

1 **The Development of a Novel Nanobody Therapeutic for SARS-CoV-2**

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39 Impact statement: Potent and low-cost *Nanosota-1* drugs block SARS-CoV-2 infections
40 both *in vitro* and *in vivo* and act both preventively and therapeutically.

41

42 **Abstract**

43 Combating the COVID-19 pandemic requires potent and low-cost therapeutics.
44 We identified a novel series of single-domain antibodies (i.e., nanobody), *Nanosota-1*,
45 from a camelid nanobody phage display library. Structural data showed that *Nanosota-1*
46 bound to the oft-hidden receptor-binding domain (RBD) of SARS-CoV-2 spike protein,
47 blocking out viral receptor ACE2. The lead drug possessing an Fc tag (*Nanosota-1C-Fc*)
48 bound to SARS-CoV-2 RBD with a K_d of 15.7picomolar (~3000 times more tightly than
49 ACE2 did) and inhibited SARS-CoV-2 infection with an ND_{50} of
50 0.16microgram/milliliter (~6000 times more potently than ACE2 did). Administered at a
51 single dose, *Nanosota-1C-Fc* demonstrated preventive and therapeutic efficacy in
52 hamsters subjected to SARS-CoV-2 infection. Unlike conventional antibody drugs,
53 *Nanosota-1C-Fc* was produced at high yields in bacteria and had exceptional
54 thermostability. Pharmacokinetic analysis of *Nanosota-1C-Fc* documented a greater than
55 10-day *in vivo* half-life efficacy and high tissue bioavailability. *Nanosota-1C-Fc* is a
56 potentially effective and realistic solution to the COVID-19 pandemic.
57

58 **Introduction**

59 The novel coronavirus SARS-CoV-2 has led to the COVID-19 pandemic,
60 devastating human health and the global economy (1, 2). Anti-SARS-CoV-2 drugs are
61 urgently needed to treat patients, save lives, and revive economies. Yet daunting
62 challenges confront the development of such drugs. Though small molecule drugs could
63 theoretically target SARS-CoV-2, they can take years to develop and their use is often
64 limited by poor specificity and off-target effects. Repurposed drugs, developed against
65 other viruses, also have low specificity against SARS-CoV-2. Therapeutic antibodies can
66 be identified and generally have high specificity; however, their expression in
67 mammalian cells often leads to low yields and high production costs (3, 4). A realistic
68 therapeutic solution to COVID-19 must be potent and specific, yet easy to produce.

69 Nanobodies are unique antibodies derived from heavy chain-only antibodies
70 found in members of the camelidae family (llamas, alpacas, camels, etc.) (Fig. S1) (5, 6).
71 Because of their small size (2.5 nm by 4 nm; 12-15 kDa) and unique binding domains,
72 nanobodies offer many advantages over conventional antibodies including the ability to
73 bind cryptic epitopes on their antigen, high tissue permeability, ease of production and
74 thermostability (7, 8). Although small, nanobodies bind their targets with high affinity
75 and specificity due to an extended antigen-binding region (7, 8). Furthermore, it has been
76 documented that they have low toxicity and immunogenicity in humans, if any (7, 8).
77 One drawback of nanobodies is their quick clearance by kidneys due to their small size;
78 this can be overcome by adding tags to increase the molecular weight to a desired level.
79 Underscoring the potency and safety of nanobodies as human therapeutics, a nanobody
80 drug was recently approved for clinical use in treating a blood clotting disorder (9).

81 Additionally, due to their superior stability, nanobodies can be inhaled to treat lung
82 diseases (10) or ingested to treat intestine diseases (11). Nanobodies are currently being
83 developed against SARS-CoV-2 to combat COVID-19 (12, 13). However, to date, none
84 of the reported nanobodies have been evaluated for therapeutic efficacy *in vivo*.

85 The receptor-binding domain (RBD) of the SARS-CoV-2 spike protein is a prime
86 target for therapeutic development (14). The spike protein guides coronavirus entry into
87 host cells by first binding to a receptor on the host cell surface and then fusing the viral
88 and host membranes (15, 16). The RBDs of SARS-CoV-2 and a closely related SARS-
89 CoV-1 both recognize human angiotensin-converting enzyme 2 (ACE2) as their receptor
90 (14, 17-19). Previously, we showed that SARS-CoV-1 and SARS-CoV-2 RBDs both
91 contain a core structure and a receptor-binding motif (RBM), and that SARS-CoV-2
92 RBD has significantly higher ACE2-binding affinity than SARS-CoV-1 RBD due to
93 several structural changes in the RBM (20, 21). We further showed that SARS-CoV-2
94 RBD is more hidden than SARS-CoV-1 RBD in the entire spike protein as a possible
95 viral strategy for immune evasion (22). Hence, to block SARS-CoV-2 binding to ACE2,
96 a nanobody drug would need to bind to SARS-CoV-2 RBD more tightly than ACE2.

97 Here, we report the development of a novel series of anti-SARS-CoV-2 nanobody
98 therapeutics, *Nanosota-1*. Identified by screening a camelid nanobody phage display
99 library against the SARS-CoV-2 RBD, the *Nanosota-1* series bound potently to the
100 SARS-CoV-2 RBD and were effective at inhibiting SARS-CoV-2 infection *in vitro*. The
101 best performing drug, *Nanosota-1C-Fc*, demonstrated preventative and therapeutic
102 efficacy in a hamster model of SARS-CoV-2 infection. *Nanosota-1C-Fc* was produced at
103 high yields easily scalable for mass production and was also found to have a

104 pharmacologically relevant *in vivo* half-life and excellent bioavailability. Our data
105 suggest that *Nanosota-1c-Fc* may provide an effective solution to the COVID-19
106 pandemic.

107

108 **Results**

109 ***Nanosota-1* was identified by phage display**

110 For the rapid identification of virus-targeting nanobodies, we constructed a naïve
111 nanobody phage display library using B cells isolated from the spleen, bone marrow, and
112 blood of nearly a dozen non-immunized llamas and alpacas (Fig. 1). Recombinant SARS-
113 CoV-2 RBD, expressed and purified from mammalian cells, was screened against the
114 library to identify RBD-targeting nanobodies. Select nanobody clones were tested in a
115 preliminary screen for their ability to neutralize SARS-CoV-2 pseudovirus entry into
116 target cells (see below for more details about the assay). The nanobody that demonstrated
117 the highest preliminary neutralization potency was named *Nanosota-1A* and then
118 subjected to two rounds of affinity maturation. For each round of affinity maturation,
119 random mutations were introduced to the whole gene of *Nanosota-1A* through error-
120 prone PCR, and mutant phages were selected for enhanced binding to SARS-CoV-2
121 RBD. Nanobodies contain four framework regions (FRs) as structural scaffolds and three
122 complementarity-determining regions (CDRs) for antigen binding. The nanobody after
123 the first round of affinity maturation, named *Nanosota-1B*, possessed one mutation in
124 CDR3 and two other mutations in FR3 (near CDR3). Affinity maturation of *Nanosota-1B*
125 resulted in *Nanosota-1C*, which possessed one mutation in CDR2 and another mutation

126 in FR2. We next made an Fc-tagged version of *Nanosota-1C*, termed *Nanosota-1C-Fc*, to
127 create a bivalent construct with increased molecular weight.

128 ***Nanosota-1* tightly bound to the SARS-CoV-2 RBD and completely blocked out**
129 **ACE2**

130 To understand the structural basis for the binding of *Nanosota-1* drugs to SARS-
131 CoV-2 RBD, we determined the crystal structure of SARS-CoV-2 RBD complexed with
132 *Nanosota-1C*. The structure showed that *Nanosota-1C* binds close to the center of the
133 SARS-CoV-2 RBM (Fig. 2A). When the structures of the RBD/*Nanosota-1C* complex
134 and the RBD/ACE2 complex were superimposed together, significant clashes occurred
135 between ACE2 and *Nanosota-1C* (Fig. 2B), suggesting that *Nanosota-1C* binding to the
136 RBD blocks ACE2 binding to the RBD. Moreover, trimeric SARS-CoV-2 spike protein
137 is present in two different conformations: the RBD stands up in the open conformation
138 but lies down in the closed conformation (22-24). When the structures of the
139 RBD/*Nanosota-1C* complex and the closed spike were superimposed together, no clash
140 was found between RBD-bound *Nanosota-1C* and the rest of the spike protein (Fig.
141 S2A). In contrast, severe clashes were identified between RBD-bound ACE2 and the rest
142 of the spike protein in the closed conformation (Fig. S2B). Additionally, neither RBD-
143 bound *Nanosota-1C* nor RBD-bound ACE2 had clashes with the rest of the spike protein
144 in the open conformation (Fig. S2C, S2D). Thus, *Nanosota-1C* can access the spike
145 protein in both its open and closed conformations, whereas ACE2 can only access the
146 spike protein in its closed conformation. Overall, our structural data reveal that *Nanosota-1C*
147 *IC* is an ideal RBD-targeting drug that not only blocks virus binding to its receptor, but
148 also accesses its target in the spike protein in different conformations.

149 To corroborate our structural data on the *Nanosota-1*/ACE2 interactions, we
150 performed binding experiments between *Nanosota-1* drugs and SARS-CoV-2 RBD using
151 recombinant ACE2 for comparison. The binding affinity between the nanobodies and the
152 RBD were measured by surface plasmon resonance (Table 1; Fig. S3). *Nanosota-1A*, *-1B*,
153 *and -1C* bound to the RBD with increasing affinity (K_d - from 228 nM to 14 nM),
154 confirming success of the stepwise affinity maturation. *Nanosota-1C-Fc* had the highest
155 RBD-binding affinity (K_d - 15.7 pM), which was ~3,000 times tighter than the RBD-
156 binding affinity of ACE2. Moreover, compared with ACE2, *Nanosota-1C-Fc* bound to
157 the RBD with a higher k_{on} and a lower k_{off} , demonstrating significantly faster binding and
158 slower dissociation. Next, we investigated the competitive binding among *Nanosota-1C*,
159 ACE2, and RBD using protein pull-down assay (Fig. S4A). ACE2 and *Nanosota-1C*
160 were mixed together in different ratios in solution, with the concentration of ACE2 kept
161 constant; RBD-Fc was added to pull down ACE2 and *Nanosota-1C* from solution. The
162 result showed that as the concentration of *Nanosota-1C* increased, less ACE2 was pulled
163 down by the RBD. Thus, ACE2 and *Nanosota-1C* bound competitively to the RBD. We
164 then analyzed the competitive binding using gel filtration chromatography (Fig. S4B).
165 ACE2, *Nanosota-1C*, and RBD were mixed, with both ACE2 and *Nanosota-1C* in molar
166 excess over the RBD. Analysis by gel filtration chromatography documented that no
167 ternary complex of ACE2, *Nanosota-1C*, and RBD formed; instead, only binary
168 complexes of RBD/ACE2 and RBD/*Nanosota-1C* were detected. Hence, the bindings of
169 ACE2 and *Nanosota-1C* to the RBD are mutually exclusive.
170 ***Nanosota-1C-Fc* potently neutralized SARS-CoV-2 infection *in vitro* and *in vivo***

171 The ability of the *Nanosota-1* drugs to neutralize SARS-CoV-2 infection *in vitro*
172 was investigated next. Both a SARS-CoV-2 pseudovirus entry assay and authentic SARS-
173 CoV-2 infection assay were performed (Fig. 3). For the pseudovirus entry assay,
174 retroviruses pseudotyped with SARS-CoV-2 spike protein (i.e., SARS-CoV-2
175 pseudoviruses) were used to enter human ACE2-expressing HEK293T cells in the
176 presence of an inhibitor. The efficacy of the inhibitor was expressed as the concentration
177 capable of neutralizing 50% of the entry efficiency (i.e., 50% Neutralizing Dose or
178 ND₅₀). *Nanosota-1C-Fc* had an ND₅₀ for the SARS-CoV-2 pseudovirus of 0.27 µg/ml,
179 which was ~10 times more potent than monovalent *Nanosota-1C* (2.52 µg/ml) and over
180 100 times more potent than ACE2 (44.8 µg/ml) (Fig. 3A). Additionally, *Nanosota-1*
181 drugs potently neutralized SARS-CoV-2 pseudovirus bearing the D614G mutation in the
182 SARS-CoV-2 spike protein (Fig. S5), which has become prevalent in many strains (25).
183 For the authentic virus infection assay, live SARS-CoV-2 was used to infect Vero cells in
184 the presence of an inhibitor. Efficacy of the inhibitor was described as the concentration
185 capable of reducing the number of virus plaques by 50% (i.e., ND₅₀). *Nanosota-1C-Fc*
186 had an ND₅₀ of 0.16 µg/ml, which was ~20 times more potent than monovalent
187 *Nanosota-1C* (3.23 µg/ml) and ~6000 times more potent than ACE2 (980 µg/ml) (Fig.
188 3B; Fig. S6). Overall, both *Nanosota-1C-Fc* and *Nanosota-1C* are potent inhibitors of
189 SARS-CoV-2 pseudovirus entry and authentic SARS-CoV-2 infection.

