

Novel Ankyrin Repeat Binding Proteins And Their Uses

Field of the invention

The present invention relates to recombinant binding proteins comprising one or more designed ankyrin repeat domains with binding specificity for coronavirus spike proteins, nucleic acids encoding such proteins, pharmaceutical compositions comprising such proteins or nucleic acids, and the use of such proteins, nucleic acids or pharmaceutical compositions in the treatment of coronavirus diseases, particularly diseases caused by SARS-CoV-2.

Background of the Invention

With a positive-stranded RNA genome of 28 to 32 kb, the *Coronaviridae* are the largest enveloped RNA viruses. Coronaviruses infect many different mammalian and avian species. They are responsible for a variety of acute and chronic diseases of the respiratory, hepatic, gastrointestinal, and neurological systems. The common cold is an example of a mild form of coronavirus infection. The 2003 SARS outbreak and the 2012 MERS outbreaks were both caused by coronaviruses. SARS-CoV-2 (also called 2019-nCoV) is the virus strain that causes COVID-19.

Coronaviruses have four structural proteins, known as the spike protein, envelope protein, membrane protein, and nucleocapsid protein. The spike protein is the viral membrane protein responsible for cell entry.

Coronaviruses make use of a densely glycosylated spike protein to gain entry into host cells. The spike protein consists of three subunits and is a trimeric class I fusion protein that exists in a metastable prefusion conformation that undergoes a substantial structural rearrangement to fuse the viral membrane with the host cell membrane. This process is triggered when the S1 subunit binds to a host cell receptor. Receptor binding destabilizes the prefusion trimer, resulting in shedding of the S1 subunit and transition of the S2 subunit to a stable post-fusion conformation. To engage a host cell receptor, the receptor-binding domain (RBD) of S1 undergoes hinge-like conformational movements that transiently hide or expose the determinants of receptor binding. These two states are referred to as the "down" conformation and the "up" conformation, where down corresponds to the receptor-inaccessible state and up corresponds to the receptor accessible state, which is thought to be less stable. Once the spike protein is in the "up" conformation, binding to the angiotensin-converting enzyme 2 (ACE2) receptor in the host cell can occur, allowing the virus into the cell. "Activation" of the spike protein to the "up" conformation can be carried out by enzymes such as furin or TMPRSS2 which act by opening the spike protein, allowing the nucleocapsid protein out of the viral capsid and into the cell, resulting in infection.

Once the cell is infected with the coronavirus, treatment options become more difficult as the immune system (or therapeutic agent) must only target virus-infected cells, without damaging non-infected cells.

Because of the indispensable function of the spike protein, it represents a target for antibody-mediated neutralization. Thus, one approach to coronavirus therapy is to inhibit binding of the virus to the cell by neutralizing the spike proteins, preventing infection of the cell.

5 DARPin® proteins are genetically engineered ankyrin repeat proteins, which can function like antibody mimetic proteins, typically exhibiting highly specific and high-affinity target binding. DARPin® proteins comprise one or more designed ankyrin repeat domains. Designed ankyrin repeat domains are derived from natural ankyrin repeat proteins and each designed ankyrin repeat domain typically binds a target protein with high specificity and affinity. Due to their high specificity, stability, potency and affinity and due to their flexibility in formatting to generate mono-, bi- or multi-specific proteins, DARPin® proteins are attractive therapeutic agents for a wide variety of clinical applications. For example, WO 2011/135067 describes DARPin® proteins for use in the treatment of cancer and other pathological conditions including eye diseases such as age-related macular degeneration. DARPin® is a registered trademark owned by Molecular Partners AG.

15 The technical problem underlying the present invention is identifying novel recombinant binding proteins comprising one or more designed ankyrin repeat domains with binding specificity for coronavirus, preferably SARS-CoV-2. Such recombinant binding proteins may be useful for inhibiting binding of the coronavirus to cells and for preventing viral infection of cells. Such recombinant binding proteins and pharmaceutical compositions comprising such proteins may further be useful for methods of preventing, treating or diagnosing coronavirus diseases, such as coronavirus diseases caused by SARS-CoV-2, and/or for methods of detecting coronavirus, preferably SARS-CoV-2.

Summary of the Invention

25 Based on the disclosure provided herein, those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following embodiments (E).

30 1. In a first embodiment, the present invention relates to a recombinant binding protein comprising a first ankyrin repeat domain, wherein said first ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85.

35 1a. In embodiment 1a, the present invention relates to a recombinant binding protein comprising a first ankyrin repeat domain, wherein said first ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11.

- 1b. In embodiment 1b, the present invention relates to a recombinant binding protein comprising a first ankyrin repeat domain, wherein said first ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76 and 77.
2. In a second embodiment, the present invention relates to the recombinant binding protein according to embodiment 1, wherein said first ankyrin repeat domain comprises an amino acid sequence that has at least about 95% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85.
- 2a. In embodiment 2a, the present invention relates to the recombinant binding protein according to embodiment 1a, wherein said first ankyrin repeat domain comprises an amino acid sequence that has at least about 95% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11.
- 2b. In embodiment 2b, the present invention relates to the recombinant binding protein according to embodiment 1b, wherein said first ankyrin repeat domain comprises an amino acid sequence that has at least about 95% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76 and 77.
3. In a third embodiment, the present invention relates to the recombinant binding protein according to embodiment 1, wherein said first ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85.
- 3a. In embodiment 3a, the present invention relates to the recombinant binding protein according to embodiment 1a, wherein said first ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11.
- 3b. In embodiment 3b, the present invention relates to the recombinant binding protein according to embodiment 1b, wherein said first ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11, 76 and 77.
4. In a fourth embodiment, the present invention relates to the recombinant binding protein according to any one of embodiments 1 to 3 further comprising a second ankyrin repeat domain, wherein said second ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85.

4a. In embodiment 4a, the present invention relates to the recombinant binding protein according to any one of embodiments 1a, 2a or 3a further comprising a second ankyrin repeat domain, wherein said second ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11.

4b. In embodiment 4b, the present invention relates to the recombinant binding protein according to any one of embodiments 1b, 2b or 3b further comprising a second ankyrin repeat domain, wherein said second ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76 and 77.

5. In a fifth embodiment, the present invention relates to the recombinant binding protein according to embodiment 4, wherein said second ankyrin repeat domain comprises an amino acid sequence that has at least about 95% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85.

5a. In embodiment 5a, the present invention relates to the recombinant binding protein according to embodiment 4a, wherein said second ankyrin repeat domain comprises an amino acid sequence that has at least about 95% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11.

5b. In embodiment 5b, the present invention relates to the recombinant binding protein according to embodiment 4b, wherein said second ankyrin repeat domain comprises an amino acid sequence that has at least about 95% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76 and 77.

6. In a sixth embodiment, the present invention relates to the recombinant binding protein according to embodiment 4, wherein said second ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85.

6a. In embodiment 6a, the present invention relates to the recombinant binding protein according to embodiment 4a, wherein said second ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11.

6b. In embodiment 6b, the present invention relates to the recombinant binding protein according to embodiment 4b, wherein said second ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11, 76 and 77.

7. In a seventh embodiment, the present invention relates to the recombinant binding protein according to any one of embodiments 4 to 6 further comprising a third ankyrin repeat domain, wherein said third ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85.

7a. In embodiment 7a, the present invention relates to the recombinant binding protein according to any one of embodiments 4a, 5a or 6a further comprising a third ankyrin repeat domain, wherein said third ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11.

7b. In embodiment 7b, the present invention relates to the recombinant binding protein according to any one of embodiments 4b, 5b or 6b further comprising a third ankyrin repeat domain, wherein said third ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76 and 77.

8. In an eighth embodiment, the present invention relates to the recombinant binding protein according to embodiment 7, wherein said third ankyrin repeat domain comprises an amino acid sequence that has at least about 95% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85.

8a. In embodiment 8a, the present invention relates to the recombinant binding protein according to embodiment 7a, wherein said third ankyrin repeat domain comprises an amino acid sequence that has at least about 95% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11.

8b. In embodiment 8b, the present invention relates to the recombinant binding protein according to embodiment 7b, wherein said third ankyrin repeat domain comprises an amino acid sequence that has at least about 95% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76 and 77.

9. In a ninth embodiment, the present invention relates to the recombinant binding protein according to embodiment 7, wherein said third ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85.

5 9a. In embodiment 9a, the present invention relates to the recombinant binding protein according to embodiment 7a, wherein said third ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11.

10 9b. In embodiment 9b, the present invention relates to the recombinant binding protein according to embodiment 7b, wherein said third ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11, 76 and 77.

15 10. In a tenth embodiment, the present invention relates to the recombinant binding protein according to embodiment 7, 7a or 7b, wherein said first, second and third ankyrin repeat domains comprise amino acid sequences and are arranged, from the N-terminus to C-terminus, as follows:

- (i) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 1 and 3;
- (ii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 4, 2 and 1;
- (iii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 4, 6 and 3;
- (iv) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 3 and 6;
- 20 (v) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 7, 3 and 6;
- (vi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 8, 4 and 1;
- (vii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 6 and 7;
- (viii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 4, 1 and 8;
- (ix) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 6 and 9;
- 25 (x) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 9, 3 and 6;
- (xi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 1, 6 and 9;
- (xii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 9, 6 and 1;
- (xiii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 9 and 10;
- (xiv) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 9 and 11;
- 30 (xv) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 10, 9 and 6;
- (xvi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 11, 9 and 3;
- (xvii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 5, 1 and 3;
- (xviii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 1, 2 and 5;
- (xix) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 5 and 6;
- 35 (xx) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 3 and 5;
- (xxi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 7, 3 and 5;
- (xxii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 8, 5 and 6;

- (xxiii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 10 and 11;
(xxiv) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 10 and 10;
(xxv) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 5, 6 and 9;
(xxvi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 9, 3 and 5;
5 (xxvii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 9, 6 and 5;
(xxviii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 5, 9 and 10;
(xxix) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 9 and 11;
(xxx) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 10, 9 and 5;
(xxxi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 11, 9 and 6;
10 (xxxii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 76 and 77;
or (xxxiii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 85 and 77.

10a. In embodiment 10a, the present invention relates to the recombinant binding protein according to
15 embodiment 10 (xx).

10b. In embodiment 10b, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 68.
20

10c. In embodiment 10c, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with SEQ ID NO: 68.

25 10d. In embodiment 10d, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 68.

10e. In embodiment 10e, the present invention relates to the recombinant binding protein according to embodiment 10 (xxviii).
30

10f. In embodiment 10f, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 69.

35 10g. In embodiment 10g, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with SEQ ID NO: 69.

- 10h. In embodiment 10h, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 69.
- 5 10i. In embodiment 10i, the present invention relates to the recombinant binding protein according to embodiment 10 (xxxii).
- 10j. In embodiment 10j, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence
10 identity with SEQ ID NO: 79.
- 10k. In embodiment 10k, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence
15 identity with SEQ ID NO: 79.
- 10l. In embodiment 10l, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 79.
- 10m. In embodiment 10m, the present invention relates to the recombinant binding protein according to
20 embodiment 10 (xxxiii).
- 10n. In embodiment 10n, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence
25 identity with a sequence selected from the group consisting of SEQ ID NOs: 89 to 91.
- 10o. In embodiment 10o, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence
identity with a sequence selected from the group consisting of SEQ ID NOs: 89 to 91.
- 30 10p. In embodiment 10p, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence selected from the group consisting of SEQ ID NOs: 89 to 91.
11. In an eleventh embodiment, the present invention relates to the recombinant binding protein
35 according to any one of embodiments 1 to 10p, wherein said binding protein binds to a coronavirus spike protein.

12. In a twelfth embodiment, the present invention relates to the recombinant binding protein according to embodiment 11, wherein said spike protein is SARS-CoV-2 spike protein.
13. In a thirteenth embodiment, the present invention relates to the recombinant binding protein according to any one of embodiments 11 and 12, wherein said first, second and/or third ankyrin repeat domain binds said coronavirus spike protein with a dissociation constant (K_D) of or below about 100 nM.
14. In a fourteenth embodiment, the present invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain, wherein said ankyrin repeat domain binds a coronavirus spike protein with a dissociation constant (K_D) of or below about 100 nM.
15. In a fifteenth embodiment, the present invention relates to the recombinant binding protein according to any preceding embodiment further comprising at least one serum albumin binding domain.
16. In a sixteenth embodiment, the present invention relates to the recombinant binding protein according to embodiment 15, wherein said serum albumin binding domain comprises an amino acid sequence that has at least about 90% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 47-49.
- 16a. In embodiment 16a, the present invention relates to a recombinant binding protein according to any one of embodiments 15 and 16, wherein said recombinant binding protein has a terminal half-life in mice of at least about 30 hours, preferably at least about 35 hours, at least about 40 hours, or at least about 45 hours.
17. In a seventeenth embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 12-42, 75, 84, 87 and 88.
- 17a. In embodiment 17a, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 31.
- 17b. In embodiment 17b, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 39.

- 17c. In embodiment 17c, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 75.
- 5 17d. In embodiment 17d, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 84.
- 10 17e. In embodiment 17e, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 87.
- 15 17f. In embodiment 17f, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 88.
- 20 17g. In embodiment 17g, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 12-42.
- 25 17h. In embodiment 17h, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 12-42 and 75.
- 30 18. In an eighteenth embodiment, the present invention relates to the recombinant binding protein according to embodiment 17, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 12-42, 75, 84, 87 and 88.
- 35 18a. In embodiment 18a, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with SEQ ID NO: 31.
- 18b. In embodiment 18b, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with SEQ ID NO: 39.

- 18c. In embodiment 18c, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with SEQ ID NO: 75.
- 5 18d. In embodiment 18d, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with SEQ ID NO: 84.
- 10 18e. In embodiment 18e, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with SEQ ID NO: 87.
- 15 18f. In embodiment 18f, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with SEQ ID NO: 88.
- 20 18g. In embodiment 18g, the present invention relates to the recombinant binding protein according to embodiment 17g, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 12-42.
- 25 18h. In embodiment 18h, the present invention relates to the recombinant binding protein according to embodiment 17h, wherein said polypeptide has an amino acid sequence that has at least about 95% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 12-42 and 75.
- 30 19. In a nineteenth embodiment, the present invention relates to the recombinant binding protein according to embodiment 17, wherein said polypeptide has an amino acid sequence that is selected from the group consisting of SEQ ID NOs: 12-42, 75, 84, 87 and 88.
- 35 19a. In embodiment 19a, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 31.
- 19b. In embodiment 19b, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 39.
- 19c. In embodiment 19c, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 75.

- 19d. In embodiment 19d, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 84.
- 5 19e. In embodiment 19e, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 87.
- 19f. In embodiment 19f, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 88.
- 10 19g. In embodiment 19g, the present invention relates to the recombinant binding protein according to embodiment 17g, wherein said polypeptide has an amino acid sequence that is selected from the group consisting of SEQ ID NOs: 12-42.
- 15 19h. In embodiment 19h, the present invention relates to the recombinant binding protein according to embodiment 17h, wherein said polypeptide has an amino acid sequence that is selected from the group consisting of SEQ ID NOs: 12-42 and 75.
- 20 20. In a twentieth embodiment, the present invention relates to the recombinant binding protein according to any one of embodiments 17 to 19h, wherein said binding protein binds to a coronavirus spike protein.
21. In a twenty-first embodiment, the present invention relates to the recombinant binding protein according to embodiment 20, wherein said spike protein is SARS-CoV-2 spike protein.
- 25 22. In a twenty-second embodiment, the present invention relates to the recombinant binding protein according to any one of embodiments 20 and 21, wherein said binding protein binds said coronavirus spike protein with a dissociation constant (K_D) of or below about 100 nM.
- 30 23. In a twenty-third embodiment, the present invention relates to the recombinant binding protein according to any one of embodiments 1 to 22, wherein said binding protein is capable of inhibiting infection of cells by a coronavirus.
- 35 24. In a twenty-fourth embodiment, the present invention relates to the recombinant binding protein according to any one of embodiments 1 to 22, wherein said binding protein is capable of inhibiting infection of cells by SARS-CoV-2.

25. In a twenty-fifth embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to any one of embodiments 1 to 24.

5 25a. In embodiment 25a, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 70 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 70.

10 25b. In embodiment 25b, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 71 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 71.

15 25c. In embodiment 25c, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 72 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 72.

20 25d. In embodiment 25d, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 73 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 73.

25 25e. In embodiment 25e, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 74 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 74.

30 25f. In embodiment 25f, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 80 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 80.

35 25g. In embodiment 25g, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 81 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 81.

25h. In embodiment 25h, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 82 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 82.

- 25i. In embodiment 25i, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 83 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 83.
- 5 25j. In embodiment 25j, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 78 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 78.
- 10 25k. In embodiment 25k, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 86 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 86.
- 15 25l. In embodiment 25l, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 92 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 92.
- 20 25m. In embodiment 25m, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 93 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 93.
- 25 25n. In embodiment 25n, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 94 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 94.
- 25 25o. In embodiment 25o, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 95 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 95.
- 30 26. In a twenty-sixth embodiment, the present invention relates to a host cell comprising the nucleic acid molecule of any one of embodiments 25 to 25o.
- 35 27. In a twenty-seventh embodiment, the present invention relates to a method of making the recombinant binding protein according to any one of embodiments 1 to 24, comprising culturing the host cell of embodiment 26 under conditions wherein said recombinant binding protein is expressed.

28. In a twenty-eighth embodiment, the present invention relates to a pharmaceutical composition comprising the binding protein of any one of embodiments 1 to 24 or the nucleic acid of any one of embodiments 25 to 25o, and a pharmaceutically acceptable carrier or excipient.

5 29. In a twenty-ninth embodiment, the present invention relates to a method of treating a coronavirus infection in a subject, the method comprising the step of administering an effective amount of at least one binding protein according to any one of embodiments 1 to 24, or of the nucleic acid of any one of
10 embodiments 25 to 25o, or of the pharmaceutical composition according to embodiment 28, to a subject in need thereof.

29a. In embodiment 29a, the present invention relates to a method of treating according to embodiment 29, wherein said method is a therapeutic treatment method.

15 29b. In embodiment 29b, the present invention relates to a method of treating according to embodiment 29, wherein said method is a prophylactic treatment method.

29c. In embodiment 29c, the present invention relates to a method of preventing a coronavirus infection in a subject, the method comprising the step of administering an effective amount of at least one binding protein according to any one of embodiments 1 to 24, or of the nucleic acid of any one of embodiments 25
20 to 25o, or of the pharmaceutical composition according to embodiment 28, to a subject in need thereof.

29d. In embodiment 29d, the present invention relates to at least one binding protein according to any one of embodiments 1 to 24, or the nucleic acid of any one of embodiments 25 to 25o, or the pharmaceutical composition according to embodiment 28 for use in a method of diagnosing a coronavirus infection in a
25 subject.

29e. In embodiment 29e, the present invention relates to a method of diagnosing a coronavirus infection in a subject comprising the steps of contacting a sample from the subject *in vitro* or *ex vivo* with at least one binding protein according to any one of embodiments 1 to 24.

30 29f. In embodiment 29f, the present invention relates to a method of detecting a coronavirus infection in a subject, said method comprising:
a) obtaining a sample from a subject;
b) contacting said sample with at least one binding protein according to any one of embodiments 1 to 24;
35 and
c) detecting the presence of a coronavirus infection.

29g. In embodiment 29g, the present invention relates to at least one binding protein according to any one of embodiments 1 to 24, or of the nucleic acid of any one of embodiments 25 to 25o, or of the pharmaceutical composition according to embodiment 28 for use in treating or preventing a coronavirus infection in a subject.

5

30. In a thirtieth embodiment, the present invention relates to the method according to any one of embodiments 29 to 29g, wherein the coronavirus infection is caused by SARS-CoV-2.

31. In a thirty-first embodiment, the present invention relates to the method according to any one of embodiments 29, 29a, 29b, 29c, 29e, 29f, 29g and 30, or the use according to embodiment 29d wherein said subject is a human.

10

Brief Description of the Figures

15

Figure 1: 2019-nCoV spike protein protomer showing the proposed binding sites for different ankyrin repeat proteins (DARPin® proteins).

Figure 2: 2019-nCoV spike protein protomer in down conformation.

20

Figure 3: 2019-nCoV spike protein protomer in up conformation, showing the hACE2 binding site elevated. hACE2 is thought to bind to the up conformation of the spike protein, but not to the down conformation.

25

Figure 4: 2019-nCoV spike protein protomer, indicating the location of the hACE2 binding site, S1/S2 cleavage site and S2' cleavage site. During molecular maturation, the spike protein trimerizes and is cleaved at the S1/S2 site. It is displayed at the membrane as a non-covalent complex. A concerted action of receptor-binding and proteolytic processing of the spike protein is required for membrane fusion. An initial energy barrier for conformational transition is necessary. Without wishing to be bound by theory, this energy barrier is overcome by (i) binding to the hACE2 receptor; and (ii) proteolytic priming at the S2' site. The interaction with ACE2 at the host cell surface is believed to trigger the cleavage of the S2' site. This cleavage has been proposed to activate the protein for membrane fusion via extensive irreversible conformational changes.

30

Figure 5: SARS-CoV-2 VSV pseudotype virus inhibition at 100 nM of various recombinant binding proteins comprising a single ankyrin repeat domain that binds to the spike protein (mono-domain and mono-paratopic DARPin® binding proteins). Shorter bars are indicative of stronger virus inhibition.

35

Figure 6: Representative SPR (surface plasmon resonance) trace of a recombinant binding protein comprising a single ankyrin repeat domain that binds to the spike protein (mono-domain and mono-paratopic DARPin® binding protein).

5 **Figure 7:** SARS-CoV-2 VSV pseudotype virus inhibition at 100 nM of various recombinant binding proteins comprising three ankyrin repeat domains that bind to the spike protein (multi-domain and multi-paratopic DARPin® binding proteins). Shorter bars are indicative of stronger virus inhibition.

10 **Figure 8:** SARS-CoV-2 VSV pseudotype virus inhibition at 1 nM of various recombinant binding proteins comprising three ankyrin repeat domains that bind to the spike protein (multi-domain and multi-paratopic DARPin® binding proteins). Shorter bars are indicative of stronger virus inhibition.

15 **Figures 9a-c:** SPR (surface plasmon resonance) trace of recombinant binding proteins comprising a single ankyrin repeat domain that binds to the spike protein. Four or five concentration SPR fitted curves confirm the high binding affinity of these mono-domain, mono-paratopic DARPin® binding proteins (e.g. in the double-digit pM range). In Figure 9c, the upper panel represents SEQ ID NO: 9 and the lower panel represents SEQ ID NO: 10.

20 **Figure 10:** Fluorescence microscopy image showing GFP positive Vero E06 cells which were infected with the GFP-labeled VSV pseudotype SARS-CoV-2 virus. DARPin® constructs ALE043 (SEQ ID NO: 25) and vS07_M101E04 do not show any infected cells in well 1 (at 100 nM concentration) and well 6 (at 3.125 nM) while there is infection of the Vero E06 cells with the GFP-labeled VSV pseudotype SARS-CoV-2 virus visible in wells 1 and 6 for the isotype negative control (his-tagged MP0250). At lower DARPin® protein concentrations in well 12 (0.049 nM) infected Vero E06 cells (GFP positive) are visible for all constructs.

25 **Figure 11:** Neutralization of VSV pseudotype SARS-CoV-2 virus by multi-domain DARPin® binding proteins. The names of the tested constructs (ALE030, ALE031, etc.) are indicated in the Figure.

