

Structure-Guided Design of Temporin-Derived Peptides Reveals Potent Dual-Mechanism Inhibitors of SARS-CoV-2

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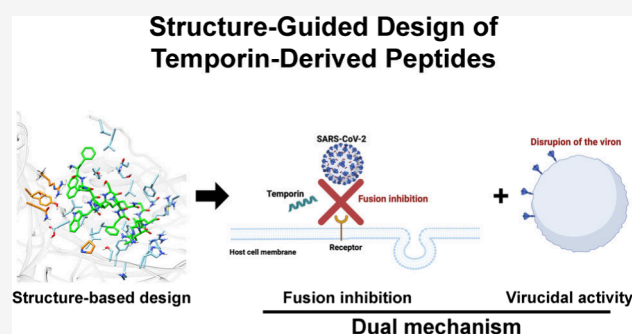


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ABSTRACT: The study investigates temporin-derived antimicrobial peptides as broad-spectrum antiviral candidates against SARS-CoV-2. Surface plasmon resonance screening shows that temporin G (TG), temporin L (TL), and the nonhemolytic analog Pro³-TL bind the trimeric Spike protein with high affinity, following a two-state binding model. NMR confirms TG–Spike interactions and identifies Phe² as critical for binding. Docking simulations place the interaction at the NTD–RBD interface and reveal a cation– π interaction between TG Phe² and Spike Arg³⁵⁷. These insights informed the design of the chimeric analog RB-142 and four derivatives (RB-143 to RB-146) incorporating bulkier aromatic residues. All analogs exhibit submicromolar binding affinities. Biological assays show low cytotoxicity and potent virucidal activity, with RB-146 demonstrating the strongest effect and a high therapeutic index. Mechanistic analyses indicate that RB-146 disrupts viral attachment and damages the viral envelope. The findings position RB-146 as a promising dual-mechanism antiviral candidate.



INTRODUCTION

The COVID-19 pandemic has highlighted the urgent need for the development of novel antiviral strategies that can complement vaccines and currently approved therapeutics.¹ Although vaccines have significantly mitigated the burden of SARS-CoV-2 worldwide, the continued emergence of viral variants with increased transmissibility, immune escape, or drug resistance poses a serious public health threat.^{2,3} This scenario emphasizes the necessity of exploring innovative, broad-spectrum antiviral agents capable of counteracting evolving viral pathogens.⁴

Among emerging antiviral strategies, antimicrobial peptides (AMPs) have attracted increasing attention.⁵ These are small, cationic peptides that constitute a fundamental part of the innate immune defense across various organisms. Initially discovered for their antibacterial properties, AMPs have been shown to exhibit remarkable antiviral activity against both DNA and RNA viruses.^{6,7} AMPs with antiviral properties (also called AVPs) have emerged as an important class of host-derived molecules capable of inhibiting enveloped viruses through multiple mechanisms,^{7–10} including direct disruption of viral membranes and interference with virus–host membrane fusion. Several envelope-targeting AMPs have been shown to bind lipid components of viral envelopes, alter membrane curvature, or destabilize bilayer integrity, thereby impairing viral attachment and entry.^{11–13} In addition to direct virucidal

effects, some peptides inhibit conformational changes in viral fusion proteins or prevent the close apposition of viral and cellular membranes required for fusion.^{14,15} A particularly promising subclass of AVPs is represented by the **temporins**, a group of short peptides first isolated from the skin secretions of the European red frog *Rana temporaria*.^{16,17}

Our previous studies have demonstrated that temporins are active against enveloped viruses such as the influenza virus and herpes simplex virus type 1 (HSV-1). For instance, **temporin G (TG)** was found to significantly inhibit early stages of the influenza virus life cycle.¹⁸ Indeed, TG was found to prevent virus-induced agglutination of red blood cells, suggesting that this peptide impairs viral replication by blocking entry into host cells. **Temporin B (TB)** showed a potent virucidal effect against HSV-1.¹⁹ Pretreatment of virions with TB resulted in a dramatic reduction in viral titers (up to 5-log), an effect attributed to the disruption of the viral envelope as visualized through transmission electron microscopy. In parallel,

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temporin L (TL) has been found to act as a membrane-targeting broad-spectrum antiviral compound.¹⁶

These observations support the notion that temporins, by targeting common features of viral envelopes and entry mechanisms, may be broadly effective against multiple enveloped viruses. Considering that SARS-CoV-2—like HSV-1—is an enveloped virus relying on membrane fusion for host cell entry, it is plausible that temporins could exert comparable antiviral effects against SARS-CoV-2.

SARS-CoV-2 is a positive-sense single-stranded RNA virus with a lipid envelope that protects its genome and facilitates infection.²⁰ The virus initiates host cell invasion through its trimeric Spike (S) glycoprotein, which engages the human ACE2 receptor, predominantly expressed in respiratory tract epithelia.²¹ Upon binding, conformational changes in the Spike protein enable membrane fusion, allowing viral RNA to enter the cytoplasm, where it hijacks the host machinery for replication and assembly of new virions. The critical dependence of SARS-CoV-2 on membrane fusion and lipid envelope integrity presents an opportunity to target the virus with AMPs that either block Spike–ACE2 interactions or directly compromise the viral membrane.

Based on these premises, the present study investigated the antiviral potential of selected temporin peptides against SARS-CoV-2, starting from the natural temporins TA, TB, TG, TL, and from a TL nonhemolytic analog Pro³-TL which was previously developed by some of us (Table 1).²²

Table 1. Sequences of Pre-existing Peptides

Peptide	Sequence
TA	F ¹ -L ² -P ³ -L ⁴ -I ⁵ -G ⁶ -R ⁷ -V ⁸ -L ⁹ -S ¹⁰ -G ¹¹ -I ¹² -L ¹³ -NH ₂
TB	L ¹ -L ² -P ³ -I ⁴ -V ⁵ -G ⁶ -N ⁷ -L ⁸ -L ⁹ -K ¹⁰ -S ¹¹ -L ¹² -L ¹³ -NH ₂
TG	F ¹ -F ² -P ³ -V ⁴ -I ⁵ -G ⁶ -R ⁷ -I ⁸ -L ⁹ -N ¹⁰ -G ¹¹ -I ¹² -L ¹³ -NH ₂
TL	F ¹ -V ² -Q ³ -W ⁴ -F ⁵ -S ⁶ -K ⁷ -F ⁸ -L ⁹ -G ¹⁰ -R ¹¹ -I ¹² -L ¹³ -NH ₂
Pro ³ -TL	F ¹ -V ² -P ³ -W ⁴ -F ⁵ -S ⁶ -K ⁷ -F ⁸ -L ⁹ -G ¹⁰ -R ¹¹ -I ¹² -L ¹³ -NH ₂

In parallel, the direct interaction between selected temporins and the SARS-CoV-2 Spike protein was explored using surface plasmon resonance (SPR). The results were further validated through nuclear magnetic resonance (NMR) spectroscopy and docking studies, revealing key interactions between the peptides and specific regions of the Spike protein.

