**Griffithsin Inhibits Nipah Virus Entry and Fusion, and Can Protect Syrian Golden Hamsters from Lethal Nipah Virus Challenge**

**(Supplemental Materials and Methods)**

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**MATERIALS AND METHODS**

**Biosafety**

All work with infectious virus or infected animals was conducted in a biosafety level 4 (BSL-4) laboratory at Centers for Disease Control (CDC) following established BSL-4 standard operating procedures approved by the Institutional Biosafety Committee.

**Cloning, expression and purification of GRFT, monomeric GRFT (mGRFT), Q-GRFT and mGRFT tandemers**

All restriction enzymes were purchased from New England Biolabs. All chemicals were obtained from American Bioanalytical. All primers were purchased from Integrated DNA Technologies. GRFT, monomeric GRFT (mGRFT), mGRFT tandemers, and Q-GRFT were expressed and purified as described previously for the monomeric forms of GRFT [1], except that buffer A contained 50 mM maltose.

**Plasmids**

Codon-optimized mCherry, Malaysian NiV-F tagged with AU1 and NiV-G tagged with either HA or His6× were synthesized and cloned into a modified pCAGGS mammalian expression plasmid (Genscript) [2]. A pTM1-based plasmid encoding enhanced green fluorescent protein (eGFP) under the control of a T7 polymerase promoter (pT7-eGFP) was a kind gift from Dr. Mark E. Peeples.

**Cells**

HeLa (ATCC CCL-2), HEK293T/17 (ATCC CRL-11268), HT-1080 (ATCC CCL-121), Vero (ATCC CCL-81), and BSRT7/5 cells [3] were cultivated in DMEM supplemented with 10% fetal bovine serum (FBS). CHO-K1 cells (ATCC CCL-61) were maintained in F-12K medium supplemented with 10% FBS. All cells were incubated at 37°C in 5% CO2.

**Viruses**

Nipah viruses (NiV-M (passage 3), NiV-B (passage 3), rNiV-RLuc (formerly named NiV-Luc2AM)(passage 2); rNiV-ZsG (formerly named NiV-GFP2AM))(passage 3) [4], Hendra virus (HeV) (passage 7), and recombinant reporter recombinant Measles vaccine virus (rMVEZEGFP(3))(passage 5) [5], Mumps virus (rMuV-EGFP, Iowa 2006 strain) (passage 3) [6], human parainfluenza virus 3 (hPIV3-GFP, JS strain) (passage 3)[7], human metapneumovirus (hMPV-GFP, CAN97-83 strain) (passage 2) [8], Lassa virus (rLASV-ZsG) (passage 3)[9], and Crimean Congo Hemorrhagic Fever virus (rCCHFV-ZsG) (passage 2)[10] were propagated in either Vero E6 (ATCC CRL-1586) or Vero (ATCC CCL-81) cells, and were quantitated by 50% tissue culture infections dose (TCID50) assay using the Reed and Muench method [11]. Both hMPV-GFP (CAN97-83) and hPIV3-GFP (JS) were obtained from ViraTree. Unless stated otherwise, all infections were performed at multiplicity of infection (MOI) of 0.25.

**Reporter virus and minigenome assays**

Recombinant reporter virus assays were performed as previously described [4, 12]. 104 cells were seeded into 96-well opaque white plates (Corning 3917) overnight and were pre-treated with 3-fold serial dilutions of GRFT for 1 h before being infected with rNiV-RLuc without washing at multiplicity of infection (MOI) = 0.25. At 24 h post-infection (hpi), Renilla luciferase activity was measured using Renilla-Glo reagent (Promega) in a Biotek HD1 synergy instrument and was normalized to untreated cells infected with rNiV-RLuc. Recombinant reporter viruses expressing green fluorescent proteins were assayed between 48-72 hpi either for total fluorescence or for numbers of GFP+ cells using respective size and fluorescence parameters of ≥ 10 µm and ≥ 5000 fluorescence units. Total fluorescence was measured using a Biotek HD1 Synergy instrument, while cell-counting assays were performed using a Cytation5 instrument. 50% effective concentrations (EC50) were calculated using four-parameter variable slope non-linear regression fitting of mean values of assays performed in quadruplicate. For time of addition assay, GRFT was either pre-incubated with rNiV-RLuc for 2 hours before being applied to HT-1080 cells, or was added to cells at 0, 2, 6, or 12 hpi. For assays using increasing amounts of rNiV-RLuc, 1, 3, or 9 µg/mL of GRFT was added to cells 1 h prior to infection. A Nanoluciferase-based NiV minigenome assay was conducted as previously described [13]. BSRT7/5 cells [3] (104 per well) were seeded in 96-well plates, and transfected with appropriate amounts of NiV support plasmids (N (50 ng/well), P (32 ng) and L (50 ng)), NiV minigenome (120 ng) prepared in RNase-free TE buffer, mixed with 0.8 μL/well LT-1 transfection reagent (Mirus Bio, Madison, WI) and 10 μL Opti-MEM/well. Complexes were incubated for 30 min at room temperature before adding to cells. GRFT was added directly to the cells 4 h post-transfection. At 48 h post-transfection, 50 μL of Nanoluciferase assay buffer solution (Promega) was added directly to each well. Well contents were transferred to 96-well opaque white plates, and after three minutes, luminescence was read on a plate reader (HD1-Synergy, Biotek). Reporter Nanoluciferase activity for each well was then normalized to the levels of activity detected in untreated transfected cells.

