registered, with 8 deaths confirmed in laboratory and 10,039 cases of microcephaly were reported. Considering the high prevalence of ZIKV infection in certain areas, the risks that the virus has for the developing brain, and the fact that there are currently no licensed vaccines or specific prophylaxis, an effective treatment capable of regressing the infection would be be extremely desirable. Therefore, in this work the antiviral activity of xanthenedione compounds against ZIKV was evaluated for the first time, where the compound was able to completely inhibit infection in Vero cells, as well as reduced the viral load in the brain of newborn Swiss mice, by interaction of the compound with the viral particle, through the E protein, blocking the adsorption of the virus.

SUMÁRIO

INTRODUÇÃO GERAL

1 EPIDEMIOLOGIA DO VÍRUS ZIKA

O vírus Zika (ZIKV) foi isolado pela primeira vez em 1947, de macacos Rhesus (Macaca mulatta) sentinelas, na floresta Zika, Uganda, durante pesquisa sobre Febre Amarela, e também à partir de mosquitos Aedes africanus em 1948 (Dick et al., 1952). Em 1952, também em Uganda, foi obtido o primeiro isolado de ZIKV proveniente de humanos (Smithburn, 1952). Apenas em abril de 2007, ZIKV disseminou de seu alcance geográfico habitual e foi detectado fora da África e Ásia, com a epidemia da Ilha de Yap (Hayes, 2009). Houve um aumento exponencial no número de notificações em 2009, sendo detectado o vírus na Nigéria, Uganda, Egito, Índia, Paquistão, Norte do Vietnã, Tailândia, Malásia, Indonésia, Filipinas, África do Sul e no Sul da Ásia (Duong et al., 2009).

O ZIKV não havia sido documentado na América do Sul até a primeira transmissão autóctone (com origem no próprio país) em 2015 no Brasil (Zanluca et al., 2015). Em janeiro de 2016, casos autóctones de infecção por ZIKV foram notificados em 28 países das Américas (Samarasekera e Triunfol, 2016). A disseminação rápida do vírus nas Américas ocorreu por duas razões: a população não ser imunologicamente protegida do ZIKV e a presença do mosquito vetor Aedes aegypti. O vetor pode ser encontrado da região sul dos EUA até o norte da Argentina (excluindo o Chile), uma área abrangendo cerca de 500 milhões de pessoas (Samarasekera e Triunfol, 2016).

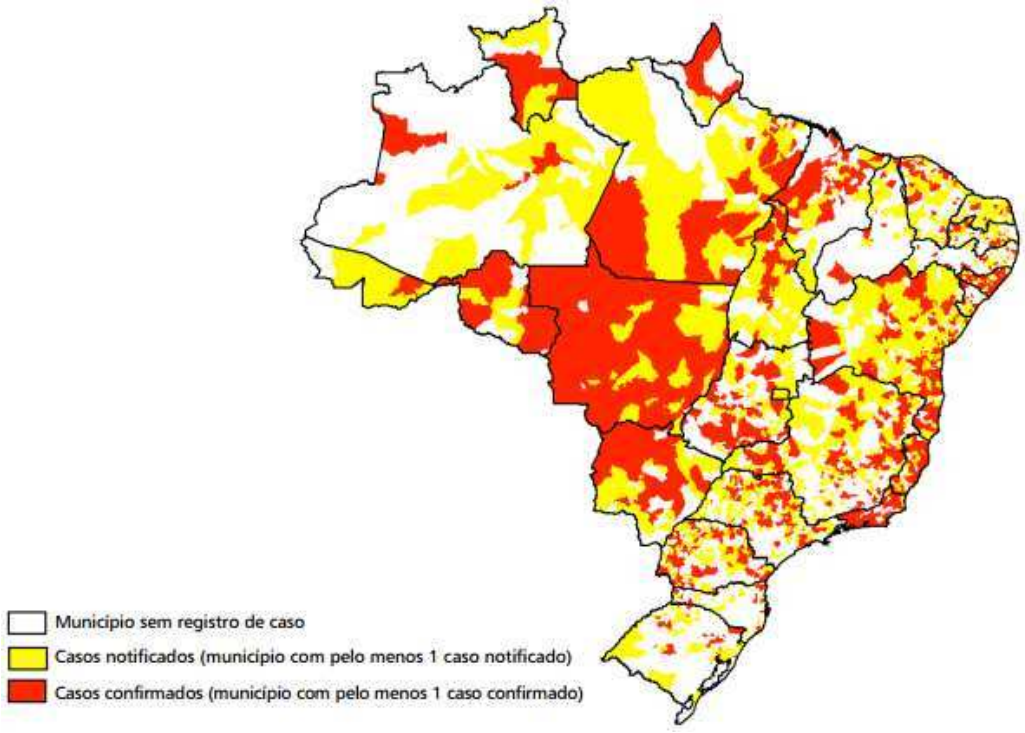


Figura 01: Unidades da Federação com casos autóctones de febre pelo vírus Zika com confirmação laboratorial, até a semana epidemiológica 37 (Svs/Ms, 2016).

Outra possível razão para esse grande número de casos, pode se o grande número de subnotificações, o que explicaria a falta de notificações de surtos anteriores, especialmente por conta das similaridades clínicas associadas a outras doenças causadas por Arbovírus, como Dengue (DENV) e Chikungunya (CHIKV), e a frequente co-circulação dos três vírus (Paixao et al., 2016).

Em 2016, entre as Semanas Epidemiológicas 1 e 52, foram registrados 216.207 casos prováveis de febre pelo ZIKV, sendo 8 óbitos confirmados laboratorialmente. Já em 2017, até a Semana Epidemiológica 49, 17.321 prováveis casos foram registrados no país, sendo 8.703 (50,2%) confirmados laboratorialmente incluindo dois óbitos (Svs/Ms, 2017b).

2 O VÍRUS ZIKA

O ZIKV pertence à família Flaviviridae, gênero Flavivirus (Kuno et al., 1998), e pode ser dividido em duas ou três linhagens principais: linhagem Africana, linhagem Asiática (que incluem as cepas das Américas) e linhagem Africana II, esta última

constituiria um grupo relacionado as outras duas, sendo uma linhagem circulante negligenciada na África (Gong et al., 2016; Wang et al., 2016; Li et al., 2017).

Trata-se de um vírus envelopado cujo genoma consiste de RNA fita simples polaridade positiva de aproximadamente 11 kilobases (Kuno e Chang, 2007), codifica três proteínas estruturais (capsídeo (C), pré membrana/membrana (prM/M) e envelope (E)) e sete proteínas não-estruturais (NS1, NS2A, NS2B, NS3, NS4A, NS4B E NS5) (Kuno e Chang, 2007; Dai et al., 2016).

O núcleo interno da partícula viral, o capsídeo, é composto do RNA genômico complexado com a proteína C, envolto com uma membrana lipídica derivada do retículo endoplasmático da célula hospedeira, que por sua vez ancora 180 cópias das proteínas E e M, com tamanhos que variam entre 40-43 nm para partículas virais maduras (Kostyuchenko et al., 2016).

2.1 PROTEÍNAS VIRAIS

De maneira similar à descrita para outros Flavivirus, a poliproteína codificada pelo genoma viral é clivada em 10 proteínas maduras principalmente por proteases virais na célula (Flint et al., 2015). As proteínas estruturais participam da montagem do vírus: a proteína C na formação do núcleo dos vírions com o RNA, a proteína E media a ligação entre receptor celular e vírus, promovendo a fusão do vírus com a membrana alvo, e a clivagem de prM em M promove a maturação da partícula viral (Flint et al., 2015; Dai et al., 2016).

A camada externa da partícula viral é basicamente composta de uma concha proteica icosaédrica formada de proteína E, que oculta a proteína M por baixo. O ectodomínio da proteína E é organizado em três diferentes domínios (D): DI, DII e DIII. DI age como uma ponte entre os domínios DII e DIII. No DII existe uma extremidade hidrofóbica que forma o loop de fusão, o qual interage com o receptor da membrana promovendo a entrada do vírus na célula (Kostyuchenko et al., 2016).

As sete proteínas não estruturais (NS) participam de uma variedade de funções dentre as quais incluem: replicação do genoma viral, morfogênese das partículas virias, modulação das respostas imunes do hospedeiro, rearranjo da membrana e atividade de protease (Flint et al., 2015).

A proteína NS1 é largamente utilizada para propósitos de diagnóstico, pois é secretada de células infectadas logo no início da infecção e induz grande produção de

anticorpos (Steinhagen et al., 2016). A NS3 é uma protease envolvida no processamento da poliproteína viral e utiliza NS2B como cofator (Gruba et al., 2016). Possui também atividade helicase, tendo importante papel na replicação do RNA (Lennemann e Coyne, 2017) e é capaz ainda de modular a resposta autofágica em células infectadas pelo ZIKV (Tian et al., 2016). Devido a grande relevância das funções do complexo NS2B-NS3, a busca por inibidores da atividade enzimática deste complexo se tornou um dos principais objetivos na descoberta por antivirais contra ZIKV (Rut et al., 2017).

NS5 tem função de RNA polimerase RNA-dependente sendo responsável pela replicação do genoma (Xu et al., 2017) e também contribui para a multiplicação viral inibindo as respostas celulares antivirais mediados por interferons (Grant et al., 2016). Por suas funções, a proteína NS5 também provê novas oportunidades para o desenvolvimento de antivirais (Lu et al., 2017).

2.2 TRANSMISSÃO

O meio primário de transmissão do ZIKV entre humanos é através da picada do mosquito fêmea do gênero Aedes (Who, 2016). Desde o primeiro isolamento do ZIKV em Aedes africanus em Uganda (1948), que ocorreu um ano após ter sido isolado dos macacos sentinelas, o vírus foi encontrado infectando outras espécies de Aedes ao longo dos anos (1958, 1964 e 1969) ainda restritos ao território africano (Haddow et al., 1964; Vorou, 2016). Fora da África, Ae. hensillii foi associado à epidemia da Ilha Yap (Schmitz e Chew, 2008) enquanto Ae. polynesium pode ter contribuído para as transmissões na Polinésia Francesa juntamente com o Ae. aegypti (Richard et al., 2016).

No Brasil, Ae. aegypti e Ae. albopictus foram os principais responsáveis pela epidemia, e alarmou a comunidade mundial sobre a capacidade desses mosquitos serem vetores para o ZIKV (Ferreira-De-Brito et al., 2016). Porém, essa competência já tinha sido demonstrada anteriormente, sendo associada a surtos no Gabão em 2007, várias áreas da África e em Singapura (Wong et al., 2013; Grard, 2014).

A grande disseminação das espécies de Ae. aegypti e Ae. albopictus por locais no mundo, incluindo as Américas, a alta capacidade de reprodução mesmo em condições desfavoráveis, resistência a mudanças climáticas e de estações e a dispersão passiva facilitada por ações antrópicas fazem do mosquito o vetor ideal, o que, junto com a tendência da urbanização, viagens, processos migratórios e mudanças climáticas, proporciona o atual momento de risco epidêmico (Saiz et al., 2017).

A hipótese de transmissão não vetorial foi levantada em 2008, quando dois cientistas americanos contraíram ZIKV enquanto trabalhavam no Senegal. No retorno aos EUA um deles transmitiu o vírus para sua esposa (Foy et al., 2011), assim como outros estudos mostraram evidências de transmissão sexual por relação vaginal, anal ou oral sem proteções (Moreira et al., 2017). O material genético do ZIKV já foi isolado de sêmen humano (Musso et al., 2015) e encontrado no trato genital feminino em amostras de pacientes em viremia (Visseaux et al., 2016).

