UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

Tamara de Carvalho

ESTUDO DA AÇÃO *IN VITRO* DE PEPTÍDEOS SINTÉTICOS COMO ANTIVIRAIS CONTRA O SARS-CoV-2

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

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Dedido este trabalho à meus pais Sueli Silva de Carvalhoe Wilson de Carvalho Júnior, e meus irmãos Tamires de Carvalho e Raphael de Carvalho, por sempre me apoiareme incentivarem.

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"A cachorra Baleia acompanhou-o naquela hora difícil. Repousava junto à trempe, cochilando no calor, à espera de um osso. Provavelmente não o receberia, mas acreditava nos ossos, e o torpor que a embalava era doce. Mexia-se de longe em longe, punha na dona as pupilas negras onde a confiança brilhava. Admitia a existência de um osso graúdo na panela, e ninguém lhe tirava esta certeza, nenhuma inquietação lhe perturbava os desejos moderados. Às vezes recebia pontapés sem motivo. Os pontapés estavam previstos e não dissipavam a imagem do osso.“

VIDAS SECAS, 1938, Graciliano Ramos.

RESUMO

O coronavírus da síndrome respiratória aguda grave 2 (SARS-CoV-2) é o agente causador da Covid-19, e os primeiros casos de pacientes diagnosticados com essa doença foram na China, em dezembro de 2019. Em março de 2020, devido ao aumento expressivo no número de infectados por todo o mundo, a Organinzação Mundial da saúde declarou que estávamos vivendo um quadro pandêmico. Apesar de já existir vacina para esse vírus, ainda há necessidade de desenvolvimento de tratamento no caso de pacientes graves ou imunocomprometidos. Diante do grande problema de saúde pública mundial que o vírus SARS-CoV-2 representa, juntamente com o potencial de estudos com peptídeos antivirais, o objetivo do presente trabalho foi identificar e investigar o potencial de peptídeos inibidores de infecção por SARS-CoV-2. Para isso, três questões foram eladoradas em torno do dímero (MR1903): 1) Como a dimerização dos peptídeos está relacionada com a ação anti-SARS-CoV-2? Para responder essa questão, outros três peptídeos foram elaborados com diferentes padrões de dimerização (PE1940, MR2024, EMC2109); 2) A adição de peptídeos com característica de penetração celular pode resultar num aumento da atividade antiviral contra SARS-CoV-2? E a partir disso,outros dois peptídeos foram desenvolvidos (MC1937 e MC1947); 3) A dextrorotação do peptídeo dímerico mantêm sua atividade? E por fim, obteve-se o peptídeo contedo D-amino ácidos (NB2080). Inicialmente, foi realizado um screening com todos os peptídeos em células Vero infectadas com SARS-CoV-2. Os peptídeos MR1903, PE1940, MC1937, MC1947 e NB2080 apresentaram atividade anti-SARS-CoV-2 e foram investigados quanto ao seus mecanismos de ação, e atividade contra duas variantes (Omicron e Delta) desse vírus. Resultados demonstraram que a dimerização está relacionada com a atividade contra esse vírus.O peptídeo tetrâmero (PE1940) apresentou atividade nos estágios de entrada e pós-entrada do ciclo de replicação do SARS-CoV-2, e o dímero (MR1903), demonstrou efeito profilático e emetapas após a entrada. Já os peptídeos MC1937 e MC1947, característicos pela adição de peptídeos de penetração celular, resultaram em inibição de todas as etapas testadas do ciclo replicativo (entrada, pré tratamento, virucida e pós-entrada) do SARS-CoV-2. Por fim, o peptídeo D-amino ácido (NB2080) possui um mecanismo de ação muito semelhante a MR1903usado nesse trabalho, porém não apresentou efeito contra as variantes testadas desse vírus.

Palavras-chave: SARS-CoV-2. Antiviral. Peptídeos. Mecanismo de ação.

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for causing Covid-19, and the first patients diagnosed with this disease were reported in China, in December of 2019. In March of 2020, because of the high number of infected in the whole world, the World health organization declared it was a pandemic case. Even though, there is a vaccine approved for this virus, there is still the needed to develop a treatment for grave and immunosuppressed patients. In reason of the great world health problem that SARS-CoV-2 still represents, along with the potential of studies using antiviral peptides, the aim of this work was to identify and investigate the potential of peptides against SARS-CoV-2 infection. For that purpose, three questions were elaborated in relation to a main peptide (MR1903): 1) How can dimerization be related to anti-SARS-CoV-2 action? To answer that, three other peptides were elaborated, with different pattern of dimerization (PE1940, MR2024, EMC2109); 2) Can the addition of cell-penetrating peptide to the structure result in an enhance of antiviral activity against SARS-CoV-2? Other three peptide were developed for this question (MC1937 e MC1947); 3) Peptide dextrorotation can be related to similar or high activity against SARS-CoV-2? And for that, one peptide with D-amino acids was elaborated (NB2080). Initially, it was performed a screening with all peptides using Vero infected with SARS-CoV-2. Peptides MR1903, PE1940, MC1937, MC1947 e NB2080 showed anti-SARS-CoV-2 activity, and they were investigated for their mechanism of action and effect against two variants (Omicron and Delta) of this virus. Results showed that dimerization is related to action against this virus. The tetrameric peptide (PE1940) has activity on entry and post-entry of the replication cycle of SARS-CoV-2, and the dimeric (MR1903) showed a prophylactic effect and action on steps after virus entering cells. Furthermore, peptides MC1937 and MC1947, with characteristics of cell penetrating peptides addition, resulted in inhibition of all steps of the replication cycle (entry, pretreatment, virucidal, and post-entry) of SARS-CoV-2. Finally, the D-amino acid peptide (NB2080) has a mechanism of action very similar to MR1903 used in the present work, however it did not present action against the variants tested.

Key-words: SARS-CoV-2. Antiviral. Peptides. Mechanism of action.

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LISTA DE ABREVIAÇÕES E SIGLAS

(pBthTX-I)2K - peptídeo (KKYRYHLKPF)2K

3Clpro - Protease semelhante à quimiotipsina, do inglês 3C-like protease

ACE2 - enzima conversora de angiotensina 2, do inglês Angiotensin-converting enzyme 2

BSL-3 - Sala de biossegurança de nível 3, do inglês Biosafety Level 3

cDNA - DNA complementar

CoVs – Coronavírus

CC50 – Concentração de citoxicidade 50%, do Inglês 50% Cytotoxic concentration

DMEM - Meio Dulbecco MEM

DMSO - Dimeltisulfóxido

ECMO - Oxigenação por membrana extracorpórea, do inglês Extracorporeal membrane oxygenation

FDA - do inglês Food and Drug Administration

HCoV-NL63 - Coronavírus humano NL63

HCoV-229E - Coronavírus humano 229E

HCoV-OC43 - Coronavírus humano OC43

HCV - Vírus da hepatite C, do inglês Hepatite C vírus

HIV - Vírus da imunodeficiência humano, do inglês Human immunodeficiency vírus

HKU1 - Coronavírus humano HKU1

ICTV - Comitê Internacional de Taxonomia de Vírus

IC50 – Concentração de inibição 50%, do Inglês Half-maximal inhibitory concentration

SI – Índice de seletividade, do Inglês Selective index

MEM - Meio essencial mínimo

MERS - Síndrome respiratória do Oriente Médio

MOI - multiplicidade de infecção, do inglês multiplicity of infection

Mpro - Principal protease do SARS-CoV-2, do inglês Main proteaseMTT: (3-(4, 5-dimethylthiazolyl-2)-2, 5- diphenyltetrazolium bromide)

nsPs - Proteínas não estruturais, do inglês non structural proteins

OMS - Organização Mundial da Saúde

ORFs - quadros de leitura aberta, do inglês Open Reading Frames

PCR - Reação em cadeia da polimerase, do inglês Polymerase Chain Reaction

PFU - Unidades formadoras de placa por mL, do inglês plaque forming unit

Plpro - Protease semelhante à papaína, do inglês Papain-like protease

PPCs - Peptídeos de penetração celular

RaTG13 - Betacoronavirus

RE - Retículo endoplasmático

RNA - Ácido ribonucleico, do inglês Ribonucleic acid

RTC - Complexo replicase-transcriptase, do inglês Replicase Transcription Complex

RdRp - Polimerase dependente de RNA, do inglês RNA-dependent RNA polymerase

SARS - Síndrome respiratória aguda grave

SFB - Soro Fetal Bovino

TMPRSS2 - Serino-protease transmembranica 2, do inglês Transmembrane protease, serine 2

VOCs - Variantes de preocupação

VOIs - Variantes de interesse

VUMs - Variantes sob monitoramento

ZIKV - Zika vírus

SUMÁRIO

CAPÍTULO I –
CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

1.1. Coronavírus

Os coronavírus (CoVs) são característicos por causar doenças associadas à problemas respiratórios e infecções intestinais em animais e no homem (CUI; LI; SHI, 2019a). Esse grupo está incluso na família Coronaviridae, composto por vírus de RNA envelopado, e que são capazes de infectar diversos hospedeiros, dentre estes aves, suínos e humanos (LIM et al., 2016). Porém, a maioria dos vírus pertencentes a esse grupo são os coronavírus causadores de doenças em humanos, como resfriados ou até broquite e pneumonia .(PENE et al., 2003).

As principais características dos coronovírus englobam sua morfologia, de maneira que estes são esféricos e apresentam cerca de 125 nm de diâmetro, e devido a aparência da superfície do vírus se assemelhar a uma coroa solar, resultou sua nomenclatura de coronavírus (BÁRCENA et al., 2009; MALIK, 2020; NEUMAN et al., 2006). No interior do envelope, o nucleocapsídeo se dispõem em forma de hélice simetricamente, característica inclusive que não é comum entre os vírus que possuem RNA de sentido positivo (MALIK, 2020).

Somente após o surto de 2002 e 2003, causado pela síndrome respiratória aguda grave (SARS-CoV), que ocorreu na província de Guangdong, na China, esse grupo foi considerado como de elevada patogenicidade para os seres humanos, pois, anteriormente, os coronavírus que estavam em circulação só causavam infecções leves (CUI; LI; SHI, 2019b). Posteriormente, no ano de 2012, ocorreram relatos em países do Oriente Médio do surgimento do coronavírus causador da síndrome respiratória do Oriente Médio (MERS-CoV), que também demonstrou características patogênicas graves, como deficiência respiratória e insuficiência renal (CUI; LI; SHI, 2019b; ZAKI et al., 2012).

O SARS-CoV foi caracterizado como um vírus de alta infectividade, após uma notificação de um surto em um hospital em Hong Kong, em que 138 pessoas se infectaram após duas semanas de contato com um paciente positivo para o vírus (LEE et al., 2003). Até o controle da pandemia por esse vírus, que foi em julho de 2003, foram notificados relatos do SARS-CoV em 29 países e regiões, totalizando 8098 casos e 774 mortes (ZUMLA et al., 2016).

Até o início de outubro do ano de 2015, já haviam sido notificados à Organização Mundial da Saúde cerca de 1593 casos de pacientes infectados pelo vírus MERS-CoV, e desses casos 568 levaram a morte. O MERS-CoV possui um potencial pandêmico menor que o SARS-CoV, porém o MERS possui maior rapidez de desenvolvimento dos sintomas clínicos e maior

taxa de letalidade, fazendo com que esse vírus seja ainda considerado de alto risco (ZUMLA et al., 2016).

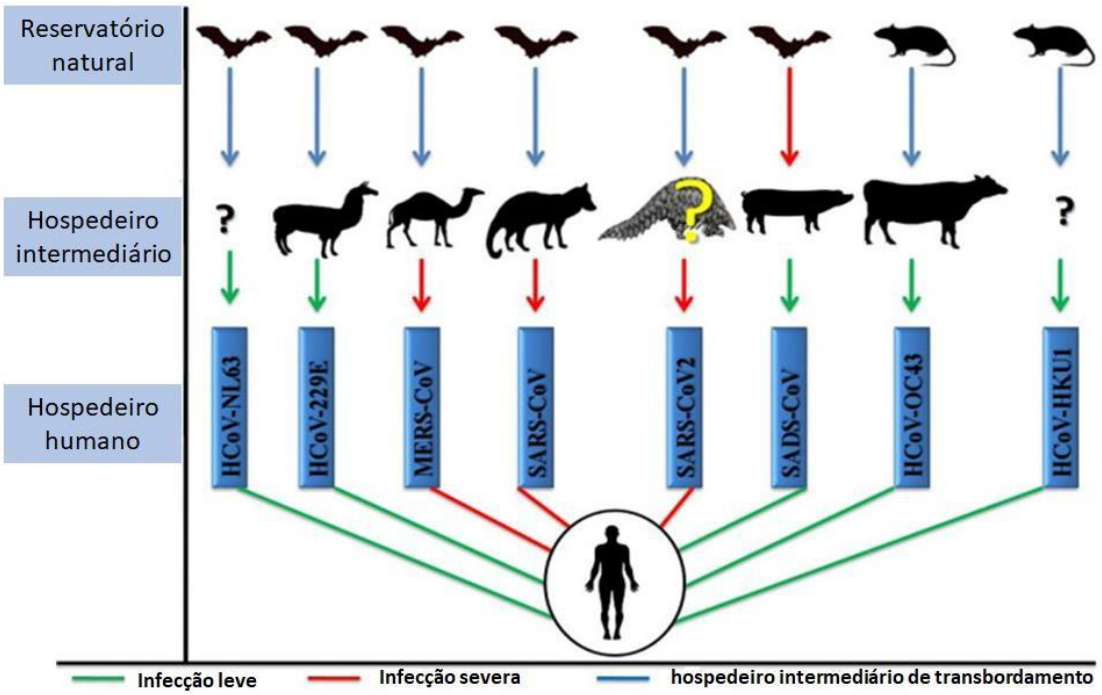
A transmissão desses vírus para os seres humanos ocorreu potencialmente por meio de camelos dromedários e civetas de mercado. Mas há ainda, estudos que apontam que a principal hipótese é da transmissão do vírus de morcegos para seres humanos, sendo assim, de origem zoonótica (CUI; LI; SHI, 2019a).

HCoV-NL63, HCoV-229E, HCoV-OC43 e HKU1 são outros exemplos de coronavírus que causam doenças em humanos. Estes possuem como característica o desenvolvimento de doenças respiratórios superiores leves em hospedeiros que estão imunocompetentes. Além disso, esses coronavírus também podem causar infecções graves em bebês, crianças e idosos (CUI; LI; SHI, 2019a; FORNI et al., 2017; PENE et al., 2003).

Em relação à origem desses coronavírus (Figura 1), todos que são causadores de doenças humanas, possuem como origem os animais, e precisaram de um hospedeiro intermediário de trasbordamento (JUNEJO et al., 2020). A origem dos coronavírus citados anteriormente SARS-CoV, MERS-CoV, HCoV-NL63 e HCoV-229E são os morcegos, já HCoV-OC43 e HKU1 possuem como origem provável os roedores (CUI; LI; SHI, 2019b).

Ainda sobre a origem desse vírus, além de estudos demonstrarem semelhanças entre os genomas do SARS-CoV-2 e o coronavírus RaTG13 presente em morcego (CHEN; LIU; GUO, 2020a; GUO et al., 2020a; SINGHAL, 2020), ainda foram encontrados segmentos que consistem com relações filogenéticas de diferents cepas de Sarbecovirus, em relação a proteína S do SARS-CoV-2 (SINGH; YI, 2021).

Figura 1 - Origem dos coronavírus causadores de doenças humanas.



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

1.2. COVID-19

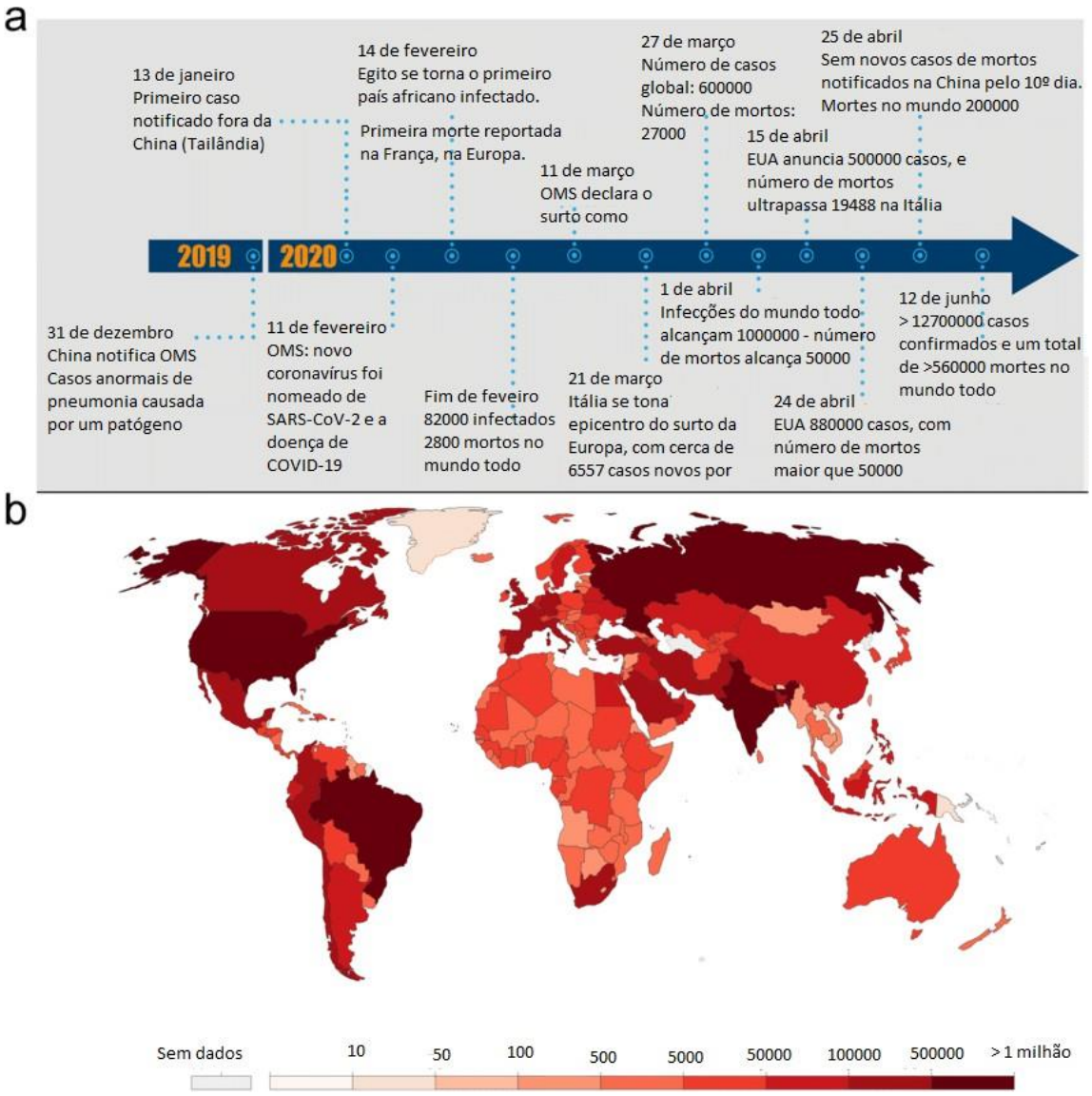
Em dezembro de 2019, diversos casos de problemas respiratórios de origem desconhecida foram notificados na província de Wuhan, na China. Relatos apontavam que os pacientes que apresentavam sintomas parecidos, semelhantes a uma pneumonia, teriam frequentado um mercado local da província, conhecido como Huanan Seafood WhosaleMarket. Tal descoberta foi importante para rastrear e detectar a causa da doença nos infectados: o coronavírus SARS-CoV-2 (síndrome respiratória aguda grave coronavírus 2) (CIOTTI et al., 2019).

A identificação desse novo coronavírus ocorreu em 4 de janeiro pela Organização Mundial da Saúde (OMS), e a nomeação como SARS-CoV-2 ocorreu em 11 de fevereiro pelo Comitê Internacional de Taxonomia de Vírus (ICTV), em razão da similaridade genética com o SARS-CoV (MACHHI et al., 2020). No final do mês de fevereiro de 2020, já haviam sido relatados 82.000 infecções e um número de mortes de 2.800 por todo o mundo. Diante a isso, a OMS declarou, no dia 11 de março, um surto de pandemia, fazendo com que houvesse a implementação de medidas pelo mundo todo, buscando maneiras de “achatar a curva” de infecção, e assim tentando desacelerar a disseminação do vírus (MACHHI et al., 2020).

Porém, no final do mês de março, enquanto a Itália se tornou um epicentro, em decorrência do rápido avanço no número de casos, os EUA notificou pelo menos 100.000 casos

e cerca de 2.700 mortes, e o número de casos no mundo alcançou mais de 600.000, com cerca de 29.000 mortes (Figura 2a). No início de abril, alcançou-se a marca de 1 milhão de infectados e 50.000 mortes. Apenas em meados do fim do mês de abril, que se observou uma diminuição nos casos de infectados para os primeiros países que notificaram situação mais alarmante no início da pandemia, como era o caso da Itália e China. Porém, nesse período foi contabilizado um total de 3 milhões de infectados pelo SARS-CoV-2 no mundo (Figura 2b) (MACHHI et al., 2020).

Figura 2 - a: Linha do tempo da pandemia causada pela COVID-19 da indicação dos primeiros casos e metado do ano de 2020. b: Distribuição global e a incidência de casos de COVID-19.



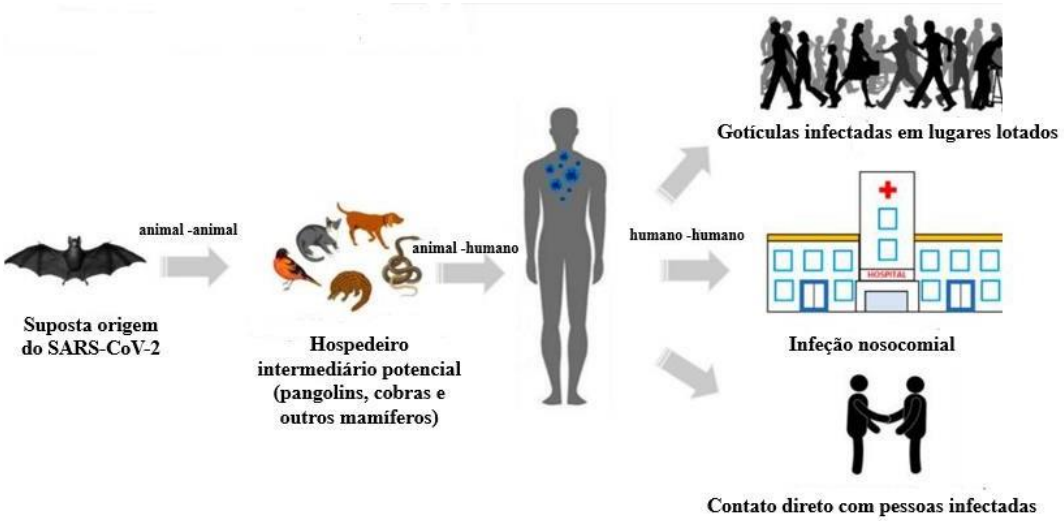
Fonte: Adaptado de (MACHHI et al., 2020).

A última coleta de dados para o número de infectados por SARS-CoV-2 foi realizado em 03 de outubro de 2023, pela Universidade John Hopkins, e o resultados mostraram até essa

data, um total de 676.609.955 de infectados no mundo todo, contando com 6.881.955 mortes (CENTER FOR SYSTEMS SCIENCE AND ENGINEERING (CSSE), 2023).

A transmissão do SARS-CoV-2 entre humanos ocorre principalmente através das gotículas salivares de pacientes infectados, e também é possível através do toque em superfícies contaminadas e depois no nariz, olhos e boca, denominada de transmissão de fômite, além da transmissão via oral-fecal (Figura 3) (CHEN et al., 2020; GUO et al., 2020b; SINGHAL, 2020). Também é conhecida a denominada infecção nosocomial, na qual ocorre a contaminação em quartos de hospital que abrigam pacientes positivos para o vírus. O estudo de Santarpia et al (2020) confirmou a presença do RNA viral do SARS-CoV-2 em itens de locais do hospital e das instalações sanitárias, assim como também em amostras de ar (Santarpia et al., 2020; Sharma et al., 2021).Em relação a transmissão vertical intrauterina, um estudo demonstrou testes negativos para recém-nascidos, em que as mães grávidas estavam positivas para o SARS-CoV-2, assim como também, análises do líquido amniótico, sangue do cordão umbilical e leite materno demonstraram resultados negativo para o SARS-CoV-2 (SANTARPIA et al., 2020; SHARMA; AHMAD FAROUK; LAL, 2021). Porém, um estudo mais recente, demonstrou que há a possibilidade de 5,7% de transmissão vertical, baseado principalmente na transmissão placentária, mas o estudo enfatiza a importância de ainda serem realizados mais estudos clínicos (WANG; DONG, 2022). Ainda, também há evidências de detecção do RNA viral em amostras de tecido de placenta, o que corrobora para uma potencial transmissão vertical (DENIZ; TEZER, 2022).

Figura 3 - Modos zoonóticos de transmissão do SARS-CoV-2, vírus causador da COVID-19.



Fonte: Adaptado (SHARMA; AHMAD FAROUK; LAL, 2021).

A transmissão desse vírus pode iniciar de 1 a 2 dias antes da apresentação dos sintomas, sendo que nos estágios de início da infecção, o título viral apresenta maiores valores, e logo após isso, começa a decair (OCHANI et al., 2021). Os testes mais comumente utilizados para a confirmação do diagnóstico de infecção por SARS-CoV-2 são: detecção de anticorpos humanos, antígenos virais e genes virais, sendo este último, considerado a técnica com resultados de maior confiabilidade (YÜCE; FILIZTEKIN; ÖZKAYA, 2021). Porém, existem outros métodos de identificação do vírus, como testes baseados em CRISPR, que é uma técnica mais sensível, sequenciamento, tomografia computacional por raio-x, e biosensores (RONG et al., 2023).

A detecção de genes virais é realizada por meio do PCR em tempo real, utilizando uma amostra de swab nasal ou garganta (CHEN; LIU; GUO, 2020b). Ainda, já foi comprovado na literatura ser possível a detecção do SARS-CoV-2 utilizando amostras de saliva, sendo um método menos invasivo para a testagem de pacientes (TAKEUCHI et al., 2020).

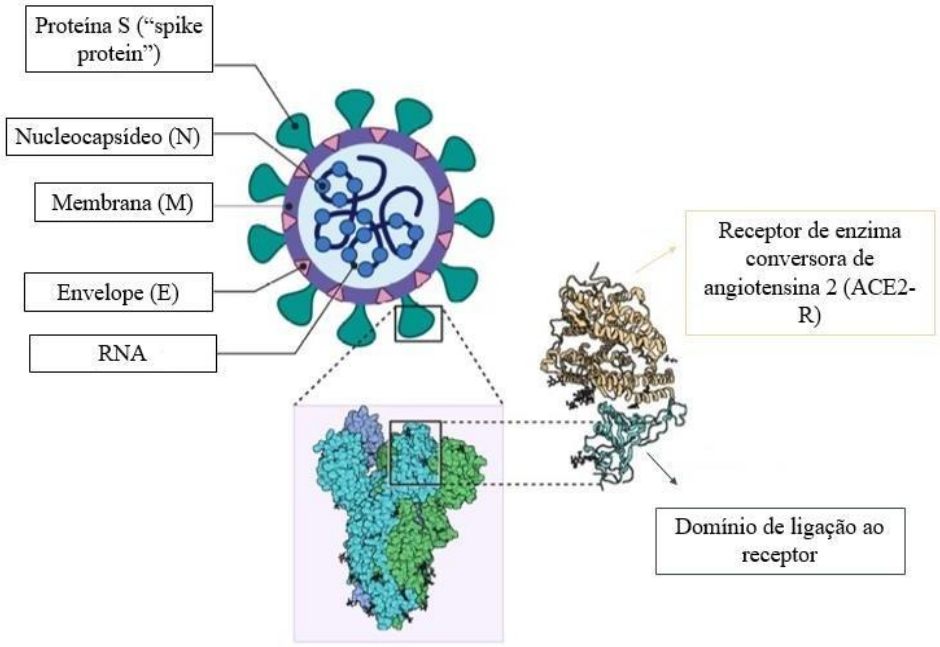
Os sintomas mais comuns apresentados por pacientes envolvem: febre, dores no corpo e musculares, cansaço, nariz congestionado e com corrimento, tosse seca, dor de garganta, e alguns pacientes apresentaram perda do apetite. Outros sintomas que também já foram relatados incluem: náuseas e vômitos, produção de escarro, dor de cabeça, arrepio, dispneia, dores no peito, e até mesmo expectoração de sangue (MADABHAVI; SARKAR; KADAKOL, 2020). Ainda, foi descrito em estudos, que pacientes que desenvolveram síndrome de desconforto

respiratório agudo podem levar a um quadro de piora acelerada, vindo a óbito por falência de múltiplos órgãos (SINGHAL, 2020; WANG; DONG, 2022). Além disso, existem casos de pacientes assimtomáticos, que estão infectados porém não apresentam nenhum sintoma durante todo o período de incubação do vírus (OCHANI et al., 2021).

1.3. SARS-CoV-2

O vírus SARS-CoV-2 é envelopado, possui um RNA de fita simples e senso positivo. Dentre os vírus de RNA, os coronavírus possuem os maiores genomas, com 27 a 32 kb (RAVI; SAXENA; PANDA, 2022a). O nucleocapsídeo protege o genoma viral dos coronavírus, sendo que, esse nucleocapsídeo possui como característica um formato helicoidal, quando relaxado, e quando dentro do vírus possui um formato esférico (Figura 4). Os coronavírus fazem parte de uma família da ordem Nidovirales, que possui vírus com característica de utilizar um conjunto de mRNA durante seu processo replicação (LAUXMANN; SANTUCCI; AUTRÁN-GÓMEZ, 2020a; YAO et al., 2020).

Figura 4 -O vírus SARS-CoV-2 e suas principais proteínas estruturais.



Fonte: Adaptado de (CASCELLA et al., 2024).

O genoma do SARS-CoV-2 contém seis quadros de leitura aberta de codificação (ORFs) (Figura 5). A ORF1a/b que representa aproximadamente um terço de todo o genoma e está em 5', é responsável por codificar as proteínas não estruturais (nsp) (poliproteína 1a,b (pp1ab)) (RAVI; SAXENA; PANDA, 2022b). Sendo a poliproteína 1a correspondente as proteínas nsp1

a nsp11, e a poliproteína 1ab correspondente as proteínas nsp11 a nsp16. Já as ORFs localizadas na região 3', codificam as proteínas estruturais: proteínas S ("spike protein"), de membrana (M), de envelope (E) e nucleocapsídeo (N) (CHEN; LIU; GUO, 2020a; DU et al., 2009; RAVI; SAXENA; PANDA, 2022b; TU et al., 2020a). Além de também possuir proteínas acessórias que são codificadas por ORF3a, ORF6, ORF7a, ORF7b e ORF8 (KHAILANY; SAFDAR; OZASLAN, 2020).

A proteína M tem papel principalmente na montagem de novas partículas virais, em que fatores do hospedeiro se juntam aos do vírus e tem-se a formação dessas novas partículas. Já a proteína E tem função de canal iônico, sendo importante desde a montagem da partícula viral até sua liberação. A proteína N é importante para a patogênese viral, coopera com a proteína M, de maneira a transcrição e a montagem do vírus ocorrerem mais eficientemente (KAUL et al., 2021a).

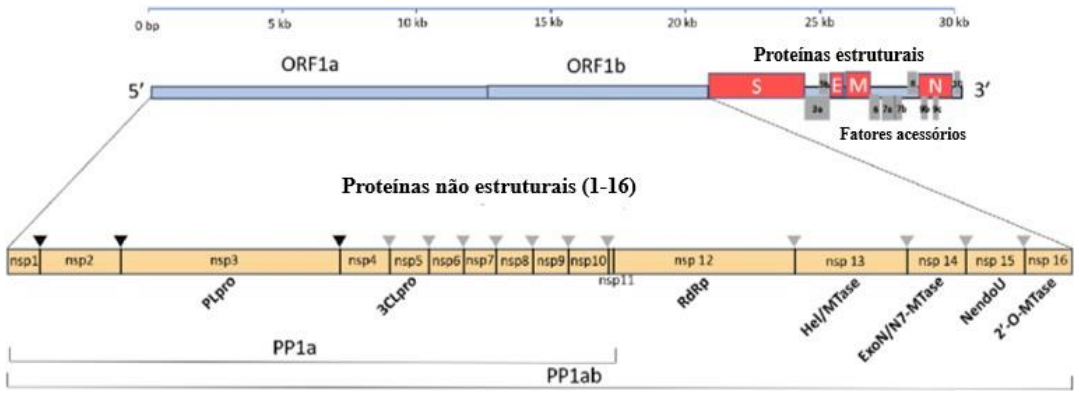
A proteína estrutural S é a responsável pela ligação do vírus à célula hospedeira, mediando então a entrada do vírus na célula, sendo essa proteína reconhecida pelo receptor da célula, que é a enzima conversora de angiotensina 2 (ACE2). A proteína S possui um domínio de ligação ao receptor (RBD), um domínio de fusão e um domínio transmembranar (ZHANG et al., 2021). O RBD da proteína S se liga à ACE2 para que então se inicie a entrada celular. A ligação da proteína S à ACE2 resulta em uma exposição dos locais de clivagem a proteases celulares. A clivagem da proteína S pela serino-protease transmembranica 2 (TMPRSS2) e outras proteases celulares inicia o processo de fusão e a endocitose. Dessa maneira, a entrada desse vírus na célula envolve a ligação ao receptor, o processamento proteolítico dessa proteína S, que leva então a fusão célula-vírus (PILLAY, 2020).

Estudos demonstraram que a entrada do SARS-CoV-2 na célula ocorre pela sequência de dois processos de clivagem da proteína S. Primeiramente, a ligação do vírus na ACE2 leva a modificações conformacionais na subunidade S1, expondo o sítio de clivagem na subunidade S2. O sítio S2' (interno a subunidade S2) é clivado por diversas proteases, sendo que a ativação das mesmas dependerá da maneira que o SARS-CoV-2 entra na célula, ou seja, a rota utilizada por esse vírus. A primeira rota está relacionada com a célula hospedeira não expressar TMPRSS2 suficiente ou se o complexo formado por vírus-ACE2 não encontrar TMPRSS2, de maneira que esse complexo será internalizado por via endocitose mediada por clatrina nos endolisossomos, e a clivagem do sítio S2' é feito pelas catepsinas, que necessitam de um ambiente ácido para seu funcionamento. Já na outra rota utilizada, a serino-protease transmembranica 2 (TMPRSS2) está presente, e então a clivagem de S2' ocorre na superfície

da célula. Independente da via, essa clivagem de S2’ deixa o peptídeo de fusão exposto, além da dissociação de S1 de S2 acarretar mudanças conformacionais na subunidade S2, o que estimula o peptídeo de fusão para dentro da membrana da célula do hospedeiro. A partir dessa fusão entre a membrana do vírus e da célula, ocorre a formação de um poro, no qual o RNA é liberado no citoplasma para ocorrer as próximas etapas do ciclo de replicação do vírus SARS-CoV-2 (JACKSON et al., 2022; ZHANG et al., 2021)

Apesar de ACE2 ser o principal receptor utilizador por esse vírus para entrar nas células, outros estudos já relataram que outros possíveis receptores utilizados, como o estudo de Liu et al (2022), que utilizou um pseudovirus, analisou uma via alternativa de entrada do SARS-CoV-**2.** Os autores demonstraram que esse vírus também possui afinidade pela a integrina α5β1, interagindo com o domínio extracelular dessa integrina, e desse modo, a internalização desse vírus também seria possível por outra via, que não seja utilizando o ACE2 .(LIU et al., 2022).

Figura 5 -Genoma do SARS-CoV-2 19, com organização em ORFs individuais e a poliproteína 1ab (PP1ab), que incorpora as 16 proteínas não estruturais.

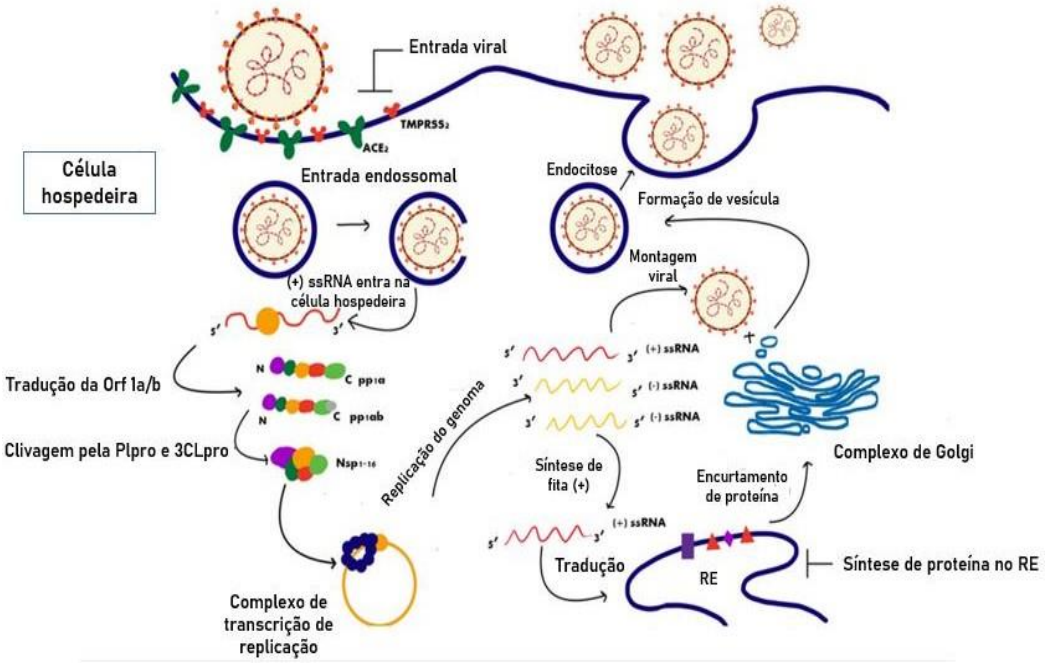


Fonte: Adaptado de (ROMANO et al., 2020).

O processo de infecção por SARS-CoV-2, de maneira geral, envolve as etapas de ligação, entrada, indução de proteínas replicase, replicação, transcrição, montagem das partículas virais e, por fim, a liberação dessas partículas (Figura 6). Mais detalhadamente, o vírus se liga à célula hospedeira e libera seu material genético no citosol, a partir disso então, ocorre a tradução do RNA em duas poliproteínas (pp1a e pp1ab, mencionadas anteriormente). Essas poliproteínas são então clivadas pelas proteases de cistéina, protease semelhante à papaína (PLpro) e a protease semelhante à quimiotipsina (3CLpro), de maneira a gerar as proteínas não estruturais (nsPs), e dessas uma parte irá se juntar para a formação do complexo replicase-transcriptase (RTC). As nsPs são importantes no direcionamento da síntese e

processamento do RNA, na degradação do mRNA da célula, na supressão da resposta imune da célula infectada, e também na formação de vesículas de membrana dupla. O domínio RNA polimerase dependente de RNA (RdRp) faz parte do RTC, e este domínio tem como função auxiliar na replicação do RNA de sentido positivo, de maneira a ter a formação do RNA de fita negativa intermediário, que possui dois destinos diferentes. O primeiro destino está relacionado com esse RNA ser transcrito descontinuamente, e como resultado cria segmentos de RNA subgenômicos mais curtos, e quando traduzido este produz as proteínas M, S e E. Essas proteínas passaram por processamentos e serão empacotadas, formando o vírion. O segundo destino desse RNA de fita negativa é a replicação adicional, que forma o RNA de fita positiva, e este será inserido na partícula viral, que sofrerá exocitose da célula para infectar outras células. No retículo endoplasmático (RE), a proteína do nucleocapsídeo é combinada com o RNA genômico de polaridade positiva para se tornar o complexo de nucleoproteína. No compartimento do retículo endoplasmático-aparato de Golgi, ocorre a fusão da partícula viral completa, sendo então os vírions transportados por vesículas, e por fim, liberados para o meio extracelular (DU et al., 2009; KAUL et al., 2021b; KIM et al., 2020; SHEREEN et al., 2020).

