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Efficient inhibition of emerging SARS-CoV-2 variants including NB.1.8.1, XFG and BA.3.2 by a fusion inhibitor HY3000 peptide

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Dear Editor,

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants have caused a serious threat to global public health due to their enhanced transmissibility and immune evasion. However, the identification of six serotypes highlights the continued evolution of the virus, posing additional obstacles to effective vaccine and therapy development.[1]

Since late 2021, Omicron variants with multiple spike receptor-binding domain (RBD) mutations have emerged, compromising RBD-ACE2 targeting and reducing the efficacy of existing vaccines and therapeutics.[2] To overcome the immune evasion of SARS-CoV-2 variants, several strategies have been explored for developing broad neutralizing antibodies, including multispecific (e.g., Dia-19), multivalent (e.g., MR14) and tandem antibody (e.g., bn03) designs.[3-5]Additionally, antibodies targeting conserved epitopes in the S2 or N-terminal domain (NTD) of the S protein, such as our previously identified S102 and N235, have demonstrated broad but weak neutralizing activity against major SARS-CoV-2 variants.[6,7] Currently, several broad antibodies, including bn03 (BM219) and our Dia-19, are under clinical evaluation. Notably, the SA55 monoclonal antibody, which targets the RBD, has advanced to Phase II clinical trials (https://clinicaltrials.gov/study/NCT06042764).

Beyond neutralizing antibodies, peptide fusion inhibitors derived from the conserved heptad repeat (HR) regions in S2 have shown broad antiviral activity by blocking the formation of the viral six-helix bundle (6-HB) structure, which is essential for membrane fusion. We previously developed HY3000, an HR2-derived fusion inhibitory peptide targeting the HR1 domain, which demonstrated potent inhibitory activity against all tested SARS-CoV-2 variants, including Alpha, Beta, Gamma, Delta and Omicron variants from BA.1 to BA.2.86 and EG.5.1.[8,9] Currently, the peptide has been approved for phase III clinical trials in human populations. Additionally, YKYY017 and EK1 peptide inhibitors are also undergoing clinical evaluation. In this study, we further assess the inhibitor ability of HY3000 against the recently emerging SARS-CoV-2 variants.

MATERIALS AND METHODS

Cells

HEK293T (ATCC, CRL-3216) and Vero E6 (ATCC CRL-1586) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1‰ penicillin, and 1‰ streptomycin, under standard conditions (37°C, 5% CO_2).

Pseudovirus preparation

The generation of SARS-CoV-2 pseudoviruses and the subsequent inhibition assay were conducted following the procedure reported by Wu,[9] with minor adjustments. In brief, spike protein genes of each tested variant, truncated by 18 residues at the C-terminus, were cloned into the pCAGGS vector and transiently expressed in HEK293T cells. After 24 h, cells were inoculated with VSV-ΔG-G-GFP particles. Following 2 h adsorption period, the inoculum was replaced with DMEM supplemented with 10 μg/mL anti-VSV-G monoclonal antibody to neutralize residual parental VSV virus. After an additional 30 h, culture supernatants were collected, clarified by centrifugation (3 000 rpm, 10 min), passed through a 0.45 μm filter, and stored at −80 °C.

Pseudovirus inhibition assay

HY3000 peptide (VDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQEL-linker-Chol) synthesized at >99% purity was dissolved in DMSO to prepare a 1 mM stock solution. For the inhibition assay, Vero E6 cells were seeded into 96-well plates one day before infection. Test peptides were prepared as 3-fold serial dilutions in DMEM containing 2% FBS and mixed with an equal volume of pseudovirus. To ensure a consistent infection, we titrated our pseudovirus stocks on Vero E6 cells and adjusted the working dilution to achieve approximately 1 000 RLU signals in virus control wells. After 1 h incubation at 37 °C, the mixtures were added to the cells. Eighteen hours later, GFP-positive cells were measured using a CQ1 confocal image cytometer (Yokogawa, Tokyo, Japan), and inhibitory activity was calculated with GraphPad Prism 10.

