In vitro functional testing of candidate drugs against dengue fever

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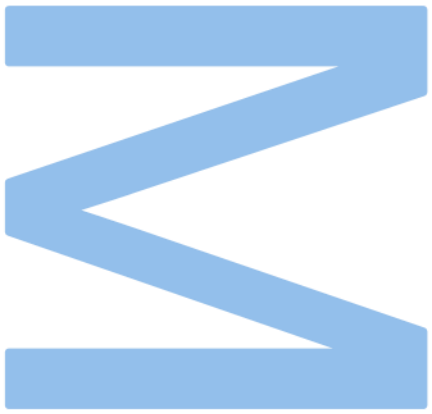

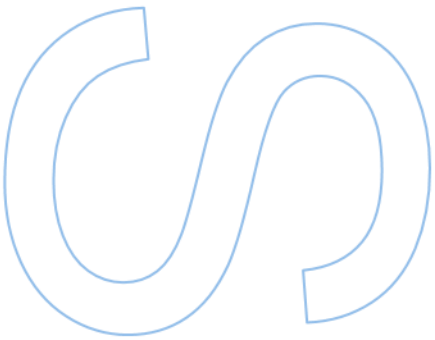

Dedicated to my grandparents

Sworn Statement

I, Ana Rita Neto Oliveira, enrolled in the Master Degree in Biochemistry at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

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22/06/2023

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Resumo

O vírus da dengue (DENV, de *dengue virus*) é um vírus transmitido por mosquitos e que causa a febre da dengue (DF). A DF é endémica em zonas tropicais e subtropicais, afetando 128 países em todo o mundo. De momento, outras zonas como a Europa estão em risco de sofrer surtos da dengue, devido ao alargamento das zonas de distribuição dos vetores, causado pela globalização e pelas alterações climáticas. Estima-se que 3,97 biliões de pessoas estão em risco de ter dengue, sendo que 100 milhões de infeções são sintomáticas e 10000 mortes ocorrem anualmente. Não existem tratamentos específicos para a dengue. A profilaxia da doença é feita essencialmente pelo controlo da população de vetores e, ainda em aplicações limitadas, pela administração da vacina Dengvaxia. Deste modo, a dengue é um problema crescente de saúde pública.

O estudo de fatores de suscetibilidade genética à dengue, em diferentes populações, permitiu a identificação de genes associados a três vias que envolvem o fator de transcrição nuclear RXR (de *retinoid X receptor*): a via de ativação VDR (de *vitamin D receptor*)/RXR, a via de ativação LXR (de *liver X receptor*)/RXR e a via de ativação PPARA (de *peroxisome proliferator activated receptor alpha*)/RXRA (de *retinoid X receptor alpha*). Já existem fármacos que modulam estes recetores e vias associadas. O antineoplásico bexaroteno é um retinoide que ativa seletivamente o RXR, potencialmente interferindo com todas as vias relacionadas com este fator de transcrição. A vitamina D3, um precursor da vitamina D, ativa o VDR. O 25-hidroxicolesterol, um metabolito do colesterol, é um agonista do LXR. A rosiglitazona, um fármaco antidiabético, ativa o PPAR. Estas vias estão envolvidas no metabolismo lipídico, que é relevante para a infeção por diversos vírus. Tendo isto em conta, colocámos a hipótese de a modulação do metabolismo lipídico por estas vias relacionadas com o RXR poder interferir com a infeção por DENV. Sendo assim, o objetivo deste trabalho foi testar *in vitro* o efeito destes quatro fármacos que modulam vias relacionadas com o RXR na infeção pelo DENV.

Começámos por avaliar o impacto dos fármacos na viabilidade das linhas celulares Vero (células epiteliais de rim de macaco verde africano) e Huh-7 (células de fígado humano), identificando o intervalo de viabilidade [0,5 a 100μM]. Nos ensaios de infeção, as células Vero e Huh-7 foram inicialmente cultivadas e expostas a seis concentrações dos fármacos. Depois, sob condições Nível de Biossegurança 3 (BSL3, de *Biosecurity Level 3*), as células foram infetadas com o serotipo DENV2. A infeção foi avaliada por imunofluorescência indireta do DENV2. Os fármacos bexaroteno, vitamina D3 e 25-

hidroxicolesterol reduziram a infeção por DENV2 em ambas as linhas celulares, enquanto que a rosiglitazona não teve esse efeito.

Estes resultados sugerem que as vias relacionadas com o RXR possam ter papéis essenciais na dengue, apoiando a continuação do seu estudo.

Palavras-chave: dengue; vírus da dengue; vias relacionadas com o receptor X do retinóide; testes de fármacos; bexaroteno; vitamina D3; 25-hidroxicolesterol; rosiglitazona; nível de biossegurança 3.

Abstract

The dengue virus (DENV) is an arthropod-borne virus responsible for the dengue fever (DF) disease. DF is endemic in tropical and subtropical regions, affecting 128 countries worldwide. Currently, other regions as Europe are at risk of DF disease outbreaks due to the increasing spreading of the vectors, caused by globalization and climate change. It is estimated that 3.97 billion people are at risk of developing DF, with 100 million symptomatic infections and 10000 deaths occurring annually. There are no specific treatments to DF disease, and prophylaxis is mainly achieved through vector control methods, plus the Dengvaxia vaccine administration in limited cases. Due to this, DF poses an increasing global health challenge.

The study of genetic susceptibility factors to DF, in several populations, has identified associated genes in three related pathways involving the nuclear transcription factor retinoid X receptor (RXR): the vitamin D receptor (VDR)/RXR pathway, the liver X receptor (LXR)/RXR pathway and the peroxisome proliferator activated receptor alpha (PPARA)/retinoid X receptor alpha (RXRA) pathway. Some drugs that modulate these receptors and related pathways are available. The antineoplastic bexarotene is a retinoid that selectively activates RXRs, potentially interfering with all the RXR-related pathways. Vitamin D3, a precursor of vitamin D, activates the VDR. 25-hydroxycholesterol, an intermediate in cholesterol metabolism, is an agonist of the LXR. Rosiglitazone, an antidiabetic drug, binds to and activates the PPAR. These pathways are involved in lipid metabolism, which is relevant to infection by various viruses. Therefore, we hypothesized if the modulation of lipid metabolism by RXR-related pathways may interfere with DENV infection. Hence, this work aimed to test *in vitro* the effect of these four known pathway modulators against DENV infection.

First, we evaluated the drugs impact on Vero (African green monkey kidney) and Huh-7 (human liver) cell lines' viability, identifying a range of safe drug concentrations [0.5 to 100μM]. In the infection assays, the Vero and Huh-7 cells were cultured and exposed to the six drug concentrations. Then, under Biosafety Level 3 (BSL3) conditions, the cells were infected with DENV2 serotype. The infection was evaluated through indirect immunofluorescence of DENV2. Bexarotene, vitamin D3 and 25-hydroxycholesterol reduced DENV2 infection of both cell lines, while rosiglitazone seemed to have no effect on infection.

These results provide useful insights into which RXR-related pathways may have key roles in DF, so their further study is encouraged.

Keywords: dengue fever disease; dengue virus; retinoid x receptor related pathways; drug tests; bexarotene; vitamin D3; 25-hydroxycholesterol; rosiglitazone; biosecurity level 3 conditions.

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List of Abbreviations

+ssRNA	Positive-sense, single-stranded RNA
ADE	Antibody-Dependent Enhancement
AHRR	Aryl Hydrocarbon Receptor Repressor
BMI	Body-Mass Index
BSA	Bovine Serum Albumin
BSL3	Biosafety Level 3
C protein	Capsid protein
CDC	Centers for Disease Control and prevention
CH25H	Cholesterol 25-Hydroxylase
CHIKV	Chikungunya Virus
CHST10	Carbohydrate Sulfotransferase 10
CMC	Carboxymethyl Cellulose
CNS	Central Nervous System
CTCL	Cutaneous T Cell Lymphoma
CTD	C-Terminal Domain
DAMPs	Damage-Associated Molecular Patterns
DAPI	4’,6-diamidino-2-phenylindole
DBD	DNA-Binding Domain
DENV	Dengue Virus
DENV1	Dengue Virus Serotype 1
DENV2	Dengue Virus Serotype 2
DENV3	Dengue Virus Serotype 3
DENV4	Dengue Virus Serotype 4
DF	Dengue Fever
DHF	Dengue Haemorrhagic Fever
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DMT2	Diabetes Mellitus Type 2
DSS	Dengue Shock Syndrome
E protein	Envelope protein
EBOV	Ebola Virus
EMA	European Medicines Agency
ER	Endoplasmatic Reticulum
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration

FFA	Focus Forming Assay
FFU	Fluorescent Focus Units
FXR	Farnesoid X Receptor
GRIP1	Glutamate Receptor Interacting Protein 1
GWAS	Genome-Wide Association Study
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HSP70	Heat Shock Protein 70
HSP90	Heat Shock Protein 90
HSV-1	Herpes Simplex Virus Type 1
IC_{50}	Half Maximal Inhibitory Concentration
IDOL	Inducible Degrader of the LDL-receptor
IL-10	Interleukin-10
ISG	Interferon-Stimulated Gene
JNK	c-Jun N-terminal Kinase
kb	Kilobase
LBD	Ligand-Binding Domain
LD	Lipid Droplets
LDLR	Low-Density Lipoprotein Receptor
LXR	Liver X Receptor
LXREs	LXR-Responsive Elements
M protein	Membrane protein
MAPKs	Mitogen-Activated Protein Kinases
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MHC	Major Histocompatibility Complex
MICA	Major Histocompatibility Complex class I polypeptide-related sequence A
MICB	Major Histocompatibility Complex class I polypeptide-related sequence B
miRNAs	microRNAs
MOI	Multiplicity Of Infection
MR	Mannose Receptor
ND	Non-Determinable
NRs	Nuclear Receptors
NSAIDs	Non-Steroid Anti-Inflammatory Drugs

NS proteins	Non-Structural proteins
NTD	N-Terminal Domain
OAS3	2'-5'-oligoadenylate Synthetase 3
ORF	Opening Reading Frame
OSBPL10	Oxysterol Binding Protein Like 10
P/S	Penicillin/Streptomycin
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PLCB4	Phospholipase C Beta 4
PLCE1	Phospholipase C Epsilon 1
PPAR	Peroxisome Proliferator-Activated Receptor
PPREs	Peroxisome Proliferator Response Elements
PPP2R5E	Protein Phosphatase 2 Regulatory subunit B'Epsilon
prM protein	Pre-membrane protein
RAR	Retinoic Acid Receptor
RC	Replication Complex
Rdrp	RNA-dependent RNA polymerase
RER	Rough Endoplasmatic Reticulum
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RXR	Retinoid X Receptor
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SD	Standard Deviation
SREBP	Sterol-Responsive Element Binding Protein
STAT1	Signal Transducer and Activator of Transcription 1
TG	Triglycerides
TLRs	Toll-Like Receptors
TNFα	Tumor Necrosis Factor α
TR	Thyroid hormone Receptor
USA	United States of America
VDR	Vitamin D Receptor
VDREs	Vitamin D Response Elements
VSV	Vesicular Stomatitis Virus
WBC	White Blood Cells
WHO	World Health Organization

WNV	West Nile Virus
YFV	Yellow Fever Virus
ZIKV	Zika Virus

Introduction

1. Dengue, an Overview

1.1 Epidemiology

Dengue, also known as “water poison”, “cramp-like seizure” and “break-bone fever”, is an arthropod-borne disease caused by the dengue virus (DENV) (1-3). DENV is mainly transmitted by mosquitoes of the *Aedes* genus, especially by *Aedes aegypti* and *Aedes albopictus* (4). Besides DENV, both *Aedes aegypti* and *Aedes albopictus* can transmit the Chikungunya virus (CHIKV) (5), Yellow Fever virus (YFV) (6) and Zika virus (ZIKV) (7). Currently, the dengue disease is endemic in Africa, Western Pacific, Southeast Asia, the Americas and East Mediterranean, affecting 128 countries (**Figure 1**) (8, 9). Globalization and climate change are enlarging the *Aedes* mosquitoes distribution across the globe (10), increasing the risk of dengue disease outbreaks in non-endemic parts of the world, namely in the United States of America (USA) (11) and in Europe (12). A dengue outbreak in the Portuguese Madeira archipelago in 2012 is an example of this reality (13). It is estimated that 3.97 billion people are at risk of developing dengue disease (8). Annually, about 100 million symptomatic infections occur (14) and approximately 10000 people die of dengue disease (15). However, the actual numbers of dengue cases and deaths may be substantially higher, as underdiagnosis and non-reported cases are frequent (15). These facts make the dengue disease an increasing public health concern that not only affects the well-being of the world population, but also causes a huge economic burden on countries and their health care systems (9, 16).

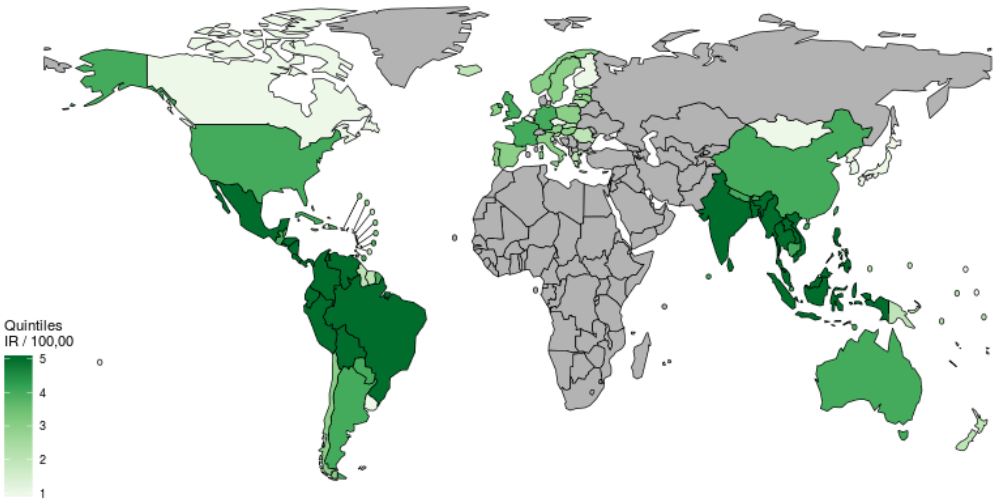

Figure 1 - Reported dengue cases to the World Health Organization (WHO) between 1990 and 2017. From WHO’s online dengue data application, available from https://ntdhq.shinyapps.io/dengue5/, accessed on 22.05.23.

1.2. DENV Structure, Replication Cycle and Transmission

Taxonomically wise, DENV is included in the *Flaviviridae* family and its Flavivirus genus, together with the related ZIKV, YFV and West Nile virus (WNV) (17). DENV is also related to the Hepatitis C Virus (HCV), belonging to the same family (17). DENV can be sub-classified in four serotypes (DENV1, DENV2, DENV3, DENV4) (4), based on their genome and antigens (9). The viruses in the same serotype group can be further divided into different genotypes and clades, and they usually differ 6% and 3% on their nucleotide and amino acid sequence, respectively (18, 19).

DENV has the shape of a spherical particle with approximately 50nm of diameter (20). This virion has a lipid envelope, consisting of a membrane bilayer derived from the host cell, an icosahedral nucleocapsid and an 11 kilobase (kb) long, positive-sense, single-stranded RNA genome (+ssRNA) (**Figure 2A**) (21, 22). This viral genome has only one Opening Reading Frame (ORF) (23), coding for ten proteins in the following order: 5'-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' (21, 24). Three of these are structural proteins: capsid protein (C protein), pre-membrane/membrane protein (prM/M protein) and envelope protein (E protein) (24). The other seven are non-structural proteins (NS proteins): NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (24). The M and E proteins are anchored externally on the membrane bilayer (9).

DENV can infect a wide range of different cell types: epithelial, endothelial, muscle, dendritic, mast, B and T cells, hepatocytes, monocytes and macrophages (25). In fact, dendritic cells, monocytes and macrophages are often the first to be infected when the mosquito bites a person (9), while hepatocytes and the liver are quite damaged during DENV infection (26). The replication cycle of DENV (**Figure 2B**) starts with the binding of its E protein to host cell receptors, while the M protein facilitates this virus-cell contact (9). For example, DENV2 can infect HepG2 cells by using the receptor GRP78 (27), Huh-7 cells by using the receptor claudin (28), and Vero cells by using the receptor heparan sulfate (29). Through this step, the clathrin-dependent, receptor-mediated endocytosis of the viral particle is induced (9, 30). There are different reports suggesting that the virus may be able to enter the host cell through other pathways like direct fusion (31), macropinocytosis (32), or in a clathrin-independent manner (33). Moreover, lipid rafts, which are plasma membrane microdomains enriched in cholesterol and sphingolipids, are relevant for DENV entry, namely by localization of the DENV entry receptors Heat Shock Protein 70 (HSP70) and HSP90 in monocytic/macrophage cell lines (34). After this, as the formed endosome containing the virus matures and its pH decreases, the E protein undergoes conformational changes that allow the fusion of the viral lipid envelope with the endosomal membrane (9). Therefore, the nucleocapsid containing the viral

genome is released into the cytosol. This nucleocapsid disassembles to release the viral genome (35). The dengue virus genome (+ssRNA) is equivalent to a mRNA (22), enabling direct translation by the cellular machinery to synthesize viral proteins. This translation of the viral genome occurs in the Rough Endoplasmatic Reticulum (RER) and results in the production of a viral polyprotein, which is cleaved into the individual proteins by the viral proteases NS2B or NS3 and by the host proteases (9). The synthetized NS proteins are targeted to the Endoplasmatic Reticulum (ER) to form the viral Replication Complex (RC) (35), where the +ssRNA is used as a template for the transcription of an antisense RNA and the subsequent production of more viral genomes (22, 35).

The interaction between the synthetized viral genome and the C protein leads to the assembly of the nucleocapsid (35). The viral particle acquires its membrane bilayer through the budding from the cytosol into the ER, with the synthetized prM and E proteins localizing in the ER membrane and being integrated in the new viral envelope (9, 35). After this, the viral particle follows the secretion pathway and transitions to the Golgi Complex, where a more acidic environment promotes the maturation of some viral particles (protein prM is cleaved by cellular furin proteases, hereby originating the M protein, its mature form, but the pr portion only detaches from the M protein when the viral particle undergoes exocytosis) (9). At the end of the DENV replication cycle, the new viral particles exit the cell through exocytosis (9, 35). The released viral particles may be in different stages of maturation upon their release (24).

The NS proteins have important roles in viral replication and immune evasion, even though they are not packed into the new virions (23). NS1 is the most relevant because it is both present at the surface of and secreted by infected host cells, reaching the blood circulation of dengue patients (9). Thus, the soluble form of NS1 can be detected by laboratory tests, making it an important diagnostic biomarker of DENV infection (9, 23).

The DENV propagates through a cycle of infecting mosquitoes and humans (**Figure 3**). A female mosquito becomes infected with DENV when it feeds on the blood of a person that is in the viraemic phase of the DENV infection. This virus infects the midgut first and, in 8 to 10 days, eventually reaches the salivary glands of the mosquito (4). Then, the mosquito becomes infected for life and transmits DENV by stinging humans (9). Not all people that get stung will experience DENV infection symptoms, but both asymptomatic and symptomatic patients can transmit DENV to new mosquitoes in the viraemic phase, repeating the infection cycle (9). Besides this horizontal transmission, if DENV infects the eggs of the female mosquito, it is possible that the virus gets vertically transmitted to the mosquito progeny (4).