**Virus yield reduction assay**

104 HT-1080 or Vero cells were infected with 0.25 TCID50 of either NiV-M, NiV-B, or rNiV-ZsG per cell for 1 h. Virus inoculum was then removed, cells were washed once with phosphate buffered saline (PBS), and replaced with culture medium containing GRFT or 3mG in a 10-point 3-fold dilution series. At 24-48 hpi supernatants were harvested, 10-fold serially diluted, and mixed with 104 Vero cells per well in 96-well plates. At day 5 post-infection, plates were assayed for cytopathic effect (CPE), and then viral titers were quantitated by 50% tissue TCID50 assay using the Reed and Muench method [11]. EC50 values were calculated using four-parameter variable slope non-linear regression fitting of mean values derived from quadruplicate samples. Fluorescent micrographs of rNiV-ZsG infected cells were captured with the EVOS FL Cell Imaging System (Thermo Fisher) at 4× magnification using the GFP fluorescence filter. Infectious virus titer reduction and fluorescence micrographs were performed using 96-well plates with black opaque sides and clear bottoms (Corning 3603).

**NiV-F and NiV-G glycoprotein expression, purification, and detection by Western blot**

6-well plates seeded with 3×105 HEK293T cells/well were transfected with 3 µg of eukaryotic expression plasmid encoding either an AU1-tagged codon-optimized NiV F protein, a His6×-tagged codon-optimized NiV-G protein, or a codon-optimized mCherry protein. At 48 h post-transfection, supernatants were discarded, cells were washed once in PBS before being harvested in 250 µL Radioimmunoprecipitation (RIPA) buffer/well. AU1-tagged NiV-F protein (NiV-F AU1) was purified from cell lysates using an anti-AU1 affinity bead column according to manufacturer’s recommendations (BioLegend). NiV-G His6× was purified using nickel-nitrilotriacetic acid affinity resin. Cell lysates and purified proteins were run on SDS-PAGE gels (Supplemental Figure 1A), transferred onto PVDF membranes, and probed with either anti-AU1 (Bethyl) or anti-His6x (Cell Signaling Technology) rabbit antibodies, or with GRFT along with anti-GRFT polyclonal rabbit sera, and detected using goat anti-rabbit 680RD secondary antibodies (LI-COR) (Supplemental Figure 1B-C). Western blots were visualized using LI-COR imaging.

**Enzyme linked immunosorbent Assay (ELISA) against purified NiV F and NiV G glycoproteins**

GRFT binding ELISAs were performed as previously described [14]. Purified NiV-F AU1 and NiV-G His6× protein concentrations were estimated using absorbance at 280 nm and converted to molarity using the recombinant protein theoretical MW. As NiV-F AU1 is present in the purified pool as at least two products (F0, F1), the MW used for molar conversion was 55 kDa. The MW of NiV-G His6× was estimated at 70 kDa. For GRFT binding saturation ELISA studies comparing NiV-F and NiV-G binding, a quantity of 1 pmol of purified recombinant NiV-F AU1 or NiV-G His6× was immobilized in triplicate on a Griener 96-well plate overnight. The plate was washed with PBS including 0.05% tween-20 (WB) and blocked using 5% BSA in PBS. Wells were washed three times with WB and incubated with serial 100.5 log dilutions of recombinant His-tagged GRFT in PBS for one hour at room temperature. Wells were washed three times with WB and incubated for one hour at room temperature with a 1:1000 dilution of rabbit anti-GRFT polyclonal antibody in WB supplemented with 0.5% BSA. Wells were washed three times with WB and incubated for one hour at room temperature with a 1:1000 dilution of goat anti-rabbit IgG-HRP in WB supplemented with 0.5% BSA. HRP activity was detected using a chromogenic TMB development solution. Reactions were quenched with 1M HCl prior to reading at ABS 450 nm. For comparative binding studies between GRFT and 3mG, 50 ng of either NiV-F AU1 or NiV-G His6× was used in each well.