A transmissão vertical, sendo o primeiro trimestre de gestação o de maior risco (Oliveira Melo et al., 2016), se tornou um dos maiores desafios de saúde pública, já que pode causar complicações neurológicas no feto. Partículas infecciosas de ZIKV também foram isoladas de leite materno de uma paciente, e o material genético viral foi detectado no leite de três mães que apresentavam sintomas da febre Zika (Besnard et al., 2014; Dupont-Rouzeyrol et al., 2016). Como grande parte dos casos de infecção por ZIKV são assintomáticos (Who, 2016), a transmissão por transfusão de sangue não pode ser descartada, sendo dois casos reportados por pacientes que receberam doação de plaquetas (Motta et al., 2016).

3 MANIFESTAÇÕES CLÍNICAS

É estipulado que infecções por ZIKV sejam sintomáticas em 18%-57% dos casos, causando sintomas brandos, e perdurando por um período de incubação de até 10 dias (Who, 2016; Aubry et al., 2017). Sintomas comuns a outras infecções arbovirais taiscomo artralgia (dores nas juntas), febre, exantema (erupções cutâneas), dor de cabeça e mialgia, podem se desenvolver no curso da infecção aguda (Ahmad et al., 2016; Svs, 2016). Desde a epidemia na Polinésia Francesa, infecções por ZIKV têm sido associadas a complicações neurológicas e oftalmológicas, que incluem microcefalia em fetos e recém-nascidos e síndrome de Guillain-Barre em adultos (Oehler et al., 2014; Svs/Ms, 2017a).

3.1 COMPLICAÇÕES NEUROLÓGICAS

Dois casos de complicações neurológicas em adultos foram relacionados ao ZIKV na ilha de Martinica. Dois pacientes (um adulto jovem e um idoso) apresentaram síndromes de encefalopatia, com convulsões ou mudanças eletroencefalográficas, provavelmente devido à encefalite, tendo resultados positivos de RT-PCR (reação da

transcriptase reversa, seguida de reação em cadeia da polimerase) para ZIKV em amostras de sangue, urina e fluido cerebrospinal (Roze et al., 2016). Na França, um idoso de 81 anos, o qual apresentava febre e perda de consciência, foi diagnosticado com meningoencefalite e seu fluido cerebrospinal foi testado por RT-PCR para ZIKV, sendo o resultado positivo (Carteaux et al., 2016). O paciente teve acompanhamento pós-alta, e depois de 38 dias ainda apresentava fraqueza residual no braço direito, mas com suas funções cognitivas totalmente recuperadas.

No Brasil, uma paciente grávida de 47 anos foi internada na UTI apresentando confusão, disartria e fraqueza dos membros inferiores. Quatro dias antes, apresentava exantema e artralgia. Foi diagnosticada com encefalite, amostras de urina foram PCR positivas para ZIKV (indicando a presença de material genético do vírus), ZIKV IgM foi positivo nos fluídos cerebroespinhal e soro. A paciente veio a óbito 11 dias após a internação (Soares et al., 2016).

Análises em sítios do Ministérios da Saúde e órgãos de saúde internacionais mostraram comparativamente o índice de ocorrência da Síndrome de Guillain-Barré (SGB) antes e depois dos surtos de ZIKV. Percebe-se que houve um aumento do número de casos de 887% na Venezuela, 400% no Suriname, 211% na Colômbia, 150% na República Dominicana, 144% em Honduras, 100% em El Salvador. No Brasil, só no estado da Bahia 172% de aumento (Dos Santos et al., 2016). De maneira similar, um aumento de pacientes apresentando SGB foi visto durante a epidemia na Polinésia Francesa, além dos relatos de microcefalia em recém-nascidos (Paploski et al., 2016).

Com o risco da infecção vertical e a correlação entre o ZIKV e complicações neurológicas, a possibilidade de má formação congênita devido a infecção com o vírus é evidente. Infecção por ZIKV já foi confirmada em recém-nascidos com microcefalia na Polinésia Francesa, Brasil e em outros países da América Latina, onde o índice de notificação de anormalidades cerebrais aumentou mais de 20 vezes durante os períodos de surto (Brasil et al., 2016; Paploski et al., 2016; Ventura et al., 2016).

No período de setembro de 2015 a maio de 2016, 345 gestantes que apresebtaran exantema até 5 dias antes dos testes foram acompanhadas durante estudo, 53% tiveram positivo para ZIKV em amostras de sangue e/ou urina. Houve nove óbitos de fetos e 116 das gestantes ZIKV-positivo deram a luz a 117 bebês, destes, 42% apresentaram sinais clínicos e/ou de neuroimagem alterados além de quatro bebês com microcefalias (Brasil et al., 2016). Onze recém-nascidos de gestação de 6 meses com síndrome ZIKV congênita no Brasil foram acompanhados, identificando injúrias neurológicas que incluem

lisencefalia, hidrocefalia, ventriculomegalia, microcefalia, redução do volume cerebral, hipoplasia cerebelar e artrogripose (Melo et al., 2016).

Além das complicações neurológicas, complicações oftalmológicas também foram relatadas. Três recém-nascidos de mães infectadas por ZIKV que apresentavam microcefalia e calcificações cerebrais foram diagnosticados com lesões oculares (Ventura et al., 2016). Um estudo conduzido com 40 crianças com microcefalia mostrou que lesões oculares eram mais frequentes em crianças cuja mãe apresentou os sintomas da febre Zika durante a gestação. Dentro do mesmo estudo, uma criança também apresentou lesões oculares mesmo não tendo microcefalia, mas a mãe pertencia ao grupo de gestantes que apresentaram sintomas (Ventura et al., 2016). Lesões oculares e danos ao nervo óptico por infecções congênitas por ZIKV também foram relatados em outro trabalho, onde recém-nascidos de um grupo de risco foram avaliados por meio de exames oftalmológicos (De Paula Freitas et al., 2016).

Estas observações em relação aos danos neurológicos só foram descritas recentemente, pouco se sabe sobre a frequência e infecção direta do ZIKV ao Sistema Nervoso Central (SNC). Além do fato de ocorrência simultânea de arbovirus, como DENV e CHIK, atrapalharem uma melhor elucidação dessas infecções, já que esses vírus podem infectar diretamente o SNC causando mielite, encefalite e meningite (Moulin et al., 2016).

3.2 DANOS AO SISTEMA NERVOSO CENTRAL

Desde o primeiro caso de infecção por ZIKV em humanos, desordens neurológicas foram associadas a essa infecção, mostrando o tropismo do vírus pelo sistema nervoso (Dick et al., 1952; Bell et al., 1971). Estes estudos descrevem, de maneira breve, a neuropatologia induzida por inoculação intracerebral do ZIKV em camundongos adultos e recém-nascidos, assim como a neurodegeneração e reatividade dos astrócitos no hipocampo (Bell et al., 1971).

Bem como DENV, o ZIKV também é capaz de infectar células tronco neuronais (NSC- Neuronal Stem Cells). Porém, o ZIKV leva à uma desregulação do ciclo celular e consequente apoptose, enfraquece a formação de neuroesferas, diminui a taxa de crescimento de organoides de cérebro humano (Tang et al., 2016), reduz a capacidade de NSC se diferenciar em neurônios e astrócitos (Liang et al., 2016), além de inibir a

proliferação de células progenitoras neuronais ((NPC) Neural Progenitor Cells) (Liang et al., 2016; Qian et al., 2016).

Organoides cerebrais, que são modelos tridimensionais originados a partir de células tronco, em diferentes estágios de neurogenese, foram expostas ao ZIKV por 24 horas. Foi observado que a maior parte das células infectadas eram NPC. Porém, o vírus também foi detectado em neurônios imaturos, células progenitoras intermediárias e astrócitos mesmo que em uma menor proporção (Qian et al., 2016). Ainda, a infecção por ZIKV em organoides além de causar os efeitos acima citados induz apoptose e a redução da proliferação, não só de células infectadas, mas também das não infectadas (Qian et al., 2016).

No SNC, o ZIKV pode se ligar à célula hospedeira utilizando diferentes fatores de aderência como DC-SIGN e receptores de ligação de fosfatidilserina, onde AXL parece ser o principal receptor para entrada em fibroblasto de pele humana (Hamel et al., 2015), micróglia e astrócitos (Meertens et al., 2017), e células endoteliais da barreira hematoencefálica (BHE) (Liu et al., 2016).

Estudos em cultura primária de cérebro humano demostram que o ZIKV infecta não só células tronco neuronais como também células precursoras de oligodendrócitos (OPC- Oligodendrocitos Precursor Cells), astrócitos e micróglia (Retallack et al., 2016). Micróglia, oligodendrócitos e astrócitos fazem parte da neuróglia, constituem aproximadamente 70% do total das células do SNC, e são reconhecidas como importantes elementos neuromoduladores, neurotrópicos e neuroimunes (Chao et al., 1996).

4 VACINAS

Algumas vacinas contra Flavivirus já estão inseridas no mercado, como os casos de sucesso das vacinas contra Febre Amarela, Encefalite do Carrapato e Febre do Nilo. Estas vacinas são produzidas utilizando diferentes estratégias como; (i) vírus atenuados ou inativados, (ii) proteínas recombinantes e (iii) partículas subvirais expressas em diferentes sistemas (Martin-Acebes e Saiz, 2012), o que poderia ser reprodutível com outros Flavivirus.

Vacinas de ZIKV inativado e com adjuvante de alúmem mostraram proteção completa em macacos Rhesus (Abbink et al., 2016) e camundongos (Larocca et al., 2016). No entanto, mesmo vacinas de vírus atenuados sendo utilizadas em vários casos contra diferentes infecções virais, este tipo de vacina é normalmente contra-indicada pela

OMS (Organização Mundial de Saúde) para mulheres grávidas, e algumas vezes para crianças (Who, 2015).

No que se trata de vacinas de DNA, até o momento nenhuma vacina com esse tipo de tecnologia foi licenciado para uso em humanos. Porém, vacina de DNA contra ZIKV expressando o comprimento total da proteína prM-E induz completa proteção contra viremia tanto em camundongos (Larocca et al., 2016) quanto em macacos Rhesus (Abbink et al., 2016), bem como uma vacina recombinante com um híbrido ZIKV/JEV de prM-E, mostra um alto nível de proteção contra a viremia em macacos Rhesus (Dowd et al., 2016). Até mesmo anticorpos IgG purificado de camundongos vacinados com plasmídeo contendo sequencias das proteínas prM- E do ZIKV em uma vacina de DNA, são capazes de conferir uma proteção passiva em camundongos e macacos Rhesus (Larocca et al., 2016).

Por conta das características das infecções flavivirais, vários pontos específicos devem ser levados em consideração. Existe uma grande preocupação da possível interação da imunidade pré-existente contra outros Flavivirus com a neutralização e/ou aumento da infecção, já que muitos Flavivirus co-circulam em áreas endêmicas de ZIKV, incluindo DENV que é capaz de causar Aumento da infecção Dependente de Anticorpo (ADE- Antibody Dependent Enchancement) e a relação entre ZIKV e infecções anteriores por DENV já foi demonstrada (Dejnirattisai et al., 2016).

Até o presente momento, não existe vacina licenciada para a prevenção do ZIKV. Várias vacinas estão em fase de teste pré-clínico ou de desenvolvimento (aproximadamente 40 vacinas) (Soriano-Arandes et al., 2018). Uma vacina de DNA contra ZIKV desenvolvida pelo NIH (Instituto Nacional de Saúde-EUA), encontra-se na fase II de testes clínicos (Nih, 2017).