190 After the *in vitro* studies, we next evaluated the therapeutic efficacy of the lead drug
191 *Nanosota-1C-Fc* in a hamster model challenged with SARS-CoV-2 via intranasal
192 inoculation. In addition to an untreated control group, three groups of animals were
193 injected with a single dose of *Nanosota-1C-Fc*: (i) 24 hours pre-challenge at 20 mg/kg

194 body weight, (ii) 4 hours post-challenge at 20 mg/kg, and (iii) 4 hours post-challenge at
195 10 mg/kg. As previously validated in this model (26), body weight, tissue pathology and
196 virus titers in nasal swabs were used as metrics of therapeutic efficacy. In the untreated
197 control group, weight loss was precipitously starting on day 1 post-challenge with the
198 lowest weight recorded on day 6 (Fig. 4A). Nasal virus titers were high on day 1 and
199 remained high on day 5 before a decline (Fig. S7). Pathology analysis on tissues collected
200 on day 10 revealed moderate hyperplasia in the bronchial tubes (i.e., bronchioloalveolar
201 hyperplasia) (Fig. 4B), with little hyperplasia in the lungs. These data are consistent with
202 previous reports showing that SARS-CoV-2 mainly infects the nasal mucosa and
203 bronchial epithelial cells of this hamster model (26). In contrast, hamsters that received
204 *Nanosota-1C-Fc* 24-hours pre-challenge were protected from SARS-CoV-2, as
205 evidenced by the metrics of no weight loss, no bronchioloalveolar hyperplasia, and
206 significantly reduced nasal virus titers (Fig. 4, Fig. S7). When administered 4 hours post-
207 challenge, *Nanosota-1C-Fc* also effectively protected hamsters from SARS-CoV-2
208 infections at either dosage (20 or 10 mg/kg), as evidenced by the favorable therapeutic
209 metrics (Fig. 4, Fig. S7). Overall, *Nanosota-1C-Fc* was effective at combating SARS-
210 CoV-2 infections both preventively and therapeutically.

211 ***Nanosota-1C-Fc* is stable *in vitro* and *in vivo* with excellent bioavailability**

212 With the lead drug *Nanosota-1C-Fc* demonstrating therapeutic efficacy *in vivo*,
213 we characterized other parameters important to its clinical translation. First, we expressed
214 *Nanosota-1C-Fc* in bacteria for all the experiments carried out in the current study (Fig.
215 5A). After purification on protein A column and gel filtration, the purity of *Nanosota-1C-*
216 *Fc* was nearly 100%. With no optimization, the expression yield reached 40 mg/L of

217 bacterial culture. Second, we investigated the *in vitro* stability of *Nanosota-1C-Fc*
218 incubated at four temperatures (-80°C, 4°C, 25°C or 37°C) for one week and then
219 measured the remaining SARS-CoV-2 RBD-binding capacity by ELISA (Fig. 5B). With
220 -80°C as a baseline, *Nanosota-1C-Fc* retained nearly all of its RBD-binding capacity at
221 the temperatures surveyed. Third, we measured the *in vivo* stability of *Nanosota-1C-Fc*
222 (Fig. 5C). *Nanosota-1C-Fc* was injected into mice via tail vein. Sera were obtained at
223 different time points and measured for their SARS-CoV-2 RBD-binding capacity by
224 ELISA. *Nanosota-1C-Fc* retained most of its RBD-binding capability after 10 days *in*
225 *vivo*. Antithetically, *Nanosota-1C* was stable for only several hours *in vivo*. (Fig. S8A).
226 Last, we examined the biodistribution of *Nanosota-1C-Fc* in mice (Fig. 5D). *Nanosota-*
227 *1C-Fc* was radiolabeled with zirconium-89 and injected systemically into mice. Tissues
228 were collected at various time points and biodistribution of *Nanosota-1C-Fc* was
229 quantified by scintillation counter. After three days, *Nanosota-1C-Fc* remained at high
230 levels in the blood, lung, heart, kidney, liver and spleen, all of which are targets for
231 SARS-CoV-2 (27); moreover, it remained at low levels in the intestine, muscle and
232 bones. In contrast, *Nanosota-1C* had poor biodistribution documenting high renal
233 clearance (Fig. S8B). Overall, our findings suggest that *Nanosota-1C-Fc* is potent SARS-
234 CoV-2 therapeutic with translational values applicable to the world's vast population.

235

236 **Discussion**

237 Nanobody therapeutics derived from camelid antibodies potentially offer a
238 realistic solution to the COVID-19 pandemic compared to conventional antibodies.
239 Currently, there have only been a few reports of nanobody drugs that specifically target

240 SARS-CoV-2 (12, 13). Those reported were developed against SARS-CoV-2 RBD,
241 either blocking out ACE2 or locking the RBD in the closed inactive state on the spike
242 protein (12, 13). None of the nanobodies have been evaluated in animal models for their
243 anti-SARS-CoV-2 therapeutic efficacy. From our novel library, we developed a series of
244 nanobody drugs, named *Nanosota-1*, that specifically target the SARS-CoV-2 RBD. Two
245 rounds of affinity maturation yielded *Nanosota-1C* which bound to the RBD with high
246 affinity. Addition of an Fc tag to make a bivalent construct with increased molecular
247 weight and picomolar RBD-binding affinity resulted in the best performing drug
248 *Nanosota-1C-Fc*. Our structural and biochemical data showed that binding of *Nanosota-*
249 *1C* to the RBD blocked virus binding to viral receptor ACE2. A unique feature of the
250 SARS-CoV-2 spike protein is that it is present in two different conformations, an RBD-
251 up open conformation for receptor binding and an RBD-down closed conformation for
252 immune evasion (20, 22, 23). Due to its small size as well as its ideal binding site on the
253 RBD, *Nanosota-1* can bind to the spike protein in both conformations. In contrast, ACE2
254 can only bind to the spike protein in its open conformation. Thus, *Nanosota-1* drugs are
255 ideal RBD-targeting therapeutics - they can chase down and inhibit SARS-CoV-2 viral
256 particles whether they are infecting cells or hiding from immune surveillance. As a result
257 of this unique property, both *Nanosota-1C* and *Nanosota-1C-Fc* exhibited a profound
258 therapeutic effect *in vitro* against SARS-CoV-2 pseudovirus and authentic SARS-CoV-2.
259 *Nanosota-1C-Fc* was also found to be the first anti-SARS-CoV-2 camelid nanobody-
260 based therapeutic reported in the literature to demonstrate efficacy in an animal model.
261 Additionally, *Nanosota-1C-Fc* was the first anti-SARS-CoV-2 nanobody to have been
262 characterized for ease of production and purification, *in vitro* and *in vivo* stabilities, and

263 biodistribution. These features are critical for the implementation of *Nanosota-1C-Fc* as a
264 COVID-19 therapeutic.

265 When evaluating the anti-SARS-CoV-2 potency of the nanobody therapeutics, we
266 used recombinant ACE2 as a comparison. Recombinant ACE2 was selected because
267 *Nanosota-1* series directly compete with cell-surface ACE2 for the same binding site on
268 the RBD. Our study showed that compared with ACE2, the best performing drug
269 *Nanosota-1C-Fc* bound to the RBD ~3000 fold more strongly, blocking out ACE2
270 binding to the RBD. Furthermore, compared with ACE2, *Nanosota-1C-Fc* inhibited
271 SARS-CoV-2 pseudovirus entry ~100 fold more effectively and inhibited authentic
272 SARS-CoV-2 infections ~6000 fold more effectively. Note that recombinant ACE2 has
273 been shown to be a potent anti-SARS-CoV-2 inhibitor (28) and is currently undergoing
274 clinical trials in Europe as an anti-COVID-19 drug. Compared with ACE2, the much
275 higher anti-SARS-CoV-2 potency of *Nanosota-1C-Fc* was due to both its much higher
276 RBD-binding affinity and its better access to the oft-hidden RBD in the spike protein. As
277 a result, *Nanosota-1C-Fc* was a potent therapeutic *in vivo*. Remarkably, a single dose of
278 *Nanosota-1C-Fc* effectively prevented SARS-CoV-2 infection in hamsters and also
279 effectively treated SARS-CoV-2 infection in the same model. The hamster model is one
280 of the best non-primate models available for studying anti-SARS-CoV-2 therapeutic
281 efficacy, but it is limited by a short virus infection window; hence, repeated dosing was
282 not evaluated. As a result, we were only able to dose the mice once via intraperitoneal
283 injection. Because SARS-CoV-2 is fast acting in hamsters, the time points and dosages
284 for drug administration in hamsters are difficult to directly translate to humans. Our
285 supporting data document that *Nanosota-1c-Fc* is easy to produce in bacteria and has

286 excellent bioavailability and pharmacokinetics when administered intravenously in mice.
287 This suggests that *Nanosota-IC-Fc* may have therapeutic potential when administered
288 intraperitoneal, intravenous or even intramuscular. These parameters will need to be
289 determined in future studies in anticipation of clinical trials. Overall, *Nanosota-IC-Fc*
290 has proven to be an effective therapeutic in the model that we currently have available.

291 How can the novel nanobody therapeutics help to end the COVID-19 pandemic?
292 First, as evidence by our animal study, *Nanosota-IC-Fc* can be used to prevent SARS-
293 CoV-2 infection. Because of its long *in vivo* half-life (>10 days), a single injected dose of
294 *Nanosota-IC-Fc* can theoretically protect a person from SARS-CoV-2 infection for days
295 or weeks in the outpatient setting, reducing the spread of SARS-CoV-2 in human
296 populations. Second, we also learned from our *in vivo* study that *Nanosota-IC-Fc* can
297 potentially be used to treat SARS-CoV-2 infections, thus, saving lives and alleviating
298 symptoms in infected patients in the clinical setting. Third, though ephemeral in nature
299 given its short half-life and rapid clearance from the blood, *Nanosota-IC* could be used
300 as an inhaler to treat infections in the respiratory tracts (*I0*) or as an oral drug to treat
301 infections in the intestines (*I1*). Overall, the novel series of *Nanosota-I* therapeutics can
302 help minimize the mortality and morbidity of SARS-CoV-2 infections and help restore
303 the economy and daily human activities. Given the wide distribution of SARS-CoV-2 in
304 the world, large quantities of anti-SARS-CoV-2 therapeutics would need to be
305 manufactured to provide for the world's populations. This is only feasible with easy to
306 produce and scalable molecules, such as *Nanosota-I* drugs, that are produced at high
307 yields and have long *in vitro* and *in vivo* half-life. Therefore, if further validated in
308 clinical trials, *Nanosota-I* therapeutics can provide a realistic and effective solution to

309 help end the COVID-19 global pandemic.

