INSTITUTO CARLOS CHAGAS

Doutorado em Biociências e Biotecnologia

EFEITO ANTIVIRAL DO FLAVONÓIDE NARINGENINA SOBRE CÉLULAS HUMANAS INFECTADAS COM ZIKA VÍRUS

ALLAN HENRIQUE DEPIERI CATANEO

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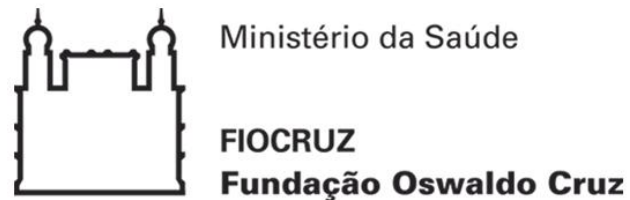

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ALLAN HENRIQUE DEPIERI CATANEO

Efeito antiviral do flavonóide naringenina sobre células humanas infectadas com Zika vírus

Tese apresentada ao Programa de Pós-Graduação em Biociências e Biotecnologia do Instituto Carlos Chagas/Fiocruz/PR como parte dos requisitos para obtenção do título de Doutor em Biociências

Orientadores: Dr. Juliano Bordignon
Drª Pryscilla Fanini Wowk

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À minha esposa Keyla e

aos meus pais Eduardo e Marcia

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"…the truth of which is emphasized by every advance in science, that "Great are the Works of the Lord.""

Sir Joseph John Thomson

Nobel Prize in Physics 1906

RESUMO

O Zika vírus (ZIKV) é o agente causador de uma arbovirose emergente transmitido principalmente através da picada de mosquitos do gênero *Aedes* spp. O vírus pertence ao gênero *Flavivirus*, da família *Flaviviridae*, da qual fazem parte outros vírus de relevância para a saúde pública, como o vírus da dengue, febre amarela e vírus do oeste do Nilo. A infecção pelo ZIKV em humanos é caracterizada por uma doença autolimitada apresentando como sintomas mais frequentes febre baixa, mialgia, exantema, artralgia, dor de cabeça, dor de garganta e vômito. No entanto, manifestações neurológicas após infecção por ZIKV foram observadas nas recentes epidemias da Polinésia Francesa (2013) e do Brasil (2015-2016). Em adultos, síndrome de *Guillain-Barré*, e em crianças nascidas de mães infectadas anormalidades no desenvolvimento do sistema nervoso central e microcefalia, posteriormente denominada de Síndrome Congênita do Vírus Zika. Apesar da redução nos casos de febre do ZIKV nos últimos anos, o vírus continua em circulação no Brasil, podendo causar novas epidemias. Adicionalmente, cerca de 61 países estão sob risco de novos surtos, uma vez que o inseto vetor está presente nessas regiões. Considerando o exposto, a busca por moléculas com atividade antiviral é de grande importância. Entre os compostos naturais, os flavonóides representam uma classe de compostos com ampla atividade biológica/farmacológica, incluindo atividade antiviral. Portanto, neste estudo foi realizada a avaliação *in vitro* da atividade antiviral do flavonóide naringenina (NAR) contra ZIKV. Adicionalmente, neste trabalho foi reportado a identificação de um vírus inseto-específico contaminando cepas de ZIKV. Ainda, foram descritos protocolos *in vitro* e *in vivo* para eliminar o contaminante dos isolados de ZIKV, uma vez que estes podem interferir com os estudos de atividade antiviral. Com base na atividade antiviral da NAR contra os quatro sorotipos do vírus da dengue, e levando-se em conta a similaridade genética entre dengue e ZIKV, foi avaliada a atividade anti-ZIKV da NAR *in vitro*. Através de técnicas como citometria de fluxo, imunodetecção por foco, imunofluorescência e RT-qPCR demonstrou-se que a NAR exerce atividade anti-ZIKV de forma dose-dependente. Os resultados demonstram que o efeito anti-ZIKV da NAR é linhagem-independente, tendo efeito contra cepas da linhagem Asiática e Africana do vírus. Além disso, o efeito anti-ZIKV da NAR foi demonstrado em diferentes tipos celulares, incluindo uma linhagem de célula humana neuronal (A172) e células dendríticas derivadas de monócitos humanos (*hmdDCs*). Os ensaios de tempo de adição da droga sugerem que a NAR atue na inibição da replicação viral, uma vez que o efeito foi observado apenas quando o tratamento foi realizado após a infecção. A análise por *docking* molecular reforça essa hipótese ao demonstrar que a NAR é capaz de se ligar à protease viral (NS2B-NS3). Ainda, observou-se que adição de cadeias de hidrocarbonetos na molécula de NAR apesar de aumentar a atividade anti-ZIKV mostrou-se mais tóxica para as células, sem efeito aditivo ao índice de seletividade. Desta forma, os dados sugerem que a NAR pode ser uma molécula promissora no desenvolvimento de moléculas anti-ZIKV e futuros estudos pré-clínicos devem ser realizados. Por fim, moléculas sintéticas derivadas da NAR foram testadas na tentativa de reduzir a toxicidade e melhorar o efeito anti-ZIKV, no entanto, dentre as 18 moléculas testadas nenhuma foi mais eficaz que a NAR.

Palavras-chave: antiviral, arbovírus, compostos naturais, flavonóides, Zika vírus

ABSTRACT

Zika virus (ZIKV) is an emerging arbovirus transmitted through the bites of *Aedes* spp mosquitoes. The virus belongs to the genus *Flavivirus*, from the *Flaviviridae* family, which includes other viruses of public health relevance, such as dengue virus, yellow fever and West Nile virus. A self-limiting disease with incubation of approximately 10 days characterizes ZIKV infection in humans. The most characteristic signs and symptoms are low fever, myalgia, rash, arthralgia, headache and less often edema, sore throat and vomiting. Unlike dengue infection, ZIKV fever appears less severe, with a milder head and body ache than dengue, and no bleeding and hypovolemic shock are reported. However, cases of Guillain-Barré Syndrome have been reported in ZIKV-infected patients in French Polynesia. Recently, ZIKV has been associated with cases of abnormalities in central nervous system development and microcephaly in newborns, especially in Brazil, later known as Congenital Zika Virus Syndrome. Despite the reduction in ZIKV fever cases in Brazil, around 61 countries are at risk of further outbreaks, since the vector insect is present in these regions. Given the above, the search for antiviral molecules is extremely important. Among natural compounds, flavonoids have been studied in a variety of pathological conditions, including as potential antiviral molecules. Therefore, the *in vitro* antiviral potential of the flavonoid naringenin (NAR) against ZIKV was performed. Additionally, this work reports the identification and elimination of an insect-specific virus contaminant in ZIKV laboratory strains that would be used in antiviral assays. The contamination of ZIKV strains was detected using molecular biology and immunofluorescence techniques. Since contaminants may influence the results obtained, we propose protocols to eliminate the contaminating virus from ZIKV strains. The elimination of the contaminant was performed by successive passage in vertebrate cell line or by inoculation in mice, aiming to guarantee the quality of the strains before the antiviral tests. Based on the antiviral activity of NAR against the four serotypes of dengue virus, and taking into account the genetic similarity between dengue and ZIKV, the *in vitro* anti-ZIKV effect of NAR was evaluated. Through techniques such as flow cytometry, focus immunodetection, immunofluorescence and RT-qPCR we demonstrated that NAR exerts anti-ZIKV activity in a dose-dependent manner. The results demonstrate that the effect remains on different strains and strains of ZIKV. In addition, the anti-ZIKV effect of NAR has been demonstrated in different cell types, including a human neuronal cell line (A172) and human monocyte-derived dendritic cells (hmdDCs). The time of drug-addition assays suggested that NAR acts inhibiting viral replication, as the effect was observed only when treatment was performed after infection. Molecular docking analysis reinforces this hypothesis by demonstrating that NAR is capable of binding to NS2b-NS3 viral protease. Thus, the data suggest that NAR may be a promising molecule in the development of antivirals, and further studies in preclinical trials are needed. Finally, we decided to test synthetic molecules derived from NAR in an attempt to reduce toxicity and improve the anti-ZIKV effect, however, among the 18 molecules tested none was more effective than NAR.

Keywords: antiviral, arbovirus, flavonoids, natural compounds, Zika virus

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1 INTRODUÇÃO

1.1 CAPÍTULO 1

Flavonóides como fonte de moléculas com atividade anti-ZIKA vírus

Flavonoids as source of molecules with anti-Zika virus activity

Autores: Allan Henrique Depieri Cataneo, Juliano Bordignon, Pryscilla Fanini Wowk

Artigo de revisão a ser submetido para publicação.

O Zika vírus (ZIKV) é um arbovírus transmitido principalmente através da picada de mosquitos do gênero *Aedes* spp. O vírus pertence ao gênero *Flavivirus*, da família *Flaviviridae*, da qual também fazem parte outros vírus de relevância para a saúde pública, como o vírus da dengue, febre amarela e vírus do oeste do Nilo. O primeiro isolamento do ZIKV ocorreu a partir de um macaco Rhesus na floresta de Zika em Uganda no ano de 1947 (DICK; KITCHEN; HADDOW, 1952). Os primeiros casos de infecção em seres humanos datam da década de 1950 (MACNAMARA, 1954; SMITHBURN, 1952). Por mais de 50 anos, o vírus esteve relacionado à infecções esporádicas na África e na Ásia, contudo, no ano de 2007, uma epidemia de ZIKV se instalou nas ilhas Yap na Micronésia (DUFFY et al., 2009).

A infecção por ZIKV em humanos geralmente é caracterizada por febre baixa, mialgia, exantema, artralgia, dor de cabeça e menos frequentemente edema, dor de garganta e vômito, ou seja, sintomas semelhantes à outras viroses e geralmente sem grandes complicações (ZANLUCA et al., 2015a). No entanto, casos de Síndrome de *Guillain Barré* foram reportados em pacientes infectados com ZIKV na Polinésia Francesa no ano de 2013 (OEHLER et al., 2014). Recentemente o ZIKV tem sido implicado em casos de anormalidades no desenvolvimento do sistema nervoso central e microcefalia em recém-nascidos, especialmente no Brasil (MLAKAR et al., 2016b). Portanto, o desenvolvimento de alternativas terapêuticas torna-se uma prioridade em saúde pública.

Grande parte das drogas aprovadas pela *Food and Drug Administration* (FDA) são derivadas, ou tem como origem produtos naturais (PATRIDGE et al., 2016). Dentre os compostos naturais, os flavonóides se destacam por exercerem diversas

atividades biológicas, tais como, atividade antioxidante, anti-inflamatória, antitumoral e antiviral.

Desta forma, como capítulo introdutório da tese (Capítulo 1) apresentamos um artigo de revisão sobre os mais recentes avanços no uso de flavonóides como potenciais moléculas com atividade anti-ZIKV. A revisão inicia com uma apresentação do ZIKV, a recente associação entre a infecção por ZIKV e síndromes congênitas e neurológicas, fontes, estrutura e atividades biológicas dos flavonóides culminando nas pesquisas em flavonóides como moléculas anti-ZIKV.

**Cabe salientar que esta revisão está atualmente em análise pelos pesquisadores/professores colaboradores neste projeto e que serão autores da versão final deste artigo, a saber: Prof. Dr. Waldiceu Aparecido Verri Junior (Universidade Estadual de Londrina), Profa. Dra. Sandra Fabrasile (Universidad de La Republica, Uruguai), Prof. Dr. Mauro Almeida (Universidade Federal de Juiz de Fora) e Dra. Claudia Nunes Duarte dos Santos (Instituto Carlos Chagas, ICC/Fiocruz).*

Artigo de revisão

Flavonoids as source of molecules with anti-Zika virus activity

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Abstract

The Zika virus (ZIKV) is an arthropod born virus mainly transmitted to humans by mosquitoes of the genus *Aedes spp*. After the first isolation in 1947, only a few human cases had been described until large outbreaks occurred on Yap Island in 2007, French Polynesia in 2013 and Brazil in 2015. Most of ZIKV-infected individuals are asymptomatic or presented a self-limiting disease and non-specific symptoms such as fever, myalgia, and headache. However, in French Polynesia and Brazil outbreaks cases of Zika virus related congenital malformations and microcephaly in newborns and Guillain-Barré Syndrome in adults were diagnosed. The new clinical presentations raised a concern of the public health authorities and highlighted the need for anti-Zika treatments and vaccines to control neurological damage caused by the virus. Despite many efforts on the search for an effective treatment, neither vaccine nor antiviral drugs have become available to control ZIKV replication. Flavonoids, a class of natural compounds, well known for several biological properties, have shown antiviral activity against different viruses. Also, the fact that flavonoids are used in some countries as food supplemental indicates that these molecules are not toxic for human use. However, this issue should be better addressed in future studies together with the potential mechanism of the antiviral action and pharmacodynamics of the different flavonoids. Thus, here we summarized the knowledge on the use of flavonoids as source of anti-ZIKV molecules and discuss the gaps and challenges in the area before its consideration for pre-clinical and clinical trials.

Introduction

The Zika virus (ZIKV) is an arthropod born virus belonging to the *Flaviviridae* family and *Flavivirus* genus. The first isolation of ZIKV occurred from a serum sample of a sentinel rhesus monkey in 1947 in the Zika forest in Uganda, when a surveillance program for Yellow fever was in progress (DICK; KITCHEN; HADDOW, 1952). One year later the virus was successfully isolated from *Aedes africanus* mosquitoes (DICK; KITCHEN; HADDOW, 1952). Later, was demonstrated the susceptibility of *Aedes spp* mosquitoes to ZIKV and its potentially to transmit the virus (LI et al., 2012). Recently, putative non-vector transmission was also reported, via contaminated blood, sexual contact and vertical transmission (MLAKAR et al., 2016; MUSSO et al., 2014, 2015).

Since the first isolation in the 1940s, human infections occurs mainly across Asia and Africa, always associated with mild clinical manifestations (KINDHAUSER et al., 2016). Most of ZIKV-infected individuals are asymptomatic, while around 20-25% develop a self-limiting and mild illness with symptoms from a flu-like disease, such as fever, myalgia, headache, maculopapular rash and lower back pain (CALVET; DOS SANTOS; SEQUEIRA, 2016).

Until 2007, only few cases of ZIKV infection had been reported worldwide, however, this year an outbreak was reported on Yap Island, Federated States of Micronesia. It was estimated that 5005 of the 6892 residents were infected (DUFFY et al., 2009). In October 2013, ZIKV infection was confirmed in French Polynesia and caused a massive outbreak, with almost 11% of the population seeking for medical care (CAO-LORMEAU et al., 2014; MUSSO; NILLES; CAO-LORMEAU, 2014). Previously from the French Polynesia outbreak, there were no reports of severe disease related to ZIKV infection, however, an increasing in neurological symptomatology, including cases of Guillain-Barré Syndrome (GBS) was associated to ZIKV infection (MUSSO; NILLES; CAO-LORMEAU, 2014; CALVET et al., 2016; DE NORONHA et al., 2016; KRAUER et al., 2017). In the early 2015, the first autochthonous transmission of ZIKV in Brazil was reported in the North region of the country (ZANLUCA et al., 2015). Figure 1 infographic timeline summarizes the most important landmarks of ZIKV since the first detection of the virus.

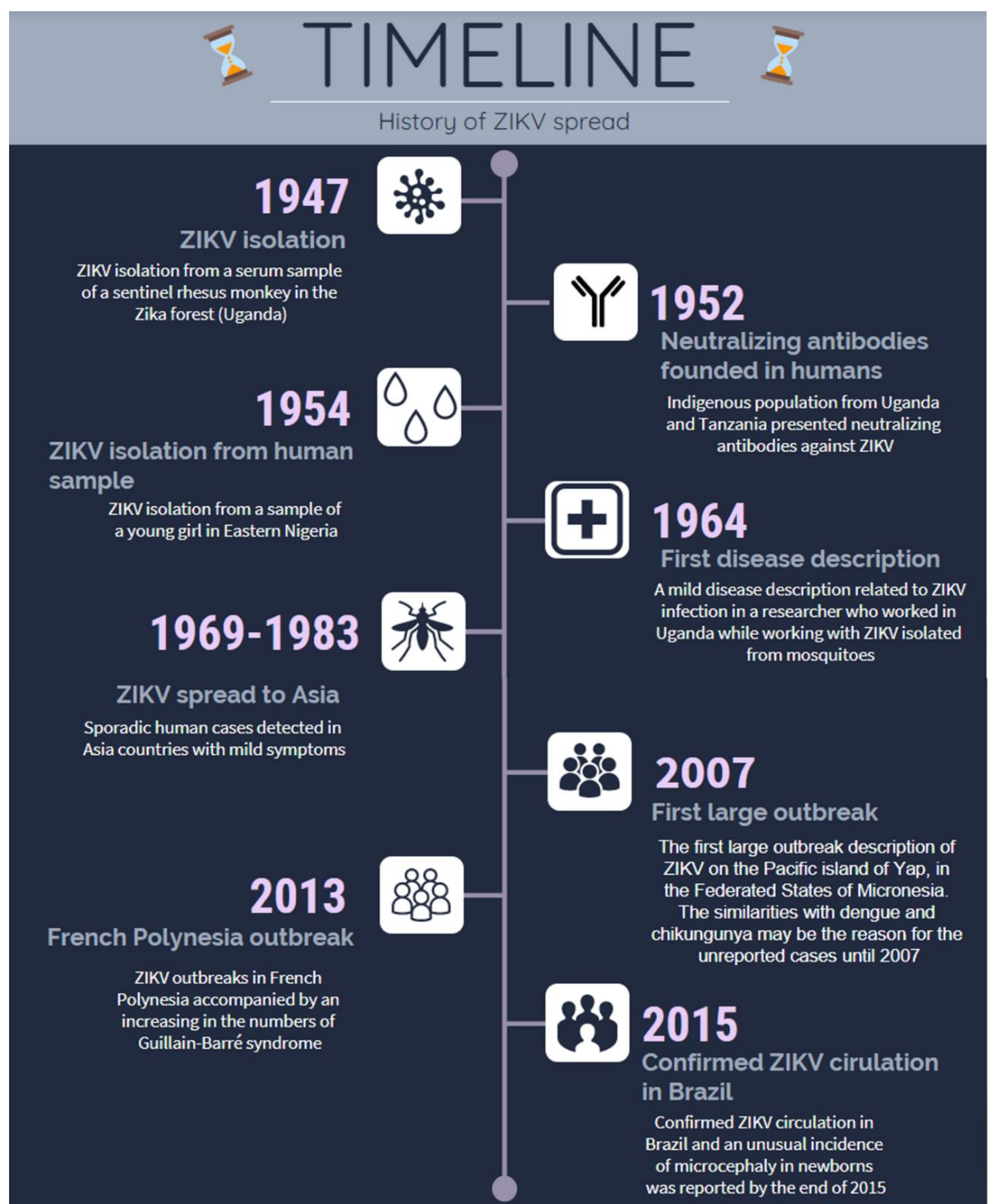

Figure 1 – ZIKV spread since isolation until the outbreak in Brazil in 2015. 1 – ZIKV isolation in the Zika forest at Uganda from a serum sample from rhesus monkey (DICK; KITCHEN; HADDOW, 1952). 2 – Neutralizing antibodies were founded in indigenous populations from Uganda and Tanzania (SMITHBURN, 1952). 3 – The first isolation of ZIKV from a human serum sample in Nigeria (MACNAMARA, 1954). 4 – A mild disease description related to ZIKV infection in a researcher who worked in Uganda while working with ZIKV isolated from mosquitoes. 5 – Sporadic human cases detected in Asia countries with mild symptoms (SIMPSON, 1964). 6 – The first large outbreak description of ZIKV on the Pacific island of Yap, in the Federated States of Micronesia. The similarities with DENV and chikungunya may be the reason for the unreported cases until 2007(DUFFY et al., 2009). 7 – ZIKV outbreaks in French Polynesia accompanied by an increasing in the numbers of Guillain-Barré syndrome (OEHLER et al., 2014). 8 – Confirmed ZIKV circulation in Brazil and an unusual incidence of microcephaly in newborns was reported by the end of 2015 (MINISTÉRIO DA SAÚDE, 2016; ZANLUCA et al., 2015).

Therefore, the rapidly spread of this emerging arbovirus and recent evidences of severe disease of the nervous system caught the attention of the scientific community. Since the first detection of ZIKV circulation in Brazil in 2015, more than 240.000 cases were reported (MINISTÉRIO DA SAÚDE, 2019a) and approximately 3000 cases of microcephaly and other congenital malformations associated to ZIKV in newborns were confirmed (MINISTÉRIO DA SAÚDE, 2019b). The children with microcephaly present health disorders, such as seizures, brain and eye damage that restricts its development and has both health and social impact. Thus, based on the impact of the ZIKV epidemic on newborns, it is mandatory to pursue on the research in the strategies to prevent and treat infected patients, as anti-ZIKV molecules and vaccines, to avoid or reduce damage caused by the infection.

ZIKV biology

ZIKV was demonstrated to infect a variety of human cells, such as skin fibroblasts, endometrial stromal cells, neural progenitor cells, monocytes, dendritic cells and Hofbauer cells (BOWEN et al., 2017; HAMEL et al., 2015; MICHLMAYR et al., 2017; PAGANI et al., 2017; QUICKE et al., 2016; TANG et al., 2016). C-type lectin receptors such as DC-SIGN as well transmembrane phosphatidylserine receptors such as TIM (TIM1, TIM3 and TIM4) and TAM receptors (TYRO3, AXL and MER) may mediated ZIKV entry in target cells (HAMEL et al., 2015). After cell attachment, *Flavivirus* entry occurs through clathrin-mediated endocytosis, followed by endosome acidification, exposure of fusion loop that allows the fusion between viral envelope and endosome membrane followed by release of viral RNA into cell cytoplasm (MODIS et al., 2004; PERSAUD et al., 2018).

The ZIKV genome is a positive single strand RNA of approximately 11,000 bases that encode a polyprotein, which is processed by host and viral proteases to three structural proteins and seven nonstructural proteins (NS). Envelope (E), premembrane/membrane (prM/M) and Capsid (C) are the structural proteins and NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 are the NS proteins (KUNO; CHANG, 2007).

The *Flavivirus* E protein allows the viral particle to bind cell receptors and is an important target for neutralizing antibodies (DAI et al., 2016). The C protein is mainly associated to virus assembly, by packing the RNA genome, however, it was suggested that this protein may play a role in the fusion process (FREIRE et al., 2015). The prM/M

protein is essential in the late steps of viral replication cycle with central role in virus maturation and effective production of infective particles. Moreover, prM mutation was suggested to be involved in the emergence of fetal microcephaly associated to ZIKV infection (LI et al., 2008; YUAN et al., 2017a).

The NS proteins are important players in the *Flavivirus* replication cycle as well as in immune response evasion and pathogenesis. The *Flavivirus* NS1 protein was demonstrated to play an important role helping the viral replication and is associated with pathogenesis by increasing vascular permeability (PUERTA-GUARDO et al., 2019; YOUN et al., 2012). The NS2B is the cofactor for the NS3 protease; therefore, the NS2B-NS3 complex is essential for optimal catalytic activity and cleavage of specific regions in the precursor polyprotein (NATARAJAN, 2010). The NS3 is a bipartite protein with a protease and a helicase domain. This protein plays important role cleaving the polyprotein and unwinding the RNA secondary structure to allows the synthesis of viral RNA copies (BOLLATI et al., 2010). The NS4A and NS4B, together with NS2A and NS2B act as a scaffold for the replication complex essential to viral replication (ZOU et al., 2015). The NS5 protein is a RNA-dependent RNA polymerase that is necessary to RNA replication and has a methyltransferase domain required to the RNA capping process (EGLOFF et al., 2007; TAN et al., 1996). Additionally, to essential role in viral replication, the NS proteins were described to have ability to overcome the immune response, which is essential for viral replication success. The *Flaviviridae* NS proteins were suggested to inhibit the RIG-I signaling (DALRYMPLE; CIMICA; MACKOW, 2015), suppress the type I interferon response (GRANT et al., 2016) as well NF-κB activation (CHEN et al., 2015). Altogether, the viral proteins play important roles during infection, viral replication and immune response evasion; therefore, they are important targets for antiviral compounds design (MOTTIN et al., 2018a).

Drug discovery research against ZIKV

There are successful strategies to support the drug discovery against emerging viruses, such as the drug repurposing, the computational-based drug discovery and search for natural products (DA SILVEIRA OLIVEIRA et al., 2017; PUSHPAKOM et al., 2018; SINIGAGLIA et al., 2018).

The drug repurposing is the method used to find new use of a previous approved

drug. This is an interesting strategy, since the drug is already approved to safety use in humans (ASHBURN; THOR, 2004). As examples of this strategy it was recently shown that chloroquine, an anti-malaria drug, and sofosbuvir, an antiviral used for hepatitis C virus treatment, both FDA-approved compounds, exerts anti-ZIKV activity (DELVECCHIO et al., 2016; SACRAMENTO et al., 2017).

A successful example of a computational-based technique used in drug discovery against ZIKV is the OpenZika project, where *in silico* analysis searched for anti-ZIKV drug among millions of compounds (EKINS; PERRYMAN; HORTA ANDRADE, 2016). The OpenZika used compounds from the ZINC database, FDA-approved drugs and National Institutes of Health (NIH) clinical collection in collaboration with the IBM's World Community Grid (WCG – worldcommunitygrid.org) to virtually screen millions of compounds against crystal structures of ZIKV proteins or related viruses. Mottin and colleagues (2018) summarizes different computational-based strategies used to look for anti-ZIKV molecules, such as Structure-based drug design (SBDD), Ligand-based drug design (LBDD) and In silico fragment-based drug discovery (FBDD) (MOTTIN et al., 2018b).

In addition, the natural products derived compounds play important role as protagonist within drug discovey against pathogens and development for the treatment of human diseases (NEWMAN; CRAGG, 2012). Natural products have been used to discovery new molecules to overcome antibiotic resistance, to prevent drug resistance in cancer and to find antiviral drugs (LIN; HSU; LIN, 2014; ROSSITER; FLETCHER; WUEST, 2017; YUAN et al., 2017b).

Flavonoids: Biosynthesis, structure and sources

Natural products and their derivates are an important source of bioactive compounds and represent more than one-third of all FDA-approved new molecules (PATRIDGE et al., 2016). Among natural products, flavonoids are one of the major secondary metabolites naturally occurring compounds ubiquitous found in plants (HAVSTEEN, 1983; YONEKURA-SAKAKIBARA; HIGASHI; NAKABAYASHI, 2019). Flavonoids can be found as glycosides conjugates, therefore contributing to the complexity of this class of metabolites, with over than 6,000 flavonoids identified (BEECHER, 2003; KOZŁOWSKA; SZOSTAK-WĘGIEREK, 2018). The structure of this

polyphenolic compounds is general characterized by a skeleton structure containing 15-carbons, a heterocyclic ring (ring C) and two phenyl rings (rings A and B).

The beginning of flavonoid biosynthesis allows the condensation of one molecule of p-coumaryl-CoA, derived from chiquimate (ring B), with three molecules of malonyl-CoA, of polyketide origin (ring A), mediated by the enzyme chalcone synthase (CHS) giving rise to a chalcone. The isomerization of flavanone occurs by the enzyme flavanone isomerase (CHI). The pathway separates into lateral branches, under the action of different enzymes such as isomerases, hydroxylases, reductases, giving different classes of flavonoids (SANTOS et al., 2017).

The classification of flavonoids in subgroups is generally based on modifications of the C ring, whereas within the same group, the classification is based on modifications in the A and B rings. Moreover, some carbons of the skeleton structure can be replaced for an hydroxyl, methoxyl and others groups (KOZŁOWSKA; SZOSTAK-WĘGIEREK, 2018). Therefore, it can be classified in six major groups, which are: flavonols, flavanones, flavones, flavanols, isoflavones and anthocyanins (Figure 2). The flavones, flavonols and isoflavolones are characterized by a planar structure due the presence of a double bond in central ring (C) (NIJVELDT et al., 2018). Flavanones and flavanols, present at least one stereogenic center, with a tetrahedrical geometry due the presence of two sp^3 carbons.

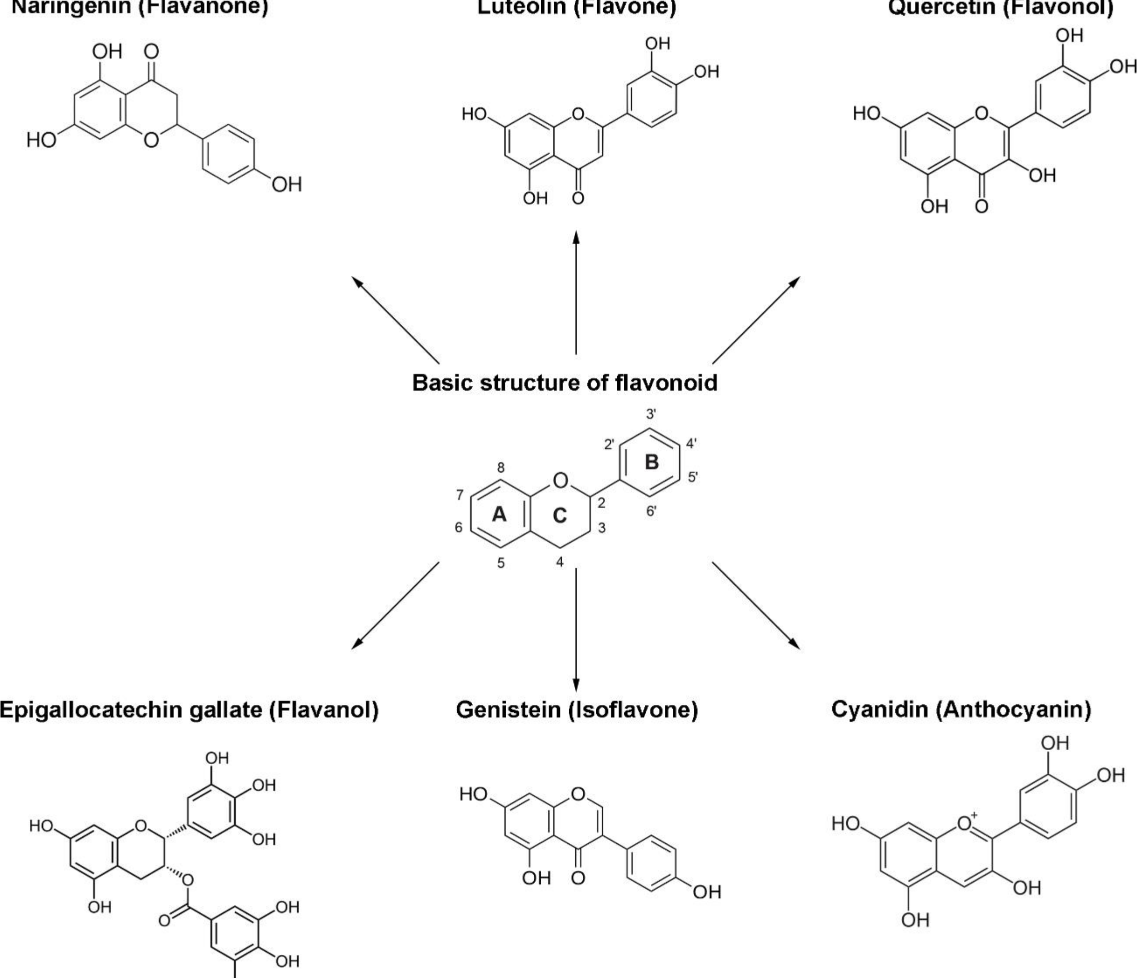

Figure 2 – Chemical structures of flavonoids and subclasses.

The presence of chiral centers is extremely important for a molecule to be a candidate for a particular drug, since most of targets enzymes or proteins are stereo-, regio- and chemoselectives (CHAPLIN; BUCKE, 1990). It is worth mentioning that the majority of virus are inactivated through interaction of enzymes to an active molecule specifically.

Flavonoids are widely encountered in dietary sources, mainly vegetables, fruits, seeds roots and cereals. (BHAGWAT; HAYTOWITZ; HOLDEN, 2014; FERRAZ et al., 2019). The quantity of these compounds range from 3.34mg/100g in beer to 4854.49mg/100g in dried parsley. Subgroups of flavonoids also may vary depending on the plant, where one group can be more prominent compared to others. Most of

flavonoids founded in dried parsley are from flavones group (4523.25mg/100g). Flavonols are most founded in fresh capers with 493.03mg/100g, whereas dried mexican oregano is an important source of flavanones (412.13mg/100g). Soy flour and soybeans mature seeds are sources of isoflavones (166.66mg/100g) and 103.56mg/100g, respectively). Berries are important sources of flavonoids and elderberry juice concentrate presents high amount of anthocyanins (411.40mg/100g)(KOZŁOWSKA; SZOSTAK-WĘGIEREK, 2018). Despite widely distributed among different sources, the presence of flavonoids in plants may vary depending on numerous factors, such as soil composition, climate, season, storage conditions and plant species (PATIL; PIKE; HAMILTON, 1995; VAN DER SLUIS et al., 2001). In addition, the food processing, like cooking, may result in transformation or losses of the flavonoid content (BEECHER, 2003).

Flavonoids biological properties

The first demonstration of biological/pharmacological activity of the flavonoids was made almost 100 years ago, in 1936. It was demonstrated that ascorbic acid was not effective in the treatment of purpura condition, unless if used together with extracts of Hungarian red pepper or lemon juice, which restored the normal vascular permeability. Authors had fractionated the extracts and describe the active molecule as substance P due to its action on vascular permeability. Later, the substance P was called citrin and it was demonstrated that hesperidin, a flavonoid, was its major compound (BRUCKNER; SZENT-GYÖRGYI, 1936; RUSZNYÁK; SZENT-GYÖRGYI, 1936). The main biological properties of flavonoids will be reviewed below.

Antioxidant

The antioxidant mechanisms of flavonoids include: i) quenching free radical components, ii) chelating metal, iii) inhibiting the activity of enzymes associated with the generation of free radical, and iv) inducing the expression of endogenous antioxidant enzymes(KUMAR; PANDEY, 2013). Of note, flavonoids may also act as

pro-oxidants accordingly to concentration, structure and hydroxide substitutions (CAO; SOFIC; PRIOR, 1997).

Flavonoids deactivate reactive species by donating hydrogen and an electron to radicals of hydroxyl, peroxyl and peroxynitrite. The resulting radical is more stable than those reactive species (CAO; SOFIC; PRIOR, 1997; PIETTA, 2000). Flavonoids are thermodynamically capable of removing strongly oxidizing free radicals such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation due to their lower redox potential. Free metal ions boost the production of ROS by reducing hydrogen peroxide and producing the hydroxyl radical, which is highly reactive. Thus, flavonoids also inhibit free radical production due to their ability to chelate metal ions (MISHRA; KUMAR; PANDEY, 2013).

The hydroxyl groups in the catechol moiety (B ring) play an essential role in scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS). The heterocycle of flavonoids contributes to the antioxidant activity due to the presence of a free hydroxyl group on position 3 and by allowing conjugation between the aromatic rings. Conjugation between the rings A and B enables the aromatic nucleus to have a resonant effect and provides stability to the formed radical flavonoid. Furthermore, removal of the 3-OH or O-methylation of flavonoid hydroxyl groups compromises the flavonoid ability to scavenge free-radicals (BORS et al., 1990; RICE-EVANS; MILLER; PAGANGA, 1996). The presence, location, composition, and the total number of flavonoid sugar moieties (flavonoids glycosides) also impact the antioxidant effect of these molecules. Flavonoid aglycones are more potent antioxidants than flavonoid glycosides (RATTY; DAS, 1988). In combination with a 4-oxo structure, flavonoids with an unsaturated 2-3 bond are more effective antioxidants than flavonoids with either or both functions (RICE-EVANS; MILLER; PAGANGA, 1996).