RESULTS

Screening of Known Peptides for Their Ability to Bind the Spike Protein by SPR

Binding Experiments. Given the potential for temporin peptides to interact with the SARS-CoV-2 Spike protein, a biosensor with the Spike protein trimer immobilized was prepared to be used in SPR experiments. The sensor chip was setup according to the standard amine coupling protocol. The Spike trimer was immobilized on the active channel of a gold biosensor, using in parallel one reference channel to remove any undesired nonspecific interaction. Subsequently, selected known temporin peptides were dissolved in pure water, diluted in running buffer HBS-EP+ at concentrations 100 μ M, and flowed over the sensor chip surface. Results are summarized in Figure 1. The results demonstrate the binding of temporin peptides to the immobilized Spike protein trimer. Notably, peptides TG, TL, and Pro³-TL exhibited higher binding

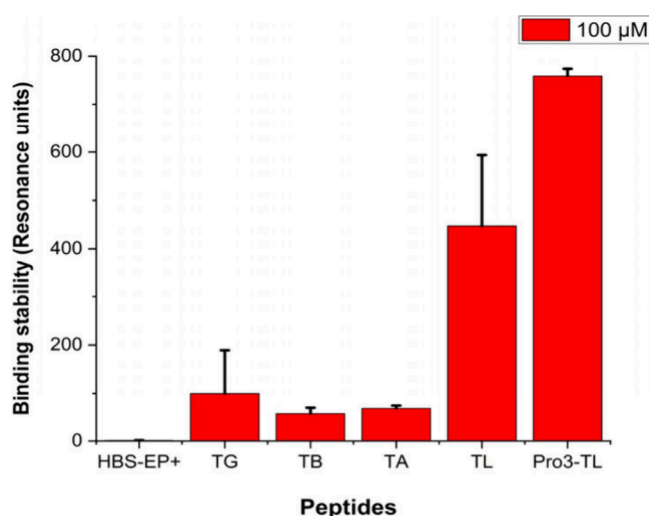


Figure 1. Binding experiments to the immobilized SARS-CoV-2 Spike protein trimer of running buffer HBS-EP+ and temporin peptides TG, TB, TA, TL, and Pro³-TL at the concentration of 100 μ M.

signals and they were selected for further investigation in kinetic experiments.

Kinetic Experiments. Peptides TG, TL, and Pro³-TL were flowed over the immobilized Spike protein separately at different concentrations (6.25, 12.5, 25, 50, and 100 μ M). The injections were performed in a single cycle analysis without intermediate regeneration steps over the duration of 110 s, obtaining concentration-dependent binding signals that were then evaluated using the theoretical fitting models. We achieved a high-quality fit with both the Binding 1:1 kinetic model and the Two-state reaction model. Precise K_D values comparison was obtained with the two-state model, which has been validated using three statistical parameters: the residuals closely distributed along the zero, the χ^2 values, and the standard errors (below 10% of the corresponding parameter values). According to this latter model, after temporin peptides interaction, eventual conformational change or engagement is assumed. In any case, it does not contribute to SPR response because there is no change in the refractive index or in mass over the chip, but only a sort of rearrangement of the bounded analyte. Calculated kinetic constants values are summarized in Table 2.

Table 2. Calculated Kinetic Constants for Peptides TG, TL, and Pro³-TL

Curve	k_{a1} (1/Ms)	k_{d1} (1/s)	k_{a2} (1/s)	k_{d2} (1/s)	K_D (M)
TG	6.18	0.0268	0.00674	5.35E ⁻⁴	3.19E ⁻⁴
TL	4.69E ²	0.292	9.18E ⁻⁴	4.43E ⁻³	5.16E ⁻⁴
Pro ³ -TL	1.97	0.0862	0.00180	1.70E ⁻⁵	4.08E ⁻⁴

Interaction Studies by NMR and Docking: Rational Design of Novel Analogs

SPR kinetic experiments indicated that the peptides Pro³-TL and TG exhibited the best K_D values among the peptides studied. Consequently, Pro³-TL and TG were selected for further investigation of their binding modes to the Spike protein using NMR spectroscopy. In particular, ligand-based NMR techniques—Saturation Transfer Difference (STD),²³ WaterLogsy NMR²⁴ and transferred-NOESY (tr-NOESY)²⁵—were employed to gain additional insight into the interaction.

The STD, WaterLogsy NMR, and tr-NOESY spectra of TG (Figure 2), recorded in the presence of a soluble portion of the

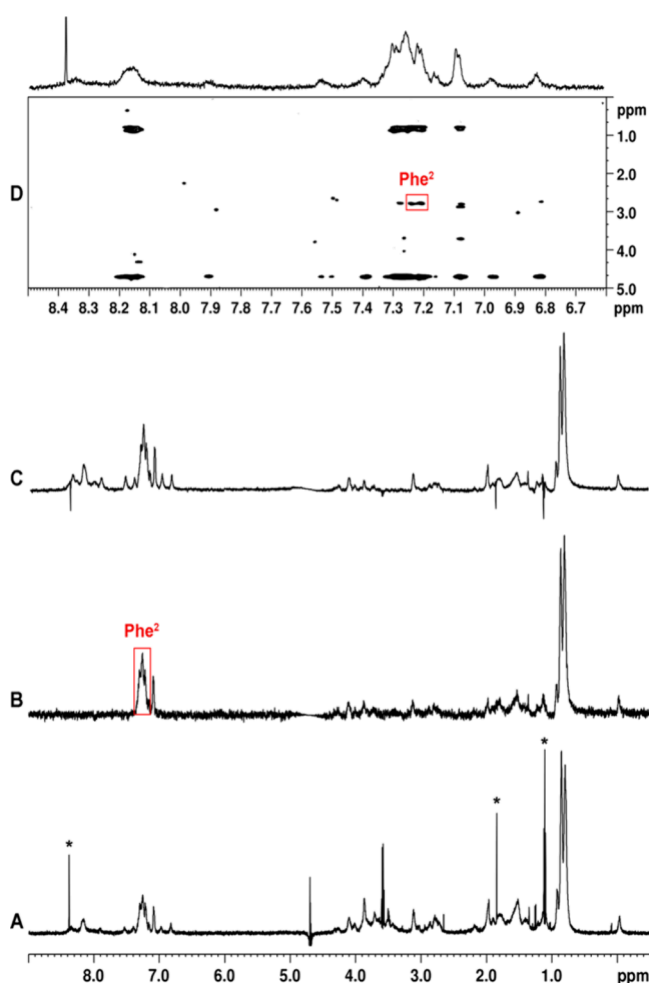


Figure 2. ^1H NMR spectrum of the TG (0.10 mM)/Spike (1 μM) complex in PBS (A). Corresponding STD-NMR recorded upon saturation at -1 ppm (B), WaterLogsy-NMR (C), Partial tr-NOESY spectrum (D). Aromatic Phe² signals are evidenced. Sample impurities are labeled with stars.

Spike protein (1 μM), showed several positive signals from the peptide, confirming that binding occurs. The most intense STD signals (Figure 2B) could be assigned to the aromatic protons of Phe² (See Table S1, Supporting Information, for NMR proton assignment), as corroborated by the tr-NOESY spectrum (Figure 2D), which indicates that the phenyl ring of Phe² serves as a key anchoring point in the interaction.

In contrast, for the peptide Pro³-TL, no signals were detected in its STD NMR and tr-NOESY spectra (data not shown). Combined with the results of the SPR experiment, this suggests that the dissociation rate constant (k_d) of the complex Pro³-TL/Spike is too low for STD and tr-NOE signal detection under the present experimental conditions.