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320 University of Minnesota has filed a patent on *Nanosota-1* drugs with F.L., G.Y., A.M.L.,
321 J.P.G., J.S., and Y.W. as inventors. We thank Professor Yuhong Jiang for consultation on
322 the design of animal testing and statistical analysis and for editing the manuscript.
323 Coordinates and structure factors have been deposited to the Protein Data Bank with
324 accession number XXXX.

325 **Methods**

326 *Ethics statement*

327 This study was performed in strict accordance with the recommendations in the
328 Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.
329 All of the animals were handled according to approved institutional animal care and use
330 committee (IACUC) protocols of the University of Texas Medical Branch (protocol
331 number 2007072) and of the University of Minnesota (protocol number 2009-38426A).

332

333 *Cell lines, plasmids and virus*

334 HEK293T cells (American Type Culture Collection) were cultured in Dulbecco's
335 modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-
336 glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Life Technologies).
337 ss320 *E. coli* (Lucigen), TG1 *E. coli* (Lucigen), SHuffle T7 *E. coli* (New England
338 Biolabs) were grown in TB medium or 2YT medium with 100 mg/L ampicillin. Vero E6
339 cells (American Type Culture Collection) were grown in Eagle's minimal essential
340 medium (EMEM) supplemented with penicillin (100 units/ml), streptomycin (100
341 µg/ml), and 10% fetal bovine serum (FBS). SARS-CoV-2 spike (GenBank accession
342 number QHD43416.1) and ACE2 (GenBank accession number NM_021804) were
343 described previously (20). SARS-CoV-2 RBD (residues 319-529) was subcloned into
344 Lenti-CMV vector (Vigene Biosciences) with an N-terminal tissue plasminogen activator
345 (tPA) signal peptide and a C-terminal human IgG4 Fc tag or His tag. The ACE2
346 ectodomain (residues 1–615) was constructed in the same way except that its own signal
347 peptide was used. *Nanosota-1A*, *-1B* and *-1C* were each cloned into PADL22c vector

348 (Lucigen) with a N-terminal PelB leader sequence and C-terminal His tag and HA tag.
349 *Nanosota-1C-Fc* was cloned into pET42b vector (Novagen) with a C-terminal human
350 IgG₁ Fc tag. SARS-CoV-2 (US_WA-1 isolate) from CDC (Atlanta) was used throughout
351 the study. All experiments involving infectious SARS-CoV-2 were conducted at the
352 University of Texas Medical Branch and University of Iowa in approved biosafety level 3
353 laboratories.

354

355 *Construction of camelid nanobody phage display library*

356 The camelid nanobody phage display library was constructed as previously
357 described (29, 30). Briefly, total mRNA was isolated from B cells from the spleen, bone
358 marrow and blood of over a dozen non-immunized llamas and alpacas. cDNA was
359 prepared from the mRNA. The cDNA was then used in nested PCR reactions to construct
360 the DNA for the library. The first PCR reaction was to amplify the gene fragments
361 encoding the variable domain of the nanobody. The second PCR reaction (PCR2) was
362 used to add restriction sites (SFI-I), a PelB leader sequence, a His₆ tag, and a HA tag. The
363 PCR2 product was digested with SFI-I (New England Biolabs) and then was ligated with
364 SFI-I-digested PADL22c vector. The ligated product was transformed via electroporation
365 into TG1 *E. coli* (Lucigen). Aliquots of cells were spread onto 2YT agar plates
366 supplemented with ampicillin and glucose, incubated at 30°C overnight, and then scraped
367 into 2YT media. After centrifugation, the cell pellet was suspended into 50% glycerol
368 and stored at -80°C. The library size was 7.5×10^{10} . To display nanobodies on phages,
369 aliquots of the TG1 *E. coli* bank were inoculated into 2YT media, grown to early
370 logarithmic phase, and infected with M13K07 helper phage.

371

372 *Camelid nanobody library screening*

373 The above camelid nanobody phage display library was used in the bio-panning
374 as previously described (31). Briefly, four rounds of panning were performed to obtain
375 the SARS-CoV-2 RBD-targeting nanobodies with high RBD-binding affinity. The
376 amounts of the RBD antigen used in coating the immune tubes in each round were 75 µg,
377 50 µg, 25 µg, and 10 µg, respectively. The retained phages were eluted using 1 ml 100
378 mM triethylamine and neutralized with 500 µl 1 M Tris-HCl pH 7.5. The eluted phages
379 were amplified in TG1 *E. coli* and rescued with M13K07 helper phage. The eluted
380 phages from round 4 were used to infect ss320 *E. coli*. Single colonies were picked into
381 2YT media and nanobody expressions were induced with 1 mM IPTG. The supernatants
382 were subjected to ELISA for selection of strong binders (described below). The strong
383 binders were then expressed and purified (described below) and subjected to SARS-CoV-
384 2 pseudovirus entry assay for selection of anti-SARS-CoV-2 efficacy (described below).
385 The lead nanobody after initial screening was named *Nanosota-1A*.

386

387 *Affinity maturation*

388 Affinity maturation of *Nanosota-1A* was performed as previously described (32).
389 Briefly, mutations were introduced into the whole gene of *Nanosota-1A* using error-prone
390 PCR. Two rounds of error-prone PCR were performed using the GeneMorph II Random
391 Mutagenesis Kit (Agilent Technologies). The PCR product was cloned into the PADL22c
392 vector and transformed via electroporation into the TG1 *E. coli*. The library size was 6 x
393 10⁸. Three rounds of bio-panning were performed using 25 ng, 10 ng and 2 ng RBD-Fc,

394 respectively. The strongest binder after affinity maturation was named *Nanosota-1B*. A
395 second round of affinity maturation was performed in the same way as the first round,
396 except that three rounds of bio-panning were performed using 10 ng, 2 ng and 0.5 ng
397 RBD-Fc, respectively. The strongest binder after the second round of affinity maturation
398 was named *Nanosota-1C*.

399

400 *Production of Nanosota-1 drugs*

401 *Nanosota-1A*, *1B* and *1C* were each purified from the periplasm of ss320 *E. coli*
402 after the cells were induced by 1 mM IPTG. The cells were collected and re-suspended in
403 15 ml TES buffer (0.2 M Tris pH 8, 0.5 mM EDTA, 0.5 M sucrose), shaken on ice for 1
404 hour and then incubated with 40 ml TES buffer followed by shaking on ice for another
405 hour. The protein in the supernatant was sequentially purified using a Ni-NTA column
406 and a Superdex200 gel filtration column (GE Healthcare) as previously described (20).

407 *Nanosota-1C-Fc* was purified from the cytoplasm of Shuffle T7 *E. coli*. The induction of
408 protein expression was the same as above. After induction, the cells were collected, re-
409 suspended in PBS and disrupted using Branson Digital Sonifier (Thermofisher). The
410 protein in the supernatant was sequentially purified on protein A column and
411 Superdex200 gel filtration column as previously described (20).

412

413 *Production of SARS-CoV-2 RBD and ACE2.*

414 HEK293T cells stably expressing SARS-CoV-2 RBD (containing a C-terminal
415 His tag or Fc tag) or human ACE2 ectodomain (containing a C-terminal His tag) were
416 made according to the E and F sections of the pLKO.1 Protocol from Addgene

417 (<http://www.addgene.org/protocols/plko/>). The proteins were secreted to cell culture
418 media, harvested, and purified on either Ni-NTA column (for His-tagged proteins) or
419 protein A column (for Fc-tagged protein) and then on Superdex200 gel filtration column
420 as previously described (20).

421

422 *ELISA*

423 ELISA was performed to detect the binding between SARS-CoV-2 RBD and
424 *Nanosota-1* drugs (either purified recombinant drugs or drugs in the mouse serum) as
425 previously described (33). Briefly, ELISA plates were coated with recombinant SARS-
426 CoV-2 RBD-His or RBD-Fc, and were then incubated sequentially with nanobody drugs,
427 HRP-conjugated anti-llama antibody (1:5,000) (Sigma) or HRP-conjugated anti-human-
428 Fc antibody (1:5,000) (Jackson ImmunoResearch). ELISA substrate (Invitrogen) was
429 added to the plates, and the reactions were stopped with 1N H₂SO₄. The absorbance at
430 450 nm (A₄₅₀) was measured using a Synergy LX Multi-Mode Reader (BioTek).

431

432 *Determination of the structure of SARS-CoV-2 RBD complexed with Nanosota-1C*

433 To prepare the RBD/*Nanosota-1C* complex for crystallization, the two proteins
434 were mixed together in solution and purified using a Superdex200 gel filtration column
435 (GE Healthcare). The complex was concentrated to 10 mg/ml in buffer 20 mM Tris pH
436 7.2 and 200 mM NaCl. Crystals were screened at High-Throughput Crystallization
437 Screening Center (Hauptman-Woodward Medical Research Institute) as previously
438 described (34), and were grown in sitting drops at room temperature over wells
439 containing 50 mM MnCl₂, 50 mM MES pH 6.0, 20% (W/V) PEG 4000. Crystals were

440 soaked briefly in 50 mM MnCl₂, 50 mM MES pH 6.0, 25% (W/V) PEG 4000 and 30%
441 ethylene glycol before being flash-frozen in liquid nitrogen. X-ray diffraction data were
442 collected at the Advanced Photon Source beamline 24-ID-E. The structure was
443 determined by molecular replacement using the structures of SARS-CoV-2 RBD (PDB
444 6M0J) and another nanobody (PDB 6QX4) as the search templates. Structure data and
445 refinement statistics are shown in Table S1.

446

447 *Surface plasmon resonance assay*

448 Surface plasmon resonance assay using a Biacore S200 system (GE Healthcare)
449 was carried out as previously described (20). Briefly, SARS2-CoV-2 RBD-His was
450 immobilized to a CM5 sensor chip (GE Healthcare). Serial dilutions of purified
451 recombinant *Nanosota-1* drugs were injected at different concentrations: 320 nM – 10
452 nM for *Nanosota-1A*; 80 nM - 2.5 nM for *Nanosota-1B* and *Nanosota-1C*; 20 nM - 1.25
453 nM for *Nanosota-1C-Fc*. The resulting data were fit to a 1:1 binding model using Biacore
454 Evaluation Software (GE Healthcare).

455

456 *Protein pull-down assay*

457 Protein pull-down assay was performed using Immunoprecipitation kit
458 (Invitrogen) as previously described (20). Briefly, 10 µl protein A beads were incubated
459 with 1 µg SARS-CoV-2 RBD-Fc at room temperature for 1 hour. Then different amounts
460 (7.04, 3.52, 1.76, 0.88, 0.44, 0.22, or 0 µg) of *Nanosota-1C* (with a C-terminal His tag)
461 and 4 µg human ACE2 (with a C-terminal His tag) were added to the RBD-bound beads.
462 After one-hour incubation at room temperature, the bound proteins were eluted using

463 elution buffer (0.1 M glycine pH 2.7). The samples were then subjected to SDS-PAGE
464 and analyzed through Western blot using an anti-His antibody.

465

466 *Gel filtration chromatography assay*

467 Gel filtration chromatography assay was performed on a Superdex200 column.
468 500 µg human ACE2, 109 µg *Nanosota-1C* and 121 µg SARS-CoV-2 RBD were
469 incubated together at room temperature for 30 min. The mixture was subjected to gel
470 filtration chromatography. Samples from each peak off the column were then subjected to
471 SDS-PAGE and analyzed through Coomassie blue staining.

