



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE CIÊNCIAS BIOMÉDICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA E PARASITOLOGIA
APLICADAS

**AVALIAÇÃO DOS EFEITOS DO LAPACHOL NO CICLO REPLICATIVO DO ZIKA
VÍRUS**

STEPHANIE PAOLA BORJAS REYES

UBERLÂNDIA, MG

Setembro de 2024

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STEPHANIE PAOLA BORJAS REYES

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

Orientadora: Prof^a. Dr^a. Ana Carolina Gomes Jardim

Coorientador: Prof. Dr. Diego Pandeló José

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LISTA DE ABREVIATURAS E SIGLAS

ADMET	Absorção, Distribuição, Metabolismo, Excreção e Toxicidade
Cryo-EM	Cryogenic electron microscopy (Crio-microscopia eletrônica)
DENV	Dengue virus (Vírus da Dengue)
E/M	Proteína do envelope (E) e de membrana (M)
EBV	Epstein-Barr Virus (Vírus Epstein-Barr)
ECA2	Enzima conversora da angiotensina 2
E/M	Proteína do envelope (E) e de membrana (M)
EGCG	Epigallocatequina galato
EMC	Endocitose mediada por clatrina
ERO	Espécies Reativas de Oxigênio
ESPII	Emergência Sanitária de Importância Internacional
EVA71	Enterovirus A71
FDA	Órgão governamental dos EUA que faz o controle dos alimentos
Gas6	Growth arrest specific gene-6 (Gene do parada de crescimento específico-6)
HCV	Hepatitis C Virus (Vírus da Hepatite C)
HSV-1	Herpes simplex virus-1 (Herpesvírus simples 1)
IFN-1	Interferon-1
JAK/STAT	Janus quinase e transdutor de sinal/ativador da transcrição
mRNA	RNA mensageiro
NSC	Neural Stem Cell (Células Tronco-Neurais)
Nts	Nucleotídeos
OMS	Organização Mundial da Saúde
ORF	Open Reading Frame (Quadro Aberto de Leitura)
PCR	Polymerase Chain Reaction (Reação em cadeia da polimerase)
RdRp	RNA polimerase dependente de RNA (RNA dependent RNA polymerase)
RRP	Receptores de Reconhecimento de Padrões
SCZ	Síndrome Congênita associada à infecção pelo vírus Zika
SGB	Síndrome de Guillain-Barré
ssRNA	Single-stranded RNA (RNA de fita simples)
TAM	Receptores tirosina quinase (TYRO3, AXL, MER)
TBEV	Tick-borne Encephalitis Virus (Vírus da encefalite transmitida por carrapatos)

TIM	T-cell/transmembrane, Immunoglobulin, Mucin (Célula T/transmembrana, Imunoglobulina, Mucina)
UTR	Untranslated Regions (Regiões Não Traduzidas)
WNV	West Nile Virus (Virus do Oeste do Nilo)
YFV	Yellow Fever Virus (Vírus da Febre-Amarela)
ZIKV	Zika Virus (Vírus da Zika)

LISTA DE FIGURAS

Figura 1. Árvore filogenética do gênero Orthoflavivirus	11
Figura 2. Disseminação do ZIKV pelo mundo ao longo do tempo	12
Figura 3. Manifestações clínicas da infecção pelo ZIKV	16
Figura 4. Microcefalia em recém-nascidos diagnosticados com SCZ	17
Figura 5. Partícula viral do ZIKV.	18
Figura 6. Representação esquemática do genoma do ZIKV e dos sítios de clivagem da poliproteína viral.	19
Figura 7. Ciclo Replicativo do ZIKV	21
Figura 8. Estrutura tridimensional da NS2B-NS3^{Pro} do ZIKV.	23
Figura 9. Estrutura da NS5 do ZIKV.	24
Figura 10. Posições dos grupamentos carbonila das quinonas	28
Figura 11. Classificação das Quinonas	29
Figura 12. Representação do Ciclo Redox induzido por quinonas.	30
Figura 13. Estrutura química do Lapachol	31

RESUMO

A reemergência do *Orthoflavivirus zikaense* (vírus da Zika, ZIKV) em 2015 causou uma crise de saúde pública devido à rápida disseminação e sua associação com complicações clínicas graves, como a Síndrome de Guillain-Barré e a Síndrome Congênita pelo vírus Zika (SCZ). Apesar das recentes pesquisas, não existem atualmente tratamentos eficazes contra o ZIKV, o que torna urgente o desenvolvimento de terapias antivirais. O lapachol, uma 1,4-naftoquinona, tem demonstrado potencial como antiviral, com atividade já relatada contra outros vírus RNA e patógenos humanos. Neste estudo a atividade antiviral do lapachol foi avaliada na inibição da replicação do ZIKV em células Vero E6. As células foram tratadas com lapachol, utilizando concentrações entre 0,78 μM a 100 μM , na presença ou ausência da infecção com ZIKV_{PE243}, e ensaios de imunofluorescência foram realizados para avaliar os títulos virais 72 horas após infecção, por meio da quantificação de unidades formadoras de foco (FFU). Ensaios de adição de composto em diferentes tempos de infecção foram empregados para identificar as etapas do ciclo replicativo viral que o lapachol é capaz de inibir. Adicionalmente, estudos sobre o mecanismo de ação incluíram ensaios de interação do lapachol com proteínas virais e análises de ancoragem molecular, além da avaliação da geração de espécies reativas de oxigênio (EROs). Os resultados demonstraram que o lapachol apresentou potente inibição da replicação viral, com índice de seletividade de 50,7 e EC_{50} de 3,0 μM , e demonstrou agir principalmente nas etapas após a entrada do vírus, interferindo com a atividade da protease NS2B-NS3 e da polimerase NS5, sugerido como mecanismo de ação. Além disso, um efeito protetor também foi evidenciado quando as células foram tratadas antes da infecção, bem como a capacidade do lapachol em induzir a formação de ERO *in vitro*, sugerindo que outros mecanismos podem contribuir para sua atividade antiviral, como desencadear uma resposta ao estresse oxidativo que danifica as partículas virais. Estes resultados indicam que o lapachol tem grande potencial como candidato para o desenvolvimento de inibidores do ZIKV.

ABSTRACT

Since its re-emergence in 2015, *Orthoflavivirus zikaense* (Zika virus - ZIKV) has been a cause of public health concern due to its propensity to cause widespread epidemics and its sudden association with serious complications such as Guillain-Barré and Congenital Zika Syndrome (CSZ), despite the efforts made there still is a lack of effective treatments against it, making imperative the need to develop antiviral therapies. Lapachol, a 1,4-naphthoquinone compound, seems to be a promising candidate as an antiviral agent since its activity against other RNA viruses as well as other human pathogens has been previously reported. Here we demonstrated the activity of lapachol in inhibiting ZIKV replication *in vitro*. Vero E6 cells were treated with lapachol at concentrations ranging from 0.78 μM to 100 μM in the presence or absence of infection with ZIKV_{PE243}. Time-of-addition assays were used to identify the steps of ZIKV life cycle that lapachol was able to inhibit. Immunofluorescence assays were employed to assess virus titers by quantifying focus formation units (FFU). Its mechanism of action was studied through protein-based substrate digestion assays and target-specific molecular docking analysis. The formation of intracellular reactive oxygen species (ROS) was also assessed. The results demonstrate that lapachol acts as a strong inhibitor of viral replication with a strong selectivity index of 50.7 and an $\text{EC}_{50} = 3.0 \mu\text{M}$, exhibiting its greater effect after virus entry to the cell. Further assays suggest that its mechanism of action may be through the blockage of the catalytic site of NS2B-NS3 protease and NS5 polymerase. A protective effect over the cells was also evidenced when treated before infection, as well as the capacity of lapachol to induce ROS formation *in vitro*, suggesting further mechanisms may be contributing to its antiviral activity, such as triggering an oxidative stress response that damages the viral particles. These findings shed light on the potential of lapachol to be employed for the development of ZIKV inhibitors.

Keywords: Zika virus, lapachol, antiviral, natural compounds, polymerase inhibitors

SUMÁRIO

CAPÍTULO I.....	11
<i>Fundamentação Teórica</i>	<i>11</i>
1. INTRODUÇÃO	11
1.1 Classificação do <i>Orthoflavivirus zikaense</i>	11
1.2 Histórico e epidemiologia do ZIKV	11
1.3 Transmissão e tratamento do ZIKV	15
1.4 Febre Zika: manifestações clínicas e patogênese	15
1.5 A partícula viral do ZIKV	18
1.6 Ciclo Replicativo do ZIKV	20
1.7 Proteína NS3 e protease NS2B-NS3Pro do ZIKV.....	22
1.8 Proteína NS5 do ZIKV	24
1.9 Propriedades biológicas e bioativas dos compostos isolados de plantas: compostos naturais com atividade antiviral.....	25
1.10 Naftoquinonas	28
1.11 Lapachol	30
2. OBJETIVOS	32
2.1 Objetivos Específicos	32
REFERÊNCIAS.....	33
CAPÍTULO II	45
<i>Manuscrito</i>	<i>45</i>
Abstract.....	47
CAPÍTULO III.....	71
<i>Considerações Finais</i>	<i>71</i>

CAPÍTULO I

Fundamentação Teórica

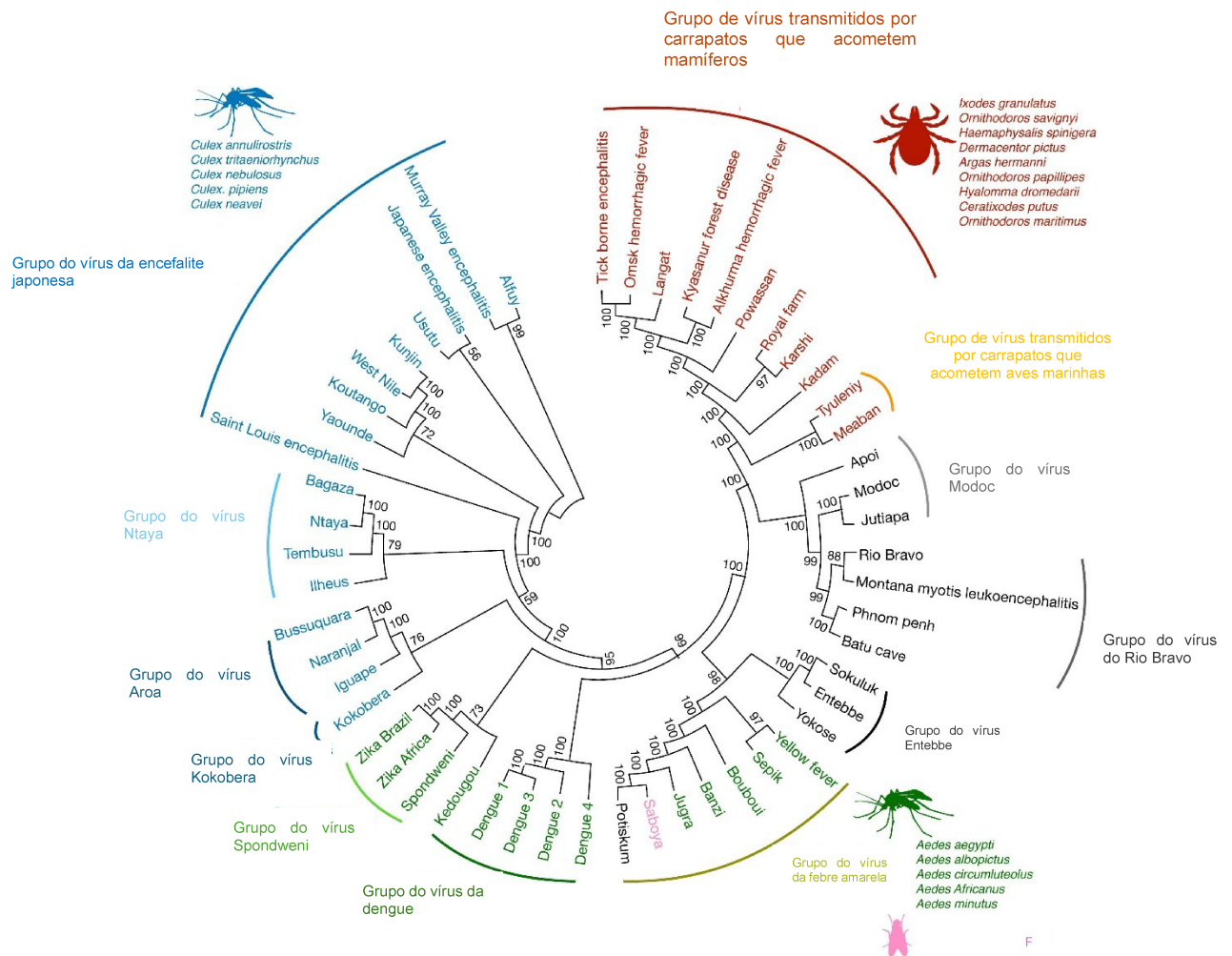
1. INTRODUÇÃO

1.1 Classificação do *Orthoflavivirus zikaense*

O termo arbovírus abrange cerca de 500 vírus, dos quais aproximadamente 100 são patógenos que afetam os seres humanos (VIGLIETTA et al., 2021). Eles são classificados em diferentes famílias virais, que incluem as famílias *Togaviridae*, *Flaviviridae*, *Bunyaviridae* e *Rhabdoviridae* (“Bunyaviridae | ICTV,” [2011]; “Family: Rhabdoviridae | ICTV,” [2023]; CHEN et al., 2018; SIMMONDS et al., 2017). Apesar de possuírem características morfológicas e bioquímicas distintas, estes vírus são agrupados sob esse termo devido à capacidade de se manterem na natureza através da transmissão entre vertebrados suscetíveis e artrópodes hematófagos (TADEU; FIGUEIREDO, 1990).

A família *Flaviviridae* é classificada em quatro gêneros, de acordo com estudos filogenéticos baseados na comparação de um domínio altamente conservado na RNA polimerase dependente de RNA (*RNA dependente RNA polimerase*, RdRp): 1) *Orthoflavivirus*; 2) *Pestivirus*; 3) *Hepacivirus*; e 4) *Pegivirus* (SIMMONDS et al., 2017b). A maioria dos arbovírus desta família está classificada no gênero *Orthoflavivirus*, o qual se destaca pela sua ampla distribuição geográfica e a diversificação de cepas virulentas, que se estabeleceram em regiões com condições climáticas que favorecem sua disseminação (AMORIM et al., 2020). O gênero *Orthoflavivirus* inclui patógenos virais humanos de importância médica, como *Orthoflavivirus denguei* (vírus da dengue, DENV), *Orthoflavivirus nilense* (vírus do oeste do Nilo, WNV), *Orthoflavivirus encephalitidis* (vírus da encefalite transmitida por carrapatos, TBEV), *Orthoflavivirus flavi* (vírus da febre amarela, YFV) e *Orthoflavivirus zikaense* (vírus da Zika, ZIKV) (“Genus: Orthoflavivirus | ICTV”, 2017) (**Figura 1**).