RESULTS

Following BA.2.86, several Omicron variants have emerged, including JN.1, LB.1, KP.2, KP.3, XEC, LP.8.1, LP.8.1.1, NB.1.8.1 and XFG. Among them, JN.1 has been designated as a variant of interest (VOI) by the World Health Organization (WHO), while KP.3, XEC, LP.8.1, NB.1.8.1 and XFG have been classified as variants under monitoring (VUM) (https://www.who.int/activities/tracking-SARS-CoV-2-variants). Additionally, a recent report highlighted the potential outbreak of the BA.3.2, which carries over 50 mutations relative to its ancestral BA.3 and 44 mutations distinct from the LP.8.1 variant. This variant has drawn global attention and exhibits significant immune escape from vaccine-elicited neutralizing antibodies, despite showing reduced ACE2 binding affinity and infectivity.[10] These findings highlight the ongoing antigenic evolution of the S protein and the urgent need for effective vaccines and therapeutics.

We analyzed the amino acid mutations present in these emerging Omicron variants and found that the S2 subunit of the S protein remains relatively conserved among them (Figure 1A). Five amino acid substitutions are shared across all the variants. Notably, all these variants share three conserved HR1 mutations, S939F, Q954H and N969K, as observed in BA.2.86. In the HR2 region, only the BA.3.2 carries a D1184E mutation. LP.8.1.1 carries an additional K679R mutation compared to LP.8.1, while the rest of the S2 region remain conserved. Based on this relatively conserved pattern, we hypothesized that the HY3000 peptide would maintain potent inhibitory activity against these variants. To validate this hypothesis, we experimentally assessed the inhibitory activity of the peptide using the pseudotyped viruses. Consistent with our previous findings, HY3000 exhibited potent inhibitory activity against the SARS-CoV-2 prototype (PT) and BA.2.86 strains, with half-maximal effective concentration (EC_{50}) values (41.5 nM and 34.0 nM, respectively) similar to our earlier results (38.8 nM and 28.3 nM, respectively) (Figure 1B).[8] Notably, HY3000 also demonstrated comparable inhibitory potency against the JN.1, LB.1, KP.2, KP.3, XEC, LP.8.1.1, NB.1.8.1 and XFG variants, with EC_{50} values ranging from 11 nM to 35 nM. Even for the BA.3.2 lineage, HY3000 maintained effective inhibitory activity, with an EC_{50} of 25.8 nM. These results indicate that HY3000 retains broad-spectrum inhibitory efficacy despite the ongoing evolution of the SARS-CoV-2. However, due to the unavailability of the live SARS-CoV-2 variants, we have not yet evaluated the inhibitory potency of the HY3000 peptide against them. Furthermore, the cytotoxicity values of HY3000, as previously reported,[8] is much higher than the effective antiviral concentrations (>150-fold), thereby indicating a favorable selectivity index. Based on our previous *in vivo* studies,[8] we speculate that HY3000 would similarly demonstrate effective inhibition of live virus infection *in vivo*, which should be investigated in future studies.

A

		PT	BA.2.86	JN.1	LB.1	KP.2	KP.3	XEC	LP.8.1.1	NB.1.8.1	XFG	BA.3.2
S2	688	A	-	-	-	-	-	-	-	-	-	D
	704	S	-	-	-	-	-	-	-	-	-	L
	764	N	K	K	K	K	K	K	K	K	K	K
	795	K	-	-	-	-	-	-	-	-	-	T
	796	D	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	852	A	-	-	-	-	-	-	-	-	-	K
	939	S	F	F	F	F	F	F	F	F	F	F
HR1	954	Q	H	H	H	H	H	H	H	H	H	H
	969	N	K	K	K	K	K	K	K	K	K	K
	1086	K	-	-	-	-	-	-	R	-	-	-
	1104	V	-	-	-	L	L	L	L	-	-	-
	1143	P	L	L	L	L	L	L	L	L	L	-
HR2	1184	D	-	-	-	-	-	-	-	-	-	E