**Pseudotyped Vesicular Stomatitis virus (VSV) entry assay**

A replication deficient VSV expressing enhanced green fluorescent protein in place of the VSV attachment glycoprotein (VSV-ΔG-eGFP) was pseudotyped with either VSV G (VSVpt) or NiV F and NiV G glycoproteins (NiVpt) as previously described [15]. 50 particles of VSVpt or NiVpt were used to either infect Vero cells in conjunction with GRFT/3mG treatment for 24 h, or to infect Vero cells that were pretreated with GRFT/3mG for 1 h and then removed prior to infection. At 24 hpi, numbers of eGFP expressing cells were visualized and counted using an Olympus CK-40 microscope equipped with epifluorescence [16].

**Immune Fluorescence Assay**

104 HT-1080 cells in 96-well clear plates were infected with wild-type recombinant NiV (rNiV) for 1 h. Virus inoculum was removed, cells were washed once with PBS, and replenished with growth media containing 3-fold dilutions of GRFT. At 24 hpi, supernatants were removed, and cells were fixed in 10% formalin supplemented with 0.2% Triton-X 100 detergent for 15 min. Fixed cells were stained using GRFT at 1 µg/mL and a monoclonal anti-NiV nucleoprotein antibody [17] (1:5000 dilution in 5% skim milk in PBS) for 1 h at 37° C. After 3 washes with WB, cells were stained with anti-GRFT polyclonal rabbit antibody (1:100 dilution in 5% skim milk in PBS) for 1 h at 37° C. After another 3 washes with WB, cells were stained with Dylight488 and anti-mouse Dylight550 secondary detection antibodies (Bethyl Laboratories, 1:1000 dilution) along with 4′,6-diamidino-2-phenylindole (DAPI) (1:1000 dilution) for 1 h 37° C. After 3 final washes with WB, 100 µL of PBS was added to each well, and fluorescence micrographs were taken using a Nikon Axioscope Ti inverted fluorescence microscope at 4× magnification.

**Cytopathic effect (CPE) inhibition assays**

Inhibition of virus-induced CPE was assayed using CellTiter-Glo 2.0 reagent (Promega) in a Biotek HD1 Synergy plate reader [13]. Briefly, 104 Vero cells pre-treated with serial dilutions GRFT, Q-GRFT, mGRFT, 2mG, or 3mG in 96-well opaque white plates were infected with NiV-M, NiV-B, or HeV. At 72 hpi, 100 µL CellTiter-Glo 2.0 reagent was added to each well and the plate was incubated at room temperature for at least 10 minutes prior to luminescence measurement. Luminescence values were normalized to uninfected cell controls according to % viability as follows: % viability = [(specific value-reference value)/(DMSO control value – reference value)] X 100. Reference values were derived from control wells without cells. Uninfected cell control values (after subtraction of reference values) were set at 100% inhibition of CPE. EC50 values were calculated using four-parameter variable slope non-linear regression fitting of values.

**Fusion Assay**

104 HT-1080 cells were seeded in 96-well black plates with clear bottoms (3603 Costar) overnight. Mirus LT-1 reagent (MirusBio) was used to transfect cells with 0.2 µg of different combinations of expression plasmids either encoding AU1-tagged codon-optimized NiV F protein or HA-tagged codon optimized NiV G protein. At 4 h post-transfection, cells were washed once with PBS, and replenished with growth media containing 3-fold serial dilutions of either GRFT or 3mG. At 24 h post-transfection, cells were fixed using 10% formalin solution for 15 minutes, washed once with PBS, and stained with CellMask Green (Thermo Fisher) and DAPI according to manufacturer’s instructions. Cells were viewed and micrographs were taken using a Nikon Axioscope Ti inverted fluorescence microscope using 4× magnification.

**Quantitative fusion assay**

2×104 CHO-K1 cells seeded in 96-well black plates with clear bottoms were transfected with 0.1 µg each of NiV-F AU1, NiV-G HA, and pT7-eEGFP plasmids with Mirus LT-1 transfection reagent at a DNA to LT-1 reagent ratio of 1:2. At 4 h post-transfection, transfection mixes were removed, cells were washed once with PBS, and were replenished with growth media containing 2-fold serial dilutions of GRFT or 3mG. After addition of GRFT/3mG conditioned media, 2×104 of BSRT7/5 cells were overlaid onto the transfected CHO-K1 cells. At ~ 16 h post-overlay, NiV F/G-induced syncytia were visualized using a 2.5× objective, and total numbers were counted from photograph montages of each complete well using Gen5 Software on a Cytation5 instrument (Biotek). Green fluorescent objects that were both greater than 75 µm in diameter and that had a relative fluorescence reading above 5000 were classified as positive syncytia. Negative control CHO-K1 cells were transfected with 0.2 µg of NiV-F AU1 and 0.1 µg pT7-eGFP.