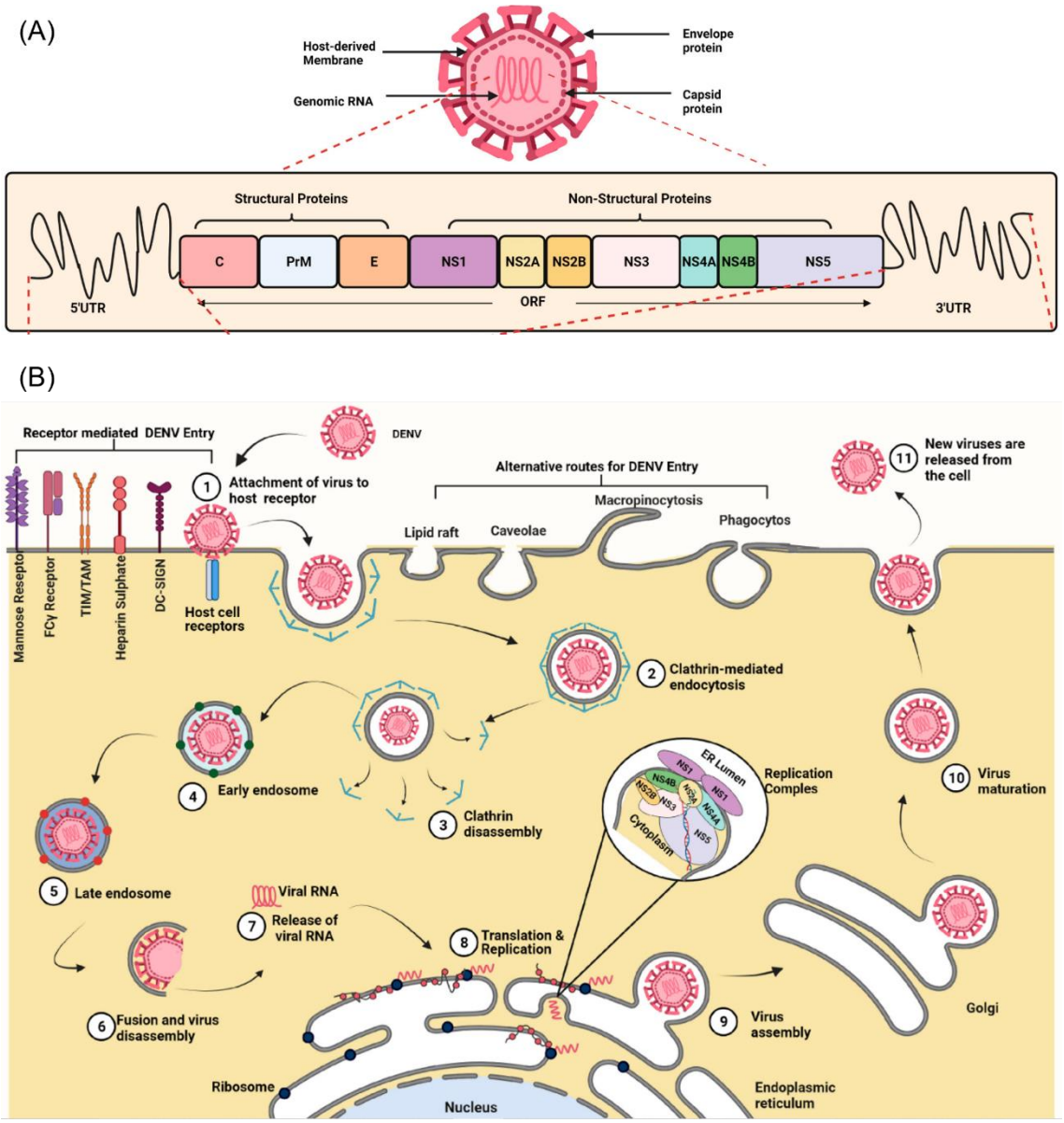

Figure 2 - The structure, genome and replication cycle of DENV. (A) DENV is a 50nm diameter spherical particle containing a host-derived lipid envelope, an icosahedral nucleocapsid and a +ssRNA. The RNA codes for ten proteins, three of which are structural proteins called capsid protein (C protein), pre-membrane/membrane protein (prM/M protein) and envelope protein (E protein), and seven non-structural proteins (NS proteins): NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. **(B)** The DENV replication cycle starts with the binding of its E protein to host cell receptors (1), inducing the clathrin-dependent receptor-mediated endocytosis of the viral particle, the main DENV entry route (2, 3). The formed endosome containing the virus (4) matures and its pH decreases (5). Then, the viral lipid envelope fuses with the endosomal membrane, which leads to the release and the disassembly of the nucleocapsid containing the +ssRNA (6). Thus, the +ssRNA is released (7), enabling its direct translation and replication by the cellular machinery in the ER (8), with the produced NS proteins forming the viral Replication Complex (RC). The virus assembly occurs through the interaction of the synthetised viral genome and proteins, and the lipid envelope is acquired through the budding from the cytosol into the ER (9). After this, the virus follows the secretion pathway and transitions to the Golgi Complex, where a more acidic environment promotes its maturation (10). At the end, the new virus exits the cell through exocytosis (11). From Nanaware *et al* (2021).

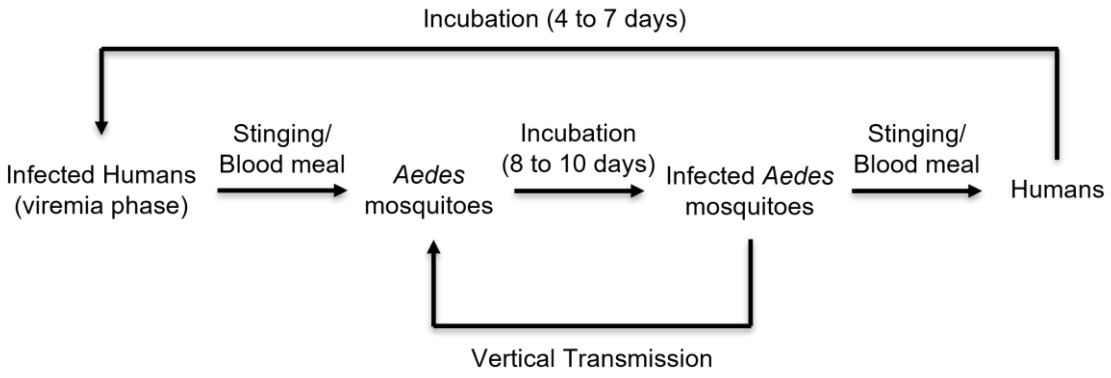

Figure 3 - The DENV infection cycle between *Aedes* mosquitoes and humans. A female mosquito becomes infected with DENV when it feeds on the blood of a person that is in the viraemic phase. After 8 to 10 days of incubation, the mosquito becomes infected for life and transmits DENV to humans by stinging and feeding on the blood. Additionally, if DENV infects the eggs of the female mosquito, the virus can be vertically transmitted to the mosquito progeny. Adapted from Guzman *et al* (2016) and Gubler *et al* (1998).

1.3. Dengue Phenotypes and Diagnosis

The dengue disease has a diverse range of phenotypes that include asymptomatic forms, mild disease forms and aggravated illness that may lead to death (36). The virus incubation usually takes 4 to 7 days, but it can also range from 3 to 14 days (9). Overall, the symptomatic dengue disease can be divided in three phases, consisting of the febrile, critical and recovery phases (**Figure 4**) (10). The febrile stage happens after the virus incubation and is characterized by the sudden start of a fever that can last from 2 to 7 days, as well as other symptoms like myalgia and headaches (36). Even though the febrile phase of the dengue infection may be non-distinguishable from other diseases, a positive result for the tourniquet test and a decrease in the leukocyte count may alert for a dengue infection. It is also important to follow the evolution of the clinical situation of the person to identify early signs of worsening of the disease (10). After the febrile phase comes the critical phase. The critical phase starts around the 3rd or 7th day of the disease, usually with a decrease of the body temperature accompanied by plasma leakage that can last 1 or 2 days, and an increase in the haematocrit levels. The non-severe dengue patients are those whose fever decreases or disappears and that experience no plasma leakage. On the other hand, the patients who experience plasma leakage with or without defervescence are said to have dengue with warning signs. If enough plasma volume leaks, the patient can enter shock, which compromises organ perfusion and consequent function. Besides this, abnormal coagulation may occur, leading to bleeding problems. During the critical phase, it is important to rehydrate the patient by administering fluids intravenously (10). After the critical phase, there may be an improvement of the overall clinical situation of the patient that marks the beginning of the recovery phase. In this phase the plasma leakage reverts with the reabsorption of extravascular fluids and

intravenous fluid administration should be ended (10). On the other hand, the patient may not improve and end up dying.

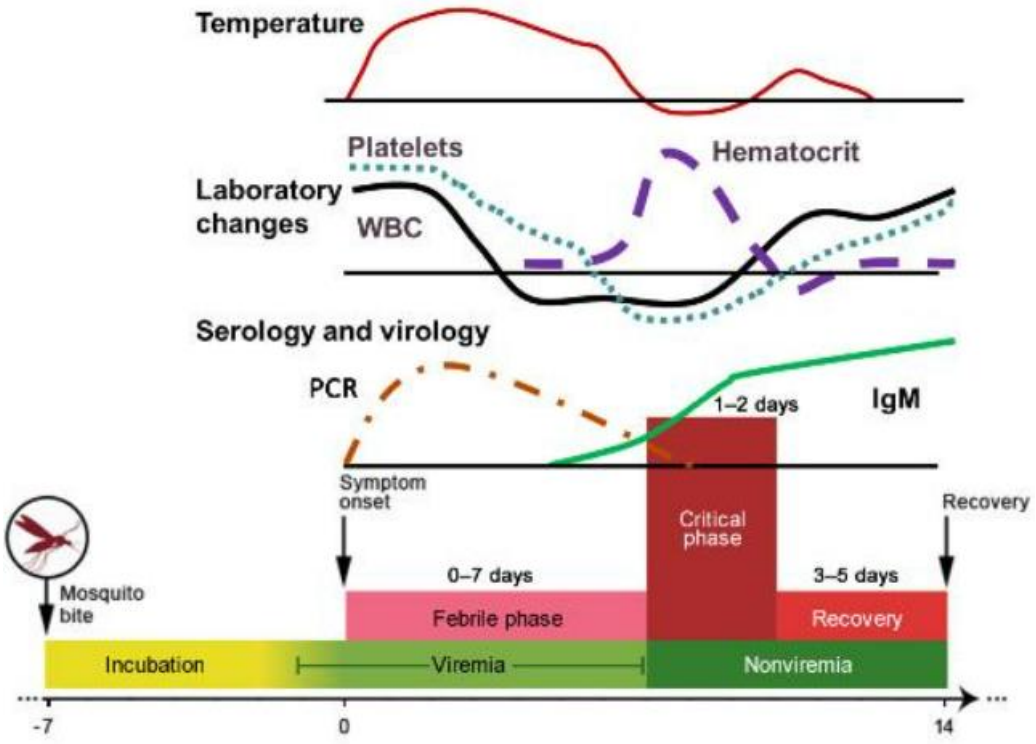

Figure 4 - The dengue disease phases and laboratory findings. The virus incubation usually takes 4 to 7 days. The febrile stage happens after the virus incubation and can last from 2 to 7 days, being characterized by the sudden start of a fever accompanied by the decrease in the white blood cells (WBC) and platelets. Then, the critical phase starts, usually lasting 1 or 2 days and being characterized by a decrease of the body temperature, possible plasma leakage and an increase in the haematocrit levels. After the critical phase, if there is an improvement of the overall clinical situation of the patient, the recovery phase starts. In this phase, the plasma leakage reverts with the reabsorption of extravascular fluids. DENV viremia can be detected between the onset of the symptoms till around the 6th day of the disease by Polymerase Chain Reaction (PCR), or by anti-DENV IgM detection after this timepoint. From Centers for Disease Control and Prevention (CDC)'s Dengue Clinical Case Management Training, available from https://www.cdc.gov/dengue/training/cme/ccm/page69981.html, accessed in 4/6/2023.

The current clinical classification of dengue cases was introduced by the World Health Organization (WHO) in 2009. Dengue cases can be sorted into dengue without warning signs, dengue with warning signs or severe dengue. Dengue without warning signs can manifest through pain, rash and leukopenia. On the other hand, symptoms like haemorrhage of the mucosa and constant vomiting are warning signs that indicate the need of close patient observation and careful clinical management. Severe dengue is characterized by aggravated bleeding, plasma leakage that may lead to shock, and organ dysfunction. Both dengue patients with and without warning signs can evolve to severe dengue, which may be deathly (10). These new categories overlap with previously used categories of Dengue Fever (DF), Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) (21).

The dengue disease diagnosis is quite challenging due to the similarities of its symptoms with other febrile and infectious diseases. Clinical suspicion is important and the diagnosis can be confirmed by DENV detection (37). DENV viremia is detectable by several methods in the time frame of 2 days before symptom onset to 6 days after (9). One of the most used techniques is the detection of anti-DENV IgM antibodies in patient's serum around the 6th day of acute symptoms (37). When the patient enters the convalescence phase, the detection is done by comparing quantities of both anti-DENV IgM and IgG present in two serum samples, one from the acute phase and the other from the convalescent phase, due to the antibody seroconversion. There are commercially available tests for the detection of these antibodies. DENV infection can also be detected through mosquito inoculation or by virus isolation in mosquito cell lines. Another way to identify DENV infection is through its RNA detection either by RT-PCR (Reverse Transcription Polymerase Chain Reaction) or real time RT-PCR (9).

1.4. Risk Factors and Genetic Susceptibility to Severe Dengue

Most dengue infections are self-resolving, but about 5% of dengue patients progress to severe dengue (38). The risk factors associated with worse dengue disease manifestations are linked with the interplay between the host and virus (9).

When it comes to the DENV, its ability to infect both vector and human cells, to replicate and to evade the immune system may lead to worse disease outcomes (9, 25). In some cases, infection with the DENV2 serotype has been associated with a higher risk of severe dengue compared to the other serotypes (39).

The infection with one DENV serotype usually confers life-lasting immunity against that serotype and temporary protection against other serotypes (cross-immunity) (1). Noticeably, subsequent infection with another DENV serotype often results in severe disease. In fact, severe dengue is more frequent in individuals experiencing a second infection with a heterologous DENV serotype, or in the first infection of children whose mothers are immune to dengue (1). Some explanations have been proposed, namely the antibody-dependent enhancement (ADE) phenomenon (23). The first DENV infection leads to the development of antibodies and memory cells against that serotype. When the second infection occurs with another serotype, the antibodies developed during the first infection can still bind to the new serotype, but they are unable to nullify it due to antigenic differences. The formed antibody-virus complexes can undergo phagocytosis through Fcγ receptors, even in cells not usually infected by the DENV. This ADE phenomenon increases the viral infection, replication and consequent disease severity

due to the presence of non-neutralizing antibodies against DENV, either developed during the first infection or passively passed from the immune mother to the child (25). The longer the time interval between the infections, the higher the risk of severe disease and mortality (1). Interestingly, some population surveillance reports indicate that third and fourth infection symptomatic cases are uncommon, suggesting that protection against other DENV serotypes may be induced by the secondary infection (35).

When it comes to the host, studies suggest that pre-existing conditions like diabetes mellitus and hypertension (40), high body-mass index (BMI) and obesity (41), cardiac disorders (42), sickle-cell anaemia (10) and asthma (42) are associated with severe dengue. Most of these co-morbidities are characterized by a state of increased inflammation or by an impairment in normal vascular function (38), which may explain the aggravation of the dengue disease in these patients. The dengue disease is often more severe and lethal in young patients (43), possibly due to the inability of young children to counter capillary leakage (10). The female biological sex has also been associated with worst dengue disease outcomes (9).

The host genetic factors are also relevant in the dengue disease manifestations. Evidence has shown that different populations have different responses to DENV infections, and genetic studies have identified several genes as conferring susceptibility or resistance against dengue phenotypes. Some alleles of the Major Histocompatibility Complex (MHC) class I polypeptide-related sequence A (*MICA*) and B (*MICB*) genes have been associated with symptomatic infections in Cuban patients (44). In 2011, a Genome-Wide Association Study (GWAS) identified variants of the *MICB* and phospholipase C epsilon 1 (*PLCE1*) genes to be associated with the development of severe dengue disease in Vietnamese patients (45). Additionally, the phospholipase C beta 4 (*PLCB4*) gene has been associated with the DSS in Thai patients (46). The genes *PLCE1* and *PLCB4* belong to the same family and are relevant in the Peroxisome Proliferator-Activated Receptor (PPAR)/RXRA activation pathway, which is related to lipid metabolism (46, 47). In addition, DF phenotype in Thai patients has been associated with four genes of the xenobiotic (environmental chemicals) metabolism signalling pathway: Carbohydrate Sulfotransferase 10 (*CHST10*), Aryl Hydrocarbon Receptor Repressor (*AHRR*), Protein Phosphatase 2 Regulatory Subunit B'Epsilon (*PPP2R5E*) and Glutamate Receptor Interacting Protein 1 (*GRIP1*) (46). In another study, the gene 2'-5'-Oligoadenylate Synthetase 3 (*OAS3*) has been associated with DSS (48). It was also demonstrated that African ancestry has a protective role against severe dengue development when compared to European ancestry in Cuban admixed habitants, namely due to lower Oxysterol Binding Protein Like 10 (*OSBPL10*) gene expression in

African descendants and differential expression of the Retinoid X Receptor α (*RXRA*) gene between African and European descendants (47). The OSBPL10 and RXRA are key players in the LXR (Liver X Receptor)/RXR activation pathway. This pathway is important in cholesterol metabolism for both hepatocytes and macrophages, and in cytokine production for macrophages (47). The differential expression of *OSBPL10* and *RXRA* genes has been associated with differences in lipid metabolism between dengue patients and controls (47). Additionally, an allele polymorphism in the Vitamin D Receptor (*VDR*) gene has been associated with a protective effect against DHF in Vietnamese patients (49). The VDR interacts with the RXRA in the VDR/RXR activation pathway, which regulates immune system genes (47). Overall, these previous findings suggest that RXR-related pathways may be relevant in the dengue disease, owing to their role in lipid metabolism and immune system function.

2. Dengue Prevention and Treatment

There have been different approaches to prevent and treat dengue cases, ranging from the application of vector control methods to the research on vaccine and drug development.

2.1. Vector Control

Due to the role of *Aedes* mosquitoes in dengue transmission and their proximity to domestic environments, often laying their eggs on containers with still water and trash, vector control has been the most used strategy, so far, to prevent dengue (4). To cut off the DENV transmission chain, infected patients should use bed nets and mosquito repellent (9). Furthermore, some countries have applied methods to reduce larvae, approved fines against individuals whose properties were infested with *Aedes* mosquitoes, administered low doses of pesticides (e.g.: pyrethroid, organophosphates) to kill adult mosquitoes, while also raising population awareness and literacy about dengue (4, 50-53). Additionally, *Aedes aegypti* mosquitoes infected with *Wolbachia* have been released into the environment (54). *Wolbachia* is a bacterium that infects insects, making them more resistant to DENV infection and decreasing their lifespan. This, allied with the fact that *Wolbachia* is transmitted to other mosquitoes through breeding, reduced mosquito population and dengue transmission. Another method with a similar purpose is the genetic engineering of male *Aedes aegypti* mosquitoes carrying a dominant lethal gene and their release into the environment (55). When these male mosquitoes mate with wild-type females, their offspring ends up dying before reaching reproductive age, therefore decreasing the mosquito population. Despite these efforts,

the vector control methods could not eliminate dengue outbreaks in the following decades, especially because neighbour countries with endemic dengue weren't applying vector control policies (4, 9). Taking this into account, vector control is an important way to reduce dengue transmission, but it should be performed simultaneously with other strategies and in a collective effort of neighbour countries with endemic dengue (9).

2.2. Vaccines against DENV

Besides dengue disease prophylaxis through vector control, there have been efforts to develop effective vaccines against DENV. The ideal DENV vaccine should be able to induce long-term protection against all four serotypes (9). There are five types of DENV vaccines under development based on the method of viral presentation: live attenuated virus, inactivated virus, virus subunits, virus-vectored, and DNA vaccines (56, 57).

