



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

**COMPOSTOS DE COORDENAÇÃO COMO UMA ABORDAGEM INOVADORA
CONTRA VÍRUS DOS GÊNEROS ALFAVÍRUS E BETACORONAVÍRUS**

IGOR DE ANDRADE SANTOS

UBERLÂNDIA

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Tese apresentada ao Colegiado do Programa de Pós-Graduação em Imunologia e Parasitologia Aplicadas da Universidade Federal de Uberlândia como requisito parcial a obtenção do título de Doutor.

Financiadora: CNPq, FAPEMIG e CAPES.PrInt

Discente: MsC. Igor de Andrade Santos

Orientadora: Profa. Dra. Ana Carolina Gomes Jardim

Co-orientador: Prof. Dr. Pedro Paulo Corbi e Prof. Dr. Mark Harris

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Em 24 de novembro de 2023, às 13 horas, reuniu-se na sala 2B213 - UFU - Campus Umuarama, e também de forma remota via Microsoft Teams, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas, assim composta pelos membros titulares: Profa. Dra. Ana Carolina Gomes Jardim, DEMIC/UFU (Presidente) orientadora do candidato; Prof. Dr. Thiago Moreno Lopes e Souza, Fiocruz Rio de Janeiro; Prof. Dr. Lindomar José Pena, Fiocruz Pernambuco; Prof. Dr. Gustavo Von Poelhsitz, UFU; Prof. Dr. Celso de Oliveira Rezende Junior, UFU.

Iniciando os trabalhos a presidente da mesa, Profa. Dra. Ana Carolina Gomes Jardim, apresentou a Comissão Examinadora e o candidato, agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação, o tempo de arguição e resposta foram conforme as normas do Programa.

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Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.



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Lista de Abreviações

COVID-19	Doença do Coronavírus 2019 - <i>coronavirus disease 2019</i>
SARS-COV-2	Síndrome Respiratória Aguda Grave Por Coronavírus 2 - <i>severe acute respiratory syndrome coronavirus 2</i>
CHIKV	Vírus da chikungunya - <i>chikungunya virus</i>
RNA	Ácido Ribonucleico - <i>Ribonucleic acid</i>
HCOV-229E	Coronavírus Humano 229e - <i>human coronavirus 229e</i>
HCOV-NL63	Coronavírus Humano NL63 - <i>human coronavirus NL63</i>
HCOV-OC43	Coronavírus Humano OC43 - <i>human coronavirus OC43</i>
HCOV-HKU1	Coronavírus Humano HKU1 - <i>human coronavirus HKU1</i>
SARS-COV	Síndrome Respiratória Aguda Grave Por Coronavírus - <i>severe acute respiratory syndrome coronavirus</i>
MERS-COV	Síndrome Respiratória do Oriente Médio Por Coronavírus - <i>middle east respiratory syndrome coronavirus</i>
ARDS	Síndrome Respiratória Aguda Grave - <i>Acute Respiratory Distress Syndrome</i>
NPIs	Intervenções Não Farmacêuticas - <i>Non-Pharmaceutic Interventions</i>
OMS	Organização Mundial Da Saúde
RBD	Domínio De Ligação Ao Receptor - <i>Receptor Binding Domain</i>
ECA2	Receptor Celular Humano da Enzima Conversora de Angiotensina 2
N	Proteína de Nucleocapsídeo
E	Proteína de Envelope
M	Proteína de Membrana
S	Proteína Spike
ORF	Região Aberta de Leitura - <i>Open Reading Frame</i>
UTR	Região Não Codificante - <i>Non Codifying Region</i>
NSPs	Proteína Não Estrutural – <i>non structural protein</i>
mRNA	Ácido Ribonucleico mensageiro - <i>messenger ribonucleic acid</i>
PL^{PRO}	Protease <i>Papain-Like</i>
DMV	Vesículas de Membrana Dupla - <i>Double Membrane Vesicles</i>
Mpro	Cisteína Protease
mTOR	Alvo da Rapamicina em Mamíferos - <i>Mammalian Target of Rapamycin</i>
NendoU	Endoribonuclease
vRNA	RNA Viral
RdRp	RNA-Polimerase Dependente de RNA
IFN	Interferon
MAVS	Proteína Mitocondrial de Sinalização Antiviral - <i>Mitochondrial Antiviral Signaling Protein</i>
TMPRSS2	Receptor Acessório Serina Protease 2
RTC	Complexo de Replicação e Transcrição Viral
TRS	Sequências Regulatórias de Transcrição - <i>Transcription Regulatory Sequences</i>
sgRNA	RNA Subgenômico
ERGIC	Região Intermediária Retículo Endoplasmático-Complexo de Golgi
SMV	Vesículas de Membrana Simples
R₀	Taxa de Reprodução Viral

MRCA	Ancestral Comum Mais Recente
GISAID	<i>Global Initiative on Sharing All Influenza Data</i>
VMU	Variantes Sob Monitoramento - <i>Variant Under Monitoring</i>
VOI	Variantes de Interesse - <i>Variant Of Interest</i>
VOC	Variantes de Preocupação - <i>Variant Of Concern</i>
DSRNA	Dupla de Fita De Rna
MADA5	Proteína Associada a Diferenciação de Melanoma 5 - <i>Melanoma Differentiation-Associated Protein 5</i>
Rig-1	Gene Indutor de Ácido Retinóico I - <i>Retinoic Acid-Inducible Gene</i>
IRFS	Fatores Regulatórios de Interferon - <i>Interferon Regulatory Fator</i>
IL	Isoleucinas
JAK-STAT1/2	Janus Kinase 2
AT2	Células Alveolares do Tipo 2
AT1	Células Alveolares do Tipo 1
NK	Células Natural Killers
DAD	Danos Alveolares Difusos
GABA	Ácido Gama-Aminobutírico
VVR	Vetor Viral Replicante
VVRn	Vetor Viral Não Replicante
FDA	Administração de Alimentos e Medicamentos - <i>Food And Drug Administration</i>
ANVISA	Agência Nacional de Vigilância Sanitária
CDC	Centro De Controle e Prevenção de Doenças Infecciosas do Estados Unidos da América - <i>U.S. Centers For Disease Control And Prevention</i>
USAMRIID	Instituto Médico de Pesquisa em Doenças Infecciosas do Exército dos Estados Unidos da América - <i>U.S. Army Medical Research Institute of Infectious Diseases</i>
EBOV	Vírus do ebola – <i>ebola virus</i>
YFV	Vírus da febre amarela – <i>yellow fever virus</i>
DENV-2	Vírus da dengue tipo 2 – <i>dengue virus 2</i>
VEEV	Vírus da encefalite equina venezuelana – <i>venezuelan encephalitis equine virus</i>
IAV	Vírus da influenza A – <i>influenza virus A</i>
RSV	Vírus sincicial respiratório – <i>respiratory syncytial virus</i>
HIV-1	Vírus da imunodeficiência humana 1 – <i>human immunodeficiency virus 1</i>
HIV-2	Vírus da imunodeficiência humana 2 - <i>human immunodeficiency virus 2</i>
DNA	Ácido Deoxiribonucleico - <i>Deoxyribonucleic Acid</i>
WA	Linhagem Oeste Africano do CHIKV
ECSA	Linhagem Leste/Centro/Sul da África do CHIKV
AUL	Subgenótipo Linhagem Urbana Asiática do CHIKV
IOL	Subgenótipo Linhagem do Oceano Índico do CHIKV
E1	Glicoproteína de Envelope 1
TF	Proteína Transfame
TIM-1	Proteína de Domínio de Imunoglobulina e Mucina de Célula T 1 - <i>T-Cell Immunoglobulin and Mucin Domain Protein 1</i>
DC-SIGN	Receptor de Lectina Tipo C - <i>C-Type Lectin Receptor</i>
MXRA8	Limitrin
gRNA	RNA Genômico – <i>genomic RNA</i>

PPRs	Receptores de Reconhecimento Padrão
CCL2	Proteína Quimiotática de Monócitos
MHC	Complexo de Histocompatibilidade Humano
Treg	Células Tregulatórias
Atd	Amantadina
Mtn	Memantina
Rtd	Rimantadina
DMEM	Meio Eagle's Modificado de Dulbecco - <i>Dulbecco's Modified Eagle's Medium</i>
TCID₅₀	Dose Infecciosa de 50% em Cultura De Células - <i>50% Tissue Culture Infectious Dose</i>
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyl Tetrazolium Bromide
DMSO	Dimetilsulfóxido
MOI	Multiplicidade de Infecção - <i>Multiplicity of Infection</i>
EC₅₀	Concentração Efetiva de 50% - <i>Effective Concentration of 50%</i>
CC₅₀	Concentração Citotóxica de 50% - <i>Cytotoxic Concentration of 50%</i>
A549-AT	Células de Adenocarcinoma Humano Expressando ECA2 e TMPRSS2
PBS	Tampão Fosfato-Salino - <i>Phosphate-Buffered Saline</i>
GCU	Intesidade Verde Média - <i>Green Mean Intensity</i>
RCU	Intesidade Vermelha Média - <i>Red Mean Intensity</i>
TAE	Tampão Tris-Acertato-EDTA
SI	Índice de Seletividade - <i>Selectivity Index</i>
SNPs	Mutações de único nucleotídeo - <i>Single Nucleotide Mutations</i>
HCV	Vírus da Hepatite C - <i>Hepatitis C Virus</i>
HBV	Vírus da Hepatite B - <i>Hepatitis B Virus</i>
BHK-21	Fibroblastos do Rim de Hamster Bebê - <i>Fibroblasts from Kidney Golden Hamster</i>
μM	Micromolar
RAW	Macrófagos de Camundongo - <i>Mouse Macrophage Cell Line</i>
FBS	Soro Fetal Bovino - <i>Fetal Bovine Serum</i>
ROS	Espécies Reativas de Oxigênio - <i>Reactive Oxygen Species</i>

LISTA DE FIGURAS

Figura 1: Estrutura da partícula viral do SARS-CoV-2.	21
Figura 2: Genoma viral do SARS-CoV-2 e as proteínas não-estruturais expressas pelas ORF1a e ORF1b.	22
Figura 3: Genoma do SARS-CoV-2 e as proteínas estruturais e acessórias expressas por suas respectivas ORFs.	23
Figura 4: Figura esquemática do ciclo replicativo do SARS-CoV-2.	26
Figura 5: Esquema representativo das VOCs com local e ano de emergência	30
Figura 6: Estrutura química do remdesivir e seus análogos, e sua fosforilação em sistemas biológicos.	34
Figura 7: Estrutura química do Molnupiravir e suas formas fosforiladas	36
Figura 8: Estrutura química do Nirmatrelvir e Ritonavir (Paxlovid).	37
Figura 9: Mapa mundi representando a disseminação do CHIKV através dos anos	39
Figura 10: Representação esquemática da partícula e genoma viral do CHIKV	41
Figura 11: Esquema representativo do ciclo replicativo do CHIKV	44
Figura 12: Representação gráfica da infecção e patogênese do CHIKV	46
Figura 13: Esquema das geometrias de coordenação de acordo com alguns dos números de coordenação	50

SUMÁRIO

RESUMO	15
ABSTRACT	16
CAPÍTULO 1: Fundamentação teórica	17
1. Coronavírus 2 da Síndrome Respiratória Aguda Grave (SARS-CoV-2)	18
1.1 Histórico e Epidemiologia.....	18
1.2 SARS-CoV-2: partícula viral, genoma e proteínas virais	20
1.3 Ciclo replicativo do SARS-CoV-2	25
1.4 Transmissão.....	27
1.5 Variantes do SARS-CoV-2	28
1.6 COVID-19, Resposta Imune e Síndrome Pós-COVID	30
1.7 Profilaxia e Tratamento	33
2. Vírus da Chikungunya (CHIKV)	38
2.1. Histórico e Epidemiologia.....	38
2.2. Transmissão.....	40
2.3. Vírus da chikungunya.....	41
2.4. Febre chikungunya e Patogênese	45
2.5. Resposta Imune ao CHIKV	47
3. Compostos de Coordenação	49
3.1. Histórico e Desenvolvimento	49
3.1 52	
3.2 Atividades antivirais dos Compostos Metálicos	52
REFERÊNCIAS.....	53
OBJETIVOS.....	69
Objetivos específicos	69
CAPÍTULO 2: NEW Pd(II) AMINOADAMANTINE COMPLEXES STRONGLY IMPAIRS SARS-CoV-2 INFECTION	70
CAPÍTULO 3: Cu(I) N-HETEROCYCLICARBENES AS POTENT INHIBITORS OF SARS-CoV-2 REPLICATION.....	102
CAPÍTULO 4: COPPER(II) BENZYLOXYCHALCONE ANALOGUES AS NEW POTENTIAL METALLODRUGS AGAINST SARS-CoV-2 REPLICATION	125
CAPÍTULO 5: REPURPOSING POTENTIAL OF RIMANTADINE HYDROCHLORIDE AND DEVELOPMENT OF A PROMISING PLATINUM(II)-RIMANTADINE METALLODRUG FOR THE TREATMENT OF CHIKUNGUNYA VIRUS INFECTION	
150	
CAPÍTULO 6: TRIPHENYLPHOSPHINE GOLD(I) DERIVATIVES PROMOTE ANTIVIRAL EFFECTS AGAINST THE CHIKUNGUNYA VIRUS	163

CAPÍTULO 7: MEMANTINE HYDROCHLORIDE: A DRUG TO BE REPURPOSED AGAINST CHIKUNGUNYA VIRUS?	176
CAPÍTULO 8: CONSIDERAÇÕES FINAIS.....	185
ANEXOS: Artigos publicados	188
Lista de Artigos Publicados:	189

RESUMO

Doenças causadas por vírus emergentes representam importantes causas de surtos e epidemias, impactando a vida dos indivíduos, os serviços de saúde e a economia. Como exemplo, a pandemia de COVID-19, causada pelo coronavírus 2 da síndrome respiratória aguda grave por coronavírus 2 (SARS-CoV-2), tem acentuado a demanda por antivirais e desafiado o mundo no controle da disseminação viral. O Brasil também enfrenta um cenário crítico relacionado ao controle de arboviroses, como as infecções causadas pelo vírus da chikungunya (CHIKV), causador da febre chikungunya. Atualmente, não existe terapia antiviral contra o CHIKV, e apenas três fármacos licenciados para casos severos da COVID-19, tornando essencial e emergencial o desenvolvimento de fármacos antivirais contra essas viroses. Neste contexto, complexos metálicos têm atraído atenção pela eficácia e versatilidade, visto que íons metálicos como ouro (Au), platina (Pt), paládio (Pd) e cobre (Cu), quando complexados a ligantes específicos, podem gerar compostos estáveis, biologicamente ativos e não citotóxicos. Por isso, aqui avaliamos uma série de compostos de coordenação pela associação de íons de Au(II) Pt(II), Pd(II) e Cu(I) com moléculas orgânicas, como os aminoadamantanos memantina (mtn), rimantadina (rtd) e amandatadina (atd), carbenos heterocíclicos e/ou chalconas, contra o SARS-CoV-2 e CHIKV. Os compostos coordenados apresentaram índices de seletividade entre 1,7 e 36,2, e diferentes perfis de inibição, interferindo com a replicação viral por diferentes modos de ação, seja pela inibição da entrada dos vírus em células hospedeiras, ou interagindo com dsRNA em ambos os vírus e interrompendo a replicação viral, e/ou diminuindo a transmissão célula a célula do SARS-CoV-2. Portanto, os trabalhos descritos aqui permitiram a identificação de antivirais que inibem vírus de diferentes famílias, e que potencialmente possam ser capitalizados no tratamento da COVID-19 e febre chikungunya. Dessa forma, os resultados aqui apresentados poderão contribuir para o desenvolvimento de novos fármacos antivirais, e, portanto, melhorar a qualidade de vida da população. Adicionalmente, esperamos que os resultados deste trabalho estimulem estudos adicionais com compostos de coordenação metálica e desenvolvimento de novas moléculas inéditas com potencial de serem aplicadas para doenças atuais, mas também para futuros surtos, endemias e epidemias.

ABSTRACT

Diseases caused by emerging viruses represent important causes of outbreaks and epidemics, impacting individuals' lives, healthcare services, and the economy. For example, the COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has heightened the demand for antivirals and challenged the world in controlling viral spread. Brazil also faces a critical scenario related to the control of arboviruses, such as infections caused by the chikungunya virus (CHIKV), the agent responsible for chikungunya fever. Currently, there is no antiviral therapy against CHIKV, and only three drugs licensed for severe cases of COVID-19, making the development of antiviral drugs against these viruses essential and urgent. In this context, metal complexes have drawn attention due to their efficacy and versatility, as metal ions such as gold (Au), platinum (Pt), palladium (Pd), and copper (Cu), when complexed with specific ligands, can generate stable, biologically active, and non-cytotoxic compounds. Therefore, we evaluated a series of coordination compounds formed by the association of Au(II), Pt(II), Pd(II), and Cu(I) ions with organic molecules, such as the aminoadamantanes memantine (mtn), rimantadine (rtd), and amantadine (atd), heterocyclic carbenes, and/or chalcones, against SARS-CoV-2 and CHIKV. The coordinated compounds showed selectivity indices (SI) between 1.7 and 36.2, and different inhibition profiles, interfering with viral replication through different modes of action, either by inhibiting virus entry into host cells, interacting with dsRNA in both viruses and interrupting viral replication, and/or reducing cell-to-cell spread of SARS-CoV-2. Hence, the work described here allowed for the identification of antivirals that inhibit viruses from different families, potentially being capitalized in the treatment of COVID-19 and Chikungunya Fever. Therefore, the results presented here may contribute to the development of new antiviral drugs, thereby improving the population's quality of life. Additionally, we hope that the results of this work will stimulate further studies with metal coordination compounds and the development of new, unprecedented molecules with the potential to be applied not only for current diseases but also for future outbreaks, endemics, and epidemics.

CAPÍTULO 1: Fundamentação teórica

1. Coronavírus 2 da Síndrome Respiratória Aguda Grave (SARS-CoV-2)

1.1 Histórico e Epidemiologia

Os coronavírus foram identificados em 1960 e classificados como membros da família *Coronaviridae* (KAHN; MCINTOSH, 2005; PAYNE, 2017). Existem 7 coronavírus conhecidos por causar doenças em humanos: Coronavírus humano 229E (HCoV-229E), Coronavírus humano NL63 (HCoV-NL63), Coronavírus humano OC43 (HCoV-OC43), Coronavírus humano HKU1 (HCoV-HKU1), Síndrome respiratória aguda grave por Coronavírus (SARS-CoV), Síndrome respiratória do Oriente Médio por Coronavírus (MERS-CoV) e mais recentemente, o coronavírus 2 da síndrome respiratória aguda (SARS-CoV-2) (CUI; LI; SHI, 2019; GRAHAM; DONALDSON; BARIC, 2013). Os CoVs HCoV-229E, HCoV-NL63, HCoV-OC43 e HCoV-HKU1 causam sintomas leves similares à um resfriado comum, e são mais comuns em crianças (PAYNE, 2017). Os SARS-CoV e MERS-CoV emergiram em meados de 2002 e 2012, respectivamente (SHARIF-YAKAN; KANJ, 2014; ZHONG et al., 2003). Os casos de infecção por esses vírus permaneceram isolados a alguns países, mantendo a doença controlada (ABDELRAHMAN; LI; WANG, 2020).

O SARS-CoV-2 foi primeiramente detectado em 18 de dezembro de 2019, como um vírus respiratório em 5 pacientes hospitalizados com síndrome respiratória aguda grave (*acute respiratory distress syndrome*, ARDS) e se disseminou rapidamente pelo mundo (REN et al., 2020; THE LANCET, 2020), identificado como o agente etiológico da COVID-19 (Doença por Coronavírus 2019 (THE LANCET, 2020). Os primeiros casos identificados no final de dezembro de 2019 foram associados ao mercado alimentício na província de Hubei, Wuhan, China (LI et al., 2020b). Após sua identificação, os casos progrediram para 41 casos em 2 de janeiro, 571 em 22 de janeiro e 31.161 casos em 7 de fevereiro de 2020 na China (WU; CHEN; CHAN, 2020; ZHU et al., 2020). Os primeiros casos fora da China foram identificados ainda em janeiro de 2020, na Tailândia e Estados Unidos da América (ASGHAR et al., 2022; WOROBEY et al., 2020), demonstrando que a transmissão entre humanos do SARS-CoV-2 acontecia de forma rápida e eficiente. Ao final de janeiro de 2020, os casos confirmados de SARS-CoV-2, dentro e fora da China, indicaram que a contenção regional do vírus seria improvável (ASGHAR et al., 2022; WU; CHEN; CHAN, 2020; ZHU et al., 2020). Ao final de fevereiro de 2020, o SARS-CoV-2 havia se disseminado por todos os continentes e a transmissão foi classificada como comunitária em diversos países (ASGHAR et al., 2022; WOROBEY et al., 2020). Devido a projeções do aumento da curva de transmissão, criou-se a necessidade de restringir a transmissão viral, e por isso, intervenções não farmacêuticas (*non pharmaceutical intervention* – NPIs) foram impostas, sendo essas distanciamento social, uso de

máscaras faciais e confinamento (LAI et al., 2020a, 2020b). Essas medidas resultaram no achatamento da curva de transmissão na primeira onda de infecções, e desta forma, ajudaram a prevenir alguns sistemas públicos de ficarem sobrecarregados (LAI et al., 2020a, 2020b).

O aumento significativo de casos de COVID-19 no planeta, desde a identificação do SARS-CoV-2, e a detecção do vírus em quase todos os países, levou a Organização Mundial da Saúde (OMS) a declarar a COVID-19 como uma pandemia em 11 de março de 2020 (W.H.O., 2020) e a sugerir o aderimento a medidas NPIs. Entretanto, apesar da eficácia das NPIs em escala mundial, a redução equitativa dos riscos de infecção não foi alcançada em todos os países (ASGHAR et al., 2022; KOELLE et al., 2022; LAI et al., 2020b; WU; CHEN; CHAN, 2020). Adicionalmente, diferentes ocupações laborais possuíam diferentes riscos de infecção, sendo aqueles em locais com pouca ventilação e superlotados mais propícios de infecção, como hospitais, fábricas, casas de repouso e abrigos (BHALA et al., 2020; FRANCO-PAREDES et al., 2020; KOELLE et al., 2022). Os perfis de infecção se agravaram com o surgimento de novas ondas de infecção marcados pela emergência de variantes virais com mutações adaptativas que resultaram em maior transmissibilidade e severidade da doença (ASGHAR et al., 2022; KOELLE et al., 2022; LAI et al., 2020a).

No Brasil, o primeiro caso do SARS-CoV-2 foi confirmado em 26 de fevereiro de 2020 na cidade de São Paulo em paciente com histórico de viagem para Itália e após algumas semanas todos os estados já havia casos confirmados (LAI et al., 2020a; SERDAN et al., 2021). No mês de março houve a confirmação da transmissão comunitária no país, e após um mês, iniciou-se o colapso das redes públicas e privadas com aumento substancial no número de casos e mortes relacionados à COVID-19, sendo que em julho, os índices diários de infecção atingiram 69.074 casos/dia (SERDAN et al., 2021). A incidência de casos permaneceu alta até o fim de 2020, porém, se tornou mais crítica com a emergência na região de Manaus de uma nova variante com três mutações (K417T, E484K, and N501Y) na região genômica viral da proteína *spike* (S), chamada naquele momento de linhagem P.1 (FARIA et al., 2021; MONTAGUTELLI et al., 2021; WANG et al., 2021b). Com o aumento de transmissibilidade devido a linhagem P.1, mais de 13 milhões de pessoas foram infectadas e mais de 300.000 mortes foram reportadas até março de 2021 (ASGHAR et al., 2022; LAI et al., 2020a). Desde então, mais de 771 milhões de casos confirmados e 6,9 milhões de mortes associadas à COVID-19 foram relatados em todo o mundo, com 37.7 milhões de casos e 704.000 mortes relatadas no Brasil até o momento (W.H.O., 2023a).

Assim como outros vírus causadores de síndrome respiratória aguda, o SARS-CoV-2 apresenta similaridade genômica com coronavírus isolados em morcegos, e, nesse caso, possui 94.6% de

identidade em aminoácidos com o vírus RaTG13, encontrado em morcegos da província chinesa de Yunnan (LAM et al., 2020). A similaridade genômica entre esses vírus sugere evidência de origem zoonótica do SARS-CoV-2, que possivelmente aconteceu por meio de um hospedeiro intermediário entre morcegos e humanos.

A teoria mais aceita atualmente sobre a origem do SARS-CoV-2 é a associação com Pangolins, o quais são mamíferos comercializados de forma ilegal em mercados alimentícios chineses, como os da província de Hubei (XIAO et al., 2020; ZHANG; WU; ZHANG, 2020). Essa hipótese é embasada pela similaridade de 97.4% em aminoácidos da região genômica do domínio de ligação ao receptor (RBD, *Receptor-binding domain*) na glicoproteína S, entre o coronavírus presente em pangolins e o SARS-CoV-2 (ANDERSEN et al., 2020). Mais especificamente, os 6 aminoácidos essenciais para o RBD se ligar ao receptor celular humano da enzima conversora de angiotensina 2 (ECA2) são iguais entre estes coronavírus, enquanto somente 1 aminoácido é semelhante ao RBD do RaTG13, demonstrando a maior afinidade e adaptação ao ECA2 humano (ANDERSEN et al., 2020). Outro ponto a ser abordado é a presença dos aminoácidos prolina, arginina e alanina, os quais formam o sítio de clivagem da protease celular furina na glicoproteína S, que realiza a pré-clivagem dos sítios ativos das subunidades 1 e 2 (S1 e S2, respectivamente) para mediar a fusão entre a membrana viral e celular (HOFFMANN; KLEINE-WEBER; PÖHLMANN, 2020; PEACOCK et al., 2021). Esse mecanismo é descrito para outros betacoronavírus, como o MERS-CoV, HCoV-OC43 e HCoV-HKU1, mas não foi identificado em vírus filogeneticamente similares ao SARS-CoV-2, como o RaTG13 e coronavírus do pangolin, o que dificulta a descrição exata da origem do SARS-CoV-2 (PEACOCK et al., 2021). Desta forma, mesmo após quase 4 anos de sua emergência, é difícil determinar especificamente a origem correta do vírus.

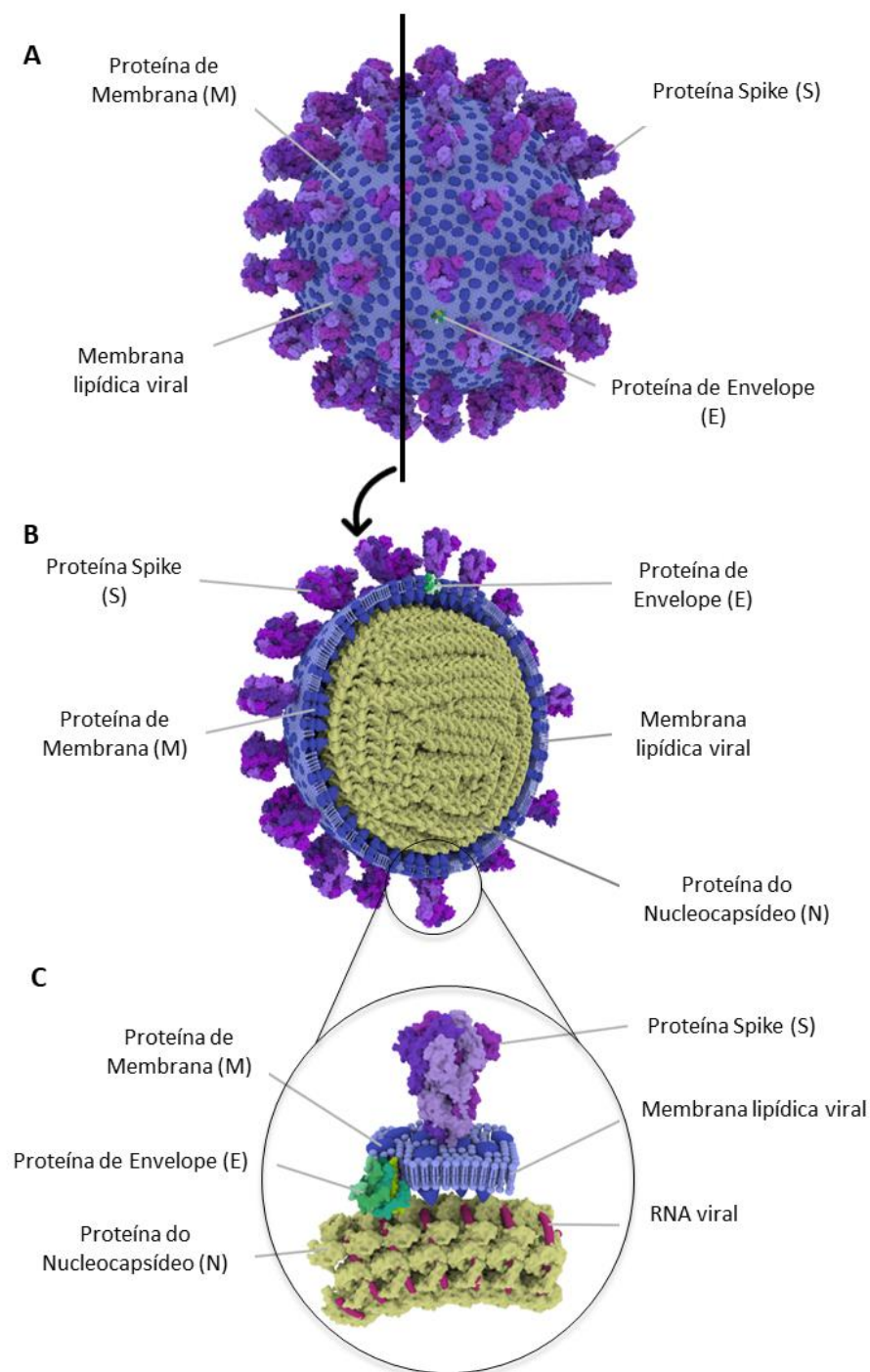
1.2 SARS-CoV-2: partícula viral, genoma e proteínas virais

O SARS-CoV-2 é um vírus da família *Coronaviridae* e gênero *Betacoronavirus* (ROTHER et al., 2020; THE LANCET, 2020; WIERSINGA et al., 2020). O SARS-CoV-2, assim como outros CoVs, possuem um RNA de fita simples polaridade positiva de aproximadamente 30 kb, associado a uma nucleocapsídeo (N), envolto por uma membrana viral derivada das membranas da célula hospedeira contendo as proteínas de envelope (E) e membrana (M), e inserida das glicoproteínas S (PAYNE, 2017; SANTOS et al., 2020; V'KOVSKI et al., 2021a) (**Figura 1**).

O genoma do SARS-CoV-2 é dividido em 14 regiões abertas de leitura (*Open Reading Frames – ORFs*) flanqueadas por duas regiões não codificantes, sendo uma na região 5'UTR e outra na 3'UTR (BAI; ZHONG; GAO, 2022; KIM et al., 2020). A região 5' UTR contém as ORF1a e ORF1b, as

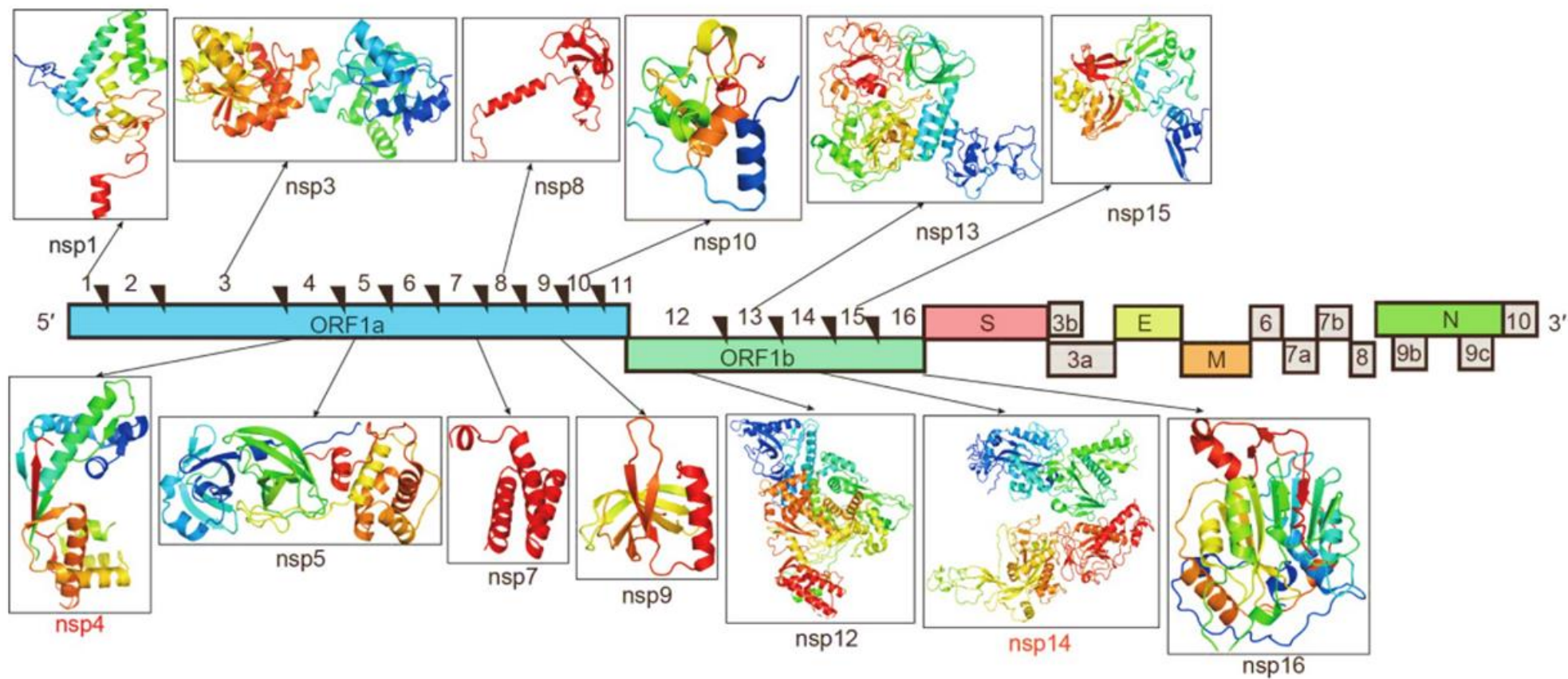
quais são cerca de 70% do genoma viral e são traduzidas em proteínas não estruturais (nsps) nsp1-nsp11 e nsp12-nsp16, respectivamente, seguindo pelas 12 ORFs codificantes das proteínas estruturais S, E, M e N, e as proteínas acessórias 3a, 3b, 6, 7a, 7b, 8, 9b, 9c e 10 (BAI; ZHONG; GAO, 2022) (Figuras 2 e 3).

Figura 1: Estrutura da partícula viral do SARS-CoV-2.



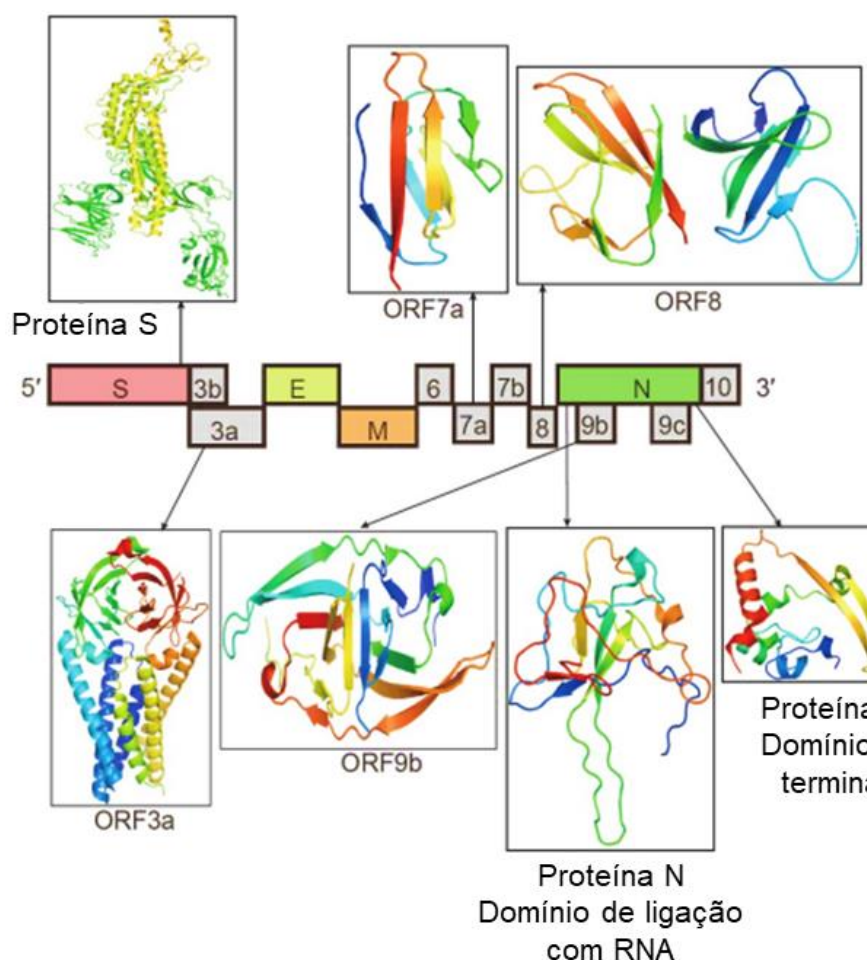
Fonte: Adaptado de “Getting to know the new coronavirus” (2020), disponível em <https://coronavirusexplained.ukri.org/en/article/cad0010/>.

Figura 2: Genoma viral do SARS-CoV-2 e as proteínas não-estruturais expressas pelas ORF1a e ORF1b



Fonte: Adaptado de (BAI; ZHONG; GAO, 2022)

Figura 3: Genoma do SARS-CoV-2 e as proteínas estruturais e acessórias expressas por suas respectivas ORFs



Fonte: Adaptado de (BAI; ZHONG; GAO, 2022)

As nsps expressas a partir genoma do SARS-CoV-2 possuem diversas atividades enzimáticas, dentre elas: a nsp1 está relacionada a supressão de genes no hospedeiro, pela ligação com a região 40S do ribossomo humano, obstruindo a entrada do mRNA e acelerando a degradação de mRNAs (KAMITANI et al., 2006; THOMS et al., 2020); a nsp2 não possui função específica determinada, entretanto foi relacionada com tráfego endossomal e tradução viral, e sua deleção resulta na atenuação da síntese de RNA (GRAHAM et al., 2006; ZHENG et al., 2021); a nsp3 possui 7 domínios, sendo a protease *papain-like* (PLpro) o mais caracterizado devido a hidrólise do peptídeo nsp1-4 do SARS-CoV-2; a nsp4 é uma proteína transmembrânica essencial para formação de redes retículo-vesiculares e vesículas de membrana dupla (*double membrane vesicles*, DMV) (BAI; ZHONG; GAO, 2022; WOLFF et al., 2020); a nsp5 possui atividade cisteína protease processando as poli proteínas do SARS-CoV-2, conhecida como Mpro (BAI; ZHONG; GAO, 2022; ZHANG et al., 2020); a nsp6

também é uma proteína transmembranica responsável pela formação de autofagossomos e inibição da via de sinalização mTOR (BAI; ZHONG; GAO, 2022; BENVENUTO et al., 2020); a nsp9 é uma proteína ligante de mRNA viral (BAI; ZHONG; GAO, 2022; LITTLER et al., 2020); a nsp15 é uma enzima do tipo endoribonuclease (NendoU) responsável pela degradação do RNA viral (vRNA), para não detecção do sistema imune (GAO et al., 2020). As proteínas nsp7, nsp8, nsp12 e nsp13 formam o complexo de polimerase, sendo a nsp12 a RNA-polimerase dependente de RNA (RdRP), a nsp7 uma molécula estabilizadora da nsp8, a nsp8 é uma catalizadora de iniciadores de RNA e a nsp13 uma helicase e ATPase (BAI; ZHONG; GAO, 2022). Adicionalmente, as proteínas nsp10, nsp14 e nsp16 formam o complexo nsp16-10, sendo que em complexo apresentam atividade 3'–5' exoribonuclease e 2'-O-metiltransferase, funcionando como um mecanismo de correção (*proofreading*) (BAI; ZHONG; GAO, 2022; DONG et al., 2020; VISWANATHAN et al., 2020) (**Figura 2**).

Por sua vez, as proteínas estruturais são expressas cada uma por sua própria ORF, sendo a proteína S responsável pela entrada do vírus na célula, mediando a ligação e fusão entre membranas (BAI; ZHONG; GAO, 2022; PLANTE et al., 2021; WALLS et al., 2020; WATANABE et al., 2020). A proteína M é uma glicoproteína responsável pela conformação do envelope viral, sendo essencial para organização da montagem de novos vírions (BAI; ZHONG; GAO, 2022; FU et al., 2021). Já a proteína E, é uma proteína transmembrânica que formam um canal iônico não seletivo em membranas do compartimento intermediário entre retículo endoplasmático e Golgi (ER-Golgi), sendo poucas delas inseridas no envelope viral (ABDELMAGEED et al., 2020; BAI; ZHONG; GAO, 2022). A última proteína estrutural é a N a qual sua função primária é se ligar ao vRNA, formar o nucleocapsídeo e garantir a organização do RNA para replicação e transcrição (BAI; ZHONG; GAO, 2022; KANG et al., 2020; LIANG et al., 2020). Todas as proteínas estruturais também apresentam funções de regulação e evasão do sistema imune do hospedeiro (BAI; ZHONG; GAO, 2022) (**Figuras 1 e 3**).

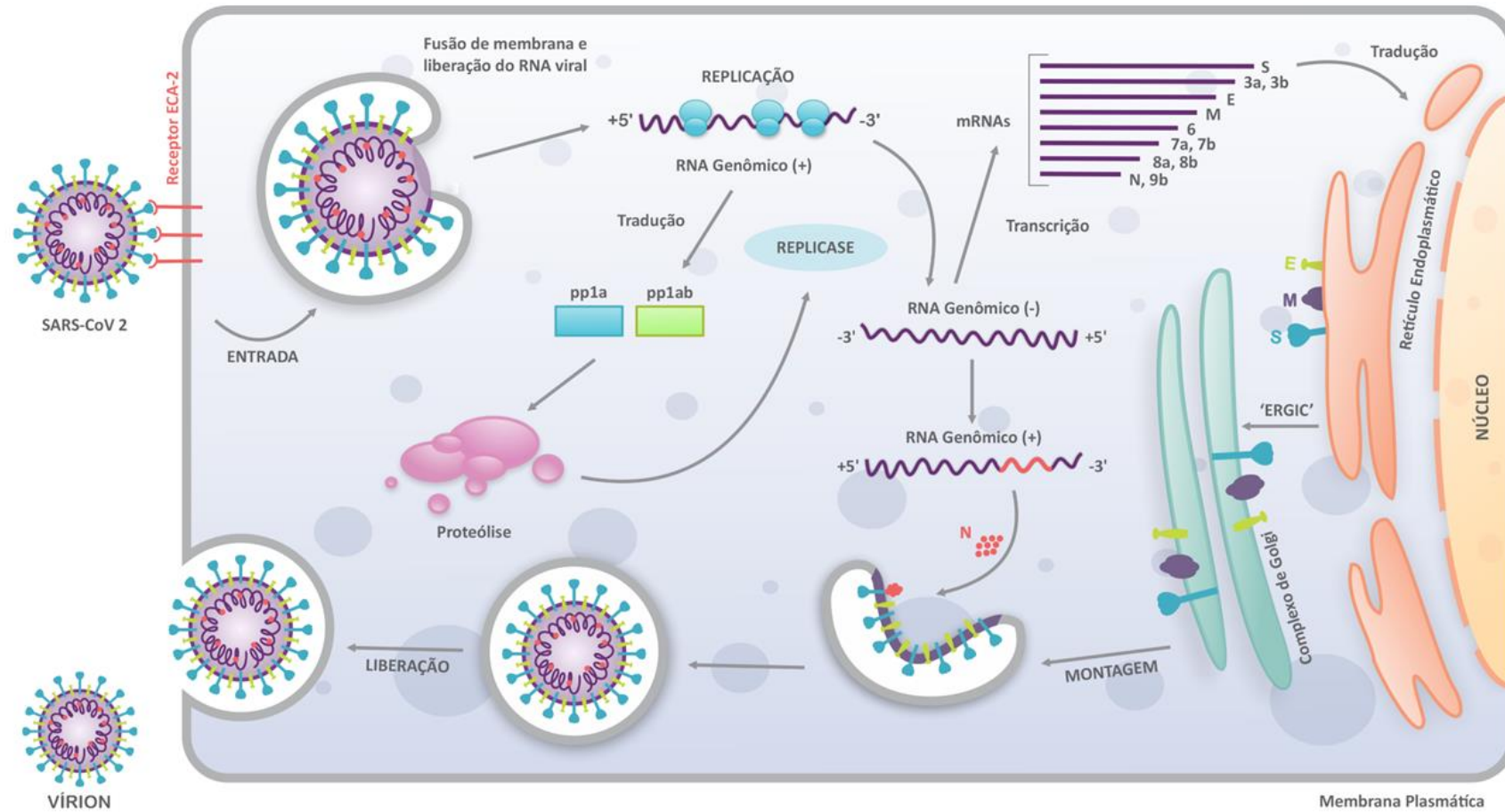
As proteínas acessórias têm funções essenciais para interação e evasão do sistema imune, assim como aumento da virulência viral (BAI; ZHONG; GAO, 2022). As ORF3a e ORF3b codificam as proteínas 3a e 3b, respectivamente, sendo a 3a uma viroporina essencial para a liberação do vírus, e a 3b um peptídeo supressor de interferon (IFN) (KONNO et al., 2020). As proteínas 6, 7a e 7b são expressas a partir da ORF6, ORF7a e ORF7b, respectivamente. A proteína 6 é responsável por interação com proteínas exportadoras de mRNA inibindo a tradução de proteínas do hospedeiro pela formação de híbridos de RNA-DNA (BAI; ZHONG;

GAO, 2022), enquanto a proteína 7a interage com proteínas estruturais M, E e S, e regula a formação de autofagossomos essenciais para replicação do SARS-CoV-2 (BAI; ZHONG; GAO, 2022; GORDON et al., 2020; HOU et al., 2023). A proteína acessória 7b é indutora de moléculas de IFN tipo I e é um componente essencial na estrutura dos vírions (BAI; ZHONG; GAO, 2022; SCHAECHER; MACKENZIE; PEKOSZ, 2007; YANG et al., 2021). A ORF8 é responsável pela expressão da proteína acessória 8, a qual é essencialmente uma molécula de interferência no sistema imune (BAI; ZHONG; GAO, 2022; FLOWER et al., 2021). As ORFs ORF9b, ORF9c e ORF10 codificam as proteínas acessórias 9b, 9c e 10, respectivamente, sendo a 9b uma molécula mediadora do sistema imune, a 9c molécula de interferência na mitocôndria da célula hospedeira, e a 10 supressora de IFN-I por degradação da proteína mitocondrial de sinalização antiviral (*mitochondrial antiviral signaling protein* - MAVS) (BAI; ZHONG; GAO, 2022; LI et al., 2022) (**Figura 3**).

1.3 Ciclo replicativo do SARS-CoV-2

A infecção do SARS-CoV-2 se inicia pela interação da glicoproteína S com o receptor ECA2 em células humanas (LAN et al., 2020). A glicoproteína S é dividida funcionalmente em duas partes: S1, a qual é exposta na superfície viral contendo a RBD; e S2, que possui domínio transmembrânico e peptídeo de fusão, responsável pela fusão em membranas virais e celulares durante o processo de entrada do vírus nas células (LAN et al., 2020; LETKO; MARZI; MUNSTER, 2020; LI, 2016; TORTORICI; VEESLER, 2019). Adicionalmente, entre a S1 e S2 existe um sítio de clivagem de furina, o qual é essencial para o pré-processamento da proteína S, permitindo a liberação do sítio de fusão da proteína S2 (PEACOCK et al., 2021). Para a penetração viral nas células hospedeiras, o SARS-CoV-2 também é dependente do receptor acessório serina protease (*transmembrane serine protease 2* - TMPRSS2) (CHIKHALE et al., 2020; HOFFMANN et al., 2020; LUKASSEN et al., 2020; V'KOVSKI et al., 2021b). Dessa forma, a entrada do vírion ocorre pela sua interação da glicoproteína S viral, especificamente pelo domínio RBD da S1, com os receptores ECA2 e TMPRSS2, seguido pela clivagem do sítio de furina, liberando o domínio S2 para permear a fusão de membranas virais e celulares e liberação do RNA viral no citoplasma celular (HOFFMANN et al., 2020; LAN et al., 2020; LETKO; MARZI; MUNSTER, 2020; LUKASSEN et al., 2020). É importante ressaltar que a interação ECA2-TMPRSS2-Spike pode também desencadear a formação de endossomo, e posteriormente promover a fusão das membranas do endossomo e do vírus (LETKO; MARZI; MUNSTER, 2020; V'KOVSKI et al., 2021b) (**Figura 4**).

Figura 4: Figura esquemática do ciclo replicativo do SARS-CoV-2.



Fonte: Adaptado de SANTOS, et al. (2020).

No citoplasma celular, o RNA viral é inicialmente traduzido em duas poli proteínas, pp1a e pp1ab, através das ORF1a e ORF1b, respectivamente (KLEIN et al., 2020; SANTOS et al., 2020; V'KOVSKI et al., 2021b). A poli proteína pp1a é clivada em nsp1-11, e a pp1ab em nsp1-10 e nsp12-16, por meio das cisteína proteases PL^{pro} e protease semelhante à quimotripsina (3CL^{pro}) (KLEIN et al., 2020; V'KOVSKI et al., 2021b). O complexo de replicação e transcrição viral (RTC) é formado por dois complexos, sendo um com nsp7, nsp8, nsp12 e nsp13, que é responsável pela transcrição e replicação do vRNA, e o complexo contendo nsp10, nsp14 e nsp16 realiza o processamento do RNA, modificações enzimáticas do RNA e correção/revisão do RNA, garantindo a integridade do seu genoma. As outras nsps, nsp1-6, nsp9, nsp11, nsp13 e nsp15, de forma individual, são responsáveis por modular membranas intracelulares, evasão imunológica e produção de cofatores de replicação viral (V'KOVSKI et al., 2019, 2021b). Na presença do RTC uma fita de RNA negativa do genoma completo é sintetizada como molde de novas fitas de RNA polaridade positiva (IRIGOYEN et al., 2016; KLEIN et al., 2020; V'KOVSKI et al., 2021b). Durante a síntese de fitas de RNA negativa, o RTC também interrompe a transcrição nas sequências regulatórias de transcrição (*Transcription Regulatory Sequences* - TRSs), e reinicia na região 5' UTR do genoma, permitindo a formação de fitas de RNA subgenômico (sgRNA) de polaridade negativa que serão usadas como molde para síntese de sg mRNA, que serão traduzidas em proteínas estruturais e acessórias (KIM et al., 2020; SAWICKI; SAWICKI, 1995; SOLA et al., 2015) **(Figura 4)**.

A replicação viral acontece dentro de DMVs ou esférulas de membrana dupla mediadas pelas nsps3, 4 e 6, que possuem características semelhantes ao retículo endoplasmático na região ER-Golgi (KNOOPS et al., 2008; OUDSHOORN et al., 2017; V'KOVSKI et al., 2021b). Ao serem produzidas, as fitas de sg mRNA e as novas fitas de vRNA são transportadas para fora das DMVs, para a região intermediária ER-Golgi (ERGIC), onde serão traduzidas em proteínas E, M, S e N, sendo as proteínas S, E e M inseridas nas membranas das vesículas de membrana simples (SMVs). Na ERGIC, a proteína N recém traduzida se associa com novas fitas vRNA formando o nucleocapsídeo e dando início ao brotamento através das SMVs (MENDONÇA et al., 2021). Os vírions produzidos serão liberados por exocitose ou por meio da via de tráfego lisossomal (DE HAAN; ROTTIER, 2005; KLEIN et al., 2020; KNOOPS et al., 2008; STERTZ et al., 2007; V'KOVSKI et al., 2021b) **(Figura 4)**.

1.4 Transmissão

A transmissão do SARS-CoV-2 ocorre por meio de gotículas respiratórias e aerossóis, sendo que outras rotas de transmissão como a fecal-oral, descrita para SARS-CoV, ainda não foram confirmadas para SARS-CoV-2 (KANG et al., 2020; VAN DOORN et al., 2020). A replicação do SARS-CoV-2 acontece principalmente nos tratos respiratórios superior e inferior, entretanto, também foi detectada no trato gastrointestinal, além da detecção de RNA viral na corrente sanguínea periférica (CORREIA et al., 2020; DING; LIANG, 2020; ROTHE et al., 2020; WIERSINGA et al., 2020).

A alta taxa de transmissão do SARS-CoV-2 está relacionada à replicação no trato respiratório superior, baixa dose infectante, e alta carga viral mesmo em indivíduos não assintomáticos (ENDO et al., 2020; LI et al., 2020b). Isso é confirmado pela taxa de reprodução viral (R_0), que varia entre 2 e 3, chegando em algumas estimativas a 14.8 (LI et al., 2020b, 2020c; ROCKLÖV; SJÖDIN; WILDER-SMITH, 2020; SALZBERGER et al., 2021). Isso significa que um indivíduo infectado pode infectar 2 a 3 pessoas, sendo que esses valores são variáveis dependendo das variantes do SARS-CoV-2 (HOTEIT; YASSINE, 2022). Adicionalmente, é importante enfatizar que a taxa de transmissão do SARS-CoV-2 é mais alta do que outros vírus respiratórios, o que facilitou sua disseminação global, e, embora o SARS-CoV-2 tenha uma taxa de letalidade variando entre 2% e 4%, a sua alta transmissibilidade resultou em números de indivíduos infectados e mortes mais elevados do que os vistos anteriormente para SARS-CoV e MERS-CoV (ABDELRAHMAN; LI; WANG, 2020; CECCARELLI et al., 2020; LAI et al., 2020a). Após a transmissão, os indivíduos infectados apresentam um período de incubação médio de 5.7 dias (intervalo de confiança de 2-14 dias) (LAUER et al., 2020; SALZBERGER et al., 2021).

1.5 Variantes do SARS-CoV-2

O genoma de RNA do SARS-CoV-2 propicia o surgimento de mutações e substituições de nucleotídeos e aminoácidos, o que acarretou a emergência de variantes virais, acumulando mutações com aumento em transmissão e/ou severidade da COVID-19 em curto período (DUFFY, 2018; GROSCHE et al., 2021; JUSTO AREVALO et al., 2021; WANG et al., 2020).

Inicialmente, as variantes do SARS-CoV-2 foram classificados em linhagens A e B, baseado na variabilidade observada nas sequências dos genomas virais. A linhagem B foi identificada no início da pandemia, sendo a única identificada em humanos associados com o mercado Hubei, Wuhan, na China, onde foi sugerida a emergência do SARS-CoV-2 (ANDERSEN et al., 2020; RAMBAUT et al., 2020). A linhagem A foi identificada em 5 de

janeiro de 2020 e apresenta dois nucleotídeos semelhantes aos vírus RaTG13 e RmYN02 na ORF1ab e ORF8, enquanto esses nucleotídeos são diferentes na linhagem B de Wuhan-Hu-1 (GenBank nº de acesso MN908947) coletada em 26 de dezembro de 2019 (ZHU et al., 2020). Embora os vírus da linhagem B tenham sido sequenciados e publicados primeiro, é provável (com base nos dados atuais) que o ancestral comum mais recente (MRCA) da filogenia do SARS-CoV-2 compartilhe a mesma sequência genômica que as primeiras sequências da linhagem A (por exemplo, Wuhan/WH04/2020).

A linhagem B se tornou predominante em relação a linhagem A, porém, com a alta taxa de mutações e evolução viral, atualmente, existem diversas linhagens com centenas de variantes classificadas no *Global Initiative on Sharing All Influenza Data* (GISAID) (RAMBAUT et al., 2020). Por isso, a OMS passou a classificar as variantes em: variantes sob monitoramento (*Variant Under Monitoring* - VUM), variantes de interesse (*Variant of Interest* - VOI) e variantes de preocupação (*Variant of Concern* - VOC) (KONINGS et al., 2021; W.H.O., 2023b). A classificação leva em consideração a severidade da doença causada, mudança na epidemiologia da doença, impacto nos sistemas de saúde e a diminuição da eficácia das vacinas disponíveis (KONINGS et al., 2021; W.H.O., 2023b).

As VOCs foram nomeadas com letras gregas para facilitar comunicação e informação populacional, e entre elas se destacam a VOC Alfa (B.1.1.7), identificada primeiramente no Reino Unido em 2020, Beta (B.1.351) na África do Sul em 2020, Gamma (P.1) no Brasil em 2020, Delta (B.1.617.2) na Índia em 2020, e a Ômicron (B.1.1.529) na África do Sul em 2021 (FARIA et al., 2021; HOTEIT; YASSINE, 2022; JOHNSON et al., 2022; MONTAGUTELLI et al., 2021; WANG et al., 2021b). Atualmente, não existem VOCs classificadas no manual da OMS, existindo somente as VOIs XBB.1.16, XBB.1.5 e EG.5, sendo todas essas sublinhagens da ômicron, e VUMs BA.2.75, CH.1.1, XBB, XBB.1.9.1, XBB.1.9.2 e XBB.2.3 (W.H.O., 2023b) (**Figura 5**).

Figura 5: Esquema representativo das VOCs com local e ano de emergência



Fonte: Adaptado de “SARS-CoV-2 Variants: Evading the Immune System” (disponível em <https://bpsbioscience.com/sars-cov-2-variants>)

1.6 COVID-19, Resposta Imune e Síndrome Pós-COVID

A infecção por SARS-CoV-2 pode se apresentar de forma assintomática, entretanto a maior parte dos pacientes infectados desenvolvem a COVID-19, apresentando sintomas leves a moderados como febre, tosse, dispneia, dores de cabeça, dores musculares e fadiga (CAO; LI, 2020; ESAKANDARI et al., 2020; GRASSELLI et al., 2020a; TROUGAKOS et al., 2021a). Os pacientes podem exibir também sintomas relacionados a infecções do trato respiratório superior e inferior como pneumonia, ARDS e sepse, os quais representam casos mais graves e usualmente levam à morte (CAO; LI, 2020; ESAKANDARI et al., 2020; GRASSELLI et al., 2020a; LAMERS; HAAGMANS, 2022; TROUGAKOS et al., 2021a).

O SARS-CoV-2 infecta inicialmente células multiciliadas presentes no epitélio da nasofaringe e traqueia ou células da mucosa olfatória (AHN et al., 2021; KHAN et al., 2021; LAMERS; HAAGMANS, 2022). Ao iniciar o processo de replicação, a formação de moléculas de dupla fita de RNA (dsRNA) como intermediários de replicação são reconhecidos por receptores de reconhecimento padrão como a proteína associada à diferenciação de melanoma 5 (*melanoma differentiation-associated protein 5*, MDA5) e o gene indutor de ácido retinóico

I (*retinoic acid-inducible gene*, RIG-I), e dá início a cascata de sinalização via fatores regulatórios de interferon (*interferon regulatory fator*, IRFs) IRF3 e IRF7, para iniciar a transcrição e tradução de citocinas inflamatórias do IFN tipo I e tipo III (LAMERS; HAAGMANS, 2022; SAMPAIO et al., 2021; YIN et al., 2021).

