Effect of a Neuropilin-1-Derived Virus Receptor Trap on Enterovirus A71 Infection In Vitro

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ABSTRACT We discovered that neuropilin 1 (NRP1) is a new receptor candidate to mediate enterovirus A71 (EVA71) into cells. In the engineered form as a decoy receptor, NRP1 was able to recognize and neutralize EVA71 but not enterovirus D68 or coxsackievirus B3 (CVB3). NRP1 recognizes EVA71 through a novel domain on the VP3 capsid protein. The principle in the design, engineering, and refinement of the NRP1-based decoy receptor described in this study represents a general and well-suited antiviral strategy.

KEYWORDS enterovirus A71, neuropilin 1, virus receptor trap, antiviral research, decoy receptor

Enterovirus A71 (EVA71), a positive-stranded RNA virus in the Picornaviridae family, can cause viral encephalitis and severe hand, foot, and mouth disease in young children. Although vaccines have been successfully developed, only two strains of inactivated EVA71 have been approved and available in China. Because of the severity of EVA71 infection and the absence of effective therapeutics for treating EVA71 infection, it is important to emphasize drug discovery for EVA71 (1).

Currently, seven EVA71 receptors have been identified, and some were successfully applied in animal models for neuropathogenesis studies and therapeutic development (Table 1) (2–10). However, no decoy strategy from the known receptors has yet been shown to be effective for EVA71 treatment (11). In this study, we demonstrated that neuropilin 1 (NRP1) can bind to EVA71 through the soluble extracellular domain (ECD), NRP1-expressing BHK21 cells susceptible to EV71 infection, and NRP1 decoy receptor can neutralize viral infectivity.

NRP1 is a 130- to 140-kDa glycoprotein consisting of an ECD, a transmembrane region, and an intracellular PDZ-domain binding motif at the C’ terminus. NRP1 is expressed in many cell types, and it is involved in the development of the nervous and vascular systems (12). In addition, the modular structure of the NRP1 ECD can bind to diverse ligands; the a1-a2 domain (NRP1-ECD-[a1a2]) and the b1-b2 domain (NRP1-ECD-[b1b2]) confer selective binding to semaphorin 3 (SEMA3) and vascular endothelial growth factor 165 (VEGF165), respectively (Fig. 1A) (13, 14). Notably, NRP1 is not only an Epstein-Barr virus entry factor but is also involved in human T-cell lymphotropic virus

Citation Wang H-C, Huang P-N, Hung H-C, Tseng S-N, Chang C-C, Tsai Y-R, Wang Y-M, Shih S-R, Hsu JT-A. 2021. Effect of a neuropilin-1-derived virus receptor trap on enterovirus A71 infection in vitro. Antimicrob Agents Chemother 65:e00695-20. https://doi.org/10.1128/AAC.00695-20.

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Received 15 April 2020
Returned for modification 5 May 2020
Accepted 23 October 2020
Accepted manuscript posted online 2 November 2020
Published 16 December 2020
Recent studies identified that NRP1, highly expressed in endothelial cells, may facilitate severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) cell entry (17, 18). To determine whether NRP1 expression can enhance permissiveness to EVA71, we transfected Chinese hamster ovary (CHO) cells with a plasmid encoding full-length human NRP1 cDNA followed by infection of the CHO-NRP1 cells with recombinant EVA71 encoding a green fluorescence protein (GFP) (EVA71-GFP). We found that the CHO-NRP1 cells stably expressing human NRP1 were permissive to EVA71 infection, whereas EVA71 permissiveness in CHO control cells were almost undetectable (Fig. 1B). Additionally, through a similar strategy, we observed that only NRP1-expressing or scavenger receptor B2 (SCARB2)-expressing, but not nonmodified control BHK21 cells, could be more permissive with EVA71-GFP viruses (Fig. 1C). Because there were more GFP-positive cells in SCARB2-expressing BHK21, SCARB2 was determined to play a more important role for EVA71. To examine the effect of NRP1 expression for EVA71 viral growth kinetics, we infected BHK21, BHK21-SCARB2, and BHK21-NRP1 cells with EVA71 TW/4643/98 (genotype C2) at a multiplicity of infection of 0.01. The viral loads from one run replication cycle (16 h postinfection) of EVA71-infected BHK21, BHK21-NRP1, and BHK21-SCARB2 cells were 620 ± 35, 3,267 ± 2,532, and 36,667 ± 2,082 PFU/ml, respectively (Fig. 1D). Significantly, BHK21-NRP1 cells could support EV71 infection and replication as well as BHK21-SCARB2 cells, and the yields of progeny viruses from these modified cells were >5-fold greater than those from nontransfected cells. Besides, viral proteins in EVA71-infected BHK21-NRP1 or BHK21-SCARB2 were more abundant in Western blot analysis (Fig. 1E). In summary, NRP1 confers susceptibility to EVA71 in BHK21 and CHO cells.