These structural properties bestowing direct antioxidant actions to flavonoids are only part of their mechanisms of action. Flavonoids also stimulate endogenous antioxidant defenses or inhibit enzymes responsible for the formation of superoxides, such as oxidases (GONZÁLEZ-PARAMÁS et al., 2018). Flavonoids activate endogenous antioxidant enzymes such as Glutathione Peroxidase (GPx), Catalase (CAT) and Superoxide Dismutase (SOD). SOD transforms superoxide to H_2O_2, and CAT or peroxidase (e.g., glutathione peroxidase or peroxiredoxins) consequently transforms H_2O_2 to water. Peroxidase activity may be restored further by either thioredoxin (TRX) or glutaredoxin (MASELLA et al., 2005; NAGATA et al., 1999).

To sum up, the antioxidant functions that have been demonstrated for flavonoids include the scavenging of free radicals, chelation of metal ions, enzyme activation/induction, gene expression regulation, and interference with cell signaling pathways. The catechol moiety is essential not only to the scavenging of free radicals and the sequestration of metal ions, but also to the inhibition of pro-oxidant enzymes. Combining all these properties into a single class of molecules makes flavonoids extremely promising multifunctional antioxidants.

Immunomodulatory

Flavonoids can modulate the immune response with actions on immune cells populations, modulating cytokine production and pro-inflammatory genes expression (FERRAZ et al., 2020; YAHFOUFI et al., 2018), which are discussed below.

Luteolin, quercetin and rutin seem to change macrophage polarization towards M2 phenotype. Depending on disease context, this macrophage phenotype plays a role preventing tissue repair and limiting inflammation (KIM; PARK, 2016; NADELLA et al., 2019; WANG et al., 2019).. Quercetin (GUAZELLI et al., 2018), apigenin (CARDENAS et al., 2016), diosmin (FATTORI et al., 2020; TAHIR et al., 2013), rutin (CARVALHO et al., 2019), and hesperidin methyl chalcone (RASQUEL-OLIVEIRA et al., 2020) inhibit the activation of NF-κB, important inflammatory transcription factor, in varied models of disease. Interestingly, quercetin inhibits NF-κB by preventing its binding to DNA as well as by inducing its phosphorylation (VICENTINI et al., 2011).

During inflammatory responses, cells use molecules such as cytokines to communicate and orchestrate the immune response. Flavonoids such as quercetin (BORGHI et al., 2018; GUAZELLI et al., 2013), naringenin (MARTINEZ et al., 2015) and vitexin (NIKFARJAM et al., 2017) reduce cytokine production.. As a result of inhibiting TNF-α and IL-1β production there is a reduction of neutrophil recruitment (BORGHI et al., 2013; CHO et al., 2003; PINHO-RIBEIRO et al., 2016)

Cytokines and other molecules have their production also regulated by MAP kinase signaling cascades (KAMINSKA, 2005). Inflammasomes such as NLRP3 are important in the maturation of cytokines such as IL-1β and IL-18 (SEGOVIA et al., 2018). Quercetin (LEE et al., 2018), vitexin (JIANG et al., 2019), and naringenin (ZHANG et al., 2019) inhibit MAP kinase signaling cascades in macrophages, and

quercetin (DOMICIANO et al., 2017) and trans-chalcone (STAURENGO-FERRARI et al., 2018) inhibit NLRP3 inflammasome activation.

Lipid mediators such as prostaglandins E2 (PGE_2),e derived from the metabolization of arachidonic acid by the peroxidase and cyclooxygenase actions of cyclooxygenases (COX) 1 and 2 (NASRY; RODRIGUEZ-LECOMPTE; MARTIN, 2018) can also be modulated by several flavonoids. Apigenin inhibits COX-2, cytokines (IL-1β, IL-2, IL-6, IL-8, and TNF-α), iNOS and the Activation protein 1 proteins (AP-1) production in the context of LPS cellular activation (PATIL et al., 2016). Vitexin inhibits the production of NO and PGE_2 in human osteoarthritis chondrocytes (KIM et al., 2018). Epigallocatechin-3-gallate decreases COX-2 and PGE_2 production in human osteoarthritis chondrocytes induced by IL-1β (BOSCH-MOLA et al., 2017). Hesperidin decreases the levels of NF-κB, iNOS, and COX-2 resulting in inhibition of neuroinflammation induced by an infusion of streptozotocin in the coronal brain (JAVED et al., 2015).

Thus, flavonoids are multi-target molecules that modulate the immune system by targeting, cytokines, chemokines, lipid mediators, MAP kinases, and nitric oxide production (CHEN et al., 2005; KIM et al., 1999; RIBEIRO et al., 2015).

Anti-tumoral

Developing novel anti-tumoral drugs is still a challenge. The flavonoids anti-tumoral effects have also multi-target. Thus, selecting the right flavonoids to the correct type of cancer might be an essential step for a successful therapeutic intervention in the future. The targets of flavonoids in cancer include cell cycle, heat-shock protein, tyrosine kinase, p53 protein, Ras protein (BATRA; SHARMA, 2013; CHAHAR et al., 2011), which are addressed below.

Mutations in the p53 gene occur at multiple stages of the cycle of malignant development, thereby leading differently to tumor creation, growth, aggressiveness and metastasis (RIVLIN et al., 2011). The p53 protein may be downregulated by flavonoid intake (LAMSON; BRIGNALL, 2000), which is interesting for therapeutic purposes since inhibition of p53 expression may result in the arrest of the cancer cells in G2-M step of the cell cycle.

Tyrosine kinases are a class of proteins that are involved in transducing signals from the growth factors to the nucleus. Drugs inhibiting the function of tyrosine kinase

are known to be potential antitumor agents without the side effects seen with traditional chemotherapy (KUMAR; PANDEY, 2013). Quercetin displayed great antitumor activity against tyrosine kinase without the cytotoxicity effects in a phase I clinical trial (FERRY et al., 1996). In many malignant cell types such as leukemia, colon cancer and breast cancer cells, flavonoids suppress heat-shock proteins (LAMSON; BRIGNALL, 2000). Flavonoids inhibit tumour cell growth also by interacting with nuclear estrogen binding sites (type II) (MARKAVERICH et al., 1988) and reducing the protein levels and half-life of oncogenic Ras proteins *in vitro* (PSAHOULIA et al., 2007; RANELLETTI et al., 2000).

Chen and colleagues (2018) tested the total flavonoids content of an extract from *Diospyros kaki* leaves in H22 tumor-bearing mice model. The *Diospyros kaki* leaves extract presents several flavonoids like, rutin, quercetin, myricitrin, kaempferol, and myricetin (CHEN et al., 2018). This extract suppresses tumor growth (49.35% inhibition) in a dose-dependent manner. This antitumor activity is possible due to the immunomodulatory effect of the extract, which increases serum levels of IL-18, monocyte/macrophage phagocytic activity and NK cell cytotoxicity. Quercetin also reduces metastasis by inhibiting migration and invasion pathways on gastric cancer cells models (LI; CHEN, 2018). Moreover, the flavanone hesperetin induces the apoptosis of gastric cancer cells by reactive oxygen species, promoting changes in the mitochondrial membrane potential and decrease in the antiapoptotic/proapoptotic (Bcl-2/Bax) protein ratio (ZHANG et al., 2015). Similar mechanisms of cancer cell death were observed when gastric cancer SGC-7901 cells were treated with the flavanone naringenin (BAO et al., 2016).

Flavonoids are strong candidates in the treatment of various types of cancer via modulating cell cycle, heat-shock protein, tyrosine kinase, p53 protein, and Ras protein.

Antibacterial activity

Because of microbial resistance to antibiotics, there are still special need to development new antibacterial agents (MCEWEN; COLLIGNON, 2018). Flavonoids are considered good candidates (SARBU et al., 2019) due to antibacterial effects that include, nucleic acid synthesis inhibition, alteration in cytoplasmic membrane function, energy metabolism inhibition, reduction in cell attachment and biofilm formation, inhibition of the porin

on the cell membrane, changing of the membrane permeability, attenuation of the pathogenicity (FARHADI et al., 2019; XIE et al., 2014).

The antibacterial study on flavones isolated from *Scutellaria barbata* aerial parts (apigenin and luteolin) against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) demonstrated that apigenin and luteolin are active against all strains of MRSA and MSSA with a minimum inhibitory concentration (MIC) of 3.9–15.6 µg/ml and 62.5–125 µg/ml respectively (SATO et al., 2000). Galangin purified from propolis ethanol extract shows bactericidal activity against MRSA, *Enterococcus* spp. and *Pseudomonas aeruginosa* with MIC of 0.16, 0.24 and 0.17 mg/ml, respectively (PEPELJNJAK; KOSALEC, 2004). Extract of the leaves of *Oncoba Spinosa* Forssk and as well as compounds quercetin, apigenin-7-O-β-D-glucuronopyranoside, quercetin 3-O-β-D-galactopyranoside and quercetin 3-O-α-L-rhamnopyranosyl (1 → 6) β-D-glucopyranoside demonstrated antimicrobial effects against *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (DJOUOSSI et al., 2015).

The chrysoeriol-7-O-β-D-xyloside, luteolin-7-O-β-D-apiofuranosyl-(1 → 2)-β-D-xylopyranoside, chrysoeriol-7-O-β-D-apiofuranosyl-(1 → 2)-β-D-xylopyranoside, chrysoeriol-7-O-α-L-rhamnopyranosyl-(1 → 6)-β-D-(4″-hydrogeno sulfate) glucopyranoside and isorhamnetin-3-O-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside from *Graptophyllum grandulosum* present bactericidal activity due to cell lysis and disruption of the cytoplasmic membrane by action upon the membrane permeability leading to leakage of cellular components and eventually cell death (TAGOUSOP et al., 2018). A study demonstrates antimicrobial mechanism of kaempferol and hesperetin against *Escherichia coli* is due to interaction between the polar head-group of the model membrane and the hydrophobic regions may damage *Escherichia coli* membrane (HE et al., 2014). The catechins are able to inhibit bacterial DNA gyrase by binding to the ATP binding site of the gyrase B subunit. Molecular docking showed that the benzopyran ring of epigallocatechin penetrates deeply into the active site while the galloyl moiety anchors it to the cleft through interactions with its hydroxyl groups (GRADIŠAR et al., 2007).

These findings and many others show that of flavonoids are promising candidates to find and develop new antibiotics.

Antiviral

The first description of the antiviral activity of flavonoids was done by Béládi, Pusztai and Bakai in 1965. The authors showed the possible virucidal effect of quercetin and morin against herpesvirus (BÉLÁDI; PUSZTAI; BAKAI, 1965). A few years later, the same authors demonstrated the antiviral effect of quercetin, rutin,

morin, luteolin, apigenin and fisetin on herpes simplex virus and parainfluenza virus type 3 (BÉLÁDI et al., 1977). Also, the data suggested that the antiviral activity was associated with the flavonoid molecular structure. The number and position of hydroxyl groups and glycosides could modulate the antiviral activity probably by interfering in the binding to their targets by modulating electrostatic interactions such as hydrogen bonds and ionic interactions (BÉLÁDI et al., 1977; LIU et al., 2008). Since then, numerous studies have evaluated the antiviral activity of flavonoids against several viruses. Quercetin and morin provided protection in mice infected with Mengo virus (VECKENSTEDT; BÉLÁDI; MUCSI, 1978). Quercetin, hesperetin, catechin and naringin were tested against herpes simplex virus type 1, parainfluenza virus type 3, poliovirus type 1 and respiratory syncytial virus with promisor results, except for naringin that did not showed any antiviral activity (KAUL; MIDDLETON; OGRA, 1985). Some flavonoids were also described as inhibitors of HIV infection and HIV reverse transcriptase (MAHMOOD et al., 1993; SPEDDING; RATTY; MIDDLETON, 1989). Silymarin and derivates have been described as promising antiviral compounds, which exert effects against HIV, Hepatitis B and C and Influenza A (DAI et al., 2013; MCCLURE et al., 2012; UMETSU et al., 2018; WAGONER et al., 2010).

The effect of flavonoids against arboviruses of medical importance has been extensively studied in the past few years, mainly due to the emerging and re-emerging of arbovirus, like DENV, ZIKV, chikungunya (CHIKV) and yellow fever (YFV) (ESPINAL et al., 2019). Frabasile and colleagues (2017) recently demonstrated the ability of the flavanone naringenin (NAR) to inhibit the replication of DENV virus in Huh7.5 cells and primary human monocytes (FRABASILE et al., 2017). The inhibition of DENV replication by flavonoids is in agreement with previous studies that demonstrated that these compounds acts as noncompetitive inhibitors of proteases involved in viral replication, such as NS2B-NS3 from DENV virus (DE SOUSA et al., 2015). Therefore, this class of compounds could impact the viral replication suggesting a possible mechanism of antiviral action. Baicalein and baicalin were able to inhibit replication of DENV-2 in Vero cells (ZANDI et al., 2012b). Furthermore, *in silico* analysis suggested the ability of both compounds to bind with DENV nonstructural proteins, and once again suggesting a possible interference in the viral replication (HASSANDARVISH et al., 2016; MOGHADDAM et al., 2014; ZANDI et al., 2012b).

Further, the flavonoids apigenin, chrysin, NAR and silybn seems to impair the viral entry and replication of the CHIKV and Semliki Forest virus in BHK cells

(POHJALA et al., 2011b). Recently, Murali and colleagues (2015) demonstrated the anti-CHIKV effect of the ethanolic extract of *Cynodon dactylon*. By reverse phase-HPLC and GC-MS they observed that the major constituent of ethanolic extracts are luteolin and apigenin (MURALI et al., 2015).

In silico prediction

The *in vitro* and *in vivo* evaluation of a drug candidate has been the predominantly process for antiviral drug discovery in the past years. Recently, the computational-based strategy to rational selected of compounds has grown in the field of drug-discovery (SLIWOSKI et al., 2014). The *in silico* approach may operate to reduce the number of compounds to be tested, once it works as a filter screening to predict the molecules efficacy (TERSTAPPEN; REGGIANI, 2001). Thus, computational-based strategies applied for drug discovery has the potential to reduce the costs and time until a new drug reaches the market.

In the road of *in silico* analysis, the screenings for potential anti-ZIKV molecules were not left out, mainly in the evaluation of interactions of varied compounds with viral structures and molecules, like the OpenZika project (EKINS; PERRYMAN; HORTA ANDRADE, 2016).

Understanding the structure of the flavivirus as well as the respective receptors are crucial for therapeutic interventions (GORSHKOV et al., 2019; LUO et al., 2008). Thus, docking, modeling studies are extremely important for the development of new drug candidates through the simulation of interaction between target molecules with specific receptors, are extremely important for the discovery of new drugs against epidemic and emerging viruses (SAW et al., 2017).

Indeed, flavonoids have been used in docking analyses to determine potential interactions with viral proteins. The epigallocatechin gallate (EGCG) compound was demonstrated, by docking strategy, to impair the fusion of ZIKV with the cellular membrane (SHARMA et al., 2017). Despite an interesting strategy, the docking analyses depends on the characterization of protein conformation to evaluate possible interactions with compound libraries. Cox and colleagues (2015) predicted for the first time, based on closely related viruses, the structure of ZIKV proteins, making possible the *in silico* analyses to help in vaccine and drug discovery development (COX; STANTON; SCHINAZI, 2015).

Saw and colleagues (2017) demonstrated the molecular characterization of the essential nonstructural proteins -3, and -5 (NS3 and NS5) of ZIKV (SAW et al., 2017). Both NS3 and NS5 are indispensable to viral replication, with protease/helicase and RNA-dependent RNA-polymerase activity, respectively. The viral replication depends on viral proteases, and it makes them key targets for drug development (BABÉ; CRAIK, 1997). As already described for DENV virus by enzyme inhibition kinetic assays and docking studies, a set of natural flavonoids was demonstrated to be NS2B-NS3 protease inhibitors (DE SOUSA et al., 2015). Given the similarities between DENV and ZIKV, it is plausible that the same flavonoids would also inhibit ZIKV proteases. Roy and colleagues (2017) worked in the structural characterization of ZIKV NS2B-NS3 protease and tested the NS2B-NS3 inhibitory ability of some flavonoids. The authors identified five compounds (myricetin; quercetin; luteolin; isorhamnetin and apigenin) that act as inhibitors of viral protease (ROY et al., 2017). Thus, using *in silico* methods authors demonstrated the structure-activity relationship between the effective compounds and NS2B-NS3 protease. Interestingly, the docking analyses showed the compounds binding in pockets on the back of the active site of the enzyme (ROY et al., 2017). Some examples of flavonoids against E protein of DENV type 2 (DENV-2) were developed for Ismail and Jusoh (2017). Active sites docked amino acids Ile4, Gly5, Asp98, Gly100 and Val151 showed interactions to flavonoids obtaining E protein-flavonoids complexes. For example, hydroxyl groups (6-OH and 7-OH) of baicalein formed hydrogen bonds with different amino acid residues such as Asp 98, Gly100, Ile4 and Val151. The hydrogen bonds were observed among the amino groups of Gly100, carbonyl oxygen of Gly 5 to the 7-OH (ISMAIL; JUSOH, 2017).

De Sousa and colleagues (2015) reported good inhibition of quercetin, which presented high binding energy against NS2B-NS3 protease. Docking studies shown the evident formation of six hydrogen bonds with the amino acid residues at the binding site of the receptor. In general, the inhibition of viral RNA metabolism occurs through flavonoid binding, both NS2B-NS3 complex of DENV and ZIKV. Studies suggest the ability of hydroxyl groups whose are able to bind with amino acid sites through hydrogen bonds. Consequently, flavonoids are good candidates as antiviral therapy (DE SOUSA et al., 2015). Indeed, all computational approaches have been extremely useful in the drug discovery, rushing the process and reducing the cost of biological assays. However, *in silico* data needs to be followed by *in vitro* and *in vivo* validation.

Anti-ZIKV activity of flavonoids

Despite the reduction in the incidence of ZIKV infection in Brazil, from 200,000 suspected cases in 2016 to 9,000 cases in 2019, ZIKV remains circulating and affecting human healthy in South America. During 2018, changes in growth and development of 3,332 children were related to ZIKV or other infectious etiology. Between January and August 2019, 2 deaths by ZIKV infection and 447 cases of ZIKV-infected pregnant women were confirmed by the Brazilian Ministry of Health (MINISTÉRIO DA SAÚDE, 2019). In spite of the global effort to develop specific anti-ZIKV drugs and a vaccine, only one vaccine has reached phase 2 clinical trial until now (U.S. NATIONAL INSTITUTES OF HEALTH, 2019).

Studies have been carried out in order to search drug candidates against Zika by using strategies such as, screening of compounds libraries or repurposing of approved drugs. In this scenario, the screening of natural flavonoids has been an interesting source to find new anti-ZIKV drugs due to numerous beneficial biological effects already described for these molecules. The recent advances in the flavonoid-based anti-ZIKV molecules and the probable mechanisms of action were summarized in table 1 and figure 3, respectively.

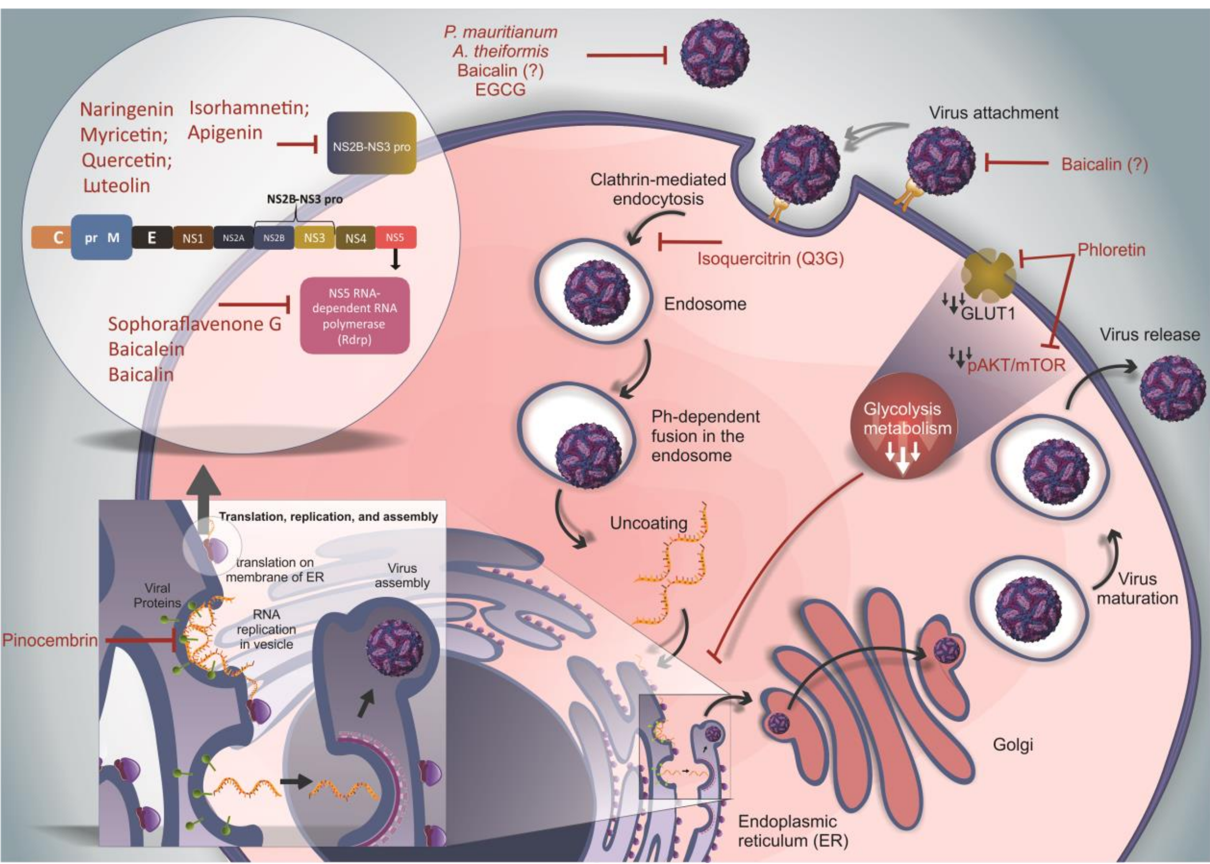

Figure 3 – Replication cycle of ZIKV and action of different flavonoids. The free viral particles can be disrupted by the action of curcumin, *P. mauritianum* extract, *A. theiforms* extract, baicalin and epigallocatechin gallate (EGCG) before virus binding to cell receptors. ZIKV attach to host receptors and is internalized by clathrin-mediated endocytosis. This phase can be inhibited by curcumin and baicalin, but the inhibitory mechanism needs to be clarified. Isoquercitrin (Q3G) inhibits the internalization and trafficking of viral particles. The acid microenvironment within endosome allows the fusion between viral and endosome membrane, resulting in viral RNA release into cytoplasm. The viral RNA is translated in a polyprotein (The inset in the upper left corner shows the sequential and structural organization of the ZIKV polyprotein) containing three structural proteins (capsid (C), pre-membrane (prM), and envelope (E) proteins) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The viral polyprotein is cleaved by host and viral proteases. NAR, myricetin, quercetin, luteolin, isorhamnetin, apigenin and curcumin can inhibit NS2B-NS3 protease (NS2B-NS3 pro). On the other hand, sophoraflavenone G, baicalein and baicalin can inhibit the NS5-RNA polymerase (Rdrp). Meanwhile, The NS3 protein interacts with Rdrp to allow the viral genome replication and pinocembrin can impair viral RNA synthesis. Phloretin was demonstrated to decrease glycolysis metabolism by downregulation of pAKT/mTOR pathway and GLUT1 blockade, impairing ZIKV replication by downmodulation of energetic metabolism. Note: (?) highlights molecules with inconsistent results.

Table 1 – *In vitro* activity of flavonoids against ZIKV.

Compound	Chemical structure	Proposed antiviral mechanism	Ref.
Epigallocatechin gallate (EGCG)		Blockage of viral-host membrane fusion.	(CARNEIRO et al., 2016; SHARMA et al., 2017)

Quercetin-3-β-O-D-glucoside (Q3G)		Inhibition on internalization and trafficking of viral particles.	(GAUDRY et al., 2018; WONG et al., 2017)
Sophoraflavenone G		Inhibition of viral RNA dependent RNA polymerase (Rdrp).	(SZE et al., 2017)
Baicalein		Unknown; docking analysis suggested NS5 binding	(OO et al., 2019)
Baicalin		Unknown; docking analysis suggested NS5 binding	(OO et al., 2019)
Phloretin		Interference with glucose metabolism required for ZIKV replication	(LIN et al., 2019)
Pinocembrin		Inhibition of viral RNA and envelope protein synthesis	(LEE et al., 2019)
Naringenin		Unknown; docking analysis suggested NS2B-NS3 protease binding	(CATANEO et al., 2019)

Flavonoids extracted from plants

Considering the potential antiviral properties of flavonoids and the high content of these natural compounds in *Psiloxylon mauritianum*, its anti-ZIKV activity was tested (Clain et al., 2019). In a time-of-drug addition experiment, the *P. mauritianum* polyphenolic aqueous extract showed to be effective only at the initial steps of the infection. Binding and internalization specific assays displayed a 10-fold inhibition in ZIKV-infected Vero cells compared to untreated control. Moreover, the results evidenced that the extract was selective to the viral particle, once pre-incubation of the

virus with the extract reduced the infectivity by ~80% (CLAIN et al., 2019). Despite promising, the polyphenol extract derived from *P. mauritianum* is composed by several flavonoids, then, the exact active compound is unknown.

Another aqueous plant extract, derived from the aerial parts of *Aphloia theiformis,* also presented similar antiviral activity against ZIKV, inhibiting early steps in the viral infection. The phytochemical analysis demonstrated that the *Aphloia theiformis* extract was rich in polyphenol content (CLAIN et al., 2018), but, the active compounds against ZIKV in the extract remained unknown. Testing isolated compounds from an active extract may give key answers in understanding if the compounds are effective alone or acts in synergy with other molecules.

In a study carried out with the hydroalcoholic extract of *Doratoxylum apetalum*, a medicinal plant common in the Mascarene Islands, rich in polyphenols, suggested activity against ZIKV, targeting the initial stages of infection. The study suggested that the extract may bind to the viral particles causing the inhibition of ZIKV internalization. However, it was not possible to determine which compounds are responsible for the loss of viral infectivity, and this is an issue that still needs to be investigated (HADDAD et al., 2019).

Finally, despite presenting the potential for discovery of new molecules/activities, antiviral research using plant extracts presents some limitations, such as the fact that the composition of secondary metabolites in plant extracts may vary depending on soil characteristics, climate, radiation and period of plant collection (WANG et al., 2012). It is also essential to determine the bioactive compounds, which represents a challenge for researchers working on purification and characterization of compounds. Those are common issues when studying biological/pharmacological properties of plant extracts and should be taken account. We discuss below the current data on the anti-ZIKV effects from isolated flavonoids

Sophoraflavenone G

The *Sophora flavescens* is one of the medicinal herbs with long history among Asian countries, presenting hundreds of compounds in its composition, including a variety of flavonoids derived from the roots. The roots of this herbal has been used to treat fever, pain and several other sickness symptoms (HE et al., 2015). Sze and colleagues (2017) identified the sophoraflavenone G derived from the roots of *Sophora*

flavescens as an antiviral compound against DENV and ZIKV. The isolated molecule, Sophoraflavenone G, 5,7,2',4'-tetrahydroxy-8-lavandulylflavanone is a flavonone, analogue of naringerin with two stereogenic centers, bearing an additional hydroxyl substituent at position 2', a (2R)-5-methyl-2-(prop-1-en-2-yl)hex-4-en-1-yl (lavandulyl) substituent at position 8'. The sophoraflavenone G treatment after infection lead to a reduction in the frequency of infected cells for both DENV and ZIKV. These data suggest that sophoraflavenone G affects later steps of the virus replication cycle. To address the anti-ZIKV mechanism of the compound, the authors performed RNA-dependent RNA-polymerase (RdRP) activity assay and observed the inhibition of this enzyme. Despite a low selective index (SI) of sophoraflavenone G, the specific inhibition of ZIKV-RdRP has the potential to present less side effects *in vivo* (SZE et al., 2017). The inhibition of viral RdRP was suggested to be effective against dengue, Middle East respiratory syndrome coronavirus (MERS-COV) and severe acute respiratory syndrome coronavirus 2 (SARS-COV2) (GORDON et al., 2020; LUNG et al., 2020; NIYOMRATTANAKIT et al., 2010). Against ZIKV, similarly to sophoraflavenone G, the clinically approved drug Sofosbuvir was shown to block ZIKV RdRP activity (SACRAMENTO et al., 2017). However, the *in vivo* efficacy of both sophoraflavenone G and sofosbuvir against ZIKV needs to be determined.

Baicalein and baicalin

The *Scutellaria baicalensis* is a Chinese medicinal herb that presents high concentration of the flavonoid baicalein in their roots, and its glucoside form, baicalin, is the most abundant glucoside flavonoid isolated. Baicalein, 5,6,7-trihydroxy-2-phenyl-chromen-4-one is a trihydroxyflavone with hydroxyl groups at positions C-5, C-6 and C-7. Its derivative baicalin is a flavone glycoside with a glucuronide portion at 7-OH.

Baicalin has low bioavailability and it could limit its use and result in failure of the clinical efficacy (HUANG; LIU; ZHANG, 2019). Due to its high polarity, the transportation is limited through lipid bilayer via simple diffusion.(HUANG; LIU; ZHANG, 2019). Baicalein presents good permeability due to its good lipophilicity. Thus, some strategies to increase the efficacy of these compounds, for example, modifying the structure with groups in which could improve the bioavailability.

Baicalein exert a potent antiviral activity against DENV due to its virucidal activity (ZANDI et al., 2012a). Additionally, baicalin was also effective against DENV probably by interfering in the binding of viral particles to the host cells (MOGHADDAM et al., 2014). The anti-ZIKV activity of baicalein and baicalin was assessed by Oo and colleagues (2019). Using a time-of-drug addition experiments a 50% reduction in ZIKV-RNA levels in cell culture supernatant was observed for both baicalein and baicalin-treated Vero cells. Both compounds were able to impair the infection at different steps of viral cycle. However, baicalein was shown to be more potent when added after infection, while baicalin seemed to be most effective in the early steps of viral cycle. Due to their low toxicity, higher doses could be promising in treatments of ZIKV through their higher affinity to NS5 RNA-Polymerase (Oo *et al.* 2019), which are corroborated by docking analyses. Docking analysis showed high binding affinity of both compounds with ZIKV NS5 (Oo et al., 2019). It was predicted binding afinities as baicalein (-8.4 kcal/mol) as baicalin (-10 kcal/mol) with the ZIKV NS5, who presented strong interactions while glycoprotein virus membrane weaker interactions (-7.3 kcal/mol and -7.2 kcal/mol for baicaleina and baicalin respectively). It sugests the intermolecular bonds of amino acids residues of active sites ligand-receptor through hydrogen bonds and hydrophobic interactions between Lys 105 alkylic chain and π system. As results, this kind of afinity towards ZIKV NS5 protein is corroborate with their biological efficiency, since the protein’s polymerase is the main responsible for replication of virus (Oo et al., 2019).

Interestingly, the molecular docking analyses for baicalin were not consistent with the *in vitro* findings that showed baicalin action in the initial steps of viral cycle. It was already demonstrated that ZIKV NS5 protein antagonizes the type I interferon response by degradation of STAT2 (GRANT et al., 2016). Therefore, molecules able to bind and inhibits the NS5 function may be suitable candidates to drug development, nevertheless, more studies are necessary to fully understand the impact on the viral replication.

Epigallocatechin gallate

The EGCG is the main natural flavonoid found in green tea, has shown to exert antiviral activity against herpes simplex virus, hepatitis C virus and DENV (CIESEK et al., 2011; LYU; RHIM; PARK, 2005b; RAEKIANSYAH et al., 2018). EGCG comprises

catechin group, similar to epicatechin, with a benzopyran moiety similar to the catechol and two stereocenters being the relative and absolut stereochemistry *cys* and (2*R*,3*R*) respectively. Its synthesis was reported for the first time by Li and Chan (2001) obtaining exclusively one stereoisomer. It's worth mention that the key step consisted of sharpless asymmetric hihydroxilation achieving the control of stereoselectivity with a stereospecific step (LI; CHAN, 2001).

To access the antiviral activity of EGCG against ZIKV, Carneiro and colleagues (2016) pre-incubated for 1 hour different concentrations of EGCG with viral particles and then challenge Vero E6 cells (CARNEIRO et al., 2016). A 1-log reduction in the number of viral foci was observed, with an EC_{50} of 21.4 μM. ZIKV African lineage seemed to be more susceptible to EGCG activity, once lower concentrations of the compound were needed to decrease the foci number at same extent of the Brazilian isolate from the Asian lineage of ZIKV. The results suggested a direct action of the drug in the viral particle, due to the destruction of the phospholipid envelop (YAMAGUCHI et al., 2002), which could inhibit the viral adsorption to the cell surface and impairs the viral entry into the cells. The direct action of EGCG in the virus was reinforced when no reduction in foci number was observed with Vero E6 pretreatment before infection. In agreement with these data, docking studies proposed that several residues from ZIKV E protein were involved in the interaction with EGCG. The molecular dynamics simulation and docking studies showed that EGCG blocked the fold back of domain III, which is an important step to form the hairpin structure (SHARMA et al., 2017). Therefore, this interaction between EGCG and ZIKV E protein blocks the viral host membrane fusion and the virus entry. The residues Thr40, Gly145, Glu162, Asn163, Tyr305, Lys340 and Asn362 show strong hydrogen bond interactions throughout the simulation (SHARMA et al., 2017). It was observed hydrogen bonds and hydrophobic interactions. Kumar and colleagues (2019) developed mechanistic studies with NS3 helicase where NS3 with 618 amino acid residues being the second bigger protein. This interaction NS3-EGCG was strong, with an binding energy of 7,8 kcal/mol regards to amino acids residues of ATPase. The enzymatic inhibition has shown IC_{50} of 295.7 µM, thus being a starting material for the research and development of broad spectrum inhibitors (KUMAR et al., 2019). Vázquez-Calvo and colleagues (2017) also observed similar results testing EGCG against ZIKV, DENV and West Nile Viruses (WNV) (VÁZQUEZ-CALVO et al., 2017). In contrast, Raekiansyah and colleagues (2018) observed minimal effect of EGCG against ZIKV,

despite the high activity against the four serotypes of DENV. These opposite results may be explained by the differences on the viral strains used, which reinforces the need for testing as many virus isolates/strains as possible (RAEKIANSYAH et al., 2018).