Considerando as limitações acima citadas, incluindo a restrição do uso de alguns tipos de vacinas em gestantes (as quais seriam um dos principais públicos-alvo devido a possibilidade de transmissão vertical), em crianças recém-nascidas e também o risco de ADE ou neutralização da vacina devido a imunidade pré-existente oriunda de outros Flavivirus, a busca por antivirais contra o ZIKV tem se tornado o foco de estudo de muitas pesquisas.

5 ANTIVIRAIS

Atualmente, não existe antiviral específico contra qualquer tipo de Flavivirus, o tratamento é geralmente direcionado ao alívio dos sintomas e manutenção do bem estar do paciente com analgésicos e antitérmicos. Desde a epidemia global do ZIKV, e principalmente da América do Sul, vários candidatos a antivirais vem sendo testados in vitro e in vivo (Saiz e Martin-Acebes, 2017). Estes candidatos incluem: análogos de nucleotídeos, inibidores de síntese de nucleosídeos e de polimerases, imunomoduladores, antibióticos, anti-inflamatórios, antimaláricos, anti-helmínticos, dentre várias outras classes de compostos que vem sendo testadas, incluindo ou não as que são baseadas em uma biblioteca de compostos que normalmente já são aprovados pelo FDA (Food and Drugs Administration) como tratamento para outras doenças.

Como mencionado anteriormente, a proteína NS5 do ZIKV é uma RNA polimerase RNA-dependente responsável pela replicação do genoma viral, e por sua importância e estrutura bem caracterizada tem sido um alvo valioso na busca por antivirais. Análogos e derivados de nucleosídeos capazes de ter como alvo a polimerase viral e não a da célula hospedeira são normalmente seguras para uso em humanos (De Clercq e Neyts, 2009).

Vários análogos e derivados de nucleosídeos tiveram sua capacidade de serem incorporados na replicação da cadeia de RNA nascente do ZIKV testada, cinco deles ((7-deaza-2=-C methyladenosine [7-deaza-2=-CMA], 2=-Cmethyladenosine [2=-CMA], 2=-C methylcytidine [2=-CMC], 2=-C-methylguanosine [2=-CMG], e 2=-C methyluridine [2=-CMU]) mostraram uma efetiva redução da replicação viral em células Vero (Eyer et al., 2016). De maneira similar, o medicamento Sofosbuvir se mostrou eficaz em inibir a replicação do ZIKV in vitro (Sacramento et al., 2017) e também in vivo, utilizando como modelo camundongos imunodeficientes (Bullard-Feibelman et al., 2017). Além dos citados acima, vários outros compostos que utilizam do mesmo mecanismo foram testados com resultados promissores contra ZIKV, porém nenhum deles ainda foi licenciado para tal propósito (Saiz e Martin-Acebes, 2017).

Assim como a importância da proteína NS5 foi destacada, a protease NS2B-NS3 também tem papel fundamental, uma vez que é responsável por clivar a poliproteína viral (Gruba et al., 2016), tem participação na replicação do RNA (Lennemann e Coyne, 2017) e na modulação da resposta autofágica da célula hospedeira (Tian et al., 2016). Essa protease vem se destacando como o principal alvo terapêutico na busca por novas drogas antivirais (Rut et al., 2017). De fato, pequenas moléculas não peptídicas (um total de 71),

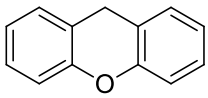
tendo NS2B-NS3 como alvo, mostraram significativa redução da replicação viral (Lee et al., 2017).

Anticorpos já foram usados como antivirais de maneira satisfatória. A transferência passiva de anticorpos humanos neutralizantes para camundongos gestantes suprimiu a replicação do ZIKV e a microcefalia dos filhotes (Sapparapu et al., 2016). Outro exemplo de uso foi de anticorpos monoclonais contra o DIII da proteína E do ZIKV, mostrando que esse bloqueio era suficiente para proteger os camundongos de uma infecção letal de ZIKV (Stettler et al., 2016).

Compostos presentes em vários produtos de origem natural também podem ter como alvo partículas virais, como o galato de epigalocatequina de polifenol ([EGCG] comumente encontrado no chá verde) (Carneiro et al., 2016) e cloreto de delfinidina (Vázquez-Calvo, 2017), que possuem atividade anti-ZIKV provavelmente por conta de um efeito virucida.

6 XANTENODIONAS

O xanteno (9H-xanteno) possuem diversas propriedades biológicas e por isso tem atraído a atenção de muitos grupos de pesquisa. Estas propriedades incluem usos como corantes (Bhowmik e Ganguly, 2005), fotossensibilizantes para terapia fotodinâmica (Ion et al., 1998), antiviral, antibacteriana, anti-inflamatória, antidepressivo e antimalárica (Negi et al., 2013).



xanteno (9*H*-xanteno)

Figura 02: Estrutura do xanteno.

Uma diversidade de xantenos tem sido isolada de fontes naturais, como por exemplo, as xantonas (9H-xanten-9-onas), que possuem como característica estrutural a presença de dois anéis aromáticos fundidos a uma *γ*-piranona. A primeira xantona de origem natural, *α*-mangostina, foi isolada em 1855, a partir da casca de frutos de mangostão (Garcinia mangostana Linn.) pelo químico alemão Dr W. Schmid. Hoje existem relatos de isolamentos destes compostos em outras famílias de plantas superiores, além de fungos, liquens e bactérias (Masters e Bräse, 2012).

As xantonas podem ser classificadas como: diidro- (3,4), tetraidro- (5,6) e hexaidro-xantona (2) em função do número de insaturações presentes na estrutura, sendo as duas primeiras raramente encontradas na natureza (Negi et al., 2013).

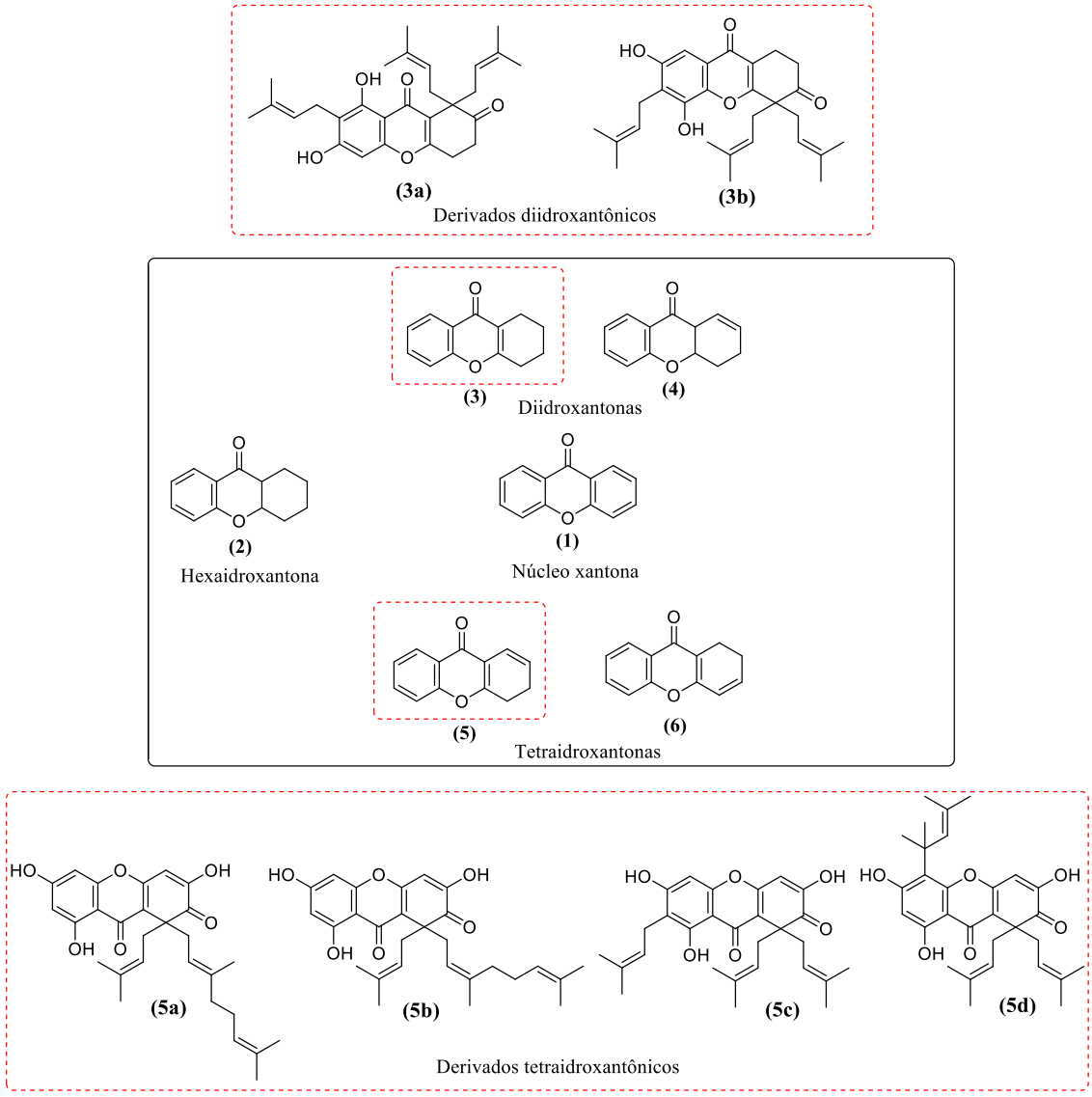
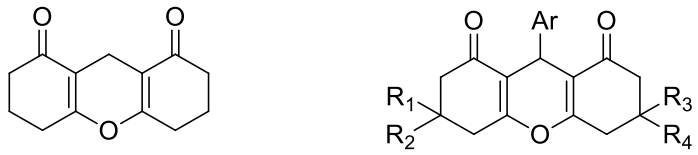


Figura 03: Subclasses das xantonas e derivados naturais diidroxantona (3) e tetraidroxantona (5), adaptado de (M. et al., 1991; Pinto et al., 2011; Masters e Bräse, 2012)

As xantenodionas, também conhecidas como 1,8-dioxo-octaidroxanteno, são derivadas de xanteno que tem como característica a presença de um núcleo pirano fundido a dois anéis cicloexen-2-ona. Este derivado sintético por sua vez tem atraído à atenção de químicos orgânicos e sintéticos devido suas atividades antiproliferativas (Mulakayala et al., 2012), leishmanicida (Nisar et al., 2013), antibacteriana e antifúngica (Rahimifard et al., 2016).



Núcleo xantenodiona Típico padrão de substituição

Figura 04: Estrutura do núcleo xantenodiona e padrão de substituição tipicamente descrito na literatura.

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ANTIVIRAL EFFECT OF XANTHENEDIONES (AND INTERMEDIATES INVOLVED IN THEIR SYTHESIS) AGAINST ZIKA VIRUS

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ABSTRACT

Zika Virus (ZIKV), an arbovirus that belongs to the *Flaviviridae* family, has become a global concern since its outbreak in the Americas in 2015. With symptoms similar to other *Flavivirus* as Dengue and Yellow Fever viruses, infections by ZIKV have also been related to several neurological complications such as microcephaly in newborns and Guillain-Barre syndrome. Considering the high prevalence of ZIKV infection in certain areas, the risks the virus poses to fetal brain development, and the fact that there is no vaccine or specific prophylaxis available, an effective treatment capable of preventing the infection is of potential interest. Therefore, in the present investigation, the antiviral activity on ZIKV of a group of xanthenodiones and intermediate ketones involved in their synthesis was evaluated for the first time. It was found that the compound 2-(2,6-dichlorobenzylidene)cyclohexane-1,3-dione **27** was able to complete inhibit the viral infection of Vero cells as well as to significantly reduce viral load in the brains of newborn Swiss mice. These effects are related to a direct interaction of the compound with the viral particle, blocking the viral adsorption.