The interaction between TG and the Spike protein observed by NMR was further supported by docking experiments. AlphaFold3 was employed to generate the initial structures of the Spike protein and of the different peptides. This approach was necessary because, although structures of the Spike protein are available in the PDB, none cover the full-length sequence used in our biophysical assays. Thus, AlphaFold3 allowed us to

model the complete protein sequence used in our experiments and ensure high structural accuracy.²⁶ Blind docking simulations of the peptides were thus performed on the Spike model with the HPEPDOCK program.²⁷ The best-ranked pose was then optimized by unrestrained energy minimization (Figure 3). Docking result indicated that binding

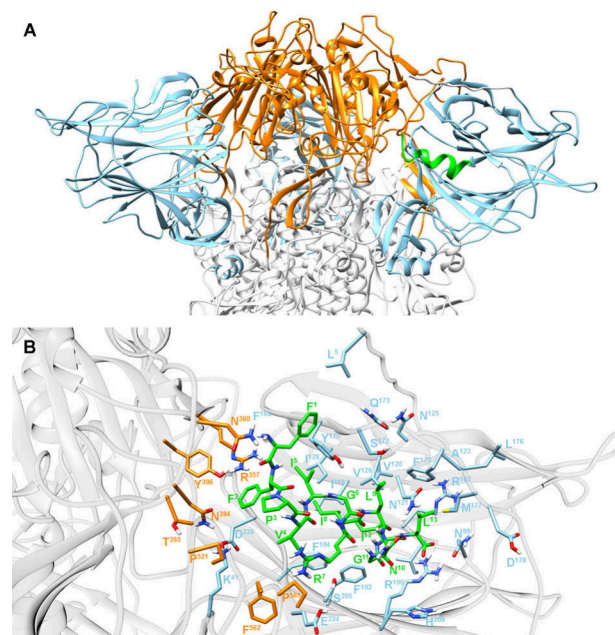


Figure 3. Model of the Spike protein complexed with peptide TG. (A) Ribbon representation of the complex (TG is reported in green, Spike NTD in cyan, Spike RBD in orange, Spike remaining domains in light gray). (B) TG within the binding pocket of Spike. Carbon atoms and the residue type/number maintain the same color code as in (A), other atoms are colored by atom type (nitrogen, blue; oxygen, red; sulfur, yellow, hydrogen white). For clarity, only polar hydrogens are shown.

of TG predominantly occurs at the N-terminal domain (NTD, residues 14–305) of the Spike protein (Figure 3A), with partial extension into the Receptor Binding Domain (RBD, residues 319–541). Notably, the first three residues of the peptide are positioned at the interface between the NTD and RBD. In agreement with NMR data, Phe² of TG emerged as a key residue for binding, being deeply inserted into a cleft formed by the neighboring residues (Figure 3B).

A noteworthy cation– π stacking interaction was observed between the phenyl ring of Phe² and the guanidinium group of Arg³⁵⁷ within the RBD. Similarly, Pro³-TL was predicted to bind the Spike protein at the same site as TG, with comparable docking energies despite the absence of the Phe² residue (Figure S1, and Table S2, Supporting Information). Both peptides adopt an amphipathic α -helical conformation when bound to the Spike protein along most of their length, with the exception of the N-terminal region, which displays a kink induced by the presence of the Pro³ residue. This structural feature is consistent with previously reported NMR data.^{22,28} The amphipathic α -helix engages the protein surface through its hydrophobic face, whereas the hydrophilic face, comprising the side chains of Arg⁷ and Asn¹¹ in TG and Lys⁷ and Arg¹¹ in Pro³-TL, is oriented toward the bulk solvent.

This new peptide, termed RB-142 (Table 3), was docked to the Spike protein, confirming a binding pose similar to its

Table 3. Sequences of Novel-Designed Analogs of Pro³TL

Peptide	Sequence
Pro ³ -TL	F ¹ -V ² -P ³ -W ⁴ -F ⁵ -S ⁶ -K ⁷ -F ⁸ -L ⁹ -G ¹⁰ -R ¹¹ -I ¹² -L ¹³ -NH ₂
RB-142	F ¹ -F ² -P ³ -W ⁴ -F ⁵ -S ⁶ -K ⁷ -F ⁸ -L ⁹ -G ¹⁰ -R ¹¹ -I ¹² -L ¹³ -NH ₂
RB-143	F ¹ -(4Cl)F ² -P ³ -W ⁴ -F ⁵ -S ⁶ -K ⁷ -F ⁸ -L ⁹ -G ¹⁰ -R ¹¹ -I ¹² -L ¹³ -NH ₂
RB-144	F ¹ -(3,4diCl)F ² -P ³ -W ⁴ -F ⁵ -S ⁶ -K ⁷ -F ⁸ -L ⁹ -G ¹⁰ -R ¹¹ -I ¹² -L ¹³ -NH ₂
RB-145	F ¹ -Nal(2) ² -P ³ -W ⁴ -F ⁵ -S ⁶ -K ⁷ -F ⁸ -L ⁹ -G ¹⁰ -R ¹¹ -I ¹² -L ¹³ -NH ₂
RB-146	F ¹ -Nal(1) ² -P ³ -W ⁴ -F ⁵ -S ⁶ -K ⁷ -F ⁸ -L ⁹ -G ¹⁰ -R ¹¹ -I ¹² -L ¹³ -NH ₂

parent peptides and preserving the cation- π interaction between Phe² and Arg³⁵⁷ (Figure 4A, and Table S2).

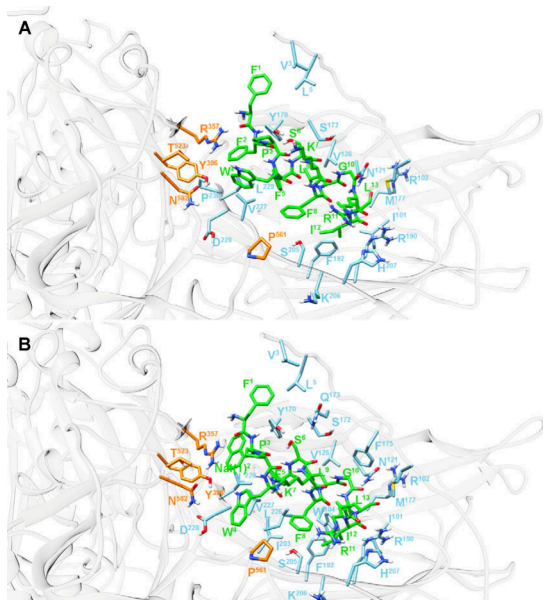


Figure 4. Model of the Spike protein complexed with peptides RB-142 (A) and RB-146 (B). Color codes are the same used in Figure 3.

To further enhance this interaction and considering that the corresponding binding pocket could accommodate bulkier residues, the phenylalanine was systematically replaced by 4'-chlorophenylalanine, 3',4'-dichlorophenylalanine, 2-naphthylalanine, and 1-naphthylalanine, yielding the corresponding derivatives RB-143/146 (Table 3). The docking analysis of RB-146 with the Spike protein (Figure 4B, and Table S2) confirmed the design hypothesis, revealing that the naphthalene moiety of RB-146 fits well within a hydrophobic pocket and engages in a favorable interaction with Arg³⁵⁷.

SPR of the Novel Analogs

The novel designed peptides RB-142/146 were synthesized as reported in the Experimental section and the analytical data are reported in the Supporting Information (Table S3 and Figures S2–S11). The novel analogs RB-142/146 demonstrate a strong binding affinity for the Spike protein, with a dissociation constant under the millimolar range ($K_D \approx 10^{-4}$ M, Table 4).

More importantly, peptides maintained the ability to bind the Spike protein even at concentrations as low as 0.5 μ M (Figure 5).