472

473 *SARS-CoV-2 pseudovirus entry assay*

474 The potency of *Nanosota-1* drugs in neutralizing SARS-CoV-2 pseudovirus entry
475 was evaluated as previously described (20, 22). Briefly, HEK293T cells were co-
476 transfected with a plasmid carrying an Env-defective, luciferase-expressing HIV-1
477 genome (pNL4-3.luc.R-E-) and pcDNA3.1(+) plasmid encoding SARS-CoV-2 spike
478 protein. Pseudoviruses were collected 72 hours after transfection, incubated with
479 individual drugs at different concentrations at 37°C for one hour, and then were used to
480 enter HEK293T cells expressing human ACE2. After pseudoviruses and target cells were
481 incubated together at 37°C for 6 hours, the medium was changed to fresh medium,
482 followed by incubation of another 60 hours. Cells were then washed with PBS buffer and
483 lysed. Aliquots of cell lysates were transferred to plates, followed by the addition of
484 luciferase substrate. Relative light units (RLUs) were measured using an EnSpire plate

485 reader (PerkinElmer). The efficacy of the drug was expressed as the concentration
486 capable of neutralizing 50% of the entry efficiency (Neutralizing Dose 50 or ND₅₀).

487

488 *SARS-CoV-2 plaque reduction neutralization test*

489 The potency of *Nanosota-1* drugs in neutralizing authentic SARS-CoV-2
490 infections was evaluated using a SARS-CoV-2 plaque reduction neutralization test
491 (PRNT) assay. Specifically, individual drugs were serially diluted in DMEM and mixed
492 1:1 with 80 pfu SARS-CoV-2 at 37°C for 1 hour. The mixtures were then added into
493 Vero E6 cells at 37°C for an additional 45 minutes. After removing the culture medium,
494 cells were overlaid with 0.6% agarose and cultured for 3 days. Plaques were visualized
495 by 0.1% crystal violet staining. The efficacy of each drug was calculated and expressed
496 as the concentration capable of reducing the number of virus plaques by 50% compared
497 to control serum-exposed virus (i.e., ND₅₀).

498

499 *SARS-CoV-2 challenge of hamsters*

500 Equal sex Syrian hamsters (n=24) were obtained from Envigo (IN) and
501 challenged via intranasal inoculation with SARS-CoV-2 (at a titer of 1×10^6 Median
502 Tissue Culture Infectious Dose or TCID₅₀) in 100 μ L DMEM (50 μ L per nare). Sample
503 size was comparable to previous animal challenge studies (33) and constrained by the
504 availability of resources. At a sample size of 6 animals per group, G*Power analysis
505 indicates that we can detect an effect size of 1.6 with a power of .80 (alpha = .05 one-
506 tailed). Four groups of hamsters (n=6 each randomly assigned) were treated with
507 *Nanosota-1C-Fc* via intraperitoneal injection at one of the following time points and

508 dosages: (1) 24 hours pre-challenge at 20 mg/kg body weight of hamsters; (2) 4 hours
509 post-challenge at 20 mg/kg body weight of hamsters; (3) 4 hours post-challenge at 10
510 mg/kg body weight of hamsters. Hamsters in the control (negative) group were
511 administered PBS buffer 24 hours pre-challenge. An additional group was tested for a
512 different hypothesis and the data were not included in the current study. Body weights
513 were collected daily beginning prior to challenge. Nasal swabs were collected prior to
514 challenge and additionally 1 day, 2 days, 3 days, 5 days and 10 days post-challenge for
515 quantitative real-time RT-PCR (nasal swabs collected on day 2 and day 3 were lost due to
516 Hurricane Laura). Hamsters were humanely euthanized 10 days post-challenge via
517 overexposure to CO₂. The lungs and bronchial tubes were collected and fixed in formalin
518 for histopathological analysis. This experiment was performed in accordance with the
519 guidelines set by the Institutional Animal Care and Use Committee at the University of
520 Texas Medical Branch (UTMB).

521

522 *Half-life of Nanosota-1 drugs in mice*

523 Male C57BL/6 mice (3 to 4 weeks old) (Envigo) were intravenously injected (tail-
524 vein) with *Nanosota-1C* or *Nanosota-1C-Fc* (100 µg in 100 µl PBS buffer). At varying
525 time points, mice were euthanized and whole blood was collected. Then sera were
526 prepared through centrifugation of the whole blood at 1500xg for 10 min. The sera were
527 then subjected to ELISA for evaluation of their SARS-CoV-2 RBD-binding capability.

528

529 *Biodistribution of Nanosota-1 drugs in mice*

530 To evaluate the *in vivo* biodistribution of *Nanosota-1C-Fc* and *Nanosota-1C*, the
531 nanobodies were labeled with Zirconium-89 [⁸⁹Zr] and injected into male C57BL/6 mice
532 (5 to 6 weeks old) (Envigo). Briefly, the nanobodies were first conjugated to the
533 bifunctional chelator p-SCN-Bn-Deferoxamine (DFO, Macrocyclic) as previously
534 described (35), and [⁸⁹Zr] (University of Wisconsin Medical Physics Department) was
535 then conjugated as previously described (36). [⁸⁹Zr]-labeled nanobodies (1.05 MBq, 1-2
536 µg nanobody, 100 µl PBS) were intravenously injected (tail-vein). Mice were euthanized
537 at different time points. Organs were collected and counted on an automatic gamma-
538 counter (Hidex). The total number of counts per minute (cpm) for each organ or tissue
539 was compared with a standard sample of known activity and mass. Count data were
540 corrected to both background and decay. The percent injected dose per gram (%ID/g) was
541 calculated by normalization to the total amount of activity injected into each mouse.
542

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628

629 **Table 1. Binding affinities between *Nanosota-1* drugs and SARS-CoV-2 RBD as**
630 **measured using surface plasmon resonance.** The previously determined binding
631 affinity between human ACE2 and RBD is shown as a comparison (20).
632

	K_d with SARS-CoV-2 RBD (M)	k_{off} (s^{-1})	k_{on} ($M^{-1}s^{-1}$)
<i>Nanosota-1A</i> (before affinity maturation)	2.28×10^{-7}	9.35×10^{-3}	4.10×10^4
<i>Nanosota-1B</i> (after 1 st round of affinity maturation)	6.08×10^{-8}	7.19×10^{-3}	1.18×10^5
<i>Nanosota-1C</i> (after 2 nd round of affinity maturation)	1.42×10^{-8}	2.96×10^{-3}	2.09×10^5
<i>Nanosota-1C-Fc</i> (after 2 nd round of affinity maturation; containing a C-terminal human Fc tag)	1.57×10^{-11}	9.68×10^{-5}	6.15×10^6
ACE2	4.42×10^{-8}	7.75×10^{-3}	1.75×10^5

633
634

635 **Figure legends:**

636 **Figure 1: Construction of a camelid nanobody phage display library and use of this**
637 **library for screening of anti-SARS-CoV-2 nanobodies.** A large-sized (diversity $7.5 \times$
638 10^{10}), naïve nanobody phage display library was constructed using B cells of over a
639 dozen llamas and alpacas. Phages were screened for their high binding affinity for SARS-
640 CoV-2 RBD. Nanobodies expressed from the selected phages were further screened for
641 their potency in neutralizing SARS-CoV-2 pseudovirus entry. The best performing
642 nanobody was subjected to two rounds of affinity maturation.

643

644 **Figure 2: Crystal structure of SARS-CoV-2 RBD complexed with *Nanosota-1C*.** (A)
645 Structure of SARS-CoV-2 RBD complexed with *Nanosota-1C*, viewed at two different
646 angles. *Nanosota-1C* is in red, the core structure of RBD is in cyan, and the receptor-
647 binding motif (RBM) of RBD is in magenta. (B) Overlay of the structures of the
648 RBD/*Nanosota-1C* complex and RBD/ACE2 complex (PDB 6M0J). ACE2 is in green.
649 The structures of the two complexes were superimposed based on their common RBD
650 structure. The *Nanosota-1C* loops that have clashes with ACE2 are in blue.

651

652 **Figure 3. Efficacy of *Nanosota-1* drugs in neutralizing SARS-CoV-2 infections *in***
653 ***vitro*.** (A) Neutralization of SARS-CoV-2 pseudovirus entry into target cells by one of
654 three inhibitors: *Nanosota-1C-Fc*, *Nanosota-1C*, and recombinant human ACE2.
655 Retroviruses pseudotyped with SARS-CoV-2 spike protein (i.e., SARS-CoV-2
656 pseudoviruses) were used to enter HEK293T cells expressing human ACE2 in the
657 presence of the inhibitor at various concentrations. Entry efficiency was characterized via

658 a luciferase signal indicating successful cell entry. Data are the mean \pm SEM (n = 4).
659 Nonlinear regression was performed using a log (inhibitor) versus normalized response
660 curve and a variable slope model ($R^2 > 0.95$ for all curves). The efficacy of each inhibitor
661 was expressed as the 50% Neutralizing Dose or ND₅₀. The assay was repeated three times
662 (biological replication: new aliquots of pseudoviruses and cells were used for each
663 repeat). (B) Neutralization of authentic SARS-CoV-2 infection of target cells by one of
664 two inhibitors: *Nanosota-1C-Fc* and *Nanosota-1C*. The potency of *Nanosota-1* drugs in
665 neutralizing authentic SARS-CoV-2 infections was evaluated using a SARS-CoV-2
666 plaque reduction neutralization test (PRNT) assay. 80 pfu infectious SARS-CoV-2
667 particles were used to infect Vero E6 cells in the presence of the inhibitor at various
668 concentrations. Infection was characterized as the number of virus plaques formed in
669 overlaid cells. Images of virus plaques for each inhibitor at the indicated concentrations
670 are shown. Each image represents data from triplications. The efficacy of each inhibitor
671 was calculated and expressed as the concentration capable of reducing the number of
672 virus plaques by 50% (i.e., ND₅₀). The assay was repeated twice (biological replication:
673 new aliquots of virus particles and cells were used for each repeat).

674

675 **Figure 4. Efficacy of *Nanosota-1* drugs in protecting hamsters from SARS-CoV-2**
676 **infections.** Hamsters (6 per group) were injected with a single dose of *Nanosota-1C-Fc* at
677 the indicated time point and the indicated dosage. At day 0 all groups (experimental and
678 control) were challenged with SARS-CoV-2 (at a titer of 10⁶ Median Tissue Culture
679 Infectious Dose or TCID₅₀). (A) Body weights of hamsters were monitored on each day
680 and percent change in body weight relative to day 0 was calculated for each hamster.