Figura 1. Árvore filogenética do gênero *Orthoflavivirus*

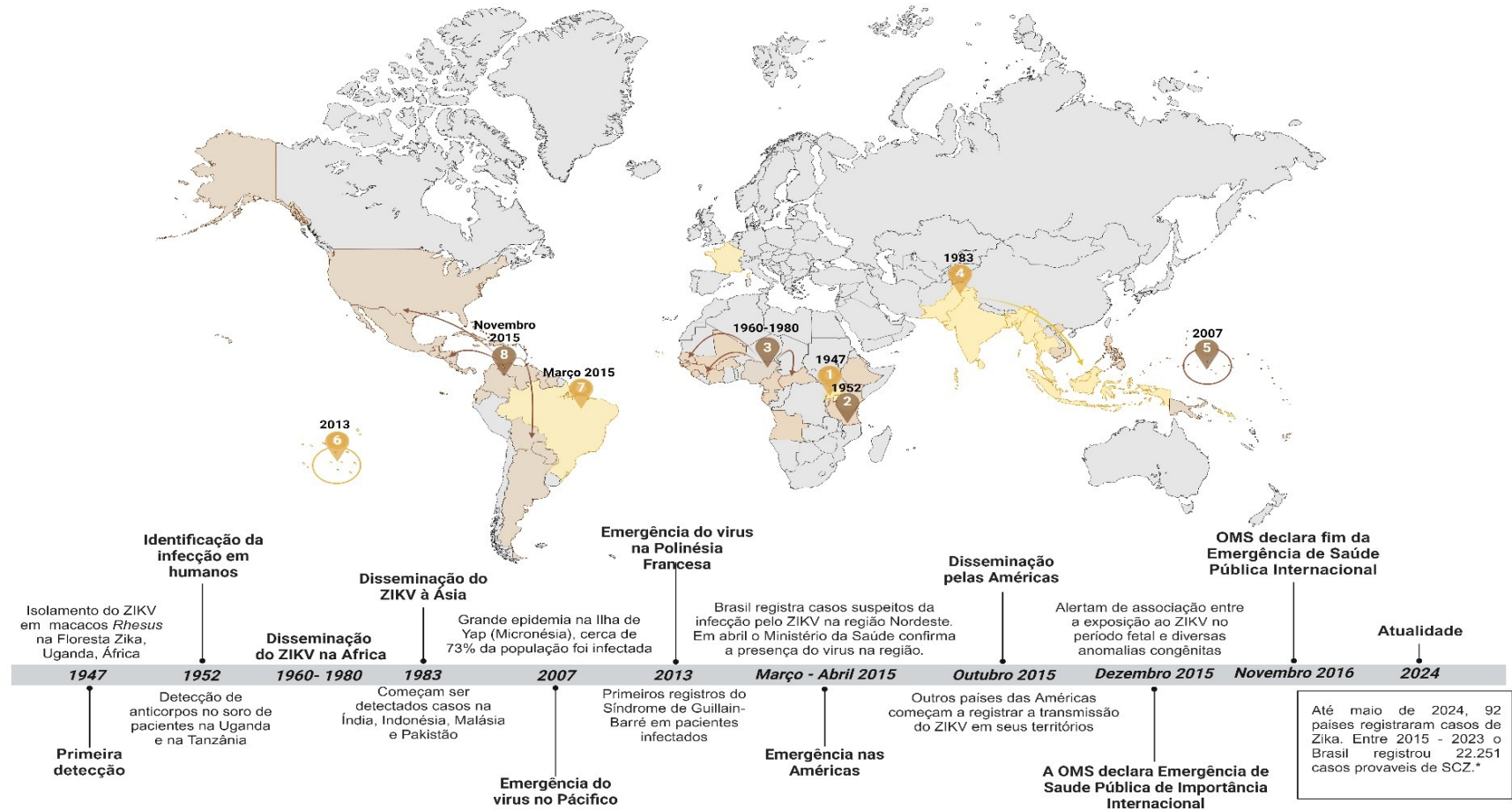


Fonte: adaptada de (RATHORE; ST. JOHN, 2020)

1.2 Histórico e epidemiologia do ZIKV

O ZIKV foi identificado pela primeira vez em uma amostra de macaco *Rhesus* em Kampala, Uganda, na floresta Zika, em 1947 (SONG et al., 2017). Um ano depois, foi isolado na mesma localidade, porém, de mosquitos *Aedes africanus* capturados na floresta (DICK; KITCHEN; HADDOW, 1952). Cinco anos após a sua primeira identificação, em 1952, foram reportados os primeiros casos em humanos, localizados na Uganda e na República Unida da Tanzânia (KINDHAUSER et al., 2016a) (**Figura 2**).

Figura 2. Disseminação do ZIKV pelo mundo ao longo do tempo



*SCZ: Síndrome Congênita Associada à Infecção pelo vírus *Orthoflavivirus zikaense* (ZIKV)

Fonte: imagem feita pela autora com BioRender.com (2024)

Nas décadas de 1960 a 1980, o vírus continuou a ser isolado de macacos *Rhesus* e mosquitos, como parte do monitoramento das arboviroses na região da Uganda (BRÈS, 1970; HAMMON; SCHRACK; SATHER, 1958; MACNAMARA; HORN; PORTERFIELD, 1959; SMITHBURN, 1954). Além disso, casos esporádicos foram reportados em humanos em diferentes países da África; no entanto, apenas casos com sintomas leves foram documentados, sem registros de mortes ou hospitalização pela infecção (MUSSO; GUBLER, 2016). Em 1983, o ZIKV foi detectado pela primeira vez no continente asiático, e estudos filogenéticos demonstram que o vírus detectado representava uma variante diferente da isolado na África, resultando em sua classificação em duas linhagens distintas: a linhagem asiática e a linhagem africana (BASU; TUMBAN, 2016) **(Figura 2)**.

A variante asiática foi responsável pelo primeiro grande surto de ZIKV, que ocorreu nas Ilhas Yap em 2007, onde infetou aproximadamente 73% da população dessa região (DUFFY et al., 2009). Disseminou-se para Polinésia Francesa em 2013, causando surtos em 2014 e 2015 (PLOURDE; BLOCH, 2016; POLAND; OVSYANNIKOVA; KENNEDY, 2019; RANDOLPH; ROGERS, 2010). O surto de 2015 foi o mais proeminente devido à coincidência com aumento do número de casos da síndrome de Guillain-Barré (SGB) na região e o nascimento de bebês com complicações neurológicas graves, como a microcefalia congênita (CAUCHEMEZ et al., 2016) **(Figura 2)**.

Em março de 2015, foi notificado no Brasil um aumento de casos de uma doença caracterizada por um exantema de etiologia desconhecida nos estados do Nordeste, registrando 7.000 casos entre fevereiro e abril, com a suspeita que se tratava da infecção pelo ZIKV (KINDHAUSER et al., 2016b). Em abril de 2015, a presença do ZIKV no Brasil foi confirmada através da detecção do material genético viral, por reação em cadeia da polimerase (PCR), em amostras de casos suspeitos nos estados da Bahia e no Rio Grande do Norte (ZANLUCA et al., 2015). Entre janeiro e julho do mesmo ano, foram notificados 121 casos de SGB em adultos em diversas regiões do Nordeste do Brasil (CIPRIANO; MONTEIRO, 2015). Adicionalmente, um estudo realizado no país em 2015 investigou a associação de mais de 4 milhões de casos de microcefalia com a infecção pelo ZIKV. Os resultados demonstraram um risco absoluto de ocorrência de microcefalia em 40,8% para cada 10.000 partos, e um risco relativo de 16,8%, com a infecção ocorrendo no primeiro ou segundo trimestre da gestação (VASCONCELOS, 2015) **(Figura 2)**.

Nos meses seguintes, outros países da América do Sul começaram a relatar a presença do vírus, estendendo-se até os países da América do Norte (KINDHAUSER et al., 2016). Em fevereiro de 2016, devido à rápida propagação do ZIKV nas Américas, a ocorrência da microcefalia em recém-nascidos, e as suspeitas da sua associação com ZIKV, a Organização Mundial da Saúde (OMS, 2016) declarou Emergência Sanitária de Importância Internacional (ESPII), alertando sobre a possível associação entre a infecção pelo ZIKV e malformações congênitas nos embriões de grávidas infectadas durante a gestação (OMS, 2016) (**Figura 2**).

Nos anos subsequentes, esta associação foi corroborada por evidências epidemiológicas e sorológicas (DE ARAÚJO et al., 2018; VISSOCI et al., 2018). Com isso, a condição congênita resultante da infecção pelo ZIKV no período gestacional passou a ser denominada de síndrome congênita associada à infecção pelo vírus Zika (SCZ). A SCZ é caracterizada por um conjunto de anomalias congênitas, estruturais e funcionais, com repercussões no crescimento e no desenvolvimento dos embriões ou dos fetos expostos ao vírus durante a gestação (MINISTERIO DA SAÚDE, 2023).

Portanto, o surto de 2015-2016 marcou a história do ZIKV, mudando a percepção de uma doença caracterizada por sintomas leves e pouco frequentes, para uma crise de saúde pública que requereu medidas de mitigação. Em 2016, o número de casos da febre Zika mundial foi estimado entre 440.000 e 1.300.000, além de mais de 4.000 casos prováveis de SCZ (GYAWALI; BRADBURY; TAYLOR-ROBINSON, 2016). No Brasil foram reportados 213.350 casos prováveis segundo ao Ministério da Saúde, em contraste com 2017, onde houve a redução do número de casos prováveis para 17.593. Desde então, o número de casos reportados no território brasileiro varia entre 18.000 e 31.000 por ano. O último boletim epidemiológico de 2023 relatou 7.292 casos prováveis (Ministério da Saúde, 2024). Estes números demonstram um caráter endêmico da doença, que corresponde ao fato do território brasileiro possuir os fatores suficientes para disseminação da infecção, ou seja, condições ambientais, vetores e população suscetível (LOWE et al., 2018).

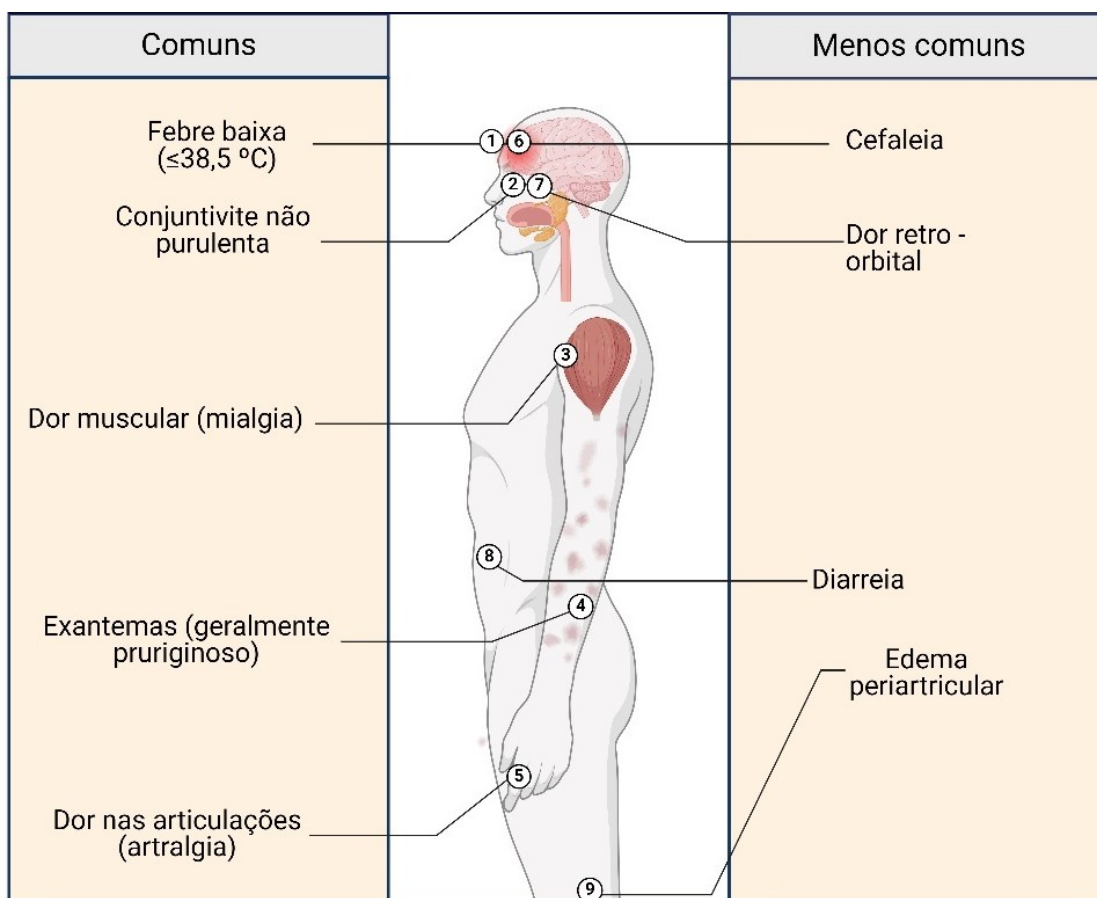
Atualmente, a incidência de infecções pelo ZIKV é menor, mas ainda representa um desafio devido à endemia de variantes virulentas em países vulneráveis e à capacidade de causar complicações clínicas graves como a SCZ (BERNARDO-MENEZES et al., 2022). Segundo a OMS, até maio de 2024, o ZIKV era endêmico em 92 países, 49 destes localizados nas Américas (OMS, 2024).

1.3 Transmissão e tratamento do ZIKV

O ciclo de transmissão do ZIKV pode ser classificado em dois: o ciclo silvestre e o ciclo urbano. No ciclo silvestre, os mosquitos atuam como hospedeiros invertebrados enquanto os primatas não humanos como os hospedeiros vertebrados. Já no ciclo urbano, os mosquitos funcionam como vetores, porém a infecção dos hospedeiros invertebrados é feita pela picada dos mosquitos a outros humanos infectados (FERRARIS; YSSEL; MISSÉ, 2019). Além da transmissão vetorial, o ZIKV pode ser transmitido verticalmente (de mãe para filho), por secreções, pela via sexual e por transfusões (DAIANE; DAL PONTE, 2005). Nos últimos anos, as implicações da transmissão pelas secreções têm sido investigadas. Estudos conduzidos por Lustig e colaboradores em 2016 demonstraram que o vírus pode ser detectado no sangue periférico por dois meses após o início dos sintomas (LUSTIG et al., 2016). Como resultado, os múltiplos modos de transmissão do vírus dificultam o desenvolvimento de estratégias para o controle efetivo da infecção (RANDOLPH; ROGERS, 2010).

1.4 Febre Zika: manifestações clínicas e patogênese

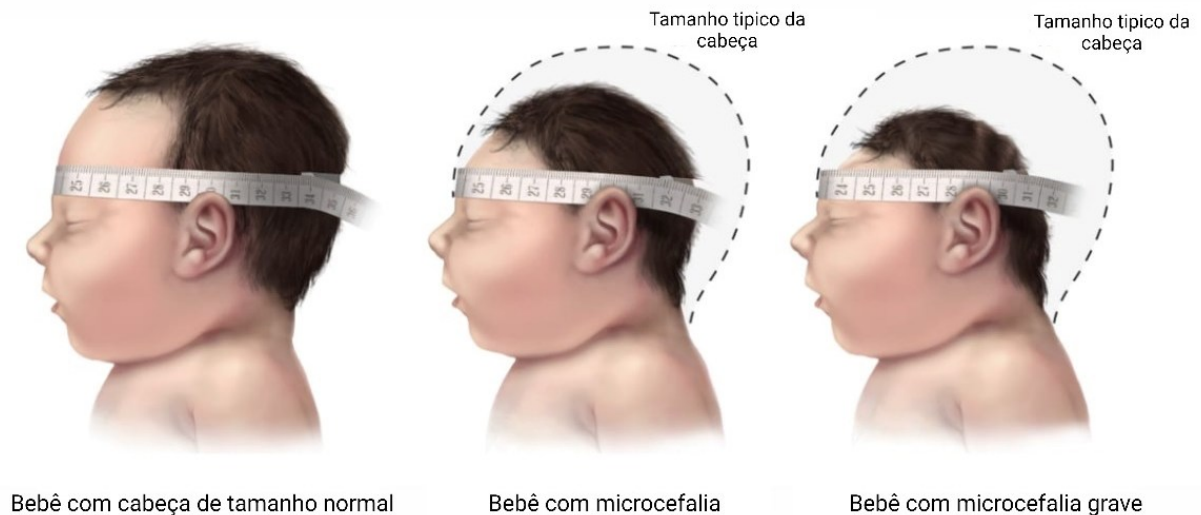
A febre Zika é uma doença assintomática em 80% dos casos. Nos casos sintomáticos, as manifestações clínicas são caracterizadas por febre baixa, artralgia, mialgia, cefaleia, astenia, erupção macopapular, conjuntivite, diarreia e edema periarticular, sendo o curso da infecção autolimitado (MWALIKO et al., 2020) (**Figura 3**). Em contraposição, um aumento da associação da infecção pelo ZIKV e malformações congênitas em recém-nascidos de mulheres infectadas durante a gestação começou a ser reportado desde o surto de 2015-2016 (ANTONIOU et al., 2020a). Uma revisão sistemática realizada por Freitas e colaboradores (2020), a qual avaliou os sinais clínicos e sintomas associados com a SCZ relatados nos últimos anos, reportou os principais sinais associados: microcefalia, calcificações parenquimatosas ou cerebelares, ventrículo-megalia, hipoplasia ou atrofia do sistema nervoso central, malformações oftalmológicas e baixo peso ao nascer para a idade gestacional (FREITAS et al., 2020).