The live attenuated virus vaccines consist of administering weakened DENV forms to elicit a safer immune response that is still similar to the natural infection (58). This weakening of DENV and its ability to replicate may be obtained through several passages in cell cultures or by inducing specific mutations (58). The live attenuated vaccines against DENV are the furthest in development. Two tetravalent vaccines, Dengvaxia® (59) and Qdenga® (60), were already approved. The Dengvaxia® (CYD-TDV), produced by Sanofi Pasteur, uses the YFV 17D vaccine strain and substitutes its E and prM proteins by each DENV serotype's corresponding proteins (35, 56). This vaccine is approved by the WHO (59), Food and Drug Administration (FDA) (61) and European Medicines Agency (EMA) (62). While the FDA allows the use of Dengvaxia® in the prevention of the dengue disease in 9 to 16 years-old individuals with a prior DENV infection confirmed by laboratory tests and living in a dengue-endemic region (61), the EMA enlarges the group to individuals aged from 6 to 45 years also with a confirmed previous DENV infection (62). Dengvaxia® efficiency is age-dependent, decreasing slightly in individuals younger than 9 years (62). The vigilance studies showed that dengue-naive individuals (seronegative) before Dengvaxia® administration had an increased risk of severe disease and hospitalisation following natural DENV infection, when compared to individuals already exposed to dengue (seropositive) at the moment of vaccination (63). Additionally, the vaccine was less effective in protecting against DENV-confirmed infection in seronegative individuals than seropositive individuals (64). Therefore, Dengvaxia® administration is restricted to previously DENV-infected individuals. As most people in naïve populations have not been infected, the use of this vaccine in these regions, including Europe, is quite limited at the moment. More recently, a live attenuated dengue vaccine called Qdenga® (TAK-003), produced by the Takeda Pharmaceutical Company, has been approved by the EMA to be used in the prophylaxis

of the dengue disease in people older than 4 years, presenting the advantage that it can be administered to both DENV-naïve and previously infected individuals (60). This vaccine uses an attenuated DENV2 strain as a backbone for the prM and E proteins of the four serotypes (65), protecting against severe forms of dengue and hospitalisation in both seronegative and seropositive individuals (66, 67). Nonetheless, continuous surveillance studies are needed to keep evaluating both vaccines' efficacy and safety.

The inactivated virus vaccines use chemical substances (e.g.: formaldehyde, formalin) to inactivate or reduce the infectivity of DENV and its components, before their administration (57). An example is the TDENV-PIV vaccine, in which four DENV serotypes are inactivated with formalin and administered with different adjuvants to boost the immune response (68). In phase I clinical trials, this vaccine was well tolerated and induced neutralizing antibody production against all serotypes in healthy adults (68).

The virus subunits vaccines contain and deliver DENV viral proteins to induce protection (57). An example is the V180 vaccine, which has a truncated form of the E protein of the four DENV serotypes and an adjuvant (69). This vaccine was already tested in phase I clinical trials, being well tolerated in healthy, flavivirus-naïve adults (69).

In the virus-vectored vaccines, DENV antigens are delivered through other well-studied, immunogenic and low pathogenic viruses such as vaccinia virus, adenovirus and alphavirus (57). An example is the CAd-Vax-Den, in which an adenovirus was genetically edited to code for and deliver the prM and E proteins of the four DENV serotypes (70). This vaccine was tested in rhesus monkeys, leading to promising results (70).

The DNA vaccines use DNA plasmids to code for and deliver DENV antigens, but they are less immunogenic so they may need to be administered with an adjuvant (57). An example is the TVDV-Vaxfectin® vaccine, in which monovalent DNA plasmids coding for the prM and E protein of each of the four DENV serotypes are administered with the adjuvant vaxfectin (71). In phase I clinical trials, this vaccine was tested in healthy, flavivirus-naïve adults, leading to good tolerance, but low immunogenic responses (71).

2.3. Drug Development and Repurposing against DENV

There are currently no specific treatments or even cure for dengue. Most of the disease treatment is based on clinical surveillance and symptoms management, achieved by fluid reposition (either orally or intravenously), bed rest and hospital admission if there is a suspicion on disease worsening (9, 72). Acetaminophen/paracetamol can be prescribed as an analgesic and antipyretic drug, but Non-Steroid Anti-Inflammatory Drugs (NSAIDs) should be avoided (56).

There are also no approved DENV antivirals. Clinical trials have been ongoing to find suitable drugs. Due to DENV replication cycle, both viral and host components are being studied and considered as possible therapeutic targets (73). Regarding the viral part, mainly its E protein, NS3pro/NS2B protease, NS3 helicase, NS5 methyltransferase and NS5's RNA-dependent RNA polymerase (Rdrp) are being studied to find specific inhibitors (73). Recently, Janssen's JNJ-AO7 compound showed promising pan-serotype antiviral activity in mice models by inhibiting the interactions between the DENV's NS4B and NS3 proteins, interfering with the viral RC (74). Following this, another compound with a similar mechanism of action called JNJ-1802 was identified. JNJ-1802 presents better pre-clinical safety than JNJ-AO7 and has already demonstrated *in vitro* and *in vivo* antiviral activity against DENV and other flavivirus like the WNV and the ZIKV. Currently, JNJ-1802 is on the phase 2 of clinical trials (75).

Another approach to identify possible dengue therapies is drug repurposing. Drug repurposing consists of finding new applications for medications already discovered and used to treat other diseases. This strategy has the advantage of taking less time and money than the usual drug discovery and development process, because the drugs pharmacokinetics and toxicity profiles are already known (76, 77). Some already used drugs like chloroquine, ribavirin, prednisolone, ivermectin and lovastatin were tested against DENV, but the results showed no benefit of their administration (78). Ribavirin is a synthetic guanosine analogue used as an antiviral against DNA and RNA viruses, namely the HCV (79).

3. Putative Target Pathways and Drug Candidates against DENV

As mentioned before, the research of susceptibility genes to the dengue disease led to the identification of the *VDR* (49), *OSBPL10* (47), *RXRA* (47), *PLCE1* (45) and *PLCB4* (46) genes. These genes interact in RXR-related activation pathways involving the nuclear receptor heterodimers VDR/RXR, LXR/RXR and PPARA/RXRA (47).

Nuclear receptors (NRs) are a superfamily of transcription factors with the ability to regulate gene expression. Their structures consist of a N-terminal domain (NTD), a conserved DNA-binding domain (DBD), a hinge region, a conserved Ligand-binding domain (LBD) and a C-terminal domain (CTD). The DBD binds to the promoter region of target genes. The LBD of nuclear receptors is conserved regarding its function but variable when it comes to the specific ligands that activate it (80, 81). Nuclear receptors can be divided into four classes based on their DNA-binding motifs, ligand binding and dimerization characteristics: Class I (steroid receptors), Class II (RXR heterodimers),

Class III (homodimeric orphan receptors) and Class IV (monodimeric orphan receptors). From these, the Class II (RXR heterodimers) are especially relevant to this work, as these nuclear receptors need to dimerize with RXR to perform their function. RXR heterodimerizes with the mentioned LXR, VDR and PPAR, as well with Farnesoid X Receptor (FXR), Retinoic Acid Receptor (RAR) and Thyroid hormone Receptor (TR) (80). These heterodimers have crucial roles in regulating physiological functions, which explains their involvement in conditions like inflammation and cancer (80, 81).

The LXR/RXR pathway is important in lipid metabolism, immune cell function and inflammation regulation (82). LXRs belong to the superfamily of NRs, acting as transcription factors to regulate cholesterol and lipid homeostasis (83). There are two LXRs isoforms: LXRα, more expressed in myelomonocytic lineage cells, adipose tissue, intestine and the liver, as well as LXRβ, more ubiquitously expressed. LXRs are activated by endogenous ligands like oxidized forms of cholesterol (oxysterols) and cholesterol precursors (83). The LXRs activation induces their heterodimerization with RXRs, followed by binding to LXR-responsive elements (LXREs) through target gene promoters. When it comes to target genes, most of them are related to glucose and lipid metabolism. In fact, LXRs induce the expression of sterol transporters, transcription factors that regulate the lipogenic pathways and the inducible degrader of the LDL-receptor (IDOL), responsible for the Low-Density Lipoprotein Receptor (LDLR) degradation and consequent less cholesterol uptake by the cell (83). Regarding LXRs' role in inflammation, they have anti-inflammatory action as they inhibit the expression of pro-inflammatory genes induced by transcription factors like the NF-kB (82), for example, in addition to inhibiting the maturation of pro-inflammatory cytokines (e.g.: IL-18) and inducing certain endogenous inhibitors (83). Due to these properties, the pharmacological targeting of LXRs has been considered as an option in cancer treatment and immunotherapy (83).

The VDR/RXR pathway also regulates gene transcription (84). VDR is activated through the binding to the active form of vitamin D, 1α,25-$(OH)_2$ D_3 (85), and to bile acids metabolites (86). Then, VDR heterodimerises with RXR and the complex binds to Vitamin D Response Elements (VDREs) in the promoters of target genes. This activity is regulated by co-activators and co-repressors that interact with the VDR/RXR heterodimer, so VDR can lead to gene expression or repression (84). When it comes to the biological effects of vitamin D, these are highly variable depending on the tissue type. Vitamin D and its interaction with the VDR may lead to the inhibition of B cells differentiation (87) and of the adaptive immunity (88), while conversely stimulating part of the innate immunity responses (89). It is hypothesized that vitamin D may have non-

genomic effects too, activating signalling cascades by inducing the opening of ion channels (84).

The PPAR/RXR heterodimer regulates gene transcription by binding to Peroxisome Proliferator Response Elements (PPREs) in target genes. PPAR has three isoforms: PPARα, PPARβδ and PPARγ (90). These isoforms have differential expression across tissues and specific functions. Nonetheless, all of them are involved in glucose and lipid metabolism regulation, in addition to being expressed in the liver (91).

NRs agonists are defined as ligands that induce the active conformation of the NR, while antagonists lead to a different configuration and effect (92). The RXR, LXR, VDR and PPAR receptors and related pathways are already being modulated by drugs in the treatment of diverse diseases, other than dengue:

3.1. Bexarotene

Bexarotene, also known as Targretin, is a synthetic analogue of retinoic acid/retinoid, binding selectively to and activating the RXRs (93, 94). This leads to altered gene expression and cell differentiation, as well as decreased cell proliferation, cancer cell apoptosis and subsequent tumour regression *in vitro* and *in vivo* (93, 94). Due to its antineoplastic effect, this drug is used in the treatment of skin manifestations of cutaneous T cell lymphoma (CTCL) in adults that do not respond to other systemic treatments (93). However, bexarotene's exact mechanism of action in CTCL treatment is unknow (93). Additionally, bexarotene has reported *in vitro* antiviral action against the Hepatitis B Virus (HBV) (95), HCV (96), Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (97), so it is possible it may affect other viral infections.

3.2. Vitamin D3

Vitamin D3, also known as cholecalciferol, is the biologically inactive precursor of the steroid hormone vitamin D, also known as calcitriol (84). Vitamin D3 can be enzymatically synthetized in the skin through exposure to sun light or obtained from the diet. After this, it is hydroxylated by the liver's 25-hydroxylase (CYP2R1) and the proximal renal tubule's 1α-hydroxylase (CYP27B1), forming vitamin D ($1\alpha,25\text{-}(OH)_2 D_3$) (84, 98). Vitamin D3 and vitamin D's active form can bind to and activate the VDR (84). Vitamin D has a key role in bone metabolism, maintaining calcium and phosphorous blood concentrations by modulating the activity of osteoblasts, chondrocytes, as well as intestine and kidney epithelial cells (84). Vitamin D3 is used to treat vitamin D insufficiency and deficiency, as well as related diseases like osteoporosis (98), hypoparathyroidism, familial hypophosphatemia and rickets (99),

and chronic kidney disease (100). There have been several reports of the antiviral effect of vitamin D3 against DENV infection *in vitro* (101, 102). This may be explained by the immune modulator action of vitamin D3 (103).

3.3. 25-Hydroxycholesterol

25-hydroxycholesterol is a naturally occurring metabolite formed by the hydroxylation of cholesterol by the cholesterol 25-hydroxylase (CH25H) (104). Oxysterols bind to the LXR, increasing the expression of the cholesterol 7α-hydroxylase gene and leading to the conversion of cholesterol into bile acids (104). 25-hydroxycholesterol inhibits the cleaving of the sterol-responsive element binding protein (SREBP), thus supressing cholesterol biosynthesis and uptake (104, 105). CH25H is an interferon-stimulated gene (ISG) involved in innate immunity (106-108). It has been reported that CH25H and 25-hydroxycholesterol have *in vitro* antiviral activity against several viruses, namely ZIKV (109, 110), Herpes Simplex Virus Type 1 (HSV-1) (111), Human Immunodeficiency Virus (HIV), Ebola Virus (EBOV) and Vesicular Stomatitis Virus (VSV) (112, 113). Currently, 25-hydroxycholesterol is only used as an experimental molecule (114).

3.4. Rosiglitazone

Rosiglitazone is a selective PPARγ agonist. This drug is used to treat Diabetes Mellitus Type 2 (DMT2) due to its ability to reduce blood glucose levels by increasing insulin sensitivity in tissues (115). Additionally, rosiglitazone has anti-inflammatory properties as it can inhibit the NF-kB pathway (116). Rosiglitazone has antiviral activity against enterovirus and coronavirus *in vitro* (117), as well against HIV in some infection models (118). Conversely, in a study of the infection of the Central Nervous System (CNS) by the WNV, rosiglitazone increased the viral load of the infected *ex vivo* brain slice cultures and the disease severity of the infected mice (119).

The RXR-related pathways and their pharmacological targeting with bexarotene, vitamin D3, 25-hydroxycholesterol and rosiglitazone are represented in **Figure 5**.

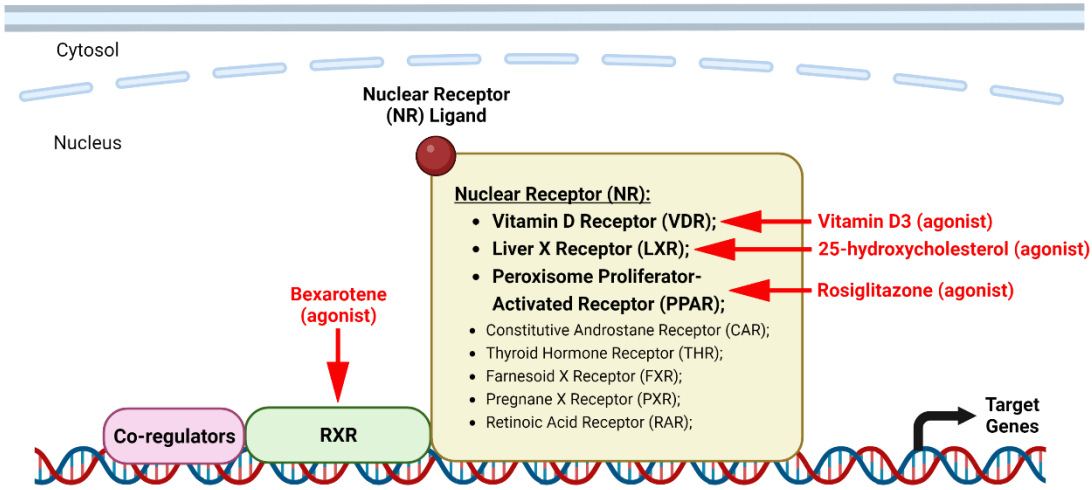

Figure 5 - Schematic representation of the RXR-related pathways and their pharmacological modulation with bexarotene, vitamin D3, 25-hydroxycholesterol and rosiglitazone. Bexarotene is a selective agonist of the RXR. Vitamin D3 activates the VDR, 25-hydroxycholesterol is an agonist of the LXR, and rosiglitazone is an agonist of the PPARγ. Created with Biorender.com.

Aims

The dengue disease poses an increasing public health challenge, and the lack of specific treatments and the difficult prophylaxis are still obstacles to overcome. The study of the genetic susceptibility factors to the dengue disease offers some enlightenment on possible host characteristics that may have key roles in DENV infection and subsequent disease. The RXR-related pathways, together with their importance in lipid metabolism and immune functions, may be one of these key players. Taking all this knowledge into account, the aim of this work is to test four known RXR-related pathway modulators - bexarotene, vitamin D3, 25-hydroxycholesterol, rosiglitazone - against DENV infection *in vitro*, in a prophylactic manner.

To perform the *in vitro* experiments, two cell types were cultured: Vero and Huh-7 cells. The Vero cells are widely used for virus culture (120). The human hepatocyte cell line Huh-7 aimed to model the liver, one of the most affected organs in the dengue disease (26). Then, the following specific objectives were proposed:

1. **Evaluation of the drugs impact on cell viability** – The four drugs were administered to the cell lines to evaluate their impact on cell viability and infer a range of safe concentrations for the following experiments.
2. **Optimisation of the DENV infection kinetics** – The infection conditions of DENV, namely the virus concentration and incubation time, were studied, so that the infection could be quantified and analysed.
3. **Evaluation of the drugs impact on the DENV infection of cells** – The drugs were prophylactically added to the cell lines, followed by DENV infection in optimised conditions, so that the drugs effect on DENV infection could be examined and compared.

Materials and Methods

Cell lines and drugs

The cell lines used in this work were Vero cells from the kidney of African green monkey (ATCC, #CCL-81; ATCC, Manassas, VA, USA) and Huh-7 cells from human hepatocellular carcinoma (kindly gifted by Tiago Duarte, i3S - Instituto de Investigação e Inovação em Saúde da Universidade do Porto). These cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Scientific, Waltham, MA, USA), supplemented with 10% Fetal Bovine Serum (FBS; Labclinics, Barcelona, Spain), 100 U/mL of penicillin and 100 µg/mL of streptomycin (1% P/S; Thermo Scientific, Waltham, MA, USA). Cells were maintained at 37°C, under a 5% CO_2 humified atmosphere, and regularly tested for mycoplasma presence. Besides the four drug candidates, the antiviral ribavirin was selected as a positive control for the experiments. Bexarotene (#11571), vitamin D3 (#11792), 25-hydroxycholesterol (#11097), rosiglitazone (#71740) and ribavirin (#16757) were purchased from Cayman Chemical Company (Cayman Chemical Co.; Ann Arbor, MI, USA). The drugs bexarotene, vitamin D3, rosiglitazone and ribavirin were prepared as 10mM stock solutions in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany), while the drug 25-hydroxycholesterol was prepared as a 5mM stock solution in DMSO.

Viral stock preparation and viral titration

DENV2 was acquired from ATCC (strain New Guinea C, #VR-1584, ATCC, Manassas, VA, USA). For the preparation of viral stocks, Vero cells were cultured in T75 flasks till 90% confluency was obtained. Following this, the medium was removed and the viral inoculum of DENV2 was prepared in DMEM without FBS and added to the cells (multiplicity of infection, MOI, of 0.01). After 1h incubation at 37°C and 5% CO_2, DMEM supplemented with 2% FBS was added to the flasks. The cells were incubated for 10-11 days and then the supernatant was collected, divided into freezing vials and stored at -80°C. DENV2 viral titters were determined by Focus Forming Assay (FFA) (121). Briefly, ten-fold dilutions of DENV2 viral supernatants were independently inoculated in Vero cells, seeded 24h before infection in 96-well plates. After 1h incubation, an overlay medium of DMEM with 4% FBS and 3% carboxymethyl cellulose (CMC) in a ratio of 1:1 was added to the inoculum. Then, the infected monolayer was incubated for 48h at 37°C, under a 5% CO_2 humidified atmosphere. Following this, an immunofluorescence protocol was performed to visualize and quantify the viral particles. Briefly, cells were fixed with

4% paraformaldehyde (4% PFA; Fisher Scientific, Waltham, MA, USA) at room temperature (RT) during 30 min, permeabilized with 0.2% Triton-X 100 (Sigma, St. Louis, MO, USA) for 10min, blocked with 1% Bovine Serum Albumin (1% BSA; Sigma, St. Louis, MO, USA) for 10min and incubated with a mouse DENV E protein antibody (4G2 antibody, anti-Flavivirus E protein, GTX57154, GeneTex, Irvine, CA, USA) for 1h at RT. Then, cells were incubated with an anti-mouse Alexa 488-conjugated secondary antibody for 1h (Thermo Scientific, Waltham, MA, USA), followed by nuclear staining with 4',6-diamidino-2-phenylindole (DAPI; BioRad, Hercules, CA, USA) for 1h, before analysis. Images were acquired as described below, and the Fluorescent Focus Units (FFU) were counted. All experiments involving infectious DENV2 were performed under Biosafety Level 3 (BSL3) conditions in accordance with the institutional guidelines.