KEYWORDS: Zika Virus, Xanthenodiones, Antiviral, Ketones, Flavivirus

1 INTRODUCTION

The Zika Virus (ZIKV) was first isolated in 1947 from the serum of a sentinel monkey in the Zika Forest in Uganda (Dick et al., 1952). Even though epidemics of this virus were reported at Yep Island and other countries outside Africa (Duffy et al., 2009), ZIKV only became a global concern with its introduction into the Americas in 2015 (Zanluca et al., 2015). In January 2016, cases of ZIKV infection were notified in twenty-eight countries (Samarasekera and Triunfol, 2016). Only in Brazil, 216.207 probable cases of Zika fever were registered in 2016 and eight deaths confirmed by laboratory analyses (SVS/MS, 2017b).

ZIKV belongs to the family *Flaviviridae*, genus *Flavivirus* (Kuno et al., 1998). It can be separated in two lineages, namely African and Asian, being the latter one responsible for the outbreak in the Americas (Wang et al., 2016). It is an enveloped virus, its genome is composed of a single-stranded RNA molecule of positive polarity about 11 kb in length (Donald et al., 2016; Kuno and Chang, 2007) coding three structural proteins [capsid (C), premembrane/membrane (prM/M), and envelope (E) proteins] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B E NS5) (Dai et al., 2016; Kuno and Chang, 2007)

ZIKV is mainly transmitted to humans through the bite of the female mosquito of the genus *Aedes* (WHO, 2016) and the symptoms are alike to other arboviral infections, which include joint pain, fever, rash, headache and myalgia (Ahmad et al., 2016). Since the outbreak in France Polynesia, ZIKV has been associated with neurological and ophthalmological complications, including microcephaly in newborns and Guillain-Barre syndrome in adults (Oehler et al., 2014; SVS/MS, 2017a). Along with the risk of the vertical infection (Oliveira Melo et al., 2016), the possibility of congenital malformation

is remarkable. In fact, the ZIKV infection was already confirmed in newborns with microcephaly in France Polynesia, Brazil and other countries in Latin America. In these countries, the rate of cerebral abnormalities increased twenty times during the outbreaks (Brasil et al., 2016; Paploski et al., 2016; Ventura et al., 2016).

To date, there is no licensed vaccine for the prevention of ZIKV infection. Many of the vaccine candidates are under development or pre-clinical phase (Soriano-Arandes et al., 2018). In addition, there is no specific antiviral drug approved against any *Flavivirus*. The treatment of the infection is usually directed to the relief of the symptoms and maintenance of patient well-being with analgesics and antipyretics (Saiz and Martin-Acebes, 2017). Considering the importance that an antiviral drug would have during an outbreak, a variety of compounds has been screened for their antiviral effect both *in vitro* and *in vivo.* These compounds include nucleoside analogs/derivatives, polymerase and nucleoside inhibitors, immunomodulators, antibiotics, anti-inflammatory substances, antimalarials, anthelminthic drugs, among others (Saiz and Martin-Acebes, 2017).

The xanthenodiones (1,8-dioxooctahydroxanthenes) are a group of heterocyclic synthetic compounds that present as structural feature a pyran nucleus fused on either side with cyclohex-2-enone rings. These substances display several biological activities including antifungal, antibacterial, anti-inflammatory, leishmanicidal, and antitumor (da Silva et al., 2017). The xanthenodiones resemble the xanthones which also display important pharmacological activities including antiviral, antibacterial, anti-inflammatory, antidepressant and antimalarial (Negi et al., 2013).

Considering the importance of an antiviral for the treatment of ZIKV and the already described biological activities both for xanthenodiones and xanthones, in this paper it is described the evaluation of the antiviral activity of xanthenodiones against ZIKV. During

the preparation of the xanthenodiones, intermediate ketones obtained and their antiviral profile was also evaluated.

2 MATERIAL AND METHODS

2.1 Synthesis

2.1.1 Generalities

All reagents were purchased from commercial sources (Sigma Aldrich - St. Louis, MO, US and Vetec - Rio de Janeiro, Brazil) and were employed as received. Solvents were procured from Vetec (Rio de Janeiro, Brazil) and used as received. The ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Varian Mercury 300 instrument (Varian, Palo Alto, California, US), using $CDCl_3$ and DMSO-d_6 as solvents. Hydrogen nuclear magnetic resonance (NMR) data are presented as follows: chemical shift (δ) in ppm, number of hydrogen atoms, multiplicity, J values in Hertz (Hz). Multiplicities are shown as the following abbreviations: s (singlet), d (doublet), d_{ap} (apparent doublet), t_{ap} (apparent triplet), dd_{ap} (apparent double of a doublet) m (multiplet). Infrared spectra (IR) were obtained employing the equipment Agilent 660-IR (Santa Clara, California) with accessory GladiATR. Melting points are uncorrected and were determined using MQAPF-301 melting point apparatus (Microquimica, Palhoça, Santa Catarina, Brazil). Low resolution mass spectra were obtained on a SHIMADZU GCMS-QP5050A instrument (Kyoto, Japan) by direct injection using the following temperature program: 40 °C min^{-1} until temperature reaches 60 °C, and then 80 °C min^{-1} until temperature reaches 300 °C; the detector temperature was 280 °C. Analytical thin layer

chromatography analyses were carried out on TLC plates recovered with 60GF254 silica gel.

2.1.2 General procedure for the preparation of xanthenodiones

A round-bottomed flask (25 mL) was charged with a 1,3-diketone (2.00 mmol), aldehyde (1.00 mmol) and $ZrOCl_2 \cdot 8H_2O$ (12 mg, 2 mol%). The mixture was stirred at 85 °C and the progress of the reaction was monitored by TLC analysis. After completion of the reaction, the mixture was cooled down to room temperature. Thereafter, it was added 50 mL of dichloromethane and the mixture was kept under stirring for about 30 minutes. Then, the catalyst, which is insoluble in dichloromethane, was separated by filtration. After that, 50 mL of ethanol was added to the filtrated and the system was kept undisturbed for crystallization process. The structures of xanthenodiones are supported by the following data.

2.2 Cell Culture and Viral Stock

A confluent culture flask (75 cm^2) with C6/36 cells (ATCC® CRL-1660™) was inoculated with 1 mL of the ZIKV strain (ZIKV/sapiens/Brazil/PE243/2015). The flask was allowed to stand on a shaker for 1 hour for viral adsorption. After that, 9 mL of the L-15 medium (Leibovitz's L-15 Medium) with 2% FBS (Fetal Bovine Serum) was add to the flask which was incubated at 28 ºC up to 7 days. The medium was removed from the flask, centrifuged at 800 x g for 10 min at 4 ºC. To the supernatant, FBS was added so that a final concentration of 10% was achieved and it was stored at -80 ºC. Vero cells (ATCC® CCL-81™), kept at 37 °C and 5% CO_2 atmosphere with Dulbecco's Modified

Eagle's Medium (DMEM) supplemented with 10% FBS, were used in the viral titration and Plaque Reduction Neutralization Tests (PRNT). The cell line was low in passage number for reproducible results.

2.3 Viral Titration

The Vero cells ($1x10^6$ cells/well) were seeded on 24-well plates and kept at 37 ºC and 5% CO_2 for 24 hours or until confluent. A volume of 100 µL of tenfold dilution of the viral suspension was added to the cell monolayer (triplicate) and the plate was allowed to stand on an orbital shaker for 1 h for adsorption. The media was removed and replaced by the overlay solution (carboximetilcelulose 3% + DMEM supplemented with 2% FBS in a 1:2 ratio). The plate was incubated at 37 ºC and 5% CO_2 up to 7 days and then fixed with formaldehyde 10% and stained with 5% violet crystal. The lysis plates were counted and viral titer expressed in Plate Forming Unit per microliter (PFU/mL).

2.4 Cytotoxicity Assay

To determine the 50% cell cytotoxic concentration (CC_{50}), the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) Invitrogen™] method was performed. The Vero cells ($5x10^3$/well) were seeded on 96-well plates and incubated until confluence. The cells were exposed to a serial two-folder dilution of the compounds, starting at 1000 µmol L^{-1} until .1 µmol L^{-1}, during 24 hours. After that, the medium was replaced by DMEM containing MTT in a final concentration of 0.5 mg/mL and incubated for 4 hours. The supernatant was removed and 100 µL of DMSO (dimethyl sulfoxide) per well were added to dissolve the formazan. After shaking, absorbance was measured at

540 nm and the result expressed in a non-linear regression in which the negative control was considered 100%.

2.5 Antiviral assay

The antiviral activity of twenty-eight xanthenodiones and three ketones on infected Vero cells was carried out using the CC_{50} concentration determined as described in section 2.4. The cell viability was measured by the MTT method. After 80% of cell confluence on 96-well plates, the virus was previously incubated for 1 hour at 37 ºC with the evaluated compounds and then placed on the cell monolayer for 1.5 h. Thereafter, the content of the wells was replaced by DMEM and the plates incubated up to 7 days at 37 ºC and 5% CO_2. Negative control group (cells with DMEM only) was considered 100% viability and positive control group (cells infected with ZIKV) was considered 0% viability. Compounds able to maintain viability equal to or higher than 50% were selected for further experiments.

2.6 Virucide assay

In order to evaluate the direct effect of a compound on the viral particles, plaque-forming assay was performed. Thus, ZIKV solution (200 PFU/mL) was mixed with equal volume of different concentrations of the tested compound starting from CC_{50} value found to the cells, incubated for 1 hour, inoculated on Vero Cells monolayer on 24-well plates, and incubated for additional 1 hour under constant shaking. After that, the viral suspensions containing the compounds were aspirated and 1 mL of the overlay solution was added to each well. After 5 to 7 days of incubation, according to the presence of cytopathic effects,

the cells were fixed and stained as previously described (section 2.2). Based on the lysis plate, a dose-response viral activity reduction was used to determine the effective concentration (EC_{50}) value.

As recommended by FDA (Evaluation and Research, 2006), specific antiviral activity is determined using a quantitative measurement of the virus replication in the presence of increasing concentration of the evaluated compound compared to replication in the absence of it. The effective concentration (EC_{50}) is the concentration of a compound at which viral activity is inhibited by 50%. The dose-response viral activity reduction was used to determine the EC_{50} value through a non-linear regression. The ratio between CC_{50} value and EC_{50} is defined as the Selectivity Index (SI). It is desirable to have a high SI, which means a maximum antiviral activity with minimal cell toxicity.

2.7 Mechanism of action assays

Plaque-forming assay was conducted at different stages of infection, using different conditions, in order to verify how a compound acts on the viral particle or if it acts directly on the cell.

2.7.1 Pre infection treatment – assessment of the viral infection inhibition by cell

To investigate whether a compound blocks the viral infection protecting the cells from the virus, the compound is preincubated with the cells. In this regard, Vero cells were incubated, at 37 ºC, with different concentrations of the compound under evaluation for 3 h, with periodical shake every 20 minutes. After that, the compound was removed, the cells were washed with DMEM, 100 PFU of the ZIKV suspension was added, and the

plates incubated 1 h under constant shaking. Overlay solution was added after 1 h of the incubation with the virus. The plates were kept at 37 °C and 5% CO_2 atmosphere up to 7 days, being fixed and stained as previously described (section 2.2). The lysis plaques were compared with the lysis from control group (virus without treatment).

2.7.2 Post infection Treatment – viral replication inhibition assessment

To assess whether a compound could act on the cell cytoplasm inhibiting the viral replication, confluent Vero cells were incubated with 100 PFU of the virus suspension on 24-well plates. After 1 hour of adsorption, cells were treated with different concentrations of the compound under evaluation for 2 hours. Subsequently, the media with the compound was removed and replaced by the overlay solution, and the plate incubated up to 7 days, fixed and stained as previously mentioned (section 2.2).