Soluble decoy receptors have become an attractive approach to develop biopharmaceuticals for treatment of autoimmune diseases and abnormal angiogenesis. Prominent examples are soluble tumor necrosis factor-alpha (TNF-α) receptors, such as etanercept, or VEGF trap, such as aflibercept (19). To improve the pharmaceutical properties of ectodomains of membrane proteins, fragment crystallizable (Fc), derived from immunoglobulin, is often employed as a fusion partner to produce chimeric decoy receptor fusion proteins with a prolonged serum half-life in vivo through the Fc receptor-mediated recycling mechanism (20). During the preparation of this work, recombinant angiotensin-converting enzyme 2 (ACE2), as a virus receptor trap, was shown to be effective in controlling SARS-CoV-2 infection and viral growth (21). Currently, human recombinant soluble ACE2 is being tested as a drug candidate to treat SARS-CoV-2 infection in a phase 2 trial (ClinicalTrials registration no. NCT04335136). In this study, we designed and produced NRP1-ECD-(a1b2)-hFc fusion proteins (where hFc is human IgG fragment crystallizable) (Fig. 2A). Recombinant NRP1-ECD-(a1b2)-hFc fusion protein was successfully expressed and purified, and its molecular weight and purity was revealed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2B).

NRP1-ECD-(a1b2)-hFc showed remarkable binding affinity to EVA71 by enzyme-linked immunosorbent assay (ELISA) (Fig. 2C), highlighting the unique interaction between NRP1-ECD-(a1b2) and EVA71. To evaluate a possible binding epitope of NRP1, the recombinant human SCARB2 fusion protein was applied in a competitive binding assay. NRP1-ECD-(a1b2)-hFc exhibited receptor-competing activity in the assay

### Table 1: EVA71 receptor structure and function

| Receptor | Structural feature | Function (internalization) | Transgenic mouse model |
|----------|-------------------|-----------------------------|------------------------|
| SCARB2   | Type III double-transmembrane protein | Functional receptor (clathrin dependent) | Confirmed in vivo infection |
| PSGL-1   | Single-pass type I transmembrane protein | Functional receptor (caveolin dependent) | Failed to support in vivo infection |
| Anx2     | Cellular adherent factor | Alternative receptor | Not reported |
| Sialylated glycans | Cellular adherent factor | Alternative receptor | Not reported |
| Heparan sulfate | Cellular adherent factor | Alternative receptor | Not reported |
| Vimentin  | Cellular adherent factor | Alternative receptor | Not reported |
| Nucleolin | Cellular adherent factor | Alternative receptor | Not reported |
| NRP1     | Single-pass type I transmembrane protein | Functional receptor | Not confirmed |

*PSGL-1, P-selectin glycoprotein ligand-1; Anx2, annexin II.*
Because VP3 of enterovirus may be involved in SCARB2-EVA71 interaction (22), we sought to examine whether NRP1 interacts with VP3.