As citocinas isoleucinas (IL) IL-1, IL6, IL-8 e o fator de necrose tumoral (TNF) são liberadas no meio extracelular e serão empregadas de forma autóctone e parácrina, sendo reconhecidos por receptores de membrana IFN tipo I e III (KHANMOHAMMADI; REZAEI, 2021; SARIOL; PERLMAN, 2021; YIN et al., 2021). A ativação dos receptores leva a fosforilação da Janus Kinase 2 (JAK-STAT1/2) e produção de genes estimuladores de interferon para ativação de células vizinhas e atração de células imune (KHANMOHAMMADI; REZAEI, 2021; SARIOL; PERLMAN, 2021; YIN et al., 2021). As células T são ativadas para eliminação de células infectadas por SARS-CoV-2 e para ativação e diferenciação de células B em plasmócitos secretores de anticorpos (KHANMOHAMMADI; REZAEI, 2021; LAMERS; HAAGMANS, 2022; TROUGAKOS et al., 2021a). Quando a infecção não é eliminada, o vírus se dissemina para o trato respiratório inferior e alvéolos pulmonares, principalmente células alveolares do tipo 2 (AT2) (KATSURA et al., 2020; LAMERS; HAAGMANS, 2022; YOUK et al., 2020). As células AT2 são células produtoras de surfactante que é necessário para diminuição de tensão superficial facilitando a troca gasosa feita por células alveolares do tipo I (AT1) (KATSURA et al., 2020; LAMERS; HAAGMANS, 2022; YOUK et al., 2020).

Após o estabelecimento da infecção e o recrutamento de células imune, é iniciado um processo de dano epitelial causado pela inflamação e infiltração de monócitos, macrófagos ativados, células dendríticas e natural killers (NK) atraídas por quimiocinas CCL7, CCL8 e CCL13 (CHUA et al., 2020; LAMERS; HAAGMANS, 2022). Clinicamente, o processo de inflamação se desenvolve para ARDS e pneumonia, e histologicamente apresentam danos alveolares difusos (DAD), em que há o acúmulo de fluidos e exsudatos celulares que levam a fibrose intersticial, edema, trombose microvascular e a morte de pneumócitos (CARDINAL-FERNÁNDEZ et al., 2017; LAMERS; HAAGMANS, 2022). Adicionalmente, os DADs levam ao desequilíbrio entre coagulação e fibrinólise que: i) desencadeiam a formação de membranas hialinas para diminuir o acúmulo de fluidos nos alvéolos, mas também resulta na limitação de oxigênio disponível; e ii) formam trombos de fibrina em pequenas artérias e altas nos níveis de dímeros D (fator indicador de fatalidade, sendo altos níveis correlacionados a severidade da doença) (C. SEBAG; A. BASTARACHE; B. WARE, 2011; CARDINAL-FERNÁNDEZ et

al., 2017; GRASSELLI et al., 2020b; IBA et al., 2020; KLOK et al., 2020). Outro problema associado ao desenvolvimento de SARS em pacientes com SARS-CoV-2 é a hiperativação de células imunes, com células T CD8⁺ e NK liberando grânulos citotóxicos para desencadear apoptose e piroptose de células infectadas gerando um inflamossoma e a “tempestade de citocinas” (elevados níveis de IL-1beta, IL-6, IL-2, IL-10) (GRASSELLI et al., 2020a; LAMERS; HAAGMANS, 2022; REMY et al., 2020). A continuidade do processo inflamatório pode levar a eliminação da infecção viral e recuperação do paciente, mas na maioria dos casos severos da doença levam à morte (GRASSELLI et al., 2020a; LAMERS; HAAGMANS, 2022; REMY et al., 2020).

Atualmente, a maioria das pessoas não desenvolve a forma severa da COVID-19, apresentando somente sintomas leves e moderados, sendo capazes de eliminar a infecção em algumas semanas (MALTEZOU; PAVLI; TSAKRIS, 2021a). Entretanto, pacientes infectados podem desenvolver a COVID-longa ou Síndrome Pós-COVID meses após a infecção (MALTEZOU; PAVLI; TSAKRIS, 2021a; ORONSKY et al., 2021; YONG, 2021). A síndrome Pós-COVID foi descrita pela primeira vez em 2020, com pacientes apresentando sintomas como fadiga, dispneia, disfunção olfatória, dor no peito e problemas mentais e do sono, que podem permanecer por meses, impactando a qualidade de vida dos indivíduos (AUGUSTIN et al., 2021; YONG, 2021). De acordo com Logue e colaboradores, em 2021, cerca de 30% dos pacientes infectados não recuperaram seu estado de saúde inicial e apresentaram sintomas persistentes em até 9 meses pós-infecção (LOGUE et al., 2021). Nos últimos meses da Síndrome Pós-COVID, outros sintomas mais graves como complicações neurológicas, tromboembolismo e arritmias cardíacas foram descritos (MALTEZOU; PAVLI; TSAKRIS, 2021a). A patogênese da síndrome ainda não foi completamente descrita, porém a inflamação prolongada possui um papel principal na maioria dos casos pós-COVID (STRATTON; TANG; LU, 2021). A inflamação com elevados níveis de IL-6 foi associada com uma diminuição do ácido gama-aminobutírico (GABA), resultando em fadiga neuro motora e cognitiva (GARCIA-OSCOS et al., 2012; MALTEZOU; PAVLI; TSAKRIS, 2021b). Adicionalmente, os coronavírus podem infectar a região do hipocampo no sistema nervoso central, levando diretamente a complicações neurológicas (MALTEZOU; PAVLI; TSAKRIS, 2021b).

1.7 Profilaxia e Tratamento

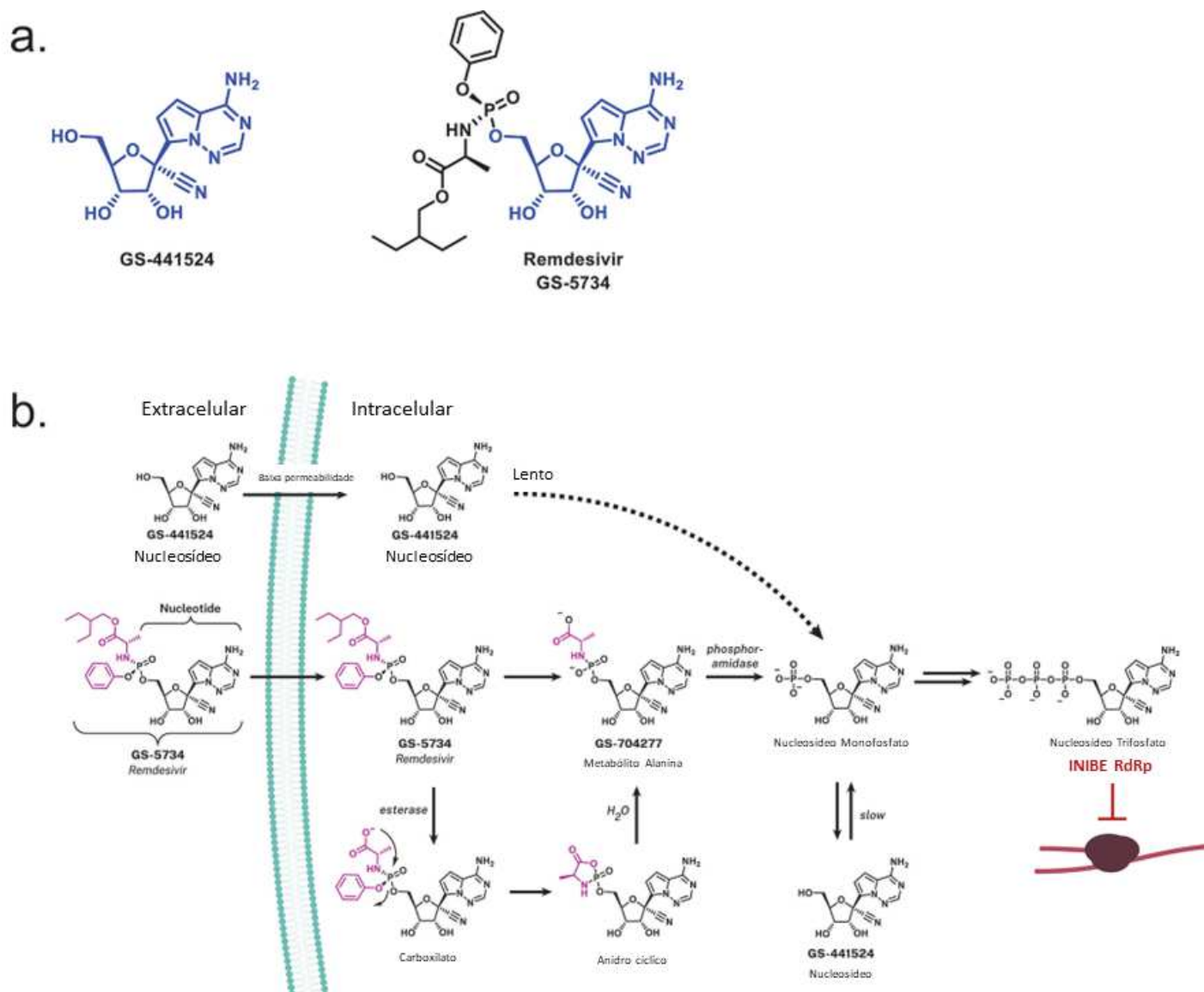
De acordo com a OMS, existe 199 vacinas em estágio pré-clínico de desenvolvimento e 183 vacinas em estágio clínico, sendo essas de tecnologia de subunidade proteica, vetor viral não-replicante (VVnr), DNA, RNA, vírus inativado, vírus atenuado, vetor viral replicante (VVr), VVr com célula apresentadora de antígeno, VVnr com célula apresentadora de antígeno e vetor bacteriano de expressão de antígeno-esporo. Atualmente, existem 10 vacinas aprovadas para profilaxia da COVID-19 (W.H.O., 2023c). Entre elas estão: i) Sinovac-Coronavac, Sinopharm, Bharat e Valneva, utilizando a tecnologia de vírus inativado; ii) Novavax/Serum Institute of India utilizando a tecnologia de subunidades proteicas virais; iii) AstraZeneca/Oxford, Janssen e CanSino, com tecnologia de vetor viral não replicante; e iv) Pfizer/BioNTech e Moderna, com tecnologias de RNA/DNA (W.H.O., 2023c).

A emergência e necessidade de tratamentos para a COVID-19 resultaram em avanços para identificação de moléculas capazes de inibir a replicação do SARS-CoV-2 em pacientes infectados, almejando a melhora de sinais clínicos (SANTOS et al., 2020; TROUGAKOS et al., 2021b). Atualmente aprovados pela Administração de Alimentos e Medicamentos (*Food and Drug Administration* – FDA) e Agência Nacional de Vigilância Sanitária (Anvisa), estão o Remdesivir, Molnupiravir e o Paxlovid (CROSS et al., 2021; EASTMAN et al., 2020; FDA, 2020; SAÚDE., 2022).

O Remdesivir (GS-5734) (**Figura 6**), desenvolvido pela Gilead Sciences em parceria com o Centro de Controle e Prevenção de Doenças Infecciosas do Estados Unidos da América (*U.S. Centers for Disease Control and Prevention*, CDC) e o Instituto Médico de Pesquisa em Doenças Infecciosas do Exército dos Estados Unidos da América (*U.S. Army Medical Research Institute of Infectious Diseases*, USAMRIID), é um análogo de nucleosídeo para o tratamento de doenças causadas por vírus de RNA que possuem um grande potencial pandêmico (BEIGEL et al., 2020; BROWN et al., 2019; EASTMAN et al., 2020). Foi identificado a partir de uma biblioteca de aproximadamente 1000 análogos de nucleosídeo, tendo atividade amplo-espectro contra o vírus do ebola (EBOV), vírus da febre amarela (YFV), vírus da dengue tipo 2 (DENV-2), e SARS-CoV (EASTMAN et al., 2020). O remdesivir (GS-5734) é uma pró-droga, que ao ser metabolizada no citoplasma celular é transformada no análogo de nucleosídeo ativo fosforilado (radical trifosfato) (EASTMAN et al., 2020; SLUSARCZYK; SERPI; PERTUSATI, 2018; TOBIAS; BORCH, 2001). Sua molécula central GS-441524 possui baixa permeabilidade celular e um lento processo de fosforilação, e por isso apresenta uma baixa

atividade antiviral (**Figura 6**) (SLUSARCZYK; SERPI; PERTUSATI, 2018; TOBIAS; BORCH, 2001). Adicionalmente, o remdesivir é uma molécula de baixa afinidade para as RNA polimerases humanas, e por isso, age de forma seletiva contra a replicação de vírus de RNA (EASTMAN et al., 2020; FENG et al., 2016).

Figura 6: Estrutura química do remdesivir e seus análogos, e sua fosforilação em sistemas biológicos



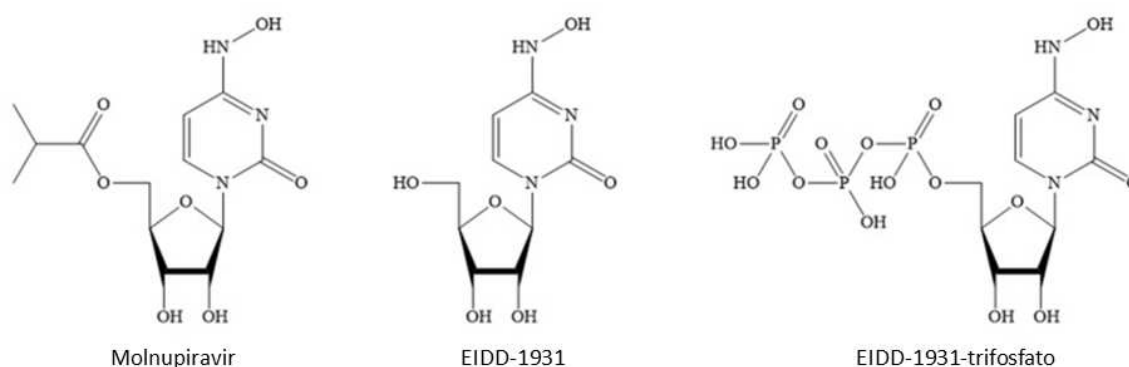
Fonte: Adaptado de EASTMAN, et al. (2020)

O remdesivir foi descrito como um potente inibidor do EBOV em primatas não humanos. Um estudo clínico foi realizado na República do Congo (NCT02818582) durante o surto de EBOV em 2014 e, apesar de demonstrar diminuição de sintomas clínicos e morte de pacientes, teve um menor efeito quando comparado a terapias baseadas em anticorpos (Mab114 e REGN-

EB3), e por isso, seu processo de intervenção foi descontinuado para esse fim (EASTMAN et al., 2020; MULANGU et al., 2019). Para as infecções causadas pelo SARS-CoV-2, ao início do mês de abril de 2020, sete ensaios clínicos de fase 3 estavam registrados para avaliar o tratamento de pacientes SARS-CoV-2 positivos internados em redes hospitalares com o remdesivir no mundo todo (EASTMAN et al., 2020; MULANGU et al., 2019). A partir de resultados atuais, o tratamento com remdesivir nas primeiras 48h foi associado com recuperação mais rápida, deixando o hospital cerca de 3 a 4 dias antes que o grupo controle, além de ter poucos casos de efeitos adversos (hepatocitotoxicidade e nefrocitotoxicidade em 6.9% e 2.1%, respectivamente), se mostrando um perfil de segurança para uso em pacientes com COVID-19.

O Molnupiravir (FIDD-2801) (**Figura 7**), por sua vez, desenvolvido pela associação *Emory University, Ridgeback Biotherapeutics e Merck Research Laboratories*, é também um análogo de nucleosídeo, mais especificamente, um análogo de ribonucleosídeo de pirimidina (**Figura 7**) (FISCHER et al., 2022; JAYK BERNAL et al., 2022a). O Molnupiravir é uma pró-droga, convertida em EIDD-1931, e posteriormente fosforilada por quinases do hospedeiro em EIDD-1931-trifosfato (**Figura 7**), sendo essa sua forma ativa, um substrato competidor/alternativo para a RdRp de vírus de RNA (IMRAN et al., 2021; JAYK BERNAL et al., 2022a; PAINTER et al., 2021). O Molnupiravir foi desenvolvido para o tratamento de infecções causados por Alfavírus, mais especificamente o vírus da encefalite equina venezuelana (VEEV) e o CHIKV, em 2013, devido aos níveis seguros de toxicidade do EIDD-1931 in vitro (IMRAN et al., 2021; PAINTER et al., 2021). Posteriormente, sua atividade também foi reportada contra IAV, vírus sincicial respiratório (RSV), SARS-CoV, MERS-CoV e EBOV (IMRAN et al., 2021; JAYK BERNAL et al., 2022a; PAINTER et al., 2021). Entretanto, quando administrado oralmente, o EIDD-1931 era rapidamente metabolizado em primatas não-humanos, o que levou ao desenvolvimento em sua forma de pró-droga Molnupiravir, o qual possui um ótimo perfil de biodisponibilidade e com baixa citotoxicidade a níveis sistêmicos. Com a emergência da COVID-19 e a existência de dados mostrando a eficácia contra outros vírus respiratórios, inclusive outros betacoronavírus, o Molnupiravir foi rapidamente implementado para realização de ensaios clínicos de fase 1 (IMRAN et al., 2021; PAINTER et al., 2021). No final de 2022, as conclusões do ensaio clínico de fase 3 demonstraram que o tratamento com Molnupiravir levaram ao menor risco de hospitalização e de morte em pacientes internados, sendo esses não vacinados para COVID-19 (JAYK BERNAL et al., 2022a; WAN et al., 2023).

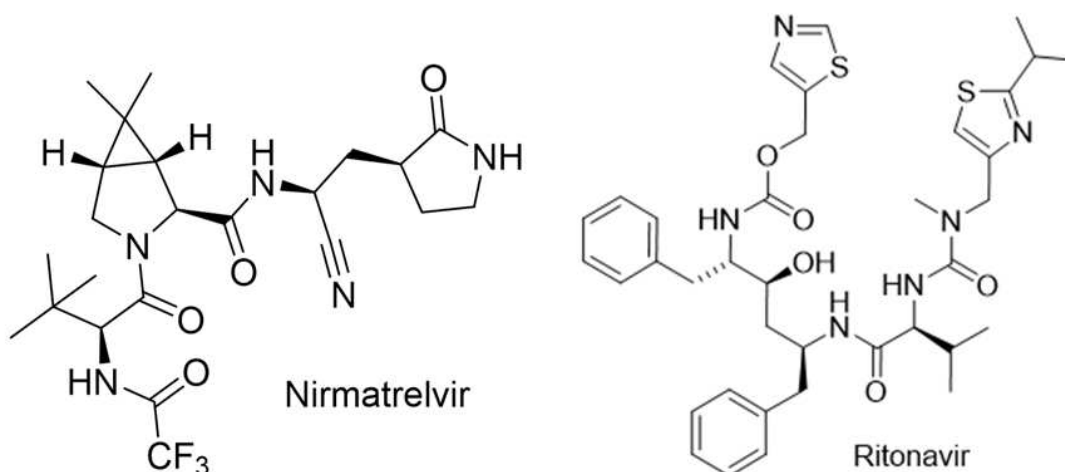
Figura 7: Estrutura química do Molnupiravir e suas formas fosforiladas



Fonte: Adaptado de IMRAN, et al (2021)

Adicionalmente, o Paxlovid (PF-07321332) (**Figura 8**), desenvolvido pela Pfizer, é constituído pelas moléculas Nirmatrelvir, um inibidor de protease 3CL^{pro} ou M^{pro} do SARS-CoV-2, e o Ritonavir, um inibidor de protease do vírus da imunodeficiência 1 e 2 (HIV-1 e HIV-2) (OWEN et al., 2021). O Nirmatrelvir foi desenvolvido como um inibidor da proteólise da poli proteína viral do SARS-CoV-2, antes do início da replicação do RNA, dessa forma impedindo a maturação das proteínas não estruturais, essenciais para formação do complexo RTC (HAMMOND et al., 2022; LIU et al., 2023a). Adicionalmente, o Nirmatrelvir não foi capaz de induzir mutações no DNA celular e se apresenta como um fármaco de administração oral, demonstrando ser um bom candidato para o tratamento de COVID-19. A associação com Ritonavir, também um inibidor de protease viral, ajuda na maior biodisponibilidade do Nirmatrelvir devido à diminuição da velocidade do seu metabolismo, permanecendo em sua forma ativa por maior tempo (HAMMOND et al., 2022; LIU et al., 2023a). Sua utilização foi aprovada pelo FDA, devido aos resultados de ensaio clínico de fase 3 reduzindo a progressão clínica da COVID-19, com menor carga viral após 5 dias de tratamento (HAMMOND et al., 2022).

Figura 8: Estrutura química do Nirmatrelvir e Ritonavir (Paxlovid).



Fonte: Adaptado de Lowe Derek (2022) (“*Discovering Paxlovid*” – disponível em <https://www.science.org/content/blog-post/discovering-paxlovid>)

Adicionalmente, atualmente existem cerca de 194 ensaios clínicos com moléculas como potenciais inibidores do SARS-CoV-2 em pacientes infectados (<https://clinicaltrials.gov/>). Apesar do alto número de ensaios clínicos como potenciais tratamentos contra o SARS-CoV-2, é importante enfatizar a necessidade da contínua busca por novas fontes de moléculas que possam futuramente serem capitalizadas para o tratamento de pacientes infectados com SARS-CoV-2. Isso se deve a diversos motivos, dentre eles: i) a alta evolução viral do SARS-CoV-2, podendo sofrer mutações ao longo do tempo, o que pode resultar em variantes mais resistentes e, por isso, é necessário desenvolver antivirais que possam ser eficazes contra essas variantes emergentes; ii) a inacessibilidade de vacinas e terapias contra a COVID-19 para todos os países no mundo; iii) a prevenção de futuras pandemias, devido ao fato que desenvolver antivirais eficazes contra o SARS-CoV-2 pode fornecer *insights* valiosos e ferramentas para combater futuros surtos virais ou pandemias, contribuindo para a preparação e resposta a possíveis ameaças à saúde pública; e iv) promover uma variedade de abordagens terapêuticas, visto que a diversidade de antivirais com mecanismos de ação diferentes podem ser usados em conjunto para o manejo das diferentes fases da doença e para atender às necessidades de diversos grupos de pacientes.

2. Vírus da Chikungunya (CHIKV)

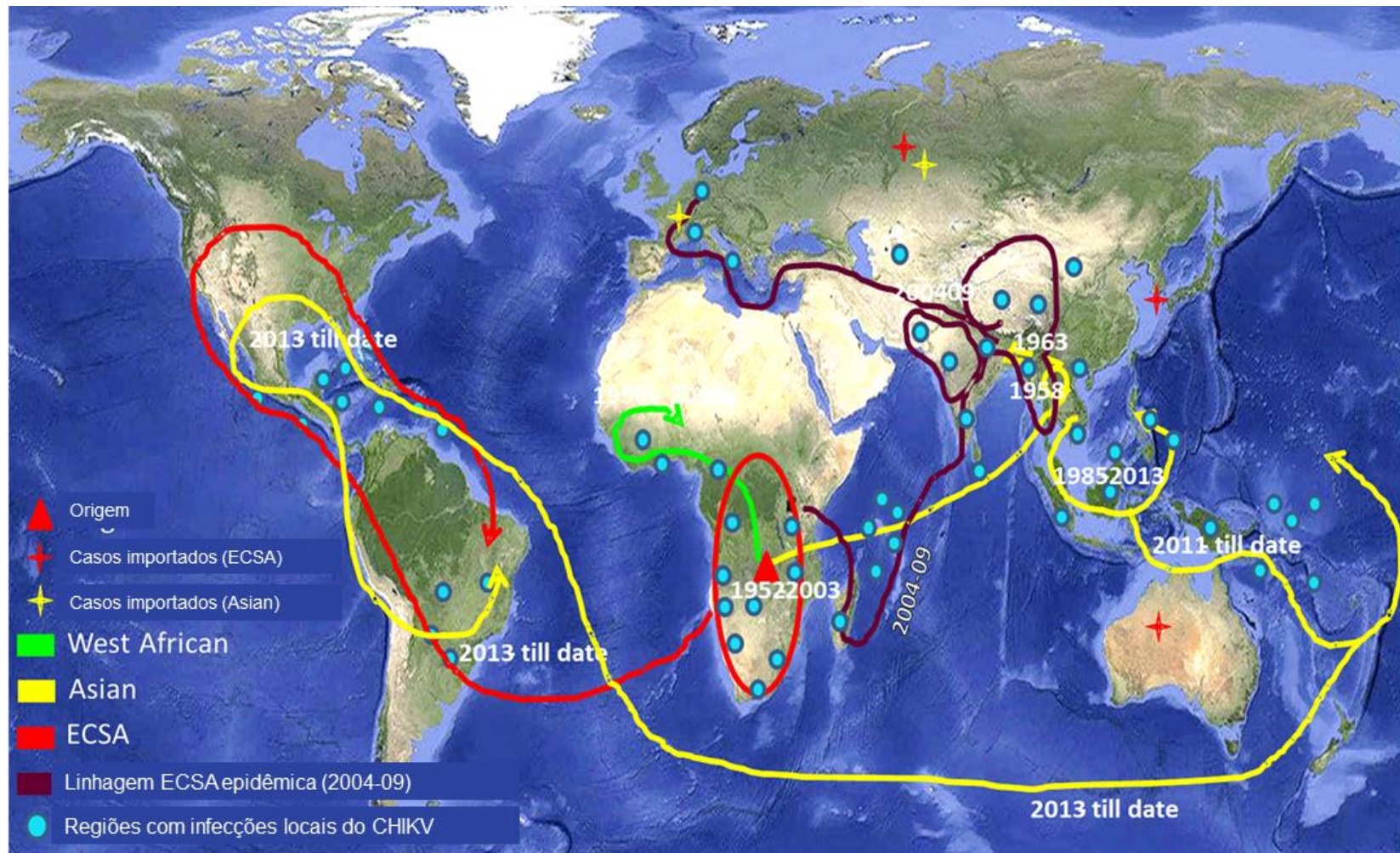
2.1. Histórico e Epidemiologia

O vírus da chikungunya (CHIKV) foi descoberto em 1950 na região da Tanzânia (HALSTEAD, 2015; LUMSDEN, 1955) e sua identificação como o agente etiológico da Febre do Chikungunya ocorreu somente em 1955 (ROSS, 1955; WEAVER; FORRESTER, 2015). O nome “chikungunya” originou-se do idioma Makonde, e significa ‘aqueles que se dobram’, referindo aos pacientes que possuíam uma aparência curvada durante a primeira epidemia documentada entre 1952 e 1953 (MONATH, 2019).

Durante os 50 anos após sua descoberta, casos esporádicos de CHIKV permaneceram restritos às regiões da África e Ásia, com baixa mortalidade, porém, alta morbidade e impacto na qualidade de vida dos infectados (COHEN et al., 1969; NJENGA et al., 2008). Em 2005-2006, um surto global se iniciou com alguns casos em ilhas do oceano Índico, infectando cerca de 775.000 pessoas na ilha de Réunion, com 237 mortes e notificações posteriores em países como Itália e França, associados à intensa circulação de pessoas nestas regiões (RAVI, 2006; SAMBRI et al., 2008). O surto de febre chikungunya atingiu também as Américas, onde o vírus se espalhou com grande facilidade, em consequência do clima favorável e a presença de vetores suscetíveis (GRATZ, 2004; PAGÈS et al., 2009; RASHAD; MAHALINGAM; KELLER, 2013).

A partir de estudos filogenéticos identificou-se que o CHIKV se originou na África cerca de 500 anos atrás e foi introduzido na Ásia, se dividindo inicialmente em três genótipos distintos, originados a partir de uma linhagem comum, sendo eles: Oeste Africano (*West African*, WA), Leste/Centro/Sul da África (*East/Central/South Africa*, ECSA), e a Asiática (VOLK et al., 2010; WEAVER; FORRESTER, 2015). A ocorrência de um surto na Ásia cerca de 150 anos atrás, causado pela cepa ECSA, possivelmente propiciou a sua diferenciação em um subgenótipo conhecido como a Linhagem Urbana Asiática (*Asian Urban Lineage*, AUL) (BURT et al., 2017; VOLK et al., 2010), e a inserção de uma linhagem ECSA nas regiões do Oceano Índico deu origem ao subgenótipo Oceano Índico (*Indian Ocean Lineage*, IOL), entretanto, estima-se que essa linhagem tenha se extinguido (BURT et al., 2017; VOLK et al., 2010) (**Figura 9**).

Figura 9: Mapa *mundi* representando a disseminação do CHIKV através dos anos



Fonte: Adaptado de DEEBA, et al. (2020).

No Brasil, os primeiros casos da febre do chikungunya foram relatados no Amapá e na Bahia em 2014, causados pelas cepas ECSA e AUL, as quais foram rapidamente difundidas para os demais estados do território brasileiro (BRASIL., 2015; SILVA et al., 2017). Desde então, essas cepas foram responsáveis por mais de 900 mil casos de febre chikungunya entre os anos de 2014 e 2022 (MINISTÉRIO DA SAÚDE BRASIL, 2023; SILVA et al., 2018). No ano de 2022, foram registrados até a semana epidemiológica 52, 174.517 casos prováveis de febre chikungunya, com uma incidência de 81,8 casos por 100 mil habitantes, e 94 óbitos confirmados (MINISTÉRIO DA SAÚDE BRASIL, 2023). No ano de 2023, até a semana epidemiológica 35, 143.739 casos prováveis de CHIKV foram reportados, sendo 82 mortes confirmadas (MINISTÉRIO DA SAÚDE BRASIL, 2023).

2.2. Transmissão

O CHIKV é transmitido por meio da picada de mosquitos fêmeas infectados, sendo que os principais vetores no ciclo urbano são *Aedes aegypti* e *Aedes albopictus* (BARTHOLOMEEUSEN et al., 2023; NGOAGOUNI et al., 2017; POWERS et al., 2000). O *A. albopictus* como vetor de transmissão para o CHIKV ocorreu por uma microevolução genômica em 2006, que identificou uma mutação de alanina para valina na posição 226 na proteína E1 do envelope viral, que aumenta de transmissão e melhora a replicação em insetos (BARTHOLOMEEUSEN et al., 2023; DE LIMA CAVALCANTI et al., 2022).

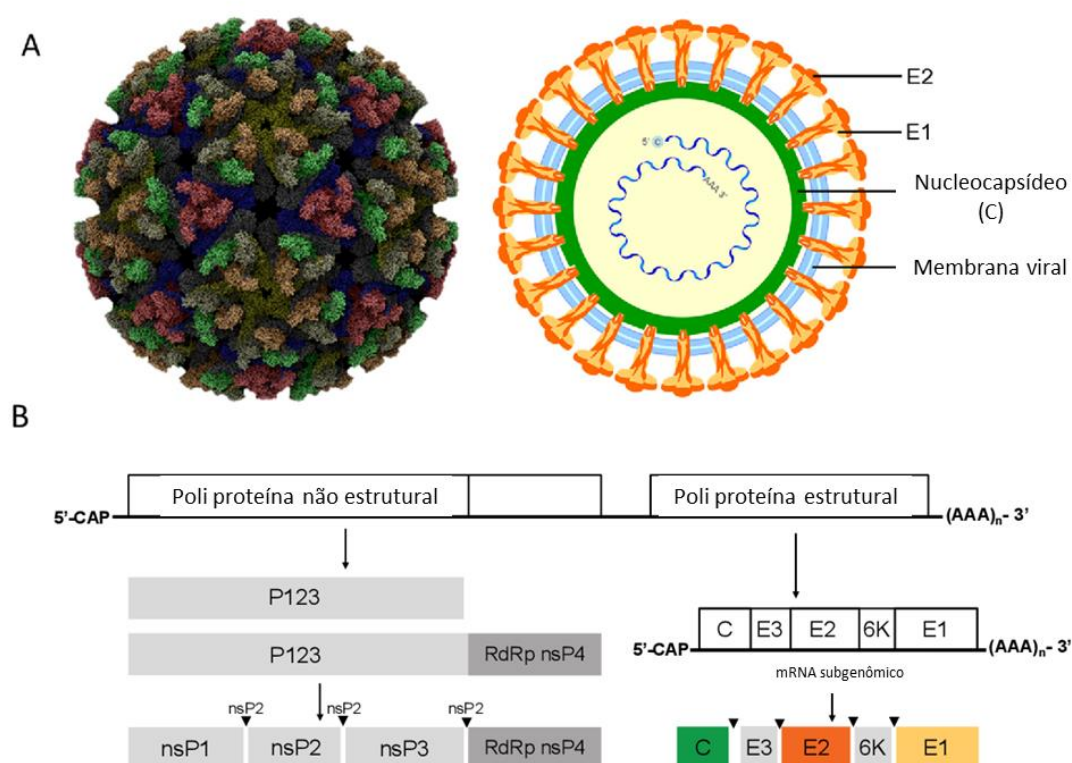
Regiões com clima tropical facilitam o aumento da densidade de vetores e, consequentemente, a transmissão do vírus, especialmente durante períodos chuvosos (DE LIMA CAVALCANTI et al., 2022; MAVALE et al., 2010). O CHIKV possui também um ciclo silvestre transmitido por outros vetores, como *Haemagogus leucocelaenus*, *A. terreus*, *A. furcifer* e *A. africanus*, que possuem alta susceptibilidade pelo vírus, podendo se tornar potenciais vetores transmissores, e assim, facilitar novos surtos da infecção (LOURENÇO-DE-OLIVEIRA; FAILLOUX, 2017). Adicionalmente, o CHIKV apresenta reservatórios em primatas não humanos, favorecendo a manutenção e disseminação viral (BARTHOLOMEEUSEN et al., 2023; DE LIMA CAVALCANTI et al., 2022).

A transmissão vertical do CHIKV é classificada como rara, mas devido à patologia, pode provocar abortos em fases iniciais, bem como altas taxas de morbidade infantil, o que pode impactar diretamente na documentação desses casos (AGARWAL et al., 2014; GÉRARDIN et al., 2008). Entretanto, em 2018 foi relatado o primeiro caso de morte de recém-nascido no Brasil após infecção por CHIKV (OLIVEIRA et al., 2018).

2.3. Vírus da chikungunya

O CHIKV pertence à família *Togaviridae* e gênero *Alfavirus* (CHEN et al., 2018; GAY et al., 2016; ICTV, 2019; ROSS, 1955). É um vírus envelopado, com aproximadamente 70 nm de diâmetro e uma fita simples de RNA de polaridade positiva com aproximadamente 12 kB (BARTHOLOMEEUSEN et al., 2023; ROBERTS et al., 2017; SOLIGNAT et al., 2009). O genoma viral apresenta uma região não-codificante 5', seguida de uma ORF codificante das 4 proteínas não estruturais (nsP1 a nsP4), relacionadas ao complexo replicativo, seguida da ORF codificante das 5 proteínas estruturais: capsídeo (C), proteínas de envelope (E) E1, E2, E3, proteína *transfame* (TF) e vioporina (6K), presentes no capsídeo ou envelope do vírus, e por fim de uma região não-codificante 3' (DE LIMA CAVALCANTI et al., 2022; LUM; NG, 2015) (Figura 10).

Figura 10: Representação esquemática da partícula e genoma viral do CHIKV



Fonte: Adaptado de Metz, Stefan W. “Chikungunya virus-like particle vaccine.” (2013).

A proteína nsP1 é uma proteína que realiza o *capping* de novas fitas de RNA viral e possui a habilidade de se ligar a membranas, enquanto a nsP2 possui a atividade helicase e cisteína protease, que realiza a clivagem da poliproteína formada pela ORF1. A nsP3 é uma ADP-ribosil hidrolase responsável por interagir com proteínas do hospedeiro, e a nsP4 é a

RNA-polimerase dependente de RNA, responsável pela síntese de novas fitas de vRNA (LAURENT et al., 2022; SINGH et al., 2018) (**Figura 10**).

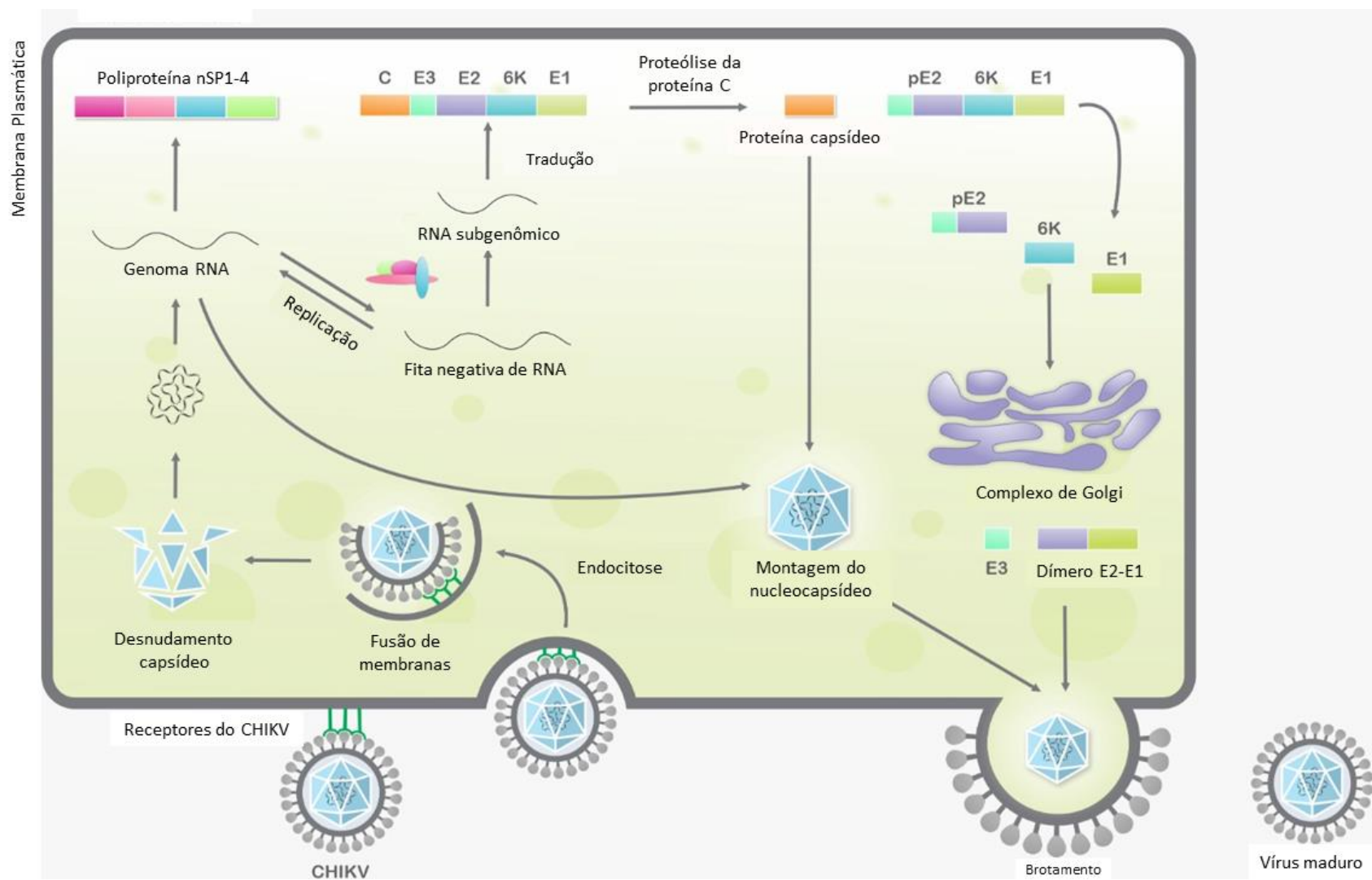
Dentre as proteínas estruturais, a proteína do capsídeo (C) é responsável por interagir com as novas fitas de RNA viral a partir de sua região N-terminal, e formar o nucleocapsídeo viral; as proteínas de envelope E1, E2, e E3 são glicosiladas no ER, sendo que o trímero E1-E2-E3 formam uma *spike*, responsável pelo reconhecimento e ligação a receptores celulares e fusão de membranas na entrada do vírus nas células hospedeiras (DEEBA et al., 2020; LAURENT et al., 2022; SILVA et al., 2014; SINGH et al., 2018). A proteína 6K é uma viroporina, responsável pela troca iônica e permeabilização da membrana, enquanto a proteína TF também possui atividade de canal iônico, porém sua deleção parece não afetar a replicação viral (LAURENT et al., 2022; MELTON et al., 2002; SINGH et al., 2018; TAYLOR et al., 2016) (**Figura 10**).

O ciclo replicativo do vírus ocorre em hospedeiros vertebrados e células de artrópodes, sendo assim classificada como uma arbovirose (BORGHERINI et al., 2007). A replicação viral se inicia pela adsorção do vírus à célula hospedeira, através da ligação da glicoproteína E2 do envelope viral com os glicosaminoglicanos e receptores proteína de domínio de imunoglobulina e mucina de célula T 1 (*T-cell immunoglobulin and mucin domain protein 1*, TIM-1), receptor de lectina tipo C (*C-type lectin receptor*, DC-SIGN) e, principalmente, limitrina (MXRA8) da membrana da célula hospedeira, promovendo a entrada do vírus por endocitose dependente de clatrina (BARTHOLOMEEUSEN et al., 2023; DE LIMA CAVALCANTI et al., 2022; SONG et al., 2019; ZHANG et al., 2018). Ao interagir com o receptor celular, o vírus é internalizado por um endossoma, onde por alteração do pH intraendossomal para um ambiente ácido são induzidas mudanças conformacionais que expõe a glicoproteína E1, e que inicia a fusão entre as membranas do endossomo e do envelope viral (BARTHOLOMEEUSEN et al., 2023; DE LIMA CAVALCANTI et al., 2022). Após a fusão das membranas, o capsídeo viral é liberado no citoplasma da célula infectada, com posterior liberação do RNA viral (AHOLA; MCINERNEY; MERITS, 2021) (**Figura 11**).

O CHIKV possui um RNA de polaridade positiva, em que inicialmente a ORF 5'UTR é rapidamente traduzida em uma poliproteína nsP1234, a qual é clivada pela atividade protease da nsP2 em nsP123 e nsP4 (AHOLA; MCINERNEY; MERITS, 2021; BARTHOLOMEEUSEN et al., 2023; HARDY; STRAUSS, 1989). Estas, formam um complexo conhecido como Complexo de Replicase Viral que é translocado para a membrana plasmática e formam esférulas de replicação, sendo a base das esférulas ocupadas pelo

complexo de replicase viral mediado pela nsP1 e nsP2 (JONES et al., 2021; LEMM et al., 1994). Nesse local, a nsP4 é responsável por sintetizar uma fita de RNA polaridade negativa, a partir do RNA de polaridade positiva, e produzir RNAs genômico (gRNA) e subgenômico (sgRNA), a partir da ORF 3'UTR (AHOLA; MCINERNEY; MERITS, 2021; BARTHOLOMEEUSEN et al., 2023; LEMM et al., 1994). Este sgRNA é expulso da esférula de replicação e é traduzido em uma poli proteína estrutural C-E3-E2-6K/TF-E, que será clivada por auto proteólise em proteína C do capsídeo (proteínas de automontagem), enquanto as proteínas do complexo E2-6K-E1 serão processadas por proteases tipo furina, glicosiladas no retículo endoplasmático e posteriormente inseridas na membrana da célula hospedeira (BARTHOLOMEEUSEN et al., 2023). Finalmente, os capsídeos se associam com o gRNA no citoplasma formando um nucleocapsídeo viral. O nucleocapsídeo interagem com a região C-terminal da E2 inserida na membrana celular, e coordena o brotamento dos vírus, levando a região da membrana celular com as proteínas E1 e E2 como envelope viral (ABDELNABI; NEYTS; DELANG, 2015; AHOLA; MCINERNEY; MERITS, 2021; BARTHOLOMEEUSEN et al., 2023; SOLIGNAT et al., 2009) (**Figura 11**).

Figura 11: Esquema representativo do ciclo replicativo do CHIKV



Fonte: Adaptado de MARTINS et al., 2020

2.4. Febre chikungunya e Patogênese

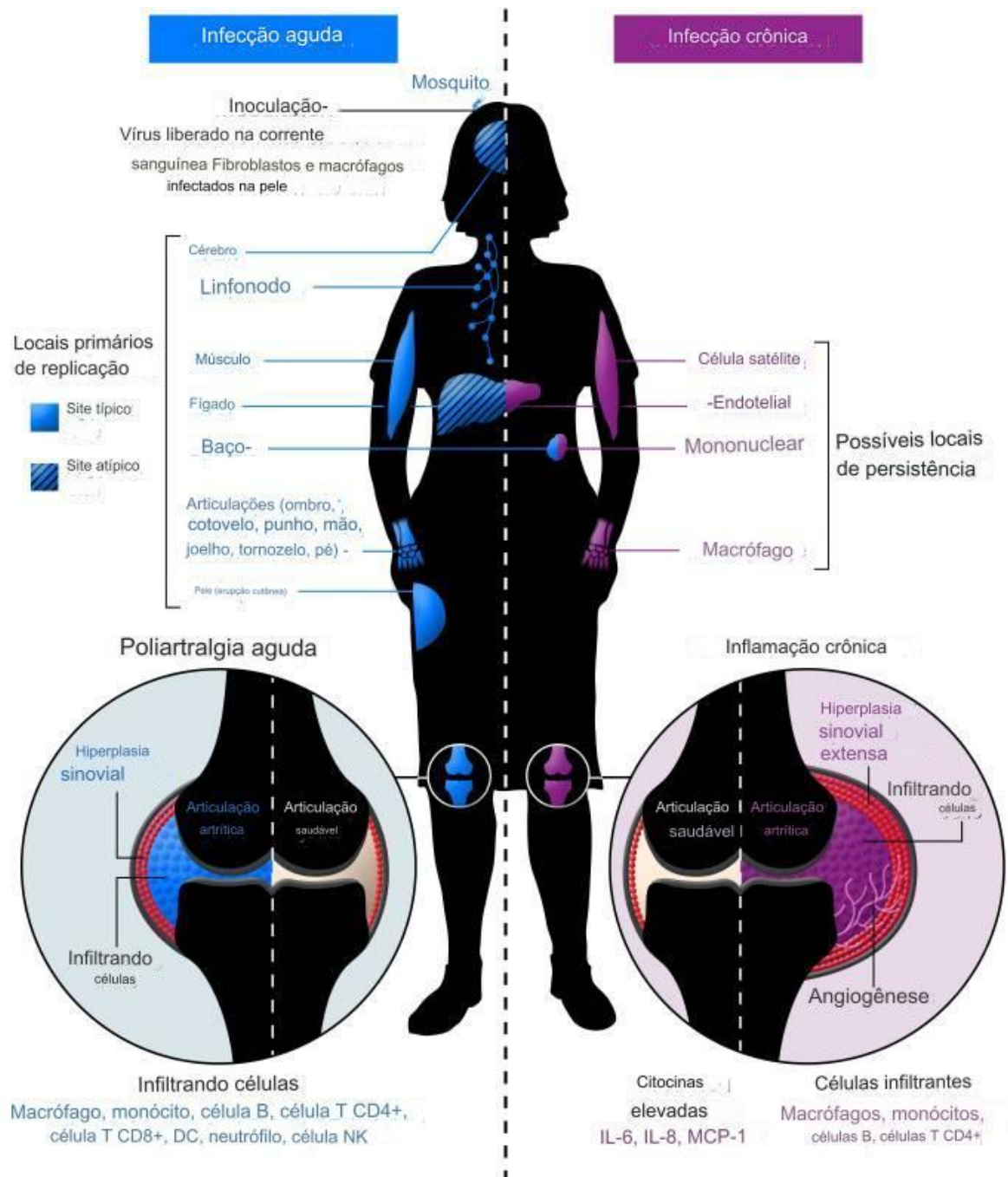
A febre chikungunya é uma doença viral com período de incubação ≤ 3 dias e fase aguda com duração entre 5 a 7 dias (WHO, 2019). Os sintomas gerais são muito semelhantes aos de outras arboviroses como a dengue e incluem febre, dores musculares, fadiga e manchas vermelhas na pele (MOIZÉIS et al., 2018). Entretanto, as fortes dores nas articulações são características da infecção pelo CHIKV (LEE et al., 2012). Em alguns casos raros são observadas encefalopatias, hepatites e miocardites, podendo também levar a óbito (DAS et al., 2010). Apesar de ser uma infecção majoritariamente aguda e ocasionalmente assintomática, cerca de 40% dos pacientes diagnosticados com CHIKV desenvolvem a infecção crônica, permanecendo de alguns meses até anos (ALESSANDRA LO PRESTI et al., 2014; HOARAU et al., 2010; PAIXÃO et al., 2018; PIALOUX G, GAÜZÈRE BA, JAURÉGUIBERRY S, 2007).

O CHIKV é capaz de infectar diferente tipos celulares, dentre eles células dendríticas, macrófagos, fibroblastos sinoviais, células endoteliais, miócitos e osteoblastos (DE LIMA CAVALCANTI et al., 2022; THON-HON et al., 2012). Após a picada por um mosquito infectado, o CHIKV é liberado na derme e na corrente sanguínea do hospedeiro vertebrado (BARTHOLOMEEUSEN et al., 2023; MOLONEY et al., 2014). O vírus se dissemina em linfonodos por meio de células migratórias infectadas e/ou como vírus livres dando início à fase aguda da doença (BARTHOLOMEEUSEN et al., 2023; WAGGONER et al., 2016). A replicação viral em células locais e em células no linfonodo propiciam níveis altos de viremia, que facilitam a disseminação viral, atingindo sítios de infecção secundários por meio da circulação linfática e sanguínea, como fígado, articulações e tecidos musculares (DUPUIS-MAGUIRAGA et al., 2012; WAGGONER et al., 2016). A associação de processos inflamatórios causados pela replicação viral persistente e a resposta imune do hospedeiro resultam em manifestações relacionadas à mialgia e poliartralgia em articulações distais, provocando fortes dores articulares e musculares (DA SILVA-JÚNIOR et al., 2017; DE LIMA CAVALCANTI et al., 2022; SILVA et al., 2017).

Após a eliminação da viremia, cerca de 8 dias após o início dos sintomas, alguns pacientes podem apresentar dores nas articulações de 21 dias a 3 meses, fase caracterizada como pós-aguda (SIMON et al., 2015). A permanência dos sintomas por mais de 3 meses indica o desenvolvimento da doença crônica (HOARAU et al., 2010; SIMON et al., 2015). Cerca de 40% dos indivíduos infectados podem progredir para a fase crônica (variando de acordo com a idade) (CHOPRA et al., 2012; HEATH et al., 2018; HOARAU et al., 2010). Atualmente,

sabe-se que pacientes com cargas virais altas, dores no corpo e fraqueza durante a fase aguda possuem maior risco de progredir para doença crônica, mas sem a detecção do vírus em sangue ou fluido sinovial (CHANG et al., 2018). Em muitos pacientes, a artralgia crônica é acompanhada de artrite reumatóide, e a presença de níveis elevados de IL-6 e IL-17 (BARTHOLOMEEUSEN et al., 2023; CHOPRA et al., 2012; MANIMUNDA et al., 2010) (Figura 12).

Figura 12: Representação gráfica da infecção e patogênese do CHIKV



Fonte: Adaptado de SILVA; DERMODY, (2017)

2.5. Resposta Imune ao CHIKV

A resposta imune contra a infecção pelo CHIKV é baseada inicialmente em uma resposta imune inata (WAUQUIER et al., 2011). No local de inoculação viral, as células infectadas identificam o RNA viral por meio de receptores de reconhecimento padrão (PPRs), como os receptores *toll-like* 3 e 7 (BARTHOLOMEEUSEN et al., 2023; DE LIMA CAVALCANTI et al., 2022; VAN DUIJL-RICHTER et al., 2015), e produzem quimiocinas, como a proteína quimiotática de monócitos (CCL2), que estimulam a migração e diferenciação de monócitos em macrófagos no local de replicação viral (BARTHOLOMEEUSEN et al., 2023). Apesar dos macrófagos residentes e infiltrados serem importantes para a produção de citocinas e para a eliminação da infecção, esses também são suscetíveis à infecção pelo CHIKV e podem funcionar como um reservatório em pacientes com infecção crônica, facilitando a disseminação do vírus (BARTHOLOMEEUSEN et al., 2023; DE LIMA CAVALCANTI et al., 2022).

A resposta imune adaptativa é baseada no reconhecimento das células T CD4⁺, as quais estão presentes na primeira semana de infecção (WAUQUIER et al., 2011). As células infectadas apresentam o antígeno viral por meio do complexo de histocompatibilidade humano (MHC) tipo II para os receptores de células T (BURT et al., 2017). Essas também migram para capsulas sinoviais em articulações, sendo responsáveis pela produção de interferon gama (IFN- γ) para controle da viremia (BARTHOLOMEEUSEN et al., 2023; DE LIMA CAVALCANTI et al., 2022). As células T CD4⁺ junto com as células T regulatórias (Treg) são responsáveis pela diferenciação da resposta em células Th1, essencial para a ativação de células B produtoras de imunoglobulinas anti-CHIKV (BARTHOLOMEEUSEN et al., 2023; DE LIMA CAVALCANTI et al., 2022). Em alguns indivíduos pode haver a produção de citocinas em ambas as vias Th1 e Th2, possivelmente comprometendo a eliminação viral e recuperação clínica (FOX; DIAMOND, 2016). Além disso, as células T CD4⁺ participam ativamente no recrutamento e ativação de células T CD8⁺ citotóxicas nos períodos iniciais da infecção (BARTHOLOMEEUSEN et al., 2023; DE LIMA CAVALCANTI et al., 2022; WAUQUIER et al., 2011).

Em relação às células B, essas são ativadas pelas células T CD4⁺ e estimuladas a produzir imunoglobulinas para neutralização viral (FOX; DIAMOND, 2016). Os anticorpos do tipo IgM são detectáveis no início da fase aguda, e após alguns dias ocorre o desenvolvimento de anticorpos do tipo IgG, permanecendo após o fim da fase aguda. Nos casos de cronificação,

os níveis de IgG permanecem altos durante toda a fase crônica (FOX; DIAMOND, 2016). Isso demonstra que os níveis de anticorpos são necessários para o controle, mas não são capazes de eliminar a infecção (BARTHOLOMEEUSEN et al., 2023; FOX; DIAMOND, 2016).

2.6. Profilaxia e Tratamento

Atualmente, não existe nenhuma vacina aprovada e licenciada para profilaxia da população contra a febre Chikungunya (BARTHOLOMEEUSEN et al., 2023). Entretanto, existem 4 vacinas que estão em fase três de desenvolvimento: VLA1553 (ROQUES et al., 2022), PXVX0317 (BENNETT et al., 2022), V184 (REISINGER et al., 2018) e BBV87 (CEPI, 2020), sendo que a VLA1553 já recebeu licença para aplicação em 2022 pelo FDA nos Estados Unidos da América (BARTHOLOMEEUSEN et al., 2023; ROQUES et al., 2022).

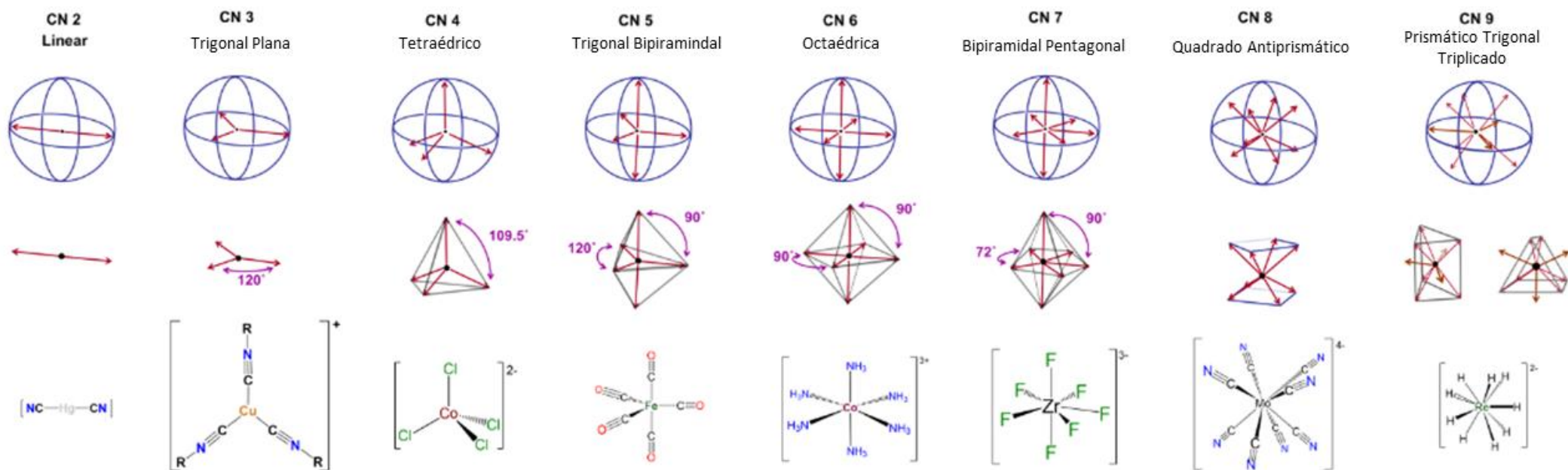
A inexistência de antivirais para uso clínico contra o CHIKV resulta no uso de tratamentos paliativos para mitigação dos sintomas, baseando-se na administração de analgésicos, antitérmicos, repouso e hidratação (BARTHOLOMEEUSEN et al., 2023; DE LIMA CAVALCANTI et al., 2022). Nesse contexto, a identificação de moléculas capazes de inibir a replicação do CHIKV e diminuir os efeitos da doença em humanos são de extrema importância. Por isso, os compostos de coordenação se apresentam como uma fonte de informações para o desenvolvimento de tratamentos futuros contra a febre chikungunya, o que poderá resultar na melhora da qualidade de vida da população infectada, e redução dos gastos públicos devido aos tratamentos paliativos de longo prazo e a incapacitação dos indivíduos infectados.

3. Compostos de Coordenação

3.1. Histórico e Desenvolvimento

A Química Inorgânica Medicinal, dentre as suas áreas, possui a estratégia terapêutica de compostos de coordenação, os quais são moléculas orgânicas e/ou inorgânicas que possuem um íon metálico ligado à sua estrutura (**Figura 13**) (KARGES; STOKES; COHEN, 2021; VON ZELEWSKY, 1999). A coordenação metálica é conhecida desde século 18-19, porém apresentava uma química controversa e complicada devido ao fato que a descrição de estado de oxidação e número de coordenação eram descritos como iguais, diferentemente dos dias de hoje (CONSTABLE; HOUSECROFT, 2013; ERNST et al., 2011). Em 1913, Alfred Werner ganhou o prêmio Nobel de Química por postular que os íons metálicos possuem dois estados de valência: estado de oxidação, correspondendo à carga positiva do íon metálico, e número de coordenação que seria a quantidade total de ligações entre ligante-metal no íon metálico. Atualmente, existem mais de nove números de coordenação que variam entre os metais e seus números de oxidação, sendo algum desses números de coordenação descritos na **Figura 13** (CONSTABLE; HOUSECROFT, 2013; ERNST et al., 2011; KARGES; STOKES; COHEN, 2021).