Cell Viability Assays

Firstly, the effect of the drugs on cell viability was evaluated (**Figure 6**). Vero and Huh-7 cells were seeded in 96-well imaging plates at densities of $1x10^4$/well and $2x10^4$/well, respectively, 24h before drug testing, and cultured overnight at 37°C and 5% CO_2. Then, eight final concentrations (0.5μM, 1μM, 5μM, 10μM, 50μM, 100μM, 150μM and 300μM) of each compound, resuspended in DMEM containing 1% P/S, were tested. DMSO (tested volumes were equivalent to the ones used in the drug dilutions) and non-treated cells were used as controls. To the 100μL of seeded cells in the plates (one for each cell line), 100μL of each compound were added, for the eight tested concentrations. Each concentration had two replicates. Following a 48h incubation with the drug, cells were fixed with 4% PFA for 10min, permeabilized with 0.2% Triton-X 100 for 10min and stained with the nuclear marker DAPI for 1h. After this, images of each well were acquired and analysed as described below. Three independent experiments were performed (N=3), and data were presented as percentage compared with no drug administration control wells. The half maximal inhibitory concentration (IC_{50}) values in the absence of infection were calculated with Prism software as described below.

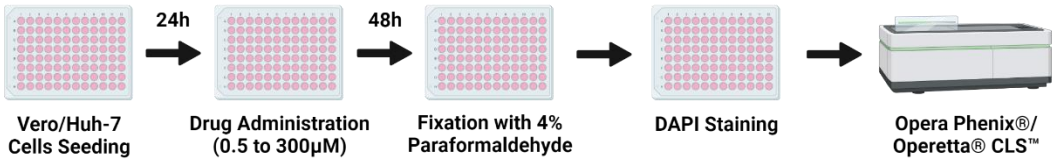

Figure 6 - Schematic representation of the workflow for the cell viability assays. Created with Biorender.com.

Infection Kinetics Assays - optimization

After the cell viability assays, tests were conducted to find the optimal DENV2 concentration and incubation time to be used in the infection assays. For this, Vero cells were seeded in 96-well plates at the density of $1x10^4$/well and cultured for 24h at 37°C and 5% CO_2. Then, to the 100µL of seeded cells in each well, 100µL of six dilutions of each compound, resuspended in DMEM containing 1% P/S, were added, resulting in six final concentrations (0.5µM, 1µM, 5µM, 10µM, 50µM and 100µM). Each drug concentration had three replicates. Non-treated infected cells and infected cells treated only with DMSO were used as controls. After 1h incubation with the drugs, Vero cells were infected with the DENV2 strain in a MOI of 0.1 or 1, by adding 50µL of viral solution to each well. Vero cells were incubated with the virus either during 24h or 48h for both MOI. Following these, the cells were fixed with 4% PFA for 30min, immunofluoresced against DENV2 E protein using a mouse anti DENV E protein primary antibody and an anti-mouse Alexa 488-conjugated secondary antibody, and stained with DAPI for 1h at RT. After this, images of each well were acquired as described below.

Effects of drugs on viral infection

After the optimization of the conditions for the DENV2 infection, the effect of the different drugs on infection was evaluated in Vero and Huh-7 cells (one plate for each cell line), following all the steps described in the above section, but only for MOI=1 and 48h incubation (**Figure 7**). Three independent experiments were performed for each cell line (N=3). Data is presented as percentage compared with DMSO only infected control wells and IC_{50} values were calculated with Prism software as described below.

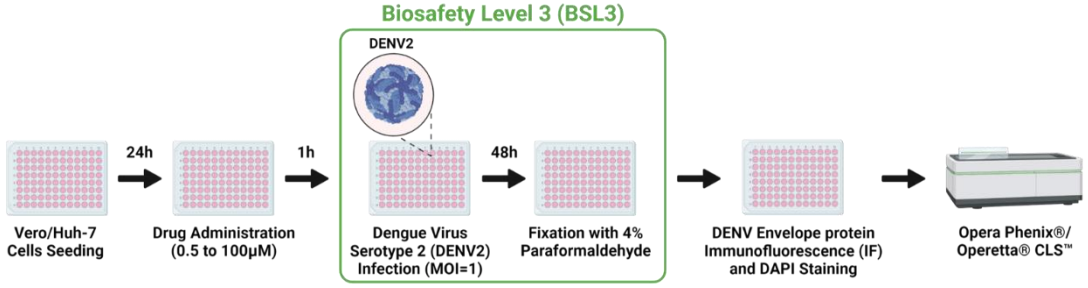

Figure 7 - Schematic representation of the workflow for the infection assays. Created with Biorender.com.

Image Analysis and Statistics

The images of the 96-well plates from the different experiments were acquired on the Opera Phenix®/Operetta® CLS™ high-content analysis systems (PerkinElmer, Waltham, MA, USA) with a 10x air magnifying objective. For the cell viability assays, a protocol to count the total Vero and Huh-7 cell number based on the nuclei DAPI staining was created on the Harmony® high-content analysis software (PerkinElmer, Waltham, MA, USA), following image acquisition. For this, several settings were optimised, namely the thresholds for the software-recognised objects size, roundness and DAPI signal intensity, which allowed to identify cell nuclei. Only after this, the cell nuclei per well were automatically counted by the software, based on the DAPI output. For the infection assays, another protocol was created in the same software. This protocol identified the cell nuclei in the same way, plus detected the immunofluorescence of the secondary antibody, allowing the identification of infected cells. The thresholds for signal intensity of the secondary antibody were also adjusted to eliminate background noise. After this, the software was used to count the total number of infected cells, based on the immunofluorescence output. All the results were reviewed to check for errors originated from the automatic method, and to change settings to improve reliability.

Following the automatic counting of the total number of cells and the number of infected cells per condition, the results were normalised as percentage compared to the controls (no drug administration for the cell viability assays and DMSO-only treated and infected cells for the infection assays). Then, the Prism software GraphPad was used to calculate the mean values and their standard deviation (SD). These values were plotted as dose-response curves, from which the respective IC_{50} of each drug for cell viability and DENV2 infection was determined using GraphPad non-linear regressions.

Results

1. Cell Viability Assays

The effects of the different drug concentrations on the cell viability of Vero and Huh-7 cells are presented in **Figures 8** and **9**, respectively. The number of Vero and Huh-7 cells was markedly affected by bexarotene and vitamin D3 at concentrations between 50μM and 300μM. The drugs rosiglitazone and ribavirin affected both cell lines viability at the highest tested concentration, 300μM. 25-hydroxycholesterol caused a marked reduction of Huh-7 viability at concentrations between 50μM and 300μM, while Vero viability was most affected at 150μM and 300μM.

The mean cell viability values and determined IC_{50} are represented in **Figure 10**. Overall, the viability of Vero and Huh-7 cells declined in a similar way when they were exposed to crescent concentrations of each of the drugs bexarotene (**Figure 10A**), vitamin D3 (**Figure 10B**), rosiglitazone (**Figure 10D**) and ribavirin (**Figure 10E**). The Huh-7 cells had higher IC_{50} than the Vero cells to these four drugs (**Figure 10**), hence showing less vulnerability. Conversely, the Huh-7 cells were more sensitive to 25-hydroxycholesterol than Vero cells, presenting an IC_{50} of 24.25μM (**Figure 10C**), the lowest determined IC_{50} in the cell viability assays.

Overall, the cell viability assays showed that the 100 to 300μM concentrations of bexarotene, vitamin D3 and 25-hydroxycholesterol caused a marked reduction on the viability of both cell lines. At 150μM and 300μM, rosiglitazone reduced both cell lines viability, while ribavirin only presented such effect at 300μM. Taking these results into account, the two highest tested concentrations for each drug, 150μM and 300μM, were considered unsafe and excluded from the following infection experiments.

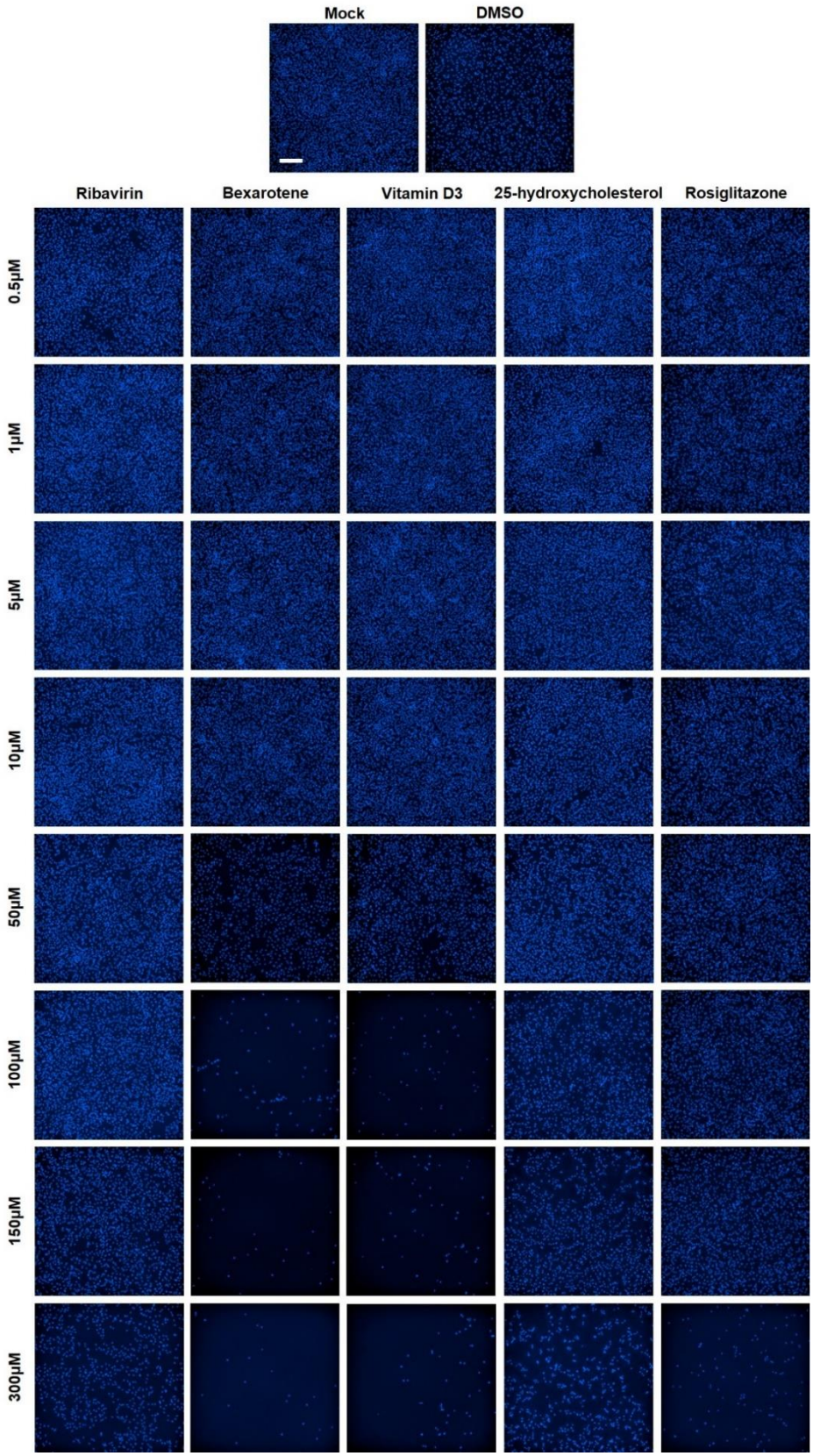

Figure 8 - Representative images of the effect of bexarotene, vitamin D3, 25-hydroxycholesterol, rosiglitazone and ribavirin on Vero cell viability. Vero cells were exposed to eight different concentrations [0.5, 1, 5, 10, 50, 100, 150 and 300µM] of each of the tested drugs during 48h, followed by fixation and staining with DAPI. DMSO represents the highest volume used in the drugs dilution. Images of the DAPI staining of Vero cell nuclei were obtained in the Opera Phenix® with a 10x air magnifying objective and one representative field per condition was chosen. Three independent experiments were performed (N=3). Scale bar equals 200µm.

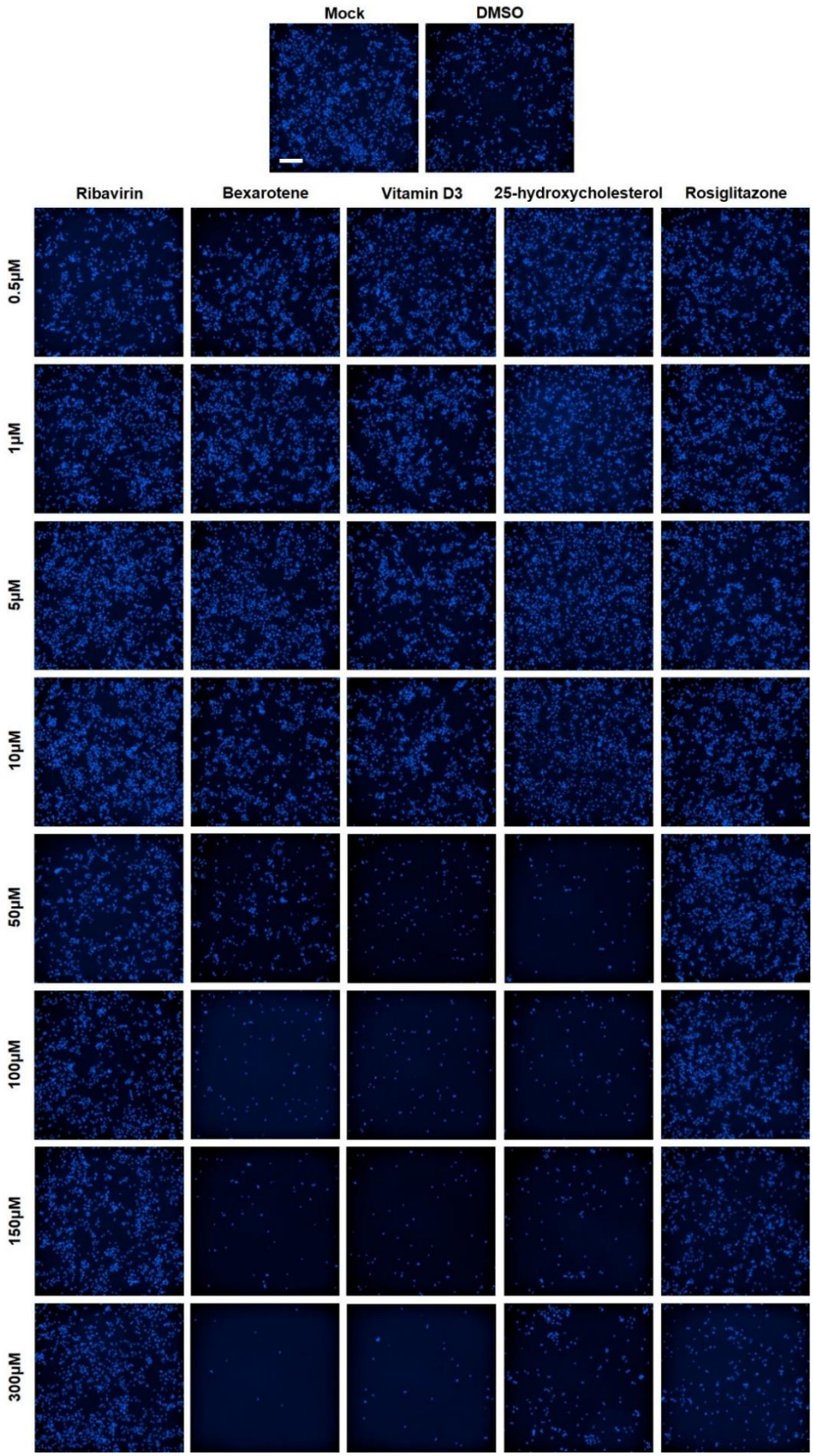

Figure 9 - Representative images of the effect of bexarotene, vitamin D3, 25-hydroxycholesterol, rosiglitazone and ribavirin on Huh-7 cell viability. Huh-7 cells were exposed to eight different concentrations [0.5, 1, 5, 10, 50, 100, 150 and 300µM] of each of the tested drugs during 48h, followed by fixation and staining with DAPI. DMSO represents the highest volume used in the drugs dilution. Images of the DAPI staining of Huh-7 cell nuclei were obtained in the Opera Phenix® with a 10x air magnifying objective and one representative field per condition was chosen. Three independent experiments were performed (N=3). Scale bar equals 200µm.

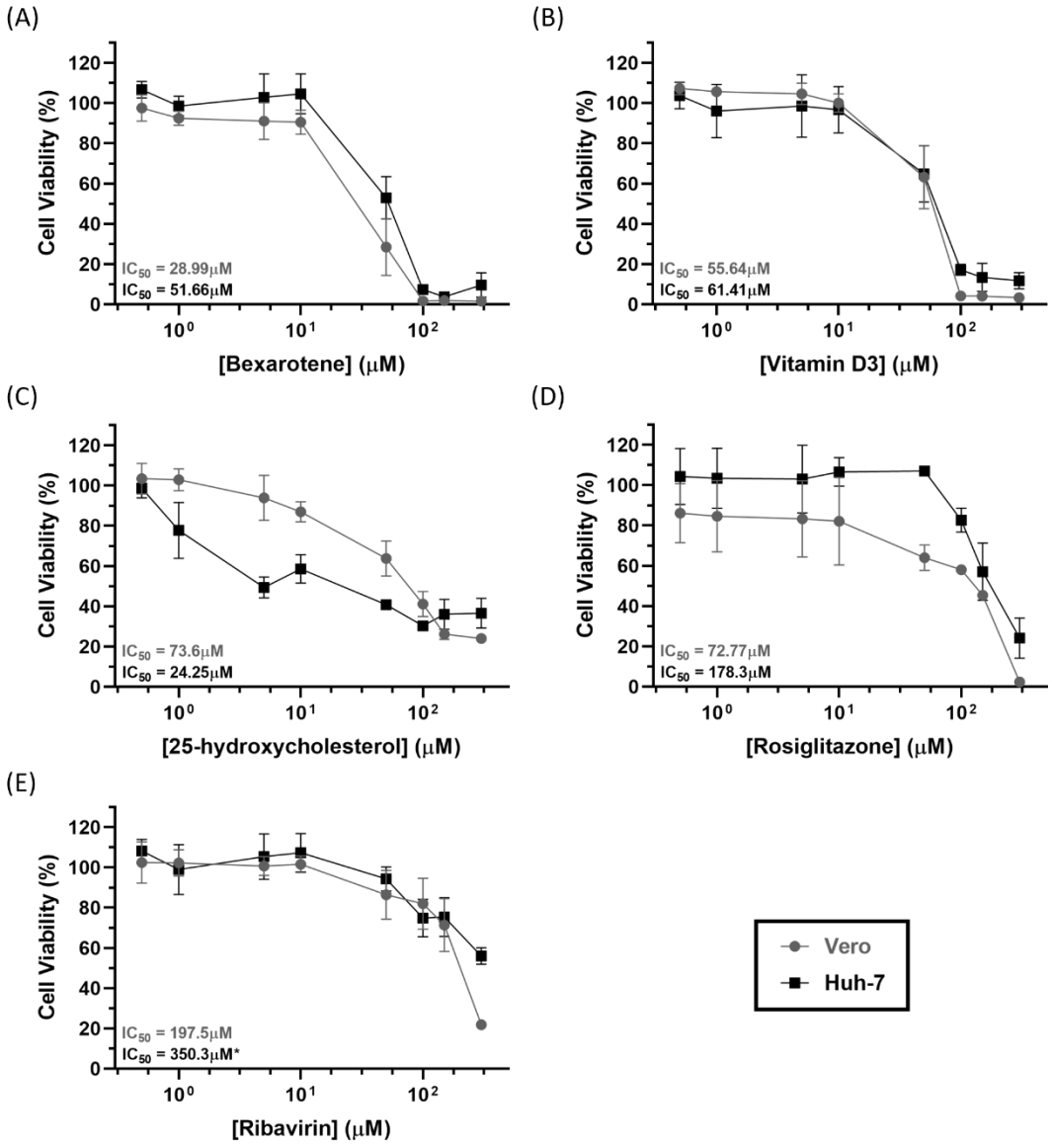

Figure 10 - The effect of bexarotene, vitamin D3, 25-hydroxycholesterol, rosiglitazone and ribavirin on Vero and Huh-7 cell viability. Plots represent the dose-response curves of the effect of eight different concentrations [0.5, 1, 5, 10, 50, 100, 150 and 300μM] of each of the tested drugs, **(A)** bexarotene, **(B)** vitamin D3, **(C)** 25-hydroxycholesterol, **(D)** rosiglitazone and **(E)** ribavirin, on the cell viability of Vero (grey) and Huh-7 (black) cells, with the IC_{50} values. Data are plotted as percentage compared with no drug administration control wells. Values represent mean and SD of three independent experiments for each cell line (N=3). Graphs and IC_{50} were determined with Prism software. *estimated IC_{50} (not included in the interval of tested drug concentrations).