30 **Figure 12:** Neutralization of VSV pseudotype SARS-CoV-2 virus by multi-domain DARPin® binding proteins. The names of the tested constructs (ALE030, ALE033, etc.) are indicated in the Figure.

Figure 13: A map of the test plates used in Example 4 with border zones around the edge and triplicate wells for each dilution value from 0.0064 to 100 nm, and control wells.

35 **Figures 14a-f:** Photographs of the test plates obtained from Example 4.

Figures 15a-b: Characterization of ALE033 (see Table 5, sample 3). Figure 15a is an SPR (surface plasmon resonance) trace showing high affinity binding to the coronavirus spike protein. No loss of target binding was observed over time. Figure 15b shows a size exclusion chromatography (SEC) profile (molar mass vs time). No aggregates or oligomers were observed. No unfolding was detectable up to 85°C on CD (circular dichroism) spectra (not provided).

Figure 16: SPR (surface plasmon resonance) trace for ALE030 (see Table 5, sample 1).

Figure 17: SPR (surface plasmon resonance) trace for ALE038 (see Table 5, sample 7).

Figures 18a-d: Photographs of the test plates obtained from Example 5.

Figure 19: Cell protection as measured with CellTiter-Glo® luminescent cell viability assay (Promega), see Example 7.

Figure 20: Photographs of the test plates obtained following violet crystal staining, see Example 8.

Figure 21a-c: Cell protection as measured with CellTiter-Glo® luminescent cell viability assay (Promega), see Example 8.

Figures 22a-b: (A) Molecular model of ALE049 (yellow: HSA-binding domains; cyan, blue and magenta: RBD-binding domains) bound to the spike ectodomain (gray) of SARS-CoV-2. (B) Molecular model of ALE058 (yellow: HSA-binding domains; blue: RBD-binding domain; green: S1-NTD-binding domain; red: S2-binding domain) bound to the spike ectodomain (grey) of SARS-CoV-2.

Figure 23: Neutralization of SARS-CoV-2 VSV pseudotype virus with multi-specific binding proteins ALE049 and ALE058, see Example 8.

Figure 24: ELISA method as used in Example 9.

Figure 25: Mean serum concentration data for ALE033, ALE048 and ALE049, see Example 9.

Figures 26a-e: Efficacy of ALE049 in treating SARS-CoV-2 infection in a preventative Syrian gold hamster model, see Example 10.

Figure 27: Representative histopathology microscopic pictures of hamster lung tissue taken at day 4. Left panel: healthy hamster lung tissue of an animal treated with 1600 µg ALE049 (group 1); right panel: diseased lung tissue of an animal which received the placebo injection (group 4).

5 **Figure 28:** Structural visualization of mutations of the SARS-CoV-2 spike protein evaluated in Examples 11 and 12. A) Representation of the full trimeric SARS-CoV-2 spike protein with all residues analyzed in the Pseudovirus neutralization assay visualized as blue spheres. Binding regions for the individual DARPin® domains incorporated in ALE049 and ALE109 are colored in blue (RBD), green (NTD) and red (S2); B) monomeric spike protein structure representing the variant first identified in the UK B.1.1.7 (del69-
10 70, del145, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H); C) monomeric spike protein structure representing the variant first identified in South Africa B.1.351 (D80A, D215G, E484K, N501Y, A701V). The PDB file 6xcn was used for generating the figures with PyMol version 2.1.1 (Schrödinger, LLC). In order to visualize all mutations, the loops 518-520, 676-689, 811-813 and the regions of the NTD domain missing in the cryo-EM structure, were modelled with MODELLER included in the BIOVIA Discovery Studio software
15 using the PDB file 6zge as template for the NTD domain (BIOVIA, Dassault Systèmes, BIOVIA Discovery Studio 2021).

Figure 29: (A) A visual representation of the ALE109 constructs generated for knock out experiments. For each knock out (k.o.) construct, the indicated SARS-CoV-2-binding DARPin® domain was replaced with a
20 non-binding DARPin® domain. HSA: HSA-binding DARPin® domain, RBD: RBD-binding DARPin® domain, NTD: NTD-binding DARPin® domain, S2: S2-binding DARPin® domain, see Example 11. (B) Neutralization profiles of ALE109 and k.o. constructs against VSV-SARS-CoV-2 pseudoviruses expressing the wild-type spike protein. (C) Upper panel: protective effect of DARPin® molecules against SARS-CoV-2 (100 pfu)-mediated cytopathic effect. Depicted are the percentage of cell protection conferred by ALE109
25 or the k.o. constructs. Cell protection was determined after 3 days of incubation by measuring intracellular ATP levels in a cell viability assay using Cell Titer-Glo. Lower panel: inhibition of SARS-CoV-2 viral replication quantified by real-time RT-PCR and expressed as percentage of viral genome equivalents present in the supernatant of Vero E6 cells exposed to 100 pfu SARS-CoV-2 with increasing amounts of ALE109 or k.o. constructs. (D) IC₅₀/EC₅₀ values and potency ranking of the constructs analyzed.
30

Figure 30: Schematic representation of the procedure of Example 12.

Figure 31: Tables showing the cytopathic effects observed in Example 12. The DARPin® binding protein R1b is called RBD-2 in this Figure.

35

Figure 32: Neutralization of VSV pseudotype SARS-CoV-2 virus by multi-domain DARPin® binding proteins. The names of the tested constructs (ALE049, ALE058, etc.) are indicated in the Figure.

Figure 33: Mean serum concentration-time profile of ALE058 in BALB/c mice following administration of 1 mg/kg.

5 **Figure 34:** Mean serum concentration-time profile of ALE109, ALE126, ALE129, and ALE133 in BALB/c mice following administration of 1 mg/kg.

Figure 35. Schematic study outline. Body weight and temperature were measured daily and swabs, blood and tissues were collected from 3 animals for each group, which were euthanized at day 3 and day 5,
10 respectively.

Figure 36. Average and SEM of body weight measurements of all five study groups over the time course from day 0 to day 5.

15 **Figure 37a to 37d:** Virus quantification by live virus titration of lung homogenate at day 3 (A) and at day 5 (B) and by qPCR measurement of genome copies in the lung at day 3 (C) and at day 5 (D), of three animals for each of the time points.

Figure 38a to 38d: Sum of the averaged histopathological scores grouped into four categories for signs of inflammation (A), affected blood vessels (B), alveoli (C) or bronchi (D).
20

Detailed Description of the Invention

Overview

25 Disclosed herein are recombinant binding proteins comprising one or more designed ankyrin repeat domains with binding specificity for coronavirus spike proteins, particularly SARS-CoV-2 spike proteins. Also disclosed are nucleic acids encoding the binding proteins, pharmaceutical compositions comprising the binding proteins or nucleic acids, and methods of using the binding proteins, nucleic acids, or pharmaceutical compositions.

30 The recombinant binding proteins according to the present invention bind to the coronavirus spike protein at one or more binding sites, thereby neutralizing the virus. These binding sites are illustrated in Figure 1. In one embodiment, the recombinant binding proteins bind to three sites on the spike protein.

35 Without wishing to be bound by theory, the designed ankyrin repeat proteins of the present invention are believed to act by (i) inhibiting receptor binding; (ii) providing allosteric inhibition of spike protein conformational change; and/or (iii) blocking protease sites needed for spike protein activation. As shown in

Figure 1, designed ankyrin repeat domain 1 (DARPin® 1) is understood to act by blocking angiotensin-converting enzyme 2 (ACE2) receptor binding. Designed ankyrin repeat domains 1 and 2 (DARPin® 1 and 2) are further understood to act by preventing conformational change in the spike protein, effectively locking the spike protein in the closed configuration. Designed ankyrin repeat domain 3 (DARPin® 3) is understood to further inhibit conformational change and to block protease binding. These designed ankyrin repeat domains can bind and/or inhibit the spike protein as individual proteins. Multi-epitope targeting by multi-domain, multi-specific proteins is believed to provide even more potent neutralization of the spike proteins, and to minimise the likelihood of escape mutations.

Further advantages to the described designed ankyrin repeat proteins are that they may reduce the incidence of Acute Lung Inflammation (ALI) due to lack of Fc-mediated macrophage or complement activation (as described by Liu et al., JCI Insight, 2019 4(4):e123158). Designed ankyrin repeat proteins may also address epitopes which are not accessible with monoclonal antibodies.

Further advantages to the described designed ankyrin repeat proteins are that they have low immunogenic potential and no off-target effects. DARPin® candidates also display favorable development properties including rapid, low-cost and high-yield manufacturing and up to several years of shelf-life at 4°C.

Definitions

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art.

The terms "comprising", "having", "including" and "containing" are to be construed as open-ended terms unless otherwise noted. If aspects of the invention are described as "comprising" a feature, embodiments also are contemplated "consisting of" or "consisting essentially of" the feature. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illustrate the disclosure and does not pose a limitation on the scope of the disclosure unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the disclosure. Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about" as that term would be interpreted by the person skilled in the relevant art. The term "about" as used herein is equivalent to $\pm 10\%$ of a given numerical value, unless otherwise stated.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range and each endpoint, unless otherwise indicated herein, and each separate value and endpoint is incorporated into the specification as if it were individually recited herein.

The term "nucleic acid" or "nucleic acid molecule" refers to a polynucleotide molecule, which may be a ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) molecule, either single stranded or double stranded, and includes modified and artificial forms of DNA or RNA. A nucleic acid molecule may either be present in isolated form or be comprised in recombinant nucleic acid molecules or vectors.

In the context of the present invention the term "protein" refers to a molecule comprising a polypeptide, wherein at least part of the polypeptide has, or is able to acquire, a defined three-dimensional arrangement by forming secondary, tertiary, and/or quaternary structures within a single polypeptide chain and/or between multiple polypeptide chains. If a protein comprises two or more polypeptide chains, the individual polypeptide chains may be linked non-covalently or covalently, e.g. by a disulfide bond between two polypeptides. A part of a protein, which individually has, or is able to acquire, a defined three-dimensional arrangement by forming secondary and/or tertiary structure, is termed "protein domain". Such protein domains are well known to the practitioner skilled in the art.

The term "recombinant" as used in recombinant protein, recombinant polypeptide and the like, means that said protein or polypeptide is produced by the use of recombinant DNA technologies well known to the practitioner skilled in the art. For example, a recombinant DNA molecule (e.g. produced by gene synthesis) encoding a polypeptide can be cloned into a bacterial expression plasmid (e.g. pQE30, QIAGEN), yeast expression plasmid, mammalian expression plasmid, or plant expression plasmid, or a DNA enabling in vitro expression. If, for example, such a recombinant bacterial expression plasmid is inserted into appropriate bacteria (e.g. *Escherichia coli*), these bacteria can produce the polypeptide(s) encoded by this recombinant DNA. The correspondingly produced polypeptide or protein is called a recombinant polypeptide or recombinant protein.

In the context of the present invention, the term "binding protein" refers to a protein comprising a binding domain. A binding protein may also comprise two, three, four, five or more binding domains. Preferably, said binding protein is a recombinant binding protein. More preferably, the binding proteins of the instant invention comprise an ankyrin repeat domain with binding specificity for a coronavirus spike protein.

The term "target" refers to an individual molecule such as a nucleic acid molecule, a peptide, polypeptide or protein, a carbohydrate, or any other naturally occurring molecule, including any part of such individual

molecule, or to complexes of two or more of such molecules, or to a whole cell or a tissue sample, or to any non-natural compound. Preferably, a target is a naturally occurring or non-natural polypeptide or protein, or a polypeptide or protein containing chemical modifications, for example, naturally occurring or non-natural phosphorylation, acetylation, or methylation.

5

In the context of the present invention, the term "polypeptide" relates to a molecule consisting of a chain of multiple, i.e. two or more, amino acids linked via peptide bonds. Preferably, a polypeptide consists of more than eight amino acids linked via peptide bonds. The term "polypeptide" also includes multiple chains of amino acids, linked together by S-S bridges of cysteines. Polypeptides are well-known to the person skilled in the art.

10

Patent application WO2002/020565 and Forrer et al., 2003 (Forrer, P., Stumpp, M.T., Binz, H.K., Plückthun, A., 2003. FEBS Letters 539, 2-6), contain a general description of repeat protein features and repeat domain features, techniques and applications. The term "repeat protein" refers to a protein comprising one or more repeat domains. Preferably, a repeat protein comprises one, two, three, four, five or six repeat domains. Furthermore, said repeat protein may comprise additional non-repeat protein domains, polypeptide tags and/or peptide linkers. The repeat domains can be binding domains.

15

The term "repeat domain" refers to a protein domain comprising two or more consecutive repeat modules as structural units, wherein said repeat modules have structural and sequence homology. Preferably, a repeat domain also comprises an N-terminal and/or a C-terminal capping module. For clarity, a capping module can be a repeat module. Such repeat domains, repeat modules, and capping modules, sequence motives, as well as structural homology and sequence homology are well known to the practitioner in the art from examples of ankyrin repeat domains (Binz et al., J. Mol. Biol. 332, 489-503, 2003; Binz et al., Nature Biotech. 22(5): 575-582 (2004); WO2002/020565; WO2012/069655), leucine-rich repeat domains (WO2002/020565), tetratricopeptide repeat domains (Main, E.R., Xiong, Y., Cocco, M.J., D'Andrea, L., Regan, L., Structure 11(5), 497-508, 2003), and armadillo repeat domains (WO2009/040338). It is further well known to the practitioner in the art, that such repeat domains are different from proteins comprising repeated amino acid sequences, where every repeated amino acid sequence is able to form an individual domain (for example FN3 domains of Fibronectin).

20

25

30

The term "ankyrin repeat domain" refers to a repeat domain comprising two or more consecutive ankyrin repeat modules as structural units, wherein said ankyrin repeat modules have structural and sequence homology.

35

The term "designed" as used in designed repeat protein, designed repeat domain and the like refers to the property that such repeat proteins and repeat domains, respectively, are man-made and do not occur in nature. The binding proteins of the instant invention are designed repeat proteins and they comprise at

least one designed repeat domain. Preferably, the designed repeat domain is a designed ankyrin repeat domain.

5 The term "target interaction residues" refers to amino acid residues of a repeat module, which contribute to the direct interaction with a target.

10 The terms "framework residues" or "framework positions" refer to amino acid residues of a repeat module, which contribute to the folding topology, i.e. which contribute to the fold of said repeat module or which contribute to the interaction with a neighboring module. Such contribution may be the interaction with other residues in the repeat module, or the influence on the polypeptide backbone conformation as found in α -helices or β -sheets, or the participation in amino acid stretches forming linear polypeptides or loops. Such framework and target interaction residues may be identified by analysis of the structural data obtained by physicochemical methods, such as X-ray crystallography, NMR and/or CD spectroscopy, or by comparison with known and related structural information well known to practitioners in structural biology and/or bioinformatics.

20 The term "repeat modules" refers to the repeated amino acid sequence and structural units of the designed repeat domains, which are originally derived from the repeat units of naturally occurring repeat proteins. Each repeat module comprised in a repeat domain is derived from one or more repeat units of a family or subfamily of naturally occurring repeat proteins, preferably the family of ankyrin repeat proteins. Furthermore, each repeat module comprised in a repeat domain may comprise a "repeat sequence motif" deduced from homologous repeat modules obtained from repeat domains selected on a target, e.g. as described in Example 1, and having the same target specificity.

25 Accordingly, the term "ankyrin repeat module" refers to a repeat module, which is originally derived from the repeat units of naturally occurring ankyrin repeat proteins. Ankyrin repeat proteins are well known to the person skilled in the art.

30 Repeat modules may comprise positions with amino acid residues which have not been randomized in a library for the purpose of selecting target-specific repeat domains ("non-randomized positions" or "fixed positions" used interchangeably herein) and positions with amino acid residues which have been randomized in the library for the purpose of selecting target-specific repeat domains ("randomized positions"). The non-randomized positions comprise framework residues. The randomized positions comprise target interaction residues. "Have been randomized" means that two or more amino acids were allowed at an amino acid position of a repeat module, for example, wherein any of the usual twenty naturally occurring amino acids were allowed, or wherein most of the twenty naturally occurring amino acids were allowed, such as amino acids other than cysteine, or amino acids other than glycine, cysteine and proline.

- The term "repeat sequence motif" refers to an amino acid sequence, which is deduced from one or more repeat modules. Preferably, said repeat modules are from repeat domains having binding specificity for the same target. Such repeat sequence motifs comprise framework residue positions and target interaction residue positions. Said framework residue positions correspond to the positions of framework residues of the repeat modules. Likewise, said target interaction residue positions correspond to the positions of target interaction residues of the repeat modules. Repeat sequence motifs comprise non-randomized positions and randomized positions.
- The term "repeat unit" refers to amino acid sequences comprising sequence motifs of one or more naturally occurring proteins, wherein said "repeat units" are found in multiple copies, and exhibit a defined folding topology common to all said motifs determining the fold of the protein. Examples of such repeat units include leucine-rich repeat units, ankyrin repeat units, armadillo repeat units, tetratricopeptide repeat units, HEAT repeat units, and leucine-rich variant repeat units.
- The term "ankyrin repeat domain" refers to a domain that comprises at least one ankyrin repeat motif, which is originally derived from the repeat units of naturally occurring ankyrin repeat proteins. In general, the ankyrin repeat motif comprises about 33 residues that form two alpha helices, separated by loops. Ankyrin repeat proteins are known in the art. See, for example, International Patent Publication Nos. WO 2002/020565, WO 2010/060748, WO 2011/135067, WO 2012/069654, WO 2012/069655, WO 2014/001442, WO 2014/191574, WO 2014/083208, WO 2016/156596, and WO 2018/054971, all of which are incorporated by reference in their entireties. Ankyrin repeat domains optionally further comprise appropriate capping modules.
- Ankyrin repeat domains may be modularly assembled into larger ankyrin repeat proteins according to the present disclosure, optionally with half-life extension domains, using standard recombinant DNA technologies (see, e.g., Forrer, P., et al., FEBS letters 539, 2-6, 2003, WO 2012/069655, WO 2002/020565).
- An ankyrin repeat domain "specifically binds" or "preferentially binds" (used interchangeably herein) to a target if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular target (e.g., cell or substance) than it does with alternative targets (e.g., cells or substances). For example, an ankyrin repeat domain that specifically binds to coronavirus spike protein is an ankyrin repeat domain that binds coronavirus spike protein with greater affinity, avidity, more readily, and/or with greater duration than it binds to other non-coronavirus spike proteins. It is also understood by reading this definition that, for example, an ankyrin repeat domain which specifically or preferentially binds to a first target may or may not specifically or preferentially bind to a second target. As such, "specific

binding" does not necessarily require (although it can include) exclusive binding. In general, under designated assay conditions, an ankyrin repeat domain binds preferentially to a particular target molecule and does not bind in a significant amount to other components present in a test sample.

- 5 A variety of assay formats may be used to select or characterize an ankyrin repeat domain that specifically binds a molecule of interest. For example, solid-phase ELISA immunoassay, immunoprecipitation, BIAcore™ (GE Healthcare, Piscataway, NJ), fluorescence-activated cell sorting (FACS), Octet™ (ForteBio, Inc., Menlo Park, CA) and Western blot analysis are among many assays that may be used to identify an ankyrin repeat domain that specifically reacts with a target. Typically, a specific or selective reaction will be
- 10 at least twice background signal or noise and more typically more than 10 times background. Even more specifically, an ankyrin repeat domain is said to "specifically bind" a target when the equilibrium dissociation constant (K_D) value is $< 1 \mu\text{M}$, such as $< 100 \text{ nM}$, $< 10 \text{ nM}$, $< 1 \text{ nM}$, $< 100 \text{ pM}$, $< 10 \text{ pM}$, or $< 1 \text{ pM}$.

- The K_D value is often referred to as binding affinity. Binding affinity measures the strength of the sum total
- 15 of non-covalent interactions between contact residue(s) of one binding partner and contact residue(s) of its binding partner. Unless indicated otherwise, as used herein, binding affinity refers to binding affinity that reflects a 1:1 interaction between members of a binding pair or binding partners. In case of a binding protein comprising two binding domains for one binding partner, binding affinity may refer to binding affinity that reflects a 1:2 interaction between the binding protein and the binding partner.

- 20 A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. For example, as exemplified herein, the binding affinity can be expressed as K_D value, which refers to the dissociation rate of a particular ankyrin repeat domain and its binding target. K_D is the ratio of the rate of dissociation, also called the "off-rate (K_{off})", to the association rate, or "on-rate (K_{on})". Thus, K_D equals $K_{\text{off}}/K_{\text{on}}$ and is expressed as a molar concentration (M), and the smaller the K_D , the
- 25 stronger the affinity of binding.

- K_D values can be determined using any suitable method. One exemplary method for measuring K_D is surface plasmon resonance (SPR) (see, e.g., Nguyen et al. *Sensors* (Basel). 2015 May 5; 15(5):10481-510). K_D value may be measured by SPR using a biosensor system such as a BIAcore® system. BIAcore kinetic analysis comprises analyzing the binding and dissociation of an antigen from chips with immobilized molecules (e.g., molecules comprising epitope binding domains), on their surface. Another method for determining the K_D of a protein is by using Bio-Layer Interferometry (see, e.g., Shah et al. *J Vis Exp*. 2014; (84): 51383). K_D value may be measured using OCTET® technology (Octet QKe system, ForteBio).
- 30 Alternatively, or in addition, a KinExA® (Kinetic Exclusion Assay) assay, available from Savidyne Instruments (Boise, Id.) can also be used. Any method suitable for assessing the binding affinity between
- 35

two binding partners is encompassed herein. Surface plasmon resonance (SPR) is particularly preferred. Most preferably, the K_D values are determined in PBS and by SPR.

5 The term "PBS" means a phosphate buffered water solution containing 137 mM NaCl, 10 mM phosphate and 2.7 mM KCl and having a pH of 7.4.

10 The term "treat," as well as words related thereto, does not necessarily imply 100% or complete cure. Rather, there are varying degrees of treatment of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In this respect, the methods of treating coronavirus infections described herein can provide any amount or any level of treatment. Furthermore, the treatment provided by the method of the present disclosure can include treatment of (i.e., relief from) one or more conditions or symptoms. In exemplary aspects, the methods treat by way increasing the survival of the subject. The term "treatment" also includes prophylactic (preventive) treatment.

15 Therapeutic responses in any given disease or condition can be determined by standardized response criteria specific to that disease or condition. The subject undergoing therapy may experience the beneficial effect of an improvement in the symptoms associated with the disease.

Recombinant Binding Proteins that Target Coronavirus Spike Proteins

20 Described herein are recombinant binding proteins comprising one, two, three or more designed ankyrin repeat domains with binding specificity for coronavirus spike proteins. In a preferred embodiment, such recombinant binding proteins comprising two, three or more designed ankyrin repeat domains with binding specificity for coronavirus spike proteins target two, three or more different epitopes on coronavirus spike proteins.

25 The described recombinant binding proteins, or binding domains thereof, comprising designed ankyrin repeat motifs or modules are also referred herein as DARPin® proteins. See Stumpp et al., Curr Opin Drug Discov Devel. 10(2): 153-9 (2007); and Binz et al., Nature Biotech. 22(5): 575-582 (2004). DARPin® proteins can be considered as antibody mimetics with high specificity and high binding affinity to a target protein. In general, a DARPin® protein comprises at least one ankyrin repeat domain, for example, at least 30 1, 2, 3, 4, 5, or more ankyrin repeat domains.

