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Communication

Covalently Engineered Protein Minibinders with Enhanced Neutralization Efficacy against Escaping SARS-CoV-2 Variants

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ABSTRACT: The rapid emergence and spread of escaping mutations of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has significantly challenged our efforts in fighting against the COVID-19 pandemic. A broadly neutralizing reagent against these concerning variants is thus highly desirable for the prophylactic and therapeutic treatments of SARS-CoV-2 infection. We herein report a covalent engineering strategy on protein minibinders for potent neutralization of the escaping variants such as B.1.617.2 (Delta), B.1.617.1 (Kappa), and B.1.1.529 (Omicron) through in situ cross-linking with the spike receptor binding domain (RBD). The resulting covalent minibinder (GlueBinder) exhibited enhanced blockage of RBD-human angiotensin-converting enzyme 2 (huACE2) interaction and more potent neutralization effect against the Delta variant than its noncovalent counterpart as demonstrated on authentic virus. By leveraging the covalent chemistry against escaping mutations, our strategy may be generally applicable for restoring and enhancing the potency of neutralizing antibodies to SARS-CoV-2 and other rapidly evolving viral targets.

The development of various neutralizing reagents including antibodies,[1−3] small molecules,[4,5] peptides,[6,7] and DNA aptamers[8,9] to interfere the interaction between the spike receptor binding domain (RBD) and human angiotensin-converting enzyme 2 (huACE2) represents a critical therapeutic strategy against SARS-CoV-2 infection. However, the RNA genome of SARS-CoV-2 is more rapidly evolving than other RNA viruses,[10] and survival mutations with higher infectivity and immune evasion (e.g., the Delta variant, B.1.617.2) usually dampen or even abolish the potency of neutralizing reagents based on small molecules, peptides, or aptamers due to their moderate binding affinity or relatively low stability.[11] Furthermore, as clinical experiences indicated that effective treatments of COVID-19 require high density of inhibitory domains of neutralizing antibodies to maximize the blockage efficiency,[12] the large size and low inhibitory density of the monoclonal antibodies rendered it easier for the escaping variants to gain access and entry to host cells, casting serious concerns on our global efforts in fighting against the COVID-19 pandemic.[13] Broadly neutralizing antibodies capable of overcoming viral mutational escape are therefore in urgent need.

Protein minibinders are attractive therapeutic agents against SARS-CoV-2 due to their small size and high stability with enhanced inhibitory density. In addition, the intranasal delivery capability of these minibinders is particularly valuable for prophylactic and therapeutic treatments of virus that target the respiratory system.[14] For example, the recently reported *de novo* designed protein minibinders (LCB1 and LCB3) combined a range of binding modes with viral RBD and increased density of binding sites, with a neutralization ability of authentic viruses at the picomolar level.[12] Nevertheless, since these binding events still rely on the noncovalent interaction with specific residues in RBD, mutations on receptor binding motif (RBM) would inevitably reduce or even diminish their neutralization capacity. Inspired by the covalent small molecule drugs that irreversibly inhibit the target with enhanced on-target retention[15−18] as well as the potential for minimizing drug resistance caused by target mutations,[19,20] we envisioned that converting the noncovalent antibody−antigen interactions into covalent linkages may be a promising solution to prevent the dampened potency of neutralizing antibodies caused by viral escaping mutations. Notably, by covalently locking the antibody−antigen interactions as the recently developed covalent PD-1 protein drug[21] and covalent nanobody-based PROTAC (GlueTAC),[22] these covalent binders would have an enhanced on-target retention time to further maximize the inhibitory density on virus surface that may lead to increased potency *in vivo*.[16]

Herein, we report the covalently engineered protein minibinder, GlueBinder, to potently neutralize escaping SARS-CoV-2 variants by irreversible blockage of its RBD domain (Scheme 1) based on the *in situ* cross-linking reactions.[23−26] A pool of LCB1 and LCB3 variants (five sites for LCB1 and four sites for LCB3) bearing site-specifically incorporated proximal reactive fluorosulfate-$_L$-tyrosine (FSY)[27] were generated and our systematic survey identified the LCB3-F30FSY variant (renamed as GlueBinder) as the covalent minibinder that irreversibly bound to four RBD variants (Figure S1) with the highest efficiency, especially the newly