2. Infection Kinetics

After the cell viability assays, tests were performed to study and optimise the DENV2 infection settings, by comparing two MOIs (0.1 and 1) and two incubation times (24h and 48h). Exposing Vero cells to DENV2 in a MOI=0.1 and 24h incubation led to no visible infection (results not shown). The MOI=1 24h incubation condition led to few and dispersed infected Vero cells (**Figure 11**). Still, the higher concentrations of bexarotene, vitamin D3, 25-hydroxycholesterol and ribavirin seemed to have some effect on the DENV2 infection (**Supplementary Figures S1**, **S2**, **S3** and **S5**, respectively). The

MOI=0.1 48h incubation setting (**Figure 11**) apparently led to more infected cells than in the MOI=1 24h, and the infected cells appeared to be closely localized in groups. Despite these differences, bexarotene, vitamin D3, 25-hydroxycholesterol and ribavirin also seemed to have some effect on Vero cell infection (**Supplementary Figures S1**, **S2**, **S3** and **S5**, respectively). The MOI=1 48h incubation condition had the most infected cells, which were well spread across the well, and a similar pattern in the drug effect over infection. Overall, both the MOI=0.1 48h incubation as the MOI=1 24h incubation led to low and hard to quantify DENV2 infection of Vero cells, when compared with the MOI=1 48h incubation condition. Thus, the MOI=1 48h was selected as the setting to use for the following infection assays of Vero and Huh-7 cells.

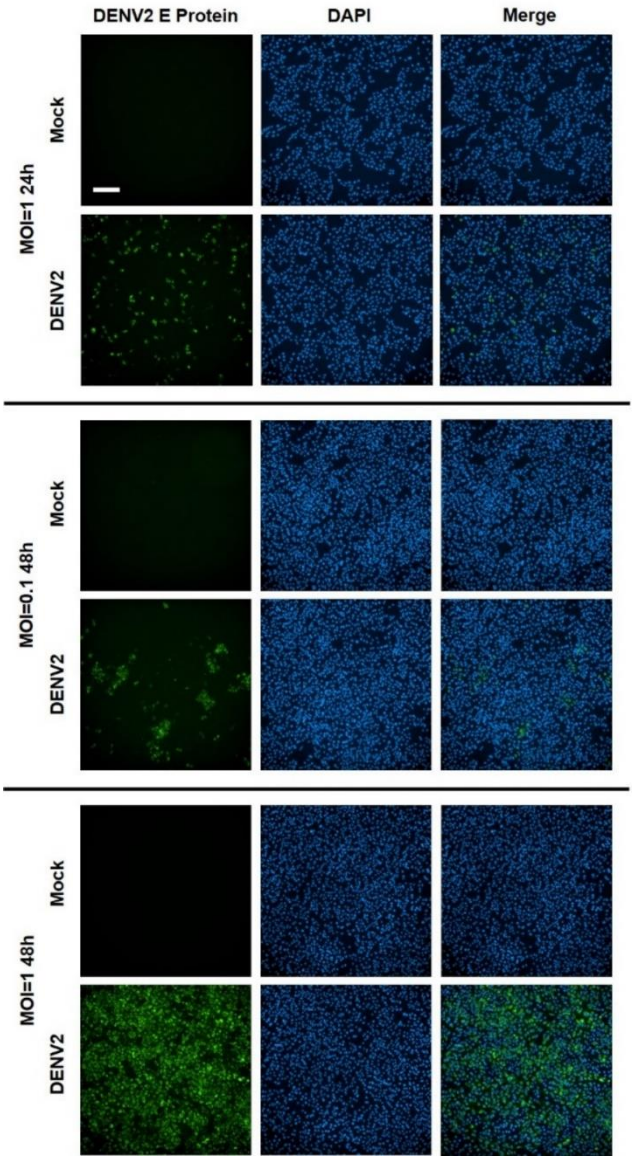

Figure 11 - The influence of different MOIs and times of incubation on the DENV2 infection of Vero cells. Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after the cells were subjected to three infection conditions: MOI=1 and a 24h incubation with the virus (MOI=1 24h), MOI=0.1 and a 48h incubation (MOI=0.1 48h), and MOI=1 and a 48h incubation (MOI=1 48h). Images were obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200µm.

3. Infection Assays

After the optimisation of the DENV2 infection conditions, the DENV2 infections assays were performed. Controls are represented in **Figure 12**, while **Figures 13** and **14** show the reduction of the DENV2 infection by the antiviral and positive control ribavirin. The results of each drug candidate will be presented in the following subsections.

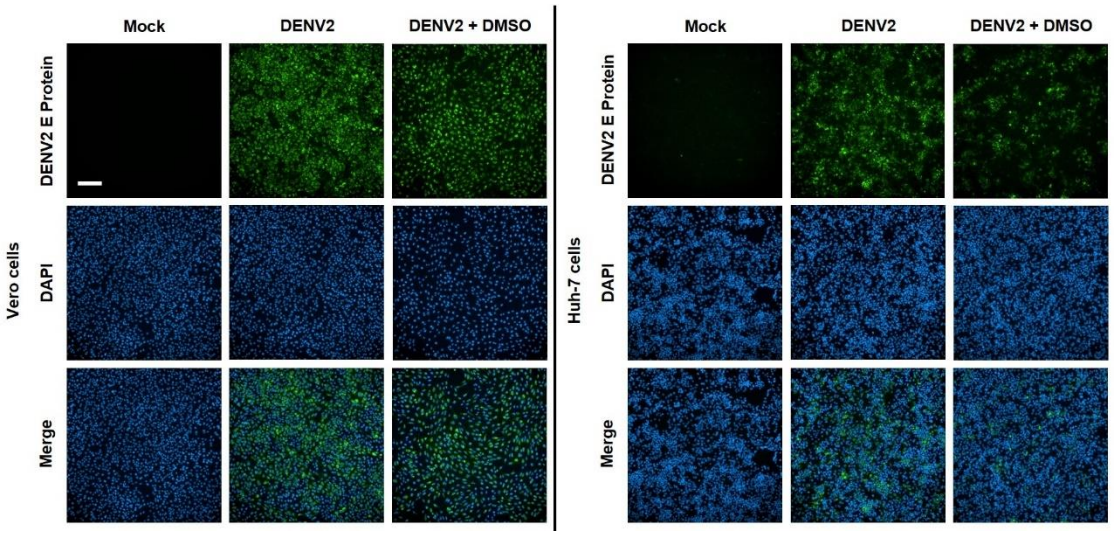

Figure 12 - **Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero and Huh-7 cell nuclei (blue) in controls - mock (no infection), DENV2 infection, DENV2 infection with DMSO.** Images obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200µm.

In the infection assays, ribavirin did not severely affect Vero viability (**Figure 13A**), leading to an estimated IC_{50} of 110.3µM (**Figure 13B**) superior to maximum tested concentration, 100µM. This finding is concordant with the cell viability assay results, in which only 300µM of ribavirin slightly reduced Vero viability (**Figure 8**). Still, the estimated IC_{50} of 110.3µM is inferior to the corresponding IC_{50} for Vero viability without infection, 197.5µM (**Figure 10E**). Ribavirin reduced the DENV2 infection of Vero cells between 10 and 50µM (**Figure 13A**), so the determined IC_{50} of 22.88µM (**Figure 13B**) was in this interval.

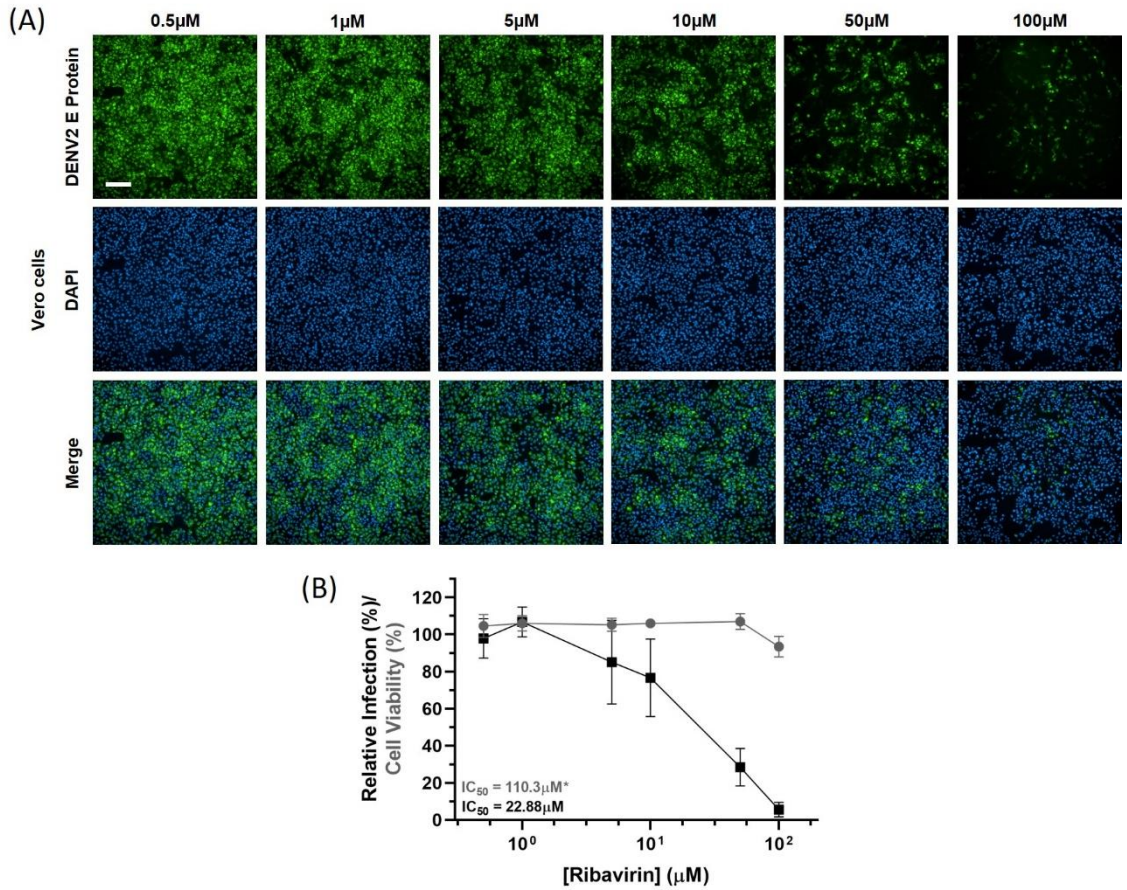

Figure 13 - Ribavirin reduces the DENV2 infection of Vero cells. (A) Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after ribavirin administration, obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200μm. **(B)** Plots representing the dose-response curves of the effect of the six different concentrations of ribavirin on the DENV2 infection (black) and cell viability in the presence of infection (grey) of Vero cells, with the respective IC_{50} values. Data are plotted as percentage compared with DMSO only control wells. Values represent mean and SD of three independent experiments (N=3). Graphs and IC_{50} were determined with Prism software. *estimated IC_{50} (not included in the interval of tested drug concentrations).

Ribavirin led to similar effects on Huh-7 cells. This antiviral did not compromise their viability at the tested concentrations (**Figure 14A**), so the IC_{50} for Huh-7 viability with infection, 121.5μM (**Figure 14B**), was estimated as well. Still, the estimated IC_{50} of 121.5μM is inferior to the corresponding estimated IC_{50} for Huh-7 viability without infection, 350.3μM (**Figure 10E**). Ribavirin also reduced the DENV2 infection of Huh-7 cells in the same concentration range of 10 to 50μM (**Figure 14A**), leading to a determined IC_{50} of 18.29μM (**Figure 14B**). Overall, ribavirin had equivalent effects on both cell lines by reducing their DENV2 infection at similar concentrations, while not compromising their cell viability.

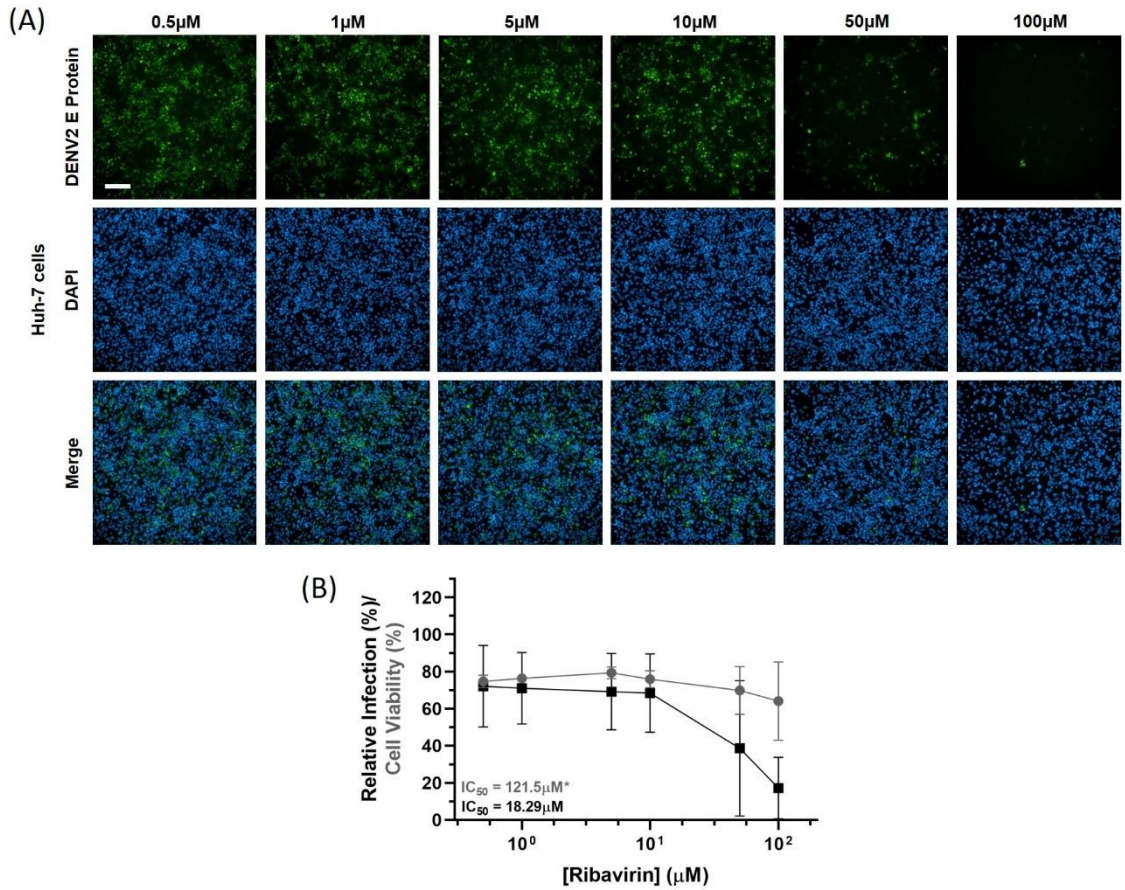

Figure 14 - Ribavirin reduces the DENV2 infection of Huh-7 cells. (A) Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Huh-7 cell nuclei (blue) after ribavirin administration, obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200μm. **(B)** Plots representing the dose-response curves of the effect of the six different concentrations of ribavirin on the DENV2 infection (black) and cell viability in the presence of infection (grey) of Huh-7 cells, with the respective IC_{50} values. Data are plotted as percentage compared with DMSO only control wells. Values represent mean and SD of three independent experiments (N=3). Graphs and IC_{50} were determined with Prism software. *estimated IC_{50} (not included in the interval of tested drug concentrations).

3.1. The effect of Bexarotene on DENV2 infection

Similarly to what occurred in the cell viability assays (**Figure 8**), 100μM of bexarotene led to the death of almost all Vero cells in the presence of infection (**Figure 15A**). The determined IC_{50} for Vero cell viability in the presence of infection was 53.44μM (**Figure 15B**), a higher value than the IC_{50} for Vero cell viability in the absence of infection - 28.99μM (**Figure 10A**). This difference may be explained by the fact that DENV2 infection may induce Vero cell division for more viral replication, therefore increased concentrations of bexarotene were needed to reduce 50% of the number of Vero cells. Bexarotene reduced the number of Vero cells infected with DENV2 between the tested

concentrations of 10μM and 50μM (**Figure 15A**). In accordance with this observation, the determined IC_{50} value for infection was 19.71μM (**Figure 15B**).

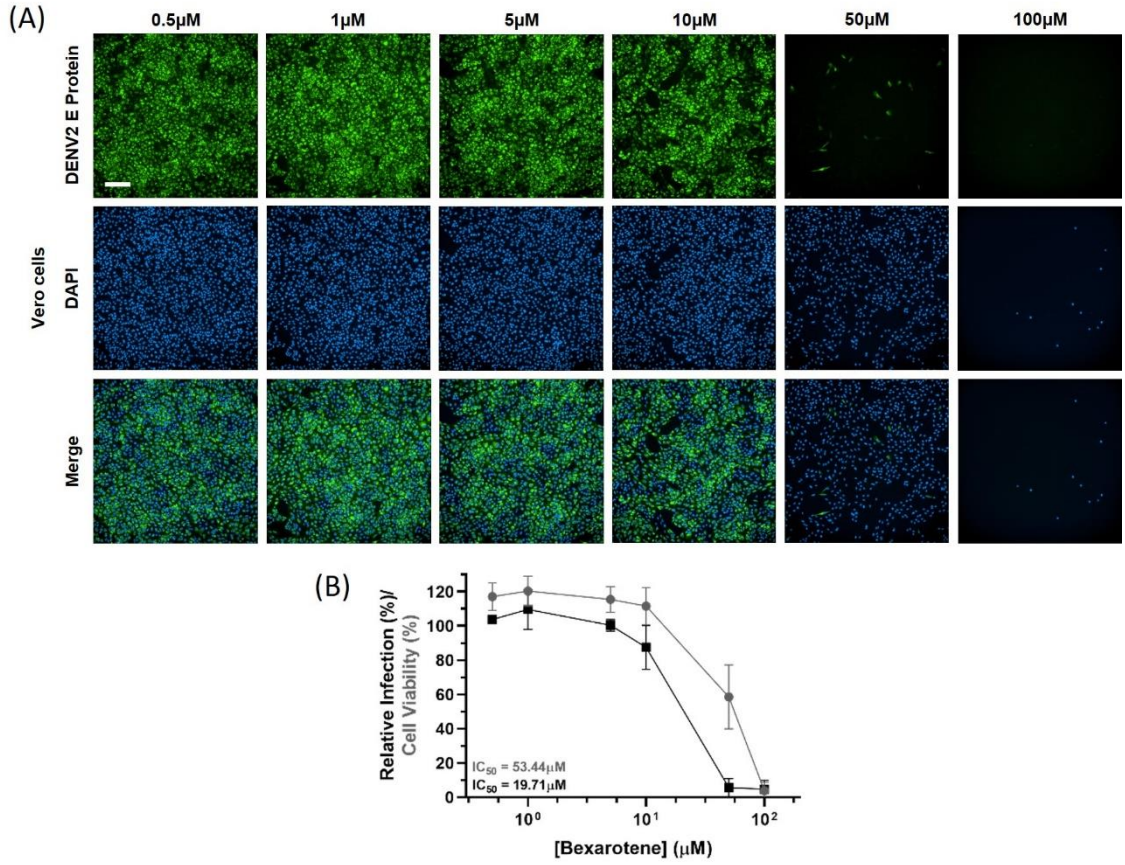

Figure 15 - Bexarotene reduces the DENV2 infection of Vero cells. (A) Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after bexarotene administration, obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200μm. **(B)** Plots representing the dose-response curves of the effect of the six different concentrations of bexarotene on the DENV2 infection (black) and cell viability in the presence of infection (grey) of Vero cells, with the respective IC_{50} values. Data are plotted as percentage compared with DMSO only control wells. Values represent mean and SD of three independent experiments (N=3). Graphs and IC_{50} were determined with Prism software.