Figura 6 - Processo de infecção por SARS-CoV-2 em uma célula hospedeira.



Fonte: Adaptado de (KAUL et al., 2021b).

Em relação à estrutura e patogenicidade, os vírus SARS-CoV e SARS-CoV-2 possuem muitas semelhanças, porém é observada uma diferença entre esses vírus na proteína S, que é

responsável pela ligação do vírus à célula hospedeira e essa diferença está relacionada com o maior potencial de disseminação do SARS-CoV-2 (LIU et al., 2020). Em razão disso, é importante ressaltar que, a proteína S se destaca como um alvo terapêutico (TU et al., 2020b; WALLS et al., 2020). A proteína S (“spike protein) possui como composição três unidades:

S1/S2 e o RBD, localizado em S1, e análises filogenéticos identificaram que a diferença entre o corononavírus SARS-CoV-2 e outros coronavírus relacionados ao SARS estaria em um local de reconhecimento de furina “RRAR” em S1 / S2. O SARS-CoV possui apenas uma arginina nesse local. Devido então à ligação mais forte formada pelo complexo ACE2-SARS-CoV-2, esse vírus se espalha de maneira mais rápida e estudos demonstraram a maior afinidade de ligação desse complexo quando comparado à ACE2-SARS-CoV (BAI; WARSHEL, 2020; XIA et al., 2020).

A linhagem original do SARS-COV-2, como outros vírus de RNA, passou por diversos processos evolutivos, de maneira a surgirem assim, diversas variantes desse vírus, que foram identificadas durante sequenciamentos genômicos periódicos. Dentre as variantes que surgiram, a OMS separou essas variantes em variantes de preocupação (VOCs), variantes de interesse (VOIs) e variantes sob monitoramento (VUMs) (CASCELLA et al., 2024; HE et al., 2021).

As variantes de preocupação possuem como algumas de suas principais características um maior potencial de transmissibilidade e também de virulência em relação à cepa original do SARS-CoV-2. Além disso, estudos demonstraram que possuem menor neutralização por anticorpos por infecção ou vacinação. Nessa categoria foram enquadradas 5 variantes : Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) e Ômicron (B.1.1.529) (ALEEM; AKBAR SAMAD; VAQAR, 2024; CASCELLA et al., 2024). Já as variantes de interesse são aquelas que possuem alterações genéticas previstas por afetar as propriedades do vírus relacionadas com sua transmissibilidade, o desenvolvimento da doença, e escapes diagnóstico e imunológico, e as variantes que entraram nessa categoria são: Epsilon (B.1.427 e B.1.429), Zeta (P.2), Eta (B.1.525), Theta (P.3), Iota (B.1.526), Kappa (B.1.617.1), Lambda (C.37) e Mu (B.1.621), segundo a Organização Mundial da Saúde. As variantes sob monitoramento são as que apresentam suspeita de causar alterar as características do vírus, mas ainda não há evidencia bem esclarecida sobre seu impacto fenotípico ou epidemiológico (CASCELLA et al., 2024; CHOI; SMITH, 2021; HE et al., 2021).

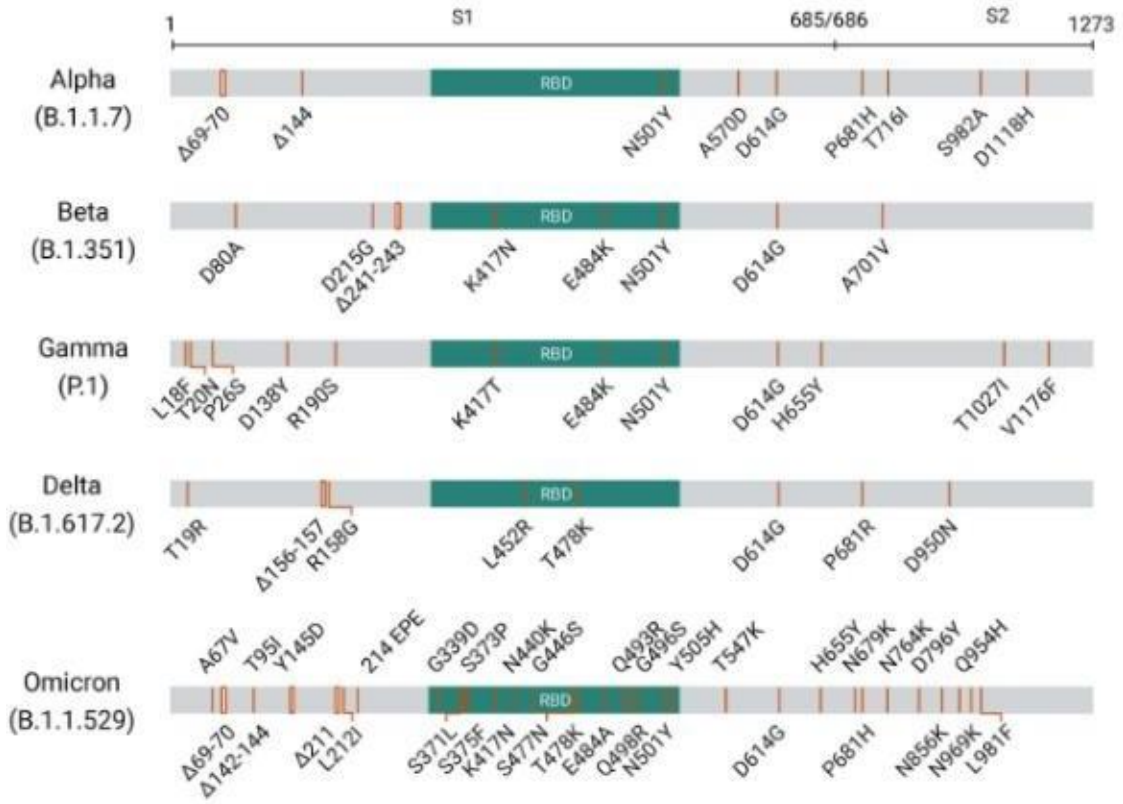
Dentre as variantes de preocupação, tem-se a variante Delta (B.1.617.2), que foi identificada pela primeira vez no final de 2020, em Maharashtra na Índia (MLCOCHOVA et

al., 2021). Essa variante prevaleceu no mundo entre o período de dezembro de 2020 a outubro de 2021, em razão de sua alta taxa de transmissão, sendo duas vezes maior que a da cepa original de Wuhan e 40-60% maior que da variante Alpha (BHATTACHARYA et al., 2023). Dentre suas características identificadas, sua proteína S (spike) se dispõem de maneira predominantemente clivada, em comparação com a variante Alpha, resultando em um aumento na eficiência de replicação nos sistemas organóides de vias aéreas e também em sistemas epiteliais das vias aéreas humana. Além disso, em comparação com a linhagem do tipo selvagem, a proteína S também demonstrou capacidade em formar um sincício de alta eficiência menos sensível a inibição por anticorpos neutralizantes (MLCOCHOVA et al., 2021).

Após o período de prevalência da variante Delta, foi a variante Ômicron que demonstrou dominância global (BHATTACHARYA et al., 2023). Identificada de uma amostra coletada em 9 de novembro de 2021 e relatada à OMS em 24 de novembro do mesmo ano. Após dois dias deste relato, a OMS já classificou essa variante como de preocupação. Essa variante se espalhou por diversos países de maneira muito rápida, o que já a caracterizou como um grande problema de saúde pública (HE et al., 2021). Com mais de 60 substituições/exclusões/inserções, essa é a variante que contém o maior número de locais de mutação de todas as variantes (HE et al., 2021). Ainda, esta é classificada em cinco linhagens (BA.1, BA.2, BA.3, BA.4, BA.5) e ainda em cinco sublinhagens (BA.1.1, BA.2.12.1, BA.2.11, BA.2.75, BA.4.6), e todas são caracterizadas pela alta taxa de mutação na proteína S (ZHOU; ZHI; TENG, 2023). Dentre suas principais características, é possível observar que esse grande número de mutações na proteína S (spike) demonstra uma potencialidade de aumento de transmissibilidade, resistênciaa terapias, e ainda, o escape parcial de imunidade induzida por vacina ou por infecção. A sua proteína S possui um grande número de alterações no domínio de ligação ao receptor e no domínio N-terminal, que são os alvos primários dos anticorpos neutralizantes (“SARS-CoV-2 B.1.1.529 (Omicron) Variant — United States, December 1–8, 2021”, 2021; SCHMIDT et al., 2022).

Como dito ao longo do texto, todas essas variantes possuem alterações na proteína S, que incluem substituições, deleções e inserções, e essas modificações podem ser observadas no quadro abaixo (Figura 7).

Figura 7 - Alterações, que incluem substituições, deleções e inserções observadas nas variantes de preocupação identificadas.



Fonte: (HE et al., 2021).

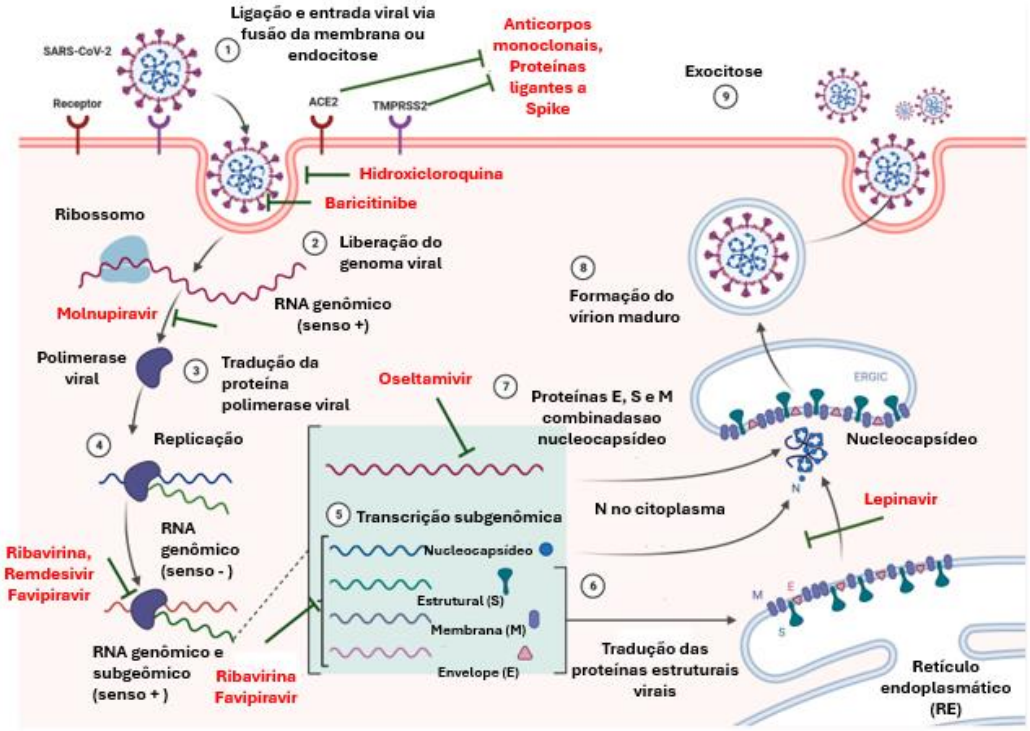
1.4. Prevenção e tratamentos disponíveis

Desde o início da pandemia de COVID-19, a comunidade cientifica reuniu esforços para a busca de alternativas para diminuir os casos de pacientes positivos para o vírus, além de também diminuir a gravidade da doença causada pelo SARS-CoV-2. Segundo dados da Organização Mundial da Saúde, sendo a última atualização até o ano de 2023, existem 183 vacinas em etapa de desenvolvimento clínico, e 199 vacinas em desenvolvimento pré-clínico (WORLD HEALTH ORGANIZATION, 2023). Diferentes tecnologias foram utilizadas para o desenvolvimento de vacinas para a COVID-19, dentre elas, tem se o vírion inteiro inativado, o vírus atenuado vivo, de ácido nucleico, proteína S recombinante e vetores do vírus (TO et al., 2021). Após a autorização de diversas vacinas pelo mundo todo e o início das campanhas de vacinação, obteve-se como resultado uma diminuição dos sintomas e dos casos severos da doença (LOPEZ BERNAL et al., 2021).

Em relação ao tratamento da doença, a maior parte dos pacientes que apresentam sintomas leves recebem tratamento sintomático, realizam o isolamento domiciliar e o

monitoramento da doença. Em casos de pacientes que já tenham problemas respiratórios, é indicado o fornecimento de oxigênio, através do uso de respiradores. A grande maioria dos pacientes aceitam oxigenoterapia, porém foi recomendada pela OMS a necessidade de oxigenação por membrana extracorpórea (ECMO) a pacientes com hipoxemia refratária (GUO et al., 2020b; SINGHAL, 2020). Além disso, existem diversas estratégias sendotestadas contra o SARS-CoV-2, e dentre essas, o uso de antimicrobianos e imunoterapia (ZOU et al., 2021a) (Figura 8).

Figure 8 – Principais alvos antivirais testados contra o SARS-CoV-2



Fonte: (Hillary & Ceasar, 2023)

O uso de antimicrobianos engloba os antivirais, antimaláricos, antibióticos e antiparasitários. Devido à similaridade em 80-90% da sequência genômica e de diversas enzimas existentes entre o SARS-CoV-2 e os coronavírus MERS e SARS, diversos compostos já testecontra MERS e SARS para a análise *in vitro* de possíveis ações contra o SARS-CoV-2 (JAMSHAID et al., 2020). Em razão disso, e de inibidores de proteases demonstrarem uma atividade de inibição contra vírus de RNA, foi realizado um estudo na China com lopinavir-ritonavir, um ensaio clínico aberto e randomizado (ChiCTR2000029539), porém não foi observada diferença de melhora entre tratamento com lopinavir-ritonavir e o tratamento padrão, para pacientes hospitalizados (CAO et al., 2020). Análogos estruturais de nucleosídeos, como Favipiravir, Remdesivir e Ribavirina, demonstraram

também uma atividade inibitória contra o SARS-CoV-2, em estudos *in vitro* (JAMSHAID et al., 2020).

Ainda são necessário mais estudos com Favipiravir para comprovar sua ação em ensaios clínicos. Já o composto Remdesivir está sendo estudado sobre sua ação conjunto com Baricitinibe, em ensaios clínicos de fase I (Hillary & Ceasar, 2023).

Além disso, também já existem estudos que demonstram a ação inibitória de compostos contra esse vírus, como um estudo que utilizou o antibiótico Ivermectina e demonstrou a ação antiviral na etapa de replicação desse vírus, sendo possível observar uma diminuição da carga viral nas células que receberam o tratamento com esse composto (CALY et al., 2020). É importante ressaltar que os pacientes infectados com SARS-CoV-2 que precisaram ser internados possuem risco de coinfecção por bactérias, representando cerca de 3,5% dos casos. Sendo o caso então de alguns estudos recomendarem o tratamento com antibióticos. Porém, esse tipo de intervenção deve ser analisada pelo médico responsável para cada paciente (ZOU et al., 2021b).

Outro estudo analisou o efeito inibitório do composto Atazanavir na etapa de replicação do vírus SARS-CoV-2, e ainda, nesse mesmo trabalho, os autores testaram a ação do composto Atazanavir em conjunto com o Ritonavir, e foi possível observar novamente a inibição dessa etapa do ciclo viral, de forma que, o estudo sugere esses compostos como candidatos para maiores estudos clínicos contra o SARS-CoV-2 (FINTELMAN-RODRIGUES et al., 2020). Além desses estudos, outro trabalho também demonstrou um significativo potencial de inibição da replicação do SARS-CoV-2 em células VERO E6, utilizando um composto denominado Cápsula de Liu Shen (LS) (MA et al., 2020). Os estudos citados sustentam a importância da análise de compostos que potencialmente podem mostrar ação antiviral contra o SARS-CoV-2.

Como citado anteriormente, antimaláricos também foram analisados quanto a sua ação inibitório contra o SARS-CoV-2, como é o caso da Cloroquina e Hidroxicloroquina. A Cloroquina demonstrou um possível mecanismo de ação que envolve a inibição da fusão e entrada desse vírus na célula hospedeira, porém esse composto possui limitações relacionadas a sua toxicidade (RABI et al., 2020). Já a Hidroxicloroquina possui mecanismo de ação relacionado à prevenção da entrada do vírus na célula, por meio de um aumento causado no pH endossomal (SAVARINO et al., 2003). Também foi realizado estudo observacional não randomizado de associação da Hidroxicloroquina e o antibiótico azitromicina, que demonstrou uma redução da carga viral e taxa de mortalidade (GAUTRET et al., 2020). Apesar disso, é importante ressaltar que nenhum estudo comprova uma potencial terapia com o uso desses compostos contra a infecção pelo vírus SARS-CoV-2 (IZCOVICH et al., 2022; JAMSHAID et al., 2020; LAUXMANN; SANTUCCI; AUTRÁN-GÓMEZ, 2020b).

Uma outra alternativa abordada é a imunoterapia, por meio do uso de anticorpos monoclonais, como Tocilizumabe, Mepolizumab e Sarilumab (ZOU et al., 2021). Apesar de estudos utilizando anticorpos monoclonais não resultarem na redução da mortalidade, foram demonstrados resultados de redução da carga viral, no caso de administração no início dos sintomas e também antes do surgimento de anticorpos anti-SARS-CoV-2 no soro de pacientes não hospitalizados (TO et al., 2021). Além dos anticorpos monoclonais, também existem estudos utilizando um tipo de terapia baseada em células-tronco (ZOU et al., 2021b). Estudos demonstraram melhora na função pulmonar de alguns pacientes infectados que foram transplantados com células-tronco mesenquimais (LENG et al., 2020). E por fim, tem-se a transfusão de plasma covalescente, porém há controvérsias em relação ao potencial de intervenção por esse tipo de tratamento contra o SARS-CoV-2 (ZOU et al., 2021b).

Como dito anteriormente, outro tipo de tratamento que está em estudo é o que envolve a medicina tradicional chinesa. Um exemplo, envolve a análise do potencial da capsúla Lianhuaqingwen juntamente com o tratamento sintomático, em que resultados demonstraram aumento na taxa de recuperação e o tempo de sintomas diminuíram, além de outros resultados positivos (HU et al., 2021; ZOU et al., 2021b).

Além desses estudos, estão sendo analisados medicamentos novos, que são moléculas pequenas, com ação de impedimento de entrada do vírus na célula. O Mesilato de camostato demonstrou uma ação de bloqueio na entrada do vírus, que está relacionada com o bloqueio de serino-protease transmembranica 2 (TMPRSS2) (HOFFMANN et al., 2020). Outro exemplo de composto é o Arbidol (umifenovir), que possui atividade de inibição da endocitose, resultando em sua inclusão em testes clínicos. Ainda, estudos que demostraram que a inibição da endocitose poderia ser realizada por meio da administração parental de ACE2 solúvel, resultando na ligação à proteína S (“spike protein”), e assim levando a endocitose celular, induziram ao desenvolvimento de um ACE2 humano recombinante (APN01), que está em ensaios clínicos para análises do potencial anti-COVID-19 (JAMSHAID et al., 2020).

Com o surgimento das variantes, muito estudos de antivirais contra o SARS-CoV-2 passaram a também testar os compostos em variantes (ABDELNABI et al., 2021; MARTINEZ et al., 2021; SINGH et al., 2022; ZAHRADNÍK et al., 2021). Como o estudo de Carter-Timofte et al (2021) que analisou o composto antimicrobiano Atovaquona contra o vírus do tipo selvagem SARS-CoV-2, e também suas variantes Alpha, Beta, e Delta, conseguindo demonstrar o efeito antiviral em todas, inclusive em células VERO e Calu-3 (CARTER-

TIMOFTE et al., 2021). Já o estudo de Wang et al (2021) analisou a ação de doze lectinas derivadas de plantas nas variantes B.1.1.7, B.1.351, e P.1 (WANG et al., 2021).

Até o momento da escrita deste trabalho, a ANVISA possui 6 medicamentos aprovados para tratamento da COVID-19: Rendesivir, Sotrovimabe, Baricitinibe, Paxlovid (nirmatrelvir + ritonavir), Molnupiravir, e Tocilizumabe (ANVISA, 2024). Dois dos medicamentos aprovados são anticorpos monoclonais, o que seria problemático, pois não necessariamente possui eficácia em todos os sistemas imunológicos, dependendo do organismo de cada indivíduo responder ao tratamento. Dessa forma, apenas três são medicamentos antivirais, o que demonstra a necessidade do desenvolvimento de novas moléculas, e ainda diante do panorama do surgimento de novas variantes, evidenciando a importância da disponibilização de novos estudos para a elaboração de tratamentos eficazes e com vantagens sobre os já disponibilizados contra esse vírus.

1.5. A ampla possibilidade de estudo envolvendo peptídeos como antivirais

Os peptídeos são compostos de pequenos fragmentos de proteínas, que vem ganhando destaque para o estudo de potenciais antivirais. Isto ocorre devido a uma possível função desses componentes proteicos em apresentar uma barreira defensiva, mostrando que peptídeos com ação antimicrobiana podem estar associados ao estudo de diversos vírus, de forma a serem denominados peptídeos antivirais. Esses peptídeos antivirais que interagem com as partículas do vírus ou em algum alvo das etapas críticas de replicação viral do ciclo de vida podem potencialmente ser candidatos para serem utilizados como tratamento ou profilaxia (ALTMANN et al., 2012; CHINCHAR et al., 2004).

O estudo de peptídeos pode ser realizado, primeiramente, por meio de estudos *in silico*, sendo possível analisar a interação desses com algum alvo determinado, como pode ser o caso de uma glicoproteína presente na superfície do vírus ou ainda de uma determinada enzima importante no ciclo de replicação de um vírus. E devido à diversidade de características que os peptídeos possuem, como carga, tipos de composições diferentes de aminoácidos e características físico-químicas, esses compostos podem demonstrar diferentes potencialidades de atividades antivirais (OKAZAKI; KIDA, 2004; VILAS BOAS et al., 2019a)

O uso de peptídeos sintéticos como antivirais apresenta diversas vantagens, em comparação com outros compostos, pois possuem uma alta especificidade e eficácia, baixa toxicidade, baixa acumulação em tecidos, ampla gama de alvos, e apresenta menos efeitos colaterais (CASTEL et al., 2011; MARQUS; PIROGOVA; PIVA, 2017). Além disso, os

peptídeos utilizados como antivirais já demonstraram ação principalmente na inibição da ligação do vírus e também na fusão da membrana celular do vírus, destruição do envelope do vírus, e inibição da replicação do vírus (HUAN et al., 2020). Existem diversos estudos que foram realizados *in vitro* que demonstraram a ação de peptídeos como antivirais, como, por exemplo, o peptídeo denominado LL-37 contra os vírus HCV (MATSUMURA et al., 2016), ZIKV (HE et al., 2018), HIV (PETER BERGMAN et al., 2007), e contra o vírus Dengue-2 (ALAGARASU et al., 2017). Além desses estudos, já foram analisados diversos peptídeos com ação antiviral contra coronavírus, como os peptídeos HR1P e HR2P contra o vírus MERS-CoV (LU et al., 2014), e ainda outro estudo utilizando o peptídeo à base HR2 que teria o potencial de inibir a fusão e entrada do vírus MERS-CoV (GAO et al., 2013). Já existem diversos estudos *in silico* na literatura, que utilizam de modelo computacional, com o intuito de demonstrar o potencial de peptídeos como inibidores contra o SARS-CoV-2, propondo os peptídeos analisados como compostos terapêuticos eficientes contra esse vírus (ÇAKIR et al., 2021; DE CAMPOS; PALERMO; CONDA-SHERIDAN, 2021; HAN; KRÁL, 2020; LING et al., 2020; LISCANO; OÑATE-GARZÓN; OCAMPO-IBÁÑEZ, 2020; SITTHIYOTHA; CHUNSRIVIROT, 2021). Isso demonstra que os peptídeos antivirais são compostos potencialmente promissores para o estudo contra o vírus SARS-CoV-2.

Estudos sugerem o uso de drogas à base de peptídeos como adjuvantes ou ainda em combinação com outros antivirais com diferentes mecanismos de ação, de maneira a resultar em uma diminuição do estabelecimento da resistência aos medicamentos e gerar menos efeitos colaterais (VILAS BOAS et al., 2019a). Além disso, a descrição de novos antivirais complementa as terapias existentes e oferece alternativas para o tratamento de doenças virais que causam graves pandemias, reduzindo a mortalidade/morbidade a elas associadas (VILAS BOAS et al., 2019b).

O principal peptídeo do presente trabalho (MR1903) possui como caractéristica ser um dimérico ((p-BthTX-I)$_2$K), que foi derivado a partir da estrutura da miotoxina Bothropstoxin-I, do veneno da cobra *Bothrops jararacussu.* Essa miotoxina já possui atividade antimicrobial demonstrada na literatura (ANDRIÃO-ESCARSO et al., 2000; BARBOSA et al., 2005; CINTRA et al., 1993). E mais recentemente, um trabalho do nosso grupo demonstrou a atividade antiviral do dimérico derivado dessa miotoxina contra os vírus Chikungunya e Zika (AYUSSO et al., 2023).

A partir do dimérico MR1903 ((KKYRYHLKPF)$_2$K) e com o intuito de explorar a relação entre a dimerização de peptídeos e o efeito antiviral, outros peptídeos foram

sintetizados, um tetrâmero (PE1940, $KKYRYHLKPF)_4$ $(K)_2K$, um monômero (EMC2109, KKYRYHLKPF), e um linear (MR2024, KKYRYHLKPFAFPKLHYRYKK).

Além disso, foram elaborados outros dois peptídeos (MC1947, $(KKYRYHLKPF)_2K$-YGRKKRRQRRR e MC1937, $(KKYRYHLKPF)_2K$-KRLRWR), com a estrutura do dímero, porém com adição de peptídeos com caractéristicas de penetração celular. Essa estratégia foi utilizada por conta da barreira presente na membrana das células, pois essa seleciona a entrada de moléculas, o que pode representar um problema durante a testagem de compostos contra microorganismos (LUNDBERG; LANGEL, 2003). Por conta disso se justifica a escolha de peptídeos com caractéristica de penetração celular, que tem a capacidade de adentrar as membranas celulares usando processos independete ou dependente de energia (NEUNDORF, 2019).

A estereoquímica de moléculas explica as duas possíveis configurações para aminoácidos em peptídeos, podendo ser levógiro ou dextrógiro (GENCHI, 2017). E diante a isso, o último peptídeo foi elaborado para o estudo (NB2080, $(KKYRYHLKPF)_2K$ (D-aa)), composto por D-aminoácidos, ao invés de L-aminoácidos, como é o caso do dímero MR1903, de maneira a comparar os resultados obtidos.

Dessa maneira, diante do atual quadro de pandemia e o grande problema de saúde pública mundial que o vírus SARS-CoV-2 representa, juntamente com o potencial de estudos com peptídeos antivirais, faz de suma importância o estudo de peptídeos sintéticos e sua ação inibitória contra células infectadas com diferentes variantes do vírus SARS-CoV-2.

2. OBJETIVOS

2.1. Objetivo geral

Identificar e investigar o potencial de peptídeos inibidores de infecção pela linhagem do tipo selvagem de Wuhan do SARS-CoV-2 e pelas variantes de preocupação Delta e Ômicron. Além disso, avaliar a atividade anti-SARS-CoV-2 dos peptídeos selecionados em diferentes fases do ciclo de replicação do vírus SARS-CoV-2.

2.2. Objetivos específicos

- Avaliar a citotoxicidade dos peptídeos sintéticos nas linhagens celulares Vero (células epiteliais renais extraídas de um macaco verde africano), A549 (linhagem celular epitelial pulmonar derivada de carcinoma de alveólos humanos) e BHK-21 (fibroblastos de rim de bebê hamster);

- Verificar a atividade antiviral dos peptídeos propostos em células Vero infectadas com a linhagem do tipo selvagem de Wuhan do SARS-CoV-2;

- Verificar a atividade antiviral dos peptídeos propostos em células infectadas com as variantes de preocupação Delta e Ômicron;

- Avaliar o efeito dos peptídeos com atividade antiviral nas etapas de entrada, atividade virucida e efeito nas etapas pós-entrada em células Vero e A549;

- Analisar a ação dos peptídeos no replicon subgenômico do SARS-CoV-2, e assim analisar seus efeitos dentro da etapa de replicação;

- Avaliar o efeito dos peptídeos no RNA dupla fita de SARS-CoV-2;

- Analisar a adaptação do vírus ao tratamento dos peptídeos.

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CAPÍTULO II – Artigos científicos

Manuscrito I

Could peptide structure dimensions be related with anti-SARS-CoV-2 activity? An in vitro evaluation of synthetic peptides

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Abstract

Given the global public health problem that SARS-CoV-2 still represents the aim of the present work was to identify and investigate the potential of peptides derived from the structure of Bothropstoxin-I (MR1903, PE1940, EMC2109, and MR2024), a myotoxin from the Bothrops jararacussu snake, as inhibitors against SARS-CoV-2 infection. Also, we analysed different analogs, so we compared the structure-activity relationship with anti-SARS-CoV-2 action. Primarily, we performed a screening of the potential peptides using infected Vero cells, which showed that linear and monomeric peptides (EMC2109 and MR2024) presented non-significant effect against SARS-CoV-2. Following by that, initial replication effect analyses showed specific action (virucidal effect, entry, and post-entry steps) of the previous selected peptides. Experiments using lung human cells demonstrated the dose dependence of both potential antiviral peptides, with higher selective index for MR1903. The tetrameric peptide (PE1940) has a potent effect on entry steps of viral replication, and the dimeric (MR1903) shows a protective action on cells before infection. Both peptides have high antiviral potential on post-entry steps, and this action can be related with their inhibition of double strand RNA (dsRNA) in vitro, and on the replication step. These peptides also showed inhibition against Delta and Omicron SARS-CoV-2 variants. Finally, we analysed if the virus could adapt to these peptides' treatment, which was confirmed after some passages for PE1940, and it was variable for MR1903 between passages. Our results demonstrated how the potential of dimerization in peptides structure result in different anti-SARS-CoV-2 effects.

Keywords: SARS-CoV-2, Antiviral, Synthetic peptides, replication cycle inhibition.

1. Introduction

In December of 2019, what started with several cases of respiratory problems with unknown source identified first in China, would turn into a pandemic caused by a coronavirus, which was declared officially by World Health Organization in March 2020 [1,2]. COVID-19 is the disease caused by severe acute respiratory syndrome coronavirus (SARS-CoV-2), that can be associated to different clinical manifestations. Patients can develop symptoms that occur more frequently like fever, fatigue, and dry cough, or more rare ones, for example, loss of smell and taste, headache, dyspnea, vomiting, among others [1]. Due to the fast mode that this virus spreads all over the world, it was necessary to develop therapeutics against it, and an effective vaccine to control the pandemic. Since then, different vaccines technologies were applied, such as live attenuated, vector, or virus-like particles [3,4]. In December of 2020, some countries already had started approving specific vaccines, but the great majority of approval and distribution happened in January of 2021 [5]. However, there is still a lack in the development of an efficient antiviral treatment against this virus, especially with the continued emergence of new variants [6]. Given the swift changes observed in SARS-CoV-2 and the potential ineffectiveness of certain vaccines against it, there is a critical need to investigate compounds or molecules that hold promise in combating this virus.

Peptides are characterized as small molecules composed by amino acids sequence, and they can have different chemistry specific features, regarding their net charge, structure, dimensions, complexity, and hydrophobic properties [7]. They can be isolated from different organisms and synthetically modified by solid phase peptide synthesis [8]. Peptides have been showing great potential and perspectives in the antiviral field [9], as we can find several studies in the literature that already showed the inhibitory effect of these compounds against viruses [10–13], as West Nile virus [14,15], Zika virus [16,17], Dengue [18–20], and HCV [21]. Including as example, different peptides with promising effects against Influenza viruses [22] and against infection caused by herpes simplex, using natural or synthetic peptides [23].Peptides have proven effective in targeting specific targets of coronaviruses. For example, peptides can impede the binding, replication, fusion, and entry steps, and they may also engage with crucial viral enzymes necessary for the virus's replication or the viral particle itself. [24]. Even more specific, antiviral peptides also have been studied against SARS-CoV-2 [25]. Studies associated the potential of these molecules to target essential proteins for viral replication or interrupting virus infection by targeting envelope or spike proteins, preventing the virus to bind on the host cells [25,26]. Interestingly, most antiviral peptides act in different

steps of virus replication, showing a broad spectrum of targets. These characteristics highlight the potential of peptides as antivirals and the importance of the studies using these molecules.

Bothropstoxin-I is a myotoxin from the *Bothrops jararacussu* snake, and it has showed antimicrobial activity in previous studies [27–31]. Derived from it, a new peptide was developed (p-BthTX-I)$_2$K (KKYRYHLKPF)$_2$K), characterised as a dimeric peptide. Previous studies from our group showed that this peptide has antiviral activity, since it was able to inhibit Chikungunya and Zika virus [32]. Based on this, other peptides have been developed to evaluate the aspects of dimerization of this peptide and their antiviral activity. For that, a monomeric peptide was elaborated, EMC2109 (KKYRYHLKPF), and this sequence was synthesized residue by residue, unlike the other dimers whose chains were obtained together, and replacing the Lys that allowed dimerization with alanine, to eliminate the positive charge that does not exist in the dimer. This sequence would allow us to obtain information on whetherdimerization by Lys is important for antiviral and enzymatic activity. Another linear peptide was developed, MR2024 (KKYRYHLKPFAFPKLHYRYKK), aiming to better elucidate antiviral activity being related to peptide dimensions. On the other hand, MR1903 ([KKYRYHLKPFCKK]2), the dimeric peptide, and the tetrameric peptide PE1940 (KKYRYHLKPF)4 (K)2K, representing non-linear structures. In this way, we could compare the different activities resulting from treatment using distinct structure of peptide. So, the present work aimed to identify and investigate the potential of peptides derived from Bothropstoxin-I as inhibitors against different strains of SARS-CoV-2.

2. Material and Methods

2.1. Peptides

Peptides were synthesized manually through solid-phase peptide synthesis (SPPS) following the methodology outlined by Merrifield in 1963 and Bittencourt in 2023 [33,34] . Basically, to obtain dimers and tetramers, Fmoc-Lys (Fmoc)-OH amino acid was employed at the initiation of synthesis as a branching point, allowing peptide chains to grow from the α-amino and ε-amino groups of lysine, as detailed by Lorenzón et al., 2012, and Santos-Filho et al., 2021 [35,36]. Post-synthesis, the peptides underwent purification via semi-preparative HPLC using a Shimadzu chromatograph (Tokyo, Japan) equipped with a C_{18} Jupiter column measuring 25 × 1 cm and featuring a particle size of 10 µm. The purity of the obtained materials (<95%) was assessed through analytical HPLC on a Shimadzu chromatograph (Kyoto, Japan) with a reverse-phase C_{18} column measuring 0.46 × 15 cm and a particle size of 5 µm (Agilent, Santa Clara, CA, USA). Verification that the desired materials were acquired involved mass

spectrometry, employing an Ion Trap MS mass spectrometer (Bruker) in direct injection and positive detection mode.

2.2. Cells

Vero (ATCC CCL-81), BHK-21 (ATCC C-13), and A549 (ATCC CCL-185) cell lines were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS, Gibco – Thermo Fisher Scientific, Waltham, MA, USA) and 1% Penicillin/Streptomycin (5,000 U/mL) (P/S, Cultilab, Campinas, SP, Brazil). A549-ACE2-TMPRSS2 isolated from pulmonary carcinoma, was transduced to express human ACE2, selected under hygromycin B, further transduced to express human TMPRSS2 under selection of geneticin. This cell line was gently donated by Prof. Arvind Patel from the NIBSC Research Reagent Repositor (United Kingdom). During the culture of A549 cells, it was also added 2mg/ml Geneticin (G418, Sigma–Aldrich, St. Louis, MO, USA) and 200 µg/ml Hygromycin B (Thermo Fisher Scientific, Waltham, MA, USA) to DMEM.

2.3. Virus

The SARS-CoV-2 Wuhan lineage used for this work was isolated from a Brazilian patient positive sample (SARS-CoV-2/SP02.2020; EPI_ISL_450506), and it was provided from Prof. Edson Luiz Durigon. The SARS-CoV-2 variants Omicron (BA.2) (hCoV/England/FCI-179/2022) and Delta (B.1.617.2) (MS066352H) were also used in this work, both conceded from Francis Crick Institute (London, United Kingdom).

The infections cDNA (icDNA) clone of SARS-CoV-2 based on the Wuhan strain, and the recombinants with replaced spike protein from Omicron and Delta, all expressing mCherry reporter [37] were also used for our analysis. To rescue these plasmids, BHK-21 cells were seeded in 12-well plate (TPP, Trasadingen, Switzerland), and were transfected with 3 µg of plasmid using Lipofectamine 2000 (Thermo Fisger Scientific, Waltham, MA, USA) and OPTI-MEM (Gibco—Thermo Fisher Scientific, Waltham, MA, USA. After 72 hours post transfection (hpi), the supernatant was collected and used to propagate virus in 6-well plate seeded with $8x10^6$ A549 cells. After 96 hpi, the supernatant was collected and stocked at -80°C as the passage 0.

To determinate the virus titer, it was performed a Tissue Culture Infectious Dose (TCID50). A549 cells ($1x10^4$) were seeded in 96-well plates (TPP, Trasadingen, Switzerland). Twenty-four hours later, virus dilution was performed, and cells were infected. Seventy-two hours after, cytopathic effect was quantified to determine viral titer, which was calculated using TCID50 calculator (Spearman–Kärber Method) [38].

2.4. Evaluation of the Cytotoxicity Profile of the Peptides

The cytotoxicity of the peptides in BHK-21, Vero, and A549 cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) protocol as described by [39]. $1x10^4$ cells per well were seeded in 96-well culture plates (TPP, Trasadingen, Switzerland). After 24 h, the cells were incubated with different ranges of concentrations of the peptides (between 0.3, 0.7, 1.5, 3.1, 6.2, 12.5, 25, 50, 100 µM) for 24 h. Then, the medium containing the peptides was removed, and 100 µL of MTT (Sigma-Aldrich, St. Louis, MO, USA) diluted in DMEM (Cultilab, Campinas, SP, Brazil) (1 mg/mL) was added to each well of the plate (1 mg/mL). After 30 min of incubation at 37 °C, the medium containing MTT (Sigma-Aldrich, St. Louis, MO, USA) was removed, and 100 µL of dimethylsulfoxide (DMSO, Synth, Diadema, SP, Brazil) was added to the cells. The plate was agitated at 200 rpm. After 5 min, the absorbance was measured at a wavelength of 572 nm on a plate reader (FLUOstar Omega/BMG LABTECH, Ortenberg, Germany). The CC_{50} values for A549 cells were calculated using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

2.5. Anti-SARS-CoV-2 primary screening of the peptides

For this experiment, $1x10^5$ Vero cells were seeded in 24-wells plate (TPP, Trasadingen, Switzerland), twenty-four hours before the experiment started. After this period, a solution containing SARS-CoV-2 at MOI 0.01 and each one of the peptides was prepared and added in duplicate to the wells. After 24 hours of treatment incubation, the supernatant was collected and frozen for further analysis using Median Tissue Culture Infectious Dose (TCID50) or qPCR real time technique to quantify virus replication.