Biological Assays

Peptides' Cytotoxicity. Before evaluating peptides' anti-SARS-CoV-2 activity, we first assessed their cytotoxicity (Figure 6). Vero cells were treated with various concentrations of each peptide, ranging from 0.78 to 100 μ M, and cell

Table 4. Calculated Kinetic Constants for Peptide Analogs RB-142/146

Curve	k_{a1} (1/Ms)	k_{d1} (1/s)	k_{a2} (1/s)	k_{d2} (1/s)	K_D (M)
RB-142	3.51	0.181	2.82×10^{-4}	8.10×10^{-8}	1.48×10^{-5}
RB-143	3.56×10^2	0.133	0.00162	0.00134	1.69×10^{-4}
RB-144	3.98×10^2	0.130	0.00130	0.00129	1.63×10^{-4}
RB-145	1.47×10^2	0.134	7.66×10^{-4}	4.68×10^{-4}	3.48×10^{-4}
RB-146	1.23×10^2	0.0932	0.00346	0.00175	2.55×10^{-4}

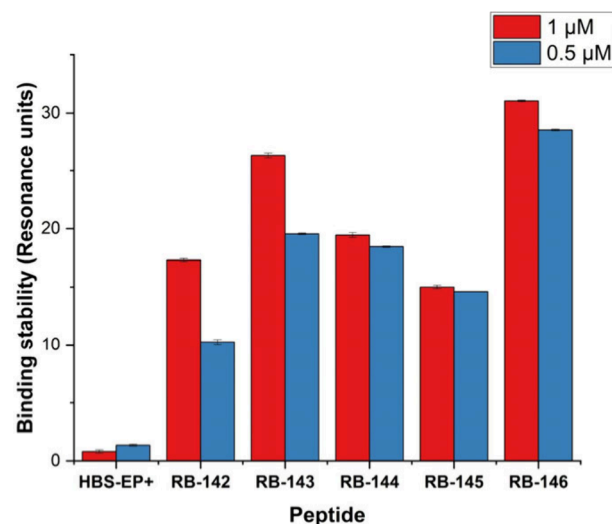


Figure 5. Binding experiments of running buffer HBS-EP+ and novel analog peptides at the concentrations 1 and 0.5 μ M to Spike protein trimer.

metabolic activity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 1 and 24 h (h).

After 1 h of treatment, the 50% cytotoxic concentration (CC_{50}) was 25 μ M for RB-142. The other modified peptides demonstrated an improved cytotoxicity profile, with CC_{50} values greater than 50 μ M. In comparison, when the peptides were added to cells for 24 h, cytotoxicity was slightly higher with CC_{50} of 12.5 μ M (RB-142), 50 μ M (RB-143), 25 μ M (RB-144), and 30 μ M (RB-145 and RB-146).

Antiviral Assays

After determining the relatively low cytotoxicity of the novel derived peptides by their CC_{50} values, we proceeded to evaluate the antiviral activity of these analogues. Temporins are widely reported to possess virucidal activity by directly targeting viral particles.¹⁶

Based on this, we conducted a virus pretreatment assay to assess whether the temporin analogues could exert a similar effect against SARS-CoV-2 (Figure 7).

This assay was conducted by incubating SARS-CoV-2 with each peptide, followed by titration of the virus-peptide mixture on cell monolayers to assess residual infectivity.

We observed that RB-142 displayed notable antiviral activity, with a 50% inhibitory concentration (IC_{50}) of 3.125 μ M. In line with these findings, all other modified peptides also exhibited a pronounced antiviral effect, with even greater potency maintained at very low concentrations. Specifically, RB-146 was the most potent, with an IC_{50} of 0.2 μ M, followed by RB-144 (IC_{50} = 0.9 μ M), RB-145 (IC_{50} = 1.8 μ M), and RB-143 (IC_{50} = 3.125 μ M). We also calculated the therapeutic

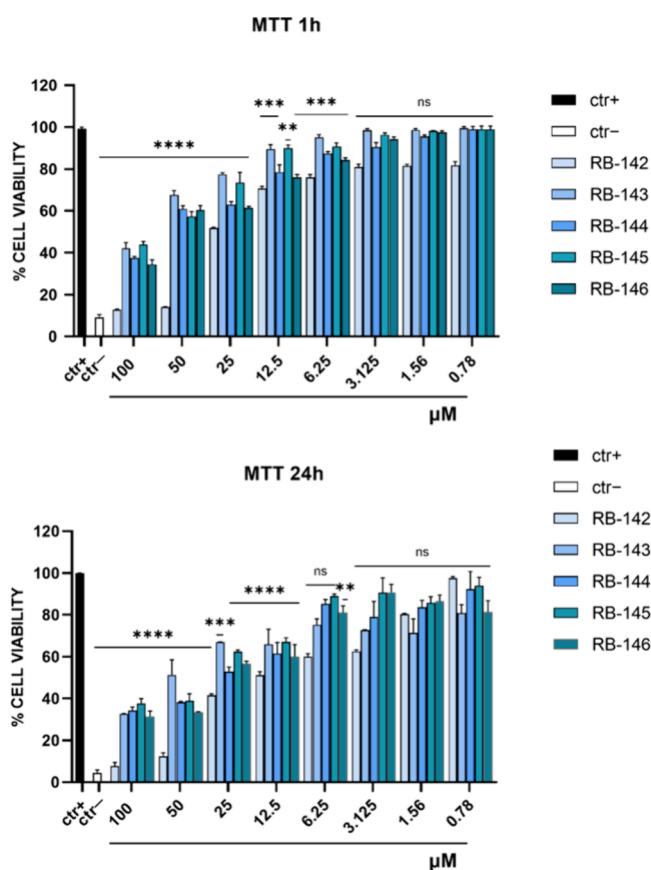


Figure 6. Cytotoxic profile of the indicated peptides on Vero-76 cells. MTT assay was performed to establish the noncytotoxic concentrations for the cell-based antiviral assays after 1 h (upper panel) and 24 h (lower panel). ctr+ refers to not-treated Vero cells, representing the positive control of viability. ctr− indicates the DMSO-treated cells, referring to the control of toxicity. **** p -value < 0.0001; *** p -value = 0.0006; ** p -value = 0.0023; ns = nonsignificant.

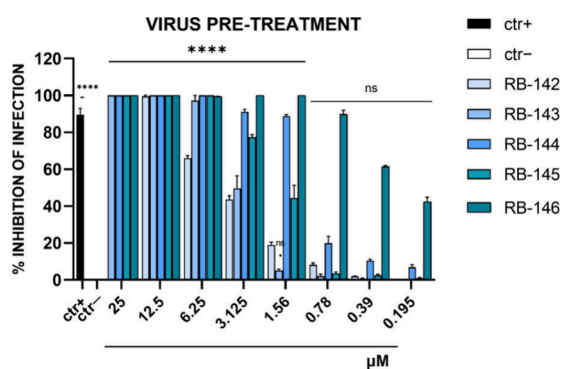


Figure 7. Antiviral activity of the indicated peptides against SARS-CoV-2. A virus pretreatment assay was performed by pretreating virions with each peptide and titrating the mixture on cell monolayers. The antiviral activity is expressed as % of inhibition. Infected and ivermectin-treated cells (12 μ M) are used as ctr+, while ctr− refers to infected cells. The data shown in each column are the means $\pm$ standard deviations (SD; error bars) from three independent experiments performed in duplicate. **** p -value < 0.0001; ns = nonsignificant.

index (TI) for each peptide (Table 5), i.e., a key parameter that defines the safety window between an effective antiviral dose and the dose that may induce toxic cellular effects.