2.7.3 Viral adsorption inhibition assessment

To evaluate the capability of a compound to act on the viral adsorption, the cells were kept at 4 ºC during the period of the assay; at this temperature, the virus is able to attach to the cells but not to internalize. Thus, 24-well plates with confluent Vero cells was cooled down to 4 ºC for 30 minutes. Afterwards, the medium was removed and 200 PFU of the virus suspension was added along with solutions of different concentrations of the compound under evaluation. The plate was maintained at 4 ºC for 2 h. After the incubation period, the cells were washed twice with cold Phosphate Buffered Saline (PBS) pH 7.4. As a control to the methodology, half of the viral controls were wash with Citrate Buffer (pH 3.0), which is capable of disestablishing the viral particles on the cell membrane

causing the elimination of the particle after washing. The wells were then filled with 1 mL of the overlay solution, incubated up to 7 days, fixed and stained in the as previously mentioned (Section 2.2).

2.7.4. Inhibition of viral internalization assessment

To evaluate the capacity of a compound to act on the viral internalization, the virus was incubated at 4 ºC, allowing the virus to attach but not internalize. Therefore, similarly to what was described in the previous assay (section 2.7.3), the plate with the Vero cell monolayer was incubated at 4 ºC for 30 min. After that, 100 PFU of the virus suspension was added and the plate was kept at 4 ºC for 2 h. Later, the cells were washed and the temperature increased to 37 ºC. Solutions of different concentrations of the compound were added, the plate was maintained at 37 ºC for 2 h and after that, the medium was removed and the cells washed with citrate buffer (pH 3.0) and PBS to normalize the pH to 7.4. Subsequently, 1mL/well overlay was added and the plate incubated up to 7 days, fixed and stained as previously described (Section 2.2).

2.8 *In Vivo* assessment

2.8.1 Ethics statement

The animals used in this study were kept under specific conditions in the Animal Research Facilities of the Universidade Federal de Viçosa, Viçosa, Minas Gerais State, Brazil. The described methodology was evaluated and approved by the Ethics Committee on Animal

Use (CEUA) of the Universidade Federal de Viçosa, process nº 21/2017. All the steps on the investigation were conducted as required by the Ethics Committee.

2.8.2 Animal Inoculation

Two groups (control and treated groups) of ten one-day-old neonatal immunocompetent Swiss mice (*mus musculus*) were inoculated intraperitoneally with 10 µL of the viral suspension at $2x10^3$ PFU/mL, previously incubated with the compound under assessment, at the higher non-cytotoxicity concentration for 1 h at 37 °C, simulating the *in vitro* virucide assay. Control group received only the virus. The animals were observed for 5 days and then euthanized starting from the day 5 post infection until day 9.

2.8.3 Animal Euthanasia and Brain removal

The animals were euthanized following the "Perfusion Surgery Protocol" (Gage et al., 2012). Prior to surgery, a ketamine/xylazine mixture (up to 80 mg/kg body weight ketamine and 10 mg/kg body weight xylazine) was administered via intraperitoneal injection. Once the animal has reached a surgical plane of anesthesia, the procedures were taken. A small incision in the diaphragm and along the entire length of the rib cage was made so that to expose the pleural cavity. The sternum was lifted, exposing the heart, and a small incision on right atrium was made. With a syringe and needle, about 6 mL of PBS was slowly inoculated trough the posterior end of the left ventricle, so that the buffer could wash all the blood carrying way the circulating virus. After the perfusion, the animal head was removed using scissors; the skull was exposed trough a midline incision

along the integument from the neck to the nose. Next, the skull was opened and the brain removed, weighted, divided in vials and stored at -80 ºC freezer for subsequent analyses.

2.8.4 Brain Tissue Titration

Part of the brain was used for the viral titration on Vero cells. The brain pieces frozen in PBS supplemented with 10% FBS were disrupted with a small pistil, centrifuged for 10 min at 800 x g at 4 ºC. The supernatant was collected, filtered in a 0,22 μm syringe filter and tittered as described in item 2.2.

2.8.5 qPCR

For the molecular analyses, part of the brain was frozen in QIAzol Lysis Reagent (QUIAGEN®), and the RNA extraction was made fallowing the manufacturer instructions. GoScript™ Reverse Transcription System (Promega®) was used for cDNA production. Absolute qPCR reaction performed in an Eco Realtime PCR System (Illumina®) using 5 μL Applied Biosystems™ TaqMan™ Fast Advanced Master Mix, 1 μL 2,5 mM TaqMan QSY Probe (sequence ZIKV1107: 6FAM-AGC CTA CCT TGA CAA GCA AGA CAC TCA A-QSY), 0.5 μL (400 nmol L^{-1}) of each primer, 500 ng of cDNA template, and water to complete 10 μL. Primer sequence: ZIKV 1086 5'-CCGCTGCCCAACACAAG-3' and ZIKV 1162c 5'-CCACTAACGTTCTTTTGCAGACAT-3' (Gupta et al., 2016).

2.9 In Silico analyses – Docking

The docking analysis was made using *Schrodinger*, LLC, New York, NY -2016-2 and *PyMol*, Molecular Graphics System, Version 1.7.4.5. Ligands were prepared in the program Ligprep employing the OPLS_2005 force field, with protonation states predicted with Epik at pH 7.0± 2.0 for E protein. The Zika virus E protein PDB code 5GJ4, chosen as the receptor, was prepared with the Protein Preparation Wizard, with removal of all waters and addition of hydrogens based on PROPKA calculations at pH 7.0. Docking calculations were performed with the software Glide, employing the Induced Fit docking and Glide SP. Two grids were used, one centered in the amino acid Alanine 264 (called central), and another in the amino acid Threonine 366 (called extremity). The receptor was considered as flexible. Docking results were ranked based on their docking score and the top ranking poses for each compound were analyzed with the software PyMOL.

2.10 Zeta Potential

Zeta Potential (surface electrical charge) was measured using a Zetasizer Nano ZS apparatus (Malvern Instruments Ltd. UK). The measurements were carried out at 24 ºC using a capillary cell with an equilibration time of 120 s. Tree measurements of 10–100 runs were performed using the automode option. A volume of 800 µL of the virus suspension (2.10^9 PFU/mL) diluted in ultrapure water and 800 µL of the virus (2.10^9 PFU/mL) preincubated with the compound (270.80 µmol L^{-1}) for 1 h at 37 ºC were submitted to the measurements. All experiment was carried out in a neutral pH environment and water was used as dispersant. Zetasizer Software version 7.03 was used to data analyses.

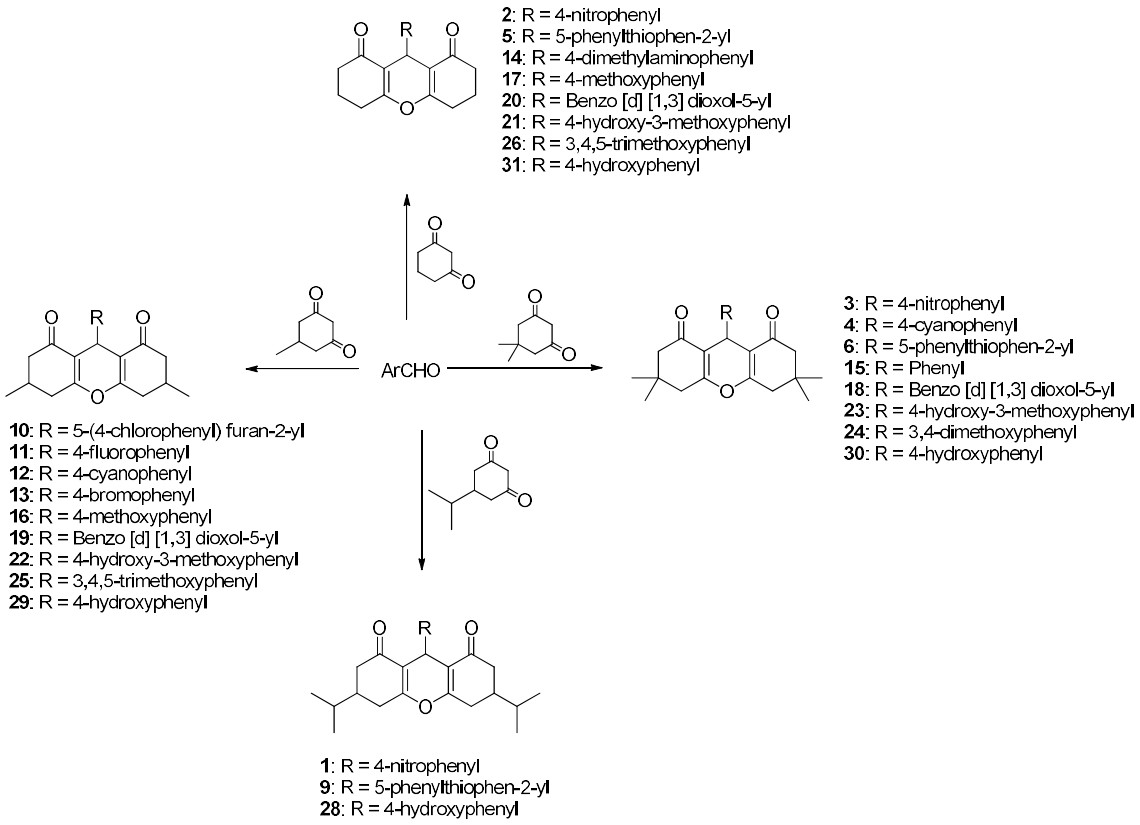
2.11 Statistical analysis

GraphPad Prism (6.0 version) was used for statistical significance of the data. Non-linear regression was used for the determination of EC_{50} and CC_{50} values. A comparison between columns in the different performed assays was carried out using analysis of variance (ANOVA).

3 RESULTS

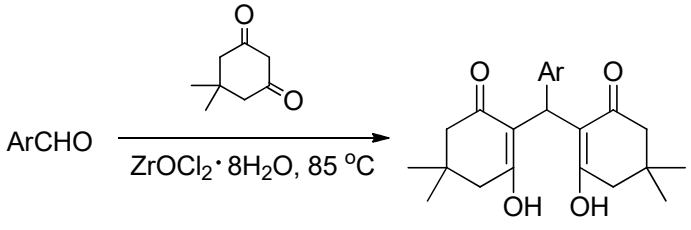
3.1. Synthesis of compounds

Our research group has been involved in the search of antiviral compounds against *Flavivirus* (Oliveira et al.). In addition, we have also become interested in the structural aspects (da Silva et al., 2017) as well as biological activities of xanthenodiones. Within this context, we decided to prepare a series of xanthenodiones and evaluated their antiviral activities. Several methods have been reported concerning the preparation of this class of compounds (da Silva, 2017). It was decided to synthesize the xanthenodiones *via* the zirconium catalyzed condensation of 1,3-diketones and different aldehydes. By using this methodology, it was possible to obtain fifty-four xanthenodiones. Because of solubility issues, however, only twenty-eighty of them were investigated concerning their antiviral effect (Scheme 1).



Scheme 1 – Preparation of xanthenodiones. Reactions were carried out at 85 °C using 1 mmol of an aromatic aldehyde (ArCHO), 2 mmol of a 1,3-diketone, and 12 mol% of $ZrOCl_2 \cdot 8H_2O$.

