POLYPEPTIDES ANTAGONIZING WNT SIGNALING IN TUMOR CELLS

FIELD OF THE INVENTION

The present invention relates to novel low-density lipoprotein receptor-like protein 5 (LRP5) binding polypeptides. The invention also relates to nucleic acids encoding such polypeptides; to methods for preparing such polypeptides; to host cells expressing or capable of expressing such polypeptides; to compositions comprising such polypeptides; and to uses of such polypeptides or such compositions, in particular for therapeutic purposes in the field of cancer diseases.

BACKGROUND OF THE INVENTION

Activation of the Wnt signaling pathway requires binding of extracellular Wnt ligands to the Frizzled receptor and to the co-receptor LRP5 (Accession number: UniProtKB - O75197 / LRP5_HUMAN). There are 19 Wnt proteins and 10 Frizzled receptors in mammalian cells. In the absence of Wnt ligand, cytoplasmic beta-catenin is phosphorylated by a protein complex consisting of the scaffolding proteins Axin and APC and the kinases GSK3beta and CK1a. Subsequent recognition by the ubiquitin ligase beta-TrcP leads to ubiquitin-mediated degradation of beta-catenin. In the presence of Wnt ligand, binding of Wnt to Frizzled and LRP5 leads to recruitment of the cytoplasmic effector protein Dvl and phosphorylation of the LRP5 cytoplasmic tail, which provides the docking site for Axin. Axin sequestration by LRP5 leads to the inactivation of the Axin-APC-GSK3beta complex and, therefore, intracellular beta-catenin stabilization and accumulation. Hence, cytoplasmic levels of beta-catenin rise, and beta-catenin migrates to the nucleus and complexes with members of the T-cell factor (TCF)/Lymphoid enhancer-binding factor (LEF) family of transcription factors. Basal transcription machinery and transcriptional co-activators are then recruited, including cAMP response element-binding protein (CREB)–binding protein (CBP) or its homolog p300, leading to expression of various target genes, including Axin2, cyclin D1 and c-Myc.

An additional level of ligand-dependent Wnt pathway regulation is mediated by the E3 ligase RNF43, and its closely related homologue ZNRF3, and by the secreted R-Spondin proteins (de Lau *et al*. “The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength”. *Genes Dev*. 2014; 28(4):305-16). RNF43 mediates the ubiquitination of the Frizzled/LRP5 receptor complex at the cell surface, leading to its degradation and, thereby, inhibiting ligand-dependent Wnt pathway activity. The activity of RNF43 is

counteracted by the R spondin family members (R-spondin 1 to 4 ligands). When R-Spondin ligand is present, it removes RNF43 from the cell surface, allowing Frizzled/LRP5 complex accumulation and enhancement of Wnt signaling in the presence of Wnt ligands.

LRP5 functions as gatekeeper of ligand dependent Wnt signaling activation and, therefore, may be considered as targets to achieve complete blockade of the pathway mediated by all 19 Wnt ligands and 10 Frizzled receptors and enhanced by R-spondin ligands. In particular, Wnt ligands can be divided into a Wnt1 class and a Wnt3a class, each binding to different epitopes/regions of LRP5 for signaling. The ectodomain of LRP5 comprises four repeating units of a beta-propeller connected to an EGF-like domain, followed by three LDLR-type A repeats. Combined structural and functional analyses of LRP5 show that Wnt1 (Wnt1-class ligand) binds to a fragment containing beta-propeller 1 and 2 and Wnt3a binds to a fragment containing beta-propeller 3 and 4.

LRP5 and agents interfering with LRP5 activity have been described in the context of various indications including bone disorders, lipid modulated disorders, Alzheimers disease, rheumatoid arthritis, and insulin-dependent diabetes (see e.g., WO2002/092015, WO2006/102070, WO2009/155055, and WO1998/046743).

Hyperactivation of Wnt signaling is further involved in the pathogenesis of various types of cancer. In some cancer types frequent mutations in downstream signaling molecules contribute to constitutively activated Wnt pathway (e.g. APC mutations in colorectal cancer; beta-catenin activating mutation in hepatocellular carcinoma). In contrast, in Triple Negative Breast Cancer (TNBC), Non Small Cell Lung Cancer (NSCLC), pancreatic adenocarcinoma and in a subset of Colo-Rectal Cancer (CRC) and endometrial cancers, Wnt signaling activation is driven by a ligand dependent mechanism (i.e. by an autocrine/paracrine Wnt activation), as detected by beta-catenin intracellular accumulation. In NSCLC, TNBC and pancreatic adenocarcinoma, ligand dependent Wnt activation is mediated by multiple mechanisms, including increased expression of the Wnt ligands and/or of LRP5 receptors, or silencing of LRP5 negative regulator DKK1 (TNBC: Khramtsov *et al*. "Wnt/beta-catenin pathway activation is enriched in basal-like breast cancers and predicts poor outcome". *Am J Pathol*. 2010; 176(6): 2911-20; NSCLC: Nakashima *et al*. "Wnt1 overexpression associated with tumor proliferation and a poor prognosis in non-small cell lung cancer patients". *Oncol Rep*. 2008; 19(1):203-9; Pancreatic cancer: Zhang *et al*. "Canonical wnt signaling is required

for pancreatic carcinogenesis". *Cancer Res*. 2013; 73(15):4909-22). Ligand dependent Wnt activation in tumors was shown to drive tumor growth and resistance to chemotherapy or immunotherapy, and is linked to recurrence in pre-clinical models.

Some LRP5-binding molecules, able to modulate the Wnt signaling pathway, are known in the art:

Dickkopf-1 (DKK1) is a LRP5 inhibitor. DKK1 associates with LRP5 and the transmembrane protein, Kremen, inhibits Wnt signaling and leads to rapid LRP5 internalization. It was shown that DKK1 inhibits both Wnt1 and Wnt3a mediated signaling.

It was further shown that DKK1 treatment *in vivo* causes severe toxicity in the gastrointestinal tract. In particular, it was shown that adenovirus-mediated expression of DKK1 in adult mice markedly inhibited proliferation in small intestine and colon, accompanied by progressive architectural degeneration, severe body weight loss and mortality from colitis and systemic infection. In particular, LRP5 is expressed in the intestine in the proliferative epithelial cells and is required for proliferation of the intestinal epithelium, suggesting that LRP5 inhibition may be toxic for this and other normal tissues (Zhong *et al*. "Lrp5 and Lrp6 play compensatory roles in mouse intestinal development". *J Cell Biochem*. 2012; 113(1):31-8). This makes it doubtful whether agents which inhibit LRP5, or which inhibit the Wnt (Wnt1 and Wnt3a) signaling pathway in general, can be used for therapeutic purposes, e.g. can be developed as anti-cancer drugs.

WO 1998/046743 A1 discloses an antibody specific for LRP5 and suggests several LRP5 peptides for raising such an antibody. However, no experimental results with respect to generating such an antibody are described, nor is such an antibody specifically disclosed.

US9175090 discloses methods of inhibiting Wnt signaling in a cancer cell with a defect in Apc expression by administering a monoclonal antibody, specifically an IgM antibody that binds LRP5.

WO2013/109819 discloses anti-LRP5 antibodies, in particular antibodies potentiating Norrin activity and/or Norrin/Fzd4 signaling, and their use in the treatment of conditions associated with angiogenesis.

However, none of the LRP5-binding molecules described in the art has so far been authorized by health authorities for the use as a medicament to treat any disease.

Specifically, such use requires very specific binding properties, the right specificity, so that such molecules do or do not bind, activate or inhibit other targets (e.g. resulting in undesired activation or inhibition of other signaling pathways, or lack of activation or inhibition with respect to target isoforms), in the case of bi- or multispecific agents the right balance between the two or more binding specificities, suitable pharmacokinetic and –dynamic properties, an acceptable toxicological profile, and of course *in vivo* efficacy.

In view of the above, there is a need for novel therapeutic agents that allow an efficient treatment of several types of cancer diseases and tumors. It is thus an object of the invention to provide such pharmacologically active agents that can be used in the treatment of several cancer diseases, including NSCLC and TNBC.

In particular, it is an object of the invention to provide such pharmacologically active agents, compositions and/or methods of treatment that provide certain advantages compared to the agents, compositions and/or methods currently used and/or known in the art. These advantages include *in vivo* efficacy, improved therapeutic and pharmacological properties, less side effects, and other advantageous properties such as improved ease of preparation or reduced costs of goods, especially as compared to candidate drugs already known in the art.