B

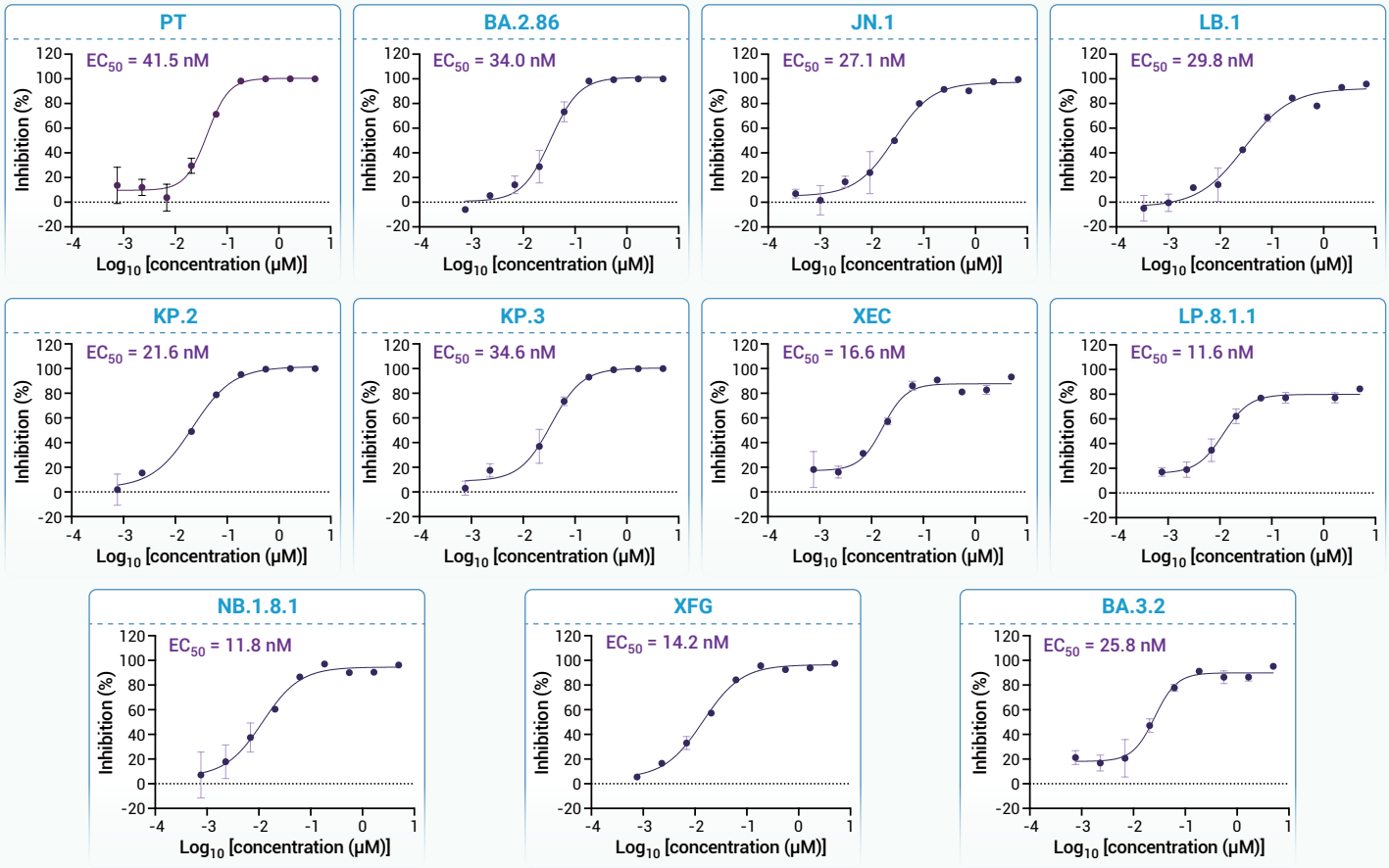

Figure 1. Amino acid mutation profiling of the S2 subunit of SARS-CoV-2 spike (S) proteins from emerging variants, and inhibitory activities of the HY3000 peptide against corresponding pseudoviruses (A) Amino acid mutations in the S2 subunit of S proteins from SARS-CoV-2 BA.2.86, LB.1, JN.1, KP.2, KP.3, XEC, LP.8.1.1, NB.1.8.1, XFG and BA.3.2 compared to the prototype (PT). Each column represents one variant, with specific amino acid mutations shown using single-letter codes. Unchanged residues relative to PT are indicated with a dash ("–"). (B) Inhibitory activities of the HY3000 peptide against pseudotypes viruses. The experiments were performed twice independently with two technical replicates (n = 2) each. One representative result was shown.

Collectively, these findings further support the HY3000 as a promising broad-spectrum candidate for combating emerging SARS-CoV-2 variants. Given the continuous viral evolution, sustained surveillance of HY3000's efficacy against future variants remains crucial. Moreover, the sequence and structural conservation of the S2 domain highlights its potential as an ideal target for antiviral drug development.

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AUTHOR CONTRIBUTIONS

This manuscript was written by W. L. and L. W., experiments and data analysis were performed by W. L. and J. L., study was designed by G. F. G. and Q. W. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

Patents (application number ZL202210333422.5) was filed containing the fusion inhibitor HY3000 peptide described in this study. G. F. G., Q. W. and L. W. are the inventors. The other authors declare that they have no competing interests.