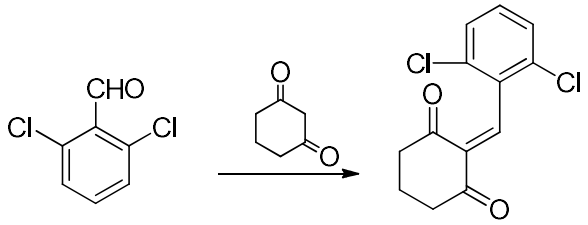
We also evaluated the tetraketones **7** and **8** which the preparation have been previously reported (da Silva et al., 2018)(Scheme 3). It should be mentioned that such tetraketones are intermediates involved in the preparation of xanthenodiones (da Silva, 2017).



7: R = 5-(4-bromophenyl) furan-2-yl
8: R = 5-(4-chlorophenyl) furan-2-yl

Scheme 3 – Reactions involved in the preparation of tetraketones **7** and **8**.

Moreover, during the attempt preparation of a xanthenodione from the 2,6-dichlorobenzaldehyde, we obtained the ketone 27, as depicted in Scheme 3, which also had their antiviral activity assessed.



27

Scheme 3 – Preparation of ketone **27**.

Therefore, a total of thirty one compounds had their antiviral activity evaluated. All the synthesized compounds were fully characterized by means of spectroscopic (IV and NMR) and spectromectric (mass spectrometry) techniques). Details about the synthesis of the compounds herein evaluated as well as spectroscopic data of them can be found in the supplementary material.

3.2 Cytotoxicity

Once prepared, the compounds **1-31** had their cytotoxicity evaluated against Vero cells. As can be seen in Table 1, the compounds present low toxicity against them (in general, CC_{50} higher than 170 μmol L^{-1}). Exceptions to this generalization are the substances **6**,

402 **7**, **8** and **21**. Concentrations used in subsequent assays do not present a reduction in cell
403 viability.

404
405
406 Table 1 - Cytotoxicity of the compounds **1-31** against Vero cells
407

Compound	CC_{50} (µmol L^{-1})	Compound	CC_{50} (µmol L^{-1})	Compound	CC_{50} (µmol L^{-1})
1	231.4	**12**	344.9	**23**	241.2
2	288.0	**13**	345.4	**24**	234.4
3	292.6	**14**	326.5	**25**	712.9
4	373	**15**	211.1	**26**	344.2
5	345.3	**16**	343.7	**27**	270.8
6	27.64	**17**	362.0	**28**	290.4
7	62.08	**18**	179.4	**29**	601.8
8	91.21	**19**	268.9	**30**	220.1
9	336.8	**20**	288.7	**31**	1173
10	338.8	**21**	53.27		
11	324.1	**22**	360.01		

408
409
410 *3.3 Antiviral Screening*
411
412 Among the thirty-one compounds screened agains ZIKV, three of them (**6**, **27** and **31**)
413 were capable of maintaining cell viability around 50%, as indicated in Figure 1. These
414 compounds were selected to be evaluated in the virucide assay.
415

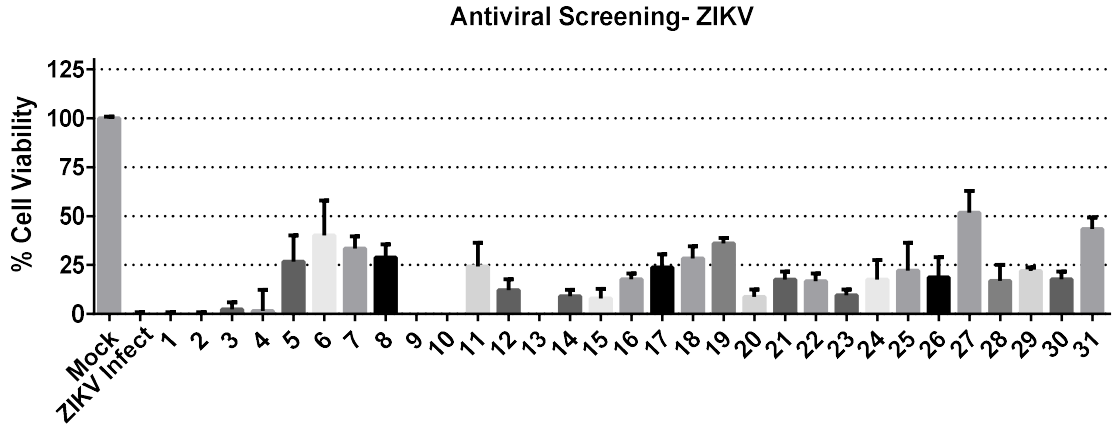


Figure 1 - Antiviral screening of compounds **1-31** against ZIKV. As a negative control, cells were infected with a MOI 1 ZIKV solution. The compounds were previously incubated with the virus for 1 hour at 37 ºC. Cell viability was measured by MTT method.

3.4 Virucide assay

The direct effect of the compounds **6, 27,** and **31** on the viral particles was evaluated ***via*** plaque-forming assay. As demonstrated in Figure 2, the compounds significant reduced the viral activity based on the lysis plate formation. Compound **31** (Fig. 2 C) was far less effective than the others, while substance **06** (Fig. 2 A) reduced lysis plate in 100% at 27.60 µmol L^{-1} and *ca.* 96% at 13.80 µmol L^{-1}. The compound **27** (Fig. 2 B) was the most effective reducing 100% of lysis plate at 33.85 µmol L^{-1}, and at the lowest tested concentration (8.46 µmol L^{-1}) the reduction was around 60%. Considering the best virucide activity of ketone **27** as well as its superior selective index (SI, Table 2), this compound was selected for subsequent assays.

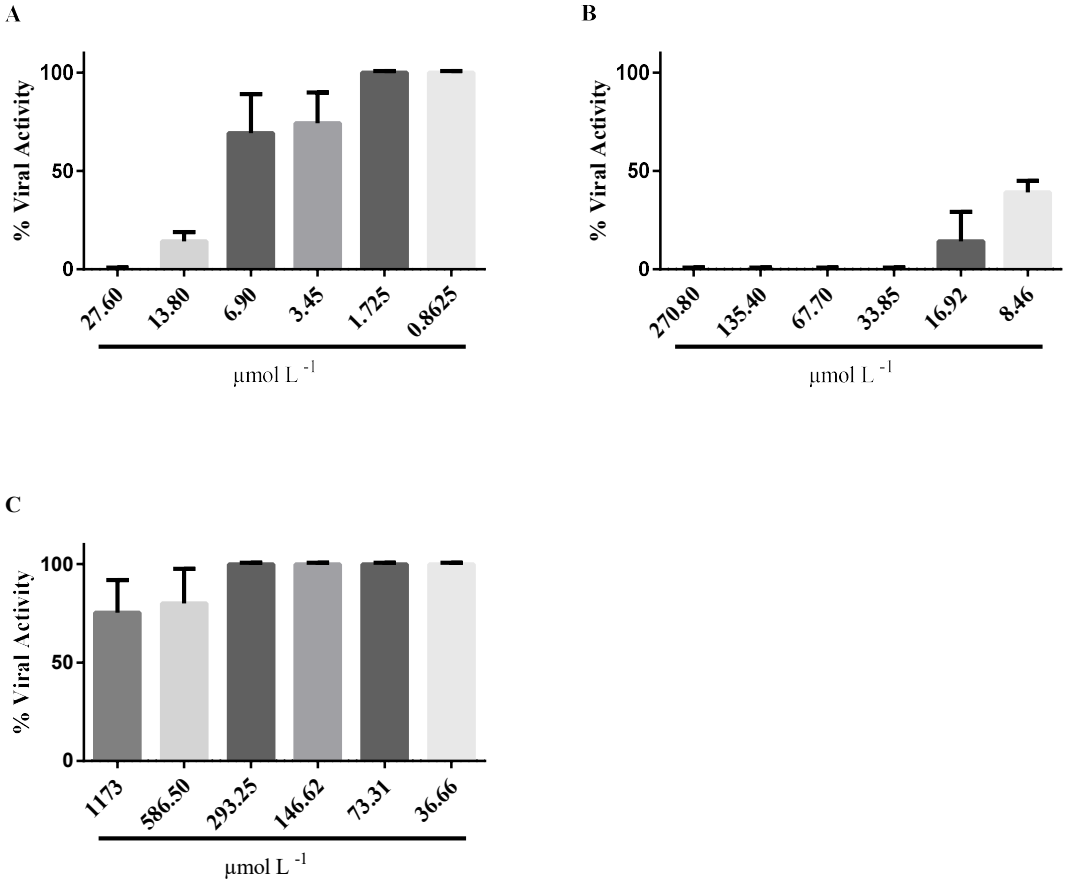


Figure 2 - Virucide Assay. The three compounds selected from the antiviral screening were used. In this assay, 200 PFU/mL of the ZIKV were mixed with equal volumes of serial dilution of the tested compounds starting from CC_{50} value, incubated for 1 hour and inoculated on Vero cells monolayer. As positive control (C+), 200 PFU/mL of the ZIKV were mixed with equal volumes of DMEM. The results of the virucide assays conducted with compounds **6**, **27** and **31** are shown in graphs (A), (B) and (C), respectively. Viral activity is based on the lysis plate formation normalized to percentage, considering positive control as 100%. **** $P \leq 0.0001$ vs (C+).

Table 2- Selective Index (SI) related to compounds **6**, **27**, and **31**

Compound	**CC_{50} (µmol L $^{-1}$)**	**EC_{50} (µmol L $^{-1}$)**	**SI**
6	27.60	7.639	3.61
27	270.8	7.038	38.48
31	1173	2479	0.473

3.5 Investigation of mechanism of action

Plaque-forming assays were conducted at different stages and conditions of infection to verify if the compound **27** acts on the viral particle or whether it acts directly on the cells.

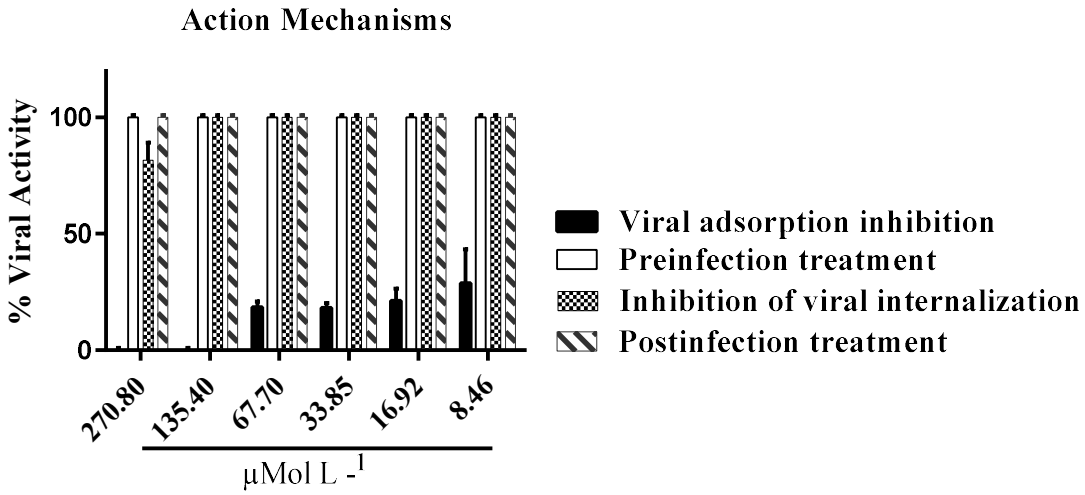


Figure 3 – Investigation of mechanism of action of compound **27**. The viral activity was measured at different stages and conditions of infection *via* the presence or absence of lysis plate formation. Viral activity was based on the lysis plate formation normalized to percentage considering (C+) as 100%. $P \leq 0.0001$ vs (C+).