2.5.1. Initial analysis of peptides potential on SARS-CoV-2 replication cycle

For this experiments, twenty-four hours before starting the experiment, $1x10^5$ Vero cells were seed in 24 wells plate. After this period, three experiments were performed. **(A)** Virucidal: Peptides and SARS-CoV-2 at MOI 0.01 were incubated for one hour at 37°C. After that, this solution was used to infect cells for one hour. Next, cells were washed twice with PBS, and fresh supplemented medium (2% FBS and 1% P/S) was added to cells. **(B)** Entry: A solution containing SARS-CoV-2 at MOI 0.01 and each peptide was added to cells and incubated for one hour. After that, cells were washed twice with PBS, and fresh supplemented medium (2% FBS and 1% P/S) was added to wells. **(C)** Post-entry: First, an infection of SARS-CoV-2 at MOI 0.01 was performed in seeded cells for one hour. After that, cells were washed twice with PBS solution, and fresh medium containing the treatment with each peptide was added to the

cells and incubated for 24 hours. After the incubation time for all described experiments, with supernatant resulting from each assay was performed RNA extraction using Trizol (Invitrogen, Waltham, MA, USA) reagent, and then cDNA was synthetized using High-Capacity kit (Applied Biosystems, Waltham, MA, USA), following instructions from handbook. After that, qPCR was performed to measure virus RNA in each experiment.

2.5.2. Analysis by real time qPCR

TaqMan mastermix kit was used to perform the experiments using qPCR analysis. The reaction was composed of 2.75 μL of ultrapure DEPEC water, primers (0.96 μM of each), and probe (0.48 μM). Viral nucleocapsid (N1) gene specify was used for this reaction: 2019-nCoV_N1-F (5'GAC CCC AAA ATC AGC GAA AT-3'), 2019-nCoV_N1-R (5'-TCT GGT TAC TGC CAG TTG AAT CTG-3') e 2019-nCoV_N1-P (5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3') (Integrated DNA Technologies, California, EUA) (CDC, 2020), e 5 μL de 2X TaqMan™ Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA), and 1,5 μL of cDNA. The reaction was submitted to the equipment QuantStudioTM 12K Flex Real Time PCR System (Applied Biosystems, Waltham, MA, USA) programmed for specific cycling.

2.6. Antiviral activity of the peptides in human lung cells

For this section of experiments, the infectious SARS-CoV-2 cDNA clone expressing mCherry gene as a marker [37] was used, so we could quantify the results by the red fluorescence using the IncuCyte® S3 live-cell equipment (Sartorius, Gottingen, Germany).

2.6.1. Dose dependence analysis

Twenty-four hours before the experiment started, $1x10^4$ cells were seeded in 96-wells plate (TPP, Trasadingen, Switzerland). After this period, solutions containing different concentrations (0.1 μM to 100 μM) of peptides were prepared and incubated in the cells with SARS-CoV-2 mCherry infectious clone at MOI 0.1 for 24 hours. After that, fluorescence levels were measured to quantify the inhibition in dose dependence using the IncuCyte® S3 live-cell equipment (Sartorius, Gottingen, Germany).

The software GraphPad was used to calculate 50% cytotoxic concentrations (CC_{50}) and Half-maximal inhibitory concentrations (IC_{50}). The selective index was calculated using the CC_{50}/IC_{50} relation.

2.6.2. Analysis of peptides action on entry step

Twenty-four hours before the experiment, $1x10^4$ cells were seeded in 96-wells plate (TPP, Trasadingen, Switzerland). In the next day, a solution containing SARS-CoV-2 at MOI 0.1 and each one of the peptides was added to cells and incubated for one hour. After that, cells were washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at 12 and 24 hpi using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.6.3. Analysis of peptide on the SARS-CoV-2 attachment to the cells

Twenty-four hours before the experiment, $1x10^4$ cells were seeded in 96 wells plate. In the following day, a solution containing SARS-CoV-2 at MOI 0.1 and each peptide was added to the cells and incubated at 4°C for one hour. After that, cells were washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. This experiment protocol was based in other studies already published in the literature [40,41]. Fluorescence percentage was measured at 12 and 24 hpi using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.6.4. Analysis of peptide on the SARS-CoV-2 internalization

Twenty-four hours before the experiment, $1x10^4$ cells were seeded in 96 wells plate. In the next day, a solution containing SARS-CoV-2 at MOI 0.1 and each peptide was added to the cells, and they were incubated at 4°C for one hour. After that, cells were incubated at 37°C for more 30 minutes so we could imitate the process of virus internalization to the cells. Finally, cells were also washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at twelve- and twenty-four-hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.6.5. Virucidal effect of peptides

Twenty-four hours before the experiment, $1x10^4$ cells were seeded in 96-wells plate (TPP, Trasadingen, Switzerland). In the following day, two solutions were prepared: one with SARS-CoV-2 at MOI 2 and other with MOI 5. In each one it was added the solutions of the peptides at previous selected concentration, and it was incubated for 1 hour at 37°C. After that, the solutions containing virus and peptide were added to the cells for additional one hour. Finally, the cells were washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at twelve and twenty-four hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.6.6. Protective effect of peptides against SARS-CoV-2 infection

Twenty-four hours before the experiment, $1x10^4$ cells were seeded in 96 wells-plate (TPP, Trasadingen, Switzerland). In the next day, cells were treated with peptides for one hour. After that, cells were washed twice with PBS solution, and they were infected with SARS-CoV-2 at MOI 0.1 for another one hour. Next, cells were washed again with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at 12 and 24 hpi using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.6.7. Study of the peptides action on the post-entry of SARS-CoV-2 in cells

Twenty-four hours before the experiment, $1x10^4$ cells were seeded in 96 wells plate. In the following day, cells were infected with SARS-CoV-2 at MOI 0.1 for one hour. After that, cells were washed twice with PBS solution, and treatment containing each one of the peptides was added to the cells. Fluorescence percentage was measured at 12 and 24 hpi using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.6.8. Evaluation of activity of the peptides against SARS-CoV-2 variants: Omicron (BA2) and Delta

$1x10^5$ A549 cells were seeded in 24 wells plate twenty-four hours before the experiment. In the following day, two separated experiments were performed, in one, a solution containing SARS-CoV-2 Omicron (BA2) at MOI 0.1 was prepared with peptides, in the second one, a solution containing SARS-CoV-2 Delta at MOI 0.1 was prepared with peptide. Both treatments were added to cells, and they were incubated for twenty-four hours. After that, the supernatant resulted from this experiment was freeze. For quantification of this experiment, a TCI50 experiment was performed with the supernatant.

2.6.9. Analysis of peptides action on the replication cycle of the Omicron and Delta variants

For the analyses of the peptides' action on replication steps of Omicron and Delta infection, methodologies from the previous experiments were repeated (Entry, Virucidal, pretreatment, post-entry), but instead of working with the Wild-type infectious clone, it was performed analysis using the recombinants infectious clone with spike protein from Omicron and Delta variants. Fluorescence percentage was measured at twelve and twenty-four hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.7. Investigation of peptides action on replication step

$5x10^4$ BHK-21 cells were seeded in 48 wells plate twenty-four hours before the experiment. After, cells were transfected with 600 ng of the plasmid of SARS-CoV-2 subgenomic replicon expressing NanoLuciferase marker (Fig. S3), using Lipofectamine™ 2000 protocol. After 4 hours of transfection, media containing the treatment with peptides wasadded and incubated for 24 and 48 hours. After, luminescence was read for quantification of difference between treatments by using FLUOstar Omega Microplate Reader (FLUOstarOmega/BMG LABTECH, Ortenberg, Germany).

2.8. Evaluation of *in vitro* inhibition of SARS-CoV-2 synthetic double strand RNA by peptides

To produce SARS-CoV-2 double strand RNA, forward and reverse oligonucleotide primers with and without T7 promoter sequences (Supplementary Figure 1) were designed. Two sets of PCRs were required to generate a DNA template for transcription of sense and antisense RNA, respectively. After that, a 1% agarose gel with 1X TAE was run to confirm PCR reaction. The PCR product was cleaned using Monarch kit (New England Biolabs) and the double strand RNA was prepared subsequently. After that, RNA was cleaned using RNA clean up kit (New England Biolabs). RNA concentration was determined, and an annealing reaction was performed with sense RNA and antisense RNA, annealing buffer (10X), and nuclease-free water. Finally, to check the integrity of the double stranded RNA, a gel was run. Then, solutions were prepared at the same concentration containing the peptides (treatment), positive control (Platine), and controls (without any compound) and all incubated for 45 minutes at room temperature. After, a 1% agarose gel was run using a 100 pb ladder.

2.9. Analysis of SARS-CoV-2 resistance to treatment with the peptides

$8x10^5$ cells were seeded in 6 wells plates twenty-four hours before the experiment. After that, a solution containing SARS-CoV-2 (Wuhan Wild-type strain) at MOI 0.05 with each peptide at the same concentration selected for the other experiments was added to the cells and incubated for twenty-four hours. After that, supernatant was kept, and it was frozen. The supernatant was quantified using TCID50, and knowing the titre of the experiment, a new infection was performed at the same MOI, and adding peptide again. This cycle of infections and quantifications was done for six passages. The last passage of each treatment was compared with the original sequence of this virus.

2.10. Sequencing

140 µl of cell culture supernatant was extracted with a Viral RNA mini kit (QIAGEN) and eluted in 60 µl of kit elution buffer. cDNA was synthesised with 20 µl of RNA using RNA to cDNA EcoDry Premix (Takara Bio) and validated by Qpcr [42] 2.5µl was then used as template for tiled amplicon whole genome sequencing following the Midnight protocol [43] except with an alternative and novel set of primers (available on request).

2.11. Statical analysis

All graphs and statistical analyses were performed using GraphPad Prism 8 and were described in figures. For quantification of band density percentage, it was used ImageJ software.

3. Results

3.1. Maximum non-toxic peptides concentrations selection for antiviral assays using Vero, BHK-21, and A549 cell lines

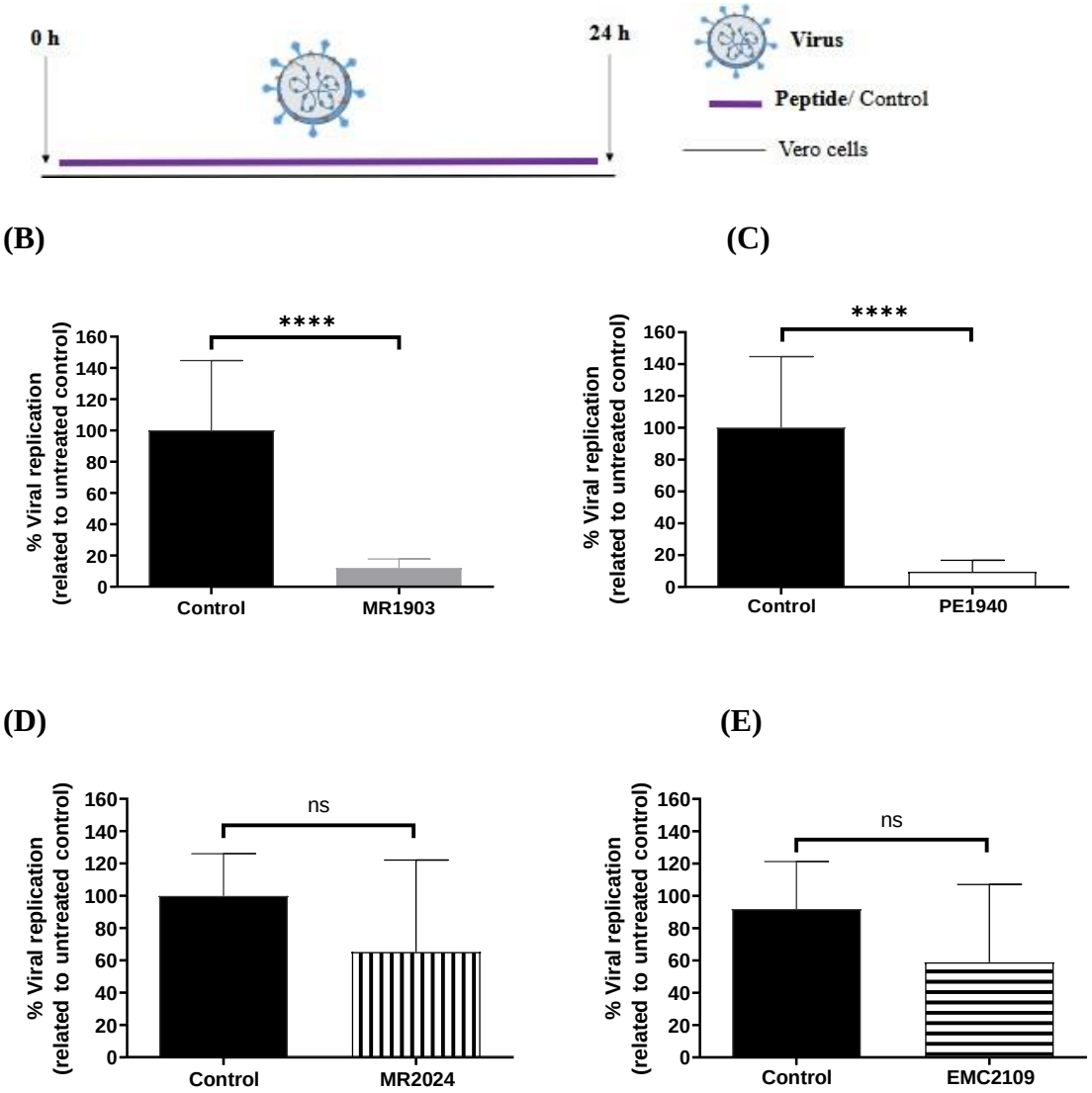
For all tested cell lines, cytotoxic experiments showed that peptides MR1903, MR2024, EMC2109, and PE1940, had up to 80% cell viability in the concentration of 100 µM for the first three peptides (Fig. S2A, C, and D), and 6.25 µM for the last one (Fig. S2B). These concentrations were selected to perform the antiviral experiments, respectively.

3.2. Screening shows that dimerization is related to anti-SARS-CoV-2 action

The first screening with the four peptides (MR1903, PE1940, EMC2109, and MR2024) showed that only the peptides MR1903 and PE1940 presented significant inhibition of 87.9% (p<0.0001) and 69.6% (p<0.0001), respectively in the replicative cycle of wild type of SARS-CoV-2 (Figure 1) in Vero cells. The peptides EMC2109 and MR2024 were excluded of the next antiviral assays.

Figure 1. Results of the inhibition resulted from an initial antiviral screening for different dimerization patterns with the peptides: (A) Scheme describing experiment protocol. (B) MR1903, (C) PE1940, (D) MR2024, and (E) EMC2109 are showed below. Results were expressed in percentage of cells treated related the control (sterile water). Experiments were carried out in three independent events, each performed in duplicate, and data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. ****: p<0.0001; ns: non-significant

(A)



3.2.1. Peptides have potential effect on SARS-CoV-2 replication cycle

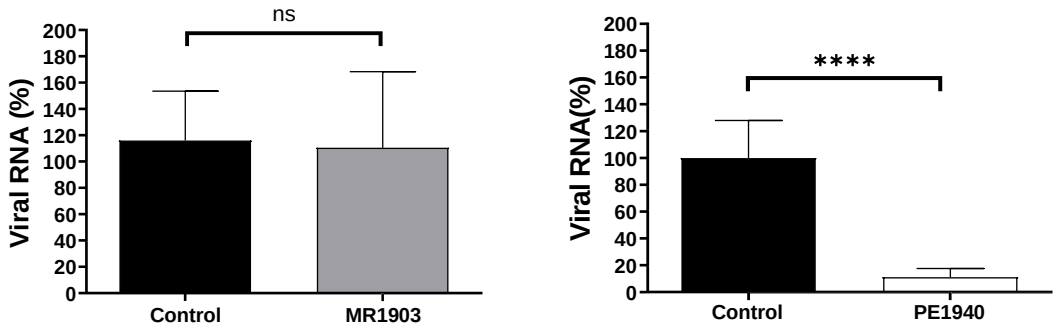
The compounds selected with activity against SARS-CoV-2 on the previous experiment were taken for two time-of-addition assays using Vero cells. For the virucidal experiment, it was observed that PE1940 presented a significant virucidal effect of around 89% of inhibition (p<0,0001). For the entry assay, it was observed that both MR1903 and PE1940 presented considerable inhibition potential, showing values of 59.8% (Fig. 2BI), and 88% (Fig 2.BII), respectively. In the post-entry assay, we also observed that both peptides showed significant activity, with an inhibition of 37.7% from MR1903 (p=0.0196) (Fig 2.BI) and 97.8% from PE1940 (p<0.0001) (Fig C.BII), which showed that PE1940 has a promisor antiviral activity in all antiviral assays.

Figure 2. Results of antiviral initial assay showed peptides MR1903 and PE1940 with inhibition against SARS-CoV-2. **(A)** Virucidal for MR1903 and PE1940, **(B)** Entry step assay

for MR1903 and PE1940, and **(C)** Post-entry assay for MR1903 and PE1940 using Vero cells. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in duplicate, and data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. *: $p<0.05$; ****: $p<0.0001$; ns: non-significant.

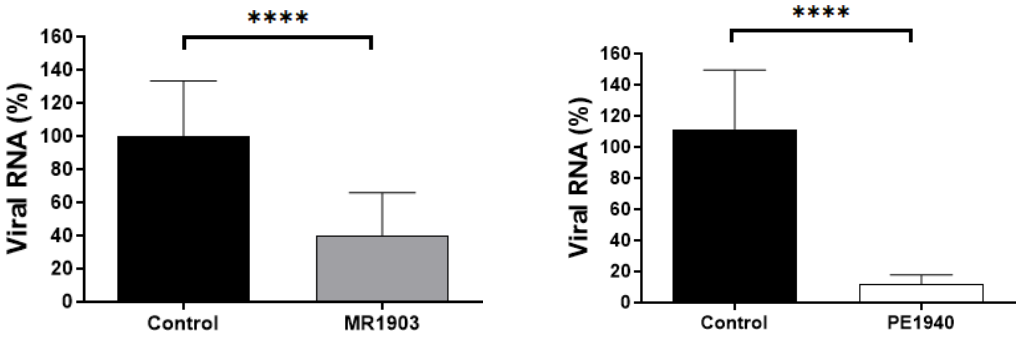
(A) Virucidal

(I) **(II)**



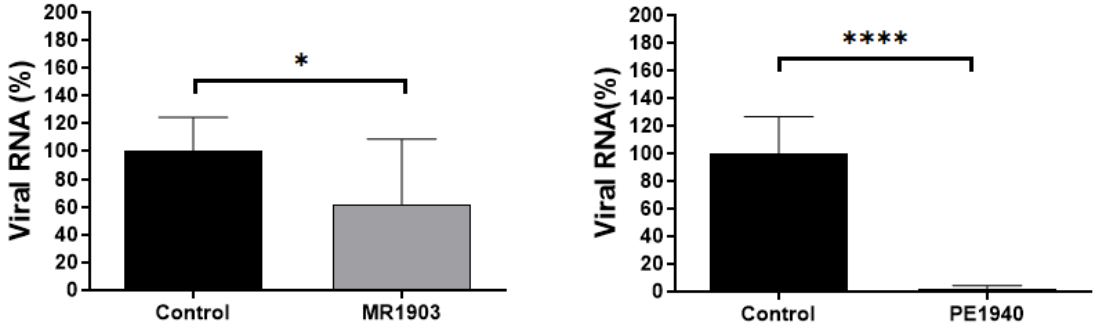
(B) Entry effect

(I) **(II)**



(C) Post-entry effects

(I) **(II)**



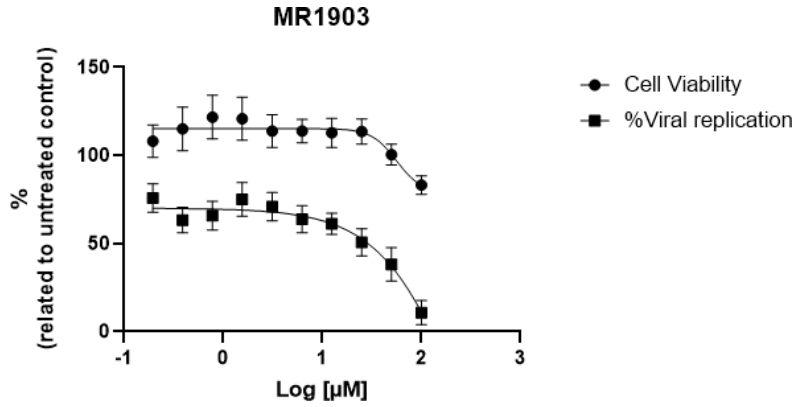
3.3. Peptides show potential antiviral effect on several steps of replication cycle in lung human cells infected with SARS-CoV-2

3.3.1. Dimeric and tetrameric peptides have dose dependence effect against SARS-CoV-2

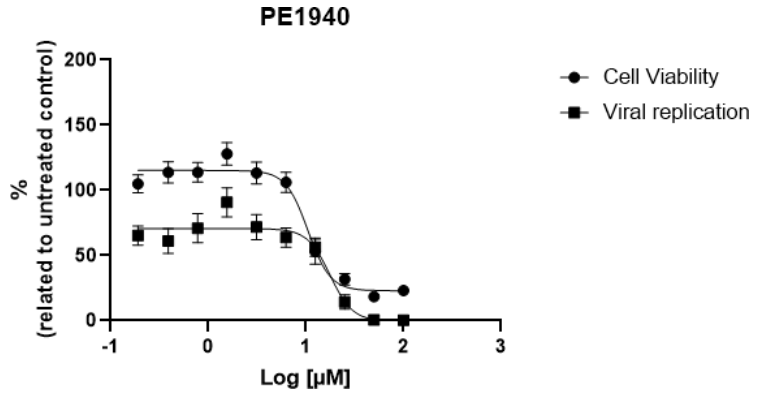
The peptides MR1903 and PE1940 were taken for further investigation of antiviral activity using A549 cells. As we can see in the following graphs, MR1903 showed a dose dependence antiviral activity, and this peptide has proven to have low levels of toxicity (Fig.3A). Meanwhile, the peptide PE1940 has dose dependence for both cell viability and antiviral effect, with a major toxicity resulted from treatment with peptide PE1940 (Fig.3B). Resulting from that, MR1903 has a higher selective index value (7.94) (Fig. 3C).

Figure 3. Analysis of cell viability and the dose dependence were performed using A549 cells for the peptides **(A)** MR1903 **(B)** PE1940. Cell viability analysis was performed using absorbance reading, and dose dependence analysis by percentage of fluorescence measure. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate. **(C)** Values of CC50, IC50, and selective index, calculated using software GraphPad 8.

(A)



(B)



(C)

Peptide	CC50 (µM)	IC50 (µM)	Selective Index (SI)
MR1903	108.5	13.83	7.84
PE1940	17.65	5.952	2.97

3.3.2. Tetrameric peptide shows effect on entry step

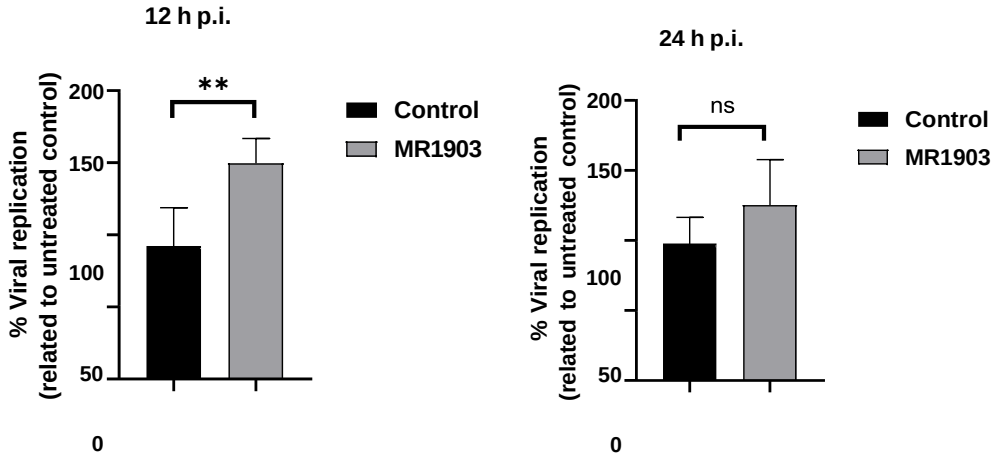
Continuing our further investigation of these peptides' effects, we performed the entry inhibition assay in A549 cells. The peptide MR1903 did not present inhibitory effect in this step of the viral replication (Fig. 4B). However, PE1940 showed action for 12 and 24 hours post infection analysis, with 47.9% (p<0,0001) decrease in related to control for 12h, and 33.6% (p=0,0017) for 24h (Fig. 4C).

Figure 4. The entry step analysis of SARS-CoV-2 replication cycle. **(A)** This experiment was performed by doing an infection with a solution containing the virus and the peptide/water(control) for one hour, and then the cells were washed two times with PBS solution, and media was added so the analysis could be performed between 12 to 24 hours with

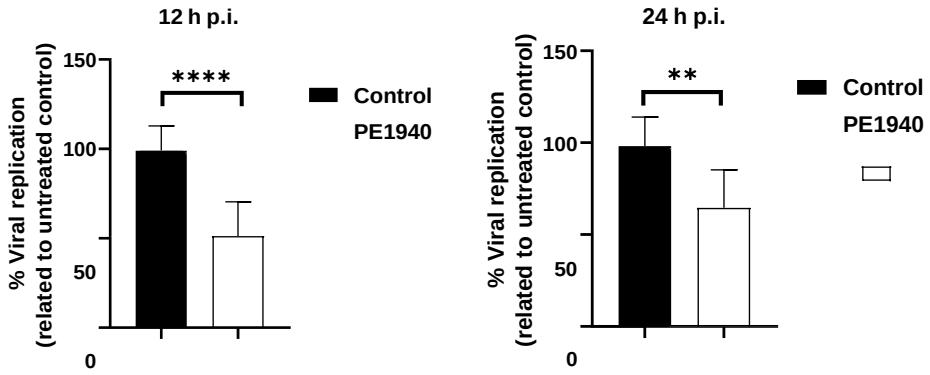
intervals of 4 hours. Percentage of inhibition for both **(B)** MR1903 results for 12 and 24 hours post infection with SARS-CoV-2 WT mCherry infectious clone. **(C)** PE1940 results for 12 and 24 hours after post infection with SARS-CoV-2 WT mCherry infectious clone. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. ns: non-significant; **: $p<0.001$; ****: $p<0.0001$.

(A)

(B)



(C)



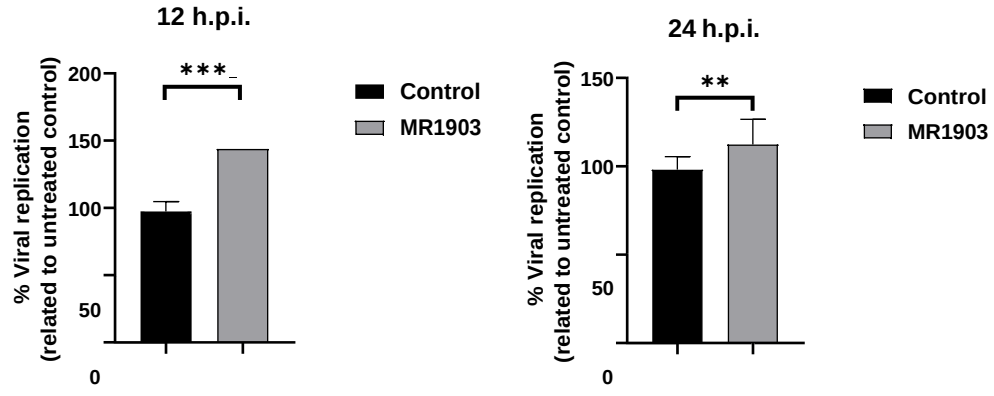
3.3.3. Peptides do not show potential in virus particle incubation prior infection

Virucidal effect caused by the peptides was tested at MOI 5 of SARS-CoV-2. Both peptides did not show any effect against SARS-CoV-2 virus particle, and it even produced the opposite effect expected for the peptide MR1903 (Fig. 5B). PE1940 did not show any significant difference (Fig. 5C).

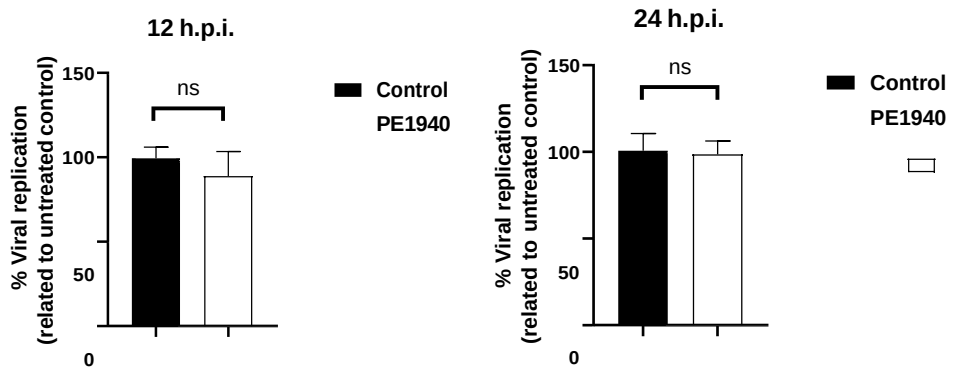
Figure 5. The virucidal effect against SARS-CoV-2. **(A)** This experiment was performed by doing an infection with a solution containing the virus MOI 2 and 5 and the peptide/water(control) for one hour after incubating this solution for prior 1 hour at 37°C. Then, the cells were washed two times with PBS solution, and media was added so the analysis could be performed between 12 to 24 hours with intervals of 4 hours. Percentage of inhibition for **(B)** MR1903 MOI 5 after 12 and 24 hours. **(C)** PE19040 MOI 5 after 12 and 24 hours, post infection with SARS-CoV-2 WT mCherry infectious clone. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standarddeviation. Statistical Paired t test was performed to check significance of data. ns: non- significant *: p<0.05; **: p<0.001; ***: p<0.0001

(A)

(B)



(C)

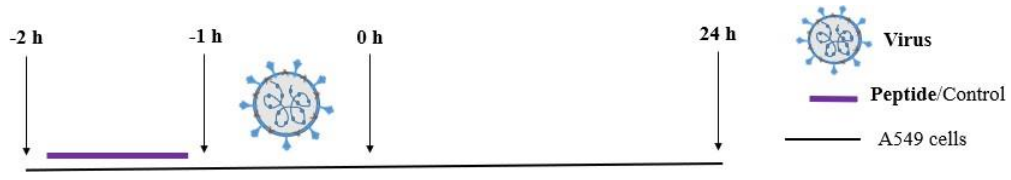


3.3.4. MR1903 causes protective effect on A549 cells

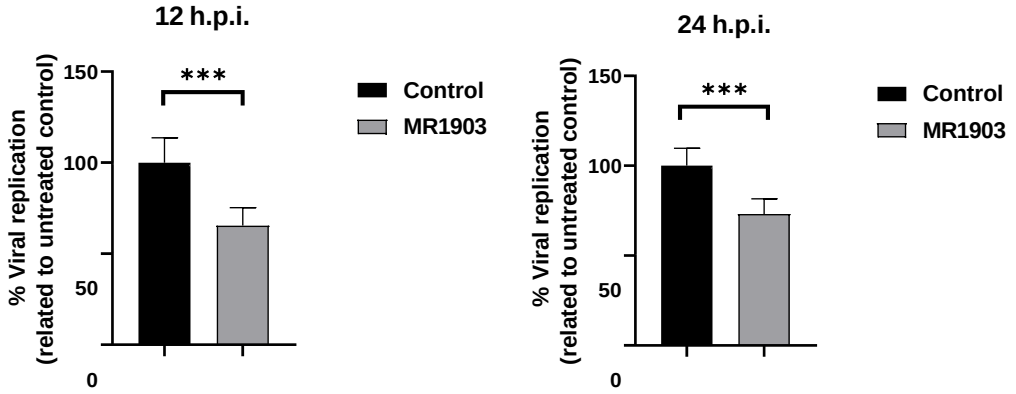
We also evaluated a possible protective effect instigated by the peptides on A549 cells. The results showed that the peptide MR1903 has a significant effect for both times post infection with SARS-CoV-2 tested, with inhibition 34.6% (p=0.0002) at 12 hours and of about 27% (p=0.0001) at 24 hours (Fig. 6B). However, PE1940 behaved completely differently and did not present any inhibitory effect (Fig. 6C).

Figure 6. Protective effect of the peptides against SARS-CoV-2 infection. **(A)** This experiment was performed by doing an initial treatment with a solution containing the peptide/water(control) for 1 hour, after that an infection was performed for another hour. Then, the cells were washed two times with PBS solution, and media was added. Percentage of inhibition for both **(B)** MR1903 and **(C)** PE1940, after 12 and 24 hours post infection with SARS-CoV-2 WT mCherry infectious clone. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. *: p<0.05; ***: p<0.0001

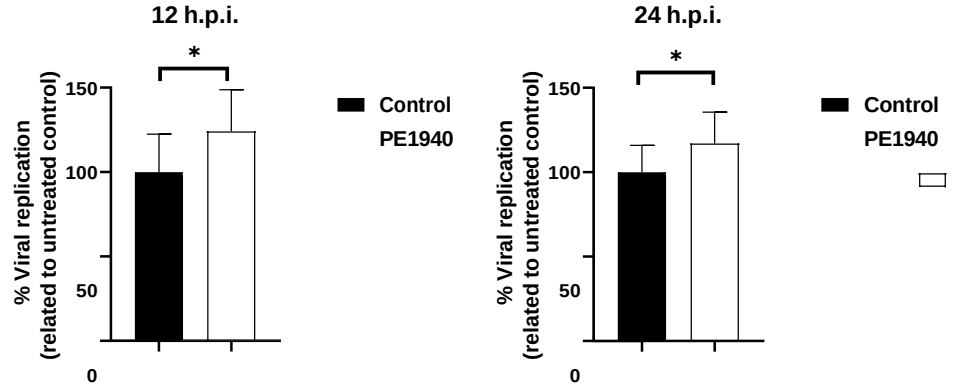
(A)



(B)



(C)



3.3.5. Further investigation of PE1940 in the entry step of the SARS-CoV-2 replication cycle

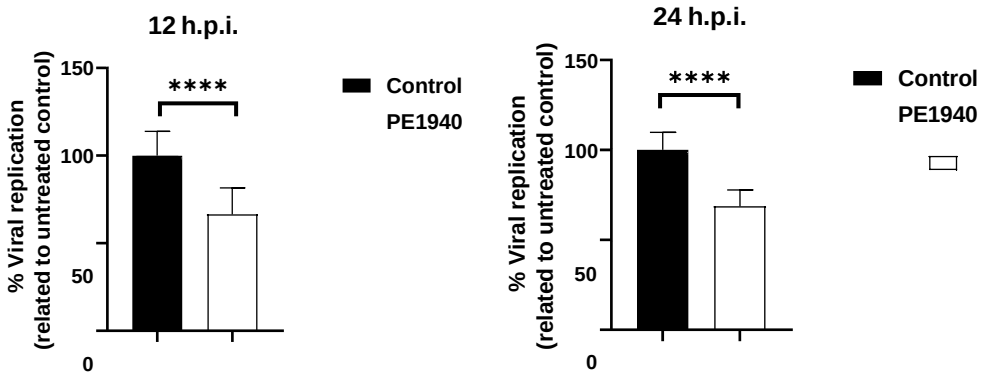
Since PE1940 was the only one that showed potential action for entry step of viral replication, it was taken for additional experiments to better understand this peptide action in this step. The first experiment performed was an attachment assay. PE1940 showed aninhibition of 33.6% (p <0.0001) in viral replication 12 hpi and 31.4% (p <0.0001) for 24 hourspost infection, so we can see only a slightly difference between both times post infection treatments (Fig. 7B). For the second experiment, we analysed the internalization step, and it ispossible to notice, PE1940 decreased in 30% (p= 0.0005) viral replication at 12 hours post infection, and only 18% (p= 0.0045) at 24 hours post infection, so its potential in this step decreased over the times tested (Fig. 7D).

Figure 7. Effects of the peptide PE1940 on SARS-CoV-2 attachment and internalization on A549 cells. **(A)** Attachment experiment was performed by doing an infection with a solution containing the virus and the peptide/water(control) for one hour at 4°C, and then the cells were washed two times with PBS solution, and fresh media was added. Results this experiment **(B)** PE1940 is demonstrated after 12 and 24 hours post infection with SARS-CoV-2 WT mCherry infectious clone. **(C)** for the internalization analysis, the cells were incubated for 0.5 hour at

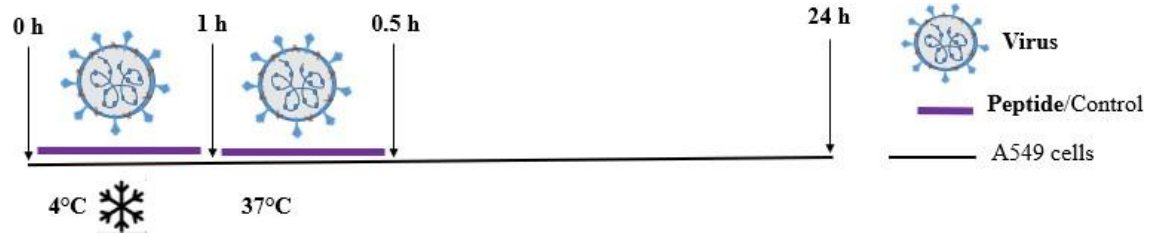
37°C prior wash and media addition for the cells. Percentage of inhibition for **(D)** PE1940 is demonstrated after 12 and 24 hours post infection with SARS-CoV-2 WT mCherry infectious clone. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. **: $p<0.001$; ***: $p<0.0001$; ****: $p<0.00001$

(A)

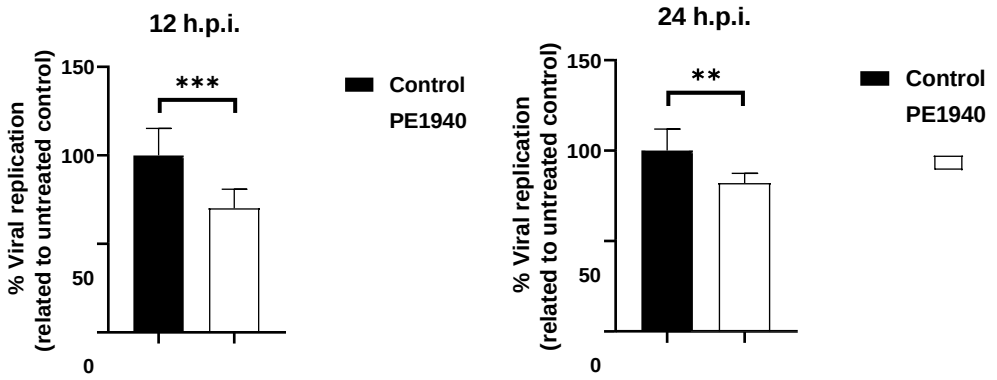
(B)



(C)



(D)

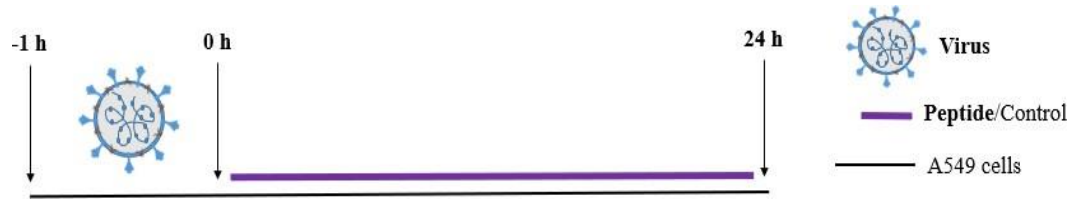


3.3.6. Peptides have high antiviral potential on post-entry step

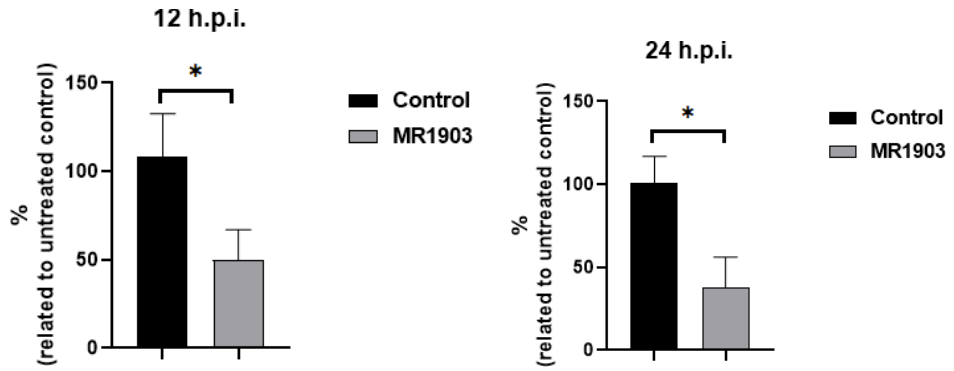
MR1903 showed an inhibition of 58.2% (p= 0.0110) at 12 h.p.i. and 63.5% (p= 0.0136) at 24 hpi compared to the control (Fig. 8B). On the other hand, we noticed that PE1940 inhibited 74.2% (p<0.0001) at 12 h and 92.4% (p<0.0001), at 24 h post infection, so it has a major difference of inhibition over time, and it has the highest inhibitory effect when compared to MR1903 for this specific step.