Quercetin-3-β-O-D-glucoside (Q3G or isoquercitrin)

Quercetin was described to exert antiviral activity against influenza, HCV and DENV (WU et al., 2015; ZANDI et al., 2011a; ROJAS et al., 2016). The anti-ZIKV activity of Quercetin-3-β-O-D-glucoside (Q3G) was recently evaluated. Q3G is also known as isoquercitrin and is one of the main glycosidic forms derivates from the flavonoid quercetin. It is noteworthy that the glycosides forms of quercetin were rapidly absorbed and exhibit higher bioavailability than the aglycones forms (ARTS et al., 2004; HOLLMAN et al., 1995, 1997), which are important features to an antiviral candidate. First, the anti-ZIKV activity of Q3G was tested *in vitro* on Vero cells. The effect of Q3G treatment before and after the ZIKV infection was evaluated by viral RNA quantification two- and four-days post-infection. The results showed a reduction in the non-structural protein 1 RNA in the supernatant and cell lysate, suggesting an effect on viral replication. Additionally, *in vivo* assays in immunocompromised mice (Type I IFN receptor knockout - *Ifnar1*$^{-/-}$) demonstrated a 50% survival up to 21 days after ZIKV infection upon Q3G treatment, which contrasted with 100% death within 7 days of infection in the vehicle control group (WONG et al., 2017). Moreover, despite initial weight loss of nearly to 20%, the surviving animals recovered the initial weight.

Additionally, Gaudry and colleagues (2018) demonstrated that Q3G treatment at a concentration of 100 μM from the beginning of infectious cycle of the virus was able to decrease the number of A549-infected cells, intracellular viral RNA and viral titers in the supernatant. Q3G antiviral effect was showed by an internalization assay, suggesting that Q3G may act as an inhibitor of internalization and trafficking of viral particles. Furthermore, the same study also evaluate the related Q3G flavonoids quercetin, hyperoside and kaemperol, however, no anti-ZIKV effect was observed for the additional compounds tested (GAUDRY et al., 2018).

Phloretin

The anti-ZIKV activity of phloretin was tested *in vitro* against both African and Asian lineages of the virus. Phloretin decreases the cell-death induction by ZIKV infection, viral progeny and intracellular ZIKV envelope protein staining. Interestingly, the antiviral effect of phloretin seems to be cell-type dependent since higher activity was observed in Vero and U87MG cells than in human umbilical vein endothelial cells. In a time-of-drug addition assay, the results showed that phloretin was able to interfere in multiple steps of viral cycle, but mainly in the RNA production and later steps of viral cycle. Moreover, it was demonstrated the importance of glucose to ZIKV replication and that phloretin induced greater reduction in viral titers in cells cultured in glucose-free medium (LIN et al., 2019). Phloretin is a glucose transporter inhibitor (GLUT1) and ZIKV effect on GLUT1 was hypothesized to be involved in the risk of congenital syndrome (BLONZ, 2016). The AKT/mTOR pathway was demonstrated to be involved in the increase of glucose uptake and metabolism (KOHN et al., 1996). In addition, mutations in viral proteins such as NS4A and NS4B were reported to induce inhibition of AKT/mTOR pathway, which is important in the development of neuropathies (JUN et al., 2017). Lin and colleagues (2019) also investigated the AKT/mTOR pathway and observed decreased phosphorylation of the AKT/mTOR pathway in infected phloretin-treated cells, suggesting a possible involvement of this pathway in virus replication. Altogether, these data suggest the positive correlation between GLUT1 transporter expression and ZIKV titers. In agreement with these data, it was demonstrated that ZIKV infection can upregulate glycolysis metabolism to support replication (TIWARI et al., 2017). Moreover, phloretin was demonstrated to exert *in vitro* neuroprotective effects in human neuroblastoma cells (BARRECA et al., 2017). Therefore, phloretin would be an interesting molecule for further studies since impairs ZIKV replication and showed neuroprotective ability. However, it is important to determine the relation between glucose metabolism and viral replication before assuming this compound as an antiviral drug, mainly in the context of pregnancy and fetal development.

Pinocembrin

Pinocembrin, (5,7-dihydroxyflavanone), is a chiral flavanone isolated from the variety of plants, mainly from *Pinus* heartwood, *Eucalyptus*, *Populus*, Euphorbia, and *Sparattosperma leucanthum*. Although there are two enantiomers, only the enantiomer

(2S) is most often found (GUO et al., 2016). It is the major flavonoid with industrial applications due to its several pharmacological activities (RASUL et al., 2013).

Following an immunofluorescence-based high throughput screening (HTS) assay of 483 flavonoid derivates, Lee and colleagues (2019) identified the flavanone pinocembrin with higher inhibitory effect of ZIKV. Pinocembrin is a flavanone widely distributed in honey and propolis with neuroprotective characteristics (GUANG; DU, 2006). The anti-ZIKV activity of pinocembrin was observed to be cell type dependent, inhibiting the infection on Huh7 (human-derived hepatoma) cells, however, presenting none reduction when tested in BHK-21 (baby hamster kidney fibroblasts) and HEK293T (human embryonic kidney). A set of experiments was performed in order to determine which step of the viral cycle the compound act and the results suggested an interference with viral RNA synthesis and envelope proteins, affecting directly the viral replication (LEE et al., 2019). Interestingly, pinocembrin was already approved in China for clinical trial phase II in ischemic stroke patients (U.S. NATIONAL INSTITUTES OF HEALTH, [s.d.]) making its repurposing as an anti-ZIKV reasonable.

Naringenin

Naringenin, (NAR; 4, 5, 7-trihydroxyflavanone) is the natural flavonoid aglycone of naringin, with one asymmetric center. The inhibitory effects of the citrus flavanone NAR has been described against herpes simplex virus, Sindbis and CHIKV replicon (LYU; RHIM; PARK, 2005a; PAREDES et al., 2003; POHJALA et al., 2011a).

Regarding anti-flavivirus effect, NAR was revealed to exert antiviral activity against all four serotypes of DENV (FRABASILE et al., 2017). Also, anti-ZIKV activity of NAR against Asian- and African-lineage of the virus was also showed (Cataneo et al., 2019). The treatment of infected-A549 cells decreased the frequency of infected cells, the amount of viral RNA inside the cells and the viral progeny in the supernatant. Furthermore, a set of time-of-drug addition assays, suggest that the antiviral activity of NAR should occur at later steps of virus live cycle, between the replication and viral release. Molecular docking studies have shown interactions between NAR and NS2B-NS3 protein through binding of actives sites with hydrogen bonds with Gln-74. Carbonyl group of NAR acts as hydrogen acceptor of acid portion of carboxylic acid Gln-167 residue; 6 and 7-hydroxyl phenolic moieties, hydrogen donators, bond with Thr-166 and Gln-167 carboxyl groups respectively. Oxygen of 4'-OH bonds to amine

hydrogen of Trp 89. On top of that, hydrophobic interactions occurs through contact of Ile-123 side chain NAR carbon atoms (CATANEO et al., 2019). Therefore, docking studies reinforced the hypothesis of viral replication inhibition of viral replication. Interesting, while Frabasile and colleagues (2017) observed that NAR impairs DENV replication with no virucidal effect (FRABASILE et al., 2017), Zandi and colleagues (2011) showed a virucidal effect and that NAR was not able to block DENV replication (ZANDI et al., 2011b). These contrasting results might be explained by differences in cell type and/or DENV-strains used in each experimental condition. Additionally, despite *in vitro* activity of NAR for DENV and ZIKV, *in vivo* assays in small animal models should be performed to confirm its potential use to treat infected patients.

Chemical structure affects the biological activity

The data presented here suggests that flavonoids could be the source of suitable molecules to struggle ZIKV infection. The potential actions of flavonoids cover several aspects of ZIKV life cycle and host-pathogen interactions such as viral replication, binding and entry, maturation and release, and antiviral immune response. These mechanisms of action would lead to the control of infection or reduce damage in the host.

Biological activity of compounds depends mainly the interaction of both active molecule and biological active site that depends of intermolecular strengths such as hydrophilic, hydrophobic, electrostatics and sterics. By introducing nonpolar groups, such as hydrocarbon chains, the solubility in lipid phases could be improved, for example, etherify ring A phenols such as methoxy and others alkyl chain, while bioavailability could be improved by increasing polyhydroxylated groups, which are responsible for improving solubility in the biological environment through hydrogen bonds with water.

Additionally, chemically-directed changes in the structure of flavonoids could be rationally used to improve activity and reduce toxicity of the compounds. Numerous substituents can be found at the rings of the flavonoids structure, such as hydroxyl, methoxy, benzyl and methyl groups, and as mentioned above, flavonoids can undergo glycosylation at different positions (FENG; HAO; LI, 2017; KOSTRZEWA-SUSŁOW; DMOCHOWSKA-GŁADYSZ; OSZMIAŃSKI, 2007). Although flavonoids are potential candidates for drugs, they present low bioavailability due to their poor solubility. To

improve biological activities and properties, structural modifications could be a good strategy.

Most flavonoid functionalizations are made on phenol, which due to its high acidity, becomes a nucleophile species on weak bases. Thus, is possible the insertion of alkyl, alkynyl, triazol and a several functional groups. Addition of alkyl chain is achieved through SN_2 reaction with alkyl halides. 1,2,3-Triazole-bridged flavonoid could be accessed through alkynyl ether through click chemistry reaction with an azide functionalized (SUM et al., 2016).

Suroengrit and colleagues (2017) has demonstrated anti-ZIKV and anti-DENV activity of halogenated derivatives of chrysin (Suroengrit et al., 2017). The electronegative substituents, bromine and iodine, in positions 6 and 8, in addition to the free hydroxyl groups in positions 5 and 7, contributed to the antiviral activity of these molecules. This result could be observed through the low viral inhibition of the other derivatives with the absence of electronegative substituents in positions 6 and 8 and with free hydroxyls in 5 and 7. In addition to the low activity of analogs containing methoxy groups in positions 3, 5 and 7. These compounds were able to inhibit virus production by up to 62% at a concentration of 10 μM, while halogenated derivatives showed an inhibiting power greater than 99% at the same concentration. The compounds halogenated derivatives showed potent activity against all serotypes of DENV and ZIKV and similar cytotoxicity, suggesting that these compounds are broad-spectrum anti-flavivirus drugs with action on multiple targets, however, the greatest efficacy was achieved with early post treatment -infection.

A study by Du and colleagues (2016) demonstrated antiviral activity of a chrysin analog, a phosphate ester, substituted in position 7 by a diisopropyl phosphate group against DENV1 and DENV2. The phosphorylated analog inhibited viral protein synthesis in cells infected with DENV1 and DENV2, with IC_{50} of 18.6 and 15.1 μmol L-1, respectively, demonstrating efficacy against the replication of the two serotypes in cell cultures without cytotoxicity (DU et al., 2016). Studies reveal that phosphorylated flavonoids have strong binding affinities, in addition to forming non-covalent complexes with proteins more easily than non-phosphorylated compounds (CHEN et al., 2004).

Motivated by the biospherical relationship between quercetin and aryl diketoacid (ADK), known for its antiviral activity, in addition to the role of arylmethyl substituents in the antiviral activity described in previous works, Park and colleagues (2012) reported the synthesis and antiviral evaluation of 7-O-benzylated quercetin derivatives

(7-O-Arylmethylquercetin) functionalized against hepatitis C (HCV) and SARS-associated coronavirus (SARS-CoV, SCV). Regarding the anti-HCV activity, among the synthesized compounds, derivatives containing strong electron withdrawing groups in the meta and para positions of the benzyl ring, such as the nitro group, proved to be the most active with EC_{50} 11.0, 1.0 and 8.9 µM, respectively, without cytotoxicity. In addition, the presence of electron donating groups such as hydrogen, methyl and methoxy in the position four (4'') of the benzyl ring also showed selective activity, presenting EC_{50} 25.7, 23.5 and 33.9 µM, respectively. These data suggest an important role for the electronic properties of these groups around the benzyl ring in the bioactivity of these molecules (PARK et al., 2012).

Lim and colleagues (2017) demonstrated the ZIKV NS2B-NS3 inhibitory activity of 22 selected flavonoids (LIM et al, 2017). It was shown that the number and position of hydroxyl groups favor the anti-ZIKV activity of flavonoids, while glycosylation, methoxy and prenyl groups seems to be responsible to lower inhibitory activity on ZIKV NS2B-NS3 (LIM et al., 2017). Thus, although glycosylation increases the absorption of flavonoids, it decreases their efficacy against ZIKV. Bhargava and colleagues (2017) used the 22 compounds tested by Lim and colleagues (2017) to perform a Monte Carlo-based QSAR simulation in order to identify structural requirements for inhibitory activity of those flavonoids on ZIKV NS2B-NS3. Some of the structural features related to inhibitory activities are the presence of sp^2 and sp^3 oxygen bonded with carbon, presence of sp^2 carbon, presence of double bond, presence of sp^2 carbon within a ring system, presence of sp^2 carbons with branching and others (BHARGAVA et al., 2017). Moreover, the presence of an oxygen within a ring seems to be crucial for the inhibitory activity. On the other side, glycone moieties were found to be detrimental for the inhibitory activity (BHARGAVA et al., 2017). Therefore, the Monte Carlo-based QSAR model could be a suitable tool in the prediction of new antiviral candidates, helping to find active molecules faster, which is essential in emerging global public health problem, as happened with ZIKV outbreaks.

Although studies of flavonoids as biological molecules are well established, biological evaluation of flavonoid-based compounds are few explored in literature as anti ZIKV. Therefore, directed structural chemical modifications in the flavonoid skeleton could be a rational approach to improve anti-ZIKV activity, optimize pharmacokinetics and reduce toxicity of potential anti-ZIKV flavonoids.

Others biologically actives molecules against ZIKV: a comparison with flavonoids

Although flavonoids are promisors against ZIKV, there is no specific drug already approved. However, recent efforts about several other molecules have been reported and these studies have been guided computationally. These molecules were evaluated against different viral targets. Nanchangmycin, NSC157058, Suramin, Sofosbuvir, Sinefungin acting against envelope glycoprotein, NS2B-NS3B protease; NS3 helicase, NS5 polymerase, NS5 methyl polymerase, respectively (Table 2) (MOTTIN et al., 2018a).

Table 2. Biologically actives molecules against ZIKV

	ZIKV structure	IC_{50} or EC_{50}	Flavonoid
Nanchangmycin	Envelope glycoprotein (E protein)	0.1 μM	EGCG (> 100 μm)
NSC157058 (NSC)	NS2B–NS3 protease	0.82 μM	Naringerin (58.79 μM)
Suramin	NS3 helicase	0.42 μM	Quercetin (150 mM);
Sofosbuvir	NS5 polymerase	7.3 μM	Baicalein (1,9 μM)
Sinefungin	NS5 methyltransferase	0.46 μM	-

Nanchangmycin, a complex molecule, inhibited infection with lower IC_{50} (0.1 μM), while EGCG presented IC_{50} >100 μM. Despite the efficient inhibition, unfortunately it may not be viable, as it presents a reactive site like Michael's acceptor, called PAINS structure (RISHTON, 2003). Thus, side reactions can occur with soft nucleophiles, such as sulfur-containing enzymes. Regarding the structure, it has a high complexity with several stereogenic centers, which makes its synthesis impossible with the control of stereoselectivity. Catechol groups presented in EGCG can also acts promiscuously many different active sites (MOTTIN et al., 2018a). EGCG can target multiple viral protein such as envelope, protease and helicase. Since EGCG has shown virucidal effect against several viruses, therefore EGCG backbone could be used to develop a broad-spectrum antiviral molecule in near future.

Some strategies for developing bioactive molecules against ZIKV through NS2B-NS3 complexes comprises the five pharmacophoric hypotheses, being one hydrogen bond acceptor (A), two hydrogen bond donors (DD) and two aromatic rings (RR) – ADDRR as are both NSC and NAR (ROHINI et al., 2019). Therefore, NSC is a

stronger NS2B-NS3 protease inhibitor than NAR (58.79 mM), however, *in vivo* assays demonstrated an unfavorable pharmacokinetic profile (SHIRYAEV et al., 2017). Thus, NAR may become a potential although *in vivo* studies must be performed (CATANEO et al., 2019). Thus, strategic functionalizations in NAR and their respective cytotoxicity studies and molecular computer simulations may be an alternative.

Suramin can bind NS3 helicase and it has good inhibition while flavonoids as quercetin and EGCG have higher IC_{50} (TAN et al., 2017). As described previously, EGCG also presents affinity with NS3.

Regarding NS5 polymerase inhibitions, baicalein presented $IC_{50} \approx 1.9$ μM, stronger inhibitor than FDA-approved sofosbuvir ($IC_{50} = 7.3$ μM) which was predicted by computational simulation. Both hydrogen bonds and attractive charges interactions between amino acids of RNA polymerase and phosphate moiety may disrupt the hydrogen bonding of incoming nucleotides to ZIKV RNA polymerase (SACRAMENTO et al., 2017). Therefore, phosphate groups present great trends and strong links with active enzyme. Thus, it suggests that the insertion of polar phosphate groups in flavonoids may be promising. In addition to presenting low toxicity, they are composed of relative complexity and simpler syntheses, leading to an atomic economy.

Finally, NS5 Methyltransferase domain is responsible for transferring mRNA cap and can prevent the virus replication when its structure is target and specific sites are deactivated. The competitive methyltransferase inhibitor sinefungin, a natural product of class of adenosine isolated from *Streptomyces griseoleus*, was suggested to be a promising inhibitor of ZIKV replication (HERCIK et al., 2017). However, the high toxicity in animal models might prevent its clinical use (ROBERT-GERO; LAWRENCE; LEDERER, 1989).

Bottlenecks and Future Directions

The potential use of flavonoids as a source of molecules with anti-ZIKV activity was addressed in this review. Even though promising, several questions remain to be answered. As flavonoids became an important class of compounds with potential biological benefits, it is essential that these molecules are well absorbed by the gastrointestinal tract and present minimal side effects. Cellular models were used to simulate the ability of flavonoids to permeate the intestinal tract and found that hydrophobic molecules had better potential to be absorbed (BARRINGTON et al.,

2009). The absorption and bioavailability of flavonoids can vary depending on different factors. As already stated before, the chemical structure is an important feature that determines the biological activity of flavonoids, however, also impacts on *in vivo* absorption and bioavailability.

Some data reviewed here demonstrated that glucosides forms of flavonoids have better absorption, however, they present reduced antiviral activity (DAI; YANG; LI, 2008). In general, several mechanisms are involved in the impaired bioavailability of flavonoids, such as poor transport due to glycoside characteristics or enhanced metabolism of the aglycones, leading to low concentrations available to exert the desired biological effect. However, some strategies can be adopted to overcome this barrier, such as improving the intestinal absorption with micro-emulsions and nano-carriers (CONSTANTINIDES, 1995; GUAZELLI et al., 2013; LEMBO; TROTTA; CAVALLI, 2018; ZHANG et al., 2011), or by structural modifications leading to better hydrophobic characteristic, improving the delivery and efficacy of molecules. Indeed, the absorption, metabolism, transport and bioavailability of flavonoids are a great challenge to overcome in the drug discovery field. Advancing in this field is essential to avoid the delay in the progress of bioactive compounds to preclinical and clinical stages of a drug development.

The identified active compounds against ZIKV were mostly evaluated by *in silico* or *in vitro* studies using computational and cell-based assays and need to be further evaluated in preclinical analysis using small animal models before redirection to clinical tests in humans. The preclinical assays using small animal models is a special challenge regarding flavivirus infection. Most of murine models for flavivirus infection are animals lacking important components of the immune response, for example, mice lacking type I and II interferons receptors, an essential pathway of innate immunity in antiviral response (DOWALL et al., 2016). Several other mice strains lacking a variety of receptors or type I IFN downstream proteins were described to be susceptible to ZIKV infection (LAZEAR et al., 2016). Besides that, the age of infected animals is determinant in the outcome of pathogenesis, while wild type animals are resistant to ZIKV infection (ROSSI et al., 2016). Despite the limitations of the *in vivo* flavivirus infection, susceptible animals presenting quantifiable viral load, viremia and measurable clinical score can be a suitable model to study the efficacy of antiviral molecules, pharmacokinetic and toxicological effects of flavonoids.

The immune deficient mouse models restrict the evaluation of compounds that affect viral cycle, but cannot concomitantly evaluate the activity of compounds that also boost the innate immune response against the virus. Flavonoids were described as modulators of immune response, therefore, data obtained with immunocompromised animal models need to be carefully interpreted. Despite this, the mouse models are widely used to screening drugs and evaluate the toxicity of synthetic or natural compounds, including flavonoids. However, the rational use of animals must be priority, running out all *in vitro* assays before preclinical tests and considering the principle of 3Rs (replacement, reduction and refinement) of animal use for research purpose.

Conclusion

An important information in drug discovery field is the determination of the mechanism of action of the drug candidates. Here, we presented several flavonoids with antiviral activity and how each compound acts on different steps of viral cycle. However, determining the entirely mechanism of action of a flavonoid is challenging since they are usually multi-target drugs (SUROENGRIT et al., 2017). Despite that, multi-target drugs can present advantages on their action since they do not fully disrupt endogenous physiological mechanisms compared to single target drugs, therefore, presenting better outcome regarding side effects (VERRI et al., 2012).

Furthermore, evidence supports that flavonoids are promising sources of candidate molecules to the development of effective anti-ZIKV drugs. However, several challenges persist such as the lack of a ZIKV-immunocompetent animal model, flavonoid purification, structural-relation activity, toxicity and pharmacokinetic properties of the molecules, and determination of precise mechanism of action needs to be fully addressed in the future. Finally, flavonoids are present in our diet and also taken as supplements, however, all questions mentioned above must be considered before a flavonoid-based anti-ZIKV molecule proceeds to clinical trials.

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2 OBJETIVOS

2.1 Objetivo geral

Avaliar o potencial efeito antiviral do flavonóide naringenina (NAR) na infecção *in vitro* de células humanas por Zika vírus (ZIKV).

2.2 Objetivos específicos

- Caracterizar as cepas virais utilizadas no estudo;
- Definir a concentração máxima não tóxica da NAR para as células A549 e determinar o CC_{50} para a NAR nesta linhagem celular;
- Caracterizar a atividade antiviral da NAR em células A549 infectadas com ZIKV;
- Calcular através de uma curva dose-resposta o IC_{50}, IC_{90} e SI para a NAR;
- Avaliar o efeito antiviral da NAR sobre células A549 infectadas com diferentes cepas da linhagem Asiática e Africana de ZIKV;
- Investigar a atividade virucida da NAR e o possível momento do ciclo de replicação viral onde a NAR atua;
- Realizar estudos *in silico* para determinar a potencial interação da NAR com proteínas virais;
- Avaliar o efeito antiviral da NAR em células humanas relacionadas à patogênese da infecção por ZIKV ou no desenvolvimento da resposta imune antiviral;
- Avaliar o efeito anti-ZIKV de diferentes compostos sintéticos derivados do flavonóide NAR.
- Definir a concentração máxima não tóxica das moléculas derivadas da NAR para as células A549, bem como, calcular o CC_{50} nesta linhagem celular;
- Avaliar a atividade antiviral dos compostos derivados de NAR em células A549 infectadas com ZIKV;
- Calcular através de curva dose-resposta o IC_{50}, CC_{50} e SI para os compostos com melhor atividade anti-ZIKV.

3 RESULTADOS

3.1 CAPÍTULO 2

"Detection and clearance of a mosquito densovirus contaminant from laboratory stocks of Zika virus"

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Estudos genéticos identificaram duas linhagens distintas de ZIKV, a Africana e a Asiática (HADDOW et al., 2012; WANG et al., 2016). Adicionalmente, foi demonstrada a existência de diferenças significativas na virulência e patogenicidade entre as duas linhagens (BEAVER et al., 2018). Recentemente foi demonstrado que as linhagens Asiática e Africana diferem entre si quanto à replicação viral e a respostas imunes de macrófagos e células dendríticas humanas induzidas pós-infecção (ÖSTERLUND et al., 2019), fatos que podem estar relacionados com a patogênese da doença. Além disso, estudos prévios objetivando caracterizar cepas de ZIKV, identificaram diferenças genéticas entre as cepas classificadas dentro da mesma linhagem, e tais aspectos podem ter impacto na capacidade de infecção e replicação de cada cepa, como demonstrado por Strottmann e colaboradores (STROTTMANN et al., 2019). Portanto, tais diferenças podem ter associação com o surgimento de sintomatologias graves, como os casos de Síndrome Congênita do Vírus Zika e Síndrome de *Guillain-Barré*.

Desta forma, previamente aos ensaios para avaliação do efeito antiviral da NAR, optamos por ampliar o número de cepas disponíveis para os ensaios além daquelas isoladas de amostras clínicas e caracterizadas pelo grupo do Laboratório de Virologia Molecular do Instituto Carlos Chagas/Fiocruz-PR. Ainda, cabe destacar que as cepas de ZIKV isoladas no Brasil são da linhagem Asiática, assim seria de grande relevância obtermos uma cepa da linhagem Africana do ZIKV para efeito de

comparação. Portanto, em colaboração com diferentes grupos de pesquisa do país, duas cepas amplamente utilizadas no meio científico foram enviadas ao nosso laboratório, a cepa Asiática ZIKV PE243 e a cepa Africana MR766. Deste modo, seria possível avaliar a atividade antiviral da NAR contra cepas das duas linhagens de ZIKV. Durante a produção dos estoques virais que seriam utilizados no estudo da NAR como molécula anti-ZIKV foi observado que ambas as cepas apresentaram efeito citopático incomum em células de *Aedes albopictus* (C6/36), fato este que levantou suspeitas sobre a presença de um possível contaminante presente nas amostras enviadas ao ICC/Fiocruz-PR. Portanto, neste segundo capítulo, abordamos a detecção da contaminação por densovírus de mosquito (MDV) em ambas as cepas virais recebidas pelo ICC/Fiocruz-PR. Além da detecção, propusemos duas estratégias para eliminação do MDV contaminante; *in vitro* através de sucessivas passagens em células de mamífero; ou *in vivo* utilizando modelo murino de infecção. Desta forma, os resultados obtidos nesta etapa foram compilados em uma primeira publicação no periódico Memórias do Instituto Oswaldo Cruz (doi:10.1590/0074-02760180432).

Neste trabalho foi confirmada através da amplificação via reação em cadeia da polimerase (PCR) de um fragmento de 324 pb, seguido de sequenciamento pela metodologia de Sanger, a contaminação pelo *Brevidensovirus,* nomeado na publicação a seguir como densovírus de mosquito (MDV). Ensaios adicionais de imunofluorescência e PCR (em diferentes amostras e condições) confirmaram a contaminação em ambas as cepas de ZIKV com MDV.

Tendo identificado o MDV como vírus contaminante dos estoques de ZIKV, executamos uma estratégia para eliminar a contaminação dos estoques, a fim de que pudéssemos utilizar as cepas nos ensaios antivirais. Sabe-se que o MDV não é capaz de infectar e replicar em células de mamíferos, enquanto que já foi demonstrado que vários tipos celulares são suscetíveis à infecção por ZIKV, inclusive células A549, uma linhagem celular derivado do epitélio pulmonar humano (ATCC: CCL185) (FRUMENCE et al., 2016). Desta forma, utilizamos estas células na tentativa de bloquear a replicação do MDV enquanto o ZIKV mantinha-se replicando normalmente. Após 3 passagens em células A549 não foi mais possível detectar o fragmento do MDV por PCR. Para garantir a total eliminação do MDV, o sobrenadante recuperado foi utilizado para infectar células C6/36, as quais são permissivas à infecção por MDV. Da mesma forma, o ensaio de imunofluorescência confirmou a eliminação dos contaminantes de ambos os estoques de ZIKV. Em paralelo aos ensaios *in vitro*, foram

realizados ensaios *in vivo* de infecção de camundongos A129 com a cepa PE243 contaminada, seguida de alimentação de *Aedes aegypti* nestes animais. Ensaios de PCR e imunofluorescência também demonstraram que a passagem em camundongos A129 acaba por eliminar o vírus contaminante (MDV).

Portanto, neste trabalho, objetivamos alertar à comunidade científica quanto à presença de contaminantes em estoques virais. O impacto que tais contaminações podem causar nas pesquisas científicas necessita de maior investigação, porém há relatos de que coinfecções podem interferir na infecção e/ou replicação de outros microrganismos (MOSIMANN et al., 2011; WEI et al., 2006). Todos os dados apresentados resumidamente acima podem ser mais detalhadamente analisados no artigo de Cataneo e colaboradores (2019) a seguir.

Detection and clearance of a mosquito densovirus contaminant from laboratory stocks of Zika virus

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BACKGROUND The Zika virus (ZIKV) epidemics that affected South America in 2016 raised several research questions and prompted an increase in studies in the field. The transient and low viraemia observed in the course of ZIKV infection is a challenge for viral isolation from patient serum, which leads to many laboratories around the world sharing viral strains for their studies. C6/36 cells derived from *Aedes albopictus* larvae are commonly used for arbovirus isolation from clinical samples and for the preparation of viral stocks.

OBJECTIVES Here, we report the contamination of two widely used ZIKV strains by *Brevidensovirus*, here designated as mosquito densovirus (MDV).

METHODS Molecular and immunological techniques were used to analyse the MDV contamination of ZIKV stocks. Also, virus passages in mammalian cell line and infecting susceptible mice were used to MDV clearance from ZIKV stocks.

FINDINGS MDV contamination was confirmed by molecular and immunological techniques and likely originated from C6/36 cultures commonly used to grow viral stocks. We applied two protocols that successfully eliminated MDV contamination from ZIKV stocks, and these protocols can be widely applied in the field. As MDV does not infect vertebrate cells, we performed serial passages of contaminated stocks using a mammalian cell line and infecting susceptible mice prior to re-isolating ZIKV from the animals' blood serum. MDV elimination was confirmed with immunostaining, polymerase chain reaction (PCR), and analysis of the mosquitoes that were allowed to feed on the infected mice.

MAIN CONCLUSIONS Since the putative impact of viral contaminants in ZIKV strains generally used for research purposes is unknown, researchers working in the field must be aware of potential contaminants and test viral stocks to certify sample purity.

Key words: arbovirus isolation - contamination - C6/36 cultures - densovirus - vertebrate cells - Zika virus

In the past few years, human Zika virus (ZIKV) infection has caused an increase in public health concerns due to an association with new clinical manifestations, such as *Guillain-Barré* syndrome and congenital neurological manifestations.[1,2] These concerns accelerated scientific research aimed at understanding the mechanisms by which the ZIKV interacts with its host to cause new clinical presentations.

Between 1947, when ZIKV was first reported in a Uganda forest, and 2015,[3] 124 articles were published regarding ZIKV. However, recent outbreaks and clinical manifestations associated with ZIKV infection resulted in more than 4,500 Zika-related published medical/scientific manuscripts during the 2016/2018 period. This increase in research was beneficial to the ZIKV field and added to our understanding of this new, emerging viral disease.

Arboviral isolation from clinical samples typically employs the use of mosquito cells, such as C6/36, from *Aedes albopictus* larvae.[4] It is well known that mosquito cell lines can harbor contaminants including insect viruses, and the presence of contaminant viruses could induce cytopathic effects in insect cells, including syncytia formation or cell lysis, depending on the contaminant virus.[5] Viruses belonging to the genus *Brevidensovirus* are among previously reported insect cell culture contaminants.[6,7] *Brevidensovirus* is a genus of the *Parvoviridae* family, *Densovirinae* sub-family, which encompasses viruses known for infecting insects of the *Diptera* order, like *Aedes aegypti* and *Ae. albopictus*, among others.[8] As far as it is known, these viruses are not able to replicate in vertebrates, however they can be pathogenic for their invertebrate hosts.[9,10,11]

In this study, we identified the presence of two different *Brevidensovirus*, here designated as mosquito densovirus (MDV), contaminating two ZIKV strains; one strain is of African origin, and the other strain is of Asian lineage. These strains were sent to our laboratory for research purposes. We also provide two simple strategies to remove MDV contamination from ZIKV strains using vertebrate cells as a bottleneck for MDV replication.

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MATERIALS AND METHODS

Virus stocks production - Two different ZIKV strains were recently sent to our laboratory. The ZIKV strain of Asian origin was named *strain A*, and the strain that resembled African origin was *strain B*. As those samples were sent to our laboratory without information on viral passage history we identified both samples of ZIKV *strains A* and *strain B* as zero (P.0). Both P.0 viral supernatants were used to infect C6/36 cells (ATCC® CRL-1660™) that were cultured in L-15 media supplemented with 5% FBS, 25 μg/mL gentamicin and 0,26% triptose (Thermo Fisher Scientific, Grand Island, New York, USA) at a multiplicity of infection (MOI) of 0.01 for viral stock production.

ZIKV titration using foci forming assay - ZIKV virus titers were determined by the foci forming immunodetection assay in C6/36 cells ($FFU_{C6/36}$/mL), as previously described.[12] Briefly, C6/36 cells were infected with 10-fold serially diluted mice sera / cell culture supernatant for 90 minutes. After inoculum was removed a CMC overlay media (L-15 plus 5% SFB, 0.26% tryptose, 25 μg/mL gentamicin, 1.6% carboxymethylcellulose) was added and plates incubated at 28ºC for seven days. The immunostaining was performed using the anti-flavivirus mouse monoclonal antibody 4G2 (anti-E protein; ATCC® HB-112™), followed by alkaline phosphatase conjugated goat anti-mouse antibody (Promega, Madison, WI, USA). The reaction was detected using NBT/BCIP substrate solution (nitroblue tetrazolium chloride/5-bromo-4-chloro-39-indolyphosphate p-toluidine salt) (Promega, Madison, WI, USA). Foci were counted and expressed as $FFU_{C6/36}$/mL.

Molecular detection of densovirus - Briefly, viral nucleic acids from C6/36 cell supernatants infected with each ZIKV strain were isolated using the RNeasy Mini kit (QIAGEN). For MDV DNA amplification (324 bp), the primers DNV3R (5'-TTTATTTCCATAGATATTGACT-GTTTCGAT-3') and DNV3F 5'-AATCGAGAAACAG-CATACTACACATTCGT-3') were used as previously described.[13] These primers amplified a viral genomic region encompassing a small segment of the NS1 and NS2 genes of MDV. As a control for MDV amplification, a plasmid containing the same target gene from the MDV BR/07 isolate was used.

Additionally, a reverse transcription polymerase chain reaction (RT-PCR) assay was used for the molecular detection of MDV. Briefly, total nucleic acids from the supernatant and pellet of C6/36 cells was extracted using TRIzol reagent (Invitrogen). Blood samples from ZIKV infected mice were collected one to four days post inoculation, and nucleic acids was extracted using TRIzol reagent (Invitrogen). A total of 500 ng of nucleic acids was reverse transcribed using 300 ng of random primers. The resulting cDNA was used as a template for PCR with the primers DensoBR07_F (5'-ATTGTTGGGAGCATGAC-GGA-3') and DensoBR07_R (5'-CAACGGTTTGAC-CAGCGAAA-3') resulting in 212 bp of amplification. To test for the presence of densovirus in the mosquitoes that fed on ZIKV infected mice, the total nucleic acids from individual mosquitoes was extracted and pooled to prepare cDNA. During the replication cycle of MDV the ssDNA genome produces mRNA,[14] thus, both RT-PCR or direct PCR could be used to detect MDV contamination (data not shown).

Zika virus detection by RT-PCR - ZIKV genomic RNA was detected by RT-PCR (364 bp) using the primer set ZIKVENVF (5'-GCTGGDGCRGACACHGGRACT-3') and ZIKVENVR (5'-RTCYACYGCCATYTGGRCTG-3') as previously described.[15,16] RNA from the ZIKV strain ZV BR2015/15261 isolate (South Brazil, 2016) was used as a control for ZIKV E gene amplification.