Table 5. TI Values of the Peptides^a

Peptide	CC ₅₀ ^a (μ M)	IC ₅₀ (μ M)	TI
RB-142	12.5	3.125	4
RB-143	50	3.125	16
RB-144	25	0.9	27.8
RB-145	30	1.8	16.7
RB-146	30	0.2	150

^aCC₅₀: 50% cytotoxic concentration referred to 24 h-treatment; IC₅₀: 50% inhibitory concentration; TI: therapeutic index calculated as IC₅₀/CC₅₀.

Elevated TI scores mean improved antiviral potency combined with reduced cytotoxic effect. As shown in Table 5, RB-146 emerged as the most promising candidate for further investigation due to its highest TI.

To gain deeper insight into the specific stage at which the temporin analogues act on the viral particle, an additional temperature shift assay was conducted. During the attachment assay, cells were coinoculated with both the virus and peptide at 4 °C, a temperature that allows viral binding to the cell membrane but prevents internalization (Figure 8).

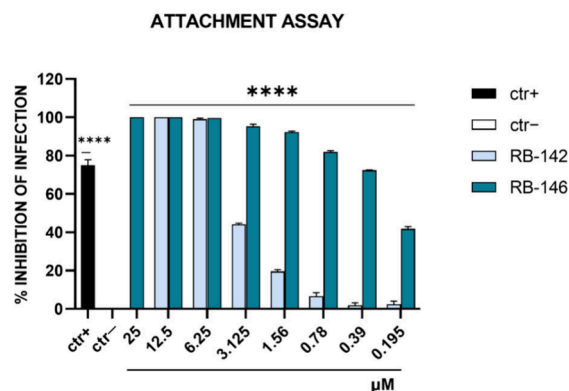


Figure 8. Attachment assay of the indicated peptides against SARS-CoV-2. Antiviral activity is presented as a percentage of inhibition. Infected cells treated with ivermectin (12 μ M) serve as the positive control (ctr+), whereas infected cells alone represent the negative control (ctr−). Each column shows the mean $\pm$ standard deviation (SD; error bars) from three independent experiments, each performed in duplicate. **** p -value < 0.0001.

RB-142 and RB-146 appear to block viral entry primarily by interfering with the attachment step, likely through direct interaction with the Spike protein. Specifically, RB-146 exhibited the highest potency, with an IC₅₀ of 0.2 μ M, consistent with findings from the virus pretreatment assay (Figure 7). In comparison, RB-142 demonstrated a less pronounced effect, with an IC₅₀ of 3.7 μ M.

Cell–Cell Fusion Assay with SARS-CoV-2 Variants

To evaluate whether RB-146 retained activity against SARS-CoV-2 variants, we extended our analysis to viral fusion assays using Spike proteins from selected variants of concern. The peptide was tested in a cell–cell fusion assay mediated by the Spike proteins of the SARS-CoV-2 Wuhan, Delta, and Omicron variants. As shown in Figure S12 (Supporting Information), RB-146 reduced spike-mediated membrane fusion in a concentration-dependent manner for all variants tested. Although the inhibitory effect was less pronounced compared to that observed with the live virus (Figures 7 and

8), **RB-146** reduced fusion efficiency, reaching a maximal inhibition of approximately 40% at the highest concentration tested. These results indicate that the peptide retains a detectable antifusogenic activity across multiple SARS-CoV-2 variants.

RBD–ACE2 Binding Inhibition

The potential of the peptides to interfere with RBD–ACE2 binding, as suggested by docking analysis indicating interaction with the RBD domain, was experimentally tested by the SARS-CoV-2 Spike-ACE2 Interaction Inhibitor Screening Assay Kit (Cayman Chemical, No. 502050) performed on peptide **RB-146**, which showed the highest antiviral activity. The peptide was tested at two different concentrations 20 and 40 μM . The results indicated that **RB-146** is unable to inhibit the binding between the Spike RBD and the receptor ACE2 as shown in Figure S13, Supporting Information.

Transmission Electron Microscopy

As AMPs, temporins, including **TL** and **Pro³-TL**, interact with the membranes of microbial hosts.^{22,28} Therefore, the effect of the most active peptide, **RB-146**, on the viral envelope was investigated using Transmission Electron Microscopy (TEM). Microscopic analysis of SARS-CoV-2 particles treated with **RB-146** was performed at three concentrations: 25, 1.56, and 0.195 μM (Figure 9).

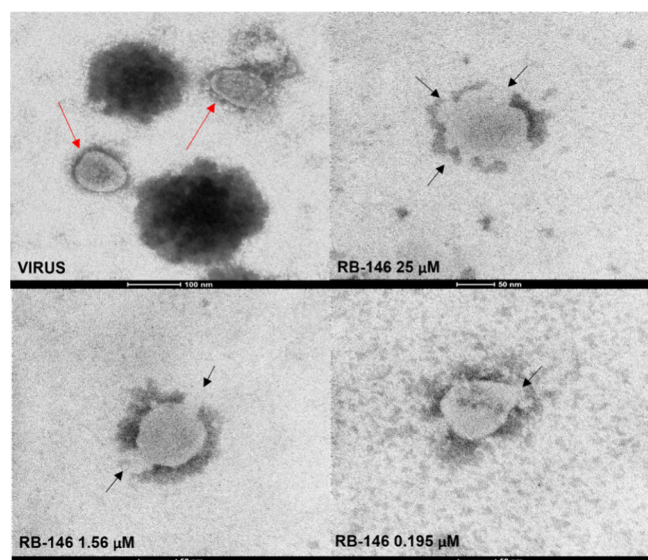


Figure 9. TEM analysis of SARS-CoV-2 virions treated with **RB-146** peptide. Images depict viral particles untreated (virus, the typical crown is indicated with red arrows) and after exposure to **RB-146** at concentrations of 25, 1.56, and 0.195 μM . Damages on the viral envelope are visible and indicated with black arrows.

Untreated virions displayed the typical spherical morphology with a distinct peripheral crown-like structure, characteristic of the viral envelope. In contrast, treatment with the highest peptide concentration (25 μM) resulted in a complete disruption of this external layer. At lower concentrations (1.56 and 0.195 μM), the envelope appeared swollen and structurally compromised, suggesting a progressive, concentration-dependent destabilization of the viral membrane. These observations suggest that **RB-146** exerts a direct, concentration-dependent virucidal effect on SARS-CoV-2 particles by compromising the integrity of the viral envelope. At higher

concentrations, the peptide induces complete disruption of the characteristic surface layer, while lower concentrations result in visible envelope structural changes. This supports the hypothesis that **RB-146** targets the viral membrane, contributing to its antiviral mechanism of action.

DISCUSSION

The current study aimed to explore the potential of temporin-derived peptides to target the SARS-CoV-2 Spike protein, with the goal of identifying novel antiviral candidates with virucidal properties. Through a combination of biophysical assays, computational modeling, and antiviral testing, our findings demonstrate that specific temporin analogs can effectively bind the viral Spike protein, disrupt the structural integrity of the viral envelope, and inhibit viral infectivity at low micromolar to submicromolar concentrations.

Initial SPR screening confirmed the ability of several known temporins to bind the trimeric Spike protein, with **TG**, **TL**, and **Pro³-TL** exhibiting elevated binding profiles. Kinetic analysis using a two-state model provided further evidence of concentration-dependent stable binding, with **TG** and **Pro³-TL** displaying favorable dissociation constants, suggesting a robust interaction with the Spike trimer. The two-state model was chosen over a 1:1 binding model due to its better fit, accounting for postbinding rearrangements that are not detectable by SPR yet are consistent with complex formation.