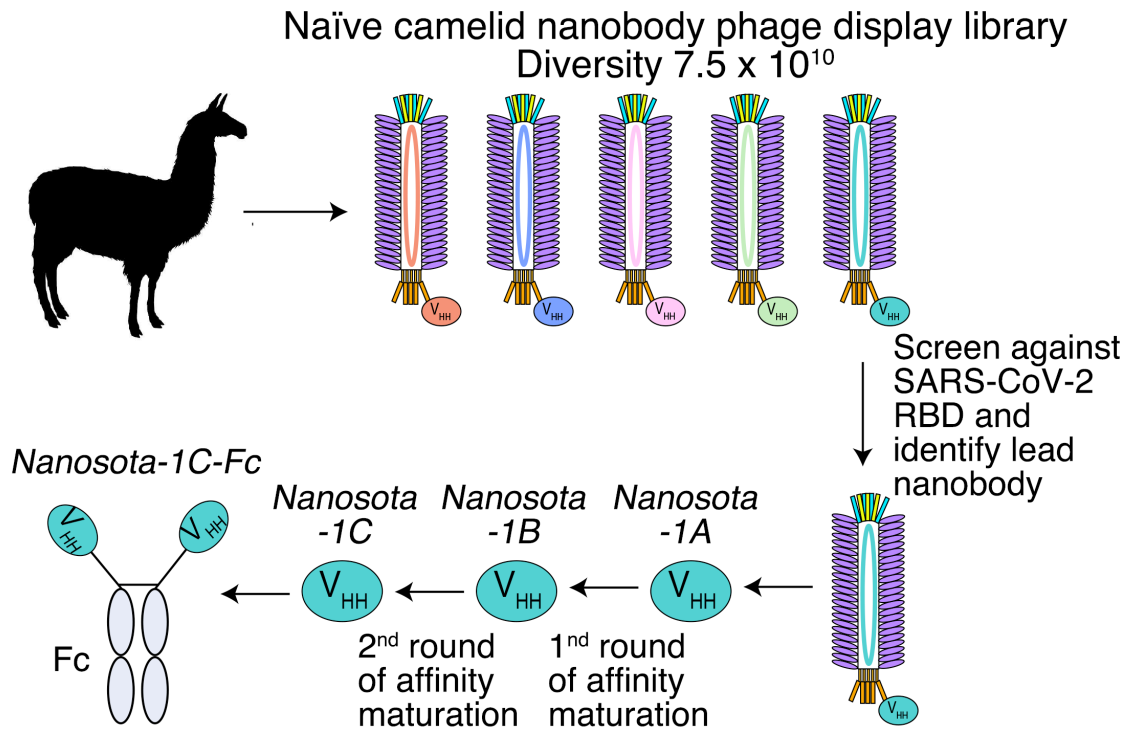
681 Data are the mean \pm SEM (n = 6). ANOVA on group as a between-group factor and day
682 (1-10) as a within-group factor revealed significant differences between the control group
683 and each of the following groups: 24 hour pre-challenge (20 mg/kg) group ($F(1, 10) =$
684 $17.80, p = .002$; effect size $\eta_p^2 = .64$), 4 hour post-challenge (20 mg/kg) group ($F(1, 10) =$
685 $5.02, p = .035$; $\eta_p^2 = .37$), and 4 hour post-challenge (10 mg/kg) group ($F(1, 10) = 7.04, p$
686 $= .024, \eta_p^2 = .41$). All p -values are two-tailed. (B) Tissues of bronchial tubes from each of
687 the hamsters were collected on day 10 and scored for the severity of bronchioloalveolar
688 hyperplasia: 3 - moderate; 2 - mild; 1 - minimum; 0 - none. Data are the mean \pm SEM (n
689 = 6). A comparison between the control group and each of other groups was performed
690 using one-tailed Student's t-test for directional tests. *** $p < 0.001$; * $p < 0.05$.

691

692 **Figure 5. Analysis of expression, purification and pharmacokinetics of *Nanosota-1C-***
693 ***Fc*.** (A) Purification of *Nanosota-1C-Fc* from bacteria. The protein was nearly 100% pure
694 after gel filtration chromatography, as demonstrated by its elution profile and SDS-PAGE
695 (stained by Coomassie blue). The yield of the protein was 40 mg/L of bacterial culture,
696 without any optimization of the expression. (B) *In vitro* stability of *Nanosota-1C-Fc*. The
697 protein was stored at indicated temperatures for a week, and then a dilution ELISA was
698 performed to evaluate its SARS-CoV-2 RBD-binding capability. Data are the mean \pm
699 SEM (n = 4). (C) *In vivo* stability of *Nanosota-1C-Fc*. *Nanosota-1C-Fc* was injected into
700 mice, mouse sera were collected at different time points, and *Nanosota-1C-Fc* remaining
701 in the sera was detected for its SARS-CoV-2 RBD-binding capability as displayed in a
702 dilution ELISA. Data are the mean \pm SEM (n = 3). (D) Biodistribution of [^{89}Zr]Zr-
703 *Nanosota-1C-Fc*. *Nanosota-1C-Fc* was radioactively labeled with ^{89}Zr and injected into

704 mice via tail vein injection. Different tissues or organs were collected at various time
705 points (n=3 mice per time point). The amount of *Nanosota-1C-Fc* present in each tissue
706 or organ was measured through examining the radioactive count of each tissue or organ.
707 Data are the mean \pm SEM (n = 3).
708
709

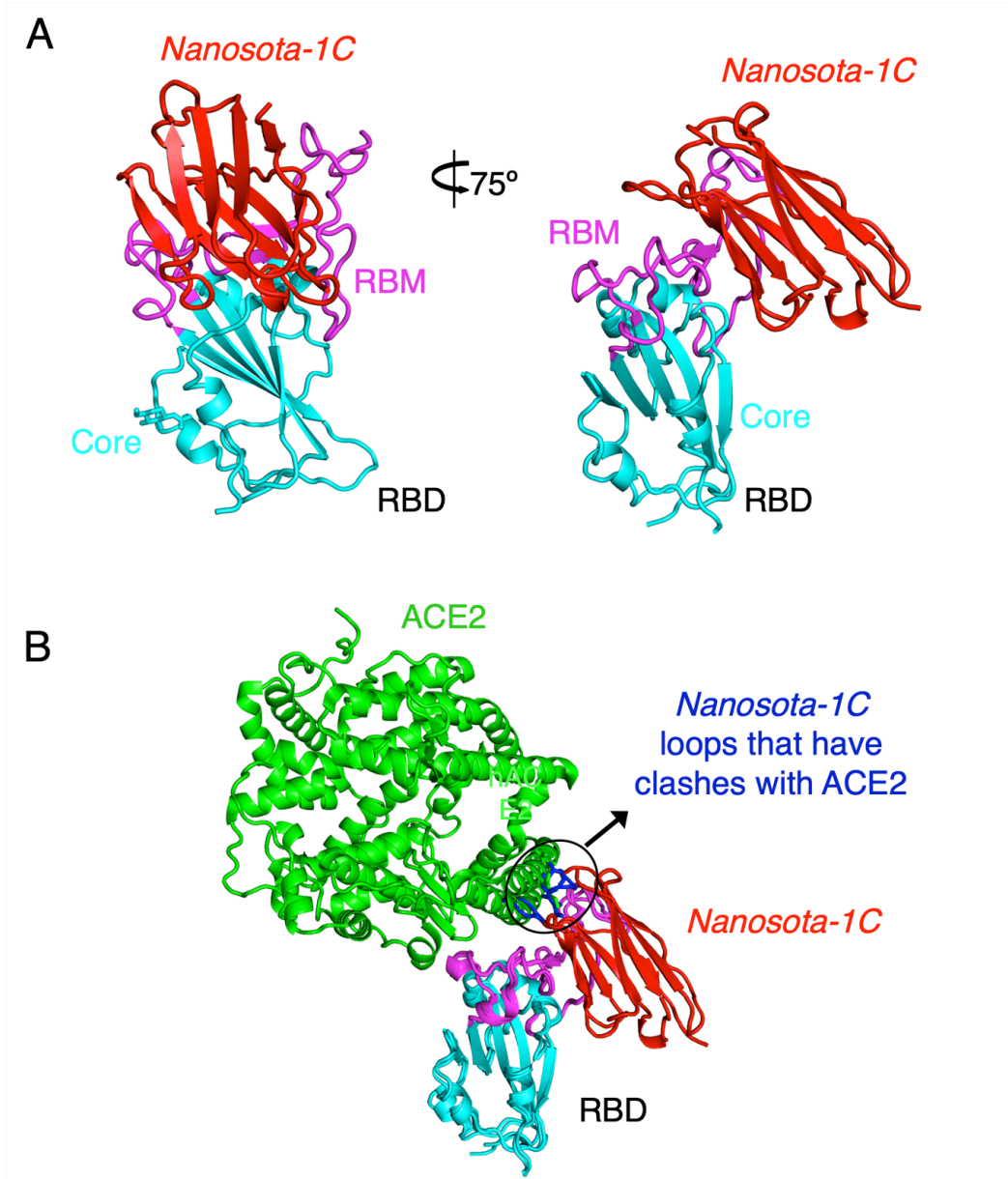
710 Figure 1



711

712

713 Figure 2

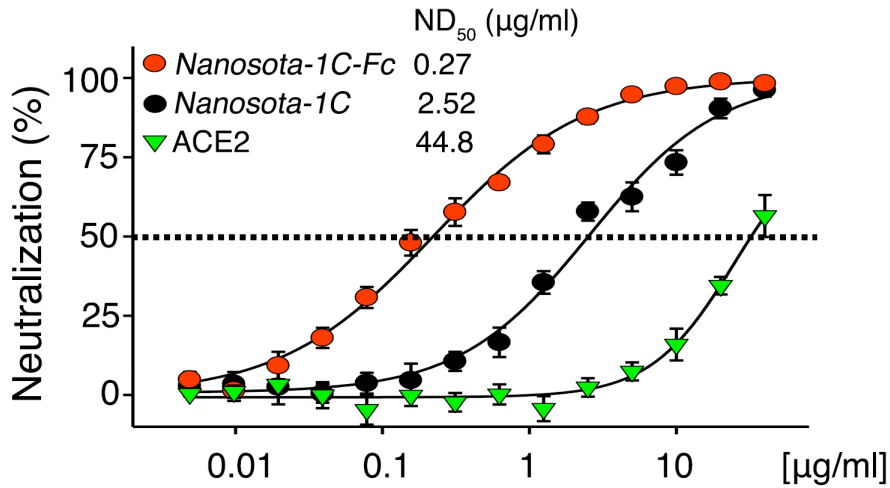


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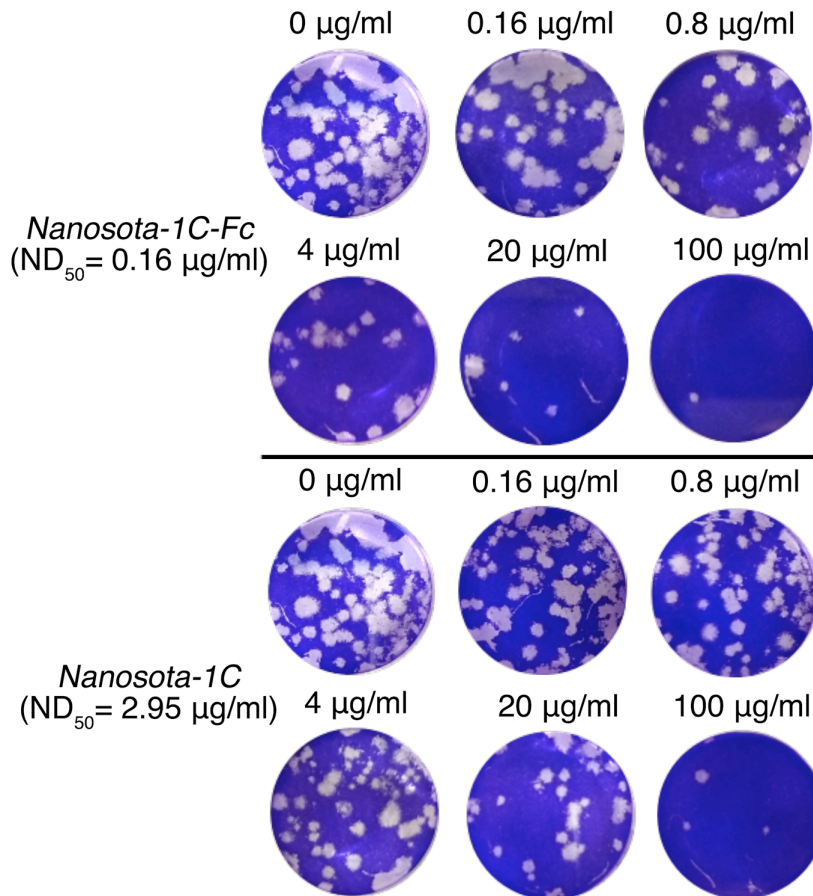
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716 Figure 3