Immunofluorescence assay for ZIKV and MDV detection - C6/36 cells (2x10^4 cells/well) were seeded in a 96-well plate and infected (in triplicate) with P.0 of ZIKV *strain A* and *strain B* at an MOI of 1. The MOI was based on the titration of ZIKV *strain A* and *strain B* in C6/36 using a pan-flavivirus monoclonal antibody that recognises the E protein (4G2; ATCC® HB-112™; see ZIKV titration using foci forming assay). After 72 h, the cells were fixed and permeabilised with methanol:acetone (v/v) as previously described.[13] For immunostaining, three different antibodies were used — an anti-flavivirus envelope (E) protein (4G2), an *in-house* mouse polyclonal antibody anti-MDV and an anti-MDV monoclonal antibody (clone 94DL1; IgG2a kappa).[13] A goat anti-mouse IgG Alexa Fluor 488 conjugate was used as secondary antibody, and digital images were taken with a fluorescence microscope (Leica DMI6000B) using LAS AF (Leica) software. As an MDV positive control, C6/36 cells were infected with MDV BR/07 (GenBank: GU452720) with a multiplicity of genome (MOG) of 0.01 or 1 for 72 h. The polyclonal and monoclonal antibodies against MDV strain BR/07 used in the immunostaining assays were generated in a previous study.[13]

Densovirus nucleic acid sequencing - PCR fragments (324 bp) from MDV detection were purified using the High Pure PCR Product Purification Kit (Roche), and nucleotide sequencing was performed with primers for DNV3R and DNV3F by dideoxynucleotide termination sequencing at Macrogen Inc. (Seoul, South Korea). The sequences were assembled using the Assembler tool (http://www.hpabioinformatics.org.uk/cgibin/assembly_tool/seq_assemble.cgi?no=2) and aligned using ClustalW[17] as implemented in BioEdit software v.7.2.5.[18] The length of nucleotide sequence used in the analysis was 265 bp due to the primer sequence removal. The consensus sequence of densovirus *strain A* and *B* were deposited in GenBank under accession numbers, MH720336 and MH720337, respectively.

MDV removal from ZIKV samples - As MDV does not infect vertebrate cells, we performed serial passages of ZIKV *strain A* P.0 and *strain B* P.0 in A549 cells (lung epithelial cells; ATCC: CCL185). Briefly, A549 cells (1x10^5 cells/well in 24 well plates) were infected with 100 μL of ZIKV *strain A* P.0 or *strain B* P.0 for 90 min. After infection, cell monolayers were washed three times with 1X PBS and incubated in culture medium (DMEM-F12, 7% FCS, 100 IU/μg/mL of penicillin/streptomycin) for 72 h. The cell culture supernatants were collected and used (100 μL) to infect a new set of A549 cell cultures (second pas-

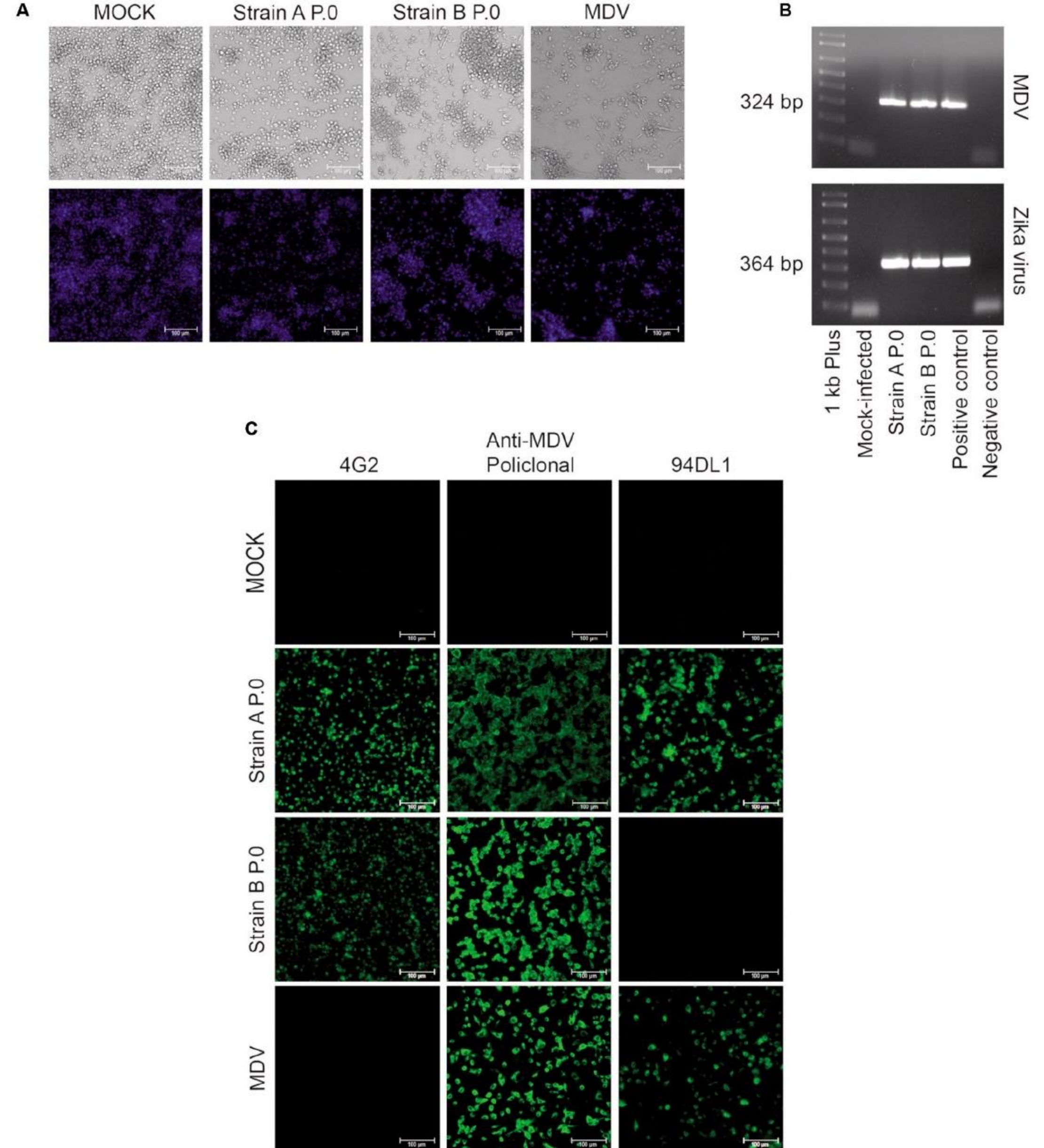

Fig. 1: mosquito densovirus (MDV) coinfection in Zika virus (ZIKV) *strains A* P.0 and *B* P.0. (A) Cytopathic effects in C6/36 MDV-contaminated ZIKV *strain A* and *strain B* cells compared to mock-infected cells and C6/36 cells infected with MDV BR/07 at an multiplicity of genome (MOG) of 1 for 72 h. (B) Agarose gel showing polymerase chain reaction (PCR) amplification of a 324 bp fragment from the MDV genome and reverse transcription-PCR (RT-PCR) amplification of a 364 bp fragment of the ZIKV E gene in *strain A* and *strain B* (P.0). (C) Immunofluorescence assay of ZIKV *strain A* and *strain B* (P.0) infected C6/36 cells stained with a 4G2 monoclonal antibody, anti-MDV mouse polyclonal serum, and monoclonal antibody (clone 94DL1) raised after immunisation with the MDV BR/07 strain. As a positive control, C6/36 cells were infected with MDV BR/07 at an MOG of 1 for 72 h.

sage). An additional passage in A549 cells was performed as previously described (third passage). To confirm the exclusion of MDV after three passages in A549 cells, the cell culture supernatant of ZIKV *strain A* P.3/A549 and *strain B* P.3/A549 was passaged three additional times in the C6/36 mosquito cell line. All A549 and C6/36 cell passages were performed as described above. Nucleic acid was extracted from cell supernatants, and RT-PCR and PCR were performed for ZIKV and MDV, respectively.

Additionally, ZIKV *strain A* was used to infect A129 mice using a dose of 4×10^6 PFU[19] per individual by the intraperitoneal route. Blood samples were collected daily from 1 to 4 days post infection (dpi), and the presence of MDV was tested as previously described. To certify that MDV was eliminated in mouse blood, 3 dpi *Ae. aegypti* females (5-7-day-old) were allowed to feed on ZIKV infected animals. MDV RT-PCR was performed on mosquitoes at four days post feeding. A total number

of 10 fed mosquitoes were used to test for the presence of MDV. Additionally, ZIKV was titrated in mice sera (using foci forming assay in C6/36 cells) three days after infection in order to quantify ZIKV recovery.

ZIKV infection to confirm MDV elimination - C6/36 cells were infected with a low [0.01] to high [10] MOI of ZIKV *strain A* P.3/C6/36 and ZIKV *strain B* P.3/C6/36. After 72 h, the supernatants and cell pellets were tested for the presence of MDV using a PCR assay as previously described.

Ethics - Experiments involving A129 mice were approved by the ethics committee at UFMG (CEUA 337/2016).

RESULTS

Two different ZIKV strains (referred to as *strain A* and *strain B*) were recently sent to our laboratory from two different sources for research purposes. During the preparation of viral stocks, visual inspection of C6/36 cell cultures revealed an atypical cytopathic phenotype that raised suspicion of potential contamination with an additional virus/microorganism (Fig. 1A). Our previous experience with MDV contamination[13] showed similar cytopathic effects, so we performed molecular and immunological assays to check for possible contamination.

Using PCR, we amplified a segment of the MDV genome in ZIKV *strains A* and *B* to confirm coinfection with MDV (Fig. 1B); an immunofluorescence assay was also performed. The immunofluorescence assay confirmed the presence of the ZIKV E-antigen in the cytoplasm and coinfection of both ZIKV *strains A* and *B* with MDV (Fig. 1C). Furthermore, the inability of an anti-MDV monoclonal antibody to recognise *strain B* P.0 suggests that different MDV strains were coinfecting the ZIKV strains. To address this, we determined the nucleotide sequence (from PCR product) of the MDV present in both ZIKV strains. Despite the short viral genomic region analysed (265 bp), the nucleotide identity was 95.4% between the MDVs present in each ZIKV strain, which confirmed different viral strains. This could be explained by 1) the different passage history of the two ZIKV strains; 2) divergent evolution of both MDV strains due to the maintenance in C6/36 cell culture. However, we could not confirm any of these hypotheses once we do not have information on the ZIKV *strain A* and *B* passage history before samples were sent to our Laboratory. Despite that, results suggest that the contamination originated from two different sources (Table).

A comparison of the new MDV isolates with the MDV previously reported by our group (BR/07; GenBank: GU452720) shows a nucleotide identity of 98.4% with the sequence amplified from *strain A* and an identity of 96.2% with the one amplified from *strain B* P.0 (Table). It is important to note that the C6/36 cell cultures in our laboratory are routinely checked for insect viral contaminations, including MDV, due to our reference laboratories activities for the Brazilian Ministry of Health.

MDV belongs to the *Parvoviridae* family and the *Brevidensovirus* genus.[14] This nonenveloped virus presents a 4kB negative-polarity, single-stranded DNA genome.[20] MDV is considered nonpathogenic for humans; however, MDV may be detrimental to mosquitoes.[21,22] Once we confirmed contamination of each ZIKV strain with MDV, we focused on strategies to eliminate it from the ZIKV samples to prevent interference with future experiments. It was previously demonstrated that MDV does not infect vertebrate cells,[6] so we performed serial passages of the ZIKV *strains A* and *B* using a ZIKV-susceptible A549 lung epithelial cell line.[23] After three passages of ZIKV *strains A* and *B* in A549 cells, the MDV coinfection was no longer detected in cell culture supernatants using PCR, while detection of a ZIKV envelope gene was successful (Fig. 2A). Additionally, PCR for MDV and RT-PCR for ZIKV were performed after each passage (P1, P2 and P3) using nucleic acids extracted from the supernatants, and the results demonstrated that fragments of the NS1 and NS2 genes of MDV were not detected for *strain A* after the first passage (P1) in A549 cells or at the second passage (P2) for *strain B* (data not shown).

Additionally, to confirm the exclusion of MDV from ZIKV strains, we performed three additional passages of ZIKV *strain A* P.3/A549 and *strain B* P.3/A549 using the C6/36 mosquito cell line, as this cell line is susceptible and permissive to MDV. After the third passage in C6/36 cells, nucleic acid was extracted from the supernatants, and RT-PCR and PCR for ZIKV and MDV, respectively, were performed (Fig. 2B). These results demonstrated that successive passages of MDV-contaminated ZIKV strains in A549 cells are effective for removing MDV contamination from ZIKV samples (Fig. 2). An immunofluorescence assay was also used to confirm MDV exclusion from each ZIKV-strain (Fig. 2C). Additionally, ZIKV titration after each passage in cell culture showed a ZIKV recovery rate between 10^4 to 10^7 FFU/mL (data not shown). After passages in A549 and C6/36 cells, the cytopathic effects observed in C6/36 cells were no longer apparent compared to previous infections prior to the removal of MDV (Figs 1A and 2D). As infection with ZIKV induces cytopathic effects on C6/36 even after the elimination of MDV, some damage on C6/36 cells could be observed when compared to mock-infected cells (Fig. 2D).

To further confirm the elimination of MDV from each ZIKV strain, C6/36 cells were infected with a different MOI of ZIKV *strain A* P.3/C6/36 and ZIKV *strain B* P.3/C6/36. After 72 h, the supernatants and cell pellets were tested for MDV using PCR. Even after infection with a high MOI [10], MDV was no longer detected in

TABLE

Nucleotide identity matrix comparing the two mosquito densovirus (MDV) presented on Zika virus (ZIKV)-isolates

	GU452720	ZIKV *strain A*	FJ805445	ZIKV *strain B*
GU452720	100%	98.4%	97.3%	96.2%
ZIKV *strain A*	98.4%	100%	96.6%	95.4%
FJ805445	97.3%	96.6%	100%	98.8%
ZIKV *strain B*	96.2%	95.4%	98.8%	100%

GU452720: mosquito densovirus BR/07; FJ805445: *Culex* densovirus 0507JS11.

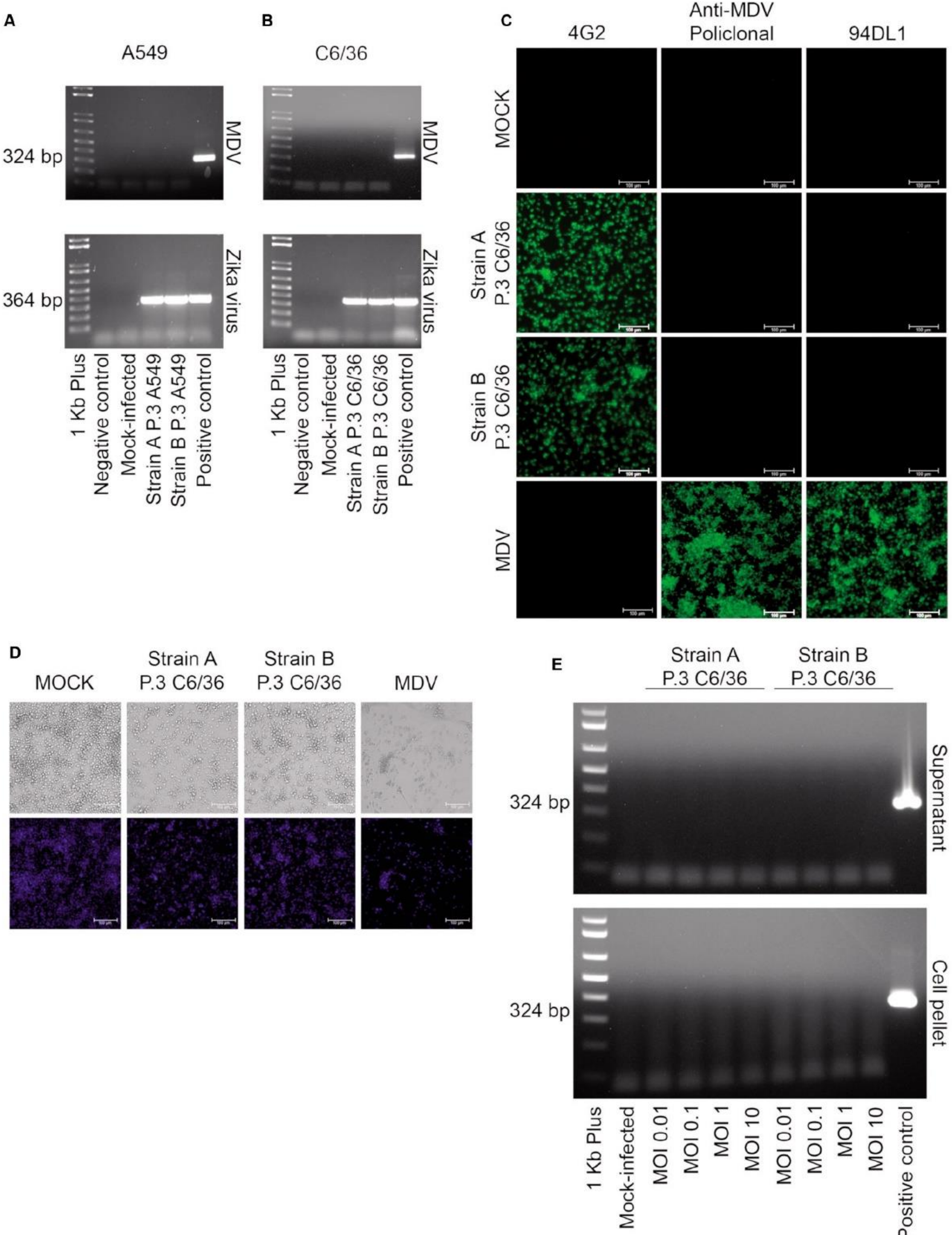

Fig. 2: mosquito densovirus (MDV) clearance from the Zika virus (ZIKV) *strains A* and *B* (P.0). Agarose gels showing polymerase chain reaction (PCR) amplification of a 324 bp fragment from MDV and reverse transcription-PCR (RT-PCR) amplification of a 364 bp ZIKV E gene fragment in ZIKV *strain A* and *strain B* after three passages in A549 cells (P.3/A549) (A) followed by three passages in C6/36 cells (P.3/C6/36) (B). (C) Immunofluorescence assay in C6/36 cells infected with ZIKV *strain A* and *strain B* (P.3/C6/36) after three passages in C6/36 cells stained with 4G2 monoclonal antibody, anti-MDV mouse polyclonal serum, and anti-MDV monoclonal antibody (clone 94DL1). (D) Cytopathic effects on C6/36 cells infected with ZIKV *strain A* and *strain B* after three passages in C6/36 compared to mock-infected cells. Infection with the MDV BR/07 strain [multiplicity of genome (MOG) 0.01 for 72 h] was used as positive control for immunofluorescence and cytopathic effect assays. (E) Agarose gel showing PCR amplification of a 324 bp fragment from MDV. C6/36 cells were infected with different multiplicity of infections (MOIs) (0.01, 0.1, 1 and 10) of ZIKV *strain A* and *strain B* after three passages in C6/36 cells (P.3 C6/36). Nucleic acid was extracted from cell pellet and cell culture supernatant of infected cells and tested for the amplification of MDV genes by PCR. As a positive control, a plasmid containing the same target gene from the isolate MDV BR/07 was used (324 bp).

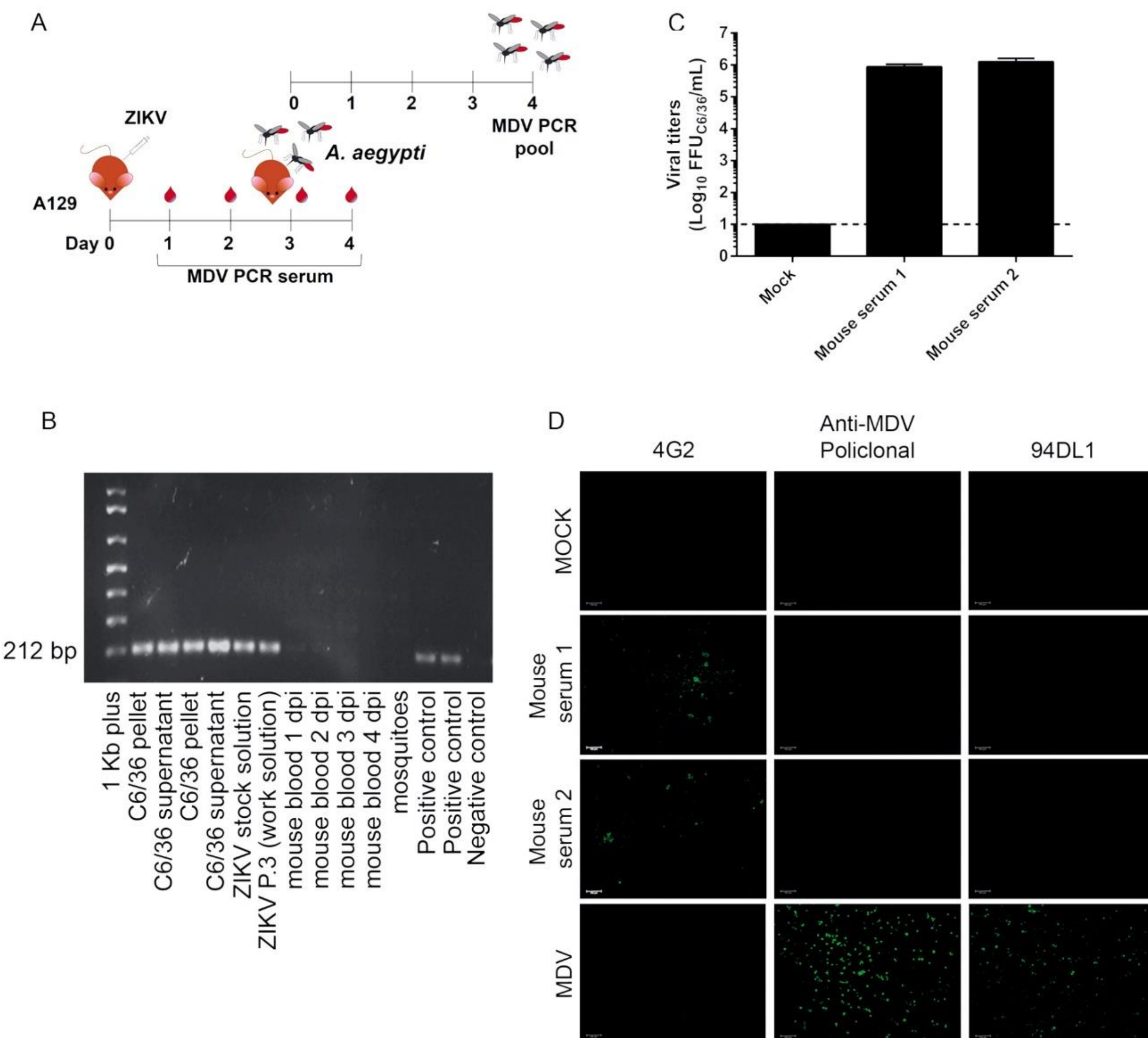

Fig. 3: infection of A129 mice provide a reliable strategy for clearance of mosquito densovirus (MDV)-contamination. (A) Experimental design for *in vivo* assays. (B) Agarose gel showing reverse transcription-PCR (RT-PCR) amplification of a 212 bp fragment from MDV in the blood of A129 mice infected with $4x10^6$ PFU of Zika virus (ZIKV) *strain A* at different days post infection (dpi) and in *Aedes aegypti* mosquitoes that fed on infected animals. As a control, ZIKV *strain A* stocks and C6/36 cells (pellets and culture supernatants were used) together with a plasmid control were tested for MDV. (C) Viral titers in mice sera three days post-infection. The sera from two mice were tested in biological replica. (D) Immunofluorescence assay of C6/36 cells infected with ZIKV *strain A* recovered from mice sera three days post-infection. C6/36 cells were infected with mice sera at a multiplicity of infection (MOI) of 1 and after three days stained with 4G2 monoclonal antibody, anti-MDV mouse polyclonal serum, and anti-MDV monoclonal antibody (clone 94DL1). As a positive control, C6/36 cells were infected with MDV BR/07 at a multiplicity of genome (MOG) of 0.01 for 72 h.

these ZIKV stocks. These results confirmed the efficiency of this protocol in the removal of MDV contamination from ZIKV strains (Fig. 2E).

As an alternative protocol, we also hypothesised that passing ZIKV isolates in susceptible mice would eliminate MDV. To test this hypothesis, type I IFN receptor KO (A129) mice were infected with contaminated stocks of ZIKV *strain A* (Fig. 3A). Blood was collected from one to four dpi and tested for MDV. As early as 1 dpi and throughout the kinetics, blood samples were negative for MDV. We also allowed *Ae. aegypti* to feed on the blood of infected mice at 3 dpi to further test for the successful elimination of MDV (since it would be amplified in mosquitoes even if present at low titers). Mosquitoes that fed on infected mice were negative for MDV (Fig. 3B). These results indicate that the passage of MDV-contaminated ZIKV-stocks in mice is also a suitable method to eliminate contamination. For all time points, the presence of MDV was tested in mice and mosquitoes, and we confirmed ZIKV RNA using RT-PCR (data not shown). Additionally, ZIKV was recovered from mice sera at higher titers three days after infection (Fig. 3C). Also,

C6/36 cells were infected with mice sera (three dpi) and an immunofluorescence assay confirmed the elimination of MDV contamination and the recovery of ZIKV *strain A* (Fig. 3D).

DISCUSSION

The exchange of biological samples, such as viral isolates and cell lines, often occurs between research laboratories. Although this is important for scientific development, the certification of the microorganism strains or cell lines shared between laboratories is essential to avoid contamination problems. One of the most notorious cases of biological contamination in research laboratories is the contamination of cell lines with HeLa cells.[24] Contamination of cell lines with MDV is not unusual, as this has already been demonstrated in the mosquito cell lines C6/36 and AP-61.[7] Although difficult to track, we suspect that the MDV contamination origin began in contaminated cell cultures used for virus propagation prior to both ZIKV strains being exhaustively shared between laboratories.

Although MDV infection can result in the development of cytopathic effects, the virus can also be unnoticed due to its ability to establish persistent infections without any clear cytopathic effects.[6,7] Multiple authors have also demonstrated the ability of MDV to affect cell growth that is likely due to arrest of the cell cycle at the G2 phase.[13,25] Thus, it has been suggested that MDV could be used to control the mosquito population and have implications for the transmission of arboviruses.[11,22]

There are no studies addressing the effects of coinfections with MDV and ZIKV, and the outcome of such a coinfection is unknown; however, the negative impact of MDV in dengue virus infection and replication was demonstrated *in vitro* and *in vivo*, further reinforcing the potential use of MDV for the biological control of arboviral infections.[11,13] Given the similarities between DENV and ZIKV, it is plausible that MDV and ZIKV coinfection may affect *in vitro* and *in vivo* ZIKV infections. Conversely, studies using the C6/36 cell line and *Ae. aegypti* mosquito models have shown that coinfections with the chikungunya and densonucleosis viruses do not impact the infection and replication of either virus.[26] Furthermore, MDV could induce the production of antibodies in BALB/C mice after immunisation with Freund's complete adjuvant (first dose) and Alu-S-Gel (doses 2 to 4).[13] Thus, the potential impact of infecting mice with flaviviruses (dengue or ZIKV) contaminated with MDV is still an open question.

Regarding the protocol used for MDV clearance from ZIKV stocks, additional care should be considered. First, it has been already shown that vertebrate cells and some supplies used in cell culture, like fetal calf serum, could also harbor contaminant viruses.[27,28] A contamination with Infectious Bovine Rhinotracheitis Virus (IBRV) has already been shown in a commercially A549 cell seed stock.[28]. Ideally, the source of cells used for virus growth and titration should be tested for the presence of contaminants. Also, for virus evolution studies, the protocols suggested here could impact on the results, as they are based in successive viral passaging in cell culture or mice, which could introduce genetic mutations in ZIKV genome. It has been shown that a single passage in cells could influence the genetic diversity of Chikungunya virus.[29] Furthermore, successive passages of ZIKV in vertebrate (Vero cells) or invertebrate (C6/36 cells) cells influence plaque sizes, kinetic and restriction to grow. In addition, four mutations were identified associated with plaque size that might have influence on ZIKV biology.[30]

Since coinfections are not limited to ZIKV strains, researchers who work in the arbovirology field should check their cell lines and viral stocks periodically to avoid contamination with arthropod viruses such as MDV. The main purpose of reporting these findings is to call the attention of the scientific community of the potential presence of mosquito virus contaminants in ZIKV strains/stocks. We also suggest two simple strategies to efficiently eliminate MDV contamination from ZIKV strains/stocks, *in vitro* and *in vivo* passages in vertebrate cell lines or mice models, respectively. Finally, the potential interference of MDV contamination in ZIKV isolates needs further analysis.

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AUTHORS' CONTRIBUTION

AC and DK performed all *in vitro* assays; AM performed the sequence analysis; ES, AF and JM performed *in vivo* assays; and PW, CS and JB participated in the experimental design. All of the authors helped to draft the manuscript and approved the final text. All authors declare that they have no conflict of interest.

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3.2 CAPÍTULO 3

"The citrus flavonoid naringenin impairs the in vitro infection of human cells by Zika virus"

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Neste terceiro capítulo são apresentados os resultados da avaliação da atividade anti-ZIKV do flavonóide NAR. Os dados aqui apresentados foram publicados no periódico *Scientific Reports* em novembro de 2019 (doi: 10.1038/s41598-019-52626-3).

As graves malformações no sistema nervoso de fetos em desenvolvimento, aborto espontâneo, e casos de síndrome de *Guilain Barré* em adultos, nas recentes epidemias de ZIKV causaram grande impacto na sociedade (CAO-LORMEAU et al., 2016; OLIVEIRA MELO et al., 2016). A falta de conhecimento sobre o agente patogênico, sua rápida expansão e novas apresentações clínicas associadas a esta infecção levaram a um grande movimento de profissionais de saúde e pesquisadores na tentativa de compreender e combater este novo problema. Entre os pontos de relevância está o desenvolvimento de drogas antivirais capazes de controlar a infecção.

Dentre as estratégias utilizadas, os produtos naturais representam rica fonte de moléculas com diferentes atividades biológicas já descritas na literatura (PANCHE; DIWAN; CHANDRA, 2016). Ademais, desde a década de 1960, os flavonóides vem sendo reportados como moléculas com atividade antiviral contra diversos vírus, incluindo arboviroses de importância médica, como dengue, chikungunya e ZIKV (BÉLÁDI; PUSZTAI; BAKAI, 1965; FRABASILE et al., 2017; MOUNCE et al., 2017).

A atividade antiviral do flavonóide NAR já foi descrita contra chikungunya, vírus da hepatite C e recentemente, contra os quatro sorotipos de dengue (AHMADI et al., 2016; FRABASILE et al., 2017; GOLDWASSER et al., 2011; WEAVER; REISEN, 2010). Levando-se em conta as similaridades entre dengue e ZIKV, a falta de alternativas terapêuticas e o surgimento de síndromes neurológicas associadas à infecção por ZIKV, decidiu-se por avaliar a atividade antiviral *in vitro* da NAR contra essa arbovirose emergente. Os ensaios de atividade antiviral demonstraram que o tratamento com NAR exerce efeito dose-dependente na redução da frequência de células infectadas, título viral e RNA viral. Embora tenha sido descrito a atividade antiviral da NAR contra os 4 sorotipos de dengue (FRABASILE et al., 2017), resultados opostos foram observados contra a cepa C de dengue-2 de Nova Guiné (ZANDI et al., 2011b), portanto, o efeito antiviral pode ser cepa-dependente. Desta forma, ampliamos a análise utilizando diferentes cepas de ZIKV. Os dados obtidos demonstram o efeito antiviral da NAR de forma cepa-independente, embora o efeito mais potente foi observado contra as cepas da linhagem Asiática do ZIKV, responsável pela recente epidemia nas Américas.

Com relação ao mecanismo de ação da NAR contra o ZIKV, ensaios de tempo de adição de droga indicaram que o efeito antiviral da NAR deve ocorrer em etapas mais tardias do ciclo viral, como replicação, montagem ou liberação das partículas virais. Além disso, o efeito antiviral foi observado mesmo quando o tratamento foi realizado até 24 horas após a infecção, sugerindo que a NAR poderia ser utilizada com a infecção estabelecida, destacando o potencial terapêutico deste composto. Ainda, a análise *in silico* por *docking* molecular demonstrou que a NAR pode se ligar à protease viral NS2B-NS3, reforçando a hipotése de que o mecanismo de ação ocorra na etapa de replicação viral.

Adicionalmente, o efeito antiviral da NAR também foi testado em diferentes linhagens de células humanas relacionadas à patogenia da infecção por ZIKV, como célula tronco embrionária (NKX2-5^{eGFP}/whESC), célula neuronal (A172 - ATCC® CRL-1620™) e célula dendrítica derivada de monócitos humanos (*hmdDCs - human monocyte-derived dendritic cells*), bem como, em linhagem de célula hepática (Huh7.5 ATCC PTA-8561), visando confirmar os dados obtidos. O efeito antiviral da NAR foi confirmado nas células Huh7.5, A172 e em *hmdDCs*. Estes dados demonstram o potencial da NAR como uma molécula antiviral promissora, embora mais estudos sejam necessários para confirmar o mecanismo de ação exato e sua eficácia *in vivo.*

Todos os dados descritos acima podem ser analisados integralmente no manuscrito a seguir que compõe o capítulo 3 da tese.

OPEN

The citrus flavonoid naringenin impairs the *in vitro* infection of human cells by Zika virus

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The Zika virus (ZIKV) is an arthropod-borne virus that belongs to the *Flaviviridae* family. The ZIKV infection is usually asymptomatic or is associated with mild clinical manifestations; however, increased numbers of cases of microcephaly and birth defects have been recently reported. To date, neither a vaccine nor an antiviral treatment has become available to control ZIKV replication. Among the natural compounds recognized for their medical properties, flavonoids, which can be found in fruits and vegetables, have been found to possess biological activity against a variety of viruses. Here, we demonstrate that the citrus flavanone naringenin (NAR) prevented ZIKV infection in human A549 cells in a concentration-dependent and ZIKV-lineage independent manner. NAR antiviral activity was also observed when primary human monocyte-derived dendritic cells were infected by ZIKV. NAR displayed its antiviral activity when the cells were treated after infection, suggesting that NAR acts on the viral replication or assembly of viral particles. Moreover, a molecular docking analysis suggests a potential interaction between NAR and the protease domain of the NS2B-NS3 protein of ZIKV which could explain the anti-ZIKV activity of NAR. Finally, the results support the potential of NAR as a suitable candidate molecule for developing anti-ZIKV treatments.

The Zika virus (ZIKV) is an arthropod-borne virus from the *Flavivirus* genus and the *Flaviviridae* family that was first isolated from a rhesus monkey from the Zika forest of Uganda in 1947[1]. The ZIKV is transmitted primarily by *Aedes spp*. mosquitoes[2]. However, transmission by sexual contact, via contaminated blood and from mother to fetus has also been described[3–5].

Infection by ZIKV progresses as a self-limiting disease like observed in dengue infection, with mild clinical manifestations such as fever, macular or papular rash, arthritis and arthralgia, nonpurulent conjunctivitis, myalgia, headache, edema, orbital pain and vomiting[6,7]. However, during Zika epidemics in French Polynesia (2013) and Brazil (2015), greater numbers of cases of *Guillain-Barré* and congenital syndrome (microcephaly) were observed[7–9]. Strong evidence suggests a link between ZIKV infection and microcephaly once the viral genome has been detected in the amniotic fluid and brains of affected fetuses[5,9].