35 The ankyrin repeat domains described herein generally comprise a core scaffold that provides structure, and target binding residues that bind to a target. The structural core includes conserved amino acid residues, and the target binding surface includes amino acid residues that differ depending on the target.

International Patent Publication No. WO 2002/020565 and Binz et al., Nature Biotech. 22(5): 575-582 (2004) describe libraries of ankyrin repeat proteins that can be used for the selection/screening of a protein that binds specifically to a target. Methods of making such libraries are also provided.

5 Multiple ankyrin repeat domains can be linked (either through a covalent bond or non-covalent association) to form bispecific or multi-specific molecules. One such molecule is shown in FIG. 1, where three separate coronavirus spike protein binding domains are linked to form a multi-specific molecule. The linkers are illustrated by dashed lines joining the three binding domains.

10 Coronavirus Spike Protein

As set out above, the coronavirus spike protein is an attractive therapeutic target. Neutralizing the coronavirus spike protein can prevent infection of mammalian cells, stopping the coronavirus disease from taking hold in a subject. The recombinant binding proteins according to the present invention are specific for a mammalian coronavirus. Preferably, the designed ankyrin repeat proteins are specific for a coronavirus of mice, rat, dog, rabbit, monkey or human origin. More preferably, the designed ankyrin repeat proteins are specific for a coronavirus of human origin. The coronavirus SARS-CoV-2 is most preferred. As used herein, the term "SARS-CoV-2" includes both wild-type virus (such as SARS-CoV-2 found in infected humans at the beginning of the COVID-19 pandemic) and mutated forms or variants thereof. In one embodiment, the term "SARS-CoV-2" includes wild type and the specific variants B.1.1.7 (the so-called "UK variant") and B.1.351 (the so-called "South African variant").

The recombinant binding protein described herein comprises an ankyrin repeat domain that specifically binds to coronavirus spike protein. In one embodiment, the recombinant binding protein described herein comprises two, three or more ankyrin repeat domains that specifically bind to coronavirus spike protein. In one embodiment, the recombinant binding protein described herein comprises one, two, three or more ankyrin repeat domains that specifically bind to SARS-CoV-2 spike protein.

The target domains of interest in this disclosure on the coronavirus spike protein include, but are not limited to, the receptor binding domain (RBD domain); the S1 NTD domain; and the S2 domain. These domains are known in the art (see, e.g. Wrapp et al., Science 367, 1260–1263 (2020)).

Ankyrin repeat domains according to the present invention that bind coronavirus spike protein are provided in Table 1:

35

SEQ ID NO	DARPin® name	protein	Abbreviation	Spike Protein Target Domain
SEQ ID NO 1	vS07_19G10		R2a	RBD
SEQ ID NO 2	vS07_06F12		R1a	RBD
SEQ ID NO 3	vS07_12C06		R1b	RBD
SEQ ID NO 4	vS07_22E12		R3a	RBD
SEQ ID NO 5	vS07_23E04		R3c	RBD
SEQ ID NO 6	vS07_29B10		R3b	RBD
SEQ ID NO 7	vS07_07F02		RN1	RBD
SEQ ID NO 8	vS07_26C03		RN2	RBD
SEQ ID NO 9	vS07_08F10		S1a	S1-NTD
SEQ ID NO 10	vS07_14G03		S2a	S2
SEQ ID NO 11	vS07_18A05		S2b	S2
SEQ ID NO 76	vS07_08F10v27			S1-NTD
SEQ ID NO 77	vS07_14G03v19			S2
SEQ ID NO 85	vS07_08F10v47			S1-NTD

Table 1

Thus, in one embodiment, the present invention relates to a recombinant binding protein comprising a first ankyrin repeat domain, wherein said first ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85, as illustrated in Table 1 above.

In one embodiment, the present invention relates to a recombinant binding protein comprising a first ankyrin repeat domain, wherein said first ankyrin repeat domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85, as illustrated in Table 1 above. In one embodiment, the present invention relates to a recombinant binding protein comprising a first ankyrin repeat domain, wherein said first ankyrin repeat domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76 and

77, as illustrated in Table 1 above. In one embodiment, the present invention relates to a recombinant binding protein comprising a first ankyrin repeat domain, wherein said first ankyrin repeat domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%,
5 at least about 89%, at least about 90%, at least about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, as illustrated in Table 1 above.

In one embodiment, the present invention relates to a recombinant binding protein comprising a first ankyrin repeat domain, wherein said first ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85, as illustrated in Table 1 above.

The ankyrin repeat domains listed in Table 1 may be combined in any manner to provide a bi-specific or multi-specific molecule. The first, second and third ankyrin repeat domains may have identical sequences.
15 The first, second and third ankyrin repeat domains may have different sequences.

Thus, in one embodiment, the present invention relates to a recombinant binding protein further comprising a second ankyrin repeat domain, wherein said second ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85, as illustrated in Table 1 above.

In one embodiment, the present invention relates to a recombinant binding protein comprising a second ankyrin repeat domain, wherein said second ankyrin repeat domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%,
25 at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85, as illustrated in Table 1 above. In one embodiment, the present invention relates to a recombinant binding protein comprising a second ankyrin repeat domain,
30 wherein said second ankyrin repeat domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1
35 to 11, 76 and 77, as illustrated in Table 1 above. In one embodiment, the present invention relates to a recombinant binding protein comprising a second ankyrin repeat domain, wherein said second ankyrin repeat domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least

about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, as illustrated in Table 1 above.

In one embodiment, the present invention relates to a recombinant binding protein comprising a second ankyrin repeat domain, wherein said second ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85, as illustrated in Table 1 above.

In one embodiment, the present invention relates to a recombinant binding protein as defined above further comprising a third ankyrin repeat domain, wherein said third ankyrin repeat domain comprises an amino acid sequence that has at least about 90% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85, as illustrated in Table 1 above.

In one embodiment, the present invention relates a recombinant binding protein as defined above further comprising a third ankyrin repeat domain, wherein said third ankyrin repeat domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85, as illustrated in Table 1 above. In one embodiment, the present invention relates a recombinant binding protein as defined above further comprising a third ankyrin repeat domain, wherein said third ankyrin repeat domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, 76 and 77, as illustrated in Table 1 above. In one embodiment, the present invention relates a recombinant binding protein as defined above further comprising a third ankyrin repeat domain, wherein said third ankyrin repeat domain comprises an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs 1 to 11, as illustrated in Table 1 above.

In one embodiment, the present invention relates to a recombinant binding protein as defined above further comprising a third ankyrin repeat domain, wherein said third ankyrin repeat domain is selected from the group consisting of SEQ ID NOs 1 to 11, 76, 77 and 85, as illustrated in Table 1 above.

- 5 The present invention further relates to specific combinations of first, second and third ankyrin repeat domains having amino acid sequences and being arranged from the N-terminus to the C-terminus as follows:
- (i) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 1 and 3;
 - (ii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 4, 2 and 1;
 - 10 (iii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 4, 6 and 3;
 - (iv) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 3 and 6;
 - (v) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 7, 3 and 6;
 - (vi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 8, 4 and 1;
 - (vii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 6 and 7;
 - 15 (viii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 4, 1 and 8;
 - (ix) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 6 and 9;
 - (x) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 9, 3 and 6;
 - (xi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 1, 6 and 9;
 - (xii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 9, 6 and 1;
 - 20 (xiii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 9 and 10;
 - (xiv) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 9 and 11;
 - (xv) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 10, 9 and 6;
 - (xvi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 11, 9 and 3;
 - (xvii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 5, 1 and 3;
 - 25 (xviii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 1, 2 and 5;
 - (xix) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 5 and 6;
 - (xx) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 3 and 5;
 - (xxi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 7, 3 and 5;
 - (xxii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 8, 5 and 6;
 - 30 (xxiii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 10 and 11;
 - (xxiv) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 10 and 10;
 - (xxv) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 5, 6 and 9;
 - (xxvi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 9, 3 and 5;
 - (xxvii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 9, 6 and 5;
 - 35 (xxviii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 5, 9 and 10;
 - (xxix) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 6, 9 and 11;
 - (xxx) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 10, 9 and 5;

(xxxi) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 11, 9 and 6;
(xxxii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 76 and 77;
or (xxxiii) amino acid sequences having at least about 90% sequence identity with SEQ ID NOs 3, 85 and 77.

5

In one embodiment, the present invention relates to the recombinant binding protein according to embodiment (xx) as listed above. In a further embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%,
10 about 98% or about 99% sequence identity with SEQ ID NO: 68. In another embodiment, the recombinant binding protein comprises a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 68.

In one embodiment, the present invention relates to the recombinant binding protein according to
15 embodiment (xxviii), as listed above. In a further embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with SEQ ID NO: 69. In another embodiment, the recombinant binding protein comprises a polypeptide, wherein said polypeptide has the amino acid
20 sequence of SEQ ID NO: 69.

In one embodiment, the present invention relates to the recombinant binding protein according to (xxxii), as listed above. In a further embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about
25 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with SEQ ID NO: 79. In another embodiment, the recombinant binding protein comprises a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 79.

In one embodiment, the present invention relates to the recombinant binding protein according to (xxxiii), as listed above. In a further embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about
30 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 89 to 91. In another embodiment, the recombinant binding protein comprises a polypeptide, wherein said polypeptide
35 has an amino acid sequence selected from the group consisting of SEQ ID NOs: 89 to 91.

In another embodiment, the recombinant binding protein of the present invention binds to a coronavirus spike protein. In another embodiment, the spike protein is SARS-CoV-2 spike protein.

5 In another embodiment, the recombinant binding protein of the invention comprising at least one ankyrin repeat domain binds to a coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM. In another embodiment, the spike protein is SARS-CoV-2 spike protein.

10 In another embodiment, the recombinant binding protein of the invention comprises first, second and/or third ankyrin repeat domains and said first, second and/or third ankyrin repeat domains bind to a coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM. In another embodiment, the spike protein is SARS-CoV-2 spike protein.

15 In exemplary embodiments, the recombinant binding protein of the invention binds coronavirus spike protein, preferably SARS-CoV-2 spike protein, with an K_D value of, or less than: about 100 nM; about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, about 5 nM, about 2 nM, about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 250 pM, about 200 pM, about 150 pM, about 100 pM, about 50 pM, about 40 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, about 10 pM, about 5 pM, or about 1 pM. In one exemplary embodiment, the recombinant binding protein binds coronavirus spike protein, preferably SARS-CoV-2 spike protein, with a K_D value of less than or equal to about 10 nM. In another exemplary embodiment, the recombinant binding protein binds coronavirus spike protein, preferably SARS-CoV-2 spike protein, with a K_D value of less than or equal to about 1 nM.

25 In certain embodiments, the coronavirus spike protein is human coronavirus spike protein. In certain embodiments, the coronavirus spike protein is human SARS-CoV-2 spike protein.

In certain embodiments, the recombinant binding protein may further comprise at least one human serum albumin binding domain. In embodiments, the at least one human serum albumin domain may be located at the N-terminus, the C-terminus, or both.

30 In certain embodiments, the serum albumin binding domain comprises an amino acid sequence that has at least 90% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 47-49. In one embodiment, the serum albumin binding domain comprises an amino acid sequence that has at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity with SEQ ID NO: 47.

In further embodiments, the recombinant binding protein of the invention has a terminal half-life in mice of at least about 30 hours, preferably at least about 35 hours, more preferably at least about 40 hours, and more preferably at least about 45 hours. Said terminal half-life is preferably determined in Balb/c mice, as described in Example 9.

5

Particularly preferred combinations of ankyrin repeat domains are listed in Table 2, wherein H denotes human serum albumin and R3b, R2a etc are as defined in Table 1 above:

#		5 Domain DARPin® Designs				
		1	2	3	4	5
1	SEQ ID NO: 12	H	H	R3b	R2a	R1b
2	SEQ ID NO: 13	H	H	R3a	R1a	R2a
3	SEQ ID NO: 14	H	H	R3a	R3b	R1b
4	SEQ ID NO: 15	H	H	R3b	R1b	R3b
5	SEQ ID NO: 16	H	H	RN1	R1b	R3b
6	SEQ ID NO: 17	H	H	RN2	R3a	R2a
7	SEQ ID NO: 18	H	H	R1b	R3b	RN1
8	SEQ ID NO: 19	H	H	R3a	R2a	RN2
9	SEQ ID NO: 20	H	H	R1b	R3b	S1a
10	SEQ ID NO: 21	H	H	S1a	R1b	R3b
11	SEQ ID NO: 22	H	H	R2a	R3b	S1a
12	SEQ ID NO: 23	H	H	S1a	R3b	R2a
13	SEQ ID NO: 24	H	H	R3b	S1a	S2a
14	SEQ ID NO: 25	H	H	R1b	S1a	S2b
15	SEQ ID NO: 26	H	H	S2a	S1a	R3b
16	SEQ ID NO: 27	H	H	S2b	S1a	R1b
17	SEQ ID NO: 28	H	H	R3c	R2a	R1b
18	SEQ ID NO: 29	H	H	R2a	R1a	R3c
19	SEQ ID NO: 30	H	H	R1b	R3c	R3b
20	SEQ ID NO: 31	H	H	R3b	R1b	R3c
21	SEQ ID NO: 32	H	H	RN1	R1b	R3c
22	SEQ ID NO: 33	H	H	RN2	R3c	R3b
23	SEQ ID NO: 34	H	H	R3b	S2a	S2b
24	SEQ ID NO: 35	H	H	R1b	S2a	S2a
25	SEQ ID NO: 36	H	H	R3c	R3b	S1a
26	SEQ ID NO: 37	H	H	S1a	R1b	R3c

27	SEQ ID NO: 38	H	H	S1a	R3b	R3c
28	SEQ ID NO: 39	H	H	R3c	S1a	S2a
29	SEQ ID NO: 40	H	H	R3b	S1a	S2b
30	SEQ ID NO: 41	H	H	S2a	S1a	R3c
31	SEQ ID NO: 42	H	H	S2b	S1a	R3b
32	SEQ ID NO: 75	H	H	R1b	SEQ ID NO 76	SEQ ID NO 77
33	SEQ ID NOs: 84, 87 and 88	H	H	R1b	SEQ ID NO 85	SEQ ID NO 77

Table 2

In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 12-42, 75, 84, 87 and 88. In one embodiment, said binding protein binds to a coronavirus spike protein. In one embodiment, said spike protein is SARS-CoV-2 spike protein. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM.

In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 12-42, 75, 84, 87 and 88. In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 12-42 and 75. In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with a sequence selected from the group consisting of SEQ ID NOs: 12-42. In one embodiment, said binding protein binds to a coronavirus

spike protein. In one embodiment, said spike protein is SARS-CoV-2 spike protein. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM.

5 In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that is selected from the group consisting of SEQ ID NOs: 12-42, 75, 84, 87 and 88. In one embodiment, said binding protein binds to a coronavirus spike protein. In one embodiment, said spike protein is SARS-CoV-2 spike protein. In one
10 embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM.

In another embodiment, the present invention relates to the recombinant binding protein as described herein, wherein said binding protein is capable of inhibiting infection of cells by a coronavirus. In another embodiment, the present invention relates to the recombinant binding protein as described herein, wherein
15 said binding protein is capable of inhibiting infection of cells by SARS-CoV-2.

In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about
20 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with SEQ ID NO: 31. In another embodiment, the recombinant binding protein comprises a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 31. In one embodiment, said binding protein binds to a coronavirus
25 spike protein. In one embodiment, said spike protein is SARS-CoV-2 spike protein. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM, of or below about 10 nM, of or below about 1 nM, of or below about 100 pM, of or below about 10 pM, or of or below about 1 pM. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 1 nM. In one embodiment, said binding protein has a terminal
30 half-life in mice of at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, or at least about 45 hours. In one embodiment, said binding protein has a terminal half-life in mice of at least about 40 hours. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 50°C, above 60°C, above 70°C, or above 80°C. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 60°C. In one embodiment, said binding
35 protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.5 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below

1 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC_{50} value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.1 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC_{50} value of or below 1 nM. In one embodiment, said binding protein has a combination of two, three, four, five or six properties selected from the properties listed in this paragraph relating to amino acid sequence, binding affinity, terminal half-life, thermal stability, IC_{50} of SARS-CoV-2 VSV pseudovirus inhibition and IC_{50} of SARS-CoV-2 inhibition. In one exemplary embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 31, and wherein said binding protein binds to SARS-CoV-2 spike protein with a binding affinity (K_D) of or below about 1 nM, wherein said binding protein has a terminal half-life in mice of at least about 40 hours, wherein said binding protein exhibits a high thermal stability with a T_m above 60°C, wherein said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below 1 nM, and/or wherein said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC_{50} value of or below 1 nM.

In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with SEQ ID NO: 39. In another embodiment, the recombinant binding protein comprises a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 39. In one embodiment, said binding protein binds to a coronavirus spike protein. In one embodiment, said spike protein is SARS-CoV-2 spike protein. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM, of or below about 10 nM, of or below about 1 nM, of or below about 100 pM, of or below about 10 pM, or of or below about 1 pM. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 1 nM. In one embodiment, said binding protein has a terminal half-life in mice of at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, or at least about 45 hours. In one embodiment, said binding protein has a terminal half-life in mice of at least about 20 hours. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 50°C, above 60°C, above 70°C, or above 80°C. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 60°C. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.5 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below 1 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an

- IC₅₀ value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.4 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC₅₀ value of or below 1 nM. In one embodiment, said binding protein has a combination of two, three, four, five or six properties selected from the properties listed in this paragraph relating to amino acid sequence, binding affinity, terminal half-life, thermal stability, IC₅₀ of SARS-CoV-2 VSV pseudovirus inhibition and IC₅₀ of SARS-CoV-2 inhibition. In one exemplary embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 39, and wherein said binding protein binds to SARS-CoV-2 spike protein with a binding affinity (K_D) of or below about 1 nM, wherein said binding protein has a terminal half-life in mice of at least about 20 hours, wherein said binding protein exhibits a high thermal stability with a T_m above 60°C, wherein said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC₅₀ value of or below 1 nM, and/or wherein said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC₅₀ value of or below 1 nM.
- In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with SEQ ID NO: 75. In another embodiment, the recombinant binding protein comprises a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 75. In one embodiment, said binding protein binds to a coronavirus spike protein. In one embodiment, said spike protein is SARS-CoV-2 spike protein. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM, of or below about 10 nM, of or below about 1 nM, of or below about 100 pM, of or below about 10 pM, or of or below about 1 pM. In one embodiment, said binding protein has a terminal half-life in mice of at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, or at least about 45 hours. In one embodiment, said binding protein has a terminal half-life in mice of at least about 30 hours. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 1 nM. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 50°C, above 60°C, above 70°C, or above 80°C. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 60°C. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC₅₀ value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.5 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC₅₀ value of or below 1 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC₅₀ value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.4 nM. In one

embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC_{50} value of or below 1 nM. In one embodiment, said binding protein has a combination of two, three, four, five or six properties selected from the properties listed in this paragraph relating to amino acid sequence, binding affinity, terminal half-life, thermal stability, IC_{50} of SARS-CoV-2 VSV pseudovirus inhibition and IC_{50} of SARS-CoV-2 inhibition. In one exemplary embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 75, and wherein said binding protein binds to SARS-CoV-2 spike protein with a binding affinity (K_D) of or below about 1 nM, wherein said binding protein has a terminal half-life in mice of at least about 30 hours, wherein said binding protein exhibits a high thermal stability with a T_m above 60°C, wherein said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below 1 nM, and/or wherein said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC_{50} value of or below 1 nM.

In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with SEQ ID NO: 84. In another embodiment, the recombinant binding protein comprises a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 84. In one embodiment, said binding protein binds to a coronavirus spike protein. In one embodiment, said spike protein is SARS-CoV-2 spike protein. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM, of or below about 10 nM, of or below about 1 nM, of or below about 100 pM, of or below about 10 pM, or of or below about 1 pM. In one embodiment, said binding protein has a terminal half-life in mice of at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, or at least about 45 hours. In one embodiment, said binding protein has a terminal half-life in mice of at least about 40 hours. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 1 nM. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 50°C, above 60°C, above 70°C, or above 80°C. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 60°C. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.5 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below 1 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC_{50} value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.4 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC_{50} value of

or below 1 nM. In one embodiment, said binding protein has a combination of two, three, four, five or six properties selected from the properties listed in this paragraph relating to amino acid sequence, binding affinity, terminal half-life, thermal stability, IC₅₀ of SARS-CoV-2 VSV pseudovirus inhibition and IC₅₀ of SARS-CoV-2 inhibition. In one exemplary embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 84, and wherein said binding protein binds to SARS-CoV-2 spike protein with a binding affinity (K_D) of or below about 1 nM, wherein said binding protein has a terminal half-life in mice of at least about 40 hours, wherein said binding protein exhibits a high thermal stability with a T_m above 60°C, wherein said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC₅₀ value of or below 1 nM, and/or wherein said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC₅₀ value of or below 1 nM.

In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with SEQ ID NO: 87. In another embodiment, the recombinant binding protein comprises a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 87. In one embodiment, said binding protein binds to a coronavirus spike protein. In one embodiment, said spike protein is SARS-CoV-2 spike protein. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM, of or below about 10 nM, of or below about 1 nM, of or below about 100 pM, of or below about 10 pM, or of or below about 1 pM. In one embodiment, said binding protein has a terminal half-life in mice of at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, or at least about 45 hours. In one embodiment, said binding protein has a terminal half-life in mice of at least about 35 hours. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 1 nM. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 50°C, above 60°C, above 70°C, or above 80°C. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 60°C. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC₅₀ value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.5 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC₅₀ value of or below 1 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC₅₀ value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.4 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC₅₀ value of or below 1 nM. In one embodiment, said binding protein has a combination of two, three, four, five or six

properties selected from the properties listed in this paragraph relating to amino acid sequence, binding affinity, terminal half-life, thermal stability, IC_{50} of SARS-CoV-2 VSV pseudovirus inhibition and IC_{50} of SARS-CoV-2 inhibition. In one exemplary embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has
5 at least about 90% sequence identity with SEQ ID NO: 87, and wherein said binding protein binds to SARS-CoV-2 spike protein with a binding affinity (K_D) of or below about 1 nM, wherein said binding protein has a terminal half-life in mice of at least about 35 hours, wherein said binding protein exhibits a high thermal stability with a T_m above 60°C, wherein said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below 1 nM, and/or wherein said binding protein inhibits
10 viral entry of SARS-CoV-2 in VeroE6 cells with an IC_{50} value of or below 1 nM.