Figure 8. Effect of the peptides on the post-entry steps of SARS-CoV-2 infection. **(A)** For this assay, cells were infected with SARS-CoV-2 WT mCherry infectious clone for one hour, after that, they were washed twice with PBS, and then, a solution containing peptide was added to cells. **(B)** Results for MR1903 after 12 and 24 hours. **(C)** Results for PE1940 after 12 and 24, post infection with SARS-CoV-2 WT mCherry infectious clone. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. Statistical Paired t test was performed to check significance of data. *: p<0.05; ****: p<0.00001

(A)



(B)



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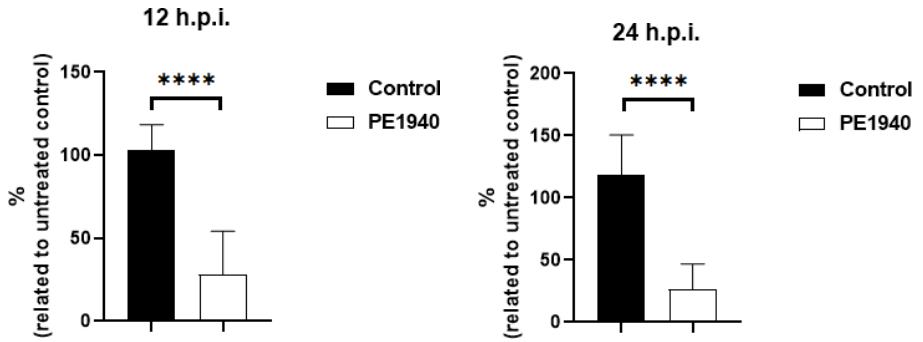


As both peptides showed effect for post-entry steps of the SARS-CoV-2 replication cycle, they were taken for study of action on replication of SARS-CoV-2, using a subgenomic replicon of this virus, containing the non-structural proteins. MR1903 showed the highest effect at 24 h time of treatment post transfection, with 32.6% (*: $p<0.05$) of inhibition at 50 µM and 77.6% ($p<0.05$) at 100 µM (Fig. S4A). The same happened with PE1940, it only showed inhibition effects for 6.2 µM, with about 43% ($p<0.001$) for also 24 h of treatment (Fig S4B).

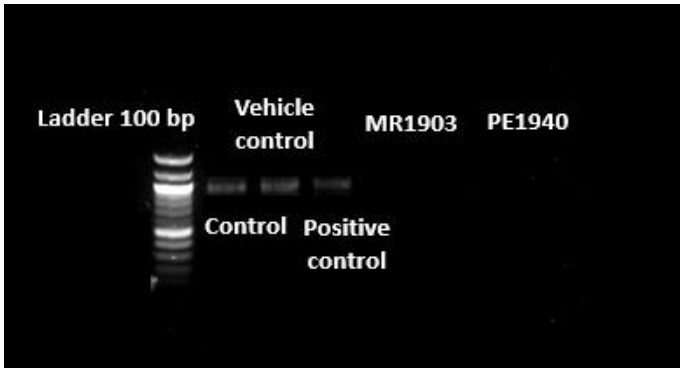
3.3.7. Peptides can inhibit double strand RNA in vitro

A double strand RNA (dsRNA) of SARS-CoV-2 was produced to better understand the post entry effects of the peptides MR1903 and PE1940 on the replication cycle of this virus. Quantification of band density, after treating this dsRNA with MR1903 and PE1940, showed an inhibitory effect of about 100% for both (MR1903 ($p<0.001$), PE1940 ($p<0.001$)), which is also about two times higher than the effect of the positive control used.

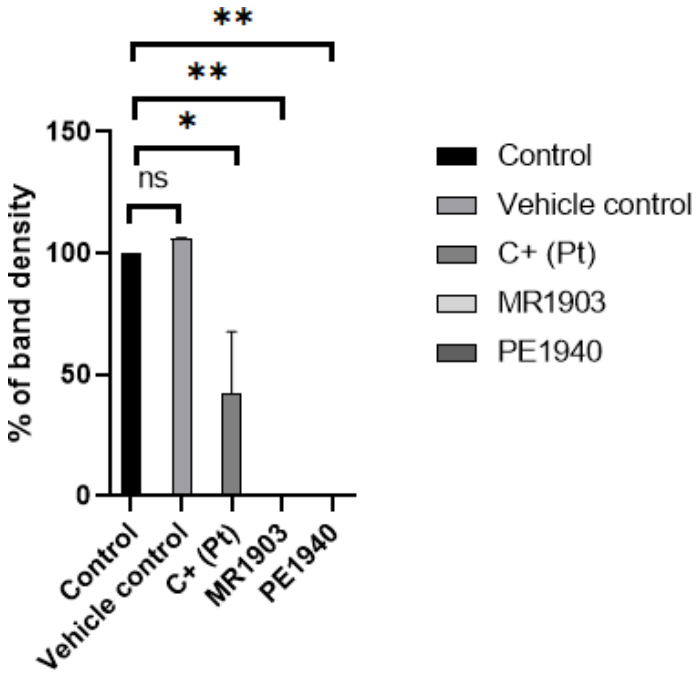
Figure 9. Double strand RNA in vitro analysis was performed by incubating the peptides with synthetised dsRNA as described in our methodology section. (A) 1% Agarose gel showing results of full inhibition with both peptides (MR1903 and PE1940) incubations. (B) Quantification of bands density expressed by percentage of cells treated related to the control.

Comparisons between control and MR1903 and control and PE1940 treatment. Platinum was used and represented as the positive control for this experiment (C+ (Pt)). Control represents the sample without any treatment under the same conditions. Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. Dunnett's multiple comparisons test was performed. *: $p<0.05$; **: $p<0.001$

(A)



(B)

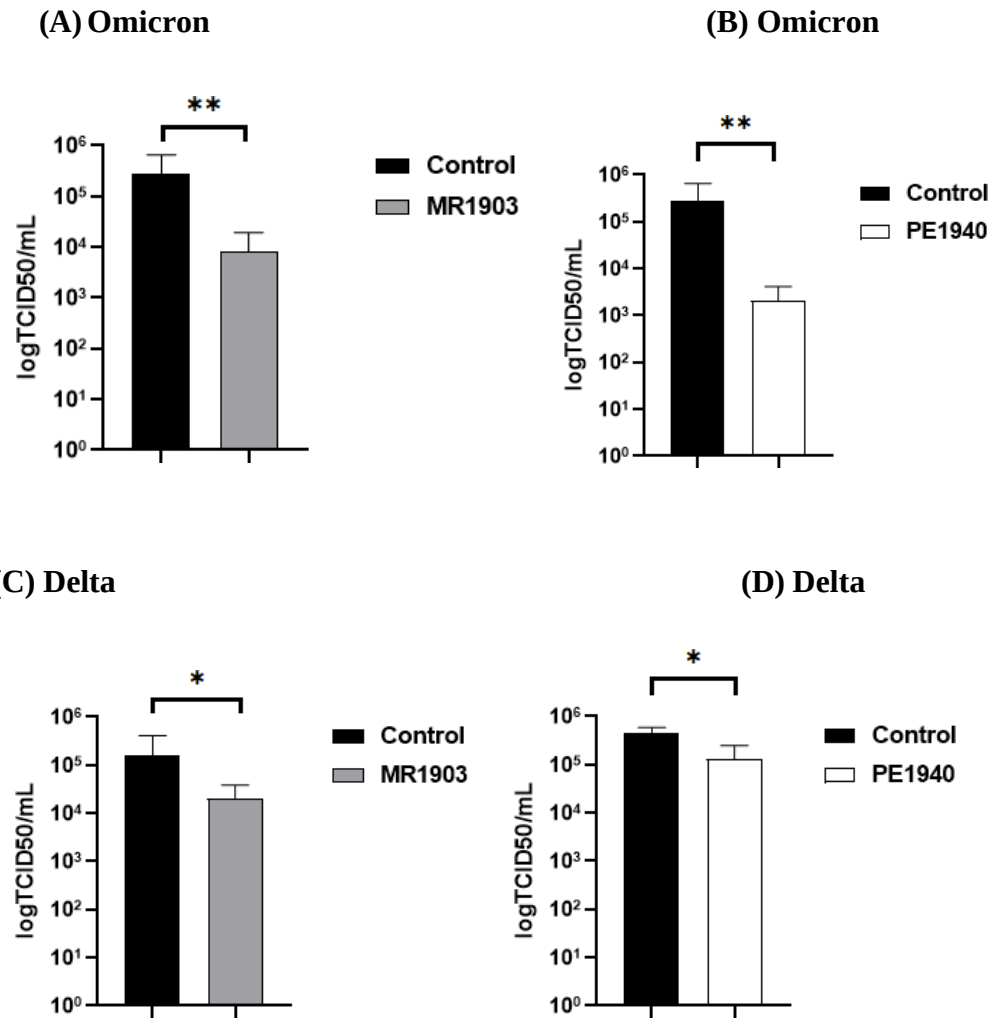


3.3.8. Peptides can also inhibit the infection with the variants omicron (BA2) and delta

We compared our results of the Wild-type strain of SARS-CoV-2 with the variants Omicron (BA2) and Delta of this virus, and as we can see bellow, both peptides revealed effect against these variants.

M1903 showed inhibition of about 97% (p= 0.0065) of the SARS-CoV-2 Omicron variant, and for PE1940 was of almost 99% of the same variant (p= 0.0022) (Fig. 11A). Regarding the delta variant, it was possible to notice that MR1903 inhibited 87.8% (p= 0.0325) of it, and PE1940 was responsible for an inhibition of 70.4% of this variant (p= 0.0238) (Fig. 11B).

Figure 10. General antiviral assay using A549 cells infected with Omicron (BA2) and Delta variants of SARS-CoV-2. **(A)** TCID50 results from treatment containing peptide MR1903 and cells infected with Omicron (BA2). **(B)** TCID50 results from treatment containing peptide PE1940 and cells infected with Omicron (BA2). **(C)** TCID50 results from treatment containing peptide MR1903 and cells infected with Delta. **(D)** TCID50 results from treatment containing peptide PE1940 and cells infected with it. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in duplicate, and data are presented as the mean ± standard deviation. Mann Whitney test was performed for this statistical analyse. *: p<0.05; **: p<0.001

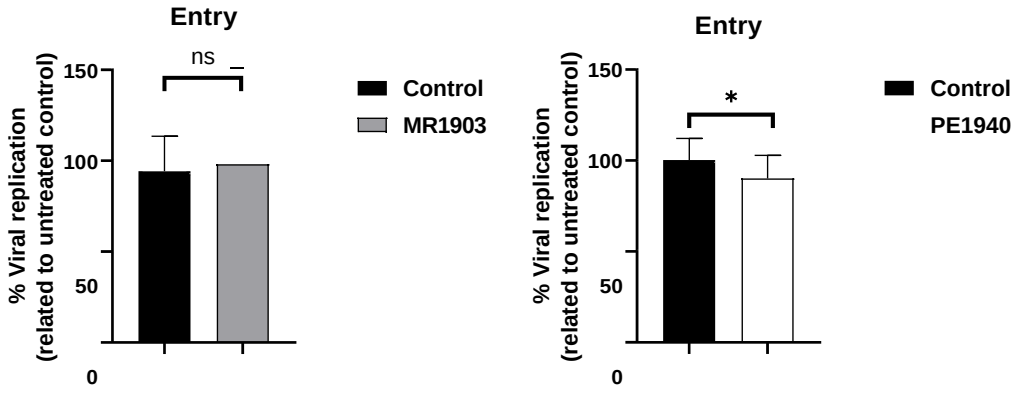


3.3.9. Peptides effects on steps of Omicron variant replication cycle

A study of inhibition on steps of viral replication, using replaced virus with S-protein from Omicron variant, showed that MR1903 presents post-entry inhibitory effect of about 17.3% (p=0.0235) (Fig 12D). However, PE1940 has effects on entry and post-entry steps. The entry action is related with about 10% (p=0.0136) inhibition, but mostly of its potential comes the post-entry which counts for almost 92% (p<0.0001) (Fig. 12 A and D).

Figura 11. Analysis of time-addition experiments with A549 cells infected with a recombinant infection clone with replaced S-protein from Omicron. **(A)** Results for entry step inhibition assay of MR1903 and PE1940 peptides **(B)** Results for protective analysis of MR1903 and PE1940 peptides. **(C)** Results of virucidal assay for MR190 and PE1940 peptides. **(D)** Results for post-entry analysis for MR1903 and PE1940 peptides. All experiments were performed using the same methodology described previously. Quantification analyses were performed by percentage of fluorescence measure at 24 hours after treatment. Results were expressed in percentage of cells treated related to the control (sterile water). Statistical Paired t test was performed to check significance of data. *: p<0.05; ***: p<0.0001

(A)



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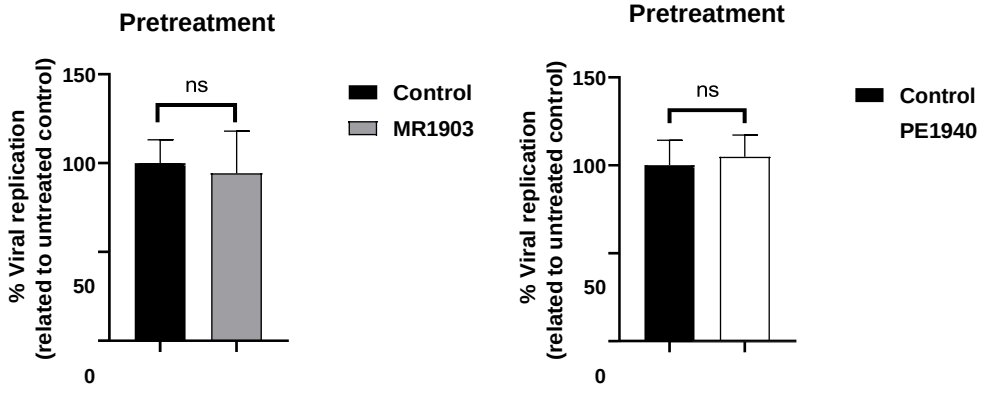

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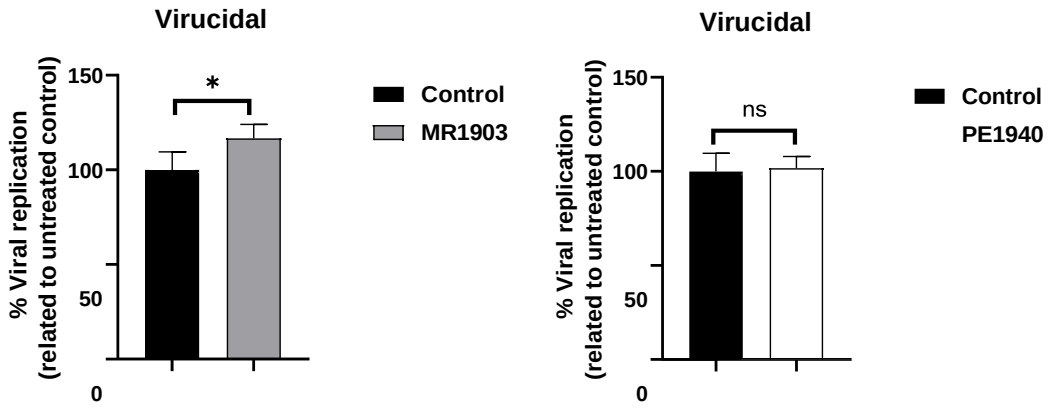

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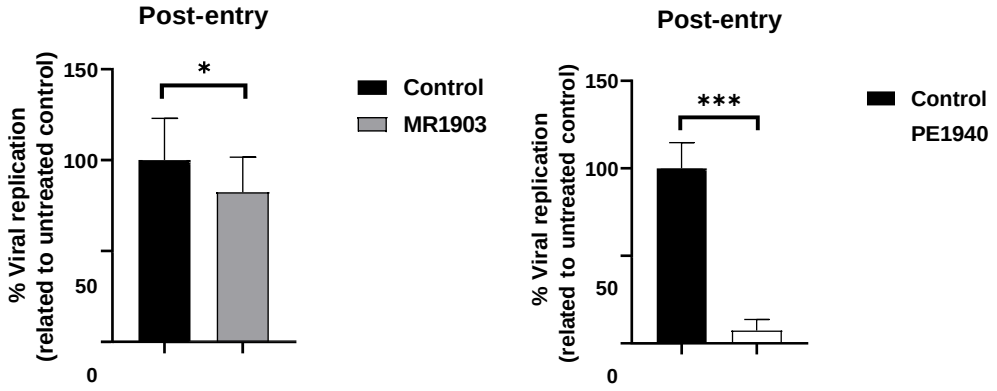


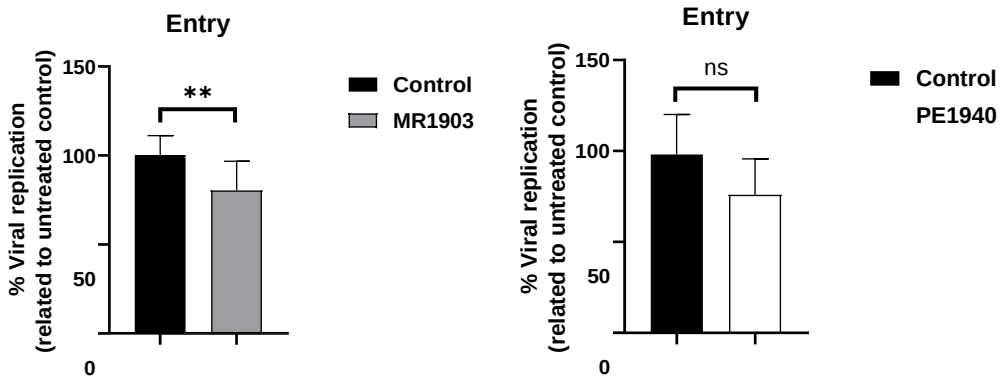
3.3.10. Peptides effects on steps of delta variant replication cycle

A study on the steps of viral replication, using replaced S-protein from Delta, was also performed, and results showed us that MR1903 has only effect for entry step for this variant, with an inhibitory action of almost 20% (p= 0.0061) compared to control cells, and PE1940 showed effects on pretreatment and post-entry analysis, inhibiting about 22% (p=0.0200) and 94% (p<0.0001), respectively. Like the previous experiment with Omicron variant, PE1940 had action in more than one step of the replication cycle.

Figure 12. Analysis of time-addition experiments with A549 cells infected with a recombinant infection clone with replaced S-protein from Delta. **(A)** Results for entry step inhibition assay of MR1903 and PE1940 peptides **(B)** Results for protective analysis of MR1903 and PE1940 peptides. **(C)** Results of virucidal assay for MR1903 and PE1940 peptides. **(D)** Results for post-entry analysis for MR1903 and PE1940 peptides. All experiments were performed using the same methodology described previously. Quantification analyses were performed by

percentage of fluorescence measure at 24 hours after treatment. Results were expressed in percentage of cells treated related to the control (sterile water). *: p<0.05; ***: p<0.0001

(A)



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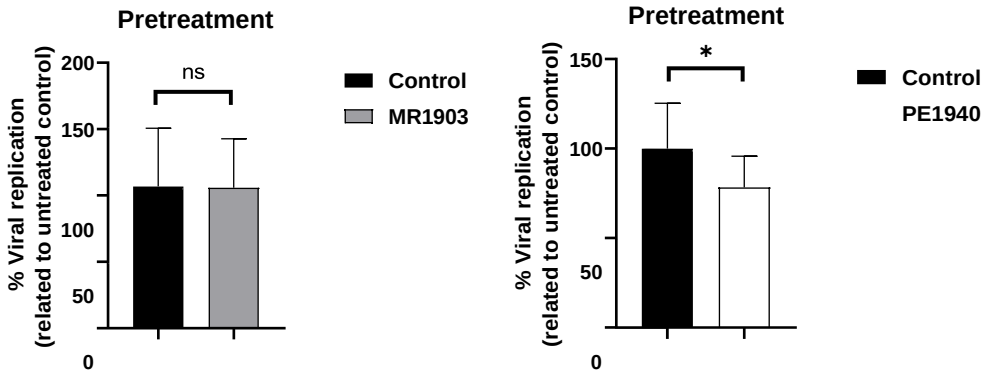
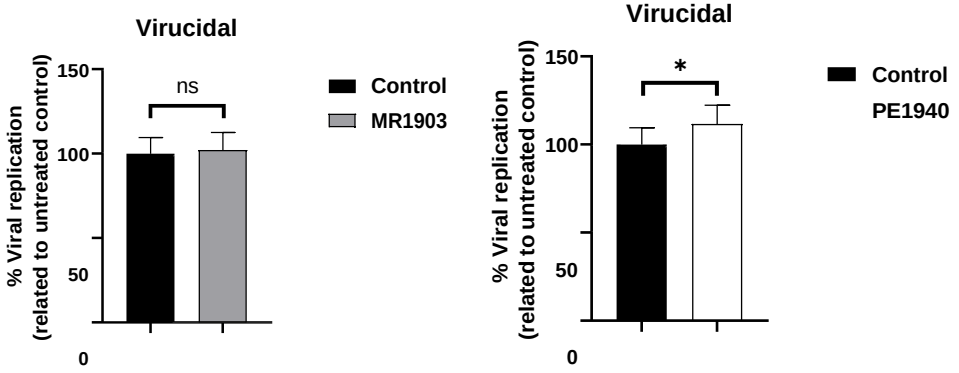
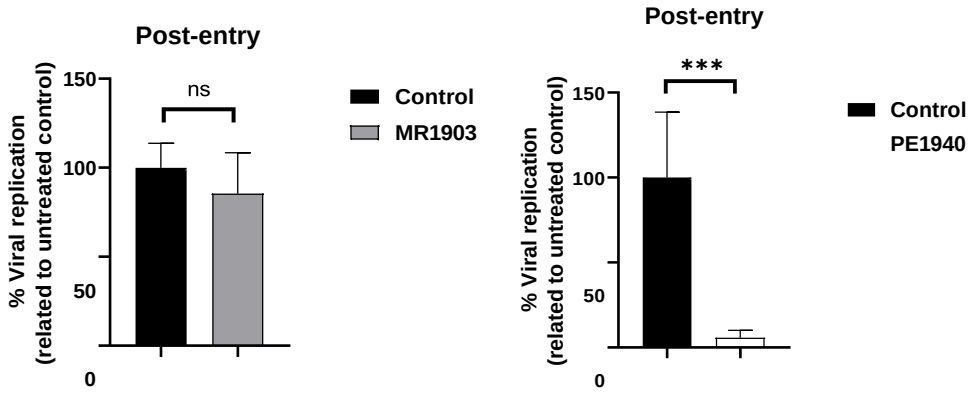


(C)



(D)



3.3.11. SARS-CoV-2 shows adaptation to peptides treatment

To analyse if SARS-CoV-2 can adapt to the peptides and become resistant to treatment, an in vitro passaging assay was performed. Results from TCID50 quantification of each passaged samples showed that after five passages, both peptides MR1903 and PE1940 did not show an inhibitory effect against the virus. Although, we had some alterations in the sixth passage for the peptide MR1903, but that can be related to experimental variations (Fig.14).

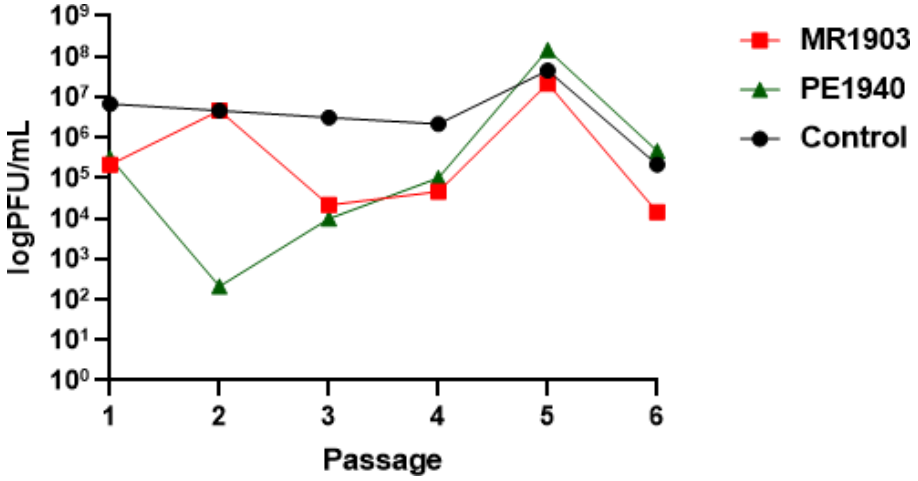
To continue our study, we sequenced the following samples resulted from in vitro passaging: virus without passaging, virus after passaging with PE1940 and with MR1903 treatments, and virus after passaging without any treatment (assay control).

The sequencing results from the last passage treated with MR1903 presented deletion on the regions 23595-23624 (Fig.S7) and 27220-27263 (Fig.S8) of SARS-CoV-2 genome when we compare to the original virus sequence. However, the exact same sequence was also deleted in the control after passage for the first region mentioned, as well as the second one in the control for mutation error, what difficulted further analysis and discussion on these results.

Regarding PE1940 treatment, first we found deletions on the region on 23595-23624 (Fig.S5) of SARS-CoV-2 genome after the treatment with PE1940, but as observed for MR1903 treatment, when we compare to the original virus sequence after passaging control, it is possible to see the same deletion, what shows that this modification could be related to viral fitness, during virus adaptation to replication in the cells [44].

The second found in PE1940 treatment was on the region 27202-27387 (Fig.S6). There is a deletion on 27224-27228 for control after passaging as well as PE1940 until 27227, so in the in treatment, there is a recover on 27228. Also, only for treatment with this peptide, it is possible to find a second deletion between 27237- 27243.

Figure 13. Results from six passages for drug resistance assay using A549 cells infected with Wild-type SARS-CoV-2. Re infections were performed after titration by TCI50 quantification. With each infection, a solution containing the treatment with peptides MR1903 and PE1940 at concentrations selected by previous cell viability assay was added to the cells for six times.



4. Discussion

The field of peptide testing against viruses is now growing very fast all over the world, since their characteristics can be related to action in several specific steps of viral replication, in a way they are able to interact with important targets that can compromise the virus replication without affect the cells [45]. Studies have shown that structures based on peptides present promising anti-SARS-CoV-2 activity [46–48]. Given that, in our study, we analysed different structures of peptides analogs to the peptide MR1903 $(KKYRYHLKPF)_2K$, to find potential inhibitors against SARS-CoV-2, and then better understand how the difference between their structures could be related to their action in the different steps of viral replication for this virus.

Higher concentrations, resulted of above 80% of Vero cell viability, were selected for our base peptide (MR1903), and for the linear and monomeric ones (EMC2109 and MR2024), but the tetrameric peptide (PE1940) showed higher levels of toxicity, so a lower concentration was selected. This demonstrates that the tetramerization of the peptide can be related to major interactions with cells, and consequently higher cytotoxicity. However, antiviral peptides can present a potential action even when it is necessary to work with them in lower concentrations [46]. Indeed, we observed this antiviral potential for PE1940, since even working with a lower concentration of it, we observed that it causes an impairment in viral replication of SARS-CoV-

2. The antiviral screening also showed that the peptide MR1903 has inhibitory effects against this virus. Interestingly, the monomeric and dimeric linear peptide did not present effect against this virus, but the ones that have as characteristics to be dimeric/tetrameric, and non-linear, showed great inhibition activity. Because of that, we can confirm that the complexity of peptide sequence is related to the anti-SARS-CoV-2 in the present work.

Both peptides MR1903 and PE1940 had the effected demonstrated on entry and post-entry steps of replication cycle during infection of SARS-CoV-2 on Vero cells. The entry potential can be associated with a prevention of ACE2 being bind to the spike protein of this virus, showed by also using peptides [49]. The peptides can interact with domains as RBD or ACE2 to result in the entry effects [49,50]. Besides those steps analysis, the virucidal effect was also evaluated, and results showed that only the peptide PE1940 had significant action on the viral particle treatment prior infection of Vero cells. This could be caused by this peptide being able to destabilize the virus particle by an interaction of its structure with virus envelope resulting in a decrease of infection, as it was observed in a study using a scorpion venom peptide against measles, SARS-CoV, and Influenza [51]

The further analysis of this work, using a tumoral pulmonary cell line (A549), showed the dose dependence of the peptides and the selective index (SI) of them. SI is related to the ratio between cell toxicity and the antiviral effect, and the higher is the value, more ideal the compound is as an antiviral candidate [52]. The higher SI of 7.84 for MR1903 in comparison to 2.97 of PE1940 is resulted of less cytotoxicity caused from treatment with MR1903. Despite PE1940 shows higher levels of toxicity, this peptide was able to inhibit the virus, even at lower concentrations. Zhang et al (2021) studied a peptide against SARS-CoV-2 spike protein, and the value of SI obtained was of 7.46, which is very similar to the one we found for MR1903 [53].

Different analysis of action on the replication cycle of SARS-CoV-2, using A549 cells, were performed, so we could explore more of the antiviral effect of each peptide. Even though MR1903 showed effect in the entry step of viral replication using Vero cells, the same did not happen when we worked with human A549 cells and a higher MOI (0.1). However, this peptide demonstrated to have a protective effect, since it inhibited levels around 30% for the two hours (12 and 24) post-infection analysed. This effect could be related to a possible interaction between the dimeric peptide and host factors, as per example Angiotensin-converting enzyme 2 (ACE2) or transmembrane protease serine 2 (TMRPRSS2), as they were already proven to be used by SARS-CoV-2 spike protein during the entry process into the cells, besides that they

are both expressed in the cell line used for this experiment [54–57]. MR1903 could be interacting with these proteins before infection, and consequently disturbing the virus interaction with them during the virus entry process.

In addition, MR1903 has demonstrated the induction of a post-entry effect, possibly associated with its interactions with antiviral targets during viral replication. [58]. Our analysis, using the subgenomic replicon, confirmed that this peptide has statistical inhibition on replication for the times of 24 and 48 h of treatment. This subgenomic replicon contains ORF1a and ORF1b (Supplementary material – Fig S3), which is translated into the polyprotein PP1ab, and then, produces the non-structural proteins (nsp) 1-16, including Helicase and Reverse transcriptase-polymerase (RdRp). These proteins are considered important targets for antivirals [59–61], which means that MR1903 could be interacting with them, resulting in the interruption of virus replication.

Moreover, MR1903 completely suppressed the in vitro production of double-stranded RNA by SARS-CoV-2 in our experiments. Coronaviruses generate double-stranded RNAs during the initial phases of the infectious cycle, originating from the replication of their genome and mRNA transcription. These double-stranded RNAs are also regarded as intermediates in the viral replication process. [62,63]. Thus, these RNAs present an additional target for antiviral agents. By examining the inhibition of double-stranded RNA by the peptides, we were able to assess their potential as RNA interferents. This was confirmed not only for MR1903 but also for PE1940.

PE1940 was also inhibited the entry step of the SARS-CoV-2 replication cycle, and, for this reason, we performed additional assays to better understand how this peptide is inhibiting SARS-Cov-2 entry in the host cells. The attachment analysis was performed at 4°C because at this temperature, the virus would not be able to enter the cell [40] so then we could elucidate possible interactions happening between PE1940, virus surface proteins, and cell receptors before the virus adsorption [64]. Besides that, an incubation at 37°C was added so we could understand if the peptide were acting in steps right after entering the cells [65]. Results showed that PE1940 inhibited both attachment and the steps involving uncoating, but most of the percentage comes from inhibition on attachment. A study in the literature showed that the molecules polysaccharides were also able to demonstrate effect against SARS-CoV-2 for this step, and the work relates the effects to the charge network of these compounds, as they are anionic, to be interacting to the positive charge of cells [64]. As already mentioned, this peptide was able to inhibit penetration and uncoating processes, and this could be associated to

interruption of the allosteric conformation of viral particle [64], since it has been proven that other study showed a compound causing that with human rhinovirus [66]. Besides that, since internalization of SARS-CoV-2 into cells happens through endocytoses via clathrin mediation, this peptide could also be affecting this process [67].

The tetrameric peptide also was responsible for a post-entry effect, with high levels of inhibition. As described for MR1903, this peptide could be acting in several stages after entering the cells. Since we performed an assay using the subgenomic replicon as well, and it showed inhibition, it could also be interacting with important nsp for viral replication [68]. Another antiviral target is nsp5 that is also named main protease (Mpro) or 3CL-like protease [69]. This is an important target for drug discovery because this protease is responsible for processing viral polyproteins, so it is related the viral replication complex generation [70,71].

Both peptides MR1903 and PE1940 also have action against the variants Omicron and Delta. This confirmation is very important since these peptides not only have effective potential against SARS-CoV-2 Wild-type, but also its variants that were tested. Omicron, per example, has modifications in the main protease and RdRp [72], what suggests that these peptides have potential to inhibit the virus even with mutations in SARS-CoV-2 important enzymes for replication.

Regarding the experiments with the recombinants, the omicron replacement of spike protein is related with modification in the affinity with hosts, and consequently the interaction with the cellular receptor ACE2 [73]. Interesting, our experiments to evaluate the effect of MR1903 for different steps of viral replication demonstrated that it only has effect for the post-entry stage, so the replacement of spike protein from Omicron did not affect other steps of viral replication like entry, as we expected. On the hand, PE1940 demonstrated an effect in entry and post-entry steps.

Analysis using the recombinant with the replacement of spike protein from Delta, showed that MR1903 causes effect only in the entry step, so the action of this peptide against this variant is directly associated to the modifications on the spike protein of this variant. Interesting, PE1940 demonstrated action on pretreatment and post-entry for this variant recombinant, and it did not demonstrate for entry step, so this peptide action is not directly associated with the spike protein from Delta.

An antiviral resistance is related to the virus potential to reduce the action of a drug [74], and it happens in consequence of an adaptive process of the virus during its treatment [75].

Against HCV, per example, it is known the treatment using the direct-acting antiviral agents (DAAs), however this virus reaches resistance to them after only some mutations, because these antivirals have the characteristic of low genetic barrier. That is why when looking for a treatment against viruses, it is of great importance to have compounds with high genetic barrier, so the virus does not reach resistance to the antiviral easily [75]. In this way, we simulated a resistance analysis for our peptides.

The deletions found in the treatment PE1940 (Fig.S5) are related to the ORF6 region of SARS-CoV-2 genome. Furthermore, ORF6 is an auxiliary protein [76], and it is involved with the nuclear transport machinery [77]. Some studies have already demonstrated that this protein can be a target for compounds against SARS-CoV-2, which suggests that it could be related to another possible mechanism of action for this peptide [78].

5. Conclusion

Amont analogs of the peptide MR1903, the nonlinear dimeric and tetrameric peptides were able to affect different stages of SARS-CoV-2 replication cycle, meaning that they probably have more than one specific target to trigger action against this virus. The peptides were able to impair mainly the steps after entry into cells, showing a strong post-entry action. Both were able to also inhibit the variants Omicron e Delta of SARS-CoV-2. Furthermore, after passages of virus being treated, we could identify some deletions on virus genome that caused adaptation of it to peptides treatment. This analysis was crucial to better understand viral fitness after treatment, by that it is still necessary further analysis of these structures to achieve more stability after a higher number of virus passages.

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Supplementary material

FS1. Primers designed for the double strand RNA experiment performed. (A) Forward and reverse oligonucleotide primers with (B) and without T7 promoter (A).

(A)

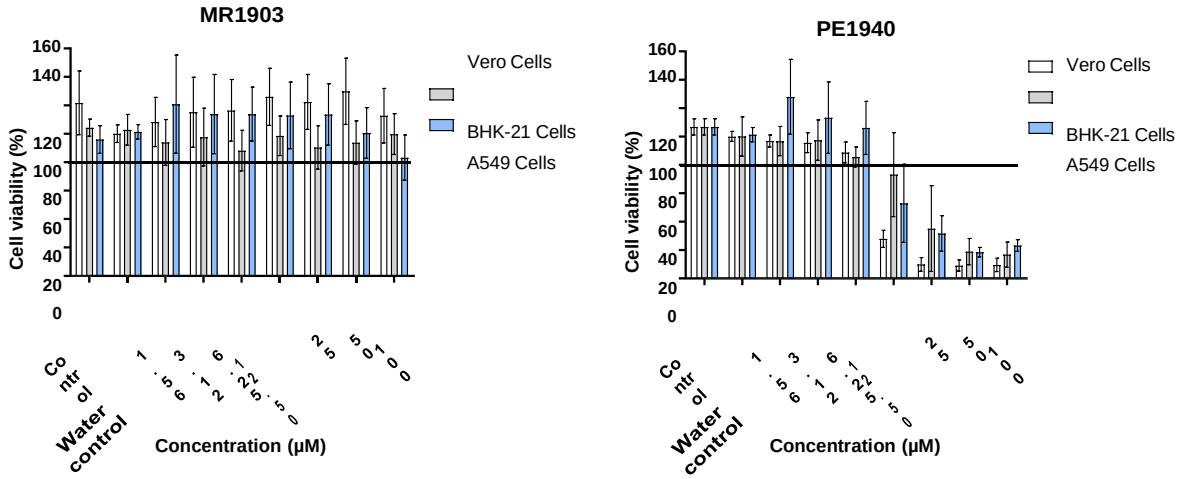
SARS-CoV-2 ORF1 Foward	GACCGAAAGGTAAGATGGAG
SARS-CoV-2 ORF1 Reverse	AAATCGCCCGTCTGCCATGAAG

(B)

SARS-CoV-2 ORF1 Foward+T7	TAATACGACTCACTATAGGGGACCGAAAGGTAAGATGGAG
SARS-CoV-2 ORF1 Reverse+T7	TAATACGACTCACTATAGGGAAATCGCCCGTCTGCCATGAAG

Fig S2. Cell viability results after treating Vero, BHK-21, and A549 cells for 24 hours with the peptides **(A)** MR1903, **(B)** PE1940, **(C)** MR2024, and **(D)** EMC2109. A line was drawn at 80% of cell viability since this the minimum necessary for the selection of the concentration determinant for antiviral assays. Control represents treatment with only DMEM. Water control represents the treatment with water, as it is the reagent used to dilute the peptides.