A SARS-CoV-2 pseudovirus neutralization

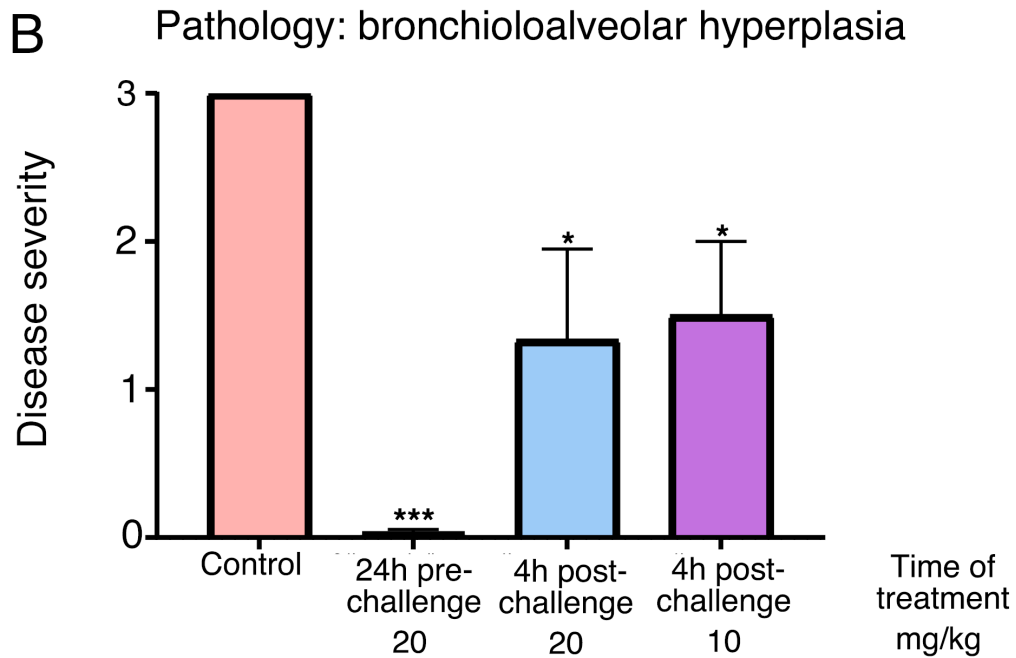
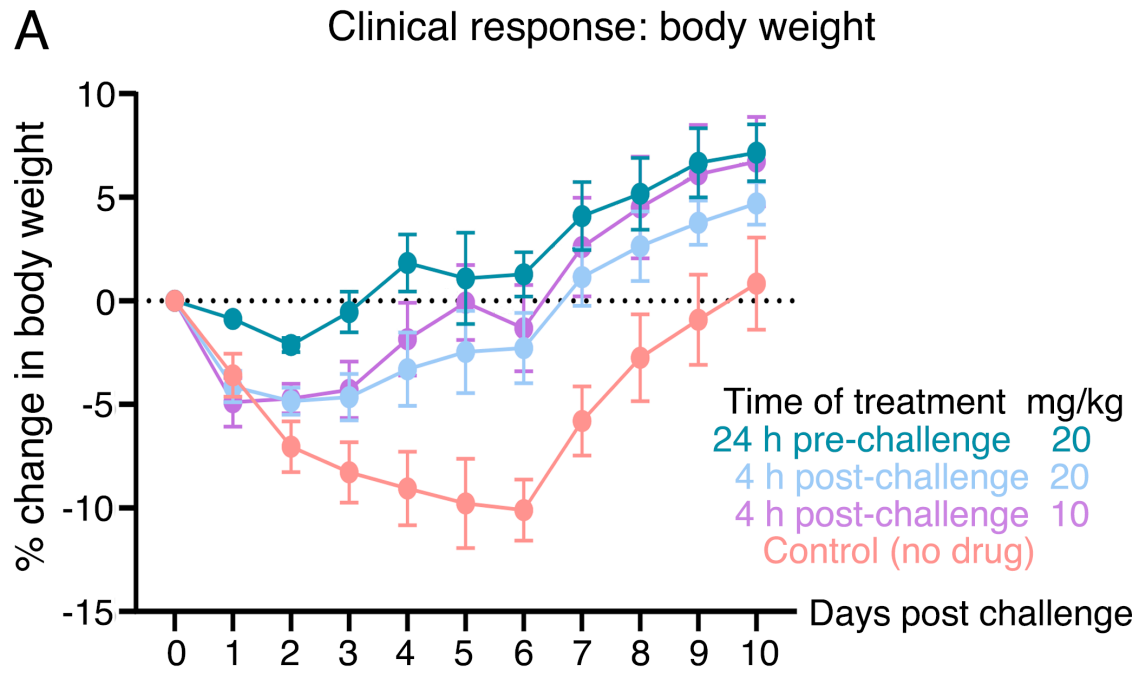


B Authentic SARS-CoV-2 neutralization



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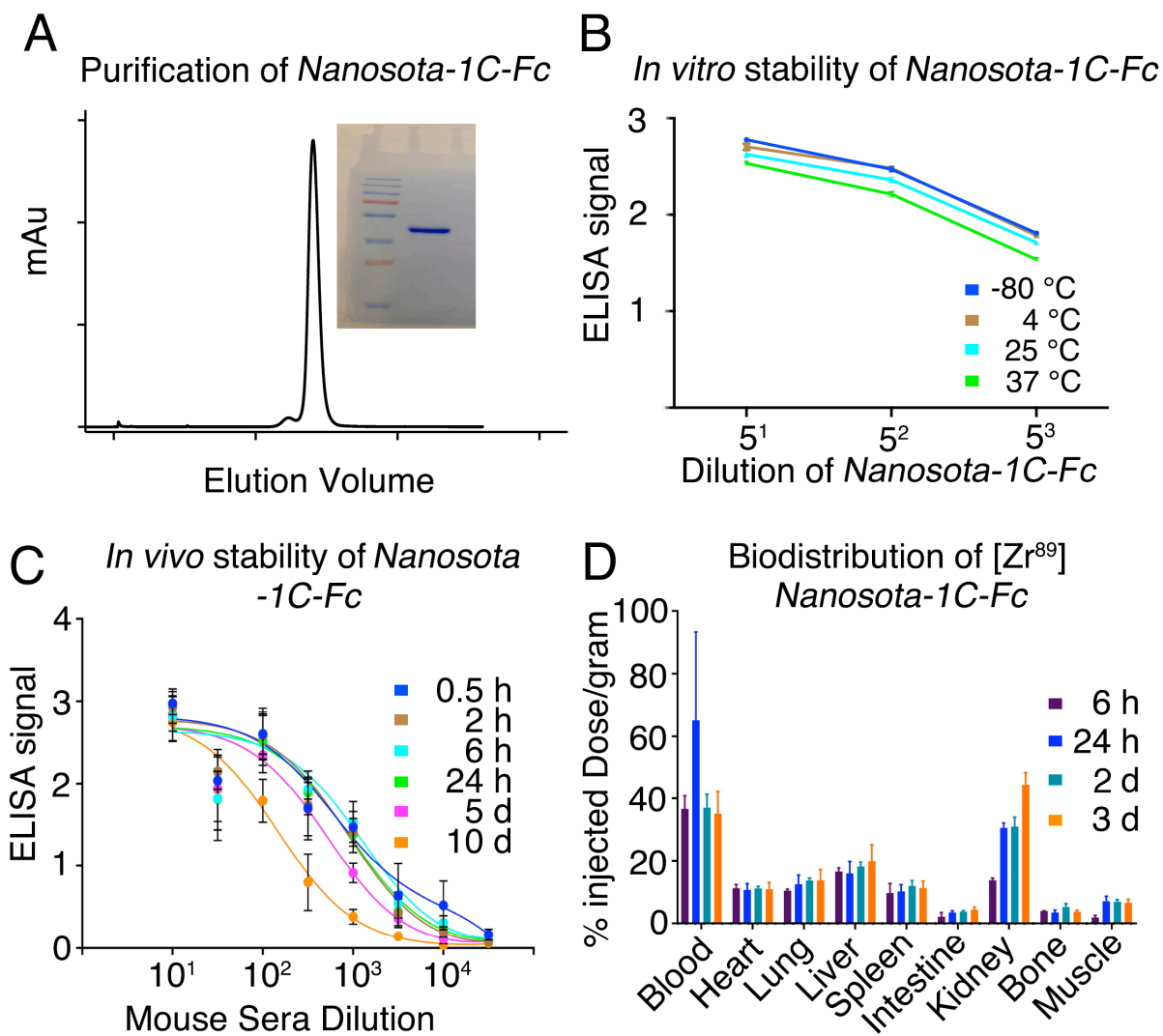
718 Figure 4



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721 Figure 5



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724 **Supplementary materials for**

725

726 **“The Development of a Novel Nanobody Therapeutic for SARS-CoV-2”**

727

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730 Aleksandra Drelich ⁷, Kempaiah R. Kempaiah ⁷, Vivian Tat ⁸, Stanley Perlman ⁶,
731 Lanying Du ⁵, Chien-Te Tseng ^{7,9}, Hideki Aihara ⁴, Aaron M. LeBeau ^{2,#}, Fang Li ^{1,#}

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733 **Table S1. X-ray data collection and structure refinement statistics**
 734 **(SARS-CoV-2 RBD/*Nanosota-1C* complex)**

Data collection

Wavelength					0.979
Resolution range	45.48	- 3.19	(3.30	- 3.19)	
Space group					P 43 21 2
Unit cell	60.849	60.849	410.701	90 90 90	
Total reflections					64167 (5703)
Unique reflections					13607 (1308)
Multiplicity					4.7 (4.4)
Completeness (%)					96.82 (97.60)
Mean I/sigma(I)					8.41 (1.80)
Wilson B-factor					83.24
R-merge					0.145 (0.928)
R-meas					0.1638 (1.053)
R-pim					0.07385 (0.4858)
CC1/2					0.995 (0.861)
CC*					0.999 (0.962)

Refinement

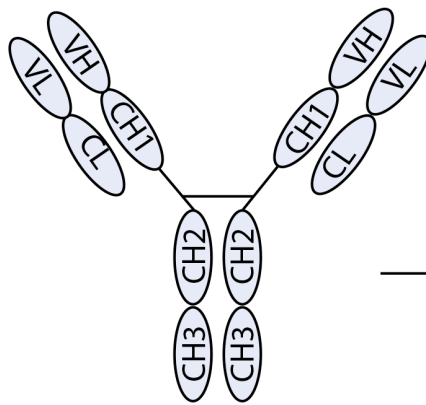
Reflections used in refinement					13567 (1301)
Reflections used for R-free					674 (62)
R-work					0.2483 (0.3521)
R-free					0.2959 (0.4153)
CC(work)					0.963 (0.819)
CC(free)					0.909 (0.615)
Number of non-hydrogen atoms					4890
macromolecules					4833
ligands					57
Protein residues					621
RMS(bonds)					0.002
RMS(angles)					0.45
Ramachandran favored (%)					93.11
Ramachandran allowed (%)					6.89
Ramachandran outliers (%)					0.00
Rotamer outliers (%)					3.23
Clashscore					5.25
Average B-factor					90.29
macromolecules					89.84
ligands					127.91

735 Statistics for the highest-resolution shell are shown in parentheses.

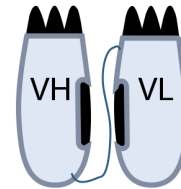
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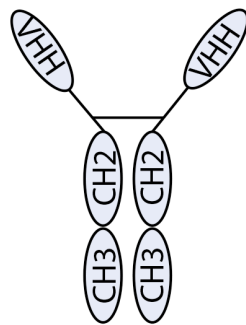
Conventional antibody (150 kDa)



scFv (30 kDa)



Heavy chain only antibody (80 kDa)