In another embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about
15 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity with SEQ ID NO: 88. In another embodiment, the recombinant binding protein comprises a polypeptide, wherein said polypeptide has the amino acid sequence of SEQ ID NO: 88. In one embodiment, said binding protein binds to a coronavirus
20 spike protein. In one embodiment, said spike protein is SARS-CoV-2 spike protein. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 100 nM, of or below about 10 nM, of or below about 1 nM, of or below about 100 pM, of or below about 10 pM, or of or below about 1 pM. In one embodiment, said binding protein has a terminal half-life in mice of at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least
25 about 40 hours, or at least about 45 hours. In one embodiment, said binding protein has a terminal half-life in mice of at least about 40 hours. In one embodiment, said binding protein binds said coronavirus spike protein with a binding affinity (K_D) of or below about 1 nM. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 50°C, above 60°C, above 70°C, or above 80°C. In one embodiment, said binding protein exhibits a high thermal stability with a T_m above 60°C. In one embodiment, said binding
30 protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.5 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC_{50} value of or below 1 nM. In one embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC_{50} value of or below 100 nM, of or below 10 nM, of or below 1 nM, or of or below 0.4 nM. In one
35 embodiment, said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC_{50} value of or below 1 nM. In one embodiment, said binding protein has a combination of two, three, four, five or six properties selected from the properties listed in this paragraph relating to amino acid sequence, binding

affinity, terminal half-life, thermal stability, IC₅₀ of SARS-CoV-2 VSV pseudovirus inhibition and IC₅₀ of SARS-CoV-2 inhibition. In one exemplary embodiment, the present invention relates to a recombinant binding protein comprising a polypeptide, wherein said polypeptide has an amino acid sequence that has at least about 90% sequence identity with SEQ ID NO: 88, and wherein said binding protein binds to SARS-CoV-2 spike protein with a binding affinity (K_D) of or below about 1 nM, wherein said binding protein has a terminal half-life in mice of at least about 40 hours, wherein said binding protein exhibits a high thermal stability with a T_m above 60°C, wherein said binding protein inhibits viral entry of SARS-CoV-2 VSV pseudovirus in VeroE6 cells with an IC₅₀ value of or below 1 nM, and/or wherein said binding protein inhibits viral entry of SARS-CoV-2 in VeroE6 cells with an IC₅₀ value of or below 1 nM.

Half-Life Extending Moieties

The “half-life extending moiety” extends the serum half-life *in vivo* of the recombinant binding proteins described herein, compared to the same protein without the half-life extending moiety. Examples of half-life extending moieties include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, an immunoglobulin domain, maltose binding protein (MBP), human serum albumin (HSA) binding domain, or polyethylene glycol (PEG). In some embodiments, the half-life extending moieties are glutathione S transferase (GST), protein A, protein G, an immunoglobulin domain, human serum albumin (HSA) binding domain, or polyethylene glycol (PEG).

In some embodiments, the recombinant binding protein described herein comprises an ankyrin repeat domain that specifically binds serum albumin (such as preferably human serum albumin), also referred herein as “serum albumin binding domain”. The recombinant binding protein described herein may also comprise more than one serum albumin binding domain, for example, two or three or more serum albumin binding domains. Thus, the recombinant binding protein described herein may comprise a first and a second serum albumin binding domain, or a first, a second and a third serum albumin binding domain. The embodiments provided below describe such a first serum albumin binding domain, second serum albumin binding domain, and/or third serum albumin binding domain.

In some embodiments, the half-life extending moiety described herein comprises a serum albumin binding domain comprising an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 47 to 49. In an exemplary embodiment, the half-life extending moiety described herein comprises an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 47 to 49. In some embodiments, the half-life extending moiety described herein comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at

least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 47. In an exemplary embodiment, the half-life extending moiety described herein comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 47.

- 5 In some embodiments, two or more serum albumin binding domains are preferred. In some embodiments, two serum albumin binding domains are located at the N-terminus. In exemplary embodiments, the recombinant binding protein comprises, from the N-terminus to C-terminus: (i) an ankyrin repeat domain that specifically binds serum albumin; (ii) an ankyrin repeat domain that specifically binds serum albumin; and (iii) one or more ankyrin repeat domains that specifically bind coronavirus spike protein. In certain
- 10 embodiments, the N-terminal serum albumin binding domain (also referred to herein as serum albumin binding domain 1) comprises an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 47. In certain embodiments, the second serum albumin binding domain (also referred to herein as serum albumin binding domain 2) comprises an amino acid sequence that is at least
- 15 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 47.

- In some embodiments, the half-life extending moiety comprises an immunoglobulin domain. In some embodiments, the immunoglobulin domain comprises an Fc domain. In some embodiments, the Fc domain
- 20 is derived from any one of the known heavy chain isotypes: IgG (γ), IgM (μ), IgD (δ), IgE (ϵ), or IgA (α). In some embodiments, the Fc domain is derived from any one of the known heavy chain isotypes or subtypes: IgG₁ (γ 1), IgG₂ (γ 2), IgG₃ (γ 3), IgG₄ (γ 4), IgA₁ (α 1), IgA₂ (α 2). In some embodiments, the Fc domain is the Fc domain of human IgG₁.

- 25 In some embodiments, the Fc domain comprises an uninterrupted native sequence (i.e., wild type sequence) of an Fc domain. In some embodiments, the immunoglobulin Fc domain comprises a variant Fc domain resulting in altered biological activity. For example, at least one point mutation or deletion may be introduced into the Fc domain so as to reduce or eliminate the effector activity (e.g., International Patent Publication No. WO 2005/063815), and/or to increase the homogeneity during the production of the recombinant binding protein. In some embodiments, the Fc domain is the Fc domain of human IgG₁ and
- 30 comprises one or more of the following effector-null substitutions: L234A, L235A, and G237A (Eu numbering). In some embodiments, the Fc domain does not comprise the lysine located at the C-terminal position of human IgG₁ (i.e., K447 by Eu numbering). The absence of the lysine may increase homogeneity during the production of the recombinant binding protein. In some embodiments, the Fc domain comprises
- 35 the lysine located at the C-terminal position (K447, Eu numbering).

Ankyrin repeat domains

In some embodiments, no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 substitution is made in any ankyrin repeat domain of a recombinant binding protein of the invention relative to the sequences of SEQ ID NOs: 1 to 11, 47, 76, 77 and 85. In some embodiments, no more than 5 substitutions are made relative to the sequences of SEQ ID NOs: 1 to 11, 47, 76, 77 and 85. In some embodiments, no more than 4 substitutions are made relative to the sequences of SEQ ID NOs: 1 to 11, 47, 76, 77 and 85. In some embodiments, no more than 3 substitutions are made relative to the sequences of SEQ ID NOs: 1 to 11, 47, 76, 77 and 85. In some embodiments, no more than 2 substitutions are made relative to the sequences of SEQ ID NOs: 1 to 11, 47, 76, 77 and 85. In some embodiments, no more than 1 substitution is made relative to the sequences of SEQ ID NOs: 1 to 11, 47, 76, 77 and 85. In some embodiments, the substitution(s) do not change the K_D value by more than 1000-fold, more than 100-fold, or more than 10-fold, compared to the K_D value of the protein comprising the sequences of SEQ ID NOs: 1 to 11, 47, 76, 77 and 85. In certain embodiments, the substitution is a conservative substitution according to Table 3. In certain embodiments, the substitution is made outside the structural core residues of the ankyrin repeat domain, e.g. in the beta loops that connect the alpha-helices.

<i>Original Residue</i>	<i>Conservative Substitutions</i>	<i>Exemplary Substitutions</i>
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Asp, Lys; Arg
Asp (D)	Glu	Glu; Asn
Cys (C)	Ser	Ser; Ala
Gln (Q)	Asn	Asn; Glu
Glu (E)	Asp	Asp; Gln
Gly (G)	Ala	Ala
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; Norleucine
Leu (L)	Ile	Norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Tyr	Leu; Val; Ile; Ala; Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr; Phe

Tyr(Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; Norleucine

Table 3. Amino Acid Substitutions

In certain embodiments, the substitution is made within the structural core residues of the ankyrin repeat domain. For example, the ankyrin domain may comprise the consensus sequence:
5 DxxGxTPLHLAxxxGxxxIVxVLLxxGADVNAx (SEQ ID NO: 50), wherein "x" denotes any amino acid (preferably not cysteine, glycine, or proline); or DxxGxTPLHLAxxGHLEIVEVLLKzGADVNAx (SEQ ID NO: 51), wherein "x" denotes any amino acid (preferably not cysteine, glycine, or proline), and "z" is selected from the group consisting of asparagine, histidine, or tyrosine. In one embodiment, the substitution is made to residues designated as "x". In another embodiment, the substitution is made outside the residues
10 designated as "x".

In addition, the second last position of any ankyrin repeat domain of a recombinant binding protein of the invention can be "A" or "L", and/or the last position can be "A" or "N". Accordingly, in some embodiments, each ankyrin repeat domain comprises an amino acid sequence that is at least 80%, at least 81%, at least
15 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 1 to 11, 47, 76, 77 and 85, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. In an exemplary embodiment, each spike protein binding domain comprises an amino
20 acid sequence that is at least 90% identical to any one of SEQ ID NOs: 1 to 11, 47, 76, 77 and 85, and wherein optionally A at the second last position is substituted with L and/or A at the last position is substituted with N. Furthermore, the sequence of any ankyrin repeat domain comprised in a binding protein of the invention may optionally comprise at its N-terminus, a G, an S, or a GS (see below).

25 In addition, each ankyrin repeat domain comprised in a recombinant binding protein of the invention may optionally comprise a "G," an "S," or a "GS" sequence at its N-terminus. Accordingly, in some embodiments, each ankyrin repeat domain comprises an amino acid sequence that is at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%,
30 at least 98%, at least 99%, or 100% identical to any one of SEQ ID NOs: 1 to 11, 47, 76, 77 and 85, and further comprises at its N-terminus a GS (as e.g. in SEQ ID NOs: 1 to 11, 47, 76, 77 and 85) or only a G or an S instead of the GS.

35 In certain embodiments, the affinity between the recombinant binding protein and its target (spike protein or serum albumin) is described in terms of K_D . In exemplary embodiments, the K_D is about 10^{-1} M or less,

about 10^{-2} M or less, about 10^{-3} M or less, about 10^{-4} M or less, about 10^{-5} M or less, about 10^{-6} M or less, about 10^{-7} M or less, about 10^{-8} M or less, about 10^{-9} M or less, about 10^{-10} M or less, about 10^{-11} M or less, about 10^{-12} M or less, about 10^{-13} M or less, about 10^{-14} M or less, from about 10^{-5} M to about 10^{-15} M, from about 10^{-6} M to about 10^{-15} M, from about 10^{-7} M to about 10^{-15} M, from about 10^{-8} M to about 10^{-15} M, from about 10^{-9} M to about 10^{-15} M, from about 10^{-10} M to about 10^{-15} M, from about 10^{-5} M to about 10^{-14} M, from about 10^{-6} M to about 10^{-14} M, from about 10^{-7} M to about 10^{-14} M, from about 10^{-8} M to about 10^{-14} M, from about 10^{-9} M to about 10^{-14} M, from about 10^{-10} M to about 10^{-14} M, from about 10^{-5} M to about 10^{-13} M, from about 10^{-6} M to about 10^{-13} M, from about 10^{-7} M to about 10^{-13} M, from about 10^{-8} M to about 10^{-13} M, from about 10^{-9} M to about 10^{-13} M, or from about 10^{-10} M to about 10^{-13} M.

In exemplary embodiments, the recombinant binding protein binds spike protein or serum albumin with an K_D value of, or less than: about 900 nM, about 800 nM, about 700 nM, about 600 nM, about 500 nM, about 400 nM, about 300 nM, about 250 nM, about 200 nM, about 150 nM, about 100 nM, about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, about 5 nM, about 2 nM, about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 200 pM, about 100 pM, about 10 pM, or about 1 pM. In one exemplary embodiment, the recombinant binding protein binds spike protein or serum albumin with a K_D value of less than or equal to 100 nM. In another exemplary embodiment, the recombinant binding protein binds spike protein or serum albumin with a K_D value of less than or equal to 10 nM.

Linkers

The recombinant binding proteins described herein may comprise a linker. A “linker” is a molecule or group of molecules that binds two separate entities (for example DARPin® 1 and DARPin 2® as shown in Figure 1) to one another and can provide spacing and flexibility between the two entities such that they are able to achieve a conformation in which they can bind their respective targets. Protein linkers are particularly preferred, and they may be expressed as a component of the recombinant binding protein using standard recombinant DNA techniques well-known in the art.

The ankyrin repeat domains can be linked either covalently, for example, by a disulfide bond, a polypeptide bond or a crosslinking agent; or non-covalently, to produce a heterodimeric protein. The recombinant binding protein can comprise linkers between the coronavirus spike binding domains, and the optional half-life extending moiety.

In some embodiments, the linker is a peptidyl linker. In some embodiments, the peptidyl linker comprises about 1 to 50 amino acid residues. Exemplary linkers includes, e.g., a glycine rich peptide; a peptide comprising glycine and serine; a peptide having a sequence $[\text{Gly-Gly-Ser}]_n$, wherein n is 1, 2, 3, 4, 5, or 6; or a peptide having a sequence $[\text{Gly-Gly-Gly-Gly-Ser}]_n$ (SEQ ID NO: 54), wherein n is 1, 2, 3, 4, 5, or 6. A

glycine rich peptide linker comprises a peptide linker, wherein at least 25% of the residues are glycine. Glycine rich peptide linkers are well known in the art (e.g., Chichili et al. Protein Sci. 2013 February; 22(2): 153-167).

- 5 In some embodiments, the peptidyl linker is a proline-threonine rich peptide linker. In an exemplary embodiment, the linker is the proline-threonine rich peptide linker of SEQ ID NO: 52. In another exemplary embodiment, the linker is the proline-threonine rich peptide linker of SEQ ID NO: 53.

- 10 In some embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 53. Examples of longer proline-threonine rich peptide linkers are found in SEQ ID NOs: 84 and 88.

N-Terminal and C-Terminal Capping Sequences

- 15 The ankyrin repeat domains of the recombinant binding protein disclosed herein may comprise N-terminal or C-terminal capping sequences. Capping sequences refers to additional polypeptide sequences fused to the N- or C-terminal end of the ankyrin repeat sequence motif(s), wherein said capping sequences form tight tertiary interactions (i.e. tertiary structure interactions) with the ankyrin repeat sequence motif(s), thereby providing a cap that shields the hydrophobic core of the ankyrin repeat domain at the side from exposing to the solvent.

- 20 The N- and/or C-terminal capping sequences may be derived from, a capping unit or other structural unit found in a naturally occurring repeat protein adjacent to a repeat unit. Examples of capping sequences are described in International Patent Publication Nos. WO 2002/020565 and WO 2012/069655, in U.S. Patent Publication No. US 2013/0296221, and by Interlandi et al., J Mol Biol. 2008 Jan 18;375(3):837-54. Examples of N-terminal ankyrin capping modules (i.e. N-terminal capping repeats) are SEQ ID NOs: 55 to 25 57 and examples of ankyrin C-terminal capping modules (i.e. C-terminal capping repeats) includes SEQ ID NO: 58.

Nucleic acids & Methods

- 30 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein as defined herein.

- In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 70 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 70.

35

In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 71 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 71.

- 5 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 72 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 72.

- 10 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 73 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 73.

- 15 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 74 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 74.

- 20 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 80 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 80.

In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 81 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 81.

- 25 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 82 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 82.

- 30 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 83 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 83.

- 35 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 78 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 78.

In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 86 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 86.

- 5 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 92 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 92.

- 10 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 93 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 93.

- 15 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 94 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 94.

- 20 In one embodiment, the present invention relates to a nucleic acid encoding a recombinant binding protein according to one of the preceding embodiments, wherein said nucleic acid comprises or consists of SEQ ID NO 95 or a variant thereof encoding the same amino acid sequence as SEQ ID NO 95.

The present invention further relates to a vector comprising said nucleic acid molecule. In one embodiment, said vector is an expression vector.

- 25 The present invention further relates to a host cell comprising said nucleic acid molecule or said vector.

In one embodiment, the present invention relates to a method of making the recombinant binding protein as defined herein, comprising culturing the host cell defined herein under conditions wherein said recombinant binding protein is expressed.

30 Compositions, Uses and Methods of Treatment

The recombinant binding proteins described herein can be used to treat a subject infected with the coronavirus. In one embodiment, the subject is infected with coronavirus SARS-CoV-2.

- 35 Thus, in one embodiment, the present invention relates to a pharmaceutical composition comprising the binding protein or nucleic acid as defined herein and a pharmaceutically acceptable carrier or excipient.

The pharmaceutical compositions may comprise a pharmaceutically acceptable carrier, diluent, or excipient. Standard pharmaceutical carriers include a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents.

5 The pharmaceutical compositions can comprise any pharmaceutically acceptable ingredients, including, for example, acidifying agents, additives, adsorbents, aerosol propellants, air displacement agents, alkalizing agents, anticaking agents, anticoagulants, antimicrobial preservatives, antioxidants, antiseptics, bases, binders, buffering agents, chelating agents, coating agents, colouring agents, desiccants, detergents, diluents, disinfectants, disintegrants, dispersing agents, dissolution enhancing agents, dyes, emollients, emulsifying agents, emulsion stabilizers, fillers, film forming agents, flavour enhancers, flavouring agents, flow enhancers, gelling agents, granulating agents, humectants, lubricants, mucoadhesives, ointment bases, ointments, oleaginous vehicles, organic bases, pastille bases, pigments, plasticizers, polishing agents, preservatives, sequestering agents, skin penetrants, solubilizing agents, solvents, stabilizing agents, suppository bases, surface active agents, surfactants, suspending agents, sweetening agents, therapeutic agents, thickening agents, tonicity agents, toxicity agents, viscosity-increasing agents, water-absorbing agents, water-miscible cosolvents, water softeners, or wetting agents. See, e.g., the Handbook of Pharmaceutical Excipients, Third Edition, A. H. Kibbe (Pharmaceutical Press, London, UK, 2000), which is incorporated by reference in its entirety. Remington's Pharmaceutical Sciences, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980), which is incorporated by reference in its entirety.

20 The pharmaceutical compositions can be formulated to achieve a physiologically compatible pH. In some embodiments, the pH of the pharmaceutical composition can be, for example, between about 4 or about 5 and about 8.0, or between about 4.5 and about 7.5, or between about 5.0 and about 7.5. In exemplary embodiments, the pH of the pharmaceutical composition is between 5.5 and 7.5.

25 In another embodiment, the present invention relates to a method of treating a coronavirus infection in a subject, the method comprising the step of administering an effective amount of at least one binding protein as defined herein, or the nucleic acid as defined herein, or of the pharmaceutical composition as defined herein, to a subject in need thereof. The subject may be exhibiting any of the symptoms associated with a coronavirus infection, with differing degrees of severity, when the method of treating is administered.

30 In some embodiments, a single administration of the method of treating may be sufficient. In other embodiments, repeated administration may be necessary. Various factors will impact on the number and frequency of administrations, such as the age and general health of the subject, as well as the state of the subject's coronavirus infection and the severity of the symptoms associated with coronavirus infection.

In some embodiments, the method is a prophylactic method, i.e. a method of preventing a coronavirus infection in a subject. In such methods, an effective amount of at least one binding protein as defined herein, or the nucleic acid as defined herein, or of the pharmaceutical composition as defined herein is administered to a subject. Typically, the subject will not be exhibiting any of the symptoms associated with a coronavirus infection when the prophylactic method is administered.

In some embodiments, a single administration of the prophylactic method may be sufficient. In other embodiments, repeated administration may be necessary. Various factors will impact on the number and frequency of administrations, such as the age and general health of the subject, as well as the subject's risk of exposure to a coronavirus.

In certain embodiments, the coronavirus infection is caused by SARS-CoV-2. In certain embodiments, the subject is a human.

The binding proteins described herein can be administered to the subject via any suitable route of administration, such as parenteral, nasal, oral, pulmonary, topical, vaginal, or rectal administration. Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. For additional details, see *Pharmaceutics and Pharmacy Practice*, J. B. Lippincott Company, Philadelphia, PA, Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Toissel, 4th ed., pages 622-630 (1986)).

The binding proteins described herein may be used in combination with another therapeutic agent, such as an analgesic. Each therapeutic agent may be administered simultaneously (e.g., in the same medicament or at the same time), concurrently (i.e., in separate medicaments administered one right after the other in any order) or sequentially in any order. Sequential administration may be useful when the therapeutic agents in the combination therapy are in different dosage forms (e.g., one agent is a tablet or capsule and another agent is a sterile liquid) and/or are administered on different dosing schedules, e.g., an analgesic that is administered at least daily and a biotherapeutic that is administered less frequently, such as once weekly or once every two weeks.

Methods of Detection or Diagnosis

In one embodiment, the present invention relates to at least one binding protein described herein for use in a method of diagnosing a coronavirus infection in a subject.

In one embodiment, the present invention relates to a method of diagnosing a coronavirus infection in a subject comprising the steps of contacting a sample from the subject *in vitro* or *ex vivo* with at least one binding protein as described herein.

5 In one embodiment, the present invention relates to a method of detecting a coronavirus in a subject, said method comprising:

- a) obtaining a sample from a subject;
- b) contacting said sample with at least one binding protein as described herein; and
- c) detecting the presence of a coronavirus.

10

In said methods and uses, the sample may be obtained from a bodily fluid such as blood, cerebrospinal fluid, plasma or urine. Samples may also be obtained from mucus (such as via nasal, oropharyngeal or vaginal swabs) or may be solid tissue samples (e.g. from biopsy).

15 Samples may be stored before use in any of these methods. For example, samples may be subject to cryogenic freezing for a suitable period of time before use in said methods.

In said methods and uses, the subject may be exhibiting symptoms associated with a coronavirus infection, with differing degrees of severity. Alternatively, the subject may be asymptomatic. The methods and uses
20 may also be carried out on samples obtained from non-living subjects to investigate cause of death.

Examples

Starting materials and reagents disclosed below are known to those skilled in the art, are commercially available and/or can be prepared using well-known techniques.

25

Materials

Chemicals were purchased from Sigma-Aldrich (USA). Oligonucleotides were from Microsynth (Switzerland). Unless stated otherwise, DNA polymerases, restriction enzymes and buffers were from New England Biolabs (USA) or Fermentas/Thermo Fisher Scientific (USA). Inducible *E. coli* expression strains
30 were used for cloning and protein production, e.g. *E. coli* XL1-blue (Stratagene, USA) or BL21 (Novagen, USA).

Molecular Biology

Unless stated otherwise, methods are performed according to known protocols (see, e.g., Sambrook J.,
35 Fritsch E.F. and Maniatis T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory 1989, New York).

Cells and viruses

Vero E6 cells (African green monkey kidney cells, ATCC® CRL1586™) purchased from ATCC (Manassas, VA 20110 USA) were passaged in cell culture medium DMEM (FG0445) containing 10% FBS and supplements (2mM L-Glutamine, Non-essential amino acids and 100 U/ml Penicillin 100 µg/ml Streptomycin and HEPES, all from Biochrom, Berlin, Germany) at 37°C without CO₂. SARS-CoV-2 (2019-nCoV/IDF0372/2020) was propagated in Vero E6 cells in MEM containing 2% FBS and supplements (2%-FBS-MEM) at 37°C. Viruses were cultured without CO₂ in non-vented flasks, 24 well-, or 96 well-plates covered with sealing foil (Biorad, microseal B-film, MSB 1001) for the duration of experiments.

10 Designed ankyrin repeat protein libraries

Methods to generate designed ankyrin repeat protein libraries have been described, e.g. in U.S. Patent No. 7,417,130; Binz et al. 2003, loc. cit.; Binz et al. 2004, loc. cit.. By such methods designed ankyrin repeat protein libraries having randomized ankyrin repeat modules and/or randomized capping modules can be constructed. For example, such libraries could accordingly be assembled based on a fixed N-terminal capping module or a randomized N-terminal capping module, one or more randomized repeat modules, and a fixed C-terminal capping module or a randomized C-terminal capping module. Preferably, such libraries are assembled to not have any of the amino acids C, G, M, N (in front of a G residue) and P at randomized positions of repeat or capping modules.