(A) **(B)**



(C) **(D)**

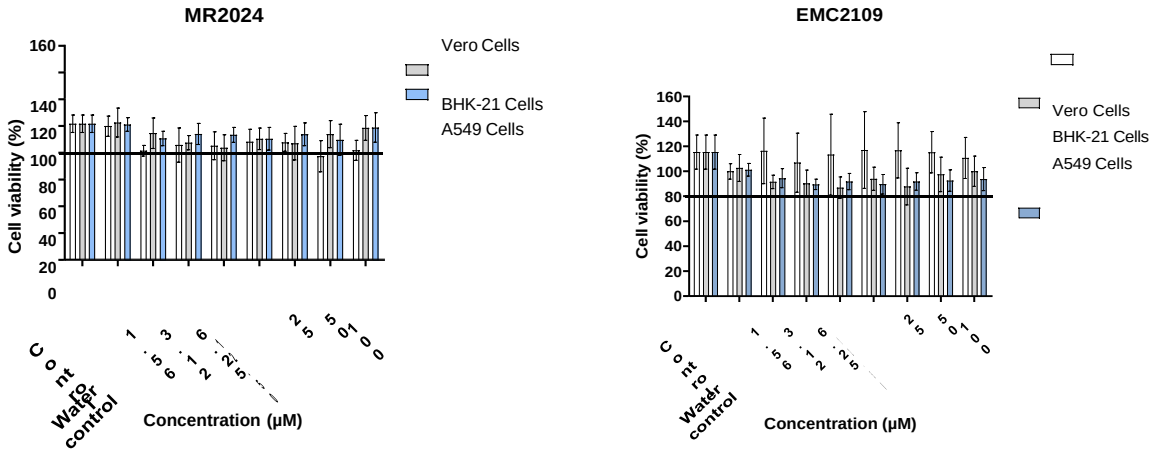


Fig S3. Subgenomic replicon backbone construction. This construction was conceded by Professor Andres Merits, University of Tartu.

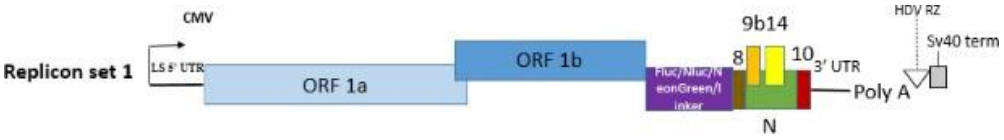
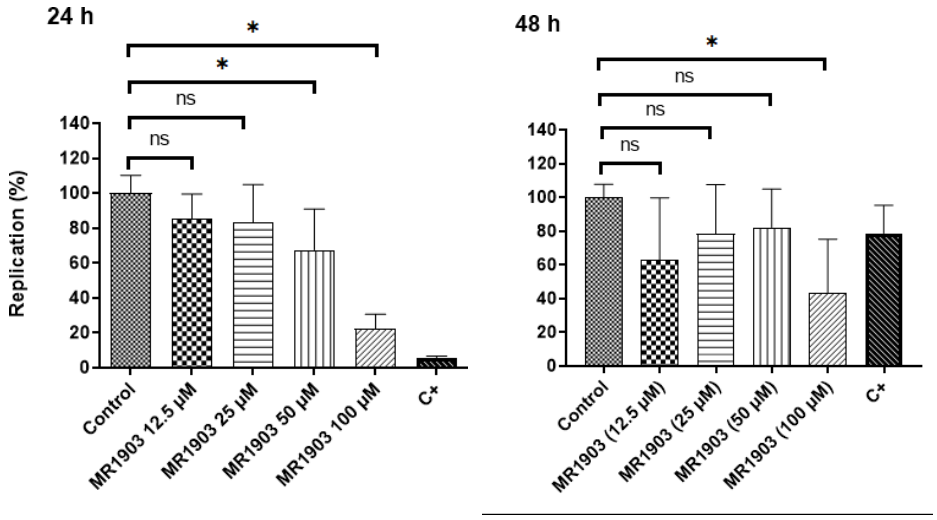


Fig.S4. The replication effect caused by the peptides was tested using a subgenomic replicon for SARS-CoV-2. Transfections were performed using BHK-21 cells, previously confirmed the cytotoxic effects on these cells (supplementary material – Fig S2) prior treatment with peptides using more than one concentration to analyses efficiency of action for them for two periods of time (24 and 48 hours). **(A)** Results for peptide MR1903 at several concentrations tested: 12.5 µM, 25 µM, 50 µM and 100 µM. **(B)** Results for peptide PE1940 at several concentrations tested: 3.1 µM and 6.2 µM. All results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. A positive control (C+, molnupiravir) for replication effect against SARS-CoV-2 was used. Tukey's Multiple Comparison Test was performed for these analyses. *: $p<0.05$; **: $p<0.001$

(A)



(B)

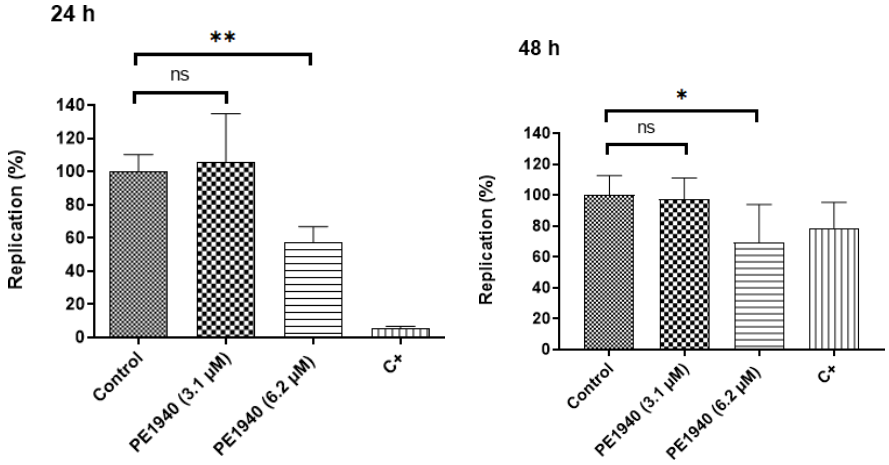


Fig.S5. Alignment of sequencing results from SARS-CoV-2 initial passage, after passages, and after treatment with peptide PE1940. Representation of spike protein region.

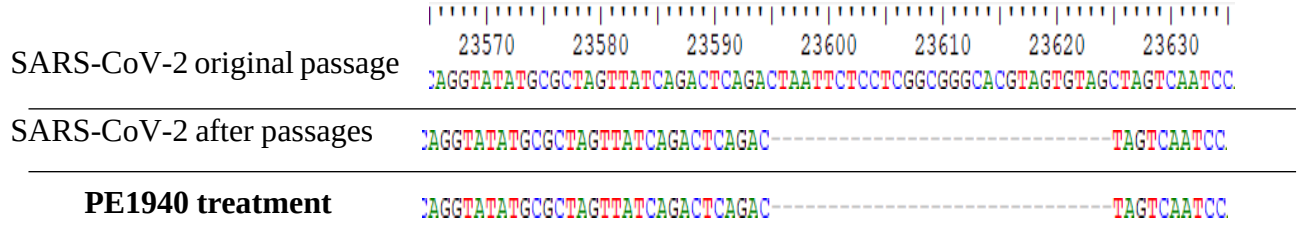


Fig.S6. Alignment of sequencing results from SARS-CoV-2 initial passage, after passages, and after treatment with peptide PE1940. Representation of ORF6 region.

Fig.S7. Alignment of sequencing results from SARS-CoV-2 initial passage, after passages, and after treatment with peptide MR1903.

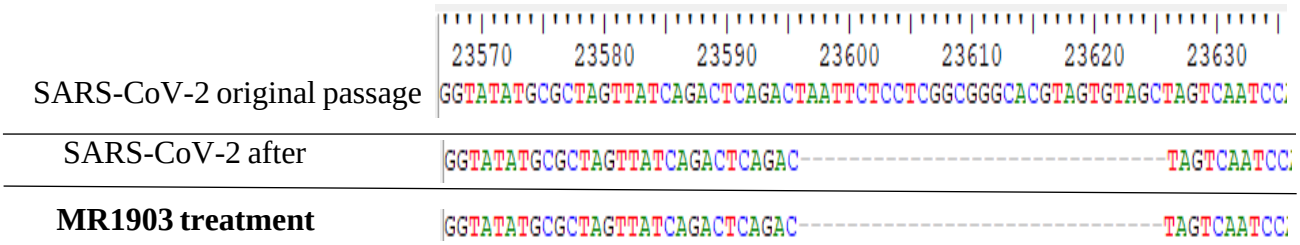
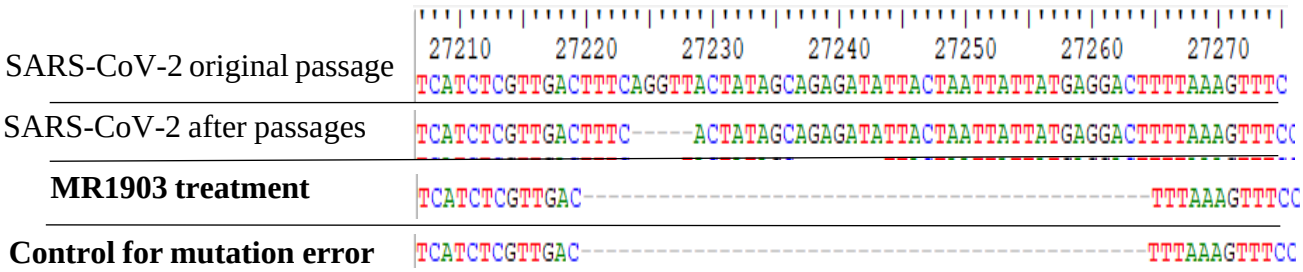


Fig.S8. Alignment of sequencing results from SARS-CoV-2 initial passage, after passages, and after treatment with peptide MR1903.



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CAPÍTULO III – Artigos científicos

Manuscrito II

Cell-penetrating peptides addition to Dimeric Peptide (KKYRYHLKPF)2K results in multiple steps inhibition against SARS-CoV-2

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Abstract

The dimeric peptide has already showed potential against SARS-CoV-2, and since this class of cell-penetrating peptides is a promising area in development, our work put together the two types of structures and that resulted in two peptides (MC1937 and MC1947), with different amino acids added, so we can elucidate how they could impair SARS-CoV-2 infection. We confirmed their cytotoxicity in three different cell lines (Vero, BHK-21, and A549), and after that used the selective concentrations for the antiviral assays. First, we confirmed theirinhibition action against the Wuhan original strain of SARS-CoV-2 using Vero cells. The nextstep was to understand their effects on A549 infected with this virus, by a dose dependence assay, that allowed us to obtain the peptides selective index. Our work also demonstrated that these peptides were potent inhibitors in different steps of viral replication, with similar action. Besides that, we also demonstrated these peptides can impair two variants of SARS-CoV-2 (Omicron and Delta) infection. Analyses of the Wuhan original strain resistance to their treatment was also evaluated, and sequencing of the samples showed a point mutation. In this way, the present work was able to demonstrate the potential of using cell-penetrating peptides against SARS-CoV-2.

Key-words: SARS-CoV-2, variants, antivirals, peptides, and cell-penetrating peptides.

1. Introduction

After the COVID-19 pandemic caused by SARS-CoV-2, several studies were developed to find an effective antiviral against this virus [1]. Some works, aimed to identify an inhibitor by targeting a specific protease, like the Main protease (Mpro) [2,3] or 3-chymotrypsin-like protease (3CLpro) [4], both essential for viral replication [5]. Literature shows the most diverse types of compounds tested against SARS-CoV-2, with that different aspects of replication that they were able to impair [6]. Some molecules found to have activity on entry step, an important target since it is a process mediated by the spike protein which can interact with some compounds, resulting in viral replication inhibition [7]. The antiviral action can also be related to other different steps of the replication cycle, like a post-entry effect [8].

Related to the diversity of potential antivirals against SARS-CoV-2 used in these studies, there are the interferons [9,10], repurposing compounds [11,12], or even natural compounds [13–15]. Peptides are another type of molecule that has recently showed antiviral potential [16], since it has also demonstrated before antimicrobial and anti-inflammatory effects [17–19]. Peptides can be originated from natural source or synthetized [20,21]. Between the characteristics of peptides, it is known they have several advantages over other molecules, like less chances of side effects, specific action, and they are usually tolerable [22,23]. Besides that, virus can present less resistance to them [22].

Besides those characteristics, peptides can have other properties that increase their biological activities. The cell penetrating potential is one of them [24]. Cells have a barrier to select what goes inside of them as a mechanism of defence [25]. But this can be problematic during the process of screening new compounds against microorganisms. In front of that, this family of peptides presenting cell penetrating peptides characteristic, that can be obtained naturally or synthetized, are able to get through the membrane of cells using independent or dependent of energy processes [26].

Apart from being able to be used as vectors to deliver different molecules into the cells, this presented type of peptides have also shown antiviral effect previously [27]. One study showed the potential of cell-penetrating peptides to inhibit the entry step of infection with Herpes Simplex virus [28]. Another study demonstrated the potential against the Human Papillomavirus [29]. Furthermore, these peptides were also used as carriers of antivirals besides acting as one, like studies showed against Hepatitis B virus [30,31].

Based on that and our previous works with the dimeric peptide $(KKYRYHLKPF)_2K$, it was developed a new study with different peptides, in which we added peptides with characteristic of cell-penetrating to the structure of the dimeric one and tested against SARS-CoV-2 and two important variants of this virus.

2. Material and methods

2.1. Peptides

Peptides were synthesized manually through solid-phase peptide synthesis (SPPS) following the methodology outlined by Merrifield in 1963 and Bittencourt in 2023 [33,34] . Basically, to obtain dimers and tetramers, Fmoc-Lys (Fmoc)-OH amino acid was employed at the initiation of synthesis as a branching point, allowing peptide chains to grow from the α-amino and ε-amino groups of lysine, as detailed by Lorenzón et al., 2012, and Santos-Filho et al., 2021 [35,36]. Post-synthesis, the peptides underwent purification via semi-preparative HPLC using a Shimadzu chromatograph (Tokyo, Japan) equipped with a C_{18} Jupiter column measuring 25 × 1 cm and featuring a particle size of 10 µm. The purity of the obtained materials (<95%) was assessed through analytical HPLC on a Shimadzu chromatograph (Kyoto, Japan) with a reverse-phase C_{18} column measuring 0.46 × 15 cm and a particle size of 5 µm (Agilent, Santa Clara, CA, USA). Verification that the desired materials were acquired involved mass spectrometry, employing an Ion Trap MS mass spectrometer (Bruker) in direct injection and positive detection mode.

2.2. Cells

To cultivate the cell lines A549 (ATCC CCL-185), Vero (ATCC CCL-81), and BHK-21 (ATCC C-13), Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS, Gibco – Thermo Fisher Scientific, Waltham, MA, USA) and 1% Penicillin/Streptomycin (5,000 U/mL) (P/S, Cultilab, Campinas, SP, Brazil) was used. The A549 cell line (isolated from pulmonary carcinoma) that was transduced to express human ACE2, selected under 200 µg/ml Hygromycin B (Thermo Fisher Scientific, Waltham, MA, USA), and human TMPRSS2 under selection of 2mg/ml Geneticin (G418, Sigma–Aldrich, St. Louis, MO, USA). A549 cell line was gently donated by Prof. Arvind Patel from the NIBSC Research Reagent Repositor (United Kingdom).

2.3. Virus

The SARS-CoV-2 Wuhan lineage used for this work was isolated from a Brazilian patient positive sample (SARS-CoV-2/SP02.2020; EPI_ISL_450506), and it was provided from Prof. Edson Luiz Durigon. The SARS-CoV-2 variants Omicron (BA.2) (hCoV/England/FCI-179/2022) and Delta (B.1.617.2) (MS066352H) were also used in this work, both conceded from Francis Crick Institute (London, United Kingdom).

The infections cDNA (icDNA) clone of SARS-CoV-2 based on the Wuhan strain, and the recombinants with replaced spike protein from Omicron and Delta, all expressing mCherry reporter (SARS-CoV-2-mCherry) [32] were also used for our analysis. To rescue these plasmids, BHK-21 cells were seeded in 12-well plate (TPP, Trasadingen, Switzerland), and were transfected with 3 µg of plasmid using Lipofectamine 2000 (Thermo Fisger Scientific, Waltham, MA, USA) and OPTI-MEM (Gibco—Thermo Fisher Scientific, Waltham, MA, USA. After 72 hours of post transfection, the supernatant was collected and used to propagate virus in 6-well plate seeded with 8x10 6 A549 cells. After four days, the supernatant was collected and stocked at -80°C as the passage 0.

To determinate the virus titer, it was performed a Tissue Culture Infectious Dose (TCID50). A549 cells ($1x10^4$) were seeded in 96-well plates (TPP, Trasadingen, Switzerland). Twenty-four hours later, virus dilution was performed, and cells were infected. Seventy-two hours after, cytopathic effect was quantified to determine viral titre, which was calculated using TCID50 calculator (Spearman–Kärber Method).

2.4. Evaluation of the Cytotoxicity Profile of the Peptides

The cytotoxicity of the peptides MC1937 and MC1947 in all cell lines cited before was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) protocol as described previously [33]. $1x10^4$ cells per well were seeded in 96-well culture plates (TPP, Trasadingen, Switzerland). After 24 h, the cells were incubated with different ranges of concentrations of the peptides (between 0.3, 0.7, 1.5, 3.1, 6.2, 12.5, 25, 50, 100 µM) for 24 h. Then, the medium containing the peptides was removed, and 100 µL of MTT (Sigma-Aldrich, St. Louis, MO, USA) diluted in DMEM (Cultilab, Campinas, SP, Brazil) (1 mg/mL) was added to each well of the plate (1 mg/mL). After 30 min of incubation at 37 °C, the medium containing MTT (Sigma-Aldrich, St. Louis, MO, USA) was removed, and 100 µL of dimethylsulfoxide (DMSO, Synth, Diadema, SP, Brazil) was added to the cells. The plate was agitated at 200 rpm. After 5 min, the absorbance was measured at a wavelength of 572 nm on a plate reader (FLUOstar Omega/BMG LABTECH, Ortenberg, Germany). The CC_{50} values for A549 cells

were calculated using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

2.5. Initial screening for anti-SARS-CoV-2 action using Vero cells

For this experiment, $1x10^5$ Vero cells were seeded in 24-wells plate (TPP, Trasadingen, Switzerland), twenty-four hours before the experiment started. After this period, a solution containing SARS-CoV-2 at MOI 0.01 and each one of the peptides was prepared and added in duplicate to the wells. After 24 hours of treatment incubation, the supernatant was collected and frozen for further analysis using Median Tissue Culture Infectious Dose (TCID50) or real time qPCR to quantify virus replication.

2.5.1. Analysis of antiviral effect of peptides on replication cycle using Vero cells

$1x10^5$ Vero cells were seeded in 24 wells plate, twenty-four hours prior the start of this experiment. After that, three types of analysis were performed to elucidate the effects on replication cycle. **(A)** Virucidal: Each peptide (MC1937 or MC1947) and SARS-CoV-2 at MOI 0.01 were incubated for one hour at 37°C. After that, this solution was used to infect cells for one hour. Next, cells were washed twice with PBS, and fresh supplemented medium was added to cells. **(B)** Entry: A solution containing SARS-CoV-2 at MOI 0.01 and each peptide was added to cells and incubated for one hour. After that, cells were washed twice with PBS, and fresh supplemented medium was added to wells. **(C)** Post-entry: First, an infection of SARS- CoV-2 at MOI 0.01 was performed in seeded cells for one hour. After that, cells were washed twice with PBS solution, and fresh medium containing the treatment with each peptide was added to the cells and incubated for 24 hours. After the incubation time for all described experiments, with supernatant resulting from each assay was performed RNA extraction using Trizol (Invitrogen, Waltham, MA, USA) reagent, and then cDNA was synthetized using High-Capacity kit (Applied Biosystems, Waltham, MA, USA), following instructions from handbook. After that, qPCR was performed to quantify the quantity of virus RNA in this experiment.

2.5.2. Analysis by real time qPCR

For qPCR analysis, the taqman assay was used to perform the experiments. The reaction was composed of 2.75 μL of ultrapure DEPEC water, primers (0.96 μM of each), and probe (0.48 μM). Viral nucleocapsid (N1) gene specify was used for this reaction: 2019-nCoV_N1-F (5'GAC CCC AAA ATC AGC GAA AT-3'), 2019-nCoV_N1-R (5'-TCT GGT TAC TGC CAG TTG AAT CTG-3') e 2019-nCoV_N1-P (5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3') (Integrated DNA Technologies, California, EUA) (CDC, 2020), e 5 μL

de 2X TaqMan™ Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA), and 1,5 μL of cDNA. The reaction was submitted to the equipment QuantStudioTM 12K Flex Real Time PCR System (Applied Biosystems, Waltham, MA, USA) programmed for specific cycling. The cycling program was 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 1 minute (45 times).

2.7. Antiviral activity of the peptides in human lung cells

The experiments using the A549 cell lines were performed using the infectious SARS-CoV-2 cDNA clone expressing mCherry gene as a marker (SARS-CoV-2-mCherry) [32]. In this way, we were able to quantify the results by the red fluorescence using the IncuCyte® S3 live-cell equipment (Sartorius, Gottingen, Germany).

2.7.1. Dose dependence analysis

For the first part of this section, twenty-four hours prior the experiment, $1x10^4$ cells were seeded in 96-wells plate (TPP, Trasadingen, Switzerland). Then, solutions containing different concentrations (0.1 μM to 100 μM) of peptides were prepared and incubated in the cells with (SARS-CoV-2-mCherry) infectious clone at MOI 0.1 for 24 hours. After that, the fluorescence was measured for the quantification of the inhibition in the dose dependence matter by using the IncuCyte® S3 live-cell equipment (Sartorius, Gottingen, Germany).

Using the software GraphPad it was possible to calculate 50% cytotoxic concentrations (CC50) and Half-maximal inhibitory concentrations (IC50), and by that, it was possible to calculate the selective index.

2.7.2. Associated treatment analysis

For this experiment, a solution containing both peptides at the concentration selected before was prepared. This solution was added to cells previously seeded in a 96-well plate (TPP, Trasadingen, Switzerland). Then, cells were incubated with SARS-CoV-2-mCherry at MOI 0.1 for twenty-four hours at the incubator. The fluorescence percentage was measured after this period of incubation, using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.7.3. Analysis of peptides action on entry step

Twenty-four hours before the experiment, $1x10^4$ A549 cells were seeded in 96-wells plate (TPP, Trasadingen, Switzerland). In the next day, a solution containing SARS-CoV-2-mCherry at MOI 0.1 and each one of the peptides was added to cells and incubated for one hour. After that, cells were washed twice with PBS solution, and fresh media supplemented

with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at twelve and twenty-four hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.7.4. Analysis of peptides on the SARS-COV-2 attachment to the cells

Twenty-four hours before the experiment, $1x10^4$ cells were seeded in 96 wells plate. In the following day, a solution containing SARS-CoV-2-mCherry at MOI 0.1 and each peptide was added to the cells and incubated at 4°C for one hour. After that, cells were washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. This experiment protocol was based in other studies already published in the literature [34,35]. Fluorescence percentage was measured at twelve and twenty-four hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany.

2.7.5. Analysis of peptide on the SARS-CoV-2 internalization

First, $1x10^4$ A549 cells were seeded in 96 wells plate, and in the next day, a solution containing SARS-CoV-2-mCherry at MOI 0.1 and each peptide was added to the cells, and they were incubated at 4°C for one hour. After this period, cells were incubated at 37°C for 30 minutes, aiming to imitate the process of virus internalization to the cells. At last, cells were also washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at twelve- and twenty-four-hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.7.6. Virucidal effect of peptides

$1x10^4$ A549 cells were seeded in 96-wells plate (TPP, Trasadingen, Switzerland), twenty-four hours before the experiment started. In next day, two solutions were prepared: one containing SARS-CoV-2-mCherry at MOI 2 and other with MOI 5. In each one of them, it was added the solutions of the peptides at the previous selected concentration by cytotoxic assay, and it was incubated for 1 hour at 37°C. After that, the solutions with virus and peptide were added to the A549 cells for another one hour. For the last part of the experiment, the cellswere washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at twelve and twenty-fourhours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.7.7. Protective effect of peptides against SARS-CoV-2 infection

$1x10^4$ of the A549 cells were seeded in 96 wells-plate (TPP, Trasadingen, Switzerland), one day before the experiment. In the following day, cells were treated with each one of the peptides for one hour. Then, cells were washed twice with PBS solution, and they were infected with SARS-CoV-2-mCherry at MOI 0.1 for another one hour. Next, cells were washed again with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at twelve and twenty-four hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.7.8. Study of the peptides action on the post-entry of SARS-CoV-2 in cells

One day before the experiment, $1x10^4$ A549 cells were seeded in 96 wells plate. In the next day, cells were infected with SARS-CoV-2-mCherry at MOI 0.1 for one hour. Then, cells were washed twice with PBS solution, and treatment contained of each one of the peptides was added to the cells. The fluorescence percentage was measured at twelve and twenty-four hours post infection by the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.7.9. Evaluation of activity of the peptides against SARS-CoV-2 variants: Omicron (BA2) and Delta

$1x10^5$ A549 cells were seeded in 24 wells plate twenty-four hours before the experiment. In the following day, two separate experiments were performed. For the first experiment, a solution containing SARS-CoV-2 Omicron (BA2) at MOI 0.1 was prepared containing each peptide, while the second one, a solution containing SARS-CoV-2 Delta at MOI 0.1 was prepared with each peptide. Both treatments were added separately to the cells, and they were incubated for twenty-four hours. After that, the supernatant resulting from this experiment was frozen. The quantification of this experiment was performed by TCI50 experiment was performed with the supernatant.

2.7.10. Analysis of peptides action on the replication cycle of the Omicron and Delta variants

For the analyses of the peptides' specific effects on replication steps of Omicron and Delta infection, all the methodologies from the previous experiments were repeated (Entry, Virucidal, pretreatment, post-entry), but using for infection the recombinants infectious clone with spike protein from Omicron. Fluorescence percentage was measured at twelve and twenty-four hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.8. Investigation of peptides action on replication step

$5x10^4$ of the BHK-21 cells were seeded using a 48 wells plate, one day before the experiment. In the following day, cells were transfected with 600 ng of the plasmid of SARS-CoV-2 subgenomic replicon expressing NanoLuciferase marker, using Lipofectamine™ 2000 protocol. After 4 hours of transfection, media containing the treatment with peptides was added and incubated for 24 and 48 hours. After this period, luminescence levels were read to quantify the difference between treatments. It was used the FLUOstar Omega Microplate Reader (FLUOstar Omega/BMG LABTECH, Ortenberg, Germany) to make the readings.

2.9. Evaluation of *in vitro* inhibition of SARS-CoV-2 synthetic double strand RNA by peptides

The production of SARS-CoV-2 double strand RNA was performed as described in our previous work. To produce SARS-CoV-2 double strand RNA, forward and reverse oligonucleotide primers with and without T7 promoter sequences (Supplementary Figure 1) were designed. Two sets of PCRs were necessaire to produce a DNA template for transcription of sense and antisense RNA, respectively. Then, a 1% agarose gel with 1X TAE was run to confirm PCR reaction. The PCR product was cleaned using Monarch kit (New England Biolabs), following instructions of the manual. The next step was to prepare the double strand RNA. Next, the RNA was cleaned using RNA clean up kit (New England Biolabs). RNA concentration was determined, and an annealing reaction was performed with sense RNA and antisense RNA, annealing buffer (10X), and nuclease-free water. For last, to check the integrity of the double stranded RNA, a gel was run. After that, solutions were prepared at the same concentration containing the peptides (treatment), positive control (Platine), and controls (without any compound). All solution were incubated for 45 minutes at room temperature. After this period, a 1% agarose gel was run using a 100 pb ladder.

2.10. Analysis of SARS-CoV-2 resistance to treatment with the peptides

$8x10^5$ of A549 cells were seeded in the 6 wells plates, one day before the experiment had been performed. Next, a solution with SARS-CoV-2 (Wuhan Wild-type strain) at MOI 0.05 with each peptide was added to the cells and incubated until the following day. After that, the supernatant was collected, and frozen. The supernatant was quantified using TCID50, and knowing the titre of the experiment, a new infection was performed at the same MOI, and adding peptide again. This cycle of infections and quantifications was done for six passages. The last passage of each treatment was compared with the original sequence of this virus.

2.11. Sequencing

140 µl of cell culture supernatant was extracted with a Viral RNA mini kit (QIAGEN) and eluted in 60 µl of kit elution buffer. cDNA was synthesized with 20 µl of RNA using RNA to cDNA EcoDry Premix (Takara Bio) and validated by qPCR [36]. 2.5µl was then used as template for tiled amplicon whole genome sequencing following the Midnight protocol [37] except with an alternative and novel set of primers (available on request).

2.11. Statical analysis

All graphs and statistical analyses were performed using GraphPad Prism 8 and were described in figures. For quantification of band density percentage, it was used ImageJ software.

3. Results

3.1. Cytotoxicity profile of peptides

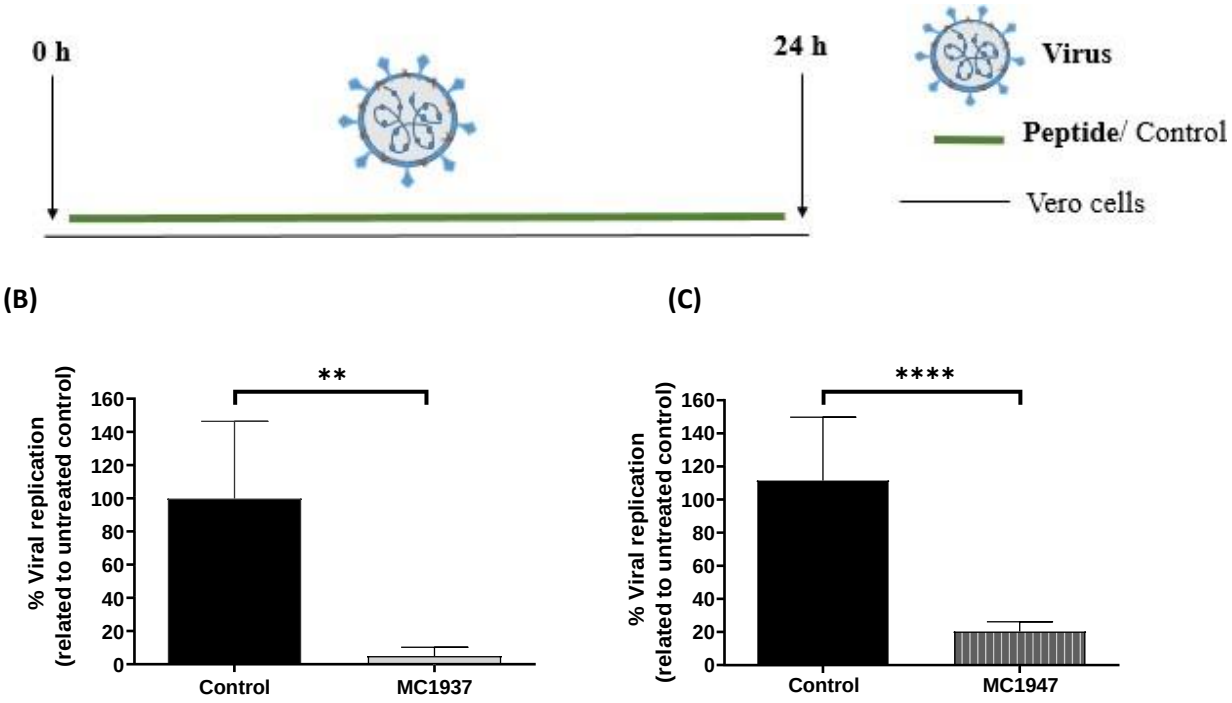
The cell lines (Vero, BHK-21, and A549) were treated with the peptides MC1937 and MC1947 for 24 hours to select a concentration that represents cytotoxicity above 80%, so those concentrations could be used for the antiviral assays. For the peptide MC1937, the concentration of 25 µM was selected for all cell lines (Fig.S2A), and for MC1947, concentrationof 12.5 µM was able to keep similar cell viability levels (Fig.S2B) for cell lines. These peptides'concentrations were used for the next experiments.

3.2. Initial screening for anti-SARS-CoV-2 action using Vero cells

An initial trial was performed to analyse if the peptides have any action against SARS-CoV-2, so together with the treatment of each peptide, Vero cells were infected with the Wuhan original strain. Our results showed that both peptides can significantly inhibit this virus. Detection of RNA levels demonstrated that MC1937 inhibited about 95% of the virus (p= 0.0035), and MC1947 was able to decrease viral replication in about 90% (p <0.0001).

Figure 1. Results of the inhibition resulted from an initial antiviral screening for two different peptides with cell-penetrating characteristics added to their structure. **(A)** Scheme describing experimental protocol. Graphs of comparison between treatment with peptides: **(B)** MC1937 **(C)** MC1947 are shown below. Results were expressed in percentage of cells treated related the control (sterile water). Experiments were carried out in three independent events, each performed in duplicate, and data are presented as the mean ± standard deviation. The Statistical Paired t test was performed to check significance of data. **: p<0.01; ****: p<0.0001.

(A)



3.2.1. Analysis of antiviral effect of peptides on replication cycle using Vero cells

Since both peptides showed their antiviral potential against SARS-CoV-2, they were taken to further analyses of the action of these peptides in each step of the viral replication.

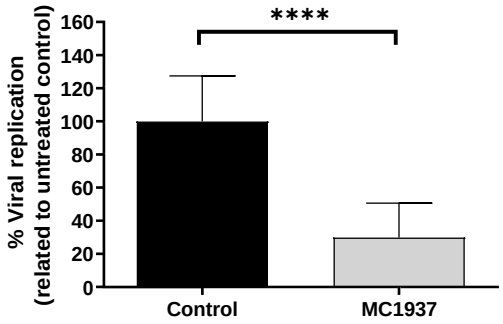
MC1937 and MC1947 demonstrated to be able to inhibit all the steps tested. Analysis of entry step showed that MC1937 inhibited 70% of viral replication (p<0.0001, Fig.2AI), meanwhile, MC1947 treatment resulted in a decrease of 90% (p<0.0001, Fig.2AII).

The potential to inhibit the post-entry steps is of about 85% for MC1937 (p<0.0001), and 90% for MC1947 (p=0.0006). Regarding the virucidal action, both peptides were capable to cause effect, inhibiting the viral particle in 85% (p<0.0001) and 77% (p<0.0001) for MC1937 and MC1947, respectively.

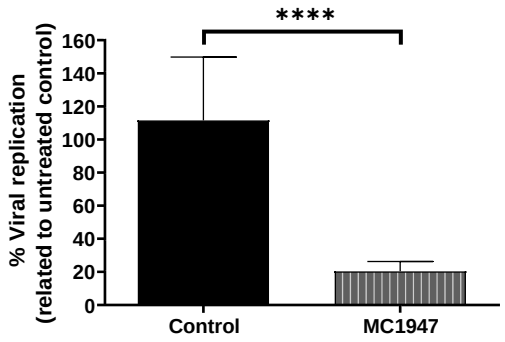
Figure 2. Results of antiviral initial assay showed peptides MR1903 and PE1940 with inhibition against SARS-CoV-2, so they were taken for a time addition analysis to test for **(A)** Entry step inhibition, **(B)** Post-entry effects, and **(C)** Virucidal effect for MC1937 and MC1947 using Vero cells. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in duplicate, and data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. **: p<0.01; ***: p<0.001; ****: p<0.0001.

(A) Entry step inhibition

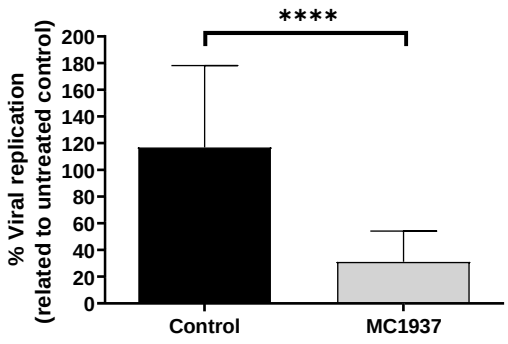
(I)

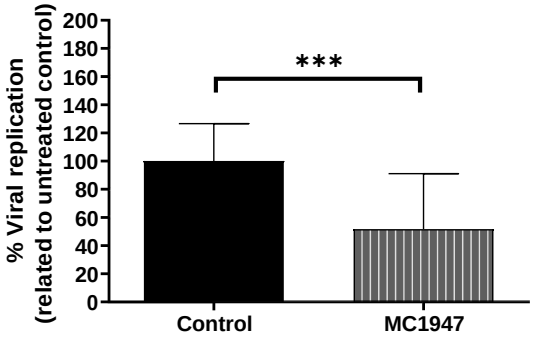


(II)

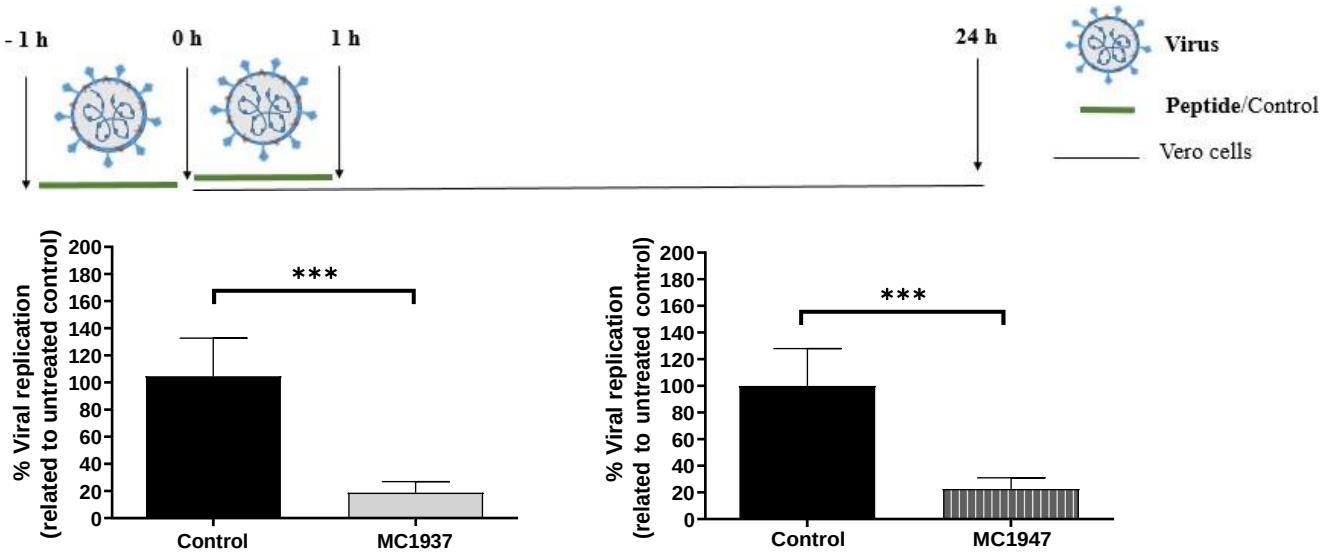


(B) Post-entry inhibition





(C) Virucidal effect



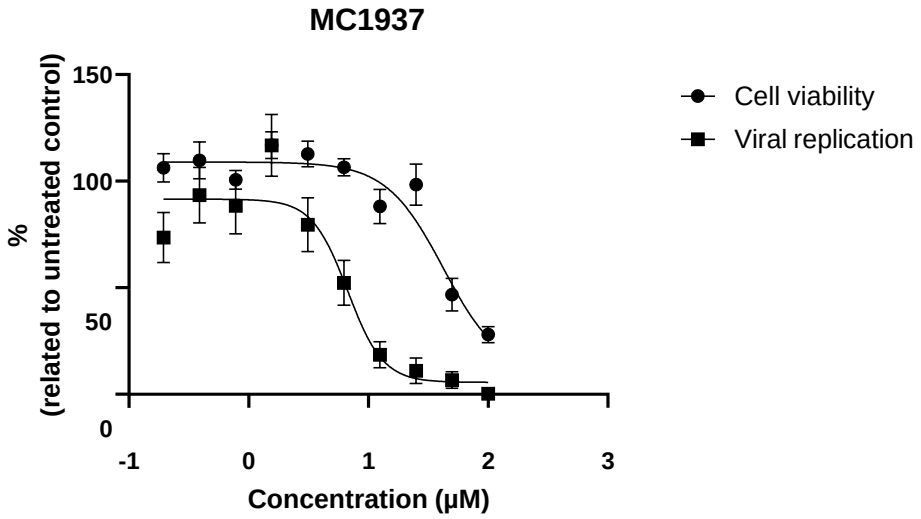
3.3. MC1937 and MC1947 show dose dependence effect against SARS-CoV-2 in A549 cells

A pulmonary cell line was used to analyze the dose dependence of the peptides against the Wuhan original strain of SARS-CoV-2. Those results also allowed us to calculate the selective index (SI) of these compounds.