BRIEF SUMMARY OF THE INVENTION

According to a first aspect of the invention, the present invention provides a polypeptide which binds to low-density lipoprotein receptor-like protein 5 (LRP5), the polypeptide comprising an immunoglobulin single variable domain (ISVD) selected from the group consisting of the following LRP5-binding ISVDs (i) to (iv):

(i) an ISVD with the following complementarity-determining region (CDR) sequences:

CDR1: TYVMG (SEQ ID NO:1)

CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)

CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3),

(ii) an ISVD with the following CDR sequences:

CDR1: RYAVA (SEQ ID NO:4)

CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)

CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6),

(iii) an ISVD with the following CDR sequences:

CDR1: IGAMG (SEQ ID NO:7)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9), and

(iv) an ISVD with the following CDR sequences:

CDR1: INAMG (SEQ ID NO:10)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9).

In this aspect, the polypeptide of the present invention preferably comprises a first ISVD (a) selected from ISVDs (i) and (ii) as defined above and a second ISVD (b) selected from ISVDs (iii) and (iv) as defined above. Even more preferably, one or more of ISVDs (i)-(iv) are defined by comprising the following sequences, respectively:

(i)

AVQLVESGGGLVQPGGSLRLSCAASGRTFSTYVMGWFRQAPGKEREFVAAISWSGGS TYYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCAASRGTSTPSRASGVSRYD YWGQGTLVTVSS (SEQ ID NO:11), or

EVQLVESGGGLVQPGGSLRLSCVASGRTFSTYVMGWFRQAPGKEREFVAAISWSGGS TYYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCAASRGTSTPSRASGVSRYD YWGQGTLVTVSS (SEQ ID NO:23),

(ii)

EVQLVESGGGLVQPGGSLRLSCAASGLTFSRYAVAWFRQAPGKEREFVAAITWSSGRI DYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCAADRRPRSTGRSGTGSPSTY DYWGQGTLVTVSSA (SEQ ID NO:12),

(iii)

AVQLVESGGGLVQPGGSLRLSCAASGSIFRIGAMGWYRQAPGKQRELVAAVSSGGST YYVDSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCNRETGPYGPPKRDYWGQGT LVTVSS (SEQ ID NO:13), or

EVQLVESGGGLVQPGGSLRLSCAASGSIFRIGAMGWYRQAPGKQRELVAAVSSGGST YYVDSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCNRETGPYGPPKRDYWGQGT LVTVSSA (SEQ ID NO:22), and

(iv)

EVQLVESGGGLVQPGGSLRLSCAASGSIFRINAMGWYRQAPGKQRELVAAVSSGGST YYVDSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCNRETGPYGPPKRDYWGQGT LVTVSS (SEQ ID NO:14).

The terms “first” and “second” with respect to such ISVDs or domains in general, as used herein, is solely intended to indicate that these domains are two different domains (as they will at least include different CDR sequences). Thus, these terms shall not be understood to refer to the exact order or sequence of the domains within such polypeptide chain. In other words, the above ISVDs (a) and (b) may either be arranged in the order (a)-(b) or in the order (b)-(a) within the polypeptides described herein.

In some embodiments, the polypeptide described herein comprises
- a first ISVD (a) comprising the following CDR sequences:
  CDR1: TYVMG (SEQ ID NO:1)
  CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)
  CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3), and
- a second ISVD (b) comprising the following CDR sequences:
  CDR1: IGAMG (SEQ ID NO:7)
  CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
  CDR3: ETGPYGPPKRDY (SEQ ID NO:9).

In some embodiments, the polypeptide described herein comprises
- a first ISVD (a) comprising the following CDR sequences:
  CDR1: TYVMG (SEQ ID NO:1)
  CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)
  CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3), and
- a second ISVD (b) comprising the following CDR sequences:
  CDR1: INAMG (SEQ ID NO:10)
  CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
  CDR3: ETGPYGPPKRDY (SEQ ID NO:9).

In some embodiments, the polypeptide described herein comprises
- a first ISVD (a) comprising the following CDR sequences:
  CDR1: RYAVA (SEQ ID NO:4)
  CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)
  CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6), and
- a second ISVD (b) comprising the following CDR sequences:
  CDR1: IGAMG (SEQ ID NO:7)
  CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
  CDR3: ETGPYGPPKRDY (SEQ ID NO:9).

In some embodiments, the polypeptide described herein comprises
- a first ISVD (a) comprising the following CDR sequences:
CDR1: RYAVA (SEQ ID NO:4)
CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)
CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6), and
- a second ISVD (b) comprising the following CDR sequences:
CDR1: INAMG (SEQ ID NO:10)
CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3: ETGPYGPPKRDY (SEQ ID NO:9).

Specifically, the ISVDs of the polypeptides described herein (e.g. ISVDs comprising the CDR sequences as defined above) are VHH domains, preferably humanized VHH domains.

In some embodiments, ISVD (i) of the polypeptides described herein comprises the sequence of SEQ ID NO:11 or the sequence of SEQ ID NO:23. In some embodiments, ISVD (ii) comprises the sequence of SEQ ID NO:12. In some embodiments, ISVD (iii) comprises the sequence of SEQ ID NO:13 or the sequence of SEQ ID NO:22. In some embodiments, ISVD (iv) comprises the sequence of SEQ ID NO:14.

Specifically, the polypeptides described herein comprise a first ISVD (a) and a second ISVD (b), said first ISVD comprising a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:23, and said second ISVD comprising a sequence selected from the group consisting of SEQ ID NO:13, SEQ ID NO:14 and SEQ ID NO:22. In some embodiments, the first ISVD comprises the sequence of SEQ ID NO:11 and the second ISVD comprises the sequence of SEQ ID NO:22. In some embodiments, the first ISVG comprises the sequence of SEQ ID NO:12 and the second ISVD comprises the sequence of SEQ ID NO:13. In some embodiments, the first ISVD comprises the sequence of SEQ ID NO:23, and the second ISVD comprises the sequence of SEQ ID NO:14. In some embodiments, the first ISVD comprises the sequence of SEQ ID NO:11 and the second ISVD comprises the sequence of SEQ ID NO:14. In some embodiments, the first ISVD comprises the sequence of SEQ ID NO:11 and the second ISVD comprises the sequence of SEQ ID NO:13. In some embodiments, the first ISVD comprises the sequence of SEQ ID NO:12 and the second ISVD comprises the sequence of SEQ ID NO:14. In some embodiments, the first ISVD comprises the sequence of SEQ ID NO:12 and the second ISVD comprises the

sequence of SEQ ID NO:22. In some embodiments, the first ISVD comprises the sequence of SEQ ID NO:23 and the second ISVD comprises the sequence of SEQ ID NO:22. In some embodiments, the first ISVD comprises the sequence of SEQ ID NO:23 and the second ISVD comprises the sequence of SEQ ID NO:13.

According to one aspect, the first ISVD and the second ISVD of the polypeptides described herein are covalently linked by a linker peptide, wherein said linker peptide optionally comprises or consists of a third ISVD; e.g. an albumin binding ISVD.

According to a further aspect, the polypeptides described herein further comprise a half-life extending moiety, which is covalently linked to said polypeptide and is optionally selected from the group consisting of an albumin binding moiety, such as an albumin binding peptide or an albumin binding immunoglobulin domain, a transferrin binding moiety, such as an anti-transferrin immunoglobulin domain, a polyethylene glycol molecule, human serum albumin, and a fragment of human serum albumin. Specifically, the half-life extending moiety is an albumin binding moiety, preferably an albumin binding ISVD, even more preferably the Alb11 domain comprising the following sequence:

EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSIS GSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSRSS QGTLVTVSS (SEQ ID NO:21)

According to a preferred aspect, the polypeptides described herein comprise a first (a) and a second (b) LRP5 binding ISVD and a third ISVD (c), e.g., an albumin binding ISVD (c);

- said first LRP5 binding ISVD (a) selected from the group consisting of ISVDs (i) and (ii):

(i) an ISVD comprising the following CDR sequences:

CDR1: TYVMG (SEQ ID NO:1)

CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)

CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3), and

(ii) an ISVD comprising the following CDR sequences:

CDR1: RYAVA (SEQ ID NO:4)

CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)

CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6),

-said second ISVD (b) selected from the group consisting of ISVDs (iii) and (iv):

(iii) an ISVD comprising the following CDR sequences:

CDR1: IGAMG (SEQ ID NO:7)
CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3: ETGPYGPPKRDY (SEQ ID NO:9), and

(iv) an ISVD comprising the following CDR sequences:
CDR1: INAMG (SEQ ID NO:10)
CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3: ETGPYGPPKRDY (SEQ ID NO:9), and

-said albumin binding ISVD (c) defined by comprising the following CDR sequences:
CDR1: SFGMS (SEQ ID NO:15)
CDR2: SISGSGSDTLYADSVKG (SEQ ID NO:16)
CDR3: GGSLSR (SEQ ID NO:17).