20 Furthermore, such randomized modules in such libraries may comprise additional polypeptide loop insertions with randomized amino acid positions. Examples of such polypeptide loop insertions are complement determining region (CDR) loop libraries of antibodies or de novo generated peptide libraries. For example, such a loop insertion could be designed using the structure of the N-terminal ankyrin repeat domain of human ribonuclease L (Tanaka, N., Nakanishi, M, Kusakabe, Y, Goto, Y., Kitade, Y, Nakamura, K.T., EMBO J. 23(30), 3929-3938, 2004) as guidance. In analogy to this ankyrin repeat domain where ten amino acids are inserted in the beta-turn present close to the border of two ankyrin repeats, ankyrin repeat protein libraries may contain randomized loops (with fixed and randomized positions) of variable length (e.g. 1 to 20 amino acids) inserted in one or more beta-turns of an ankyrin repeat domain.

30 Any such N-terminal capping module of an ankyrin repeat protein library preferably possesses the RILLAA, RILLKA or RELKA motif (e.g. present from position 21 to 26 in SEQ ID NO: 55) and any such C-terminal capping module of an ankyrin repeat protein library preferably possesses the KLN, KLA or KAA motif (e.g. present at the last three amino acids in SEQ ID NO: 58).

35 The design of such an ankyrin repeat protein library may be guided by known structures of an ankyrin repeat domain interacting with a target. Examples of such structures, identified by their Protein Data Bank

(PDB) unique accession or identification codes (PDB-IDs), are 1WDY, 3V31, 3V30, 3V2X, 3V2O, 3UXG, 3TWQ-3TWX, 1N11, 1S70 and 2ZGD.

Examples of designed ankyrin repeat protein libraries, such as N2C and N3C designed ankyrin repeat protein libraries, have been described (U.S. Patent No. 7,417,130; Binz et al. 2003, loc. cit.; Binz et al. 2004, loc. cit.). The digit in N2C and N3C describes the number of randomized repeat modules present between the N-terminal and C-terminal capping modules.

The nomenclature used to define the positions inside the repeat units and modules is based on Binz et al. 2004, loc. cit. with the modification that borders of the ankyrin repeat modules and ankyrin repeat units are shifted by one amino acid position. For example, position 1 of an ankyrin repeat module of Binz et al. 2004 (loc. cit.) corresponds to position 2 of an ankyrin repeat module of the current disclosure and consequently position 33 of an ankyrin repeat module of Binz et al. 2004, loc. cit. corresponds to position 1 of a following ankyrin repeat module of the current disclosure.

Example 1: Selection of binding proteins comprising an ankyrin repeat domain with binding specificity for SARS-CoV-2 spike protein

Summary

Using ribosome display (Hanes, J. and Plückthun, A., PNAS 94, 4937-42, 1997), multiple ankyrin repeat domains with binding specificity for different domains of the SARS-CoV-2 spike protein (RBD domain; S1 NTD domain; S2 domain) were selected from DARPIn® libraries in a way similar to the one described by Binz et al. 2004 (loc. cit.), with specific conditions and additional de-selection steps. The binding and specificity of the selected clones towards recombinant SARS-CoV-2 spike protein target domains were assessed by *E. coli* crude extract Homogeneous Time Resolved Fluorescence (HTRF), indicating that multiple SARS-CoV-2 spike protein specific binding proteins were successfully selected. For example, the ankyrin repeat domains of SEQ ID NOs: 1 to 11 constitute amino acid sequences of selected binding proteins comprising an ankyrin repeat domain with binding specificity for SARS-CoV-2 spike protein.

Spike protein domains as target and selection material

Spike protein domains were used as target and selection material. Proteins used for selections comprised SARS-CoV-2 S protein ectodomain (SARS2-Secto-d72-GCN4-Streptag), SARS-Cov-2 S protein (S1+S2 ECT, His-tag; Sinobiological 40589-V08B1), Bio-COVID-19_S1 protein_His_Avitag (Acro Biosystems), SARS2-S1-Flag-3Streptag, COVID-19_S_protein_RBD_Fc (Acro Biosystems), and SARS2-S1B-2Streptag. Such target proteins were selected from the polypeptides of SEQ ID NOs: 43 to 45 and 59 to 67. Proteins were biotinylated using standard methods.

Selection of SARS-CoV-2 spike protein-specific ankyrin repeat proteins by ribosome display

Designed ankyrin repeat protein libraries (N2C and N3C) were used in ribosome display selections against the SARS-CoV-2 spike protein fragments (see Binz *et al.*, *Nat Biotechnol* **22**, 575-582 (2004); Zahnd *et al.*, *Nat Methods* **4**, 269-279 (2007); Hanes *et al.*, *Proc Natl Acad Sci USA* **95**, 14130-14135 (1998)).

5

Four selection rounds were performed per target and library. The four rounds of selection employed standard ribosome display selection, using decreasing target concentrations and increasing washing stringency to increase selection pressure from round 1 to round 4 (Binz *et al.* 2004, loc. cit.). The number of reverse transcription (RT)-PCR cycles after each selection round was continuously reduced, adjusting to the yield due to enrichment of binders. The 12 resulting pools were then subjected to a binder screening.

10

Selected clones bind specifically to the RBD, S2 and S1-NTD domains of the spike protein of SARS-CoV-2 as shown by crude extract HTRF

Individually selected ankyrin repeat proteins specifically binding to the RBD, S2 and S1-NTD domains of the spike protein of SARS-CoV-2 in solution were identified by a Homogeneous Time Resolved Fluorescence (HTRF) assay using crude extracts of ankyrin repeat protein-expressing *Escherichia coli* cells using standard protocols. Ankyrin repeat protein clones selected by ribosome display were cloned into a derivative of the pQE30 (Qiagen) expression vector, transformed into *E. coli* XL1-Blue (Stratagene), plated on LB-agar (containing 1% glucose and 50 µg/ml ampicillin) and then incubated overnight at 37°C. Single colonies were picked into a 96 well plate (each clone in a single well) containing 165 µl growth medium (LB containing 1% glucose and 50 µg/ml ampicillin) and incubated overnight at 37°C, shaking at 800 rpm. 150 µl of fresh LB medium containing 50 µg/ml ampicillin was inoculated with 8.5 µl of the overnight culture in a fresh 96-deep-well plate. After incubation for 120 minutes at 37°C and 850 rpm, expression was induced with IPTG (0.5 mM final concentration) and continued for 6 hours. Cells were harvested by centrifugation of the plates, supernatant was discarded and the pellets were frozen at -20°C overnight before resuspension in 8.5 µl µl B-PERII (Thermo Scientific) and incubation for one hour at room temperature with shaking (600 rpm). Then, 160 µl PBS was added and cell debris was removed by centrifugation (3220 g for 15 min).

20

25

The extract of each lysed clone was applied as a 1:200 dilution (final concentration) in PBSTB (PBS supplemented with 0.1% Tween 20® and 0.2% (w/v) BSA, pH 7.4) together with 20 nM (final concentration) biotinylated spike protein domain, 1:400 (final concentration) of anti-6His-D2 HTRF antibody – FRET acceptor conjugate (Cisbio) and 1:400 (final concentration) of anti-strep-Tb antibody FRET donor conjugate (Cisbio, France) to a well of a 384-well plate and incubated for 120 minutes at 4°C. The HTRF was read-out on a Tecan M1000 using a 340 nm excitation wavelength and a 620 ±10 nm emission filter for background fluorescence detection and a 665 ±10 nm emission filter to detect the fluorescence signal for specific binding.

30

35

The extract of each lysed clone was tested for binding to the biotinylated spike protein domains, in order to assess specific binding to the spike protein.

Further analysis and selection of binding proteins

- 5 A total of 909 binders and inhibitors were identified. Based on binding profiles, 360 candidates were selected to be expressed in 96-well format and purified to homogeneity in parallel to DNA sequencing. Candidates were characterized biophysically by size exclusion chromatography, Sypro-Orange thermal stability assessment (see Niesen et al., *Nat Protoc* **2**, 2212-2221, (2007)), ProteOn surface plasmon resonance (SPR) target affinity assessment, ELISA, hACE2-competition HTRF experiments, SDS-PAGE, and/or SARS-CoV-2 pseudotype virus inhibition assay. Based on these data, 11 candidates (SEQ ID NOs: 1 to 11), binding to the RBD, S1-NTD or the S2 domain, were chosen for further analysis. This analysis also included 31 combinations in multi-domain formats (SEQ ID NOs: 12 to 42), exploring novel modes of action, determining inhibition potency, epitope and target diversity, sequence diversity, and/or biophysical properties. Multi-domain constructs were prepared using Gibson assembly as described previously (see Binz, H. K. et al. *MAbs* **9**, 1262-1269, (2017)). Binding proteins of the invention were expressed with a His-tag (SEQ ID NO: 46) at their N-terminus for ease of purification or detection and tested in this His-tagged form in the experiments described below.

Engineering of additional binding proteins

- 20 In further development of the initially identified binding proteins, binding domains with improved properties, such as increased affinity to and/or reduced off-rate from target protein or improved pharmacokinetic characteristics in mouse, were generated using various methods. In one approach, an initially identified binding protein (the "parental" binding protein) was selected as a suitable starting point for affinity maturation. The affinity maturation procedure entailed saturation mutagenesis of each randomized position of the ankyrin repeat domain used as a starting point. Sequences generated by the affinity maturation procedure were screened for lower off-rates by competition HTRF. Beneficial mutations identified thereby were combined in binding proteins by protein engineering. The binding properties of affinity matured and engineered binding proteins were validated by surface plasmon resonance (SPR). In another approach, certain amino acid residues in the N-terminal and/or C-terminal capping modules of the ankyrin repeat domain were altered in order to achieve improved pharmacokinetic properties, including a prolonged terminal half-life, of the ankyrin repeat domain and of proteins comprising the ankyrin repeat domain. Such altered amino acid residues were mostly surface exposed residues (see, e.g., PCT/EP2020/085855). In one example, ankyrin repeat domains with binding specificity for the S1-NTD domain of the SARS-CoV-2 spike protein, namely vS07_08F10v27 (SEQ ID NO: 76) and vS07_08F10v47 (SEQ ID NO: 85), were generated by introducing a number of mutations in ankyrin repeat domain vS07_08F10 (SEQ ID NO: 9), in order to reduce hydrophobicity and/or increase binding affinity to and/or reduce off-rate from its target. Reduction of hydrophobicity (e.g. by altering residues in the N-terminal and C-terminal capping modules)

reduced the amount of any multimerization detected by SEC, reduced viscosity and/or improved the pharmacokinetic properties in mouse. Several mutated residues were identified in an affinity maturation process using a single site mutagenesis approach on “parental” binding protein, whereby potential binding residues were randomized to all 20 amino acids by PCR, using degenerated primers. Individual variants

5 were tested for an improved off-rate by using a competitive HTRF screening. Some individual mutations increased the HTRF signal at least up to 2 to 3-fold. As examples, mutations found in vS07_08F10v47 (SEQ ID NO: 85) include the following:

IR1_V11T: In the first internal repeat module, Valine at position 11 was mutated to Threonine based on a 2 to 3-fold higher signal in a HTRF competition assay indicating an improved off-rate;

10 IR2_S3K: In the second internal repeat module, Serine at position 3 was mutated to Lysine based on a 1.5 to 2-fold higher signal in a HTRF competition assay indicating an improved off-rate;

IR2_I4V: In the second internal repeat module, Isoleucine at position 4 was mutated to Valine based on a 1.5 to 2-fold higher signal in a HTRF competition assay indicating an improved off-rate, and reduced multimerization of the protein compared to parental protein;

15 IR2_R14Q: In the second internal repeat module, Arginine at position 14 was mutated to Glutamine based on a > 3-fold higher signal in a HTRF competition assay indicating an improved off-rate;

IR2_V15S: In the second internal repeat module, Valine at position 15 was mutated to Serine based on a 1.5 to 2-fold higher signal in a HTRF competition assay indicating an improved off-rate;

20 C_W3V: In the C-terminal capping module, Tryptophan at position 3 was mutated to Valine based on a 1.2 to 1.5-fold higher signal in a HTRF competition assay indicating an improved off-rate, and reduced multimerization of the protein compared to parental protein;

C_I4V: In the C-terminal capping module, Isoleucine at position 4 was mutated to Valine based on a 2 to 3-fold higher signal in a HTRF competition assay indicating an improved off-rate; and

25 C_I6V: In the C-terminal capping module, Isoleucine at position 6 was mutated to Valine based on a 2 to 3-fold higher signal in a HTRF competition assay indicating an improved off-rate.

In another example, an ankyrin repeat domain with binding specificity for the S2 domain of the SARS-CoV-2 spike protein having improved properties, namely the ankyrin repeat domain of SEQ ID NO: 77, was generated by introducing a number of mutations in ankyrin repeat domain vS07_14G03 (SEQ ID NO: 10).

30 Engineered binding proteins, such as SEQ ID NOs: 76, 77 and 85, were characterized biophysically similarly as described above for SEQ ID NOs: 1 to 11. Furthermore, combinations in multi-domain formats comprising one or more of such engineered binding domains were generated (e.g. SEQ ID NOs: 75, 84, 87 and 88), exploring novel modes of action, determining inhibition potency, epitope and target diversity, sequence diversity, and/or biophysical properties, similarly as described above for SEQ ID NOs: 12 to 42.

35 **Example 2: SPR binding assays**

Surface plasmon resonance (SPR) assays were used to determine the binding affinity of the binding proteins of the invention to the spike protein of SARS-CoV-2.

All SPR data were generated using a Bio-Rad ProteOn XPR36 instrument with PBS-T (0.005% Tween20) as running buffer. A new neutravidin sensor chip (NLC) was air-initialized and conditioned according to Bio-Rad manual.

- 5 Mono-domain DARPin proteins: In-house chemically biotinylated (via lysines) SARS-CoV-2 Spike Protein (Sino Biologicals, cat. 40589-V08B1, Lot MF14MA0701) was captured to ~3400 RUs (30ug/ml, 30ul/min, 300s). Two buffer injections (100ul/min, 60s) followed by two 12.5mM NaOH regeneration steps (100ul/min, 18s) were applied before the first injections. Mono-domain DARPin proteins were injected (at 50/16.7/5.6/1.9/0.6nM (or at 16.7/5.6/1.9/0.6nM for SEQ ID NO: 9 and 10)) for 180s at 100ul/min for association and dissociation was recorded for 3600s (at 100ul/min). The ligand was regenerated with a 12.5mM NaOH pulse (100ul/min, 18s). The data was double referenced against the empty surface and a buffer injection and fitted according to the 1:1 Langmuir model.

- 15 Multi-domain DARPin proteins: In-house chemically biotinylated (via lysines) SARS-CoV-2 (COVID-19) S protein RBD (cat. SPD-C5255, lot. BV3539b-203FF1-203K) was captured to ~1000 RUs (775ng/ml, 30ul/min, 300s). Two buffer injections (100ul/min, 60s) followed by two 12.5mM NaOH regeneration steps (100ul/min, 18s) were applied before the first injections. One single concentration of 25nM of each multi-domain DARPin construct (including, e.g. ALE033, ALE030, ALE038, ALE049, ALE058) was injected for 180s at 100ul/min for association and dissociation was recorded for 36000s (at 100ul/min). The data was double referenced against the empty surface and a buffer injection. Due to avidity gain, no significant dissociation can be recorded during the measured time.

Exemplary results of SPR assays are shown in Figures 6, 9a-c, 15a, 16 and 17 and in Table 4. See also Example 4.

- 25 Ankyrin repeat domains according to SEQ ID Nos 1-11 were tested for their binding affinity to specific coronavirus spike protein domains using SPR (multi trace, unless indicated). In addition, other biophysical and functional properties were also tested, using methods described herein in the Examples, such as size exclusion chromatography (SEC), thermal stability measurements (T_m), and SARS-CoV-2 VSV pseudovirus neutralization assays.

- 30 Results are provided in Figures 9a-c and in Tables 4a and 4b below:

bio-S ecto Sino (SEQ ID NO: 44)	
SEQ ID NO	K_D [M]
1	2.6E-10

2	2.5E-10
3	2.1E-11
4	2.4E-10
5	9.0E-11
6	8.1E-11
7	1.4E-08 *
8	2.3E-08 *

*single trace

Table 4a

SEQ ID NO	SEC	T _m [°C]	VSV-SARS-CoV-2 IC ₅₀ [10 ⁻⁹ M]
3	Monomer	>85°C	<2
5	Monomer	>85°C	<2
6	Monomer	>85°C	<2
9	Monomer	>85°C	
10	Monomer		<100

5

Table 4b

- SEQ ID NOs: 1 to 8 were shown by SPR (single trace) to bind to the RBD domain or the S1 domain of the spike protein with similar affinities as indicated in Table 4a, using the bio-RBD Fc Acro (SEQ ID NO: 45) and the bio-S1 Acro (SEQ ID NO: 43) as target materials. SEQ ID NO: 9 was shown to bind to the S1 domain of the spike protein with a K_D of 2.0E-08 M (single trace), using the bio-S1 Acro (SEQ ID NO: 43) as target material. SEQ ID NO: 9 and SEQ ID NO: 10 were shown to bind to the ecto-domain of the spike protein with a K_D of 1.2E-09 M and 7.9E-10 M, respectively, using the S ecto U (SEQ ID NO: 61) as target material (see Figure 9c). SEQ ID NO: 76 was shown to bind to the S1 domain or the ecto-domain of the spike protein with about the same K_D as observed for SEQ ID NO: 9, while SEQ ID NO: 85 was found to bind to the S1 domain or the ecto-domain of the spike protein with an even higher binding affinity (i.e. a lower K_D) than SEQ ID NO: 9 or SEQ ID NO: 76. SEQ ID NOs: 10, 11 and 77 were shown to bind to the S2 domain of the spike protein, e.g. by HTRF assay. Table 4b shows that each of SEQ ID NOs: 3, 5, 6, 9 and 10 was monomeric in size exclusion chromatography. Furthermore, high thermal stability (>85°C) and IC₅₀ values in the nanomolar range (e.g. <2 nM) when tested against SARS-CoV-2 VSV pseudovirus are indicated for several of the SEQ ID Nos.
- For the multi-domain DARPin proteins, no significant dissociation could be recorded during the measured time due to avidity gain (see, e.g., Figures 15a, 16 and 17). The apparent affinity of the multi-domain

proteins (including, e.g., of ALE049 and ALE058) was beyond the limit of SPR, indicating sub-pM target affinity (data not shown).

Example 3: Functional screening

- 5 This Example describes functional screening of mono-domain and multi domain proteins using the SARS-CoV-2 VSV pseudotype virus assay. The results of this assay are provided in Figures 5 to 8.

10 Infection inhibition was assessed using a vesicular stomatitis virus (VSV) pseudovirus assay (psVSV), where the glycoprotein of VSV was replaced by the Wuhan variant of the SARS-CoV-2 spike glycoprotein tagged with an enhanced green fluorescent protein (EGFP) and firefly luciferase (LUC). Inhibition of infection following addition of 1 nM, 10 nM, or 100 nM of candidate was measured by simple quantification of EGFP and LUC activity (see Torriani, G. et al., *Virology* 531, 57-68 (2019)).

15 Figures 5 and 7 show pseudotype SARS-CoV-2 virus inhibition at 100 nM of various recombinant binding proteins that bind to a single site on the spike protein (mono-domain DARPin® proteins) and three sites on the spike protein (multi-domain DARPin® proteins), respectively. Shorter bars are indicative of stronger virus inhibition. Figure 8 repeats Figure 7 but at 1 nM. Figure 6 shows a representative SPR trace of a mono-domain recombinant binding protein. This data shows that the Applicant was able to rapidly establish the structures of multi-domain DARPin® proteins having sub-nanomolar antiviral activity. Further rational
20 design of the recombinant binding proteins further increased potency.

Example 4: Neutralization assay using SARS-CoV-2 VSV pseudovirus (PsV nCoV)

Cells

Vero E6, plated in 9 Costar 3610, clear bottom, white plate

25

Pseudo SARS-CoV-2 (PsV nCoV)

2000 IU/well (25 µL)

80'000 IU/mL = 8×10^4 IU/mL

4000 IU/well made 1.6×10^5 IU/mL

30 Per plate 100 * 35 µL. Prepared 4 mL of virus x 8 plates = 32 mL.

Took C15 at about 1×10^6 IU/mL

6 ml stock into 26 mL medium 2%FCS (fetal calf serum). Total 32 mL

35

Recombinant Binding Proteins

Sample no.	Sample name	5 Domain Multi-Specific DARPIn® Designs					Stock (μM)	Vol (μL)
		1	2	3	4	5		
1	ALE030	H	H	R3b	R2a	R1b	20	100
2	ALE031	H	H	R3a	R1a	R2a	20	100
3	ALE033	H	H	R3b	R1b	R3b	20	100
4	ALE034	H	H	RN1	R1b	R3b	20	100
5	ALE035	H	H	RN2	R3a	R2a	20	100
6	ALE037	H	H	R3a	R2a	RN2	20	100
7	ALE038	H	H	R1b	R3b	S1a	20	100
8	ALE039	H	H	S1a	R1b	R3b	20	100
9	ALE040	H	H	R2a	R3b	S1a	20	100
10	ALE041	H	H	S1a	R3b	R2a	20	100
11	ALE042	H	H	R3b	S1a	S2a	20	100
12	ALE043	H	H	R1b	S1a	S2b	20	100
13	ALE044	H	H	S2a	S1a	R3b	20	100
14	ALE045	H	H	S2b	S1a	R1b	20	100
15*	ACO268						167	
16**	vS07_M101E04						10	50

*negative control; ** positive control

Table 5: Samples

5 Human Serum Albumin

A 3.0 mM stock solution of human serum albumin (HSA) was used to prepare a 10 μM solution of HSA.

The medium for this solution comprised DMEM (Dulbecco's Modified Eagle Medium) 2% FCS (fetal calf serum) and 20 μM HEPES buffer solution (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid).

10

Sample dilutions

2-fold dilutions were prepared. Each dilution was mixed with one volume (PsV nCoV).

Samples 1-14: stock at 20 μM

15 Prepared 300 μL (quadruplicates, 4 x 70 μL) at 100 nM

Dilution 1: /1/10:

Took 15 μ L of the stock 20 μ M + 135 μ L PBS Final conc. 2 μ M

Dilution 2: 1/20:

Took 15 μ L of the dilution 1 + 285 μ L de milieu DMEM 2% FCS, with 20 μ M HSA.

5 Final conc. 100 nM

Negative control:

Dilution 1 neg control.

Dilution 1: /1/16.5:

10 Took 10 μ L of the stock 167 μ M + 157 μ L PBS Final conc. 10 μ M

Dilution 2: 1/10:

Took 15 μ L of the stock 10 μ M + 135 μ L PBS Final conc. 1 μ M

15 Dilution 3: /1/10:

Took 30 μ L of the dilution 2 + 270 μ L de milieu DMEM 2% FCS, with 20 μ M HSA.

Positive control:

Prepared 300 μ L (quadruplicates, 4 x 70 μ L) at 100 nM

20 Dilution 1: /1/10:

Took 15 μ L of the stock 10 μ M + 135 μ L PBS Final conc. 1 μ M

Dilution 2: 1/10:

Took 30 μ L of the dilution 1 + 270 μ L de milieu DMEM 2% FCS, with 20 μ M HSA.

