Infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) requires membrane fusion between the viral envelope and the host cell, at either the cell surface or the endosomal membrane. The fusion process is mediated by the viral transmembrane spike glycoprotein (S). Upon viral attachment or uptake, host factors trigger large-scale conformational rearrangements in S, including a refolding step that leads directly to membrane fusion and viral entry (1–5). Peptides corresponding to the highly conserved heptad repeat (HR) (Fig. 1A) domain at the C terminus of the S protein (HRC peptides) (Fig. 1B) can prevent this refolding and inhibit fusion, thereby preventing infection (4–8). The HRD peptides form six-helix bundle-like assemblies with the extended intermediate form of the S protein trimer, disrupting the structural rearrangement of S that drives membrane fusion (4) (see also movie S1).

Our approach in designing SARS-CoV-2 S-specific fusion inhibitors builds on our previous work that demonstrated that lipid conjugation of HRD-derived inhibitory peptides markedly increases antiviral potency and in vivo half-life (9, 10). These peptides successfully inhibit human parainfluenza virus type 3 (HPV-3), measles virus, influenza virus, and Nipah virus infection (9, 11–13). Furthermore, the combination of dimerization and lipidopeptide integration into cell membranes protects the respiratory tract and prevents systemic lipopeptide dissemination (14). Lipid-conjugated peptides administered intranasally to animals reached high concentrations both in the upper and lower respiratory tract, and the lipid could be designed to modulate the extent of transit from the lung to the blood, but themonomeric peptide remained at high concentrations in the lung with minimal entry into the blood, and the monomeric peptide entered the circulation and the lung concentration decreased (Fig. 2A). The dimeric [SARS-HRC-PEG4]2-chol lipopeptide was distributed throughout the lung after intranasal administration (Fig. 2B). A cellular toxicity (MTT) assay in primary human airway epithelial cells showed minimal toxicity even after 6 days at the highest concentrations tested (<20% at 100 μM) and no toxicity at its 90% maximal inhibitory concentration (IC90) (~35 nM) (fig. S5). The longer respiratory tract persistence of [SARS-HRC-PEG4]2-chol, in concert with its in vitro efficacy, led us to advance this dimeric lipopeptide.
**Fig. 1. Peptide-lipid conjugates that inhibit SARS-CoV-2 spike (S)-mediated fusion.** (A) The functional domains of SARS-CoV-2 S protein, the receptor binding domain (RBD) and heptad repeats (HRN and HRC), are indicated. (B) Sequence of the peptides derived from the HRC domain of SARS-CoV-2 S. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (C) Monomeric and dimeric forms of lipid-tagged SARS-CoV-2 inhibitory peptides that were assessed in cell–cell fusion assays. (D) Cell–cell fusion assays with different inhibitory peptides. The percentage inhibition is shown for six different SARS-CoV-2 specific peptides and a control HPIV-3-specific peptide at increasing concentrations. Percent inhibition was calculated as the ratio of the relative luminescence units in the presence of a specific concentration of inhibitor (X) and the relative luminescence units in the absence of inhibitor, corrected for background luminescence. Percent inhibition = 100 × [1 – (luminescence at X/–luminescence in absence of inhibitor – background)]. The difference between the results for [SARSHRC-PEG4-Chol] and SARSHRC-PEG4-chol lipopeptides was statistically significant (two-way analysis of variance (ANOVA), P < 0.0001). (E) Fusion inhibitory activity of [SARSHRC-PEG4-Chol] peptide against emerging SARS-CoV-2 S variants, MERS-CoV S, and SARS-CoV S. Data in (D) and (E) are means ± standard error of the mean (SEM) from three separate experiments, with the curve representing a four-parameter dose-response model.