In Brazil, between December 2015 and March 2018 more than 230,000 cases of ZIKV-infection were reported[10]. Additionally, 3,100 cases of children with ZIKV-associated congenital and neurological syndrome were confirmed[11]. At present, neither a vaccine nor an antiviral drug is available to prevent or treat the ZIKV infection. Despite recent advances in drug discovery against ZIKV, no antiviral compound has been authorized at the phase I clinical trial level[12]. Flavonoids are polyphenolic compounds that are present in a wide range of

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fruits and vegetables as low-molecular weight secondary metabolites[13]. Flavonoids are known to have antiviral activity against HIV-1, herpes simplex 1 and 2, influenza, dengue and yellow fever[14–18]. Additionally, it was demonstrated that several flavonoids could inhibit Zika virus infection *in vitro* and *in vivo*[19–25]. The antiviral effect of flavonoids seems to occur through interactions between the phenol rings of flavonoids and viral proteins and/or RNA, or via its capacity to interfere in host cell defense by regulating MAP kinase signaling[26–29]. Naringenin (NAR) (4, 5, 7-trihydroxyflavanone), a natural flavonoid aglycone of naringin, is widely distributed in citrus fruits, tomatoes, cherries, grapefruits and cocoa[30]. NAR was shown to present a wide range of activities, including anti-inflammatory and analgesic actions[30–32]. In addition, NAR was recently shown to have anti-dengue virus activity *in vitro*[18], and the similarities between these flaviviruses prompted us to test NAR against ZIKV infection *in vitro*.

Here, NAR was shown to present *in vitro* anti-ZIKV activity against four different strains of recent clinical isolates of the Asian lineage and one classical African lineage. Additionally, the antiviral activity of NAR seems to occur in the late steps of virus life cycle. A promising characteristic of NAR is that the antiviral effect is observed even when it is added to cultures 24 hours after the establishment of the infection. Finally, *in silico* docking analysis suggests a close interaction between NAR and the protease domain of ZIKV, strengthening the data from *in vitro* assays.

Results

High doses of NAR affect A549 cell viability.

To determine the concentration of NAR to use for the *in vitro* assays, we performed a viability assay. Results indicated that NAR is toxic for A549 cells at higher concentrations (from 500 to 2,000 μM) (Fig. 1A,B). After treatment of A549 cells with 500 μM of NAR 10% of cells become apoptotic (Fig. 1A,B). Also, data demonstrated, using double-negative A549 cells (Annexin-V^-/7-AAD^-), that NAR could be safely used in the antiviral assays (Fig. 1A,B). Moreover, double-negative A549 cells were used to establish the cytotoxic concentration for 50% of the culture (CC_{50}), which was calculated as 693.6 μM (Fig. 1C). Despite 250 μM NAR being non-toxic for A549 cells (Fig. 1A,B), it was defined 125 μM of NAR as the maximal non-toxic concentration (MNTC), to avoid residual toxicity and with the aim of using a lower concentration of NAR in the antiviral assays. Moreover, the cell nuclei count using Operetta high-content imaging show that NAR (125 μM) did not reduce the number of cells compared to untreated A549 cells (Fig. 1D). Altogether, results from different assays confirm that 125 μM of NAR was safe to use in A549 cells and did not show toxic effects.

In vitro inhibition of Zika virus infection by NAR is concentration-dependent.

Based on the previous data showing the anti-dengue virus activity of NAR[18], a set of experiments were performed to evaluate the anti-ZIKV activity of NAR. A549 cells were infected with ZIKV (ZV BR 2015/15261) and treated with different concentrations of NAR (15.6, 31.25, 62.5 and 125 μM) after inoculum removal. According to the literature[33] and kinetic experiments it was observed that 48 h was the best time point for immunostaining once at 72 h we observe cytopathic effect (Supplementary Fig. 1). Thus, to access antiviral activity of NAR, FACS and the foci-forming immunodetection assay were performed in cells and cell culture supernatant after 48 hours post-infection (hpi), respectively. Results demonstrate that the anti-ZIKV activity of NAR was concentration-dependent (Fig. 2A–C). Surprisingly, IFN-α 2A-treated cells presented more viable ZIKV particles in the cell culture supernatant than NAR-treated cells did (Fig. 2C). An analysis of the mean fluorescence intensity (MFI) of infected cells indicates that IFN-α 2A controls the number of infected cells; however, the production of the virus is not reduced to the same level compared to NAR-treated cells (Fig. 2D). Additionally, the IC_{50} and IC_{90} indexes were calculated as 58.79 and 154.37 μM, respectively, with confidence of interval of 95% (Fig. 2E). The selective index (CC_{50}/IC_{50}) of NAR was 11.79.

Using an immunofluorescence assay (Operetta high-content imaging), NAR was shown to reduce the number of A549-infected cells (Fig. 3A,B). Additionally, RT-qPCR (Fig. 3C) demonstrated that NAR impairs ZIKV replication. Furthermore, results from two different assays indicate the anti-ZIKV activity of NAR was not due to a virucidal effect on the virus particles (Supplementary Fig. 2). Virus particles treated with NAR (125 μM) did not affect viral infectivity, suggesting no effect on the viral structure (Supplementary Fig. 2). Additionally, it was tested if NAR treatment (125 μM) of A549 cells could modulate cell autofluorescence. Using FACS it was demonstrated that NAR does not affected A549 cells autofluorescence compared to untreated cells (Supplementary Fig. 3).

Anti-Zika virus activity of NAR is lineage-independent.

Using phylogenetic analyses it was demonstrated the existence of two main Zika virus lineages, the African- and Asian- lineages[34]. To confirm the Asian- and African-origin of the virus a real time RT-PCR assay was performed using a set of primers and probes employed as previously described[35,36] (Supplementary Table 1 and Supplementary Fig. 4). Moreover, it was shown that the African- and Asian-lineage of ZIKV present differences in virulence/pathogenicity *in vitro* and *in vivo*[37]. Thus, to exclude a lineage-specific anti-ZIKV activity of NAR against ZV BR 2015/15261, four additional ZIKV strains were tested, three from Asian-lineage (ZV BR 2016/16288, ZV BR 2015/15098, ZIKV PE243) and one from African-lineage (ZIKV MR766). Using FACS and the foci-forming immunodetection assay, it was possible to demonstrate that NAR impairs the A549-infection with both Asian- and African-lineages of ZIKV (Fig. 4). However, despite NAR was effective to reduce the infection with both ZIKV lineages, a higher effect was observed for Asian-lineage (~4 fold reduction of infection) when compared to African-lineage (~2 fold reduction of infection) (Supplementary Fig. 5).

Time of NAR addition experiments.

To determine which step of the ZIKV life cycle NAR exerts its effects, a time of addition experiment was performed in A549 cells[18,38] (Fig. 5A). The results indicate that NAR treatment simultaneous to the addition of ZIKV-inoculum and after inoculum removal (during + after infection) or only after inoculum removal (after infection) seems to impact the ZIKV infected-A549 cells (Fig. 5B–E). Also,

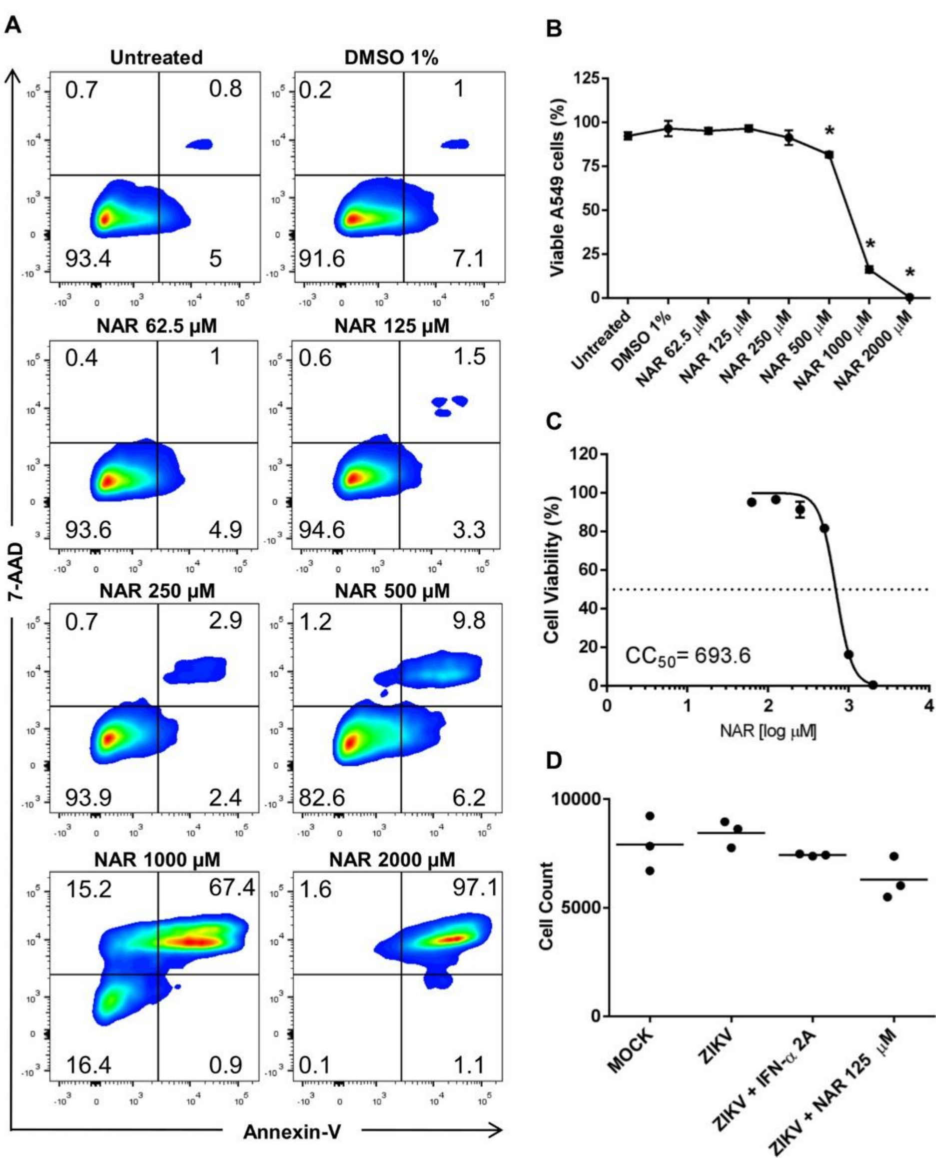


Figure 1. Toxicity of NAR to A549 cells. A549 cells were treated with different concentrations of NAR (2,000 to 62.5 µM) for 48 hours. DMSO (1%) was used as a control and vehicle for the preparation of NAR stock. The cell viability was analyzed through Annexin-V and 7-AAD staining by flow cytometry. (**A**) Representative density plot showing the Annexin-V and 7-AAD staining of one representative experiment. (**B**) Viable A549 cells (Annexin-V^-/7-AAD^-). (**C**) The NAR concentration that promoted a 50% reduction in cell viability (CC_{50}) was obtained by using nonlinear regression, a sigmoidal concentration-response curve and a variable slope (GraphPad Prism; La Jolla, CA.USA). (**D**) The average number of cells (cell nuclei count after counterstained with DRAQ5) presented in culture in each experimental condition determined by Operetta high-content imaging system. Analyses were performed using a one-way ANOVA followed by Tukey´s Multiple Comparison Test (*$p < 0.05$ *vs* untreated). All the data represent three independent experiments, each one in technical triplicate.

a slightly anti-ZIKV effect was observed if A549 cells were pretreated with NAR for long periods of time (18 h) before infection, compared to 1.5 h used in the experiment (Supplementary Fig. 6). Additionally, to confirm the efficiency of NAR treatment, we added NAR to the cell cultures at different time points after infection establishment (Fig. 6A–D). The results showed the reduction of viral titers in A549 cells even when cells were treated 24 hpi. However, when the NAR treatment was postponed to 24 hpi, we observe increased viral titers in cell culture supernatant when compared to those observed in A549 cells treated up to 6 hpi. Otherwise, IFN-α 2A showed

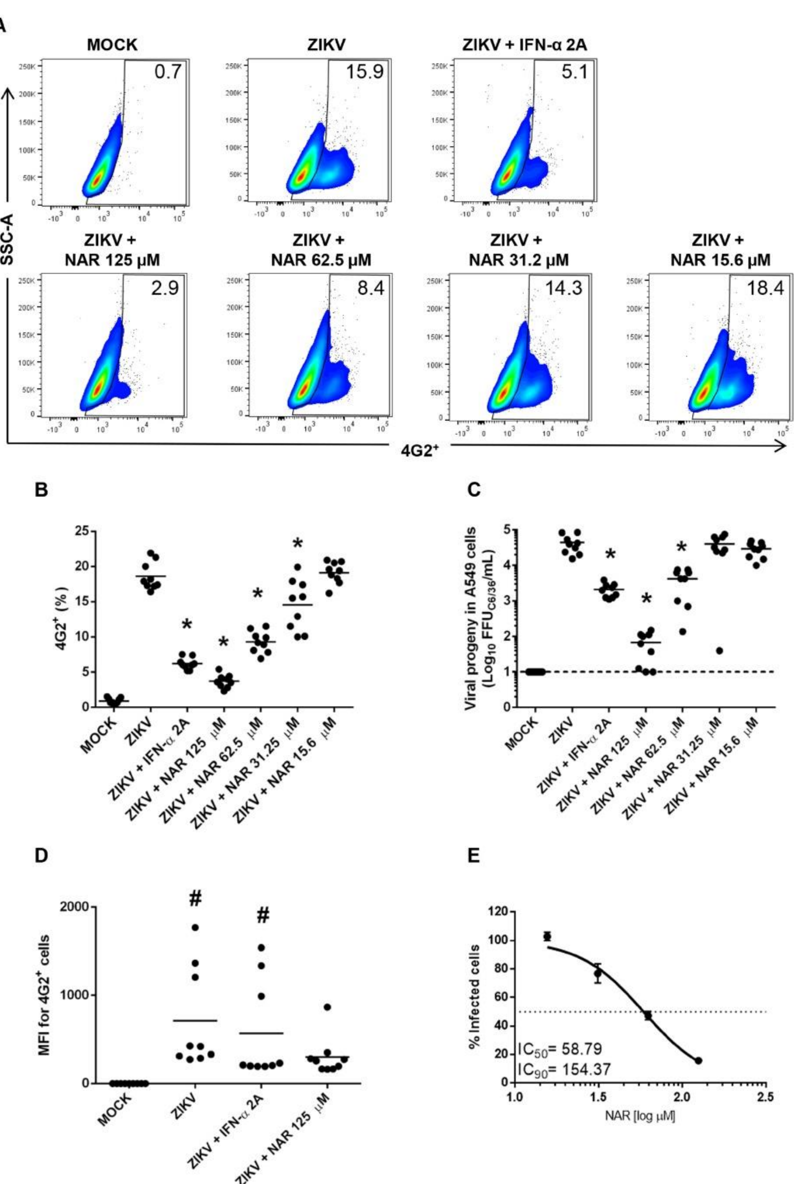


Figure 2. Anti-Zika virus activity of NAR is concentration-dependent. The A549 cells were infected with ZIKV (ZV BR 2015/15261; MOI 0.1) for 90 minutes and treated with different concentrations of NAR (125 to 15.6 μM) after inoculum removal. After 48 hours, the cells were harvested and stained for flow cytometry assay using anti-flavivirus E protein monoclonal antibody (4G2). IFN-α 2A (200 IU/mL) was used as a positive anti-viral control, and the non-infected cells (MOCK) were used as a negative control. (**A**) Representative density plot showing the frequency of A549-infected cells ($4G2^+$) after treatment with different concentrations of NAR. (**B**) The frequency of ZIKV-infected A549 cells ($4G2^+$) after treatment with NAR. (**C**) The viral titers detected by foci-forming immunodetection assay ($FFU_{C6/36}$/mL) in A549 cell culture supernatant after 48 hours of infection. (**D**) The quantification of the mean fluorescence intensity (MFI) in $4G2^+$ cells. (**E**) The concentration response curve of NAR against ZIKV. The NAR concentration that inhibited 50% and 90% of the infection (IC_{50} and IC_{90}) was defined using a sigmoidal dose response curve (variable slope). Results from three independent experiments performed in technical triplicate and analyzed by one-way ANOVA followed by Tukey´s Multiple Comparison Test (*$p < 0.05$ *vs* ZIKV infected and untreated cells and # $p < 0.05$ *vs* mock-infected cells). The dashed line represents the assay's limit of detection.

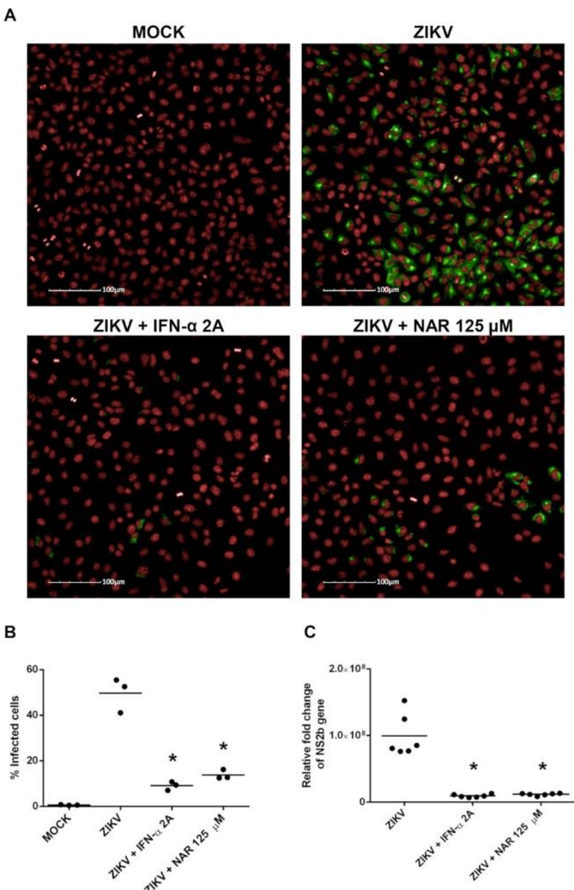


Figure 3. NAR impairs the A549 infection by Zika virus. A549 cells were infected with ZIKV (ZV BR 2015/15261; MOI 0.1) and treated with NAR (125 µM) or IFN-α 2A (200 IU/mL) after inoculum removal or uninfected (MOCK-control). After 48 hours, the numbers of infected A549 cells were quantified using a high-content imaging assay (20X magnification; Operetta High-Content Imaging System from PerkinElmer). (**A**) Representative imaging of A549 cells obtained by the Operetta System showing red cell nuclei (counterstained with DRAQ5), and in green anti-flavivirus E protein monoclonal antibody (4G2) plus goat anti-mouse Alexa Fluor 488 secondary antibody. (**B**) The average number of infected-A549 cells. (**C**) ZIKV RNA detection in A549 cells by RT-qPCR assay. The ZIKV NS2B RNA was quantified in infected cells compared to mock-infected cells (relative fold change) and normalized to the housekeeping gene *18S*. Results from three independent experiments, in technical triplicate (except the RT-qPCR, which was performed in technical duplicate), were analyzed by one-way ANOVA followed by Tukey´s Multiple Comparison Test (*$p < 0.05$ *vs* ZIKV-infected cells).

similar results for the reduction of infected cells, but it was not as efficient at reducing the viable virus particles in the cell culture supernatant as NAR (Fig. 6D). Taken together, these results suggest that NAR might act on the replication, maturation or assembly of viral particles.

Molecular docking of NAR. *In silico* analyzes, as molecular docking has been used to indicate the potential mechanism of action of flavonoids against Zika virus[24,39]. Once the experimental data indicated that NAR could act on the replication, maturation or assembly of viral particles, we employed molecular modeling to assess the hypothesis that NAR may act as a non-competitive inhibitor of the NS2B-NS3 viral protease. First, we validated a docking protocol using the available experimental data[28]. For validation, two flavonoids known as non-competitive inhibitors of the ZIKV protease were chosen, namely myricetin, because of its high activity, and apigenin, due to its close structural similarity to NAR[27]. Docking calculations show that NAR is able to interact with the ZIKV protease in a similar way as myricetin and apigenin (Supplementary Fig. 7). Thus, we speculate that NAR is able to bind to the ZIKV protease and inhibit it via a non-competitive mechanism in the same way as other flavonoids. Experimental validation is needed to prove the interaction between NAR and ZIKV-protease.

Anti-ZIKV activity of NAR in different cell lines. As ZIKV-infection could trigger neurological defects in children born from infected women[5,9,40], the *in vitro* anti-ZIKV activity of NAR was assessed in cell lines

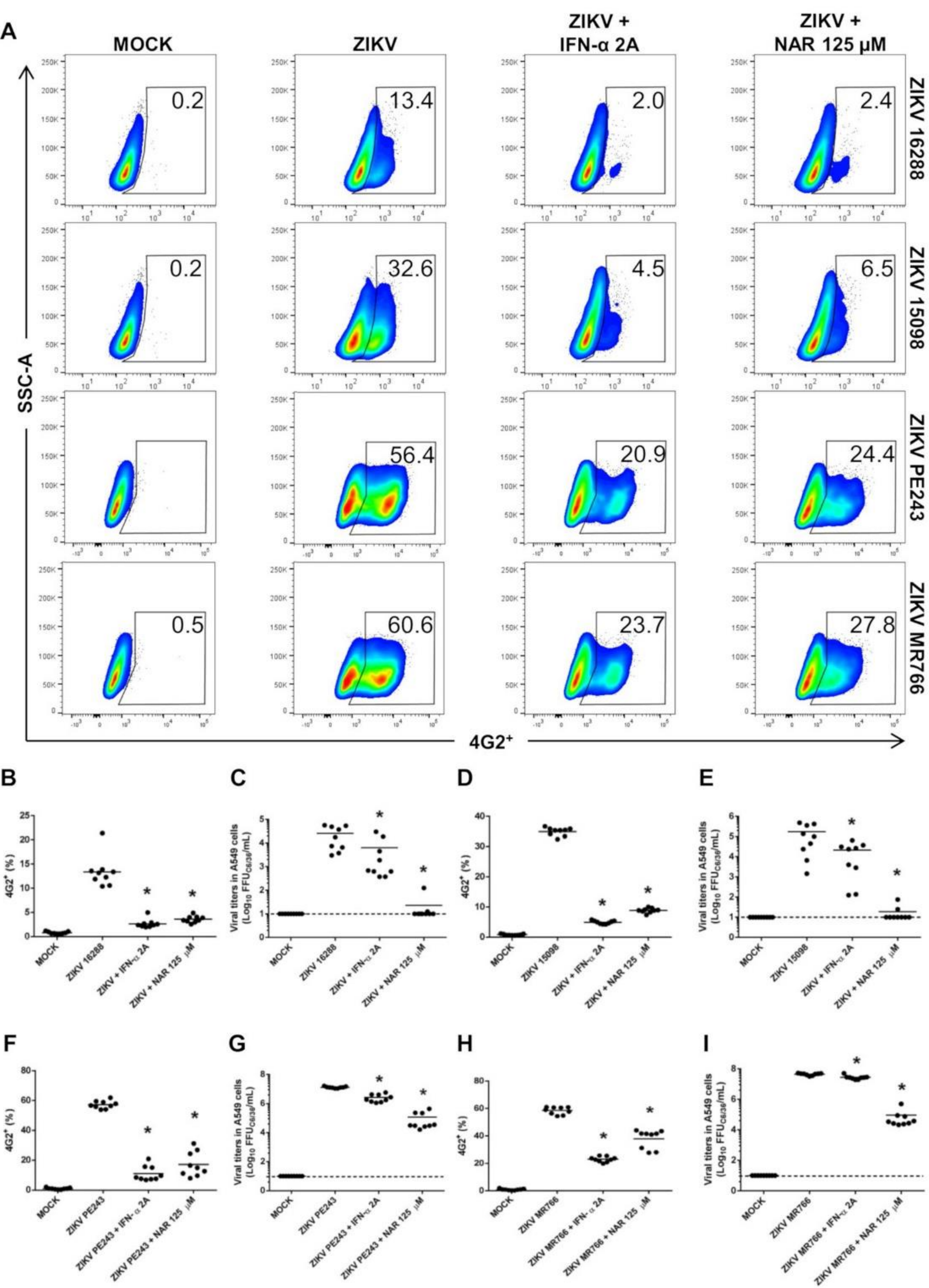


Figure 4. NAR impairs the infection of Zika virus from Asian- and African-lineage in A549 cells. A549 cells were infected with four additional strains of ZIKV (ZV BR 2016/16288, ZV BR 2015/15098, ZIKV PE243, ZIKV MR766; MOI 0.1), three from Asian- and one from African-lineage and treated with NAR (125 μM) or IFN-α 2A (200 IU/mL). After 48 hours, the cells were harvested and stained for flow cytometry assay using anti-flavivirus E protein monoclonal antibody (4G2). (**A**) A representative density plot showing the frequency of A549 cells (4G2$^+$) that were infected with the ZV BR 2016/16288, ZV BR 2015/15098, ZIKV PE243 and ZIKV MR766 strains. The frequency of A549 cells infected (4G2$^+$) with the ZV BR 2016/16288 (**B**), ZV BR 2015/15098 (**D**), ZIKV PE243 (**F**) and ZIKV MR766 (**H**) strains. Determination of viable ZIKV in the cell culture supernatant of A549 cells infected with ZV BR 2016/16288 (**C**), ZV BR 2015/15098 (**E**), ZIKV PE243 (**G**) and ZIKV MR766 (**I**) strains using the foci-forming immunodetection assay ($FFU_{C6/36}$/mL). The data represent three independent experiments, each one in technical triplicate then analyzed by a one-way ANOVA followed by Tukey´s Multiple Comparison Test (*$p < 0.05$ *vs* ZIKV infected and untreated A549 cells).

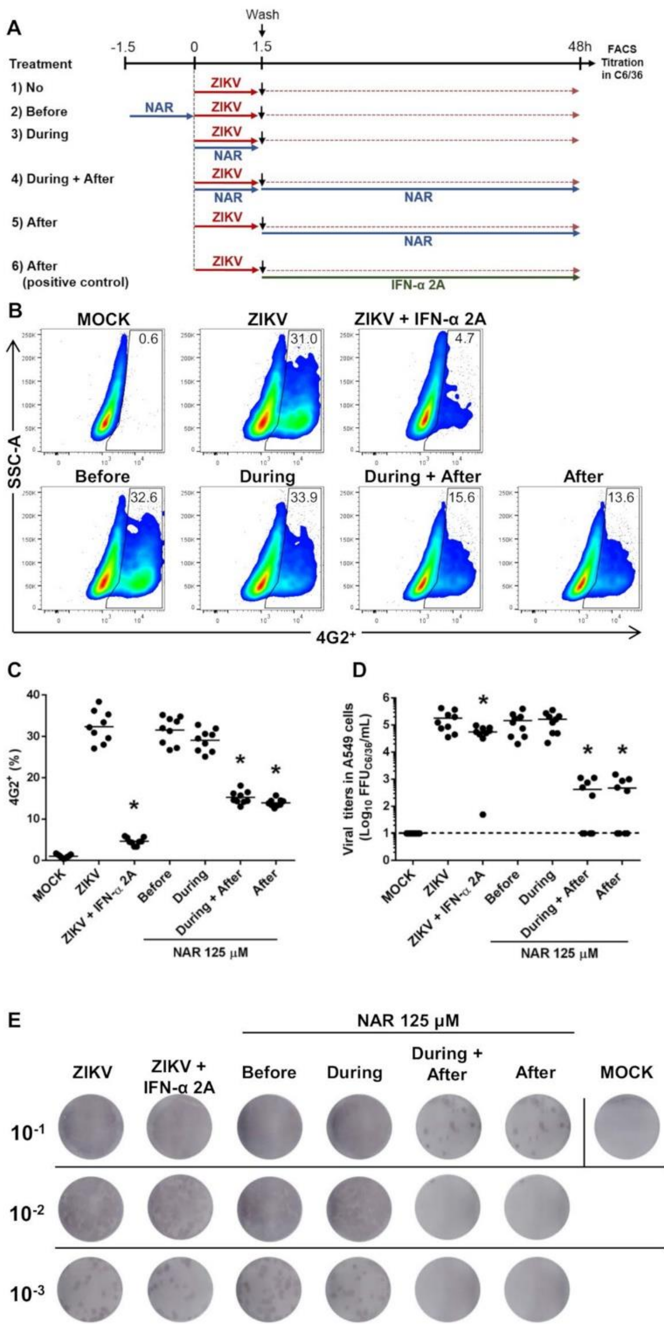
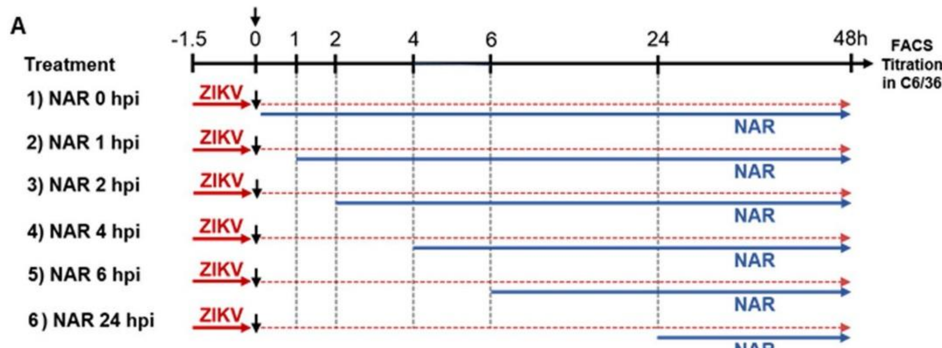


Figure 5. Time-of-drug-addition experiments of NAR in A549 cells. The A549 cells were infected with 0.1 MOI of ZIKV (ZV BR 2015/15261 strain) and cells were treated with NAR (125 μM) at different moments. (**A**) Schematic representation from the experimental design. A549 cells were submitted to different treatment regimens: (1) infected and left untreated (positive control of infection); (2) treated with NAR (125 μM) for 90 minutes prior to ZIKV infection; (3) treated with NAR during ZIKV infection; (4) treated with NAR during and after ZIKV infection; (5) treated with NAR after ZIKV infection; or (6) treated with IFN-α 2A (200 UI/ml) after ZIKV infection as a control of treatment. (**B**) Representative density plot showing the frequency of infected cells ($4G2^+$) in each experimental condition. (**C**) Frequency of ZIKV-infected cells ($4G2^+$). (**D**) The viral titers detected by foci-forming immunodetection assay ($FFU_{C6/36}$/mL) from cell culture supernatant. (**E**) Representative foci-forming immunodetection assay ($FFU_{C6/36}$/mL) from Fig. 5D. The data represent three independent experiments in technical triplicate that were analyzed by one-way ANOVA followed by Tukey´s Multiple Comparison Test (*$p < 0.05$ *vs* ZIKV-infected and untreated cells).



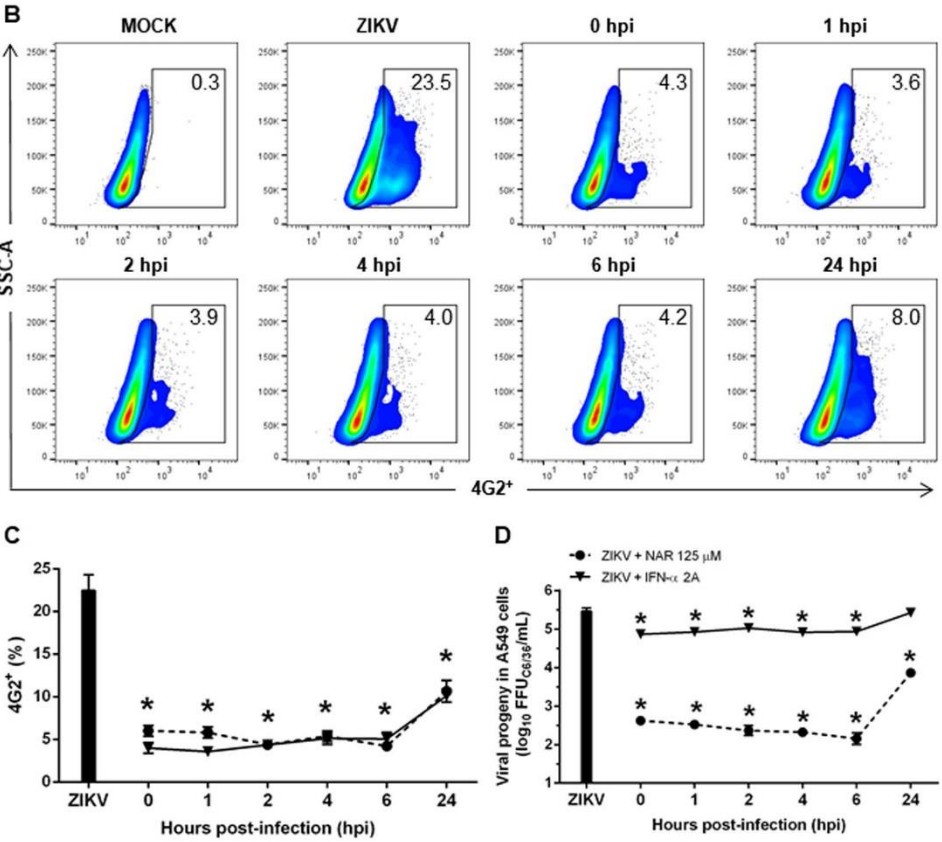


Figure 6. Naringenin treatment was effective even after the infection establishment. The A549 cells were infected with ZV BR 2015/15261 (MOI 0.1) and treated (one time) with NAR (125 μM) or IFN-α 2A (200 IU/mL) at different times points after the infection establishment, at 0, 1, 2, 4, 6, and 24 hours post-infection (hpi). After 48 h of infection, the A549 cells were harvested and stained with mouse anti-flavivirus E protein (4G2) monoclonal antibody for the flow cytometry analyses. (**A**) Schematic representation of the experimental design. (**B**) Representative density plot showing the frequency of A549-infected cells ($4G2^+$). (**C**) Frequency of ZIKV-infected A549 cells ($4G2^+$). (**D**) The viral titers detected by foci-forming immunodetection assay ($FFU_{C6/36}$/mL) in the supernatant of A549-infected cells. The dotted line represents A549 cells treated with NAR, and the continuous line represents A549 treated with recombinant IFN-α 2A. The data represent three independent experiments for which each was performed in technical triplicate then analyzed by one-way ANOVA followed by Tukey´s Multiple Comparison Test (*$p < 0.05$ *vs* ZIKV-infected and untreated cells).

potentially involved in ZIKV-pathogenesis, like human glioblastoma cell line (A172) and human embryonic stem cell line (NKX2-5$^{eGFP/w}$hESC). Thus, using ZIKV strains from Asian- and African-lineage and two different techniques (FACS and foci-forming immunodetection assay) it was shown that NAR was able to reduce the infection

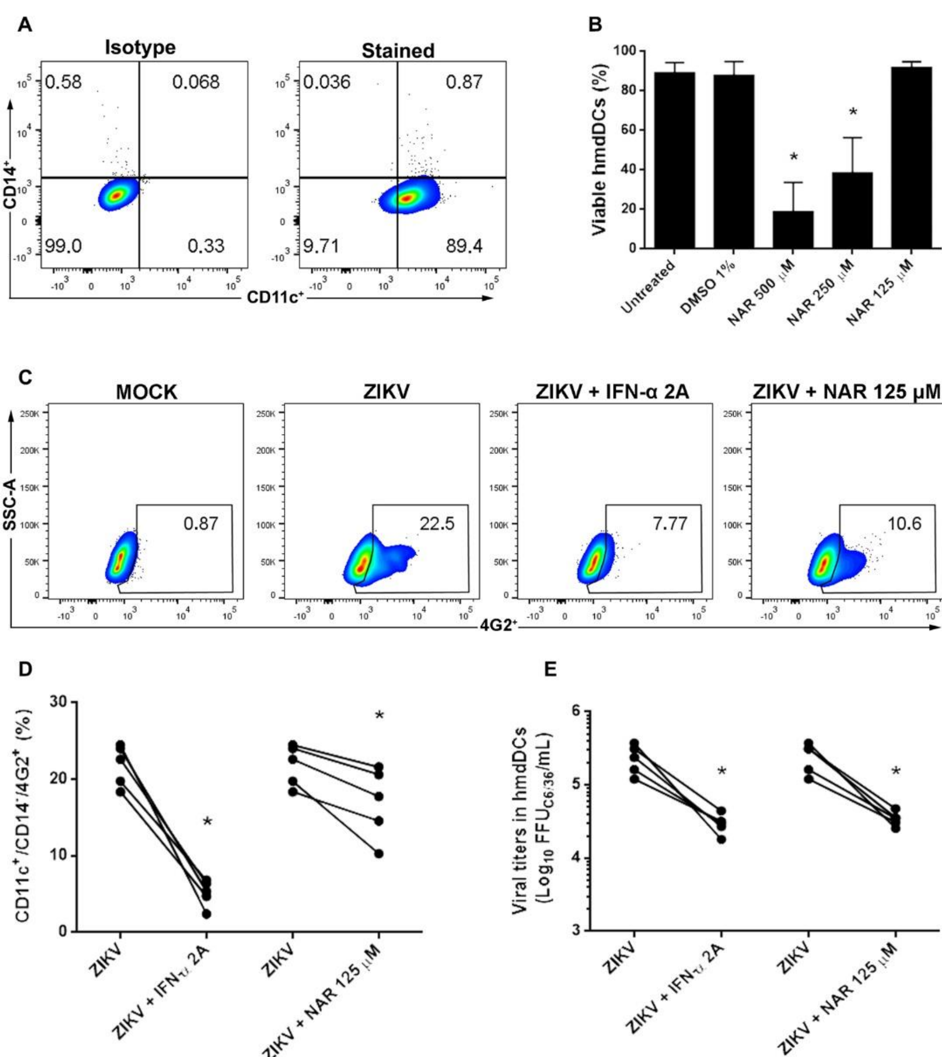


Figure 7. NAR impairs the infection of human monocyte-derived dendritic cells by ZIKV. Human monocytes derived-dendritic cells from five healthy donors were tested. (**A**) Representative density plot showing the phenotype of hmdDCs ($CD14^-/CD11c^+$) after seven days of differentiation. To determine the MNTC of NAR for hmdDCs cells were treated with different concentrations of NAR (500 to 125 µM) for 24 h. DMSO was used as a control and vehicle for the preparation of NAR stock solution. (**B**) The frequency of viable hmdDCs ($Annexin\text{-}V^-/7\text{-}AAD^-$) were shown. For antiviral activity of NAR, hmdDCs were infected with ZIKV (ZV BR 2015/15261 at MOI 10) and treated with NAR (125µM) or IFN-α 2A (200 IU/mL). After 24 hours, the cells were harvested and stained for flow cytometry assay using anti-human CD11c-PE, anti-CD14-APC and anti-flavivirus E protein monoclonal antibody (4G2-FITC). (**C**) Representative density plot showing the frequency of hmdDCs-infected cells ($CD14^-/CD11c^+/4G2^+$) after treatment with NAR. (**D**) The frequency of ZIKV-infected hmdDCs ($CD14^-/CD11c^+/4G2^+$) after treatment with NAR. (**E**) The viral titers detected by foci-forming immunodetection assay ($FFU_{C6/36}$/mL) in hmdDCs culture supernatant after 24 hours of infection. Data represent each donor measure and were evaluated by paired one-way ANOVA and Tukey's post-test ($*p < 0.05$ *vs* ZIKV-infected and untreated cells).

in glioblastoma cell line (A172) (Supplementary Fig. 9B), while no effect was observed in human embryonic stem cell line (Supplementary Fig. 8). In spite of the fact that no effect was observed in human embryonic stem cell line, the anti-ZIKV activity of NAR was confirmed using other human cell line, Huh7.5, from the hepatic origin (Supplementary Fig. 9D).