25 Final conc. 100 nM

Prepared

- In a V-bottom plate
- Prepared an initial 1/10 dilution of the samples. Volume needed 4 x 70 μ L = 280 μ L. Prepared 300 μ L media containing 2% FCS and 10 mM HEPES and
- 30 • Distributed 70 μ L in the quadruplicate samples
- Two-fold dilutions carried out in the V-bottom plate

Method & Results

- 35 One volume (35 μ L) of PsV nCOV was added to each well before incubation for one hour at 37°C. The cells were then infected with 50 μ L/well and incubated again 37°C for 90 minutes. The inoculum was then removed, and 150 μ L medium 2% FCS was added before a final incubation at 37°C for 16 hours. After the final incubation period, the assay was stopped and infected cells (EGFP+) were counted at the appropriate

dilution using an inverted fluorescence microscope. Fixation of the cells was not required. A luciferase assay was then carried out. Part of the cell media was removed (100 µl out of the 150 µl) and 50 µl of Glow (PROMEGA) was added to each well. The results were read using a Berthold® TriStar LB941 luminometer for approximately 1 sec. The data was analysed using the software Graph Pad Prism 7, and the results are provided in Table 6:

Sample no.	Sample name	Stock (µM)	IC ₅₀ (nM)
1	ALE030	20	0.11950
2	ALE031	20	0.07529
3	ALE033	20	0.12470
4	ALE034	20	0.24070
5	ALE035	20	0.23770
6	ALE037	20	0.26320
7	ALE038	20	0.26380
8	ALE039	20	0.27920
9	ALE040	20	0.41750
10	ALE041	20	0.47560
11	ALE042	20	0.09803
12	ALE043	20	1.26700
13	ALE044	20	0.14710
14	ALE045	20	0.69270
15	ACO268	167	>>250.0000
16	vS07_M101E04	10	0.35780

Table 6

Samples 1-14 have been found to be potent inhibitors of pseudo-SARS-CoV-2, showing an IC₅₀ of less 1.5 nM, and in most cases of less than 0.7 nM. Samples 2 and 11 were particularly potent, with an IC₅₀ of less than 0.1 nM. Figure 5 shows fluorescence microscopy images showing GFP positive Vero E06 cells which were infected with the GFP-labeled VSV pseudotype SARS-CoV-2 virus. ALE043 in Figure 5 corresponds to sample no. 12 in Table 6 above. ACO268 and vS07_M101E04 are the negative and positive controls respectively.

Figure 11 shows neutralization of SARS-CoV-2 VSV pseudotype virus.

Figure 12 shows neutralization of SARS-CoV-2 VSV pseudotype virus for samples 1 (ALE030), 4 (ALE033), 9 (ALE038), 13 (ALE042) and 14 (ALE043). The positive control is also included (vS07_M101E04).

In Figures 11 and 12, titration of candidates was from 50nM – 50pM (2-fold dilutions). The presence of 10µM of HSA did not seem to influence the assay (see the control M101E04 without HSA-binders). The results demonstrate that half-life extended multi-domain constructs are potent inhibitors of PsV nCoV, with IC₅₀ values around 100pM.

Example 5: Virus Neutralization activity; Microtitration assay of DARPin® proteins (open cell system)

In this example, samples were tested against SARS-CoV-2 virus samples (i.e. not pseudovirus). Samples of the compounds set out in Table 8 below were prepared in dilutions of 100 nM, 20 nM, 4 nM, 0.8 nM, 0.16 nM, 0.032 nM and 0.0064 nM.

Sample no.	Sample name	Stock (µM)	IC ₅₀ (nM)
1	ALE030	20	0.11950
3	ALE033	20	0.12470
4	ALE034	20	0.24070
5	ALE035	20	0.23770
6	ALE037	20	0.26320
7	ALE038	20	0.26380
8	ALE039	20	0.27920
9	ALE040	20	0.41750
10	ALE041	20	0.47560
11	ALE042	20	0.09803
12	ALE043	20	1.26700
13	ALE044	20	0.14710
14	ALE045	20	0.69270

Table 8

The following control samples were also prepared:

- Antibody positive serum (from a patient): 1:100, 1:500, 1: 2500, 1:625, ...
- Antibody negative serum: 1:100, 1:500, 1: 2500, 1:625, ...
- Negative control DARPin protein: ACO268, a HSA-binding DARPin protein
- ACE2

- Virus back titration

Medium: MEM, 2% FCS, L-Glut, NEAA, Neo, Pen Add: 10 μ M HSA (Human Serum Albumin) dilute stock 1:300

5

The day before the assay was carried out, a 96-well plate was prepared with confluent VeroE6 cells (open system) per two compounds to be tested. All tests were carried out in triplicate. Figure 13 shows a map of the test plates, with border zones around the edge and triplicate wells for each dilution value from 0.0064 to 100 nM, and control wells.

10

The samples were diluted to 100 nM in 1 ml medium containing 2% FCS (fetal calf serum) and 10 μ M HSA (human serum albumin). 100 μ l medium containing 2% FCS and 10 μ M HSA was added to all wells in lines 4-11. 100 μ l of diluted test compound or control (100 nM) was added to line 2, and 125 μ l was added to line 3. Starting from line 3, the serum was serially diluted 1:5, by mixing 25 μ l of the upper row with the lower row (each time, the wells were thoroughly mixed by transferring the liquid up and down the pipette 5 times) until line 10.

15

6 ml of virus suspension was prepared per plate with 1000 TCID₅₀/ml in MEM, 2% FCS, 10 μ M HSA (TCID₅₀ is the 50% tissue culture infective dose). 100 μ l virus suspension (100 TCID₅₀) was added to each well of line 3-10. The plates were incubated for 1h at 37°C. The medium was then removed from the 96 well plate containing VeroE6 cells. 200 μ l of the test compound/virus mixture was transferred to the 96 well plate with cells, and the plates were incubated for 3 days at 37°C. CPE was then determined by microscope and crystal violet staining.

20

The results of Example 4 are shown in Figures 14a to 14f. Blue colored cells indicate 100% activity; colorless cells indicate no activity. As clearly demonstrated, there was almost complete protection of the cells down to 32 pM showing that the recombinant binding proteins of the present invention are very potent inhibitors of coronavirus spike protein, and specifically SARS-CoV-2 spike protein.

25

30 **Example 6: Virus Neutralization activity; Microtitration assay of DARPIn® proteins (open cell system)**

In this example, ankyrin repeat binding domains were tested against SARS-CoV-2 virus. Samples of the ankyrin repeat binding domains set out in Table 9 below were prepared in dilutions of 200 nM, 100 nM, 20 nM, 2 nM and 0.2 nM.

35

SEQ ID NO	Sample name
3	vS07_12C06
1	vS07_19G10
6	vS07_29B10
5	vS07_23E04
10	vS07_14G03

Table 9

The following control samples were also prepared:

- ACE2 100 nM, 20 nM, 4 nM, 0.8 nM, 0.16 nM
- Virus back titration

Material used: Biotinylated human ACE-2 Fc, Acro Biosystems (cat# AC2-H82F9)

Medium: MEM, 2% FCS, L-Glut, NEAA, Neo, Pen Add: 10 μ M HSA (Human Serum Albumin) dilute stock 1:300

The day before the assay was carried out, a 96-well plate was prepared with confluent VeroE6 cells (open system) per two compounds to be tested. All tests were carried out in quintuplicate. As per Figure 13 each 96-well plate included a border zone, and then rows of differing concentration wells, from 200 nm through 0.2 nM.

The samples were diluted to 200 nM in 1 ml medium containing 2% FCS (fetal calf serum) and 10 μ M HSA (human serum albumin). 100 μ l medium containing 2% FCS and 10 μ M HSA was added to all wells. The ACE2 control wells were prepared in an analogous fashion, using the indicated concentrations of ACE2.

10 ml of virus suspension was prepared per plate with 1000 TCID₅₀/ml in MEM, 2% FCS, 10 μ M HSA. 100 μ l virus suspension (100 TCID₅₀) was added to each well, except for the wells at the edges of the plates (i.e. the border wells). The plates were sealed and incubated for 1h at 37°C. The medium was then removed from the 96 well plate containing VeroE6 cells. 200 μ l of the test compound/virus mixture was transferred to the 96 well plate with cells, and the plates were sealed and incubated for 2-3 days at 37°C. CPE was then determined by microscope or methyl blue staining.

The results of Example 5 are shown in Figures 18a to 18d. Blue colored cells indicate 100% activity; colorless cells indicate no activity. As clearly demonstrated, there was complete or almost complete protection of the cells down to 20 nM or even below, showing that the recombinant binding proteins of the

present invention are very potent inhibitors of coronavirus spike protein, and specifically SARS-CoV-2 spike protein. Specifically, full protection was observed for vS07_12C06 down to 2 nM, for vS07_29B10 and vS07_23E04 down to 20 nM, and for vS07_19G10 down to 100 nM (with almost full protection at 20 nM). For vS07_14G03, partial protection was observed between 2 and 200 nM.

5

Example 7: Virus neutralization activity; Titration of DARPin® proteins in a low concentration range

In order to further investigate the ability of recombinant binding proteins of the invention to inhibit the infection of cells with live SARS-CoV-2, two distinct assays were performed measuring cell viability of Vero E06 cells with i) CellTiter-Glo® from Promega and ii) crystal violet staining. The samples tested are listed in Table 10, and the results are shown in Figure 19.

10

SEQ ID NO	Sample name
15	ALE033
24	ALE042
30	ALE048
31	ALE049
35	ALE053
39	ALE058

Table 10

15

All samples were provided in 20 µM stock and were initially diluted to 800 pM, 400 pM, 200 pM, 100 pM, 50 pM, 25 pM, 12.5 pM, 6.25 pM, and 3.125 pM in 2% FCS medium containing 10 µM HSA, then further diluted 1:2 with the virus suspension.

20 Preparation

- 96-well plates with 80% confluent VeroE6 cells (open system) per two compounds were prepared the day before testing (compounds are tested in triplicates)
- Compounds were diluted to 800 pM in 1 ml MEM medium containing 2% FCS and 10 µM HSA.
- All compounds were serially diluted 1:2 by mixing 100 µl diluted compound in 100 µl MEM medium containing 2% FCS and 10 µM HSA.

25

- Border wells were kept free (only cells and medium) to avoid border effects in test
- To identify unspecific effects of compounds on cells a control line with only compound and cells was foreseen (control; line 2)
- From line 3 to 9 the compounds were serially diluted 1:2 from 200 pM to 3.125 pM

- In line 10 and 11 MEM medium containing 2% FCS and 10 μ M HSA was added for the virus control and cell control

Test Procedure

- 5 The plate layout was similar to the layout as shown in Figure 13, but with the compound concentrations indicated above
- Virus suspension (10 ml per plate) with 1000 TCID₅₀/ml of SARS-CoV-2 (2019-nCoV/IDF0372/2020) in MEM medium containing 2% FCS and 10 μ M HSA
 - 100 μ l of virus suspension (100 TCID₅₀) was added to each well of line 3-10, medium was added to all
- 10 other wells
- The plates were incubated for 1 hour at 37°C
 - From the 96 well plates containing the 80% confluent VeroE6 cells medium was removed and 200 μ l of the test compound/virus mixture added to the 96 well plate with cells
 - Cultures were incubated 3 days at 37°C
- 15 • For analysis of virus genome copies by qPCR: 100 μ l supernatant was inactivated in 400 μ l AVL buffer + 400 μ l 100% EtOH -> Inactivated supernatant was sluiced out of the BSL3 lab
- To determine cell viability: 100 μ l CellTiter-Glo® (Promega) substrate was prepared and added according to the manufacturers protocol, plates were shaken for 2 min and fluorescence read analysed in a GloMax® (Promega).

20

Results

The results of testing by CellTiter-Glo® luminescent viability assay are provided in Figure 19. VK: viral control; ZK: cell control. Full protection of Vero E06 cells was observed at approximately 25 pM of test compound. Complete protection of cells was observed down to 25 pM for ALE033, ALE042 and ALE048.

25 Protection was somewhat less efficient for ALE049, ALE053 and ALE058, but at least partial protection of cells was observed at 25 pM also for these compounds. In conclusion, multi-domain binding proteins of the invention are capable of inhibiting infection of cells by SARS-CoV-2 at picomolar concentrations.

- 30 **Example 8: Further characterization of multi-specific binding proteins comprising SEQ ID NO: 31 (ALE049) or SEQ ID NO: 39 (ALE058)**

- Further characterization of multi-specific binding proteins comprising the amino acid sequence of SEQ ID NO: 31 or SEQ ID NO: 39 included SDS-PAGE (result: fully intact size without degradation; data not shown), mass spectrometry (result: expected molecular weight; data not shown), size exclusion chromatography
- 35 coupled to static light scattering, circular dichroism, storage stability (result: stable at 60°C for 1 week; data not shown), serum stability (result: stable at 37°C in serum for one week; data not shown), surface plasmon

resonance, SARS-CoV-2 pseudotype virus inhibition assay, live virus inhibition assay, mouse pharmacokinetic analysis (see Example 9), and hamster efficacy model (see Example 10).

Experimental Methods and Results

5

Circular dichroism

Circular dichroism measurement was performed with a Jasco J-815 using a 1 cm pathlength cuvette (Hellma) with the monitor sensor inserted in the cuvette. The MRE at 222 nm was followed over a temperature ramp from 20°C to 90°C (heating and cooling). Spectra from 190-250 nm were taken before and after the variable temperature measurement at 20°C. The protein was measured at 0.25 µM in PBS.

10

Surface plasmon resonance affinity determination

SPR assays were used to determine the binding affinity of the multi-specific binding proteins to the spike protein of SARS-CoV-2. SPR experiments were performed as described in Example 2.

15

SARS-CoV-2 VSV pseudotype virus assay

The binding proteins were assessed for inhibition potency in a SARS-CoV-2 VSV pseudotype virus assay. This assay was performed as described in detail, e.g., in Examples 3 and 4.

20

SARS-CoV-2 live virus assay

The binding proteins were assessed for inhibition potency in a SARS-CoV-2 virus assay, similar as described in Example 5. In brief, the binding proteins were prepared in dilutions of 100 nM, 20 nM, 4 nM, 0.8 nM, 0.16 nM, 0.032 nM and 0.0064 nM in 96-well plates as described below, using the following medium: MEM, 2% FCS, L-Glut, NEAA, Neo, Pen; with addition of 10 µM HSA (Human Serum Albumin) (dilute stock 1:300). All tests were carried out in triplicates. The day before the assay was carried out, a 96-well plate was prepared with confluent VeroE6 cells (open system) per two compounds to be tested. The test plate was designed similar as shown in Figure 13, with border zones around the edge and triplicate wells for each final dilution value from 0.0032 nM to 50 nM, and control wells. Samples were diluted to 100 nM in 1 ml medium containing 2% FCS (fetal calf serum) and 10 µM HSA (human serum albumin) (see above). 100 µl medium containing 2% FCS and 10 µM HSA was added to all wells in lines 4-11. 100 µl of diluted test compound or control (100 nM) was added to line 2, and 125 µl was added to line 3. Starting from line 3, the serum was serially diluted 1:5, by mixing 25 µl of the upper row with the lower row (each time, the wells were thoroughly mixed by transferring the liquid up and down the pipette 5 times) until line 10. 6 ml of virus suspension was prepared per plate with 1'000 TCID₅₀/ml in MEM, 2% FCS, 10 µM HSA. 100 µl virus suspension (100 TCID₅₀) was added to each well of lanes 3-10. The plates were incubated for 1h at 37°C. The medium was then removed from the 96 well plate containing VeroE6 cells. 200 µl of the test compound/virus mixture was transferred to the 96 well plate with cells, and the plates were incubated

25

30

35

for 3 days at 37°C. Cytopathic effect was then determined either by microscope and crystal violet staining, where blue colored cells indicate 100% activity and colorless cells indicate no activity (see Figure 20), or, alternatively, using a CellTiter-Glo® luminescent cell viability assay (Promega; see Figures 21a-c). For the latter, 1'000, 10'000, or 100'000 TCID₅₀ were used.

5

As clearly demonstrated in Figure 20, there was complete or almost complete protection of the cells down to 0.08 nM for both ALE049 and ALE058, showing that the recombinant binding proteins of the present invention are very potent inhibitors of coronavirus spike protein, and specifically SARS-CoV-2 spike protein, and of infection of cells by a coronavirus, and specifically by SARS-CoV-2. Corresponding results are shown in Figures 21a-c, which also demonstrate potent inhibition for both ALE049 and ALE058. The exact concentration of the recombinant binding proteins required to achieve efficient inhibition in these assays was dependent on the viral load used. For both ALE049 and ALE058, potent inhibition of SARS-CoV-2 was observed in the picomolar range, for ALE049 down to 50 pM. IC₅₀ values for ALE049 and ALE058 are shown in Table 11 below. These values of virus inhibition represent the strongest SARS-CoV-2 inhibition reported to date.

15

Molecular model of drug candidates

A molecular model for ALE049 (Figure 22A) was built based on cryogenic electron microscopy data (data not shown). In the first step, a model structure of binding domain #2 was generated. The consensus designed ankyrin repeat domain PDB:2xee was used as template. Mutations were introduced with RosettaRemodel with fixed backbone, and the structure was refined with RosettaRelax. Forty refined structures were clustered using RosettaCluster with 0.3 Å radius, and the lowest-energy model from the largest cluster served as the final model. This model was then used for fitting domain #2 into the observed electron density generated from the complex structure of the spike protein and domain #2, resulting in a PDB file with the coordinates of the trimer of domain #2:RBD. This trimeric model was used as an input structure for the conceptual modeling of ALE049 bound to the spike ectodomain as shown in Figure 22A. Similarly, a molecular model was also built for ALE058 (Figure 22B). This model for ALE058 is based on the cryogenic electron microscopy data as well as a schematic structural prediction for the S2 binding domain.

20

Multi-specific binding proteins comprising the amino acid sequence of SEQ ID NO: 31 (ALE049) or SEQ ID NO: 39 (ALE058) each comprise combinations of 3 SARS-CoV-2 spike protein binders fused C-terminally to 2 clinically validated serum albumin-binding domains for systemic half-life extension. The resulting 5-domain proteins were expressed, purified and characterized in detail regarding biophysical properties, target affinity, and virus inhibition. The multi-specific binding proteins were expressed in soluble form at high levels in the cytoplasm of *E. coli*. Purified proteins are monomeric and exhibit high thermal stability (T_m > 88°C) and reversible unfolding as assessed by circular dichroism, and high stability in accelerated

25

30

35

storage stability assays at 60°C (data not shown). Their apparent affinity is beyond the limit of SPR, indicating sub-pM target affinity (data not shown). In psVSV assays, the multi-specific binding proteins inhibited viral entry with IC₅₀ values ranging from 3 pM to 138 pM or 0.24 ng/ml to 11.04 ng/ml (see Table 11, Figure 23). The psVSV assay results correlated well to live virus assay results, where infection inhibition was observed with concentrations of 25 pM to 100 pM or 2 ng/ml to 8 ng/ml (see Table 11, Figures 21a-c).

Name	SEC	Tm [°C] (CD)	IC ₅₀ psVSV [10 ⁻¹² M]	IC ₅₀ LV* [10 ⁻¹² M]
ALE049	Monomer	>88°C	46-138	25
ALE058	Monomer	>88°C	3-99	100

*LV: Live virus cytopathic effect assay

Table 11

Figure 23 further shows neutralization of the SARS-CoV-2 VSV pseudovirus by the recombinant binding proteins of the invention tested in the presence of the RBD domain of the spike protein. Figure 23 shows that the RBD domain competes strongly with ALE049, which contains three RBD binding domains, but not with ALE058, which contains one RBD binding domain, one S1-NTD binding domain and one S2 binding domain. ALE049 lost potency when competing with the isolated RBD-domain, while competition of the single RBD-binder in ALE058 had no significant impact on the potency of ALE058. Without wishing to be bound by theory, this data appears to confirm that ALE049 and ALE058 inhibit SARS-CoV-2 by different modes of action. While ALE049 seems to rely strongly on the neutralization of the RBD/ACE-2 interaction, ALE058 seems to show multi-mode binding and a diversified mode of action, which beyond the neutralization of the RBD/ACE-2 interaction also utilizes an independent neutralization potency of the S1-NTD-S2 arm of the molecule. Thus, based on the data shown in Figure 23, ALE049 and ALE058 appear to have different modes of action, consistent with the molecular models of the two molecules shown in Figure 22.

Such high potency as observed for the binding proteins of the invention is key for the use in SARS-CoV-2 treatment and prophylaxis where very low virus titers at infection initiation are envisioned. Importantly, several spike protein variants of the most abundant SARS-CoV-2 serotypes were blocked with high potency by the multi-specific binding proteins (see Table 12), indicating robustness against viral escape and potential of use in prophylactic treatment in the current pandemic and potentially also future pandemics. In mouse experiments, no adverse events were observed up to the highest dose (50 mg/kg, i.v.) tested.

	wt	G476S	V483A	D614G	D614G x Q675H
ALE049	16.53	27.08	27.48	11.77	12.11
ALE058	5.48	14.46	32.40	4.64	22.44

Table 12: Potency of inhibition of SARS-CoV-2 spike protein variants (IC₅₀, [10⁻¹²M])

Example 9: Pharmacokinetic analysis of multi-specific binding proteins of the invention in mice

5

A pharmacokinetic (PK) study was conducted to assess the PK characteristics of multi-specific recombinant binding proteins of the invention in mice. Such PK characteristics are useful for dose predictions of multi-specific binding proteins of the invention in animal pharmacodynamic studies, in toxicology studies or in human clinical trials.

10

The investigated multi-specific binding proteins of the invention comprise – from N-terminus to C-terminus - two HSA-specific binding domains followed by three spike protein-specific binding domains (see Table 2). The HSA-specific binding domains are cross-reactive to serum albumin of the mouse.

15

For this PK study, naive female BALB/c mice received a single intravenous bolus injection at a target dose level of 1 mg/kg of the compounds. Blood samples were collected at several time points between 5 min and 165 h after compound administration. Serum concentrations were determined with ELISA-based analytical methods.

20

From 6 h onwards the concentration-time profiles indicate a slow and steady decrease of the serum concentrations resembling roughly mono-exponential declines until 165 h, the last sampling time point. From the concentration time profiles pharmacokinetic parameters were determined using non-compartmental analysis.

25

The following multi-specific binding proteins were tested in this example:

SEQ ID NO	Sample name
15	ALE033
30	ALE048
31	ALE049

Table 13

In vivo animal experiments

The test items were administered to healthy female BALB/c mice (6 mice per test item) as a single intravenous bolus injection into the tail vein. The target dose level was 1 mg/kg. For the study of each compound, the 6 mice were split into 2 groups with equal numbers of animals. For pharmacokinetic investigations, serum samples, 4 from each mouse, were collected from the saphenous vein at time points 5 min, 6 h, 24 h, 48 h, 72 h, 96 h and 165 h. The assignment of the individual animals to the respective sampling time points was according to a predetermined scheme. Blood was kept at room temperature for approx. 30 min to allow clotting followed by centrifugation (5 min/12000g/4°C). Afterwards serum was frozen and stored at -20°C pending analyses. No major issues and no drug-related adverse effects were reported for the *in vivo* experiment.