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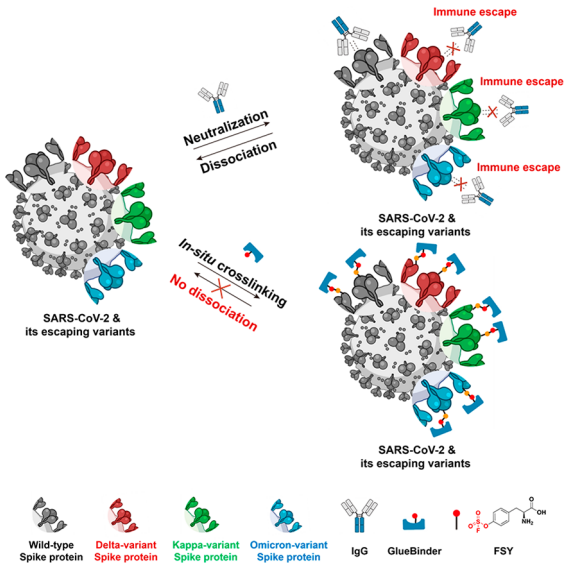
Scheme 1. Covalently Engineered Protein Minibinders (GlueBinders) Irreversibly Block SARS-CoV-2 by *In Situ* Cross-Linking with Its Spike RBD



reported Omicron variant with severe immune escaping capability.[28] Competitive ELISA verified that GlueBinder blocked the RBD-huACE2 interaction more potently *in vitro*, and the neutralization of authentic SARS-CoV-2 Delta variant showed a decreased median inhibitory concentration (IC_{50} = 20 pM), which is 6-fold lower than that of its noncovalent counterpart (WT-LCB3).

Previous studies have demonstrated that FSY has the potential to react with a variety of nucleophilic residues including Lys, His, and Tyr when incorporated into the appropriate position at the protein−protein interaction interface.[27] Since cryoelectron microscopy (Cryo-EM) analysis revealed the structures of the RBD from the wild-type strain in complex with minibinders LCB1/3[12], we chose a panel of nucleophilic residues on wild-type RBD (WT-RBD) that are oriented toward the minibinders as candidates for targeted covalent conjugation, including Lys417, Tyr421, Tyr449, Tyr453, Tyr473, Tyr489, and Tyr505. PyMOL was then utilized to mutate the amino acids throughout the entire LCB1/3 sequence into Tyr followed by optimizing the spatial orientation of the side-chain after computational mutation scanning. The distance (***O−Nu*** distance) was measured between the *Oxygen atom* from the phenol of the mutant Tyr and the *Nucleophilic atom* from the candidate residues of the RBD (nitrogen atom from the ε-amine of Lys or the imidazole of His, oxygen atom from the phenol of Tyr). Considering the cross-linking radius between FSY and