Comparably to Vero cells, 100μM of bexarotene also killed most of Huh-7 cells in both cell viability (**Figure 9**) and infection assays (**Figure 16A**). For Huh-7 cells, the determined IC_{50} for cell viability in the presence of infection was 62.97μM (**Figure 16B**), a higher value than the IC_{50} for Vero cell viability in the absence of infection – 51.66μM (**Figure 10A**). Bexarotene also reduced the DENV2 infection of Huh-7 cells between the concentrations of 10μM and 50μM (**Figure 16A**), presenting an IC_{50} of 19.00μM (**Figure 16B**). Overall, bexarotene led to similar effects on both cell lines, reducing their DENV2 infection.

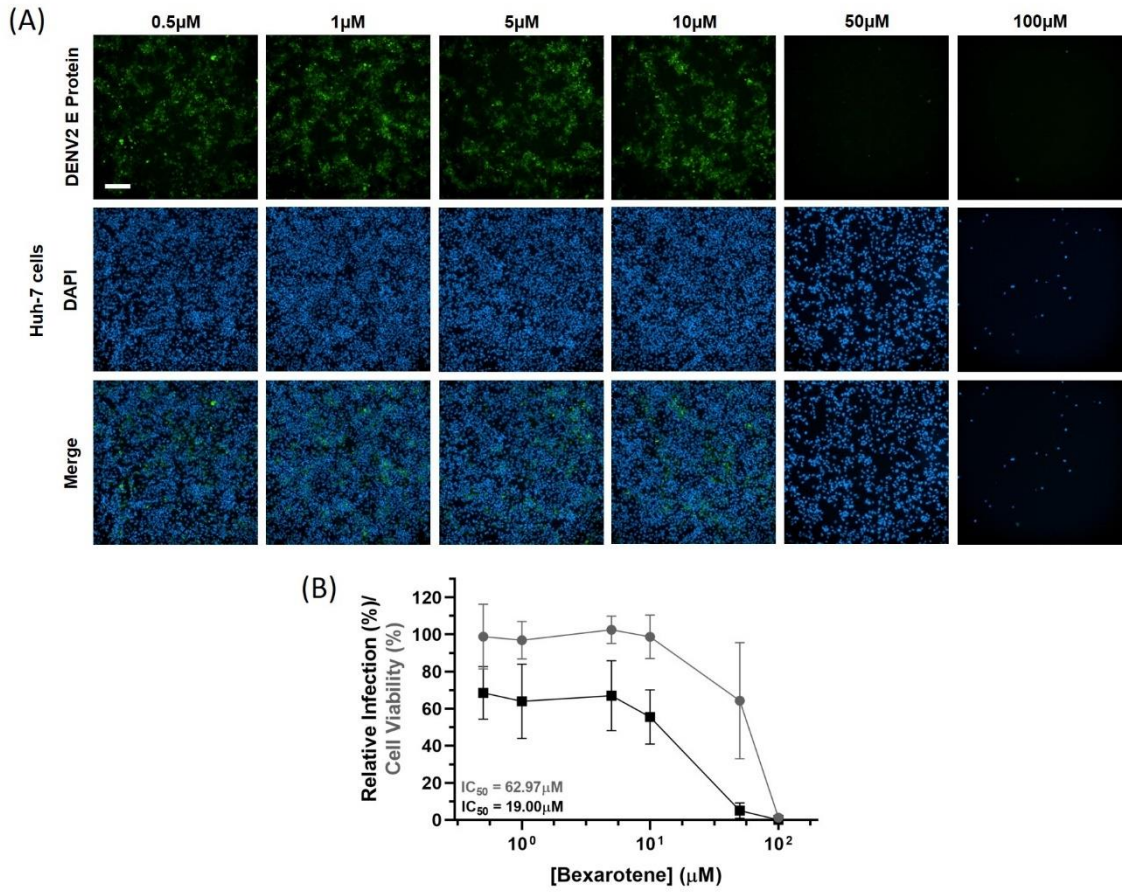

Figure 16 - Bexarotene reduces the DENV2 infection of Huh-7 cells. (A) Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Huh-7 cell nuclei (blue) after bexarotene administration, obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200μm. **(B)** Plots representing the dose-response curves of the effect of the six different concentrations of bexarotene on the DENV2 infection (black) and cell viability in the presence of infection (grey) of Huh-7 cells, with the respective IC_{50} values. Data are plotted as percentage compared with DMSO only control wells. Values represent mean and SD of three independent experiments (N=3). Graphs and IC_{50} were determined with Prism software.

3.2. The effect of Vitamin D3 on DENV2 infection

In accordance with the cell viability assays (**Figure 8**), vitamin D3 caused the death of almost all of Vero cells at 100μM in the infection assays (**Figure 17A**). The vitamin D3 IC_{50} for Vero cell viability without and with infection were very close, consisting of 55.64μM (**Figure 10B**) and 54.45μM (**Figure 17B**), respectively. Vitamin D3 reduced the DENV2 infection of Vero cells at concentrations between 10μM and 50μM (**Figure 17A**), presenting an IC_{50} of 17.72μM (**Figure 17B**).

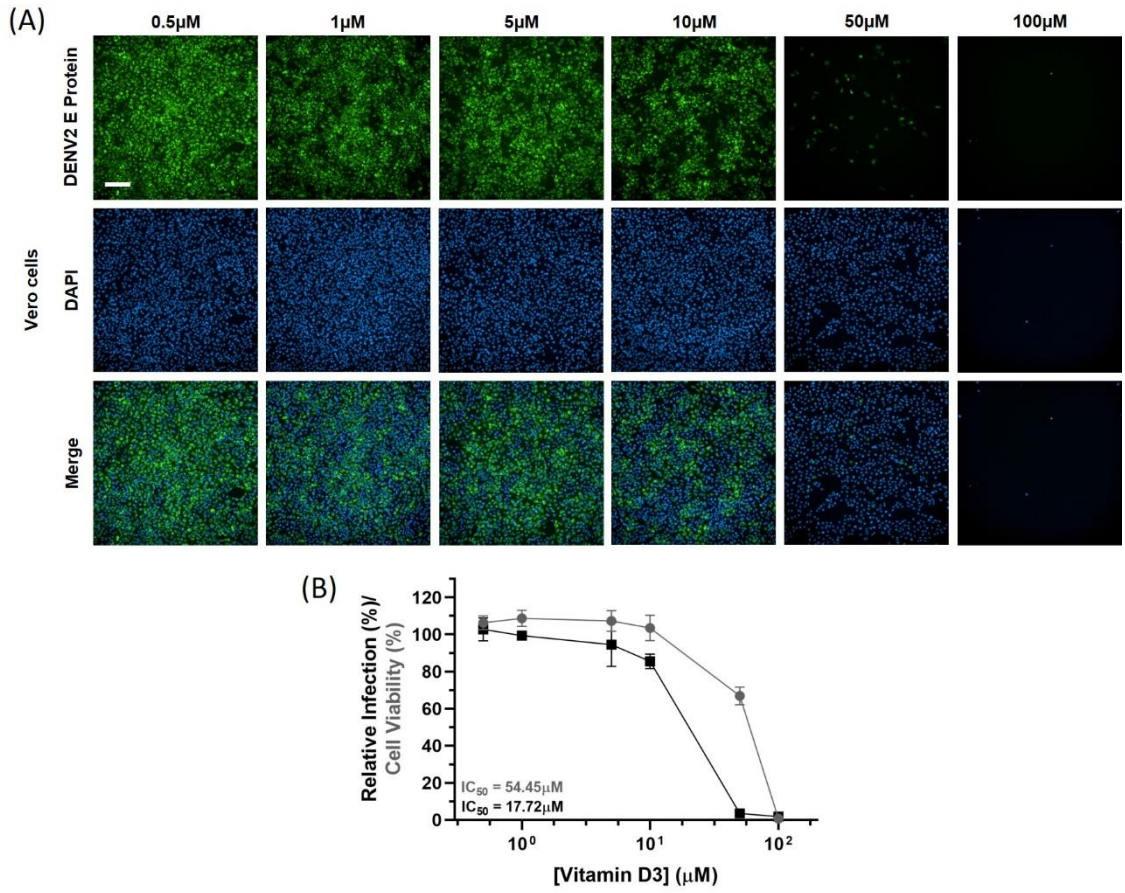

Figure 17 - **Vitamin D3 reduces the DENV2 infection of Vero cells. (A)** Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after vitamin D3 administration, obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200μm. **(B)** Plots representing the dose-response curves of the effect of the six different concentrations of vitamin D3 on the DENV2 infection (black) and cell viability in the presence of infection (grey) of Vero cells, with the respective IC_{50} values. Data are plotted as percentage compared with DMSO only control wells. Values represent mean and SD of three independent experiments (N=3). Graphs and IC_{50} were determined with Prism software.

Regarding Huh-7 cells, vitamin D3 caused the death of almost all these cells at 100μM in the infection assays (**Figure 18A**), similarly to what occurred in the cell viability assays (**Figure 9**). The vitamin D3 IC_{50} for Huh-7 cell viability without and with infection were 61.41μM (**Figure 10B**) and 53.82μM (**Figure 18B**), respectively. Vitamin D3 reduced the DENV2 infection of Huh-7 cells at concentrations between 10μM and 50μM (**Figure 18A**), presenting an IC_{50} of 45.39μM (**Figure 18B**). These results indicate that vitamin D3 is toxic to both cell lines at similar concentrations and effective in reducing their DENV2 infection.

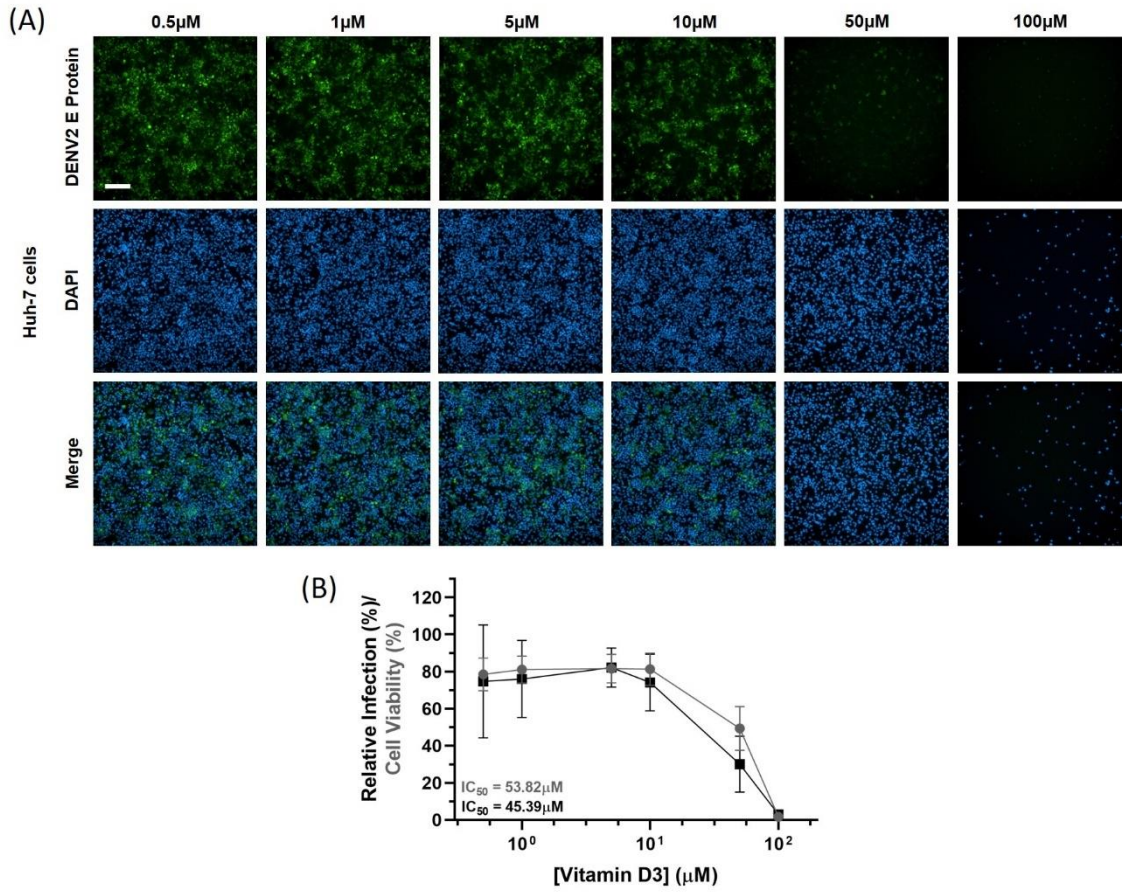

Figure 18 - Vitamin D3 reduces the DENV2 infection of Huh-7 cells. (A) Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Huh-7 cell nuclei (blue) after vitamin D3 administration, obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200μm. **(B)** Plots representing the dose-response curves of the effect of the six different concentrations of vitamin D3 on the DENV2 infection (black) and cell viability in the presence of infection (grey) of Huh-7 cells, with the respective IC_{50} values. Data are plotted as percentage compared with DMSO only control wells. Values represent mean and SD of three independent experiments (N=3). Graphs and IC_{50} were determined with Prism software.

3.3. The effect of 25-hydroxycholesterol on DENV2 infection

In the presence of infection, 25-hydroxycholesterol markedly reduced the number of Vero cells between 50 and 100μM (**Figure 19A**), and the determined IC_{50} for Vero viability in the presence of infection was 52.47μM (**Figure 19B**). This value is inferior to 73.6μM, the IC_{50} of Vero viability without infection (**Figure 10C**). Regarding DENV2 infection of Vero cells, 25-hydroxycholesterol greatly decreased it at concentrations between 1μM and 5μM (**Figure 19A**), so an IC_{50} of 1.71μM was determined (**Figure 19B**).

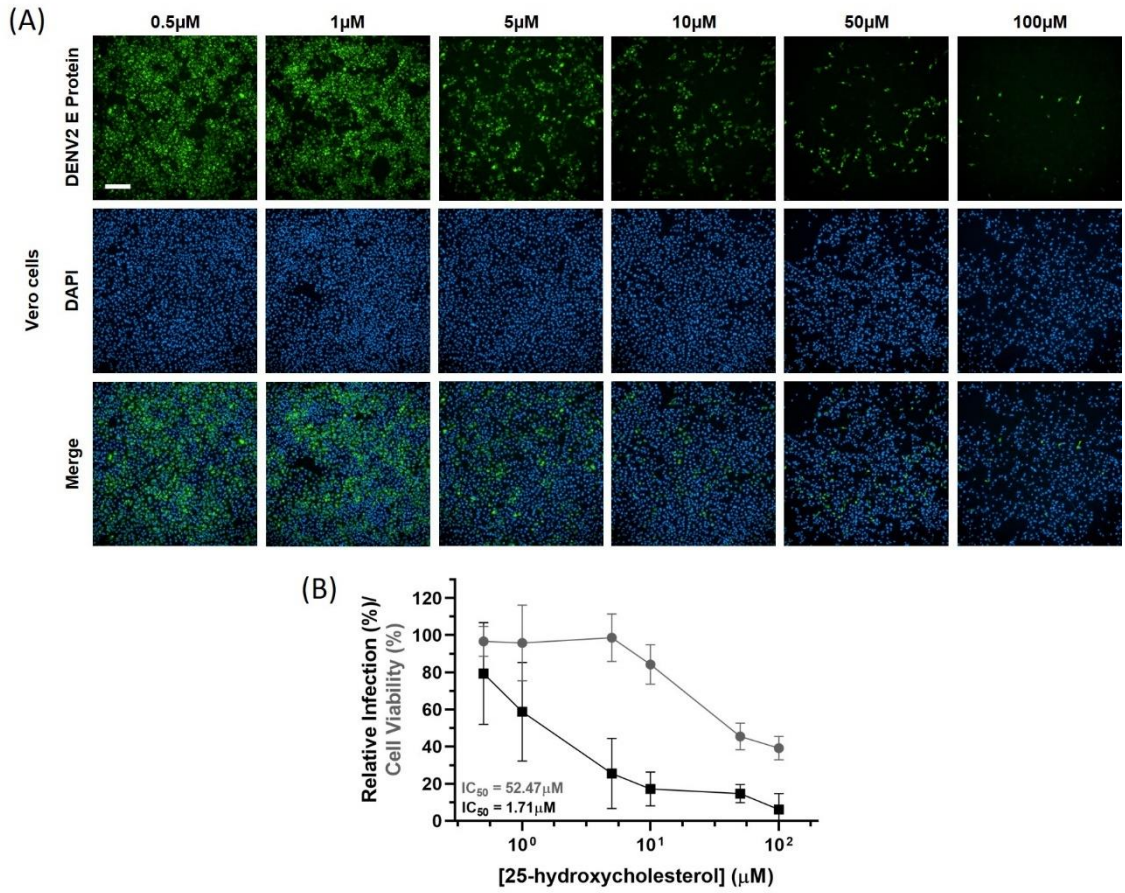

Figure 19 - 25-hydroxycholesterol reduces the DENV2 infection of Vero cells. (A) Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after 25-hydroxycholesterol administration, obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200μm. **(B)** Plots representing the dose-response curves of the effect of the six different concentrations of 25-hydroxycholesterol on the DENV2 infection (black) and cell viability in the presence of infection (grey) of Vero cells, with the respective IC_{50} values. Data are plotted as percentage compared with DMSO only control wells. Values represent mean and SD of three independent experiments (N=3). Graphs and IC_{50} were determined with Prism software.

When it came to Huh-7 cells, 25-hydroxycholesterol both greatly harmed them and reduced their DENV2 infection at low concentrations (**Figure 20A**), presenting an IC_{50} of 4.60μM for cell viability with infection and an IC_{50} of 2.31μM for infection (**Figure 20B**). Similarly to what happened to Vero cells, the 25-hydroxycholesterol IC_{50} of Huh-7 viability with infection, 4.60μM, was inferior to the corresponding IC_{50} of Huh-7 viability without infection, 24.25μM (**Figure 10C**). These results suggest that 25-hydroxycholesterol administration paired with DENV2 infection may have a synergistic effect in decreasing cell viability, with more marked effects on hepatocytes. Still, 25-hydroxycholesterol reduced DENV2 infection of both cell lines at low concentrations.