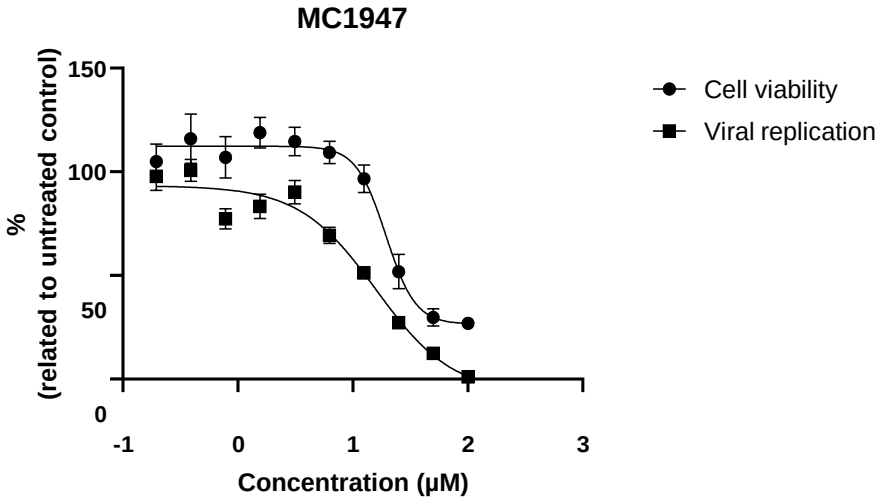
These peptides showed to have very similar profiles of dose dependence (Fig.3A and B). However, MC1937 has a higher SI than MC1947 (Fig.3C), because of its less cytotoxicity demonstrated. Besides that, MC1937 also has a 50% inhibitory concentration lower than MC1947.

Figure 3. Analysis peptides' dose dependence was performed using A549 cells for the peptides **(A)** MC1937 **(B)** MC1947. Cell viability analysis was performed using absorbance reading, and dose dependence analysis by percentage of fluorescence measure. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate. **(C)** Values of CC50, IC50, and selective index, calculated using software GraphPad.

(A)



(B)



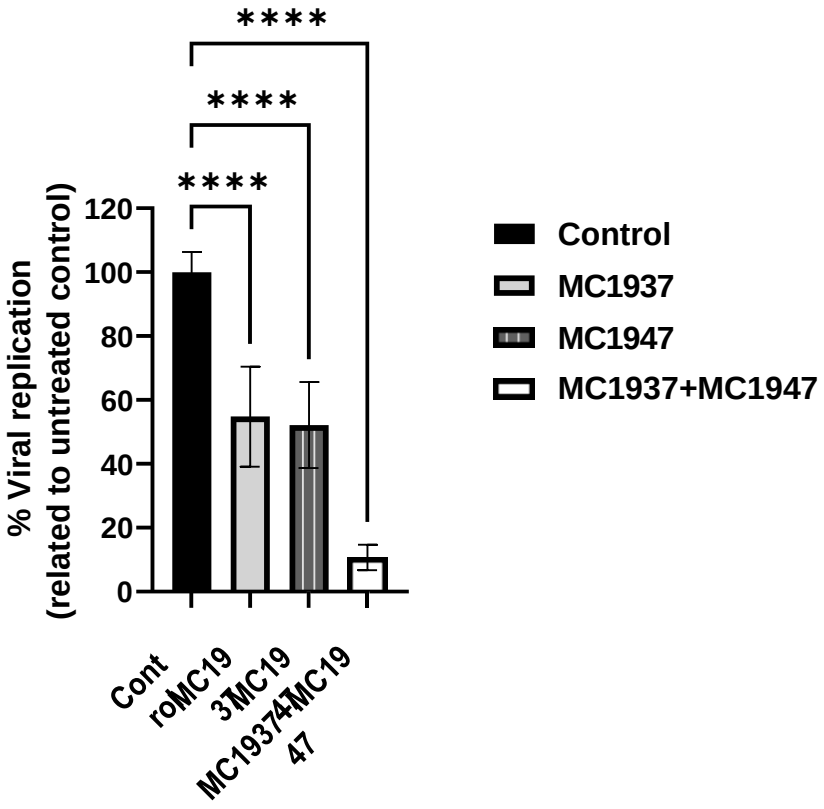
(C)

Peptide	CC50 (µM)	IC50 (µM)	Selective Index (SI)
MC1937	42.19	6.718	6.28
MC1947	19.33	15.90	1.21

3.4. Peptides potential against the virus is enhanced with associated treatment

An associated treatment with MC1937 and MC1947 in the infection with Wuhan strain of SARS-CoV-2 was performed to test if the inhibition effect would be increased. Results showed that the action was increased in 44% (p= <0.0001) compared to MC1937 alone and 42% (p=<0.0001) compared to MC1947 alone, inhibiting 90% of SARS-CoV-2 infection (p= <0.0001).

Figure 4. Results from experiment that analyze the potential of associated treatment with the peptides. Statistical difference between treatment with MC1937 and control; MC1947 and control, and both peptides and control. Statistical analysis by Dunnett's multiple comparisons test was performed. ****: p<0.0001.*: p<0.05; ****: p<0.0001; ns: non-significant.



3.5. MC1937 and MC1947 showed potential on entry step using infected A549 cells

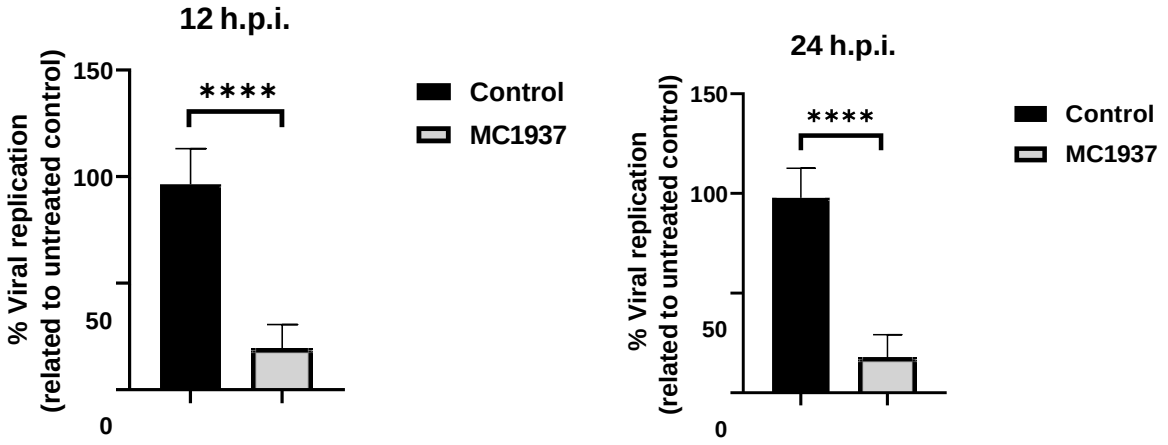
The inhibitory potential of the peptides was tested using A549 cells infected with SARS-CoV-2-mCherry. Fluorescence reading analyses showed that both peptides inhibited this step in the two times post-infection investigated. At 12 hours post infection (h.p.i.), MC1937 and MC1947inhibited 77% (p<0.0001, Fig.5B) and 84.6% (p<0.0001, Fig.5B) of the virus replication, respectively. At 24 h.p.i., the inhibition followed similar levels, representing 82.4%

(p<0.0001, Fig.5C) of decrease for MC1937 treatment, and 84.4% (p<0.0001, Fig.5C) for MC1947.

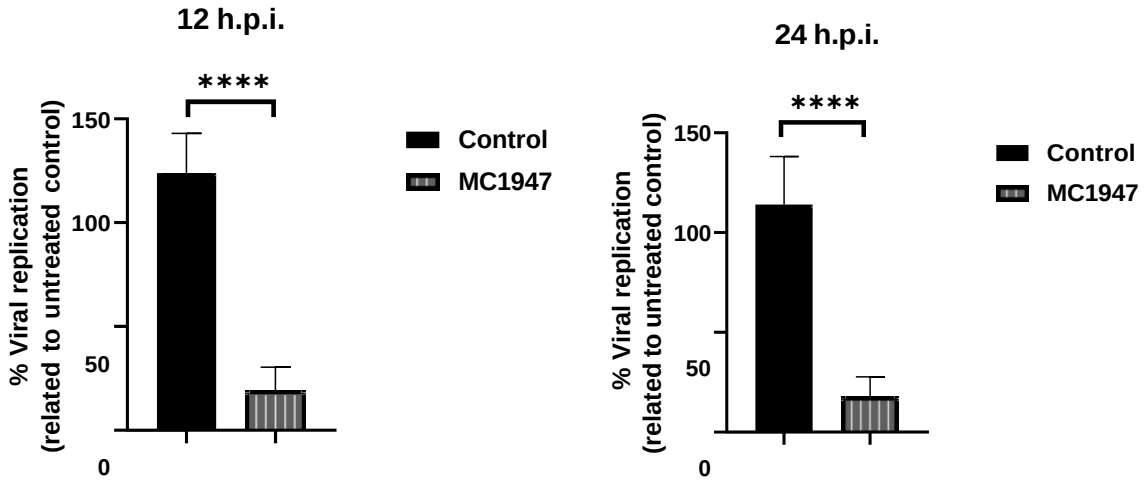
Figure 5. The effect of the peptides in entry step of SARS-CoV-2 replication cycle. **(A)** This experiment was performed by doing an infection with a solution containing the virus and the peptide/water(control) for one hour, and then the cells were washed two times with PBS solution, and media was added so the analysis could be performed in times 12 and 24 hours post infection. Percentage of inhibition for **(B)** MC1937 results for 12 and 24 hours post infection with SARS-CoV-2-mCherry. **(C)** MC1947 results for 12 and 24 hours post infection with SARS-CoV-2-mCherry. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. The Statistical Paired t test was performed to check significance of data. ****: p<0.0001. *: p<0.05; ****: p<0.0001; ns: non-significant.

(A)

(B)



(C)



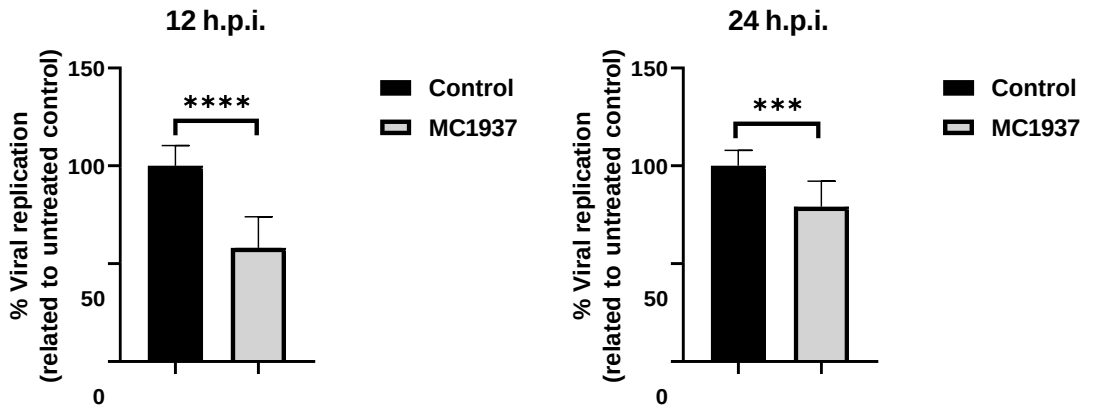
3.6. Both peptides have action on viral particle of SARS-CoV-2

An incubation of SARS-CoV-2-mCherry at MOI 5 and each peptide showed that MC1937 treatment resulted in inhibition of 42% (p<0.0001, Fig. 6B) at 12 h.p.i., and about 21.8% at 24 h.p.i. (p=0.0002, Fig. 6B). On the other hand, peptide MC1947 inhibited about 50% (p<0.0001, Fig. 6C) at the first time tested, and around 36% (p<0.0001, Fig. 6C). They both followed patterns of inhibition, in a way that they showed a higher effect for earlier hours post infection.

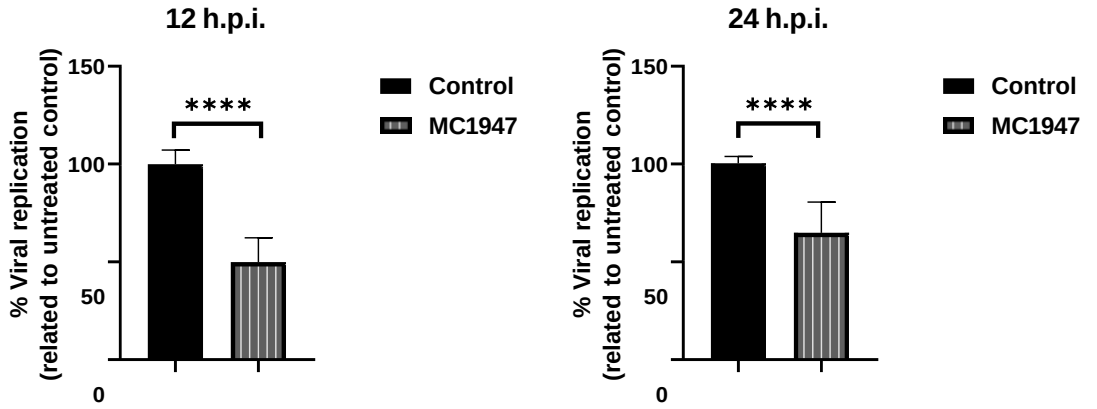
Figure 6. The virucidal effect of the peptides against SARS-CoV-2-mCherry. **(A)** This experiment was performed by doing an infection with a solution containing the virus at MOI 5 and the peptide/water(control) for one hour after incubating this solution for prior 1 hour at 37°C. Then, the cells were washed two times with PBS solution, and media was added so the analysis could be performed between 12 to 24 hours. Percentage of inhibition for both **(B)** MC1937 after 12 and 24 hours **(C)** MC1947 after 12 and 24 hours post infection with SARS-CoV-2mCherry. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. The Statistical Paired t test was performed to check significance of data. ***: p<0.001; ****: p<0.0001.; ns: non-significant.

(A)

(B)



(C)



3.7. Peptides showed potent prophylactic effect in A549 cells

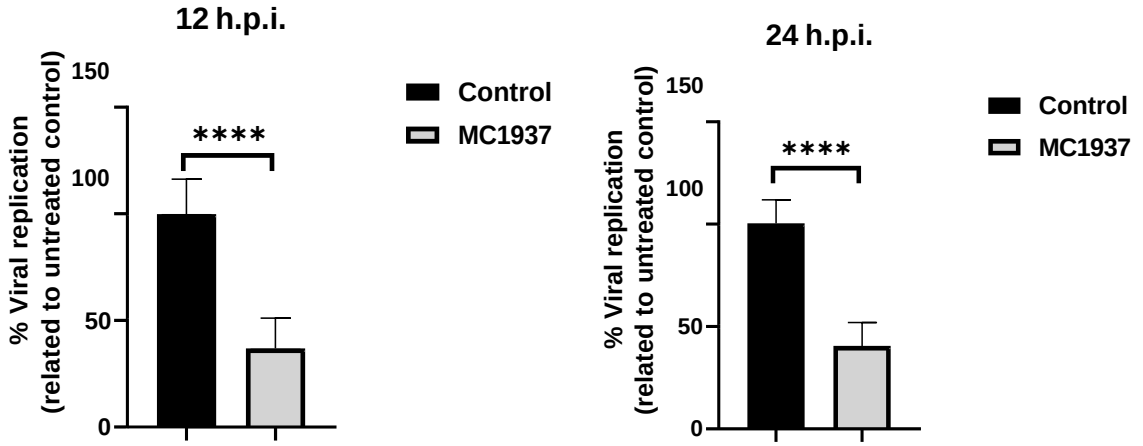
Pulmonary cells were treated before submitting to infection with SARS-CoV-2-mCherry to analyze a possible pre-treatment effect of the peptides. The results showed that MC1937 decreased viral replication in 64.1% (p<0.0001) at 12 h.p.i, and 60.5% (p<0.0001) at 24 h.p.i. MC1947 treatment was able to cause an inhibitory effect of 66% (p<0.0001) and 60% (p<0.0001) for 12 and 24 h.p.i., respectively.

Figure 7. The pre-treatment effect of the peptides against SARS-CoV-2-mCherry. **(A)** This experiment was performed by doing an initial treatment with a solution containing the peptide/water(control) for 1 hour, after that an infection with SARS-CoV-2-mCherry was performed for another hour. Then, the cells were washed two times with PBS solution, and media was added. Percentage of inhibition for **(B)** MC1937 pre-treatment 12 and 24 h.p.i., and **(C)** MC1947 pre-treatment 12 and 24 h.p.i. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation.

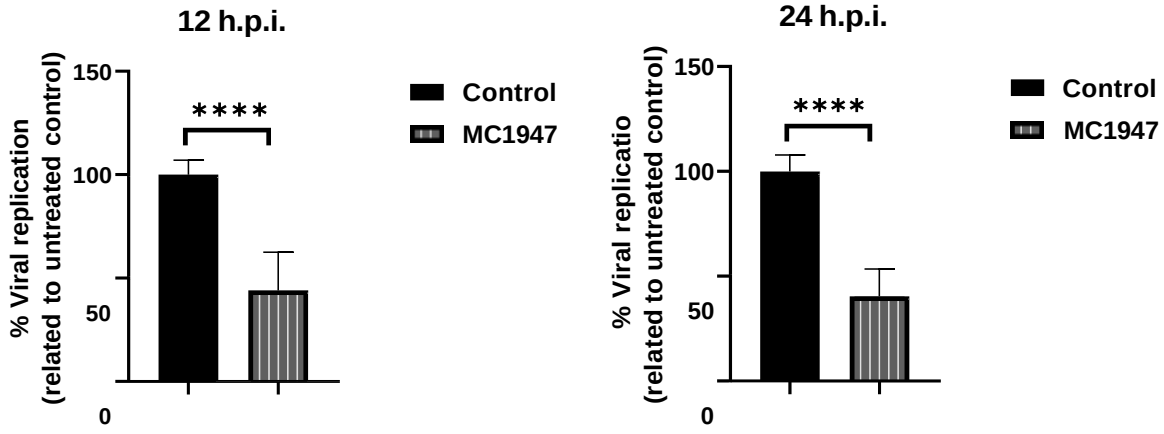
The Statistical Paired t test was performed to check significance of data. *: $p<0.05$; ****: $p<0.0001$; ns: non-significant.

(A)

(B)



(C)



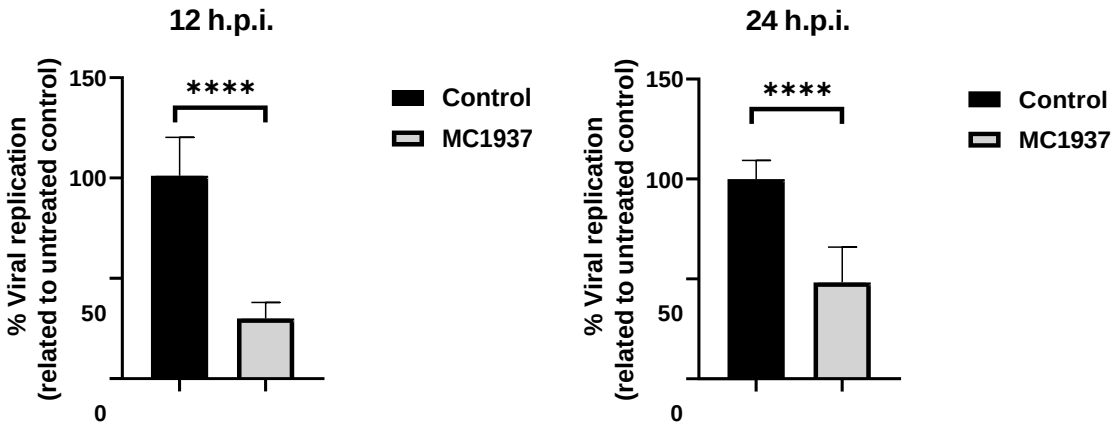
3.8. Peptides cause effect on attachment step of virus infection

A further investigation was conducted to better understand the effect of the peptides on the entry step of viral replication. The first analysis was the potential of the peptides to inhibit the attachment step of virus infection. The peptides inhibited t 70% ($p < 0.0001$) of viral replication 12 h.p.i. The inhibition of the SARS-CoV-2 entry decreased to 52% ($p<0.0001$) and 64% ($p<0.0001$) in the MC1937 and MC1947 treatments, respectively, 24 h.p.i.

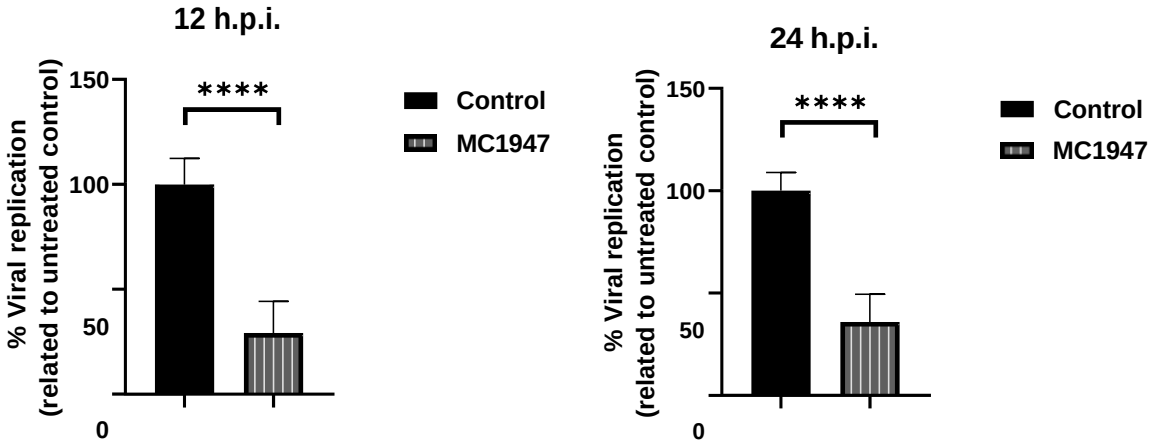
Figure 8. The effect of the peptides in the attachment step of the SARS-CoV-2-mCherry in A549 cells. **(A)** Attachment experiment was performed by doing an infection with a solution containing the virus and the peptide/water (control) for one hour at 4°C, and then the cells were washed two times with PBS solution, and fresh media was added. Results this experiment **(B)** Inhibition of the attachment step of SARS-CoV-2-mCherry with the MC1937 treatment 12 and 24 h.p.i. and **(C)** Inhibition of the attachment step of SARS-CoV-2-mCherry with the MC1947 treatment 12 and 24 h.p.i.. Results were expressed in percentage of cells treated related to the control (sterile water). Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. The Statistical Paired t test was performed to check significance of data. ****: $p<0.0001$. *: $p<0.05$; ****: $p<0.0001$; ns: non-significant.

(A)

(B)



(C)

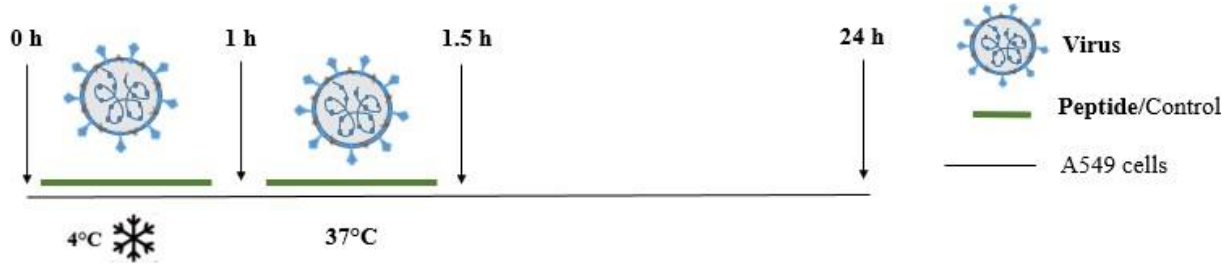


3.9. Peptides also impair internalization of virus

The internalization step was also studied, and results showed that both peptides inhibited 73-74% (p<0.0001) of replication levels at 12 h.p.i. The peptides also inhibited 66% (p<0.0001) of the internalization step of the SARS-CoV-2-mCherry when analyzed 24 h.p.i.

Figure 9. To better understand the specific effect of both peptides resulting from the entry step, the internalization analysis was performed**. (A)** First, an infection with a solution containing the virus and the peptide/water (control) was added to the cells for one hour at 4°C, and then the cells were incubated for 0.5 hour at 37°C. After that, cells were washed, and medium was added for incubation for 24 hours. Percentage of inhibition for **(B)** MC1937 is demonstrated after 12 and 24 hours **(C)** MC1947 after 12 and 24 hours post infection with SARS-CoV-2 WT mCherry infectious clone. The results are demonstrated in percentage of cells treated related to the control (sterile water). Experiments were conducted in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. The StatisticalPaired t test was performed to check significance of data. ****: p<0.0001.*: p<0.05; ****: p<0.0001; ns: non-significant.

(A)



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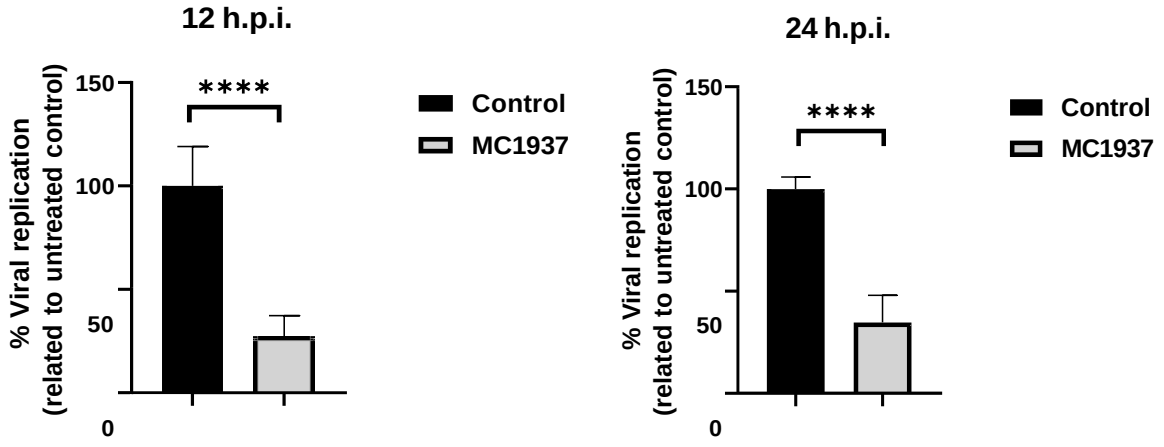

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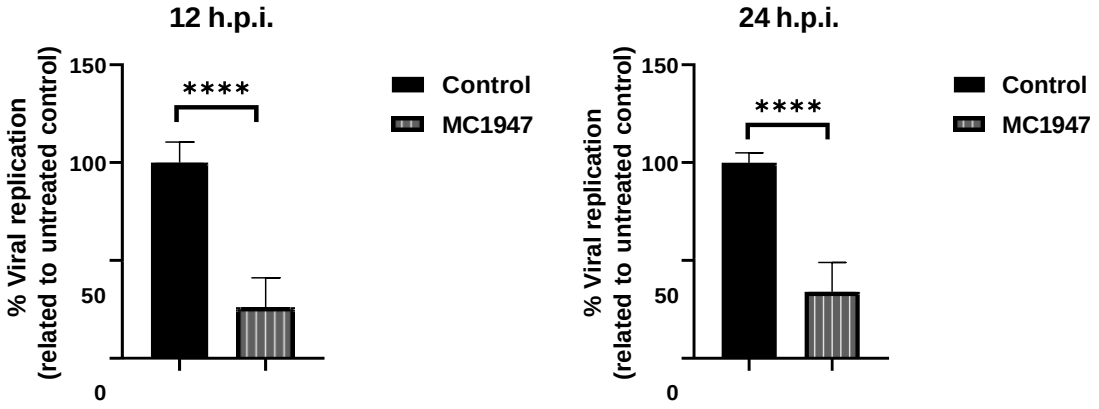


3.10. Peptides also have action on post-entry steps of viral replication

The peptides' action was also evaluated in the steps after virus entry into the cells. The results showed that both peptides have a high inhibition effect on post-entry steps. For the peptide MC1937, the effect represented 87% (p=0.0001) of decrease in viral replication, for 12 hours post infection, and 93.7% (p<0.0001) for 24 hours, so the effect was increased over the time post infection. For the peptide MC1947, the inhibition varied in 93- 94% (p=0,0001 and p<0,0001) in 12 and 24 h.p.i.

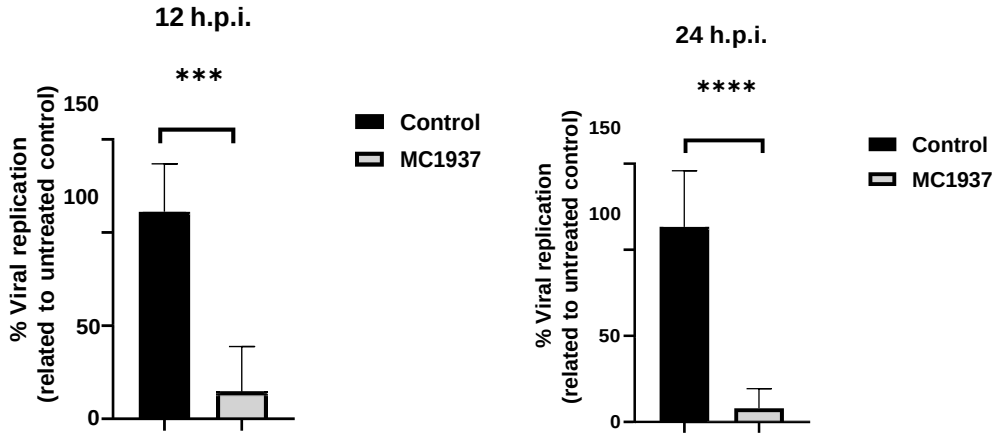
Since both peptides showed to have a strong effect on stages after entry into cells, they were tested for action against the subgenomic replicon. Results showed that peptide MC1937 was able to inhibit at both concentrations tested for twenty-four hours of treatment, and only the higher concentration for forty-eight hours (Fig.S3A). On the other hand, the peptide MC1947 demonstrated to be able to inhibit at both concentrations only for the forty-eight hours treatment (Fig.S3B).

Figure 10. Results from experiment to analyze peptides effect on steps post-entry. **(A)** For this experiment, first an infection was performed for 1 hour, and then cells were washed twice with

PBS, after that medium was added and cells were incubated for 24 hours. Percentage of inhibition for **(B)** MC1937 is demonstrated after 12 and 24 hours **(C)** MC1947 after 12 and 24 hours post infection with SARS-CoV-2 WT mCherry infectious clone. The results are demonstrated in percentage of cells treated related to the control (sterile water). Experiments were conducted in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. The Statistical Paired t test was performed to check significance of data. ***: $p<0.001$; ****: $p<0.0001$; ns: non-significant.

(A)

(B)



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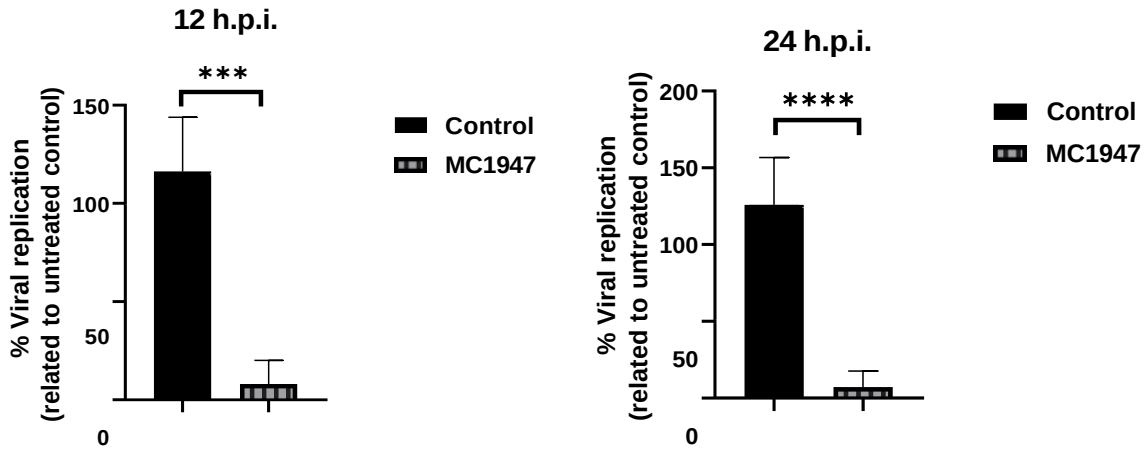
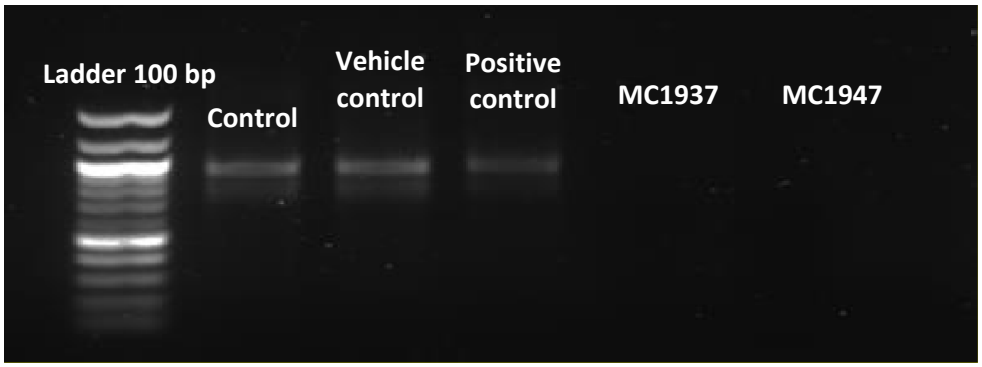


3.11. MC1937 and MC1947 cause high inhibition in double strand RNA in vitro

Treatments with peptides MC1937 and MC1947 showed very similar results in the action of them against the double strand RNA, tested *in vitro*. As it is possible to see in figure 11, there is no trail of the band resulted from each peptide treatment (Fig.11A), only the control ones and a slightly trail of the positive control. Bands density was also calculated to quantify these results, and the percentage of inhibition is around 100% (p=0.0024) for both treatments (Fig. 11B).

Figure 11. Results from a treatment of the peptides in the double strand RNA *in vitro*. **(A)** 1% agarose gel that shows control (only double strand RNA), vehicle control (treatment with water which is the dilution reagent of the peptides), positive control (C+, represented by treatment with Platinum), MC1937 (treatment with peptide MC937), and MC1947 (treatment with peptide MC1947). **(B)** Quantification of the density of the bands demonstrated by percentage of treated cells in relation to the control. The experiments were carried out in three independent events, and data are presented as the mean ± standard deviation. Ony way anova multiple comparisons test was performed, ns: non-significant; **: p<0.001. *: p<0.05; ****: p<0.0001; ns: non-significant.

(A)



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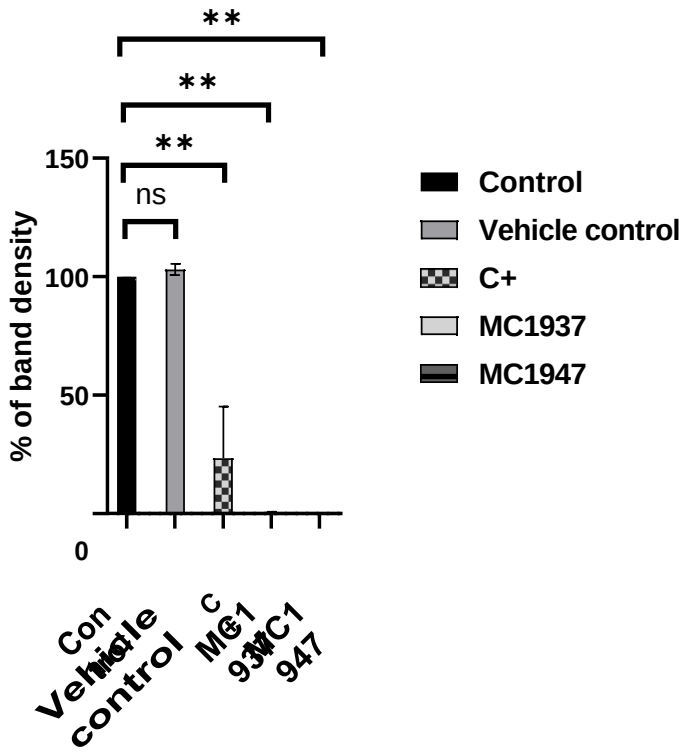
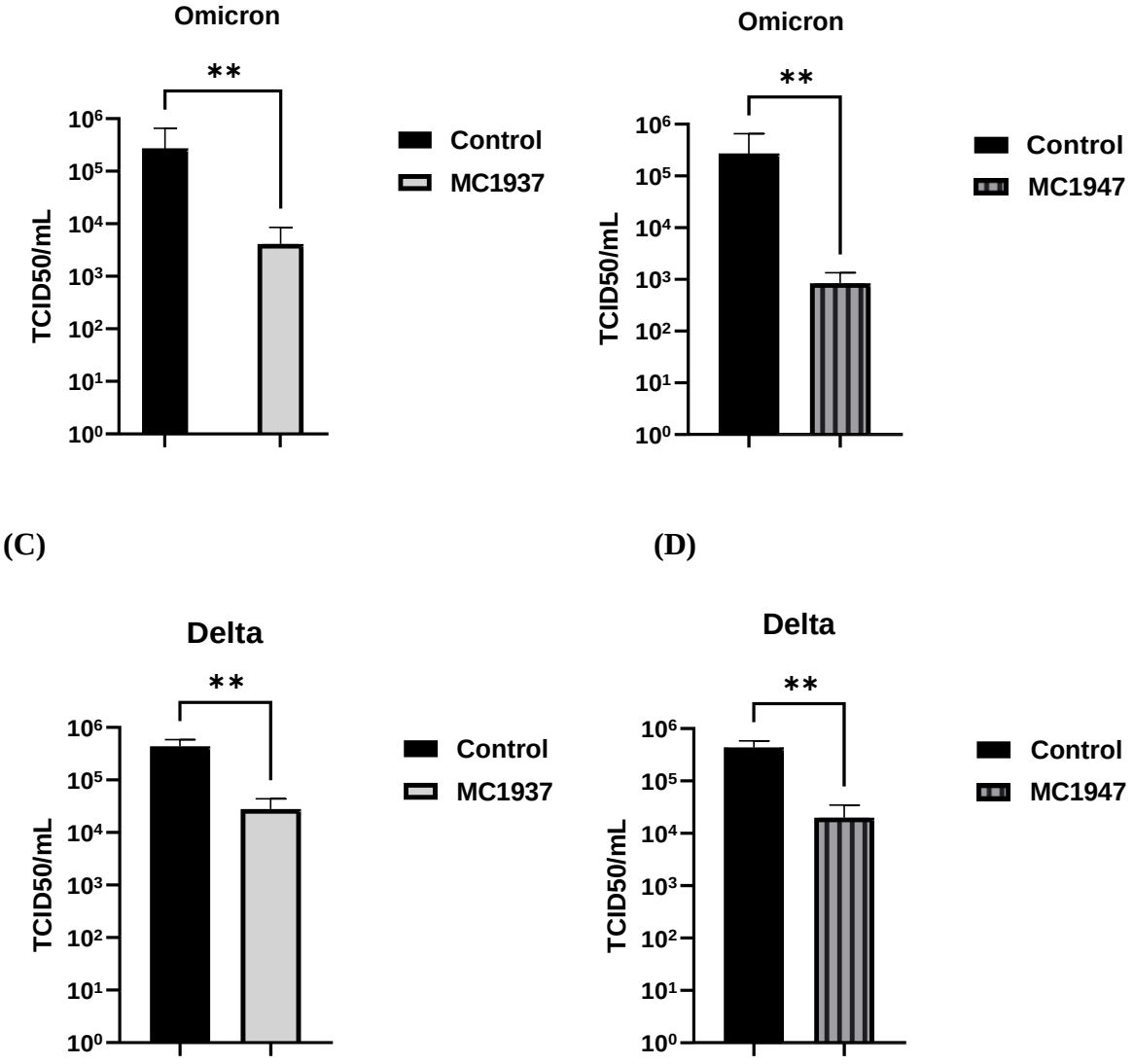


3.12. Cell penetrating peptides also showed action against SARS-CoV-2 variants

Results of the inhibition resulted from each treatment were obtained after a TCID50 of the supernatants that quantified the general assay with the variants Omicron and Delta. The peptides MC1937 and MC1947 showed to have action against both variants during the infection with each one of them. Regarding Omicron, MC1937 impaired around 95% (p=0.0041, Fig.12A) of SARS-CoV-2 infection, and MC1947 95.5% (p=0.0033, Fig.12B). For SARS-CoV-2 Delta variant, we observed that MC1937 was able to inhibit 94% (p=0.0022, Fig.12C) of this variant infection, and MC1947 t 96% (p=0.0022, Fig.12D).