Figura 13: Esquema das geometrias de coordenação de acordo com alguns dos números de coordenação



Fonte: Adaptado de Hass et al. (2023)

Desde sua caracterização e descrição, esses compostos adquiriram um papel importante em diversas áreas industriais, devidos suas aplicações para bioimagem, *drug delivery*, sensores químicos, farmacologia e química medicinal (FAYYAZ et al., 2021; KARGES; STOKES; COHEN, 2021). Os novos métodos de ligação e os novos conceitos proporcionaram avanços nos últimos anos e a produção de diversas moléculas e variadas estruturas químicas que impactaram e se tornaram essenciais em processos celulares e moleculares (DUCKETT; MURCHIE; LILLEY, 1990; KARGES; STOKES; COHEN, 2021; LIPPARD, 1993). Isso é devido ao fato que alguns desses já possuem vias metabólicas definidas e, portanto, podem apresentar menos efeitos adversos (ALVAREZ et al., 2018).

Os complexos metálicos possuem características químicas e estruturais inatingíveis apenas pelo uso de *motifs* orgânicos e por isso representam uma opção promissora e versátil para o desenvolvimento de medicamentos (FAYYAZ et al., 2021; KARGES; STOKES; COHEN, 2021). Adicionalmente, os resultados terapêuticos podem ser ajustados pela variação da natureza do metal e seu número de oxidação, características do ligante e a estereoquímica das espécies formadas (BARRY; SADLER, 2013; SANTINI et al., 2014). Por meio da seleção de ligantes, natureza de íons metálicos e seus números de oxidação, é possível desenvolver compostos com alta especificidade e efetividade, focando de forma multidimensional as doenças virais (BARRY; SADLER, 2013; SANTINI et al., 2014). Por isso, o campo da Química Inorgânica Medicinal tem sido próspero e contribuído para a concepção de complexos metálicos englobando íons como platina [Pt(II)], paládio [Pd(II)] e prata [Ag(I)], dentre outros, que, quando combinados com classes específicas de ligantes, são capazes de abordar a multidimensionalidade de doenças humanas, como o câncer (DYSON, 2007; JUNG; LIPPARD, 2007; SANTIAGO et al., 2020), Alzheimer (GOMES; BATAGLIOLI; STORR, 2020) e aquelas de origem infecciosa causadas por vírus (DOS SANTOS OLIVEIRA et al., 2023; ESQUEZARO et al., 2021; FONTES et al., 2022; OLIVEIRA et al., 2020; SANTOS et al., 2022), bactérias (incluindo gênero *Mycobacteria*) (BERGAMINI et al., 2012; DOS SANTOS PEREIRA et al., 2019; PARIS JUNIOR et al., 2020) e protozoários (FERNANDES et al., 2020a).

Os íons metálicos, quando complexados a ligantes específicos, podem gerar compostos estáveis, biologicamente ativos e não citotóxicos (CAVICCHIOLI et al., 2019; DOS SANTOS PEREIRA et al., 2019; LIU et al., 2015; MANZANO et al., 2018). Esta abordagem originou-se da descoberta da atividade anticancerígena do cisplatina, ou cis-diaminodicloridoplatina(II), há cerca de 50 anos (JOYCE et al., 2010; LI et al., 2019; MARTINHO et al., 2019). Desde

então, inúmeros complexos metálicos de Pt(II), Au(I), Ag(II) e Cu(I), dentre outros, foram sintetizados e avaliados quanto ao seu potencial efeito anticancerígeno, bem como para outras doenças (JOYCE et al., 2010; LI et al., 2019). A carboplatina, por exemplo, é um medicamento de segunda geração à base de platina que tem sido utilizado mundialmente no tratamento do câncer (JANG et al., 2020; SANTINI et al., 2014; WANG et al., 2020).

3.2 Atividades antivirais dos Compostos Metálicos

A Auranofina é um exemplo de composto de coordenação de ouro(I), já aprovado para uso como fármaco (neste caso, metalofármaco), que apresentou resultados promissores contra o vírus da ZIKA (ZIKV), o CHIKV e o VEEV (LANGSJOEN et al., 2017). Adicionalmente, a Sulfadiazina de Prata, um composto de coordenação de Ag(I) utilizado como fármaco antibacteriano (FOX, 1968), também apresentou efeito antiviral contra diversas cepas isoladas de *Herpesvirus hominis* (CHANG; WEINSTEIN, 1975). Alternativamente, os complexos metálicos podem ser direcionados para um alvo específico, como por exemplo o HIV, os quais têm sido investigados exaustivamente a fim de identificar potenciais metalofármacos. Atualmente, existem descritos pelo menos 35 compostos de coordenação com atividade antiviral contra o HIV em fase pré-clínicas e clínica (DE PAIVA et al., 2020a).

No entanto, o potencial terapêutico dos complexos metálicos como antivirais permanece pouco explorado. Portanto, se apresenta como uma fonte inovadora para o desenvolvimento de compostos capazes de combater vírus emergentes, podendo então revolucionar o desenvolvimento de medicamentos antivirais e preparar a saúde pública contra futuros surtos virais.

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OBJETIVOS

O objetivo desse trabalho foi identificar a atividade antiviral de complexos metálicos no ciclo replicativo dos SARS-CoV-2 e CHIKV.

Objetivos específicos

- 1) Avaliar a atividade antiviral de compostos coordenados com os íons Pt(II), Pd(II), Au(I), Cu(I) (complexos metálicos) na infecção pelo SARS-CoV-2 e CHIKV em cultura de células;
- 2) Analisar o efeito estrutura-atividade biológica de cada complexo metálico, comparado com seus ligantes livres;
- 3) Avaliar a atividade antiviral dos compostos contra as principais VOCs do SARS-CoV-2: B.1.617.2 (Delta) e BA.2 (Ômicron);
- 4) Determinar a concentração efetiva de 50% (EC₅₀), concentração citotóxica de 50% (CC₅₀) e índice de seletividade (SI) dos complexos ativos;
- 5) Investigar os efeitos dos complexos ativos nas diferentes etapas do ciclo replicativo do SARS-CoV-2 e do CHIKV, como os estágios anteriores a infecção viral (atividade protetora), os de entrada do vírus na célula, e estágios de replicação e pós-entrada dos vírus nas células.
- 6) Avaliar os modos de ação dos compostos ativos utilizando ensaios biofísicos *in vitro* de interação com proteínas virais estruturais e não estruturais, bem como interações com RNA de fita dupla (intermediário de replicação) do SARS-CoV-2 e do CHIKV, e ancoragem molecular para descrição de possíveis interações.
- 7) Avaliar o potencial amplo-espectro antiviral dos complexos avaliados.

**CAPÍTULO 2: NEW Pd(II)
AMINOADAMANTINE COMPLEXES
STRONGLY IMPAIRS SARS-CoV-2
INFECTION**

NEW Pd(II) AMINOADAMANTINE COMPLEXES STRONGLY IMPAIRS SARS-CoV-2 INFECTION

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ABSTRACT

The geographic advancement of the SARS-CoV-2 infection and its disease COVID-19, the high genetic variability causing new viral variants, and the increase in post-COVID syndrome instigate the discovery of new molecules that can abrogate viral infection. In this context, the aminoadamantanes amantadine (atd), memantine (mtn), and rimantadine (rtd) are commercial drugs used in the clinical treatment of Alzheimer's and influenza A diseases, possessing structures and toxicity profile previously described, and therefore are attractive candidates for metallic coordination, to increase the activity of these coordinated compounds as antivirals. Here, the metal ion palladium(II) [Pd(II)], the drugs atd, rtd, and mtn, and their coordinated derivatives Pd-atd, Pd-mtn, and Pd-rtd were screened against SARS-CoV-2 infection. With the exception of rtd, atd, mtn and Pd(II) did not inhibit SARS-CoV-2 replicative cycle, while their complexes Pd-atd, Pd-mtn, and Pd-rtd had an outstanding antiviral activity with selectivity indexes of 14.2, 36.2, and 23.5. Further assays were conducted, and all Pd(II) complexes strongly inhibited SARS-CoV-2 entry in over 75%. Additionally, these metal complexes were able to inhibit Omicron (B.2) variant, with Pd-rtd being also capable of inhibiting cell to cell transmission. Interestingly, Pd-atd significantly inhibited all stages of replicative cycle investigated here, showing multiple effects on virus infection. Furthermore, the coordination with Pd(II) resulted in more active and less capable of inducing resistance compounds. The obtained results highlight the synthesis of promising Pd(II) metallodrugs with potential application for the treatment of SARS-CoV-2 infections.

Keywords: Aminoadamantanes, Antiviral, Pd(II) complexes, SARS-CoV-2

INTRODUCTION

In late 2019, the emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the global pandemic known as Coronavirus Disease 2019 (COVID-19), unleashed a worldwide crisis (GRASSELLI et al., 2020a; WU et al., 2020; ZHANG; WU; ZHANG, 2020; ZHOU et al., 2020b).

The SARS-CoV-2 belongs to the *Coronaviridae* family and possesses a single-stranded positive-sense RNA associated with a protein nucleocapsid (N), surrounded by an envelope derived from the host cell membrane and the membrane protein (M), inserted of the glycoprotein E and the protein S (Spike) (ANDERSEN et al., 2020; KIM et al., 2020). The virus RNA genome is susceptible to high genetic variability, which contributed to the global emergence of variants in a short time, such as the Variants of Concern (VOCs) Alpha, Beta, Gamma, Delta, and Omicron (DUFFY, 2018; JOHNSON et al., 2022; MONTAGUTELLI et al., 2021; THAKUR; RATHO, 2021; WANG et al., 2021b).

As of November 17th, 2023, it has over 771 million confirmed cases and a staggering 6.9 million COVID-19-related fatalities reported globally, being in 37.7 million cases and of 704,000 deaths reported in Brazil (W.H.O., 2023c). The clinical spectrum of symptomatic COVID-19 cases encompasses a wide range, from mild presentations resembling common cold symptoms like fever, cough, and dyspnea, to more moderate and severe conditions that affect both the upper and lower respiratory tracts, including pneumonia (GRASSELLI et al., 2020b; KHANMOHAMMADI; REZAEI, 2021; REMY et al., 2020; STEIN, 2020; XU et al., 2020). The severe manifestation of this disease is the severe acute respiratory syndrome (SARS), resulting in the most critical cases that can ultimately lead to mortality (TROUGAKOS et al., 2021a; V'KOVSKI et al., 2021a).

In late 2020 and early 2021, regulatory agencies approved the emergency use of vaccines for prophylaxis (COLLIER et al., 2021; GARCIA-BELTRAN et al., 2021; SINGH; CHATTU, 2021; W.H.O., 2023c). Since then, a decrease in the number of deaths and severe diseases was observed. Despite high coverage worldwide (58%), vaccination still faces problems of equality in distribution and population resistance, due to the anti-vaccination movement and misinformation (DE SOUZA et al., 2021; SINGH; CHATTU, 2021). Aggravatingly, patients infected with SARS-CoV-2 can develop the post-COVID syndrome, in which infected individuals, even in mild cases, present long-term symptoms (up to 1 year after infection), such as loss of up to 30% of lung capacity (XU et al., 2020), memory loss and concentration problems, cardiovascular diseases, depression and anxiety, hair loss, and

worsening of pre-existing illness (MALTEZOU; PAVLI; TSAKRIS, 2021b, 2021a; ORONSKY et al., 2021; YONG, 2021).

The drugs Remdesivir, Molnupiravir, and Paxlovid® were approved by WHO/FDA/ANVISA to be used in severe COVID-19 cases (BEIGEL et al., 2020; FISCHER et al., 2022; JAYK BERNAL et al., 2022a; SAÚDE., 2022). However, those therapeutic options were not designed to directly address the viral replication and are prescribed only for moderate and severe cases of COVID-19, which might interfere with the efficacy in controlling the current pandemic (MARTINEZ, 2022). Consequently, the treatment of COVID-19 has been mainly based on palliative drugs and/or biological products to alleviate symptoms of patients, which can result in complications as the post-COVID disease, causing an overload of health systems and high costs to the worldwide economy, and threatening the quality of life of populations worldwide (BIERWIAZONEK; GUNDERSEN; KUNST, 2022; JEON et al., 2022; XAVIER et al., 2022). Therefore, it is essential to develop a variety of antiviral molecules that can abrogate SARS-CoV-2 infection and can be also used in combined therapies, as the current therapy against other RNA viruses, such as the human immunodeficiency virus (HIV) and hepatitis C virus (HCV) (CERRONE et al., 2020; JANG et al., 2020).

In this context, coordinated compounds presents represent a new therapeutic strategy, given their potential to act in several stages of the viral replication process (VON ZELEWSKY, 1999). Metal complexes have chemical and structural characteristics unachievable only using organic motifs, and therefore represent a promising and versatile option for drug development (DE PAIVA et al., 2020b; KARGES; STOKES; COHEN, 2021). When combined with specific classes of ligands, metal ions are able to address the multidimensionality of diseases such as cancer (DYSON, 2007; JUNG; LIPPARD, 2007; SANTIAGO et al., 2020), Alzheimer's (GOMES; BATAGLIOLI; STORR, 2020), bacteria (BERGAMINI et al., 2012; DOS SANTOS PEREIRA et al., 2019), and protozoa (FERNANDES et al., 2020b) infections. This class of compounds has been poorly explored against viruses, however, few studies demonstrated that coordination resulted in improved activity of compounds, or even making them active (IKOTUN et al., 2010; SODHI; PAUL, 2019). In addition, metal complexes have been reported to possess activity against HIV (DE PAIVA et al., 2020a), dengue virus (DENV) (SAINI et al., 2016), herpes simplex virus (HSV) (EPSTEIN et al., 2006), influenza virus H1N1 (KIRIN et al., 2016), and of particular interest SARS-CoV-2 (CHUONG et al., 2021).

The aminoadamantanes amantadine (atd), rimantadine (rtd), and memantine (mtn) are FDA-approved molecules to treat Alzheimer's disease and influenza virus A (IAV) infection

in humans (ALVES GALVÃO; ROCHA CRISPINO SANTOS; ALVES DA CUNHA, 2014; BANTIA et al., 2010; INTHARATHEP et al., 2008a; JEFFERSON et al., 2002; KOOIJMANS et al., 2012). The adamantane structure, characterized by a singular diamond-like carbon cage, undergoes a transformative evolution with the introduction of an amine group to produce the amantadine, or memantine when associated with the dimethyl group, or rimantadine from the fusion of adamantane with an ethanamine group, exemplifying the versatility and potential of these structural modifications (GARCIA et al., 2010; PEREIRA et al., 2020). Zhou and colleagues described the activity of atd, rtd, and mtn against SARS-CoV-2 infection with low selectivity index (ZHOU et al., 2021), which could be attractive for metal coordination, creating new molecules with enhanced activity and lower cytotoxicity, as was previously reported by us for chikungunya virus (CHIKV) replication (SANTOS et al., 2022). To this, the transition metal palladium(II) [Pd(II)] emerge as interesting ion to be coordinated to the aminoadamantanes. The Pd(II) is a soft Lewis acid forming a strong bonds with nitrogen and sulfur donors, and undergo through ligand exchange faster than other metals such as Pt(II), which make them less cytotoxic (BUTOIR et al., 1997). Considering the chemical characteristics of this metal, here were evaluated new species of Pd(II) coordinated with the aminoadamantanes against the SARS-CoV-2 as a new therapeutic approach in drug discovery.

METHODS

CELL CULTURE AND COMPOUNDS

The adenocarcinoma human alveolar basal epithelial cells expressing the receptors ACE2 and TRPMSS2 (A549-ACE2-TRPMSS2 or A549-AT, ATCC#CCL-185) were cultivated as previously described (DOS SANTOS OLIVEIRA et al., 2023)(DOS SANTOS OLIVEIRA et al., 2023), using Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 100 U/mL penicillin (Gibco Life Technologies), 100 mg/mL streptomycin (Gibco Life Technologies), 1% (v/v) non-essential amino acids (Gibco Life Technologies) and 10% (v/v) fetal bovine serum (FBS; Hyclone) at 37 °C in a humidified 5% CO₂ incubator. The cells were cultivated in the presence of Geneticin (G418) and Hygromycin B at 1 mg/mL and 200 µg/mL, respectively.

The compounds amantadine (atd), memantine (mtn), rimantadine (rtd), and Li₂PdCl₄ (**Figure 1**) were purchased from Sigma-Aldrich Laboratories. The complexes Pd-atd, Pd-mtn, and Pd-rtd (**Figure 1**) were prepared by the reaction of Li₂PdCl₄ and aminoadamantanes under stirring for 2 hours and at room temperature. The pH was adjusted with a solution of KOH. The yellowish solid obtained was collected by filtration, washed with methanol, and dried. The

metal:ligand molar composition was confirmed by elemental analysis. Mass spectrometric data permitted to confirm the composition of the complexes with Pd(II). The complexes are soluble in dimethylsulfoxide. Thermal analysis confirmed the proposed composition of the complexes. Infrared spectroscopy and nuclear magnetic resonance data confirmed coordination of ligands to Pd(II). All the compounds were dissolved in dimethyl sulfoxide (DMSO 99.8%) prior to the assays, and immediately diluted in culture media to the antiviral assays to a final concentration of 0.1%.

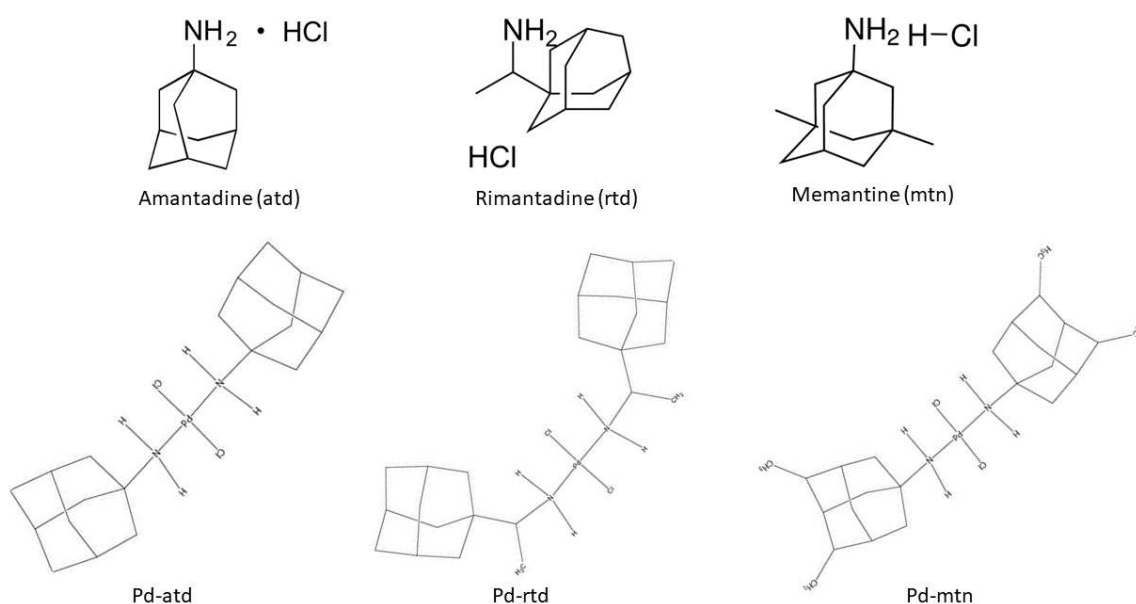


Figure 1. Chemical structure of atd, rtd, mtn and their Pd(II)-complexes Pd-atd, Pd-rtd, Pd-mtn.

VIRUSES

The viruses were isolated and characterized by The Francis Crick Institute, and kindly provided by Prof. Stephen Griffin from the University of Leeds, being those Wuhan-like (hCov-119/England/02/2020), B.1.617.2 - Delta (MS066352H), and B.2 - Omicron (hCoV/England/FCI-179/2022). The viruses were amplified by infecting Vero E6-ACE2 cells in 175 cm² flask until 80% of cytopathic effect. Supernatant was collected and the infectious titre was determined using the 50% tissue culture infectious dose (TCID₅₀) method, calculated by the Spearman & Kärber algorithm as described by Killington and Hierholzer (KILLINGTON; HIERHOLZER, 1996)(KILLINGTON; HIERHOLZER, 1996).

RESCUE OF SARS-CoV-2 REPORTER VIRUS

The rescue of SARS-CoV-2 reporter virus based on the NCBI sequence NC_045512.2 was performed as previously described (DOS SANTOS OLIVEIRA et al., 2023), by transfecting a total of 1 µg of the infectious clone pCCI-4K-SARS-CoV-2-mNeonGreen under the CMV promoter (RIHN et al., 2021) (**Figure 2A**) into BHK-21 (3×10^5 cells/ well in a 6-well plate) using the lipofectamine 2000 (1:1). After 3 days, the supernatant was collected and transferred to A549-AT cells in a 75 cm² flask. The A549AT-supernatant was harvested after 3 days as P0 stock. The infectious titer was determined using the 50% tissue culture infectious dose (TCID₅₀) method and calculated by the Spearman & Kärber algorithm as described by Killington and Hierholzer (KILLINGTON; HIERHOLZER, 1996).

CYTOTOXICITY SCREENING AND INITIAL ANTIVIRAL ACTIVITY

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich) assay as previously described (DOS SANTOS OLIVEIRA et al., 2023). A549-AT cells were cultured in 96-well plate at 1×10^4 cells/well and treated with atd, mtn, rtd, Pd(II), Pd-atd, Pd-mtn, and Pd-rtd at 50, 10, and 2 µM for 24h at 37°C with 5% of CO₂. After treatment, media containing compound was removed from cells and MTT at 1 mg/mL solution was added to each well, incubated for 30 minutes and replaced with 100 µL of DMSO to solubilize the formazan crystals. The absorbance was measured at 570 nm on FLUOstar OPTIMA microplate reader (BMGLabTech). Cell viability was calculated according to the equation $(T/C) \times 100\%$, which T and C represented the optical density of the treated well and control groups, respectively. DMSO 0.1% was used as untreated control. The highest non-cytotoxic concentration with cell viability $\geq 80\%$ of each compound was selected to antiviral screening.

For the antiviral assays, A549-AT cells were seeded at density of 1×10^4 cells per well into 96-well plates 24 h prior to the infection. SARS-CoV-2-mNeongreen at a multiplicity of infection (MOI) of 0.1 in the presence of each compound at the highest non-cytotoxic concentration was simultaneously added to cells. After 24h, the plates were placed on the IncuCyte® S3 Live-Cell Analysis System (Sartorius) and the green fluorescence light was observed at 10x objective. The photos were analysed employing the basic analyser from the IncuCyte S3 system and the total integrated intensity of the fluorescence (GCU x µm²/well) was collected. Viral replication was calculated according to the equation $(T/C) \times 100\%$, which T and C represented the optical density of the treated well and control groups, respectively. DMSO 0.1% was used as untreated control.

DOSE-RESPONSE ASSAY

To evaluate the cytotoxicity and activity profile of Pd-atd, Pd-mtn, and Pd-rtd on cell viability and viral replication, A549-AT cells were seeded at a density of 1×10^4 cells per well into 96-well plates 24 h prior to the infection. Then, cells were treated with each compound in a two-fold serial dilution with concentrations ranging between 0.58 $\mu\text{g/mL}$ to 300 $\mu\text{g/mL}$ in the presence or absence of SARS-CoV-2-mNeonGreen in a MOI of 0.1 (RIHN et al., 2021). Cell viability assays and viral inhibition were analysed following the methods above. DMSO was used as untreated control. The effective concentration of 50% (EC_{50}) and cytotoxic concentration of 50% (CC_{50}) were calculate employing a non-linear regression. Selective Index (SI) was calculated using the equation $\text{SI} = (\text{CC}_{50}/\text{EC}_{50})$.

TIME OF DRUG ADDITION ASSAYS

For all of time of drug addition assays, A549-AT cells at the density of 1×10^4 cells per well were seeded in 96 well plates 24h before infection. Treatment was performed with Pd-atd, Pd-mtn, or Pd-rtd at 50 μM , and virus replication was assessed by the green fluorescence light in 10x objective. The photos were analyzed employing the basic analyzer from the IncuCyte S3 system by the collection of total integrated intensity of the fluorescence ($\text{GCU} \times \mu\text{m}^2/\text{well}$).

In pretreatment assay, cells were treated for 1h with the compound prior to the viral infection, extensively washed with PBS and added of SARS-CoV-2-mNeonGreen (MOI of 0.1) for 1h. Then, cells were washed with PBS and incubated to remove unbound virus and added of fresh medium for 24h (**Figure 3A**).

In entry inhibition assay, cells were infected using media containing compound and virus (MOI of 1) for 1h, washed with PBS to remove the inoculum, and incubated with fresh medium for 24h (**Figure 4A**). The virucidal activity was assessed using the same protocol, except that the inoculum containing compound and virus (MOI of 5) was incubated for 1h before it was added to the cells (**Figure 4C**). On the other hand, the impact of the compounds on attachment step was analyzed using the same setting as in entry inhibition assay, except cells were incubated with virus (MOI of 1) and compound at 4°C (**Figure 4E**). A variation of this assay was that the incubation at 4°C was followed by incubation for 30 min at 37 °C, used to analyze the effect of compound on post-attachment steps of infection (**Figure 4G**).

In post-entry assay, cells were infected with SARS-CoV-2-mNeonGreen (MOI of 0.1) for 1h, washed extensively with PBS, and the incubated in compound-containing media for 24h (**Figure 5A**). Additionally, a variation of this assay was performed by infecting cells with

SARS-CoV-2-mNeonGreen (MOI of 0.1) for 6h and then incubating with the compound for the remaining time to assess the effect after establishment of viral replication (**Figure 5B**).

BROAD-SPECTRUM ASSAYS USING SARS-COV-2 VARIANTS

To assess the effect the metal compounds against Delta and Omicron variants, the SARS-CoV-2 infectious clone, based on Wuhan strain carrying the MCherry reporter gene (**Figure 6A-1**), was modified by molecular cloning inserting the sequence of the spike gene derivative from the variants B.1.617.2 (delta) or B.2 (omicron) (**Figure 6A, 2 and 3**). The rescue of the virus was carried out as described above. For the antiviral screening, Pd-atd, Pd-mtn, or Pd-rtd at 50 μ M was added to media containing the Wuhan-B.1.617.2-Spike or Wuhan-B.2-Spike viruses at an MOI of 0.1, and the inoculum was added to the cells for 24h. The total integrated intensity of the fluorescence (RCU x μ m²/well) was analyzed using the IncuCyte S3 microscope.

For the antiviral assays employing the wild-type viruses B.1.617.2 - Delta (MS066352H) and B.2 – Omicron (hCoV/England/FCI-179/2022), A549-AT cells were plated at concentration of 8x10⁵ cells in 12 well plates, and infected with each virus at a MOI of 0.1 in the presence or absence of Pd-atd, Pd-mtn, or Pd-rtd at 50 μ M for 24h. Supernatant was collected and the infectious titer was determined using the 50% tissue culture infectious dose (TCID₅₀) method and calculated by the Spearman & Kärber algorithm as described by Killington and Hierholzer (KILLINGTON; HIERHOLZER, 1996).

dsRNA INTERACTION ASSAY

To investigate the potential to interact with dsRNA, a migration retardation assay based on previously described works was performed (CAMPOS et al., 2017; KRAWCZYK et al., 2009; SANTOS et al., 2022). Through PCR, the SARS-CoV-2 ORF1a was amplified employing primers flanked by a T7 promoter site (forward: TAATACGACTCACTATAGGGGACCGAAAGGTAAGATGGAG; reverse: TAATACGACTCACTATAGGGAAATCGCCCGTCTGCCATGAAG; T7 promoter regions are underlined). The reaction product of 499 bp was purified by Monarch® PCR & DNA Cleanup Kit (New England BioLabs®) and used for in vitro transcription by the HiScribe™ T7 High Yield RNA Synthesis Kit (New England BioLabs®). The dsRNA molecule was obtained by complementary annealing in annealing buffer (Potassium acetate 2M, Hepes-KOH 1M, and Magnesium acetate 1M) at 95°C for 1 min. The SARS-CoV-2 dsRNA was quantified

in Nanodrop (ThermoScientific) and incubated at 65 nM with each Pd(II) complex at 50 μ M for 45 min and analyzed in 1% agarose 1X TAE gel stained with SYBRTM Safe DNA Gel Stain (Invitrogen). The lack or decrease of band intensity in the agarose gel suggests the compound interaction activity since it competes with SYBR. *cis*-[PtCl₂(dmsO)₂] (Pt, 10 μ M) was used as a positive control of interaction (SANTOS et al., 2022). The band quantification was performed using ImageJ.JS version 1.53j.

DISSEMINATION ASSAY

To evaluate the effect of the compound on focus formation and cell to cell spread, 5x10⁴ cells/well were plated in 48 well plates for 24h. Cells were infected with SARS-CoV-2-mNeongreen at an MOI of 0.05 for 1h, washed with PBS to remove the unbound viruses, and added of media containing methylcellulose 0.8% and Pd-atd, Pd-mtn, or Pd-rtd at 50 μ M. After 48h, the wells were photographed using the IncuCyte S3 microscope. The quantity of green focus and its size were analysed in each image using ImageJ software.

IN VITRO SELECTION OF SARS-CoV-2-RESISTANT VIRUS

Continual passages of SARS-CoV-2 (hCov-119/England/02/2020) were performed in the presence of Pd-atd, Pd-mtn or Pd-rtd at 50 μ M in A549-AT cells. Assays were undertaken in a 1x10⁶ cells/well in a 6 well plate at an MOI of 0.05, in one replicate, and controls for cell culture adaption of the virus including DMSO were undertaken in parallel (**Figure 9**). These concentrations were kept constant throughout the experiment. For each passage, supernatant was harvested 48h after infection, tittered by TCID₅₀, stored in -80°C, and used to infect the next passage maintaining virus MOI and the concentration of each compound.

STATISTICAL ANALYSIS

Antiviral assays were performed a minimum of three technical repeats in quadruplicate to confirm the reproducibility of the results. Differences between means of readings were compared using analysis of variance (One-way or Two-way ANOVA) and Student *t*-test. *p* values of <0.05 (indicated by asterisks) were considered statistically significant. For the establishment of EC₅₀ and CC₅₀ values, the data were transformed into Log(X), where X is the concentration, and submitted into a non-linear regression with four parameters in variable slope. All analyses were performed using GraphPad Prism 9.

RESULTS

The coordination with Pd(II) result in active molecules against SARS-CoV-2

Aiming to assess the cytotoxicity, A549-AT cells were treated with each compound at concentrations of 50, 10, and 2 μM , being the DMSO used as untreated control. Cell viability was assessed by MTT assay 24h later. As result, the precursor Li_2PdCl_4 was viable at 50 μM (92.5%), while their ligands amantadine (atd), rimantadine (rtd), and memantine (mtn) were viable at 10 μM (84.9%), 50 μM (93.8%), and 50 μM (84.5%), respectively. The coordinated compounds Pd-atd was viable at 2 μM (83.4%), while Pd-mtn and Pd-rtd were viable at 10 μM (83.7% and 93.2%, respectively). The specific values of cell viability for each compound in the concentrations are shown in **Supplementary Figure 1**. Then, the highest non-cytotoxic concentration was selected to analyze the antiviral activity on the SARS-CoV-2 replication cycle. To this, the SARS-CoV-2 carrying the Neongreen fluorescence gene reporter (SARS-CoV-2-mNeongreen) (RIHN et al., 2021) (**Figure 2A**), which the fluorescence intensity is proportional to the virus replication *in vitro*, was used to infect A549-AT cells at a multiplicity of infection (MOI) of 0.1, in the presence or absence of each compound. After 24h, the total integrated intensity of the fluorescence ($\text{GCU} \times \mu\text{m}^2/\text{well}$) was analyzed using the IncuCyte S3 microscope. As a result, the precursor Li_2PdCl_4 (50 μM) and the ligands atd (10 μM) and mtn (50 μM) did not inhibit viral replication (**Figure 2B**). Alternatively, rtd (50 μM) inhibited 60.1% ($p < 0.0001$) (**Figure 2B**). On the other hand, the coordinated compounds Pd-atd, Pd-mtn, and Pd-rtd inhibited the SARS-CoV-2 replication in 50.8% (2 μM), 97.2% (10 μM), and 71.1% (10 μM) ($p < 0.0001$), respectively (**Figure 2B**).

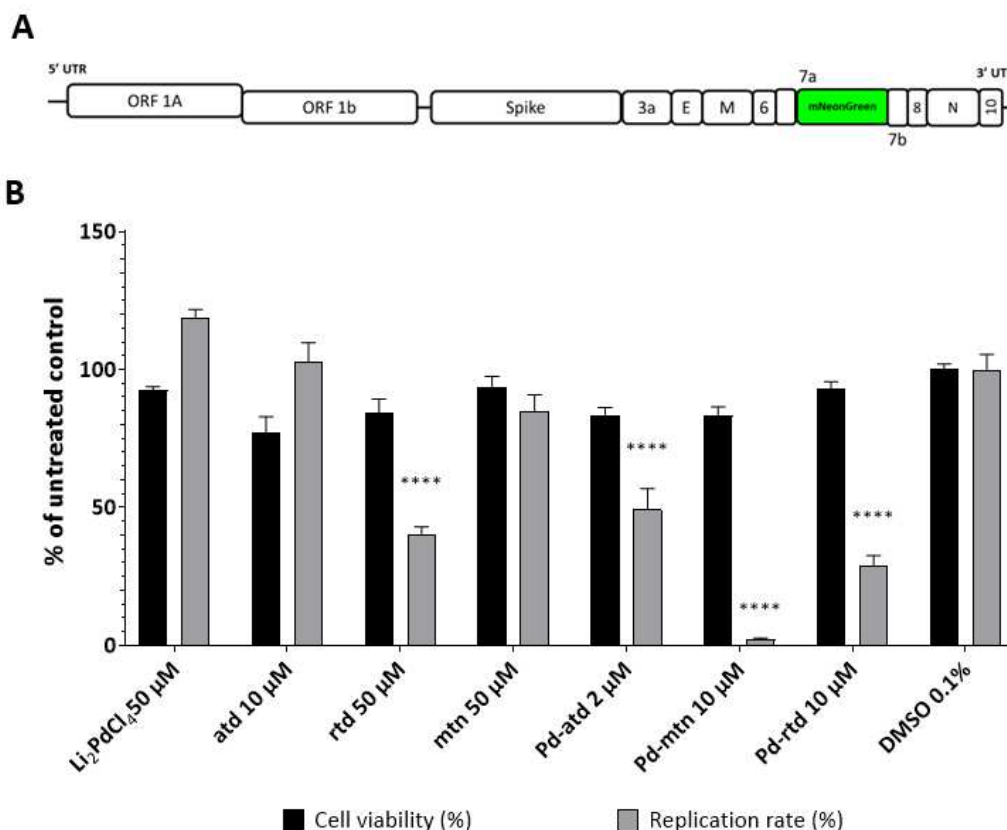


Figure 2: Effect of precursors, ligands, and coordinated compounds on A549-AT cells and SARS-CoV-2-Neongreen replication. A549-AT cells were infected with SARS-CoV-2-Neongreen and simultaneously treated with compounds at the highest non-cytotoxic concentration of each compound. After 24h, the total integrated intensity of the fluorescence (GCU x $\mu\text{m}^2/\text{well}$) was analyzed using the IncuCyte S3 microscope. Cell viability was accessed treating A549-AT cells with the compounds the same concentrations. After treatment, the medium with compound was removed and replaced with medium with MTT at 1 mg/mL and incubated for 30 min. After incubation, the medium was removed, and the crystals solubilized with DMSO. Surviving cells were measured by absorbance (490 nm). DMSO or water were used as untreated control. Mean values of two independent experiment each measured in quadruplicate including the standard deviation are shown. DMSO was used as untreated control. (****) $P < 0.0001$

The Pd(II) complexes yields highly potent molecules against SARS-CoV-2 replication

Aiming to further investigate the anti-SARS-CoV-2 activity of Pd-atd, Pd-mtn, Pd-rtd complexes, A549-AT cells were infected with SARS-CoV-2-mNeongreen at a MOI of 0.1 at concentrations ranging from 0.58 μM to 300 μM , in a two-fold serial dilution. After 24h, viral replication and cytotoxicity were accessed by the fluorescence intensity and MTT assay, respectively. The effective concentration of 50% (EC_{50}) and cytotoxic concentration of 50% (CC_{50}) were calculate employing a non-linear regression. As a result, Pd-atd had an CC_{50} and EC_{50} of 119.7 μM and 8.4 μM (**Figure 3A**), respectively, while Pd-mtn had an CC_{50} and EC_{50} of 550.9 μM and 15.2 μM , in this order (**Figure 3B**). Further, Pd-rtd had CC_{50} of 767.5 μM and EC_{50} of 32.6 μM (**Figure 3C**). The calculated selective index (SI) for Pd-atd, Pd-mtn, and Pd-rtd were 14.2, 36.2, and 23.5, respectively.

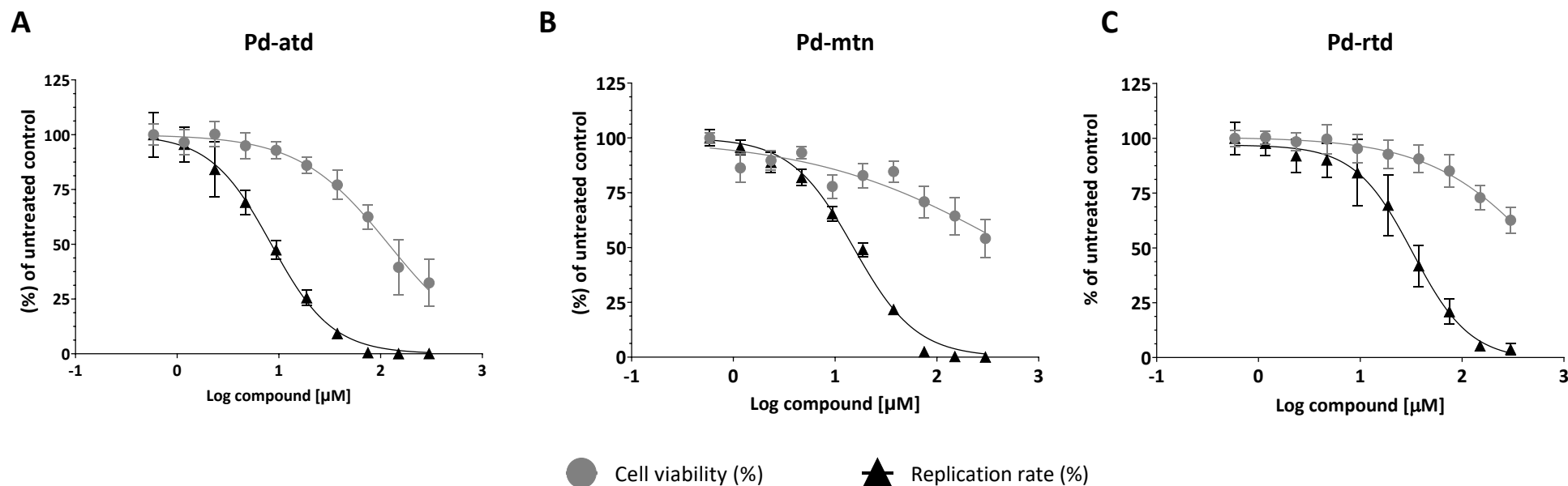


Figure 3: Dose-response curve of Pd-atd, Pd-mtn, and Pd-rtd on A549-AT cells and SARS-CoV-2-Neongreen replication. A549-AT cells were infected with SARS-CoV-2-Neongreen at a MOI of 0.1 and treated with the Pd-atd (A), Pd-mtn (B), or Pd-rtd (C) at concentrations ranging from 0.58 μM to 300 μM , in a two-fold serial dilution. After 24h, the total integrated intensity of the fluorescence (GCU $\times \mu\text{m}^2/\text{well}$) was analyzed using the IncuCyte S3 microscope. Cell viability was accessed treating A549-AT cells with the compounds at the same concentrations. After treatment, the medium with compound was removed and replaced with medium with MTT at 1 mg/mL and incubated for 30 min. After incubation, the medium was removed, and the crystals solubilized with DMSO. Surviving cells were measured by absorbance (570 nm). Mean values of two independent experiment each measured in quadruplicate including the standard deviation are shown.

Pd(II) complexes disrupt the initial stages of the SARS-CoV-2 infectious cycle

To elucidate the effect of the complexes on SARS-CoV-2 replicative cycle, a time of drug addition assay was performed employing Pd-atd, Pd-mtn, and Pd-rtd at 50 μ M. First, cells were pre-treated for 1h with each compound, followed by PBS washing to remove compound-containing media, and then were infected with the SARS-CoV-2-mNeonGreen (MOI of 0.1) for 24h (**Figure 4A**). As an outcome, Pd-atd protected the cells in 30.2% against SARS-CoV-2 NeonGreen infection ($p=0.0009$), while Pd-mtn and Pd-rtd did not show any inhibitory effect (**Figure 4B**).

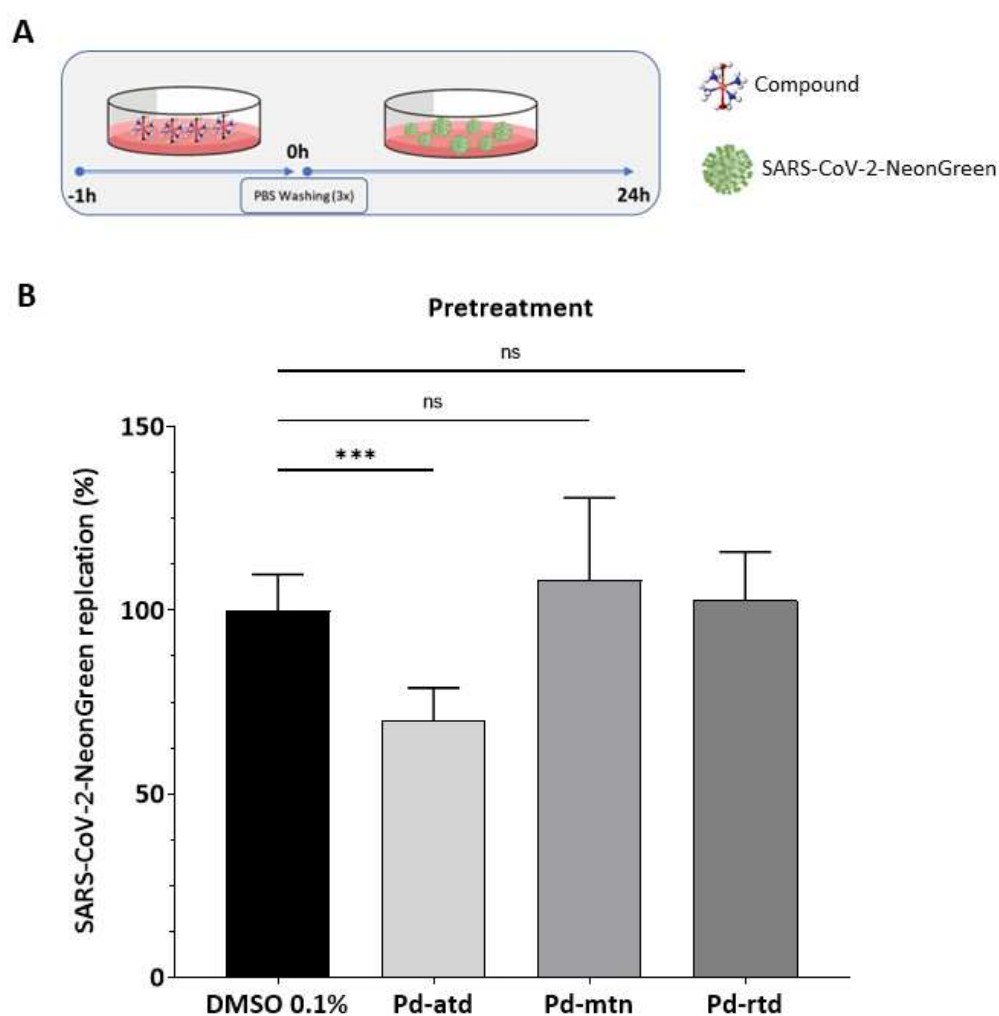


Figure 4: Metal complexes protect cells against SARS-CoV-2 infection. (A) A549-AT cells were treated with each compound at 50 μ M for 1 h. Then, cells were washed and infected with SARS-CoV-2-NeonGreen at MOI 0.1. After 24h, the total integrated intensity of the fluorescence (GCU $\times$ μ m²/well) was analyzed using the IncuCyte S3 microscope. Schematic representation of each time-based assay as indicated by A549-AT cells (blue arrows), compounds (chemical structure), and SARS-CoV-2-NeonGreen (green virus). Mean values $\pm$ SD of a minimum of three independent experiments each measured in quadruplicate. (ns) non-significant, (***) $P < 0.001$. All images were generated using GraphPad Prism 9 and GIMP 2.10.20 (www.gimp.org).

To further evaluate the effects on SARS-CoV-2-mNeonGreen entry to the host cells, virus (MOI of 1) and each compound were simultaneously added to A549-AT cells for 1 h at 37 °C, cells were washed with PBS to remove the inoculum and replaced with fresh medium (**Figure 5A**). Pd-atd inhibited 76.4% ($p < 0.0001$) of SARS-CoV-2-mNeonGreen entry, while Pd-mtn and Pd-rtd inhibited 51% and 51.2% ($p = 0.0002$), respectively (**Figure 5B**). Combining this treatment with 1 h pre-incubation of the inoculum containing each compound and SARS-CoV-2-mNeonGreen (MOI of 5) at 37 °C (**Figure 5C**) resulted in the decrease in 62.6% ($p < 0.0001$) of fluorescence levels for Pd-mtn, and 32.5% ($p = 0.0005$) for Pd-atd, while Pd-rtd did not show significant inhibition, indicating that Pd-mtn and Pd-atd possess virucidal activity (**Figure 5D**).

To analyse the effect of Pd-atd, Pd-mtn, and Pd-rtd on SARS-CoV-2 attachment, virus (MOI of 1) and each compound were first incubated with the cells at 4 °C for 1 h (**Figure 5E**). At this temperature, virus particles were able to attach to the cellular receptors, but not entry into the host cells. Cells were then washed with PBS, fresh media added, and incubated at 37°C, to allow the continuation of the entry process. Data obtained from this assay also showed strong inhibition of 69.2% and 66.5% for Pd-atd and Pd-mtn, respectively, while Pd-rtd inhibited 23% of SARS-CoV-2-mNeonGreen attachment ($p < 0.0001$) (**Figure 5F**). Post-attachment was evaluated by including an additional incubation of 30 min at 37 °C to the previous protocol (**Figure 5G**), showing that the inhibition remained strong for Pd-atd (69.8%) and Pd-mtn (66.3%) ($p < 0.0001$), and led to increase of Pd-rtd activity (37.4%) (**Figure 5H**).

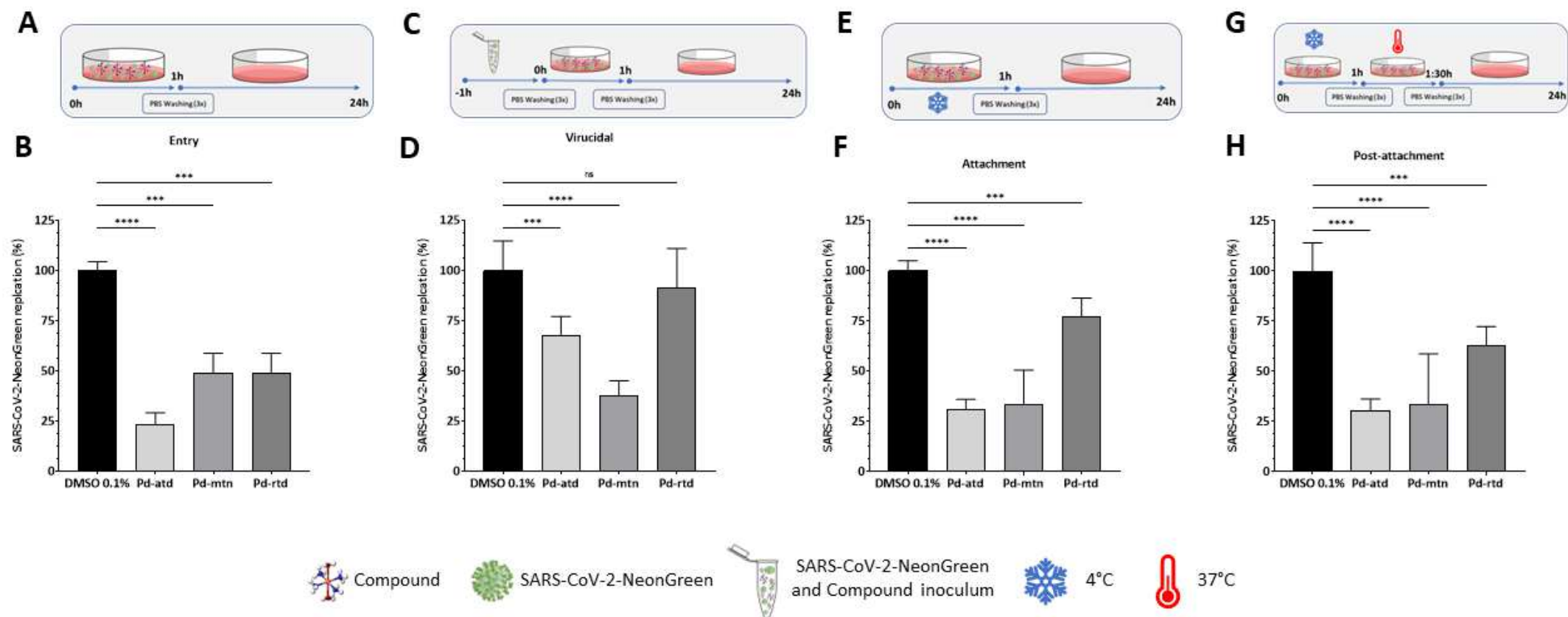


Figure 5: Metal complexes have virucidal activity and blocks SARS-CoV-2 entry to the host cells. (A and B) A549-AT cells were infected with SARS-CoV-2-NeonGreen (MOI 1) and simultaneously treated with each compound at 50 μ M for 1 h. Cells were washed and replaced by fresh medium. (C and D) SARS-CoV-2-NeonGreen (MOI 5) and each compound was incubated for 1 h at 37 °C and then for one extra hour with the cells. Then, virus and compound were removed, cells were washed with PBS, and added fresh medium. (E and F) A549-AT cells were infected with the virus and simultaneously treated with each compound for 1 h at 4 °C. The cells were washed to remove virus and compound and replaced with fresh medium. (G and H) A549-AT cells were infected with virus and simultaneously treated with the compounds for 1 h at 4 °C. Then, cells were incubated for additional 30 min with compound and virus at 37 °C, washed with PBS and replaced by fresh medium. For all assays, the total integrated intensity of the fluorescence (GCU $\times$ μ m²/well) was analyzed using the IncuCyte S3 microscope. Schematic representation of each time-based assay as indicated by A549-AT cells (blue arrows), compounds (chemical structure), and SARS-CoV-2-NeonGreen (green virus), SARS-CoV-2-NeonGreen and compound inoculum (microtube), incubation at 4°C (ice crystal) and incubation at 37°C (thermometer). Mean values $\pm$ SD of a minimum of three independent experiments each measured in quadruplicate. (ns) non-significant, (***) $P < 0.001$, (****) $P < 0.0001$. All images were generated using GraphPad Prism 9 and GIMP 2.10.20 (www.gimp.org).

Pd-atd impairs post-entry stages of SARS-CoV-2 replication

To assess the effect of the complexes on post-entry steps of SARS-CoV-2, the cells were infected with SARS-CoV-2-mNeonGreen (MOI of 0.1) for 1h, or 6h for late stages of virus replication (**Figure 6A and 6B, respectively**), and then fresh media containing the compounds was added. After 24h, Pd-atd inhibited 87.5% of SARS-CoV-2-mNeonGreen replication ($p < 0.0001$) (**Figure 6C**), but when added after 6 hours of infection it reduced the activity to 49% ($p < 0.0001$) (**Figure 6D**). On the other hand, Pd-mtn, and Pd-rtd did not show any significant activity in both protocols (**Figure 6C and 6D**).

To further investigate the effect of the Pd-atd on SARS-CoV-2 post-entry and replication, a dsRNA interaction assay was carried out. Through this assay is possible to analyse the capacity a molecule to interact with the dsRNA, an intermediate of viral replication (LI et al., 2021). To this, Pd-atd was incubated with 65nM of SARS-CoV-2 dsRNA and submitted to a agarose electrophoresis gel, where the lack or decrease of band intensity in the agarose gel suggests the compound interaction activity since it competes with SYBR. DMSO 0.1% and *cis*-[PtCl₂(dmsO)₂] (Pt, 10 μ M) were used as untreated and treated control. The assessment of band intensity in the gel demonstrated that Pd-atd decreased intensity in only 10.3% (**Supplementary Figure 2A and 2B**). The data shows a low capacity of interaction with SARS-CoV-2 dsRNA, suggesting that the activity of this complex is related to other mechanisms of RNA replication and translation.

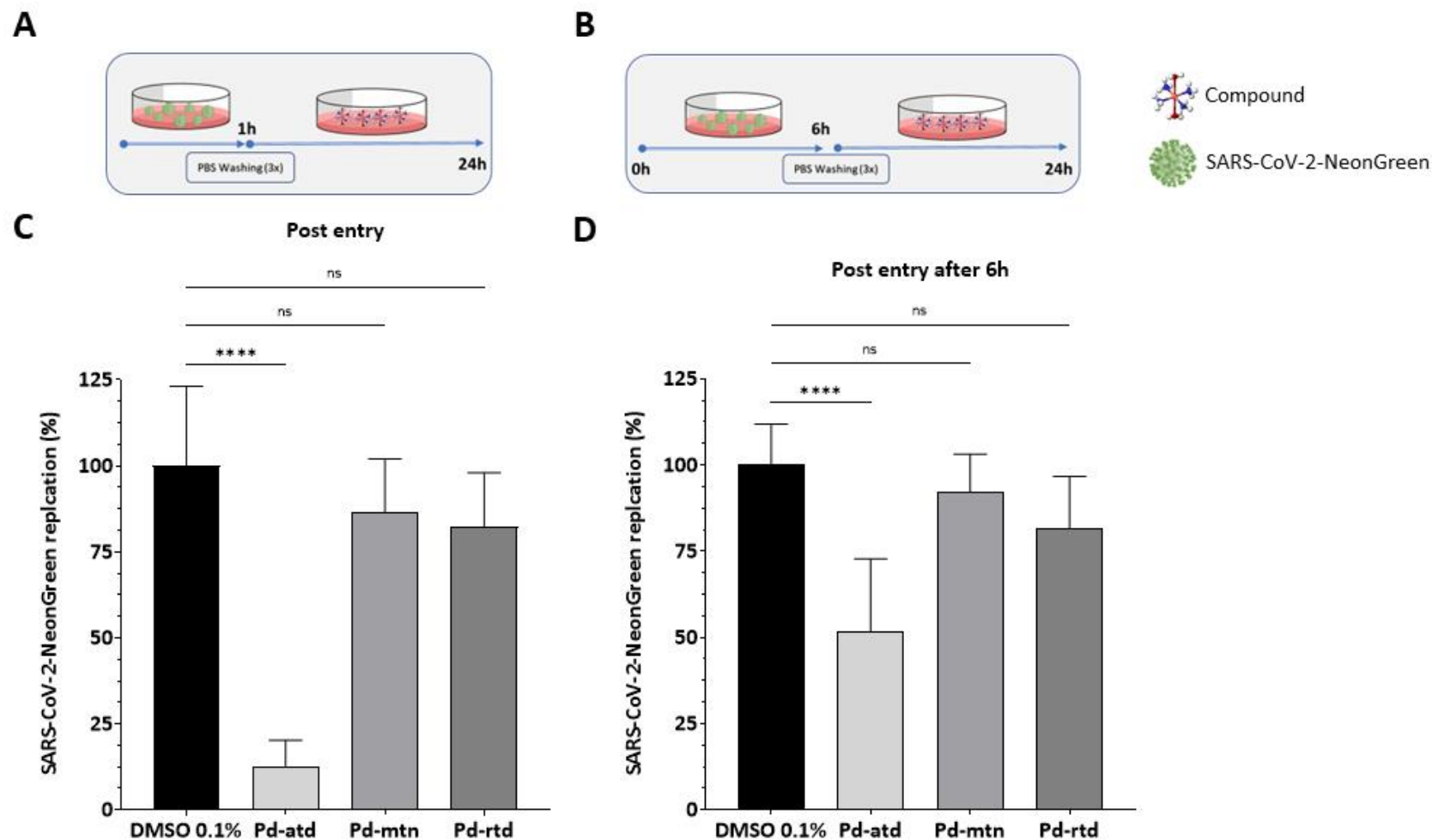


Figure 6: Pd-atd impairs post-entry stages of SARS-CoV-2 replication. A549-AT cells were infected with SARS-CoV-2-NeonGreen (MOI 1) for 1h (A) or for 6h (B) and then treated with each compound for the remaining 24h. Cells were washed and replaced by fresh medium. For all assays, the total integrated intensity of the fluorescence (GCU x $\mu\text{m}^2/\text{well}$) was analyzed using the IncuCyte S3 microscope. Results for 1h infection are shown in (C), while for 6h infection in (D). Schematic representation of each time-based assay as indicated by A549-AT cells (blue arrows), compounds (chemical structure), and SARS-CoV-2-NeonGreen (green virus). Mean values $\pm$ SD of a minimum of three independent experiments each measured in triplicate. (ns) non-significant, (****) $P < 0.0001$. All images were generated using GraphPad Prism 9 and GIMP 2.10.20 (www.gimp.org).

Effect of Pd(II) complexes against the Delta (B.1.17.2) and Omicron (B.2) variants

To assess the effect the Pd(II) complexes against the delta and omicron variants, the infectious clone of the Wuhan strain carrying the spike genome sequence of the variants B.1.617.2 (delta) or B.2 (omicron) and the MCherry gene (**Figure 7A**) was used. To that, Pd-atd, Pd-mtn, or Pd-rtd at 50 μ M was added to media containing the Wuhan-Delta-Spike or Wuhan-Omicron-Spike viruses at an MOI of 0.1 and added to the cells for 24h. The total integrated intensity of the fluorescence (RCU x μ m²/well) was analyzed using the IncuCyte S3 microscope. The complexes Pd-atd and Pd-rtd decreased SARS-CoV-2 Wuhan-Delta-Spike in 17.5% and 16.2%, respectively, while Pd-mtn did not present any effect (**Figure 7B**). On the other hand, against the SARS-CoV-2 Wuhan-Omicron-Spike virus Pd-atd, Pd-mtn, and Pd-rtd were able to decrease viral replication in 47.7%, 15.1%, and 29.1%, respectively (**Figure 7C**).