Figura 3. Manifestações clínicas da infecção pelo ZIKV

Fonte: imagem feita pela autora com BioRender.com (2024)

Um dos achados mais frequentes nos casos de SCZ é a microcefalia (**Figura 4**), representando um dos sinais clínicos mais estudados. Porém, os mecanismos moleculares envolvidos na neuro-patogênese do Zika congênito ainda não foram totalmente elucidados (FUJIMURA et al., 2023). Evidências mostram que o RNA viral é detectado no tecido neural fetal, assim como a capacidade do vírus de se replicar em diferentes tipos de células progenitoras neurais, e até mesmo causar apoptose das células infectadas (MLAKAR et al., 2016; QIAN et al., 2016; TANG et al., 2016b).

Figura 4. Microcefalia em recém-nascidos diagnosticados com SCZ



Fonte: adaptado de (ROTH et al., 2022)

Diversos estudos indicam que o principal mecanismo de patogênese está relacionado à infecção das células-tronco neurais (*neural stem cell*, NSC) do embrião durante o primeiro trimestre de gestação (SUN et al., 2017; TANG et al., 2016a; YI; PIMENTEL; PACHTER, 2017). As NSCs são células auto-regenerativas, que originam os principais fenótipos do sistema nervoso, sendo precursoras dos neurônios e da neuroglia (KUBIS; CATALA, 2015). A supressão da sua proliferação ou a diminuição do seu número pode levar a anomalias no desenvolvimento do sistema nervoso central no embrião (BINDU; PANDEY; SETH, 2023). Fujimura e colaboradores realizaram um estudo utilizando proteômica e sequenciamento de RNA para a análise de modelos murinos imunocompetentes infectados com ZIKV, no qual descobririam que a infecção das NSCs poderia ativar o sistema imunológico através da via de sinalização Janus Quinase (JAK) e transdutor de sinal/ativador da transcrição (STAT) (JAK/STAT), resultando na fagocitose das NSCs e morte celular. Além disso, devido ao aumento da transcrição de interferon-1 (IFN-1), os autores propõem que o IFN-1 pode causar a supressão da proliferação dos NSCs (FUJIMURA et al., 2023).

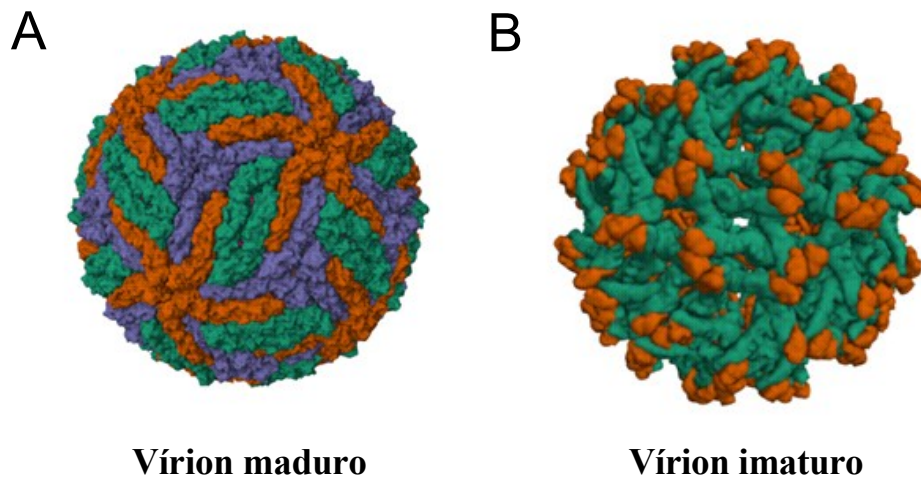
Outra complicação grave da infecção pelo ZIKV, a SGB é uma doença autoimune, caracterizada por paralisia flácida ascendente que resulta em inflamação aguda dos nervos periféricos e cranianos, levando à fraqueza progressiva e simétrica dos membros (BENETI;

LUCE; DA SILVA, 2006). Os mecanismos pelos quais a infecção pelo ZIKV pode ser um fator desencadeador para o SGB ainda não são totalmente compreendidos, no entanto, pensa-se que pode ser devido ao mimetismo molecular entre gangliosídeos e moléculas presentes na superfície do vírus, assim como a toxicidade para as células progenitoras neuronais (NICO et al., 2018).

1.5 A partícula viral do ZIKV

O vírion maduro possui uma estrutura semelhante a outros orthoflavivírus, com tamanho de 50 nm e simetria icosaédrica do capsídeo. Possui 90 dímeros da glicoproteína do envelope viral (E) e da proteína de membrana (M) em sua superfície, associados em um padrão de ‘espinha de peixe’, ancorados pelos seus domínios transmembrana na bicamada lipídica derivada do hospedeiro, que forma o envelope viral. Estes dímeros estão dispostos paralelamente à superfície, resultando em uma partícula viral lisa (**Figura 5A**) (KOSTYUCHENKO et al., 2016).

Figura 5. Partícula viral do ZIKV.

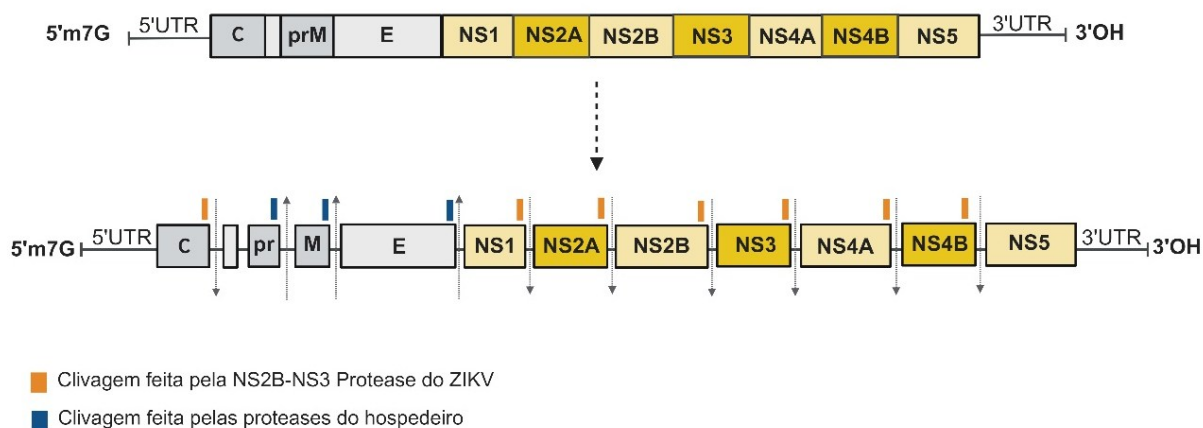


Fonte: adaptado de (SEVVANA et al., 2018; TAN et al., 2020)¹

¹ A) Estrutura tridimensional da partícula madura obtida por microscopia eletrônica (Cryo-EM) em uma resolução de 3,1 Armstrong (Å). A estrutura foi obtida do PDB (Protein Data Bank) número de acesso: 6CO8. B) estrutura tridimensional da partícula imatura do ZIKV em uma resolução de 9.0 Å, PDB: 6LNU. As imagens foram editadas pela autora com Chimera X ([UCSF ChimeraX: Structure visualization for researchers, educators, and developers](https://www.csb.pitt.edu/chimeraX/), Pettersen EF, Goddard TD, Huang CC, Meng EC, Couch GS, Croll TI, Morris JH, Ferrin TE. *Protein Sci.* 2021 Jan;30(1):70-82.)

O genoma do ZIKV é semelhante à de outros orthoflavivírus, consistindo em RNA de fita simples (*Single-Stranded RNA*, ssRNA), não segmentado, de polaridade positiva, com apenas uma região aberta de leitura (*Open Reading Frame*, ORF) e duas regiões não traduzidas (*Untranslated Regions*, UTR) nas suas extremidades 5' e 3' (VALENTE; MORAES, 2019a). O RNA viral possui 10.700 nucleotídeos (nts), que codificam para uma poliproteína viral, posteriormente processada em diferentes componentes estruturais e enzimáticos essenciais para o ciclo replicativo do vírus (**Figura 6**) (VAN HEMERT; BERKHOUT, 2016).

Figura 6. Representação esquemática do genoma do ZIKV e dos sítios de clivagem da poliproteína viral.



Fonte: imagem feita pela autora com Biorender.com (2024)

Umas das principais características do genoma dos orthoflavivírus, não observada em outros gêneros da família *Flaviviridae*, é a presença de uma estrutura de proteção do RNA denominada CAP, do tipo 1 AUG, na extremidade 5' (7-metilguanossina, 5'm7G) (**Figura 6**) (SIMMONDS et al., 2017a). As UTRs possuem funções muito importantes para a replicação viral do ZIKV, visto que a 5'UTR medeia o início da leitura do RNA viral a partir da estrutura CAP, e a 3'UTR possui uma cadeia de poli(A) que funciona como um sinal de parada na etapa final do processamento da poliproteína (BERNARDO-MENEZES et al., 2022).

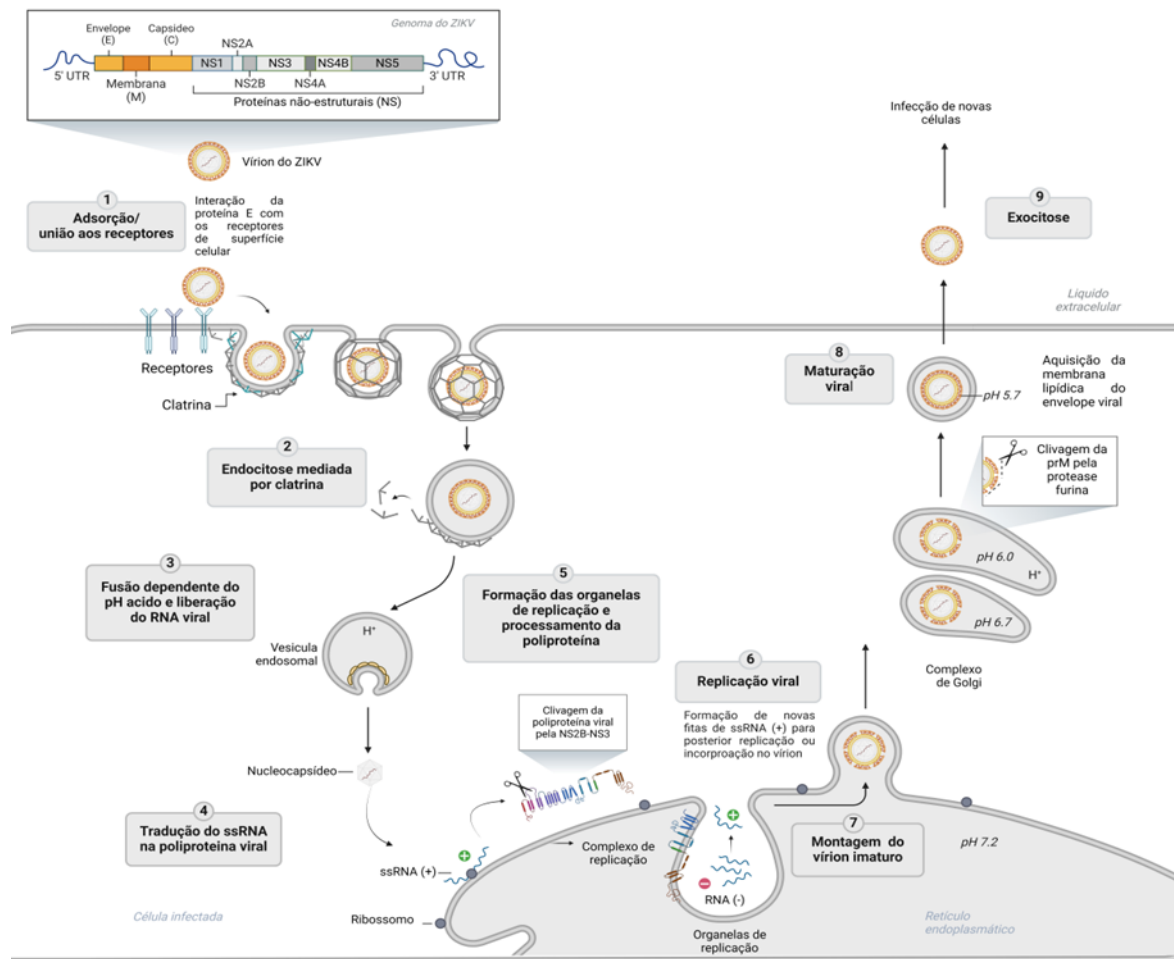
1.6 Ciclo Replicativo do ZIKV

O ciclo replicativo do ZIKV começa pela entrada e internalização do vírion maduro na célula susceptível. Para a adsorção do vírion, a proteína E, que é o principal componente da superfície do ZIKV, primeiro reconhece os receptores da célula hospedeira (CARRO et al., 2020) (**Figura 7**).

Evidências recentes sugerem alguns receptores específicos como essenciais para a entrada do ZIKV na célula hospedeira, que inclui os receptores lipídicos TIM (*T-cell/transmembrane, immunoglobulin, and mucin*) e os receptores tirosina quinase TAM (T: TYRO3, A: AXL, M: MER, que são os genes que codificam para cada um desses receptores) (AGRELLI et al., 2019). O receptor tirosina quinase codificado pelo gene AXL é um dos mais estudados, sugerindo a interação mediada pelo seu ligante Gas6 (*growth arrest specific gene-6*) com a fosfatidilserina na superfície do vírion (PERSAUD et al., 2018). No entanto, os relatos são contraditórios, já que alguns estudos apoiam seu papel central como receptor em certas linhagens celulares, como células micróglias, glioblastomas e células epiteliais (ZWERNIK et al., 2021). Por outro lado, estudos usando outras linhagens celulares conhecidas por serem suscetíveis à infecção por ZIKV, como as células progenitoras neuronais humanas e placentárias, mostram que o silenciamento do AXL não impede a infecção (XIE et al., 2021).

Depois de se ligar aos receptores específicos das células hospedeiras, o ZIKV precisa ser transportado para compartimentos com um pH mais ácido para sua internalização. Assim como muitos orthoflavivírus, as evidências sugerem que esse processo é realizado através da endocitose mediada por clatrina (EMC) (RINKENBERGER; SCHOGGINS, 2019; ZWERNIK et al., 2021). A EMC envolve a formação de uma invaginação da membrana plasmática do hospedeiro, a qual, por mecanismos coordenados por clatrina e outras proteínas adaptadoras, resulta na formação de vesículas revestidas de clatrina que englobam a partícula viral, sendo capazes de transportá-la para o endossomo (CARRO et al., 2020). Uma vez no endossomo, o pH ácido provoca mudanças conformacionais na partícula viral, onde ela adota sua forma fusogênica, que consiste no rearranjo dos dímeros da proteína E e M em 90 trímeros para expor os peptídeos de fusão, permitindo a fusão com a membrana endossomal e liberando o nucleocapsídeo no citoplasma (HASAN et al., 2017) (**Figura 7**).

Figura 7. Ciclo Replicativo do ZIKV.



Fonte: feito pela autora com BioRendercom. Reyes (2024)²

O nucleocapsídeo é então dissociado, liberando o RNA viral no citoplasma celular. O vírus induz a formação de pequenas organelas, chamadas de organelas de replicação, que estão associadas ao retículo endoplasmático do hospedeiro (VAN DEN ELSEN; QUEK; LUO, 2021). Como o genoma do ZIKV é semelhante ao RNA mensageiro (mRNA) da célula hospedeira, ele é capaz de utilizar os ribossomos do retículo endoplasmático (RE) para traduzir imediatamente a poliproteína viral (VAN DEN ELSEN; QUEK; LUO, 2021). A poliproteína é ancorada na membrana do RE, onde é clivada pela protease viral NS2B-NS3Pro e por proteases do hospedeiro, resultando nas suas proteínas constituintes. A clivagem pós-traducional da poliproteína resulta na produção de três proteínas estruturais: a glicoproteína E, a proteína C do capsídeo viral (C) e a proteína precursora de membrana (prM); assim como sete proteínas não

² Disponível em <<http://www.BioRender.com/p97y153.com>>

estruturais (NS): NS1, NS2A, NS2B NS3, NS4A, NS4B e NS5 (VALENTE; MORAES, 2019a) (**Figura 7**).

