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Supplementary Materials

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Materials and Methods

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Materials and Methods

Ethics statement. Influenza virus, SARS-CoV-2 and Aleutian Disease Virus seronegative female ferrets (*Mustela putorius furo*), weighing 900-1200g, were obtained from a commercial breeder (Triple F Farms, PA, USA). Animals were housed and experiments were performed in compliance with the Dutch legislation for the protection of animals used for scientific purposes (2014, implementing EU Directive 2010/63). Research was conducted under a project license from the Dutch competent authority (license number AVD1010020174312) and the study protocol was approved by the institutional Animal Welfare Body (Erasmus MC permit number 17-4312-07). Animal welfare was monitored on a daily basis.

Lipopeptide synthesis. The peptide ($SARS_{HRC}$) corresponding to residues 1168–1203 of SARS-CoV-2 S with a C-terminal -GSGSGC spacer sequence was prepared by solid phase peptide synthesis (SPPS). The $SARS_{HRC}$ peptide was acetylated at the N-terminus and amidated at the C-terminus. The crude peptide was purified by reversed-phase HPLC chromatography and characterization by MALDI-TOF MS. The $SARS_{HRC}$-PEG_4-chol, $[SARS_{HRC}]_2$-PEG_{11}, and $[SARS_{HRC}$-$PEG_4]_2$-chol were synthesize d via chemoselective thiol- Michael addition reactions between the terminal thiol group on the peptide cysteine residue and maleimide functional PEG linkers or PEG-cholesterol linkers as previously

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described (*1*). HPLC purification and lyophilization yielded the peptide-lipid conjugates as white powders. The identity of the conjugates was verified by MALDI-TOF MS (**Fig. S5).**

Dissolving [$SARS_{HRC}$-PEG_4]$_2$-chol for use in experiments. [$SARS_{HRC}$-PEG_4]$_2$-chol was supplied as a white powder in aliquots of 10 mg. For *in vitro* experiments with live virus and *in vivo* experiments in ferrets, 10 mg of [$SARS_{HRC}$-PEG_4]$_2$-chol was dissolved in 33.3 ul DMSO, which was subsequently added to 1632.7 ul de-ionized H_2O. This yielded a final aqueous solution of lipopeptide dissolved at a concentration of 6 mg/mL containing 2% DMSO. To obtain peptide dissolved in aqueous solution without DMSO , 100 mg/ml of the [$SARS_{HRC}$-PEG_4]$_2$-chol peptide in DMS0 (10 mg of peptide in 100 ul of DMSO) and 1 mg/ml of sucrose in sterile water were prepared. 10 ul of the peptide solution (1mg) was added to 100µl of sucrose (0.1 mg). Lyophilisation of the peptide solution (DMSO + sucrose) was performed over-night and dry powder was resuspended in 50µl of sterile water to a final concentration is 20 mg/ml in water without any DMSO.

Cells. Human kidney Epithelial (HEK) 293T and Vero (African green monkey kidney) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific) supplemented with 10% foetal bovine serum (FBS) and antibiotics in 5% CO_2 at 37°C. VeroE6 (ATCC CRL-1586) and VeroE6-TMPRSS2 cells were grown in DMEM (Gibco) with 10% FBS, 2 mM L-glutamine (Gibco), 10 mM Hepes (Lonza), 1.5 mg/ml sodium bicarbonate (NaHCO3, Lonza), penicillin (10,000 IU/mL) and streptomycin (10,000 IU/mL)(*2*).

HAE cultures. The EpiAirway AIR-100 system (MatTek Corporation) consists of normal human-derived tracheal/bronchial epithelial cells that have been cultured to form a pseudostratified, highly

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differentiated mucociliary epithelium closely resembling that of epithelial tissue *in vivo*. Cultures were transferred to six-well plates containing 1.0 ml medium per well (basolateral feeding, with the apical surface remaining exposed to air) and acclimated at 37°C in 5% CO2 for 24h prior to experimentation (*3*).