Figure 12. Results from a general effect experiment to test the action of the peptides against two SARS-CoV-2 variants. A549 cells were infected with SARS-CoV-2 Omicron variant and treated with **(A)** peptide MC1937 and **(B)** MC1947. These cells were also infected with SARS-CoV-2 Delta variant and treated with **(C)** MC1937 and **(D)** MC1947. The experiments were carried out in three independent events, and data are presented as the mean ± standard deviation. Mann-Whitney and Paired t test were performed, **: p<0.001.

(A) **(B)**



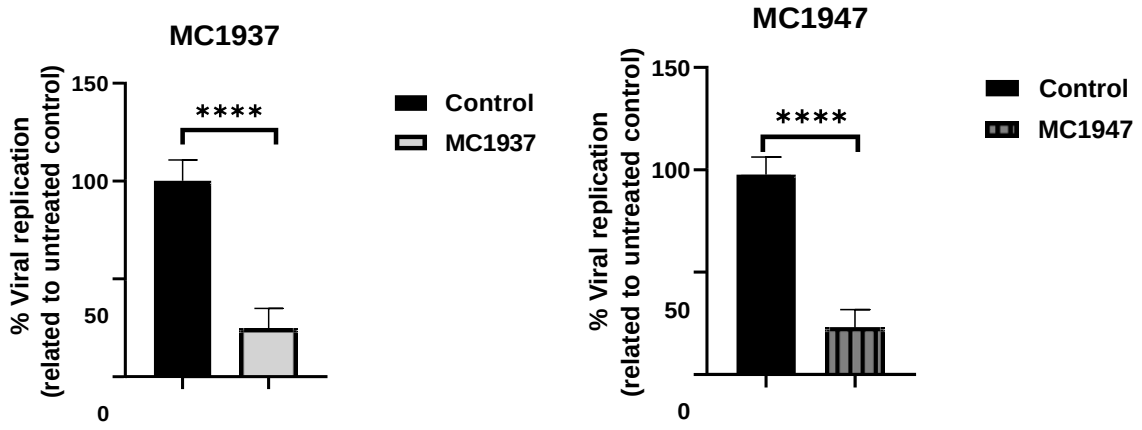
3.13. Cell penetrating peptides have effect in different steps of viral replication during SARS-CoV-2 Omicron variant infection

Each peptide was also tested for the understanding of the action in each step of the replication cycle of the Omicron variant.

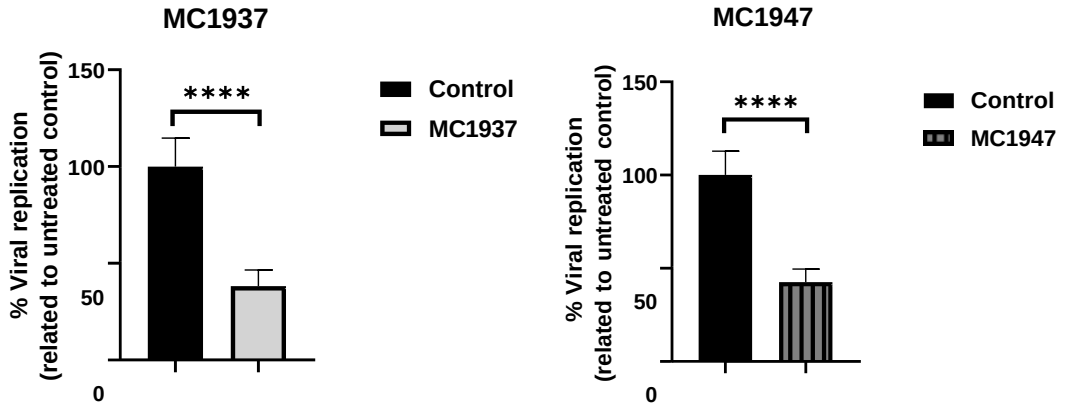
Results showed that both peptides were able to inhibit the entry step. MC1937 caused an inhibition of 65% (p<0.0001, Fig.13A), and MC1947 75% (p<0.0001, Fig.13A). Regarding a possible protective effective these peptides might be provoking prior infection with the variants, it is possible to notice that for SARS-CoV-2 Omicron variant, both peptides were able to cause this effect by 62% (p<0.0001, Fig. 13B) for MC1937 treatment and 56% (p<0.0001, Fig. 13B) for MC1947. The step with majority of effect caused by the peptides was the post-entry, representing an inhibition of 96% (p<0.0001, Fig. 13C) by MC1937 (p<0.0001, Fig. 13C) and 100% by MC1947. A virucidal was also observed, in a way that MC1937 showed 61% (p= 0.0002, Fig. 13D) of inhibition and MC1947 70% (p<0,0001, Fig. 13D).

Figure 13. Results from experiments of action of peptides in the replication cycle, using A549 cells infected with a recombinant infection clone with replaced S-protein from Omicron. (A) Entry step inhibition assay of MC1937 and MC1947 peptides (B) Protective analysis of MC1937 and MC1947 peptides. (C) Virucidal assay for MC1937 and MC1947 peptides. (D) Post-entry analysis for MC1937 and MC1947 peptides. All experiments were performed using the same methodology described previously. Quantification analyses were performed by percentage of fluorescence measure at 24 hours after treatment. Results were expressed in percentage of cells treated related to the control (sterile water). Statistical Paired t test was performed to check significance of data. ***: p<0.001; ****: p<0.0001.

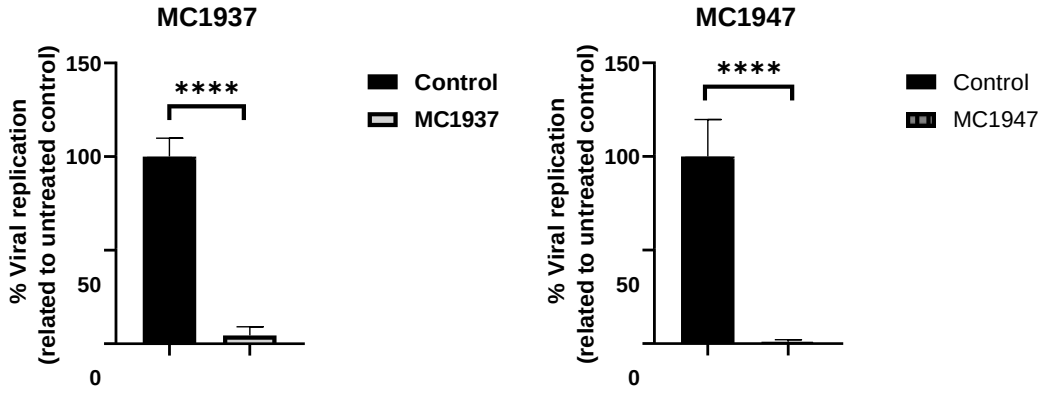
(A) Entry



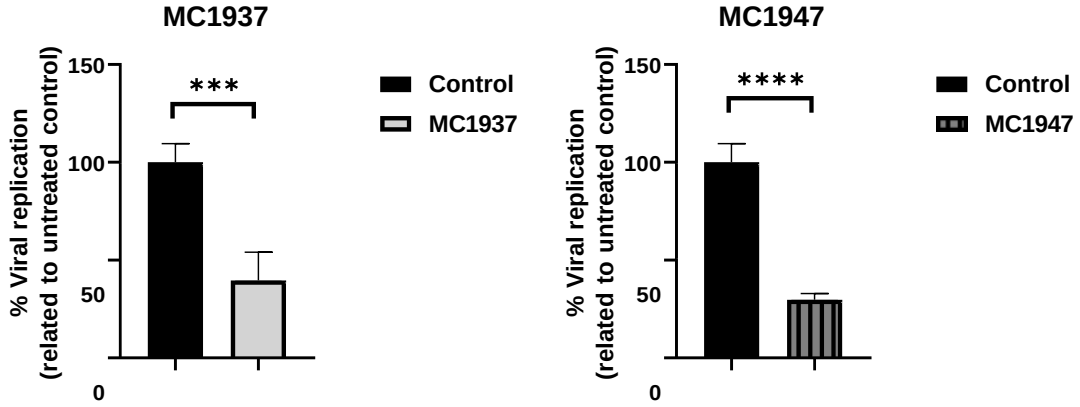
(B) Pretreatment



(C) Post-entry



(D) Virucidal



3.13. Cell penetrating peptides have effect in different steps of viral replication during Delta variant infection

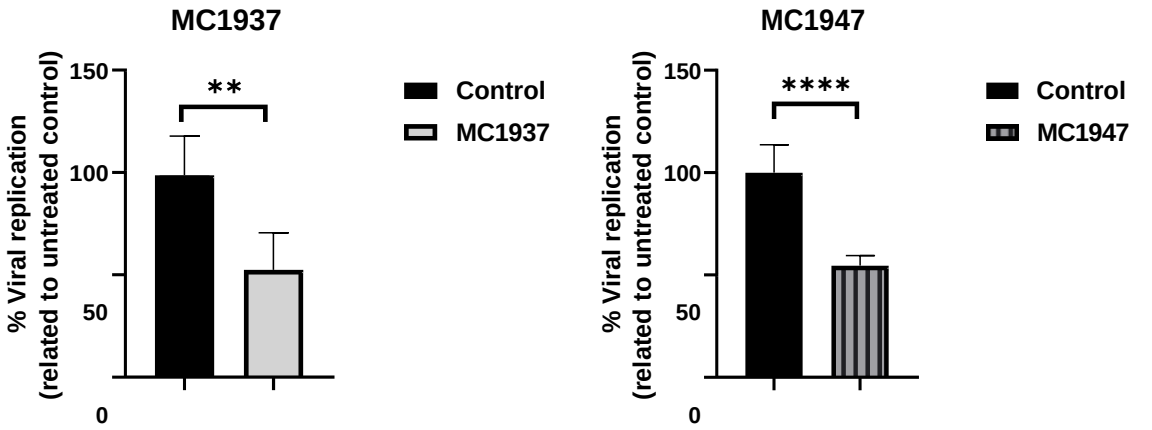
The same analysis described in the previous item was also performed for Delta variant. It was observed that the results were very similar to the Omicron variant, except for virucidal effect.

MC1937 inhibited in 46% (p= 0.0024, Fig. 14A) the entry step and MC1947 was able to inhibit in 45.5% (p<0.0001, Fig. 14A). Regarding the protective effect, the inhibition percentage was of 30% for MC1937 (p= 0.0044, Fig. 14B) and 40% (p= 0.0051, Fig. 14B) for MC1947. The post-entry was also impaired high for this variant, MC1937 caused an effect of 100% (p <0.0001, Fig. 14C) and MC1947 of 94% (p <0.0001, Fig. 14C) of inhibition. Finally, peptide MC1937 was not able to cause a statistically significant difference for the virucidal analysis, but MC1947 could cause a minimal effect of 10% (p=0.0222, Fig. 14D), by that we can conclude that they do not have a representative virucidal action against the variant delta.

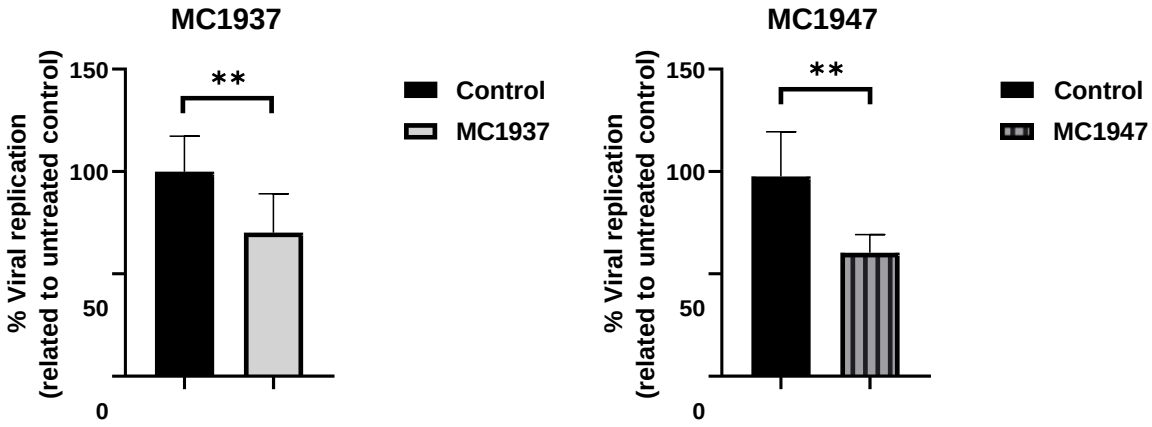
Figure 14. Results from experiments of action of peptides in the replication cycle, using A549 cells infected with a recombinant infection clone with replaced S-protein from Delta. (A) Entry step inhibition assay of MR1903 and PE1940 peptides (B) Protective analysis of MR1903 and

PE1940 peptides. (C) Virucidal assay for MR190 and PE1940 peptides. (D) Post-entry analysis for MR1903 and PE1940 peptides. All experiments were performed using the same methodology described previously. Quantification analyses were performed by percentage of fluorescence measure at 24 hours after treatment. Results were expressed in percentage of cells treated related to the control (sterile water). Statistical Paired t test was performed to check significance of data. ns: non-significant; *: $p<0.05$; **: $p<0.01$; ****: $p<0.0001$.*: p<0.05; ***: p<0.0001

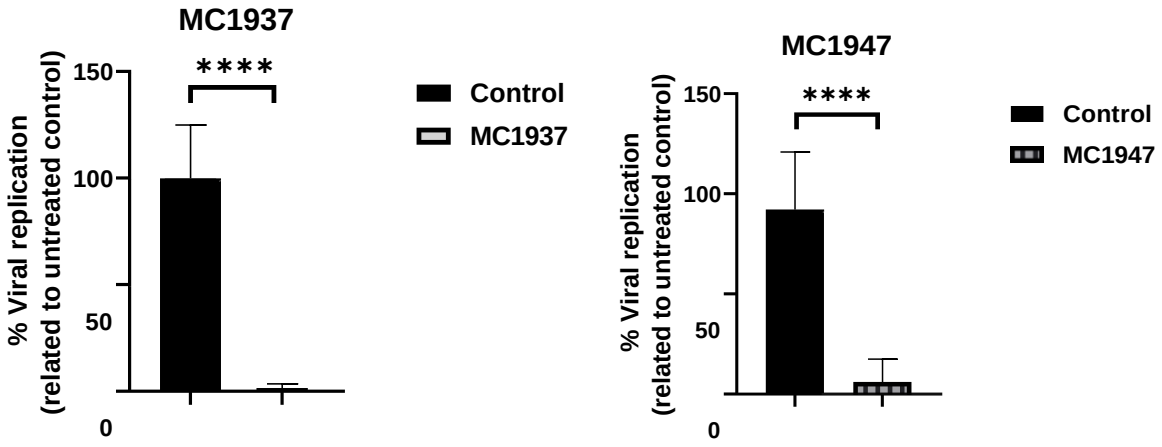
(A) Entry



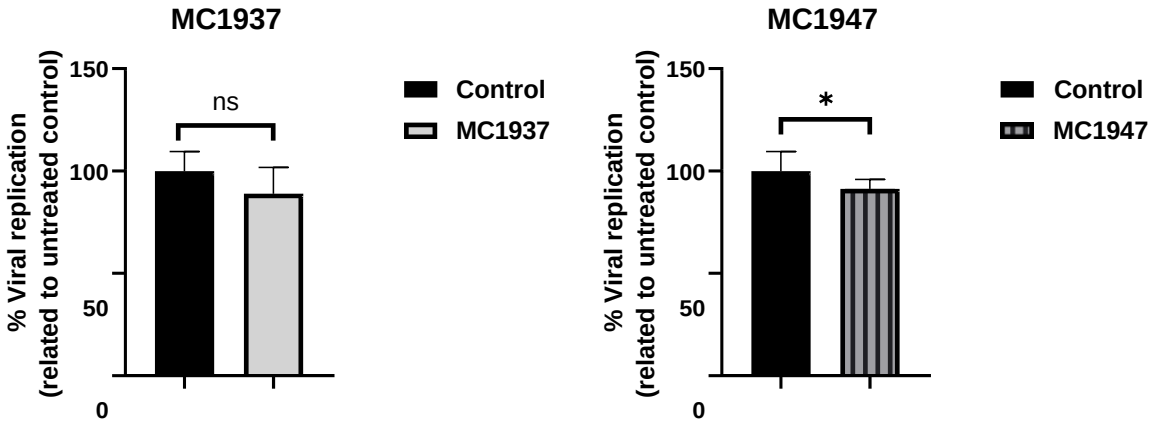
(B) Pretreatment



(C) Post-entry



(D) Virucidal



3.14. Virus can achieve resistance to treatment with peptides after six passages

Aiming to understand if the virus would become resistant to treatment with the peptides, a drug resistance assay was performed. For that, a treatment with each peptide was added to the cells, associated to the infection with the Wuhan original strain. This procedure was repeated until a different result was observed on the titer of the virus, in other words, up to the treatment with the peptide does not result in inhibition.

After six passages of the virus being treated with each peptide, it was observed that the value of PFU/mL was the same for control and treatments (Fig.15), which means that there is no inhibition happening anymore. To confirm this result, a seventh passage was performed, and results were confirmed.

The next step performed was to analyze how this resistance was affecting the virus genome. For that, the samples from the last passage were taken to sequencing.

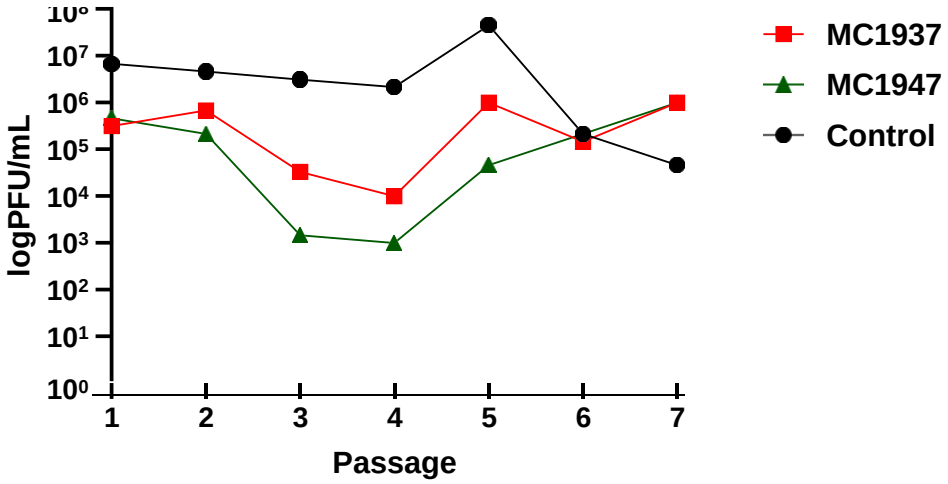
Whole genome sequencing showed a synonymous single nucleotide polymorphism (SNP) T>G at position 23605 (AA 681) of the SARS-CoV-2 genome, for both peptide

treatments immediately preceding the furin cleavage site [38] (Fig.S4). However, compared to the virus passaged without any treatment, a control with regard to any adaptations of the virus during replication in the cells, we observed a 30 base/10 amino acid deletion of the furin cleavage site, which can make it hard to better understand how this nucleotide change can be related to virus resistance.

An additional non-synonymous T>A SNP was seen at position 26354 (Fig.S5), effecting a L37H E protein mutation in both peptides and the passage control.

A final deletion of 45 nucleotides / 15 AA (delta 7-21) was observed in ORF6 in both peptide treatments (Fig.S6), as well as in control for mutation error in this case.

Figure 15. Results from seven passages after drug resistance of the peptides using A549 cells infected with Wild-type SARS-CoV-2. A TCI50 experiment was performed for quantification using each sample resulted from treatments. With each infection, a solution containing the treatment with peptides MC1937 and MC1947 at concentrations selected by previous cell viability assay was added to the cells for seven times.



4. Discussion

Despite the cell penetrating peptides have demonstrated potential as a carrier for molecules for drug testing against microorganisms or cancer [39], in this work, we demonstrated their antiviral effects in different steps of viral replication of SARS-CoV-2, including two variants, Omicron and Delta.

The inclusion of cell penetrating characteristic to the peptide structure was expected to bring more specific action on the several steps of virus replication, since they can cross the cell

membrane easier by endocytosis or direct penetration into cells [40]. Interestingly, bothpeptides were able to strongly impair SARS-CoV-2 infection using both cell lines Vero and A549 and show potential as inhibitors in different stages of the replication cycle, but mostly inthe post-entry step.

The peptides showed a dose dependence effect, with MC1937 demonstrating slightly less cytotoxicity than MC1947, and that also resulted in a higher value of selective index. This difference between the effects observed could be related to the variation of sequence of peptide conjugated to the dimeric one, since MC1937 and MC1947 vary their structure mainly on this. The amino acid added to the molecule can lead to different results to the cells [41].

It was also possible to confirm in our study that, as it shows in the literature, more than one compound treatment can result in an effectiveness therapy [42], since when it wascombined both peptides in a treatment, the response was a higher effect in the associated therapythan the individual one.

Regarding the entry step of viral replication, both peptides were able to impair this stage, for all strains of SARS-CoV-2 tested, so besides the Wuhan one, also Omicron and Delta. The ability to inhibit this step can be related to the potential against specific receptors and proteins of this virus, as per example the spike protein, the main know drug target studied [43]. This virus can entry into cells via a process very similar to SARS-CoV [44]. The two domains (S1 and S2) of the spike protein play roles during entry, that can be followed by two pathways for virus internalization to the cells (endosomal or endosomal) [45]. Interestingly, MC1937 and MC1947 were taken to investigation for the entry action, and besides acting on attachment, they were also able to inhibit internalization of the virus, then representing a potential to disturb one of the pathways cited.

These peptides also showed a protective action against SARS-CoV-2 infection. This means they are interacting with important receptors presented in the cells, like angiotensin-converting enzyme 2 (ACE2) [44], or even others as Glucose regulated protein 78 and Ezrin, that demonstrated importance during viral infection [46]. However, ACE2 represents the most present receptor used for SARS-CoV-2 [47]. And, since these peptides are acting in entry and as protectors of cells prior infection, they may be interacting specifically with this enzyme.

Both peptides also showed virucidal potential. They were able to disrupt viral particle for the Wuhan and Omicron strains, however MC1937 did not show effect for Delta and MC1947 was also had a minor action in Delta. The delta variant has mutations on the spike

protein that configures it to be more transmissible than the others, and some of these mutations can even be related to a higher affinity to the ACE2 receptor [48,49]. Here, we hypothesized that the modifications on spike protein of this variant resulted in less interactions between peptides and this variant before infection.

Even though these peptides showed antiviral effects against all these steps of SARS-CoV-2 replication cycle, we observed the major effect in the post-entry stages. They were able to cause high levels of inhibition after the virus entered the cells. There are different parts of the virus replication process after inside the cell that MC1937 and MC1947 could be acting on, like already stablished targets, that are essential during virus replication, as the proteases: papain-like (PLpro) and main (Mpro), also the RNA-dependent RNA polymerase (RdRp) [50,51]. The dimeric peptide has already showed a specific effect on the papain-like protease, so these peptides with addition of cell-penetrating characteristic could be even increasing this effect [52]. Our work also tested the peptides against a subgenomic SARS-CoV-2 replicon to better understand the post-entry effect. This replicon contains the ORF1a and ORF1b of this virus genome, that constitute the polyproteins pp1a and pp1ab, and it will have as result the formation of very important structural and accessory proteins for this virus [53]. By that, it is possible to see how this represents a drug target, and both peptides were effective against it. Furthermore, MC1937 and MC1947 showed a strong inhibition of the double strand RNA of SARS-CoV-2 that was tested, what characterizes these peptides as RNA interferents.

An effective treatment can continue showing action even after several passages of the virus. And the peptides demonstrated to be able to impair SARS-CoV-2 infection until six passages of SARS-CoV-2 infection. After that, the virus started to adapt to the therapy, and it is no longer able to decrease viral replication.

Although the after passaging sample showed a deletion on the region at position 26354 of modification for treatments with peptides, a study in the literature shows that mutations on F26L, L39M, and L37H is associated with blocking of E protein, ion channel activity, and induces viral attenuation [54]. Interesting, other studies also reported aa mutations in 10 sites (aa positions: 26,36, 37, 39, 46, 58, 68, 71, 72, and 73) of the E protein, what corroborates with our current findings [55,56].

Another observation was at position 23605, this region is part of spike protein (21563-25384). A study showed that P681 mutations are associated with the spike protein replacement from Alpha to Delta variants, and this modification in this protein is related to increase of viral

infection [57,58] Furthermore, another study demonstrated that this mutation causes the reduction of O-glycosylation, and consequently it is associated with improvement of furin cleavage and the formation of syncytia [59]. However, the sample that represents the virus being passaged without the treatment has a deletion in this region, so it is not possible to confirmhow this mutation can be related to virus resistance to these peptides treatment, for that recombinants clones would have to be constructed and treatment comparison would have to bemade.

5. Conclusion

In conclusion, the present work showed this additional cell-penetrating peptides to the dimeric peptide resulted in multiple effects on virus replication of SARS-CoV-2. The peptides MC1937 and MC1947 have very similar actions, and their associated treatment increases inhibition even more. Besides being able to impair Wuhan original strain infection, they are also effective against two important variants (Omicron and Delta). Their mode of action is related to several already established drug targets for SARS-CoV-2. In this way, the presented peptides showed to be potential anti-SARS-CoV-2 compounds.

Acknowledgement

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Funding

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Supplementary material

FS1. Primers designed for the double strand RNA experiment performed. (A) Forward and reverse oligonucleotide primers with (B) and without T7 promoter (A).

(A)

SARS-CoV-2 ORF1 Foward	GACCGAAAGGTAAGATGGAG
SARS-CoV-2 ORF1 Reverse	AAATCGCCCGTCTGCCATGAAG

(B)

SARS-CoV-2 ORF1 Foward+T7	TAATACGACTCACTATAGGGGACCGAAAGGTAAGATGGAG
SARS-CoV-2 ORF1 Reverse+T7	TAATACGACTCACTATAGGGAAATCGCCCGTCTGCCATGAAG

FS2. Cell viability of Vero, BHK-21, and A549 after treatment with peptides **(A)** MC1937 and **(B)** MC1947 for 24 hours. Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. The line was traced in 80% of cell viability to represent the concentration choice for the antiviral experiments. Control is the treatment with only DMEM. Water control is the treatment with water, as it is the dilution for the peptides.

(A) **(B)**

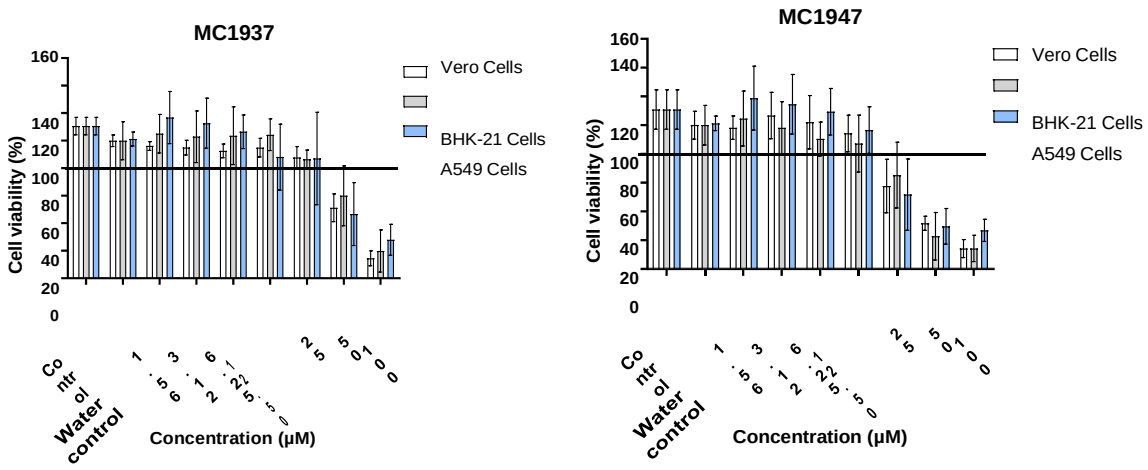
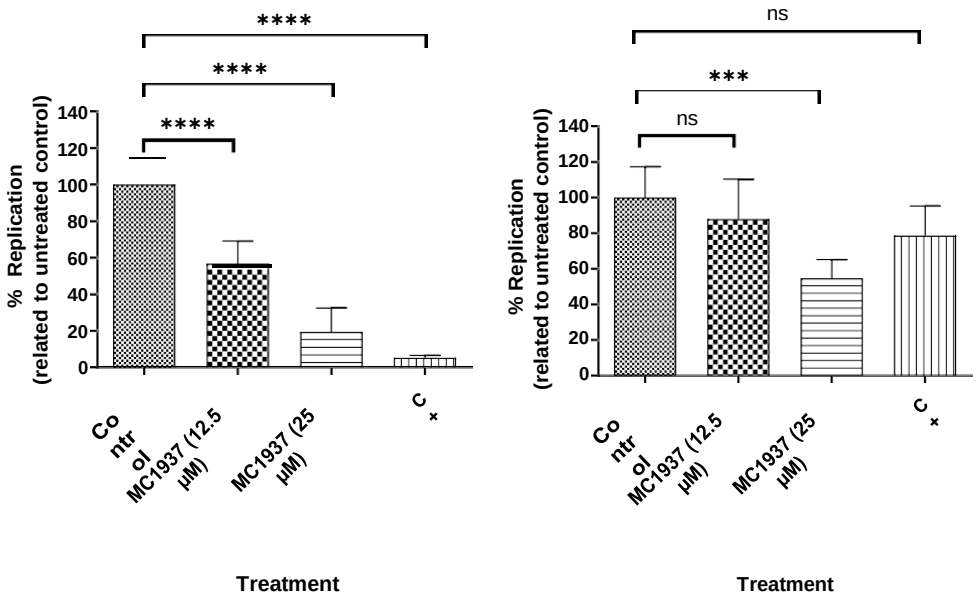


Fig S3. Results from the replication effect caused by both peptides, using the subgenomic replicon for SARS-CoV-2. Cells BHK-21 were transfected, and then treated with more than one concentration to analyses efficiency of action for them for two periods of time (24 and 48 hours). **(A)** Results for peptide MC1937 at several concentrations tested: 12.5 µM and 25 µM. **(B)** Results for peptide MC1947 at several concentrations tested: 12.5 µM and 25 µM. The

results observed are expressed in percentage of cells treated related to the control (sterile water). All experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. A positive control (C+, molnupiravir) for replication effect against SARS-CoV-2 was used. Tukey's Multiple Comparison Test was performed for these analyses. *: $p<0.05$; **: $p<0.001$

(A)



(B)

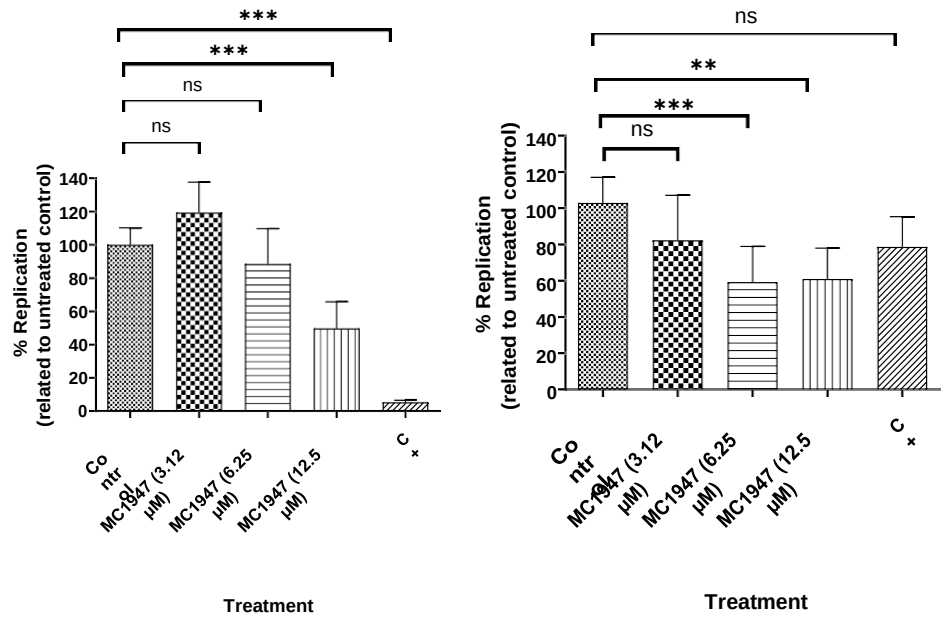


Fig.S4. Alignment of sequencing results from SARS-CoV-2 initial passage, after passages, and after treatment with peptides MC1937 and MC1947.

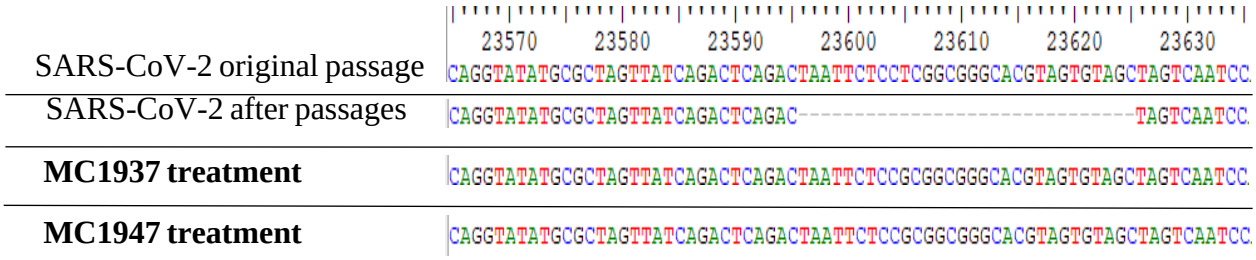


Fig.S5. Alignment of sequencing results from SARS-CoV-2 initial passage, after passages, and after treatment with peptides MC1937 and MC1947.

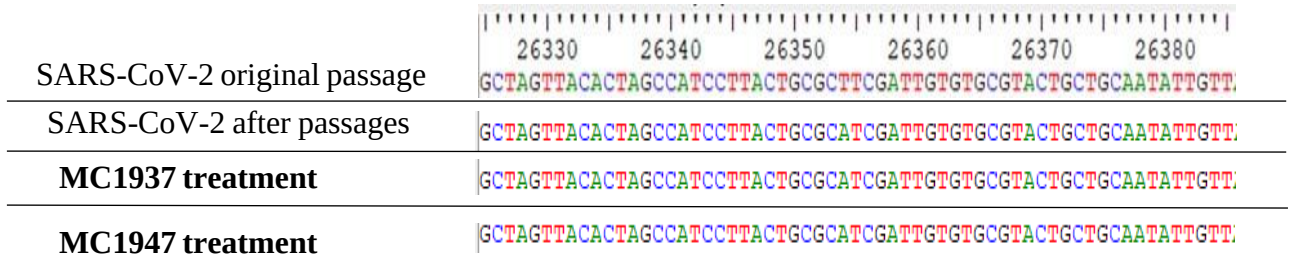
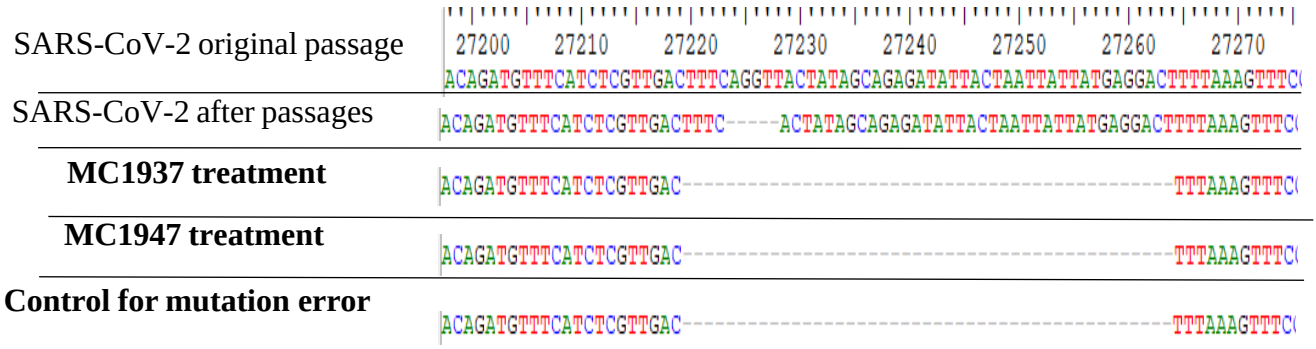


Fig.S6. Alignment of sequencing results from SARS-CoV-2 initial passage, after passages, and after treatment with peptides MC1937 and MC1947.



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CAPÍTULO IV – Artigos científicos

Manuscrito III

In vitro study reveals Dextrogyre of dimeric peptide as a promising antiviral against SARS-CoV-2

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Abstract

In December of 2019, the first cases of patients infected with a new coronavirus were identified. Later named as SARS-CoV-2, this virus rapidly spread worldwide and caused the infection of millions of people and the death of a great part of the population. Because of that, there is still an urgency of studies to analyse the potential of antivirals against SARS-CoV-2, and peptides have showed to be an interesting field to explore. Peptides can be formed by different conformations of amino acids, like D-amino acids and L-amino acids, and that can result in different results of antimicrobial activity. Because of that, here we tested an isomer D-amino acid of the dimeric peptide $(KKYRYHLKPF)_2K$, with already confirmed action against SARS-CoV-2. The peptide was able to cause a prophylactic effect and action in the inhibition of steps post-entry of the SARS-CoV-2 replication cycle, also being to degrade double strand RNA of SARS-CoV-2, demonstrating a very similar mechanism of action as the dimeric peptide. However, experiments performed using SARS-CoV-2 constructions with replacement of the spike protein from Omicron and Delta variants, as well as with the SARS-CoV-2 virus from these variants, showed that the dextrogyre peptide has no action against these variants. By that, we can confirm that this peptide is able to inhibit the Wuhan original strand of SARS-CoV-2.