3.4.1 Preinfection treatment

In this assay, even at the highest concentration of 27, there was no statistically significant reduction in the viral activity (Fig. 3), indicating that the compound **27** protection against the infection was not related to the interaction with the Vero cell receptors.

3.4.2 Postinfection Treatment

There is no statistically significant reduction of viral replication (Fig. 3) at all evaluated concentrations of compound **27**. This indicates that the compound acts directly on the viral particle at the early stages of infection.

3.5.3 Viral adsorption inhibition

The Viral adsorption inhibition assay for ZIKV showed a reduction of 100% on the viral activity (Fig. 3) at 224.4 µmol L^{-1} and 112.2 µmol L^{-1} respectively; a reduction of 80% was observerd at the lowest tested concentration (7.012 µmol L^{-1}). With these results in hands, it is possible to infer about mechanism of action of **27**, which may act directly on the viral proteins blocking the viral adsorption.

3.5.4 Inhibition of viral internalization assessment

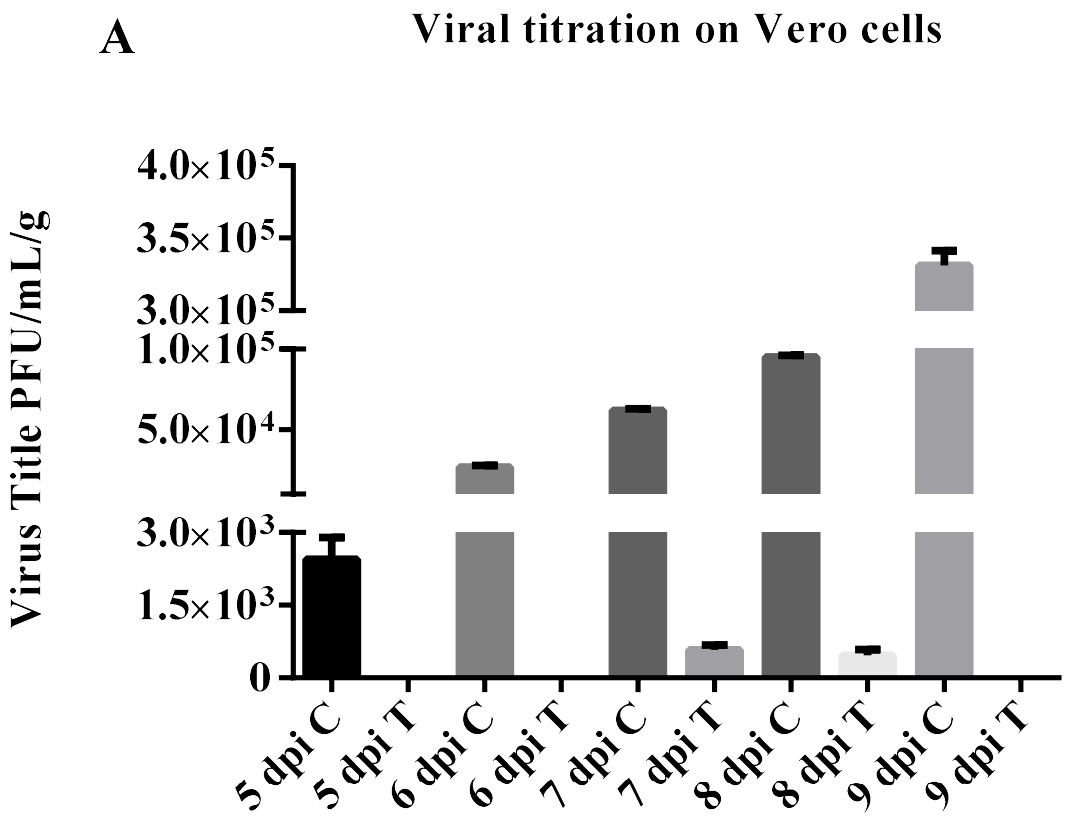
It was assessed whether compound **27** could cause the viral internalization inhibition.

3.6 *In Vivo* assessment

One-day-old mice brains represent a developmental stage equivalent to the early second trimester-stage of humans Central Neural System (CNS) development (van den Pol et al., 2017). The vertical transmission of ZIKV is possible at all stages of pregnancy; however, the highest risk period for CNS damage is during the first trimester (Cauchemez et al., 2016). Therefore, these mice were used to evaluate the virucide capacity of compound **27** on the developing brain. The animals were sedated and the perfusion method was chosen for the euthanasia to ensure that the virus found on the brains were not from the particles circulating on blood but from virus present on the brain tissue.

The brain viral titration on Vero cells presented a significant reduction on the infective viral particle load of the treated groups, when compared to the control groups (Fig. 4 A). There was no visible formation of lysis plate, showing a complete viral inhibition by the compound **27** on treated days 5, 6 and 9.

Similarly, absolute qRT-PCR analyses (Fig. 4 B) revealed a significant reduction on the number of ZIKV RNA copies when comparing treated with untreated control groups along the days post infection (dpi). This find corroborates the results obtained in the viral titration on Vero cells.



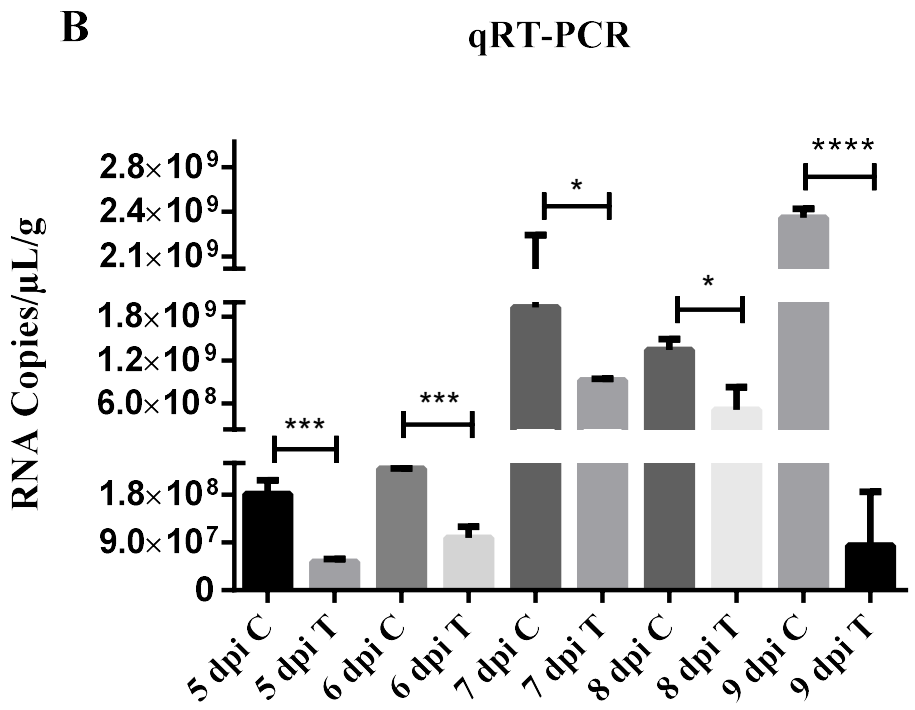


Figure 4 - *In vivo* assay. The viral titration on Vero cells (A) presets statistically significant reduction on the viral load of the animals which were inoculated with 10 µL of ZIKV ($2x10^3$ PFU/mL) preincubated 1h with compound **27** (treated group 'T') when compared with the control group (ZIKV only 'C'). Absolute qRT-PCR (B) also presents a significant reduction on the number of RNA viral copies on the treated group. (C) Control group; (T) treated group; (dpi) days post infection. **** $P \leq 0.0001$ vs (C); *** $P \leq 0.001$ vs (C); ** $P \leq 0.01$ vs (C); *$P \leq 0.05$ vs (C).

3.7 In Silico analyses – Docking

As the compound **27** presented direct effect on ZIKV particles, it was hypothesized that ketone **27** could interact with E protein of ZIKV. To shed light on this hypothesis, a docking investigation was carried out. In this *in silico* investigation, two grids were used, one centered in the amino acid Threonine 267 (called central), and the other in the amino acid Histidine 323 (named extremity). The docking results revealed that compound **27** presents higher binding affinity for the central region (energy score -4.30) of E protein

519 compared to the extremity (energy score -1.40) as shown in Table 3. The compound
520 probably binds to the junction region of the monomers of this protein, which corresponds
521 to amino acids 258-270 as previously described (Dai et al., 2016). Figure 5A shows the
522 interaction diagram of the compound.

523

524 Table 3 - Predicted hydrogen bond interactions between ZIKV protease and E protein
525 with compound **27**. Proteins crystallography structures were obtained from PDB. (H-Bond)
526 hydrogen bond

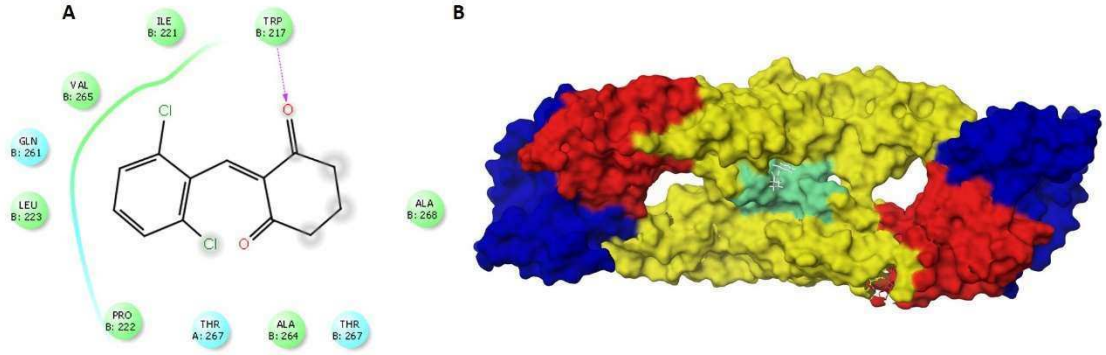
527

Target (PDB Code)	Bound		Aminoacid	Energy (Score)
	Type of interaction	Distance (Å)[a]		
E. Prot. ZIKA Extremity (5JHM)	Pi-pi stacking	5,48	HID (323)	-1,40
	H-bond	2,14	HID (323)	
E. Prot. ZIKA Central (5JHM)	H-bond	1,98	THR (267)	-4,30

528 [a] distances between donor and acceptor atoms.

529

530 As can be seen from Table 3 and Figure 5A, the carbonyl grouping of **27** plays an
531 important role in the binding to the stabilizing regions of the ZIKV E protein dimers.
532 Figure 5B shows the binding position of the compound.