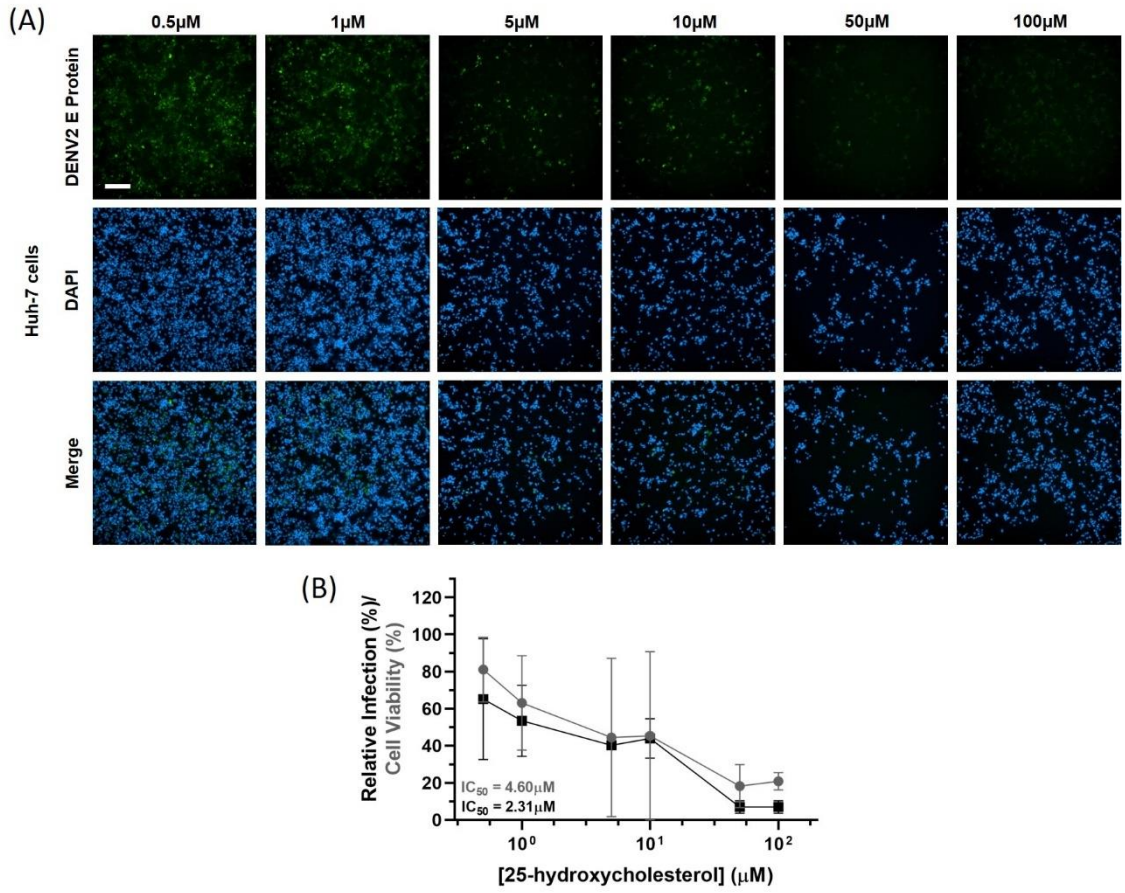

Figure 20 - 25-hydroxycholesterol reduces the DENV2 infection of Huh-7 cells. (A) Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Huh-7 cell nuclei (blue) after 25-hydroxycholesterol administration, obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200μm. **(B)** Plots representing the dose-response curves of the effect of the six different concentrations of 25-hydroxycholesterol on the DENV2 infection (black) and cell viability in the presence of infection (grey) of Huh-7 cells, with the respective IC_{50} values. Data are plotted as percentage compared with DMSO only control wells. Values represent mean and SD of three independent experiments (N=3). Graphs and IC_{50} were determined with Prism software.

3.4. The effect of Rosiglitazone on DENV2 infection

In accordance with the cell viability assays (**Figure 8**), the rosiglitazone concentrations used in the infection assays did not markedly reduce the number of Vero cells (**Figure 21A**). The estimated rosiglitazone IC_{50} for Vero cell viability in the presence of infection was 343.3μM (**Figure 21B**), a superior value to the highest tested concentration, 100μM. This estimated 343.3μM is much higher than the corresponding IC_{50} for Vero cell viability without infection, 72.77μM (**Figure 10D**). Rosiglitazone did not reduce the DENV2 infection of Vero cells (**Figure 21A**), so no IC_{50} could be determined.

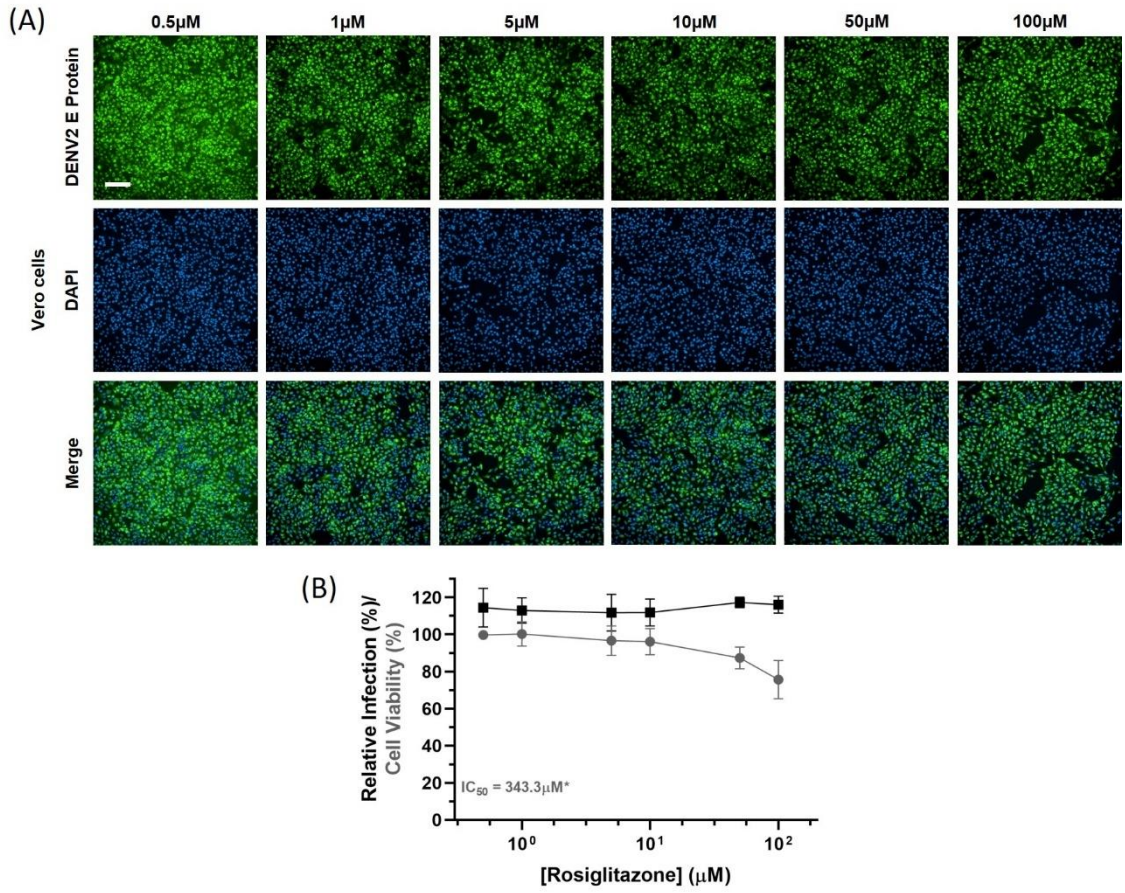

Figure 21 - Rosiglitazone doesn't reduce the DENV2 infection of Vero cells. (A) Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after rosiglitazone administration, obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200μm. **(B)** Plots representing the dose-response curves of the effect of the six different concentrations of rosiglitazone on the DENV2 infection (black) and cell viability in the presence of infection (grey) of Vero cells, with the respective IC_{50} values. Data are plotted as percentage compared with DMSO only control wells. Values represent mean and SD of three independent experiments (N=3). Graphs and IC_{50} were determined with Prism software. *estimated IC_{50} (not included in the interval of tested drug concentrations).

Analogously to the Vero cells, rosiglitazone did not severely compromise Huh-7 cell viability in the presence of infection (**Figure 22A**), leading to the estimated IC_{50} of 224.5μM (**Figure 22B**), also superior to 100μM. This estimated 224.5μM was superior to 178.3μM, the corresponding rosiglitazone IC_{50} for Huh-7 viability without infection (**Figure 10D**)**.** Rosiglitazone did not decrease the DENV2 infection of Huh-7 cells at the tested concentrations (**Figures 22A**), so it was not possible to determine an IC_{50}. Overall, rosiglitazone neither compromised the cells viability nor reduced their DENV2 infection.

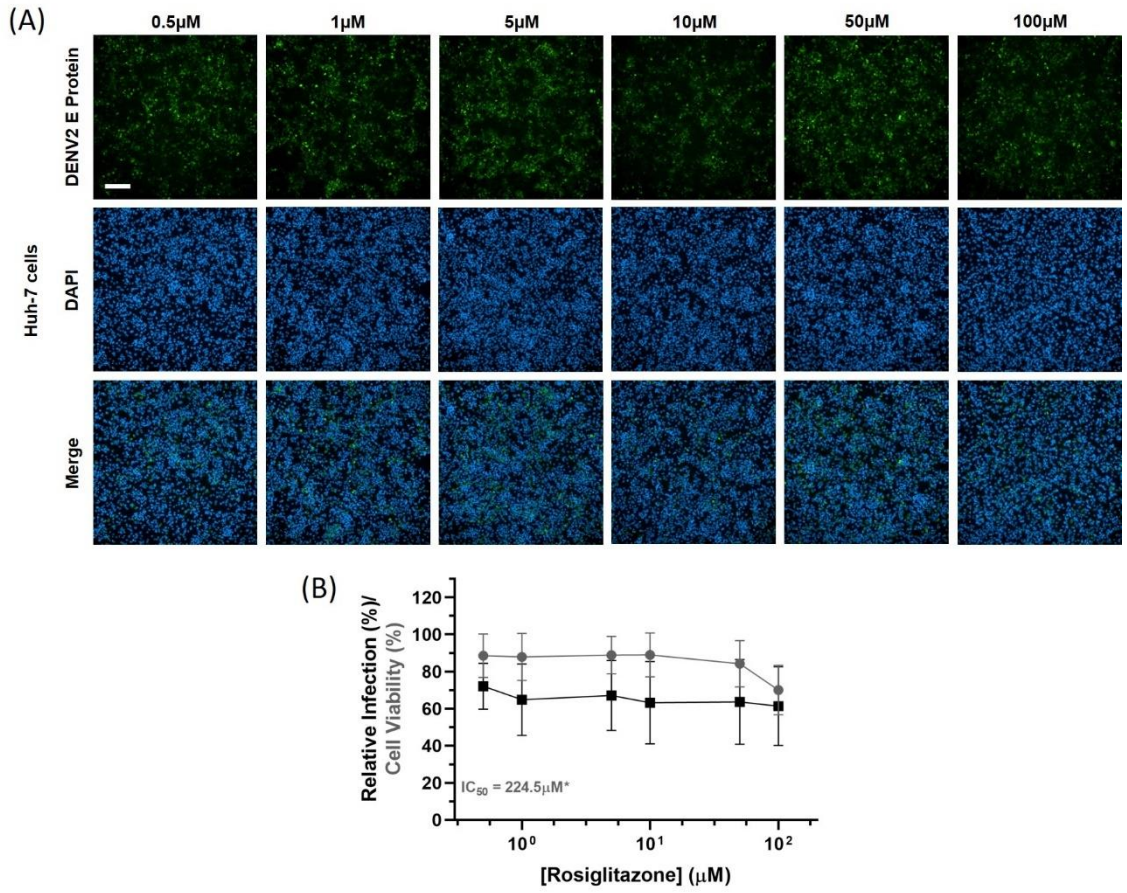

Figure 22 - Rosiglitazone doesn't reduce the DENV2 infection of Huh-7 cells. (A) Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Huh-7 cell nuclei (blue) after rosiglitazone administration, obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200μm. **(B)** Plots representing the dose-response curves of the effect of the six different concentrations of rosiglitazone on the DENV2 infection (black) and cell viability in the presence of infection (grey) of Huh-7 cells, with the respective IC_{50} values. Data are plotted as percentage compared with DMSO only control wells. Values represent mean and SD of three independent experiments (N=3). Graphs and IC_{50} were determined with Prism software. *estimated IC_{50} (not included in the interval of tested drug concentrations).

Altogether, the results for the four candidate drugs and ribavirin's IC_{50} for Vero and Huh-7 cell viability, as well as the IC_{50} for DENV2 infection, are summed up in **Table 1**.

Table 1 - IC_{50} of each drug for Vero and Huh-7 cell viability with and without infection, and for DENV2 infection. ND – Non-Determinable. *estimated IC_{50} (not included in the interval of tested drug concentrations).

	Vero cells			Huh-7 cells		
	Cell Viability (without infection)	Cell Viability (with infection)	DENV2 Infection	Cell Viability (without infection)	Cell Viability (with infection)	DENV2 Infection
Ribavirin	197.5	110.3*	22.88	350.3*	121.5*	18.29
Bexarotene	28.99	53.44	19.71	51.66	62.97	19.00
Vitamin D3	55.64	54.45	17.72	61.41	53.82	45.39
25-hydroxycholesterol	73.6	52.47	1.27	24.25	4.60	2.31
Rosiglitazone	72.77	343.3*	ND	178.3	224.5*	ND

Discussion

Lipids play roles of significant biological importance in cells. They have structural functions as they integrate membranes, energy storing functions through lipid droplets (LD) formation, signalling functions as second messengers, as well as cell to cell transport as lipoproteins (122). Pathogens like viruses need to hijack host machinery to replicate, and that includes cell lipid metabolism (122). The viral envelope is constituted by lipids and it is crucial to infection. The viral envelope allows virus fusion with the host cell membrane, as well as the release of new virions through membrane budding (123). Besides this, DENV induces the formation of certain ER-derived membrane structures, rich in lipids, to facilitate its replication (124, 125). Additionally, DENV activates autophagy, leading to free fatty acids release by the transformation of LD and triglycerides (TG) (126). These free fatty acids are used to obtain ATP for RNA replication by mitochondrial β-oxidation. The results obtained in this thesis, of the impact that drugs modulators of the RXR-related pathways have in DENV infection, seem to reinforce the important role of lipid metabolism in viral infection, and specifically in DENV.

In the cell viability assays, it was possible to conclude, in general, that these host-biology controlling drugs must be provided in relatively low concentrations (bellow 100μM) in order to be safe for cell viability. Ribavirin, an antiviral chosen to be used as positive control, had negligible impact on both cell lines viability in most tested concentrations, only affecting Vero cells viability at the high concentration of 300μM. This may be attributed to ribavirin specifically targeting viral replication (79), thus not interfering with host cellular processes. Also, we confirmed that DENV (serotype DENV2) infection assays work better with a MOI of 1 and 48h of incubation than lower concentrations and times. Even though the DENV infection depends on the characteristics of the strain and the cell type (25), different reports show that 48h are enough for quantifiable DENV replication in the mosquito C6/36 (127) and Huh-7 cells (128). Thus, the 48h time-assay is enough to interfere with entrance and beginning of replication of the virus in the cell.

Interestingly, differences were observed in two drugs effects on Huh-7 and Vero cell viability depending on DENV2 infection occurrence. Rosiglitazone had an increase in its IC_{50} for Vero and Huh-7 cell lines with infection, meaning that higher concentrations of this drug were needed to reduce 50% of the cell populations when DENV2 was present. On the other hand, 25-hydroxycholesterol's IC_{50} for only the Huh-7 cell line decreased even more with infection. This IC_{50} decrease may be explained by a synergistic hepatotoxic effect of 25-hydroxycholesterol and DENV2. It is known that the liver is one of the most affected organs in the dengue disease (26), so the impact of DENV2 infection

in hepatocytes is considerable, leading to damage and death. The cell viability IC_{50} of the two other drugs, bexarotene and vitamin D3, were not affected by DENV2 infection.

In the infection assays, bexarotene reduced identically the DENV2 infection of Vero and Huh-7 cells (IC_{50} - 19.72μM and 19μM, respectively), which is in line with the previously described antiviral effect against another *Flaviviridae* virus, the HCV (96). In that study, the determined bexarotene IC_{50} against HCV was 0.19nM (96), a notably lower value. Such variation may be explained by several reasons. The studied virus and the infection conditions (MOI and incubation time) were different, the experiments were conducted on a slightly different cell line (Huh 7.5.1 cells), bexarotene was both administered before and after the HCV infection, and the antiviral activity against HCV was evaluated through different methods (HCV RNA quantification intra and extracellularly, HCV core protein and NS5A detection) (96). Even though bexarotene mechanism of action was not characterised in that work, the study hinted that bexarotene interferes with the HCV replication and post-replication steps. Furthermore, the study showed that agonists and antagonists of the RAR and the RXR had anti-HCV activity (96). Both that study and our results provide evidence that bexarotene reduces the infection of two *Flaviviridae* viruses, therefore further research to better understand this drug antiviral effect is encouraged. Additionally, AM580, another retinoid and a RARα agonist, was able to reduce *in vitro* plaque formation by MERS-CoV, SARS-CoV, ZIKV, influenza virus, enterovirus A71 and adenovirus (129). For AM580 antiviral mechanism of action, the study proposed that this retinoid is involved in the inhibition of the SREBP pathways and in the reduction of LD and cholesterol accumulation. SREBP pathways are important for the cholesterol metabolism, and one of the SREBP isoforms, SREBP-1c, is activated by LXR receptors (130). It is hypothesised that SREBP-1c and LXR/RXR may interact to regulate lipid synthesis, as SREBP-1c is also activated through RXR and its natural ligand 9-cis-retinoic acid (130). Bexarotene's antiviral effect may be pleiotropic, acting on RXR and consequently altering the function of LXR/RXR and/or RAR/RXR heterodimers, thus modulating lipid metabolism to limit virus infection.

Vitamin D3 reduced the DENV2 infection of both cell lines, even though the IC_{50} for Huh-7 cells was higher than for Vero cells. These results are concordant with other *in vitro* studies where it has been demonstrated that vitamin D3 reduces the DENV4 serotype infection of hepatocytes (Huh-7 cells), monocytes and monocyte-derived macrophages (U937 cells), with more effective results in macrophages than hepatocytes (131). This suggests that vitamin D3 effects may vary depending on the cell type, which could explain the higher IC_{50} for Huh-7 cells in our results. Similarly, in another experiment two groups of healthy individuals were constituted, and one of the groups did vitamin D

supplementation for 10 days (132). Then, monocyte-derived macrophages were isolated from the peripheral blood samples of the individuals from both groups and infected with DENV2. The macrophages from individuals who received vitamin D supplementation were more resistant to DENV2 infection. In a kidney cell line, it has been shown that the administration of VDR agonists after DENV infection is able to inhibit infection (133). Despite the evidence of vitamin D3/D antiviral effect against DENV, the corresponding mechanism of action is still poorly understood. In a study of the *in vitro* effect of vitamin D3 in the DENV2 infection of monocyte-derived macrophages, it was shown that this drug modulates the expression of small non-coding RNAs, microRNAs (miRNAs) (102). These miRNAs regulate the mRNA translation of immunity-related genes (102, 134), namely of the transcription factor NF-kB in a VDR-dependent manner (135). Vitamin D3 treatment of macrophages leads to lower expression of the mannose receptor (MR), also known as CD209 and used by DENV during cell entry, which led to decreased DENV infection and cytokine release (e.g.: TNFα; IL-1β; IL-10) (136). Vitamin D3 reduces the expression of the innate immunity components Toll-Like Receptors (TLRs), specifically TLR2 and TLR4 (137), while vitamin D modulates TLR3 and TLR9 (132). The TLR activation by Pathogen-Associated Molecular Patterns (PAMPs) or Damage-Associated Molecular Patterns (DAMPs) leads to a signalling cascade involving Mitogen-Activated Protein Kinases (MAPK), namely the p38 and c-Jun N-terminal Kinases (JNK), culminating in the activation of the transcription factor NF-kB and the production of pro-inflammatory cytokines like TNFα and IL-8 (137). It has been reported that DENV activates the p38 and JNK kinases during macrophage infection, and the viral yield and protein secretion were reduced when the cells were treated with p38 and JNK inhibitors pre-infection (138). This raises the hypothesis that DENV may activate these kinases to aid its replication, while vitamin D3 may indirectly counter this activation. Considering all this, vitamin D3 antiviral effect against DENV may be explained by its regulation of the host inflammatory response in macrophages, but we still need to establish the molecular mechanisms of regulation in hepatocytes.