As proteínas não-estruturais se associam para criar um complexo de replicação. As organelas de replicação fornecem um suporte estrutural para a montagem da maquinaria da replicação viral. Nela, o RNA viral, a NS3 helicase e a polimerase NS5 estão associadas ao lado citoplasmático da membrana, e as proteínas integrais NS2A, NS2B, NS4A e NS4B estão ancoradas à bicamada lipídica. Os dímeros da proteína NS1 estabilizam o complexo de replicação no lúmen do RE através da interação com a NS4A e NS4B (MUKHOPADHYAY; KUHN; ROSSMANN, 2005). O complexo de replicação transcreve inicialmente o RNA genômico numa fita negativa complementar, que serve de molde para produzir múltiplas cópias de ssRNA (+) progenitoras. Estas novas fitas podem ser utilizadas para posterior replicação e transcrição ou serem incorporadas no vírion imaturo (VAN DEN ELSEN; QUEK; LUO, 2021).

O vírion imaturo (**Figura 5B**) conterá proteínas E e prM associadas em heterodímeros, resultando em 60 cópias das glicoproteínas em uma conformação trimérica de spike. A prM tem como função o impedimento de um rearranjo estrutural prematuro, evitando assim a secreção precoce da partícula viral (SEVVANA et al., 2020). Os vírions imaturos acumulados no RE são secretados através de vesículas e entram no complexo de Golgi, onde a protease furina do hospedeiro cliva o peptídeo 'pr' da prM, transformando a partícula viral na sua forma madura (**Figura 5A**) (THERKELSEN et al., 2018). Todos estes processos de tráfego da partícula nascente são dependentes da rota secretora. Os vírions maduros são liberados por exocitose, mediante a fusão das membranas das vesículas carregadoras e a membrana citoplasmática (MUKHOPADHYAY; KUHN; ROSSMANN, 2005) (**Figura 7**).

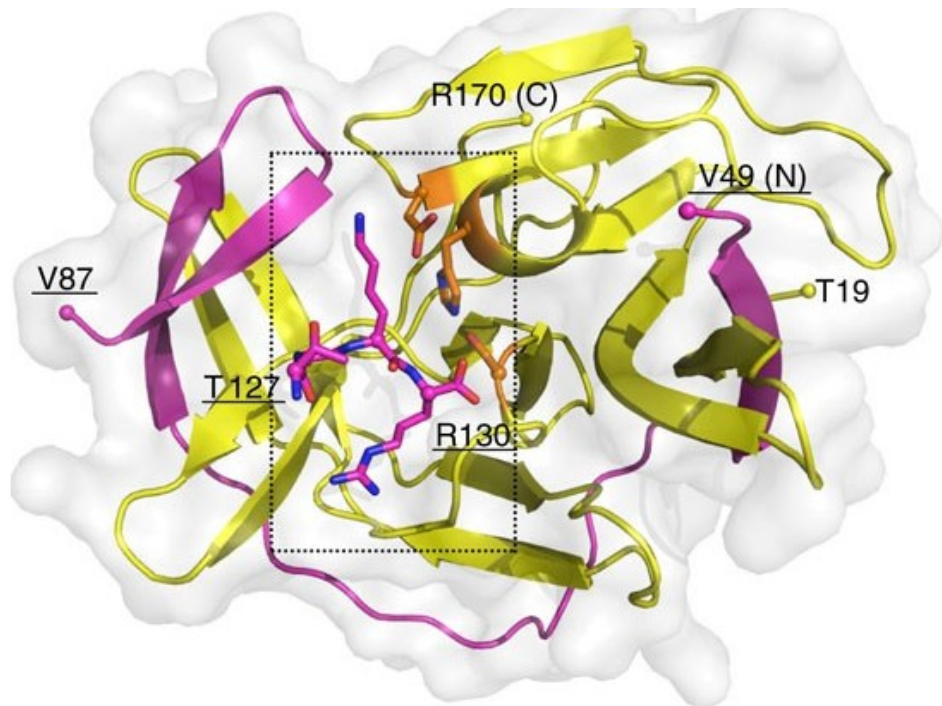
1.7 Proteína NS3 e protease NS2B-NS3Pro do ZIKV

Uma das proteínas mais estudada dos orthoflavivírus é a NS3, devido aos seus domínios promissores que podem ser usados como alvos para a inibição da replicação viral (VALENTE; MORAES, 2019a). Em seu C-terminal, possui domínios com função helicase e NTPase. A atividade de helicase é responsável pelo desenrolamento dos intermediários de RNA de dupla fita produzidos durante a replicação viral, enquanto a atividade trifosfatase fornece a energia química necessária para sua atividade enzimática (TIAN et al., 2016).

A região N-terminal da NS3, em conjunto com a NS2B, forma a protease viral NS2B-NS3^{Pro} (**Figura 8**), uma serino-protease composta por 180 resíduos da NS3 e aproximadamente 40 resíduos da região citoplasmática da NS2B. O sítio ativo da NS3 é composto por 4 domínios

(D): D1 (D129, S135, Y150, Y161), D2 (D75, D82, G83, N84, N152), D3 (F85, Q86, L87) e o D4 (V154), além da tríade catalítica formada pelos resíduos H51, D75 e S135 (ABDURAMAN et al., 2018). A NS2B atua como um cofator da NS2B-NS3^{Pro}, ajudando no dobramento da proteína e em sua ação enzimática. Sua região transmembrana ancora a proteína à membrana lipídica do RE, enquanto sua região hidrofóbica interage com a extremidade N-terminal da NS3 (PHOO et al., 2016).

Figura 8. Estrutura tridimensional da NS2B-NS3^{Pro} do ZIKV.



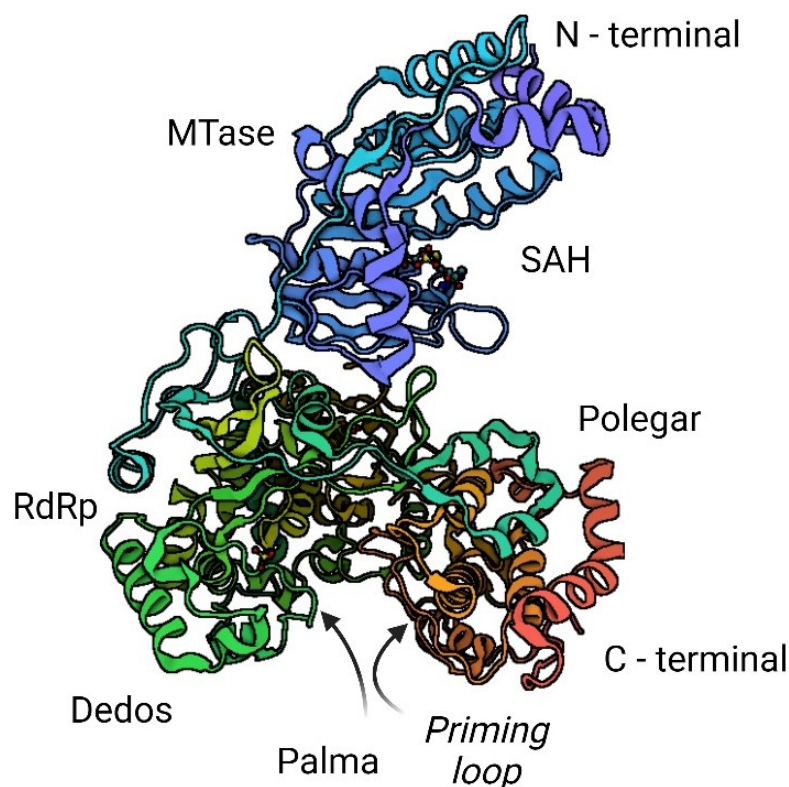
Fonte: (PHOO et al., 2016).

A estrutura da NS2B-NS3^{Pro} tem sido de difícil de resolução devido à natureza hidrofóbica da NS2B. No entanto, diferentes construções têm sido desenvolvidas para determinar a sua estrutura. Atualmente, três das principais construções utilizados para prever as interações com possíveis inibidores são: o eZiPro (**Figura 8**), que possui uma sequência de ligação TGKR entre NS2B e a NS3; o gZiPro, que utiliza um 'linker' composto por Gly4-Ser-Gly4 e o bZiPro que é a forma não ligada de NS2B-NS3 (LI et al., 2017; PHOO et al., 2016; ZHANG et al., 2016).

1.8 Proteína NS5 do ZIKV

A NS5 representa outro potencial alvo terapêutico, pois exerce atividades catalíticas essenciais para a replicação e a estabilização do genoma viral, integrando o complexo replicativo viral. É a maior e mais conservada proteína codificada pelo genoma dos orthoflavivírus, possuindo dois domínios proteicos funcionais e estruturais (VALENTE; MORAES, 2019a). A região N-terminal da NS5 do ZIKV possui domínio de RNA-metiltransferase (MTase) e domínio de RdRp na região C-terminal (GODOY et al., 2017a) (Figura 9).

Figura 9. Estrutura da NS5 do ZIKV.



Fonte: adaptada de (DUAN et al., 2017)³

O domínio MTase é essencial para a estabilização e proteção do RNA genômico, pois é responsável pela adição do CAP na extremidade 5', ou seja, a metilação da guanosina na posição 7, ligada ao primer nucleotídeo do RNA genômico. A adição do CAP estabiliza o RNA e confere semelhança com o mRNA da célula hospedeira, impedindo a ativação do sistema imune pela interação com os receptores de reconhecimento de padrões (RRP) (UPADHYAY et

³ Conformação que assemelha uma mão direita. A estrutura tridimensional da NS5 de ZIKV foi obtida de PDB:5U0B e editada com BioRender.com pela autora

al., 2017). Dentre dos sítios relevantes no domínio MTase que podem ser usados como alvos para o desenvolvimento de inibidores, estão o sítio de ligação do CAP ao mRNA (resíduos K13, L16, N17, M19, F24, K28, S150, R213 e S215) e o sítio de ligação à s-adenosil-L-metionina (SAM), estabilizado pelos resíduos S56, G86, W87, K105, H110, D131, V132, D146 e I147, que serve como doador de grupo metil (VALENTE; MORAES, 2019a).

O segundo domínio da NS5 corresponde à NS5^{RdRp}, que é a polimerase orthoflaviviral responsável pela replicação tanto da fita negativa complementar quanto do RNA genômico (UPADHYAY et al., 2017). A estrutura tridimensional da NS5^{RdRp} do ZIKV mostra uma conformação semelhante à observada para outros flavivírus, adotando uma estrutura que se assemelha a uma mão direita, com três subdomínios: os dedos (resíduos 321-488 e 542-608); o polegar (resíduos 715-903); e a palma (resíduos 489-541) (GODOY et al., 2017a) (**Figura 9**). Seu sítio catalítico está localizado na interseção de dois túneis entre os dedos e o polegar. Assim como em outros flavivírus, dois resíduos de ácido aspártico no sítio catalítico e dois íons de zinco (Zn^{2+}) são essenciais para catalisar a transferência de nucleotídeos realizada pela NS5^{RdRp} (VALENTE; MORAES, 2019a). Esses dois resíduos estão localizados nas posições Asp535 e Asp665, e os íons Zn^{2+} são coordenados pelos resíduos G439, H443, C448 e C451, do subdomínio dos dedos, e H714, C730, C849, do subdomínio do polegar. A NS5^{RdRp} também possui um *priming loop* (**Figura 9**), formado pelos resíduos V785 e D810, que é responsável pelo posicionamento alostérico da extremidade 3' do RNA molde no sítio catalítico (UPADHYAY et al., 2017).

1.9 Propriedades biológicas e bioativas dos compostos isolados de plantas: compostos naturais com atividade antiviral

O Reino *Plantae* apresenta uma elevada biodiversidade, possuindo cerca de 400.000 espécies diferentes, com apenas 10% destas estudadas em termos da sua atividade biológica (ROMANO; LUCARIELLO; CAPASSO, 2021). Esta grande diversidade oferece inúmeras possibilidades de identificar substâncias com potencial terapêutico, já que um terço das novas moléculas aprovadas pela FDA (*United States Food and Drug Administration*) são compostos naturais ou seus derivados (NASIM; SANDEEP; MOHANTY, 2022; PATRIDGE et al., 2016). Isso se deve ao fato de que as plantas, assim como outras espécies, além de produzirem metabólitos primários necessários para seu desenvolvimento, também são capazes de produzir metabólitos secundários (TEOH, 2016), relacionados com funções ecológicas, como atrair polinizadores (através de cor, odor e sabor) e proteger contra o ataque de herbívoros e

patógenos. Essas propriedades antipatogênicas os tornam alvos interessantes para o desenvolvimento de fármacos (RASKIN et al., 2002).

Os metabólitos secundários podem ser classificados em três grandes classes: os terpenos; compostos contendo nitrogênio; e compostos fenólicos (OWEN; LAIRD; SHIVKUMAR, 2022). Os terpenos, conhecidos por sua diversidade estrutural, representam mais de 55.000 moléculas descobertas e apresentam uma ampla gama de atividade biológicas, incluindo propriedades antivirais (KORTBEEK et al., 2018). Um exemplo é a hederagenina e o ácido oleanólico, derivados da β -amirina, isolada das folhas de *Hedera helix*. Yu e colaboradores (2014) reportaram a capacidade de ambas moléculas em bloquear a entrada do vírus da hepatite C (HCV) nas células hospedeiras, por meio do bloqueio da glicoproteína 2 do envelope viral. Além disso, outro estudo relatou as propriedades anti-ZIKV de alguns terpenos. Cirne-Santos e colaboradores descobriram que as frações FAc-2 e F-6 isolados da alga marinha *Dictyota menstrualis* inibem a adsorção viral, diminuindo a replicação viral durante o tratamento (CIRNE-SANTOS et al., 2019).

Os compostos contendo nitrogênio, como os alcaloides, também são conhecidos pela sua atividade biológica e potencial terapêutico, incluindo a atividade antiviral (Faisal et al., 2023; Özçelik et al., 2011). Como exemplo, a camptotecina, um alcalóide proveniente do arbusto *Camptotheca acuminata*, apresentou capacidade em inibir o Enterovirus A71 (EVA71), observando que tanto a tradução quanto a transcrição do RNA viral nas células infectadas eram suprimidas na presença do composto (WU; CHU, 2017). Adicionalmente, Jardim e colaboradores descreveram a capacidade do alcaloide APS isolado da planta *Maytrenus ilicifolia* de inibir a replicação do HCV *in vitro* com um $EC_{50} = 2,3 \mu M$ (JARDIM et al., 2015). Os compostos fenólicos são substâncias que possuem o grupo hidroxila (OH) ligado ao carbono de um anel aromático (DE LA ROSA et al., 2019). Estes compostos são reconhecidos por suas propriedades bioativas documentadas no tratamento de doenças infecciosas e não infecciosas (MIKLASÍNSKA-MAJDANIK et al., 2018; RICE-EVANS; MILLER; PAGANGA, 1997). Dentre os subgrupos dos compostos fenólicos, os flavonoides têm demonstrado eficácia contra diversos vírus como EVA71, e herpes-vírus simples 1 (HSV-1) e o vírus da Chikungunya (CHIKV) (Dai et al., 2019; Lani et al., 2016; Šudomová & Hassan, 2023). Como exemplo, a quercetina, derivada de *Caesalpinia pulcherrima*, foi relatada recentemente por sua atividade contra o SARS-CoV-2, por meio do bloqueio do receptor enzima conversora da angiotensina 2 (ECA2), impedindo adsorção viral e subsequente entrada dos vírus na célula hospedeira (LIU et al., 2020).

Os efeitos inibitórios dos compostos fenólicos em diferentes etapas do ciclo replicativo do ZIKV também foram reportados, demonstrando que alguns flavonoides se apresentaram como candidatos promissores na inibição da entrada do vírus nas células hospedeira. Como exemplo, a epigallocatequina galato (EGCG), um flavonol abundante no chá verde, demonstrou capacidade em inibir a entrada do ZIKV nas células hospedeiras, apresentando uma concentração efetiva em 50% (EC_{50}) de $21,4 \mu M$ (CARNEIRO et al., 2016). Análises *in silico* sugeriram que o EGCG interage com a proteína E do vírus, bloqueando sua adesão celular (SHARMA et al., 2017). Além disso, o EGCG também possui efeitos pós-entrada, como a inibição da atividade NTPase da NS3, uma proteína essencial na replicação viral (KUMAR et al., 2020).