**Fig. 2. Biodistribution of [SARSHRC-PEG4-Chol] and SARSHRC-PEG4 peptides after intranasal administration to mice.** (A) The concentration of lipopeptides (y axis) was measured by ELISA in lung homogenates and plasma samples (n = 4 mice, with the exception of [SARSHRC-PEG4-Chol] IN, for which n = 3 at 8 and 24 hours, and n = 1 for vehicle treatment). Median is indicated by horizontal bar. (B) Lung sections of [SARSHRC-PEG4-Chol]-treated (or vehicle-treated) mice were stained with anti-SARS-HRC antibody (red) confirming broad distribution of [SARSHRC-PEG4-Chol] in the lung (8 hours post inoculation, 8HPI). Scale bar, 500 μm in lung tile scan and 50 μm in magnification; representative images and a full tile scan are shown. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue).

The lead peptide, [SARSHRC-PEG4-Chol], was assessed for its ability to block entry of SARS-CoV-2 in VeroE6 cells or VeroE6 cells overexpressing transmembrane serine protease 2 (TMPRSS2), one of the host factors thought to facilitate viral entry at the cell membrane (2). Whereas viral fusion in VeroE6 cells predominantly occurs after endocytosis, the virus enters TMPRSS2-overexpressing cells by fusion at the cell surface, reflecting the entry route in airway cells (18). This difference is highlighted by chloroquine’s effectiveness against SARS-CoV-2 infection in Vero cells but its failure in TMPRSS2-expressing Vero cells and the human lung (19). The [SARSHRC-PEG4-Chol] peptide dissolved in an aqueous buffer containing 2% dimethyl sulfoxide (DMSO) inhibited virus entry after 8 hours with a half-maximal inhibitory concentration (IC50) of ~300 nM in VeroE6 and ~5 nM in VeroE6-TMPRSS2 cells (Fig. 3A). To strengthen translational potential toward human use, the lipopeptide was reformulated in sucrose instead of DMSO, resulting in equivalent in vitro potency (Fig. 3B). A control dimeric fusion-inhibitory lipopeptide directed against HPIV-3 blocked infection by HPIV-3 but did not inhibit SARS-CoV-2 infection. The in vitro efficacy data are summarized in table S1.

Ferrets are an ideal model for assessing respiratory virus transmission, either by direct contact or by aerosol transmission (20, 21). Mustelids are highly susceptible to infection with SARS-CoV-2, as also illustrated by frequent COVID-19 outbreaks at mink farms. Direct-contact transmission of SARS-CoV in ferrets was demonstrated in 2003 (22), and both direct-contact and airborne transmission of SARS-CoV-2 have been shown in ferrets (20, 23). Direct-contact transmission in the ferret model is highly reproducible (100% transmission from donor to acceptor animals), but ferrets display limited clinical signs. After infection via direct inoculation or transmission, SARS-CoV-2 can readily be detected in and isolated from the throat and nose, and viral replication leads to seroconversion.

To assess the efficacy of [SARSHRC-PEG4-Chol] in preventing SARS-CoV-2 transmission, naïve ferrets were dosed prophylactically with the lipopeptide before being cohoused with SARS-CoV-2–infected ferrets. In this setup, transmission via multiple routes can theoretically occur (aerosol, orofecal, and scratching or biting), and ferrets are continuously exposed to infectious virus during the period of cohousing, providing a stringent test for antiviral efficacy. The study design is shown in fig. S6. Three donor ferrets (gray in fig. S6) were inoculated intranasally with 5 × 10⁵ 50% tissue culture infective dose (TCID50) SARS-CoV-2 on day 0. Twelve recipient ferrets were dosed prophylactically with the lipopeptide before being cohoused with SARS-CoV-2–infected ferrets.
housed separately were treated by nose drops with a mock preparation or [SARSHRC-PEG4]2-chol peptide (red and green, respectively, in fig. S6) 1 and 2 days post inoculation (dpi) of the donor animals. The [SARSHRC-PEG4]2-chol peptides for intranasal administration were dissolved to a concentration of 6 mg/ml in an aqueous buffer containing 2% DMSO, and ferrets were administered a final dose of 2.7 mg/kg of body weight (450 μl divided equally in both nostrils). Peptide stocks and working dilutions had similar IC50 values, confirming potency of triplicates is shown; dotted lines show 50% inhibition. Additionally, the potency of [HPIV-3HRC-PEG4]2-chol was confirmed by infection inhibition (%)