Subsequent NMR studies deepened our understanding of the molecular interactions at play. Ligand–receptor interaction studies using ligand-based NMR experiments have been widely employed, particularly in the field of antiviral peptide design and structure–activity analysis.^{29–34}

The **TG** peptide generated significant STD and tr-NOESY signals in the presence of the Spike protein, particularly highlighting the involvement of the Phe² aromatic side chain, which appeared to be a key determinant of binding. Conversely, **Pro³-TL** did not yield detectable STD signals, likely due to a slow off-rate, underscoring the limitations of STD-NMR for tightly bound ligands.

Complementary docking studies supported the experimental data, pinpointing the NTD and parts of the RBD as primary interaction sites. Notably, both **TG** and **Pro³-TL** were predicted to bind within a cleft formed between these domains, with Phe² of **TG** establishing a cation– π interaction with Arg³⁵⁷, a residue situated within the RBD. In this context, Pro³ of **TG** seems structurally important since it introduces a kink in the peptide backbone, positioning Phe² in the right orientation for its cation– π interaction. These insights informed the rational design of a chimeric peptide, **RB-142**, integrating the Phe² residue into the **Pro³-TL** backbone to optimize binding.

The design of **RB-142** was further refined to enhance interaction with the binding pocket by incorporating bulkier aromatic side chains, yielding a small series of analogs (**RB-143** to **RB-146**). SPR analysis confirmed the high-affinity binding of these peptides to the Spike trimer, with measurable interaction at submicromolar concentrations, underscoring their potential as potent viral binders.

Importantly, biological evaluation revealed that these analogs maintain low cytotoxicity in mammalian cells, with most CC₅₀ values exceeding 25 μM . Among the tested compounds, **RB-146** emerged as the most promising candidate, showing a remarkably low IC₅₀ of 0.2 μM in viral pretreatment assays. The calculated TI for **RB-146** was

substantially higher than for the other peptides, highlighting its favorable balance of potency and safety. Notably, **RB-146** outperformed even its precursor, **RB-142**, suggesting that the substitution of Phe² with bulkier hydrophobic moieties confers improved antiviral efficacy.

Mechanistic insights into peptide action were gained through temperature-shift assays, which revealed that **RB-146** and **RB-142** likely act at the viral attachment stage. The pronounced inhibition observed during coinubation at 4 °C indicates that these peptides interfere with the virus's ability to bind to host cell receptors, consistent with a direct interaction with the Spike protein. However, ELISA-based ACE2-binding assays revealed that **RB-146** does not block the RBD–ACE2 interaction, suggesting that its mode of action does not involve competitive inhibition at the canonical ACE2 binding site. This observation is consistent with previous studies on AMPs, such as defensins and HR2-mimetic peptides, which do not directly block ACE2 interaction but instead inhibit viral fusion by likely targeting alternative regions of the Spike protein. These regions include the heptad repeat domains HR1/HR2 within the S2 subunit, which are essential for the viral fusion process and membrane insertion into the target cell.³⁵ For example, fusion-inhibitory peptides like EK1 target HR1 and prevent the formation of the six-helix bundle essential for membrane fusion, thereby stopping infection postattachment without affecting ACE2 engagement.³⁶ In previous studies, we also found the peptide **TG** to be effective against RNA and DNA viruses with similar mechanisms: by preventing the conformational rearrangements of the hemagglutinin, an event essential for the viral envelope fusion with intracellular endocytic vesicles,¹⁸ by acting during the earliest stages of the HSV-1 life cycle and affecting the earliest phases of the naked John Cunningham polyomavirus (JCPyV) life cycle, likely through an interaction with the viral capsid protein VP1.³⁷

Our findings suggest that the temporin-derived peptides may act similarly, inducing conformational destabilization of the Spike protein or interfering with structural transitions necessary for fusion activation. Consistent with this mechanism, it was demonstrated that NTD-targeting antibodies block proteolytic activation of SARS-CoV-2 Spike within fusion domains (FDs), at sites over 10 nm from the NTD, indicating an allosteric interdomain control of fusion activation.³⁸

Several Spike residues contacting the peptide (e.g., W104, N121, V126, R190, F192) overlap with the biliverdin-binding pocket in the NTD,³⁹ indicating that the peptide engages a functionally relevant and previously characterized region. In contrast to small molecules that occupy well-defined cavities, the peptide establishes extended, multivalent interactions over a shallow surface, consistent with its size and conformational flexibility. Other reported Spike binders include small molecules targeting the RBD or the S1/S2 interface, as well as fusion-inhibitory peptides acting on the S2 subunit (HR1/HR2).⁴⁰ Relative to these sites, the NTD/RBD pocket identified here represents a distinct binding mode that may modulate Spike stability or conformational dynamics rather than directly interfering with ACE2 binding or fusion core assembly.

Further supporting a multifaceted mode of action, TEM revealed progressive damage to the viral envelope upon peptide treatment. At the concentration of 25 μ M, **RB-146** completely disrupted the crown-like morphology of the virion, while lower concentrations led to visible membrane swelling.

These observations are consistent with a dual mechanism of action in which the peptide both targets the Spike protein and directly perturbs the viral membrane. This latter is in accordance with the known membrane-disruptive activity of amphipathic cationic AMPs, such as defensins, LL-37, AR-23, and melittin, which physically destabilize viral lipid bilayers and lead to virion inactivation.^{6,41} Taken together, our data strongly support a dual mechanism of action for **RB-146**, involving both: (i) fusion inhibition, likely through Spike protein destabilization or interference with S2 domain function; and (ii) virucidal activity, via direct disruption of the viral envelope. Only some AVPs have been reported to exert dual mechanisms of action by simultaneously interfering with viral fusion processes and compromising membrane integrity. For example, some defensins, such as the θ -defensin 1 (RTD-1), can target specific viral components, but also inactivate viral particles.^{42–44}

Interestingly, **RB-146** retained broad antiviral activity against multiple SARS-CoV-2 variants of concern, including Delta and Omicron sublineages BA.1, BA.5, and BQ1.1 (Figure S12). This finding can be mechanistically rationalized by its interaction with conserved residues in the spike NTD and RBD, which remain unaltered in the variants tested. This suggests that targeting nonmutable regions of the spike represents an effective strategy to achieve broad and variant-independent antifusogenic activity.

CONCLUSIONS

In conclusion, this study highlights the utility of structure-guided peptide engineering to enhance Spike-binding and envelope-targeting antiviral functions, providing compelling evidence that temporin-derived peptides, particularly those structurally optimized through rational design, represent a promising class of dual-action antiviral agents against SARS-CoV-2. The identification of **RB-146** as a potent, low-toxicity antiviral agent reinforces the potential of modified temporins as therapeutics capable of inhibiting viral fusion and compromising envelope integrity, while retaining activity across multiple SARS-CoV-2 variants through interactions with conserved Spike residues, with favorable safety profiles that underscore their promise as broad-spectrum agents against current and future viral threats, including other enveloped viruses.

Future work will focus on in vivo validation, pharmacokinetic profiling, and assessment against emerging viral variants. Moreover, resolving the peptide–Spike interaction at atomic resolution should enable the rational design of even more potent next-generation virucidal agents.