For example, the polypeptide comprises a first and second ISVD as defined by the CDR sequences above and a third ISVD, which is an albumin binding ISVD as defined by the CDR sequences above, and which directly or indirectly links the first and second ISVD. In some embodiments, the first ISVD is covalently linked via a peptide linker to the third ISVD which is covalently linked to the second ISVD via a peptide linker. The terms “first” and “second” as noted above do not indicate their position within the polypeptide, thus from N to C terminus the ISVD sequences within the polypeptide can be arranged either in the order ISVDs (a)-(c)-(b), (a)-[linker]-(c)-[linker]-(b), (b)-(c)-(a). (b)- [linker]-(c)-[linker]-(a).

In some embodiments, the polypeptide comprises
- a first ISVD comprising the following CDR sequences:
CDR1: TYVMG (SEQ ID NO:1)
CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)
CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3),

-a second ISVD comprising the following CDR sequences:
CDR1: IGAMG (SEQ ID NO:7)
CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3: ETGPYGPPKRDY (SEQ ID NO:9), and

-an albumin binding ISVD (a third ISVD) defined by comprising the following CDR sequences:
CDR1: SFGMS (SEQ ID NO:15)
CDR2: SISGSGSDTLYADSVKG (SEQ ID NO:16)
CDR3: GGSLSR (SEQ ID NO:17).

In some embodiments, the polypeptide comprises

- a first ISVD comprising the following CDR sequences:

CDR1: TYVMG (SEQ ID NO:1)

CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)

CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3),

-a second ISVD comprising the following CDR sequences:

CDR1: INAMG (SEQ ID NO:10)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9), and

-an albumin binding ISVD defined by comprising the following CDR sequences:

CDR1: SFGMS (SEQ ID NO:15)

CDR2: SISGSGSDTLYADSVKG (SEQ ID NO:16)

CDR3: GGSLSR (SEQ ID NO:17).

In some embodiments, the polypeptide comprises

- a first ISVD comprising the following CDR sequences:

CDR1: RYAVA (SEQ ID NO:4)

CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)

CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6),

-a second ISVD with the following CDR sequences:

CDR1: IGAMG (SEQ ID NO:7)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9), and

-an albumin binding ISVD defined by comprising the following CDR sequences:

CDR1: SFGMS (SEQ ID NO:15)

CDR2: SISGSGSDTLYADSVKG (SEQ ID NO:16)

CDR3: GGSLSR (SEQ ID NO:17).

In some embodiments, the polypeptide comprises

- a first ISVD comprising the following CDR sequences:

CDR1: RYAVA (SEQ ID NO:4)

CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)

CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6),

-a second ISVD comprising the following CDR sequences:

CDR1: INAMG (SEQ ID NO:10)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9), and

-an albumin binding ISVD defined by comprising the following CDR sequences:

CDR1: SFGMS (SEQ ID NO:15)

CDR2: SISGSGSDTLYADSVKG (SEQ ID NO:16)

CDR3: GGSLSR (SEQ ID NO:17).

In some embodiments, the ISVDs as defined by their CDR sequences in the above polypeptides are arranged such that the albumin binding ISVD directly or indirectly (e.g. via a linker peptide) links the first and the second ISVD.

In some embodiments, the polypeptide described herein comprises or consists of a sequence selected from SEQ ID NOs: 18, 19 and 20.

In yet a further aspect, the present invention relates to a polypeptide which binds to LRP5, comprising an ISVD which competes for binding to LRP5 with a reference ISVD, wherein the reference ISVD has the sequence identified by SEQ ID NOs: 11, 12, 13, 14, 22 or 23.

In another aspect, the present invention relates to a polypeptide which binds to LRP5, comprising a first ISVD (a) which competes for binding to LRP5 with a first reference ISVD, wherein the first reference ISVD has the sequence identified by SEQ ID NOs: 11, 23 or 12, and a second ISVD (b) which competes for binding to LRP5 with a second reference ISVD, wherein the second reference ISVD has the sequence identified by SEQ ID NOs: 13, 22 or 14.

In yet another aspect, the present invention relates to a polypeptide which binds to LRP5, comprising a first LRP5-binding domain, preferably an ISVD, and a second LRP5-binding domain, preferably an ISVD, wherein the first domain binds a region of LRP5 that is distinct from the LRP5 region which the second domain binds to. According to a preferred embodiment, the first LRP5-binding domain blocks a Wnt3a binding site of LRP5, and preferably inhibits Wnt3a-driven target gene transcription, and/or wherein the second LRP5-binding domain blocks a Wnt1 binding site of LRP5, and preferably inhibits Wnt1-driven target gene transcription.

According to further aspects, the invention relates to nucleic acid molecules, expression vectors, host cells, and methods of manufacturing used in the production of a polypeptide of the invention. Nucleic acid molecules encoding the polypeptides of the invention can be used, in an isolated form, for constructing respective expression vectors, which then are be transfected into host cells used for biopharmaceutical

production of the polypeptides of the invention. Such method of manufacturing typically comprises the steps of culturing the host cell under conditions that allow expression of the polypeptide, recovering the polypeptide and purifying it according to methods known in the art.

Further aspects, embodiments, uses and methods involving the polypeptides of the invention will become clear from the following detailed description of the invention and from the appended claims.

The invention provides for novel molecules that allow a more efficient treatment of several cancer types, such as TNBC, CRC, and NSCLC, with less side effects. The polypeptides of the invention provide for a surprising therapeutic effect (i.e. efficacy) in the treatment of cancer patients, in that they may induce tumor regression resulting in pathological complete response (pCR). This, in turn, is expected to result in significant improvement of progression free survival and overall survival, especially in high unmet medical need indications such as e.g. in breast cancer. Thus, the polypeptides of the invention provide for novel therapeutic options in the treatment of several cancer types, esp. those showing a deregulated Wnt signaling pathway and beta-catenin accumulation.

Furthermore, the polypeptides of the invention are easy to manufacture, have a high stability and low antigenicity, and offer a variety of options regarding administration routes, in addition to injection and infusion.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of biparatopic polypeptides antagonizing Wnt1 and Wnt3a signaling. They consist of three domains, with two domains binding to distinct epitopes of LRP5 (Wnt1 and Wnt3a blocker) and one domain for half-life extension (human serum albumin binder).

Figures 2A/2B show the binding of three half-life extended biparatopic LRP5–selective VHH constructs, i.e. especially preferred polypeptides of the present invention, to human LRP5 (Figure 2A) and human LRP6 (Figure 2B) overexpressing HEK293 cell lines when compared to the negative control consisting of a non-targeting binder (VHH construct that binds to a bacterial protein which is not expressed in HEK293 cells) and compared to a LRP5/6-cross-reactive binder, MOR08168IgG1LALA 6475 scfv. The present half-life

extended biparatopic LRP5 specific VHH constructs only bind to human LRP5 with no binding detected on human LRP6, in contrast to the LRP5/6-cross-reactive binder.

Figures 3A/3B show complete DKK1 competition of three half-life extended biparatopic LRP5-selective VHH constructs, i.e. especially preferred polypeptides of the present invention, for binding to both human LRP5 (Figure 3A) and mouse LRP5 (Figure 3B) overexpressing HEK293 cell lines, as detected by a FACS-based DKK1 competition assay.

Figures 4A/4B show complete inhibition of Wnt1 (Fig. 4A) and Wnt3a pathway (Fig. 4B) by three half-life extended biparatopic LRP5-selective VHH constructs, i.e. especially preferred polypeptides of the present invention.

Figure 5 shows inhibition of Wnt signaling in cancer cells, as detected by inhibition of relative Axin2 mRNA expression (right panel), and cell proliferation (left panel) as detected by decreased percentage (%) of viable cells, after treatment with a half-life extended biparatopic LRP5-selective VHH construct, i.e. a particularly preferred polypeptide of the present invention (final concentration of 1µM).

Figures 6A/6B show *in vivo* efficacy of the half-life extended biparatopic LRP5-selective VHH constructs (F012900082 in Figure 6A and F012900141in Figure 6B), i.e. especially preferred polypeptides of the present invention, in a Wnt driven tumor models (MMTV-Wnt1 xenograft model).

Figure 7 shows Wnt pathway inhibition in tumors treated with the half-life extended biparatopic LRP5-selective VHH constructs, i.e. especially preferred polypeptides of the present invention, as detected by reduction of Axin2/RNF43/Notum mRNA expression relative to the control group.