Estudos adicionais com compostos fenólicos têm sido realizados com foco em alvos moleculares específicos do ZIKV, como as proteínas não estruturais (RAMOS et al., 2022). Roy e colaboradores avaliaram a atividade inibitória de compostos naturais contra a protease NS2B-NS3 e identificaram seis compostos eficazes em inibir sua atividade. Dentre eles estão os flavonóides: luteolina ($EC_{50} = 2.7 \pm 0.3 \mu M$), apigenina ($EC_{50} = 56.3 \pm 0.9 \mu M$), myrecitina ($EC_{50} = 1.3 \pm 0.1 \mu M$), quercetina ($EC_{50} = 2.4 \pm 0.2 \mu M$) e isorhamnetina ($EC_{50} = 15.5 \pm 0.7 \mu M$), e curcumina ($EC_{50} = 3.5 \pm 0.2 \mu M$) (ROY et al., 2017). Outro composto promissor é a narigenina, uma flavona presente em frutas cítricas. Estudos demonstraram sua capacidade de inibir a replicação do ZIKV nas células humanas de pulmão A549, de maneira dose-dependente, com um EC_{50} de $58,79 \mu M$. Estudos *in silico* indicam que a narigenina pode agir como um análogo de substrato da protease NS2B-NS3, bloqueando sua atividade (CATANEO et al., 2021; SALEHI et al., 2019).

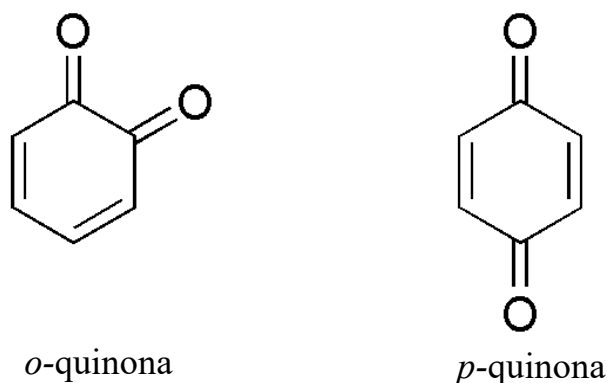
Este panorama destaca a importância dos compostos fenólicos como uma fonte relevante de compostos bioativos para o desenvolvimento de antivirais. Dentre os compostos fenólicos, as naftoquinonas são de particular interesse devido à sua ampla distribuição no ambiente e às suas propriedades farmacológicas diversas, que incluem atividades antineoplásicas, anti-inflamatórias, antiparasitárias, antimicrobianas e antivirais. (AMININ; POLONIK, 2020; DA SILVA et al., 2002b; FUTURO et al., 2018; LINZNER et al., 2020; NASIRI et al., 2013; NAVARRO-TOVAR et al., 2023; PACHE et al., 2022b; QIU et al., 2018; WU et al., 2020).

1.10 Naftoquinonas

As naftoquinonas são consideradas estruturas químicas privilegiadas na química medicinal devido à associação com diversas atividades farmacológicas e à facilidade de modificação química (QIU et al., 2018). Isso permite o uso dos compostos puros, ou como modelos para desenvolvimento de análogos, onde sejam melhoradas suas propriedades farmacológicas como a absorção, distribuição, metabolismo, excreção e toxicidade (ADMET) (PEREYRA et al., 2019). Alguns exemplos de naftoquinonas naturalmente encontradas no ambiente, estudadas pela sua versatilidade farmacológicas, são a lausona (2-hidroxi-1,4-naftoquinona), a plumbagina (5-hidroxi-2-metil-1,4-naftoquinona), a juglona (5-hidroxi-1,4-naftalenodiona), a shikonina (5,8-Dihidroxi-2-(1-hidroxi-4-metil-3-pentenil)-1,4-naftoquinona) e o lapachol (2-hidroxi-3(3-metil-2-butenil)-1,4-naftoquinona) (Guo et al., 2019; Hussain et al., n.d.; Nair et al., 2024; Padhye et al., 2012b; Strugstad & Despotovski, 2012)

Estas propriedades bioativas das naftoquinonas são atribuídas a sua estrutura química. A estrutura das quinonas apresenta dois agrupamentos carbonila em um anel insaturado de seis átomos de carbono, situados relativamente em posições ‘orto’ (grupos carbonila adjacentes) ou ‘para’ (posições opostas) (**Figura 10**).

Figura 10. Posições dos grupamentos carbonila das quinonas

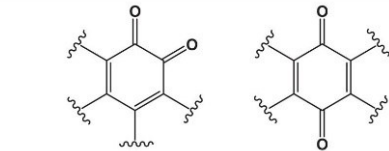
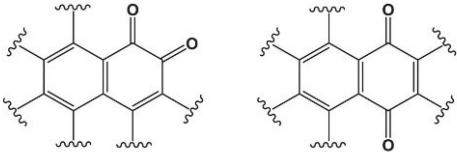
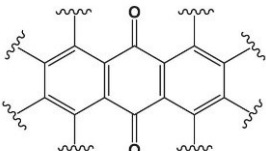
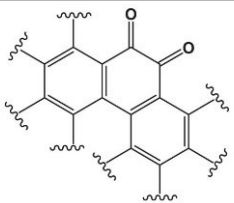


Fonte: adaptado de Wikimedia Commons⁴

⁴ Disponível em <http://commons.wikimedia.org/wiki/File:Benzoquinones.png>

Utilizando-se como critério o tipo de sistema aromático, as quinonas são classificadas como benzoquinonas, naftoquinonas, antraquinonas e fenantraquinonas (**Figura 11**) (SOUSA; LOPES; DE ANDRADE, 2016).

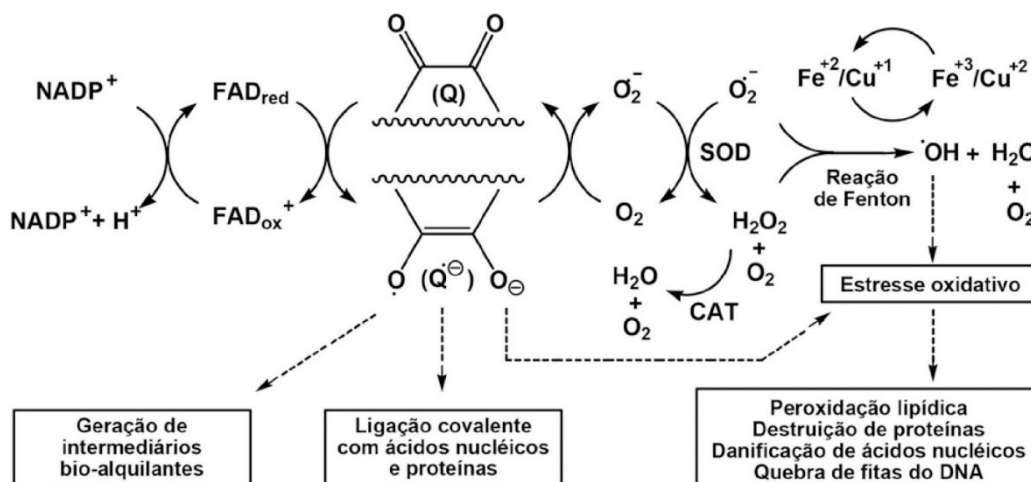
Figura 11. Classificação das Quinonas

Grupo		Sistema aromático	Estruturas Básicas
1. Benzoquinonas	→	Anel benzênico	
2. Naftoquinonas	→	Anel naftalênico	
3. Antraquinonas	→	Anel antracênico	
4. Fenantraquinonas	→	Anel fenantrênico	

Fonte: (SOUSA; LOPES; DE ANDRADE, 2016)

Em geral, as quinonas apresentam duas propriedades químicas de particular importância: são oxidantes, o que lhes permitem participar de reações do ciclo redox (**Figura 12**); e são capazes de reagir com nucleófilos por reações nucleofílicas de Michael, uma das reações mais utilizadas na formação da ligação carbono-carbono (MONKS et al., 1992). As participações das naftoquinonas em reações do ciclo redox ocorre devido a sua estrutura química aceitar um elétron, resultando na formação de semiquinonas, com uma carga negativa e um elétron desemparelhado (um ânion radical); ou aceitar dois elétrons resultando em hidroquinonas. Essas espécies formadas podem ser oxidadas uma segunda vez pelo oxigênio molecular (O_2), gerando assim espécies reativas de oxigênio (ERO) (PEREYRA et al., 2019).

Figura 12. Representação do Ciclo Redox induzido por quinonas.



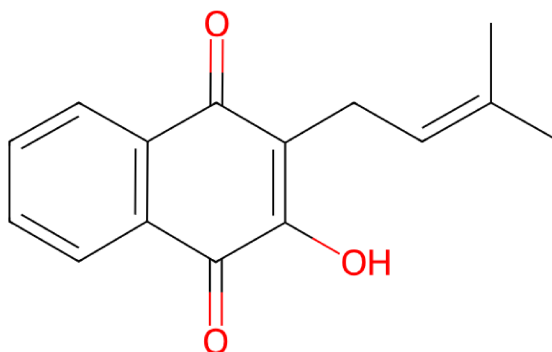
CAT = Enzima catalase; SOD = Enzima superóxido desmutase; FAD = Enzima flavina.

Fonte: (SOUSA; LOPES; DE ANDRADE, 2016)

1.11 Lapachol

O lapachol (**Figura 12**) (fórmula molecular = $C_{15}H_{14}O_3$; nome IUPAC = 2-hidroxi-3-(3-metil-butenil)-nafto-1,4-diona; massa molar = 242.2699) é uma 1,4-naftoquinona que pode ser encontrada em muitas espécies de plantas nativas da América do Sul, com maior abundância em árvores dos gêneros *Roseodendron*, *Tabebuia* e *Handroanthus*, como o *Handroanthus impertiginosus* (DE SOUZA et al., 2020). Devido à sua ampla distribuição geográfica e propriedades bioativas, o lapachol tem sido amplamente estudado, demonstrando seu papel versátil como agente antibacteriano (LINZNER et al., 2020), antifúngico (FUTURO et al., 2018), antitumoral, (DA SILVA; FERREIRA; DE SOUZA, 2003; SACAU et al., 2003a) e antiviral (LAGROTA et al., 1983).

Figura 13. Estrutura química do Lapachol



Fonte: *National Institute of Standards and Technology*⁵

O lapachol também tem sido utilizado como molécula molde para a síntese de novos derivados com potencial efeito antiviral, demonstrando sua capacidade de inibir a proliferação do CHIKV, com potencial papel como inibidor da NSP3 (PACHE et al., 2022). Além disso, a piranaftoquinona, que compartilha com o lapachol a estrutura de naftoquinona, demonstrou sua atividade contra o DENV (GUPTA; MAHAJAN, 2020).

Dessa forma, o lapachol destaca-se como um candidato promissor para o desenvolvimento de antivirais contra o ZIKV, devido à sua ampla presença em diversas espécies de plantas da América do Sul (DE SOUZA et al., 2020), facilidade de extração (VIANA et al., 2003), e seu potencial para inibir outros vírus, como o vírus Epstein-Barr (EBV) (SACAU et al., 2003a). Além disso, sua estrutura permite modificações químicas, possibilitando a síntese de múltiplos derivados como ponto de partida para aprimorar suas propriedades farmacológicas (DA SILVA et al., 2002a), conforme observado em estudos anteriores com derivados de naftoquinonas que demonstraram atividade contra outros arbovírus, como CHIKV e DENV (GUPTA; MAHAJAN, 2020; PACHE et al., 2022a). (LINZNER et al., 2020).

⁵ CSID:10770962, disponível em <<https://www.chemspider.com/Chemical-Structure.10770962.html>>, (acesso: 13 de setembro de 2024)

2. OBJETIVOS

Este projeto teve como objetivo avaliar a atividade antiviral do lapachol no ciclo de replicação do ZIKV *in vitro*.

2.1 Objetivos Específicos

- Determinar a citotoxicidade do lapachol em linhagem de células Vero E6 (células epiteliais renais extraídas de macaco verde africano, ATCC® E6™) para estabelecer as máximas concentrações não citotóxicas;
- Avaliar a atividade anti-ZIKV do lapachol em células Vero E6 infectadas com a cepa ZIKV_{PE243};
- Determinar a concentração efetiva de inibição em 50% (EC₅₀), a concentração citotóxica em 50% (CC₅₀) e calcular o Índice de Seletividade ($IS = CC_{50}/EC_{50}$) do tratamento com lapachol;
- Avaliar o efeito antiviral do lapachol nas diferentes etapas do ciclo replicativo do ZIKV_{PE243};
- Investigar potenciais modos de ação anti-ZIKV do lapachol.

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CAPÍTULO II

Manuscrito

INSIGHTS INTO INHIBITORY EFFECTS OF LAPACHOL AGAINST ZIKA VIRUS

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Abstract

Background

The reemergence of *Orthoflavivirus zikaense* (Zika virus, ZIKV) in 2015 and its association with severe neurological manifestations highlighted the urgent need for effective antiviral therapies, which, despite extensive efforts, remain unavailable. Lapachol, a 1,4-naphthoquinone compound abundant in Brazilian plant species, seems to be a promising candidate as an antiviral agent since its activity against other RNA viruses, as well as other human pathogens, has been previously reported. Here we investigated its activity in inhibiting ZIKV replication by treating Vero E6 cells infected with wild type strain ZIKV_{PE243} and quantifying focus-forming units (FFU) through immunofluorescence assays. Time-of-addition assays were used to identify the stages of its viral replication cycle that lapachol is capable of inhibiting. Mechanism insights were obtained through compound- protein interaction assays, target-specific molecular docking analysis, and evaluation of reactive oxygen species (ROS) formation.

Results

Lapachol exhibited potent inhibition of ZIKV with an EC₅₀ of 3 μ M and selectivity index of 50.7. Results suggest that its main action is exerted through post-entry inhibition of viral replication, possibly through the blockage of NS2B-NS3 protease and NS5 polymerase's catalytic site. Protective effect observed when cells were treated before infection, as well as the capacity of lapachol to induce ROS formation *in vitro*, suggest multiple antiviral mechanisms at play in addition to its enzymatic inhibition, triggering an oxidative stress response that damages the viral particles.

Conclusion

Overall, these findings shed light on lapachol's potential to be used for the development of ZIKV inhibitors, especially targeting post-entry stages of virus replication.

Keywords: Zika virus, lapachol, antiviral, natural compounds, protease and polymeraseinhibitors

Background

Among infectious diseases that cause significant health challenges in tropical countries, Zika fever stands out for its ability to cause long-lasting consequences in affected individuals (MWALIKO et al., 2020). Caused by *Orthoflavivirus zikaense* (Zika virus, ZIKV), it is an asymptomatic disease in 80% of cases, and when symptomatic, mild symptoms like low-grade fever, joint-pain, headache, asthenia, and macopapular rash are mainly observed, typically following a self-limiting course (VASCONCELOS, 2015).

ZIKV was identified for the first time in 1947 in a Rhesus monkey from the Zika forest in Uganda, proceeding to spread amongst the African continent (SILVA; SANTOS; MARTINS, 2020). Despite the low pathogenicity previously reported, ZIKV has become a cause of public health concern since its reemergence in the Americas, in 2015 (CAUCHEMEZ et al., 2016), due to its association with neural malformations in newborns and Guillain-Barré Syndrome (GBS) in adults (ANTONIOU et al., 2020b). Despite its medical significance, there is no licensed vaccine as prophylaxis or antiviral to treat ZIKV infected patients, making imperative the need to find compounds capable of impairing its replication (“Zika Vírus — Ministério da Saúde”, 2024).

ZIKV is a single-stranded positive-sense RNA arbovirus that belongs to the family *Flaviviridae*, transmitted to humans through the bite of the mosquito *Aedes aegypti* (XIE et al., 2021). ZIKV genome has one open reading frame (ORF) that codifies for a single polyprotein, that is later cleaved to produce three structural proteins, the envelope protein (E), the capsid protein (C), and the membrane protein (M), as well as seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) that are essential for virus replication into the host cells (VALENTE; MORAES, 2019b).