Also, due to the role of dendritic cells in the immune response and its highly susceptibility to ZIKV infection we decide to test the antiviral activity of NAR in human monocyte-derived dendritic cells (hmdDCs)[41–43]. First, it was shown that the concentration of 125 µM of NAR was not toxic to hmdDCs and would be used as the MNTC

(Fig. 7B). Next, it was demonstrated that NAR (125 µM) effectively reduced the number of ZIKV-infected hmdDCs as well as the viral titers in cell culture supernatants (Fig. 7C–E). Altogether, the results demonstrated that NAR is able to impair the infection of hmdDCs, a key target cell for ZIKV infection and for the immune system function[41,44].

Discussion

The Zika virus epidemic in Brazil (2015) was associated with new clinical syndromes, such as *Guillain-Barré* and neurological defects in children born from infected women[5,9–11]. Despite the many efforts to reduce the morbidity and mortality associated with this infection, to date, neither a vaccine nor an antiviral drug has been licensed for use in humans[45].

Several different compounds were already tested against ZIKV, mostly *in vitro*[12,27,28,46]. Additionally, it was already demonstrated that flavonoids could represent a source of active compounds against flaviviruses[18–26,47–49]. Recently, NAR was shown to impair the replication of dengue viruses in human cells[18].

The cytotoxicity of NAR diverges, depending on the given cell line[48,50]. A 250 µM concentration of NAR could be used in human hepatoma cell culture (Huh7.5) without toxic effects[18]. Here, the toxicity of NAR to A549 was demonstrated to be concentration-dependent, and 125 µM (MNTC) was considered safe. For the other assayed cell lines or primary cells (Huh7.5, A172, hmdDCs and NKX2-5$^{eGFP/w}$hESC), the same concentration was also non-toxic. However, higher cytotoxicity has been previously observed for primary human monocytes treated with NAR[18]. *In vivo*, NAR seems to be well absorbed and tolerated by mice, and also protects rats from dimethylnitrosamine-induced liver damage[51,52].

The antiviral effect of flavonoids has been tested against numerous viruses such as herpes simplex viruses, hepatitis B and human cytomegalovirus[14,53,54]. Additionally, the flavanone NAR presents antiviral activity against viruses from the *Flaviviridae* family such as HCV, dengue and yellow fever virus strain 17D[17,18,55]. It appears that some flavonoids are able to interact with the NS2B-NS3 protease of ZIKV through chemical structures, and they play important roles in the inhibition activities of this protease[28].

Here, four different techniques showed that NAR was able to impair the ZIKV infection in A549 cells. Additionally, we observed that NAR performed better than IFN-α 2A in reducing the virus titer in the cell culture supernatant. Thus, it seems that IFN-α 2A reduces the number of ZIKV-infected A549 cells, despite having lower impact on the secretion of viable ZIKV particles. The same phenotype was observed for A172 and Huh7.5 cells treated with IFN-α 2A. Goebel *et al.*[56], have shown that high concentration (500 IU/mL) of type I IFN is needed to reduce 1–2 log of ZIKV RNA in Vero cell culture supernatant. Finally, this effect seems to be specific for ZIKV infection, once IFN-α 2A was able to reduce the secretion of viable dengue virus particles in cell culture supernatants to the same extent as the percentage of Huh7.5-infected cells[18]. Thus, here the IFN-α 2A was used as an *in vitro* ZIKV-treatment control as already demonstrated in the literature[46]. Once type I IFN has a broad mechanism to control viral infection, through the expression of hundreds of ISGs with antiviral activity[57], we do not intend compare the anti-ZIKV effect of NAR with type I IFN.

Also, using two different techniques it was suggested that NAR does not present virucidal effect against ZIKV. In agreement, previous report shows that NAR was not virucidal to the four-dengue virus serotypes[18].

Furthermore, NAR was able to inhibit the infection of Huh7.5 cells by the four dengue serotypes[18]. However, when using the dengue-2 New Guinea C strain, Zandi *et al.*[48] were not able to show infection inhibition. Additionally, it was shown that the flavonoid isoquercitrin present anti-ZIKV activity for both African- and Asian-lineages of the virus[23]. In this context, we tested the antiviral activity of NAR against distinct ZIKV recent clinical isolates from Asian-lineage obtained from patients in Brazil, and from African-lineage isolated from a Rhesus monkey in Uganda[1,58,59]. The results showed that the anti-ZIKV activity of NAR is lineage-independent, although a higher reduction of infection was observed for Asian-lineages of ZIKV when compared to the African-lineage. Intrinsic differences in the pathogenicity and virulence of African- and Asian-lineage of ZIKV were already demonstrated[37,60]. While those differences could account for differences in clinical presentation needs to be better defined. However, Asian-lineage seems to infect cells at lower rate, produces less virus and trigger poor early cell death compared to the more virulent African-lineage[37], which could help to explain the observed results.

An additional aspect of anti-ZIKV activity of NAR that should be taken into consideration is the cell line used in the experiments. Anti-ZIKV activity of NAR seems to be cell-line dependent, once ZIKV-infection of human embryonic stem cell line was not affected neither by NAR nor by IFN-α 2A. Otherwise, for three cell lines used and a primary human cell (hmdDCs) NAR seems to be able to impair ZIKV infection. It was demonstrated that the absorption and metabolism of the flavonoids would vary between cell lines[61]. This associated with differential subcellular distribution and efficiency of NAR accumulation inside the cells might lead to differential cell-line responses observed[62].

To determine the stage of the ZIKV life cycle that is affected by NAR, a time-of-drug-addition experiment was performed[18,38]. The data indicate that NAR acts between replication and virus assembly, as already shown for the dengue and Chikungunya viruses[18,63]. In addition, the antiviral effect of NAR was evident even when NAR treatment was postponed for 24 hours after infection, and it was similar to IFN-α 2A. Similarly, treatment of ZIKV-infected Vero cells with 6-methylmercaptopurine riboside (6MMPr) 72 hpi impairs viral RNA production and demonstrated the potential use as antiviral compound[64]. Furthermore, some treatments available for flaviviruses, like IFN-α 2A for hepatitis C present several adverse effects, such as pain and depression[65]. Thus, the fact that NAR also inhibit acute inflammation and reduce pain, would represent an additional advantage of using NAR for treating ZIKV infected patients[31,32,65,66].

The inhibition of viral RNA metabolism occurs through flavonoid binding, as shown for the flavonol called kaempferol against Japanese encephalitis virus[49]. Furthermore, flavonoids seems to impair the activity of proteins related to replication, such as the NS2B-NS3 complex of dengue and ZIKV[26,28]. Flavonoids are non-competitive

inhibitors of the viral protease in both dengue and ZIKV[26–28]. A reduction in the possible hydrogen bonds of apigenin, as shown by molecular docking with the viral NS2B-NS3 protease, is consistent with the experimental data. The concentration of apigenin necessary to inhibit half the maximum activity of the protease is 43 times higher than that of myricetin, which can establish more hydrogen bonds[27,28]. According to the molecular modeling data, NAR is able to interact with the viral protease in the same way as other flavonoids, which are known allosteric inhibitors of this protein. Thus, we propose that the molecular target of NAR is the NS2B-NS3 protease. However, experimental validation is needed to confirm this hypothesis.

Conclusions

Here, we used different techniques to show that NAR presents anti-ZIKV activity. *In vitro* NAR was effective against distinct ZIKV lineages (Asian and African) and seems to act during the late phase of the viral life cycle, probably as a non-competitive inhibitor of NS2B-NS3 protease. NAR keeps its antiviral activity even when it is added several hours post-infection, supporting the idea that NAR can target virus replication. Altogether, the results demonstrated that NAR could be a suitable candidate for ZIKV therapy. Finally, further studies should be undertaken to improve the understanding of the *in vitro* antiviral activity of NAR and to establish whether it is effective *in vivo*.

Methods

Cells, Zika virus and naringenin. *Aedes albopictus* C6/36 (ATCC: CLR-1660) mosquito cells and Human-derived hepatoma cells (Huh7.5; ATCC PTA-8561) were grown as previously described[18]. Human A549 lung epithelial cells (ATCC CCL-185) were grown in Dulbecco's Modified Eagle Medium consisting of an F-12 nutrient mixture (DMEM/F-12), supplemented with 25 µg/mL of gentamicin and 7% fetal bovine serum (FBS). Human gliobastoma cell line (A172; ATCC® CRL-1620™) was grown in DMEM high glucose supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% FBS. Human embryonic stem cell line (NKX2-5$^{eGFP/w}$hESC) was seeded on matrigel-coated culture plates using the mTeSR™1 medium (StemCell Technologies) and cultured as previously described[67]. The NKX2-5$^{eGFP/w}$hESC cells were kindly provided by Dr. David Elliot from the Murdoch Childrens Research Institute (MCRI), Australia. A549, A172, Huh7.5 and NKX2-5$^{eGFP/w}$hESC were all cultured at 37 °C in a humidified, controlled 5% CO_2 atmosphere. Human monocyte-derived dendritic cells (hmdDCs) were generated from peripheral blood monocytes from healthy donors as already described[68], however using 25 ng/mL of human recombinant IL-4 (rIL-4) and 12.5 ng/mL of human recombinant GM-CSF (rGM-CSF) (PeproTech, Rocky Hill, NJ, United States). The study with human cells from healthy donors was approved by Committee of Human Experimentation from Fundação Oswaldo Cruz (Fiocruz) under the number CAAE: 60643816.6.0000.5248. All reagents, unless specified, were from Thermo Fisher Scientific (Grand Island, New York, USA).

Zika viruses were isolated from serum of infected patients in Northeast (2015) (ZV BR 2015/15261; ZV BR 2015/15098) and South Brazil (2016) (ZV BR 2016/16288). Since our laboratory is a Reference Center for the Diagnosis of Emerging Viruses of the Brazilian Ministry of Health, we obtained the waiver from the Brazilian National Ethics Committee of Human Experimentation for the written informed consent to work with these samples. However, the study with human serum samples was approved by Fundação Oswaldo Cruz (Fiocruz) and the Brazilian National Ethics Committee of Human Experimentation under the number CAAE: 42481115.7.0000.5248. Finally, the study is in compliance with all the ethical principles of the Brazilian National Ethics Committee of Human Experimentation. Additionally, the laboratory-adapted ZIKV strains ZIKV PE243[59] and ZIKV MR766[1] were tested. Viral stocks were grown in C6/36, clarified by centrifugation and titrated using a foci-forming immunodetection assay in C6/36.

Naringenin (NAR; ≥98% purity) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), prepared in a 50 mM stock solution in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and then diluted to its final concentrations in DMEM/F-12 supplemented with 100 IU/µg/mL penicillin/streptomycin and 10% FBS (Thermo Fisher Scientific, Grand Island, New York, USA).

Cell viability assay. A549 cell viability following NAR treatment was tested by Annexin-V/7-AAD (BD Biosciences, San Jose, CA, USA) cell staining procedure. A549 cells (1×10^5 cells/well in 24 well plates) were incubated for 18 hours and treated with different concentrations of NAR (2,000, 1,000, 500, 250, 125 and 62.5 µM) or 1% DMSO (vehicle). The cells were maintained for 48 hours. After incubating, the cells were trypsinized, labelled with Annexin-V (FITC) and 7-AAD, and analyzed by flow cytometry (FACS) (FACS Canto II; BD Biosciences, San Jose, CA, USA). The viable A549 cells were defined as double-negative for Annexin-V$^-$/7-AAD$^-$ and the resulting data were used to determine the maximal non-toxic concentration (MNTC) of NAR. Additionally, the concentration that inhibited viability in 50% of the cells (CC_{50}) was obtained using nonlinear regression and a sigmoidal concentration-response curve (GraphPad Prism, La Jolla, CA, USA). The same protocol was used to determine the MNTC of NAR for Huh7.5, A172, NKX2-5$^{eGFP/w}$hESC. For hmdDCs the expression of Annexin-V/7-AAD and the establishment of MNTC were performed after 24 h in culture. Finally, an immunofluorescence assay was performed to confirm that 125 µM of NAR did not affect A549 cells viability. Briefly, A549 cells (1×10^4 cells/well in 96-well plates) were treated with NAR (125 µM) in DMEM-F12 for 48 hours. The cells were than fixed and permeabilized with methanol:acetone (v/v) and cell nuclei was stained with DRAQ5 (Thermo Fisher Scientific, Grand Island, New York, USA). The cell nuclei count after treatment with NAR were determined by an Operetta High-Content Imaging System using Harmony High Content Imaging and Analysis software (4 different fields and 20x magnification; Thermo Fisher Scientific, Rockford, IL, USA).

Antiviral activity assays. The antiviral activity of NAR was assessed using immunofluorescence, FACS, one-step reverse transcriptase real time polymerase chain reaction (RT-qPCR) and foci-forming

Target gene	Primer name	Sequence (5′–3′)	Cycling
Zika virus NS2B gene[70]	Zika4481	CTGTGGCATGAACCCAATAG	50 °C-30 min 95 °C-15 min 45 cycles 95 °C-15 s 60 °C-1 min.
	Zika4552c	ATCCCATAGAGCACCACTCC	
	Probe Zika4507c-FAM	CCACGCTCCAGCTGCAAAAGG	
18S	18SF	CACGGCCGGTACAGTGAA	42 °C-30 min 95 °C-10 min 40 cycles 95 °C-15 s 60 °C-30 s 72 °C-1 min
	18SR	CCCGTCGGCATGTATTAGCT	
Zika virus envelope (E) gene[72]	ZIKVENVF	GCTGGDGCRGACACHGGRACT	95 °C-2 min 35 cycles 95 °C-20 s 55 °C-20 s 72 °C-30 s Final elongation at 72 °C-7 min
	ZIKVENVR	RTCYACYGCCATYTGGRCTG	

Table 1. Primer and probe sequences and the cycling conditions used during the RT-PCR assays.

immunodetection assays. First, the A549 cells (1×10^5 cells/well in 24 well plates) were incubated for 18 hours. The cells were then infected with ZIKV at a multiplicity of infection (MOI) of 0.1 for 90 minutes, washed and the MNTC of NAR (125 μM) was added. After a 48 hours incubation, the cell culture supernatants were removed and stored at −80 °C for virus titration in C6/36. Moreover, the cells were detached and stained for FACS using a 4G2-FITC monoclonal antibody (anti-E protein of flavivirus; ATCC® HB-112™) as previously described[18]. A BD FACS Canto II was used to quantify the number of 4G2-positive cells. Additionally, RNA extracted from the cell pellet (RNeasy Mini Kit, Qiagen, Hilden, Germany) was used to quantify the ZIKV NS2B genes using a RT-qPCR[69]. In brief, 5 μg of RNA was used to amplify the ZIKV NS2B gene using specific primers (10 pmol) and a probe (4 pmol). For normalization, an 18S gene amplification was performed using 10 pmol of the primers 18SF and 18SR. The final reaction volume was 20 μL. The gene modulation was determined by calculating the $2^{-\Delta\Delta CT}$[70]. The primer sequences and cycling conditions are listed in Table 1.

For the immunofluorescence assay, the A549 cells (1×10^4 cells/well in 96-well plates) were infected with MOI 0.1. After 90 minutes, the cells were washed and treated with NAR (125 μM) in DMEM-F12. After 48 hours, the cells were fixed and permeabilized with methanol:acetone (v/v) and the cell infection was analyzed after being labeled with 4G2 and goat anti-mouse Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific, Grand Island, New York, USA). Digital images were taken from 4 different fields (20x magnification). The infection percentage was quantified in an Operetta High-Content Imaging System using Harmony High Content Imaging and Analysis software (Thermo Fisher Scientific, Rockford, IL, USA).

For all the techniques, mock (non-infected cells) and non-treated ZIKV-infected cells were used as controls. In addition, based on the anti-ZIKV activity of type I IFN *in vitro*[46], interferon-α 2A (IFN-α 2A) (200 UI/ml; Blau Farmacêutica, Cotia, SP, Brazil) was used as a positive control for the antiviral treatment. Furthermore, concentration-response curves were obtained using a serial dilution starting from the MNTC of NAR. The concentration that inhibited 50% and 90% of the viral infection (IC_{50} and IC_{90}, respectively) was obtained using a nonlinear regression, followed by a sigmoidal concentration-response (variable slope; GraphPad, La Jolla, CA, USA). Also, the selectivity index ($SI = CC_{50}/IC_{50}$) was calculated.

To access the anti-ZIKV activity of NAR on Huh7.5 (MOI 0.1), A172 and NKX2-5$^{eGFP/w}$hESC (MOI 1) cells FACS and foci-forming immunodetection assays were employed as previously described for A549 cells using three different ZIKV-strains (ZV BR 2015/15261; ZIKV PE243 and ZIKV MR766).

The anti-ZIKV activity of NAR in hmdDCs was determined 24 hpi after infection with an MOI of 10 based on previously reported data[71]. For hmdDCs, the antiviral activity of NAR was analyzed 24 hpi, once it was demonstrated that both virus release and viral RNA loads increased over time reaching the peak at 24 hpi[43].

Foci-forming immunodetection assay. Viral titers in cell culture supernatant were determined by the foci-forming immunodetection assay in C6/36 cells ($FFU_{C6/36}$/mL). Briefly, cell culture supernatants were 10-fold serially diluted in Leibovitz's medium 15 (L-15) supplemented with 0.26% of tryptose and 25 μg/mL gentamicin and added to C6/36 (1×10^5 cells/well in 24 well plates) in duplicate. After 1h30 min, the inoculum was removed and a CMC overlay media (L-15 supplemented with 5% SFB, 0.26% tryptose, 25 μg/mL gentamicin, 1.6% carboxymethylcellulose) was added and plates incubated at 28 °C. The immunostaining was performed after seven days using the mouse monoclonal Flavivirus group-specific antibody 4G2, followed by goat anti-mouse immunoglobulin conjugated to alkaline phosphatase (Promega, Madison, WI, USA), which was detected by adding a solution of NBT/BCIP (Promega, Madison, WI, USA) as a substrate. Foci was counted and expressed as $FFU_{C6/36}$/ml.

Virucidal Assay. A virucidal assay was performed as described[18]. In brief, 2×10^6 $FFU_{C6/36}$ of ZIKV (ZV BR 2015/15261) was incubated with NAR (125 μM) in the presence or absence of RNase A (150 μg/mL) (USB-Affymetrix; Santa Clara, CA, USA) at 37 °C for 1 hour. Zika virus RNA samples that were treated with RNase or left untreated were used as controls. After incubation, the amplification of ZIKV envelope gene was performed based on a one-step RT-PCR assay previously described[72]. The primer sequences and cycling conditions are available in Table 1. Additionally, 2×10^6 $FFU_{C6/36}$ of ZIKV was incubated with NAR (125 μM) for 1 h at 37 °C and residual infectivity of ZIKV was assessed by foci-forming immunodetection assay in C6/36 as described above.

Time of drug-addition assay. The treatment of the infected cells at different moments of the infection could indicate in each step of the virus life cycle the compound act. Thus, the time of drug-addition assay was performed as previously described[18,38]. Initially, the A549 cells (1×10^5 cells/well in 24 well plate) were submitted to different treatment regimens: (1) infected and left untreated (positive control of infection); (2) treated with NAR (125 μM) for 1.5 h prior to ZIKV infection; (3) treated with NAR during ZIKV infection; (4) treated with NAR during and after ZIKV infection; (5) treated with NAR after ZIKV infection; or (6) treated with IFN-α 2A (200 UI/ml) after ZIKV infection as a control of treatment (Fig. 5A). After incubation the number of infected cells (FACS), and the virus titers at cell culture supernatant was performed for each different treatment.

Furthermore, the cells were infected with ZIKV MOI 0.1 and treated with NAR (125 μM) at different time points after ZIKV infection, namely as 0, 1, 2, 4, 6, and 24 hours post-infection (hpi) (Fig. 6A). The same experimental conditions were tested using IFN-α 2A (200 UI/ml) as a positive control for the antiviral treatment. The number of infected cells was determined by FACS, and the virus titers at cell culture supernatant was performed to evaluate the impact of NAR treatment at different time points on A549-infected cells.

Molecular docking assay of NAR. Docking calculations were performed with AutoDock Vina[73] with the value of the exhaustiveness parameter set to 1000. The 3D structure of all the compounds was initially optimized with MOPAC software[74] using the semi-empirical Hamiltonian PM7[75] in a vacuum. The receptor structure was the crystallographic structure of the NS2B-NS3 protease from the ZIKV in a complex with the peptide PDB ID 5GPI[76]. The peptide and water molecules from the experimental structure were removed and chains A and B were used as representatives of the NS2B and NS3pro subunits, respectively. Hydrogens were added using GROMACS 2016.3[77]. The search volume for the docking was focused on the allosteric cavity that was previously identified for the dengue virus protease[78]. Pictures were generated with the open source version of PyMOL 1.8.6.0 (Schrödinger, LLC). The hardware was provided by a Brazilian biotech startup called Mining Information for You (MI4U) and was composed of one machine with 32 physical cores that was hosted by the Google Cloud Platform.

Data analysis. The statistical analysis consisted in a one-way ANOVA and Tukey's post-test with a significance of $p < 0.05$, and it was performed with Prism software (GraphPad version 6.0; La Jolla, CA, USA). Flow cytometry data were analyzed using the FlowJo software version 10 (Tree Star Inc., USA).

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Author contributions

A.H.D.C., D.K., W.A.V.Jr, C.N.D.S., P.F.W. and J.B. contributed conception and design of the study. A.H.D.C., D.K., G.F.S., T.B.A., A.A.S., A.C.K. performed the infection and titration assays; C.Z. performed the RT-qPCR; L.O.B. and M.D.B. performed the molecular docking assay; A.W.R. and M.A.S. performed the stem cell assay. A.H.D.C., D.K., P.F.W. and J.B. wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Material

The citrus flavonoid naringenin impairs the *in vitro* infection of human cells by Zika virus

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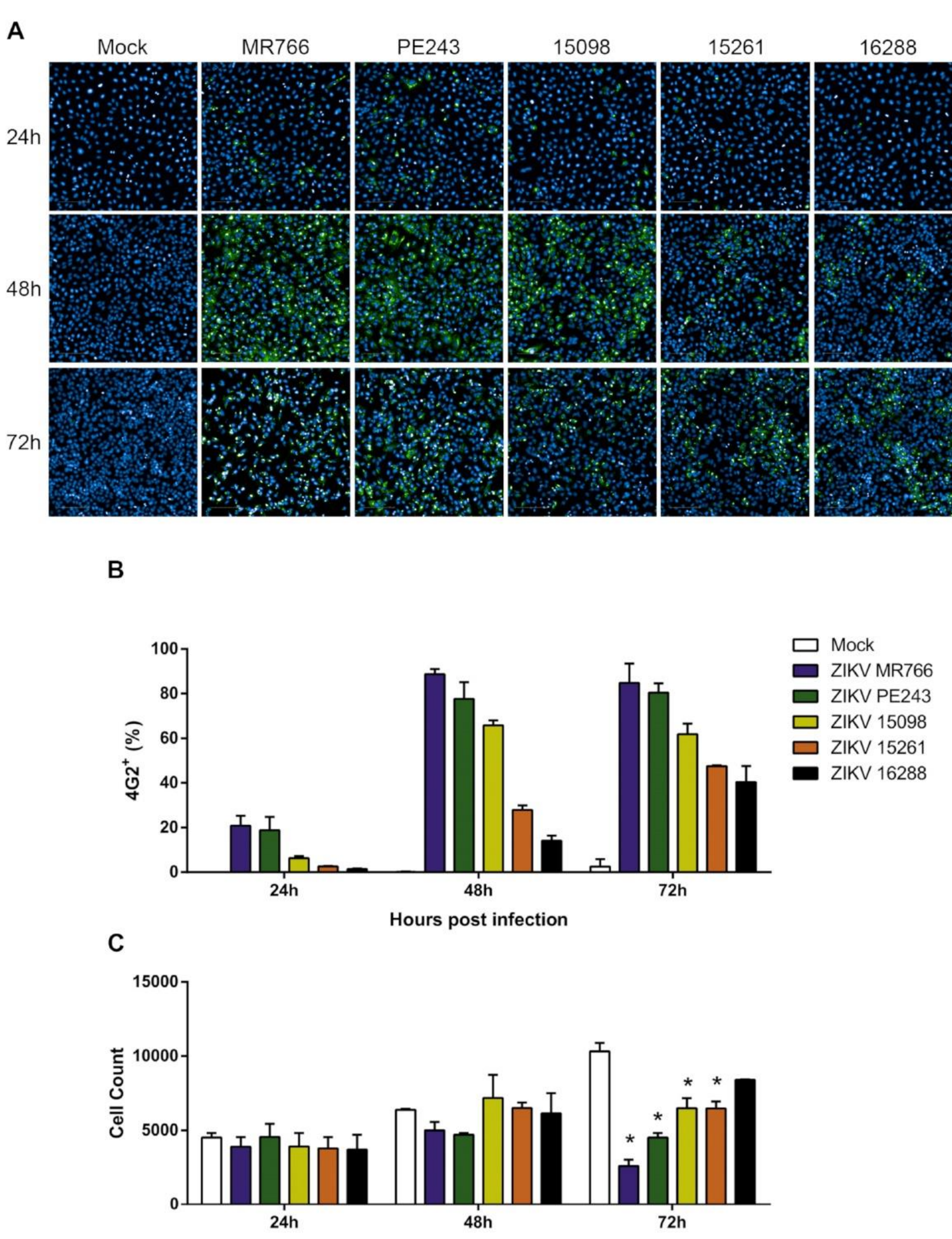
1.1 Supplementary Table

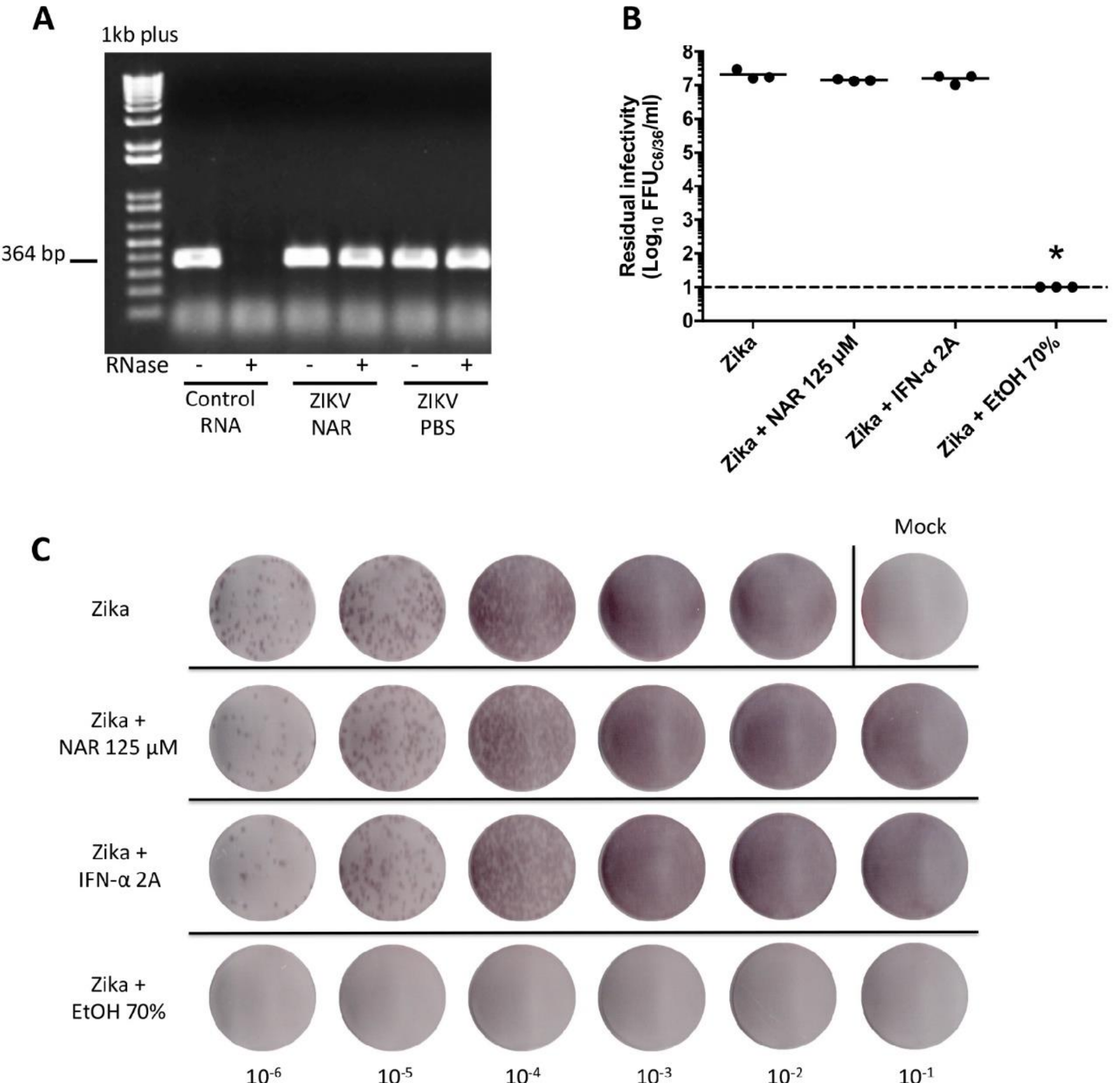
Supplementary Table 1: Primer and probe sets used for the discrimination between Asian- and African-lineage of Zika virus

Primer/Probe	Sequence (5'-3')	Genome Position	Lineage specificity	Reference
ZIKV 1086	CCGCTGCCCAACACAAG	1086-1102	Asiatic + African	Lanciotti et al., 2008 [35]
ZIKV 1162c	CCACTAACGTTCTTTTGCAGACAT	1162-1139		
ZIKV 1107-FAM	AGCCTACCTTGACAAGCAGTCAGACACTCAA	1107-1137		
Zika 4481	CTGTGGCATGAACCCAATAG	4434-4453	Asiatic	Bingham et al., 2016 [36]
Zika 4552c	ATCCCATAGAGCACCACTCC	4524-4505		
Zika 4507c-FAM	CCACGCTCCAGCTGCAAAGG	4479-4460		

1.2 Supplementary Figures



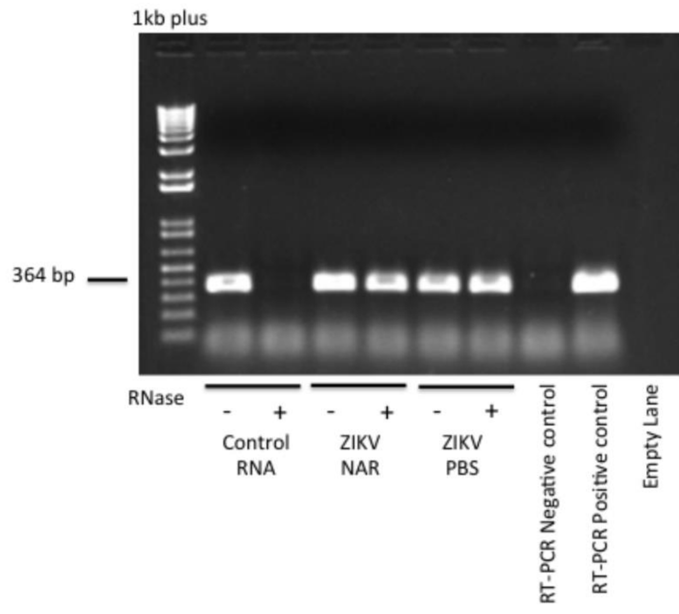
Supplementary Figure 1. Zika virus growth curve on A549 cells. A549 cells ($1x10^4$ in 96-well plate) were infected with five strains of ZIKV (ZIKV MR766, ZIKV PE243, ZV BR 2015/15098, ZV BR 2015/15261 or ZV BR 2016/16288; MOI 0.1). After 24, 48 and 72 hours post-infection, the numbers of infected A549 cells were quantified using a high-content imaging assay (20X magnification; *Operetta High-Content Imaging System from PerkinElmer*). **(A)** Representative imaging of A549 cells obtained by the Operetta System showing cell nuclei (blue; counterstained with DAPI), and anti-flavivirus E protein monoclonal antibody (green; 4G2 staining) plus goat anti-mouse Alexa Fluor 488 secondary antibody. **(B)** The average number of infected-A549 cells. **(C)** The average number of cells (cell nuclei count) presented in culture in each experimental condition and time-point. Data are the representative of 9 different fields at each well (*$p<0.05$ ZIKV-infected *vs* MOCK).