Figure 8 shows that LRP5 is higher expressed than LRP6 in human monocyte-derived dendritic cells (left panel). Furthermore, the suppressive effect of Wnt3a on T-cell activation, as determined by interferon-gamma release, is strongly mitigated by treatment with half-life extended biparatopic LRP5–selective VHH constructs (center and right panels), i.e. preferred peptides of the present invention. Each symbol represents a unique dendritic cell (DC) donor. Data shown are normalized to TNFalpha levels of the untreated control and each symbol represents a unique donor pair for DC and T cells.

Figure 9 shows selective inhibition of *RNF43* mutant CRC organoid proliferation (*RNF43* mut1 and *RNF43* mut2) when compare to no significant effect on *RNF43* wild type CRC

organoid proliferation (RNF43 WT), as detected by decreased percentage (%) of viable cells, after treatment with a half-life extended biparatopic LRP5-selective VHH construct, i.e. a particularly preferred polypeptide of the present invention. IC50 nM values of inhibition of *RNF43* mutant CRC organoid proliferation are also reported.

Figure 10 shows selective inhibition of Wnt signaling in *RNF43* mutant CRC organoids (*RNF43* mut1 and *RNF43* mut2) when compare to no significant effect in a *RNF43* wild type CRC organoid (RNF43 WT), as detected by inhibition of relative hAxin2 mRNA expression, after treatment with a half-life extended biparatopic LRP5-selective VHH construct, i.e. a particularly preferred polypeptide of the present invention (final concentration of 62nM).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The above and other aspects and embodiments of the invention will become clear from the further description herein, in which:

a) Unless indicated or defined otherwise, all terms used have their usual meaning in the art, which will be clear to the skilled person. Reference is for example made to the standard handbooks, such as Sambrook et al, "Molecular Cloning: A Laboratory Manual" (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory Press (1989); Lewin, "Genes IV", Oxford University Press, New York, (1990), and Roitt *et al.*, "Immunology" (2nd Ed.), Gower Medical Publishing, London, New York (1989), as well as to the general background art cited herein. Furthermore, unless indicated otherwise, all methods, steps, techniques and manipulations that are not specifically described in detail can be performed and have been performed in a manner known *per se*, as will be clear to the skilled person. Reference is for example again made to the standard handbooks, to the general background art referred to above and to the further references cited therein.

b) Unless indicated otherwise, the terms "*immunoglobulin*" and "*immunoglobulin sequence*" - whether used herein to refer to a *heavy chain antibody* or to a *conventional 4-chain antibody* - are used as general terms to include both the full-size antibody, the individual chains thereof, as well as all parts, domains or fragments thereof (including but not limited to antigen-binding domains or fragments such as VHH domains or VH/VL domains, respectively). In addition, the term "sequence" as used herein (for example in

terms like "immunoglobulin sequence", "antibody sequence", "(single) variable domain sequence", "VHH sequence" or "protein sequence"), should generally be understood to include both the relevant amino acid sequence as well as nucleic acid sequences or nucleotide sequences encoding the same, unless the context requires a more limited interpretation;

c) The term "*domain*" (of a polypeptide or protein) as used herein refers to a folded protein structure which has the ability to retain its tertiary structure independently of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins, and in many cases can be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain.

d) The term "*immunoglobulin domain*" as used herein refers to a globular region of an antibody chain (such as e.g. a chain of a conventional 4-chain antibody or of a heavy chain antibody), or to a polypeptide that essentially consists of such a globular region. Immunoglobulin domains are characterized in that they retain the immunoglobulin fold characteristic of antibody molecules, which consists of a 2-layer sandwich of about 7 antiparallel beta-strands arranged in two beta-sheets, optionally stabilized by a conserved disulphide bond.

e) The term "*immunoglobulin variable domain*" as used herein means an immunoglobulin domain essentially consisting of four "framework regions" which are referred to in the art and hereinbelow as "framework region 1" or "FR1"; as "framework region 2" or"FR2"; as "framework region 3" or "FR3"; and as "framework region 4" or "FR4", respectively; which framework regions are interrupted by three "complementarity determining regions" or "CDRs", which are referred to in the art and hereinbelow as "complementarity determining region 1"or "CDR1"; as "complementarity determining region 2" or "CDR2"; and as "complementarity determining region 3" or "CDR3", respectively. Thus, the general structure or sequence of an immunoglobulin variable domain can be indicated as follows: FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. It is the immunoglobulin variable domain(s) that confer specificity to an antibody for the antigen by carrying the antigen-binding site.

f) The term "*immunoglobulin single variable domain*" (or ISVD) as used herein means an immunoglobulin variable domain which is capable of specifically binding to an epitope of the antigen without pairing with an additional variable immunoglobulin domain. One example of ISVDs in the meaning of the present invention are "*domain antibodies*", such

as the ISVDs VH and VL (VH domains and VL domains). Another important example of ISVDs are "*VHH domains*" (or simply "VHHs") from camelids, as defined hereinafter.

In view of the above definition, the antigen-binding domain of a conventional 4-chain antibody (such as an IgG, IgM, IgA, IgD or IgE molecule; known in the art) or of a Fab fragment, a F(ab')2 fragment, an Fv fragment such as a disulphide linked Fv or a scFv fragment, or a diabody (all known in the art) derived from such conventional 4-chain antibody, would normally not be regarded as an ISVD, as, in these cases, binding to the respective epitope of an antigen would normally not occur by one (single) immunoglobulin domain but by a pair of (associating) immunoglobulin domains such as light and heavy chain variable domains, i.e. by a VH-VL pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen.

f1) "*VHH domains*" , also known as VHHs, V_HH domains, VHH antibody fragments, and VHH antibodies, have originally been described as the antigen binding immunoglobulin (variable) domain of "heavy chain antibodies" (i.e. of "antibodies devoid of light chains"; Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R.: "Naturally occurring antibodies devoid of light chains"; Nature 363, 446-448 (1993)). The term "VHH domain" has been chosen in order to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_H domains" or "VH domains") and from the light chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_L domains" or "VL domains"). VHH domains can specifically bind to an epitope without an additional antigen binding domain (as opposed to VH or VL domains in a conventional 4-chain antibody, in which case the epitope is recognized by a VL domain together with a VH domain). VHH domains are small, robust and efficient antigen recognition units formed by a single immunoglobulin domain.

In the context of the present invention, the terms VHH domain, VHH, V_HH domain, VHH antibody fragment, VHH antibody, as well as "Nanobody®" and "Nanobody® domain" ("Nanobody" being a trademark of the company Ablynx N.V.; Ghent; Belgium) are used interchangeably and are representatives of ISVDs (having the structure: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4 and specifically binding to an epitope without requiring the presence of a second immunoglobulin variable domain), and which can also be

distinguished from VH domains by the so-called "hallmark residues", as defined in e.g. WO2009/109635, Fig. 1.

The amino acid residues of a VHH domain are numbered according to the general numbering for V_H domains given by Kabat *et al.* ("Sequence of proteins of immunological interest", US Public Health Services, NIH Bethesda, MD, Publication No. 91), as applied to VHH domains from Camelids, as shown e.g. in Figure 2 of Riechmann and Muyldermans, J. Immunol. Methods 231, 25-38 (1999). According to this numbering,

- FR1 comprises the amino acid residues at positions 1-30,
- CDR1 comprises the amino acid residues at positions 31-35,
- FR2 comprises the amino acids at positions 36-49,
- CDR2 comprises the amino acid residues at positions 50-65,
- FR3 comprises the amino acid residues at positions 66-94,
- CDR3 comprises the amino acid residues at positions 95-102, and
- FR4 comprises the amino acid residues at positions 103-113.

However, it should be noted that - as is well known in the art for V_H domains and for VHH domains - the total number of amino acid residues in each of the CDRs may vary and may not correspond to the total number of amino acid residues indicated by the Kabat numbering (that is, one or more positions according to the Kabat numbering may not be occupied in the actual sequence, or the actual sequence may contain more amino acid residues than the number allowed for by the Kabat numbering). This means that, generally, the numbering according to Kabat may or may not correspond to the actual numbering of the amino acid residues in the actual sequence.

Alternative methods for numbering the amino acid residues of V_H domains, which methods can also be applied in an analogous manner to VHH domains, are known in the art. However, in the present description, claims and figures, the numbering according to Kabat and applied to VHH domains as described above will be followed, unless indicated otherwise.

The total number of amino acid residues in a VHH domain will usually be in the range of from 110 to 120, often between 112 and 115. It should however be noted that smaller and longer sequences may also be suitable for the purposes described herein.