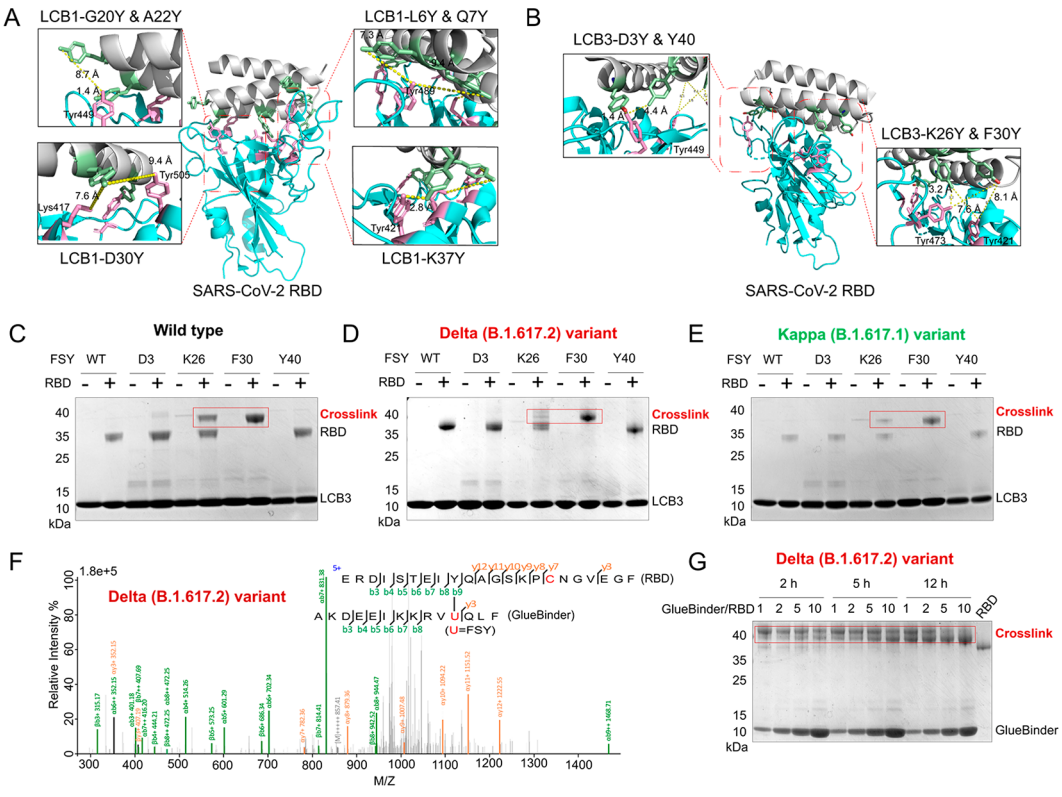


Figure 1. The development of GlueBinders for irreversible *in situ* cross-linking with escaping SARS-CoV-2 variants. (A, B) Structure of SARS-CoV-2 RBD in complex with minibinder LCB1 (A, PDB: 7jzu) or LCB3 (B, PDB: 7jzm). Candidate sites were mutated to Tyr by PyMOL followed by measuring the distance (***O−Nu*** distance) between the oxygen atom of mutated Tyr and proximal nucleophilic residues. (C−E) Four sites of LCB3 were incorporated with FSY to generate candidate covalent LCB3. After purification, four candidates were separately incubated with wild-type (C), Delta (B.1.617.2) variant (D), and Kappa (B.1.617.1) variant (E) RBDs to identify the most potent covalent LCB3. LCB3-F30FSY (renamed as GlueBinder) showed the highest cross-linking efficiency. (F) Tandem mass spectrum (MS) of the cross-linked GlueBinder/RBD complex validated that F30FSY on GlueBinder reacted with Tyr473 on Delta-variant RBD to form irreversible conjugates. (G) Studies of cross-linking efficiency between GlueBinder and Delta-variant RBD under different time and stoichiometry. The cross-linking between GlueBinder and wild-type, Kappa-variant RBD were also validated by MS.

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nucleophilic residues, sites with ***O−Nu*** distance less than 9.4 Å were selected for incorporating FSY, including Leu6, Gln7, Gly20, Ala22, Asp30, Lys37 on LCB1 (Figure 1A) and Asp3, Lys26, Phe30, Tyr40 on LCB3 (Figure 1B).

The amber codon (TAG) was introduced at these selected sites of the genes expressing LCB1 or LCB3 in *E. coli*, which was cotransformed with the plasmid for the orthogonal tRNA/chFSYRS pair, responsible for site-specific FSY incorporation. Except for Gly20 in LCB1, FSY was successfully incorporated at nine sites to afford the candidate covalent minibinders (Figure S3, Figure S4), which were respectively incubated with recombinant RBD of SARS-CoV-2 in PBS to trigger proximity-enabled cross-linking reactions. To our delight, three FSY-incorporated minibinders (LCB1-A22FSY, LCB3-K26FSY, and LCB3-F30FSY) were found to form cross-linking bands with WT-RBD as analyzed by SDS-PAGE, while the noncovalent interaction was disrupted. These results also demonstrated that the irreversible binding to RBD protein by proximity-enabled cross-linking has high selectivity due to proper FSY incorporation in the minibinders. Among these three verified covalent minibinders, LCB3-F30FSY exhibited the highest cross-linking efficiency with WT-RBD (Figure 1C, Figure S5A) and was chosen to test cross-reactivity to mutant RBD from SARS-CoV-2 Delta (Figure 1D) and Kappa variants (Figure 1E). To our delight, the results showed that LCB3-F30FSY almost completely cross-linked with all three RBD variants at the 10:1 minibinder/RBD molar ratio after 12 h.