An EVA71 VP3 peptide library harboring 47 peptides (15-mer each), with 10-mer
FIG 2 Functional study of NRP1-derived decoy receptor fusion proteins to EVA71. (A) NRP1-ECD-(a1b2)-hFc and NRP1-ECD-(b1b2)-hFc cDNAs were cloned into an expression vector in frame with and upstream from cDNA that encodes hFc, resulting in NRP1-ECD-(a1b2)-hFc (a) and NRP1-ECD-(b1b2)-hFc(b). (B) Purified NRP1-ECD-(a1b2)-hFc and NRP1-ECD-(b1b2)-hFc fusion proteins were electrophoresed using SDS-PAGE under nonreducing [DTT (dithiothreitol)] and (Continued on next page)
overlapping between neighboring peptides, was applied for binding region mapping (Fig. 2E). Interestingly, NRP1-ECD-(a1b2)-hFc was found to interact with three peptides within VP3: residues 56 to 70 (56-NNVPTNATSLMERLR-70 [VP356–70]), residues 106 to 120 (106-GYYTQWSGSLEVTFM-120 [VP3106–120]), and residues 111 to 125 (111-WSGSLEVTFMFTGSF-125 [VP3111–125]). These findings indicate that NRP1 may be a novel EVA71 coreceptor whose virus recognition regions are located within viral VP3.

Next, NRP1-ECD-(a1b2)-hFc was explored for its antiviral activity by protecting rhabdomyosarcoma (RD) cells from EVA71 infection. Although the binding affinity \(K_D\) (equilibrium dissociation constant) of NRP1-ECD-(a1b2)-hFc to EVA71 is 10.1 nM, no obvious antiviral activity was noted (Table 2). This unexpected phenomenon might reflect the fact that NRP1 interacts with heparin with higher affinity \(K_D/H11005\) 2.8 nM (23).

The engineered decoy receptor with a1a2-b1b2 domains, NRP1-ECD-(a1b2)-hFc, was also likely to dimerize with cell surfaces of other receptors present on RD cells used in this study as EVA71 host cells. All these interactions may compete with EVA71 binding with NRP1-ECD-(a1b2)-hFc. Therefore, we explored whether a truncated form of NRP1 decoy receptor, NRP1-ECD-(b1b2)-hFc, may bind EVA71 better.

Subsequently, we generated NRP1-ECD-(b1b2)-hFc containing only the b1b2 domain (partial ECD) (Fig. 2A and B). The smaller NRP1-ECD-(b1b2)-hFc not only retained its binding to the EVA71 virion, with a strong \(K_D\) of 11.6 nM (Fig. 2C), but also recognized similar epitopes located in the VP3 region (Fig. 2E). Most remarkably, the shorter NRP1-ECD-(b1b2)-hFc, in contrast to the a1b2 counterpart, exhibited antiviral activity of EVA71 in RD cells (50% inhibitory concentration, \(~420\) nM). Additionally, NRP1-ECD-(b1b2)-hFc showed no antiviral activity to other picornaviruses, such as enterovirus D68 (EVD68) or coxsackievirus B3 (CVB3) (Table 2). The prototype decoy receptor containing the b1b2 domain, but not the a1a2-b1b2 (a1b2) full-length domain, from NRP1 can be further improved by valency engineering, as shown in a recent study (24).

This proof-of-concept study demonstrated that NRP1 can mediate EVA71 binding and infection in cells. The novel decoy receptor derived from NRP1 can neutralize EVA71 infectivity in vitro. Recently, the b1b2 domain of NRP1 was found to play a role in facilitating SARS-CoV-2 cell entry (17, 18). Our findings highlight that pharmaceutical optimization of NRP1-ECD-(b1b2)-hFc, a virus receptor trap, would be a valid strategy for developing broad-spectrum antiviral agents to combat emerging virus infection.