Because of their important role in infection establishment, non-structural proteins are being widely studied as targets for new antivirals (SAIZ; MARTÍN-ACEBES, 2017). In this sense, NS3 stands out since its N-terminal domain, together with NS2B, form a trypsin-like serine protease that is responsible for cleaving the viral polyprotein, and its C-terminal domain possesses helicase and NTPase activities, that help unwind secondary RNA structures on the 5'-UTR, a function necessary for virus initial replication (VALENTE; MORAES, 2019b). Another potential target is NS5^{RdRp}, ZIKV RNA-dependent RNA polymerase (RdRp), since it functions as a key component of the virus replication complex and plays several critical roles in the viral life cycle. It is a multifunctional protein with two main domains: an N-terminal methyltransferase (MTase) domain responsible for mRNA capping, and a C-terminal RNA-

dependent RdRp domain responsible for viral RNA synthesis. Its inhibition in other viruses has been shown employing the use of nucleoside analogs and polymerase inhibitors (HAYDEN; SHINDO, 2019; ZENCHENKO et al., 2021).

An option that arises in the search for compounds with bioactive activity is the use of natural organic compounds. Compounds extracted from natural sources have become of increasing interest, with many of them showing antiviral activity against ZIKV (CATANEO et al., 2021; FONG; CHU, 2022; PEREIRA et al., 2023; RAMOS et al., 2022). Quinones are a well-studied group of compounds known for their diverse physicochemical and bioactive properties. They can be classified based on the amount of benzene rings into their structure: benzoquinones (containing one ring), naphthoquinones (containing two rings) or anthraquinones (containing three rings). Naphthoquinones, produced as secondary metabolites by a wide range of species among different kingdoms (WU et al., 2020), contain a naphthalene ring (fusion of a pair of benzene rings) with two carbonyl groups in the 1,4-positions (NAVARRO-TOVAR et al., 2023).

Lapachol is a 1,4-naphthoquinone that can be found in many plant species native to South America, with greater abundance in trees of the genus *Roseodendron*, *Tabebuia* and *Handroanthus*, such as *Handroanthus impertiginosus*. For its wide distribution and bioactive properties, lapachol has been extensively studied, demonstrating its versatile role as an antibacterial (LINZNER et al., 2020), antifungal (FUTURO et al., 2018), antitumoral (NAVARRO-TOVAR et al., 2023) and anti-inflammatory (HUSSAIN et al., 2007) agent, with reports of its activity against DNA viruses (DA SILVA et al., 2002b; SACAU et al., 2003a) and RNA viruses (LAGROTA et al., 1983). Furthermore, pyra-naphthoquinone, sharing the commonality with lapachol of a naphthoquinone moiety on its structure, has demonstrated its activity against *Orthoflavivirus denguei* (dengue virus, DENV) (GUPTA; MAHAJAN, 2020).

To the best of our knowledge, there are no reports of *in vitro* activity of lapachol against ZIKV. In this sense, the purpose of our study was to evaluate the effects of lapachol on ZIKV replication *in vitro*, with insights on its mechanism of antiviral action.

Materials and Methods

Lapachol extraction and purification

Na₂CO₃ solution (1% w/v) was added to a mass of 250 g of purple ipê (*Handroanthus impertiginosus*) sawdust. After 24 h undisturbed, the mixture was filtered and HCl (6 M) was

added until precipitation occurred. Lapachol was collected by vacuum filtration, recrystallized in ethanol and exposed to drying for 4 days away from light.

Cell culture and virus

Vero E6 cells (derived from epithelial kidney cells of African green monkey, ATCC E6) were maintained in Dulbecco's modified Eagles medium (DMEM, Sigma-Aldrich) supplemented with 100 U/mL of penicillin (Gibco Life Technologies), 100 mg/mL of streptomycin (Gibco Life Technologies), 1% non-essential amino acids (Gibco life technologies) and 5% (v/v) fetal bovine serum (FBS) (Cultilab) in a humidified 5% CO₂ incubator at 37 °C.

The isolate ZIKV_{PE243}, acquired during the 2015 Brazilian outbreak from a patient with classic Zika fever symptoms in Recife, Brazil (DONALD et al., 2016), was used for infection assays. Amplification of the virus was performed as previously described (CASSANI et al., 2023), by infecting Vero E6 cells in a 75 cm² flask, incubating for three days and collecting its supernatant. All viral aliquots were stored at -80°C. Viral titers were determined as previously described, with modifications (CASSANI et al., 2023). Briefly, 10-fold serial dilutions were performed in culture medium and used to infect previously seeded Vero E6 cells in 96 well plate at a density of 5x10³ cells/well. The infected cells were incubated for 2 hours and later overlaid with culture medium containing 2% carboxymethyl cellulose (CMC) and DMEM 2% FBS for a final 72-hour incubation. After this period, focus forming units per milliliter (FFU/mL) were quantified by immunofluorescence assay, performed by staining ZIKV NS3 protein, as previously described. All the infection assays were performed at a BSL-2 laboratory under the authorization number CBQ: 163/02 and process SEI: 01.245.006267/ 2022–14 from the CTNBio - National Technical Commission for Biosecurity from Brazil.

Cell viability assays

To determine cytotoxicity of lapachol, cultured cells at a density of 5x10³ cells/well on 96-well plates were exposed to concentrations of the compound (diluted in 5% FBS supplemented culture medium) ranging from 0.78 to 100 µM, using a two-fold serial dilution and incubating at 37°C for 72 hours. After incubation, cell viability was measured using the MTT [3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Sigma-Aldrich) assay. MTT was added at a concentration of 0.5 mg/mL, and after a 30-minute incubation at 37°C, it was replaced with dimethylsulfoxide (DMSO) to solubilize formazan crystals. Absorbance was measured by optical density (OD) at 560 nm with the Glomax microplate

reader (Promega). Cell viability was calculated as $(T/C) \times 100\%$, where T and C represent the mean optical density of the treated group and control group, respectively. The cytotoxic concentration of 50% (CC_{50}) was calculated applying non-linear regression using GraphPad Prism 8.0 (SILVA et al., 2019).

Antiviral assay

To assess antiviral activity of lapachol, a dose-dependent assay was performed, as previously described (CASSANI et al., 2023). Vero E6 cells at a density of 5×10^3 cells/well on 96-well plates were infected with ZIKV_{PE243} at a Multiplicity of Infection (MOI) of 0.01, and simultaneously treated with lapachol at concentrations ranging from 0.78 to 100 μ M in two-fold serial dilutions followed by incubation at 37°C. Cells were fixed 72 hours post-infection (h.p.i.) using 4% paraformaldehyde. FFU were determined by immunofluorescence assay (CASSANI et al., 2023). The effective concentration of 50% (EC_{50}) was calculated applying non-linear regression using GraphPad Prism 8.0. The values of CC_{50} and EC_{50} were used to calculate the selective index ($SI = CC_{50}/EC_{50}$) (SILVA et al., 2019).

Time-of-drug addition assay

For all time-of-drug addition protocols, Vero E6 cells were seeded at a density of 5×10^3 cells/well on 96-well plates and incubated overnight at 37 °C, followed by infection with ZIKV_{PE243} in the presence of lapachol at 8 μ M diluted in DMSO, therefore using DMSO alone as untreated control. Upon completion of the 72-hour incubation, the supernatant of each replicate was collected and titrated as previously described in the section above. FFU/mL were quantified through immunofluorescence assay.

For pre-treatment assessment, the cells were exposed to lapachol for 2 hours at 37°C. After this period, cells were washed with phosphate-buffered saline (PBS) [1x], to remove any remnants of the compound, and infected with the virus at a MOI of 1. The infected cells were then incubated for an additional 2 hours, followed by washing with PBS [1x] and addition of fresh 2% FBS cell culture medium for a final incubation of 72 hours at 37 °C and 5% CO₂.

For entry assays, cells were simultaneously infected with ZIKV_{PE243} (MOI = 1) and treated with the compound, incubated for 2 hours, and followed by the same washing and incubation conditions described above. For determination of virucidal activity, ZIKV_{PE243} at a MOI of 3 was incubated with lapachol at 8 μ M for 2 hours at 37 °C. Past the incubation period,

the virus pre-exposed to the compound was added to the cells, followed by the same protocol outlined for the entry assay.

For post-entry assays, seeded cells were infected with the virus at a MOI of 1 and incubated for 2 hours, then washed with PBS for unbound virus removal and incubated with compound-containing medium.

NS3 Helicase (NS3^{Hel}) ATP / GTPase activity assay

Assays for the evaluation of ATPase activity of the Helicase domain of NS3 from ZIKV NS3^{Hel} were performed using the commercial QuantiChrom™ ATPase/GTPase Assay Kit (BioAssay Systems), as described by Cao and coworkers (CAO et al., 2016). The assay was standardized for NS3^{Hel} as described in the kit manual. 200 nmol.L⁻¹ of NS3^{Hel} were incubated in 20 µl of 20 mM Bis-Tris buffer containing a pH of 7.0, 500 mM NaCl and 10% Glycerol previously supplemented with 8.0 mM MgCl₂ (Sigma) in a 96-well plate (Greiner Crystal Clear). Subsequently lapachol at 500 µM was added to the reaction. The reaction was performed with 1.0 mM of ATP and incubated for 30 min at 25 °C. Reactions were stopped with the addition of 200 µl reagent buffer from the kit itself and incubated again for 30 minutes at room temperature before absorbance measurement at $\lambda = 620$ nm, which is associated with amount of phosphate released due to ATP hydrolysis. The tests were performed in biological duplicate and technique triplicate. Reaction mix containing pure DMSO (1% vol/vol) was used as untreated control. The results were analyzed and plotted using GraphPad Prism 5.0 program.

NS2B-NS3 protease (NS2B-NS3^{Pro}) activity assay

The NS2B-NS3^{Pro} activity assay was performed as described by Li and colleagues (LI et al., 2018). For the reaction, a buffer containing 20 mM Tris pH 8.5, 10% Glycerol and 0.01% Triton X-100 was used. In each reaction Benzoyl-Nle-Lys-Arg-Arg-4-methylcoumarin-7amide (Bz-nKRR-AMC) substrate and NS2B-NS3^{Pro} were used at a final concentration of 30 µM and 4 nM, respectively. During the reaction, the temperature was held at a constant temperature of 37°C and fluorescence measurements were performed over a 30-minute interval, measuring activity every 60 seconds. Reactions were performed on 96-well black Corning® plates. The wavelengths used for excitation and emission were 380 nm and 460 nm, respectively.

For inhibition assay, lapachol was diluted in DMSO to a final concentration of 50 mM. For unique point reaction, lapachol was added at a final concentration of 500 µM, resulting in 1% of DMSO in the reaction. Furthermore, to determine the percentage of inhibition by

lapachol, protease activity was measured after a period of 20 minutes and compared to a positive control reaction containing the same amount of DMSO as the reaction containing the ligand. All tests were performed in duplicate.

For the concentration-response assay a similar approach was used, however, two-fold serial dilutions were performed with concentrations ranging from 500 μ M to 0.98 μ M, the highest concentration containing 1% of DMSO in the solution. The dose-response curves were analyzed on GraphPad Prism using Hill fitting to obtain IC₅₀ values.

ZIKV NS5^{RdRp} transcription elongation activity assay

ZIKV NS5^{RdRp} activity assay was performed as described by Fernandes and collaborators (FERNANDES et al., 2021). Briefly, transcription elongation activity of NS5^{RdRp} was measured by quantification of SYBR green signal using the synthetic biotinylated self-priming template 3'UTR-U30 (5'-biotin-U30-ACUGGAGAUCGAUCUCCAGU-3'). The endpoint assays were performed with lapachol at 500 μ M. The results were analyzed and plotted using the GraphPad Prism v. 8.0 program.

Molecular docking

To obtain the crystal structure of ZIKV NS3 and NS5 proteins, a search was carried out in the Protein Data Bank (PDB). Regarding the NS3 protein, the solved structure for the NS2B-NS3^{Pro} was selected (Accession number PDB:5GPI).

For the NS5^{RdRp}, the model with the best coverage was selected (Accession number PDB:5U04). The final model was subjected to validation regarding its structure in SAVES V6.0 (LASKOWSKI et al., 1996), by PROCHEK. The GOLD 2022.2.0 *software* was used to calculate the docking between lapachol and ZIKV NS3 and NS5 proteins. To visualize the docking solution, the *software* PyMOL (PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) was used. Additionally, for better understanding of the bonds and their chemical nature, the results were submitted to the LigPlot+ *software* (JONES; WILLETT; GLEN, 1995), obtaining the 2D diagram.

Assessment of ROS production

Total amount of intracellular levels of ROS were determined using 2', 7'-dichlorofluorescein diacetate (DCFH-DA) fluorescent probe, as previously described with modifications (KIM; XUE, 2020). Briefly, Vero E6 cells were seeded on a black 96 well plate

at a density of 5×10^3 cell/well. 24-hours later, the assay was performed with cells divided into different treatment groups: 1) cells infected with ZIKV_{PE243} at an MOI of 1 in the presence of lapachol at 8 μ M; 2) cells infected with ZIKV_{PE243} at an MOI of 1 in the presence DMSO 0.1%; 3) naïve uninfected cells treated with lapachol at 8 μ M; 4) naïve non-infected cells treated with DMSO 0.1%; and 5) untreated and uninfected cells was used as blank to establish baseline ROS levels in the cells. After a 72-hour incubation period at 37 °C, the media was removed and cells were washed with PBS, adding 50 μ L of probe per well at a concentration of 10 μ M and incubating for 40 minutes in dark conditions at 37 °C. Later the probe was removed, and the cells were washed two times with PBS 2% Bovine Serum Albumin (BSA), later adding 50 μ L of PBS 1x. Fluorescence at $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 525$ nm was measured in a plate fluorimeter (M2e, Molecular Devices). The results were presented as the ratio of fluorescence intensity at 525 nm normalized to cell density (OD₇₅₀) compared to the untreated control.

Statistical analysis

All antiviral experiments in cell culture were performed in triplicates with the repetition of the same conditions a minimum of three times for reproducibility reassurance. For the statistical analysis, GraphPad Prism 8.0 was used. Data was analyzed for normal distribution to demonstrate the applicability of the parametric or the nonparametric test. Two-way ANOVA test was employed to compare the treatment of each compound with the untreated control (DMSO 0.1%). P values differences < 0.05 were considered significant. EC₅₀ and CC₅₀ were calculated using a non-linear regression considering log(inhibitor) vs. response, with variable slope (four parameters).

Results

Lapachol exhibits potent efficacy and low cytotoxicity

Aiming to explore eligibility of lapachol as an antiviral candidate against ZIKV, we evaluated its cytotoxicity profile and antiviral activity using Vero E6 cells in the presence or absence of infection with ZIKV_{PE243}. To assess the cytotoxic concentration of 50% (CC₅₀), two-fold serial dilutions were performed, with a starting concentration of 100 μ M and a final concentration of 0.78 μ M, which were added to the cells for a 72-hour incubation period. MTT assays determined the percentage of viable cells. To calculate the Effective Concentration of 50% (EC₅₀), infected cells were treated with the same two-fold serially diluted concentrations of the compound (0.78 – 100 μ M). Viral replication rates were measured by quantification of FFU after immunofluorescence assay (CASSANI et al., 2023). The results revealed that a low

compound concentration was sufficient to achieve 50% of its maximum effect as indicated by an EC_{50} of 3.0 μ M. In contrast, a high concentration was required to induce toxicity in 50% of the cell population, showed by a CC_{50} of 154.7 μ M, resulting in a SI of 50.7 (**Figure 1**). These results suggest that lapachol acts as a strong inhibitor at low concentrations with a great antiviral index.

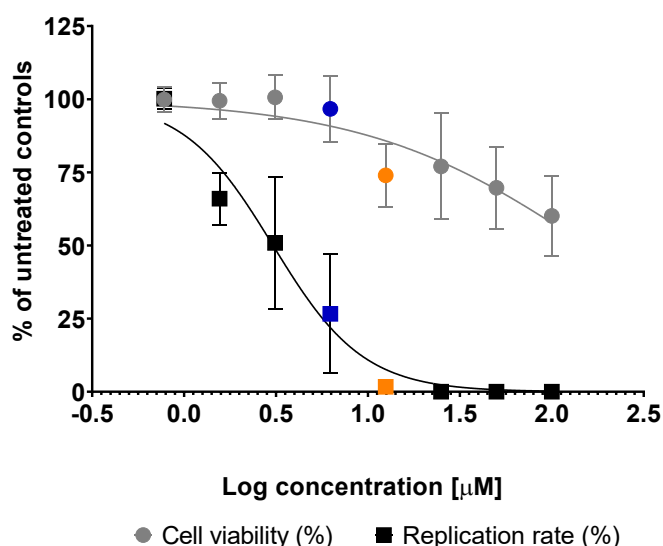


Figure 1. Dose-dependent effect of lapachol on ZIKV replication. Vero E6 cells were treated with two-fold serial dilutions ranging from 0.78 – 100 μ M in the presence or absence of ZIKV_{PE243} infection at an MOI of 0.01. The CC_{50} was measured by MTT assays (gray circles) and EC_{50} was calculated by quantification of ffu (black squares). The concentrations of 6.25 and 12.5 μ M are represented in blue and yellow, respectively. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. EC_{50} and CC_{50} values were calculated by non-linear regression using GraphPad Prism 8.0.