To interpret the irreversible binding of LCB3-F30FSY (renamed as GlueBinder) to RBDs from different strains, the cross-linking bands were subjected to in-gel digestion with chymotrypsin followed by LC-MS/MS analysis. Indeed, we found that the covalent cross-linking occurred between residue 30 (FSY) on GlueBinder and Tyr473 on RBDs (Figure 1F, S6A, and S6C). As described above, noncovalent drugs with small molecular weight usually suffer from short half-life *in vivo* due to the glomerular filtration and we therefore examined the time window for cross-linking reaction of GlueBinder. To mimic the *in vivo* conditions, we chose three time points (2, 5, 12 h) to explore the cross-linking efficiency between GlueBinder and Delta-variant RBD (Figure 1G). Encouragingly, we observed abundant cross-linking at 2 h with equal ratio of GlueBinder and RBD. Furthermore, the irreversible conjugates reached ∼85% yield at 5 h. Therefore, the cross-linking efficiency was compatible with the pharmacokinetic characteristics of minibinders *in vivo*.[29,30] RBD variants from wild-type strain (Figure S6B) and Kappa strain (Figure S6D) showed similar cross-linking efficiency as the Delta strain, indicating that GlueBinder has the potential to potently neutralize SARS-CoV-2 strains regardless of escaping mutations.

Since GlueBinder covalently cross-linked with RBD variants, we characterized its binding affinity and blocking activity against RBD-huACE2 interaction. Biolayer interferometry (BLI) assay exhibited that GlueBinder bound three RBDs with high affinities (∼0.2 nM, Figure 2A, 2C, and 2E), which were consistent with the affinities of WT-LCB3 (Figure S7A, S7B, and S7C). A competitive ELISA strategy was employed in which huACE2 was used to compete with GlueBinder or WT-LCB3 at room temperature within 3.5 h for binding to the immobilized RBDs. As expected, GlueBinder showed a more potent blockage of huACE2's interaction with RBD variants than WT-LCB3 (e.g., 8−10 fold decrease of IC_{50}, Figure 2B, 2D, and 2F), largely due to the covalently cross-linked

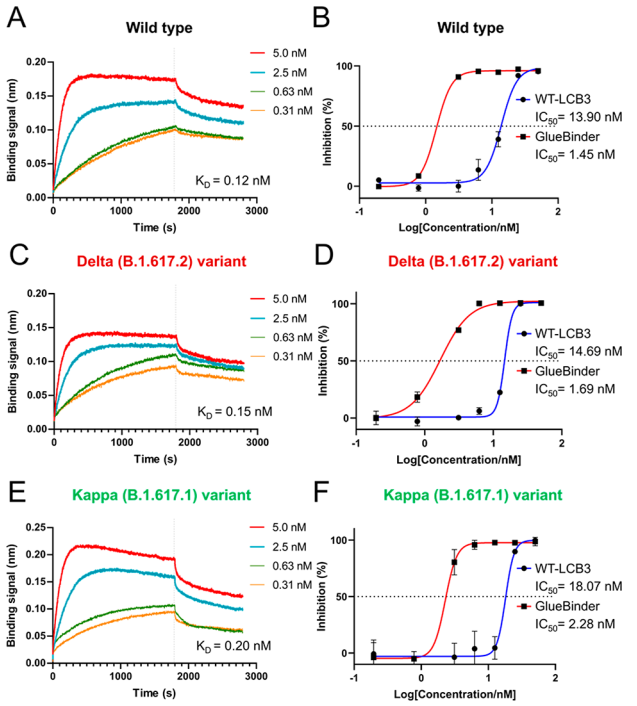

Figure 2. GlueBinder showed potent antagonization of huACE2-RBD interaction regardless of escaping mutations *in vitro*. (A−C) BLI assay of GlueBinder showed that FSY incorporation in WT-LCB3 did not influence its binding to wild-type (A), Delta-variant (B), and Kappa-variant (C) RBDs as indicted by negligible difference between their binding affinities. (D−F) GlueBinder showed accumulated, irreversible blockage of wild-type (D), Delta-variant (E), and Kappa-variant (F) RBDs. Competitive ELISA indicated that GlueBinder more potently inhibited the RBD-huACE2 interaction than WT-LCB3.