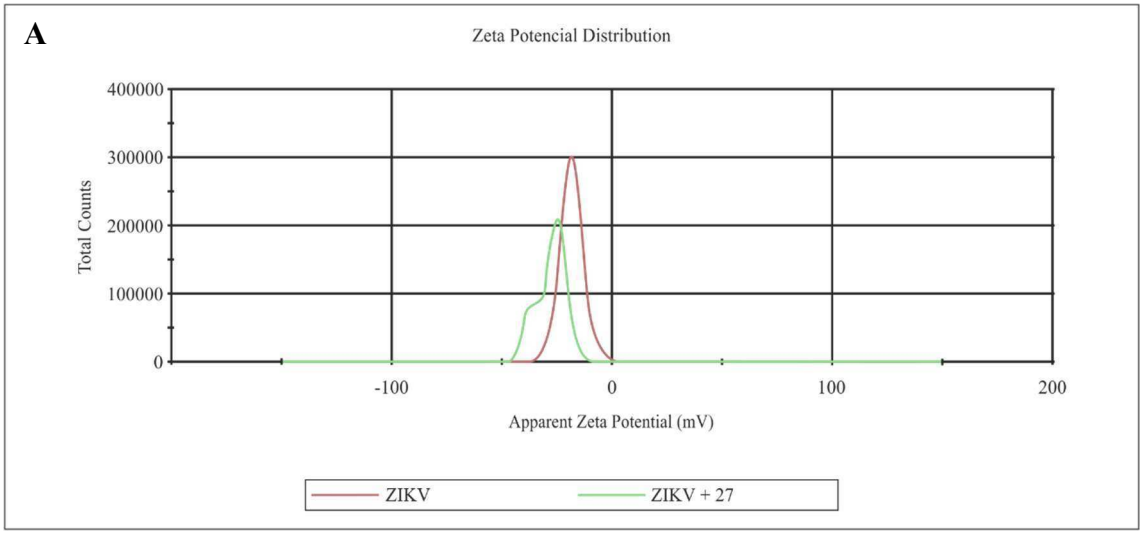
533

534 Figure 5 - (A) Bound diagram between compound **27** and ZIKV E protein (PDB code
535 5JHM). (B) Over view of the protein showing the compound interaction with the central

region (ciano) used on the *in silico* analyses (red: I domain; yellow: II domain; blue: III domain; white: compound **27**).

3.7 Zeta Potential Analysis

The Zeta Potential measurement showed a reduction in the charge of ZIKV treated with compound **27** (-27,5 mV) when compared with the ZIKV alone (-18,3 mV), indicating an interaction between ZIKV and compound **27**.



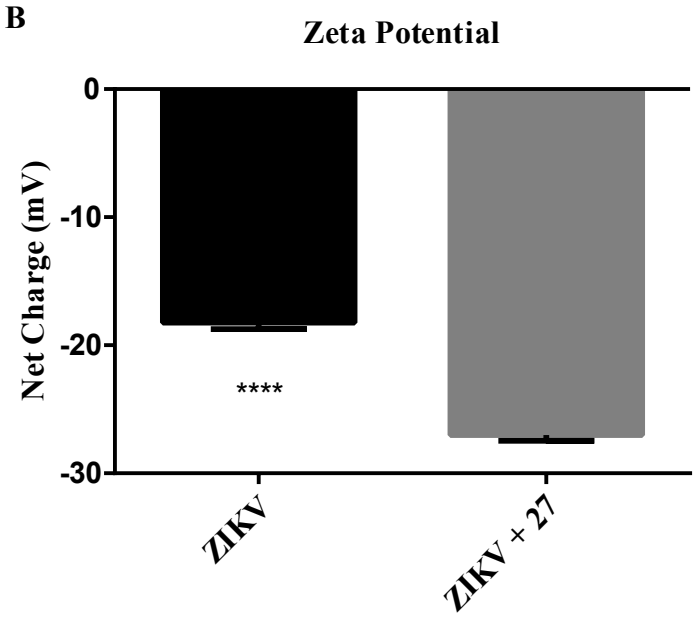


Figure 6 - Zeta Potential measurement. (A) Zeta Potential Distribution; (B) Zeta Potential (Net Charge) and statistical significance. ZIKV: Viral particles dispersed in ultra-pure water and without treatment; ZIKV + 27: Viral particles treated with the compound **27** 1 hour at 37 ºC prior to the measurements. **** $P \leq 0.0001$.

4 DISCUSSION

In this study, we prepared a series of twenty-eight xanthenodiones and three ketones and investigated their antiviral activity on ZIKV. Within this context, we initially evaluated the cytotoxicity of the synthesized compounds against Vero cells. The obtained CC_{50} values (Table 1) revealed, that in general, the compounds were barely toxic to the cells. It is important to highlight that the higher the CC_{50} value the greater the concentration of the compound that can be used with minimal cell toxicity (Evaluation and Research, 2006).

Next, the antiviral screening of the thirty-one compounds was conducted on ZIKV as the viral agent. In this assay, the viability of the cells infected with the virus was compared with the mock group as well as with the groups in which the ZIKV was previously incubated with the compounds **1-31** for 1 h at 37 ºC. Vero cell viability was determined by the MTT method. The aim of this bioassay was to select promising candidates to be used in further assays. It was selected compounds which were capable of maintain the cell viability at least near to or higher than 50%. The compounds **6**, **27** and **31** fit this criterion. However, because of its superior selective index (SI, Table 2) compound **27** was the only one chose to be used in the subsequent assays.

From the preinfection treatment assay (Fig. 3), it was possible to conclude that the compound **27** does not act directly on the cell membrane. It interacts and blocks the cell receptors or targets cellular factors directly involved in the viral life cycle. This fact has been observed for other compounds, such as chloroquine, an anti-inflammatory FDA-

approved drug. Chloroquine presents antiviral activity against several viruses, including ZIKV, through the inhibition of pH-dependent steps of viral replication (Delvecchio et al., 2016).

The NS5 enzyme and the complex NS2B-NS3 are important targets to antiviral design due to their importance to the viral cycle and replication. NS5 is a RNA-dependent RNA polymerase (RdRp) in charge of viral genome replication with a methyltransferase domain (Xu et al., 2017). The NS2B-NS3 plays a key role in virus replication by contributing to viral polyprotein processing (Gruba et al., 2016), as far as NS3 has helicase activity (Lennemann and Coyne, 2017).

Sofosbuvir, a nucleotide analog inhibitor that is commercially available for the treatment of chronic Hepatite C virus infection, was considered an option for the treatment of ZIKV. Its active metabolite is 2′-fluoro-2-C-methyl-UTP, binding to the active site of NS5 (Reznik and Ashby, 2017). Thus, Sofosbuvir efficiently inhibits the viral replication and infection of ZIKV in different cell lines such as hepatoma (Huh-7), human placental choriocarcinoma, cerebral cortex-derived neural stem cells (NSCs) and also in mices (Bullard-Feibelman et al., 2017), in SH-Sy5y neoroblastoma cells and in Baby Hamster Kidney cells (BHK) (Sacramento et al., 2017).

Taking the virus proteases as a target, our group has recently described 2-arylidene indan-1,3-diones of low cytotoxicity and high efficiency that inhibits the activity of NS2B-NS3 WNV protease *in vitro*. The same compounds were able to reduce the viral activity in cell based assays for the four DENV serotypes (DENV1-4) and ZIKV, probably due to the protease inhibition mechanism (Oliveira et al.).

In the postinfection assay conducted with compound **27**, no significant reduction of viral replication (Fig. 3 B) was noticed, demonstrating that the compound acts directly on the viral particle upon the early stages of infection instead of acting on the viral cycle in the

601 cell as would be the case if compound **27** targeted any internal viral enzymes. This
602 observation allows us to infer that there is no interference of ketone **27** after viral infection
603 and, therefore, it does not inhibit the activity of NS5 or NS2B-NS3 proteins.
604 Even though compound **27** presented a statistically significant reduction on the ZIKV
605 viral activity concerning the inhibition of viral internalization (Fig. 3) at the highest
606 concentration (270.8 μmol L^{-1}), the effect corresponded to only *ca.* 20%.
607 In the viral adsorption inhibition assay (Fig. 3), however, a reduction of 100% on the viral
608 activity was noticed at 270.80 μmol L^{-1} and 135.40 μmol L^{-1}; at the lowest concentration
609 (8.46 μmol L^{-1}) 80% reduction was observed. This trend is similar to what was observed
610 in the virucide assay and indicates that the compound **27** acts on the viral particle,
611 blocking ZIKV adsorption on the cell surface. This could be associated with a specific
612 interaction between the compound and ZIKV envelop, or more specifically, with the E
613 protein, changing the protein charge and/or conformation.
614 In Fact, E protein is also an important target for *flavivirus* drug development, since
615 monoclonal antibody binds and neutralizes circulating ZIKV *in vitro* and in mice (Dai et
616 al., 2016). Moreover, there is a hydrophobic region called fusion loop between the E
617 protein domains I and III. This region mediates the binding to the cellular receptor of the
618 virus, promotes the fusion of the virions with the target membranes, and constitutes the
619 main target for the induction of antibodies (Kostyuchenko et al., 2016).
620 Based on this data and trying to get more information about the mode of action of
621 compound **27**, *in silico* analysis was made considering the central and the extremity of
622 ZIKV E protein.
623 The obtained scores, resulted from the predicted interactions (Table 3), corroborates the
624 hypothesis that the compound may act directly on the E protein, binding to the central
625 region and blocking the viral adsorption. As represented in the *docking* diagram (Fig. 5),

the compound is able to interact with the amino acid tryptophan on the central region; more specifically, in a stabilizing region of the ZIKV E protein dimers. According to the diagram, there is a hydrogen bond between the hydroxyl group from the amino acids and the carbonyl group of the compound **27**, resulting in a more negative charge, as the amino acid Threonine has neutral charge group, it becomes negative. The cancelation of positive charges on the protein surface, making the virus more negative, leads to a viruses surface charge alteration, and this charge plays a major role in adsorption processes (Michen and Graule, 2010), adhesion and transport phenomena (Schijven and Hassanizadeh, 2000). This data became even more relevant when confronted with the Zeta Potential measurements. The result from the measurements showed that the compound **27** made the viral particle even more negative charged (Fig 6A and B). The results from Zeta Potential measurements and docking analysis show that compound **27** is able to interact with the viral particle.

According to Basak and Nandy (2016), there are tree essential phases on drug research before clinical studies: *in silico* design and analyses, *in vitro* drug testing and *in vivo* drug test (Basak and Nandy, 2016). Taking this as consideration along with the results that compound **27** showed in the *in vitro* and *in silico* studies, *in vivo* experiments were conducted using immunocompetent one-day-old Swiss mice (*mus musculus*), simulating the *in vitro* virucide assay. Swiss neonatal mice are not only susceptible to ZIKV infection but also represent a developmental stage equivalent to the early second trimester-stage of human CNS (van den Pol et al., 2017). Intraperitoneal inoculation was chosen as it would be possible to consider the ZIKV migration to the brain as occur in CNS infection by the virus.

The viral titration on Vero cells (Fig. 4 A) shows a very significant reduction on the lysis plate when control and treated groups are compared. Some treated groups had no infective

651 viral particle detected. This reduction is also very clear on the qRT-PCR (Fig. 4 B),
652 corroborating the efficacy of the compound **27** to inhibit the viral replication *in vivo* as
653 demonstrated in the lysis plate assay. At this point, it is important to highlight that the
654 detection of the viral genome does not necessarily indicate the presence of an infectious
655 virus particle, making the lysis plaque assay a more accurate method to evaluate the
656 presence of infective viral particles (Duggal et al., 2017). This explains the detection of
657 viral genome in some days post infection, but not the presence of lysis plates. With these
658 results, it is possible to infer that compound **27** is also efficient to act via a viral neutralizer
659 mechanism *in vivo* as well as *in vitro*.
660

5 CONCLUSIONS

In the present investigation, thirty-one compounds (twenty-eight xanthenodiones and three ketones) were synthesized and had their antiviral activity on ZIKV evaluated. Among the assessed compounds, the ketone 2-(2,6-dichlorobenzylidene)cyclohexane-1,3-dione **27** was able to inhibit the ZIKV viral replication *in vitro* an *in vivo* trough a virucide mechanism. The compound acts straight on the viral particles, due an interaction of the compound with the viral E protein, as it was indicated by the action mechanism assays, docking analysis and the reduction of the Zeta Potential charge. Altogether, these data allow us to conclude that the compound **27** is a promising antiviral against ZIKV, being able to be used to reduce the number of infective viral particles, preventing the replication on the CNS, reducing the risk of the virus has to cause neurological complications such as microcephaly.

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