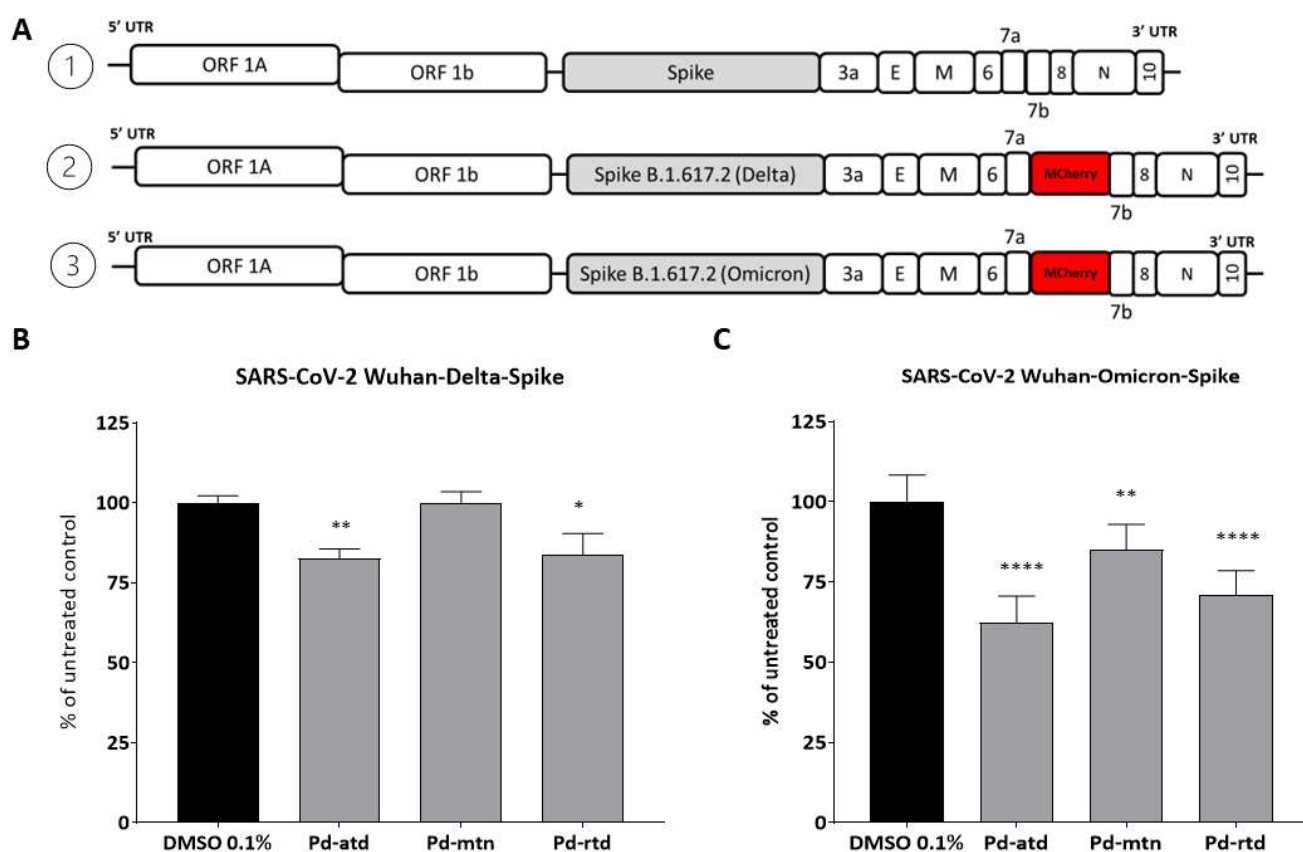


Figure 7: Effect of Pd-atd, Pd-mtn, and Pd-rtd on the replication of the infectious clones SARS-CoV-2-Wuhan-DELTA-Spike or SARS-CoV-2-Wuhan-Omicron-Spike. (A) Schematic representation of the infectious clone genome, being the original sequence of Wuhan (NCBI: NC_045512.2) (1), SARS-CoV-2-Wuhan-Delta-Spike in (2) and SARS-CoV-2-Wuhan-Omicron-Spike (3), being the last two carries of the MCherry reporter gene. A549-AT cells were infected with SARS-CoV-2-Wuhan-Delta-Spike (B) or SARS-CoV-2-Wuhan-Omicron-Spike (C) at an MOI of 0.1 in the presence of each molecule for 24h. SARS-CoV-2 replication was measured by the total integrated intensity of the fluorescence (RCU x μ m²/well) using the IncuCyte S3 microscope. All images were generated using GraphPad Prism 8 and GIMP 2.10.20. Mean values $\pm$ SD of a minimum of three independent experiments each measured in quadruplicate. (*) P < 0.05, (**) P < 0.01, (****) P < 0.0001.

To further confirm the activity, the wild-type virus variants Delta or Omicron were also employed to identify their effects on viral replication. To this, cells were infected with Delta or Omicron strains in an MOI of 0.1 in the presence or absence of each compound for 24h, supernatants were harvested and tittered by TCID₅₀. As result, against Delta variant, Pd-atd decreased the viral titer in 0.5 Log₁₀, while Pd-mtn and Pd-rtd did not present any effect (**Figure 8A**). Differently, for the Omicron variant, Pd-atd, Pd-mtn, and Pd-rtd impaired viral replication in in 2, 1, and 2 Log₁₀ (**Figure 8B**).

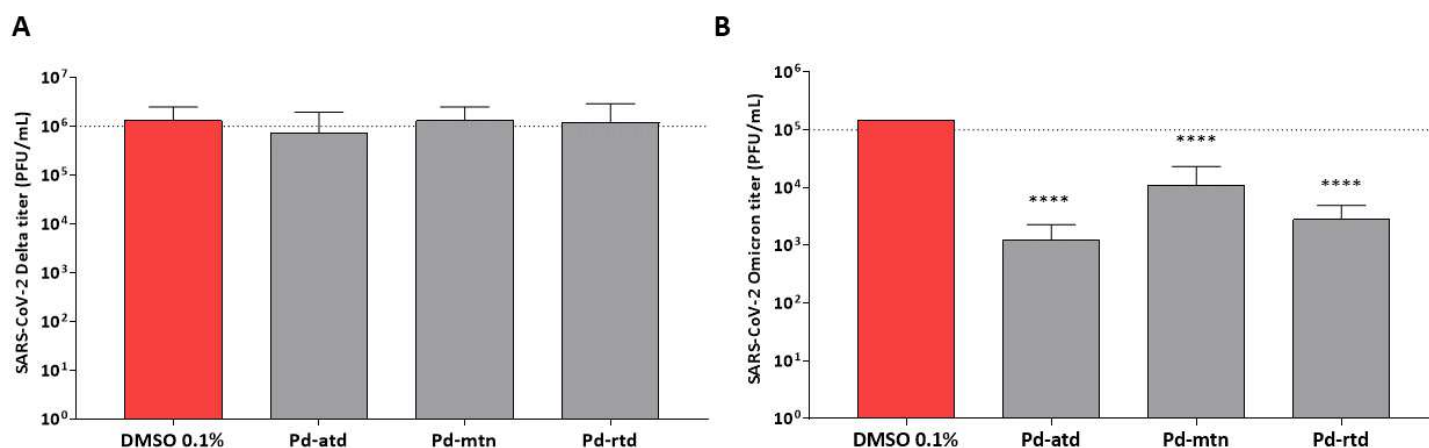


Figure 8: Effect of the Pd(II) complexes on the wild-type SARS-CoV-2-Delta and SARS-CoV-2-Omicron replication. A549-AT cells were infected with SARS-CoV-2-Delta (A) or SARS-CoV-2-Omicron (B) at an MOI of 0.1 in the presence of each substance for 24h. Then, supernatant was collected and tittered by TCID₅₀. All images were generated using GraphPad Prism 8 and GIMP 2.10.20. Mean values $\pm$ SD of a minimum of three independent experiments each measured in replicate. (****) $P < 0.0001$.

Pd-rtd decreases cell to cell spread of SARS-CoV-2 *in vitro*

During viral infection cell to cell spread is an important feature to guarantee viral spread (KIM; YOON; PARK, 2022; ZENG et al., 2022), therefore, the dissemination of SARS-CoV-2-mNeonGreen was evaluated in the presence of each complex. To that, A549-AT cells were infected with SARS-CoV-2-mNeonGreen (MOI of 0.05) for 1h and then treated with each compound in the presence of Methylcellulose 0.8%. As an outcome, Pd-rtd was able to decrease size focus of infected cells in 0.06 μm^2 (DMSO control 0.30 μm^2 , while Pd-rtd treated 0.24 μm^2) (**Figure 9A and 9B**). Pd-atd and Pd-mtn were not able to decrease viral spread (**Figure 9A and 9B**).

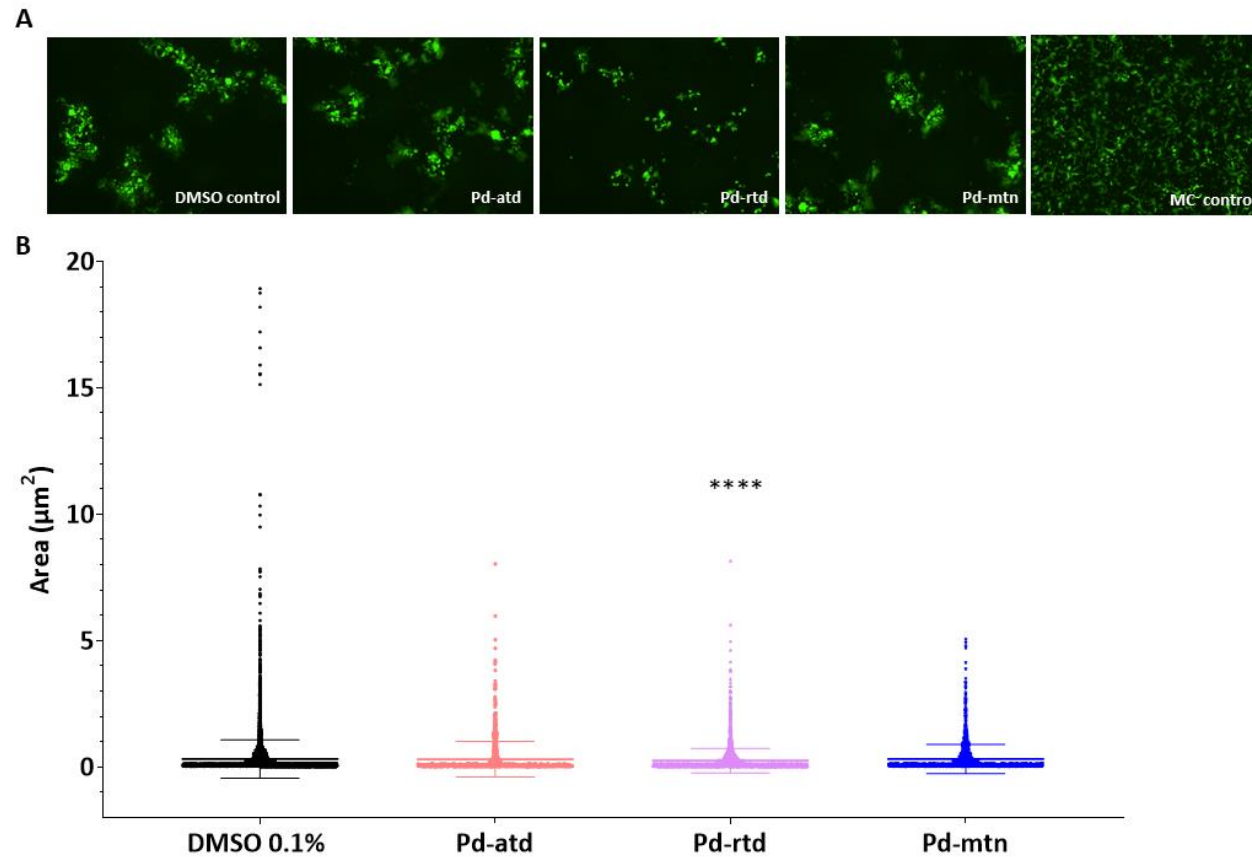


Figure 9: Pd-rtd decreases SARS-CoV-2 cell to cell spread. A549-AT cells were infected with SARS-CoV-2-mNeonGreen (MOI 0.05) for 1h, cells were washed, and replaced by fresh medium containing each compound and Methylcellulose 0.8% for the remaining 48h. The photos were taken by the IncuCyte S3 microscope in the objective 10x under the green fluorescence light and focus size analysis were performed in ImageJ. (A) Representative images of SARS-CoV-2-mNeonGreen infection in the presence of DMSO control, Pd-atd, Pd-mtn, Pd-rtd, and methylcellulose negative (MC⁻) control, in this order. (B) The average focus size in area (μm^2) of three technical repeats is shown. (****) $P < 0.0001$

Pd(II)-complexes selection of SARS-CoV-2-resistant to treatment

To evaluate the capacity of Pd(II) complexes to induce resistance on SARS-CoV-2 *in vitro*, a resistance assay utilizing SARS-CoV-2 wild-type (hCov-119/England/02/2020) was conducted. The virus underwent serial passaging in cell culture under the influence of Pd-atd, Pd-mtn, or Pd-rtd at concentrations of 50 μ M. Remarkably, Pd-atd treatment successfully suppressed viral titres until the 3rd passage, reaching a level of 3×10^6 pfu/mL (**Figure 10**). However, in the 4th passage, a noteworthy drop occurred, with titres plummeting to 1.45×10^5 pfu/mL. Interestingly, as we continued passaging, the viral titres rebounded significantly by the 6th passage, exceeding those of the control group (**Figure 10**). The sustained levels of SARS-CoV-2 in the Pd-atd-treated group mirrored those of the control through the 7th and 8th passages, hinting at the emergence of resistance mutations (**Figure 10**). Conversely, Pd-mtn treatment maintained consistently lower viral titres up to the 6th passage before equating to the untreated control. In the 7th passage, a resurgence in titres was observed, reaching 3.13×10^6 pfu/mL (**Figure 10**). Intriguingly, Pd-rtd treatment exhibited a distinctive pattern, inducing resistance as early as the 2nd passage, with viral titres soaring to 9.9×10^6 pfu/mL, and this resistance effect persisted until the 4th passage (**Figure 9**).

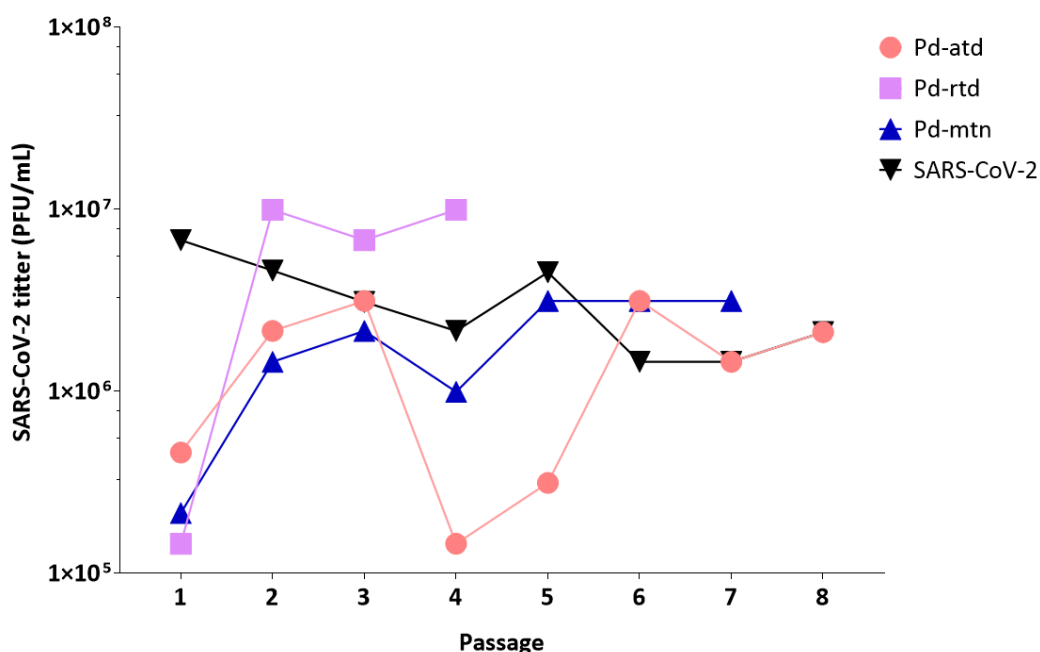


Figure 10: *In vitro* selection of SARS-CoV-2-resistant virus to the pd(II)-complexes treatment. Continual passages of SARS-CoV-2 (NCBI: OX905858.1) in an MOI of 0.05 were performed in the presence of Pd-atd, Pd-mtn, or Pd-rtd at 50 μ M in A549-AT cells. Controls for viral adaptation in cell culture and DMSO untreated control were undertaken in parallel. These concentrations were kept constant throughout the experiment. For each passage, supernatant was harvested 48h after infection, tittered by TCID₅₀, and used to infect the next passage maintaining virus MOI and the concentration of each compound. The titer of each passage is shown in pfu/mL.

DISCUSSION

The SARS-CoV-2 infection still poses as a health problem worldwide due to the emergence of variants and low adherence to vaccination in some countries (DE SOUZA et al., 2021; SINGH; CHATTU, 2021). Up to date, there are a few drugs available to treat only severe cases of COVID-19 (BEIGEL et al., 2020; FISCHER et al., 2022; JAYK BERNAL et al., 2022a; SAÚDE., 2022), therefore, discovery of new therapeutic approaches to address SARS-CoV-2 infection is still needed.

The data presented here show the potent effect of amantadine (atd), rimantadine (rtd), and memantine (mtn) coordinated with Pd(II) against SARS-CoV-2 infection *in vitro*. The ligands atd, rtd, and mtn were previously described with selectivity indexes (SI) of 17.9, 8.4, and 19.6 in A549-ACE2 cells, with EC₅₀ ranging around 70-80 μ M (ZHOU et al., 2021). Contrastingly, the coordinated compounds described here had SI of 14.2, 36.2, and 23.5 for Pd-atd, Pd-rtd, and Pd-mtn, respectively, while the initial ion salt Li₂PdCl₄ and the ligands atd and mtn did not inhibit SARS-CoV-2 in A549-AT cells. Alternatively, the ligand rtd at 50 μ M significantly inhibited 60% of SARS-CoV-2 infection. Furthermore, the EC₅₀ for Pd(II) complexes ranged from 8.4 to 32.6 μ M, much lower than what was previously described. With that, it is possible to suggest that the Pd(II) metal coordination resulted in compounds with a better antiviral profile and more active than their ligands. This was expected since Pd(II) is more stable than other metal ions, and undergo ligation exchange more faster resulting in lower cytotoxic and more active compounds (BUTOIR et al., 1997; FIORI-DUARTE et al., 2019; GENOVA et al., 2004; OLIVEIRA et al., 2019).

The Pd-atd showed to possess activity on post-entry stages of SARS-CoV-2 replication. The Pd(II) was described as non-active against DNA and RNA virus, and here the Li₂PdCl₄ also did not show any activity when administered alone, being difficult to suggest that Pd(II) could be having a synergic activity with the aminoadamantanes (GAROUFIS; HADJIKAKOU; HADJILIADIS, 2009; KARAKÜÇÜK-İYIDOĞAN et al., 2011; TUŠEK-BOŽIĆ et al., 1998). In this context, it is possible to suggest that into the complexes describe here, the Pd(II) might be functioning as drug carrier, decreasing cytotoxicity and increasing antiviral activity. In this case, the impair of SARS-CoV-2 post-entry might also be related to the inhibition of second rounds of infection in cell culture due to the strong entry effect and the low MOI employed in the time of addition assays (FROLOV et al., 2012; WILKE; REISSIG; NOVELLA, 2004). Additionally, here the capacity of impairing RNA replication was assessed

by the interaction with the SARS-CoV-2 dsRNA, which in Pd-atd treatment, low interaction was observed, suggesting the lack of effect in this mechanism of the replication stage. However, post-entry mechanisms of action for Pd-atd must be further investigated.

The aminoadamantanes are compounds described with strong activity against IAV, HCV, and CHIKV, blocking the viroporins, which are highly selective pH-regulated ion channels present in viral envelope, that transport protons necessary for viral uncoating (DEY et al., 2019; INTHARATHEP et al., 2008b; OUYANG; CHOU, 2014; PEREIRA et al., 2021; SANTOS et al., 2022). Similarly, SARS-CoV-2 also possess a viroporin expressed by ORF3a (XU et al., 2022). Here, all Pd(II) complexes are affecting SARS-CoV-2 entry, mainly in attachment and post-attachment steps over 50%, and therefore, is possible to hypothesize that these molecules are blocking the ORF3a viroporin in SARS-CoV-2 entry. Another target could be the M protein membrane, that is the most abundant viral structural protein in SARS-CoV-2 replication and play a central role in morphogenesis and virus assembly (ZHANG et al., 2022). The M protein dimer was found to be similar the ORF3a viral ion channel, suggesting that the M protein could also function as a viroporin (ZHANG et al., 2022), and therefore, could be the target of the Pd(II) complexes as well. This can further be emphasized by the effect of Pd-rtd in cell-cell transmission since M and S protein are essential to membrane fusion and syncytia formation (BUCHRIESER et al., 2020).

The C-terminus domain of M protein in SARS-CoV was found to interact with S protein in cell culture, determine fluidity of the viral membrane and distribution of S spikes in virions (HSIEH et al., 2008; ZHANG et al., 2022). What is more interesting, when the infectious clone of the Wuhan strain carrying the spike genome sequence of the variants B.1.617.2 (delta) or B.2 (omicron) was employed, a different profile of inhibition was visualized, which might be related to the different conformations of the Spike protein and how the process of infection is happening in these new virions. This was further confirmed by the evaluation of the metal complexes activity against the wild-type variants delta or omicron, which resulted in no inhibition of delta variant, but a modest inhibition of omicron. In fact, the inhibition profile of omicron was more similar to the data when Wuhan strain was employed, which is expected since both are phylogenetic closer than with delta (GUEYE et al., 2022; SUN et al., 2022).

Here, an *in vitro* resistance to Pd(II) complexes was conducted with SARS-CoV-2 to identify the potential of accumulating single nucleotide mutations (SNPs) caused by antigenic-drift. First, all metal complexes induced the selection of resistant SARS-CoV-2 virions after

cell culture passaging, being the Pd-rtd the one that induced faster resistance than Pd-atd and Pd-mtn. The aminoadamantanes were described as inducing resistance mutations in IAV-infected mice after 3rd passage under treatment (BELSHE et al., 1988; OXFORD; POTTER, 1973; SCHMIDTKE et al., 2006). In our case, only Pd-rtd induced resistance before the 3rd round of infection. However, sequencing of the virions should be carried out to confirm the acquiring of SNPs or other mutations that characterize the resistance. Additionally, through the sequencing it will be able to identify the probable mechanism of action of these compounds.

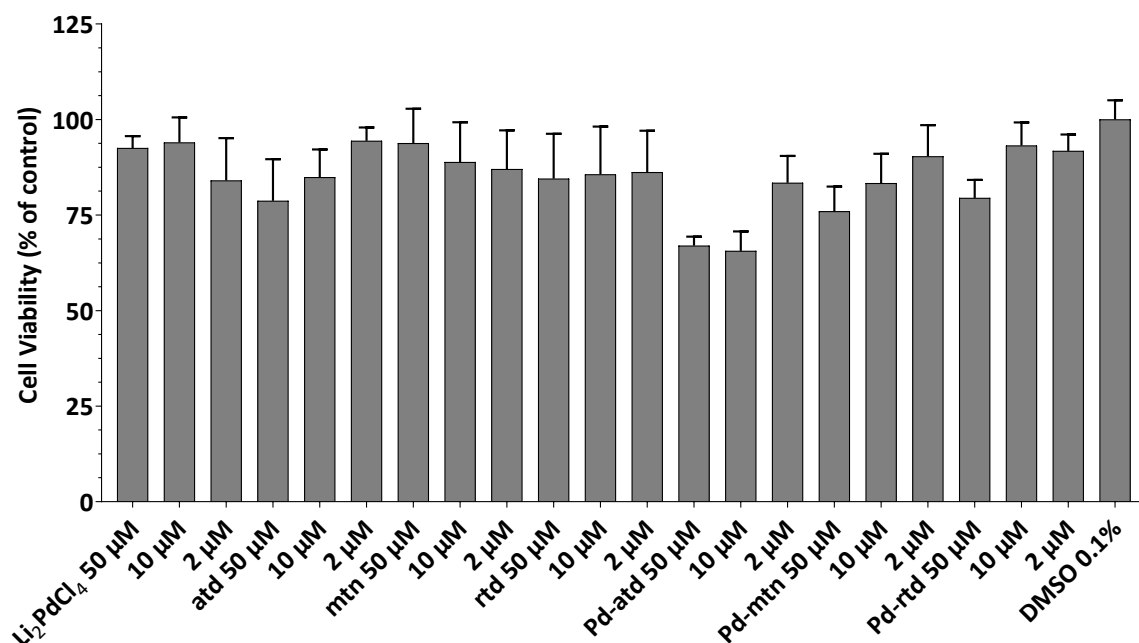
CONCLUSION

From the data showed here, the Pd(II) complexes Pd-atd, Pd-rtd, and Pd-mtn strongly inhibited early stages of SARS-CoV-2 infection cycle, all with higher SI and lower EC₅₀ than their ligands alone. Additionally, Pd-atd inhibited all stages of virus replicative cycle and induced resistance slower the other compounds investigated here. Our data warrants further investigation *in vivo*, holding the promise of expediting an effective treatment for COVID-19 patients. It strongly implies that the metal ion within these compounds plays a pivotal role in their potent antiviral action, hinting at a compelling avenue for advancing novel antiviral strategies against SARS-CoV-2. This insight reinforces the vast potential of metallodrugs in reshaping the landscape of antiviral therapeutics for diseases like COVID-19.

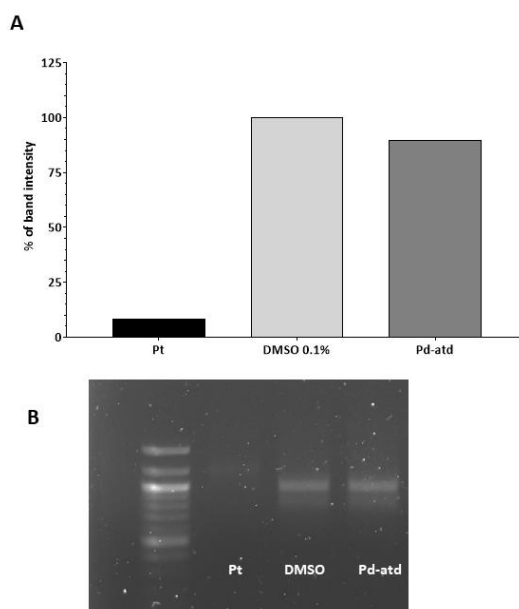
Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Files



Supplementary Figure 1: Cell viability of precursors, ligands, and coordinated compounds in A549-ACE2-TRPMSS2 cells. A549-ACE2-TRPMSS2 cells were treated with the compounds at 50μM, 10μM and 2μM. After 24h treatment, the medium with compound was removed and replaced by fresh medium with MTT at 1 mg/mL and incubated for 30 min. After incubation, the medium was removed, and the crystals solubilized with DMSO. Surviving cells were measured by absorbance (490 nm). Mean values of three independent experiments each measured in quadruplicate including the standard deviation are shown. DMSO was used as untreated control.



Supplementary figure 2; Pd-atd complexes shows low interaction with SARS-CoV-2 dsRNA. Sixty-five nanomoles of SARS-CoV-2 ORF1a dsRNA were incubated with Pd-atd at 50 μM or *cis*-[PtCl₂(dmsO)₂] at 10 μM or the DMSO 0.1% control for 45 min at room temperature. The reaction products were subjected to 1% agarose 1X TAE electrophoresis gel containing SYBR safe followed by densitometry analysis on ImageJ.JS version 1.53j.

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**CAPÍTULO 3: Cu(I) N-
HETEROCYCLICARBENES AS POTENT
INHIBITORS OF SARS-CoV-2
REPLICATION**

Cu(I) N-HETEROCYCLICCARBENES AS POTENT INHIBITORS OF SARS-COV-2 REPLICATION

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ABSTRACT

COVID-19 still poses as a threat to health system despite the vaccination and the use of emergency repurposing drugs. Therefore, the development of novel anti-SARS-CoV-2 is still need. Copper ions coordinated to N-heterocyclic-carbenes [Cu(NHC)] are organometallic complexes that pose as alternative compounds for drug development due to their variety of geometries and charges, tunability by ligand design, using their rich coordination chemistry. Also, we previously demonstrated the antiviral activity of these metal-based compounds against chikungunya virus (CHIKV). Here we evaluated the activity of Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ molecules against SARS-CoV-2 infection. Through a dose-response assay using A549-AT cells and the SARS-CoV-2-Wuhan infectious clone expressing mNeonGreen (SARS-CoV-2-mNeonGreen), Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ inhibited SARS-CoV-2 replication with a selectivity index (SI) of 8, 11.2, and 4.4, respectively. The complexes Cu(Imes)Cl and Cu(Imes)₂BF₄ inhibited all stages of viral replication (higher inhibition in post-entry steps), while Cu(Ipr)Cl impaired only entry and post-entry steps. What is more, Cu(Imes)Cl and Cu(Imes)₂BF₄ complexes decreased both Delta and Omicrons' titres, while Cu(Ipr)Cl only inhibited Omicron's, all capable of inhibiting the infectious clones SARS-CoV-2-MCherry-S-Delta and SARS-CoV-2-MCherry-S-Omicron. In addition, Cu(Imes)₂BF₄ was able to decrease cell to cell transmission. In this context, we strongly encourage further investigations involving Cu(I) and Cu(II) complexes and propose that Cu(Ipr)Cl and Cu(Imes)Cl to be considered for utilization in pre-clinical assays as a promising alternative for the treatment of COVID-19.

Keywords: Antivirals, Copper complexes, Organometallic compounds, SARS-CoV-2

INTRODUCTION

The search for new molecules that can impair viral replication and be capitalized into drugs are increasing over the years due to the emergence of new viral species and strains, causing outbreaks that can evolve to epidemics and pandemics (ADAMSON et al., 2021). However, from 2012 to 2021 the US Food and Drug Administration (FDA) approved the licensing of only about 35 antiviral drugs, to the treatment of hepatitis C virus (HCV), hepatitis B virus (HBV), influenza A virus (IAV), and human immunodeficiency virus (HIV) (ADAMSON et al., 2021; VON DELFT et al., 2023).

The Coronavirus Disease 2019 (COVID-19) pandemic, caused by SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) also highlighted the urgency of developing antivirals against current and future viral outbreaks (SHI et al., 2020; VON DELFT et al., 2023; ZHANG; WU; ZHANG, 2020). SARS-CoV-2, a member of the *Coronaviridae* family, possesses a single-stranded positive-sense RNA genome, associated with a protein nucleocapsid (N). This RNA is enclosed by an envelope derived from the host cell membrane inserted of the envelope protein (E) and embedded with S glycoproteins (Spike) (ANDERSEN et al., 2020; KIM et al., 2020). Notably, the viruses with RNA genome are highly susceptible to genetic variability, contributing to the rapid emergence of global variants, including the Variants of Concern (VOCs) such as Alpha, Beta, Gamma, Delta, and Omicron (DUFFY, 2018; JOHNSON et al., 2022; MONTAGUTELLI et al., 2021; THAKUR; RATHO, 2021; WANG et al., 2021b).

The SARS-CoV-2 infection can cause moderate to severe illness, related to upper and lower respiratory tract infection, in addition to fever, cough, dyspnea, pneumonia, and severe acute respiratory syndrome (SARS), which represents more severe cases and can lead to death. SARS-CoV-2 has a higher transmission rate than other respiratory viruses, which facilitated its worldwide spread (GRASSELLI et al., 2020b; LAMERS; HAAGMANS, 2022; STRATTON; TANG; LU, 2021; YANG et al., 2020; ZHOU et al., 2020a). Additionally, even after vaccination, infected patients can develop long-lasting chronic symptoms described as the Post-COVID Syndrome or Long-COVID, directly impacting quality of life of population around the world (AUGUSTIN et al., 2021; MALTEZOU; PAVLI; TSAKRIS, 2021b, 2021a; ORONSKY et al., 2021; YONG, 2021).

So far, there are only a few drugs licensed to the treatment of COVID-19, being them remdesivir, molnupiravir, and Paxlovid® (BEIGEL et al., 2020; IMRAN et al., 2021; JAYK BERNAL et al., 2022b; LIU et al., 2023a, 2023b; PANTAZIS et al., 2023; SAÚDE., 2022). The lack of the of active molecules poses as delay in combating COVID-19, and the rapid

resistance of omicron variants against Paxlovid and molnupiravir also emphasizes the need of a variety of therapeutic approaches that can abrogate viral infection (LIU et al., 2023b; SANDERSON et al., 2023). Additionally, the development of new molecules active against RNA viruses can help with important insights to combat and prepare health researchers for future outbreaks and emergence of new viruses.

The metal-based compounds are the association of organic or inorganic molecules with a metal ion (BOROS; DYSON; GASSER, 2020; DE PAIVA et al., 2020c; MESSERSCHMIDT et al., 2004). Metal complexes possess chemical and structural attributes that cannot be solely replicated by organic motifs which makes them a promising and versatile avenue for drug development (BOROS; DYSON; GASSER, 2020; DE PAIVA et al., 2020c; KARLIN, 1993; SHAHABADI; MIRZAEI KALAR; HOSSEINPOUR MOGHADAM, 2012; ZORODDU et al., 2019). Among the metal ions, copper (Cu) is an essential element in mammalian cells, and therefore in human homeostasis (KARLIN, 1993; ZORODDU et al., 2019). The Cu ion can be found as Cu(I) and Cu(II), which can be coordinated with different geometries, becoming attractive for medicinal purposes (ALVAREZ et al., 2018; HAAS; FRANZ, 2009; LINDER, 1991; SAGRIPANTI et al., 1997).

In this context, our group previously reported the synthesis of three Cu complexes (**Figure 1**) and their activity against chikungunya virus (CHIKV) (FONTES et al., 2022)(FONTES et al., 2022). These organometallic molecules were synthesized based on Cu(I) Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄, with all being coordinated to N-heterocyclic-carbenes (NHCs) (AL NASR et al., 2023; TEYSSOT et al., 2009). What is interesting is that NHCs possess low toxicity in biological systems and are highly easy to undergo structural variations to achieve drug-like properties, such as lipophilicity, water stability, and reactivity (AL NASR et al., 2023; FONTES et al., 2022; TEYSSOT et al., 2009). Cu(I) complexes showed an noteworthy anti-CHIKV and leishmanicidal activity (*L.amazonensis*), with low lipophilicity and high stability in solution. This one was the first description of Cu-NHC complexes as antivirals against RNA viruses, however, the compounds were not evaluated towards their mode of action (FONTES et al., 2022).

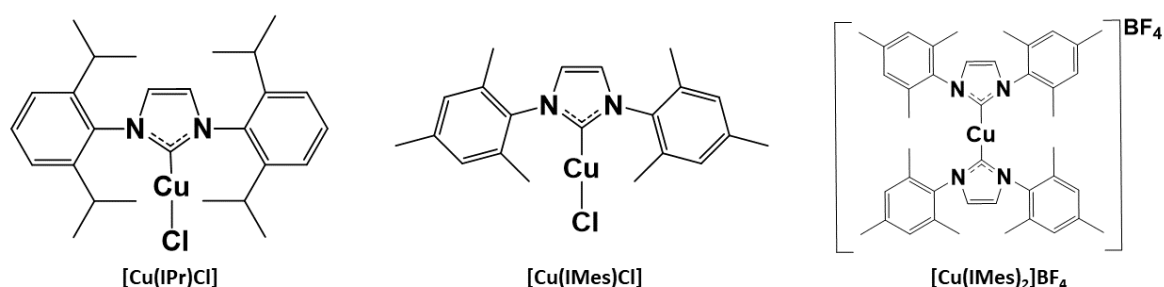


Figure 1: Chemical structure of Cu(Ipr)Cl, Cu(Imes)Cl and, Cu(Imes)₂BF₄ (FONTES et al., 2022)

Considering the data described previously and the potential of this compounds to be used as a broad-spectrum antiviral, here we evaluated the effects of Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ on SARS-CoV-2 replication cycle. Further, the effect against Delta and Omicron variants was characterized and their possible mode of action suggested.

METHODS

CELL CULTURE AND COMPOUNDS

The Adenocarcinoma human alveolar basal epithelial cells expressing the receptors ACE2 and TRPMSS2 (A549-ACE2-TRPMSS2 or A549-AT, ATCC#CCL-185) were cultivated as previous described (DOS SANTOS OLIVEIRA et al., 2023), using Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 100 U/mL penicillin (Gibco Life Technologies), 100 mg/mL streptomycin (Gibco Life Technologies), 1% (v/v) non-essential amino acids (Gibco Life Technologies) and 10% (v/v) fetal bovine serum (FBS; Hyclone) at 37 °C in a humidified 5% CO₂ incubator. The cells were cultivated in the presence of Geneticin (G418) and Hygromycin B at 1 mg/mL and 200 µg/mL, respectively.

The compounds Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ were synthesized as previously described (FONTES et al., 2022) and their structures are presented in **Figure 1**. All the compounds were dissolved in DMSO and stored in -20°C for maximum of two days. Dilutions in cell culture media were made immediately prior the assays at final concentration of 0.1%.

VIRUSES

Viruses used in this study were obtained and characterized by The Francis Crick Institute and were generously provided by Prof. Stephen Griffin from the University of Leeds. The variants include B.1.617.2 - Delta (MS066352H) and B.2 – Omicron (hCoV/England/FCI-179/2022). To amplify these viruses, Vero E6-ACE2 cells were cultured in 175 cm² flasks and was infected with each variant until approximately 80% cytopathic effect. Following the cytopathic effect, the supernatant was collected, and the infectious titre was determined using the 50% tissue culture infectious dose (TCID₅₀) method. The calculation of this titre was performed using the Spearman & Kärber algorithm, as outlined by Killington and Hierholzer (KILLINGTON; HIERHOLZER, 1996).

RESCUE OF SARS-COV-2 REPORTER VIRUS

The rescue of the SARS-CoV-2 reporter virus (SARS-CoV-2-mNeongreen), based on the NCBI sequence NC_045512.2, was conducted following a previously established protocol (DOS SANTOS OLIVEIRA et al., 2023). This process involved transfecting a total of 1 µg of the infectious clone pCCI-4K-SARS-CoV-2-mNeonGreen under the control of the CMV promoter (RIHN et al., 2021) into BHK-21 cells, with a seeding density of 3×10^5 cells per well in a 6-well plate. The transfection was carried out using lipofectamine 2000 at a 1:1 ratio. After a period of 3 days, the supernatant was collected and subsequently transferred to A549-AT cells cultured in a 75 cm² flask until the complete cell lysis occurred. The supernatant was then collected after 3 days and was designated as P0 stock. The infectious titer was determined using the 50% tissue culture infectious dose (TCID₅₀) method, and the calculation was based on the Spearman & Kärber algorithm, following the description provided by Killington and Hierholzer (KILLINGTON; HIERHOLZER, 1996).

DOSE-RESPONSE ASSAY

In order to assess the impact of Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ on both, cell viability and viral replication, A549-AT cells were initially plated at a density of 1×10^4 cells per well in 96-well plates 24 hours prior to the viral infection. Subsequently, the cells were subjected to treatment with each compound in concentrations ranging from 0.58 to 300 µg/mL, employing a two-fold serial dilution scheme. This treatment was conducted in the presence or absence of SARS-CoV-2-mNeonGreen, with a multiplicity of infection (MOI) set at 0.1 (RIHN et al., 2021). Cell viability was analyzed after 24h by removing the compound-containing media from cells and MTT solution at 1 mg/mL was added to each well, incubated for 30 minutes and replaced with 100 µL of DMSO to solubilize the formazan crystals. The absorbance was measured at 570 nm on FLUOstar OPTIMA microplate reader (BMGLabTech). Viral inhibition was analyzed after 24h by placing the plates in the IncuCyte® S3 Live-Cell Analysis System (Sartorius) and the green fluorescence light was observed at 10x objective. The photos were analysed employing the basic analyser from the IncuCyte S3 system and the total integrated intensity of the fluorescence (GCU x µm²/well) was collected. Cell viability and viral replication were calculated according to the equation $(T/C) \times 100\%$, which T and C represented the optical density of the treated well and control groups, respectively. DMSO was used as untreated control. The effective concentration of 50% (EC₅₀) and cytotoxic concentration of 50% (CC₅₀) were calculate employing a non-linear regression. The selectivity index (SI) was calculated by dividing the CC₅₀ value by the EC₅₀ ($SI = CC_{50} / EC_{50}$).

TIME OF DRUG ADDITION ASSAYS

For all time of drug addition assays A549-AT cells at the density of 1×10^4 cells per well were seeded in 96 well plates 24h before infection, treatment was performed with Cu(Ipr)Cl (10 μ M), Cu(Imes)Cl (2 μ M), and Cu(Imes)₂BF₄ (2 μ M), and virus replication was assessed by the green fluorescence light in 10x objective. The photos were analyzed employing the basic analyzer from the IncuCyte S3 system by the collection of total integrated intensity of the fluorescence (GCU x μ m²/well).

In pretreatment assay, cells were initially treated for 1h with each compound, then extensively washed with PBS to the removal of the compounds and added of SARS-CoV-2-mNeonGreen (MOI of 0.1). After 1h, cells were again washed with PBS to the removal of unbound viruses, and fresh medium was added for a 24-hour incubation period (**Figure 3A**).

In entry inhibition assay, cells were infected with a combination of each compound and the virus (MOI of 1) for a 1 hour, washed with PBS to remove this inoculum, and incubated with fresh medium for 24h (**Figure 4A**). To assess virucidal activity, a similar setup was employed, with the exception that the inoculum, containing both the compound and the virus (MOI of 5), was incubated for 1 hour before the introduction to the cells (**Figure 4B**).

Alternatively, each compound impact on the attachment step was investigated using the same conditions as the entry inhibition assay, except that the cells were infected with the virus (MOI of 1) in the presence of the compound at a temperature of 4°C (**Figure 4C**). A variation of this assay involved an initial incubation at 4°C, followed by a subsequent 30-minute incubation at 37°C to examine the compound's effect on post-attachment steps of infection (**Figure 4D**).

In the post-entry assay, cells were infected with SARS-CoV-2-mNeonGreen (at an MOI of 0.1) for a duration of 1 hour. Subsequently, the cells underwent extensive washing with PBS to remove unbound virions and were then incubated in a compound-containing media the compound for a period of 24 hours (**Figure 5A**). Additionally, a variation of this assay was performed by infecting cells with SARS-CoV-2-mNeonGreen (MOI of 0.1) for 6h, followed by incubation with the compound for the remaining duration, allowing the assessment of the compound's effect after the establishment of viral replication (**Figure 5B**).

BROAD-SPECTRUM ACTIVITY AGAINST SARS-COV-2 VARIANTS

To assess the effect of the metal compounds against the Delta and Omicron variants, the SARS-CoV-2 infectious clone based on Wuhan strain carrying the MCherry reporter gene (**Figure 6A-1**) was modified by molecular cloning, inserting the sequence of the spike gene derivative from the variants B.1.617.2 (delta) or B.2 (omicron) (**Figure 6A, 2 and 3**). The rescue of the virus was conducted in accordance with the previously mentioned procedure. Antiviral screening were performed with media containing Cu(Ipr)Cl (10 μ M), Cu(Imes)Cl (2 μ M) and Cu(Imes)₂BF₄ (2 μ M), in the presence of the Wuhan-B.1.617.2-Spike or Wuhan-B.2-Spike viruses at a multiplicity of infection (MOI) of 0.1. These compound-virus solutions were then added to the cells and incubated for 24 hours. Subsequently, the total integrated intensity of fluorescence (measured in RCU x μ m² per well) was assessed using the IncuCyte S3 microscope.

For the antiviral assays involving the wild-type viruses, specifically B.1.617.2 - Delta (MS066352H) and B.2 – Omicron (hCoV/England/FCI-179/2022), A549-AT cells were seeded at a concentration of 8×10^5 cells in 12-well plates. These cells were then infected with each virus at an MOI of 0.1 in the presence of Cu(Ipr)Cl (10 μ M), Cu(Imes)Cl (2 μ M), and Cu(Imes)₂BF₄ (2 μ M) for 24-hour incubation period. Following incubation, the supernatant was collected, and the infectious titer was determined using the 50% tissue culture infectious dose (TCID₅₀) method. The TCID₅₀ values were calculated using the Spearman & Kärber algorithm, following the procedure described by Killington and Hierholzer (KILLINGTON; HIERHOLZER, 1996).

DISSEMINATION ASSAY

To evaluate the effect of the compound in focus formation and cell to cell spread, 5×10^4 cells were plated in each well of 48 well plates for 24h. Then, cells were infected with SARS-CoV-2-mNeongreen at an MOI of 0.05 for 1h, washed with PBS and added of media containing methylcellulose 0.8% and the Cu(Ipr)Cl (10 μ M), Cu(Imes)Cl (2 μ M), and Cu(Imes)₂BF₄ (2 μ M). After 48h, the wells were photographed using the IncuCyte S3 microscope. The quantity of green focus and its size were analysed in each image using ImageJ software.

RESULTS

Cu(I) and Cu(II) complexes show a selective profile in A549-AT and SAR-CoV-2 replication

Aiming to investigate the anti-SARS-CoV-2 activity of Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ complexes, A549-AT cells were infected with SARS-CoV-2-mNeongreen at a MOI of 0.1 at concentrations ranging from 0.58 μ M to 300 μ M, in a two-fold serial dilution. After 24h, the total integrated intensity of the fluorescence (GCU x μ m²/well) was analysed using the IncuCyte S3 microscope. Simultaneously, cytotoxicity of the complexes was also evaluated through a dose-response assay. The cell viability was assessed by MTT assay 24h later. DMSO was used as untreated control. The effective concentration of 50% (EC₅₀) and cytotoxic concentration of 50% (CC₅₀) were calculate employing a non-linear regression, and Selective Index (SI) was determed by $SI = CC_{50} / EC_{50}$. As a result, Cu(Ipr)Cl had an CC₅₀ and EC₅₀ of 43.1 μ M and 5.4 μ M, respectively, while Cu(Imes)Cl had an CC₅₀ and EC₅₀ of 12.3 μ M and 1.1 μ M, in this order (**Figure 2A, 2B and 2D**). For Cu(Imes)₂BF₄ the CC₅₀ and EC₅₀ were 2.2 μ M and 0.5 μ M (**Figure 2C and 2D**). The calculated SI for Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ were 8, 11.2, and 4.4, respectively (**Figure 2D**).

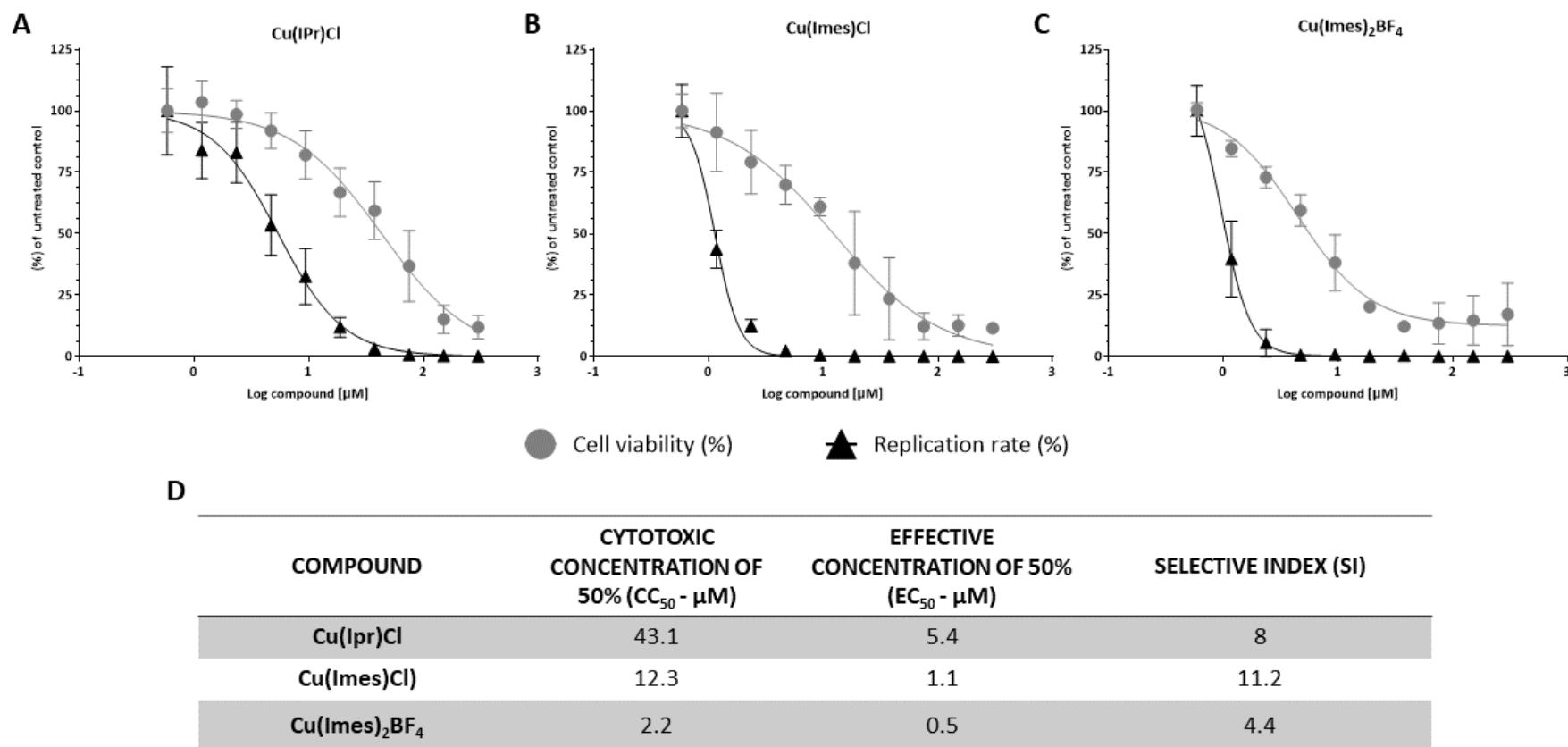


Figure 2. Dose-response curve for Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ treatment of A549-AT cells and SARS-CoV-2-mNeongreen replication. A549-AT cells were infected with SARS-CoV-2-mNeongreen at a MOI of 0.1 and treated with the Cu(Ipr)Cl (A), Cu(Imes)Cl (B), and Cu(Imes)₂BF₄ (C) at concentrations ranging from 0.58 μ M to 300 μ M, in a two-fold serial dilution. After 24h, the total integrated intensity of the fluorescence (GCU x μ m²/well) was analyzed using the IncuCyte S3 microscope. Cell viability was accessed treating A549-AT cells with the compounds the same concentrations. After treatment, the media with compound was removed and replaced with media with MTT at 1 mg/mL and incubated for 30 min. After incubation, the media was removed, and the crystals solubilized with DMSO. Surviving cells were measured by absorbance (570 nm). Mean values of two independent experiment each measured in quadruplicate including the standard deviation are shown.

Cu(Imes)Cl and Cu(Imes)₂BF₄ protects A549-AT cells against SARS-CoV-2 infection

To elucidate the effect of the complexes on SARS-CoV-2 replicative cycle, a time of drug addition assay was performed employing Cu(Ipr)Cl at 10 μ M, while Cu(Imes)Cl and Cu(Imes)₂BF₄ at 2 μ M. First the cells were pre-treated for 1h with each complex, followed by PBS washing and infection with the SARS-CoV-2-mNeonGreen (MOI of 0.1) for additional 1h (**Figure 3A**). Then cells were washed 3x with PBS to remove unbound viruses and fresh media was added to the cells. As an outcome, Cu(Imes)Cl and Cu(Imes)₂BF₄ pretreatment significantly decreased 99.9% and 87.7% of SARS-CoV-2-mNeonGreen infection ($p < 0.0001$), respectively (**Figure 3B**). On the other hand, Cu(Ipr)Cl did not show any protection effect (**Figure 3B**).

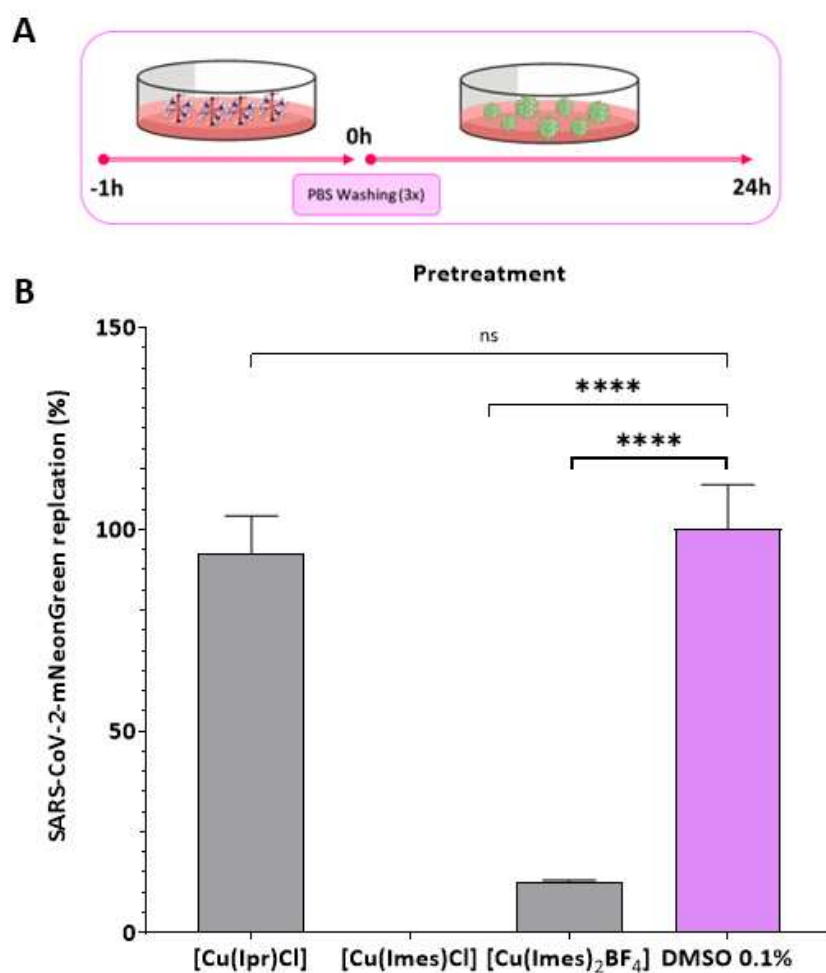


Figure 3. Cu(Imes)Cl and Cu(Imes)₂BF₄ protects A549-AT cells against SARS-CoV-2 infection. (A) A549-AT cells were treated with Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ at 10, 2, and 2 μ M, respectively, for 1 h. Then, cells were washed and infected with SARS-CoV-2-mNeonGreen at MOI 0.1. After 24h, the total integrated intensity of the fluorescence (GCU $\times$ μ m²/well) was analyzed using the IncuCyte S3 microscope. Schematic representation of each time-based assay as indicated by A549-AT cells (blue arrows), compounds (chemical structure), and SARS-CoV-2-mNeonGreen (green virus). Mean values $\pm$ SD of a minimum of three independent experiments each measured in quadruplicate. (ns) non-significant, (***) $P < 0.001$. All images were generated using GraphPad Prism 9 and GIMP 2.10.20 (www.gimp.org).

Copper complexes impair several stages of SARS-CoV-2 entry to the host cells

To evaluate the effects of Cu complexes on SARS-CoV-2-mNeonGreen entry to the host cells, virus (MOI of 1) and each complex were simultaneously added to A549-AT cells for 1 h at 37°C, cells were washed with PBS to remove the inoculum, and replaced with fresh media (**Figure 4A**). The Cu(Ipr)Cl inhibited 48.1% of SARS-CoV-2-mNeonGreen entry, while Cu(Imes)Cl and Cu(Imes)₂BF₄ inhibited 99.6% and 74% ($p < 0.0001$), respectively (**Figure 4B**). A modification of this protocol by including 1 h pre-incubation of the inoculum containing each complex and SARS-CoV-2-mNeonGreen (MOI of 5) at 37 °C, prior to the infection and treatment of the cells (**Figure 4C**), decreased fluorescence intensity in 58.5%, 100%, and 90.7% for Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄, respectively, indicating that these complexes also possess virucidal activity (**Figure 4D**).

To analyse the effect of Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ on SARS-CoV-2 attachment, virus (MOI of 1) and complexes were first incubated with the cells at 4 °C for 1 h. At this temperature, virus particles were able to attach to the cellular receptors, but not entry into the host cells. Cells were then washed with PBS to remove the inoculum, fresh media added, and incubated at 37 °C (**Figure 4E**), to allow the continuation of the entry process. Data obtained from this assay also showed strong inhibition SARS-CoV-2-mNeonGreen attachment by Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ by reducing virus entry by 67%, 99.4%, 72.5%, in this order ($p < 0.0001$) (**Figure 4F**). Post-attachment was evaluated by including an additional incubation of 30 min at 37°C to the previous protocol (**Figure 4G**), showing that the inhibition remained strong for Cu(Imes)Cl, reaching 99.9% ($p < 0.0001$), while Cu(Ipr)Cl and Cu(Imes)₂BF₄ presented similar levels (53.3% and 79.2%, respectively) (**Figure 4H**).