Plasmids. The cDNAs coding for hACE2 fused to the fluorescent protein Venus, dipeptidyl peptidase 4 (DPP4) fused to the fluorescent protein Venus, SARS-CoV-2 S, SARS-CoV S, and MERS-S (codon optimized for mammalian expression) were cloned in a modified version of the pCAGGS (with puromycin resistance for selection).

β-Gal complementation-based fusion assay. We previously adapted a fusion assay based on alpha complementation of β-galactosidase (β-Gal)(*4*). In this assay, hACE2 receptor-bearing cells (or dipeptidyl peptidase 4 (DPP4) receptor-bearing cells for MERS-CoV-2 experiments) expressing the omega peptide of β-Gal are mixed with cells co-expressing glycoprotein S and the alpha peptide of β-Gal, and cell fusion leads to alpha-omega complementation. Fusion is stopped by lysing the cells and, after addition of the substrate (®The Tropix Galacto-Star™ chemiluminescent reporter assay system, Applied Biosystem), luminescence is quantified on a Tecan M1000PRO microplate reader.

Cell toxicity assay. HEK293T or Vero cells were incubated with the indicated concentration of the peptides or vehicle (dimethyl sulfoxide) at 37 °C. Cell viability was determined after 24h using the Vybrant MTT Cell proliferation Assay Kit according to the manufacturer's guidelines. The absorbance was read at 540 nm using Tecan M1000PRO microplate reader. HAE cultures were incubated at 37°C in the presence or absence of 1, 10, or 100 μM concentrations of the peptide, and peptide was added to the feeding medium every 2 days for 7 days. Cell viability was determined on day 7 as above.

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Virus. SARS-CoV-2 (isolate BetaCoV/Munich/BavPat1/2020; kindly provided by Prof. Dr. C. Drosten) was propagated to passage 3 on VeroE6 cells in OptiMEM I (1X) + GlutaMAX (Gibco), supplemented with penicillin (10,000 IU/mL, Lonza) and streptomycin (10,000 IU/mL, Lonza) at 37°C. VeroE6 cells were inoculated at a multiplicity of infection (MOI) of 0.01. Supernatant was harvested 72 hours post inoculation (HPI), cleared by centrifugation and stored at -80°C. All live virus work was performed in a Class II Biosafety Cabinet under BSL-3 conditions at Erasmus MC.

***In vitro* potency of HRC dimer-chol.** Potency of $[SARS_{HRC}\text{-}PEG_4]_2$-chol was determined in an *in vitro* live virus fusion assay. Original stocks and working dilutions for animal experiments were tested in triplicate in VeroE6 and VeroE6-TMPRSS2 cells at concentrations of 0.06 nM to 5 μM. Peptide was pre-incubated with the cells for 1 hr at 37°C. After pre-incubation, virus (600 $TCID_{50}$) was added. After 8 hrs at 37°C, cells were washed and fixed with 4% PFA for 20 min at room temperature (RT). Plates were submerged in 70% ethanol and stained in a BSL-2 laboratory. In short, cells were washed with PBS and blocked with 10% normal goat serum (NGS) for 30 min at RT. Primary mouse-anti-SARS-CoV nucleocapsid antibody (1:1000, Biorad) was incubated for 1 hr at RT in 10% normal goat serum (NGS). After washing, secondary goat-anti-mouse IgG/FITC antibody (1:1000, Invitrogen) was incubated for 45 min at RT in 10% NGS. Fluorescent spots were visualized with an Amersham Typhoon Biomolecular Imager (GE Healthcare) and counted with ImageQuant TL 7.0 software (GE Healthcare).