GlueBinder-RBD complex that prevented the dissociation or release of RBDs from huACE2. Taken together, our site-specific FSY incorporation did not affect LCB3's stability or binding to RBD variants, and instead covalently locked the LCB3-RBD complex with enhanced potency.

Encouraged by the enhanced inhibition potency of GlueBinder against RBD-huACE2 interaction, we further investigated the capability of GlueBinder to prevent the infection of host cells by authentic viruses. Carrying two mutants in the receptor binding motif of RBD that confer higher infection ability and stronger immune escaping capability, the SARS-CoV-2 Delta variant has been widely spread as one of the dominant strains around the world in recent months. We examined whether GlueBinder could neutralize the Delta variant of SARS-CoV-2 by incubating varying concentrations of GlueBinder with live virus for 2 h and then adding to Vero E6 monolayers at a multiplicity of infection (MOI) of 0.05. After coculture for 24 h, quantitative real-time PCR assay was performed to quantify viral yields upon treatments of varying concentrations of GlueBinder (Figure 3A). The IC_{50} value of WT-LCB3 against the Delta mutant strain was measured as 0.12 nM, which was higher than the reported IC_{50} value (0.048 nM) against the original virus strain,[12] indicating that the Delta variant indeed escaped the noncovalent minibinders to some extent. In contrast, the GlueBinder exhibited a significant enhanced neutralization activity against the Delta variant, with an IC_{50} value as low as 0.02 nM (Figure 3C), which is 6-fold lower than that of WT-LCB3 (Figure 3B). Finally, the stability of GlueBinder was tested after the treatments of heat,

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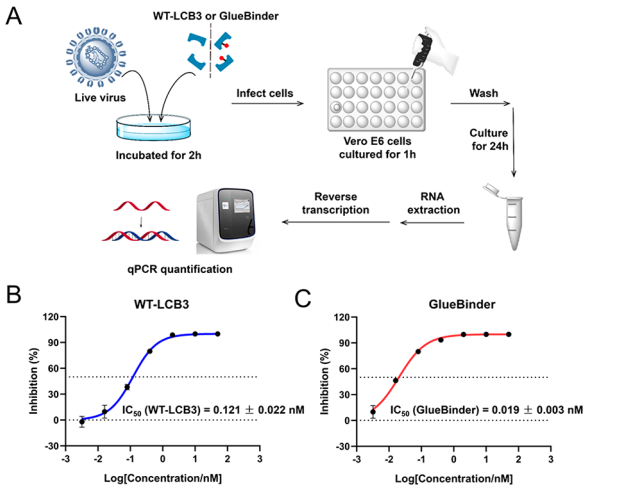

Figure 3. GlueBinder potently neutralized the infection of authentic SARS-CoV-2 Delta variant. (A) Schematic illustration of neutralization of authentic SARS-CoV-2 virus. (B, C) The neutralization activity of WT-LCB3 (B) and GlueBinder (C) were measured with a microneutralization test. Indicated concentrations of minibinders were incubated with SARS-CoV-2 Delta variant and used to infect Vero-E6 cells. Viral RNA copies in the supernatant were quantified at 24 h post infection. Assays were repeated as two independent replicates.