EXPERIMENTAL SECTION

Materials

α -Fmoc-protected amino acids, Fmoc-Phe, Fmoc-Pro, Fmoc-Trp(Boc), Fmoc-Ser(*t*Bu), Fmoc-Lys(Boc), Fmoc-Leu, Fmoc-Gly, Fmoc-Ile, Fmoc-Arg(Pbf) were purchased from GL Biochem Ltd. (Shanghai, China). Fmoc-Phe(4-Cl)-OH, Fmoc-Phe(3,4-diCl)-OH, Fmoc-2-Nal-OH, Fmoc-1-Nal-OH, were acquired from Iris-Biotech GmbH. Coupling reagents, *N,N,N',N'*-tetramethyl-O-(1H-benzotriazol-1-yl) uranium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt), as well as the Rink amide resin used were commercially obtained by GL Biochem Ltd. (Shanghai, China). *N,N*-diisopropylethylamine (DIEA), Triisopropylsilane (TIS), piperidine, and trifluoroacetic acid (TFA) were purchased from Iris-Biotech GmbH. Moreover, peptide synthesis solvents and reagents, such as

N,N-dimethylformamide (DMF), dichloromethane (DCM), diethyl ether (Et₂O), water and acetonitrile (MeCN) for HPLC, were reagent grade acquired from commercial sources (Sigma-Aldrich and VWR) and used without further purification.

Peptide Synthesis

All peptides were synthesized by employing the ultrasound-assisted solid-phase peptide synthesis combined with Fmoc/*t*Bu strategy. The resin Rink amide (loading 0.1 mmol; 0.72 mmol/g as loading, 100–200 mesh as particle size) was used as solid support to achieve the C-terminal amidated peptide. The elongation of the peptide sequence was obtained through repeated cycles of Fmoc deprotections and coupling reactions under ultrasound irradiations.⁴⁵ The Fmoc removal was carried out using a solution of 20% piperidine in DMF and placing the tube reactor in an ultrasonic bath performing two cycles of 0.5 and 1 min. Before the coupling reaction, the resin was washed with DMF and DCM. Then, each coupling was performed using Fmoc-AA (3 equiv), HBTU (3equiv), HOBt (3 equiv), and DIPEA (6 equiv), for 5 min. The solution was discarded and the coupling reaction is repeated under the same condition. After the assembly of the peptide sequence, each peptide was removed from the resin along with the protecting groups on side-chain amino acids, by preparing a cocktail of TFA:TIS:H₂O (95:2.5:2.5, *v:v:v*), for 3 h at room temperature. Then, the resin was removed by filtration and each peptide was precipitated with chilled diethyl ether and centrifuged (2 × 15 min, 6000 rpm). The supernatants were removed, while the crudes were freeze-dried, and then dissolved in 10% MeCN in H₂O to be purified by RP-HPLC. Finally, peptides were characterized by RP-HPLC and Electrospray Ionization Mass Spectrometry (ESI-MS). All compounds are >95% pure by HPLC analysis. Analytical data for the novel peptides RB-142/146 are reported in Table S3 and Figures S2–S11, whereas those for previously described peptides are reported elsewhere.^{18,19,22}

Surface Plasmon Resonance Experiments

Experiments were performed using the Biacore X100 instrument from Cytiva (Milano, Italy). All binding analyses were made at 25 °C using HBS-EP+ as running buffer. Reference channel 1 was activated and then was directly blocked with ethanolamine and used as blank to remove the nonspecific signal depending on interactions between molecules present in the samples and gold on sensor chip surface. The soluble trimeric Spike protein was obtained by removing the transmembrane domain and the C terminal intracellular tail and replacing them by a T4 foldon sequence as describe by Pino et al.⁴⁶ Spike protein was immobilized following the amine coupling standard procedure using a 10 μg/mL solution in a 10 mM acetate buffer at pH 5.0 as immobilization buffer.

SPR Binding Experiments. Peptides (1 mg/mL stock solutions) were prepared in water and then diluted in running buffer HBS-EP+. Peptides were then independently flowed at 100 μM over the immobilized Spike during 120 s. Interactions were recorded as separate sensorgrams. Responses were measured in Resonance Units (RU) as the difference between active and reference channels. Surface was regenerated with a 20 s pulse of 50 mM Glycine-HCl pH 2.5 followed by one injection of a 15 s pulse of a solution 10 mM NaOH allowing the complete removal of specifically and nonspecifically bounded material from the surface.

Kinetic Experiments. Single cycle kinetic experiments were conducted following the previously described SPR protocol.⁴⁷ Peptides were tested at different initial calculated concentrations 100, 50, 25, 12.5, and 6.25 μM and solutions were injected in duplicate over the immobilized Spike protein for 120 s. Running buffer was then flushed for 600 s during the dissociation phase and finally the chip surface was regenerated by injecting a glycine solution 50 mM pH 2.5 for 20 s and sodium hydroxide solution 10 mM for 15 s both at a flow rate of 10 μL/min. Kinetic experiments were elaborated with Biacore Evaluation Software 2.0.

NMR Spectroscopy

All the spectra were acquired at 298 K with a Bruker Avance NMR spectrometer operating at 700 MHz ¹H Larmor frequency, equipped

with a z-gradient 5 mm triple-resonance probe head. The spectra were processed with the Bruker TOPSPIN software packages. ¹H NMR chemical shift assignments of peptide TG were effectively achieved according to the procedure of Wüthrich⁴⁸ via the usual systematic application of DQF-COSY,⁴⁹ TOCSY,⁵⁰ and NOESY⁵¹ experiments (Table S1). For NMR studies of peptides with Spike, a soluble portion of the protein⁴⁶ was dissolved in 540 μL of buffer (50 mM KH₂PO₄, 50 mM Na₂HPO₄, pH 7.5, 150 mM NaCl) and 60 μL of ²H₂O. The protein final concentration was 1 μM while the peptide final concentration was 100 μM. Saturation Transfer Difference spectra were acquired with 1024 scans with on-resonance irradiation at −1.0 for selective saturation of protein resonances and off-resonance irradiation at 200 ppm for reference spectra. A train of 40 Gaussian shaped pulses of 50 ms with 1 ms delay between pulses were used, for a total saturation time of 2 s. WaterLOGSY NMR experiments employed a 20 ms selective Gaussian 180° pulse at the water signal frequency and a NOE mixing time of 1 s. Both for STD and WaterLOGSY, the FIDs were multiplied by an exponential weighting (lb = 5 Hz) before Fourier transformation. tr-NOESY experiments were run with mixing time of 100 ms. The water signal was suppressed by gradient echo.⁵²

Docking Studies

AlphaFold3 was employed to generate the initial structures of the Spike protein. This approach was necessary because, although structures of the Spike protein are available in the PDB, none cover the full-length sequence used in our biophysical assays. Thus, AlphaFold3 allowed us to model the complete protein sequence used in our experiments.²⁶ Docking calculations were performed with the HPEPDOCK software.²⁷ HPEPDOCK is an innovative online server for investigating protein-peptide docking based on the hierarchical algorithm. It performs a blind and flexible peptide docking by fast modeling of peptide conformations and sequent global sampling of binding orientations. The best model, obtained by HPEPDOCK for each complex peptide/Spike was subjected to refinement using the by molecular mechanics (MM) energy minimization without restraints (Steepest Descent algorithm; ε = 1*k) until the maximum RMS derivative was less than 0.1 kcal/Å (Module Discover; Insight 2005). Atomic potentials and partial charges were assigned using the consistent valence force field.⁵³ Protein structural quality in the resulting complex was then checked using Procheck.⁵⁴ Energy terms of the refined models (vdW and electrostatic energy contribution) are reported in Table S2 of the Supporting Information.

Cell and Virus

Vero cells (ATCC CCL-81, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose (Microtech, Naples, Italy), supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin (Himedia, Naples, Italy), and 10% fetal bovine serum (Microtech). SARS-CoV-2 (strain VR PV10734), kindly provided by Lazzaro Spallanzani Hospital in Rome, Italy, was propagated using Vero cells. All experimental work involving SARS-CoV-2 was performed in a biosafety level 3 (BSL-3) laboratory by qualified personnel holding certified documentation (C.Z., 2022_SPP_UniCA_20; A.D.F., UNICA_SPP_CA_BSL3_2024_0005).