Ferret transmission experiment. All animal handlings were performed under anaesthesia with a mixture of ketamine/medetomidine (10mg/kg and 0.05mg/kg, respectively) antagonized by atipamezole (0.25 mg/kg). Three donor ferrets were inoculated intranasally with 5.4×10^5 $TCID_{50}$ of SARS-CoV-2 in 450μl (225μl instilled dropwise in each nostril) and were housed together in a negatively pressurized

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HEPA-filtered ABSL-3 isolator. This was considered the start of the experiment (0 days post inoculation, DPI). At the same time, twelve direct contact ferrets were divided over three other isolators. Ferrets were either mock-treated (vehicle, 2%DMSO in distilled water) or treated with $[SARS_{HRC}\text{-}PEG_4]_2$-chol on 1-4 DPI. The peptide was inoculated intranasally in 450µl (225µl instilled dropwise in each nostril), HRC dimer-chol treated ferrets received a peptide dose of ~2.7 mg/kg. Leftover batches were stored at -80°C for later use in *in vitro* potency assays. At 2 DPI, six hours after the second treatment, one donor ferret was placed in the same isolators as two mock-treated and two peptide-treated ferrets, in three separate isolators (**Fig. 3a**). Each isolator now contained five ferrets, the donor ferret, the mock-treated recipient ferrets and the $[SARS_{HRC}\text{-}PEG_4]_2$-chol-treated recipient ferrets. At 3 DPI, 18 hours after onset of co-housing, the animals received a third mock or peptide treatment, Six hours later, i.e. 24 hours after the start of the co-housing, the donor animals were moved back to their original isolator and the mock-treated and peptide-treated ferrets were housed in two groups of six animals in clean isolators.

Throat and nose swabs were collected from the animals on 0, 1, 2, 3, 4, 5, 6, 7, 14 and 21 DPI. Samples were always obtained prior to dosing with mock or peptide. Swabs were stored at -80°C in virus transport medium (Minimum Essential Medium Eagle with Hank's BSS (Lonza), 5 g L−1 lactalbumine enzymatic hydrolysate, 10% glycerol (Sigma-Aldrich), 200 U/ml of penicillin, 200 mg/ml of streptomycin, 100 U/ml of polymyxin B sulfate (Sigma-Aldrich), and 250 mg/ml of gentamicin (Life Technologies)). Blood samples were obtained from ferrets on 0, 7, 14 and 21 DPI by vena cava puncture. Blood was collected in serum-separating tubes (Greiner), processed, heat-inactivated and sera were stored at -80°C. The study was stopped at 21 DPI. All animal experiments were performed in class III isolators in a negatively pressurized ABSL3 facility, all handlings were performed under general anaesthesia.

RNA isolation and RT-qPCR on throat and nose swabs. Sixty ul sample (virus transport medium in which swabs are stored) was added to 90 ul of MagNA Pure 96 External Lysis Buffer (Roche). A known

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concentration of phocine distemper virus (PDV) was added to the sample as internal control for the RNA extraction (*5*). The 150 ul of sample/lysis buffer was added to a well of a 96-well plate containing 50 ul of magnetic beads (AMPure XP, Beckman Coulter). After thorough mixing, the plate was incubated for 15 min at room temperature. The plate was then placed on a magnetic block (DynaMag™-96 Side Skirted Magnet (ThermoFisher Scientific)) and incubated for 3 min to allow the displacement of the beads towards the side of the magnet. Supernatants were carefully removed and beads were washed three times for 30 sec at room temperature with 200 ul/well of 70% ethanol. After the last wash, a 20 ul multi-channel pipet was used to remove residual ethanol. Plates were air-dried for 2 min at room temperature. Plates were removed from the magnetic block and 50ul of PCR grade water was added to each well and mixed. Plates were incubated for 5 min at room temperature and then placed back on the magnetic block for 2 min to allow separation of the beads. Supernatants were pipetted in a new plate and RNA was stored at -80°C. The RNA was directly used for RTqPCR using primers and probes targeting the E gene of SARS-CoV-2 as previously described (*6*).

Virus neutralization of ferret sera. Seroconversion of ferrets was tested with ferret sera from 0, 7, 14 and 21 DPI. Duplicates of ferret sera were incubated with 100 $TCID_{50}$ of virus in a 2-fold dilution series starting at a concentration of 1:8 for 1 hr at 37°C. Virus-sera mix was added to VeroE6 cells and incubated for 5 days at 37°C. Cytopathic effect was used as readout to determine the minimal serum concentration required to inhibit CPE formation.