Further structural characteristics and functional properties of VHH domains and polypeptides containing the same can be summarized as follows:

VHH domains (which have been "designed" by nature to functionally bind to an antigen without the presence of, and without any interaction with, a light chain variable domain) can function as a single, relatively small, functional antigen-binding structural unit, domain or polypeptide. This distinguishes the VHH domains from the VH and VL domains of conventional 4-chain antibodies, which by themselves are generally not suited for practical application as single antigen-binding proteins or ISVDs, but need to be combined in some form or another to provide a functional antigen-binding unit (as in for example conventional antibody fragments such as Fab fragments; in scFv's, which consist of a VH domain covalently linked to a VL domain).

Because of these unique properties, the use of VHH domains - either alone or as part of a larger polypeptide - offers a number of significant advantages over the use of conventional VH and VL domains, scFv's or conventional antibody fragments (such as Fab- or F(ab')2-fragments):

- only a single domain is required to bind an antigen with high affinity and with high selectivity, so that there is no need to have two separate domains present, nor to assure that these two domains are present in the right spacial conformation and configuration (i.e. through the use of especially designed linkers, as with scFv's);

- VHH domains can be expressed from a single gene and require no post-translational folding or modifications;

- VHH domains can easily be engineered into multivalent and multispecific formats (as further discussed herein);

- VHH domains are highly soluble and do not have a tendency to aggregate (as with the mouse-derived antigen-binding domains described by Ward *et al.*, Nature 341: 544-546 (1989));

- VHH domains are highly stable to heat, pH, proteases and other denaturing agents or conditions and, thus, may be prepared, stored or transported without the use of refrigeration equipments, conveying a cost, time and environmental savings;

- VHH domains are easy and relatively cheap to prepare, even on a scale required for production. For example, VHH domains and polypeptides containing the same can be produced using microbial fermentation (e.g. as further described below) and do not

require the use of mammalian expression systems, as with for example conventional antibody fragments;

- VHH domains are relatively small (approximately 15 kDa, or 10 times smaller than a conventional IgG) compared to conventional 4-chain antibodies and antigen-binding fragments thereof, and therefore

-- show high(er) penetration into tissues and

-- can be administered in higher doses

than such conventional 4-chain antibodies and antigen-binding fragments thereof;

- VHH domains can show so-called cavity-binding properties (inter alia due to their extended CDR3 loop, compared to conventional VH domains) and can therefore also access targets and epitopes not accessible to conventional 4-chain antibodies and antigen-binding fragments thereof.

Methods of obtaining VHH domains binding to a specific antigen or epitope have been described earlier, e.g. in WO2006/040153 and WO2006/122786. As also described therein in detail, VHH domains derived from camelids can be "humanized" by replacing one or more amino acid residues in the amino acid sequence of the original VHH sequence by one or more of the amino acid residues that occur at the corresponding position(s) in a VH domain from a conventional 4-chain antibody from a human being. A humanized VHH domain can contain one or more fully human framework region sequences, and, in an even more specific embodiment, can contain human framework region sequences derived from DP-29, DP-47, DP-51, or parts thereof, optionally combined with JH sequences, such as JH5.

f2) "*Domain antibodies*", also known as "Dab"s, "Domain Antibodies", and "dAbs" (the terms "Domain Antibodies" and "dAbs" being used as trademarks by the GlaxoSmithKline group of companies) have been described in e.g. Ward, E.S., *et al.*: "Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli"; Nature 341: 544-546 (1989); Holt, L.J. *et al.*: "Domain antibodies: proteins for therapy"; TRENDS in Biotechnology 21(11): 484-490 (2003); and WO2003/002609.

Domain antibodies essentially correspond to the VH or VL domains of non-camelid mammalians, in particular human 4-chain antibodies. In order to bind an epitope as a single antigen binding domain, i.e. without being paired with a VL or VH domain,

respectively, specific selection for such antigen binding properties is required, e.g. by using libraries of human single VH or VL domain sequences.

Domain antibodies have, like VHHs, a molecular weight of approximately 13 to approximately 16 kDa and, if derived from fully human sequences, do not require humanization for e.g. therapeutical use in humans. As in the case of VHH domains, they are well expressed also in prokaryotic expression systems, providing a significant reduction in overall manufacturing cost.

Domain antibodies, as well as VHH domains, can be subjected to affinity maturation by introducing one or more alterations in the amino acid sequence of one or more CDRs, which alterations result in an improved affinity of the resulting ISVD for its respective antigen, as compared to the respective parent molecule. Affinity-matured ISVD molecules of the invention can be prepared by methods known in the art, for example, as described by Marks *et al.*, 1992, Biotechnology 10:779-783, or Barbas, *et al.*, 1994, Proc. Nat. Acad. Sci, USA 91: 3809-3813.; Shier *et al.*, 1995, Gene 169:147-155; Yelton *et al.*, 1995, Immunol. 155: 1994-2004; Jackson *et al.*, 1995, J. Immunol. 154(7):3310-9; and Hawkins *et al.*, 1992, J. Mol. Biol. 226(3): 889 896; KS Johnson and RE Hawkins, "Affinity maturation of antibodies using phage display", Oxford University Press 1996.

f3) Furthermore, it will also be clear to the skilled person that it is possible to "graft" one or more of the CDRs mentioned above onto other "scaffolds", including but not limited to human scaffolds or non-immunoglobulin scaffolds. Suitable scaffolds and techniques for such CDR grafting are known in the art.

g) The terms "*epitope*" and "*antigenic determinant*", which can be used interchangeably, refer to the part of a macromolecule, such as a polypeptide, that is recognized by antigen-binding molecules, such as conventional antibodies or the polypeptides of the invention, and more particularly by the antigen-binding site of said molecules. Epitopes define the minimum binding site for an immunoglobulin, and thus represent the target of specificity of an immunoglobulin.

The part of an antigen-binding molecule (such as a conventional antibody or a polypeptide of the invention) that recognizes the epitope is called a *paratope*.

h) The term "*biparatopic*" (antigen-)binding molecule or "biparatopic" polypeptide as used herein shall mean a polypeptide comprising a first ISVD and a second ISVD as herein defined, wherein these two variable domains are capable of binding to two different

epitopes of one antigen, which epitopes are not normally bound at the same time by one monospecific immunoglobulin, such as e.g. a conventional antibody or one ISVD. The biparatopic polypeptides according to the invention are composed of variable domains which have different epitope specificities, and do not contain mutually complementary variable domain pairs which bind to the same epitope. They do therefore not compete with each other for binding to LRP5.

i) A polypeptide (such as an immunoglobulin, an antibody, an ISVD, a polypeptide of the invention, or generally an antigen binding molecule or a fragment thereof) that can "*bind*", "*bind to*", "*specifically bind*", or "*specifically bind to*", that "*has affinity for*" and/or that "*has specificity for*" a certain epitope, antigen or protein (or for at least one part, fragment or epitope thereof) is said to be "*against*" or "*directed against*" said epitope, antigen or protein or is a "*binding*" molecule with respect to such epitope, antigen or protein.

k) Generally, the term "*specificity*" refers to the number of different types of antigens or epitopes to which a particular antigen-binding molecule or antigen-binding protein (such as an immunoglobulin, an antibody, an ISVD, or a polypeptide of the invention) can bind. The specificity of an antigen-binding protein can be determined based on its affinity and/or avidity. The affinity, represented by the equilibrium constant for the dissociation of an antigen with an antigen-binding protein (K_D), is a measure for the binding strength between an epitope and an antigen-binding site on the antigen-binding protein: the lesser the value of the K_D, the stronger the binding strength between an epitope and the antigen-binding molecule (alternatively, the affinity can also be expressed as the affinity constant (K_A), which is $1/K_D$). As will be clear to the skilled person (for example on the basis of the further disclosure herein), affinity can be determined in a manner known *per se*, depending on the specific antigen of interest. Avidity is the measure of the strength of binding between an antigen-binding molecule (such as an immunoglobulin, an antibody, an ISVD, or a polypeptide of the invention) and the pertinent antigen. Avidity is related to both the affinity between an epitope and its antigen binding site on the antigen-binding molecule and the number of pertinent binding sites present on the antigen-binding molecule.