**Transduction efficiencies in CHO cells expressing NRP1.** The CHO-S cell line was purchased from Thermo Fisher Scientific, Inc. A commercial expression vector-encoding hNRP1 (Sino Biological) was used to establish CHO-NRP1 cells. Stable NRP1-CHO cells were selected after hygromycin B treatment. EVA71 (strain C2 4643/TW/98) with GFP (EVA71-GFP), which expressed GFP on infection, was kindly supplied by Hung-Yao Ho from the Department of Medical Biotechnology and Laboratory Science, Chang Gung University. The reporter virus was generated by engineering the GFP gene between the

**FIG 2 Legend (Continued)**

reducing [DTT(+)1] conditions. (C) An indirect ELISA was performed by coating wells with 20 μg/ml formalin-inactivated EVA71, and the bound fusion proteins were detected using HRP-conjugated goat anti-hFc antibody. Both NRP1-ECD-(a1b2)-hFc and NRP1-ECD-(b1b2)-hFc bound to EVA71 with similarly high affinities (10 to 11 nM). All data were calculated from the averaged duplicate values and analyzed using GraphPad Prism. (D) An ELISA-based competitive binding assay was performed by coating wells with 10 μg/ml formalin-inactivated EVA71. Then, 0.2 μM recombinant scavenger receptor B2 (SCARB2) fusion protein was competitively bound with or without NRP1-ECD-hFc (0.04 to 1 μM), and binding was detected by using an HRP assay. (E) An EVA71 VP3 peptide library containing 47 peptides was used in an overlapping-peptide ELISA for epitope mapping, and bound antibodies were detected using an HRP assay.
untranslated region and VP4 gene of the EVA71 genome. We detached the CHO-NRP1 or CHO cells in phosphate-buffered saline (PBS) and mixed them with EVA71-GFP (1 × 10^6 PFU/ml) for 1 h at 37°C. We washed the cells twice with PBS, suspended them in CDM4PerMab medium plus 2% fetal bovine serum (FBS), and incubated them at 37°C. After 72 h, we observed the GFP-positive cells under a microscope.

**Transduction efficiencies in BHK21 cells expressing NRP1 or SCARB2.** The BHK21 cell line was purchased from Bioresource Collection and Research Center (BCRC, Taiwan). A commercial expression vector-encoding NRP1 with an N-Myc tag (Sino Biological) was used to establish BHK21-NRP1 cells, and a commercial expression vector-encoding hSCARB2 with qa C-OFPSpark tag (Sino Biological) was used to establish BHK21-SCARB2 cells. GFP-positive cells were observed under a microscope 72 h after EVA71-GFP infection.

**Production of recombinant fusion proteins.** All recombinant proteins with Fc domains of human IgG1 (GenBank accession no. AEV43323.1) were produced from CHO cells and purified by using a protein A-derived affinity medium (GE Healthcare). A synthetic CHO codon-optimized target-protein cDNA sequence was cloned into a mammalian cell expression vector and then transfected into CHO cells (Thermo Fisher Scientific). Culture of CHO cells was performed using serum-free CDM4PerMab medium (GE Healthcare). The pSecTag2/Hygro mammalian expression vector (Thermo Fisher Scientific) containing the hinge and hFc was used to express NRP1-ECD-hFc fusion proteins. The NRP1-ECD-(a1b2) region encodes both the a1a2 (amino acids [aa] 22 to 272) and b1b2 (aa 273 to 586) domains, whereas NRP1-ECD-(b1b2) contains only the b1b2 domain (aa 273 to 586) (NCBI reference sequence NP_001019799.1).

**Binding of NRP1-ECD-hFc proteins to EVA71.** The binding affinities of NRP1-ECD-hFc proteins were determined using ELISA with immobilized virions (20 μg/ml formalin-inactivated EVA71). Binding of fusion proteins was revealed with a goat anti-human IgG-Fc fragment cross-adsorbed antibody (Bethyl Laboratories, Inc.) conjugated with horseradish peroxidase (HRP) and HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB) solution (U.S. Biological). Data were analyzed using GraphPad Prism software.