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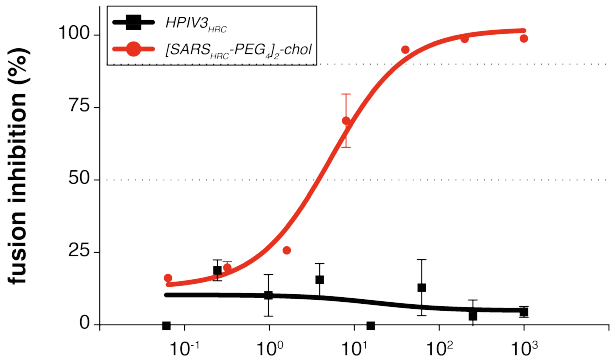

Figure S1. Specificity of SARS-CoV-2 inhibition by [SARS-CoV-2-HRC-peg$_4$]$_2$-chol. A lipopeptide based on the human parainfluenza virus type 3 (HPIV3) F protein HRC domain, used as a negative control, did not inhibit fusion at any concentration tested.

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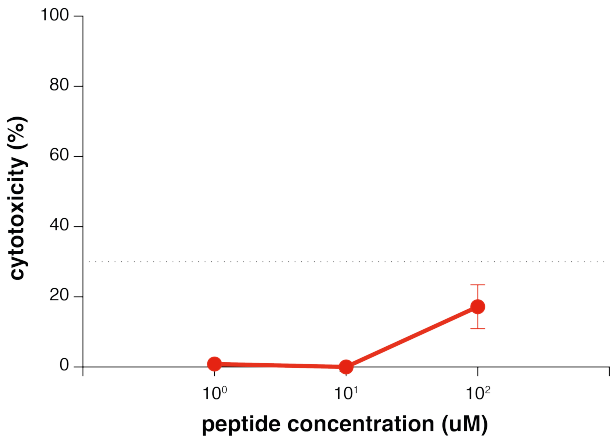

Figure S2. ***Ex vivo*** **cytoxicity assessment.** An MTT assay was used to determine the toxicity of the [SARS-CoV-2-HRC-peg_4]$_2$-chol in human airway epithelium (HAE). No toxicity was observed for the peptide at the concentrations of 1 and 10 μM. Toxicity was minimal (<20%) at the highest concentration tested (100 μM).

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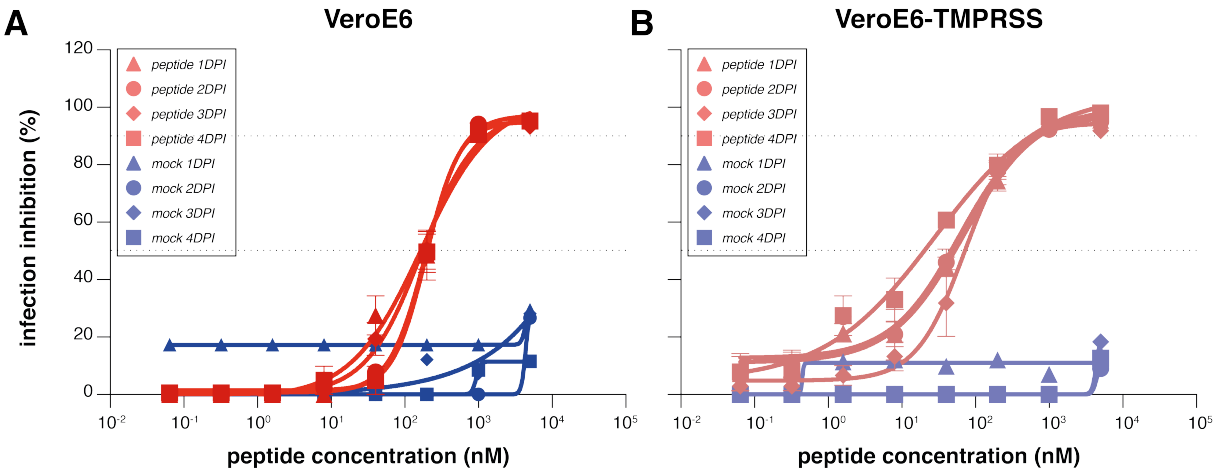

Figure S3: Potency of peptide stocks used *in vivo*. The potency of peptide dilutions used on 1-4 DPI in the *in vivo* experiments was confirmed with a live virus infection assay. The percentage infection events is shown on (A) VeroE6 and (B) VeroE6-TMPRSS with increasing concentrations of [SARS-CoV-2-HRC-peg$_4$]$_2$-chol (red) or mock (blue). Inhibitory concentrations 50% and 90% are indicated (dotted lines). Data are means ± standard error of the mean (SEM) from one experiment.