Typically, antigen-binding proteins (such as the polypeptides of the invention) will bind with a dissociation constant (K_D) of 10E-5 to 10E-14 moles/liter (M) or less, and preferably 10E-7 to 10E-14 moles/liter (M) or less, more preferably 10E-8 to 10E-14 moles/liter, and even more preferably 10E-11 to 10E-13 (as measured e.g. in a Kinexa

assay; known in the art), and/or with an association constant (K_A) of at least 10E7 ME-1 , preferably at least 10E8 ME-1, more preferably at least 10E9 ME-1, such as at least 10E11 ME-1. Any K_D value greater than 10E-4 M is generally considered to indicate non-specific binding. Preferably, a polypeptide of the invention will bind to the desired antigen with a K_D less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as less than 500 pM. Specific binding of an antigen-binding protein to an antigen or epitope can be determined in any suitable manner known *per se*, including, for example, the assays described herein, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known *per se* in the art.

l) The term "*cross-reactive*" in connection with binding molecules which are able to bind to LRP5 as well as to LRP6 ("*LRP5/LRP6 cross-reactive*") is intended to mean that such binding molecules can specifically bind to an epitope comprised in the LRP5 molecule, and can, alternatively, also specifically bind to an epitope comprised in the LRP6 molecule.

m) Amino acid residues will be indicated according to the standard three-letter or one-letter amino acid code, as generally known and agreed upon in the art. When comparing two amino acid sequences, the term "*amino acid difference*" refers to insertions, deletions or substitutions of the indicated number of amino acid residues at a position of the reference sequence, compared to a second sequence. In case of substitution(s), such substitution(s) will preferably be conservative amino acid substitution(s), which means that an amino acid residue is replaced with another amino acid residue of similar chemical structure and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example from WO 98/49185, wherein conservative amino acid substitutions preferably are substitutions in which one amino acid within the following groups (i) - (v) is substituted by another amino acid residue within the same group: (i) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (ii) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (iii) polar, positively charged residues: His, Arg and Lys; (iv) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (v) aromatic residues: Phe, Tyr and Trp. Particularly preferred conservative amino acid substitutions are as follows:

Ala into Gly or into Ser;

Arg into Lys;
Asn into Gln or into His;
Asp into Glu;
Cys into Ser;
Gln into Asn;
Glu into Asp;
Gly into Ala or into Pro;
His into Asn or into Gln;
Ile into Leu or into Val;
Leu into Ile or into Val;
Lys into Arg, into Gln or into Glu;
Met into Leu, into Tyr or into Ile;
Phe into Met, into Leu or into Tyr;
Ser into Thr;
Thr into Ser;
Trp into Tyr;
Tyr into Trp or into Phe;
Val into Ile or into Leu.

n) A nucleic acid or polypeptide molecule is considered to be "*(in) essentially isolated (form)*" - for example, when compared to its native biological source and/or the reaction medium or cultivation medium from which it has been obtained - when it has been separated from at least one other component with which it is usually associated in said source or medium, such as another nucleic acid, another protein/polypeptide, another biological component or macromolecule or at least one contaminant, impurity or minor component. In particular, a nucleic acid or polypeptide molecule is considered "essentially isolated" when it has been purified at least 2-fold, in particular at least 10-fold, more in particular at least 100-fold, and up to 1000-fold or more. A nucleic acid or polypeptide molecule that is "in essentially isolated form" is preferably essentially homogeneous, as determined using a suitable technique, such as a suitable chromatographical technique, such as polyacrylamide-gelelectrophoresis;

o) "*Sequence identity*" between e.g. two ISVD sequences indicates the percentage of amino acids that are identical between these two sequences. It may be calculated or determined as described in paragraph f) on pages 49 and 50 of WO2008/020079.

"*Sequence similarity*" indicates the percentage of amino acids that either are identical or that represent conservative amino acid substitutions.

Target specificity

The polypeptides of the invention have specificity for LRP5, in that they typically comprise ISVDs specifically binding to epitopes of LRP5. In a preference, the polypeptides of the invention have an affinity and/or avidity for LRP5, especially human LRP5, that is at least 10 times, preferably at least 100 times, more preferably at least 1000 times, even more preferably at least 10000 times, yet even more preferably at least 100000 times or at least 1000000 times stronger than their affinity and/or avidity for LRP6, in particular human LRP6 (Accession number: UniProtKB - O75581 / LRP6_HUMAN). Most preferably, the polypeptides are not cross-reactive to LRP6, in particular human LRP6 (see Example 7.1).

The molecules of the invention shall bind to the human forms of LRP5, and preferably also to counterparts in other species relevant for drug development, i.e. cynomolgus and mouse LRP5.

Polypeptides of the invention

In its broadest sense, the invention provides novel pharmacologically active agents for the treatment of cancer diseases. Typical agents according to the invention belong to a novel class of binding molecules, namely biparatopic polypeptides, comprising two or more ISVDs binding to LRP5 at different epitopes. The term “biparatopic” is explained above, so that biparatopic molecules can be defined as molecules being able to bind to LRP5 at two different epitopes comprised in the LRP5 protein.

In an aspect, the polypeptide of the invention comprises a first LRP5-binding domain, and a second LRP5-binding domain, wherein the first domain binds a region of LRP5 that is distinct from the LRP5 region which the second domain binds to. Such LRP5-binding domain may be any immunoglobulin domain, e.g. any immunoglobulin domain described herein. Preferably, such LRP5-binding domain is an ISVD.

In an embodiment, the first domain blocks a Wnt3a binding site of LRP5, and preferably inhibits Wnt3a-driven target gene transcription, and/or wherein the second domain blocks a Wnt1 binding site of LRP5, and preferably inhibits Wnt1-driven target gene transcription.

In other words, polypeptides of the invention may include:

- a first ISVD which is able to specifically bind to LRP5 via an epitope / in a manner that results in inhibition of the Wnt3a signaling pathway, so that Wnt3a-driven target gene transcription is inhibited, and

- a second ISVD which is able to specifically bind to LRP5 via an epitope / in a manner that results in inhibition of the Wnt1 signaling pathway, so that Wnt1-driven target gene transcription is inhibited.

Due to the two ISVDs present in polypeptides described above, wherein the two domains are binding to different epitopes (Wnt1 / Wnt3a signaling related), these molecules are biparatopic binding molecules. This biparatopic binding mode is schematically shown in Figure 1.

In this context, it should be noted that it is assumed that the polypeptides of the invention can bind to one single LRP5 molecule via both of its LRP5 binding domains, as shown in Fig. 1 (intramolecular binding mode). However, other binding modes may occur as well.

Finally, it is assumed that the polypeptides of the invention are able to compete with DKK1 – a natural ligand of LRP5, and interfering with Wnt1 and Wnt3a signaling – for binding to LRP5, thereby inhibiting the Wnt1 as well as the Wnt3a signaling pathway. However, also this theory should not be understood as limiting the scope of the invention.

Specifically, the present invention provides a polypeptide which binds to low-density lipoprotein receptor-like protein 5 (LRP5), the polypeptide comprising an ISVD selected from the group of the following LRP5-binding ISVDs:

(i) an ISVD having the following complementarity-determining region (CDR) sequences:

CDR1: TYVMG (SEQ ID NO:1)

CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)

CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3),

(ii) an ISVD having the following CDR sequences:

CDR1: RYAVA (SEQ ID NO:4)

CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)

CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6),

(iii) an ISVD having the following CDR sequences:

CDR1: IGAMG (SEQ ID NO:7)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9), and

(iv) an ISVD having the following CDR sequences:

CDR1: INAMG (SEQ ID NO:10)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9).

The polypeptide of the present invention preferably comprises a first ISVD (a) selected from ISVDs (i) and (ii) as defined above and a second ISVD (b) selected from ISVDs (iii) and (iv) a defined above. In an embodiment, the first ISVD is ISVD (i) and the second ISVD is ISVD (iii). In a further embodiment, the first ISVD is ISVD (i) and the second ISVD is ISVD (iv). In yet a further embodiment, the first ISVD is ISVD (ii) and the second ISVD is ISVD (iii). In another embodiment, the first ISVD is ISVD (ii) and the second ISVD is ISVD (iv). Preferably, ISVD (i) is further defined by having the sequence identified by SEQ ID NO:11 or SEQ ID NO:23, ISVD, (ii) is further defined by having the sequence identified by SEQ ID NO:12, ISVD (iii) is further defined by having the sequence identified by SEQ ID NO:13 or SEQ ID NO:22, and/or ISVD (iv) is further defined by having the sequence identified by SEQ ID NO:14.

The use of the terms “first” and “second” with respect to such ISVDs is solely intended to indicate that these domains are different domains, as they will include different CDR sequences and will bind to different epitopes. These terms shall, however, not be understood to refer to the exact order or sequence of the domains within such polypeptide chain. In other words, the above ISVDs may either be arranged in the order (i)/(ii) – (iii)/(iv) or in the order (iii)/(iv) – (i)(ii) within such polypeptide of the invention.

ISVDs typically essentially consist of four framework regions (FR1 to FR4, respectively) and three complementarity determining regions (CDR1 to CDR3, respectively). To be located within one polypeptide, or polypeptide chain, said first and said second ISVDs need to be covalently linked, either directly or by a linker peptide (e.g. a linker sequence derived from the hinge region of heavy chain antibodies, poly-alanine linker sequences, Gly/Ser linkers of different length or the like).