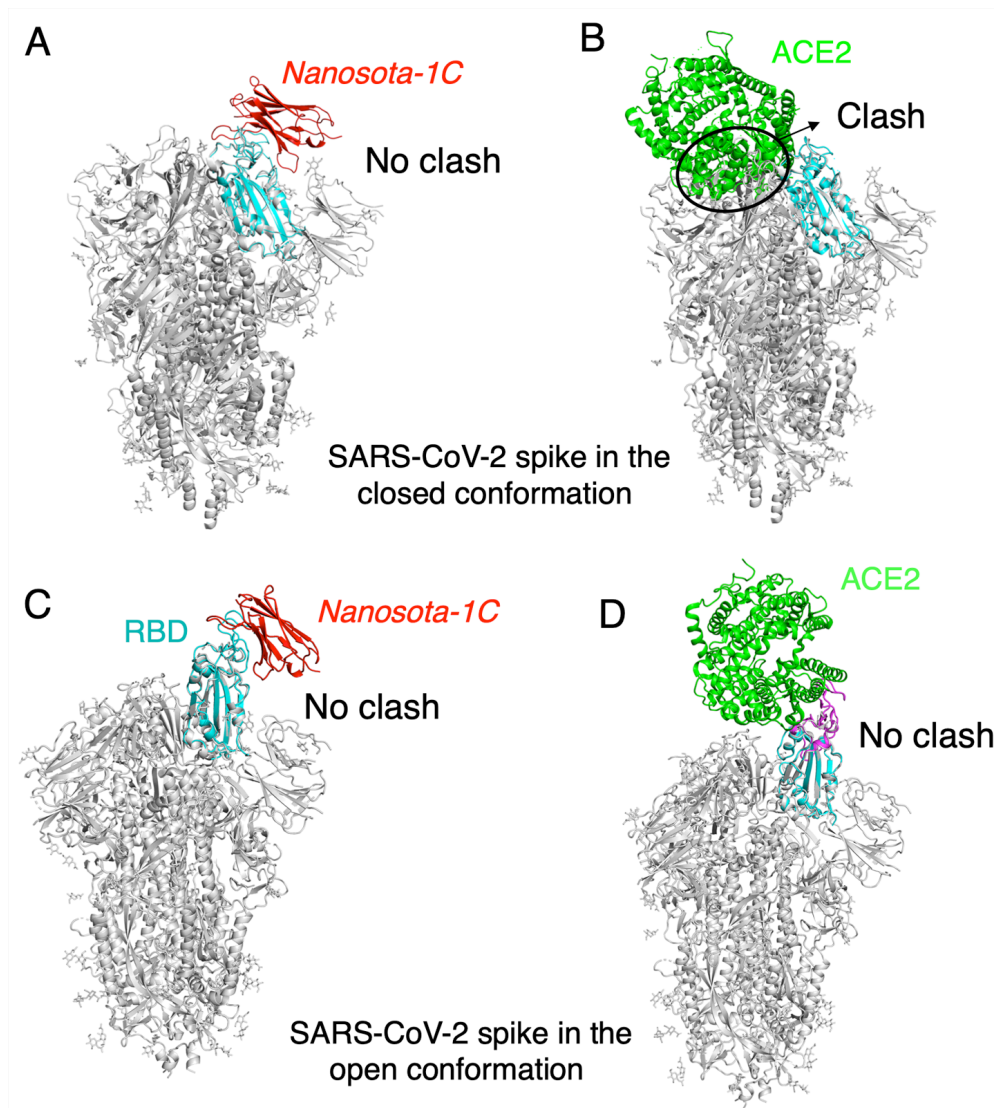
Nanobody (15 kDa)



2.5 nm x 4 nm

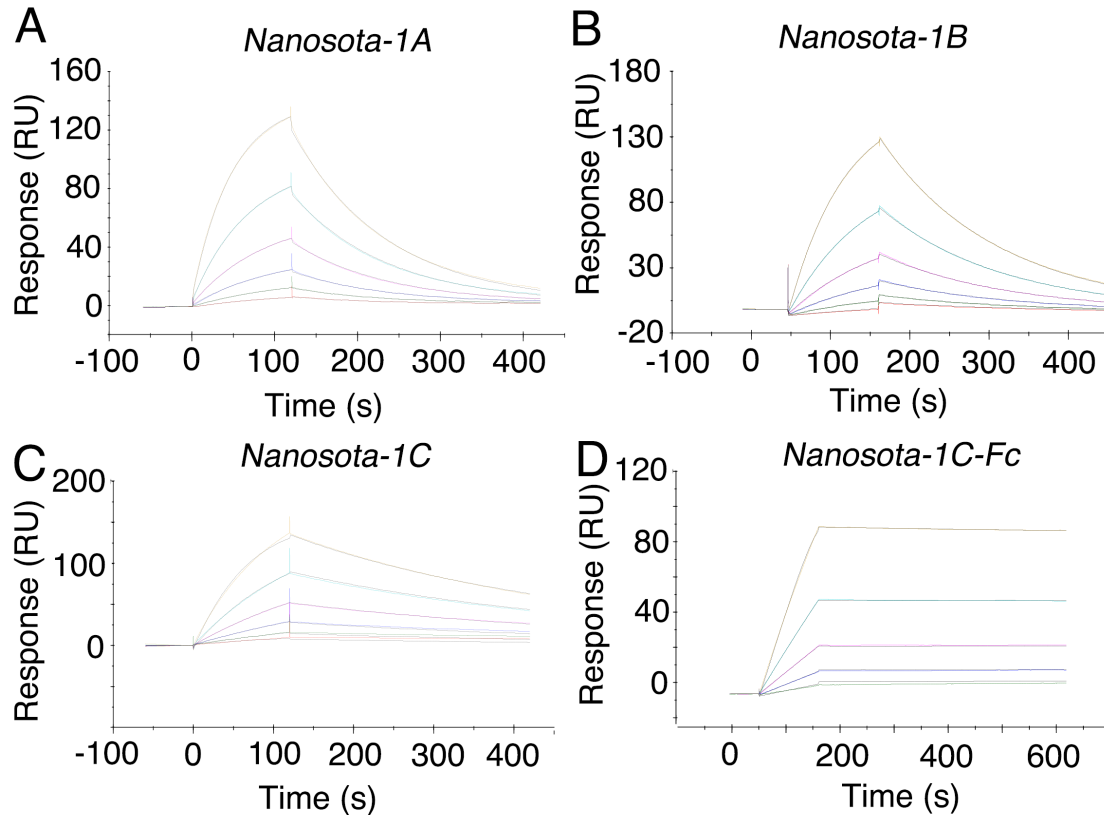
738

739 **Figure S1. Schematic drawings of nanobodies and conventional antibodies.** VH:
740 variable domain of heavy chain. CH: constant domain of heavy chain. VL: variable
741 domain of light chain. CL: constant domain of light chain. VHH: variable domain of
742 heavy-chain only antibody. scFv: single-chain variable fragment.
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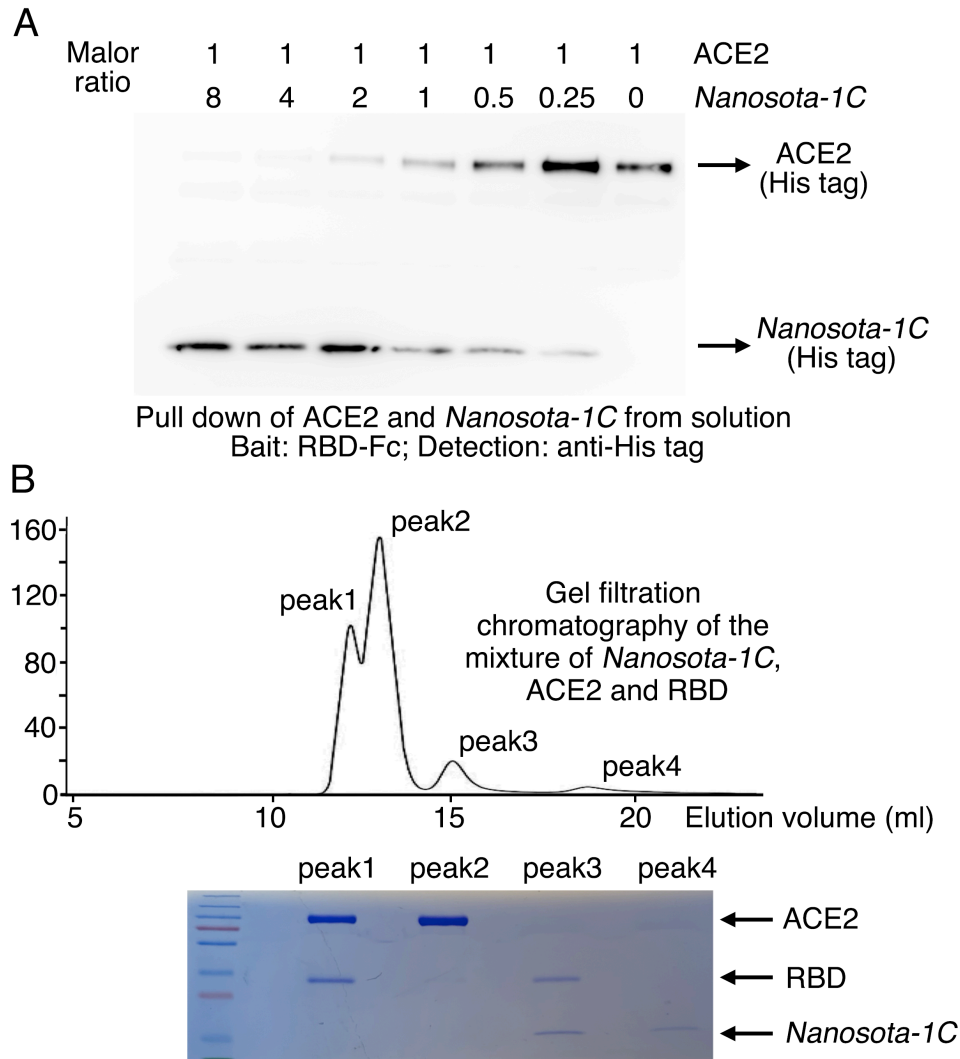
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746 **Figure S2. The binding of *Nanosota-1C* to SARS-CoV-2 spike protein in different**
747 **conformations.** (A) The binding of *Nanosota-1C* to the spike protein in the closed
748 conformation. The structures of the RBD/*Nanosota-1C* complex and SARS-CoV-2 spike
749 protein in the closed conformation (PDB: 6ZWV) were superimposed based on their
750 common RBD structure (in cyan). *Nanosota-1C* is in red. The rest of the spike protein
751 is in gray. (B) The binding of ACE2 to the spike protein in the closed conformation.
752 The structures of the RBD/ACE2 complex (PDB 6M0J) and SARS-CoV-2 spike protein
753 in the closed conformation (PDB: 6ZWV) were superimposed based on their common RBD
754 structure. ACE2 is in green. Clashes between ACE2 and the rest of the spike protein
755 were circled. (C) The binding of *Nanosota-1C* to the spike protein in the open
756 conformation (PDB: 6VSB). (D) The binding of ACE2 to the spike protein in the open
757 conformation (PDB: 6VSB).
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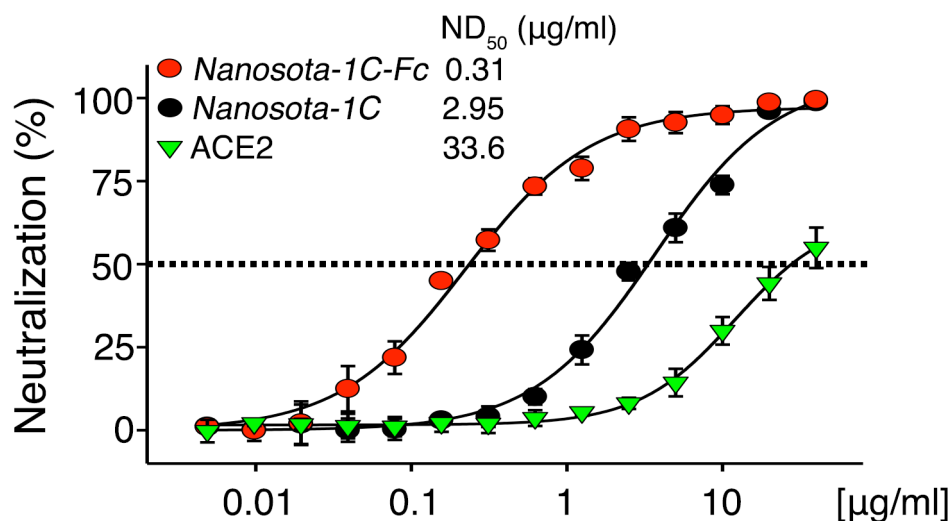
760 **Figure S3. Measurement of the binding affinities between *Nanosota-1* drugs and**
761 **SARS-CoV-2 RBD by surface plasmon resonance assay using Biacore.** Purified
762 recombinant SARS-CoV-2 RBD was covalently immobilized on a sensor chip through its
763 amine groups. Purified recombinant nanobodies flowed over the RBD individually at one
764 of five different concentrations. The resulting data were fit to a 1:1 binding model and the
765 value of K_d was calculated for each nanobody. The assay was repeated three times
766 (biological replication: new aliquots of proteins and new sensor chips were used for each
767 repeat).
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770 **Figure S4. Binding interactions between *Nanosota-1* drugs and SARS-CoV-2 RBD.**
771 (A) Binding interactions between SARS-CoV-2 RBD, *Nanosota-1C*, and ACE2 as
772 evaluated using a protein pull-down assay. Various concentrations of *Nanosota-1C* and a
773 constant concentration of ACE2 (all His tagged) were combined in different molar ratios.
774 SARS-CoV-2 RBD (Fc tagged) was used to pull down *Nanosota-1C* and ACE2. A
775 western blot was used to detect the presence of *Nanosota-1C* and ACE2 following pull
776 down by SARS-CoV-2 RBD. The assay was repeated three times (biological replication:
777 new aliquots of proteins were used for each repeat). (B) Binding interactions between
778 SARS-CoV-2 RBD, *Nanosota-1C*, and ACE2 as examined using gel filtration
779 chromatography. *Nanosota-1C*, ACE2 and SARS-CoV-2 RBD (all His tagged) were
780 mixed together in solution (both *Nanosota-1C* and ACE2 in molar excess of SARS-CoV-
781 2 RBD) and purified using gel filtration chromatography. Protein components in each of
782 the gel filtration chromatography peaks were analyzed with SDS-PAGE and stained by
783 Coomassie blue. The assay was repeated three times (biological replication: new aliquots
784 of proteins were used for each repeat).
785