ELISA method

An ELISA method (see Figure 24) was used for measuring serum concentrations of the multi-specific binding proteins making use of a common epitope of DARPin® moieties recognized by the anti-DARPin® antibody 1-1-1 for capturing and of the N-terminal His-tag, which is present in the tested binding proteins, to facilitate detection. The ELISA setup scheme illustrated in Figure 24 (showing ALE049 as a binding protein example) uses monoclonal goat anti-rabbit-IgG immobilized on the ELISA plate, which binds rabbit anti-DARPin® antibody 1-1-1, capturing the multi-specific binding proteins via DARPin® scaffold epitopes in serum sample. The captured DARPin® molecule is detected using mouse anti-RGS-His-IgG-HRP conjugate. aSA: anti serum albumin, aRBD: anti receptor binding domain (RBD)

Test Procedure

One hundred µL per well of 10 nmol/L polyclonal goat anti-rabbit IgG antibody (Ab18) in PBS was coated onto a NUNC Maxisorb ELISA plate overnight at 4°C. After washing with 300 µL PBST (PBS supplemented with 0.1% Tween20) per well five times, the wells were blocked with 300 µL PBST supplemented with 0.25% Casein (PBST-C) for 1 h at room temperature (RT) on a Heidolph Titramax 1000 shaker (450 rpm). Plates were washed as described above. One hundred µL per well of 5 nmol/L rabbit anti-DARPin® 1-1-1 antibody in PBST-C was added and the plates were incubated at RT (22°C) with orbital shaking (450 rpm) for 1 h. Plates were washed as described above.

One hundred µL per well of diluted serum samples (1:20 – 1:312500, in 1:5 dilution steps), multi-specific binding protein quality control samples (100, 10 and 1 nmol/L) or multi-specific binding protein standard curve samples (0 and 50 - 0.001 nmol/L in 1:3 dilution steps) diluted in PBST-C (supplemented with naive mouse serum to result in a final serum concentration of 1 % (initial 1:20 dilution final serum concentration of 5%)) were applied for 2 h, at RT, shaking at 450 rpm. Plates were washed as described above.

Wells were then incubated with 100 μ L murine anti-RGS-His-HRP IgG (Ab06) 1:2000 in PBST-C and incubated for 1 h, at RT, 450 rpm. Plates were washed as described above. The ELISA was developed using 100 μ L per well TMB substrate solution for 5 minutes and stopped by the addition of 100 μ L per well 1 mol/L H₂SO₄. The difference between the absorbance at 450 nm and the absorbance at 620 nm was calculated. Samples were measured in duplicate on two different plates.

Quality control samples of known concentrations were included in the measurements in order to monitor the performance of the assay.

- 10 Pharmacokinetic data analysis was performed using Phoenix WinNonlin™ 8.0 program from Certara. Calculation of the pharmacokinetic parameters of the study based on the mean concentration-time data of the animals dosed via intravenous bolus injection was performed with non-compartmental analysis (NCA model 200-202, IV bolus, linear trapezoidal linear interpolation).
- 15 The calculated pharmacokinetic parameters included at least the following: AUCinf_pred, AUClast, AUC_%extrapol, AUC_%Back_Ext_pred, Cmax, Tmax, Cl_pred, Vss_pred, t1/2 (HL_Lambda_z)

The results are shown in Table 14 and Figure 25:

Parameter	Unit	ALE033	ALE048	ALE049
AUCINF_pred	h*(nmol/L)	12428	12768	14329
AUClast	h*(nmol/L)	11439	11461	12949
Cmax	nmol/L	244	230	291
Tmax	h	0.083	0.083	0.083
Cl_pred	L/(h*kg)	0.00094	0.00091	0.00081
Vss_pred	L/kg	0.058	0.063	0.055
HL_Lambda_z	h	45.8	50.8	49.6
AUC_%Extrap_pred	(%)	8	10	10
AUC_%Back_Ext_pred	(%)	0	0	0

Table 14

Results and Conclusions

In the mono-exponential elimination phases, serum concentrations of ALE033, ALE048 and ALE049 declined with half-life values of 45.8 h, 50.8 h and 49.6 h, respectively. Clearance of ALE033, ALE048 and ALE049 was determined to be 0.00094, 0.00091 and 0.00081 L/(h*kg), respectively, and volume of distribution (Vss) of ALE033, ALE048 and ALE049 was calculated to be 0.058, 0.063 and 0.055 L/kg,

respectively. The values determined for Vss indicate that ALE033, ALE048 and ALE049 are largely confined to the systemic circulation of the animals, similarly to monoclonal antibodies.

In conclusion, following intravenous administration at a dose level of 1 mg/kg the three tested multi-specific binding proteins of the invention display a systemic half-life in the range of the half-life of albumin in mice.

- 5 Considering the half-life of albumin in mouse and human as well as previous data (Binz *et al.*, *MAbs* **9**, 1262-1269 (2017)), the terminal half-life of ALE049 in humans is expected to extrapolate to around 3 weeks. The terminal half-lives of ALE033 and ALE048 in humans are expected to extrapolate similarly.

Example 10: SARS-CoV-2 inhibition efficacy experiments in Syrian hamster

10

The efficacy of ALE049 was further assessed in a Syrian hamster model of preventive treatment of SARS-CoV-2 infection.

15

Syrian hamsters were divided into 4 groups of 6 female animals each. The groups were treated with of 16 µg, 160 µg, or 1600 µg of multi-specific binding protein having the amino acid sequence of SEQ ID NO: 31 or with placebo in a blinded manner. Treatment injection (i.p., intraperitoneal) was done 24 h prior (Day -1) to intranasal infection (Day 0) of the animals with 5×10^4 TCID₅₀ (in 100 µl) of SARS-CoV-2 (BetaCoV/Munich/BavPat1/2020). At Day -2, body weight was measured, blood was taken, and the first throat swab performed. Animals were euthanized on Day 4 and tissue was taken and gross pathology was performed. Throat swabs were collected daily in virus transport medium, aliquoted and stored. At the time of necropsy, gross pathology was performed. Lung lobes were inspected and an estimation of the percentage of affected lung tissue from the dorsal view was performed. Left lung lobes and nasal turbinates were preserved in 10% neutral buffered formalin for histopathology. The right side of these tissues was homogenised and subjected to Taqman PCR and virus titration. Additionally, other organs were collected.

20

25

Tissue samples were frozen for virological analysis, weighed, homogenized in infection medium and centrifuged briefly before titration. Histopathology was performed on lung and nasal turbinates for all animals. After fixation with 10% formalin, sections from left lung and left nasal turbinate were embedded in paraffin and the tissue sections were stained by H&E for histological examination. For virological analyses, quadruplicate 10-fold serial dilutions were used to determine the virus titers in confluent layers of Vero E6.

30

To this end, serial dilutions of the samples (throat swabs and tissue homogenates) were made and incubated on Vero E6 monolayers for 1 h at 37°C. Vero E6 monolayers were washed and incubated for 4-6 days at 37°C after which plates were scored WST8. Viral titers (TCID₅₀) were calculated using the method of Spearman-Kärber. Readout included observation of body weight, lung lesions, virus titers, and histopathology.

35

Histopathology

After fixation with 10% neutral-buffered formalin, sections of the left lung, left nasal turbinate and trachea were embedded in paraffin. The tissue sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation. Semi-quantitative scores of 0, 1, 2 or 3 were given when the extent of alveolitis and alveolar damage were estimated at 0%; 1–25%; 26–50% or >50%, respectively. The cumulative score for the extent and severity of inflammation of the lung provided the total score of alveolitis per animal (see Table 15, column “SUM of extent + severity”). For the severity of alveolitis, bronchiolitis, and bronchitis, semi-quantitative scores of 0, 1, 2 or 3 were given when no, few, moderate numbers or many inflammatory cells were present, respectively. For the presence of alveolar edema, alveolar hemorrhage, and type II pneumocyte hyperplasia, scores of 0 or 1 were given upon their absence or presence, respectively. In Table 16, the presence of alveolar edema, alveolar hemorrhage, and type II pneumocyte hyperplasia is indicated by “yes” and “no” instead of the numerical score.

Readout included observation of body weight, lung lesions, virus titers, and histopathology. At the 1600 µg dose, ALE049 exhibited significant reduction of the viral titers in the lung (Figure 26a). While the model exhibited high inter-animal variability, trends to a dose-dependent reduction of virus titers (Figure 26a), dose-dependent reduction of macroscopically determined lung lesions (Figure 26b), and dose-dependent reduction of body weight loss (Figure 26c) were observed, indicating both the 160 µg as well as the 1600 µg dose exhibited anti-viral activity. Virus titers in the throat swabs further showed that the 1600 µg dose, and to a lesser extent the 160 µg dose, inhibited the virus titers and/or accelerated the reduction of virus titers in the throat during the four day post-infection time period (Figure 26d). Virus titers in nasal turbinates (Figure 26e) and histopathology data (Tables 15 and 16, Figure 27) confirmed that the 1600 µg dose had the strongest anti-viral protective effects. Based on these encouraging initial findings further animal experiments are ongoing.

Animal no.	Group no.	Compound	Dose	Extent of alveolitis/ alveolar damage	Severity of alveolitis	SUM of extent + severity	Severity of bronchitis	Severity of bronchio-litis
1	1	ALE049	1600 µg	1	1	2	2	1
2				0	0	0	1	1
3				1	2	3	3	1
4				1	1	2	3	1
5				1	1	2	2	1
6				0	0	0	1	1
7	2	ALE049	160 µg	1	3	4	3	2
8				2	3	5	3	3
9				2	3	5	3	3
10				2	3	5	3	3
11				2	3	5	2	3
12				2	3	5	3	3
13	3	ALE049	16 µg	2	3	5	3	3
14				2	3	5	3	3

15				3	3	6	3	3
16				2	3	5	3	3
17				1	3	4	3	2
18				3	3	6	3	3
19	4	Placebo	N/A	2	3	5	3	3
20				2	3	5	3	3
21				2	3	5	3	3
22				2	3	5	3	3
23				2	3	5	3	3
24				2	3	5	3	3

Table 15. Histopathology results (1)

Animal no.	Group no.	Compound	Dose	Alveolar edema presence	Alveolar hemorrhage presence	Type II pneumocyte hyperplasia presence
1	1	ALE049	1600	no	no	no
2			μg	no	no	no
3				no	no	yes
4				no	no	yes
5				no	no	yes
6				no	no	no
7	2	ALE049	160	yes	yes	yes
8			μg	yes	yes	yes
9				yes	yes	yes
10				yes	yes	yes
11				yes	yes	yes
12				yes	yes	yes
13	3	ALE049	16	yes	yes	yes
14			μg	yes	yes	yes
15				yes	yes	yes
16				yes	yes	yes
17				yes	yes	yes
18				yes	yes	yes
19	4	Placebo	N/A	yes	yes	yes
20				yes	yes	yes
21				yes	yes	yes
22				yes	yes	yes
23				yes	yes	yes
24				yes	yes	yes

5

Table 16. Histopathology results (2)

Example 11: SARS-CoV-2 variant inhibition efficacy experiments

The efficacy of ALE049 (SEQ ID NO: 31) and ALE109 (SEQ ID NO: 75) was assessed against SARS-CoV-2 variants B.1.1.7 (the "UK variant") and B.1.351 (the "South African variant"), as well as against SARS-CoV-2 variants having single mutations in the spike protein.

10

The spike protein of SARS-CoV-2 mediates cell entry through binding to the human ACE2 receptor. SARS-CoV-2 is also capable of infecting non-primate hosts, such as felines and minks (Oude Munnink et al., 2021, *Science* 371, 172-177). The promiscuity of a multi-host lifestyle is often an indicator of early, still sub-optimal adaptation of the virus to its new host. This suggests inherent dynamic plasticity and potential for further human adaptation. The receptor-binding domain (RBD) in the spike protein forms the interface with ACE2. Site mutagenesis scanning and structure analysis revealed amino acid residues important for this interaction, such as L455, F456, A475, F486, F490 and Q493 (Yan et al., 2020, *Science* 367, 1444-1448; Yi et al., 2020, *Cell Mol Immunol* 17, 621-630). Notably, single amino acid substitutions N439R, L452K, N470T, E484P, Q498Y and N501T have been shown to increase the affinity for human ACE2 (Yi et al., 2020, loc. cit.). Consistent with these experimental findings, mutation N439K and mutation N501Y appeared in rapidly spreading SARS-CoV2 spike variants in association with facilitated receptor binding and increased transmissibility (Thomson et al., 2021, *Cell*, <https://doi.org/10.1016/j.cell.2021.01.037>). The RBD domain is also immunogenic, and among other residues, K444, E484, and F486 have been shown to be important for the binding of neutralizing antibodies (Ku et al., 2021, *Nat Commun* 12, 469).

In this example, we analyzed the impact of selected mutations of the spike protein on the neutralization capacity of ALE049 and ALE109 (Figure 28).

Generation of His-tagged mono-valent RBD binders, ALE049, ALE109 and the domain knockout variants of ALE109

Ankyrin repeat protein constructs selected and cloned as described in Example 1 and in Walser et al., 2020 (bioRxiv preprint doi: <https://doi.org/10.1101/2020.08.25.256339>) were transformed in *E.coli* BL21 cells, plated on LB-agar (containing 1% glucose and 50 µg/ml ampicillin) and then incubated overnight at 37°C. For each construct, a single colony was picked into TB medium (containing 1% glucose and 50 µg/ml ampicillin) and incubated overnight at 37°C, shaking at 230 rpm. Fresh TB medium (containing 50 µg/ml ampicillin) was inoculated with 1:20 of overnight culture and incubated at 37°C at 230 rpm. At OD600 = 1.1 the culture was induced by addition of IPTG (0.5mM final concentration) and incubated for further 5h at 37°C 230 rpm. Harvest was done by centrifugation (10 min 5000 x g). After cell disruption by sonication primary recovery was done by heat treatment for 30 min at 62.5°C and subsequent centrifugation (15 min, 12000 x g). 20mM Imidazole and 1% Triton X-100 was added to the supernatant and the 0.22µm centrifuged supernatant was further purified by immobilized metal affinity chromatography (HisTrap FF crude, Cytiva, Sweden) using the N-terminal His-tag including a wash step with 1% Triton X-100 and a step elution with 250 mM Imidazole. In a subsequent step, the elution fraction of the IMAC step was applied on a size exclusion chromatography (Superdex 200, Cytiva, Sweden) and fractions of interest were pooled and concentrated. Finally, the concentrated sample was filtered through a 0.22 µm Mustang E filter for Endotoxin removal and sterile filtration and quality controlled.

Generation of monoclonal reference antibodies, RA1 and RA2

Publicly available sequences of variable domains from monoclonal antibodies RA1 and RA2 (the U.S. Food and Drug Administration issued an emergency use authorization for RA1 and RA2 to be administered as a cocktail for the treatment of COVID-19) were used to synthesize the corresponding cDNA fragments and cloned into a proprietary expression vector at Evitria AG (Switzerland). Generated vectors containing the constant immunoglobulin hIgG1 chain or kappa light chain were used for transfection in Chinese hamster ovary cells by Evitria. Sterile filtered cell supernatants were purified via affinity purification with HiTrap MabSelect column followed by a size exclusion chromatography using HiLoad 26/600 Superdex 200 column in PBS pH7.4. Selected fractions were pooled and quality controlled (by SDS-PAGE, size exclusion chromatography and endotoxin measurement) before use in assays.

VSV-SARS-CoV-2 pseudotype mutation-vector generation

Plasmid pCAGGS encoding the spike protein of SARS-CoV-2 (Walser et al., 2020, loc. cit.) was used as template for generation of single and multiple spike protein mutants. Forward and reverse complementary primers encoding the mutation were synthesized by Microsynth (Balgach, Switzerland). High-fidelity Phusion polymerase (New England Biolabs, USA) was used for all DNA amplification.

Single mutations of the spike protein were generated via two PCR fragments of the spike ORF using high-fidelity Phusion polymerase (New England Biolabs, USA). The first fragment was generated via a generic forward primer (pCAGGS-5) annealing upstream of the spike ORF and the specific reverse primer encoding the mutation. The second fragment was generated using the specific forward primer encoding the mutation and a reverse primer (rbglobpA-R). The two fragments were gel-purified and used as input for an assembly PCR without addition of flanking primers.

For multi-mutation spike proteins, a complementary pair of primers (forward and reverse) encoding each mutation was designed. Fragment 1 was generated with forward primer pCAGGS-5 and reverse primer encoding mutation 1. Fragment 2 was generated using forward primer encoding mutation 1 and reverse primer encoding mutation 2. All subsequent fragments were generated analogously. DNA fragments were gel-purified and mixed in equimolar amounts. This mix was used for re-assembly of the full spike ORF using outer primers pCAGGS-5 and rbglobpA-R.

For both single as well as multi-mutation spike protein, the full-length spike ORF was isolated from an agarose gel, digested by restriction enzymes NheI/EcoRI and inserted into the pCAGGS vector backbone. The correct sequence was verified via sequencing the whole ORF of the spike protein by Microsynth (Balgach, Switzerland).

VSV-SARS-CoV-2 pseudotype neutralization assay for mutational analyses and ALE109 domain knock outs

The pseudotype viral system was based on the recombinant VSV*ΔG-Luc vector in which the glycoprotein gene (G) had been deleted and replaced with genes encoding green fluorescent protein and luciferase (Berger Rentsch and Zimmer, PLoS One. 2011;6(10):e25858). Pseudoviruses were generated as reported previously (Torriani et al., Virology. 2019 May;531:57-68; Torriani et al., J Virol. 2019 Mar 5;93(6):e01744-18). For the neutralization assay, an initial dilution of the compounds was followed by three-fold dilutions in quadruplicates in DMEM-2 % [vol/vol] FCS supplemented with 20 μM human serum albumin (CSL Behring). The mixture was mixed with an equal volume of DMEM-2 % FCS containing 250 IU per well of SARS-CoV-2 pseudoviruses and incubated for 90 min at 37°C. The mix was inoculated onto Vero E6 cells in a clear bottom white walled 96-well plate during 90 min at 37°C. The inoculum was removed and fresh medium added, and cells further incubated at 37°C for 16 h. Cells were lysed according to the ONE-Glo™ luciferase assay system (Promega, Madison, US) and light emission was recorded using a Berthold® TriStar LB941 luminometer. The raw data (relative light unit values) were exported to GraphPad Prism v8.01, and the % neutralization values were normalized to the untreated PsV signal. IC₅₀ with 95% confidence interval were estimated by model of nonlinear regression fit with settings for log (inhibitor) vs normalized response curves.

Cells and viruses

Vero E6 cells were passaged in Minimum Essential Medium (MEM) (Cat N° M3303) containing 10% fetal bovine serum (FBS) and supplements (2 mM L-Glutamine, 1% Non-essential amino acids, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 0.06% Sodium bicarbonate, all from Bioswisstec, Schaffhausen, Switzerland) at 37°C, >85% humidity and 5% CO₂. SARS-CoV-2 (2019-nCoV/IDF0372/2020) was propagated in Vero E6 cells in MEM containing 2% FBS and supplements (2%-FBS-MEM) at 37°C, >85% humidity and 5% CO₂. Viral titer was determined by standard plaque assay as described elsewhere.

Virus neutralization of authentic SARS-CoV-2 determined by CellTiter-Glo and real-time RT-PCR

Virus neutralization capacity of mono-domain and multi-domain ankyrin repeat binding proteins was determined for 100 TCID₅₀ SARS-CoV-2 by measuring ATP levels of protected cells in a cell viability assay. DARPIn® proteins were serially diluted 1:4 from 40 nM to 2.4 pM (in triplicates) in 100 μl cell culture medium (2%-FBS-MEM) enriched with 10 μM HSA in 96 well plates. The diluted DARPIn® proteins were mixed with 100 TCID₅₀ SARS-CoV-2 in 100 μl 2%-FBS-MEM + HSA and incubated for 1 h at 37°C. DARPIn® protein/virus mixtures (200 μl) were transferred onto 80% confluent Vero E6 cells. The controls consisted of Vero E6 cells exposed to virus suspension only, to determine maximal cytopathic effect and of cells incubated with medium only, to determine baseline state of cells. The plates were incubated for 3 days at 37°C, >85% humidity and 5% CO₂. Cell viability was determined by removing 100 μl supernatant from all wells and adding 100 μl CellTiter-Glo reagent as described in the manufacturers protocol (CellTiter-Glo® Luminescent Cell Viability Assay, Promega, Madison, USA). Luminescence was read after 2 minutes

- shaking on an orbital shaker, transferring the mixture to an opaque-walled plate and 10 min incubation at room temperature using the GloMax instrument (Promega). To determine inhibition of virus replication, the previously removed supernatant (100 µl) was inactivated in 400 µl AVL-buffer (Qiagen, Hilden, Germany) and 400 µl 100% Ethanol and extracted and eluted in 100 µl using the MagNAPure 96 system (Roche, Basel, Switzerland). Viral RNA was quantified by real-time RT-PCR targeting the E gene (Ref. Eurosurveillance | Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR) using 5 µl RNA and 45 µl TaqMan Fast Virus 1-Step Master Mix (Life Technologies, Zug, Switzerland). Viral genome equivalents (ge) were calculated using a regression analysis and an internal standard.
- 10 The results of the neutralization tests with multi-specific DARPin® molecules ALE049 and ALE109 or reference antibodies 1 or 2 (RA1 and RA2 respectively) are shown in Table 17. Table 18 shows the activity of the three spike protein-binding domains of ALE049 (SEQ ID NO: 31) as individual binders against spike protein variants.

Variants	Rational	VSV Pseudotype Neutralization Assay IC ₅₀ [ng/mL]			
		ALE049	ALE109	RA1	RA2
wild type	(Wuhan)	1.0	3.1	3.9	6.1
B.1.351	(SA, Δ5)*	3.0	2.4	19	6.2
B.1.1.7	(UK, Δ9)**	1.7	70	2.4	3.5
Individual Mutations	Residues in variants				
N501Y	in UK, SA, BRA variants; increases RBD/ACE2 interaction ¹	0.5	1.4	4.3	5.8
E484K	in SA, BRA variants; increases RBD/ACE2 interaction ¹	2.7	1.8	17	5.8
K417E	residue mutated to N/T in SA, BRA variants	0.5	1.2	>100	1.5
Y453F	key residue evolved in Danish mink farms variants	3.2	2.0	>100	12
Individual Mutations	Highly frequent mutations				
D614G	Wide global spread	2.4	2.8	n.d.	n.d.
S477N	Wide global spread	1.9	0.8	n.d.	n.d.
N439K	Widespread in Northern America, UK; increases RBD/ACE2 interaction ¹	1.3	2.5	2.8	30
A222V	Wide European spread	2.2	3.1	7.0	2.9
Individual Mutations	Within RBD epitope of DARPin® binder or reported resistance mutation for other therapeutics				
G446V		1.7	1.0	1.5	>100
G476S		1.5	3.1	n.d.	n.d.

T478I		2.7	2.8	4.0	7.0
P479S		2.1	1.5	3.7	9.8
V483A		2.3	1.9	n.d.	n.d.
F486V	key residue for DARPin® RBD binder ² ; reduces RBD/ACE2 interaction ¹	>100	7.7	>100	4.4
Q493K		7.9	2.4	>100	10
F490S	Reduces RBD/ACE2 interaction ¹	3.8	1.6	3.1	9.2

n.d.: not determined

*Mutations (SA): D80A, D215G, E484K, N501Y, A701V

**Mutations (UK): del69-70, del145, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H

¹ Influence of residue mutations on spike protein binding to human ACE2 (Yi et al., 2020, loc. cit.)