**Ethics statement and animals**

All animal experiments were approved by the CDC Institutional Animal Care and Use Committee and were performed in an AAALAC-international approved facility. Groups of 5–10 female HsdHan: AURA Syrian hamsters (Envigo #8903F; 6 weeks of age) were inoculated intranasally with DMEM (mock control) or a target dose of NiV-B (target dose: 107 TCID50; backtiter dose: 1.17×107). Hamsters were housed in a climate-controlled laboratory with a 12 h day/night cycle; provided Teklad global 18% protein rodent diet (Envigo) and water ad libitum; and group-housed on corn cob bedding (Bed-o'Cobs® ¼”, Anderson Lab Bedding) with cotton nestlets and crinkle paper, in an isolator-caging system (Thoren Caging, Inc., Hazleton, PA, USA) with a HEPA-filtered inlet and exhaust air supply. On 1 and 2 dpi and/or -1 and -2 dpi, animals were treated IN with vehicle control (PBS), GRFT (10 mg/kg), or 3mG (10 mg/kg). Weight, temperature and clinical signs were assessed daily for 27 days post infection (dpi). Clinical signs were quantified by the following scoring system: 2 points each for quiet/ dull/responsive disposition, hunched back, ruffled coat, hypoactivity/abnormal huddling; 3 points for dehydration/decreased skin turgor; 5 points each for dyspnea, anemia, or moderate neurological signs (moderate head tilt, tremors, ataxia, and/or circling); and 10 points each for inability to bear weight, frank hemorrhage, severe neurological signs (severe head tilt, seizures), or weight loss >25% baseline (at -1 dpi). Animals with a score of ≥ 10, or at completion of study (28 dpi) were humanely euthanized with an isoflurane vapor overdose.

**Quantitative RT-PCR**

RNA was extracted from blood and homogenized tissue samples using the MagMAX-96 Total RNA Isolation Kit (Thermo Fisher) on a 96-well ABI MagMAX extraction platform with a DNaseI treatment step according to manufacturer’s instructions. RNA was quantitated using a one-step real-time RT-PCR targeting the N gene sequence using in vitro transcribed NiV N gene RNA standards [18] , and was standardized to 18S with a SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher Scientific) according to manufacturer’s instructions (primer and probe sequences available on request).

**Plasma antibody analysis**

Total NiV-specific IgG and neutralizing antibodies were measured as previously described [19]. Plasma samples, separated from whole blood collected in lithium heparin tubes by centrifuging 3 min at 10000 rpm, were inactivated using gamma irradiation (5 million rads from a 60Co source). High protein-binding plates (Costar 2797) plates were coated overnight at 4°C, with the bottom half of the plate being coated with Vero control cell lysate, and the top half being coated with inactivated NiV-infected Vero cell lysates. After 3× washes with PBS supplemented with Tween-20 detergent (0.1%) (PBS-T), plates were blocked with 100 µL plasma diluent (5% skim milk in PBS-T). Hamster plasma were serially diluted 1:100, 1:400, 1:1600, and 1:6400 and incubated for 1 hour at 37°C. After 3× washes with PBS-T, plates were incubated with antibody detector protein A/G conjugated with HRP (Thermo Fisher) for 1 hour at 37°C. After 3× washes with PBS-T, plates were incubated with ABTS substrate (KPL) for ½ hour at 37°C. Absorbance was read at 410 nm and 490 nm. Adjusted absorbance was calculated by first subtracting 490 absorbance from 410 nm absorbance values. Then, absorbance values from the respective hamster plasma incubated with Vero control lysate were subtracted from the absorbance values of their corresponding well in which the same plasma was incubated with NiV-infected lysates. Cutoff values for positivity were determined by adding the mean of 6 negative control plasma (2 from GRFT treatment control group, 2 from 3mG treatment control group, and 2 from no treatment control group) to 3 times the standard deviation of the mean. Final adjusted absorbance values above this cutoff (the average cutoff value across the 4 dilutions was used = 0.09672) were considered positive at the respective dilution. Samples were only designated as true NiV IgG positives if their values were above the cutoff for both 1:100 and 1:400 dilutions.

To determine levels of NiV specific neutralizing antibodies, two-fold dilutions of hamster plasma were incubated with 200 TCID50 of NiV-B in duplicate for 1 h, and then transferred onto Vero cells for 6 days at 37°C. NiV neutralizing titers were recorded as the lowest reciprocal dilution in which both wells were protected from cytopathic effect.

**Statistical analyses**

All statistical analyses were performed using GraphPad Prism 8.1.2. Quantitative fusion assay and virus yield comparisons between GRFT and 3mG were analyzed using multiple t-tests with adjusted P values indicated next to respective asterisks in Figure 4. Animal survival curves were analyzed by Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon test, and were significantly different by both tests, with P values of < 0.0001.

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