Thus, the general structure of the molecules of the invention can also be depicted as follows:

FR(a)1 - CDR(a)1 - FR(a)2 - CDR(a)2 - FR(a)3 - CDR(a)3 - FR(a)4 – [linker peptide] - FR(b)1 - CDR(b)1 - FR(b)2 - CDR(b)2 - FR(b)3 - CDR(b)3 - FR(b)4

wherein
FR(a) denotes a framework region of the first ISVD,
FR(b) denotes a framework region of the second ISVD,
CDR(a) denotes a CDR of the first ISVD,
CDR(b) denotes a CDR of the second ISVD,
[linker peptide] denotes a linker peptide that may optionally be present,
preferably wherein the CDRs are having the sequences as set out above (i.e. CDRs of (a) may be CDRs of (i) or (ii) and CDRs of (b) may be CDRs of (iii) or (iv),).

Again, it shall be understood that (a) and (b) can be exchanged, i.e. that molecules having the general structure

FR(b)1 - CDR(b)1 - FR(b)2 - CDR(b)2 - FR(b)3 - CDR(b)3 - FR(b)4 – [linker peptide] - FR(a)1 - CDR(a)1 - FR(a)2 - CDR(a)2 - FR(a)3 - CDR(a)3 - FR(a)4

shall also be encompassed by the present invention.

The linker peptide may comprise an amino acid sequence, e.g. having a length of 9 or more amino acids, preferably at least 17 amino acids, such as about 20 to 40 amino acids. The linker sequence may be a naturally occurring sequence or a non-naturally occurring sequence. Exemplary linker sequences include but are not limited to linker sequences derived from the hinge region of heavy chain antibodies, poly-alanine linker sequences, and Gly/Ser linkers of different length (e.g., $(gly_xser_y)_z$ linkers, such as $(gly_4ser)_3$, $(gly_4ser)_5$, $(gly_4ser)_7$, $(gly_3ser)_3$, $(gly_3ser)_5$, $(gly_3ser)_7$, $(gly_3ser_2)_3$, $(gly_3ser_2)_5$, and $(gly_3ser_2)_7$).

In a preference, the polypeptide further comprises an albumin-binding ISVD (e.g., as a third ISVD), preferably and wherein the albumin-binding ISVD covalently links a segment of the polypeptide, which segment comprises or consists of the first ISVD (e.g. ISVD (i) or (ii) as defined above), to a segment of the polypeptide, which segment comprises or consists of the second ISVD (e.g. ISVD (iii) or (iv) as defined above). In other words, a linker peptide may covalently link said first ISVD to said second ISVD, wherein said linker peptide preferably comprises or consists of a further ISVD, more preferably an albumin-binding ISVD, especially an albumin-binding ISVD as set forth herein.

In some embodiments the linker peptide comprises or consists of a third domain, such as e.g. an albumin binding ISVD, such as the Alb11 domain, comprising the following CDRs:

CDR(Alb11)1: SFGMS (= SEQ ID NO:15)
CDR(Alb11)2: SISGSGSDTLYADSVKG (= SEQ ID NO:16)
CDR(Alb11)3: GGSLSR (= SEQ ID NO:17)

This results in a group of polypeptides of the invention having the following general structure:

FR(a)1 - CDR(a)1 - FR(a)2 - CDR(a)2 - FR(a)3 - CDR(a)3 - FR(a)4 – [linker peptide] - FR(Alb11)1 - CDR(Alb11)1 - FR(Alb11)2 - CDR(Alb11)2 - FR(Alb11)3 - CDR(Alb11)3 - FR(Alb11)4 - [linker peptide] - FR(b)1 - CDR(b)1 - FR(b)2 - CDR(b)2 - FR(b)3 - CDR(b)3 - FR(b)4, preferably wherein the CDRs are having the sequences as set out above (i.e. CDRs of (a) may be CDRs of (i) or (ii) and CDRs of (b) may be CDRs of (iii) or (iv).

Again, the order of the three ISVDs (a), (b), and Alb11 is not fixed but polypeptides in which the above domains are arranged in the order:
(b) – Alb11 – (a)
shall be encompassed as well.

Furthermore, polypeptides having the Alb11 domain at the N- or C-terminal end of the polypeptide (e.g. Alb11 – (a) – (b), Alb11 – (b) – (a), (a) – (b) – Alb11, or (b) – (a) – Alb11) shall also be encompassed by the invention.

In three preferred embodiments, the polypeptides of the invention include ISVDs defined as follows:

First preferred embodiment: Polypeptides comprising a first ISVD having the following CDR sequences:

CDR1: TYVMG (SEQ ID NO:1)
CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)
CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3),
and a second ISVD having the following CDR sequences:
CDR1: INAMG (SEQ ID NO:10)
CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3: ETGPYGPPKRDY (SEQ ID NO:9).

Second preferred embodiment: Polypeptides comprising a first ISVD having the following CDR sequences:

CDR1: RYAVA (SEQ ID NO:4)
CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)
CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6).

and a second ISVD having the following CDR sequences:
CDR1: IGAMG (SEQ ID NO:7)
CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3: ETGPYGPPKRDY (SEQ ID NO:9)

Third preferred embodiment: Polypeptides comprising a first ISVD having the following CDR sequences:

CDR1: TYVMG (SEQ ID NO:1)
CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)
CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3)
and a second ISVD having the following CDR sequences:
CDR1: IGAMG (SEQ ID NO:7)
CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3: ETGPYGPPKRDY (SEQ ID NO:9).

Of course, the variants as set out above – i.e. optionally including linker peptides (e.g. a linker sequence derived from the hinge region of heavy chain antibodies, poly-alanine linker sequences, Gly/Ser linkers of different length or the like) and/or further domains, esp. including an Alb11 domain, different orders of the ISVDs – shall apply to these three preferred embodiments as well.

In a specifically preferred embodiment, the albumin binding ISVD (e.g., Alb11) is located between the two LRP5 binding ISVDs (ISVD (a) and (b)). Thus, such preferred

polypeptides may have the structure (a) - Alb11 - (b) or (b) – Alb11 – (a)). Specifically, the albumin binding ISVD (e.g., Alb11) may be covalently bound to, either directly or by a linker peptide (e.g. a linker sequence derived from the hinge region of heavy chain antibodies, poly-alanine linker sequences, Gly/Ser linkers of different length or the like) the LRP5 binding ISVD domains (a) and (b) with its N- and C-terminal ends (e.g. N-terminal end of albumin binding domain bound to ISVD (a) and C-terminal end of albumin binding domain bound to ISVD (b) or vice versa, preferably wherein the ISVDs comprise the CDRs or full length ISVD sequences as set out above (i.e. CDRs/ISVD sequences of (a) may be CDRs/ISVD sequences of (i) or (ii) and CDRs/ISVD sequences of (b) may be CDRs/ISVD sequences of (iii) or (iv)).

Three specifically preferred embodiments can be envisaged as follows:

First specifically preferred embodiment: Polypeptides comprising a first (LRP5 binding) ISVD having the following CDR sequences:

CDR1: TYVMG (SEQ ID NO:1)
CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)
CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3)

an albumin binding ISVD having the following CDR sequences:

CDR1: SFGMS (= SEQ ID NO:15)
CDR2: SISGSGSDTLYADSVKG (= SEQ ID NO:16)
CDR3: GGSLSR (= SEQ ID NO:17);

and a second (LRP5 binding) ISVD having the following CDR sequences:

CDR1: INAMG (SEQ ID NO:10)
CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3: ETGPYGPPKRDY (SEQ ID NO:9);

either in this order, or the order of the above domains being changed.

Second specifically preferred embodiment: Polypeptides comprising a first (LRP5 binding) ISVD having the following CDR sequences:

CDR1: RYAVA (SEQ ID NO:4)
CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)
CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6);

an albumin binding ISVD having the following CDR sequences:

CDR1: SFGMS (= SEQ ID NO:15)
CDR2: SISGSGSDTLYADSVKG (= SEQ ID NO:16)

CDR3: GGSLSR (= SEQ ID NO:17);

and a second (LRP5 binding) ISVD having the following CDR sequences:

CDR1: IGAMG (SEQ ID NO:7)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9);

either in this order, or the order of the above domains being changed.

Third specifically preferred embodiment: Polypeptides comprising first (LRP5 binding) ISVD having the following CDR sequences:

CDR1: TYVMG (SEQ ID NO:1)

CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)

CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3);

an albumin binding ISVD having the following CDR sequences:

CDR1: SFGMS (= SEQ ID NO:15)

CDR2: SISGSGSDTLYADSVKG (= SEQ ID NO:16)

CDR3: GGSLSR (= SEQ ID NO:17);

and a second (LRP5 binding) ISVD having the following CDR sequences:

CDR1: IGAMG (SEQ ID NO:7)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9);

either in this order, or the order of the above domains being changed.