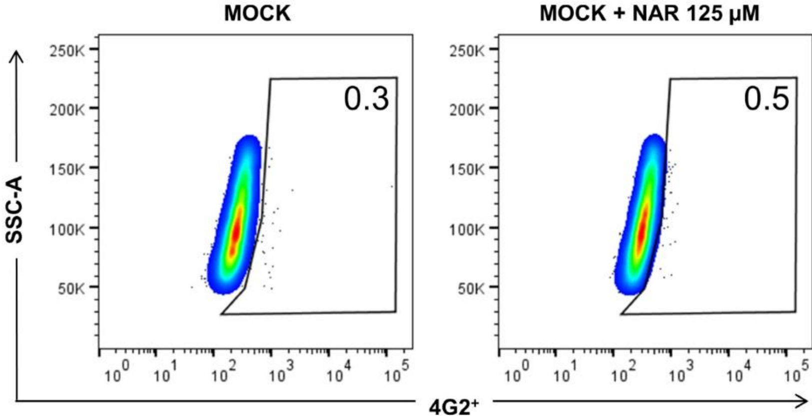


Supplementary Figure 2. NAR does not present virucidal activity against Zika virus. The ZV BR 2015/15261 was incubated with 125 µM of NAR in the presence or absence of RNase. Following a 1h incubation, the RNA samples were extracted and tested for the amplification of a fragment of the envelope gene (E) from the ZIKV using the RT-PCR technique. **(A)** An agarose gel (1%) stained with ethidium bromide demonstrated the amplification of a 364 bp fragment of the E gene from ZIKV. The figure shows a representative result from three independent experiments. As a control of the amplicon size 1kb Plus (ThermoFisher) was used. The original gel is shown below. **(B)** The ZV BR 2015/15261 was incubated with 125 µM of NAR for 1 hour. After the incubation, the residual infectivity was measured by titration in C6/36 cells as stated in material and methods ($FFU_{C6/36}$/mL). As a control, ZV BR 2015/15261 was incubated for the same time with IFN-α 2A or 70% ethanol. **(C)** Representative picture of a titration of ZV BR 2015/15261 in C6/36 cells after different treatments. Data represent three independent experiments performed in technical triplicate and analyzed by one-way ANOVA followed

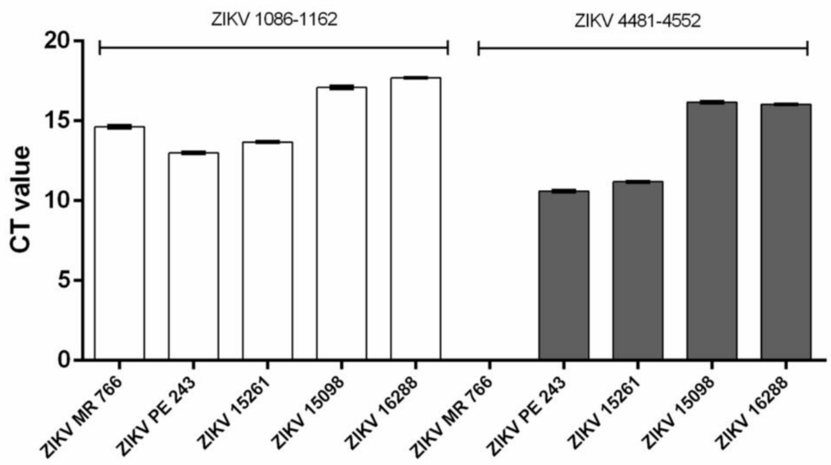
by Tukey´s Multiple Comparison Test (*$p<0.05$ *vs* ZIKV-infected and untreated cells; *vs* ZIKV-infected and treated with NAR or IFN-α 2A).

Original agarose gel image for Supplementary Figure 2A.

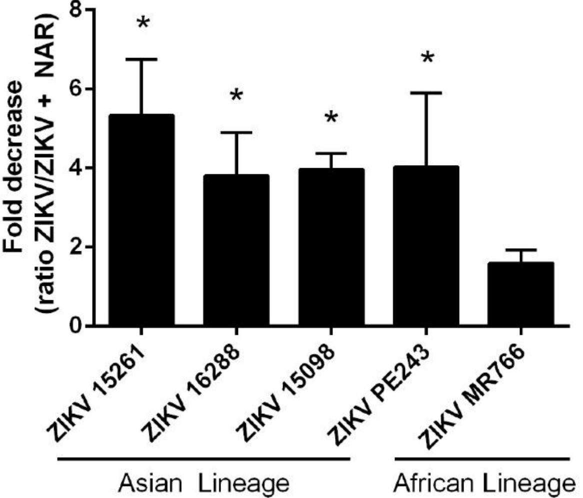




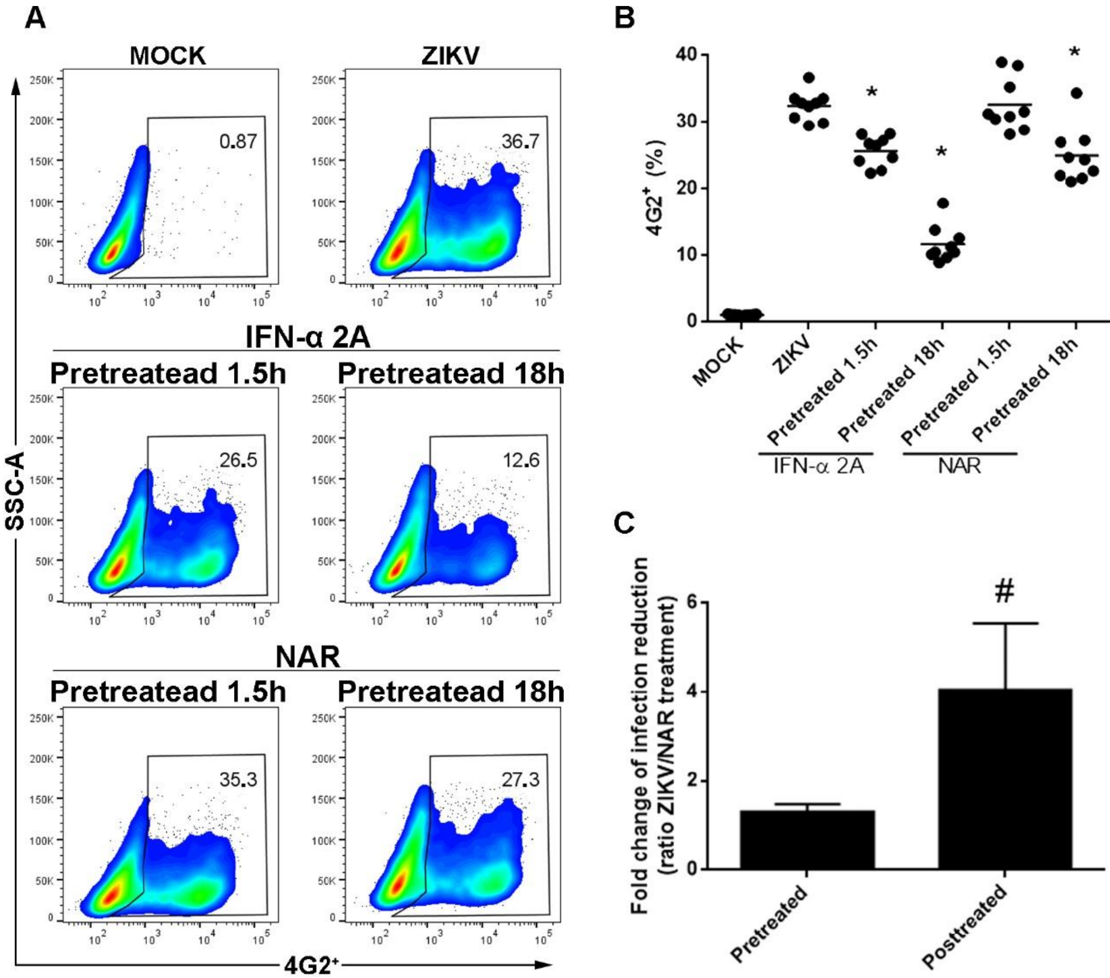
Supplementary Figure 3. NAR does not affect A549 autofluorescence during flow cytometry. The A549 cells were treated with NAR (MOCK + NAR 125 μM) or left untreated (MOCK). The impact of NAR on A549 cell autofluorescence was analyzed by flow cytometry after staining with anti-flavivirus E protein monoclonal antibody (4G2-FITC). The representative results from three independent experiments, with each one in technical triplicate.



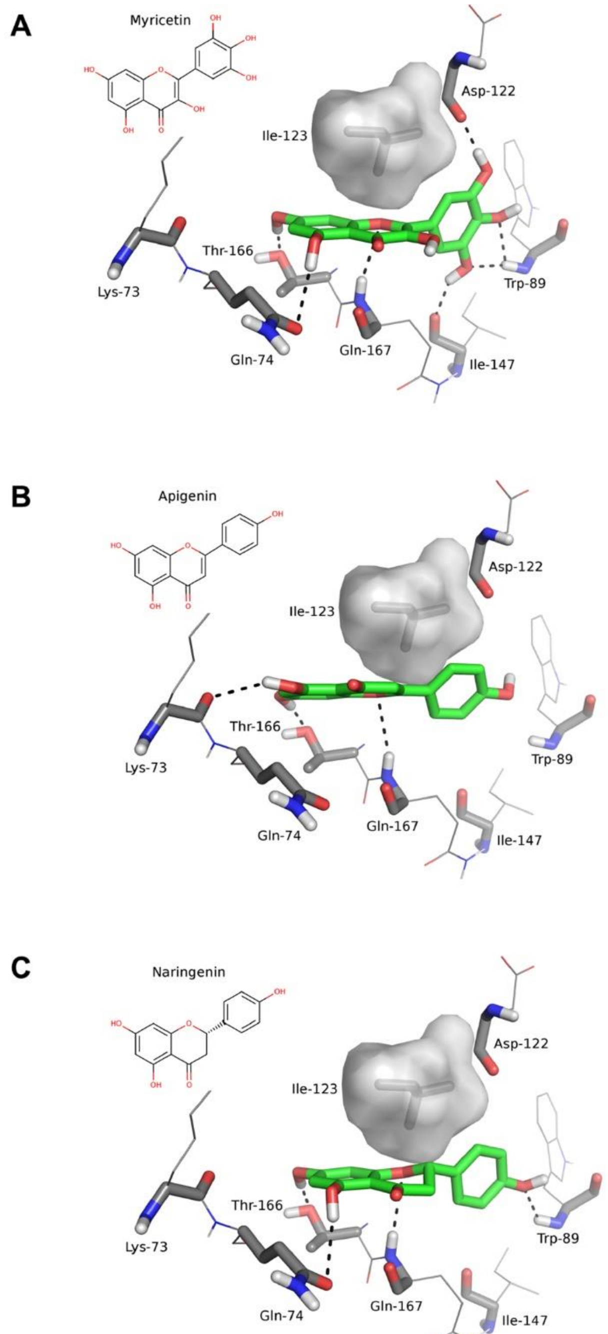
Supplementary Figure 4. Real time PCR for ZIKV lineage determination. Viral RNA was extracted from the five strains of ZIKV cell culture supernantants using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and used for real time PCR. Primers and probes for discrimination between Asian- and African-lineages of ZIKV were used following Lanciotti et al., 2008 and Bingham et al., 2016. The threshold cycle for each amplification was used to confirm the amplification of each RNA sample with different primer and probes sets. Result represent the mean plus standard deviation of three technical replicate for one real time PCR amplification.



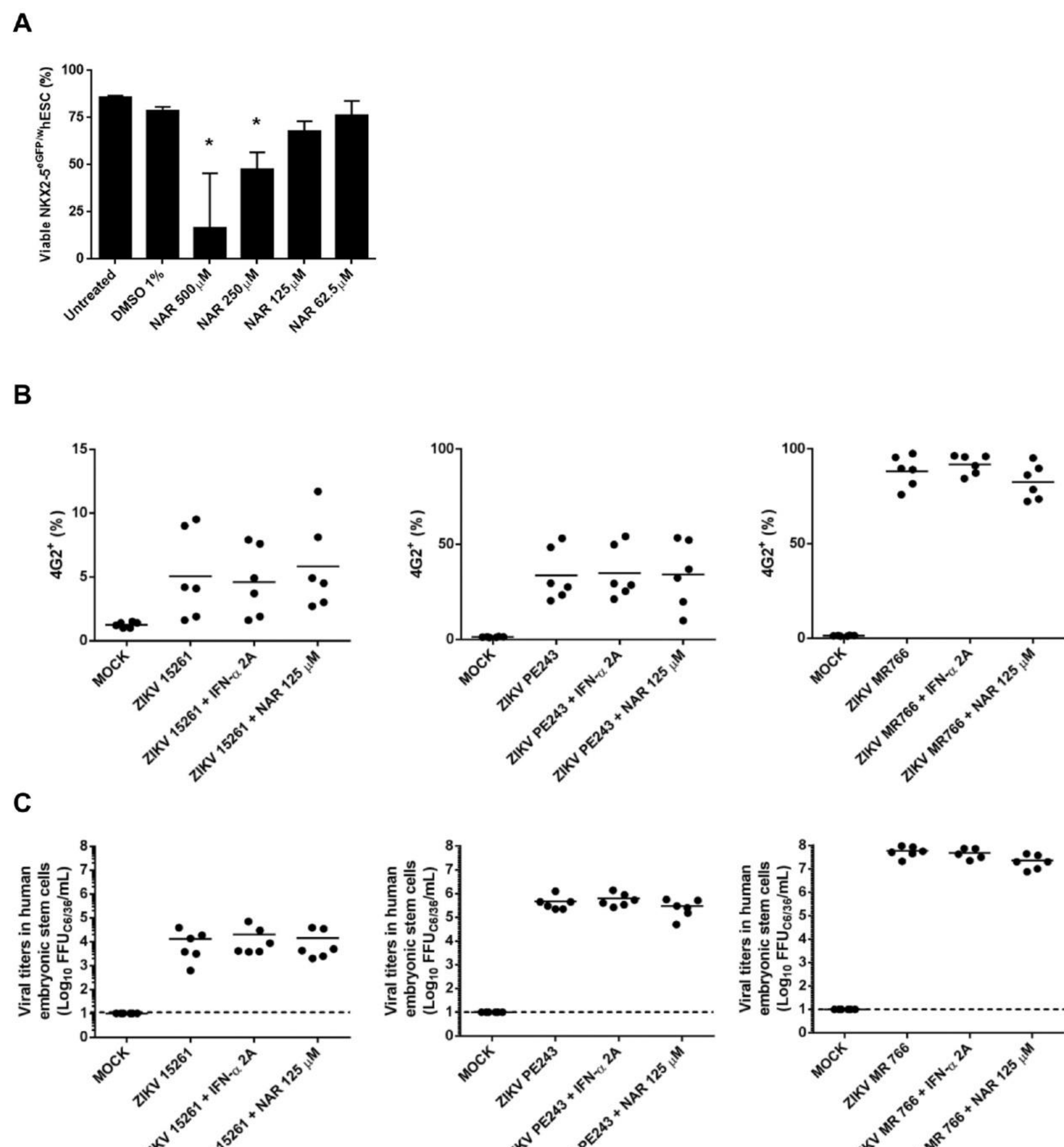
Supplementary Figure 5. NAR treatment was more effective against Asian than African lineages of ZIKV. The data from flow cytometry of Figures 2 and 4 were used to calculate the ratio between the frequency of untreated ZIKV-infected cells and the frequency of NAR-treated ZIKV-infected cells. Data represent three independent experiments performed in technical triplicate and analyzed by one-way ANOVA followed by Tukey´s Multiple Comparison Test (*p<0.05 *vs* ZIKV MR766).



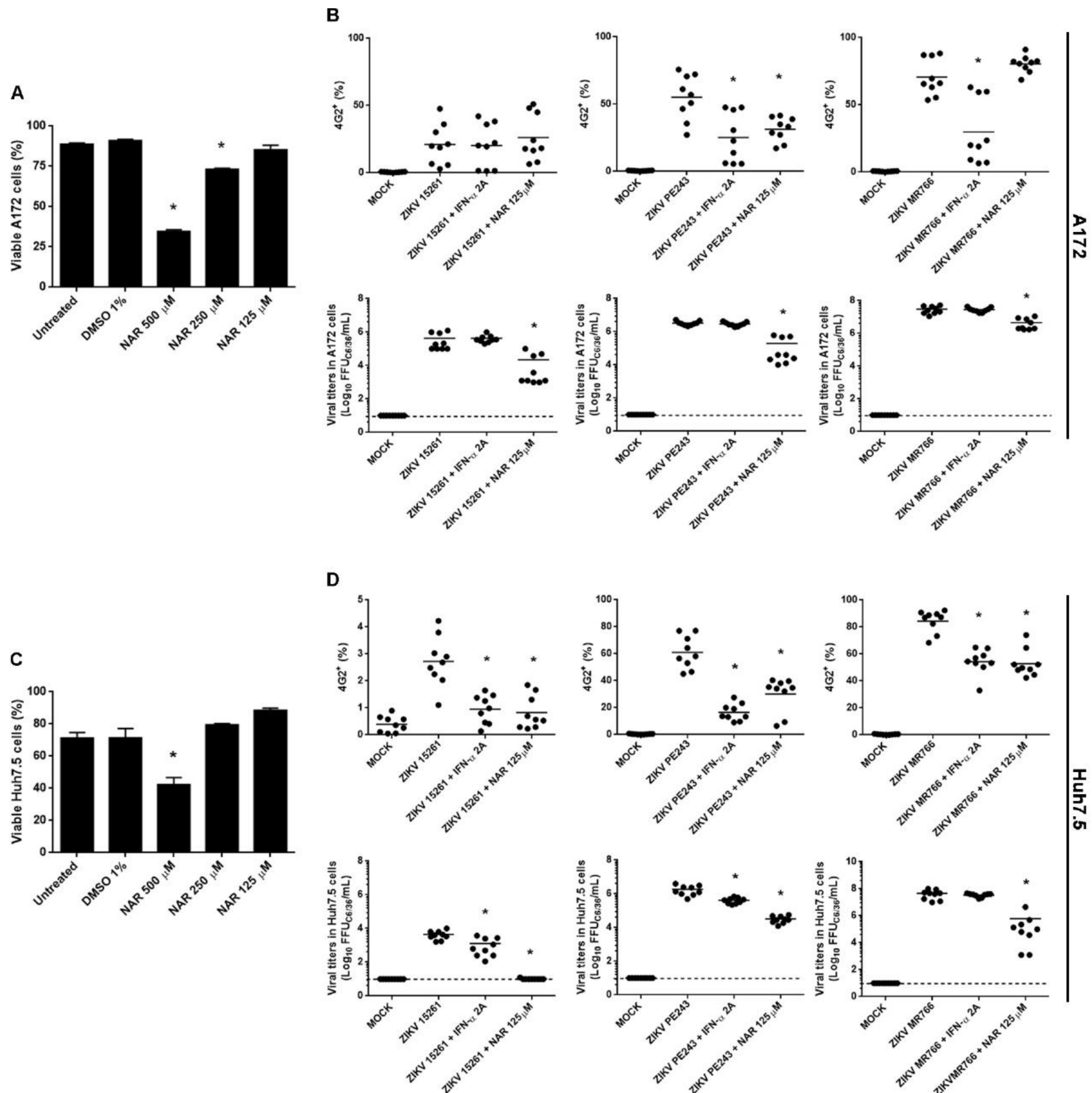
Supplementary Figure 6. Comparison of anti-ZIKV activity of NAR pretreatment and posttreatment on A549 cells. The A549 cells were pretreated with NAR (125 µM) for 1.5h or 18h prior to ZIKV infection with 0.1 MOI of ZIKV (ZV BR 2015/15261 strain). Pretreatment with IFN-α 2A (200 UI/ml) was used as control. **(A)** Representative density plot showing the frequency of infected cells ($4G2^+$) in each experimental condition. **(B)** Frequency of NAR pretreated ZIKV-infected cells ($4G2^+$). **(C)** Comparison of ZIKV-infected cells ($4G2^+$) frequency between NAR pretreatment (18h) and posttreatment. Data from three independent experiments in technical triplicate that were analyzed by one-way ANOVA followed by Tukey´s Multiple Comparison Test (*p<0.05 *vs* ZIKV-infected cells; #p<0.05 *vs* NAR pretreated cells).



Supplementary Figure 7. Proposed molecular interaction mode between the Zika Virus protease with different flavonoids. (A) Myricetin**, (B)** Apigenin**, (C)** Naringenin**.** The relevant residues of the protease are labelled. Hydrogen, Nitrogen and Oxygen atoms are displayed in white, blue and red, respectively. Carbon atoms from the protease and flavonoids are shown in gray and green, respectively. Ile-123 is shown in surface representation to emphasize its involvement in hydrophobic contacts with the flavonoids. Hydrogen bonds are shown as dashed lines.



Supplementary Figure 8. Anti-Zika virus activity of NAR in human embryonic stem cells. The NKX2-5$^{eGFP/w}$hESC cells were treated with different concentrations of NAR (500 to 62.5 μM) over 48 hours and cell viability analyzed by flow cytometry by Annexin-V and 7-AAD staining. Also, cells were infected with ZIKV MOI 1 (ZV BR 2015/15261; ZIKV PE243 and ZIKV MR766) and treated with NAR (125 μM) and analyzed by flow cytometry. **(A)** Frequency of viable NKX2-5$^{eGFP/w}$hESC cells (Annexin-V$^-$/7-AAD$^-$) after treatment with different NAR concentrations. **(B)** Flow cytometry analyses showing the frequency of ZIKV-infected cells stained with anti-E monoclonal antibody (4G2$^+$). **(C)** Viral titers in cell culture supernatant detected by focus-forming assay ($FFU_{C6/36}$/mL). DMSO was used as a control and vehicle for the preparation of NAR stock. IFN-α 2A (200 IU/mL) was used as a positive anti-viral control, and the non-infected cells (MOCK) were used as a negative control. Results from three independent experiments performed in technical triplicate and analyzed by one-way ANOVA followed by Tukey´s Multiple Comparison Test (*$p<0.05$ *vs* ZIKV infected and untreated cells).



Supplementary Figure 9. Anti-Zika virus activity of NAR in different cell lines. A172 (MOI of 1) and Huh7.5 (MOI 0.1) cells were infected with ZIKV (ZV BR 2015/15261; ZIKV PE243 and ZIKV MR766) and treated with NAR (125 µM). After 48 hours, the cells were harvested and stained for a flow cytometry assay using anti-flavivirus E protein monoclonal antibody (4G2$^+$) and viral titers in cell culture supernatant were determined using foci-forming immunodetection assay (FFU$_{C6/36}$/mL). IFN-α 2A (200 IU/mL) was used as a positive anti-viral control, and the non-infected cells (MOCK) were used as a negative control. **(A)** The frequency of viable A172 cells (Annexin-V$^-$/7-AAD$^-$). **(B)** Frequency of ZIKV-infected A172 cells (4G2$^+$) and viral titers (FFU$_{C6/36}$/mL) in cell culture supernatant after 48 hours of infection. **(C)** Frequency of viable Huh7.5 cells (Annexin-V$^-$/7-AAD$^-$). **(D)** Frequency of ZIKV-infected Huh7.5 cells (4G2$^+$) and viral titers (FFU$_{C6/36}$/mL) in cell culture supernatant after 48 hours of infection. Results from 3 independent experiments performed in technical triplicate and analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test (*p<0.05 *vs* ZIKV-infected and untreated cells).

3.3 CAPÍTULO 4

Avaliação do efeito anti-ZIKV de moléculas sintéticas derivadas do flavonóide NAR.

Conforme apresentado anteriormente no capítulo 3, o flavonóide NAR demonstrou atividade antiviral contra diferentes cepas de ZIKV, bem como em diferentes tipos celulares. De maneira geral, a triagem de compostos na descoberta de novas drogas é realizada utilizando concentrações que variam entre 1 a 10 µM, enquanto que moléculas consideradas promissoras apresentam atividade entre 100 nM e 5 µM (HUGHES et al., 2011). Apesar do efeito anti-ZIKV da NAR, a concentração utilizada nos experimentos pode ser considerada alta, uma vez que os experimentos foram conduzidos na ordem de micromolar, mais especificamente 125 µM. Embora os ensaios de viabilidade celular não tenham demonstrado toxicidade nos tipos celulares testados, decidiu-se por trabalhar com moléculas sintéticas derivadas da NAR, na busca por compostos com menor toxicidade e melhor atividade antiviral, os quais poderiam ser utilizados em menores concentrações.

Em colaboração com o Dr. Mauro Vieira de Almeida do Departamento de Química da Universidade Federal de Juiz de Fora, 20 moléculas derivadas do flavonóide NAR foram sintetizadas. As novas moléculas são compostas pela estrutura primária da NAR, com adição de cadeias éteres ou ésteres (Tabela 4), os quais poderiam aumentar a capacidade de ligação dos compostos à proteases virais, e consequentemente inibir a atividade enzimática, levando a um melhor efeito antiviral. Desta maneira, buscamos identificar moléculas derivadas da NAR com efeito anti-ZIKV superior à NAR.

3.3.1 METODOLOGIA

3.3.1.1 Linhagens de células e vírus

Células C6/36 provenientes de *Aedes albopictus* (ATCC CRL-1660) foram cultivadas em meio Lebovitz-L15 suplementado com 0.26% de triptose (Sigma-Aldrich, EUA), 5% de soro fetal bovino e 25 µg/mL de antibiótico gentamicina (Gibco/Invitrogen, EUA) a 28ºC. A linhagem de células A549 (ATCC CCL-185) foi cultivada em meio DMEM-F12 (*Dulbecco's Modified Eagle Medium-F12)*, suplemento com 25 µg/mL de antibiótico gentamicina e 7% de soro fetal bonivo (Gibco/Invitrogen, EUA) e mantidas em ambiente umidificado a 37ºC e 5% de CO_2.

O vírus ZIKV PE243 foi isolado em 2015 a partir do soro de um paciente em Recife que apresentou sintomatologia sugestiva de infecção por ZIKV (DONALD et al., 2016). As amostras virais recebidas no laboratório foram utilizadas para produzir novos estoques virais. Para tal, células C6/36 foram infectadas com ZIKV PE243 (MOI 0.01) e mantidas em cultura por 7 dias. Após este período, o sobrenadante foi coletado, centrifugado, aliquotado e o título viral determinado pelo ensaio de imunodetecção viral por de foco.

3.3.1.2 Moléculas derivadas da NAR

Um total de 20 moléculas derivadas da NAR foram gentilmente cedidas pelo Dr. Mauro Vieira de Almeida do Departamento de Química da Universidade Federal de Juiz de Fora para triagem contra o ZIKV. Os compostos foram solubilizados em 100% de dimetilsulfóxido (DMSO) a 20 mM, aliquotados em microtubos e armazenados a -80ºC. Previamente aos experimentos, os compostos foram diluídos em meio DMEM-F12 para as concentrações a serem testadas (62.5 a 7.5 µM). A concentração de DMSO nas culturas não ultrapassou 0.5%.

3.3.1.3 Triagem de compostos derivados da NAR

Células A549 (1×10^4 células/poço em placas de 96 poços) foram infectadas com ZIKV (ZIKV PE243; MOI 0.1). Após 90 minutos de incubação, as culturas foram

lavadas com PBS 1X e tratadas com diferentes concentrações dos derivados de NAR (variando de 62.5 a 7.5 μM). Após 48 horas de incubação, as células foram fixadas e permeabilizadas com metanol:acetona (v/v) e a infecção foi analisada após a marcação com anticorpo anti-proteína E de envelope de flavivírus (4G2 - anticorpo primário) e anti-*mouse* Alexa Fluor 488 (anticorpo secundário) (Thermo Fisher Scientific, Grand Island, New York, USA) e os núcleos marcados com 4′,6-Diamidine-2′-phenylindole dihydrochloride - DAPI (1 μg/mL). A determinação da frequência de células infectadas, bem como a contagem do número de núcleos para determinar a concentração máxima não tóxica foi realizada utilizando o microscópio *Operetta High-Content Imaging System* e o *software Harmony High Content Imaging and Analysis* (Thermo Fisher Scientific, Rockford, IL, USA). Como controles foram utilizadas células não infectadas (MOCK), células infectadas e não tratadas (ZIKV), células infectadas e tratadas com NAR 125 μM e células infectadas e tratadas com IFN-α 2A (200 UI/mL). Os dados obtidos a partir da frequência de células infectadas foram utilizados para determinar o IC_{50}, enquanto a contagem de núcleos foi utilizada para determinar o CC_{50}. O índice de seletividade (SI) foi calculado pela razão do valor de CC_{50} sobre o IC_{50} (CC_{50}/IC_{50}=SI).

3.3.2 RESULTADOS

Uma vez observado efeito promissor da NAR na concentração de 125 μM como molécula anti-ZIKV (CATANEO et al., 2019), decidiu-se por testar se o tratamento com moléculas derivadas da NAR em concentrações inferiores também apresentariam atividade anti-ZIKV. Do total de 20 moléculas sintetizadas, 18 foram testadas quanto à atividade antiviral, uma vez que não foi possível solubilizar 2 moléculas em DMSO mesmo sob aquecimento, a saber, LLA12A e CSP7A. Dentre os 18 compostos avaliados, os resultados demonstraram atividade antiviral de 6 compostos que receberam adiçao de cadeias éters, a saber CSP2B, CSP3B, CSP4B, CSP5B, LLA7B e LLA8B (FIGURA 1). Os valores de IC_{50} calculados variaram entre 7 e 15 μM, exceto para o composto LLA8B (IC_{50} = 68.33 μM), enquanto o IC_{50} para NAR foi calculado em 58.79 μM (Tabela 1). A contagem de núcleos demonstrou que a concentração máxima não tóxica para os 6 compostos foi de 31.25 μM, enquanto a concentração de 62.5 μM apresentou redução de aproximadamente 2 logs no número de células viáveis (FIGURA 1). Os valores de CC_{50} obtidos variaram entre 29 e 35 μM, enquanto a NAR apresentou CC_{50} de 693.6 μM. Os índices de seletividade variaram de 0.43 a

4.39 para os compostos derivados da NAR, e a NAR apresentou 11.79 (Tabela 2). Logo, os resultados demonstram que dentre as 18 moléculas testadas, 6 compostos apresentaram atividade antiviral com valores de IC_{50} até 8 vezes menores que a NAR. Por outro lado, a redução no número de células nas culturas evidenciou que estes compostos se apresentaram mais tóxicos que a NAR, resultando em baixos índices de seletividade. Desta forma, a utilização destas moléculas para o tratamento anti-ZIKV desencoraja sua utilização.

Tabela 1 – Códigos e estruturas moleculares da NAR e seus derivados.

Código	Estrutura	Solubilidade e MM (*aquecimento)
Naringenina		Acetona DMSO 272,2528 g/mol
LLA4 A		Diclorometano DMSO* 636,8577 g/mol
LLA4 B		Diclorometano DMSO 454,5553 g/mol
LLA5 A		Diclorometano DMSO* 580,7514 g/mol
LLA5 B		Diclorometano DMSO 426,5021 g/mol
CSP1 A		DMSO 468,5388 g/mol
CSP1 B		DMSO 370,3958 g/mol
CSP7A		DMSO* 777,2097 g/mol
LLA7 B		DMSO 440,5717 g/mol
LLA8 B		DMSO 412,5186 g/mol
LLA9 A		DMSO 496,6781 g/mol
LLA10 A		DMSO* 692,9641 g/mol
LLA10 B		DMSO 482,6084 g/mol
LLA11 A		DMSO 524,6451 g/mol
LLA11 B		DMSO 398,4489 g/mol
LLA12 A		DMSO* 749,0704 g/mol
LLA12 B		DMSO 510,6616 g/mol
CSP2 B		DMSO 398,492 g/mol
CSP3 B		DMSO 384,4654 g/mol
CSP4 B		DMSO 356,4123 g/mol
CSP5 B		DMSO 328,3591 g/mol

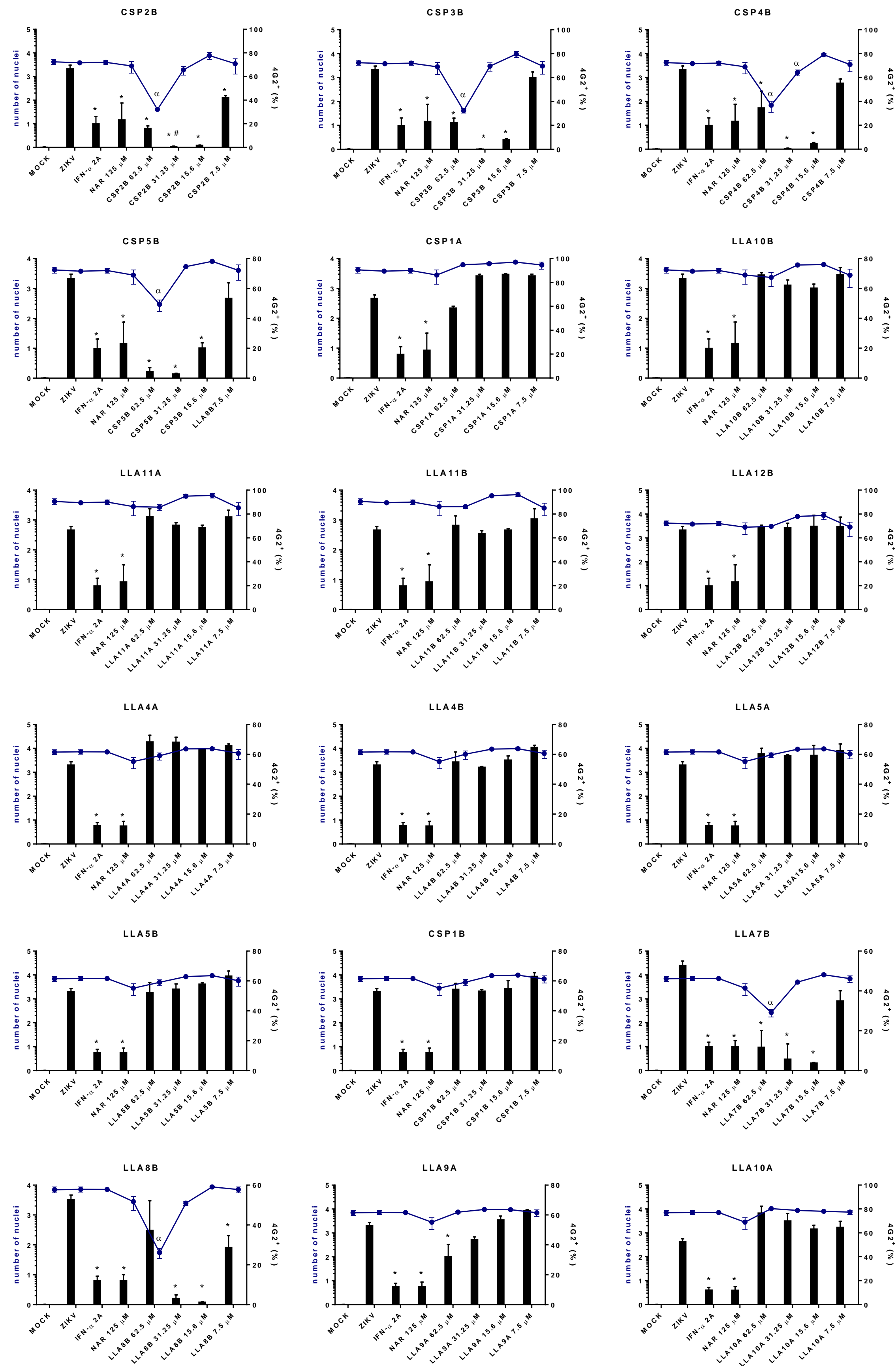


Figura 1 – Efeito anti-ZIKV de moléculas sintéticas derivadas do flavonóide NAR. Células A549 foram infectadas com ZIKV (ZIKV PE243; MOI 0.1) e tratadas com diferentes concentrações dos derivados de NAR (7.5 a 62.5 µM) após a retirada do inóculo viral. Após 48 horas, a porcentagem de

células infectadas e o número de núcleos foram quantificados utilizando o sistema *Operetta High-Content Imaging System* pelo *software Harmony High Content Imaging and Analysis*. As condições IFN-α 2A (200 UI/mL) e NAR (125 µM) foram utilizadas como controle de atividade antiviral. Os resultados representam dois experimentos independentes realizados em duplicata técnica e foram analisados por *one-way* ANOVA seguido pelo pós-teste de *Tukey* para múltiplas comparações (* $p < 0.05$ *vs* ZIKV; # *vs* NAR 125 µM e α *vs* MOCK). O eixo *Y* à esquerda representa a contagem de núcleos (linhas azuis – *number of nuclei*) e o eixo Y à direita representa a frequência de células infectadas (barras pretas - $4G2^+$).