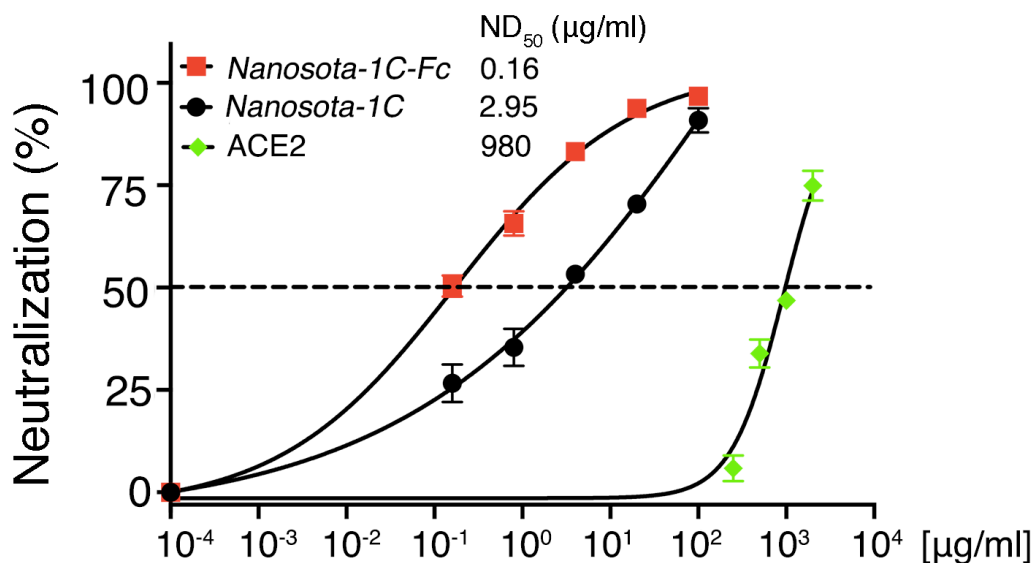
SARS-CoV-2 (D614G) pseudovirus neutralization



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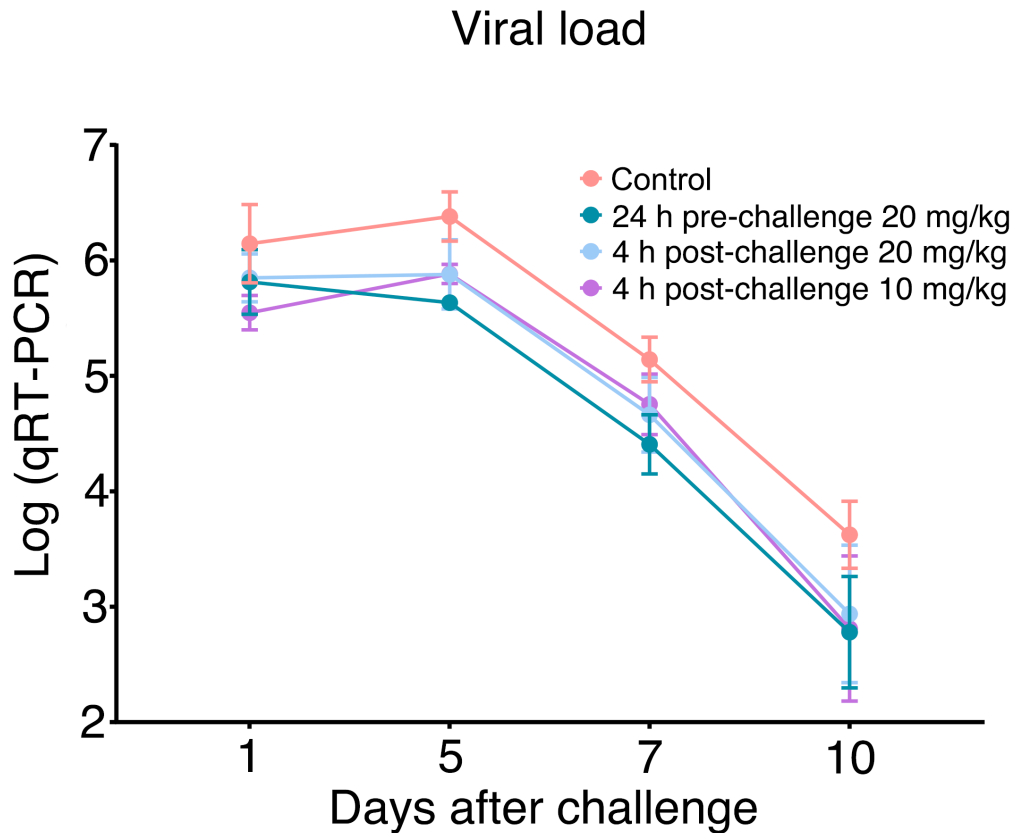
787 **Figure S5. Neutralization of SARS-CoV-2 pseudovirus, which contains the D614G**
788 **mutation in the spike protein, by *Nanosota-1* drugs.** The procedure was the same as
789 described in Fig. 3A, except that the mutant spike protein replaced the wild type spike
790 protein. The assay was repeated three times (biological replication: new aliquots of
791 pseudoviruses and cells were used for each repeat).
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Authentic SARS-CoV-2 neutralization



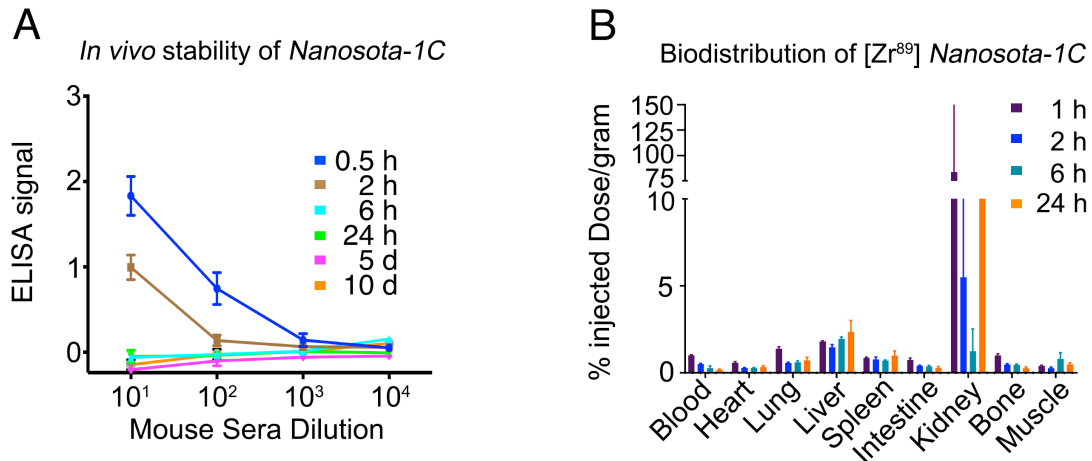
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794 **Figure S6. Detailed data on the neutralization of authentic SARS-CoV-2 infection of**
795 **target cells by *Nanosota-1* drugs.** Data are the mean \pm SEM (n = 3). Nonlinear
796 regression was performed using a log (inhibitor) versus normalized response curve and a
797 variable slope model ($R^2 > 0.95$ for all curves). The assay was repeated twice (biological
798 replication: new aliquots of virus particles and cells were used for each repeat).
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801 **Figure S7. Additional data on the efficacy of *Nanosota-1* drugs in protecting**
802 **hamsters from SARS-CoV-2 infections.** Nasal swabs were collected from each hamster
803 on days 1, 2, 3, 5, 7, and 10. Nasal swab samples from day 2 and day 3 were lost due to
804 Hurricane Laura. qRT-PCR was performed to determine the virus loads in each of the
805 samples. The qRT-PCR results are displayed on a log scale (since qRT-PCR amplifies
806 signals on a log scale). Data are the mean \pm SEM ($n = 6$). Missing data from one animal
807 in the 4-hour post-challenge (10mg/kg) group on Day 7 were replaced by the average of
808 that animal's days 5 and 10 data. ANOVA analysis using group as a between-group
809 factor and day (1, 5, 7, and 10) as a within-group factor revealed significant differences
810 between the control group and each of the following groups: 24 hour pre-challenge (20
811 mg/kg) group ($F(1, 10) = 6.02, p = .017$, effect size $\eta_p^2 = .38$), 4 hour post-challenge (20
812 mg/kg) group ($F(1, 10) = 5.38, p = .037$, $\eta_p^2 = .31$), and 4 hour post-challenge (10 mg/kg)
813 group ($F(1, 10) = 3.40, p = .048$, $\eta_p^2 = .25$). All p -values are one-tailed for directional
814 tests.
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Figure S8. Pharmacokinetics of *Nanosota-1C*. *In vivo* stability and biodistribution of *Nanosota-1C* were measured in the same way as described in Fig. 5C and Fig. 5D, respectively, except that time points for *Nanosota-1C* differed from those for *Nanosota-1C-Fc* due to pharmacokinetic differences of the small molecular weight nanobody versus the larger Fc tagged nanobody.