Cytotoxicity

Vero cells (5 × 10³ cells/well) were plated in 96-well microplates and incubated for 24 h at 37 °C with 5% CO₂. Temporin-derived peptides were tested across a concentration range (0.78 to 100 μM) following 1- and 24-h treatments. Cytotoxicity was evaluated using the (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). In brief, after peptide exposure, 100 μL of MTT solution (5 mg/mL) was added to each well and incubated for 3 h at 37 °C. The medium was then removed, and the resulting formazan crystals were solubilized with 100 μL of 100% DMSO. Absorbance was measured at 540 nm. Cell viability was expressed as a percentage relative to untreated controls.

Antiviral Screening

Cells were seeded into 12-well plates at a density of 2.5×10^5 cells per well and incubated for 24 h at 37 °C. For all assays, peptides were administered in serum-free medium and tested exclusively at concentrations previously determined to be noncytotoxic. Ivermectin was used as a positive control (ctr+). Virus pretreatment assay: Peptides were incubated with the virus (1×10^4 PFU/mL) for 1 h at 37 °C. Following incubation, the virus–peptide mixtures were diluted, applied to cell monolayers, and incubated for 1 h to allow viral adsorption. After this step, cells were overlaid with a carboxymethylcellulose (CMC) medium and incubated for 48 h. Subsequently, the cells were fixed, stained, and the number of plaques was quantified to assess residual infectivity. Attachment assay: Vero cells were seeded in 12-well plates at a density of 2.5×10^5 cells/mL and allowed to adhere for 24 h. Following this, the cells were simultaneously exposed to SARS-CoV-2 (MOI 0.01) and varying concentrations of peptides (ranging from 25 μ M to 0.195 μ M) for 1 h at 4 °C to permit viral attachment. After treatment, the monolayers were washed and incubated with fresh culture medium at 37 °C for an additional hour. The cells were then rinsed with PBS, and a DMEM/CMC overlay was applied for 48 h under standard incubation conditions (37 °C, 5% CO₂). Afterward, the cells were fixed and stained, and plaque counts were performed. *Statistics.* All assays were conducted in triplicate, and results are presented as mean $\pm$ standard deviation. CC₅₀ and IC₅₀ values were calculated using nonlinear regression analysis with GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Plasmids for Cell–Cell Fusion Assay

The cDNAs coding for hACE2 fused to the fluorescent protein Venus (GS66539), SARS-CoV-2 Spike Wuhan (GS66929-1), Delta (B.1.617.2; GS69020-2), Omicron BA.1 (GS70109), Omicron BA.5 (GS72070-1), and Omicron BQ.1.1 (GS72070-2), were codon optimized for mammalian expression and cloned in a modified version of the pCAGGS with puromycin resistance for selection (Epoch Life Science, Missouri, Texas, United States).

Gal Complementation-Based Fusion Assay

hACE2 receptor-bearing cells expressing the ω -peptide of β -Gal were mixed with cells coexpressing the Spike protein and the α -peptide of β -Gal, and cell fusion led to α - ω complementation. **RB-146** was tested starting from 25 μ M in 2-fold dilutions. Fusion was stopped by lysing the cells, and after the addition of the substrate (Tropix Galacto-Star chemiluminescent reporter assay system, Applied Biosystem, Waltham, Massachusetts, United States), luminescence was quantified by a Tecan M1000PRO microplate reader.

Elisa Assay

To detect any inhibitory effects of **RB-146** on the binding of SARS-CoV-2 Spike protein and ACE2, a SARS-CoV-2 Spike ACE2 interaction inhibitor screening assay kit was used (Cayman Chemical, No. 502050). The procedure was performed following the protocol of the product. Briefly, for the background samples 100 μ L of Immunoassay Buffer C (1 $\times$) and 50 μ L of ACE2 inhibitor screening reagent were added to the 96-well plate. For the control sample, 50 μ L of buffer, 50 μ L of ACE2 inhibitor screening reagent, and 50 μ L of Spike inhibitor screening reagent were added to the 100% initial activity wells. For the tested samples, 50 μ L of the peptide at two different concentrations (i.e., 20 and 40 μ M), 50 μ L ACE2 inhibitor screening reagent, and 50 μ L of Spike inhibitor screening reagent were added to the wells. The plate was covered and incubated for 60 min at room temperature on an orbital shaker. After incubation, each well was emptied and rinsed five times with $\sim$ 300 μ L of wash buffer (1 $\times$); then, 150 μ L of anti-His HRP conjugate was added. The plate was covered and incubated for 30 min at room temperature on an orbital shaker, and then, the washing procedure was repeated. TMB substrate solution (175 μ L) was added to each well, and the plate was incubated again for 15 min at room temperature on an orbital shaker. To terminate the reaction, 75 μ L of HRP stop solution was added to each well. Absorbance was read at 450 nm using a microplate reader

(Infinite M200; Tecan, Salzburg, Austria). The experiment was run in triplicates.

TEM Analysis

The analysis was carried out using an FEI TECNAI G2 S-twin electron microscope. Sample preparation involved incubating 5 μ L of purified SARS-CoV-2 with 5 μ L of **RB-146** for 1 h at 37 °C. As a control, the virus was incubated under identical conditions with 5 μ L of culture medium instead of the peptide. After incubation, 5 μ L of each suspension was placed onto carbon-coated copper grids (Merck, Readington Township, NJ, USA) and allowed to air-dry overnight. Negative staining was performed using 2% phosphotungstic acid (pH 6.5). A 5 μ L drop of the stain was applied to the grids for 30 s, after which the excess was removed with filter paper to enhance image contrast.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.5c03196>.

NMR data of peptide **TG** (Table S1); Energy of interaction in the Spike/peptide complexes (Table S2); Model of the Spike protein complexed with peptide **Pro³-TL** (Figure S1); Analytical data of peptides **RB-102/106** (Table S3 and Figures S2–S11); Cell-cell fusion assay with SARS-CoV-2 variants (Figure S12); RBD–ACE2 binding inhibition (Figure S13) (PDF) Molecular formula strings of novel peptides (CSV) Coordinate files of models of the Spike protein complexed with **TG** (PDB) Coordinate file of models of the Spike protein complexed with **Pro³-TL** (PDB) Coordinate file of models of the Spike protein complexed with **RB-102** (PDB) Coordinate file of models of the Spike protein complexed with **RB-106** (PDB)

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Notes

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ABBREVIATIONS

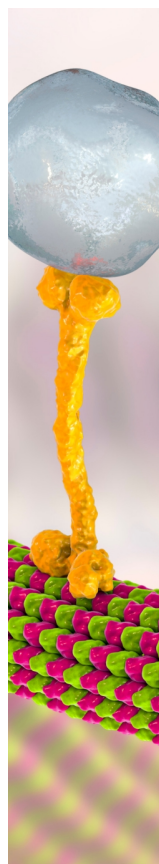
AMPs, Antimicrobial peptides; AVPs, Antiviral peptides; FDs, Fusion domains; HSV-1, Herpes simplex virus type 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NTD, N-terminal domain; RBD, Receptor binding domain; STD, Saturation Transfer Difference; TEM, Transmission Electron Microscopy; TI, Therapeutic Index; tr-, NOESY transferred-NOESY; JCPyV, John Cunningham polyomavirus

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