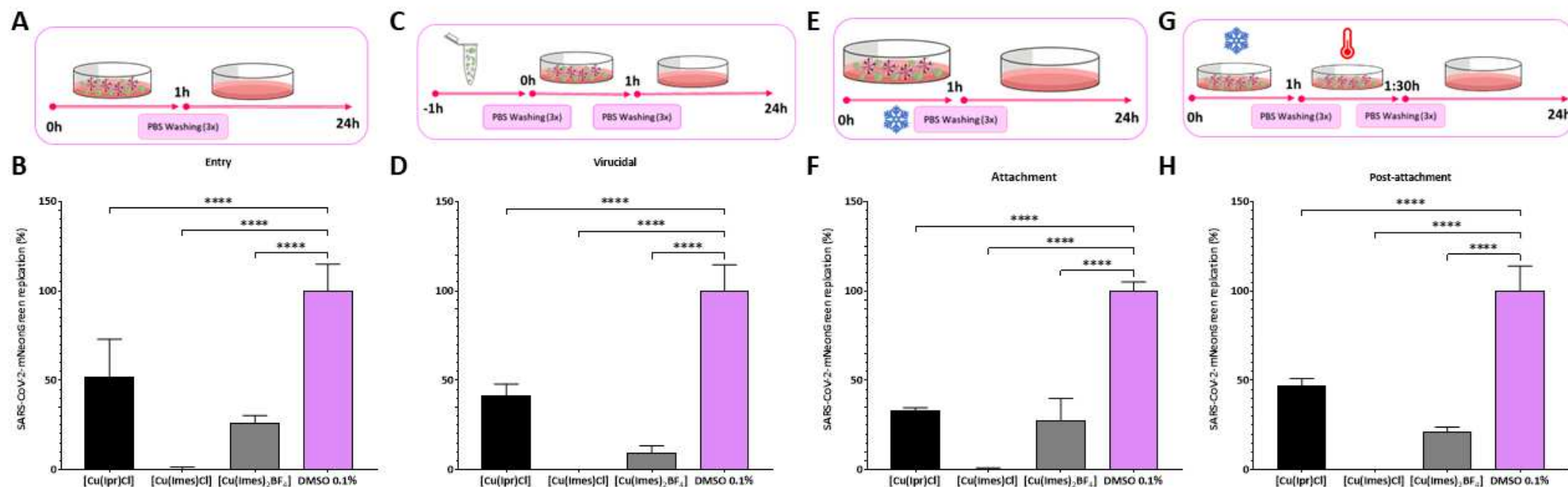


Figure 4. Copper complexes impair several stages of SARS-CoV-2 entry to the host cells. (A and B) A549-AT cells were infected with SARS-CoV-2-mNeonGreen (MOI 1) and simultaneously treated Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ at 10, 2, and 2 μ M, respectively, for 1 h. Cells were washed and replaced by fresh medium. (C and D) SARS-CoV-2-mNeonGreen (MOI 5) and each compound was incubated for 1 h at 37 °C and then for one extra hour with the cells. Then, virus and compound were removed, cells were washed with PBS, and added fresh medium. (E and F) A549-AT cells were infected with the virus and simultaneously treated with each compound for 1 h at 4 °C. The cells were washed to remove virus and compound and replaced with fresh medium. (G and H) A549-AT cells were infected with virus and simultaneously treated with the compounds for 1 h at 4 °C. Then, cells were incubated for additional 30 min with compound and virus at 37 °C, washed with PBS and replaced by fresh medium. For all assays, the total integrated intensity of the fluorescence (GCU $\times \mu$ m²/well) was analyzed using the InCuCyte S3 microscope. Schematic representation of each time-based assay as indicated by A549-AT cells (blue arrows), compounds (chemical structure), and SARS-CoV-2-mNeonGreen (green virus), SARS-CoV-2-mNeonGreen and compound inoculum (microtube), incubation at 4°C (ice crystal) and incubation at 37°C (thermometer). Mean values $\pm$ SD of a minimum of three independent experiments each measured in quadruplicate. (****) $P < 0.0001$. All images were generated using GraphPad Prism 9 and GIMP 2.10.20 (www.gimp.org).

Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ strongly impair post-entry stages of SARS-CoV-2 replication

To assess the effect of the complexes in post-entry steps of SARS-CoV-2, the cells were infected with SARS-CoV-2-mNeonGreen (MOI of 0.1) for 1h, or 6h for late-stage replication, and then fresh media containing each complex was added (**Figure 5A and 5B**). After 24h, all the complexes inhibited the viral replication in over 98% when added after 1h infection ($p < 0.0001$) (**Figure 5A and 5C**). Interestingly, when added 6h after viral infection (**Figure 5B**), the complexes were still able to maintain strong inhibition of SARS-CoV-2-mNeonGreen replication, being Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ capable of decreasing it in 95%, 99.9%, and 87.6%, respectively ($p < 0.0001$) (**Figure 5D**).

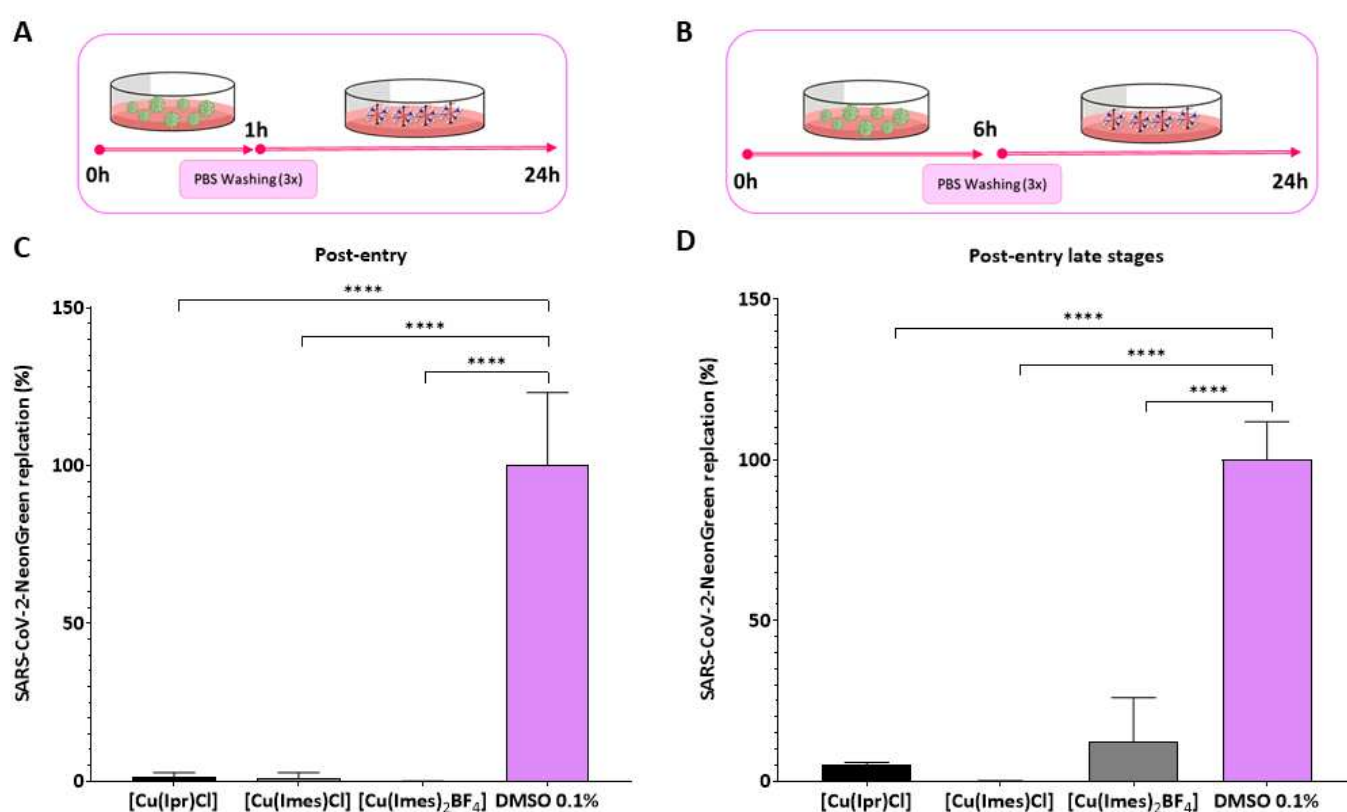


Figure 5. Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ strongly impair post-entry stages of SARS-CoV-2 replication. A549-AT cells were infected with SARS-CoV-2-mNeonGreen (MOI 0.1) for 1h (A) or for 6h (B), and then treated with each complex for the remaining 24h. Cells were washed and replaced by fresh medium. For all assays, the total integrated intensity of the fluorescence (GCU $\times \mu\text{m}^2/\text{well}$) was analyzed using the IncuCyte S3 microscope. Schematic representation of each time-based assay as indicated by A549-AT cells (blue arrows), compounds (chemical structure), and SARS-CoV-2-mNeonGreen (green virus). Mean values $\pm$ SD of a minimum of three independent experiments each measured in triplicate. (****) $P < 0.0001$. All images were generated using GraphPad Prism 9 and GIMP 2.10.20 (www.gimp.org).

Copper complexes possess a broad-spectrum activity against Delta (B.1.17.2) and Omicron (B.2) variants

Due to the remarkable effect of the complexes Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ on SARS-CoV-2 Wuhan strain, we evaluated their effect on the two most recently circulating and impactful variants of SARS-CoV-2, B.1.617.2 (delta) and B.2 (omicron). To that, an infectious clone of Wuhan strain carrying the spike genome sequence of the variants delta or omicron, and the MCherry gene (**Figure 6A, 1-3**), was produced through molecular cloning, and then used to infect the cells in the presence or absence of Cu(Ipr)Cl (10 μ M), Cu(Imes)Cl (2 μ M), and Cu(Imes)₂BF₄ (2 μ M) for 24h. As a result, Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ decreased viral replication in 61%, 94.6%, and 92.1% ($p < 0.0001$), respectively, when employed the SARS-CoV-2 Wuhan-delta-Spike (**Figure 6B**). Similarly, against SARS-CoV-2 Wuhan-omicron-Spike these complexes inhibited 69.8%, 99.7%, and 97.3% ($p < 0.0001$), in this order (**Figure 6C**).

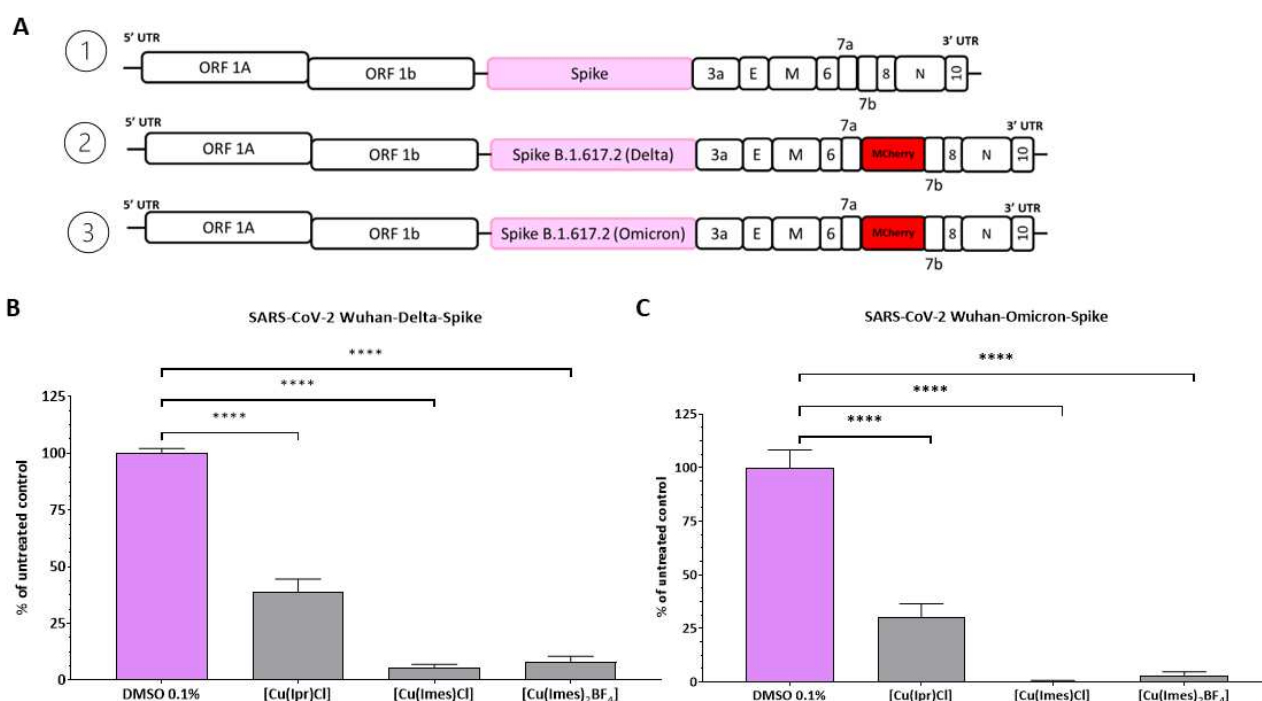


Figure 6. Effect of Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ against SARS-CoV-2-Wuhan-delta-Spike and SARS-CoV-2-Wuhan-omicron-Spike infectious clones replication (A) Schematic representation of the infectious clone genome, being the original sequence of Wuhan (NCBI: NC_045512.2) (1), SARS-CoV-2-Wuhan-delta-Spike in (2) and SARS-CoV-2-Wuhan-omicron-Spike (3), being the last two carries of the MCherry reporter gene. A549-AT cells were infected with SARS-CoV-2-Wuhan-delta-Spike (B) or SARS-CoV-2-Wuhan-omicron-Spike (C) at an MOI of 0.1 in the presence of each molecule for 24h. SARS-CoV-2 replication was measured by the total integrated intensity of the fluorescence (RCU $\times \mu\text{m}^2/\text{well}$) using the IncuCyte S3 microscope. All images were generated using GraphPad Prism 8 and GIMP 2.10.20. Mean values $\pm$ SD of a minimum of three independent experiments each measured in quadruplicate. (*) $P < 0.05$, (**) $P < 0.01$, (****) $P < 0.0001$.

We also evaluated the effect of Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄ against the SARS-CoV-2 variants delta and omicron. When the complexes were added to cells in the presence of each variant, the inhibitory profile of those viruses was different. Against the delta variant, Cu(Ipr)Cl decreased the viral titre in 0.5 Log₁₀, but with no statistical significance, while Cu(Imes)Cl and Cu(Imes)₂BF₄ significantly decreased titres in 3 Log₁₀ ($p < 0.001$) and 1 Log₁₀ ($p < 0.01$), respectively (**Figure 7A**). On the other hand, all compounds when evaluated against the omicron demonstrated capacity of decreasing significantly viral titres in 1.2 Log₁₀, 2.1 Log₁₀, and 2 Log₁₀ for Cu(Ipr)Cl, Cu(Imes)Cl, and Cu(Imes)₂BF₄, respectively (**Figure 7B**).

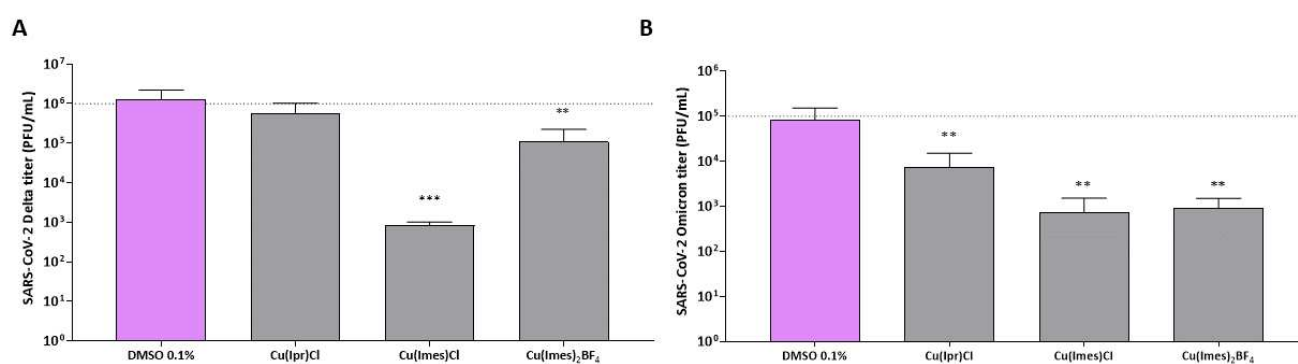


Figure 7. Effect of the Cu(I) and Cu(II) complexes against the wild-type SARS-CoV-2-B.1.617.2 and SARS-CoV-2-B.2 replication. A549-AT cells were infected with SARS-CoV-2-B.1.617.2 (A) or SARS-CoV-2-B.2 (B) at an MOI of 0.1 in the presence of each compound for 24h. Then, supernatant was collected and tittered by TCID₅₀. All images were generated using GraphPad Prism 8 and GIMP 2.10.20. Mean values $\pm$ SD of a minimum of three independent experiments each measured in replicate. (****) $P < 0.0001$.

SARS-CoV-2 cell to cell spread can be impaired by Cu(Imes)₂BF₄ treatment

The cell-to-cell spread was described as an important feature of SARS-CoV-2 dissemination and pathogenesis, and compounds that can inhibit it are widely required. Therefore, here we evaluated the SARS-CoV-2 cell to cell spread by the size of fluorescence focus on the presence of each compound, employing the SARS-CoV-2-mNeonGreen. To that, A549-AT cells were infected with virus at an MOI of 0.05 for 1h and then treated with each complex in the presence of methylcellulose 0.8%. After treatment, only Cu(Imes)₂BF₄ decreased size focus of infected cells in 0.08 μm^2 (control 0.30 μm^2 , while Cu(Imes)₂BF₄ treated 0.22 μm^2) (**Figure 8A and 8B**).

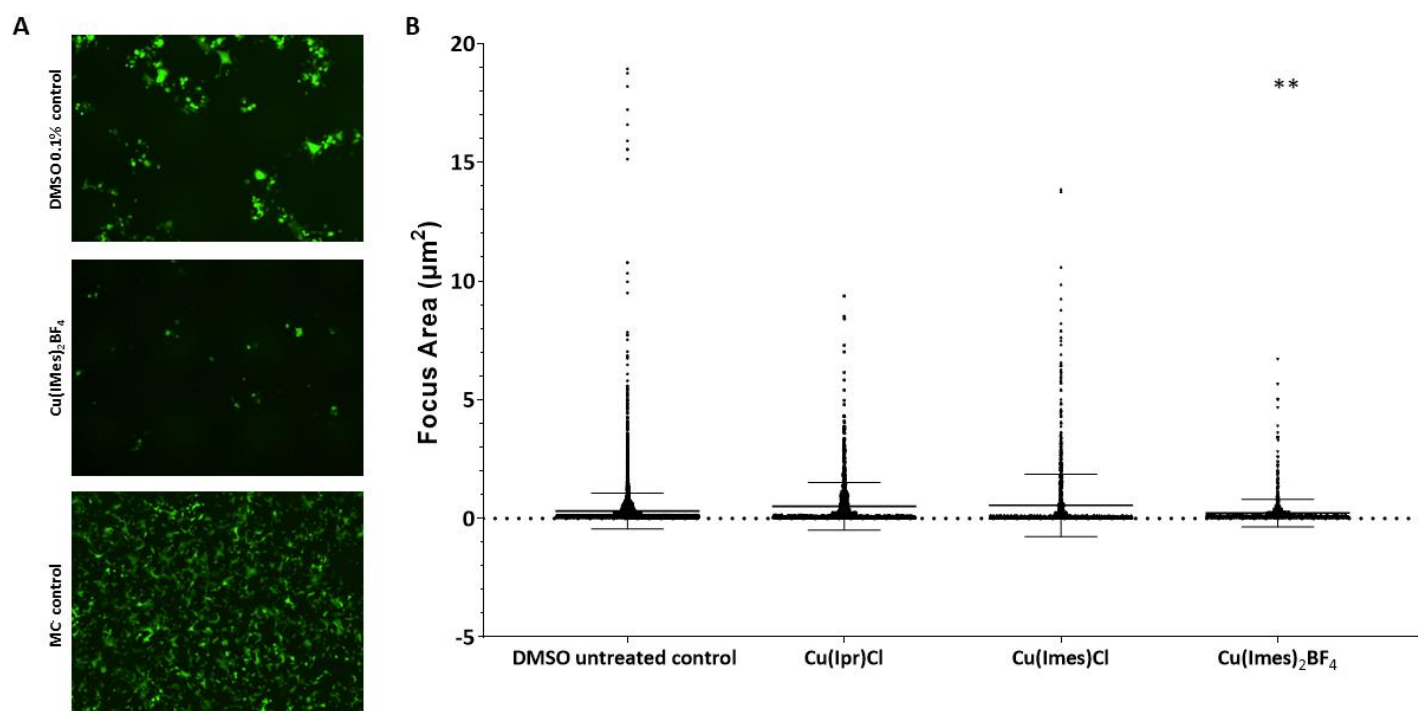


Figure 8. Cu(IMes)₂BF₄ decreases SARS-CoV-2 cell to cell spread. A549-AT cells were infected with SARS-CoV-2-mNeonGreen (MOI 0.05) for 1h, cells were washed, and replaced by fresh medium containing each compound and Methylcellulose 0.8% for the remaining 48h. The photos were taken by the IncuCyte S3 microscope in the objective 10x under the green fluorescence light and focus size analysis were performed in ImageJ. (A) Representative images of SARS-CoV-2-mNeonGreen infection in the presence of DMSO control, Cu(IMes)₂BF₄, and methylcellulose negative (MC⁻) control, in this order. (B) The average focus size in area (μm²) of three technical repeats is shown. (**) P < 0.01

DISCUSSION

The synthesis of Cu(Ipr)Cl, Cu(IMes)Cl, and Cu(IMes)₂BF₄ and their antiviral activity against CHIKV were previously described by our group, nevertheless, no assessment of the descriptive characteristics and potential of these novel metal complex species was conducted (FONTES et al., 2022). In this context, through different assays we identified here different cytotoxic and antiviral profile among these complexes. The Cu(Ipr)Cl, Cu(IMes)Cl, and Cu(IMes)₂BF₄ had selectivity indexes (SI) of 8, 11.2, and 4.4, respectively. The difference in SIs among these metallodrugs might be explained by their lipophilicity (BERTHOD; CARDABROCH, 2004; FONTES et al., 2022; GIAGINIS; TSOPELAS; TSANTILI-KAKOULIDOU, 2018). In this case, for Cu(Ipr)Cl it means that a better interaction with membranes and aqueous solution results in a improved cytotoxicity profile (lowest cytotoxicity) due to low lipoficity (FONTES et al., 2022; GIAGINIS; TSOPELAS; TSANTILI-KAKOULIDOU, 2018),

however, it might also resulted in lower potency of inhibition, due to the highest EC₅₀ among the molecules evaluated here. Alternatively, Cu(Imes)₂BF₄ high lipophilicity resulted in more active molecule (lowest EC₅₀), but less selective due to the higher cytotoxicity to cells. Additionally, Cu(Imes)₂BF₄ had a high cytotoxicity in BHK-21 and RAW cells, agreeing with the results described here (FONTES et al., 2022). What is more interesting, Cu(Imes)Cl was known to go under chemical speciation in solution, forming Cu(Imes)₂BF₄ in an chemical equilibrium (BERTHOD; CARDA-BROCH, 2004; FONTES et al., 2022; MEANWELL, 2018), which might result in the mixed hydrophilic/lipophilic and lower CC₅₀ than Cu(Ipr)Cl, however, also provided the highest SI among all compounds. This further confirms that these Cu complexes possess a close relationship among structure-activity. This fact is also reinforced by the remarkable activity on delta and omicron variants, in which probably are not related to the S glycoprotein, since the insertion of them in the Wuhan strain infectious clone did not cause any decrease in inhibition.

Furthermore, the metallodrug Cu(Ipr)Cl did not protect the cells against SARS-CoV-2 infection, but impaired SARS-CoV-2 entry (all steps over 65%), as well as its replication. Differently, both Cu(Imes)Cl and Cu(Imes)₂BF₄ were able to strongly impair all viral stages evaluated here, with an overall inhibition of over 70%, reaching the complete knockdown of virus infection (100% inhibition) for some protocols, specially under the treatment with Cu(Imes)Cl. The Cu ions are reactive oxygen species (ROS) inducing molecules in mammalian cells (ERXLEBEN, 2018; NOVELLO; STIRPE, 1969; SAGRIPANTI et al., 1997; SAGRIPANTI; ROUTSON; LYTLE, 1993; SHIONOIRI et al., 2012), and since other Cu(I) complexes were characterized as capable of speciation in biological systems, probably releasing the Cu(I) ions (DOS SANTOS OLIVEIRA et al., 2023), it is possible to hypothesize that the chemical speciation might also be related to their activity on viral replication cycle. The decreased capacity of chemical speciation of Cu(Ipr)Cl, with more stable molecule does not provide enough Cu(I) ions, that can result in ROS and/or other immune pathway activation, therefore agrees with the lack of protective activity (CAVICCHIOLI et al., 2019; CREAVEN et al., 2010; SCHIEBER; CHANDEL, 2014). In contrast, both Cu(Imes)Cl and Cu(Imes)₂BF₄ produces Cu(I) ions, resulting in an strong protective activity through that mechanism. Furthermore, the potent entry inhibition of Cu(I) complexes might also be related to the chemical speciation, since the coordination with Cu can result in molecules that can disrupt the viral envelope (GRASS; RENSING; SOLIOZ, 2011; SAGRIPANTI et al., 1997; SAGRIPANTI; ROUTSON; LYTLE, 1993). Another point to be considered is the high

lipophilicity of $\text{Cu}(\text{Imes})_2\text{BF}_4$, which makes it unlikely to be able to entry the cells as a complex, and might be interacting with viral envelope rather than impairing viral replication (SÁNCHEZ et al., 2016).

The strong post-entry capacity of all molecules also needs to be highlighted since it was able to even recover inhibition after SARS-CoV-2 replication complexes is established (KLEIN et al., 2020; V'KOVSKI et al., 2021b). Recently, silver coordinated to N-Heterocyclic carbene complexes were described as SARS-CoV-2 inhibitors with SI ranging between 3 to 100, and with a direct interaction with the SARS-CoV-2 PL^{pro} cysteine protease (GIL-MOLES et al., 2023). Firstly, it is important to emphasize that the SARS-CoV-2 infection was performed in an MOI 10 folds lower than the one used here on our dose response assay (GIL-MOLES et al., 2023). However, their data still supports the Cu-NHC organometallic drugs strong effect on viral post-entry, probably by impairing viral RNA replication, mainly for $\text{Cu}(\text{Ipr})\text{Cl}$ and $\text{Cu}(\text{Imes})\text{Cl}$ that are more likely to trespass cell membranes (GIL-MOLES et al., 2023; SÁNCHEZ et al., 2016). For $\text{Cu}(\text{Imes})_2\text{BF}_4$, the strong impairment of SARS-CoV-2 post-entry is probably related to the release of $\text{Cu}(\text{II})$ molecules that can influx through the cell membrane and interact with RNA (CHEN; SIGMAN, 1986; NOVELLO; STIRPE, 1969; WANG et al., 2021a). This is further sustained by the decrease in SARS-CoV-2-mNeonGreen cell to cell transmission in $\text{Cu}(\text{Imes})_2\text{BF}_4$ presence.

CONCLUSION

The data presented here advocates for the effects of Cu-NHC organometallic compounds as remarkable strong inhibitors of all stages of SARS-CoV-2 replicative cycle in vitro, effectively inhibiting not only Wuhan strain, but also the B.1.617.2 and B.2 variants, and also by the $\text{Cu}(\text{Imes})_2\text{BF}_4$ capacity of decreasing SARS-CoV-2 cell to cell spread. Based on these highly promising results, we strongly encourage further investigations involving Cu(I) complexes. Furthermore, we propose that all compounds to be considered for utilization in pre-clinical assays as a promising alternative for the treatment of COVID-19.

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**CAPÍTULO 4: COPPER(II)
BENZYLOXYCHALCONE ANALOGUES
AS NEW POTENTIAL METALLODRUGS
AGAINST SARS-CoV-2 REPLICATION**

COPPER(II) BENZYLOXYCHALCONE ANALOGUES AS NEW POTENTIAL METALLODRUGS AGAINST SARS-CoV-2 REPLICATION

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ABSTRACT

The chalcones are natural molecules found in a diversity of plants as a precursor and/or final products in biosynthesis of flavonoids, and were described by having anti-inflammatory, antitumoral, antimalarial, and antiviral activities through different mechanisms of action. The structure of chalcones enables them undergoing chemical manipulation making them attractive for metal coordination. Copper [Cu(II)] is an essential metal in dietary of living organisms that also possesses several biological activities. Our group recently described preliminary data on CuL₂phen and CuL₁phen against the SARS-CoV-2, based on 3-(4-(benzyloxy)phenyl)-1-(4-fluoro-2-hydroxyphenyl)prop-2-en-1-one (L₂) and 3-(4-(benzyloxy)phenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (L₁), respectively. Due to the previously reported activity of these complexes, here we further characterize CuL₂phen and CuL₁phen activity on SARS-CoV-2 replication cycle. The selectivity index (SI) presented by these complexes were 5.3 and 1.7, in this order. CuL₂phen presented itself as more attractive compound, being able to impair entry in overall 65% (entry: 71.3%; virucidal: 56.6%; attachment: 63.2%; post-attachment: 64.3%) and, mainly, post-entry steps with an inhibition of 99.9%, even when later stages of SARS-CoV-2 was analysed. What is more, CuL₂phen had a strong interaction with SARS-CoV-2 dsRNA, suggesting that its main mode of action is by interrupting RNA replication through specifically intercalating to the dsRNA. The obtained results show the activity of promising Cu(II) metallodrugs with potential application for the treatment of COVID-19 infections.

Keywords: Antivirals, Cu(II) complexes, COVID-19, chalcones, SARS-CoV-2,

INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its disease, Coronavirus disease 2019 (COVID-19), continues to burden health systems and impact social and economic aspects in several countries around the world (LAMERS; HAAGMANS, 2022; LIPPI; MATTIUZZI; HENRY, 2022; SUN et al., 2022; W.H.O., 2023b). Up to date, over 771 million confirmed cases and a staggering 6.9 million COVID-19-related fatalities were reported globally, being 37.7 million cases and of 704,000 deaths reported in Brazil (W.H.O., 2023c). After vaccination, the number of severe cases and deaths decreased (W.H.O., 2023a), however, the emergence of new variants continues to facilitate virus spread (LIPPI; MATTIUZZI; HENRY, 2022; W.H.O., 2023b). Aggravatingly, SARS-CoV-2 infected patients are developing the post-COVID syndrome which is characterized by long lasting symptoms that can last for years after infection (MALTEZOU; PAVLI; TSAKRIS, 2021b, 2021a; ORONSKY et al., 2021; YONG, 2021).

So far, there are three antivirals approved for regulatory agencies to treat COVID-19 patients: Remdesivir, Molnupiravir, and Paxlovid® (BEIGEL et al., 2020; FISCHER et al., 2022; JAYK BERNAL et al., 2022a; SAÚDE., 2022). However, these drugs are only administered to severe COVID-19 cases, being the clinical management of mild to moderate patients related to controlling symptoms with analgesics and anti-inflammatory drugs (BARTOLETTI et al., 2022; VARGHESE et al., 2020). In this context, the lack of direct acting antivirals and the restriction of treatment to severe cases potentially lead the untreated patients to be more susceptible in developing the post-COVID syndrome (MALTEZOU; PAVLI; TSAKRIS, 2021b; ORONSKY et al., 2021; YONG, 2021).

Chalcones are natural molecules found in a diversity of plants as a precursor and/or final products in the biosynthesis of flavonoids (NASIR ABBAS BUKHARI; JASAMAI; JANTAN, 2012). These molecules can be synthesized in large scales in laboratories and can also be easily modified in synthetic derivatives (ELKHALIFA et al., 2021; RANI et al., 2019). Their basic structure consists in two aromatic rings with three-carbon chain linking them to form a linear or planar skeleton (ELKHALIFA et al., 2021). The chalcones were described having anti-inflammatory, antitumoral, antimalarial, and antiviral activities through different mechanisms of action (BOUMENDJEL; RONOT; BOUTONNAT, 2009; DE MELLO et al., 2014; MATEEVA et al., 2017; NIELSEN et al., 2004; NOWAKOWSKA, 2007; RANI et al., 2019). Due to their previous reported

activity and possibility of undergoing chemical manipulation, the chalcones are attractive compounds for metal coordination.

The metal coordination is the association of organic or inorganic molecules with metal ion such as copper (Cu), platinum (Pt), palladium (Pd), and silver (Ag), among others (BARRY; SADLER, 2013; SANTINI et al., 2014). What is more interesting, when combined with specific classes of ligands, these new metal compounds are able to address the multidimensionality of several diseases, such as cancer (ALVAREZ et al., 2018; FLOREA; BÜSSELBERG, 2011), Alzheimer (GOMES; BATAGLIOLI; STORR, 2020), viruses (DE PAIVA et al., 2020a; IKOTUN et al., 2010), and bacterial infection (BERGAMINI et al., 2012; DOS SANTOS PEREIRA et al., 2019). This approach originated from the discovery of the anticancer activity of cisplatin, or cis-diaminodichloridoplatin(II), about 50 years ago (FLOREA; BÜSSELBERG, 2011; LI et al., 2019). Since then, countless metallic complexes of Pt, gold, ruthenium, Ag, and Cu, have been synthesized and evaluated for their potential for anticancer effect, as well as for other diseases (DE PAIVA et al., 2020a).

Among the metal ions of interest, Cu(II) is an essential metal in dietary of living organisms, with the capacity of boosting host immune system (GRASS; RENSING; SOLIOZ, 2011; KARLIN, 1993), and present antiviral, antifungal, and antibacterial activity by itself as a salt (BELAID et al., 2008; CHEN et al., 2017; CREAVER et al., 2010; KARLIN, 1993). The Cu(II) can act mainly in through damaging viral envelope (SAGRIPANTI et al., 1997; SAGRIPANTI; ROUTSON; LYTLE, 1993), generating reactive oxygen species, or interacting with DNA or RNA of the virus (ERXLEBEN, 2018; NOVELLO; STIRPE, 1969; SAGRIPANTI et al., 1997; SAGRIPANTI; ROUTSON; LYTLE, 1993; SHIONOIRI et al., 2012). With that in mind, our group recently evaluated the effect of Cu(II), the chalcones 3-(4-(benzyloxy)phenyl)-1-(4-fluoro-2-hydroxyphenyl)prop-2-en-1-one as L₁ and 3-(4-(benzyloxy)phenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one as L₂, and their complexes CuL₂phen and CuL₁phen against the SARS-CoV-2 (DOS SANTOS OLIVEIRA et al., 2023). As a remarkable result, the coordination with Cu(II) hindered two active compounds, while their precursors were not active against this virus (DOS SANTOS OLIVEIRA et al., 2023).

Here we further characterized the activity of these new Cu(II) complexes CuL₂phen and CuL₁phen against SARS-CoV-2, evaluated their action on different

stages of virus replicative cycle, as well as suggest one potential mode of action for these complexes.

METHODS

CELL CULTURE AND COMPOUNDS

The Adenocarcinoma human alveolar basal epithelial cells expressing the receptors ACE2 and TRPMSS2 (A549-ACE2-TRPMSS2 or A549-AT, ATCC#CCL-185) were cultivated as previous described (DOS SANTOS OLIVEIRA et al., 2023), using Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 100 U/mL penicillin (Gibco Life Technologies), 100 mg/mL streptomycin (Gibco Life Technologies), 1% (v/v) non-essential amino acids (Gibco Life Technologies) and 10% (v/v) fetal bovine serum (FBS; Hyclone) at 37 °C in a humidified 5% CO₂ incubator. The cells were cultivated in the presence of Geneticin (G418) and Hygromycin B at 1 mg/mL and 200 µg/mL, respectively.

The metal complexes CuL₂phen and CuL₁phen (**Figure 1**) were synthesized as previously described (DOS SANTOS OLIVEIRA et al., 2023), where L₂ is [3-(4-(benzyloxy)phenyl)-1-(4-fluoro-2-hydroxyphenyl)prop-2-en-1-one and L₁ 3-(4-(benzyloxy)phenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one. All the compounds were dissolved in dimethyl sulfoxide (DMSO) prior to the assays and stored in -20°C for maximum of two days and were diluted in cell culture media immediately prior the assays to a final concentration of 0.1%.

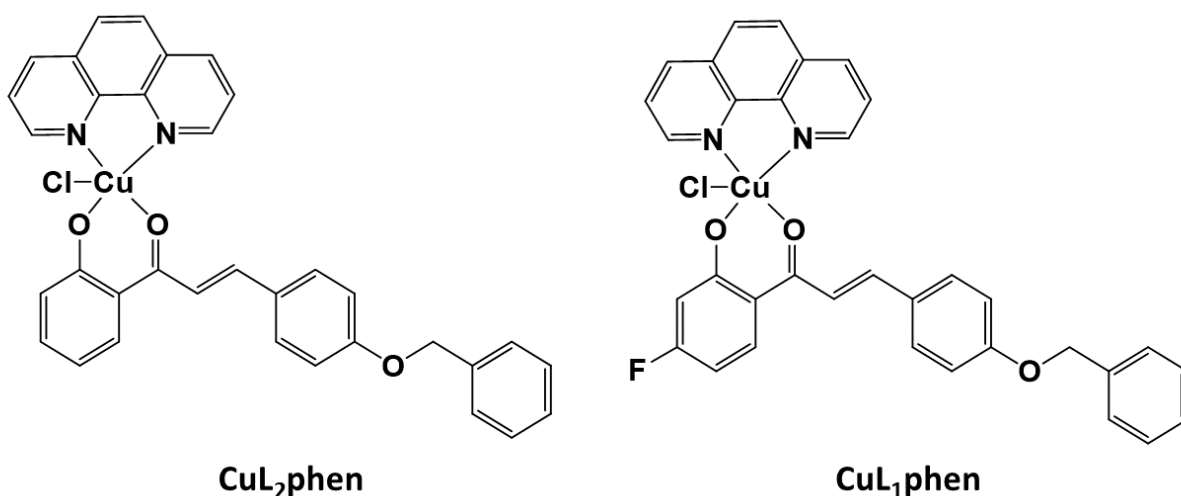


Figure 1: Chemical structure of CuL₂phen and CuL₁phen (DOS SANTOS OLIVEIRA et al., 2023).

VIRUSES

The B.1.617.2 - Delta (MS066352H) and B.2 – Omicron (hCoV/England/FCI-179/2022) viruses were isolated and characterized by The Francis Crick Institute, and kindly provided by Prof. Griffin in the University of Leeds. The viruses were amplified by infecting Vero E6-ACE2 cells in 175 cm² flask until 80% of cytopathic effect. Supernatant was collected and the infectious titer was determined using the 50% tissue culture infectious dose (TCID₅₀) method and calculated by the Spearman & Kärber algorithm as described by Killington and Hierholzer (KILLINGTON; HIERHOLZER, 1996).

RESCUE OF INFECTIOUS VIRUS

The rescue of SARS-CoV-2 reporter virus based on the NCBI sequence NC_045512.2 was performed as previous described (DOS SANTOS OLIVEIRA et al., 2023). Briefly, a total of 1 µg of the infectious clone pCCI-4K-SARS-CoV-2-mNeonGreen under the CMV promoter (RIHN et al., 2021) was transfected into BHK-21 (3x10⁵ cells/ well in a 6-well plate) using the lipofectamine 2000 (1:1). After 3 days, the supernatant was collected and transferred to A549-AT cells in a T75 flask until completely cell lysis, and the supernatant was harvested as P0 stock. The infectious titer was determined using the 50% tissue culture infectious dose (TCID₅₀) method and calculated by the Spearman & Kärber algorithm as described by Killington and Hierholzer (KILLINGTON; HIERHOLZER, 1996).

DOSE-RESPONSE ASSAY

To evaluate the cytotoxicity and activity profiles of CuL₂phen and CuL₁phen on cell viability and viral replication, A549-AT cells were seeded at a density of 1x10⁴ cells per well into 96-well plates 24 h prior to the infection. Then, cells were treated with each complex in a two-fold serial dilution, with concentrations ranging between 0.58 to 300 µg/mL, in the presence or absence of SARS-CoV-2-mNeonGreen in a MOI of 0.1 (RIHN et al., 2021). Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich) assay, as previous described (DOS SANTOS OLIVEIRA et al., 2023). After treatment, media containing compound was removed from and MTT at 1 mg/mL solution was added to each well, incubated for 30

minutes and replaced with 100 μ L of DMSO to solubilize the formazan crystals. The absorbance was measured at 570 nm on FLUOstar OPTIMA microplate reader (BMGLabTech).

For the antiviral assays, the plates were placed on the IncuCyte® S3 Live-Cell Analysis System (Sartorius) after 24h, and the green fluorescence light was observed at 10x objective. The photos were analysed employing the basic analyser from the IncuCyte S3 system and the total integrated intensity of the fluorescence ($\text{GCU} \times \mu\text{m}^2/\text{well}$) was collected.

Cell viability and viral replication were calculated according to the equation $(T/C) \times 100\%$, which T and C represented the optical density of the treated well and control groups, respectively. DMSO was used as untreated control. The effective concentration of 50% (EC_{50}) and cytotoxic concentration of 50% (CC_{50}) were calculate employing a non-linear regression. Selective Index (SI) was calculated by the equation $\text{SI} = (\text{CC}_{50}/\text{EC}_{50})$

TIME OF DRUG ADDITION ASSAYS

In all time of drug addition experimental assessments, A549-AT cells was seeded in 96-well plates at a concentration of 1×10^4 cells per well, with this seeding occurring 24 hours prior to the infection. Treatment was carried out with CuL₂phen at 2 μ M, and the extent of viral replication was quantified based on the intensity of green fluorescence when observed through a 10x objective lens. The acquired images were subsequently subjected to analysis using the basic analyzer integrated within the IncuCyte S3 system, which calculated the total integrated fluorescence intensity, measured in $\text{GCU} \times \mu\text{m}^2/\text{well}$.

In the pretreatment assay, cells were treated for 1 hour with the complexes before being infected with the SARS-CoV-2-mNeonGreen at a multiplicity of infection (MOI) of 0.1. Subsequently, the cells were thoroughly washed with phosphate-buffered saline (PBS), and the unbound virus was removed by incubation with fresh media for 24 hours (**Figure 3A**).

In the entry inhibition assay, cells were infected (MOI of 1) and simultaneously treated with the complexes for 1 hour. After that, cells were washed with PBS and then incubated with fresh media for 24 hours (**Figure 4A**). To evaluate virucidal activity, a

similar approach was used, except that the complex and virus mixture (MOI of 5) was incubated for 1 hour before being added to the cells (**Figure 4C**).

To investigate the impact of the compound on the attachment step of the viral infection, the same setting as in entry inhibition assay was employed, wherein the cells were incubated with the virus (MOI of 1) and the compound at 4°C (**Figure 4E**). Another variation of this protocol was performed by incubating the cells with the virus at 4°C for 1 hour and then at 37°C for a 30-minute incubation to assess the effect of the compound on post-attachment steps of infection (**Figure 4G**).

In the post-entry assay, cells were infected with the SARS-CoV-2-mNeonGreen virus at an MOI of 0.1 for 1 hour, followed by thorough washing with PBS. Subsequently, they were incubated in a media-containing the complex for 24 hours (**Figure 5A**). Additionally, a variation of this assay involved infecting the cells with the virus at an MOI of 0.1 for 6 hours, and then treating the infected cells with the complex for the remaining duration, to evaluate its effects after the establishment of viral replication, here described as late stages of viral replication (**Figure 5B**).

BROAD-SPECTRUM AGAINST SARS-COV-2 VARIANTS

To evaluate the impact of CuL₂phen on the Delta and Omicron variants of SARS-CoV-2, an infectious clone originated from the SARS-CoV-2 Wuhan strain, which includes a MCherry reporter gene (**Figure 6A**), was genetically modified through molecular cloning. This modification involved the insertion of the spike gene sequences derived from the B.1.617.2 (Delta) and B.2 (Omicron) variants (**Figure 6A, 2 and 3**). The rescue of these genetically altered viruses followed the protocols previously mentioned in this section. For antiviral screening purposes, CuL₂phen at 2 µM was added to the culture media-containing the Wuhan-Delta-Spike or Wuhan-Omicron-Spike viruses at a multiplicity of infection (MOI) of 0.1. This solution was then transferred to the cells for a duration of 24 hours. The quantification of viral replication was conducted by measuring the total integrated fluorescence intensity, expressed as RCU x µm²/well, employing the IncuCyte S3 microscope.

For the antiviral assays employing the wild-type viruses B.1.617.2 - Delta (MS066352H) and B.2 – Omicron (hCoV/England/FCI-179/2022), A549-AT cells were cultured at a density of 8x10⁵ cells in 12 well plates and infected with each virus at a MOI of 0.1 in the presence or absence of CuL₂phen at 2 µM for 24h. Following, the supernatant

was collected, and the infectious titre was determined using the 50% tissue culture infectious dose (TCID₅₀) method, with calculations carried out via the Spearman & Kärber algorithm, as detailed by Killington and Hierholzer (KILLINGTON; HIERHOLZER, 1996).

CUL₂PHEN INTERACTION ASSAY WITH SARS-CoV-2 dsRNA

To investigate the potential of CuL₂phen to interact with dsRNA, a migration retardation assay was performed based on previously described protocols (CAMPOS et al., 2017; KRAWCZYK et al., 2009; SANTOS et al., 2022), with modifications. Through PCR, the SARS-CoV-2 ORF1a was amplified employing primers flanked by a T7 promoter site (forward: TAATACGACTCACTATAGGGGACCGAAAGGTAAGATGGAG; reverse: TAATACGACTCACTATAGGGGAAATCGCCCGTCTGCCATGAAG; T7 promoter regions are underlined). The reaction product of 499 bp was purified by Monarch® PCR & DNA Cleanup Kit (New England BioLabs®) and used for in vitro transcription by the HiScribe™ T7 High Yield RNA Synthesis Kit (New England BioLabs®). The dsRNA molecule was obtained by complementary annealing in annealing buffer (Potassium acetate 2M, Hepes-KOH 1M, and Magnesium acetate 1M) at 95°C for 1 min. The SARS-CoV-2 dsRNA was quantified using Nanodrop (ThermoScientific) and incubated at 65 nM with CuL₂phen at 2µM for 45 min, and then samples were analyzed in 1% agarose 1X TAE gel stained with SYBR™ Safe DNA Gel Stain (Invitrogen). The lack or decrease of band intensity in the agarose gel suggests the compound interaction activity since it competes with SYBR. *cis*-[PtCl₂(dmsO)₂] (Pt, 10 µM) were used as a positive control of interaction (SANTOS et al., 2022). The band quantification was performed using ImageJ.JS version 1.53j.

STATISTICAL ANALYSIS

Antiviral assays were performed a minimum of three technical repeats in quadruplicate to confirm the reproducibility of the results. Differences between means of readings were compared using analysis of variance (One-way or Two-way ANOVA) and Student *t*-test. *p* values of <0.05 (indicated by asterisks) were considered statistically significant. For the establishment of EC₅₀ and CC₅₀ values, the data were transformed into Log(X), where X is the concentration, and submitted into a non-linear regression

with four parameters in variable slope. All analyses were performed using GraphPad Prism 9.

RESULTS

CuL₂phen possesses a higher selective index against SARS-CoV-2 replication

Since our group recently described preliminary data on the anti-SARS-CoV-2 activity of CuL₂phen and CuL₁phen (DOS SANTOS OLIVEIRA et al., 2023), here we aimed to further investigate their cytotoxicity and antiviral profiles. To that, the A549-AT cells treated with CuL₂phen and CuL₁phen at concentrations ranging from 0.58 μ M to 300 μ M in a two-serial dilution were infected with SARS-CoV-2-mNeongreen at a multiplicity of infection (MOI) of 0.1. After 24h, viral replication and cytotoxicity were accessed by the fluorescence intensity and MTT assay, respectively. The effective concentration of 50% (EC₅₀) and cytotoxic concentration of 50% (CC₅₀) were calculate employing a non-linear regression. As an outcome, CuL₂phen had an CC₅₀ of 9.5 μ M and EC₅₀ of 1.8 μ M, resulting in a selectivity index (SI) of 5.3 (**Figure 2A and 2C**). On the other hand, CuL₁phen had an CC₅₀ and EC₅₀ of 2.6 μ M and 1.5 μ M, respectively, with a SI of 1.7 (**Figure 2B and 2C**). Considering the lower SI of CuL₂phen, further assays were only conducted with CuL₂phen at 2 μ M.

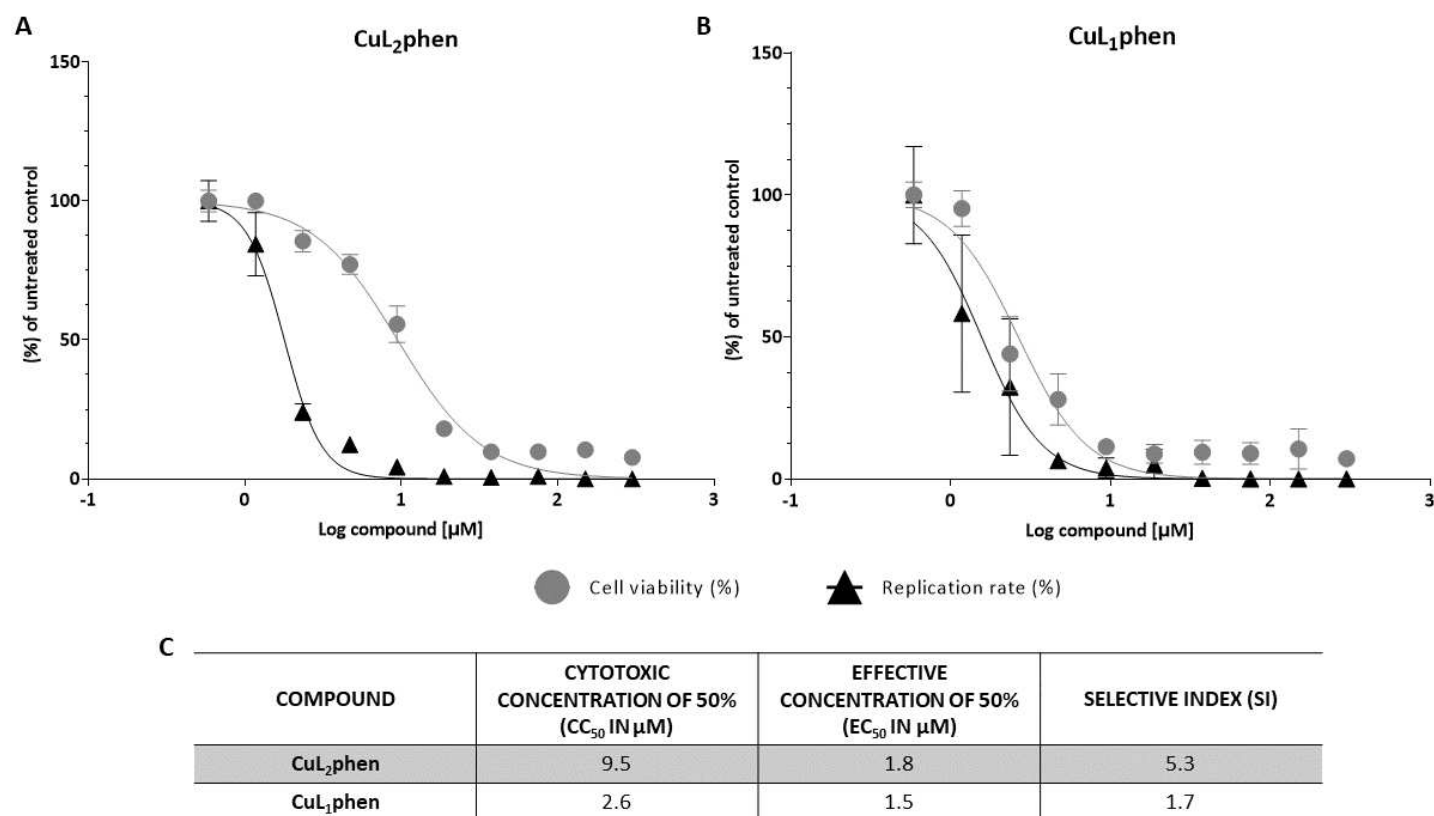


Figure 2: Dose-response curve of CuL₂phen and CuL₁phen on A549-AT cells and SARS-CoV-2-mNeongreen replication. A549-AT cells were infected with SARS-CoV-2-mNeongreen at a MOI of 0.1 and treated with the CuL₂phen (A) or CuL₁phen (B) at concentrations ranging from 0.58 μ M to 300 μ M, in a two-fold serial dilution. After 24h, the total integrated the intensity of the fluorescence (GCU x μ m²/well) was analyzed using the IncuCyte S3 microscope. Cell viability was accessed treating A549-AT cells with the compounds the same concentrations. After treatment, the media with compound was removed and replaced with media with MTT at 1 mg/mL and incubated for 30 min. After incubation, media was removed, and the crystals solubilized with DMSO. Surviving cells were measured by absorbance (570 nm). Mean values of two independent experiment each measured in quadruplicate including the standard deviation are shown. The effective concentration of 50% (EC₅₀), cytotoxic concentration of 50% (CC₅₀), and selectivity index (SI) are shown in (C).

A549-AT cells are protected against SARS-CoV-2 infection by CuL₂phen treatment

To investigate the CuL₂phen impact on the replicative cycle of SARS-CoV-2, a time-of-drug-addition assay was conducted. First, to assess its protective effects against infection, A549-AT cells were treated for 1h with CuL₂phen, washed with PBS to remove media containing the complex, and infected with SARS-CoV-2-mNeonGreen at an MOI of 0.1. After 1h, cells were washed 3x with PBS and replaced with fresh media (**Figure 3A**). As a result, CuL₂phen significantly protected cells against SARS-CoV-2 infection, decreasing fluorescence levels in 25.6% (**Figure 3B**, $p < 0.0086$).

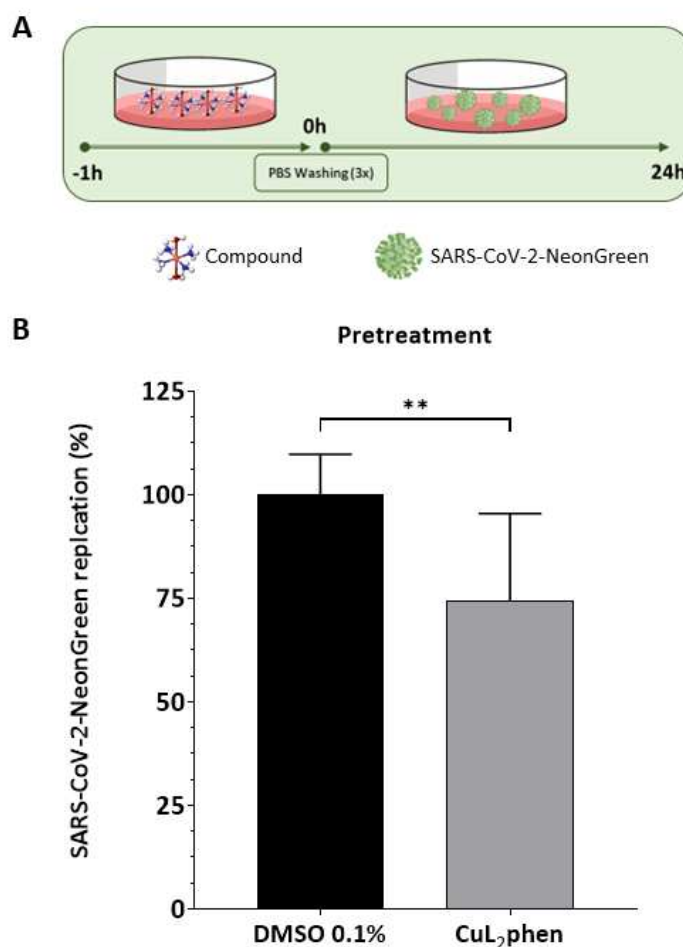


Figure 3: CuL₂phen protects cells against SARS-CoV-2 infection. (A) A549-AT cells were treated with CuL₂phen at 2 μ M for 1 h. Then, cells were washed and infected with SARS-CoV-2-mNeonGreen at MOI 0.1. After 24h, the total integrated intensity of the fluorescence (GCU $\times$ μ m²/well) was analyzed using the IncuCyte S3 microscope. Schematic representation of each time-based assay as indicated by A549-AT cells (blue arrows), compound (chemical structure), and SARS-CoV-2-mNeonGreen (green virus). Mean values $\pm$ SD of a minimum of three independent experiments each measured in quadruplicate. (**) $P < 0.01$. All images were generated using GraphPad Prism 9 and GIMP 2.10.20 (www.gimp.org).

CuL₂phen impairs SARS-CoV-2 entry into host cells

To evaluate the effects of CuL₂phen in SARS-CoV-2 entry into A549-AT cells, first SARS-CoV-2-mNeonGreen at an MOI of 0.1 and CuL₂phen was simultaneously added to the cells for 1h at 37°C, then cells were washed with PBS and added of fresh media (**Figure 4A**). The CuL₂phen was able to inhibit viral entry in 71.3% (**Figure 4B**, $p < 0.0001$). When this treatment was combined with an additional 1-hour pre-incubation of the inoculum, containing CuL₂phen and SARS-CoV-2-mNeonGreen in an MOI of 5 at 37 °C (**Figure 4C**), it led to a noteworthy decrease of 56.6% in viral infection (**Figure 4D**, $p = 0.0002$).

To assess the impact of CuL₂phen on the attachment stage of SARS-CoV-2 entry, the virus in a MOI of 1 and the compound were initially co-incubated with host cells at 4°C for 1 hour. At this lower temperature, the virus particles are capable of attaching to cellular receptors, but they are not able to enter the host cells. Subsequently, the cells were subjected to PBS washing, fresh media was added, and the temperature was raised to 37°C, allowing the continuation of the entry process (**Figure 4E**). The data derived from this experiment revealed significant inhibitory effects of CuL₂phen with inhibition of 63.2% (**Figure 4F**, $p < 0.0001$). To further investigate post-attachment events, an additional 30-minute incubation at 37°C was incorporated into the prior protocol (**Figure 4G**). This extended incubation period demonstrated sustained strong inhibition of 64.3% for CuL₂phen (**Figure 4H**, $p < 0.0001$).