![Graph](image-url)

**Fig. 3.** Inhibition of infectious SARS-CoV-2 entry by [SARSHRC-PEG4]2-chol and [HPIV-3HRC-PEG4]2-chol peptides. (A and B) The percentage inhibition of infection is shown on VeroE6 and VeroE6-TMPRSS2 cells with increasing concentrations of [SARSHRC-PEG4]2-chol (red lines) and [HPIV-3HRC-PEG4]2-chol (gray lines). DMSO-formulated (A) and sucrose-formulated (B) stocks were tested side by side. Mean ± SEM of triplicates is shown; dotted lines show 50% and 90% inhibition. Additionally, the potency of [HPIV-3HRC-PEG4]2-chol was confirmed by inhibition of infectious HPIV-3 entry (dotted green lines, performed on Vero cells).

**Fig. 4.** [SARSHRC-PEG4]2-chol prevents SARS-CoV-2 transmission in vivo. (A and B) Viral loads detected in throat (A) and nose (B) swabs by RT-qPCR. (C) Comparison of the AUC from genome loads reported in (B) for mock- and peptide-treated sentinels. (D) Viral loads detected in throat swabs by virus isolation on VeroE6. (E) Correlation between viral loads in the throat as detected via RT-qPCR and virus isolation. Presence of anti-S (F) or anti-N (G) antibodies was determined by IgG ELISA assay. Presence of neutralizing antibodies was determined in a virus neutralization assay (H). Virus neutralizing antibodies are displayed as the end-point serum dilution factor that blocks SARS-CoV-2 replication. Direct inoculation of peptide-treated or mock-treated animals with SARS-CoV-2 led to productive infection in only the previously peptide-treated animals (I), in the absence of S-specific, N-specific, and neutralizing antibodies. Donor animals shown in gray, mock-treated animals in red, peptide-treated animals in green. Symbols correspond to individual animals (defined in fig. S6). Line graphs in (A), (B), (D), and (F) to (I) connect the median of individual animals over time. Mock- and peptide-treated groups were compared using two-way ANOVA repeated measures [(A), (B), and (F) to (I)] or Mann-Whitney test (C).
promising, although the optimal formulation and dosing regimen are an area of ongoing experimentation.

The intranasal [SARSrNC-PEG4]2-chol peptide presented in this study is a successful prophylaxis that prevents SARS-CoV-2 transmission in a relevant animal model, providing complete protection during a 24-hour period of intense direct contact. Parallel approaches to prevent transmission that target the interaction between S and angiotensin-converting enzyme 2 have shown promise in vitro [e.g., the “miniprotein” approach (24)]. The lipo-

peptide described here acts on the S2 domain after shedding of S1 (fig. S2 and movie S1) and is complementary to strategies that target the functions of S1 or maintain S in its prefusion conformation, e.g., synthetic nanobodies (25). Fusion-inhibitory lipopeptides could be used for pre- and postexposure prophylaxis in combination with these strategies and in conjunction with treatments [e.g., ribonucleoside analogs (26)] that reduce replication in a treated infected individual. A combination of drugs that target different aspects of the viral life cycle is likely ideal for this rapidly evolving virus. Of note, the [SARSrNC-PEG4]2-

chol lipopeptide is equally active against several emerging SARS-CoV-2 variants, including D614G as well as the recent variants of concern B.1.1.7 and B.1.351. The [SARSrNC-PEG4]2 chol peptide has a long shelf life, does not require refrigeration, and can easily be administered, making it particularly suited to treating hard-to-reach populations. This is key in the context of COVID-19, which has affected every community, with the burden falling disproportionately on low-income and otherwise marginalized communities. This HRC lipopeptide fusion inhibitor is feasible for advancement to human use and should read-

ily translate into a safe and effective nasal spray or inhalation-administered fusion inhibitor for SARS-CoV-2 prophylaxis, supporting containment of the ongoing COVID-19 pandemic.

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SUPPLEMENTARY MATERIALS

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View/request a protocol for this paper from Bio-protocol.

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