Based on these results, a working concentration that inhibited over 80% of virus replication, while maintaining minimal toxicity was selected for further assays. As observed in **Figure 1**, this concentration is observed between 6.25 μ M (inhibition rate = 74%; cell viability = 97%), represented in blue, and 12.5 μ M (inhibition rate = 98%; cell viability = 74%), represented in yellow. Therefore, considering the dose-response profile of the compound, the concentration of 8 μ M was selected for further experiments.

Lapachol confers protection to Vero E6 cells against ZIKV infection

As an approach to elucidate the potential targets of lapachol, we exposed the virus to the compound at 8 μ M at different time points of the infection as seen in **Figure 2**. Vero E6 cells

were infected with ZIKV_{PE243} at a MOI of 1 or 3, according to the assays protocol, as detailed in **Figure 2** and in the Methods section. Virus titration of the supernatant was performed to all assays 72 h.p.i..

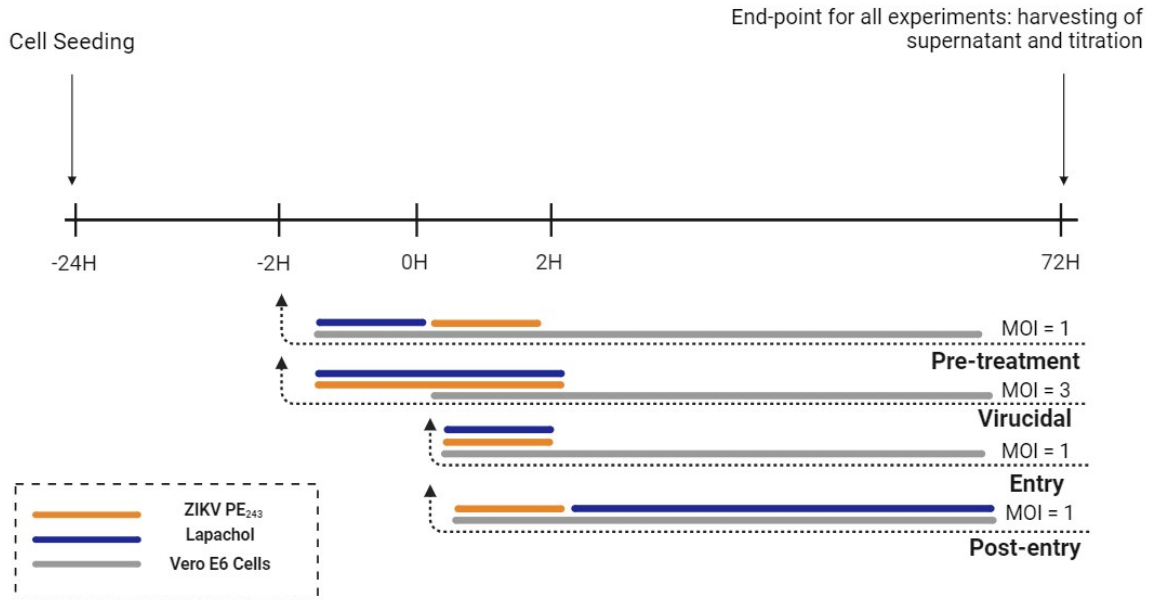


Figure 2. Schematic representation of time-of-addition assay. Vero E6 cells (gray lines) were treated with 8 μ M of lapachol (blue lines) at different time points of ZIKV (yellow lines) infection. All assays were followed by supernatant collection and titration 72 h.p.i.

An initial evaluation of the host-directed activity of lapachol was performed through pre-treatment assays, where cells were first incubated with the compound for 2 hours at 37°C prior infection (**Figure 2**). Results revealed that lapachol can confer a significant protection to the cells with a 100-fold reduction of the supernatant viral titer ($p < 0.01$) (**Figure 3A**). These results suggest that one mode of antiviral action of lapachol may be related with host elements interaction and hindering ZIKV infection.

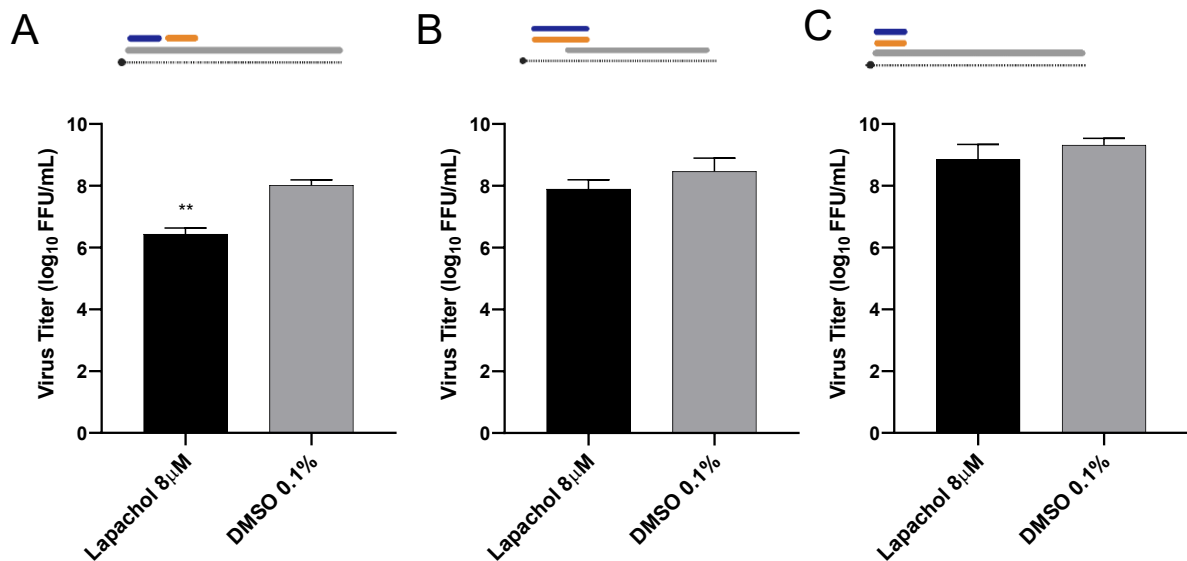


Figure 3. *Lapachol confers protection to Vero E6 cells against ZIKV infection.* A) Vero E6 cells were treated with the compound at 8 μM for 2 h at 37°C, followed by compound removal and ZIKV_{PE243} infection (MOI = 1) for 2 additional hours. Cells were washed with PBS [1x], and virus-free medium was added. B) Virucidal effect was assessed by exposing ZIKV_{PE243} (MOI = 3) to the compound at 8 μM for 2 h at 37°C with subsequent infection of Vero E6 cells with this inoculum for extra 2h. C) Entry assays were performed by adding lapachol and ZIKV_{PE243} simultaneously and incubating for 2 hours at 37°C. Schematic representation on the top of graphics as follows: Vero E6 cells (gray lines), lapachol (blue lines), ZIKV (yellow lines). Data is expressed as virus titer in logarithmic form. Mean values of three independent experiments each measured in triplicate including the standard deviation are shown. DMSO 0.1% was used as untreated control. P values < 0.05 were considered significant. (*) p < 0.05, (**) p < 0.01, (***) p < 0.001, and (****) p < 0.0001. Images were generated with BioRender.com and GraphPad Prism 8.0.

Early stages of ZIKV infection were also evaluated. An effect on ZIKV particles was assessed by incubating ZIKV with lapachol for 2 h and incubating with cells for an extra 2h. Alternatively, to investigate virus entry to host cells, ZIKV and lapachol were simultaneously added to cells for 2h. For both protocols, the inoculum was then removed, cells washed and incubated with fresh media (**Figure 2**). No virucidal effect was observed (**Figure 3B**). Additionally, entry assays revealed that the compound cannot impair virus entry to the cell (**Figure 3C**).

Lapachol strongly impairs ZIKV replication after virus entry to host cells

As an evaluation of the interference of lapachol on late stages of ZIKV replicative cycle, post-entry assays were performed by infecting cells for 2 hours, washing them to remove unbound virus and then treating the cells with the compound at 8 μM (**Figure 2**). As seen in

Figure 4, lapachol was able to effectively and strongly inhibit ZIKV replication after virus entry to the cell, leading to undetectable levels on its supernatant, in comparison to a mean viral titer of 1×10^9 FFU/mL in untreated cells ($p < 0.01$). Therefore, lapachol was able to reduce ZIKV replication in $9 \log^{10}$, when added to the cells after virus entry.

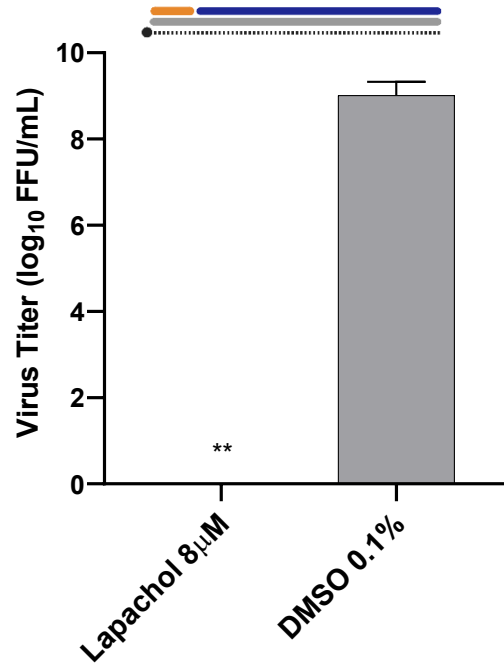


Figure 4. *Lapachol strongly inhibits post-entry stages of ZIKV replication in Vero E6 cells.* ZIKV_{PE243} at a MOI of 1 was added to cells for 2 h at 37 °C, cells were washed with PBS [1x] and the inoculum was replaced by medium containing lapachol at 8 μ M. Supernatant was titrated 72 h.p.i. Schematic representation on the top of graphics as follows: Vero E6 cells (gray lines), lapachol (blue lines), ZIKV (yellow lines). Data are presented as virus titer expressed in logarithmic form. The mean values of three independent experiments each measured in triplicate including the standard deviation were compared. DMSO 0.1% was used as untreated control. P values < 0.05 were considered significant. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, and (****) $p < 0.0001$. Images generated with and GraphPad Prism 8.0.

***In silico* data shows lapachol binds to non-structural proteins essential for virus replication**

To further comprehend lapachol targets on post-entry stages of the viral replicative cycle we performed molecular docking analysis with non-structural proteins NS3 (**Figure 5A**) and NS5 (**Figure 5B**) using lapachol as a ligand.

In regard to the analysis of the molecular docking with the NS2B-NS3^{Pro} (**Figure 5C**), it predicted five hydrophobic interactions with residues (Gly151, Asn152, Tyr161, Try130 and Ser135) and two hydrogen bonds with residues His51 (2.77Å) and Asp75 (2.94Å), these two

residues and Ser135 are components of the catalytic triad. NS5^{RdRp} analysis consisted of a total of three runs (**Figure 5D-F**), using as target three important regions for viral replication. The first run targeted a residue (His714) belonging to a subdomain necessary for coordination of Zn²⁺ ions which primary residues are His714, Cys730 and Cys849 (GODOY et al., 2017b). It resulted in five hydrophobic interactions with residues (Glu707, Glu845, Arg844, Trp848 and His714) and three hydrogen bonds with the residue Arg462 (2 hb) and Asn471 (1 hb), with a total ChemPLP score of -53.53 kJ/mol (**Figure 5D**). The second run targeted the residue Asp665 since this composes the two Aspartates residues that are fundamental for catalyzing the nucleotide transfer carried out by NS5^{RdRp} (VALENTE; MORAES, 2019a), resulting in a ChemPLP score of -42.49 kJ/mol and predicting two hydrogen bonds with the residue Arg501 (2.77Å e 3.07Å) and four hydrophobic interactions (Arg659, Ala526, Lys658 and Asp655) (**Figure 5E**). In relation to the third run, it consisted of targeting the residue Thr808 belonging to the priming loop, region responsible for allosteric positioning of the RNA template (VALENTE; MORAES, 2019a), the molecular docking predicted two hydrogen bonds with Lys758 (2.85 Å) and Val787 (3.01 Å) and seven hydrophobic interactions (Gln762, Gln765, Thr808, Asp810, Met811, Met883 and Tyr885) with a total score of -43.30kJ/mol (**Figure 5F**).

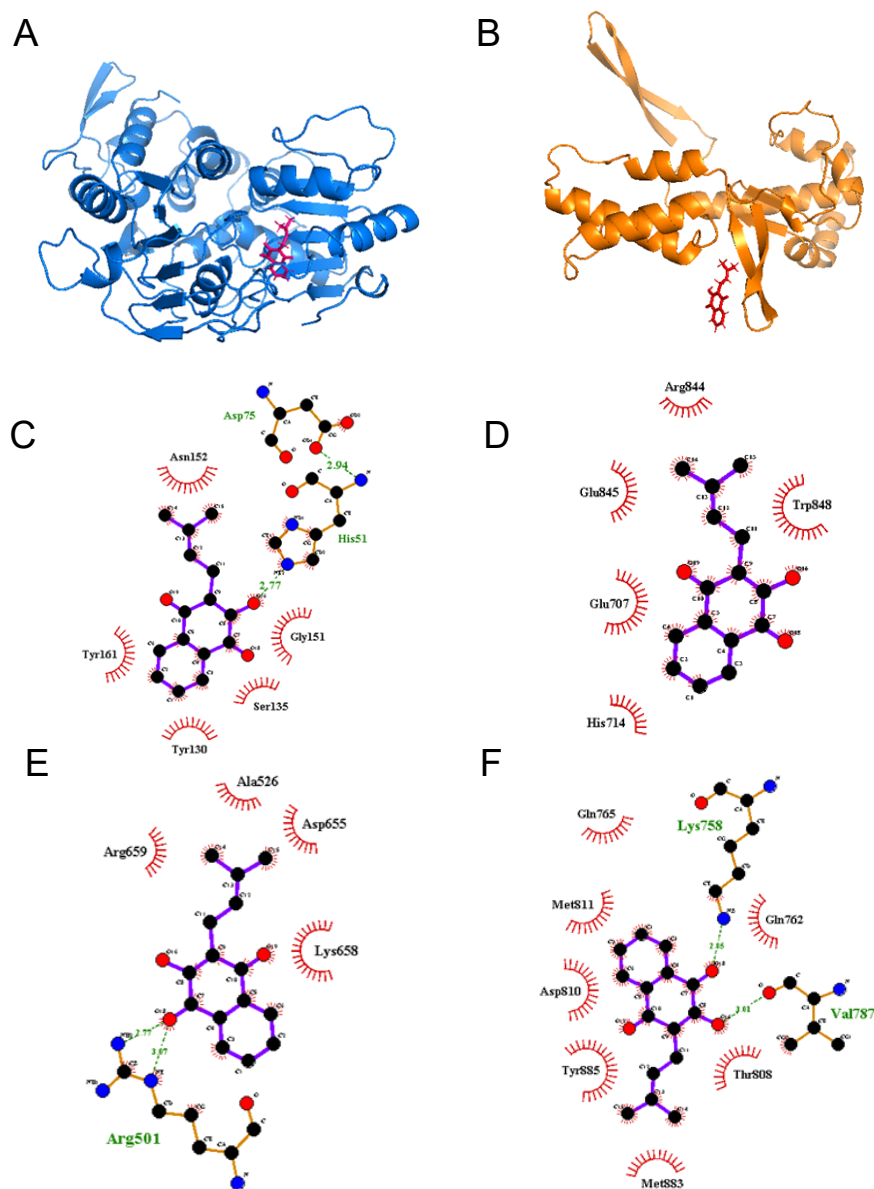


Figure 5. Molecular docking simulations using lapachol as a ligand, for ZIKV NS3 and NS5. A) PyMol representation of ZIKV NS3. B) PyMol representation of ZIKV NS5. C) LigPlot visualization of NS2B-NS3^{Pro} with a ChemPLP score of -46.2 kJ/mol. D) LigPlot visualization of NS5^{RdRp} targeting residue (His714) with ChemPLP score of -53.53kJ/mol. E) LigPlot visualization of interaction of Lapachol with NS5^{RdRp} targeting residue Arg501 with a ChemPLP score of -42.49 kJ/mol. F) LigPlot visualization of interaction of lapachol with NS5^{RdRp} targeting residue Thr808 with ChemPLP score of -43.30 kJ/mol.