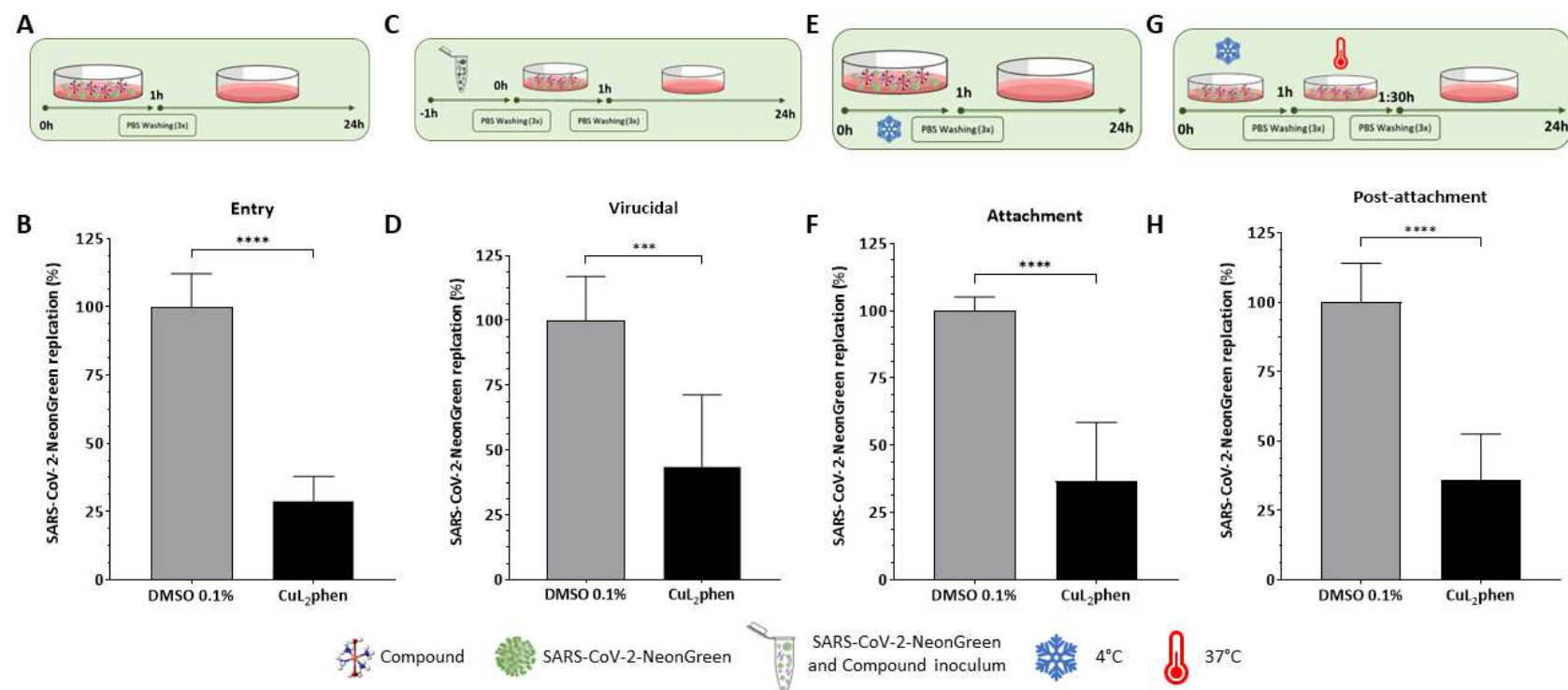


Figure 4: CuL₂phen have virucidal activity and blocks SARS-CoV-2 entry to the host cells. (A and B) A549-AT cells were infected with SARS-CoV-2-mNeonGreen (MOI 1) and simultaneously treated with CuL₂phen at 2 μ M for 1 h. Cells were washed and replaced by fresh media. (C and D) SARS-CoV-2-mNeonGreen (MOI 5) and the compound was incubated for 1 h at 37 °C and then for one extra hour with the cells. Then, virus and compound were removed, cells were washed with PBS, and added fresh media. (E and F) A549-AT cells were infected with the virus and simultaneously treated with each compound for 1 h at 4 °C. The cells were washed to remove virus and compound and replaced with fresh media. (G and H) A549-AT cells were infected with virus and simultaneously treated with the compound for 1 h at 4 °C. Then, cells were incubated for additional 30 min with compound and virus at 37 °C, washed with PBS and replaced by fresh media. For all assays, the total integrated intensity of the fluorescence (GCU x μ m²/well) was analyzed using the IncuCyte S3 microscope. Schematic representation of each time-based assay as indicated by A549-AT cells (blue arrows), compound (chemical structure), and SARS-CoV-2-mNeonGreen (green virus), SARS-CoV-2-mNeonGreen and compound inoculum (microtube), incubation at 4°C (ice crystal) and incubation at 37°C (thermometer). Mean values $\pm$ SD of a minimum of three independent experiments each measured in quadruplicate. (***) $P < 0.001$, (****) $P < 0.0001$. All images were generated using GraphPad Prism 9 and GIMP 2.10.20 (www.gimp.org).

Post-entry stages of SARS-CoV-2 infection is strongly inhibited by CuL₂phen

The effect of CuL₂phen on post-entry steps of SARS-CoV-2 infection, and consequently, in the RNA replication and translation, was assessed by infecting cells with SARS-CoV-2-mNeonGreen (MOI of 0.1) for 1h (**Figure 5A**), or 6h for late stage of replication (**Figure 5B**), and then the media containing the compound was added. As an outcome, CuL₂phen inhibited 99.6% of SARS-CoV-2-mNeonGreen when treated right after 1h infection (**Figure 5C**, $p < 0.0001$), and was still able to recover inhibition when added 6h after viral infection, decreasing the replication in 91% (**Figure 5D**, $p < 0.0001$).

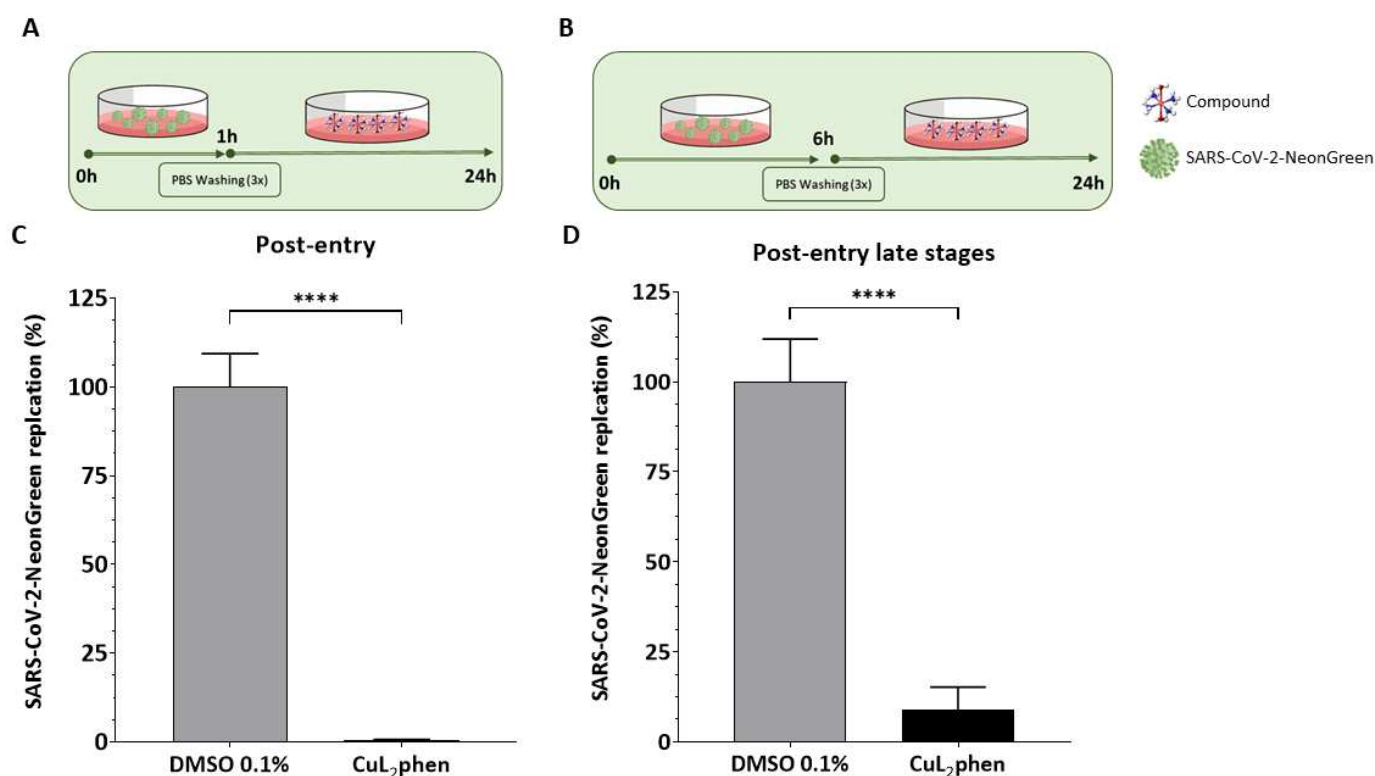


Figure 5: CuL₂phen impaired post-entry stages of SARS-CoV-2 replication. A549-AT cells were infected with SARS-CoV-2-mNeonGreen (MOI 1) for 1h (A) or for 6h (B) and then treated with CuL₂phen at 2 μ M for the remaining 24h. Cells were washed and replaced by fresh media. For all assays, the total integrated intensity of the fluorescence (GCU $\times \mu$ m²/well) was analyzed using the IncuCyte S3 microscope. Schematic representation of each time-based assay as indicated by A549-AT cells (blue arrows), compound (chemical structure), and SARS-CoV-2-mNeonGreen (green virus). Mean values $\pm$ SD of a minimum of three independent experiments each measured in triplicate. (ns) non-significant, (****) $P < 0.0001$. All images were generated using GraphPad Prism 9 and GIMP 2.10.20 (www.gimp.org).

Insights on the mode of action: CuL₂phen interacts with SARS-CoV-2 dsRNA

To further investigate the mode of action of CuL₂phen, a dsRNA interaction assay was carried out since dsRNA is essential to positive sense RNA viruses replication, such as SARS-

CoV-2 (V'KOVSKI et al., 2021b). Therefore, employing the ORF1a region of SARS-CoV-2 as a template, an amplicon flanked by a T7 promoter was produced and used for *in vitro* transcription, synthesizing a dsRNA molecule of 499 bp. Sixty-five nanomoles of dsRNA was incubated with CuL₂phen or with the controls (dimethyl sulfoxide 0.1% and *cis*-[PtCl₂(*dms*o)₂] at 10 μM). Through a migration retardation assay, it was found that CuL₂phen reduced 55% of the band intensity in the gel when compared to the negative control DMSO, also quantified by densitometry (**Figure 6**), suggesting CuL₂phen interaction with dsRNA. It is worth noting that no band can be observed in the gel for the sample treated with *cis*-[PtCl₂(*dms*o)₂], which serves as a positive control for dsRNA interaction (**Figure 6**). This result further supports the antiviral findings obtained.

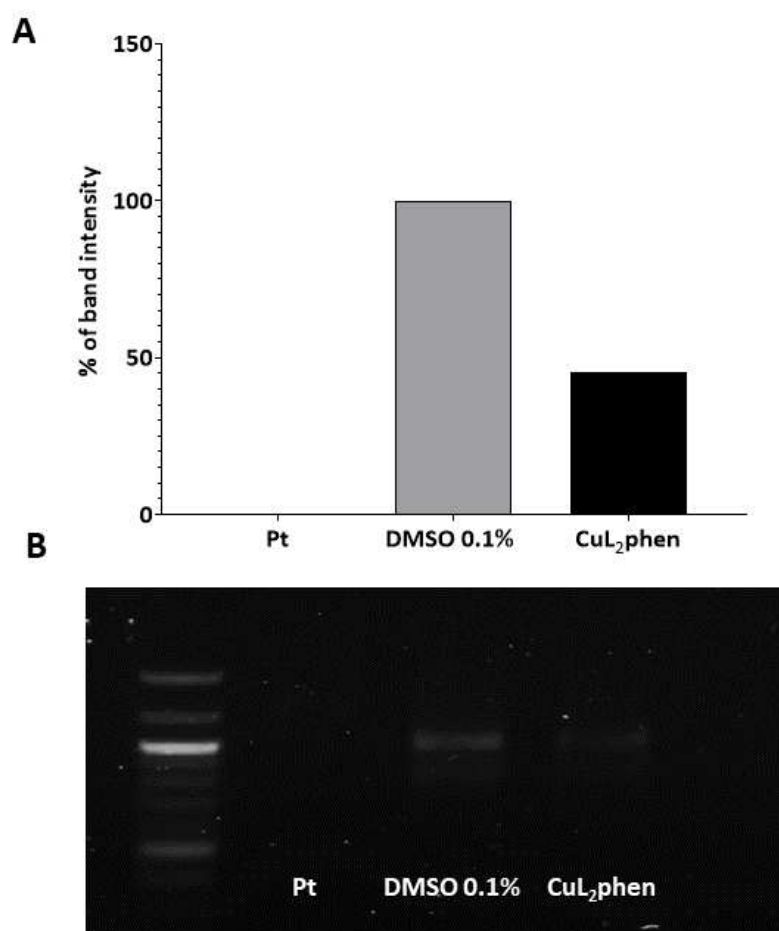


Figure 6: CuL₂phen interacts with SARS-CoV-2 dsRNA. Sixty-five nanomoles of SARS-CoV-2 ORF1a dsRNA were incubated with CuL₂phen at 2 μM or *cis*-[PtCl₂(*dms*o)₂] at 10 μM or the DMSO 0.1% control for 45 min at room temperature. The reaction products were subjected to 1% agarose 1X TAE electrophoresis gel containing SYBR safe followed by densitometry analysis on ImageJ.JS version 1.53j.

SARS-CoV-2 Delta and Omicron variants are strongly inhibited by CuL₂phen

To assess the effectiveness of CuL₂phen against the Delta and Omicron variants, we employed the infectious clones of the Wuhan strain with the spike genome sequences from the B.1.617.2 (Delta) or B.2 (Omicron) variants carrying the MCherry gene engineered by Rihn and coworkers (RIHN et al., 2021) (**Figure 7A**). Subsequently, CuL₂phen at a concentration of 2 μ M was mixed with the culture media containing the Wuhan-Delta-Spike or Wuhan-Omicron-Spike viruses at a multiplicity of infection (MOI) of 0.1. These virus-containing media was then added to the host cells for 24 hours. The quantification of viral replication was performed by measuring the total integrated fluorescence intensity, expressed as RCU x μ m² per well, using the IncuCyte S3 microscope. The results showed that CuL₂phen led to a reduction of 98.7% in SARS-CoV-2 Wuhan-Delta-Spike (**Figure 7B**, $p < 0.0001$), while when challenged against the SARS-CoV-2 Wuhan-Omicron-Spike virus, CuL₂phen was able to suppress viral replication by 99.9% (**Figure 7C**, $p < 0.0001$).

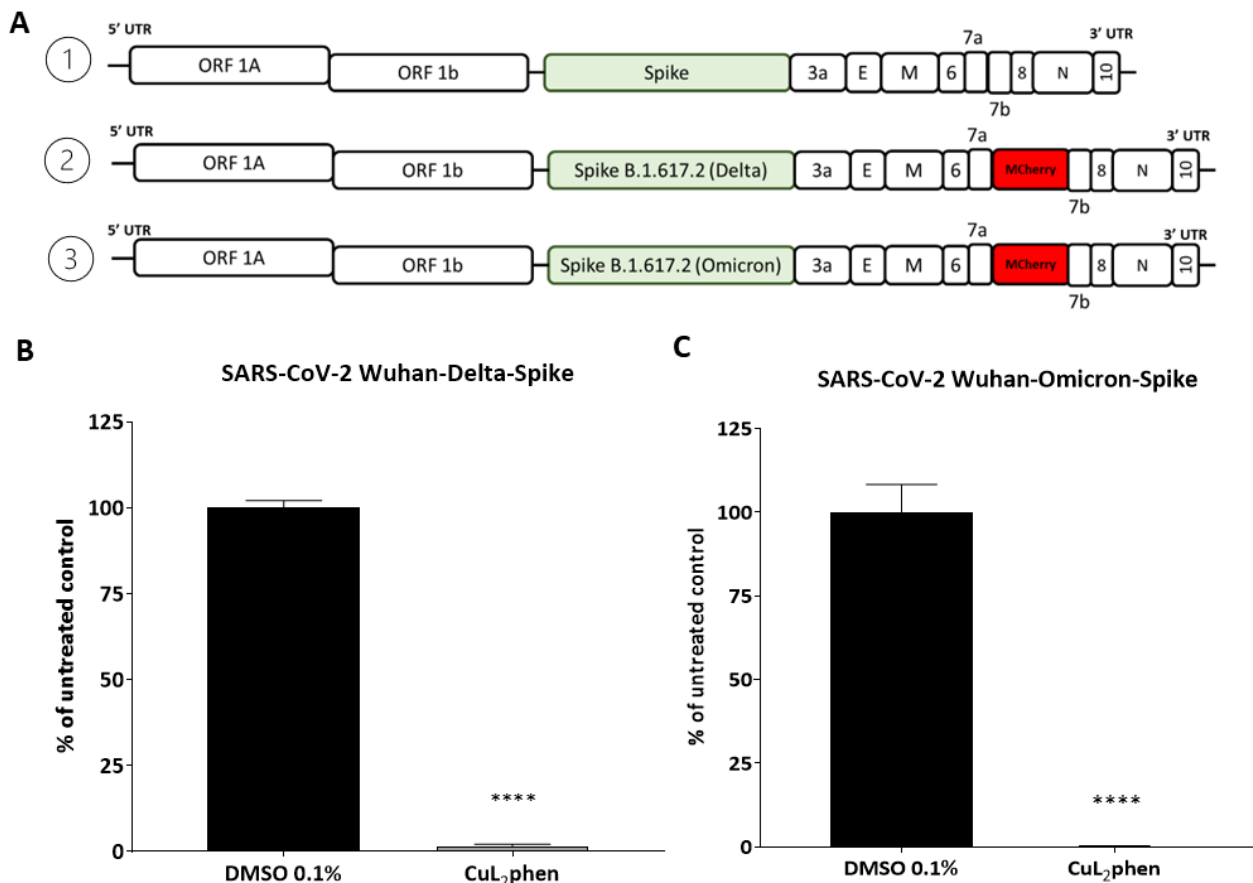


Figure 7: Effect of CuL₂phen in the infectious clones SARS-CoV-2-Wuhan-Delta-Spike or SARS-CoV-2-Wuhan-Omicron-Spike replication (A) Schematic representation of the infectious clone genome, being the original sequence of Wuhan (NCBI: NC_045512.2) (1), SARS-CoV-2-Wuhan-Delta-Spike in (2) and SARS-CoV-2-Wuhan-Omicron-Spike (3), being the last two carriers of the MCherry reporter gene. A549-AT cells were infected with SARS-CoV-2-Wuhan-B.1.617.2-Spike (B) or SARS-CoV-2-Wuhan-B.2-Spike (C) at an MOI of

0.1 in the presence of CuL₂phen at 2 μ M for 24h. SARS-CoV-2 replication was measured by the total integrated intensity of the fluorescence (RCU $\times$ μ m²/well) using the IncuCyte S3 microscope. All images were generated using GraphPad Prism 8 and GIMP 2.10.20. Mean values $\pm$ SD of a minimum of three independent experiments each measured in quadruplicate. (****) $P < 0.0001$.

To further validate these data, we employed the wild-type variants Delta and Omicron to examine their impact on viral replication. In this context, cells were infected with the Delta or Omicron variants at an MOI of 0.1 in the presence or absence of each compound for 24 hours. The supernatant was then collected and viral titers assessed using the TCID₅₀ method. The results demonstrated that against the Delta variant, CuL₂phen led to a decrease in viral titer of 4 Log₁₀ (**Figure 8A**). Similarly, for the Omicron variant, CuL₂phen hindered viral replication by approximately 2.5 Log₁₀ (**Figure 8B**).

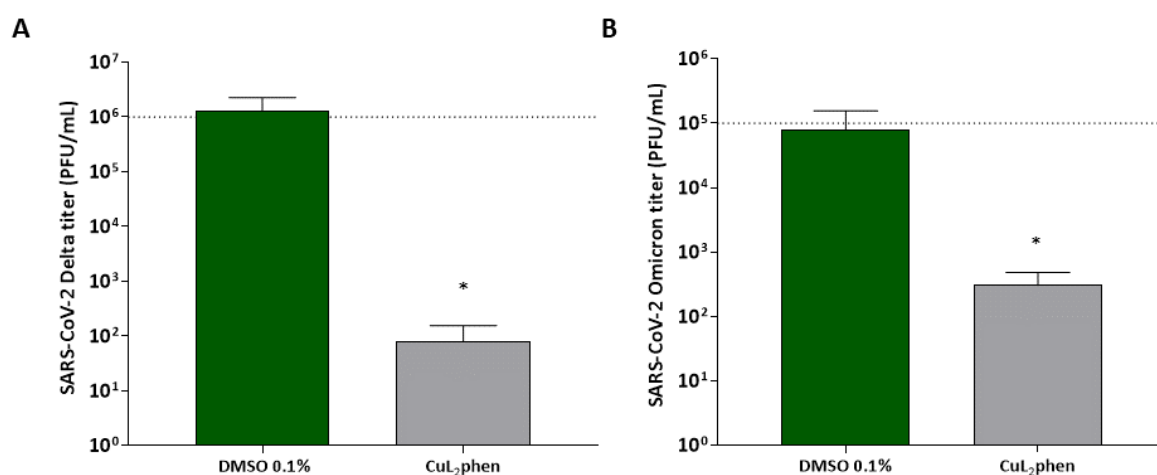


Figure 8: Effect of the CuL₂phen in the wild-type SARS-CoV-2-Delta and SARS-CoV-2-Omicron replication. A549-AT cells were infected with SARS-CoV-2-Delta (A) or SARS-CoV-2-Omicron (B) at an MOI of 0.1 in the presence of CuL₂phen at 2 μ M for 24h. Then, supernatant was collected and tittered by TCID₅₀. All images were generated using GraphPad Prism 8 and GIMP 2.10.20. Mean values $\pm$ SD of a minimum of three independent experiments each measured in replicate. (*) $P < 0.05$.

DISCUSSION

The compounds CuL₂phen and CuL₁phen were synthesized previously and were screened against SARS-CoV-2, however, the descriptive antiviral profile and potential of these new metal complexes species were not further characterized (DOS SANTOS OLIVEIRA et al., 2023). Here, our data showed that CuL₂phen presented a better selectivity index (SI) than CuL₁phen, being the values 5.3 and 1.7, respectively. The fluorination in the molecule CuL₁phen is known to decrease the ligand substitution rate in the complex, which might be causing higher cytotoxicity of the compound, impairing the speciation of the molecule in biological systems (GILLIS et al., 2015; MEANWELL, 2018). Additionally, the compound

CuL₁phen was shown to produce a stronger cytotoxic effect when evaluated against leishmania and human cells, with a less selective effect against the parasite when compared to CuL₂phen (DOS SANTOS OLIVEIRA et al., 2023). This agrees with data presented here, since CuL₁phen showed a lower CC₅₀ when compared with the non-fluorinated compound CuL₂phen.

For the CuL₂phen, the EC₅₀ and CC₅₀ resulted in a more selective activity towards A549-AT cells, however, the lower CC₅₀ for Cu(II) can be explained by possible DNA interaction, ROS production, and apoptosis induction in high concentrations (ALSHAMRANI, 2023; KASPRZAK, 1995; LIU et al., 1999; SHAO et al., 2014). It is important to emphasize that Cu(II) also plays crucial roles in various cellular processes in the human body, being an essential trace element that is required for many biological functions such as enzymatic activity, iron metabolism, DNA synthesis and repair, and cell growth and development (HARRIS, 2000; LINDER, 1991; MESSERSCHMIDT et al., 2004; RAWJI, 1997; ZORODDU et al., 2019). In this study, CuL₂phen demonstrated remarkable anti-SARS-CoV-2 activity, particularly in impairing viral entry and, more critically, the post-entry steps. This suggests the potential for achieving superior antiviral outcomes even at lower concentrations, addressing concerns about selectivity.

Notably, CuL₂phen exhibited robust effectiveness against the SARS-CoV-2 Delta and Omicron variants, reducing viral titers by over 2.5 Log₁₀ after treatment. These findings are particularly significant considering the context of COVID-19 pathogenesis, which results in increased IL-6 and decreased levels of Cu(II) in patients' blood and an increase in Cu(II)-carriers (CAO; LI, 2020; DI BELLA et al., 2017; KHANMOHAMMADI; REZAEI, 2021). Therefore, the administration of this compound *in vivo* may contribute to enhanced patient recovery by assisting various cellular processes. Another point to be considered is that the modest SI of CuL₂phen *in vitro* might be overruled by the lack of adverse effects *in vivo* when considered therapeutic significance. However, it is imperative to underscore that the effective concentration for the treatment of infected animals and/or individuals requires further evaluation. Other drugs such as Chloroquine were described with high SI *in vitro* and did not show any therapeutic significance when evaluated *in vivo* and in clinical trials (AYO BIVIGOU et al., 2022; KHAN et al., 2010; ROQUES et al., 2018; SHARMA, 2020).

The Cu(II) ions were described as elements able to generate reactive oxygen species (ROS) in mammalian cells (ERXLEBEN, 2018; NOVELLO; STIRPE, 1969; SAGRIPANTI et al., 1997; SAGRIPANTI; ROUTSON; LYTLE, 1993; SHIONOIRI et al., 2012). Previously,

the Cu(II) complexes described here were also characterized as capable of speciation in biological systems, probably releasing the Cu(II) ions (DOS SANTOS OLIVEIRA et al., 2023). In addition, the A549 cells are capable of producing ROS and inducing IL-8 mRNA, which are essential to inhibit SARS-CoV-2 dissemination in human infection (WU; KASSIE; MERSCH-SUNDERMANN, 2005; YE et al., 2009). Our data demonstrated a modest but significant protection of cells against SARS-CoV-2 infection, which might be explained by the activation of ROS pathways, decreasing the infection by degrading part of the viral particles as soon as they enter the cells. Furthermore, CuL₂phen demonstrated to be a potent entry inhibitor which might also be related to the chemical speciation, releasing Cu(II) molecules that can disrupt the viral envelope (GRASS; RENSING; SOLIOZ, 2011; SAGRIPANTI et al., 1997; SAGRIPANTI; ROUTSON; LYTLE, 1993). However, these hypotheses must be further investigated.

The strong post-entry of CuL₂phen could be expected since both chalcones and Cu(II) are known to interact with host factors and viral proteins that can impair viral replication (LI et al., 2020a; NAKAHATA et al., 2018; NOVELLO; STIRPE, 1969; SHIONOIRI et al., 2012). As a possible target, knowing the interaction of Cu(II) complexes with nucleic acids (GOVIND et al., 2021; NOVELLO; STIRPE, 1969; WANG et al., 2021a), we performed a dsRNA interaction assay to identify the ability of CuL₂phen interact with SARS-CoV-2 dsRNA. The data showed a strong and effective interaction CuL₂phen-SARS-CoV-2-dsRNA, probably by intercalating with viral dsRNA. This interaction directly impacts viral RNA replication due to the necessity of the complementary negative sense RNA to produce subgenomic mRNA that encodes structural and accessory proteins, beside the production of new viral RNA (LAMERS; HAAGMANS, 2022; V'KOVSKI et al., 2021b). What is more, it's worth noting that chalcone molecules have previously demonstrated their potential as inhibitors of SARS-CoV 3CL^{pro} cysteine proteinase and the replication of subgenomic RNA in the Hepatitis C virus (HCV) (ADIANTI et al., 2014; ELKHALIFA et al., 2021; GHOSH et al., 2006; PARK et al., 2016). This opens up the intriguing possibility that CuL₂phen could be influencing the viral replication proteins of SARS-CoV-2 and that Cu(II) could be serving as a carrier for L₂, effectively functioning as a delivery method. This theory gains support from the observation that L₂ alone did not inhibit SARS-CoV-2 (DOS SANTOS OLIVEIRA et al., 2023).

CONCLUSION

The CuL₂phen has demonstrated remarkable efficacy in suppressing SARS-CoV-2 replication in vitro, effectively inhibiting not only the Delta and Omicron variants, but also interacting with SARS-CoV-2 dsRNA and impairing post-entry viral replication. Based on these highly promising results, we strongly advocate for the initiation of further investigations involving Cu(II) complexes. Furthermore, we propose that CuL₂phen be considered for utilization in pre-clinical assays as a promising alternative for the treatment of COVID-19.

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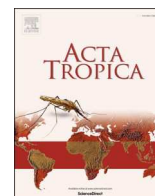
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**CAPÍTULO 5: REPURPOSING POTENTIAL
OF RIMANTADINE HYDROCHLORIDE
AND DEVELOPMENT OF A PROMISING
PLATINUM(II)-RIMANTADINE
METALLODRUG FOR THE TREATMENT
OF CHIKUNGUNYA VIRUS INFECTION**

MANUSCRITO PUBLICADO EM “ACTA TROPICA”



Repurposing potential of rimantadine hydrochloride and development of a promising platinum(II)-rimantadine metallodrug for the treatment of Chikungunya virus infection

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Platinum(II) metallodrugs

ABSTRACT

Most of the patients infected with Chikungunya virus (CHIKV) develop chronic manifestations characterized by pain and deformity in joints, impacting their quality of life. The aminoadamantanes, in their turn, have been exploited due to their biological activities, with amantadine and memantine recently described with anti-CHIKV activities. Here we evaluated the antiviral activity of rimantadine hydrochloride (rtdH), a well-known antiviral agent against influenza A, its platinum complex (Pt-rtd), and the precursor *cis*-[PtCl₂(dmsO)₂], against CHIKV infection *in vitro*. The rtdH demonstrated significant antiviral activity in all stages of CHIKV replication (29% in pre-treatment; 57% in early stages of infection; 60% in post-entry stages). The Pt-rtd complex protected the cells against infection in 92%, inhibited 100% of viral entry, mainly by a virucidal effect, and impaired 60% of post-entry stages. Alternatively, *cis*-[PtCl₂(dmsO)₂] impaired viral entry in 100% and post-entry steps in 60%, but had no effect in protecting cells when administered prior to CHIKV infection. Collectively, the obtained data demonstrated that rtdH and Pt-rtd significantly interfered in the early stages of CHIKV life cycle, with the strongest effect observed to Pt-rtd complex, which reduced up to 100% of CHIKV infection. Moreover, molecular docking analysis and infrared spectroscopy data (ATR-FTIR) suggest an interaction of Pt-rtd with CHIKV glycoproteins, potentially related to the mechanism of inhibition of viral entry by Pt-rtd. Through a migration retardation assay, it was also shown that Pt-rtd and *cis*-[PtCl₂(dmsO)₂] interacted with the dsRNA in 87% and 100%, respectively. The obtained results highlight the repurposing potential of rtdH as an anti-CHIKV drug, as well as the synthesis of promising platinum(II) metallodrugs with potential application for the treatment of CHIKV infections.

Importance Chikungunya fever is a disease that can result in persistent symptoms due to the chronic infection process. Infected patients can develop physical disability, resulting in high costs to the health system and significant impacts on the quality of life of affected individuals. Additionally, there are no licensed vaccines or antivirals against the Chikungunya virus (CHIKV) and the virus is easily transmitted due to the abundance of viable vectors in epidemic regions. In this context, our study highlights the repurposing potential of the commercial drug rimantadine hydrochloride (rtdH) as an antiviral agent for the treatment of CHIKV infections. Moreover, our data demonstrated that a platinum(II)-rimantadine metallodrug (Pt-rtd) poses as a potent anti-CHIKV molecule with potential application for the treatment of Chikungunya fever. Altogether, rtdH and Pt-

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rtd significantly interfered in the early stages of CHIKV life cycle, reducing up to 100% of CHIKV infection *in vitro*.

1. Introduction

The Chikungunya virus (CHIKV) has impacted global health in tropical and subtropical regions (Kazmi et al., 2020; Rougeron et al., 2015). CHIKV is the etiologic agent of Chikungunya Fever, a disease characterized by fever, headache, nausea, myalgia, arthralgia, and polyarthralgia during the acute infection (CDC, 2018; Thiberville et al., 2013). After the onset of symptoms, a high percentage of patients can develop a chronic infection in joints defined by severe pain and deformity, lasting for months to years (Vairo et al., 2019; World Health Organization., 2020). The chronic disease impacts the quality of life of patients, resulting in high costs to the health systems as well as impacting economically and socially both individuals and communities (Oliveira Silva Martins et al., 2020; Vu et al., 2017).

CHIKV is transmitted mainly by the bite of female mosquitos of *Aedes aegypti* and *Aedes albopictus*. Additionally, other species are capable to transmit this pathogen, such as *Haemagogus* sp and *Aedes terreus* (Artsob et al., 2016; Lourenço-de-Oliveira and Failloux, 2017). Aggravatingly, there is no specific treatment against CHIKV infections, as well as approved vaccines (CDC, 2018). In this context, the lack of effective treatment and/or vaccines in countries with susceptible vectors might allow the establishment of a successful autochthonous transmission, which can culminate in unexpected outbreaks in tropical and subtropical countries (Charrel et al., 2014; Ciocchetta et al., 2018).

The aminoadamantanes constitute a class of organic compounds that have been largely studied due to their pharmacological properties (Garcia et al., 2010). The adamantane structure consists of a single diamond-like carbon cage and its functionalization with an amine group gives rise to bioactive molecules such as amantadine (Garcia et al., 2010). Memantine is the dimethyl derivative of amantadine, while rimantadine is the product of adamantane functionalized with an ethanamine group (Garcia et al., 2010; Pereira et al., 2020). Recently, our group demonstrated the *in vitro* antiviral activity of memantine hydrochloride against CHIKV, suggesting that this drug, which is currently used in the treatment of Alzheimer (Tampi and Dyck, 2007), could be a viable candidate to be repurposed as an anti-CHIKV drug (Pereira et al., 2021). Memantine and rimantadine have been shown to possess anti-neurodegenerative (Liu et al., 2015; Spilovska et al., 2016) and antiviral effects (Intharathap et al., 2008), respectively. Rimantadine is a drug that has been applied in the treatment of influenza virus A infections (Jefferson et al., 2002). This molecule and its Au(I) (Sucena et al., 2012) and Pt(II) (Pereira et al., 2020) metal complexes demonstrated antibacterial activities *in vitro*. Nevertheless, to the best of our knowledge, rimantadine and its metal complexes have not been evaluated regarding its antiviral activity against CHIKV so far.

Since the discovery of the anticancer activities of cisplatin, or *cis*-(diaminodichlorido)platinum(II), over tumor cells at the end of the 1960s (Florea and Büsselberg, 2011), Pt(II) compounds have been explored in a multitude of potential applications such as anti(myco) bacterial (Joyce et al., 2010), antifungal (Li et al., 2019), and antiviral agents (de Paiva et al., 2020). Noticeably, Pt(II) complexes with the antiviral agents acyclovir and penciclovir were able to effectively inhibit HSV-2(G) and HSV-1 (TK-VMW1837) strains, being more active than the free ligand acyclovir (Margiotta et al., 2004). Further studies showed that some of these complexes presented strong inhibition against Cytomegalovirus strains with IC₅₀ concentrations ranging between 0.04 and 0.18 $\mu\text{mol}\cdot\text{L}^{-1}$ and best selectivity indexes than acyclovir (Kari and Gehrz, 1993; Shoup et al., 2020).

Therefore, considering the possibility to obtain a synergistic effect between the coordination of an antiviral drug to Pt(II) ions, this study evaluated, for the first time, the effects of the rimantadine hydrochloride

(rtdH) and its platinum complex (Pt-rtd) (Fig. 1) against CHIKV *in vitro*. The precursor *cis*-dichloridobis(dimethylsulfoxide)platinum(II) (*cis*-[PtCl₂(*dms*o)₂]), used in the synthesis of the Pt-rtd complex, was also evaluated for comparative purposes. The Pt-rtd presented noteworthy effects on CHIKV infectious cycle, protecting the cells against CHIKV infection, interacting with the glycoproteins on the viral particle, and inhibiting post-entry steps, probably by interacting with dsRNA. The obtained results demonstrated that the coordination of the rtdH to platinum(II) led to a new complex with improved antiviral activity, being superior to the free ligand.

2. Methods

2.1. Compounds

Rimantadine hydrochloride (rtdH, C₁₂H₂₂NCl, 99%) and *cis*-dichloridobis(dimethylsulfoxide)platinum(II) (*cis*-[PtCl₂(*dms*o)₂]) were purchased from Sigma-Aldrich Laboratories (Fig. 1). The platinum(II) complex with rimantadine (Pt-rtd) was synthesized according to the procedure described by us (Fig. 1) (Pereira et al., 2020). Briefly, the Pt-rtd complex was synthesized by the dissolution of 0.40 mmol of rtdH ligand and 0.20 mmol of the precursor *cis*-dichloridobis(dimethylsulfoxide)platinum(II) in 10 mL of methanol. The reaction was subjected to rapid heating (~ 55 °) for a complete dissolution of the reagents. Then, 0.40 mmol of potassium hydroxide (KOH), previously dissolved in 1.0 mL of methanol, was added to the solution. A yellow precipitate was obtained, collected by filtration, washed with methanol, and dried in a desiccator in the presence of P₂O₅. Subsequently, the Pt-rtd complex was sent for biological analysis.

2.2. Cell culture

BHK-21 cells (fibroblasts derived from Syrian golden hamster kidney; ATCC CCL-10), were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 100 U/mL of penicillin (Hyclone Laboratories), 100 mg/mL of streptomycin (Hyclone Laboratories), 1% dilution of stock of non-essential amino acids (Hyclone Laboratories) and 1% of fetal bovine serum (FBS, Hyclon Laboratories) in a humidified 5% CO₂ incubator at 37 °C.

2.3. Rescue of CHIKV-nanoluc reporter virus

The CHIKV expressing *nanoluciferase* reporter (CHIKV-*nanoluc*) used for the antiviral assays is based on the CHIKV LR2006OPY1 strain (East/Central/South African genotype) (Matkovic et al., 2018). The cDNA plasmid of CMV-CHIKV-*nanoluc* contains CMV promoter, SV40 terminator, and a sequence encoding *nanoluciferase* protein inserted into the region encoding the C-terminal domain of viral nsP3 protein (Matkovic et al., 2018). The production of CHIKV-*nanoluc* virions was carried out as previously described (Oliveira et al., 2020; Pereira et al., 2021; Santos et al., 2021). Briefly, BHK-21 cells were transfected with CMV-CHIKV-*nanoluc* plasmid using Lipofectamine 2000 and Opti-Mem medium. The supernatant was collected 48 h post-transfection (h.p.t.) and stored at -80 °C. The viral titers were also determined as previously described (Oliveira et al., 2020; Pereira et al., 2021; Santos et al., 2021).

2.4. Determination of cytotoxic concentration of 50% (CC₅₀)

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich) assay as previously described (Oliveira et al., 2020; Santos et al., 2021). BHK-21 cells

were plated to 48 well plates at a density of 5×10^4 cells per well and incubated overnight at 37 °C. Medium containing two-fold serial dilutions of each compound (rtdH from 1.95 to 250 μ M; *cis*-[PtCl₂(dms₂)₂] from 2.34 to 300 μ M; Pt-rtd from 1.95 to 250 μ M) was added to cells and incubated for 16 h. Then, the medium was replaced by MTT solution at 1 mg/mL, cells were incubated for 30 min, and replaced by 300 μ L of DMSO (dimethyl sulfoxide) to solubilize the formazan crystals. The absorbance was measured at 490 nm on the Glomax microplate reader (Promega®). Cell viability was calculated according to the equation $(T/C) \times 100\%$, where T and C represent the mean optical density of the treated and untreated control groups, respectively. The cytotoxic concentration of 50% (CC₅₀) was calculated using GraphPad Prism 8®.

2.5. Determination of the effective concentration 50% (EC₅₀)

BHK-21 cells were seeded at a density of 5×10^4 cells per well into 48 well plates for 24 h and infected with CHIKV-*nanoluc* at a multiplicity of infection (MOI) of 0.1 PFU/cell, in the presence of each compound (rtdH from 1.95 to 250 μ M; *cis*-[PtCl₂(dms₂)₂] from 2.34 to 300 μ M; Pt-rtd from 1.95 to 250 μ M), as previously described (Oliveira et al., 2020; Santos et al., 2021). Samples were harvested using Renilla-luciferase lysis buffer (PROMEGA®) at 16 h post-infection (h.p.i.) and virus replication levels were quantified by measuring *nanoluciferase* activity using the Renilla luciferase assay system (Promega®). The effective concentration of 50% inhibition (EC₅₀) was calculated using GraphPad Prism 8® software. The values of CC₅₀ and EC₅₀ were used to calculate the selectivity index (SI = CC₅₀/EC₅₀).

2.6. Time-of-addition assays

BHK-21 cells were seeded in 48 well plates 24 h before infection and/or treatment as previously described (Oliveira et al., 2020; Santos et al., 2021). All infections were performed using an MOI of 0.1 and virus replication levels were assessed by measurement of *nanoluciferase* activity at 16 h.p.i.

In pretreatment assay, cells were treated for 1 h with the compound prior to the CHIKV infection, extensively washed with PBS, and added of CHIKV-*nanoluc* for 1 h. Then, cells were washed with PBS to remove the unbound virus and added of fresh medium for 16 h.

To evaluate the early stages of infection, cells were infected using media containing compound and virus for 1 h, washed with PBS, and incubated with fresh medium for 16 h. The virucidal activity was also assessed, employing the same protocol, except that an inoculum containing compound and virus was incubated for 1 hour prior to the incubation with cells.

In the post-entry assay, cells were infected with CHIKV for 1 h, washed extensively with PBS, and then incubated with a medium-containing compound for 16 h.

2.7. dsRNA interaction assay

To investigate the potential to interact with dsRNA, a migration

retardation assay based on previously described works was performed (Campos et al., 2017; Krawczyk et al., 2009; Silva et al., 2019). Through PCR, the HCV JFH-1 3' untranslated region (UTR) was amplified employing primers flanked by a T7 promoter site (forward: TAA-TACGACTCACTATAGGGGGACACACTAGGTACA; reverse: TAA-TACGACTCACTATAGGGACATGATCTGCAGAGAG; T7 promoter regions are underlined). The reaction product of 273 bp was purified by ReliaPrep™ DNA Clean-Up and Concentration System (Promega®), and used for *in vitro* transcription by the HiScribe™ T7 High Yield RNA Synthesis Kit (New England BioLabs®). The dsRNA molecule was obtained by complementary annealing and incubated at 15 nM with rtdH (100 μ M), Pt-rtd (7.8 μ M), and *cis*-[PtCl₂(dms₂)₂] (100 μ M) for 45 min, and analyzed in 1% agarose 1X TAE gel stained with ethidium bromide. The lack of band in the gel confirms the compound interaction activity since it competes with ethidium bromide. Doxorubicin (100 μ M) was used as a positive control of interaction. The band quantification was performed using ImageJ.JS version 1.53j.

2.8. Infrared spectroscopy: spectral data analysis

Samples were recorded in Fourier transform infrared connected to a micro-attenuated total reflectance spectrophotometer (ATR-FTIR) system (Agilent Cary 630 FTIR, Agilent Technologies, Santa Clara, CA, USA), as previously described (Oliveira et al., 2020; Santos et al., 2021). The diamond unit in the ATR system performs an internal-reflection element to record the fingerprint infrared signature at 1800 cm⁻¹ to 800 cm⁻¹ regions. The samples were prepared as previously described (Oliveira et al., 2020; Santos et al., 2021), where rtdH (100 μ M), Pt-rtd (7.8 μ M), and *cis*-[PtCl₂(dms₂)₂] (100 μ M) were mixed with CHIKV-*nanoluc* virions (1×10^6 PFU/mL). A volume of 1.5 μ L of each sample was inserted on the diamond cell and dehydrated for 6 min using airflow, forming a thin layer on the ATR-crystal. The spectra were then recorded in triplicate (4 cm⁻¹ resolution, 32 scans). All spectra were normalized by the vector method and corrected using the rubber-band baseline correction (Caixeta et al., 2020). The second derivative spectra were created based on original data plotted in the Origin Pro 9.0 (OriginLab, Northampton, MA, USA) software, and adjusted using Savitzky-Golay algorithm with polynomial order 5 and 20 points of the window. The value heights indicated the intensity of the functional group evaluated (Oliveira et al., 2020; Santos et al., 2021). Table 1 presents the vibrational modes and proposes functional groups in each sample.

2.9. Molecular docking

The mature envelope glycoprotein complex of Chikungunya virus was obtained from PDB (entry 3N42) whereas the Pt-rtd structure was obtained from CCDC 1982409. The structure of rtdH was obtained from PubChem. The glycoprotein was preprocessed, waters with less than 3 hydrogen bonds were removed, and missing hydrogen atoms were added with Maestro®. Protonation states were defined with Epik. The docking procedure was carried out in a similar way previously described

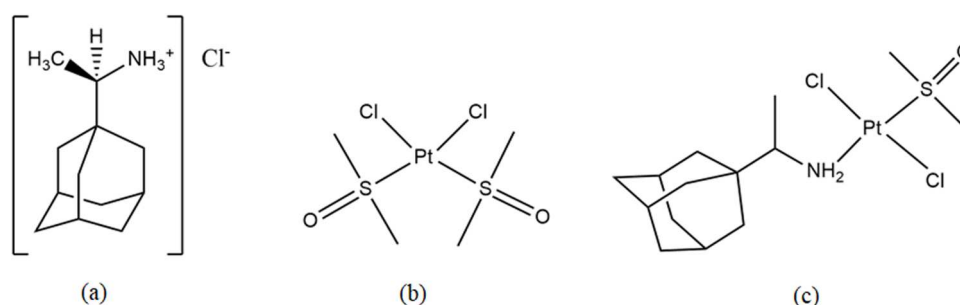


Fig. 1. Structure of (a) rimantadine hydrochloride, (b) *cis*-dichloridobis(dimethylsulfoxide)platinum(II) and (c) Pt-rtd.

Table 1

Assignments of main wavenumbers of sample ATR-FTIR spectra. Assignments based on different references. Abbreviations: ν = stretching vibrations, s = symmetric vibrations and as = asymmetric vibrations.

Vibrational mode (cm^{-1})	Proposed vibrational mode	Molecular source
1170	OH- [1] and ν as CO—O—C [2]	Pt-rtd
1162	Shift	CHIKV + Pt-rtd
1151	C—O stretching vibration (glycoprotein) [2];	CHIKV
1140	C—N [3]	rtdH
1131	Shift	CHIKV + cis-[PtCl ₂ (dmsO) ₂]
986	O—CH ₃ (saccharides)	CHIKV
980	Shift	CHIKV + cis-[PtCl ₂ (dmsO) ₂]
978	Shift	CHIKV + Pt-rtd
977	Shift	CHIKV + rtdH
973	ν C—C [4]	cis-[PtCl ₂ (dmsO) ₂]
970	ν C—C [4]	Pt-rtd
965	Shift	CHIKV + rtdH

by us for coordination compounds (Nakahata et al., 2018). Briefly, grids were defined as cubes with 36 Å edges, centered around two exposed areas of glycoprotein E2 (domains A and B), and around glycoprotein E3, which are part of the CHIKV Spike (Voss et al., 2010a). Docking of either Pt-rtd or rtdH was carried out with extra precision (XP) using Maestro 2021–2 (Schrodinger®). The compounds were sampled as flexible ligands, and the coordination bonds were treated as zero-order bonds throughout the docking procedure. No post-docking minimization was used. Three poses at most were written out per compound and inspected manually.

2.10. Statistical analyses

Individual experiments were performed in triplicates and all assays were carried out a minimum of three times to confirm reproducibility.

The EC₅₀ and CC₅₀ were calculated using a non-linear regression considering log(inhibitor) vs. response, with variable slope (four parameters), and a comparison was also performed using the F test ($p < 0.05$). Data were expressed as mean $\pm$ SD and were submitted to a normality and lognormality test. The statistical significance was determined by one-way analysis of variance (ANOVA), complemented by Dunnett's *post hoc* test, performed in comparison with the untreated control.

3. Results

3.1. Rimantadine hydrochloride (rtdH) and its platinum complex (Pt-rtd) inhibit CHIKV infection

Through the employment of BHK-21 cells and a CHIKV expressing *nanoluciferase* reporter (CHIKV-*nanoluc*), the anti-CHIKV activities of rtdH and its complex Pt-rtd were evaluated. The precursor cis-[PtCl₂(dmsO)₂] was used for comparative purposes. The antiviral investigation of each compound was assessed by performing a dose-response assay, which allows the determination of the effective concentration of 50% (EC₅₀) and cytotoxicity concentration of 50% (CC₅₀). BHK-21 cells were infected with CHIKV-*nanoluc* and simultaneously treated with each compound in two-fold serial dilutions at concentrations ranging from 1.95 to 250 μM for rtdH and Pt-rtd, and from 2.34 to 300 μM for cis-[PtCl₂(dmsO)₂]. Viral replication levels were assessed 16 h post-infection (h.p.i.) (Fig. 2A). In parallel, cell viability was assessed by the MTT assay. The results demonstrated that rtdH presented a CC₅₀ of 602 μM , EC₅₀ of 71.64 μM ($p < 0.0001$, $F_{3,160} = 152.2$), and selectivity index (SI) of 8.4 (Fig. 2B), whereas the cis-[PtCl₂(dmsO)₂] showed a CC₅₀ of 35,997 μM , EC₅₀ of 59.87 μM ($p < 0.0001$, $F_{2,112} = 173.1$), and SI of 601.2 (Fig. 2C). The Pt-rtd complex presented a CC₅₀ of 11.19 μM , EC₅₀ of 2.18 μM ($p < 0.0001$, $F_{4,120} = 118.4$), and SI of 5.1 (Fig. 2D). Although the Pt-rtd complex exhibited the lowest SI, its EC₅₀ was over 30 times lower than rtdH and cis-[PtCl₂(dmsO)₂], under the same experimental conditions what is noteworthy. This is emphasized when the EC₅₀ among the compounds is statistically compared, being the rtdH and cis-

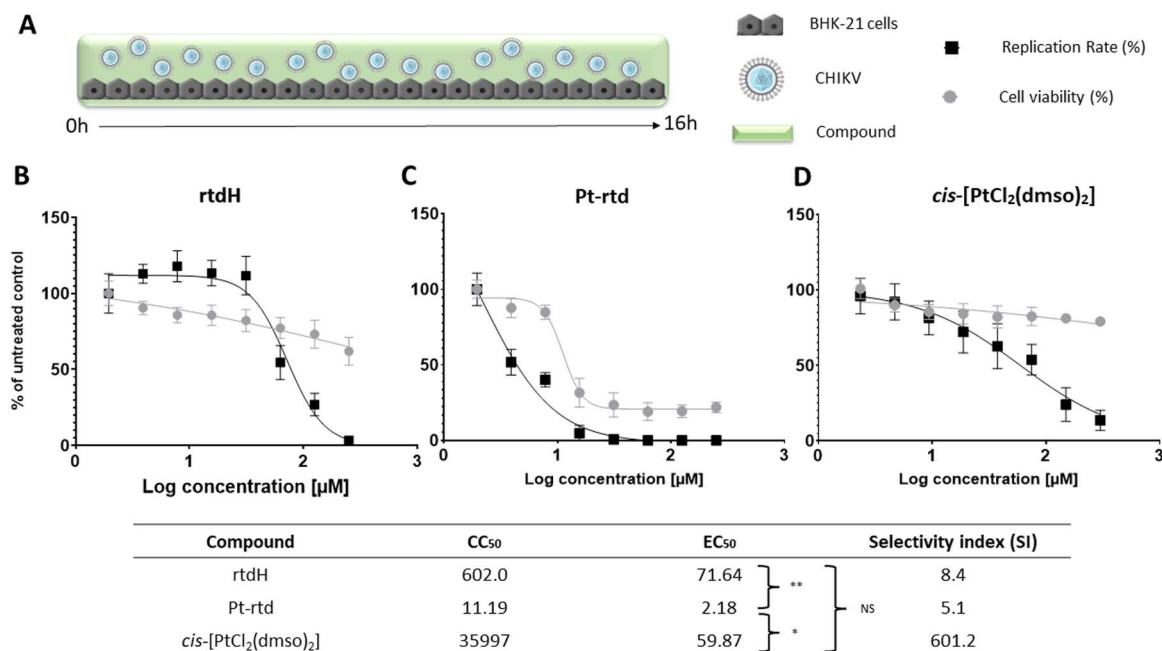


Fig. 2. The activity of rtdH, Pt-rtd, and cis-[PtCl₂(dmsO)₂] against CHIKV infection. (A) Schematic description of infectivity assay. BHK-21 cells were infected with CHIKV-*nanoluc* and simultaneously treated with each compound at concentrations ranging 1.95 to 250 μM for rtdH (B) and Pt-rtd (C), and 2.34 to 300 μM for cis-[PtCl₂(dmsO)₂] (D) in two-fold serial dilutions. CHIKV replication was quantified by measuring *nanoluciferase* activity (indicated by ■) and cellular viability was measured using an MTT assay (indicated by ●) after 16 h. The mean values $\pm$ SD of three independent experiments each measured in triplicate including the standard deviation are shown. (*) $P < 0.05$, (**) $P < 0.01$. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

[PtCl₂(dmsO)₂] significantly different from Pt-rtd ($p = 0.0035$ and $p = 0.0192$, respectively), but not from each other ($p = 0.5$).

3.2. Protective effect of rtdH and Pt-rtd against CHIKV infection

Time-of-addition assays were performed to investigate the effect of rtdH and Pt-rtd in different stages of the CHIKV replication cycle. The precursor *cis*-[PtCl₂(dmsO)₂] was used for comparative purposes. For the experiments, cells were treated with rtdH or *cis*-[PtCl₂(dmsO)₂] at 100 μM, and with Pt-rtd at 7.8 μM, based on cell viability and antiviral activity data.

The protective effect was firstly assessed by treating cells with each compound for 1 h at 37 °C prior to CHIKV infection. Then, cells were extensively washed with PBS and infected with CHIKV-*nanoluc* for 1 hour. The supernatant was removed, cells were added of fresh medium and luciferase levels were measured 16 h.p.i (Fig. 3A). The rtdH and the Pt-rtd complex significantly inhibited 29% (infection rate (IR) of 71%, $p = 0.0004$) and 92% (IR of 8%, $p < 0.0001$) of CHIKV infection, respectively (Fig. 3B). Conversely, *cis*-[PtCl₂(dmsO)₂] was not able to protect cells against CHIKV infection ($p = 0.1435$). The Pt-rtd complex showed the strongest protective effect against CHIKV infection, demonstrating that the complexation significantly affected the antiviral activity. This fact suggests that the coordination of rtdH to Pt(II) plays an important role in the antiviral action of the compound.

3.3. RtdH and Pt-rtd strongly impair CHIKV entry on BHK-21 cells

To investigate the effect of rtdH and Pt-rtd complex on the early stages of CHIKV replication cycle, BHK-21 cells were infected and simultaneously treated for 1 h at 37 °C. The precursor *cis*-[PtCl₂(dmsO)₂] was used for comparative purposes. Cells were washed and viral replication levels were assessed 16 h.p.i. (Fig. 4A). It was observed that rtdH and Pt-rtd impaired CHIKV entry in 57% and 99.7% (IR of 43% and 0.3%, respectively, $p < 0.0001$), respectively (Fig. 4B). The *cis*-[PtCl₂(dmsO)₂] precursor reduced virus entry in 72% (IR of 28%, $p < 0.0001$), and when compared to the rtdH, there was no statistical difference among them (Fig. 4B).

A further assay was performed using the same protocol, with an additional step prior to the incubation of virus and compound with cells: an inoculum containing the compound and CHIKV was incubated for 1 h at 37 °C, and then added to the cells for one extra hour (Fig. 5A). As a result, a virucidal activity against CHIKV was assigned to rtdH with 59% inhibition (IR of 41%, $p < 0.0001$), Pt-rtd in 100% ($p < 0.0001$), and the precursor *cis*-[PtCl₂(dmsO)₂] with 71% ($p < 0.0001$) (Fig. 5B). Similarly, rtdH and *cis*-[PtCl₂(dmsO)₂] treatment when compared, demonstrated no significant difference (Fig. 5B).

Altogether, the data demonstrate that either the ligand rtdH or the complexed Pt-rtd significantly interfered in the early stages of CHIKV life cycle, with the strongest effect observed to Pt-rtd, which reduced over 92% of CHIKV infection in pre-treatment, entry, and virucidal protocols.

3.4. RtdH and Pt-rtd also interfere with post-entry steps of CHIKV replicative cycle

Finally, the effects of the ligand, the complex, and the precursor were evaluated on post-entry stages of CHIKV infection, by treating cells after CHIKV infection. Both, rtdH and Pt-rtd, significantly reduced CHIKV replication in 60%, whereas *cis*-[PtCl₂(dmsO)₂] inhibited post-entry stages in 50% (IR of 40%, 40%, and 50%, respectively, $p < 0.0001$) (Fig. 6A and B), demonstrating the multiple effects of these compounds on CHIKV replicative cycle.