² Predicted interaction residue for DARPin® RBD binder (Walser et al., 2020, <https://doi.org/10.1101/2020.08.25.256339>)

5

Table 17: Efficacy Results

Variants	Rational	VSV Pseudotype Neutralization Assay IC ₅₀ [ng/mL]			
		ALE049	Mono-valent RBD Binders in ALE049		
			R3b	R1b	R3c
wild type	(Wuhan)	1.0	7.2	2.1	13.3
B.1.351	(SA, Δ5)*	3.0	76	26	>100
B.1.1.7	(UK, Δ9)**	1.7	4.6	5.4	11.7
Individual Mutations	Residues in variants				
N501Y	in UK, SA, BRA variants; increases RBD/ACE2 interaction ¹	0.5	9.1	4.8	27.8
E484K	in SA, BRA variants; increases RBD/ACE2 interaction ¹	2.7	64.2	10.2	>100
K417E	residue mutated to N/T in SA, BRA variants	0.5	1.8	1.0	3.6
Y453F	key residue evolved in Danish mink farms variants	3.2	10.9	5.9	3.3
Individual Mutations	Highly frequent mutations				
D614G	Wide global spread	2.4	11.9	6.2	23
S477N	Wide global spread	1.9	3.0	2.0	9.0
N439K	Widespread in Northern America, UK; increases RBD/ACE2 interaction ¹	1.3	7.3	5.3	12.9
A222V	Wide European spread	2.2	3.3	4.6	19.5
Individual Mutations	Within RBD epitope of DARPin® binder or reported resistance mutation for other therapeutics				
G446V		1.7	0.7	1.8	2.3

G476S		1.5	2.3	3.7	29
T478I		2.7	11.2	3.1	16.7
P479S		2.1	7.2	2.3	27.6
V483A		2.3	21.8	8.4	21.3
F486V	key residue for DARPin® RBD binder ² ; reduces RBD/ACE2 interaction ¹	>100	>100	>100	>100
Q493K		7.9	30	28.2	45.8
F490S	Reduces RBD/ACE2 interaction ¹	3.8	2.3	1.7	8.1

n.d.: not determined

*Mutations (SA): D80A, D215G, E484K, N501Y, A701V

**Mutations (UK): del69-70, del145, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H

¹ Influence of residue mutations on spike protein binding to human ACE2 (Yi et al. 2020, loc. cit.)

² Predicted interaction residue for DARPin® RBD binder (Walser et al. 2020)

Table 18: Efficacy of RBD domains of ALE049

These results show that ALE049 can neutralize variants B.1.1.7 and B.1.351 as efficiently as the wild-type form with IC₅₀ values in the low single-digit ng/mL range. ALE109 neutralized the B.1.351 variant equally efficiently as the wild-type form, with IC₅₀ values in the low single-digit ng/mL range. A slight potency loss was observed for ALE109 against the UK variant B.1.1.7 (IC₅₀ value of 70 ng/ml). Nevertheless, the potency of ALE109 against the UK variant B.1.1.7 was within the therapeutic range. It is interesting to note that the RBD binder of ALE109 (i.e. R1b) retained the same neutralization ability for B.1.1.7 as for the wild-type. The observed slight potency drop observed for ALE109 may be caused by the exposed mutations in the S2 domain (potentially P681H and T716I) alone or in combination with the NTD mutations. The structural determinants responsible for this slight potency drop are currently under investigation. Taken together, the results showed that both tested multi-specific binding proteins, ALE049 and ALE109, potently neutralized the wild-type form with IC₅₀ values in the low single-digit ng/mL range and neutralized the variants B.1.1.7 (UK) and B.1.351 (SA) with IC₅₀ values in the therapeutic range (i.e., low single-digit to double-digit ng/mL range).

Both multi-specific DARPin® molecules ALE049 and ALE109 also protected well against all individual mutations tested, with the notable exception of F486V for ALE049 and all three mono-valent DARPin® RBD binders. As F486 is a critical residue for ACE2 binding, the selective pressure on the virus favors its conservation, thus maintaining an important anchoring element for the binding of ALE049. The major impact of this mutation on ALE049 is not surprising, as previous structural analysis identified F486 as a core interacting residue for the three related but different RBD binders in ALE049 (Walser et al. 2020, loc. cit.). Consequently, the mutation F486V destabilizes the binding of the ALE049 molecule to the spike protein. Taken together, our analysis confirms that multi-specific DARPin® molecules of the invention remain highly

potent against spike proteins carrying the most frequently observed mutations, and mutations known to impact the binding of neutralizing antibodies, as expected from the multi-specific design of the DARPin® molecules.

- 5 Figure 29 shows the neutralization potency of single domain knock-out (k.o.) constructs of ALE109 against the wild type form of SARS-CoV-2. These experiments determined the contribution of each of the three spike protein-binding DARPin® domains of ALE109 to the neutralization activity against SARS-CoV-2. No potency loss compared to ALE109 was observed for the NTD knock out construct while some potency loss was observed for the RBD and S2 knock-out constructs. Without wishing to be bound by theory, the NTD
10 binding domain of ALE109 is believed to play a significant role in the neutralization activity of ALE109 against mutated forms or variants of SARS-CoV-2, e.g., by providing increased binding avidity to mutated spike protein.

Example 12: Viral passaging of SARS-CoV-2

- 15 Previous studies have shown that viral escape mutants may rapidly appear under selective pressure of a therapy (Ku et al., 2021, loc. cit.; Andreano et al., 2020, DOI: 10.1101/2020.12.28.424451). We used a viral passaging model adapted from Baum et al., Science 369, 1014–1018 (2020), to estimate the risk of viral escape from therapeutic pressure of multi-specific DARPin® proteins ALE049 and ALE109 and of a cocktail
20 of reference antibodies RA1 and RA2, in comparison to the mono-valent DARPin® binder R1b (SEQ ID NO: 3) and to the monoclonal antibodies S309, RA1 and RA2 applied as single molecules. S309 is an antibody that was isolated from a patient who recovered from severe acute respiratory syndrome (SARS) in 2003 and has been shown to be effective against SARS-CoV-2 infection in cells and in animal models (Pinto et al., Nature, Vol 583, p.290-295, 9 July 2020). S309 was prepared in the same manner as RA1 and
25 RA2 (see Example 11 above).

Experimental protocol:

- 1:5 serial dilutions of DARPin® proteins and monoclonal antibodies from 100 µg/ml to 0.032 µg/ml were prepared in Minimum Essential Medium (MEM) containing 2% FBS, supplements and 10 µM human serum
30 albumin (HSA; CSL Behring, Switzerland; 2%-FBS-MEM + HSA). 500 µl of virus suspension containing 1.5×10^6 plaque forming units (pfu) SARS-CoV-2 (a French isolate with the following differences compared to wild-type: V367F; E990A) in 2%-FBS-MEM + HSA were mixed with 500 µl of serially diluted DARPin® proteins or monoclonal antibodies and subsequently incubated for 1 hour at 37°C. The mixtures were then transferred to 80% confluent Vero E6 cells in 12 well plates and incubated for 4 days at 37°C, >85%
35 humidity and 5% CO₂. Each culture well was assessed for cytopathic effect (CPE) by microscopy. Supernatant was removed from wells with the highest DARPin® protein or antibody concentrations showing significant CPE (>20%) and used for total RNA extraction and further passaging. For subsequent rounds

of passaging, remaining 900 µl supernatant of selected wells was diluted to 4 ml in 2%-FCS-MEM + HSA and thereof 500 µl mixed with serial dilutions of DARPin® proteins or antibodies, incubated and the mixture transferred to 12 well plate with fresh Vero E6 cells as described above. Cell culture wells were assessed for CPE again after 4 days and the supernatant of wells with highest DARPin® protein or antibody concentrations with evident viral replication (CPE) harvested and used for additional passages (see Figure 30). A total of 4 passages were performed this way.

Results:

Resistant escape variants were selected by passaging the supernatant of cultures showing significant virus-induced cytopathic effect under the greatest selective pressure onto fresh cells while maintaining the selective pressure of increasing concentrations of antiviral proteins. Figure 31 shows the results obtained after the first to fourth incubation cycles (passages #1 to #4). After the first incubation cycle of four days (passage # 1) the mono-valent DARPin® binder Rb1 and the multi-specific DARPin® proteins ALE049 and ALE109, as well as the monoclonal antibody RA1 and the cocktail of the two monoclonal antibodies RA1 and RA2 conferred protection at the same concentrations of 0.4 µg/mL. The monoclonal antibody S309 was less efficient, requiring higher concentration (10 µg/mL) for protection and the monoclonal antibody RA2 as a single molecule was not protective up the highest concentration tested of 50 µg/mL. Under continuous selective pressure through passage 2 to 4, the monovalent DARPin® binder Rb1, and the individual monoclonal antibodies RA2 and RA1 lost the capacity to protect cells from virus-induced cytopathic effect, which manifested in complete CPE up to the highest selective pressure tested. In contrast, the two multi-specific DARPin® proteins ALE049 and ALE109, as single molecules or as a mixture, and the cocktail of two monoclonal antibodies (RA1 and RA2) remained effective and protected cells from CPE throughout the 4 passages.

The multi-specific DARPin® proteins ALE049 and ALE109 as single agents prevented the selection of escape mutants at concentrations of 2 µg/mL and 10 µg/mL, respectively, after 4 passages, while the combination of the two multi-specific DARPin® proteins ALE049 and ALE109 retained effectiveness even at a low concentration of 0.08 µg/mL. The antibody cocktail RA1 & RA2 prevented the selection of escape mutants at a concentration of 0.4 µg/mL after passage 4.

Example 13: Comparison of several multi-specific binding proteins in a neutralization assay using SARS-CoV-2 VSV pseudovirus (PsV nCoV)

Several multi-specific binding proteins of the invention were compared in a neutralization assay using SARS-CoV-2 VSV pseudovirus (PsV nCoV). The neutralization assay was performed similar as described in Example 4 above. The tested multi-specific binding proteins included ALE049, ALE058, ALE109, ALE126, ALE129 and ALE133. ALE049, ALE058 and ALE109 have been described above. ALE126,

ALE129 and ALE133 comprise a further engineered S1-NTD binding domain (vS07_08F10v47; SEQ ID NO: 85) as compared to ALE109, which comprises vS07_08F10v27 (SEQ ID NO: 76). ALE126, ALE129 and ALE133 differ from each other only in the length of the linker that connects the S1-NTD binding domain and the S2 binding domain (SEQ ID NO: 77).

- 5 The results of the PsV nCoV assay are shown in Figure 32, with EC50 values provided in nM. The experiment demonstrated that all the tested multi-specific binding proteins have overall comparable neutralization potencies in this SARS-CoV-2 VSV pseudovirus neutralization assay. The EC50 values of all tested constructs were in the range of 20 to 50 pM.

10 **Example 14: Pharmacokinetic analysis of multi-specific binding proteins of the invention in mice**

Another pharmacokinetic (PK) study was conducted to assess the PK characteristics of several multi-specific recombinant binding proteins of the invention in mice. Such PK characteristics are useful for dose predictions of multi-specific binding proteins of the invention in animal pharmacodynamic studies, in toxicology studies or in human clinical trials.

The PK study was performed essentially as described in Example 9.

The following multi-specific binding proteins were tested in this study:

SEQ ID NO	Sample name
39	ALE058
75	ALE109
87	ALE126
88	ALE129
84	ALE133

Table 19

20

Pharmacokinetic data analysis was performed, as also described in Example 9, using Phoenix WinNonlin™ 8.0 program from Certara. Calculation of the pharmacokinetic parameters of the study based on the mean concentration-time data of the animals dosed via intravenous bolus injection was performed with non-compartmental analysis (NCA model 200-202, IV bolus, linear trapezoidal linear interpolation).

25

The calculated pharmacokinetic parameters included at least the following: AUCinf_pred, AUClast, AUC_%extrapol, AUC_%Back_Ext_pred, Cmax, Tmax, Cl_pred, Vss_pred, t1/2 (HL_Lambda_z). The results are shown in Table 20 and Figures 33 and 34:

30

Parameter	Unit	ALE058	ALE109	ALE0126	ALE129	ALE133
AUCINF_pred	h*(nmol/L)	4261	10980	11145	12986	11909
AUClast	h*(nmol/L)	4253	10740	10726	12281	11246
Cmax	nmol/L	255	328	337	295	295
Tmax	h	0.083	0.083	0.083	0.083	0.083
Cl_pred	L/(h*kg)	0.00297	0.00115	0.00114	0.00097	0.00105
Vss_pred	L/kg	0.052	0.047	0.053	0.052	0.058
HL_Lambda_z	h	20.0	31.5	36.7	41.4	41.1
AUC_%Extrap_pred	(%)	0	2	4	5	6
AUC_%Back_Ext_pred	(%)	1	0	0	0	0

Table 20

Results and Conclusions

The results demonstrated that ALE109 has improved pharmacokinetic properties for systemic administration as compared to the precursor molecule ALE058. In the mono-exponential elimination phase of the serum concentration time profile, ALE109 serum concentrations declined with a half-life of 31.5 hours, whereas ALE058 showed a half-life of 20 hours. Moreover, the further engineered binding proteins ALE126, ALE129 and ALE133 displayed even more extended half-lives, when compared to ALE109, i.e. half-lives of 36.7 hours, 41.4 hours and 41.1 hours, respectively.

Example 15: In vivo evaluation of therapeutic efficacy of two multi-specific binding proteins, ALE049 and ALE109, in a Roborovski dwarf hamster model

In this study, a Roborovski dwarf hamster model was used to evaluate the efficacy of two multi-specific binding proteins of the invention as potential antiviral agents against SARS-CoV-2. The Roborovski dwarf hamster model is a valuable non-transgenic rodent model for SARS-CoV-2 research due to its high sensitivity to SARS-CoV-2 infections, as indicated by severe clinical signs (e.g. body weight loss or body temperature drop), viral replication in both the upper and lower respiratory tract and histopathological changes (Trimpert et al., Cell Reports 33, 108488, December 8, 2020).

Thus, the objective of this study was to investigate the therapeutic potential of ALE049 and ALE109 to inhibit or prevent body weight loss, replication of SARS-CoV-2 in the upper and lower respiratory tract and histopathological changes.

The tested binding proteins ALE049 and ALE109 are serum half-life extended with domains that bind to human serum albumin (HSA) (as well as to hamster serum albumin) to support long-acting activity. In vitro data demonstrated potent inhibition of SARS-CoV-2 virus infection in cell culture titration experiments by both binding proteins.

The study design provided that 5 groups of 6 animals each were used and treatment with tested binding protein was given either at 0, 6 or 24 hours after inoculation with SARS-CoV-2. The study design is illustrated in Figure 35 (ALE049 is also called MP0420 in this Example). Animals were treated by intraperitoneal (i.p.) administration, which served as a safe and reproducible alternative for intravenous administration. Animals in group 1 were treated with ALE049 at 20 mg/kg at 0h and animals in groups 2 to 4 were treated with ALE109 at 20 mg/kg at time points 0, 6 or 24h post-infection, respectively. Animals in the control group (group 5) were treated at time 0h with a placebo (i.e. vehicle of tested proteins only). Infection with SARS-CoV-2 was performed via the intranasal (i.n.) route, for which the dose and route of infection were based on results from earlier (model development) studies. Animals were weighed and temperatures were measured daily. Three animals for each group were euthanized on day 3 and 5 post-infection, respectively, to perform necropsy. Viral load in lung and throat tissue was determined by qPCR or virus titration and counting the plaque forming units (PFUs). Histopathological changes in selected tissues were assessed after euthanasia.

Materials

Formulation buffer and all test and control item formulations were prepared on the day of administration and were aliquoted into appropriate volumes for each group and stored at 4°C until administration. The volume of the test/control item administered was 100 µL per animal and adjusted to the animal's body weight measured on the administration day. The infection material was SARS-CoV-2, strain BetaCoV/Germany/BavPat1/2020.

Animals

Roborovski dwarf hamsters (*Phodopus roborovskii*), age 6-9 weeks, with a body weight range at the start of the study on day -2 of 20-25 gram, were used.

Procedures

Anaesthesia and analgesia

For infections and prior to euthanasia, animals were anesthetized by the injection of medetomidine, midazolam, and butorphanol at doses of 0.15, 2.0 and 2.5 mg/kg, respectively. Following infection, anaesthesia was antagonized with 0.15 mg/kg atipamezole.

Intraperitoneal administration

For intraperitoneal administration the animal was fixed by grasping the neck skin and the back skin between thumb and fingers. Subsequently, the hand was turned over so that the animal rests with its back in the palm of the hand. The head of the animal was kept downwards to prevent injection/damage in/of the organs and the needle was inserted left of the median line in the groin area, between the 4th and the 5th mammary gland/nipple. Finally, the needle was removed in a smooth motion.

Intranasal administration

For intranasal administration the animals were held on their back and the inoculum (20 µl) was equally divided over both nostrils using a pipette. Animals were held on their back until the complete inoculum was inhaled after which they were placed back in the cage to recover.

Sampling for histology

- 5 Histopathological analysis from selected tissues was performed for all animals euthanized at experimental or humane endpoints (i.e. day 2, 5 and 7). After fixation with 4% formalin for a minimum of 48 hours, sections from lung and throat were embedded in paraffin and the tissue sections were stained for histological examination.

End-point serum samples

- 10 Serum samples on day 2, 5 and 7 post-infection were collected during euthanasia and immediately transferred to appropriate tubes containing a clot activator.

Virological analysis

Detection of viral RNA

- 15 RNA was extracted from nasal washes and tracheal swabs with the RTP DNA/RNA Virus Mini Kit (Strattec, Birkenfeld, Germany) according to the manufacturer's instructions. The innuPREP Virus DNA/RNA Kit (Analytic Jena, Jena, Germany) was used for RNA extractions from tissue samples. Viral RNA was quantified using a one-step RT qPCR reaction with the NEB Luna Universal Probe One-Step RT-qPCR (New England Biolabs, Ipswich, MA, USA) and the 2019-nCoV RT-qPCR primers and probe (E_Sarbeco)
- 20 on a StepOnePlus RealTime PCR System (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Viral RNA copies were then normalized to cellular RPL18 as previously described. Standard curves for absolute quantification were generated from serial dilutions of SARS-CoV-2 RNA obtained from a full-length virus genome cloned as a bacterial artificial chromosome and propagated in E. coli.

- 25 *Detection of replication competent virus*

- Duplicate 10-fold serial dilutions were used to determine the virus titers in confluent layers of Vero E6 cells (SARS-CoV-2 titration on Vero E6 cells). To this end, serial dilutions of the samples (lung tissue homogenates) were made and incubated on Vero E6 monolayers for 2 hours at 37 degrees. Cells were washed and overlaid with semi-solid cell culture medium containing 1.5% Avicel and incubated for 48 h at
- 30 37 degrees after which plates were fixed with 4% formalin and stained with 0.75% crystal violet for plaque counting.

Histopathology

- Histopathological analysis from selected tissues was performed for all animals euthanized due to reaching an experimental or humane endpoint. After fixation with 4% formalin for 48 hours, sections from lungs were
- 35 embedded in paraffin and the tissue sections were stained for histological examination.

Results

The aim of this study was to assess the therapeutic potential of binding proteins ALE049 and ALE109 in a COVID-19 Roborovski dwarf hamster model. For this assessment, the hamsters were treated therapeutically with 20 mg/kg of binding protein at 0, 6 or 24 hours after the SARS-CoV-2 intranasal challenge with 105 PFUs per animal.

- 5 All 24 animals, treated with a binding protein of the invention at either 0, 6 or 24 hours post-infection, survived until the day of sacrifice (i.e. day 3 or 5), while 5 out of 6 animals from the placebo group had to be taken out of the study prior to the study endpoints by day 3 due to severe clinical symptoms and body weight loss. Average of body weights was determined in each of the five study groups. The placebo group showed a steady decrease in body weight until the timepoint at day 3. After this timepoint only one animal
10 from the placebo group could be taken forward to day 5 for further evaluation. All test protein-treated groups demonstrated no or only minor body weight losses. When comparing the various timepoints for treatment or when comparing ALE049 with ALE109, no significant differences were observed in terms of clinical symptoms or body weight loss (see Figure 36). Generally, there seemed to be some variation in the response of the individual animals to either the viral infection or the treatment which led to a relatively wide
15 spread in body weight loss.

- Measurement of viral titers in lung by live virus titration of lung homogenate and plaque counting demonstrated that, already at day 3, a reduction in the live virus could be observed (Figure 37A). This was especially pronounced for the timepoint where the treatment was initiated directly after the viral challenge (0 h timepoint). Still, also the treatment injections with ALE109 administered at 6 h or 24 h after the viral
20 challenge showed a considerable reduction in the load of infectious virus already at day 3. This effect seemed to be even more pronounced for the 3 animals remaining at day 5 where only 5 out of 12 binding protein-treated animals had detectable infectious virus remaining in the lung homogenates (Figure 37B). Reduction of viral RNA genome copies as detected by qPCR seemed to be considerable slower than the elimination of infectious virus. At day 3, only 1 out of 3 animals for each of the 0 h time points showed a
25 reduction of viral RNA in the lungs (Figure 37C). On viral genome level, more pronounced differences between the binding protein-treated groups and the placebo group occurred only at day 5 post infection, where again a trend for better reduction of viral genomic RNA could be observed for the earlier time points of the treatment (Figure 37D). When comparing ALE049 and ALE109 at the 0 h time point, a trend for better virus elimination could be observed for ALE049.

- 30 The histopathological assessment for various parameters in different tissues was scored with a ranking from 0 (no obvious histopathological signs) to 4 (most severe histopathological signs). All scores were averaged for the different treatment groups and categorized into four sets: i) inflammation, ii) blood vessels, iii) alveoli, and iv) bronchi. The sum graphs for all the averaged parameters are provided in Figures 38A to 38D. Generally, in all four categories, clear differences were observed between the binding protein-treated
35 hamsters and the placebo-treated hamsters. According to the histopathological assessment, all binding protein treatments had strongest effects on the reduction of tissue damage in bronchi (Figure 38D), alveoli (Figure 38C) and blood vessels (Figure 38B) and lowest impact on the reduction of inflammatory cells

(Figure 38A), when compared to the placebo group. The group treated with ALE109 at the timepoint 6 h after viral infection indicated the lowest reduction of inflammation and tissue damage amongst all binding protein-treated groups.

5 Conclusions

At viral inoculation of 105 PFUs, the Roborovsky dwarf hamster model is a well-suited COVID-19 disease model, in which non-treated animals generally develop strong clinical symptoms reaching criteria for euthanasia. The therapeutic treatment of the animals with either ALE049 at 0 hours after the viral challenge or ALE109 at 0, 6 or 24 hours after the viral challenge, led to significant reductions of severe clinical symptoms, comparable for all binding protein treatment groups, such that none of the 24 binding protein-treated animals reached euthanasia criteria prior to the official sacrifice time points at day 3 or 5, while for the 6 placebo-treated animals, 2 animals at day 2 and another 3 animals at day 3 developed strong clinical symptoms and had to be taken out of the study, with only one placebo-treated animal remaining on study until day 5.

15 In terms of viral load for infectious virus or viral genome copies, a clear reduction was observed for all binding protein treatment groups. This reduction increased from day 3 to day 5 and the treatment groups where the therapy was given earlier seemed to respond with a more pronounced reduction. When comparing ALE049 with ALE109, administered at 0h, the ALE049 treatment group responded slightly better, with respect to the rather low number of animals per treatment group.

20 Histopathological findings in the lungs showed a clear reduction of pathological scores for all binding protein treatment groups when compared to the placebo group. These findings seem to be independent of the therapeutic regimens tested in this study.

In conclusion, both ALE049 and ALE109 demonstrated therapeutic potential against SARS-CoV-2 infections, using a Roborovsky dwarf hamster model.

25 The specification is most thoroughly understood in light of the teachings of the references cited within the specification. The embodiments within the specification provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention. The skilled artisan readily recognizes that many other embodiments are encompassed by the invention. All publications, patents, and GenBank sequences cited in this disclosure are incorporated by reference in their entirety. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material. The citation of any references herein is not an admission that such references are prior art to the present invention.

30 Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.