Lapachol interferes with NS2-NS3^{Pro} and NS5^{RdRp} enzymatic activity

ZIKV NS3^{Hel}, NS2B-NS3^{Pro} and NS5^{RdRp} were initially submitted to endpoint activity assays with lapachol at 1 mM, 500 μ M and 500 μ M, respectively. As observed in **Figure 6A**, the result showcased that lapachol had a low ATPase inhibitory effect for NS3^{Hel}, with < 20%

inhibition of its activity, while strongly inhibited 87% of NS2B-NS3^{Pro} and 91% of NS5^{RdRp} activities. To determine the dose-response inhibitory effect of lapachol over NS2B-NS3^{Pro}, concentrations ranging from 500 to 0.98 μM were tested, where an IC_{50} of 143.9 μM was obtained (**Figure 6B**). Altogether, these results suggest that a mechanism of action for lapachol may be by directly interfering with viral protease and polymerase activities. It corroborates with data showing the activity of lapachol on post-entry stages, since the interference with NS2B-NS3^{Pro} and NS5^{RdRp} would jeopardize virus replication.

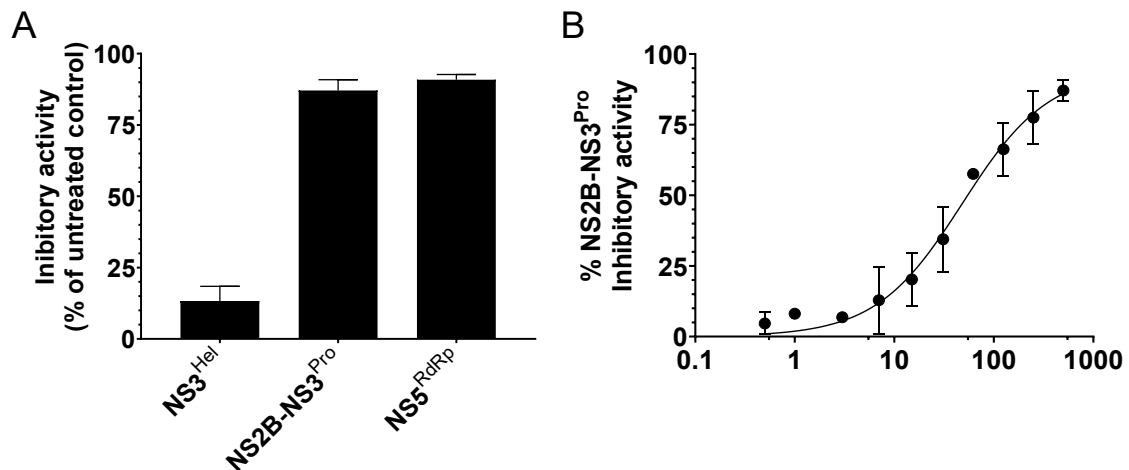


Figure 6. *Lapachol interferes with viral non-structural proteins essential for virus replication.* A) **NS3^{Hel}**: unique-point measurement of NTPase activity of 200 nmol.L⁻¹ ZIKV NS3^{Hel} in the presence of lapachol at 1 mM using 1mM of ATP as substrate. Absorbance was measured at the end of a 30-minute reaction using $\lambda = 620$ nm. **NS2B-NS3^{Pro}**: unique-point measurement of protease activity of 4 nM of purified ZIKV NS2B-NS3^{Pro} using Bz-nKRR-AAMC at 30 μM as substrate in the presence of lapachol at 500 μM . Relative fluorescence was measured against a positive control reaction containing an equal concentration of DMSO. **NS5^{RdRp}**: unique-point measurement of elongation activity of ZIKV NS5^{RdRp} by quantification of SYBR Green signal in the presence of lapachol at 500 μM . B) Dose-response evaluation of ZIKV NS2B-NS3^{Pro} inhibition in the presence of lapachol at concentration ranging from 500 μM to 0.98 μM . All tests were performed in biological duplicate and technique triplicate. Reactions containing DMSO 1% (v/v) were used as reference. Images generated with GraphPad Prism 8.0.

Lapachol induces intracellular ROS formation

Since naphthoquinones such as lapachol are prone to act as oxidants, we evaluated total levels of intracellular ROS formation in cells treated with lapachol at 8 μM or with DMSO 0.1%, in the presence or absence of ZIKV_{PE243}, and incubated for 72 hours. As observed in **Figure 7**, the infected cells, in comparison to the negative control, were already expressing elevated levels of ROS, demonstrating that one of the consequences of ZIKV infection may be

an increase in ROS production. Additionally, cells treated with lapachol in the absence of infection also showed slightly higher levels of ROS, indicating that the compound alone is capable of inducing ROS formation. Furthermore, we observed in cells that are both infected and treated with lapachol, ROS levels are significantly elevated beyond those of the three groups, suggesting a combined effect of ROS production due to the ZIKV infection and lapachol treatment.

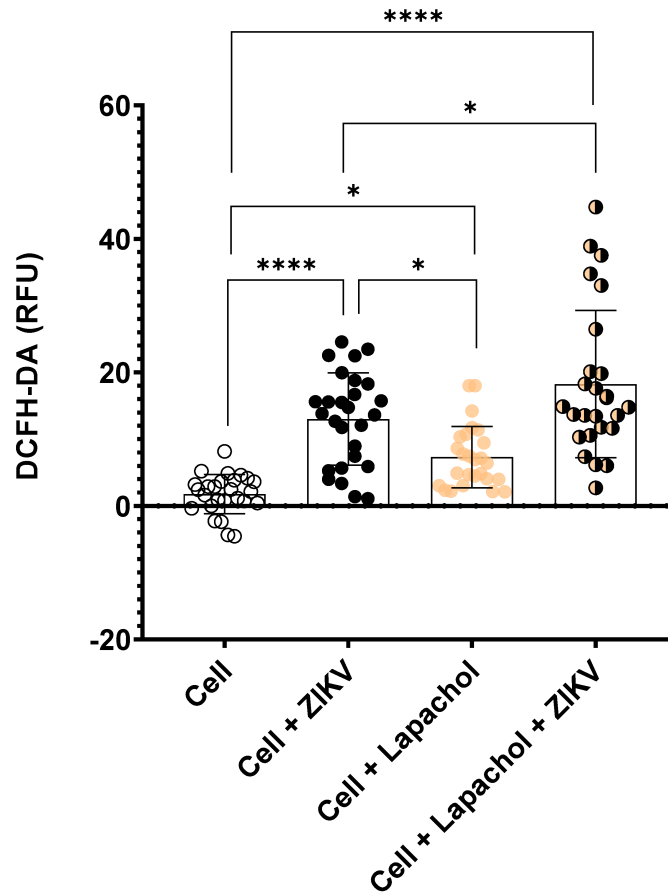


Figure 7. Determination of total intracellular ROS formation. Vero E6 cells were treated with lapachol at 8 μ M in the presence or absence of ZIKV_{PE243} infection at an MOI of 1, and incubated for 72 hours. Levels of ROS formed intracellularly were determined using a DCFH-DA (2', 7'-dichlorofluorescein diacetate fluorescent probe). Infected cells are represented in black circles and treated cells are represented in orange circles. Mean values of three independent experiments each measured in replicates of nine are shown. Bars show the mean standard deviation of each group; individual values are plotted as circles. P values < 0.05 were considered significant. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, and (****) $p < 0.0001$. Images were generated with GraphPad Prism 8.0.

Discussion

In this study we evaluated the antiviral activity of lapachol against ZIKV *in vitro* and presented evidence of potential mechanisms of antiviral action of this compound on ZIKV

replication cycle. Lapachol, a 1,4-naphthoquinone, exhibited strong inhibition of ZIKV replication, showing its main effect on post-entry stages of its cycle being able to reduce viral titers to undetectable levels, around 1×10^9 FFU/mL, in comparison with the DMSO control. In addition, it demonstrated protective activity by a 100-fold reduction in viral titer, protecting the cells from ZIKV infection. Evaluation of its mechanism of action suggests it may act by inhibiting enzymatic activity of non-structural proteins and causing oxidative stress damage to the viral particle through ROS formation.

Naturally occurring compounds containing naphthoquinones in their structure have garnered attention from the scientific community due to their wide range of pharmacological properties as well as ample distribution in nature (AMININ; POLONIK, 2020; QIU et al., 2018; WU et al., 2020). Among this group of compounds, lapachol stands out for its therapeutic versatility, acting as an anticancer agent, as well as an inhibitor of many pathogens (EPIFANO et al., 2014; GUIRAUD et al., 1994a, 1994b). Due to previously reported antiviral potentials of lapachol (LAGROTA et al., 1983; SACAU et al., 2003a), we evaluated its capacity to inhibit ZIKV replication cycle. Lapachol exhibited substantial inhibition of ZIKV replication, being able to inhibit infection at low concentrations, and presenting high tolerance by the host cells with high SI of 50.7 which demonstrates the strong antiviral index of lapachol against ZIKV *in vitro*. Therefore, our data correlates with previous studies where lapachol was able to interfere with Epstein-Barr virus (EBV) replication with a dose-dependent effect (SACAU et al., 2003b). It is also comparable to the antiviral activity reported for a synthetic 1,4-naphthoquinone structurally derived from lapachol, which could inhibit the replication of herpes simplex virus type 1 (HSV-1) with an IC_{50} of 25ng/mL and an IC_{90} of 400 ng/mL, with a therapeutic index of 2000 (DA SILVA et al., 2002).

Further evaluation of lapachol effects over ZIKV on different stages of its replication revealed that the compound exerts a more pronounced effect after ZIKV entry into host cells. This could be attributed to the interference with non-structural proteins of the virus, as observed here when enzymatic activity of NS2B-NS3^{Pro} and NS5^{RdRp} were strongly reduced in the presence of the compound, exhibiting an IC_{50} of 143.9 over NS2B-NS3^{Pro}. Further molecular docking analysis supported this data, showing that lapachol was able to form five hydrophobic interactions and two hydrogen bonds, highlighting the interactions with residues His51, Asp75 and Ser135 which make up the catalytic triad (PHOO et al., 2016). This is of great relevance since NS2B-NS3^{Pro} inhibitors seem to be a promising target for the development of ZIKV

antivirals as shown in the work of Yuan and coworkers (YUAN et al., 2017), where multiple clinically approved protease inhibitors showed significant reduction of ZIKV replication.

In addition, its effect over transcription elongation indicates lapachol inhibitory properties may be attributed, as well to direct interference with replication by hindering the activity of NS5^{RdRp}. Target-specific molecular docking analysis against this protein predicted interactions with two highly conserved regions, which are essential for enzymatic activity. Considering the three-analysis performed targeting different regions of the NS5^{RdRp} (VALENTE; MORAES, 2019a), the compound formed a total of three hydrogen bonds and 16 hydrophobic interactions. One of the main relevant findings, with the highest score, is the interactions with Asp665 and His714, since they belong to the catalytic site of the NS5^{RdRp}, suggesting that lapachol may be blocking its catalytic site (GODOY et al., 2017). Lapachol's role on viral replication was previously described by our group, where *in silico* evaluations showed its ability to bind to SARS-CoV-2 Nsp9 (JUNIOR et al., 2022), a protein whose role involves binding to genomic RNA and preventing its degradation, as well as ZIKV's NS3 protein (OGUNTADE; RAMHARACK; SOLIMAN, 2017). To date, there are reports of lapachol as an inhibitor of DNA and RNA synthesis, though by interfering with mitochondrial rate-limiting enzyme in the *de novo* synthesis of pyrimidine ribonucleotides (PERES et al., 2017).

Furthermore, our data showed that pre-treatment with lapachol protected Vero E6 cells from ZIKV infection, suggesting multiple mechanisms of action at play in addition to the ones described above. This protective effect may be explained by lapachol's ability to generate ROS, as evidenced here by the increase in intracellular ROS levels upon treatment. Lapachol can act as an oxidant because its quinone structure, during reduction, forms semiquinone anion radicals which in turn transfer electrons to molecular oxygen, leading to ROS formation (BOLTON et al., 2000; LINZNER et al., 2020). The ability of lapachol to generate ROS *in vitro* has already been documented (GOULART et al., 2003; PINK et al., 2000). For instance, the production of ROS as a mechanism of antipathogenic action for lapachol was observed by Lizner and collaborators, where the compound was able to cause an oxidative stress response, resulting in protein damage in *Staphylococcus aureus*, inhibiting its growth (LINZNER et al., 2020). Furthermore, it must be taken into consideration that ROS formation can lead to oxidative stress and be detrimental to the host cells, as well (ZHANG; RONG; LI, 2019). However, the low levels of ROS observed in uninfected cells combined with the results of the cytotoxicity assays suggest that the compound could be elevating ROS levels on infected cell, enough to act as an

inhibitor of viral replication without disrupting the balance in healthy cells. The inhibition of viral replication through ROS formation in general has already been described by Strengert and collaborators (STRENGERT et al., 2014), who demonstrated that ROS production by NADPH oxidases was key in inhibiting influenza A virus replication by damaging the viral genome. Nonetheless, more studies are needed to further assess the effects of lapachol regarding oxidative stress induction by using *in vivo* models to better evaluate its outcome on the host's metabolism. Further studies are also needed to directly link ROS formation as a mechanism of action in the inhibition of ZIKV replication.

Conclusion

Here we report the *in vitro* antiviral activity of lapachol against ZIKV, demonstrating its main effectivity on post-entry stages of virus replication. Additional studies are needed to further elucidate its mechanism of action as well as its effectiveness on alternative systems, such as *in vivo* models. Our findings highlight the potential of lapachol to be used as a bioactive compound in the development of ZIKV inhibitors, either in its original form or as template for creating analogs further enhancing its pharmacological properties.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study is included in this published article and its supplementary material.

Competing interests

The authors declare that there is no conflict of interest.

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Author's contributions: SPBR carried out antiviral and compound viability studies and drafted the manuscript. NMC carried out virus propagation, participated in the study design and edited the manuscript. MET carried out *in-silico* protein assays. BAM participated in obtaining and purifying the compound. DPJ participated in the *in-silico* protein assays, contributed in the purification and obtention of the compound and edited the manuscript. NCMRM, GO and RVCG carried out the *in vitro* protein assays. CMM and TWPM participated in ROS formation assays. ACGJ conceived the study as well as the study design and edited the manuscript. All authors read and approved the final manuscript.

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CAPÍTULO III

Considerações Finais

CONSIDERAÇÕES FINAIS

Em conclusão, este trabalho demonstrou o potencial do lapachol como um composto bioativo promissor no desenvolvimento de antivirais para inibir a replicação do ZIKV, utilizando modelos de infecção *in vitro* de células-alvo Vero E6 pela cepa brasileira ZIKV_{PE243}.

Os resultados obtidos demonstraram que o composto é capaz de inibir a replicação viral em baixas concentrações, as quais foram toleradas pelas células hospedeiras, evidenciando um significativo índice de seletividade, o qual representa a razão entre viabilidade celular e atividade antiviral do composto. Além disso, os dados demonstraram que o lapachol atua predominantemente nas etapas pós-entrada do vírus na célula hospedeira. Com isso, por meio da avaliação específica de fatores envolvidos nessa fase, demonstramos que possíveis mecanismos de ação antiviral incluem a inibição de proteínas não estruturais essenciais para a replicação viral, como a protease viral NS2B-NS3 e a RdRp NS5. Também demonstramos a capacidade do lapachol em gerar espécies reativas de oxigênio *in vitro*, sugerindo a interferência deste na indução do estresse oxidativo.

Ainda são necessários mais estudos para avaliar a eficácia do lapachol em modelos experimentais *in vivo*, bem como ensaios específicos para identificar com precisão o alvo principal do composto, e os processos específicos do seu mecanismo de ação. Isso permitirá uma melhor exploração desses mecanismos, inclusive considerando a síntese de análogos que possam potencializar essas características e melhorar suas propriedades farmacológicas.