Tabela 2 – Concentrações inibitórias (IC_{50} e CC_{50}) da NAR e moléculas derivadas e índice de seletividade para atividade anti-ZIKV em células A549. SI = CC_{50}/IC_{50}.

Molécula	*IC_{50} (µM)*	*CC_{50} (µM)*	*SI*
CSP2B	7.413	30.5	4.11
CSP3B	7.56	31.21	4.12
CSP4B	6.76	29.72	4.39
CSP5B	15.18	35.29	2.23
LLA7B	7.50	31.55	4.2
LLA8B	69.33	30.40	0.43
NAR	58.79	693.6	11.79

4 DISCUSSÃO

Os recentes surtos de ZIKV na Polinésia Francesa em 2013 e no Brasil entre os anos 2015-2016 tornaram essa arbovirose um importante problema de saúde pública, causando grande impacto sócio-econômico. No início de 2016, a Organização Mundial de Saúde (OMS) declarou o ZIKV como emergência de saúde pública internacional, levando à uma estratégia mundial coordenada no entendimento desta virose emergente. Embora o número de casos de infecções por ZIKV no Brasil tenham apresentado queda entre os anos de 2017 e 2019 (MINISTÉRIO DA SAÚDE, 2019), novos surtos não devem ser descartados, uma vez que o vírus continua em circulação. Portanto, o desenvolvimento de estratégias de combate e controle são de extrema relevância no enfrentamento desta arbovirose. Dentre as estratégias para controle de novos surtos causados por arbovírus estão o controle populacional do vetor competente, a vacinação e o desenvolvimento de drogas antivirais (ACHEE et al., 2015). O Brasil enfrenta dificuldades em controlar a população de mosquitos do gênero *Aedes* spp., principal transmissor do ZIKV (LOWY, 2017), além disso, não existe até o presente momento vacinas e nem drogas antivirais disponíveis contra o ZIKV. Neste cenário, a busca por alternativas terapêuticas tornou-se um dos pilares no enfrentamento ao ZIKV.

Em relação à busca por moléculas antivirais, os flavonóides, conforme revisado no capítulo 1, têm demonstrado potencial atividade anti-ZIKV. Tais compostos parecem inibir diferentes etapas do ciclo replicativo do ZIKV, atuando diretamente sobre a partícula viral (CARNEIRO et al., 2016), bloqueando a ligação do vírus aos receptores celulares (MOUNCE et al., 2017), na inibição de proteases virais (ROY et al., 2017) ou interferindo no metabolismo celular e indiretamente afetando a replicação viral (LIN et al., 2019). Desta forma, a associação de duas ou mais moléculas que atuem em diferentes etapas do ciclo replicativo poderiam apresentar efeito sinérgico, potencializando o efeito antiviral, conforme observado pela combinação de diferentes flavonóides contra *herpes simplex virus type 1* (AMOROS et al., 1992). Além disso, os flavonóides podem apresentar efeito antiviral sinérgico com outras classes de compostos, conforme demonstrado pela associação de isoquercetina e amantadina contra os vírus influenza A e B (KIM; NARAYANAN; CHANG, 2010).

Apesar dos flavonóides serem capazes de inibir diferentes etapas do ciclo replicativo viral, o exato mecanismo de ação pelo qual exercem tais efeitos não é completamente entendido. O provável mecanismo de ação anti-ZIKV dos flavonóides tem sido atribuído à capacidade de inibir não competitivamente proteínas virais envolvidas no ciclo replicativo, como é o caso da NS2B-NS3 e NS5 polimerase dependente de RNA (ROY et al., 2017; SZE et al., 2017; Oo et al., 2019). Além disso, alguns compostos são capazes de exercer efeito virucida diretamente sobre a partícula viral (CARNEIRO et al., 2016). Uma vez que os flavonóides são uma classe heterogênea de compostos, é plausível que os mecanismos de ação também sejam heterogêneos.

Embora os resultados, principalmente *in vitro*, sugiram atividade antiviral, os flavonóides têm limitações para aplicação clínica, uma vez que apresentam reduzida biodisponibilidade *in vivo* (WU et al., 2011). Diversos fatores impactam diretamente a biodisponibilidade dos flavonóides, tais como, peso molecular, glicosilação, conversão metabólica e interação com a microbiota (THILAKARATHNA; VASANTHA RUPASINGHE, 2013). Desta forma, deve-se interpretar com cautela os dados obtidos *in vitro*, bem como, devem ser realizados estudos *in vivo* para a validação de um flavonóide como composto promissor no tratamento de infecções virais. Portanto, faz-se necessário o desenvolvimento de estratégias para melhorar a biodisponibilidade dos flavonóides. Neste cenário, a utilização de microemulsões como sistema de entrega de drogas é capaz de aumentar a absorção intestinal destas moléculas (SHEN et al., 2011). Outra estratégia baseia-se na tentativa de impedir a metabolização dos flavonóides bloqueando os grupos hidroxila com grupos metil, melhorando tanto absorção intestinal quanto a metabolização hepática (WALLE, 2007). Portanto, uma vez que os flavonóides apresentam baixa absorção e biodisponibilidade *in vivo*, faz-se necessário a utilização de estratégias que possam melhorar este cenário antes de sua utilização clínica.

Desde 1947, quando ocorreu o primeiro isolamento, até 2015, 124 artigos haviam sido publicados em relação ao ZIKV. No entanto, devido à emergência em saúde pública causada em decorrência aos surtos de ZIKV, 4.500 artigos foram publicados entre 2016 e 2018. Neste contexto emergencial, a troca de reagentes e amostras biológicas ocorre com frequência entre laboratórios de pesquisa. Embora benéfica para a comunidade científica, tal prática deve ser acompanhada de procedimentos de certificação para garantir a qualidade dos reagentes/amostras, bem

como para evitar contaminações. No capítulo 2 da presente tese, documentou-se a contaminação de cepas laboratoriais de ZIKV com *Brevidensovirus,* posteriormente chamados de *mosquito densovirus* (MDV), obtidas em colaboração com diferentes grupos de pesquisa do País. Além de detectarmos a contaminação das cepas virais recebidas, foram propostas duas estratégias para remover a contaminação.

O isolamento de arbovírus a partir de amostras clínicas, classicamente é realizado utilizando células derivadas de insetos como a C6/36 (*A. albopictus larvae)* (TESH, 1979). A contaminação de linhagens celulares utilizadas em isolamento viral é usual, como já demonstrado anteriormente em células C6/36 e AP-61 (O'NEILL et al., 1995). Além disso, tais contaminações podem estabelecer infecções persistentes sem o aparecimento de efeito citopático (JOUSSET et al., 1993; O'NEILL et al., 1995). Portanto, é necessário que as certificações de qualidade sejam realizadas com frequência nas linhagens celulares utilizadas em virologia.

Não existem estudos avaliando o efeito da coinfecção ZIKV/MDV. No entanto, foi demonstrado que a coinfecção dengue/MDV impacta na infecção e na replicação de dengue em células C6/36 (MOSIMANN et al., 2011). Portanto, é plausível que o mesmo fenômeno ocorra com ZIKV, embora necessite comprovação. Além disso, a infecção de camundongos com MDV induz a produção de anticorpos (MOSIMANN et al., 2011), portanto, o impacto da coinfecção *in vivo* de ZIKV e MDV é desconhecida e necessita de investigação.

Neste contexto, implementou-se protocolos para eliminar o MDV dos estoques de ZIKV. Os protocolos baseiam-se na incapacidade do MDV de replicar em hospedeiros vertebrados (JOUSSET et al., 1993). Após sucessivas passagens em células de vertebrado, bem como em modelo *in vivo* de infecção em camundongo deficiente para receptor de IFN do tipo I garantiu-se a eliminação do MDV das cepas de ZIKV. Embora metodologias eficazes, algumas questões necessitam ser levadas em consideração ao utilizá-las. Em primeiro lugar, sabe-se que sucessivas passagens podem causar mutações no genoma que influenciam a biologia viral (MOSER et al., 2018), portanto, as metodologias abordadas aqui devem ser avaliadas com cautela em estudos de evolução viral. Por fim, os reagentes utilizados para a manutenção de células de vertebrados, como soro bovino fetal, podem apresentar contaminações como demonstrado anteriormente (FONG et al., 1975). Portanto, é fortemente recomendado que os estoques virais sejam constantemente avaliados quanto à presença de contaminantes antes de serem usados experimentalmente. Ademais, ao

utilizar as metodologias sugeridas, recomenda-se que os estoques sejam posteriormente sequenciados para avaliação de possíveis mutações.

Conforme abordado no primeiro capítulo da presente tese, os flavonóides são uma grande classe de compostos com diversas atividades biológicas. O flavonóide naringenina (NAR) apresentou efeito antiviral contra vírus da família *Flaviviridae*, como HCV e febre amarela (CASTRILLO et al., 2015; GOLDWASSER et al., 2011). Recentemente, foi demonstrado a capacidade da NAR em inibir, *in vitro*, a replicação dos 4 sorotipos de dengue (FRABASILE et al., 2017). No presente estudo, foi avaliado, por diferentes técnicas, o efeito antiviral da NAR sobre cepas de ZIKV. Similarmente ao demonstrado por Frabasile e colaboradores (2017) com dengue (FRABASILE et al., 2017), a NAR não apresentou efeito virucida contra ZIKV. No entanto, os ensaios de tempo de adição sugerem que a NAR atue em etapas finais do ciclo replicativo.

Os flavonóides são moléculas que parecem interagir com proteínas virais, prejudicando a capacidade replicativa, como o complexo NS2B-NS3 de dengue e ZIKV (DE SOUSA et al., 2015; ROY et al., 2017). A predição por *docking* molecular demonstrou que a NAR é capaz de interagir com o complexo NS2B-NS3 protease do ZIKV da mesma forma que outros flavonóides conhecidamente inibidores alostéricos deste complexo. Contudo, dados de predições *in silico* apesar de importantes para levantar hipóteses sobre o mecanismo de atividade antiviral necessitam de validação experimental.

Embora os ensaios *in vitro* apontem para um importante efeito anti-ZIKV da NAR, ensaios *in vivo* utilizando modelos experimentais de infecção por ZIKV são necessários. No entanto, os modelos experimentais de infecção por arbovírus comumente utilizam animais deficientes para componentes do sistema imune inato, uma vez que camundongos selvagens são conhecidamente resistentes à infecção (DOWALL et al., 2016; LAZEAR et al., 2016). Neste cenário, deve-se levar em consideração que a resposta imunológica à infecção estará prejudicada. Ademais, embora tais modelos apresentem limitações em estudos de patogênese, são úteis para a avaliação de compostos antivirais.

Como citado anteriormente, os flavonóides apresentam limitada biodisponibilidade (WU et al., 2011), portanto, é fundamental que estes parâmetros sejam avaliados em modelos experimentais. Em relação à NAR, foi demonstrado que esta apresenta baixa permeabilidade intestinal e baixa biodisponibilidade (CHABANE et al., 2009; KANAZE et al., 2007). Zhao e colaboradores (2019) abordam diversas

estratégias utilizadas para melhorar a absorção e biodisponibilidade dos flavonóides, incluindo a utilização de potencializadores de absorção, transformação estrutural, complexação com carreadores e nanotecnologia (ZHAO; YANG; XIE, 2019). Foi demonstrado que a complexação da NAR com hidroxipropoyl-β-ciclodextrina é capaz de aumentar a concentração plasmática da NAR em ratos da linhagem Sprague-Dawley (SHULMAN et al., 2011). Portanto, é possível que a utilização da NAR em modelos *in vivo* de infecção dependa da utilização de estratégias para melhorar a absorção e biodisponibilidade da molécula.

As concentrações de NAR utilizadas contra dengue e ZIKV foram de 250 e 125 μM, respectivamente, as quais podem ser consideradas altas e, portanto, uma desvantagem para o desenvolvimento da NAR como antiviral. Desta forma, a avaliação de estruturas químicas análogas à NAR, que já demonstraram efeito antiviral, podem contribuir na busca por moléculas bioativas contra ZIKV. Portanto, foram realizadas triagens para atividade anti-ZIKV de 18 moléculas sintéticas análogas à NAR. Os resultados demonstraram que 6 compostos apresentaram efeito anti-ZIKV com valores de IC_{50} até 8 vezes menores que a NAR. No entanto, a redução na contagem de núcleos mostrou que esses compostos são mais citotóxicos do que a NAR, mesmo em concentração menor (62.5 μM). As moléculas utilizadas nesta triagem foram obtidas a partir de modificações da estrutura primária da NAR, almejando obter compostos mais lipofílicos, permitindo melhor interação com membranas biológicas e tornando-as moléculas maiores e de maior peso molecular. Recentemente, foi demonstrado por análises de *docking* molecular, que o flavonóide curcumina inibe o complexo viral NS2B-NS3, e que o fato da estrutura molecular da curcumina apresentar 5 carbonos a mais na ligação entre os anéis fenil contribui para que este composto se ligue à mais resíduos do seu alvo quando comparado com flavonóides similares (ROY et al., 2017). Portanto, é plausível que as moléculas utilizadas nesta triagem sejam capazes de inibir a replicação viral e exercer efeito anti-ZIKV, porém, a alta toxicidade observada para estes compostos desencoraja sua utilização. Desta forma, novas modificações podem ser realizadas na tentativa de reduzir a toxicidade induzida por estas moléculas. No entanto, tais modificações devem levar em consideração estruturas previamente descritas como relevantes na atividade antiviral dos flavonóides, como quantidade e posição dos grupos hidroxila e o tipo de hibridização dos átomos de carbono que compõem as estruturas destes compostos (BHARGAVA et al., 2017; ROY et al., 2017).

Portanto, de maneira geral, os resultados demonstrados mantêm a NAR como molécula com melhor atividade antiviral, mesmo que utilizada em altas concentrações (125 e 62.5 µM). Porém, cabe salientar que, na concentração utilizada nos ensaios (125 µM) a NAR não apresentou efeito tóxico nas células testadas (Huh7.5, A549, A172, NKX2-5^{eGFP}/whESC e *hmdDCs*) (CATANEO et al., 2019; FRABASILE et al., 2017). Camundongos tratados diariamente com NAR (50 mg/kg) por 30 dias não apresentaram efeitos colaterais (MANCHOPE et al., 2018). Por outro lado, drogas comercialmente disponíveis, como a indometacina, só pode ser administrada por até 7 dias em doses de 5 ou 2.5 mg/kg devido aos efeitos adversos como úlcera gástrica, sangramento e hepatotoxicidade (PINHO-RIBEIRO et al., 2016). Tratamentos com 10 mg/kg de indometacina levaram os camundongos a óbito devido à intensa hemorragia gastrointestinal (SOOKVANICHSILP; PULBUTR, 2002). Assim, comparando-se indometacina com NAR, esta é mais segura em doses elevadas, evidenciando que mesmo em concentrações altas, o efeito farmacológico da NAR não apresenta efeitos colaterais (PINHO-RIBEIRO et al., 2016). Desta forma, embora novas moléculas devam ser sintetizadas e avaliadas quanto ao seu efeito antiviral e citotóxico, a NAR continua sendo um possível candidato como molécula anti-ZIKV. No entanto, ensaios *in vivo*, utilizando modelos murino são necessários para comprovar o potencial uso desta molécula na infecção por ZIKV, bem como, realizar estudos de farmacocinética deste flavonóide. Finalmente, os dados indicam o potencial da NAR como um protótipo de molécula anti-ZIKV, e reforçam a necessidade de estudos *in vitro* que detalhem seu mecanismo de ação, e *in vivo* acerca da toxicidade, farmacocinética e inibição da replicação viral.

5 CONCLUSÕES

Os flavonóides, desde a década de 1960, vem atraindo atenção como compostos com atividade antiviral contra diversos vírus. A revisão bibliográfica apresentada no capítulo 1 demonstrou o quanto esta classe de compostos é promissora na pesquisa por moléculas antivirais. Adicionalmente, os estudos citados no capítulo 1 demonstram que flavonóides podem atuar em diferentes etapas do ciclo viral. Portanto, a combinação de diferentes moléculas pode ser uma opção mais eficaz e deve ser alvo para futuros estudos sobre o potencial anti-ZIKV de flavonóides.

As recentes epidemias de ZIKV atraíram grande atenção da sociedade, mídia e da comunidade científica, especialmente com o surgimento de síndromes neurológicas associadas à infecção. Neste contexto, a corrida por respostas emergenciais à nova virose, pode ter colaborado para que etapas essenciais na investigação científica tenham sido negligenciadas. Como exemplo, reportamos no capítulo 2 deste trabalho a contaminação de cepas de ZIKV por um vírus de inseto do gênero *Brevidensovirus* (MDV). Uma vez que o efeito de tais contaminações não é conhecido e poderia causar alterações nos resultados, foi proposta uma metodologia para eliminar o contaminante antes de iniciar novos experimentos. O isolamento de uma cepa viral primariamente transmitida por insetos, como é o caso do ZIKV, pode resultar em cepas contaminadas com vírus específicos de insetos. Ademais, linhagens de células de inseto classicamente utilizadas para produção de estoques virais podem apresentar contaminações semelhantes. Portanto, torna-se altamente recomendável que os pesquisadores que trabalham nesta área analisem periodicamente os estoques virais e linhagens celulares utilizadas em seus laboratórios a fim de evitar o uso de cepas virais ou células contaminadas.

Adicionalmente, utilizando-se de diferentes metodologias, foi possível observar que a NAR exerce efeito antiviral independente da cepa ou linhagem viral utilizada, embora o efeito mais potente tenha sido observado nas cepas da linhagem Asiática. Este dado é particularmente interessante pelo fato de que os grandes surtos recentes de ZIKV tenham sido associados à esta linhagem. Os resultados de diferentes ensaios *in vitro* sugerem que o provável mecanismo de ação da NAR seja na inibição da replicação viral. Além disso, as análises *in silico* por *docking* molecular reforçam a interferência da NAR no ciclo replicativo pela potencial ligação à NS2B-NS3 protease.

Com o objetivo de potencializar o efeito antiviral e reduzir toxicidade, novas moléculas sintéticas análogas à NAR foram avaliadas quanto à capacidade anti-ZIKV no capítulo 4. Embora algumas moléculas tenham apresentado efeito antiviral mais robusto, a toxicidade dos compostos foi maior quando comparado à NAR, inviabilizando a utilização dos mesmos. Contudo, novas alterações moleculares podem contribuir para o desenvolvimento de moléculas bioativas anti-ZIKV. Adicionalmente, estas alterações devem levar em conta, além da atividade antiviral e toxicidade, uma melhor absorção pelo trato gastrointestinal, desta forma, agregando vantagens na utilização dos novos compostos.

6 PERSPECTIVAS

Avançar nos estudos do descobrimento de drogas exige avaliações em modelos pré-clínicos. Portanto, como perspectiva para a continuidade do trabalho, pretende-se estabelecer um modelo murino de infecção por ZIKV para que seja avaliado o efeito *in vivo* da NAR. Adicionalmente, a avaliação em modelos de gestação será muito relevante, uma vez que a infecção por ZIKV foi associada à síndromes congênitas. Além disso, pretende-se dar continuidade à investigação *in vitro* de novas moléculas sintéticas derivadas da NAR.

Ademais, pretende-se avaliar o efeito antiviral da NAR contra outros arbovírus de importância em saúde pública, como chikungunya e febre amarela. Adicionalmente, será interessante ampliar os estudos utilizando o reposicionamento de fármacos, através de bibliotecas de compostos, especialmente de compostos com moléculas já aprovadas pelo *Food and Drug Administration* (FDA). Para isso, será utilizado a abordagem de *High Throughput Screening* utilizando o equipamento *Operetta High-Content Imaging System*, que neste trabalho mostrou-se uma ferramenta útil para triagem de vários compostos ao mesmo tempo.

7 REFERÊNCIAS

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8 ANEXOS

8.1 Artigos publicados durante o doutorado

VIRUS-CELL INTERACTIONS

Human T Lymphocytes Are Permissive for Dengue Virus Replication

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ABSTRACT Dengue virus (DV) infection can cause either a self-limiting flu-like disease or a threatening hemorrhage that may evolve to shock and death. A variety of cell types, such as dendritic cells, monocytes, and B cells, can be infected by DV. However, despite the role of T lymphocytes in the control of DV replication, there remains a paucity of information on possible DV-T cell interactions during the disease course. In the present study, we have demonstrated that primary human naive $CD4^+$ and $CD8^+$ T cells are permissive for DV infection. Importantly, both T cell subtypes support viral replication and secrete viable virus particles. DV infection triggers the activation of both $CD4^+$ and $CD8^+$ T lymphocytes, but preactivation of T cells reduces the susceptibility of T cells to DV infection. Interestingly, the cytotoxicity-inducing protein granzyme A is highly secreted by human $CD4^+$ but not $CD8^+$ T cells after exposure to DV *in vitro*. Additionally, using annexin V and polycaspase assays, we have demonstrated that T lymphocytes, in contrast to monocytes, are resistant to DV-induced apoptosis. Strikingly, both $CD4^+$ and $CD8^+$ T cells were found to be infected with DV in acutely infected dengue patients. Together, these results show that T cells are permissive for DV infection *in vitro* and *in vivo*, suggesting that this cell population may be a viral reservoir during the acute phase of the disease.

IMPORTANCE Infection by dengue virus (DV) causes a flu-like disease that can evolve to severe hemorrhaging and death. T lymphocytes are important cells that regulate antibody secretion by B cells and trigger the death of infected cells. However, little is known about the direct interaction between DV and T lymphocytes. Here, we show that T lymphocytes from healthy donors are susceptible to infection by DV, leading to cell activation. Additionally, T cells seem to be resistant to DV-induced apoptosis, suggesting a potential role as a viral reservoir in humans. Finally, we show that both $CD4^+$ and $CD8^+$ T lymphocytes from acutely infected DV patients are infected by DV. Our results raise new questions about DV pathogenesis and vaccine development.

KEYWORDS dengue virus, replication, T lymphocytes

Dengue virus (DV) is the most prevalent arthropod-borne virus in the tropical and subtropical areas of the world. Infection by any of the four serologically related DVs (serotypes 1, 2, 3, and 4 [DV1 to DV4]) causes a wide range of clinical presentations, from a flu-like disease to severe hemorrhaging that can evolve to shock and death (1).

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Amblyomma sculptum Salivary PGE_2 Modulates the Dendritic Cell-*Rickettsia rickettsii* Interactions *in vitro* and *in vivo*

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Amblyomma sculptum is an important vector of *Rickettsia rickettsii*, causative agent of Rocky Mountain spotted fever and the most lethal tick-borne pathogen affecting humans. To feed on the vertebrate host's blood, *A. sculptum* secretes a salivary mixture, which may interact with skin resident dendritic cells (DCs) and modulate their function. The present work was aimed at depicting the *A. sculptum* saliva-host DC network and the biochemical nature of the immunomodulatory component(s) involved in this interface. *A. sculptum* saliva inhibits the production of inflammatory cytokines by murine DCs stimulated with LPS. The fractionation of the low molecular weight salivary content by reversed-phase chromatography revealed active fractions eluting from 49 to 55% of the acetonitrile gradient. Previous studies suggested that this pattern of elution matches with that observed for prostaglandin E_2 (PGE_2) and the molecular identity of this lipid mediator was unambiguously confirmed by a new high-resolution mass spectrometry methodology. A productive infection of murine DCs by *R. rickettsii* was demonstrated for the first time leading to proinflammatory cytokine production that was inhibited by both *A. sculptum* saliva and PGE_2, a result also achieved with human DCs. The adoptive transfer of murine DCs incubated with *R. rickettsii* followed by treatment with *A. sculptum* saliva or PGE_2 did not change the cytokine profile associated to cellular recall responses while IgG2a-specific antibodies were decreased in the serum of these mice. Together, these findings emphasize the role of PGE_2 as a universal immunomodulator of tick saliva. In addition, it contributes to new approaches to explore *R. rickettsii*-DC interactions both *in vitro* and *in vivo*.

Keywords: ***Amblyomma sculptum*, tick saliva, PGE_2, dendritic cells, *Rickettsia rickettsii*, immunomodulation**

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Genetic and biological characterisation of Zika virus isolates from different Brazilian regions

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BACKGROUND Zika virus (ZIKV) infections reported in recent epidemics have been linked to clinical complications that had never been associated with ZIKV before. Adaptive mutations could have contributed to the successful emergence of ZIKV as a global health threat to a nonimmune population. However, the causal relationships between the ZIKV genetic determinants, the pathogenesis and the rapid spread in Latin America and in the Caribbean remain widely unknown.

OBJECTIVES The aim of this study was to characterise three ZIKV isolates obtained from patient samples during the 2015/2016 Brazilian epidemics.

METHODS The ZIKV genomes of these strains were completely sequenced and *in vitro* infection kinetics experiments were carried out in cell lines and human primary cells.

FINDINGS Eight nonsynonymous substitutions throughout the viral genome of the three Brazilian isolates were identified. Infection kinetics experiments were carried out with mammalian cell lines A549, Huh7.5, Vero E6 and human monocyte-derived dendritic cells (mdDCs) and insect cells (Aag2, C6/36 and AP61) and suggest that some of these mutations might be associated with distinct viral fitness. The clinical isolates also presented differences in their infectivity rates when compared to the well-established ZIKV strains (MR766 and PE243), especially in their abilities to infect mammalian cells.

MAIN CONCLUSIONS Genomic analysis of three recent ZIKV isolates revealed some nonsynonymous substitutions, which could have an impact on the viral fitness in mammalian and insect cells.

Key words: Zika virus - *Flavivirus* - molecular markers - biological characterisation

ZIKV is a flavivirus (belonging to the family *Flaviviridae*, genus *Flavivirus*) that has recently emerged in many countries and has triggered an epidemic in the human population. For decades, human infections were only sporadic; however, outbreaks were noted in Yap Island in 2007 and in the Pacific Islands in 2013,[(1)] and an explosive epidemic occurred in Brazil in 2015.[(2)]

Prior to 2007, ZIKV had silently circulated in many countries from African and Asian continents with a low impact on public health. However, during the recent emergence, different transmission routes have been reported, and the epidemic profile and self-limiting character of Zika disease were dramatically altered.[(3)] After ZIKV introduction to Brazil, the virus raged on in South America and caused debilitating neurological and congenital complications, resulting in significant acute morbidity in adult patients and devastating neurological sequelae in newborns.[(4)]

As evidenced for other arthropod-borne viruses, a combination of factors ranging from environmental factors to viral genetic changes may have contributed to the selection and spread of new epidemic variants of ZIKV through a naïve population in tropical and subtropical areas. Currently, the occurrence of human ZIKV infections has dramatically decreased in Latin America. This decrease may be partially due to the population's previous experience with natural ZIKV infection and the subsequent development of immunity.[(5)] Additionally, other seasonal changes can influence the biology of vectors and can affect host-pathogen interactions; this generates a variation in the timing and severity of epidemic dynamics, as has been demonstrated by studies on other infectious agents.[(6)] Although a decrease in ZIKV transmission has been observed in the last two years, human cases are sporadically detected, which highlights the possibility of a new epidemic event. Clarifying the dynamic of ZIKV infections between animals, human hosts and vectors should aid in preventing new epidemic outbreaks and in developing strategies for controlling ZIKV infections.

The ZIKV genome consists of single-stranded, positive-sense RNA of approximately 11 kb in length. The viral RNA architecture is composed of two noncoding regions at the 5' and 3' extremities flanking a single open reading frame (ORF). The viral ORF encodes a

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Flavivirus-Mediating B Cell Differentiation Into Antibody-Secreting Cells in Humans Is Associated With the Activation of the Tryptophan Metabolism

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Patients infected with the Dengue virus (DENV) often present with a massive generation of DENV-specific antibody-secreting cells (ASCs) in the blood. In some cases, these ASCs represent more than 50% of the circulating B cells, a higher magnitude than those induced by other infections, vaccinations, and plasma cell lymphomas. However, it remains unclear how the DENV infection elicits this colossal response. To address this issue, we utilised an *in vitro* strategy to induce human PBMCs of healthy individuals incubated with DENV particles (DENV4 TVP/360) to differentiate into ASCs. As controls, PBMCs were incubated with a mitogen cocktail or supernatants of uninfected C6/36 cells (mock). The ASC phenotype and function were increasingly detected in the DENV and mitogen-cultured PBMCs as compared to mock-treated cells. In contrast to the *in vivo* condition, secreted IgG derived from the PBMC-DENV culture was not DENV-specific. Lower ASC numbers were observed when inactivated viral particles or purified B cells were added to the cultures. The physical contact was essential between B cells and the remaining PBMCs for the DENV-mediated ASC response. Considering the evidence for the activation of the tryptophan metabolism detected in the serum of Dengue patients, we assessed its relevance in the DENV-mediated ASC differentiation. For this, tryptophan and its respective metabolites were quantified in the supernatants of cell cultures through mass spectrophotometry. Tryptophan depletion and kynurenine accumulation were found in the supernatants of PBMC-DENV cultures, which presented enhanced detection of indoleamine 2,3-dioxygenase 1 and 2 transcripts as compared to controls. In PBMC-DENV cultures, tryptophan and kynurenine levels strongly correlated to the respective ASC numbers, while the kynurenine levels were directly proportional to the secreted IgG titers. Contrastingly, PBMCs incubated with Zika or attenuated

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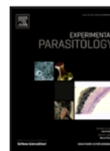

Biogenic silver nanoparticles reduce adherence, infection, and proliferation of toxoplasma gondii RH strain in HeLa cells without inflammatory mediators induction

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ABSTRACT

The highlights of biogenic silver nanoparticles (AgNp-Bio) include low toxicity – depending on size and concentration – and efficient antiparasitic activity. Therefore, the objective of this study was to assess the action of the AgNp-Bio on HeLa cells in an infection with strain of RH *Toxoplasma gondii*. Firstly, we performed a cellular viability test and characterized the AgNp-Bio to proceed with the infection of HeLa cells with *T. gondii* to be treated using AgNp-Bio or conventional drugs. Subsequently, we determined the level of standard cytokines Th1/Th2 as well as the content of nitric oxide (NO) and reactive oxygen species (ROS). Results indicated a mean size of 69 nm in diameter for the AgNp-Bio and obtained a dose-dependent toxicity. In addition, the concentrations of 3 and 6 μM promoted a significant decrease in adherence, infection, and intracellular proliferation. We also found lower IL-8 and production of inflammatory mediators. Thus, the nanoparticles reduced the adherence, infection, and proliferation of ROS and NO, in addition to immunomodulating the IL-8. Therefore, our data proved relevant to introduce a promising therapeutic alternative to toxoplasmosis.

1. Introduction

Toxoplasmosis is an infection caused by the compulsory intracellular protozoan *Toxoplasma gondii* and represents a public health issue that affects around 30% to 50% of the world population (Montazeri et al., 2017). In 90% of the causes, the infection develops without symptoms or is benign (Robert-Gangneux and Dardé, 2012; Krueger et al., 2014). However, it is possible for immunocompromised individuals to develop a severe clinical condition (Sutterland et al., 2015; Atilla et al., 2015; Alday et al., 2017). The organism does not tolerate well the association of pyrimethamine and sulfadiazine used to treat symptomatic cases, which interact indistinctly with biochemical processes of both the parasite and the host (Sepúlveda-Arias et al., 2014) generating adverse effects, such as the suppression of bone marrow, which may lead to megaloblastic anemia, leukopenia, and granulocytopenia (Petersen, 2007).

In this context, investment has focused on the study of nanomaterial, whose actions involve carrying drugs, decreasing toxicity, modulating pharmacokinetics, and increasing bioavailability, in addition to releasing the drug directly into the specific target (Khalil et al., 2013; Torres-Sangiao et al., 2016). Silver nanoparticles (AgNp) are commonly used for a variety of medical applications, especially for their anti-inflammatory and antimicrobial activities (Pourali and Yahyaei, 2016) (Shrivastava et al., 2007; Adair et al., 2010; Scandorieiro et al., 2016) found in Gram-positive and Gram-negative bacteria (Shrivastava et al., 2007; Scandorieiro et al., 2016; Durán et al., 2016), filamentous fungi (Sanguiñedo et al., 2018), some types of viruses (Park et al., 2018; Sharma et al., 2019), and protozoa of the genus Leishmania (Allahverdiyev et al., 2011; Fanti et al., 2018; Isaac-Marquez et al., 2018). Furthermore, this metallic nanoparticle has remarkable properties, such as accumulation in tissues – perhaps even reaching cysts of *T. gondii* (Adeyemi and Sulaiman, 2015) – and the production of reactive oxygen species (ROS), which are able to kill infectious agents (Butkus et al., 2004; Bhardwaj et al., 2012).

Metallic nanoparticles are known as promising anti-Toxoplasma agents (Gaafar et al., 2014; Assolini et al., 2017; Adeymi et

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