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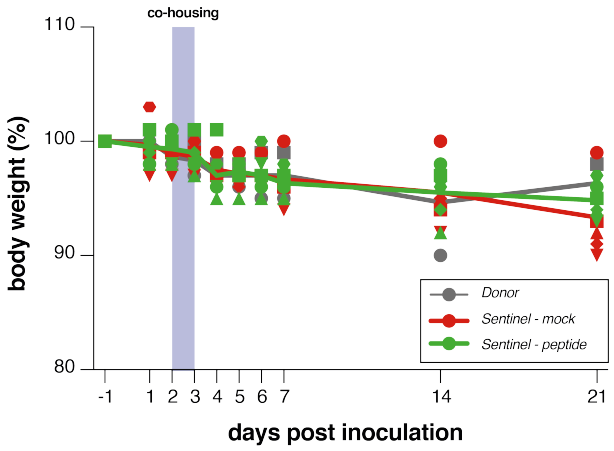

Figure S4. SARS-CoV-2-infected ferrets do not lose weight. Body weights of all ferrets remained stable over the time of the experiment. donor animals shown in grey, mock-treated animals in red, peptide-treated animals in green. Symbols correspond to individual animals and are consistent throughout figures.

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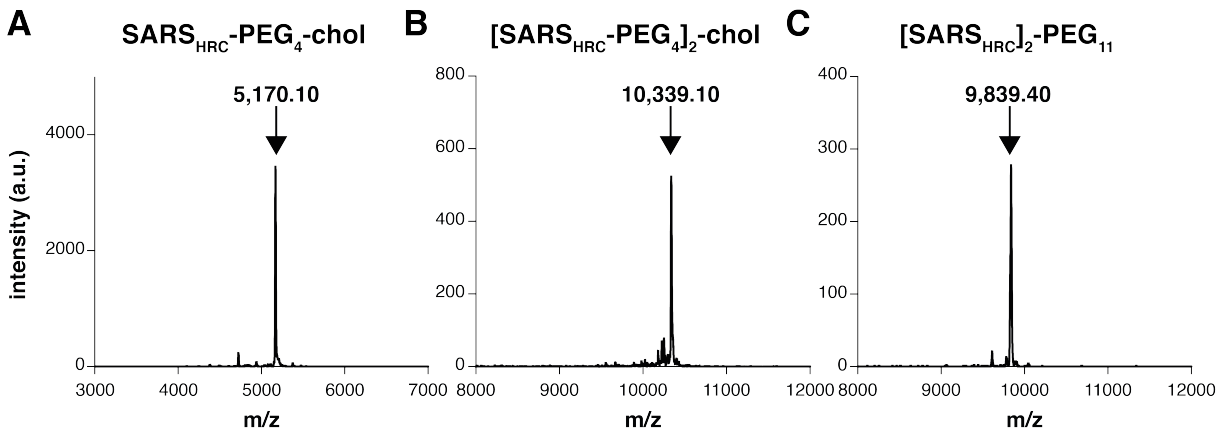

Figure S5. Identity of the conjugates was verified by MALDI-TOF MS. (A) MALDI of SARS$_{HRC}$-PEG$_4$-chol. Theoretical: 5170.8 Da; observed 5170.1 Da. (B) MALDI of [SARS$_{HRC}$-PEG$_4$]$_2$-chol. Theoretical m/z: 10,335.4 Da; observed 10,339.10 Da. (C) MALDI of [SARS$_{HRC}$]-PEG$_{11}$. Theoretical m/z: 9841.0 Da; observed m/z: 9,839.40 Da.

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