Keywords: Antiviral, SARS-CoV-2, peptide, D-amino acids.

1. Introduction

Identified in January of 2020 as SARS-CoV-2, this new coronavirus leaded to the COVID-19 pandemic [1]. It belongs to the genus Betacoronavirus, and it has a positive-sense and single-stranded RNA [2]. Even though, vaccines are already available, it is still of great importance the search of compounds that can demonstrate effeteness against this virus, as there are still the severe disease cases and immunocompromised patients [3].

As a result of the emergence of SARS-CoV-2, the search for antivirals against coronavirus have increased even more. Because of the similarities between SARS-CoV and SARS-CoV-2, some compounds like Nelfinavir that showed action against SARS-CoV was also tested for SARS-CoV-2 [4]. However, different new potent compounds were found [5], like Arbidol [6], Lopinavir [7], and Remdesivir [8]. Furthermore, the use of peptides against coronavirus has demonstrated a successful growing field [9].

Antimicrobial synthetic peptides have demonstrated advantages over other compounds, with characteristics as a lower toxicity and a high potential of interactions with pathogens drug targets [10]. Besides having action against bacteria [11,12] and fungus [13]. This class of peptide has already also shown activity against several viruses as Dengue [14], Influenza [15], HIV [16], MERS [17], and even SARS [18].

Peptides are characterised for being composed by a sequence of amino acids. The peptide designed for this work is an isomer of the dimeric peptide $(KKYRYHLKPF)_2K$, but it contains D-amino acids instead of L-amino acids, which is the case of the peptide used in our group previous work [19]. The stereochemistry of molecules explains these two configurations existent for the amino acids in peptides, and it can also be named two enantiomers of a molecule [20]. D-enantiomers demonstrated to have action against bacteria as L- enantiomer peptides [21].

Since it is reported that there are peptides containing D-amino acids with a high antimicrobial potential [20], in the present work, we aimed to analyse if the isomer of the dimeric peptide $(KKYRYHLKPF)_2K$ would also be able to inhibit SARS-CoV-2 activity, presenting then an antiviral potential.

2. Material and methods

2.1. Peptide synthesis

Peptides were synthesized manually through solid-phase peptide synthesis (SPPS) following the methodology outlined by Merrifield in 1963 and Bittencourt in 2023 [33,34] . Basically, to obtain dimers and tetramers, Fmoc-Lys (Fmoc)-OH amino acid was employed at the initiation of synthesis as a branching point, allowing peptide chains to grow from the α-amino and ε-amino groups of lysine, as detailed by Lorenzón et al., 2012, and Santos-Filho et al., 2021 [35,36]. Post-synthesis, the peptides underwent purification via semi-preparative HPLC using a Shimadzu chromatograph (Tokyo, Japan) equipped with a C_{18} Jupiter column measuring 25 × 1 cm and featuring a particle size of 10 µm. The purity of the obtained materials (<95%) was assessed through analytical HPLC on a Shimadzu chromatograph (Kyoto, Japan) with a reverse-phase C_{18} column measuring 0.46 × 15 cm and a particle size of 5 µm (Agilent, Santa Clara, CA, USA). Verification that the desired materials were acquired involved mass spectrometry, employing an Ion Trap MS mass spectrometer (Bruker) in direct injection and positive detection mode.

2.2. Cells

A549 (ATCC CCL-185) cell line was cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS, Gibco – Thermo Fisher Scientific, Waltham, MA, USA) and 1% Penicillin/Streptomycin (5,000 U/mL) (P/S, Cultilab, Campinas, SP, Brazil), it was also added 2mg/ml Geneticin (G418, Sigma–Aldrich, St. Louis, MO, USA) and 200 µg/ml Hygromycin B (Thermo Fisher Scientific, Waltham, MA, USA) to DMEM during culture. A549-ACE2-TMPRSS2 is isolated from human pulmonary carcinoma, and it was transduced to express human ACE2, selected under hygromycin B, further transduced to express human TMPRSS2 under selection of geneticin. A549 cell line was gently donated by Prof. Arvind Patel from the NIBSC Research Reagent Repositor (United Kingdom).

2.3. Virus

The infections cDNA (icDNA) clone of SARS-CoV-2 based on the Wuhan strain, and the recombinants with replaced spike protein from Omicron and Delta, all expressing mCherry reporter [22] were also used for our analysis. To rescue these plasmids, BHK-21 cells were seeded in 12-well plate (TPP, Trasadingen, Switzerland), and were transfected with 3 µg of plasmid using Lipofectamine 2000 (Thermo Fisger Scientific, Waltham, MA, USA) and OPTI-MEM (Gibco—Thermo Fisher Scientific, Waltham, MA, USA. After 72 hours post transfection (hpi), the supernatant was collected and used to propagate virus in 6-well plate seeded with $8x10^6$ A549 cells. After 96 hpi, the supernatant was collected and stocked at -80°C as the passage 0.

The SARS-CoV-2 variants Omicron (BA.2) (hCoV/England/FCI-179/2022) and Delta (B.1.617.2) (MS066352H) were also used in this work, both conceded from Francis Crick Institute (London, United Kingdom).

Determination of virus titer was performed by Tissue Culture Infectious Dose (TCID50). A549 cells ($1x10^4$) were seeded in 96-well plates (TPP, Trasadingen, Switzerland). Twenty-four hours later, virus dilution was performed, and cells were infected. Seventy-two hours after, cytopathic effect was quantified to determine viral titer, which was calculated using TCID50 calculator (Spearman–Kärber Method) [23].

2.4. Evaluation of the Cytotoxicity Profile of the Peptides

The cytotoxicity of the peptide NB2080 in A549 cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) protocol as described by [24]. $1x10^4$ cells per well were seeded in 96-well culture plates (TPP, Trasadingen, Switzerland). After 24 h. The cells were incubated with different ranges of concentrations of the peptides (0.3, 0.7, 1.5, 3.1, 6.2, 12.5, 25, 50, 100 µM) for 24 h. Then, the medium containing the peptideswas removed, and 100 µL of MTT (Sigma-Aldrich, St. Louis, MO, USA) diluted in DMEM (Cultilab, Campinas, SP, Brazil) (1 mg/mL) was added to each well of the plate (1 mg/mL). After 30 min of incubation at 37 °C, the medium containing MTT (Sigma-Aldrich, St. Louis, MO, USA) was removed, and 100 µL of dimethylsulfoxide (DMSO, Synth, Diadema, SP, Brazil) was added to the cells. The plate was agitated at 200 rpm. After 5 min, the absorbance was measured at a wavelength of 572 nm on a plate reader (FLUOstar Omega/BMG LABTECH, Ortenberg, Germany). The CC_{50} values for A549 cells were calculated using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

2.5. Dose dependence analysis

Twenty-four hours before the experiment started, $1x10^4$ cells/well were seeded in 96-wells plate (TPP, Trasadingen, Switzerland). After this period, solutions containing different concentrations (0.1 µM to 100 µM) of NB2080 were prepared and incubated in the cells with SARS-CoV-2 mCherry infectious clone at MOI 0.1 for 24 hours. After that, fluorescence levels were measured to quantify the inhibition in dose dependence using the IncuCyte® S3 live-cell equipment (Sartorius, Gottingen, Germany).

The software GraphPad was used to calculate 50% cytotoxic concentrations (CC_{50}) and Half-maximal inhibitory concentrations (IC_{50}). The selective index was calculated using the CC_{50}/IC_{50} relation.

2.6. Analysis of peptides action on entry step

Twenty-four hours before the experiment, $1x10^4$ cells/well were seeded in 96-wells plate (TPP, Trasadingen, Switzerland). In the next day, a solution containing SARS-CoV-2 mCherry infectious clone at MOI 0.1 and the peptide was added to cells and incubated for one hour. After that, cells were washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at 12 and 24 h.p.i. using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.7. Analysis of peptide on the SARS-CoV-2 attachment to the cells

Twenty-four hours before the experiment, $1x10^4$ cells/well were seeded in 96 wells plate. In the following day, a solution containing SARS-CoV-2 mCherry infectious clone at MOI 0.1 and peptide NB2080 was added to the cells and incubated at 4°C for one hour. After that, cells were washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. This experiment protocol was based in other studies already published in the literature [25,26]. Fluorescence percentage was measured at 12 and 24 h.p.i. using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.8. Analysis of peptide on the SARS-CoV-2 internalization

Twenty-four hours before the experiment, $1x10^4$ cells/well were seeded in 96 wells plate. In the next day, a solution containing SARS-CoV-2 mCherry infectious clone at MOI 0.1 and peptide NB2080 was added to the cells, and they were incubated at 4°C for one hour. After that, cells were incubated at 37°C for more 30 minutes so we could imitate the process of virus internalization to the cells. Finally, cells were also washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at twelve- and twenty-four-hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.9. Virucidal effect of peptides

Twenty-four hours before the experiment, $1x10^4$ cells/well were seeded in 96-wells plate (TPP, Trasadingen, Switzerland). In the following day, a solution of v SARS-CoV-2 mCherry infectious clone at MOI 5 was mixed with peptide NB2080 at previous selected concentration, and it was incubated for 1 hour at 37°C. After that, the solutions containing virus and peptide were added to the cells for additional one hour. Finally, the cells were washed twice with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at twelve and twenty-four hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.10. Protective effect of peptides against SARS-CoV-2 infection

Twenty-four hours before the experiment, $1x10^4$ cells were seeded in 96 wells-plate (TPP, Trasadingen, Switzerland). In the next day, cells were treated with peptide for one hour. After that, cells were washed twice with PBS solution, and they were infected with SARS-CoV-2 mCherry infectious clone at MOI 0.1 for another one hour. Next, cells were washed again with PBS solution, and fresh media supplemented with 2% FBS and 1% P/S was added to each well. Fluorescence percentage was measured at 12 and 24 h.p.i. using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.11. Study of the peptides action on the post-entry of SARS-CoV-2 in cells

Twenty-four hours before the experiment, $1x10^4$ cells were seeded in 96 wells plate. In the following day, cells were infected with SARS-CoV-2 mCherry infectious clone at MOI 0.1 for one hour. After that, cells were washed twice with PBS solution, and treatment containing peptide NB2080 was added to the cells. Fluorescence percentage was measured at 12 and 24 h.p.i. using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.12. Evaluation of activity of the peptides against SARS-CoV-2 variants: Omicron (BA2) and Delta

$1x10^5$ A549 cells were seeded in 24 wells plate twenty-four hours before the experiment. In the following day, two separated experiments were performed, in one, a solution containing SARS-CoV-2 Omicron (BA2) at MOI 0.1 was prepared with peptides, in the second one, a solution containing SARS-CoV-2 Delta at MOI 0.1 was prepared with peptide. Both treatments were added to cells, and they were incubated for twenty-four hours. After that, the supernatant resulted from this experiment was freeze. For quantification of this experiment, a TCI50 experiment was performed with the supernatant.

2.13. Analysis of peptides action on the replication cycle of the Omicron and Delta variants

For the analyses of the peptides' action on replication steps of Omicron and Delta infection, methodologies from the previous experiments were repeated (Entry, Virucidal, pretreatment, post-entry), but instead of working with the Wild-type infectious clone, it was performed analysis using the recombinants infectious clone with spike protein from Omicron and Delta variants. Fluorescence percentage was measured at twelve and twenty-four hours post infection using the equipment IncuCyte® S3 live-cell (Sartorius, Gottingen, Germany).

2.14. Evaluation of *in vitro* inhibition of SARS-CoV-2 synthetic double strand RNA by peptides

To produce SARS-CoV-2 double strand RNA, forward and reverse oligonucleotide primers with and without T7 promoter sequences (Supplementary Figure 1) were designed. Two sets of PCRs were performed to generate a DNA template for transcription of sense and antisense RNA, respectively. After that, a 1% agarose gel with 1X TAE was run to confirm PCR reaction. The PCR product was cleaned using Monarch kit (New England Biolabs) and the double strand RNA was prepared subsequently. After that, RNA was cleaned using RNA clean up kit (New England Biolabs). RNA concentration was determined, and an annealing reaction was performed with sense RNA and antisense RNA, annealing buffer (10X), and nuclease-free water. Finally, to check the integrity of the double stranded RNA, a gel was run. Then, solutions were prepared at the same concentration containing the peptide NB2080 (treatment), positive control (Platine), and controls (without any compound) and all incubated for 45 minutes at room temperature. After, a 1% agarose gel was run using a 100 pb ladder.

2.15. Statical analysis

All graphs and statistical analyses were performed using GraphPad Prism 8 and were described in figures. For quantification of band density percentage, it was used ImageJ software.

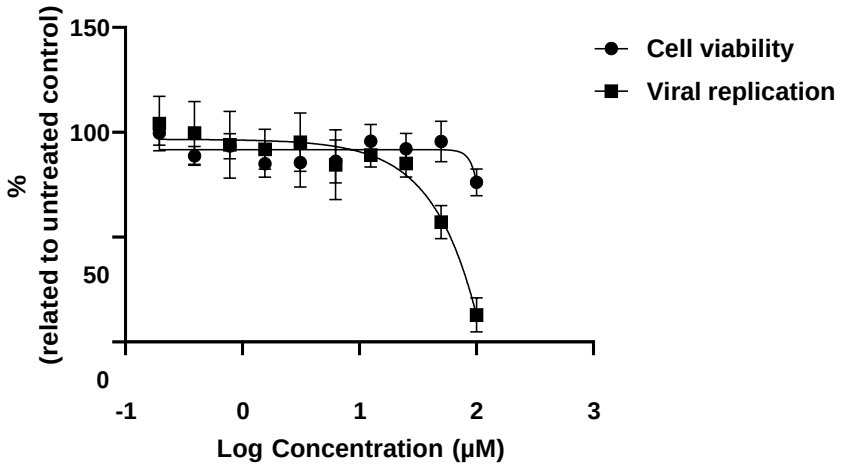
3. Results

3.1. NB2080 can inhibit SARS-CoV-2 infection in a dose dependence manner

The peptide showed a non-cytotoxic profile in A549 cells, what resulted in the selection of a concentration of 50 µM to perform the further analysis of the peptide mechanism of action against SARS-CoV-2. Furthermore, the concentration that is possible to observe the inhibition of 50% of the virus infection is 55.20 µM, which resulted in a selective index for this compound of 6.63 (Fig. 1A, B).

Figure 1. (A) Results from dose dependence of dextrogyre of dimeric peptide. Both cell viability and viral replication experiments were performed. Cell viability was calculated after analysis with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) protocol, and viral replication was performed using cDNA infection clone of SARS-CoV-2 and quantify using fluorescence by the gene reporter mCherry. This experiment was performed with three technical repeats. **(B)** Using GraphPad Software, it was possible to calculate the value of CC50, IC50, and Selective index (SI) of the peptide.

(A)



(B)

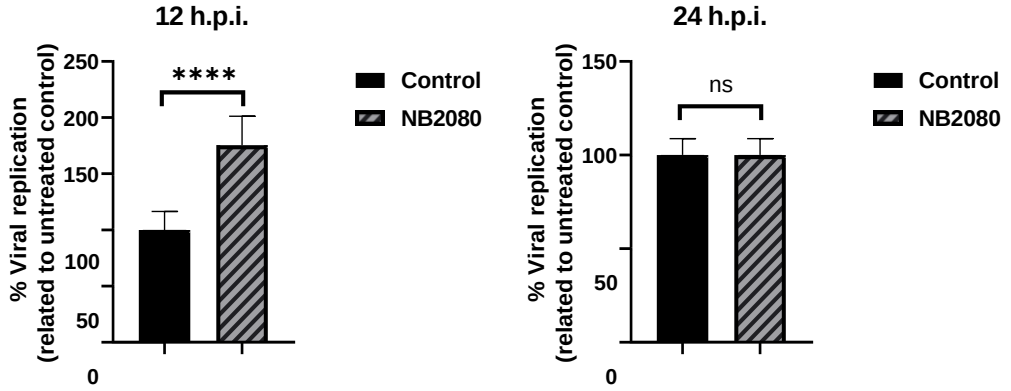
CC50	366.1 µM
IC50	55.20 µM
SI	6.63

3.2. NB2080 was not able to inhibit the entry step

After dextrogyre dimeric peptide (NB2080) was taken for analysis of possible effect during the entry of SARS-CoV-2 into the cells, it showed an increase of viral replication in 75% (Fig.2, $p<0.0001$) for the first twelve hours post infection, and a non-significant statistical difference when compared to control cells for the twenty-four hours post infection analysis.

Figure 2. Results from entry step effect of dextrogyre dimeric peptide experiments for two times post infection. Human pulmonary cell line (A549) was infected with cDNA infections clone of SARS-CoV-2 and quantify by fluorescence of gene reporter mCherry. The results represent analysis from three technical repeats. Data are presented as the mean ± standard

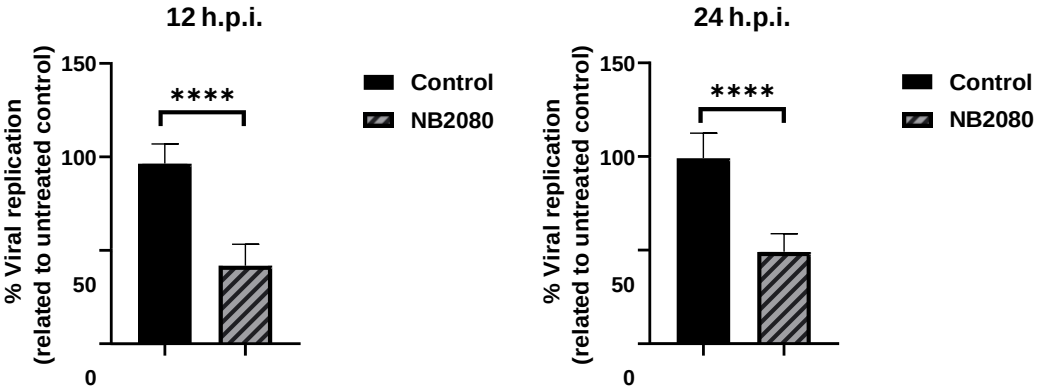
deviation. Statistical Paired t test was performed to check significance of data. ****: p<0.0001; ns: non-significant.



3.3. Peptide is responsible for causing a protective effect against SARS-CoV-2 infection

NB2080 demonstrated an action of protection of the host cells before infection with SARS-CoV-2. Results showed high levels of inhibition of the viral replication, counting for 55% (Fig. 3, p<0.0001) for twelve hours post infection, and 51% (Fig. 3, p<0.0001) for twenty-four hours.

Figure 3. Results of protective action of the peptide NB2080 after twelve and twenty-four hours post infection. Human pulmonary cell line (A549) was infected with cDNA infections clone of SARS-CoV-2 and quantify by fluorescence of gene reporter mCherry. The results represent analysis from three technical repeats. Data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. ****: p<0.0001.

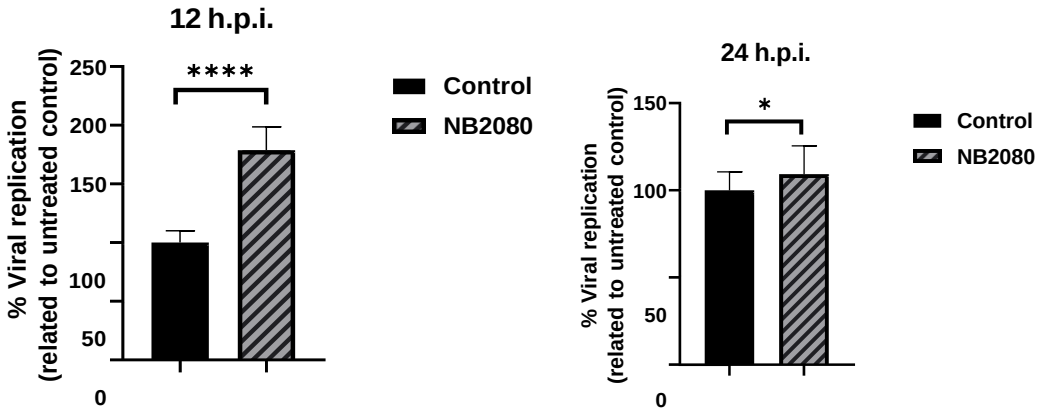


3.4. NB2080 did not impair virus particle

It was also evaluated the possible effect of the peptide NB2080 on the virus particle by incubating for one hour the virus with the peptide, before performing the infection with this solution. And it was observed a statistically significant enhance of viral replication in 78.8% for twelve hours post infection (Fig. 4, p<0.0001), and 9% for twenty-four hours (Fig. 4,

p=0.0469). In this way, this peptide does not have any direct action on the virus particle or a virucidal effect.

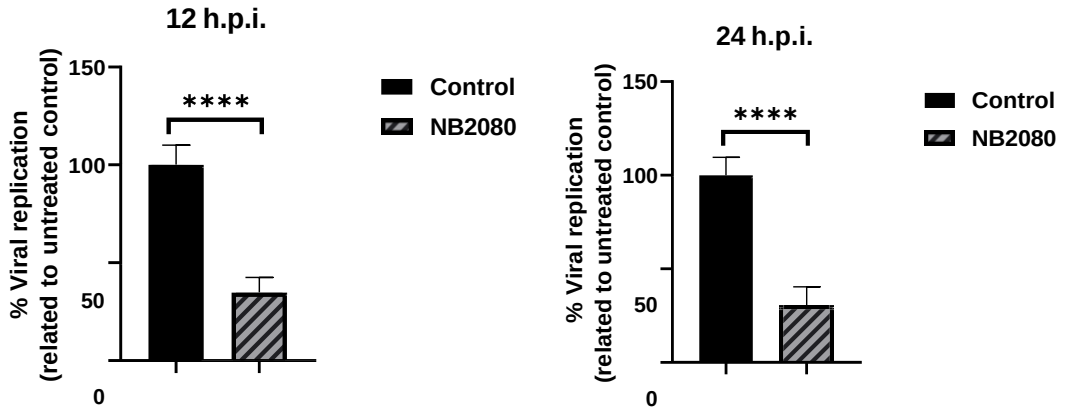
Figure 4. Results of virucidal effect of the peptide NB2080 after twelve and twenty-four hours post infection. Human pulmonary cell line (A549) was infected with cDNA infections clone of SARS-CoV-2 and quantify by fluorescence of gene reporter mCherry. The results represent analysis from three technical repeats. Data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. *: p<0.05; ****: p<0.0001.



3.5. Peptide strongly impairs post-entry step of SARS-CoV-2 replication cycle

The peptide NB2080 demonstrated to have mostly of its anti-SARS-CoV-2 action coming from post-entry steps. Results of cells being treated until twenty-hours after infection with this virus showed that NB2080 inhibited 66% (Fig. 5, $p<0.0001$) at twelve hours, and 70% (Fig. 5, $p<0.0001$) at twenty-four hours.

Figure 5. Results of post-entry action of the peptide NB2080 after twelve and twenty-four hours post infection. Human pulmonary cell line (A549) was infected with cDNA infections clone of SARS-CoV-2 and quantify by fluorescence of gene reporter mCherry. The results represent analysis from three technical repeats. Data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. ****: p<0.0001.

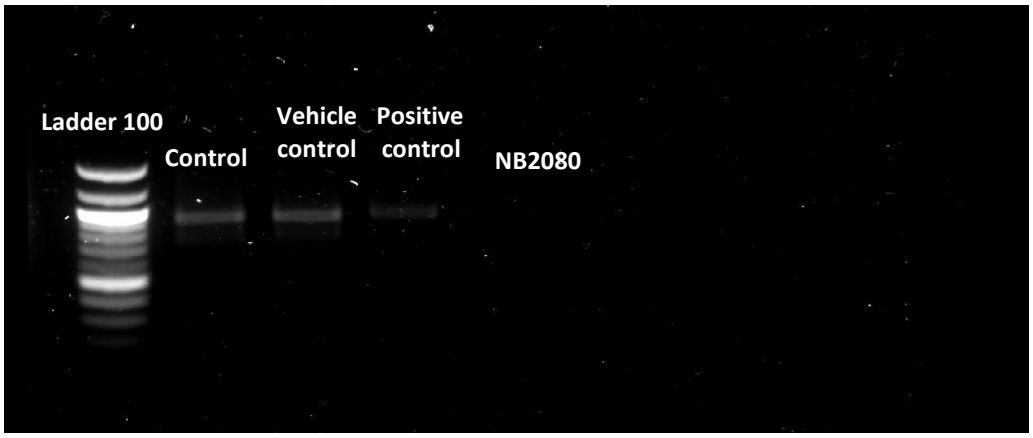


3.6. Peptide can inhibit double strand RNA of SARS-CoV-2

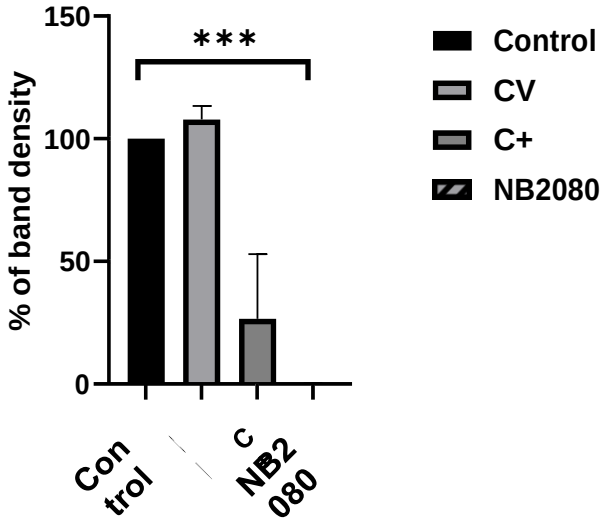
The peptide NB2080 was tested for activity on the synthetic double strand RNA of SARS-CoV-2. Results showed that NB2080 totally degraded the dsRNA, as it is not possible to see any band (Fig. 6A). Quantification of band density demonstrates that peptide decreased band density in 100% (Fig. 6B, p=0.0010).

Figure 6. Double strand RNA in vitro analysis was performed incubating a synthetic double strand RNA with peptide NB2080. (A) 1% Agarose gel showing results of full inhibition with peptide NB2080 incubation. (B) Quantification of bands density expressed by percentage of cells treated related to the control. Comparisons between control and peptide NB2080 treatment. Platinum was used and represented as the positive control for this experiment (C+ (Pt)). Control represents the sample without any treatment under the same conditions. Experiments were carried out in three independent events, each performed in triplicate, and data are presented as the mean ± standard deviation. Dunnett's multiple comparisons test was performed. ***: $p<0.001$.

(A)



(B)



3.7. Peptide does not show action against variants Omicron e Delta

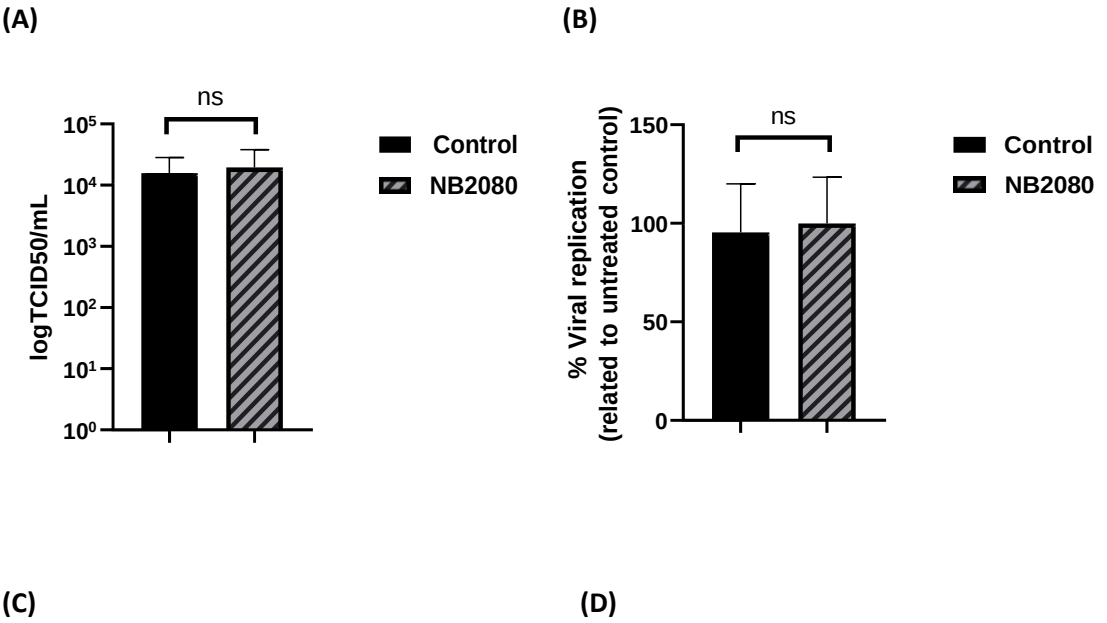
To understand better the potential of the peptide NB2080 anti-SARS-CoV-2, it was also tested against the variants Omicron and Delta.

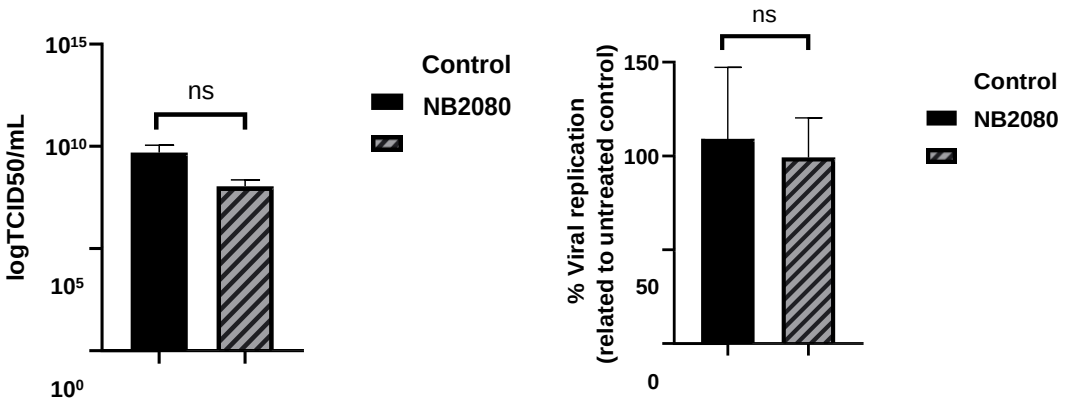
First, it was tested using Omicron variant and quantify by TCID50, but the results demonstrated that this peptide has no significant action against it (Fig. 7A). To correlate the results with the change in the spike protein of this virus, an additional experiment was performed using the cDNA infections clone with the replacement of spike protein from Delta, and no statistical difference was observed (Fig. 7B).

Next, the peptide was tested with Delta variant and the cDNA infections clone with replacement for spike protein from Delta, but again, no statistical difference could be observed (Fig. 7C, Fig. 7D).

These results show that this peptide has only action against the Wild-type strain of SARS-CoV-2, but not against the variants Delta or Omicron.

Figure 7. Results of potential of the peptide NB2080 against the variants Omicron and Delta of SARS-CoV-2. (A) A549 cells were infected with variant Omicron and treated with NB2080. (B) A549 cells were infected with cDNA infections clone of SARS-CoV-2 with replacement of spike protein from Omicron and treated with peptide NB2080. (C) A549 cells were infectedwith variant Delta and treated with NB2080. (D) A549 cells were infected with cDNA infections clone of SARS-CoV-2 with replacement of spike protein from Delta and treated withpeptide NB2080. Data are presented as the mean ± standard deviation. Statistical Paired t test was performed to check significance of data. ns: non-signficant.





4. Discussion

During the search for an antiviral against SARS-CoV-2, some *in silico* studies demonstrated the potential of the use of peptides for this virus [27], opening space for *in vitro* studies using these structures. The different mechanisms of action caused by them is one of the advantages that makes peptides overcome other compounds [28].

Peptides have showed to have different targets during SARS-CoV-2 replication in cells [29]. An example is during the entry step, targeting the Angiotensin-converting enzyme 2 (ACE2) host receptor, the transmembrane serine protease 2 (TMPRSS2), and the spike protein present on SARS-CoV-2 membrane [30]. Several peptides have the action in this step described, like by interfering during the interaction between the receptor binding domain (RBD)and ACE2 [30]. Our results demonstrated that this peptide did not show a statistical inhibitionduring entry, although it has a prophylactic effect, as the protective action analysis revealed a significant difference between cells treated and control.

This result can be corelated with an interaction with host receptors in cells that are important for the virus during infection process. Prophylactic interventions showed to be an important implementation for high-risk patients [31]. One example of compound that has this effect for SARS-CoV-2 is a small molecule called N-0385, and importantly noticed, this action can also be related to a decrease in the possibility of virus adaptation to treatment, since it is action is related to the host cell [32].

Another action of the peptide on virus replication is associated with steps after virus entry into the host cells. Other studies were also able to demonstrate the potential of compounds in the inhibition of the post-entry stage, as pantethine [33], Arteannuin B, and lumefantrine [34]. This peptide could have an action in different targets after entering cells, as replication of the genome, assembly process, or virus particle release [35]. Furthermore, it is already known

some specific targets from SARS-CoV-2 replication cycle that compounds can interact, like the viral RNA-dependent of RNA polymerase and the proteases papain-like and main one [36].

Our results showed that NB2080 has action against the synthetic double strand RNA (dsRNA), causing its degradation, what could also suggest consequences for virion assembly. This is possible because the coronavirus nucleocapsid protein is responsible for binding single and double strand RNA, and this is an important process for the virion assembly [37].

Even though we found a potential antiviral action caused by this peptide against the Wild-type strain of SARS-COV-2, it does not significantly have inhibition against variants Delta and Omicron. This can be associated with the modifications in the virus genome, even more specifically the changes on the spike protein since we tested against the recombinants with the replacement of this protein for Omicron and Delta.

5. Conclusion

In conclusion, a very similar mode of action is observed for this peptide to the dimeric isomer originated of it, since both peptides affect steps post-entry and shows a protective action. However, the peptide tested in the present work has a higher level of toxicity to cells than the dimeric isomer, what resulted in a lower value of selective index.

Furthermore, this study demonstrated that the dextrogyre is also a potential antiviral against SARS-CoV-2, but the L-amino acid peptide is more potent for use since it showed a higher selective index.

Funding

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Supplementary material

Supplementary Figure 1. Primers designed for the double strand RNA experiment performed. (A) Forward and reverse oligonucleotide primers with (B) and without T7 promoter (A).

(A)

SARS-CoV-2 ORF1 Foward	GACCGAAAGGTAAGATGGAG
SARS-CoV-2 ORF1 Reverse	AAATCGCCCGTCTGCCATGAAG

(B)

SARS-CoV-2 ORF1 Foward+T7	TAATACGACTCACTATAGGGGACCGAAAGGTAAGATGGAG
SARS-CoV-2 ORF1 Reverse+T7	TAATACGACTCACTATAGGGAAATCGCCCGTCTGCCATGAAG

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CAPÍTULO V –

Conclusão

- A análise da viabilidade celular possibilitou a seleção de uma máxima concentração não citotóxica (acima ou igual a 80% de viabilidade) para todos os peptídeos testados em linhagens Vero, A549 e BHK-21;

- Dentre os peptídeos analisados quanto ação antiviral, apenas MR1903 ($(KKYRYHLKPF)_2K$), PE1940, $(KKYRYHLKPF)_4$ $(K)_2K$, MC1947, $(KKYRYHLKPF)_2K$-YGRKKRRQRRR, MC1937, $(KKYRYHLKPF)_2K$-KRLRWR) e NB2080, $(KKYRYHLKPF)_2K$ (D-aa) apresentaram potential antiviral contra a cepa do tipo selvagem de Wuhan do SARS-CoV-2, utilizando células Vero e A549 infectadas, para análises do efeito desses peptídeos;

- Ainda, todos os peptídeos apresentaram potencial antiviral também contra as variantes Omicron e Delta de SARS-CoV-2. Com exceção do peptídeo NB2080, que não apresentou inibição contra essas variantes e seu efeito se limita ao vírus original;

- O peptídeo MR1903 demonstra potencial como um composto, principalmente, para profilaxia ou tratamento preventivo, já que apresentou efeito profilático, além de também em etapas após a entrada, em células A549 infectadas com SARS-CoV-2. Já em células Vero, esse peptídeo apresentou efeito também na etapa da entrada. O peptídeo PE1940 tem maior destaque no seu efeito para etapas mais tardias de um tratamento, já que causa efeito na entrada, mas também é responsável por inibir estágios após o vírus entrar na célula, em células A549 infectadas com o vírus. Porém, em células Vero, esse peptídeo também causou efeito virucida.

Já os peptídeos MC1947 e MC1937, devido a ação em diversas etapas do ciclo replicativo, esses compostos apresentam o potencial para um tratamento com diversos alvos, e até mesmo para o uso em ação conjunta. Essa conclusão é resultado da adição de peptídeos de penetraçãocelular ao peptídeo dimérico que resultou nos peptídeos MC1937 e MC1947 demonstrou que esses são capazes de uma inibição em todas as etapas testadas, de forma que, possuem atividadevirucida, efeito na entrada do vírus nas células, e ainda, ação nas etapas posteriores a entrada do vírus, em células Vero e A549 infectadas por SARS-CoV-2, e ainda um efeito profilático, em células A549.

O isômero (NB2080) do peptídeo dimérico MR1903 demonstrou possuir um mecanismo de ação semelhante a este, com ação no pré tratamento das células e em etapas após a infecção. Além disso, o estudo deste peptídeo concluiu que um peptídeo L-amino ácido é mais potentes do que o D-amino ácido, devido o maior valor de índice de seletividade do dimérico MR1903.

- Os peptídeos MR1903, PE1940, MC1937 e MC1947 foram capazes de inibir o replicon subgenômico, e isso afirma o potential desses peptídeos de inibir a etapa de replicação;

- Resultados da análise do RNA dupla fita sintético mostraram que todos os peptídeos apresentaram potencial de degradação dessa dupla fita, outro resultado que corrobora com o potencial desses em afetar a etapa de replicação viral;

- Além disso, foi possível identificar algumas deleções no genoma viral relacionado a adaptação do vírus ao tratamento com os peptídeos MR1903 e PE1940, porém para o peptídeo MR1903 a mesma região de deleção também foi encontrada no controle, o que impossibilitada a conclusão de uma associação entre essa alteração com a adaptação do vírus ao tratamento. Já a deleção no tratamento com PE1940 está associada com a ORF6 que já representa um alvo antiviral em estudos com SARS-CoV-2. Os tratamentos com os peptídeos MC1937 e MC1947 resultaram em adaptação do vírus após seis passagens, com os mesmo tipos de alterações no genoma para os dois peptídeos, relacionadas a proteína E e a S.

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CAPÍTULO VI –

Artigos publicados

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Effects of photodynamic therapy mediated by emodin in cervical carcinoma cells

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Effect of berberine nanoemulsion Photodynamic therapy on cervical carcinoma cell line

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Article

Long-Term Wastewater Surveillance for SARS-CoV-2: One-Year Study in Brazil

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Article

Burden of Influenza and Respiratory Syncytial Viruses in Suspected COVID-19 Patients: A Cross-Sectional and Meta-Analysis Study

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Article

The Dimeric Peptide $(KKYRYHLKPF)_2K$ Shows Broad-Spectrum Antiviral Activity by Inhibiting Different Steps of Chikungunya and Zika Virus Infection

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Abstract: Chikungunya virus (CHIKV) and Zika virus (ZIKV) are important disease-causing agents

viruses

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Mannose-Binding Lectins as Potent Antivirals against SARS-CoV-2

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Synthesis of copaiba (*Copaifera officinalis*) oil nanoemulsion and the potential against Zika virus: An *in vitro* study

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The Synthetic Peptide GA-Hecate and Its Analogs Inhibit Multiple Steps of the Chikungunya Virus Infection Cycle In Vitro

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