3.5. Insights on the mode of action: Pt-rtd interacts with the dsRNA

To further investigate the antiviral mode of action of each compound, a dsRNA interaction assay was conducted, since dsRNA is a replication intermediate of positive-strand RNA viruses. To this, employing the 3' UTR region of JFH-1 HCV as a template, an amplicon flanked by a T7 promoter was produced and used for *in vitro* transcription, synthesizing a dsRNA molecule of 273 bp. Fifteen nanomoles of dsRNA were incubated with rtdH at 100 μM, Pt-rtd at 7.8 μM, *cis*-[PtCl₂(dmsO)₂] at 100 μM, or with the controls (dimethyl sulfoxide 0.1% and doxorubicin at 100 μM). Through a migration retardation assay, it

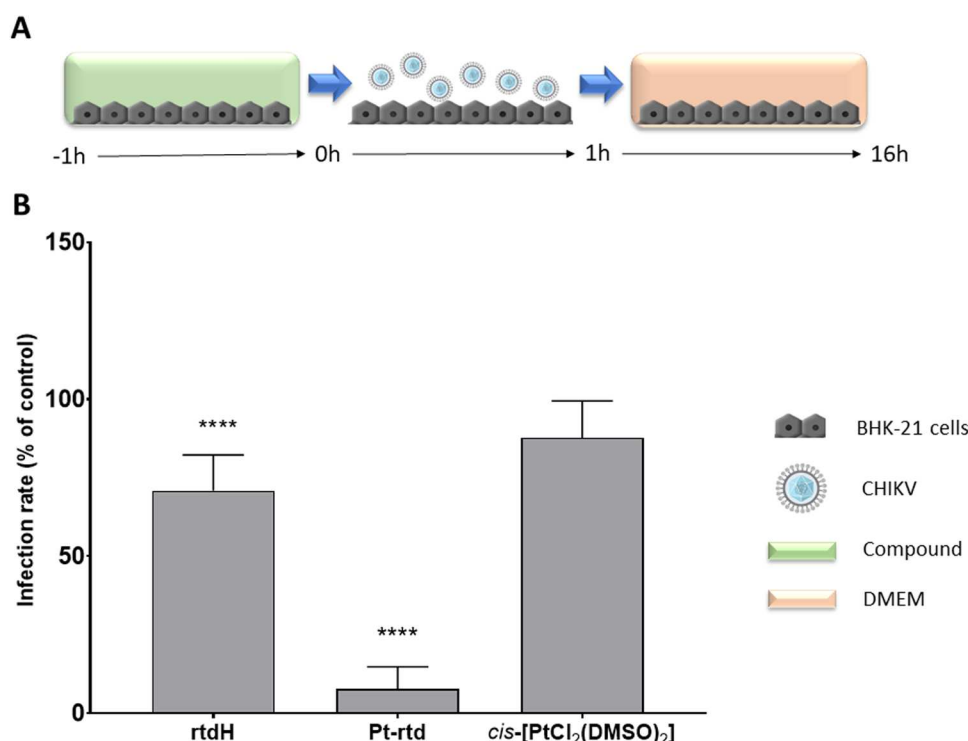


Fig. 3. rtdH and Pt-rtd protects cells against CHIKV infection *in vitro*. (A) and (B) Schematic representation of pretreatment assay as indicated by BHK-21 cells (black cells), compound (green bars), DMEM medium (orange bars), and CHIKV-*nanoluc* (viral structure). BHK-21 cells were treated with each compound at concentrations of 100 μM for rtdH and *cis*-[PtCl₂(dmsO)₂], and 7.8 μM for Pt-rtd, for 1 h. Then, cells were washed and infected with CHIKV-*nanoluc* at MOI 0.1 for 1 h. Mean values ± SD of a minimum of three independent experiments each measured in triplicate. (****) $P < 0.001$, (****) $P < 0.0001$. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

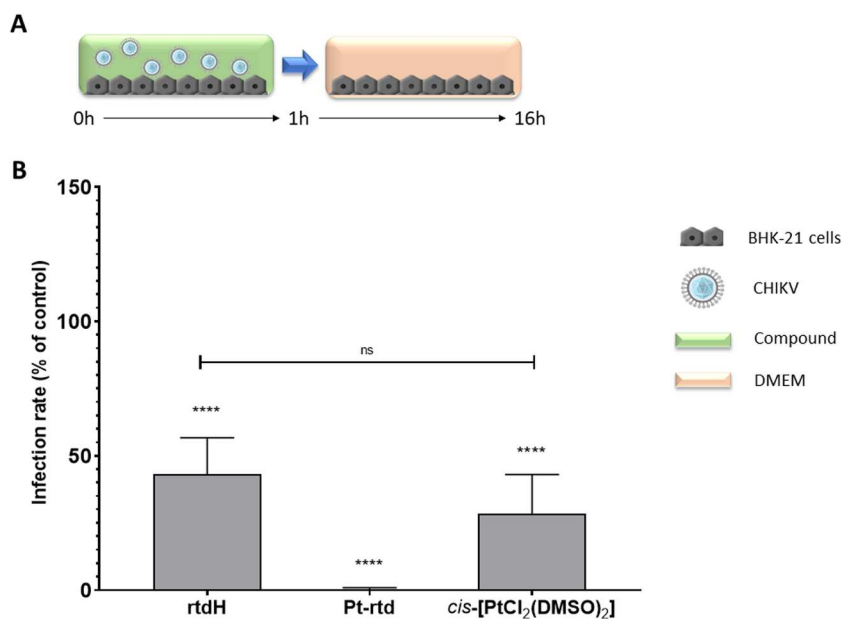


Fig. 4. Pt-rtd strongly inhibits CHIKV entry to the host cells. (A) and (B) CHIKV-*nanoluc* and BHK-21 cells were infected with CHIKV-*nanoluc* (MOI 0.1) and simultaneously treated with each compound at concentrations of 100 μ M for rtdH and *cis*-[PtCl₂(*dms*o)₂], and 7.8 μ M for Pt-rtd, for 1 h. Cells were washed and replaced by a fresh medium. CHIKV replication was measured by *nanoluc* activity at 16 h.p.i.. Schematic representation of each time-based assay as indicated by BHK-21 cells (black cells), compound (green bars), DMEM medium (orange bars), and CHIKV-*nanoluc* (viral structure). Mean values $\pm$ SD of a minimum of three independent experiments each measured in triplicate. ns: non statistical difference, (****) $P < 0.0001$. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

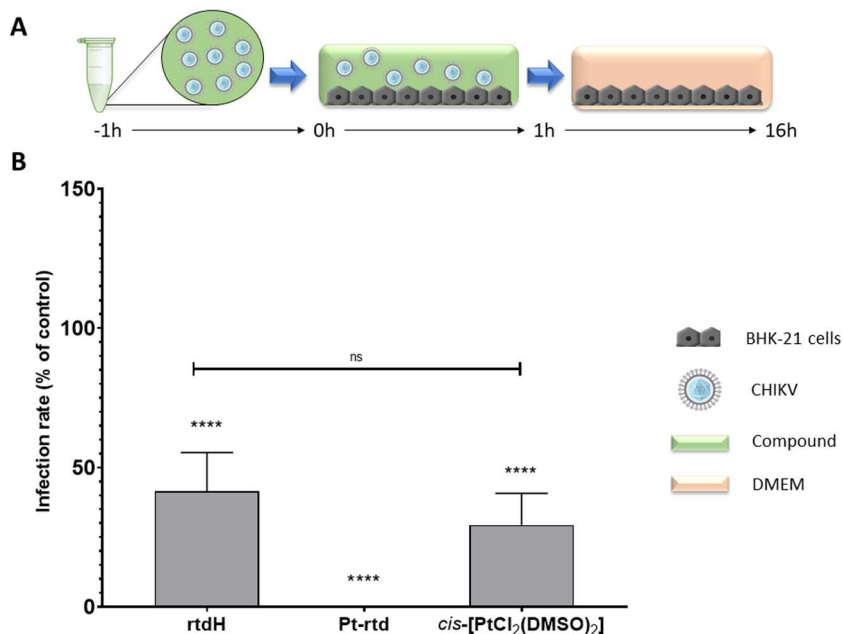


Fig. 5. Strong virucidal effect of Pt-rtd against CHIKV entry to the host cells. (A) and (B) CHIKV-*nanoluc* and each compound at concentrations of 100 μ M for rtdH and *cis*-[PtCl₂(*dms*o)₂], and 7.8 μ M for Pt-rtd, were incubated for 1 h at 37 $^{\circ}$ C and then for one extra hour with the cells. Then, the virus and compound were removed, cells were washed with PBS, and added fresh medium. CHIKV replication was measured by *nanoluc* activity at 16 h.p.i.. Schematic representation of each time-based assay as indicated by BHK-21 cells (black cells), compound (green bars), DMEM medium (orange bars), and CHIKV-*nanoluc* (viral structure). Mean values $\pm$ SD of a minimum of three independent experiments each measured in triplicate. ns: non statistical difference, (****) $P < 0.0001$. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

was found that rtdH presented 14% of dsRNA interaction when compared to the *dms*o negative control, quantified by densitometry (Fig. 7), whereas Pt-rtd and *cis*-[PtCl₂(*dms*o)₂] interacted with the dsRNA in 87% and 100%, respectively (Fig. 7). Notice that the band of the sample treated with doxorubicin (positive control of dsRNA interaction) does not appear in the gel thus reinforcing the observed result. It is important to highlight that the coordination of rtdH (without interaction activity) with *cis*-[PtCl₂(*dms*o)₂], resulted in a compound with strong interaction activity in dsRNA.

3.6. Pt-rtd and rtdH induce molecular shift in CHIKV glycoproteins

The infrared spectra of rtdH, Pt-rtd, and *cis*-[PtCl₂(*dms*o)₂] showed vibrational modes in accordance with our previous data (Pereira et al., 2020). The representatives mean infrared spectra of CHIKV prior and after the treatment with rtdH, Pt-rtd, or *cis*-[PtCl₂(*dms*o)₂] are shown in

Fig. 8A–C. The bio-fingerprint in the range of 1800–800 cm^{-1} indicates absorption bands of glycoproteins, proteins, lipids, and RNA of CHIKV and may be exploited to suggest the interaction between the virus and the compounds rtdH, Pt-rtd, or *cis*-[PtCl₂(*dms*o)₂]. The second derivative spectrum is capable to identify the accurate spatial distribution of each wavenumber referring to each biochemical component (Kohler et al., 2007; Rieppo et al., 2012). In this context, infrared spectra can also detect binding between different functional groups of materials and biological samples (Haris, 2013). We highlighted the functional group characterization in the shift interaction of each vibrational mode (Fig. 8D–E).

The binding interaction between CHIKV and rtdH is suggested by the absence of the vibrational mode at 1150 cm^{-1} in {CHIKV + rtdH}, which is related to the C–O stretching vibration present in CHIKV glycoprotein (Fig. 8G). There is an extensive overlap of vibrational modes in {CHIKV + Pt-rtd} samples when compared with CHIKV or Pt-rtd alone.

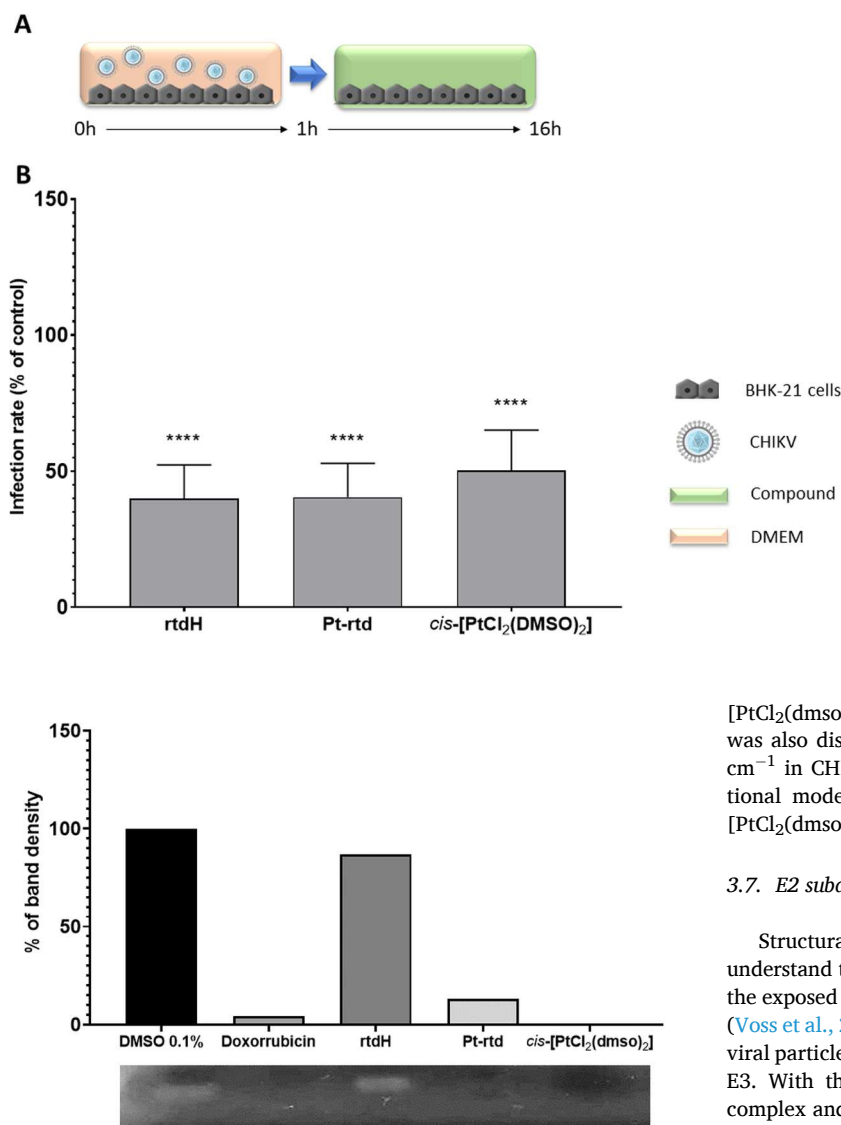


Fig. 7. Pt-rtd and *cis*-[PtCl₂(dmsO)₂] interacts with viral dsRNA. Fifteen nanomoles of JFH-1 HCV dsRNA were incubated with rtdH at 100 μ M, Pt-rtd at 7.8 μ M, or *cis*-[PtCl₂(dmsO)₂] at 100 μ M or the controls (DMSO 0.1% and doxorubicin at 100 μ M) for 45 min at room temperature. The reaction products were subjected to 1% agarose 1X TAE electrophoresis gel containing Ethidium Bromide followed by densitometry analysis on ImageJ.JS version 1.53j.

Noticeably, the vibrational mode at 1150 cm^{-1} in CHIKV was not present in {CHIKV + Pt-rtd} which suggests an interaction of Pt-rtd with C=O groups of the CHIKV glycoprotein (Fig. 8H). In addition, it was also identified a wide-ranging overlap of vibrational modes of {CHIKV + Pt-rtd} with CHIKV and Pt-rtd between 1100 and 1000 cm^{-1} . However, the {CHIKV+ Pt-rtd} vibrational mode at 978 cm^{-1} neither correlates to 986 cm^{-1} in CHIKV and nor to 970 cm^{-1} in Pt-rtd. Although within the resolution range of 978 cm^{-1} , this shift may also suggest an additional interaction between CHIKV and Pt-rtd, mainly in viral glycoprotein regions (Fig. 8K).

On the other hand, regarding the rtdH, the vibrational mode at 986 cm^{-1} in CHIKV was shifted to 977 cm^{-1} when rtdH were added to CHIKV {CHIKV + rtdH}, suggesting an interaction between CHIKV and rtdH (Fig. 8J). This vibrational mode in CHIKV can be assigned to O—CH₃, related to glycoproteins (Fig. 8J). The binding interaction between CHIKV and *cis*-[PtCl₂(dmsO)₂] is suggested by the absence of the overlap in vibrational mode at 1150 cm^{-1} in {CHIKV + *cis*-[PtCl₂(dmsO)₂]}, which was present in both CHIKV and *cis*-

Fig. 6. Effects of rtdH, Pt-rtd, and *cis*-[PtCl₂(dmsO)₂] on post-entry stages of CHIKV. (A) and (B) BHK-21 cells were infected with CHIKV-*nanoluc* (MOI 0.1) for 1 h, washed to remove unbound virus and added of medium containing each compound at concentrations of 100 μ M for rtdH and *cis*-[PtCl₂(dmsO)₂], and 7.8 μ M for Pt-rtd for 16 h. *Nanoluciferase* levels were measured to assess CHIKV-*nanoluc* replication rates. Schematic representation of each time-based assay as indicated by BHK-21 cells (black cells), compound (green bars), DMEM medium (orange bars), and CHIKV-*nanoluc* (viral structure). Mean values $\pm$ SD of a minimum of three independent experiments each measured in triplicate. (****) $P < 0.0001$. Mean values $\pm$ SD of a minimum of three independent experiments each measured in triplicate. All images were generated using GraphPad Prism 8 and GIMP 2.10.20 (www.gimp.org).

[PtCl₂(dmsO)₂] (Fig. 8I). The CHIKV interaction with *cis*-[PtCl₂(dmsO)₂] was also displayed by the minor shift in the vibrational mode at 980 cm^{-1} in CHIKV+ *cis*-[PtCl₂(dmsO)₂], which does not overlap in vibrational mode at 986 cm^{-1} in CHIKV neither with 973 cm^{-1} in *cis*-[PtCl₂(dmsO)₂] (Fig. 8L).

3.7. E2 subdomain b is a likely binding site to the intact Pt-rtd compound

Structural studies (Crystallography and CryoEM) have been used to understand the organization of the CHIKV envelope glycoproteins with the exposed regions of the glycoproteins as part of the spike determined (Voss et al., 2010b; Yap et al., 2017). In particular, upon assembly of the viral particle, E2 (subdomains A and B) remains exposed, surrounded by E3. With this structural information in mind, the compound Pt-rtd complex and the ligand rtdH were docked against the exposed regions of the CHIKV glycoprotein. A summary of the docking scores obtained with the docking procedure is shown in Table 2.

As one may observe, Pt-rtd shows a relevant binding with E2 subdomain B (docking score = -2.109), while the free rtdH returned no poses for this region of the glycoprotein. Fig. 9B shows the best ranked pose for the interaction of Pt-rtd with E2 subdomain B. The ligand interaction diagram is highlighted in Fig. 9C, in which it is possible to observe that Val216 established a hydrogen-bonding network with both the NH₂ group of rtdH of Pt-rtd and with one of the chloride ligands.

Nonetheless, for E3, rtdH presented a better docking score (-0.657 , see Fig. 10) than Pt-rtd (-0.311 , see Fig. 9D and E), although both values are much lower than that observed for the binding of Pt-rtd with E2 subdomain B. No poses returned from E2 subdomain A for either Pt-rtd or rtdH.

Collectively, the docking studies indicate that the E2 subdomain B is a likely binding site to the intact Pt-rtd compound, which correlates with the viral entry inhibition found in this study. From this initial recognition site, the interaction can evolve into a covalently bound species.

4. Discussion

As previously stated, the aminoadamantanes, which include rimantadine, are well known for their therapeutic properties (Erkulwater and Pillai, 1989; Jefferson et al., 2002; Spilovska et al., 2016; Tampi and Dyck, 2007). The results presented in this manuscript suggest that rtdH may also be considered as a potential repurposing drug to treat CHIKV

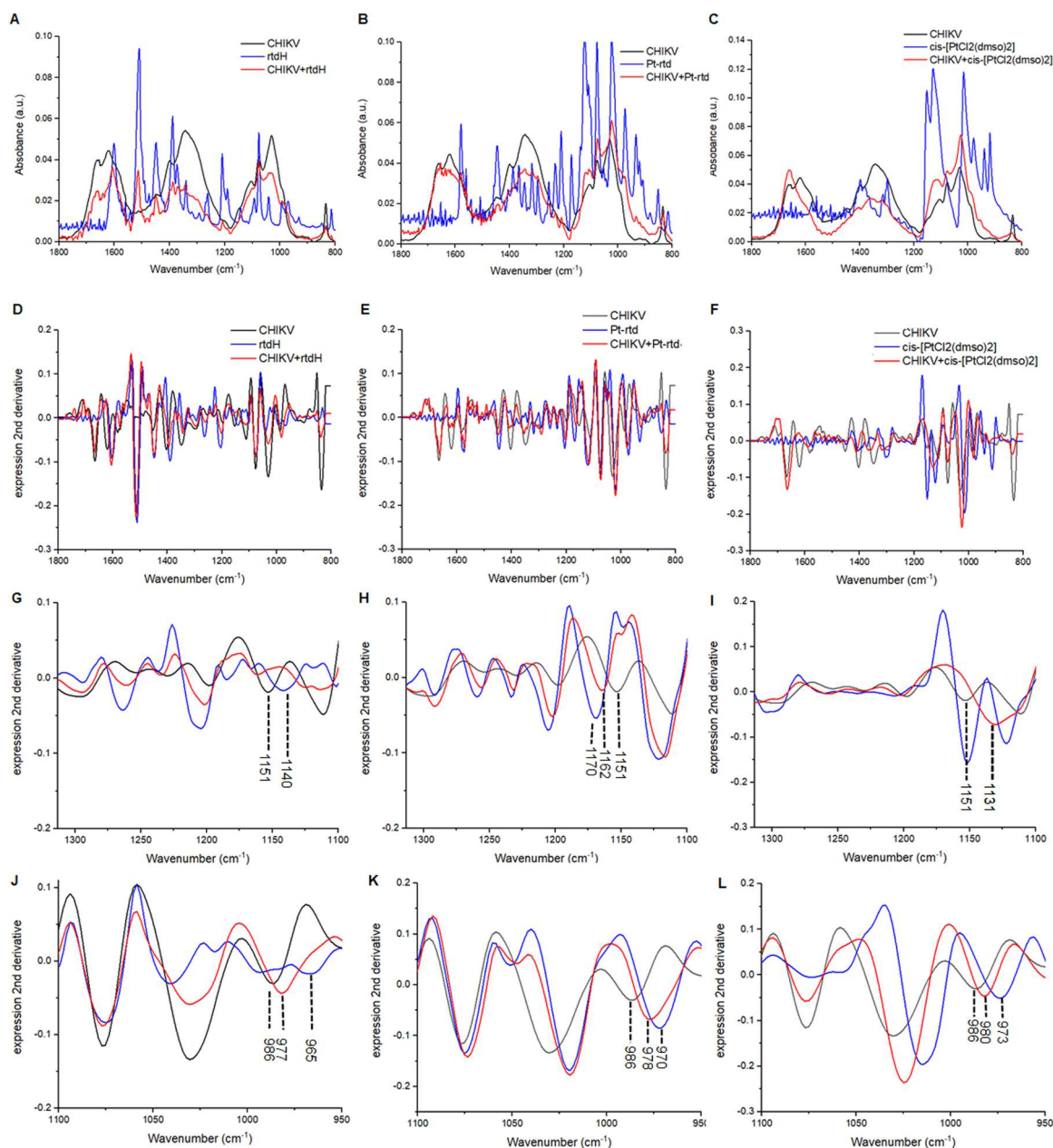


Fig. 8. ATR-FTIR indicates molecular shifts in CHIKV virion induce by rtdH, Pt-rtd, and *cis*-[PtCl₂(*dmsO*)₂]. The representative infrared average spectrum of second derivative analysis from rtdH (D), Pt-rtd (E), and *cis*-[PtCl₂(*dmsO*)₂] (F) (blue line representing the compounds), CHIKV virion (black line), and each compound plus CHIKV virion (red line) employing a Fourier Transform Infrared (FTIR) methodology, in wavenumber ranging from 1800 to 800 cm⁻¹. A second derivative analysis, which the value heights indicate the intensity of each functional group among 1300 to 1100 cm⁻¹ wavenumbers (G, H, and I to rtdH, Pt-rtd, and *cis*-[PtCl₂(*dmsO*)₂], respectively), and to 1100 to 950 cm⁻¹ wavenumbers (J, K, and L to rtdH, Pt-rtd, and *cis*-[PtCl₂(*dmsO*)₂], respectively).

Table 2

Glide docking score obtained for the best-scored poses of either the compound Pt-rtd or the free ligand rtd, docked against different exposed regions of the glycoprotein of the Chikungunya virus (PDB entry 3N42).

CHIKV glycoprotein domain	Pt-rtd	rtd
E2 subdomain A	–	–
E2 subdomain B	–2.109	–
E3	–0.311	–0.657

infections, since it presents consistent anti-CHIKV results (EC₅₀ of 71.64 μM, SI of 8.4), protecting the cells against viral infection, impairing viral entry (possibly by a virucidal effect), and inhibiting post-entry steps in viral replication. Considering that rtdH is an approved drug with a

pharmacological profile and adverse effects earlier described in the literature (Alves Galvão et al., 2014), clinical studies for repurposing may be shortened. Our data demonstrated the protective effect of rtdH and Pt-rtd, impairing CHIKV entry on cells. These results might be explained by the necessity of Ca²⁺ and Cl⁻ ions for both CHIKV entry and rtdH uptake (Kooijmans et al., 2012). As a result, a competition towards the available Ca²⁺ and Cl⁻ might be occurring. Additionally, aminoadamantanes were described as inhibitors of the clathrin reorganization into clathrin-coated pits which are necessary for endocytosis and consequently, viral internalization (Eckels et al., 2009). Therefore, is possible to hypothesize that rtdH effects in protecting cells are probably due to a disturb in clathrin reorganization. More interesting, the coordinated compound (Pt-rtd) displayed higher protection against infection, probably by a synergic outcome: preventing CHIKV to

penetrate the host cells and interacting with the viral dsRNA, impairing further viral replication.

Furthermore, it is well known that the treatment employing rimantadine against both Influenza A virus and Hepacivirus C reduce viral infection by blocking the transport of protons in the M2 and p7 viroporin ion channel, respectively, which is a highly selective pH-regulated channel present in viral envelope (Intharathep et al., 2008; OuYang and Chou, 2014). Similarly, CHIKV expresses the 6K protein, and recently, it was characterized as a viroporin (same class of M2 channel), a selective proton channel that was found to be inhibited by amantadine (Dey et al., 2019). Moreover, 6K was described with fusogenic properties in membranes (Dey et al., 2019) and, therefore, it is possible to hypothesize that rtdH also acts as a 6K viroporin blocker, which may explain the effect on multiple steps of CHIKV replication. Since 6K viroporin does not possess a crystal structure available, molecular docking studies could not be performed. Additionally, interactions with other viral biomolecules may also play an important role in rtdH activity. One may also highlight that rtdH is already employed to treat viral infections, with a well-established ADMETox profile with a safe therapeutic index for humans (Atiee et al., 2012; Bantia et al., 2010). Such data may stimulate further clinical assays regarding its antiviral effect on human subjects.

The Pt-rtd complex presented EC₅₀ and CC₅₀ with less selectivity towards BHK-21 cells. Lower CC₅₀ values for platinum(II)-based complexes are somewhat expected due to their relative cytotoxicity by DNA crosslinks, forming bifunctional adducts (Margiotta et al., 2004; Shahabadi et al., 2012). Nevertheless, the remarkable anti-CHIKV activity of Pt-rtd in the inhibition of viral entry and post-entry steps, as well as in protecting cells of infection, may overcome the lower selectivity in terms of a better antiviral outcome even at lower concentrations. In this context, Pt-rtd showed the strongest effects on the early stages of the CHIKV life cycle, over 92% (and up to 100%) inhibition of CHIKV infection in pre-treatment, entry, and virucidal protocols. In addition, when cells were treated with Pt-rtd after viral entry (Fig. 6), the antiviral effect decreased in comparison to the other treatment protocols, which might be explained by the shorter time of interaction between the compound and CHIKV particles when the early stages of the infection were evaluated. Therefore, it emphasizes that the complexation significantly contributed to the antiviral activity and probably act by a synergistic mechanism.

As possible targets, since Pt(II) compounds are known to interact with RNA and/or DNA (Zou et al., 2014), the Pt-rtd ability to interact with viral dsRNA was also investigated. The data showed that Pt-rtd effectively interacts with dsRNA, also figuring as a possible target for such complex. Moreover, due to the remarkable activity of Pt-rtd on the early stages of CHIKV life cycle and the fact that other Pt(II) complexes have also been shown to interact with lipid bilayers, culminating in effects on membrane fluidity, cell signaling pathways, as well as interacting with phospholipids and anionic lipids (Martinho et al., 2019), it is possible to speculate that the effect of Pt-rtd on viral entry and post-entry steps might be associated with direct action on the viral envelope, such as on viral envelope glycoproteins (Bailly et al., 2021). To further investigate such possibility, the ATR-FTIR technique was employed, which showed that Pt-rtd was able to interact with the envelope glycoproteins. The CHIKV vibrational mode at 1151 cm⁻¹ assigned to glycoproteins was not detected in the CHIKV+Pt-rtd sample in the same concentration. It could be related to the Pt-rtd interaction with CHIKV glycoproteins. This is further supported by molecular docking in which the E2 subdomain B is a likely binding site to the intact Pt-rtd compound. From this initial recognition site, the interaction can evolve into a covalently bound species. Additional interactions with the protein glyocalyx cannot be excluded. Nevertheless, such evaluation could not be performed since all the available crystallographic data for the envelope protein do not present the complete carbohydrate chain. The lack of the carbohydrate chain is probably due to the protein hydrolysis during crystallization (Ishchenko et al., 2017).

Metal compounds are known for alterations in transcription factors, enzymes, cell cycle through coordination (De Castro et al., 2022). The *cis*-[PtCl₂(dmsO)₂] species possess a high affinity for proteins and enzymes with thiol or selenol groups in their structure (Boros et al., 2020; De Castro et al., 2022). Therefore, it was used as a precursor for the synthesis of the Pt-rtd complex and was also evaluated against CHIKV infection. As an interesting outcome, this molecule presented an EC₅₀ of 59.87 μM and a SI of 601.2. To the best of our knowledge, there is no description of the antiviral activity of this precursor against CHIKV in the literature. The biological properties of platinum salt as *cis*-[PtCl₂(dmsO)₂] have been poorly investigated, being only the metal complexes analyzed by their biological activities. Vasic and coworkers demonstrated the interaction of [PtCl₂(dmsO)₂] with L-cysteine, a naturally occurring essential amino acid to protein synthesis (Vasić et al., 2009), and it might suggest the antiviral activity of *cis*-[PtCl₂(dmsO)₂] presented here, impairing viral replication in all steps. Although cytotoxic *in vivo* (Lakomska et al., 2020; Oliveira et al., 2020a; Shahabadi et al., 2012), complexes with similar structures of *cis*-[PtCl₂(dmsO)₂] and/or associated with nanostructures may present similar viral inhibition on entry and post-entry steps encouraging further investigations of similar compounds. Another hypothesis is the synergistic activity of rtdH and *cis*-[PtCl₂(dmsO)₂]. These compounds were previously described to possess distinct mechanisms of action (endocytose/viroporin inhibitor and dsDNA interaction, respectively). In this context, our data demonstrated that, when coordinated, the Pt-complex presented a more robust antiviral effect, as seen in Figs. 3–6. This fact relates to the intrinsic structural and functional versatility of coordinated compounds, mainly with organic ligands (Shah et al., 2015). The data here demonstrates the possibility of this complex interfering with different targets in viral replication, employing new approaches and emphasizing that metal complexes could be more efficient as antiviral than the free ligands (Shah et al., 2015).

Collectively, the obtained data suggest that the metal ion plays an important role in the antiviral action of the Pt-rtd complex, which may further stimulate the development of platinum(II)-based compounds targeting membrane and/or viral envelope dynamics.

5. Conclusions

Considering the data described here, rtdH and the Pt-rtd complex significantly interfered in the early stages of CHIKV life cycle, with the strongest effect observed to Pt-rtd, which reduced up to 100% of CHIKV infection. Our findings suggest that the commercial drug rtdH can emerge as a repurposing agent for CHIKV treatment. Such data warrants further studies *in vivo*, possibly providing a rapid and efficient treatment for patients with acute and/or chronic Chikungunya fever in a repurposing approach. In special, the Pt-rtd complex showed the strongest effects on the early stages of the CHIKV life cycle, also significantly interfering with post-entry stages of infection and in protecting cells of CHIKV infection, exhibiting a potential multiple action antiviral agent. This fact suggests that the metal ion plays an important role in the antiviral action of such compound, which might be useful for further development of new antiviral approaches against CHIKV and reinforce the potential of the use of platinum(II) metallodrugs for the treatment of antiviral diseases as the Chikungunya fever. Moreover, molecular docking analysis and ATR-FTIR spectroscopy suggest an interaction of Pt-rtd with CHIKV glycoproteins, potentially related to the mechanism of inhibition of viral entry by Pt-rtd.

CRedit authorship contribution statement

Igor Andrade Santos: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Anna Karla dos Santos Pereira:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Marco Guevara-Vega:** Conceptualization, Data curation, Formal analysis,

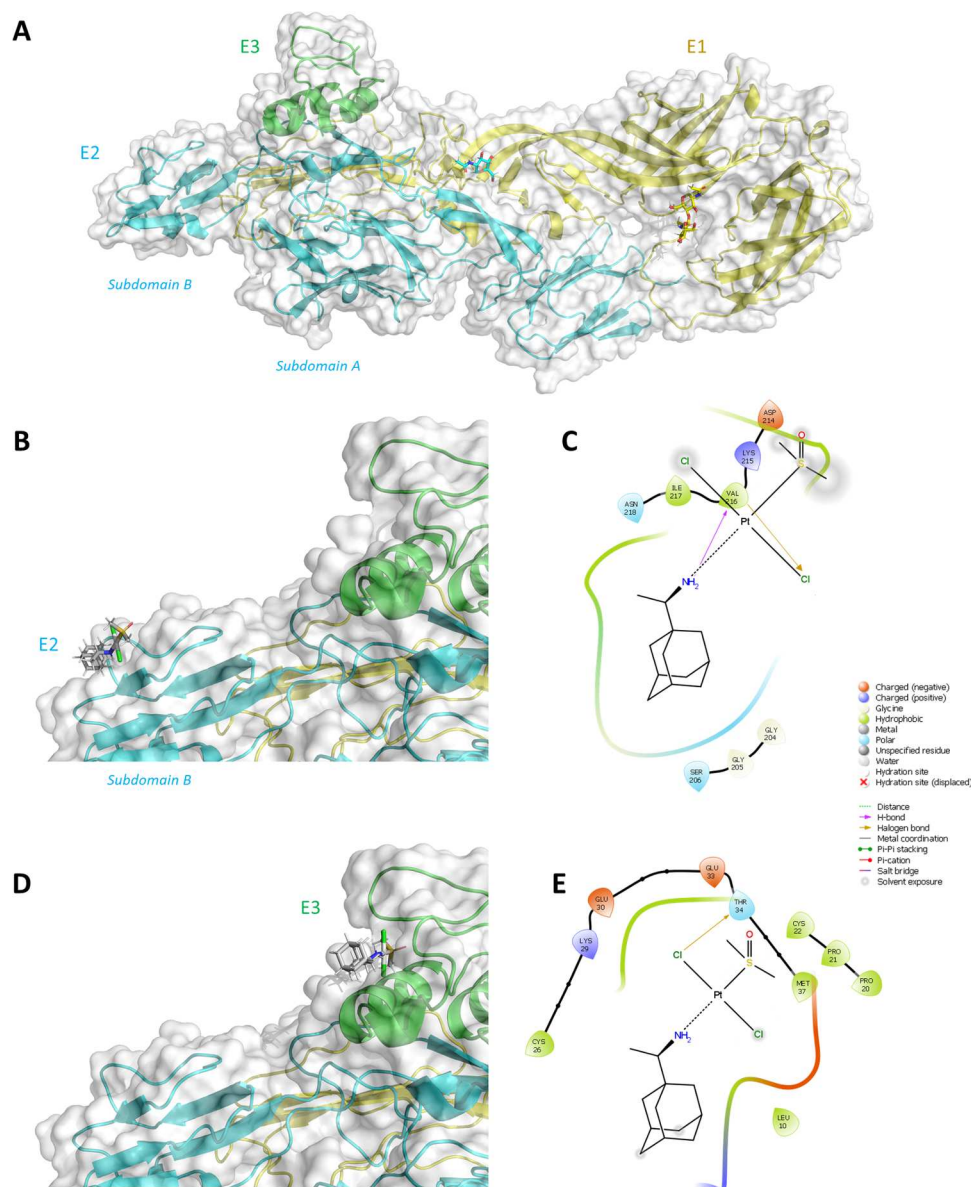


Fig. 9. (A) Structural representation of the CHIKV glycoprotein, and its exposed domains (PDB 3N42). The exposed regions are labeled, E2 (subdomains A and B) and E3. Glycans are explicitly shown as sticks. (B) Best ranked pose for the interaction of compound Pt-rtd with E2 subdomain B, with the corresponding ligand interaction diagram shown in (C). (D) Best ranked pose for the interaction of Pt-rtd with E3, with the corresponding ligand interaction diagram shown in (E). Molecular visualizations were prepared with Pymol®, while ligand interaction diagrams were prepared with Maestro®.

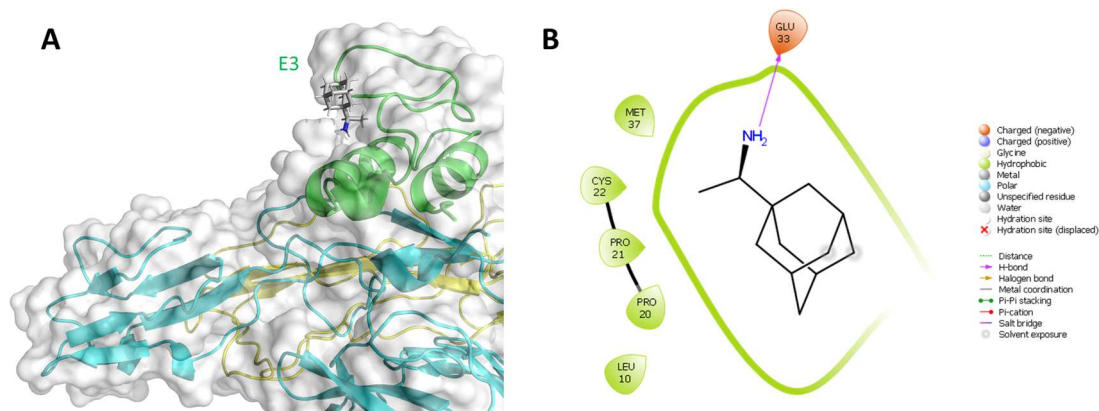


Fig. 10. A. Best-ranked pose for the interaction of rtd with the E3 region of the CHIKV glycoprotein (PDB 3N42), and corresponding ligand binding diagram (shown in B).

Writing – original draft, Writing – review & editing. **Raphael Enoque Ferraz de Paiva**: Conceptualization, Data curation, Formal analysis, Visualization, Supervision, Writing – original draft, Writing – review & editing. **Robinson Sabino-Silva**: Visualization, Supervision, Writing – original draft, Writing – review & editing. **Fernando R.G. Bergamini**: Visualization, Supervision, Writing – original draft, Writing – review & editing. **Pedro P. Corbi**: Visualization, Supervision, Writing – original draft, Writing – review & editing. **Ana Carolina G. Jardim**: Conceptualization, Data curation, Formal analysis, Visualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CAPÍTULO 6: TRIPHENYLPHOSPHINE GOLD(I) DERIVATIVES PROMOTE ANTIVIRAL EFFECTS AGAINST THE CHIKUNGUNYA VIRUS

MANUSCRITO PUBLICADO EM “METALLOMICS”

Triphenylphosphine gold(I) derivatives promote antiviral effects against the Chikungunya virus

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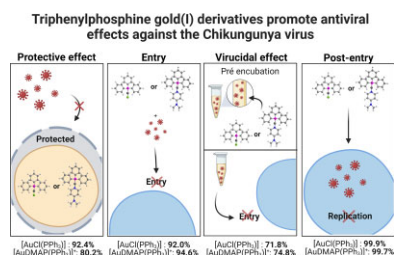
Twitter details and summary: Triphenylphosphinegold(I) complexes inhibit 99% of Chikungunya replication by promoting intracellular effects. @Gremmlenz @Cabbhausen

Abstract

Herein a systematic series of four $[\text{AuLL}']^n$ $n = 0, +1$ complexes, where $L = 1,3\text{-bis(mesityl)imidazole-2-ylidene (IMes)}$, or triphenylphosphine (PPh_3), and $L' = \text{chloride}$, or 4-dimethylaminopyridine (DMAP), had their *in vitro* antiviral activity assessed against Chikungunya virus (CHIKV). The PPh_3 derivatives inhibited viral replication by 99%, whereas the IMes derivatives about 50%. The lipophilicity of the PPh_3 derivatives is higher than the IMes-bearing compounds, which can be related to their more prominent antiviral activities. The dissociation of DMAP is faster than chloride in solution for both IMes and PPh_3 derivatives; however, it does not significantly affect their *in vitro* activities, showing a higher dependence on the nature of L rather than L' towards their antiviral effects. All complexes bind to N-acetyl-L-cysteine, with the Ph_3P -bearing complexes coordinating at a faster rate to this amino acid. The binding constants to bovine serum albumin are in the order of 10^4 , slightly higher for the DMAP complexes in both PPh_3 and IMes derivatives. Mechanistic investigations of the PPh_3 complexes showed a ubiquitous protective effect of the compounds in the pretreatment, early stages, and post-entry assays. The most significant inhibition was observed in post-entry activity, in which the complexes blocked viral replication in 99%, followed by up to 95% inhibition of the early stages of infection. Pretreatment assays showed a 92% and 80% replication decrease for the chloride and DMAP derivatives, respectively. dsRNA binding assays showed a significant interaction of the compounds with dsRNA, an essential biomolecule to viral replication.

Keywords: Chikungunya, gold, metallodrugs, antivirals

Graphical abstract



The effects of gold(I) complexes on different stages of the CHIKV replicative cycle were accessed, showing the complexes protect the cells against infection and also present a potent effect on the post-entry step. The complete inhibition of replication in viable concentrations might be due to the interference in cellular pathways or binding to viral proteins in the cytoplasm.

Introduction

Gold(I) and gold(III)-based complexes have promising antiviral, anticancer, antiparasitic, and antibacterial activities.^{1–7} The

FDA-approved antirheumatic gold(I) thiolates, especially auranofin, have also been explored as potential antiviral agents against human immunodeficiency virus (HIV), with impressive results.^{8,9} Such aurothiolates prevented the propagation of the

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virus by immunomodulation.¹⁰ Auranofin, in particular, reduced the virus reservoir in simian immunodeficiency virus infected monkeys and decreased the viral load in HIV-infected patients.¹¹ Other classes of Au(I) compounds bearing phosphor-based ligands have also presented promising results as potential anti-HIV agents, with a direct effect on the inhibition of the HIV reverse transcriptase.¹²

The current coronavirus disease 2019 (COVID-19) pandemic has stimulated the discovery of new gold-based compounds and the evaluation of the repurposing potential of auranofin against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).^{13,14} Rothan and co-workers showed that auranofin inhibits SARS-CoV-2 replication ($EC_{50} = 1.4 \mu\text{mol} \cdot \text{L}^{-1}$) and reduces cytokine production caused by the infection.¹³ Furthermore, Gil-Moles and colleagues reported that auranofin, along with a series of neutral and cationic Au(I)-N-heterocyclic-carbenes (Au-NHC), inhibited both SARS-CoV-2 spike-ACE2 interactions, and papain-like protein (PLpro).¹⁴ They also showed that the mechanism of PLpro inhibition could involve the ejection of the zinc(II) ion, in agreement with the studies of our research group comprising zinc finger inhibition.¹⁵ Ott and co-workers, on the other hand, screened a great selection of coordination chemotypes bearing a variety of metal ions, and ligands against PLpro, in which the Au(I) compounds presented striking results. The best candidates were Aurothiomalate, $[\text{AuCl}(\text{Et}_3\text{P})]$ and several Au(I)-N-heterocyclic-carbenes (NHC) of formula $[\text{AuCl}(\text{NHC})]$.^{16,17}

Although gold(I)/III complexes have been explored as potential antiviral agents against HIV and SARS-CoV-2, to our knowledge, complexes encompassing such metal ions have not been evaluated as possible antiviral compounds against other RNA viruses, such as Chikungunya virus (CHIKV). CHIKV, the etiologic agent of Chikungunya fever, belongs to the genus *Alphavirus*, the family *Togaviridae*.^{18,19} It is transmitted mainly by the bite of *Aedes aegypti* and *Aedes albopictus* female mosquitoes, figuring as an endemic disease in tropical and subtropical countries.^{20,21} Additionally, CHIKV has also been identified in the Mediterranean countries along with North America.²² The main symptoms include fever, nausea, fatigue, arthralgia, and polyarthralgia. Some patients can develop hepatitis, myocarditis, and encephalopathy, which can lead to death.²³ Furthermore, the infection can progress to a chronic condition, resulting in disability.²⁴ However, there are no antiviral drugs against CHIKV infection, resulting in health and economic impacts since the protocols to manage and treat Chikungunya fever are palliative.

The CHIKV particle contains a single-strand positive-sense RNA of approximately 12 kb, associated with a capsid protein (C), and it is enveloped by a host-derived lipid membrane containing spike glycoproteins (E1-E2-E3 trimer).^{25,26} The CHIKV RNA possesses two open reading frames (ORFs), in which ORF-1 encodes four non-structural proteins (nsP1-4) responsible for forming the replicative complex and ORF-2 that is translated into the structural proteins E1, E2, E3, C, and 6K. These proteins are essential for viral replication within the host cells, and molecules that can selectively target one or more of those proteins represent promising candidates to be further capitalized into treatment as direct-acting antivirals (DAAs).²⁷ The DAAs inhibit viral replication without interfering with host cell metabolism.^{28,29} In the CHIKV case, identifying DAAs might reduce the chronification process by impairing viral replication in the early stages of the disease, as previously explored for other viral infections such as Hepatitis C.³⁰

The soft Pearson acidity of gold increases the tendency of Au(I) complexes to interact with soft bases such as thiols and selenols. Thioredoxin reductase, a selenocysteine-bearing protein, is a valid

target for gold complexes, the cysteine-rich cathepsin, and zinc finger (ZF) domains.^{31–33} Abbehausen and colleagues have shown the ability of $[\text{AuL}'(\text{PPh}_3)]^n$, $n = 0, +1$ complexes to interact with peptide models of HIV-1 nucleocapsid protein (NCp7), a validated target for antiretroviral design. The ZFs possess multiple roles as they interact with viral genomes and host mRNAs.³⁴ In the case of CHIKV, palmitoyltransferases have two zinc finger domains that recognize nsP1 for palmitoylation, a critical reaction for viral replication.³⁵

In front of the possible application of Au(I) complexes as effective antiviral agents against RNA viruses, herein, a systematic series of four $[\text{AuLL}]^{n+}$ $n = 0, +1$ complexes, where $\text{L} = 1,3\text{-bis}(\text{mesityl})\text{imidazole-2-ylidene}$ (IMes), or triphenylphosphine (PPh_3), and $\text{L}' = \text{chloride}$, and 4-dimethylaminopyridine (DMAP) (Fig. 1) were synthesized and had the *in vitro* anti-CHIKV assessed, pin-pointing potential modes of action.

EXPERIMENTAL METHODS

Materials and equipment

Triphenylphosphine, glyoxal, mesitylamine, $\text{K}[\text{AuCl}_4]$, tetrahydrothiophene (tht), silver nitrate, 4-dimethylaminopyridine (DMAP), p-formaldehyde, and chlorotrimethylsilane were supplied by Sigma-Aldrich (Brazil) and used as received. Solvents, salts, and common lab reagents were used as received.

^1H NMR spectra were acquired in a Bruker Avance 250 MHz operating at 250.1 MHz, and ^{31}P and ^{13}C NMR were acquired in a Bruker Avance III 500 MHz operating at 125.7 MHz and 202.35 MHz, respectively. ^1H NMR and ^{13}C NMR spectra were referenced to tetramethylsilane (TMS), and ^{31}P NMR spectra were referenced to H_3PO_4 85%. Fluorescence spectra were acquired in an Agilent Cary Eclipse using a 3 ml (10 mm optical path) cuvette. The octanol/water partition coefficients were obtained by the shake flask method.³⁶ It was performed in a shaker Kasvi Basic 3D K45-4020, centrifuged in an Eppendorf centrifuge model 5804, rotor S 4-72 adapted with 15 ml conic tube holders. Atomic emission induced coupled plasma (ICP-AES) was performed in a Perkin-Elmer Optima 8300, argon flow 15 ml min^{-1} , nebulizer flow 0.75 ml min^{-1} , auxiliary gas (N_2) flow 0.5 ml min^{-1} and radiofrequency source potency 1450 W. Mass spectrometry was performed in a Waters Quattro Micro by direct infusion. Elemental analysis was performed in a Perkin-Elmer 2400 Series II.

Synthesis of $[\text{AuCl}(\text{tht})]$

The methodology was adapted from Uson and co-workers.³⁷ In a glass flask $\text{K}[\text{AuCl}_4]$ (400 mg, 1.12 mmol), deionized water (4.0 ml), and ethanol (4.0 ml) were agitated until homogenization. Tetrahydrothiophene (tht) (198 μL , 0.53 mmol) was slowly added to the mixture. The solution was stirred for 15 min, and the formed yellow precipitate was collected by filtration, washed with deionized water ($2 \times 4.0 \text{ ml}$), and vacuum dried in the dark. The dried solid was stored in the dark under an argon atmosphere at 18°C . Yield: 325 mg (96%). ^1H NMR (250 MHz, CDCl_3): δ 3.41 (s, 4H); 2.18 (s, 4H).

Synthesis of N, N'-dimesitylenediimine

In a round bottom flask, 2,4,6-trimethylaniline (14.4 ml) and 50 ml of isopropyl alcohol were mixed. Subsequently, a water solution (6.0 ml) containing 2.343 ml of glyoxal was added and kept under vigorous stirring for 24 h. The yellow solid was collected in a Büchner funnel, washed with water ($2 \times 100 \text{ ml}$), and kept under IR light until dryness. Yield: 28.9% (4.34 g). ^1H NMR (250 MHz, CDCl_3): δ 8.10 (s, 2H); 6.91 (s, 4H); 2.30 (s, 6H); 2.16 (s, 12H).

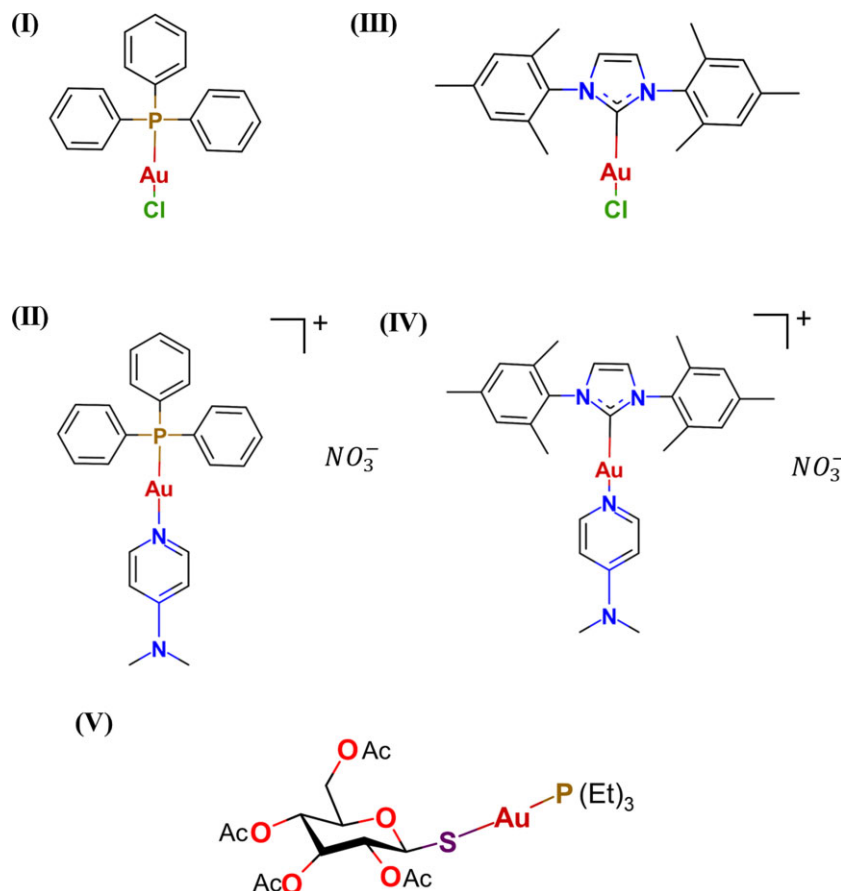


Fig. 1. Structures of the complexes I–IV and auranofin (V).

Synthesis of 1,3-Bis(mesityl)imidazolium chloride (IMes·HCl)

In a round bottom flask, ethyl acetate (EtOAc) was heated to 70°C. Then, 4.0 g N, N'-dimesitylenediimine was added, followed by 0.294 ml of p-formaldehyde. A solution of chlorotrimethylsilane (1.747 ml) in EtOAc (2.0 ml) was further added dropwise under vigorous stirring for 45 min. The solution was kept under stirring for 2 h at 70°C. After this period, a brownish suspension was formed, cooled to 6.0°C, and kept under stirring for 14 h. The solid was collected by filtration, washed with EtOAc (2 × 50 ml), diethyl ether (Et₂O, 1 × 50 ml), and dried under IR light until the yellow-brownish solid turned into a white microcrystalline material. Yield: 86% (4.47 g). ¹H NMR (250 MHz, CDCl₃): δ 10.77 (s, 1H), 7.62 (s, 2H), 7.01 (s, 4H), 2.32 (s, 6 H), 2.16 (s, 12H).

Synthesis complex I ([AuCl(PPh₃)])

The methodology was adapted from the literature.³⁸ Potassium tetrachloroaurate (K[AuCl₄], 1.00 mmol, 0.3778 g) was dissolved in a mixture ethanol:acetone 1:1 (V/V) (3.0 ml). A solution of triphenylphosphine (2.00 mmol, 0.5245 g) in CH₃Cl (6.0 ml) was added, and the mixture was kept under magnetic stirring for 10–20 min. The white precipitate was collected by filtration, washed with ethanol, acetone, Et₂O, and dried under vacuum. The solid was recrystallized from a CH₃Cl solution by adding drops of Et₂O at room temperature until the formation of transparent needles. Yield: 75% (0.3741 g). ¹H NMR (500 MHz, DMSO-d₆): δ 7.61–7.5 (m, 15H). ³¹P NMR (500 MHz, DMSO-d₆): δ 32.68. Anal. Calcd. (%) for

C₁₈H₁₅ClPAu: C, 43.70; H, 3.06; N, 0.00. Found (%): C, 43.27; H, 3.11; N, 0.06 (Table S1, ESI).

Synthesis complex III ([AuCl(IMes)])

The methodology was adapted from Visbal and co-workers.³⁹ In a round bottom flask [AuCl(tht)] (0.35 mmol, 0.1122 g) and IMes·HCl (0.35 mmol, 0.1193 g) were dissolved in CH₂Cl₂ (15.0 ml). After 15 min, K₂CO₃ (7.00 mmol, 0.9670 g) was added, and the reaction was kept under stirring for 1.5 h. The volume was reduced in a rotatory evaporator, causing the precipitation of a solid, which was collected by filtration. The solid was purified by recrystallization at –20°C in CH₂Cl₂ for 24 h. The crystals were collected and washed with Et₂O. Yield: 91% (0.1312 g). ¹H NMR (250 MHz, CD₃CN): δ 7.35 (s, 1H), 7.11 (s, 4H), 2.36 (s, 6H), 2.130 (s, 12H). Anal. Calcd. (%) for C₂₁H₂₄N₂ClAu: C, 46.98; H, 4.51; N, 5.22. Found (%): C, 47.32; H, 4.50; N, 5.22 (Table S1, ESI).

Synthesis complex II and IV ([Au(DMAP)(PPh₃)]NO₃ and [Au(DMAP)(IMes)]NO₃)

The methodology was adapted from Abbehausen and collaborators.¹⁵ In a round-bottom flask, complex I or III (1.0 mmol) was dissolved in CH₃Cl (1.0 ml). An ethanolic solution (1.0 ml) containing AgNO₃ (1.0 mmol, 0.0152 g) was then added. The white precipitate (AgCl) was removed by filtration under Celite, and DMAP (1.0 mmol, 0.0122 g) dissolved in CH₃Cl (0.5 ml) was added to the filtrated solution. The mixture was kept under stirring in the dark for 24 h. After the reaction time, the solvent was vacuum removed, and the transparent film was then dissolved in acetone

(10 ml) and drops of Et₂O. The solution was kept at −20°C for 24 h, and a white precipitate was collected, washed with Et₂O, and vacuum dried. [Au(DMAP)(PPh₃)]NO₃ **II**. Yield 89% ¹H NMR (250 MHz, CD₃CN): δ 8.11 (d, 2H), 7.60 (m, 15H), 6.77 (d, 2H), 3.08 (s, 6H) ppm. ³¹P NMR (500 MHz, DMSO-d₆): δ 29.35. Anal. Calcd. (%) for C₂₅H₂₅AuN₃O₃P: C, 46.67; H, 3.92; N, 6.53. Found (%) C, 46.67; H, 3.90; N, 6.35 (Table S2, ESI). [Au(IMes)DMAP]NO₃ **IV**. Yield: 59% ¹H NMR (250 MHz, CD₃CN): δ 7.53 (d, 2H), 7.45 (s, 2H), 7.11 (s, 4H), 6.54 (d, 2H), 2.97 (s, 6H), 2.34 (s, 6H), 2.08 (s, 12H). Anal. Calcd. (%) for [(C₂₈H₃₄N₄)Au]NO₃·H₂O (C₂₈H₃₆N₅O₄Au): C, 47.80; H, 5.16; N, 9.95. Found (%) C, 48.03; H, 5.29; N, 9.98 (Table S2, ESI).

Exchange kinetics of complexes I–IV in different solvents by ¹H NMR

Before the ¹H NMR studies, the purities of all complexes were confirmed by elemental analysis. Complexes **I–IV** (5.0 mg) were dissolved in 600 μL DMSO-d₆ or DMSO-d₆: D₂O (1:2 V/V), transferred to NMR tubes, and kept at 25°C. ¹H NMR spectra were acquired at t = 0, 3 h, 6 h, 9 h, and 24 h. Extra time points were performed when needed. Ratios were calculated following appropriate signal integrals.

NAC interaction by ¹H NMR

The reaction between complexes and NAC was followed with time by employing a similar procedure to the kinetics in different solvents. NAC was dissolved in D₂O, and the complexes **I–IV** was dissolved in DMSO-d₆. The solutions were mixed to reach a 1:1 molar ratio of NAC: complexes (0.15 mol·L^{−1}). This proportion generated a solution 1:13 D₂O: DMSO-d₆ (V/V).

Bovine serum albumin fluorescence binding assay

The interactions of the metal complexes and bovine serum albumin (BSA) were evaluated by spectrofluorimetry based on intrinsic protein fluorescence emission.^{40,41} The changes in the emission spectrum of BSA in the presence of different complexes **I–IV** concentrations were used for the binding constants calculations, using Stern-Volmer equations, according to described methods.⁴²

In brief, a PBS (10.0 mmol·L^{−1}, pH 7.4) solution (2.5 ml) containing 20.0 μmol·L^{−1} of BSA was titrated with successive additions of 2.0 μL of DMSO solutions containing 2.0 mM of the complexes reaching final concentrations from 0.0 to 24.0 μmol·L^{−1}. Fluorescence emission spectra were measured at 298 K in the 300–450 nm range at the excitation wavelength of 280 nm. Considering F₀ and F the fluorescence intensity in the absence and presence of the quencher, respectively, the Stern-Volmer constant was obtained by plotting F₀/F versus [compound] according to equation (1).⁴³

$$F_0/F = 1 + K_{SV} [\text{compound}] \quad (1)$$

In cases of both static and dynamic mechanisms being present the above correlation is not linear. To evaluate the relevance of complex formation (a static mechanism), the modified Stern-Volmer equation (2) was used.

$$F_0/\Delta F = 1/f_a K_a 1/[\text{compound}] + 1/f_a \quad (2)$$

As BSA has two binding sites, the equilibrium between a bound and a free molecule and the number of binding sites can be calculated by equation (3).

$$\log((F_0 - F)/F) = \log K_b + n \log [\text{compound}] \quad (3)$$

All curves were plotted using Origin 8.1® software, and linear fitting applied.

Lipophilicity study

The lipophilicity of compounds was determined by measuring the octanol : water partition coefficient, log P, using a shake flask method, according to OECD.³⁶ Deionized water and n-octanol were presaturated for 24 h under stirring. The stock solutions of the compounds were prepared by dissolving proper amounts in 25 ml of presaturated deionized water to a final concentration of 0.5 mmol·L^{−1}.³⁶ The samples were prepared by adding 7.5 ml of the stock solution plus 7.5 ml of presaturated 1-octanol and shaken for two hours. Then, they were centrifuged for phase separation. The aqueous phases (7.0 ml) were treated with an equivalent volume of aqua regia 50% (V/V) to remove all organic matter. The solutions were heated and concentrated to dryness. After drying the entire solution, the sample was calcined at 773,15 K for 20 min. A stock solution sample was measured to calculate the total amount of gold. For analysis, the material was solubilized in aqua regia 1.0% (V/V) and was analyzed in duplicate by ICP-OES, following the methodology adapted from Klose and colleagues.⁴⁴ The partition coefficient P was calculated by the concentration in aqueous and organic phases and expressed as log P. The samples were prepared in duplicates and analyzed in two independent experiments.

dsRNA interaction assay

A migration retardation assay was performed based on previously described works to investigate the ability of the compounds to interact with dsRNA.^{45–47} Through PCR, the HCV JFH-1 3' untranslated region (UTR) was amplified employing primers flanked by a T7 promoter site (forward: TAATACGACTCACTATAGGGGGCACACTAGGTACA; reverse: TAATACGACTCACTATAGGGACATGATCTGCAGAGAG; T7 promoter regions are underlined). The reaction product of 273 bp was purified by ReliaPrep™ DNA Clean-Up and Concentration System (Promega®) and used for *in vitro* transcription by the HiScribe™ T7 High Yield RNA Synthesis Kit (New England BioLabs®). The dsRNA molecule was obtained by complementary annealing, incubated at 30 nmol·L^{−1} with compounds **I** and **II** at 10 μmol·L^{−1} for 45 min, and analyzed in 1.0% agarose 1X TAE gel stained with ethidium bromide. The lack of bands in the gel suggests an interaction activity of the compound by competition with ethidium bromide. Doxorubicin (100 μmol·L^{−1}) was used as a positive control. The band quantification was performed using ImageJ/JS version 1.53j.

Cell viability through MTT

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich) assay as previously reported.^{48–51} Briefly, BHK-21 cells (Syrian hamster kidney; ATCC® CCL-10™) were cultured in a 48-well plate and treated with different concentrations of each compound for 16 h at 37°C with 5% of CO₂. Sixteen hours post-treatment, the media-containing compound was removed from cells, and an MTT solution (1.0 mg ml^{−1}) was added to each well, incubated for 30 min, and replaced by 300 μL of DMSO to solubilize the formazan crystals. The absorbance was measured at 490 nm on a Glomax microplate reader (Promega). Cell viability was calculated according to the equation (T/C) × 100%, in which T and C represented the optical density of the treated well and control groups, respectively. DMSO was used as non-treated control. The concentrations

of which compound capable of inducing the death of 50% of the cells (CC_{50}) were determined by performing a 2-fold serial dilution of complexes **I** and **II** at concentrations ranging from 1.17 to 300 $\mu\text{mol} \cdot \text{L}^{-1}$.