25-hydroxycholesterol reduced the DENV2 infection of Vero and Huh-7 cells at similar concentrations (IC_{50} of 1.27μM and 2.31μM, respectively), and lower than bexarotene and vitamin D3, but not without severely affecting Huh-7 cells. As mentioned, 25-hydroxycholesterol and related oxysterols have broad antiviral activity against enveloped (e.g.: ZIKV and HCV) and non-enveloped viruses (139). There are several hypotheses on how 25-hydroxycholesterol achieves this effect. For HCV, it is already known that this virus hijacks and upregulates host cell lipid metabolism, and it has been shown that 25-hydroxycholesterol administration to Huh-7 cells with HCV replicons downregulates

genes related to the mevalonate pathway, which leads to the reduction of cholesterol levels and induction of an antiviral state in the cell (140). In fact, viral infection or IFN-stimulation of macrophages leads to Signal Transducer and Activator of Transcription 1 (STAT1) binding and activation of the ISG CH25H, resulting in the production and secretion of 25-hydroxycholesterol (112). Therefore, it seems that 25-hydroxycholesterol acts as an effector molecule of innate immunity by regulating host lipid metabolism to hinder viral replication, which could explain its effect on DENV2 infection.

Rosiglitazone had no impact in the DENV2 infection of Vero and Huh-7 cells. The tested rosiglitazone concentrations may have been too low to lead to such effect. Aside from this, rosiglitazone selectively binds to one of the PPAR isoforms, PPARγ (116). This isoform regulates glucose levels, lipid biosynthesis and adipogenesis, while PPARα regulates lipid levels and PPARβ/δ regulates blood cholesterol and glucose levels, plus fatty acid oxidation (91). Since PPARα and PPARβ/δ also have roles in lipid metabolism, it is possible that their modulation could influence DENV2 infection, but it was not tested in our study. Therefore, it would be interesting to pharmacologically modulate these isoforms too by activating them using bezafibrate, for example, as this drug can bind to all PPAR isoforms (91). On the other hand, rosiglitazone was observed to increase another flavivirus infection, WNV, in *ex vivo* brain slice cultures and in mice (119), so there is a need to clarify its effect on viral infections.

In summary, the infection assays revealed that the prophylactic administration of bexarotene, vitamin D3 and 25-hydroxycholesterol reduced the DENV2 infection of Vero and Huh-7 cells, while rosiglitazone did not present such effect. Further studies are needed to confirm if the effective drugs play a role acting on the RXR-related pathways, and may have other pleiotropic effects. One thing is clear, in the design of a possible transcriptomic evaluation of the molecular impact of these drugs, the factor time is essential as infection initiates a cascade of interconnected pathways in the host response. This makes it rather difficult to disentangle what is cause and effect. Although we observed less DENV2 infection at 24h, this should be a good time point to evaluate the pathways that are activated or disrupted by the effective three drugs in the beginning of the infection process. This transcriptomic evaluation will provide useful insights about the importance of RXR-related pathways in the dengue disease.

Conclusion and Future Perspectives

This work shows that the host lipid metabolism and immune responses modulated by bexarotene, vitamin D3 and 25-hydroxycholesterol are relevant to control DENV2 infection. While rosiglitazone modulation of PPARγ did not reduce DENV2 infection, the importance of other PPAR isoforms in DENV infection cannot be ruled out yet. We still need to carefully evaluate the molecular mechanisms altered by these drugs that have impact in reducing DENV infection, such as through a transcriptomic analysis. It is also relevant to evaluate if the drugs are effective against the other three DENV serotypes. Considering the results of this work, it would be interesting to treat DENV2-infected cells with the IC_{50} of the effective drugs to perform viral titter, in order to check if the DENV exit from the cell is also affected. Other cell types like macrophages have marked importance in the dengue disease, therefore it would be relevant to also test these drugs candidates against their DENV infection. Lastly, testing drug combinations against DENV might also help to better manage the dengue disease.

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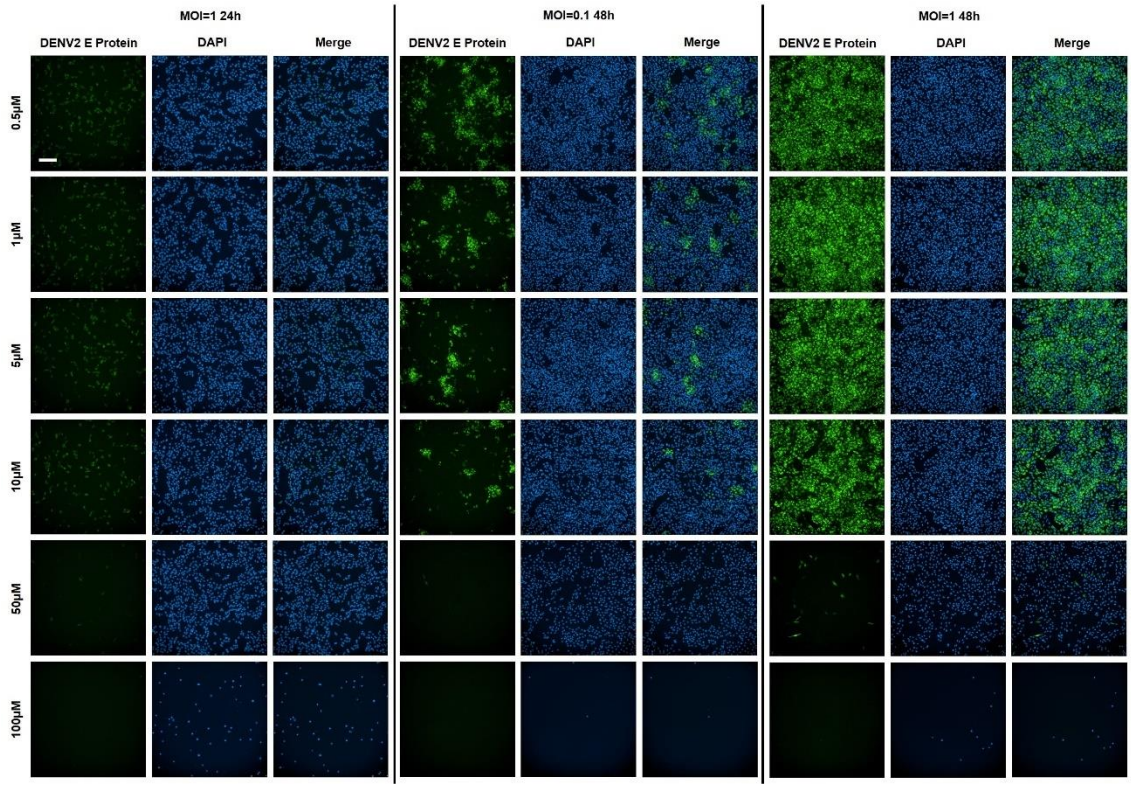
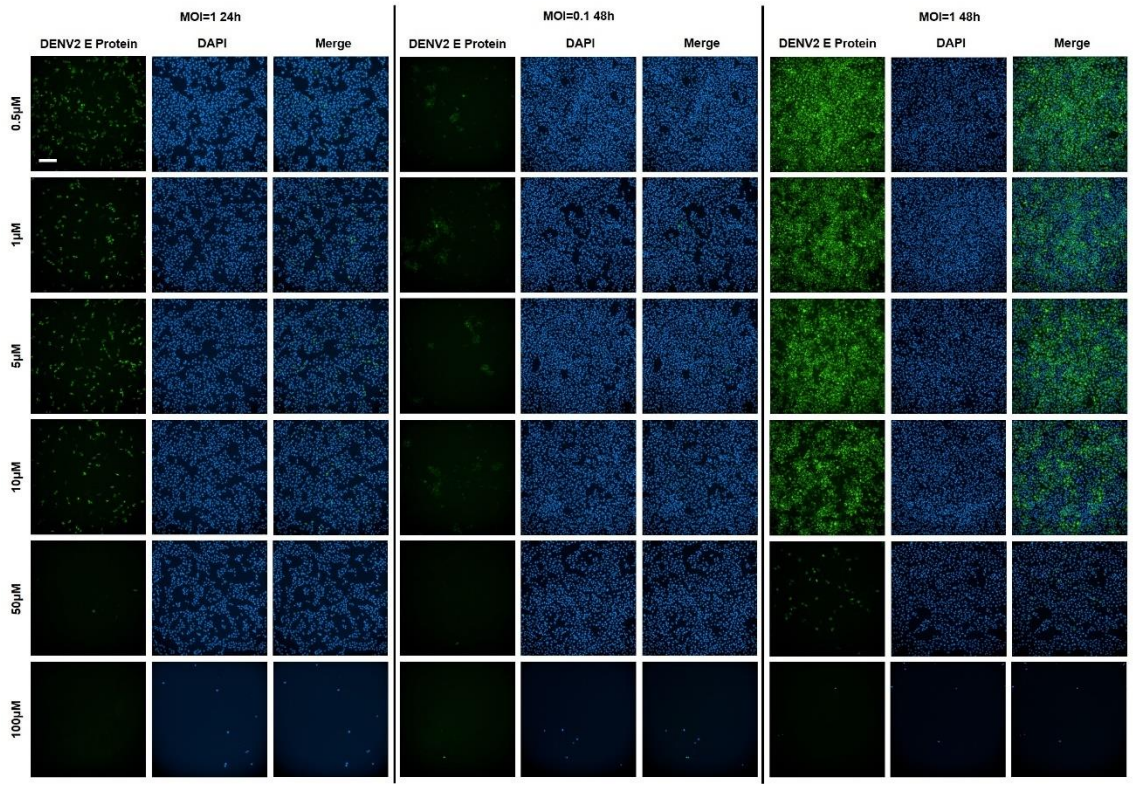
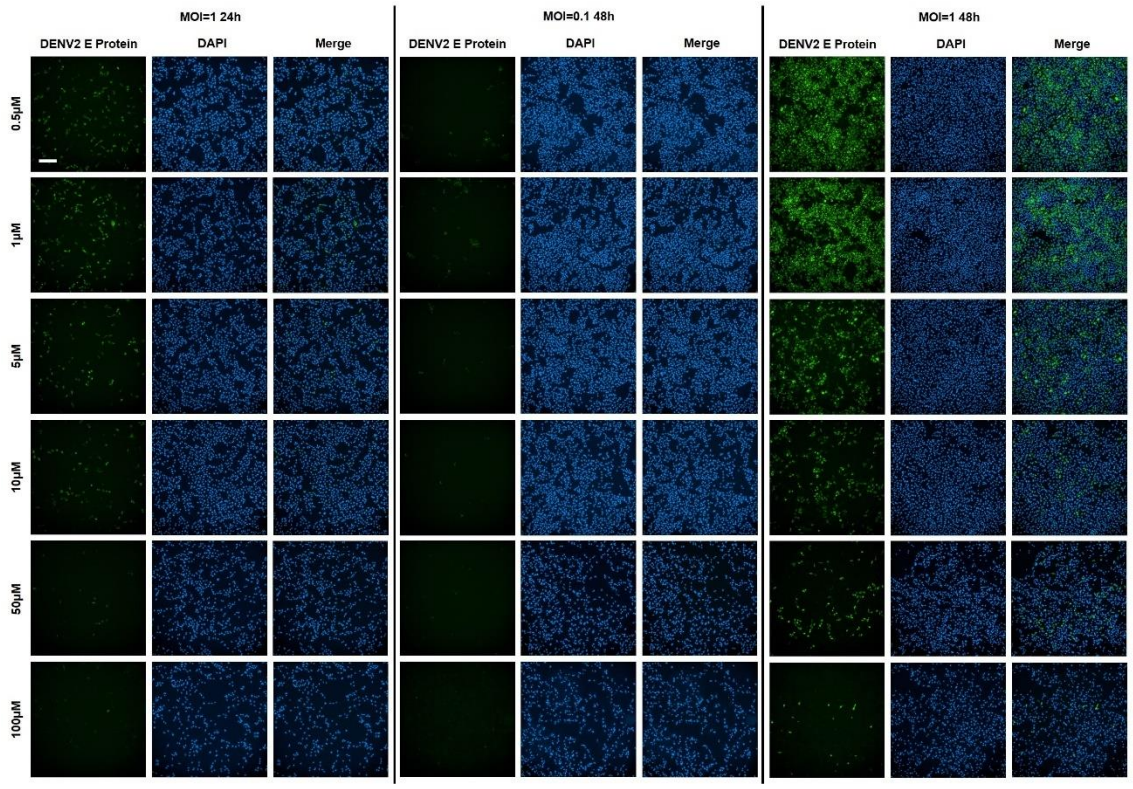
Annexes



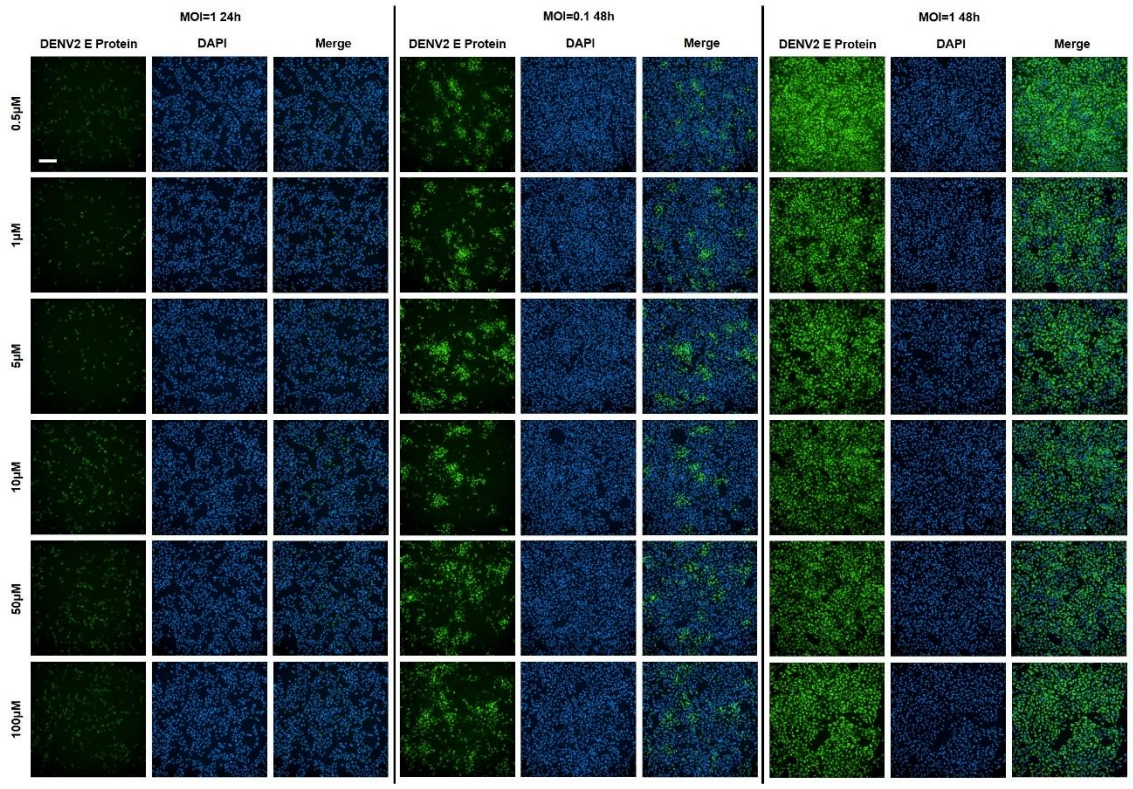
Supplementary Figure S1 - The effect of bexarotene on the DENV2 infection of Vero cells, under different MOIs and times of incubation. Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after bexarotene administration, followed by one of three infection conditions: MOI=1 and a 24h incubation with the virus (MOI=1 24h), MOI=0.1 and a 48h incubation (MOI=0.1 48h), and MOI=1 and a 48h incubation (MOI=1 48h). Images were obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200µm.



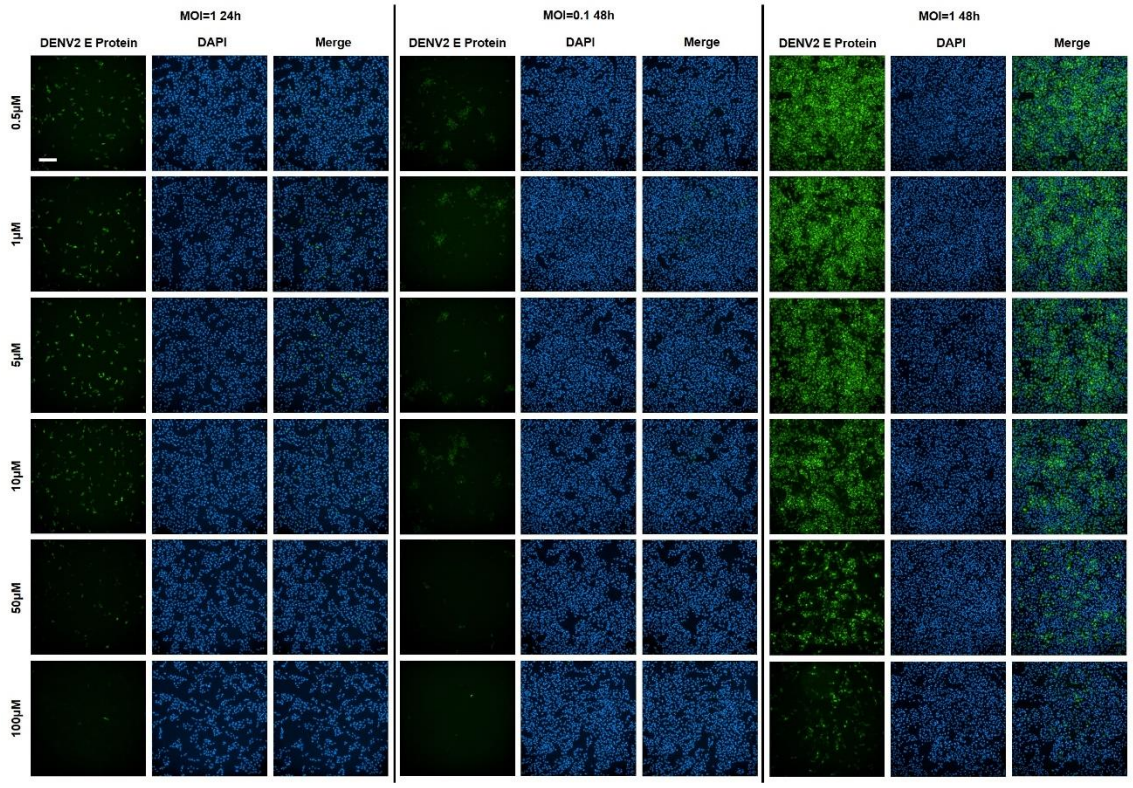
Supplementary Figure S2 - The effect of vitamin D3 on the DENV2 infection of Vero cells, under different MOIs and times of incubation. Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after vitamin D3 administration, followed by one of three infection conditions: MOI=1 and a 24h incubation with the virus (MOI=1 24h), MOI=0.1 and a 48h incubation (MOI=0.1 48h), and MOI=1 and a 48h incubation (MOI=1 48h). Images were obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200µm.



Supplementary Figure S3 - The effect of 25-hydroxycholesterol on the DENV2 infection of Vero cells, under different MOIs and times of incubation. Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after 25-hydroxycholesterol administration, followed by one of three infection conditions: MOI=1 and a 24h incubation with the virus (MOI=1 24h), MOI=0.1 and a 48h incubation (MOI=0.1 48h), and MOI=1 and a 48h incubation (MOI=1 48h). Images were obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200µm.



Supplementary Figure S4 - The effect of rosiglitazone on the DENV2 infection of Vero cells, under different MOIs and times of incubation. Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after rosiglitazone administration, followed by one of three infection conditions: MOI=1 and a 24h incubation with the virus (MOI=1 24h), MOI=0.1 and a 48h incubation (MOI=0.1 48h), and MOI=1 and a 48h incubation (MOI=1 48h). Images were obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200µm.



Supplementary Figure S5 - The effect of ribavirin on the DENV2 infection of Vero cells, under different MOIs and times of incubation. Representative images of the immunofluorescence of the DENV2 E protein (green) and the DAPI staining of Vero cell nuclei (blue) after ribavirin administration, followed by one of three infection conditions: MOI=1 and a 24h incubation with the virus (MOI=1 24h), MOI=0.1 and a 48h incubation (MOI=0.1 48h), and MOI=1 and a 48h incubation (MOI=1 48h). Images were obtained in the Operetta® CLS™ with a 10x air magnifying objective. Scale bar equals 200µm.