**ELISA analysis.** A recombinant viral receptor competitive binding assay, based on ELISA, with NRP1-ECD-hFc fusion proteins was performed by coating Nunc MaxiSorp ELISA plates with 10 μg/ml formalin-inactivated EVA71 (EVA71 TW/73/12). Subsequently, 0.2 μM recombinant SCARB2 fusion protein with tags (His tag and human Fc; Sino Biological) was prepared with or without different concentrations of NRP1-ECD-(a1b2)-hFc or NRP1-ECD-(b1b2)-hFc. Bound recombinant SCARB2 fusion protein was detected through incubation with the HRP-conjugated mouse anti-His tag antibody (BioLegend). The percentage difference in competitive inhibition was calculated using the formula 100 − ([B/A] × 100), where A represents the optical density at 450 nm (OD450) of the recombinant SCARB2 fusion protein control group without NRP1-ECD-hFc competitors, and B represents the OD450 of the experimental group with NRP1-ECD-hFc competitors.

**Overlapping-peptide ELISA-based epitope mapping.** A library of EVA71 (TW/2086/98) VP3 region overlapping synthetic peptides was kindly provided by Yen-Hung Chow and Chia-Chyi Liu (The National Health Research Institutes) (25). Forty-seven overlapping peptides were used for epitope mapping, and each peptide was 15 amino acids long, with 10-amino acid overlaps between sequential peptides. Bound antibodies were detected through incubation with the HRP-conjugated goat anti-human IgG-Fc fragment cross-adsorbed antibody (Bethyl Laboratories).

**Virus preparation and cell-based neutralization assay.** EVA71 TW/73/12 (genotype C4), EVD68 TW/02795/14, and CVB3 were isolated from the Chang Gung Memorial Hospital clinical virology laboratory. In brief, 96-well plates were seeded with 3 × 10^4 RD cells/well (ATCC CCL-136) in Dulbecco’s modified Eagle medium with 10% FBS and incubated overnight at 37°C. The NRP1-ECD-hFc fusion proteins were incubated with a 100 × 50% tissue culture infective dose (TCID<sub>50</sub>) of EVA71 for 1 h at 37°C. After
adsorption, the infected cells were covered with a medium containing 2% FBS. The infected cells were then incubated at 37°C for 64 h. The plates were fixed with formaldehyde and then stained with crystal violet. Well density at 570 nm was measured. Each experiment was performed in triplicate and repeated at least twice. The 50% effective concentration (EC₅₀) was calculated using the formula \( (Y - B)/(A - B) \times (H - L) + L \), where \( Y \) is half the mean OD at 570 nm (OD₅₇₀) of the control cells without the protein, \( B \) is the mean OD₅₇₀ of wells with the protein dilution nearest to \( Y \), \( A \) is the mean OD₅₇₀ of wells with the protein dilution nearest to and greater than \( Y \), and \( H \) and \( L \) are the protein concentrations for \( B \) and \( A \), respectively.

**Virus titration and viral protein detection in infected cells.** The EVA71 TW/4643/98 (genotype C2) used in the virus titration study was kindly provided by Jen-Ren Wang, National Cheng Kung University, Taiwan (26). Virus titers were determined by plaque assay using RD cells after a single replication cycle (16 h postinfection) in BHK21, BHK21-NRP1, and BHK21-SCARB2 cells. Intracellular viral proteins were detected by Western blotting using the anti-EVA71 monoclonal antibody MAB979 (Merck KgaA).

**ACKNOWLEDGMENTS**

We thank Li-Tsen Lin and Si-Yuan Wu for target protein purification and Yen-Hung Chow and Chia-Chyi Liu for kindly providing the synthetic peptide library corresponding to the EVA71 VP3 sequences for binding domain mapping.

The manuscript was edited by Wallace Academic Editing.

This work was financially supported by the Research Center for Emerging Viral Infections from The Featured Areas Research Center Program within the framework of the Higher Education Sprot Projekt by the Ministry of Education (MOE) in Taiwan and the Ministry of Science and Technology (MOST), Taiwan (MOST 109-2634-F-182-001, 107-2321-B-400-009, 108-2321-B-400-006), the Ministry of Economic Affairs (ROC MOE; 104-EC-17-A-22-1310), and the Council of Agriculture (ROC COA; 107AS-8.2.1-SP-H1).

We declare no competing financial interests.

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