Infection and antiviral assays

The anti-CHIKV activity of the complexes was evaluated as previously reported.⁴⁹ BHK-21 cells were seeded into 48-well plates at a density of 5×10^4 cells per well, 24 h before the infection. CHIKV expressing nanoluciferase reporter (CHIKV-nanoluc) at a multiplicity of infection (MOI) of 0.1 and the compounds **I**, **II**, **III**, or **IV** were simultaneously added to cells.⁵² Samples were harvested in Renilla luciferase lysis buffer (Promega) at 16 h post-infection (h.p.i.), and the viral replication was quantified by measuring Nanoluciferase activity using the Renilla luciferase Assay System (Promega). The concentration capable of impairing viral replication in 50% (EC_{50}) was determined by performing a 2-fold serial dilution of complexes **I** and **II** at concentrations ranging from 1.17 to 300 $\mu\text{mol} \cdot \text{L}^{-1}$ in CHIKV-nanoluc infected cells (MOI = 0.1) at 16 h.p.i. The selectivity index (SI) was calculated by $SI = CC_{50}/EC_{50}$. The SI was calculated by the ratio $SI = CC_{50}/EC_{50}$. Cell viability or viral replication percentages are obtained from non-linear regression analysis and expressed as mean values and standard deviation of three independent experiments, each measured in triplicate. The statistics of each curve were performed employing the F test, adapted from Pereira and co-workers.^{48,49}

The evaluation of the stage of viral replication in which compounds **I** and **II** are acting was performed by the time of drug-addition assays. BHK-21 cells were seeded in 48-well plates 24 h before infection and/or treatment, and the infection with CHIKV was performed at an MOI of 0.1. Levels of viral replication were assessed by measurement of the nanoluciferase activity at 16 h.p.i., as previously described.^{48,50}

In the pretreatment assay, cells were treated for 1 h with complex **I** or **II** before the infection, extensively washed with PBS, followed by incubation with CHIKV-nanoluc for 1 h. Then, cells were washed with PBS to remove the unbound virus, and a fresh medium was added.

The effect of **I** and **II** on the early stages of infection was evaluated by the incubation of cells with media containing both virus and the complex (**I** or **II**) for 1 h. Then, cells were washed with PBS and incubated with a fresh medium for 16 h. The virucidal activity was also assessed, employing the same protocol, except that, in this case, an inoculum containing complex (**I** or **II**), and the virus was preincubated for 1 h before the addition to the cells. In the post-entry test, on the other hand, cells were infected with CHIKV for 1 h, washed extensively with PBS, and then incubated with a medium-containing compound for 16 h.

RESULTS AND DISCUSSION

Characterization of complexes I–IV

The characterization data of the complexes and intermediates can be found in the experimental section and Electronic Supplementary Information (ESI) Figs S1–S3.

PPh₃ ligand generates more lipophilic Au(I) complexes than IMes

Previous reports determined that the lipophilicity of cationic Au(I)-diphosphine complexes may be correlated to their cytotoxic activity in a parabolic trend while also related linearly to drug up-

Table 1. Octanol: water partition coefficient (log P) of the complexes

Complex	log P
I	1.13
II	1.87
III	0.82
IV	0.70
Auranofin	1.6 ⁵⁷

take.⁵³ For instance, the thiosugar replacement by iodine in auranofin also improves activity by lipophilicity increment.^{54,55} Additionally, the higher lipophilicity of $[\text{Au}(\text{IMes})_2]^+$ was linearly correlated to higher cytotoxicity in tumorous and normal cells.⁵⁶

Hence, considering the effect of the lipophilicity in both drug uptake and cytotoxicity of Au(I)-based compound, log P for complexes **I** to **IV** and auranofin (**V**) were measured (Table 1).³⁶ Complete lipophilicity data of complexes **I** to **IV** can be found in Table S3, ESI.

As one may observe in Table 1, the phosphine-based Au(I) complexes **I** and **II** are more lipophilic than their IMes-based counterparts, **III** and **IV**. Despite the charge of **II**, it is significantly more lipophilic than the neutral complex **I**. Auranofin possesses intermediate lipophilicity between **I** and **II**. Complexes **III** and **IV** do not present significative differences in lipophilicity between them. The results suggest that in both cases, the ligand (phosphine or IMes, respectively) primarily affects lipophilicity rather than the charge.

PPh₃ confers slower DMAP dissociation than IMes in complexes II and IV

As ligand exchange reactions in gold(I) complexes might affect the pharmacological effects of these compounds, time-dependent NMR spectra were attained to evaluate ligand exchange kinetics in solvent conditions.^{42,58} Before the kinetics studies, ligand coordination and purity of the compounds were assigned by ¹H NMR, ³¹P NMR, MS, and elemental analysis (Figs S4–S8 and Figs S14 and S15, ESI).

In solution, these complexes are reported to undergo a ligand scramble equilibrium, as represented by equation (4).⁵⁸



The subject was recently reviewed and $[\text{Au}(\text{PPh}_3)_2]^+$ can be a relevant pharmacophore in the biological mechanism of $[\text{AuL}'(\text{PPh}_3)]^n$ $n = 0, +1$ complexes.^{42,59}

The ¹H NMR spectra of **I** and **III** in DMSO-*d*₆ (Figs S4 and S5, ESI) showed no changes during the time-dependent experiment. Complexes **II** and **IV**, ¹H NMR spectra (Figs S6 and S7, ESI), on the other hand, presented a low and constant DMAP dissociation with molar ratios [free]: [bound] as 1:20 and 1:25, respectively. After 24 h, molar ratios have not changed considerably, denoting the complex stability in DMSO-*d*₆.

Pondering that the biological media involves a high water content, the equilibria of **II**, **III**, and **IV** in DMSO-*d*₆: D₂O 1:2 (V/V) was also investigated (Fig. S8, ESI). The low solubility of complex **I** in this condition hindered such assessment for this compound. In these conditions, **II** and **IV** showed free DMAP signals in a ratio [free]: [bound] of 1:50 and 1:5, respectively. Complex **III** presents a marked shift in time towards the formation of $[\text{Au}(\text{IMes})_2]^+$ as observed in the respective spectrum (Fig. S8, ESI). The molar

Table 2. Stern-Volmer extinction constant (K_{SV}), static suppression constant (K_a), apparent binding constant (K_b), and the number of binding sites (n) for the fluorescence quenching of BSA by the Au(I) compounds

	K_{SV} $10^3 \text{ mol}^{-1} \cdot \text{L}$	$R_{K_{SV}}$	K_a $10^4 \text{ mol}^{-1} \cdot \text{L}$	n	K_b $10^4 \text{ mol}^{-1} \cdot \text{L}$
I	7.48	0.9896	1.04	1.03	1.76
II	37.9	0.9898	0.60	1.00	3.35
III	7.00	0.8914	7.53	0.95	3.50
IV	41.1	0.9934	1.00	1.28	4.10

ratio $[\text{Au}(\text{IMes})_2]^+ : [\text{AuCl}(\text{IMes})]$ is 1:25 in early time points, and 1:10 after 96 h.

[AuCl(Ph₃P)] is more reactive toward N-acetyl-Cysteine (NAC) than [AuCl(IMes)]

The study of the coordination of N-acetyl-L-cysteine (NAC) by ¹H NMR is a simple and elegant model that can reveal the speciation of complexes in the presence of thiol-bearing biomolecules (Fig. S9, ESI). Free NAC CH₃ signal is a singlet seen at 1.87 ppm in these conditions. Together with the signals of the complexes, it is a suitable probe signal to follow up the interaction with the metal center. The reaction between complex **I** and NAC is evidenced by the duplication of all NAC signals in early time points (Figs S8 and S10, ESI). The [coord] : [free] ratio is 1:1. In the case of **II**, a similar NAC signal duplication is observed (Figs S9 and S11, ESI). However, it presents a lower [coord] : [free] ratio, 4:5. Complex **III** is slowly coordinated by NAC, presenting a 1:3 [coord] : [free] molar ratio (Figs S9 and S12, ESI). The interaction of complex **IV** and NAC is demonstrated by the duplication of NAC signals in early time points with a ratio [coord] : [free] of around 1:1 (Figs S9 and S13, ESI).

DMAP increases the serum albumin binding affinity

Serum albumins are abundant proteins in the blood plasma and act as a carrier of molecules in the organism. They play essential roles in the distribution, excretion, and metabolism of a great variety of drugs, especially in the distribution of Au(I) drugs, such as auranofin.^{60,61} BSA intrinsic fluorescence emission is strong and dominated by the two tryptophan residues, the Trp-134 in the first domain and Trp-212 in the second domain.^{40,41} As these residues are positioned in the hydrophobic pockets of the binding sites, the emission is very sensitive to ligands bound nearby or structural changes caused by covalent interactions on the proteins.^{62,63} This feature allows the investigation of the interaction of drugs with BSA by fluorimetry.

The investigation of BSA interaction with gold drugs such as auranofin and aurothiomalate showed two types of interaction, one through coordination of gold to Cys-34, considered strong, and through non-covalent interactions with hydrophobic pockets.⁶⁴⁻⁶⁷

In this work, the Stern-Volmer equations were used to quantify the binding affinity between BSA and the complexes. The K_{SV} values represent the quenching constant, and values between 10^3 and $10^4 \text{ mol}^{-1} \cdot \text{L}$ demonstrate a significative interaction between the quencher and the protein. Values larger than $10^4 \text{ mol}^{-1} \text{ mmol} \cdot \text{L}^{-1}$ indicate a significative conformational change. The K_{SV} values for complexes **I-IV** are presented in Table 2, showing a significant interaction. The complexes **II** and **IV** showed higher values (order $10^4 \text{ mol}^{-1} \cdot \text{L}$) than **I** and **III** (order $10^3 \text{ mol}^{-1} \cdot \text{L}$). The

Table 3. Effects of compounds **I**, **II**, **III**, **IV**, and auranofin on BHK-21 cells and CHIKV replication

Compounds	Concentration ($\mu\text{mol} \cdot \text{L}^{-1}$)	Cell viability (%)	CHIKV inhibition (%)
I	10.0	98.3 (± 11.6)	99.4 (± 1.0)
II	10.0	91.8 (± 9.8)	90.4 (± 6.0)
III	10.0	95.7 (± 12.8)	48.5 (± 17.8)
IV	10.0	88.3 (± 6.5)	50.7 (± 15.2)
Auranofin	2.0	99.3 (± 10.4)	52.8 (± 12.5)

positive charge and the faster displacement of DMAP by thiols in **II** and **IV**, as observed in the experiments involving NAC, might explain this result. Except for complex **III**, all complexes present a linear coefficient ($R_{K_{SV}}$) close to 1.0 (Fig. S16, ESI). Complex **III** might promote dynamic and static quenching as the plot is not linear.

The binding constant, K_b , obtained by the Stern-Volmer equation (3) indicates a strong binding of all complexes. Following the K_{SV} values, compound **II** has a stronger binding than **I**, and **IV** is stronger than **III**. The n values (number of binding sites) for the complexes **I-III** are close to 1, representing an interaction with hydrophobic pockets, and higher values of n , as in complex **IV**, indicate the presence of secondary interactions.⁶²

Activity of [AuL']ⁿ⁺ n = 0, +1 complexes on cells viability and CHIKV replication

The viability of complexes **I** to **IV** towards BHK-21 cells was first measured by treating the cells with each complex at 2.0, 10, and 50 $\mu\text{mol} \cdot \text{L}^{-1}$, and performing the MTT assay. The results showed that the highest non-cytotoxic concentration tested was 10 $\mu\text{mol} \cdot \text{L}^{-1}$ for complexes **I** to **IV**, and 2.0 $\mu\text{mol} \cdot \text{L}^{-1}$ for auranofin, which was used as a control (Fig. S17, ESI). The effect of compounds on CHIKV replication was evaluated by the infection of BHK-21 cells with CHIKV-nanoluc, followed by the treatment with complexes **I**, **II**, **III**, and **IV** at 10 $\mu\text{mol} \cdot \text{L}^{-1}$.⁵² Viral replication rates were assessed by measuring the activity of the nanoluciferase reporter at 16 h.p.i. In parallel, cell viability was evaluated by an MTT. The results are presented in Table 3.

It is important to emphasize that no considerable cytotoxicity was observed for complexes **I-IV**, in contrast to the high cytotoxicity seen for other gold complexes previously reported in the literature.^{68,69} All complexes showed cell viability higher than 88% at 10 $\mu\text{mol} \cdot \text{L}^{-1}$ (Table 3). Complex **I** presented the highest cell viability rates in the assayed concentration (Table 3), which may be related to its higher stability and lipophilicity. However, complex **IV** was the most cytotoxic (Table 3), suggesting that its speciation in DMSO: H₂O solution affects the biological outcome. Complexes **I-IV** were less cytotoxic to BHK-21 cells than Auranofin, showing the significant effects of the nature of the ligands and how the coordination can modulate the biological activity of a complex. Complex **I** also presented the highest inhibition of CHIKV replication in the series, followed by **II** (Table 3), which may be correlated to the higher lipophilicity observed for these compounds and slower ligand exchange in solution. The results showed that [AuL'(Ph₃P)]ⁿ n = 0, +1 derivatives presented the best antiviral activity rates, in contrast to the [AuL'(IMes)] counterparts (Table 3). Complexes **I** and **II** were shown to be more lipophilic, reflecting that a higher cell uptake could enable further interactions with CHIKV biomolecules during viral replication and therefore an increased CHIKV inhibition when compared to **III** and **IV**.

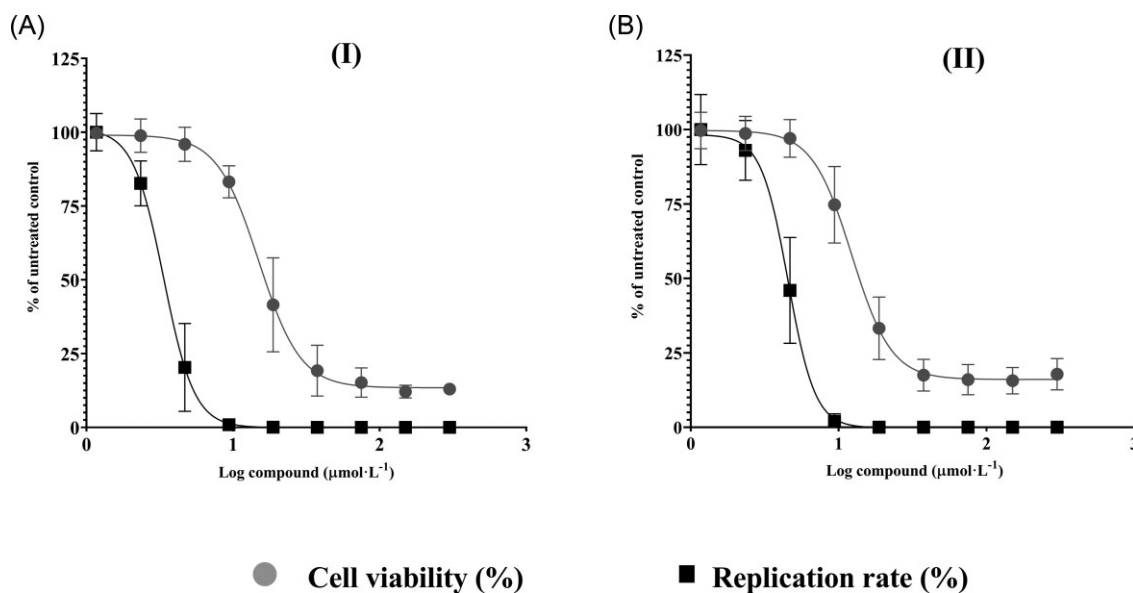


Fig. 2. Inhibition of CHIKV replicative cycle by compounds I and II. CHIKV replication (closed squares) and cell viability (closed circles) of compounds I (A) and II (B).

Table 4. Effect of compounds I and II in the time of drug-addition assay

	Protective		Entry		Virucidal		Post-entry	
	CHIKV infection (%)	Inhibition of CHIKV replication (%)	CHIKV infection (%)	Inhibition of CHIKV replication (%)	CHIKV infection (%)	Inhibition of CHIKV replication (%)	CHIKV infection (%)	Inhibition of CHIKV replication (%)
I	7.6	92.4	8.0	92.0	28.2	71.8	0.1	99.9
II	19.8	80.2	5.4	94.6	25.2	74.8	0.3	99.7

Due to the best antiviral activity of **I** and **II**, a dose-response assay was performed to determine their EC_{50} , CC_{50} , and SI values. This evaluation encompassed the infection of BHK-21 cells with CHIKV-*nanoluc* and the simultaneous treatment with **I** or **II** at concentrations ranging from 1.17 to 300 $\mu\text{mol}\cdot\text{L}^{-1}$ in 2-fold serial dilutions. The viral replication was assessed at 16 h.p.i. In parallel, cell viability was determined by an MTT assay. From this range of concentrations, complex **I** has CC_{50} of 18.2 and EC_{50} of 3.4 ($P < 0.0001$, $F_{4,133} = 397.4$). The calculated SI for **I** was 5.4 (Fig. 2A). Complex **II**, on the other hand, presented CC_{50} of 15.7, EC_{50} of 4.5, and SI of 3.5 ($P < 0.0001$, $F_{4,129} = 231.6$) (Fig. 2B). Both compounds presented a low EC_{50} , which is consistent with the therapeutic index of several described drugs and suggests the potential of these compounds to treat CHIKV infection.⁴⁹ The data herein presented shows the potential of gold-based compounds as antivirals against RNA viruses, corroborating previously observed results from Fonteh and co-workers, in which bis(thiosemicarbazone)gold(III) compounds presented low EC_{50} values against HIV.⁷⁰

Insights on the mode of action of complexes I and II

To further elucidate the complexes **I** and **II** modes of antiviral activity, the effect of these compounds on different stages of the virus replicative cycle was assessed. The protective effect was an-

alyzed by treating the cells with complex **I** or **II** for 1 h at 37°C, washing with PBS, and adding CHIKV-*nanoluc*. Luciferase levels were assessed 16 h.p.i., showing that **I** and **II** inhibited CHIKV replication in 92.4% and 80.2%, respectively (Table 4 and Fig. S18, ESI). Therefore, both compounds displayed a strong protective effect against CHIKV infection.

Experiments were performed to get further insights into the effect of the compounds on virus entry. Thus, cells were treated simultaneously with the virus and complex **I** or **II** for 1 h at 37°C, followed by cell washing with PBS and fresh medium addition (Fig. S19A, ESI). At 16 h.p.i. CHIKV replication decreased around 92% and 94.6% in the presence of **I** and **II**, respectively (Table 4 and Fig. S19B, ESI). Alternatively, the virucidal effect was assessed by preincubation of complex **I** or **II** with CHIKV for 1 h at 37°C, followed by cell treatment with the preincubation mixture for 1 h. Cells were washed with PBS, and a fresh medium was added (Fig. S19C, ESI). Complexes **I** and **II** decreased CHIKV-*nanoluc* replication by 71.8% and 74.8%, respectively (Table 4 and Fig. S19D, ESI). Altogether, these results show a significant effect of complexes **I** and **II** on the early stages of CHIKV infection (Table 4 and Figs S18 and 19, ESI). The anti-HIV effects of gold complexes were extensively studied, showing the mode of action of polymeric compounds such as aurothioglucose and aurothiomalate is due to the direct binding of cysteinyl moiety in the surface proteins of HIV (e.g. gp41 and gp160) to the complexes, and inhibition of viral entry.^{71,72} A similar result was observed for polynuclear Pt(II)

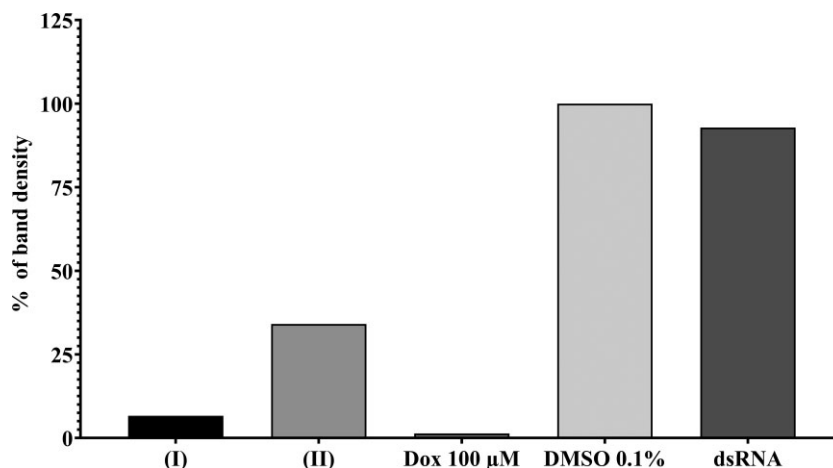


Fig. 3. Complexes I and II interact with viral dsRNA. Thirty nanomoles of JFH-1 HCV dsRNA were incubated with I or II at $10 \mu\text{mol} \cdot \text{L}^{-1}$, or the controls DMSO 0.1% and doxorubicin at $100 \mu\text{mol} \cdot \text{L}^{-1}$, for 45 min at room temperature. The reaction products were subjected to 1.0% agarose 1X TAE electrophoresis gel containing ethidium bromide, followed by densitometry analysis on ImageJJS version 1.53j. The image from the complete gel is shown in the Experimental section (Fig. S21, ESI).

complexes.^{73,74} Monomeric gold(I) complexes, on the other hand, presented a different mode of action. While polymeric compounds are slowly uptaken by cells, with only metabolites found within the cytoplasm, monomeric gold(I) compounds are more readily uptaken, therefore interacting with viral biomolecules and consequently inhibiting the virus replication.⁷⁵ Additionally, such complexes may interact with host cell biomolecules, essential to viral replication. For instance, Mathias and co-workers showed that thioredoxin 1 (Trx-1) has a role in the conformational changes in surface proteins of CD4 cells, which are crucial for HIV entry.⁷⁶ The inhibition of Trx-1 also inhibits viral entry, as previously shown for auranofin and organotin compounds.^{77,78} Hence, the protective effect of complexes I and II may be due to interactions with biomolecules within the cytoplasm.

Also, to evaluate whether complexes have a post-entry effect on viral replication, BHK-21 cells were infected with CHIKV-nanoluc for 1 h, washed with PBS, and treated with a fresh medium containing I or II (Fig. S20A, ESI). Both compounds inhibited viral replication in 99.9% and 99.7%, respectively, representing the highest rates of virus inhibition from the evaluated stages of the viral replicative cycle (Table 4 and Fig. S20B, ESI). One may suggest that complexes I and II inhibit viral replication by interacting with proteases and biomolecules related to transcription and translation processes. Although monomeric compounds can affect virus entry, previous studies demonstrated that the mechanism of monomeric complexes such as auranofin and dicyanogold(I) is assigned to the uptake of the gold(I) compound and inhibition of HIV reverse transcriptase.⁷⁵

From the analysis of the effect of I and II on the different stages of the CHIKV replicative cycle, results might suggest that either (i) the complexes I and II mainly affect the stages after post-entry, as virus replication; and/or (ii) the complexes I and II mainly interfere with the host cells, thus inhibiting virus entry and post-entry, by conferring a protective effect against CHIKV infection. The strongest effect on post-entry might also be explained by the duration of treatment (post-entry: 16 h vs. entry and protective: 1 h). Considering the literature about the anti-HIV activity of monomeric gold(I) complexes, the results are mainly consistent with the second proposal, though the first cannot be discarded.

As the data suggest that complexes I and II mainly affect the stages after virus post-entry (>99%), the interaction of these

complexes with dsRNA was investigated by performing a migration retardation assay followed by densitometry analysis (Fig. S21, ESI).^{45,77,78} Since dsRNA is an intermediary of CHIKV replication, therefore, a potential target for I and II.^{1,70} The lack of bands in the gel, confirmed by the quantification by densitometry, suggests the compound interaction by competition with ethidium bromide. As one may observe, both compounds interact with the dsRNA, represented by a decrease of band intensity of 93.4% and 66% for I and II, respectively (Fig. 3).

These results suggest that both compounds might be interfering with viral replication by dsRNA interaction. Allaudeen and coworkers described a gold(III) complex with potential interaction effect with viral DNA, whereas Che and collaborators demonstrated gold(III)-porphyrins complexes possess DNA intercalating activity.^{12,79,80} On the other hand, Traber and coworkers showed that the replication of HIV-1 was inhibited *in vitro* by aurothioglucose through the blockage of NF- κ B binding to DNA.⁸¹ However, to our knowledge, this is the first description of gold-based complexes potentially interacting with dsRNA. It corroborates with the data reported here, outlining that the main effect of both molecules is due to interactions within the cytoplasm of the host cells, interacting with viral biomolecules during replication, and/or host cell components.

Moreover, considering the ability of many metal complexes to interact with main viral proteins, concerning gold-based compounds against HIV protein machinery, it is reasonable to suggest that complexes I and II may also be interacting with CHIKV non-structural proteins.^{2,9,70}

For instance, proteins such as nsP1 (methyltransferase), nsP2 (protease/helicase), nsP3 (ADP-ribosylhydrolase), or nsP4 (RNA polymerase RNA dependent—RdRp) are essential proteins for RNA capping, proteolysis, replication complex formation, and RNA replication and may figure as potential targets to these compounds.^{82,83}

The previously reported ability of $[\text{AuL}(\text{PPh}_3)]^n$ $n = 0, +1$ complexes (similar to I and II) to inhibit HIV-1 by interaction with Zinc Fingers in NCp7 may offer a hint for further mechanistic studies.¹⁵ Considering that the CHIKV nsP1 and nsP3 proteins possess zinc finger domains, those moieties could figure as targets of compounds I and II.^{83–85} Therefore, further evaluations of the modes of action of complex I and II encompassing

such proteins and other potential targets are envisaged in future works.

Conclusions

This manuscript investigated the anti-CHIKV effects of a systematic series of four Au(I) compounds and the potential modes of action of the complexes with the best antiviral activity ratio within the series. The systematic change between the nature of L (IMes or PPh₃) and L' (Cl[−] or DMAP) in the complexes allowed the evaluation of differences between triphenylphosphine derivatives and mesityl-NHC Au(I) complexes, establishing a basis for gold-based anti-CHIKV design. At 10 μmol·L^{−1} and under the experimental conditions, all complexes were viable against BHK-21 cells (> 88%), highlighting the higher CHIKV inhibition of complexes **I** and **II** (99.4% and 90.4%, respectively) than their counterparts **III** and **IV**, presenting 48.5% and 50.7%, respectively. The higher log P values and the improved inertness in DMSO: D₂O solution might explain the results. Compounds **I–IV** are less cytotoxic to BHK-21 and more effective than auranofin. The ligand exchange of DMAP in solution is significantly faster than the chloride, which could be related to the higher SI of compound **I** in comparison with **II**. All complexes (**I–IV**) exchange the ligands L' with sulfur-bearing molecules, as demonstrated by interaction with NAC. These data suggest that ligand exchanges may occur in media containing these biomolecules, further generating the activated species. The exchange reaction is faster for **II** and **IV** (DMAP derivatives), which is corroborated by the BSA interaction assay. The phosphine derivatives **I** and **II** were selected for further *in vitro* investigations of the potential mechanisms of action, and both promoted a protective effect against infection, with the inhibition of early infection stages, and a potent antiviral effect in the post-entry step, suggesting interference in cellular pathways and/or inhibition of viral biomolecules in the cytoplasm. A significant interaction of compound **I** and **II** with dsRNA suggests that it can inhibit replication by dsRNA binding. Results suggest that **I** interacts more strongly than **II**, explaining the differences in the inhibition of the viral replication. Other targets are subject to future investigations. Considering that the main challenge in the development of effective gold(I) complexes for medicinal applications is related to the extensive ligand exchange rates in blood plasma, which generate several species and limits applications *in vivo*, the set of results presented shows important aspects of gold chemistry and biology for the development of effect anti-CHIKV drugs. Compound **I** is a promising chemotype for future developments.

CRediT authorship contribution statement

R.L.A.: methodology, investigation, writing—original draft. I.A.S.: methodology, investigation, writing—original draft. J.V.F.: investigation. F.R.G.B.: conceptualization, funding acquisition, writing—review and editing. A.C.G.J.: conceptualization, project administration, supervision, funding acquisition, writing—review and editing. C.A.: conceptualization, project administration, supervision, funding acquisition, writing—review and editing.

Supplementary Material

Supplementary data are available at [Metallomics](https://doi.org/10.1016/j.mol.2023.112722) online.

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Conflicts of interest

There are no conflicts of interest to declare.

Data Availability

The data underlying this article are available in the article and in its online supplementary material.

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CAPÍTULO 7: MEMANTINE HYDROCHLORIDE: A DRUG TO BE REPURPOSED AGAINST CHIKUNGUNYA VIRUS?

MANUSCRITO PUBLICADO EM “PHARMACOLOGICAL REPORTS”



Memantine hydrochloride: a drug to be repurposed against Chikungunya virus?

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Abstract

Background Chikungunya fever is an endemic disease caused by the Chikungunya virus (CHIKV) to which there is no vaccine or effective antiviral drug treatment so far. Our study aimed to evaluate the potential anti-CHIKV activity of memantine hydrochloride (mtnH), a drug from the class of the aminoadamantanes approved for the treatment of Alzheimer's disease, as a possible drug to be repurposed to the treatment of Chikungunya fever.

Methods MtnH antiviral activity against CHIKV was determined by infecting BHK-21 cells with CHIKV-*nanoluc*, a virus carrying the marker *nanoluciferase* reporter, in the presence or absence of mtnH at concentrations ranging from 500 to 1.45 μM . The effective concentration of 50% inhibition (EC_{50}) was calculated. Cell viability assay (determination of CC_{50}) was also performed employing BHK-21 cells. Mutagenic assays were performed by the *Salmonella* Typhimurium/microsome assay (Ames test).

Results MtnH presented a CC_{50} of $248.4 \pm 31.9 \mu\text{M}$ and an EC_{50} of $32.4 \pm 4 \mu\text{M}$ against CHIKV in vitro. The calculated selectivity index (SI) was 7.67. MtnH did not induce genetic mutation in *Salmonella* strains with or without an external metabolizing system.

Conclusion With the data herein presented, it is possible to hypothesize mtnH as a viable candidate to be repurposed as an anti-CHIKV drug. Clinical assays are, therefore, encouraged due to the promising in vitro results.

Graphic abstract

The drug memantine hydrochloride is herein personified with a doubt: as a prior regulated drug against Alzheimer, could it follow the path against Chikungunya virus too?



Keywords Chikungunya virus · Drug · Repurposing · Memantine hydrochloride · Antiviral

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Extended author information available on the last page of the article

Abbreviations

CC ₅₀	Cytotoxic concentration of 50%
CHIKV	Chikungunya virus
EC ₅₀	Effective concentration of 50% inhibition
JEV	Japanese encephalitis virus
MI	Mutagenic index
mtnH	Memantine hydrochloride
NADP	Nicotinamide adenine dinucleotide phosphate sodium salt
PFU	Plaque-forming units
RABV	Rabies virus
SI	Selectivity index

Introduction

Chikungunya fever is an endemic disease in tropical and subtropical countries, and it is characterized, during its acute phase, by *dengue-like* symptoms such as fever, skin rash, backache, headache, fatigue, and arthralgia [1–3]. However, this disease can progress to a chronic condition defined by polyarthralgia, which can last from months to years [1–3]. The potential chronic character of this disease allied to its endemic feature constitutes a case of great concern to public health and economic welfare [2–4].

The etiologic agent of Chikungunya fever is the Chikungunya virus (CHIKV), an arbovirus that belongs to the *Alphavirus* genus, which is mainly transmitted to healthy individuals by the bite of infected *Aedes aegypti* and *albopictus* mosquitoes [3]. Other mosquitoes such as *Haemagogus leucocelaenus* and *Aedes terreus* have also shown to be potential vectors for this arbovirus, which aggravates the challenges related to the surveillance, prevention, and control of CHIKV [5].

The CHIKV infection primarily affects fibroblasts, keratinocytes, and epithelial cells in the dermis by permissibility that is related mostly to Mxra8 receptors as well as the presence of T cell immunoglobulin, mucin domain-1 (TIM-1), and glycosaminoglycans [6–8]. The pathophysiology is followed by a high viremia which results in infection of several tissues such as muscles, joints, liver, heart, and nervous system [9, 10]. Moreover, some CHIKV strains were associated with higher neurovirulence, suggesting a more aggressive disease in some of the infected patients [11].

Currently, there is no available vaccine for CHIKV infection nor an effective antiviral drug for the treatment of infected patients leading only to palliative therapies [1]. In this context, drug repurposing figures as a promising short to medium terms therapeutic alternative, since a drug previously approved for a specific clinical application has the potential to be capitalized more rapidly than potential drug prototypes into the treatment of another disease [12].

CHIKV- <i>nanoluc</i>	Chikungunya virus- <i>nanoluciferase</i>
CMC	Carboxymethyl cellulose

The class of aminoadamantanes has been extensively investigated due to their diversity of pharmacological activities [13]. For instance, the drugs amantadine and rimantadine, formerly approved as anti-Influenza A virus agents, have gained attention as possible antiviral agents for other virus-driven infections [14]. In this respect, an observational study described the amantadine as a possible repurposing drug for patients with Coronavirus disease 2019 (COVID-19) [15].

Herein, we explored the potential antiviral activity of memantine hydrochloride against CHIKV. Memantine hydrochloride (mtnH; Fig. 1) is an aminoadamantane drug, employed mostly for the treatment of Alzheimer's disease (AD). This AD drug has been reported through in vitro assays as a possible antiviral agent against influenza A virus [16, 17], human coronavirus strain OC-43 (HCoV-OC43), rabies virus (RABV), Japanese encephalitis virus (JEV) [18, 19], Zika virus [20] and, most recently, SARS-CoV-2 infections [21].

Considering the impact of Chikungunya fever in tropical and subtropical countries and the severe disabilities generated by CHIKV infection, as well as the previous prospects of the mtnH for alleviating symptoms of Zika virus [20] and SARS-CoV-2 [21] infections, herein we evaluated the in vitro activity of mtnH as a promising drug to be repurposed as an anti-CHIKV drug.

Materials and methods

Reagents

Memantine hydrochloride (mtnH; purity 99%) (Fig. 1) was purchased from Acros Organics (Fisher Scientific®). The compound was dissolved in dimethylsulfoxide (DMSO) and stored at −20 °C. Dilutions of the compounds were made

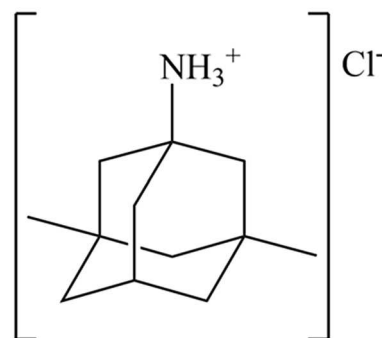


Fig. 1 Structure of memantine hydrochloride

immediately prior to the experiments. For all assays performed, DMSO was used as untreated control.

Cell culture

BHK-21 cells (ATCC® CCL-10™), derived from Syrian hamster kidney, were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich®) supplemented with 100U/mL of penicillin (Hyclone Laboratories®), 100 mg/mL of streptomycin (Hyclone Laboratories®), 1% dilution of stock of non-essential amino acids (Hyclone Laboratories®) and 1% of fetal bovine serum (FBS, Hyclone Laboratories®) in a humidified 5% CO₂ incubator at 37 °C.

Rescue of CHIKV-*nanoluc* reporter virus

The CHIKV expressing *nanoluciferase* reporter (CHIKV-*nanoluc*) (Fig. 2a) used for the antiviral assays is based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype). The cDNA of CHIKV-*nanoluc* was placed under the control of a CMV promoter [22, 23]. To produce CHIKV-*nanoluc* virions, 2.3×10^7 BHK-21 cells seeded in a T175 cm² flask were transfected with 1.5 µg of CMV-CHIKV-*nanoluc* plasmid using lipofectamine 3000® and Opti-Mem medium. Forty-eight hours post-transfection (h.p.t.) the supernatant was collected and stored at -80 °C. To determine viral titers, 1×10^5 BHK-21 cells were seeded in each of 24 wells plate 24 h prior to the infection. Then, the cells were infected with ten-fold serial

dilutions of CHIKV-*nanoluc* for 1 h at 37 °C. The inoculums were removed, cells were washed with PBS to remove the unbound virus, and added of fresh medium supplemented with 1% penicillin, 1% streptomycin, 2% FBS and 1% carboxymethyl cellulose (CMC). Infected cells were incubated for 2 days in a humidified 5% CO₂ incubator at 37 °C, followed by fixation with 4% formaldehyde and stained with 0.5% violet crystal. The viral foci were counted to determine viral titer which was presented in plaque-forming units per milliliter (PFU/mL).

Cell viability through MTT assay

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich®) assay. BHK-21 cells were plated in 48 well plates at a density of 5×10^4 cells per well and incubated overnight at 37 °C. Medium containing two-fold serial dilutions of mtnH ranging from 500 to 1.45 µM was added to the cells for 16 h at 37 °C with 5% of CO₂. After treatment, the compound-containing medium was removed and MTT solution at 1 mg/mL was added to each well, incubated for 1 h, and replaced with 300 µL of DMSO (dimethyl sulfoxide) to solubilize the formazan crystals. The absorbance was measured at 490 nm on Glomax microplate reader (Promega®). Cell viability was calculated according to the equation $(T/C) \times 100\%$, where T and C represent the mean optical density of the treated and untreated control groups, respectively. The cytotoxic concentration of 50% (CC₅₀) was calculated using GraphPad Prism 8.

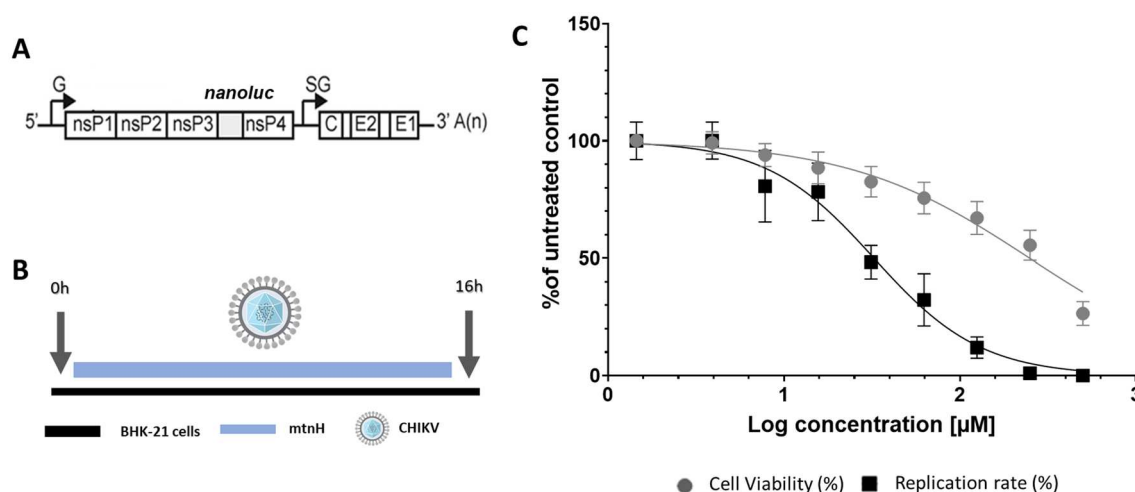


Fig. 2 Inhibition of CHIKV replicative cycle by mtnH. **a** Schematic representation of CHIKV-*nanoluc* genome. **b** Schematic representation of CHIKV infectivity assays. **c** BHK-21 cells were infected with CHIKV-*nanoluc* at MOI 0.1 in the presence or absence of mtnH ranging from 500 to 1.45 µM for 16 h.p.i.. EC₅₀ and CC₅₀ were determined and SI was calculated. CHIKV replication was measured by *nanoluciferase* assay (indicated by closed square) and cel-

lular viability measured using an MTT assay (indicated by closed circle). Results are shown as a non-linear regression analysis, indicating the percentages of the cell viability or viral replication by mtnH. Mean values $\pm$ SD of three independent experiments each measured in triplicate are shown. The comparison of each curve was performed employing the F test (confidence level of 95%), resulting in a $p < 0.0001$, and $F_{2,116} = 260.5$

Antiviral activity assays

To assess the antiviral activity of mtnH, BHK-21 cells were seeded at a density of 5×10^4 cells per well into 48 well plates 24 h prior to the infection. Cells were infected with CHIKV-*nanoluc* at a multiplicity of infection (MOI) of 0.1 in the presence or absence of mtnH at concentrations ranging from 500 to 1.45 μM . Samples were harvested using Renilla-luciferase lysis buffer (Promega®) at 16 h.p.i. (Fig. 2b) and virus replication levels were quantified by measuring *nanoluciferase* activity using the Renilla luciferase Assay System (Promega). The effective concentration of 50% inhibition (EC_{50}) was calculated using Graph Pad Prism 8 software. The values of CC_{50} and EC_{50} were used to calculate de selective index $\left(\text{SI} = \frac{\text{CC}_{50}}{\text{EC}_{50}}\right)$.

Mutagenic assay: Ames test

The Ames test was employed to evaluate the ability of mtnH to induce gene (point) mutations measured in *Salmonella* Typhimurium strains (kindly provided by B.N. Ames, Berkeley, CA) in the presence or absence of metabolic activation. The *S. Typhimurium* tester strains TA98 and TA97a detect frameshift mutations, TA100 detects base-pair substitution mutations primarily at one of the GC pairs, and TA102 normally is used to detect mutagens that cause oxidative damage and base-pair-substitution mutations (AT) [24].

For the mutation assay using a pre-incubation method, a stock solution of mtnH at 2317.4 μM was firstly prepared. Then, 0.5 mL of S9 mix or phosphate buffer (pH 7.4) was mixed with 0.1 mL of bacteria, and lastly 0.1 mL of the stock solution of mtnH, and placed in a shaking incubator at 37 °C for 20 min. After this, 2 mL of top agar was added to the mixture and poured into a plate with an agar layer. The final concentration of mtnH was 85.83 μM , corresponding to 50 $\mu\text{g}/\text{plate}$.

The activation mixture for the S9 fraction was purchased from Moltox Molecular Toxicology Inc., Boone, USA, and freshly prepared before each test. This mixture reveals whether the substance or sample is mutagenic in its original form or if it needs to be metabolized or activated to become mutagenic. The metabolic activation system consisted of 4% S9 fraction, 1% of magnesium chloride 0.4 mol L^{-1} , 1% of potassium chloride 1.65 mol L^{-1} , 0.5% of D-glucose-6-phosphate disodium 1 mol L^{-1} , and 4% of nicotinamide adenine dinucleotide phosphate sodium salt (NADP) 0.1 mol L^{-1} in 50% of phosphate buffer 0.2 mol L^{-1} and 39.5% of sterile distilled water. The plates were incubated at 37 °C for 48 h, and the His⁺ revertant colonies were counted manually.

DNA damage-inducing agents were used as positive controls: 4-nitro-*o*-phenylenediamine (10 $\mu\text{g}/\text{plate}$) for TA98 and TA97a, sodium azide (1.25 $\mu\text{g}/\text{plate}$) for TA100, and

mitomycin C (0.5 $\mu\text{g}/\text{plate}$) for TA102 in experiments without S9 mix. In experiments with S9 activation, 2-anthramine (1.25 $\mu\text{g}/\text{plate}$) was used as a positive control for TA98, TA97a, and TA100, and 2-aminofluorene (10 $\mu\text{g}/\text{plate}$) for TA102. DMSO was used as untreated control (100 $\mu\text{L}/\text{plate}$) and the spontaneous control (SC) corresponds to the rate of spontaneous reversion of each strain.

Statistical analysis

Individual experiments were performed in triplicate and all assays were carried out a minimum of three times to confirm reproducibility. The EC_{50} and CC_{50} were calculated using a non-linear regression considering log(inhibitor) vs. response, with variable slope (four parameters), and a comparison was also performed using the F test ($p < 0.05$). Data from the assay with *Salmonella* Typhimurium strains were expressed as mean $\pm$ SD and were submitted to a normality and lognormality test. The statistical significance was determined by one-way analysis of variance (ANOVA), complemented by the Dunnett's post hoc test. This test was performed in comparison with the control group (untreated control of the assay). The mutagenic index (MI) was also calculated as the average number of revertants *per* test plate divided by the average number of revertants *per* solvent (negative control plate). A sample is considered mutagenic if the ANOVA variation is significant, with $p < 0.05$ and the average number of revertants increase of the sample is a minimum of two folds of that found in the untreated control ($\text{MI} > 2$) [24, 25].

Results and discussion

Memantine hydrochloride impairs CHIKV infection in vitro

Considering the impact of Chikungunya fever in tropical countries and the previously described antiviral activities on mtnH [18, 19], we investigated the anti-CHIKV activity of mtnH employing BHK-21 cells infected with a recombinant CHIKV that expresses a *nanoluciferase* reporter (CHIKV-*nanoluc*) (Fig. 2a).

Firstly, the mtnH antiviral activity was evaluated performing a dose-response assay to determine the cytotoxicity concentration of 50% (CC_{50}) and the effective antiviral concentration of 50% (EC_{50}). For this, BHK-21 cells were infected with CHIKV-*nanoluc* and simultaneously treated with mtnH at concentrations ranging from 500 to 1.45 μM , and the viral replication was assessed 16 h post-infection (h.p.i.) (Fig. 2b). In parallel, cell viability was assessed by an MTT assay. From this range of concentrations, it was determined that mtnH has the CC_{50} of $248.4 \pm 31.9 \mu\text{M}$

and EC_{50} of $32.4 \pm 4 \mu M$ ($p < 0.0001$, $F_{2,116} = 260.5$). The calculated selectivity index (SI) was 7.67 (Fig. 2c). These results suggest mtnH as a potential alternative for the future treatment of CHIKV infection. Despite the relatively modest SI value compared to other drugs, mtnH has a dosing schedule of 20 mg daily for the treatment of mild to severe AD [26], with no severe adverse effects. One may suggest that the absence of adverse effects in vivo may thus outweigh the modest SI in vitro in terms of therapeutic significance. However, it is important to emphasize that the effective concentration for the treatment of infected animals and/or individuals must be further evaluated. In addition, SI values higher than 1.0 suggest a positive benefit and risk balance for the potential antiviral drug [27]. Other drugs such as Chloroquine, which is widely used to treat *Plasmodium falciparum* infections and presents substantial adverse effects to the patients, was reported as an alternative anti-CHIKV inhibitor in vitro with an ordinary selectivity index ranging from 15 to 26 depending on the type of treatment [28–30].

The antiviral activity of mtnH and other adamantanes against other virus strains has been suggested to be related to the inhibition of viroporins ion channels [21, 31]. Viroporins are multifunctional proteins expressed mainly by RNA viruses [32], in which the self-assembled oligomerization forms an ion conductive transmembrane pore [33, 34]. This pore is then responsible for modifying the cellular environment, unbalancing the ionic gradient as well as the charge of the membrane, which favors the viral replication [32]. The CHIKV encodes a viroporin (viral protein 6 K), which is common among alphaviruses and it is responsible for the formation of a cation-selective ion channel in the lipid bilayers [31, 35]. The lack of protein 6 K is related to a higher susceptibility to pH alterations as well as a greater sensibility to higher temperatures [36], ultimately resulting in an impairment of the viral replication. Therefore, considering the anti-CHIKV activity of amantadine, which presents a similar structure to that of mtnH, and the recent report of SARS-CoV-2 viroporin E impairment by mtnH, it is possible to suggest that mtnH also abrogates the viral replication through the interaction with CHIKV viroporin 6 K [21]. Moreover, the NMDA channel receptor blockage performed by mtnH may support this hypothesis [37], suggesting a tropism of mtnH for ion channels. However, further studies are necessary to unveil the mtnH mechanism of action against CHIKV.

Whats is more, it was observed that CHIKV can infect different cell types such as fibroblasts, dendritic cells, and macrophages [6, 10], with a possible tropism for the central nervous system (CNS) cells such as astrocytes, microglia, and neuroblastoma cells [6, 10]. This feature may induce neurological complications such as Guillan-Barré syndrome, encephalitis, and, meningitis [6, 10]. The previously

described in vivo activity of mtnH controlling inflammation and commonly associated with its neuroprotective effect [38] may, thus, indicate an additional therapeutic effect of mtnH that can be exploited seeking the minimization of the potential neurological effects triggered by CHIKV infection.

Finally, it is important to emphasize that mtnH is a worldwide approved and relatively low-cost drug with a well-established therapeutic profile both in terms of its biological outcomes (dose–response activity and potential adverse effects), as well as its physicochemical and pharmacokinetic properties [39]. In this context, from the data herein presented, and considering the lack of significant adverse effects in short-term therapies for healthy individuals (without AD, e.g., Clinical Trials NCT03121820 and NCT01999894), it is possible to hypothesize that mtnH may follow a cost- and time-reduced path in clinical trials, with a considerable potential to be capitalized as a CHIKV antiviral drug.

Effect of mtnH in cell mutagenicity

The results of mutagenicity assays are presented in Table 1 encompassing the mean and standard deviation (SD) of the number of revertants per plate, and the mutagenic indexes (MI), with (+S9) and without (–S9) metabolic activation. The results were validated using the untreated and positive controls. The positive control of each cell line produced the expected mutagenic response and was used to validate the susceptibility of the system to standard mutagens.

The strain TA98 of *S. Typhimurium* when treated with the positive control induced an increase in the revertants per plate compared to the DMSO control in the absence of S9 (–S9: 654 ± 91 vs. control 20 ± 4 , $p < 0.0001$, $F_{3,20} = 830.1$) and in the presence of S9 (+S9: 1221 ± 73 vs. control 21 ± 5 , $p < 0.0001$, $F_{3,20} = 2634$). The same results were seen in the presence of positive control for the other strains: TA100 (–S9: 1458 ± 85 vs. control 93 ± 3 , $p < 0.0001$, $F_{3,20} = 2431$; +S9: 1132 ± 85 vs. control 106 ± 5 , $p < 0.0001$, $F_{3,20} = 1904$), TA102 (–S9: 1982 ± 249 vs. control 274 ± 36 , $p < 0.0001$, $F_{3,20} = 358.9$; +S9: 2359 ± 114 vs. 270 ± 47 , $p < 0.0001$, $F_{3,20} = 803.3$), and TA97a (–S9: 1518 ± 123 vs. control 96 ± 13 , $p < 0.0001$, $F_{3,20} = 1293$; +S9: 1442 ± 152 vs. control 112 ± 18 , $p < 0.0001$, $F_{3,20} = 278.8$) (Table 1). Differently, the treatment with mtnH did not induce mutagenicity in the strains TA98 (–S9: 16 ± 1 , $p = 0.98$, and +S9: 27 ± 9 , $p = 0.96$), TA100 (–S9: 109 ± 15 , $p = 0.73$, and +S9: 113 ± 3 , $p = 0.94$), TA102 (–S9: 266 ± 47 , $p = 0.99$, and +S9: 231 ± 12 , $p = 0.8$), and TA97a (–S9: 83 ± 10 , $p = 0.94$; and +S9: 121 ± 14 , $p = 0.99$) (Table 1). In addition, the DMSO, used as the parameter to determine the mutagenic potential of the mtnH, did not statistically differ of revertants number from the spontaneous control, that yielded the spontaneous His⁺ revertants.

Table 1 Mutagenic activity expressed as the revertants/ plate, standard deviation, and mutagenicity index (in brackets) in the strains TA98, TA100, TA102, and TA97a of *S. Typhimurium* after treatment with memantine

	Number of revertants (M±SD)/plate and MI							
	TA98		TA100		TA102		TA97a	
	–S9	+S9	–S9	+S9	–S9	+S9	–S9	+S9
SC	19±2	24±8	92±10	96±12	231±27	270±38	107±7	117±13
DMSO	20±4	21±5	93±3	106±5	274±36	270±47	96±13	112±18
C+	654±91*** ^a	1221±73*** ^d	1458±85*** ^b	1132±68*** ^d	1982±249*** ^c	2359±114*** ^c	1518±123*** ^a	1442±152*** ^d
mtnH	16±1 (0.79)	27±9 (1.29)	109±15 (1.17)	113±3 (1.07)	266±47 (0.97)	231±12 (0.86)	83±10 (0.87)	121±14 (1.08)

Values shown as mean (M)±standard deviation (SD); *MI* mutagenicity index (in brackets); *SC* spontaneous control: phosphate buffer (pH 7.4) or S9 mixture (100 µL/plate), rate of spontaneous reversion; Solvent control: dimethylsulfoxide (DMSO—100 µL/plate); Memantine (mtnH—50 µg/ plate; 85.83 µM); C+ = positive control

^a4-nitro-*o*-phenylenediamine (TA98 and TA97a, 10.0 µg/plate)

^bsodium azide (TA100, 1.25 µg/ plate); ^cmitomycin (TA102, 0.5 µg/plate), in the absence of S9 and

^d2-anthramine (TA98, TA100, TA 97a, 1.25 µg/plate)

^e2-aminofluorene (TA102, 10.0 µg/ plate), in the presence of S9. The assay was performed in triplicate

*** $p < 0.0001$ with a confidence level of 95% compared to the DMSO control was considered significant through one-way ANOVA test followed by the Dunnett's post hoc test with $p < 0.05$, which yielded statistical difference of $p < 0.0001$ only for positive controls. The values of F, DFn, DFD, exact p value are described in the results

These results confirm the non-mutagenicity of this compound (Table 1), and agrees with previous studies, in which the evaluation of the genotoxic (alkaline comet assay) and mutagenic (bone marrow micronucleus test) effects of memantine on CF-1 mice showed the absence of such undesirable effects. Memantine did not cause DNA damage in the blood and brain tissues and showed antigenotoxic effects in brain tissue [40].

Final remarks and perspectives

Memantine hydrochloride has shown to be effective impairing CHIKV infection in vitro with an EC_{50} of 32.4 ± 31.9 µM, CC_{50} of 248.4 ± 4 µM, and SI of 7.67. Besides, mtnH was not mutagenic by the Ames test. With such promising data, we expect to encourage further pre-clinical and clinical assays of mtnH as a repurposing drug candidate for the treatment of Chikungunya fever.

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Author contributions Acquisition of data and results analysis: AKSP, IAS, and WWS. Drafting of the manuscript AKSP and IAS. Study design, supervision, and critical revision FARN, FRGB, ACGJ and PPC. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CAPÍTULO 8: CONSIDERAÇÕES FINAIS

Considerações finais

Os dados apresentados nesse trabalho demonstram o potencial de compostos coordenados como novas moléculas para o desenvolvimento de fármacos contra doenças virais de importância para a saúde pública, como as causados pelo SARS-CoV-2 e pelo CHIKV. Nesse contexto, os compostos Pd-atd, Pd-mtn, Pd-rtd, Pt-mtn, CuL₂phen, mtn, rtd, Cu(Ipr)Cl, Cu(Imes)Cl, Cu(Imes)₂BF₄, AuCl(PPh₃), e AuCl(Imes) avaliados aqui se apresentam como candidatos interessantes para realização de ensaios pré-clínicos em modelos animais murinos, e futuras investigações sobre o potencial como candidatos à fármacos.

Os compostos Pd-atd, Pd-mtn, Pd-rtd apresentaram uma forte inibição no ciclo replicativo do SARS-CoV-2, assim como os compostos CuL₂phen, Cu(Ipr)Cl, Cu(Imes)Cl, Cu(Imes)₂BF₄, com índices de seletividade (SI) variando entre 1,7 e 36,2. Adicionalmente, todos os compostos apresentaram uma inibição contra as variantes do delta e/ou ômicron do SARS-CoV-2. Alternativamente, os compostos Pt-mtn, mtn, AuCl(PPh₃), AuCl(Imes), Cu(Ipr)Cl, Cu(Imes)Cl e Cu(Imes)₂BF₄ se mostraram ativos contra o CHIKV, com valores de EC₅₀ entre 2.18 e 71.64 µM, resultando em valores de SI variando entre 3.5 e 8.4.

Interessantemente, os compostos apresentaram diferentes perfis de inibição, sendo os complexos associados a Pd(II) interferindo em etapas iniciais do SARS-CoV-2 (entrada do vírus nas células hospedeiras), enquanto que, os dados de tempo de adição de composto demonstram as moléculas CuL₂phen, Cu(Ipr)Cl, Cu(Imes)Cl e Cu(Imes)₂BF₄ interferindo com a replicação viral, após a entrada do vírus. De forma similar, os complexos Pt-mtn, AuCl(PPh₃), AuCl(Imes), Cu(Ipr)Cl, Cu(Imes)Cl e Cu(Imes)₂BF₄ demonstraram forte inibição na pós-entrada do CHIKV, potencialmente inibindo replicação e transcrição de RNA.

Os compostos Pt-mtn, AuCl(PPh₃), AuCl(Imes) e CuL₂phen demonstraram uma forte interação com a dsRNA viral, sugerindo um mecanismo de ação antiviral pela atividade de intercalação, e diminuindo a replicação viral do CHIKV. Os compostos mtn e Pt-mtn apresentam possuir uma forte atividade virucida contra o CHIKV, confirmada pelos ensaios de espectroscopia de infravermelho (FTIR) e demonstrada por meio de ensaios de ancoragem molecular.

Ademais, demonstramos aqui o potencial dos compostos de cobre (Cu), como por exemplo, Cu(Ipr)Cl, Cu(Imes)Cl, Cu(Imes)₂BF₄, como potente inibidores do SARS-CoV-2 e do CHIKV, apresentando uma possível atividade amplo-espectro desses compostos.

Entretanto, é necessário avaliar o efeito desses compostos contra outros vírus de RNA, para validar o efeito dos mesmo contra uma variedade de vírus.

Adicionalmente, a avaliação da atividade biológica dos complexos metálicos contra o SARS-CoV-2 e CHIKV aqui apresentados também poderão gerar *insights* para o desenvolvimento de novos complexos, com perfil farmacológico cada vez melhores e mais seletivos à inibição viral. Podendo também estimular o estudo e aplicação dessa classe de compostos contra outras doenças virais emergentes.

ANEXOS: Artigos publicados

Lista de Artigos Publicados:

1. CASSANI, NATASHA MARQUES ; **SANTOS, IGOR ANDRADE** ; GROSCHE, VICTÓRIA RIQUEÑA ; FERREIRA, GIULIA MAGALHÃES ; GUEVARA-VEGA, MARCO ; ROSA, RAFAEL BORGES ; PENA, LINDOMAR JOSÉ ; NICOLAU-JUNIOR, NILSON ; CINTRA, ADÉLIA CRISTINA OLIVEIRA ; MINEO, TIAGO PATRIARCA ; [SABINO-SILVA, ROBINSON](#) ; SAMPAIO, SUELY VILELA ; JARDIM, ANA CAROLINA GOMES . Roles of Bothrops jararacussu toxins I and II: Antiviral findings against Zika virus. INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES, v. 227, p. 630-640, 2023. <http://dx.doi.org/10.1016/j.ijbiomac.2022.12.102>
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