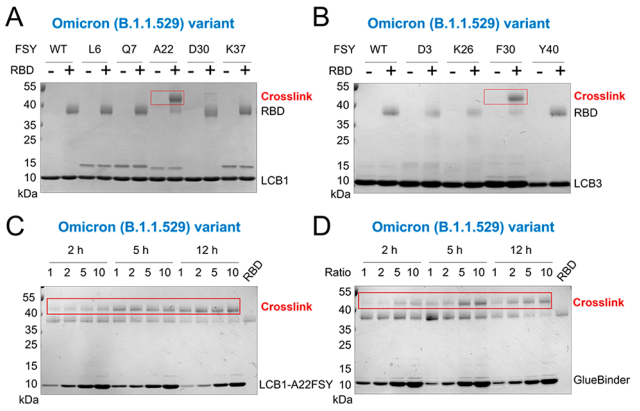

Figure 4. Covalently engineered minibinders irreversibly bound to the Omicron variant. (A, B) Nine candidates (A, LCB1 variants; B, LCB3 variants) were subjected to *in situ* cross-linking with the Omicron-variant RBD. GlueBinder retained its high cross-linking efficiency, while LCB1-A22FSY showed similar results with GlueBinder. (C, D) Studies of cross-linking efficiency between LCB1-A22FSY (C) or GlueBinder (D) and the Omicron-variant RBD under different times and stoichiometry.

aerosolization, and lyophilization, which showed the similar hyper-stability as WT-LCB3 that may be suitable for intranasal delivery (see the Supplementary Method and Figure S8–S10).

While this work was under review, the Omicron (B.1.1.529) variant of SARS-CoV-2 was rapidly emerging and has been classified as a variant of concern (VOC) by the World Health Organization (WHO), due to its severe immune escape from most neutralizing antibodies in use.[28] In particular, we found that the original noncovalent minibinder LCB3 bound poorly to this concerning variant, which serves as an excellent model to further demonstrate the strength and advantages of our covalent binders. To this end, we incubated this RBD variant with the five candidate covalent LCB1 and four candidate covalent LCB3 for 12 h. Delightfully, GlueBinder (LCB3-F30FSY, Figure 4B) retained its high cross-linking efficiency, while LCB1-A22FSY also showed comparable results with GlueBinder (Figure 4A), much higher than that against other variants. Further studies of *in situ* cross-linking between GlueBinder (Figure 4D) or LCB1-A22FSY (Figure 4C) and the Omicron-variant RBD under different times and stoichiometry showed that the efficiency was partially decreased within 2 h than longer incubation time, due to the lowered binding affinity (LCB1: K_D = 22.1 nM; LCB3: K_D > 5 μM, Figure S11). Noteworthy, most RBD formed irreversible conjugates upon extended time or increased concentration of covalent minibinders, and this retention capability could be the potential benefits of covalent engineering against virus infection. Given the more complicated *in vivo* environment, we plan to investigate the virological assays and evaluate the *in vivo* antivirus activity of our two covalent minibinders against the Omicron variant in future studies.

In summary, we have designed and prepared nine candidate covalent minibinders for SARS-CoV-2 using genetic code expansion. Structures of RBD in complex with noncovalent minibinders potentiate the rational selection of candidate sites for FSY incorporation and rapid screening for covalent minibinders. For the covalent engineering of neutralizing protein binders with an unknown structure, a library with a larger capacity of candidate covalent antibodies with FSY incorporation at more sites can also be applied for MS-based screening. The covalent neutralization process may undergo a two-step blocking mechanism: the minibinders bind to the RBD through reversible, noncovalent interactions, followed by reacting with a nucleophilic residue on RBD to form a covalent bond that irreversibly locked the complex. Our covalent blockage strategy might be broadly applicable to rescue the potency of neutralizing antibodies due to the enhanced efficacy and resistance to escaping mutations of SARS-CoV-2. To prove the concept, we have identified three covalent minibinders, and in particular, LCB3-F30FSY showed the highest cross-linking efficiency with RBDs from the wild-type strain as well as three mutant strains including the newly emerging Omicron variant with severe immune escaping ability. On the basis of its no dissociation from RBDs upon cross-linking, LCB3-F30FSY potently blocked RBD-huACE2 interaction and neutralized the live viruses including the concerning Delta variant. Given that LCB3-F30FSY irreversibly bound to both the Delta and Omicron variants, our covalently engineered minibinders could serve as a generalizable prophylactic or therapeutic strategy against SARS-CoV-2 and other rapidly evolving viruses.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c11554.

Detailed description of expression and purification of FSY-containing proteins, MS identification of the cross-linking sites, biolayer interferometry, live virus neutralization assay and stability studies, and supplementary figures (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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