The CDR sequences mentioned above are summarized in Tables IA, IB, and IC:

TABLE IA: CDR sequences of ISVDs interfering with Wnt3a signaling:

	F0129097A08	F0129093A02
CDR1	TYVMG (SEQ ID NO:1)	RYAVA (SEQ ID NO:4)
CDR2	AISWSGGSTYYADSVKG (SEQ ID NO:2)	AITWSSGRIDYADSVKG (SEQ ID NO:5)
CDR3	SRGTSTPSRASGVSRYDY (SEQ ID NO:3)	DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6)

TABLE IB: CDR sequences of ISVDs interfering with Wnt1 signaling:

	F012904 6C10(E1A,N32G)	F012904 6C10
CDR1	IGAMG (SEQ ID NO:7)	INAMG (SEQ ID NO:10)
CDR2	AVSSGGSTYYVDSVKG (SEQ ID NO:8)	AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3	ETGPYGPPKRDY (SEQ ID NO:9)	ETGPYGPPKRDY (SEQ ID NO:9)

TABLE IC: CDR sequences of ISVD binding to serum albumin (Alb11 domain):

	Alb11 domain
CDR1	SFGMS (SEQ ID NO:15)
CDR2	SISGSGSDTLYADSVKG (SEQ ID NO:16)
CDR3	GGSLSR (SEQ ID NO:17)

In addition to the CDR sequences as set out above, the ISVDs comprised in the polypeptides of the invention include immunoglobulin framework region (FR) sequences. These sequences are preferably not immunogenic in humans, and are therefore preferably human or humanized FR sequences. Suitable human or humanized FR sequences are known in the art. Specifically preferred FR sequences can be taken from the embodiments shown below, disclosing the complete ISVDs and thereby CDR sequences as well as FR sequences.

According to a more specific embodiment, the polypeptides of the invention comprise ISVDs (e.g. ISVDs as defined by the CDR sequences listed in Tables IA to IC and forming ISVDs (a), (b), or (c) as set out in the embodiments above) which are VHH domains, and preferably humanized VHH domains. Specifically, the polypeptides described herein comprise a first ISVD (a), which comprises CDR sequences of (i) or (ii) and a second ISVD (b) which comprises CDR sequences of (iii) or (iv), and a third ISVD, which is an albumin binding ISVD (e.g. Alb11), which ISVDs are VHH domains, and preferably humanized VHH domains.

According to an even more specific embodiment, the polypeptides of the invention include a first ISVD (a) selected from the group consisting of ISVDs (i) and (ii) having the following sequences:

(i)

AVQLVESGGGLVQPGGSLRLSCAASGRTFSTYVMGWFRQAPGKEREFVAAIS WSGGSTYYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCAASRGTST PSRASGVSRYDYWGQGTLVTVSS (SEQ ID NO:11), or

EVQLVESGGGLVQPGGSLRLSCVASGRTFSTYVMGWFRQAPGKEREFVAAIS WSGGSTYYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCAASRGTST PSRASGVSRYDYWGQGTLVTVSS (SEQ ID NO:23), and

(ii)

EVQLVESGGGLVQPGGSLRLSCAASGLTFSRYAVAWFRQAPGKEREFVAAIT WSSGRIDYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCAADRRPRS TGRSGTGSPSTYDYWGQGTLVTVSSA (SEQ ID NO: 12),

and a second ISVD (b) selected from the group consisting of ISVDs (iii) and (iv) having the following sequences:

(iii)

AVQLVESGGGLVQPGGSLRLSCAASGSIFRIGAMGWYRQAPGKQRELVAAV SSGGSTYYVDSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCNRETGPY GPPKRDYWGQGTLVTVSS (SEQ ID NO:13), or

EVQLVESGGGLVQPGGSLRLSCAASGSIFRIGAMGWYRQAPGKQRE VAAVSSGGSTYYVDSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYY NRETGPYGPPKRDYWGQGTLVTVSSA (SEQ ID NO:22), and

(iv)

EVQLVESGGGLVQPGGSLRLSCAASGSIFRINAMGWYRQAPGKQRELVAAV SSGGSTYYVDSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCNRETGPY GPPKRDYWGQGTLVTVSS (SEQ ID NO:14).

Preferred embodiments are polypeptides comprising

- a first ISVD having the amino acid sequence as shown in SEQ ID NO:23 and a second ISVD having the amino acid sequence as shown in SEQ ID NO:14; or
- a first ISVD having the amino acid sequence as shown in SEQ ID NO:12 and a second ISVD having the amino acid sequence as shown in SEQ ID NO:13; or

- a first ISVD having the amino acid sequence as shown in SEQ ID NO:11 and a second ISVD having the amino acid sequence as shown in SEQ ID NO:22.

Thus, the above embodiments can schematically be presented as
isvd(a) – [linker peptide] – isvd(b),
wherein "isvd" denotes the respective ISVD, and wherein otherwise the same definitions and variants shall apply as set out above, esp. with respect to the presence of optional linker peptides and/or further domains, esp. an Alb11 domain, and with respect to different orders of the ISVDs.

Specifically, the albumin binding ISVD (e.g., Alb11) may be covalently bound to, either directly or by a linker peptide (e.g. a linker sequence derived from the hinge region of heavy chain antibodies, poly-alanine linker sequences, Gly/Ser linkers of different length or the like) the LRP5 binding ISVD domains (a) and (b) with its N- and C-terminal ends; e.g. N-terminal end of albumin binding domain bound via a linker peptide to ISVD (a) and C-terminal end of albumin binding domain bound via a linker peptide to ISVD (b) or vice versa, preferably wherein the ISVDs comprise the full length ISVD sequences as set out above (i.e. ISVD sequences of (a) may be ISVD sequences of (i) or (ii) and ISVD sequences of (b) may be ISVD sequences of (iii) or (iv) as defined above).

In some embodiments, the first ISVD is linked to the albumin binding ISVD by a linker peptide and the second ISVD is linked directly to the albumin binding ISVD. In some embodiments, the second ISVD is linked to the albumin binding ISVD by a linker peptide and the first ISVD is linked directly to the albumin binding ISVD. In some embodiments, both the first and the second ISVD are linked to the albumin binding ISVD by a linker peptide, respectively.

According to specific embodiments of the invention, the above polypeptides may additionally include a half-life extending moiety, wherein said half-life extending moiety is covalently linked to said polypeptide and is optionally selected from the group consisting of an albumin binding moiety, such as an albumin binding peptide or an albumin binding immunoglobulin domain, preferably an albumin binding ISVD, more preferably the Alb11 domain, a transferrin binding moiety, such as an anti-transferrin immunoglobulin domain, a polyethylene glycol molecule, serum albumin, preferably human serum albumin, and a fragment of (human) serum albumin.

The sequence of the above-mentioned Alb11 ISVD is as follows:

EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSIS
GSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTI
GGSLSRSSQGTLVTVSS

(= Alb11 domain; = SEQ ID NO: 21)

Further examples of ISVDs binding to human serum albumin are known in the art, and are described in further detail in e.g. international patent publications WO2006/122787 and WO2008/028977. Other peptides binding to human serum albumin are described e.g. in WO2008/068280, WO2009/127691, and WO2011/095545.

Thus, three preferred specific embodiments of the invention are as follows:

First preferred specific embodiment: Polypeptides comprising
- a first (LRP5 binding) ISVD having the amino acid sequence as shown in SEQ ID NO:23;
- an albumin binding ISVD having the amino acid sequence as shown in SEQ ID NO:21;
- a second (LRP5 binding) ISVD having the amino acid sequence as shown in SEQ ID NO:14;

either in this order, or the order of the above three domains being changed.

Second preferred specific embodiment: Polypeptides comprising
- a first (LRP5 binding) ISVD having the amino acid sequence as shown in SEQ ID NO:12;
- an albumin binding ISVD having the amino acid sequence as shown in SEQ ID NO:21;
- a second (LRP5 binding) ISVD having the amino acid sequence as shown in SEQ ID NO:13;

either in this order, or the order of the above three domains being changed.

Third preferred specific embodiment: Polypeptides comprising
- a first (LRP5 binding) ISVD having the amino acid sequence as shown in SEQ ID NO:11;
- an albumin binding ISVD having the amino acid sequence as shown in SEQ ID NO:21;
- a second (LRP5 binding) ISVD having the amino acid sequence as shown in SEQ ID NO:22;

either in this order, or the order of the above three domains being changed.

In even more specifically preferred embodiments, the albumin binding ISVD is located between the two LRP5 binding ISVDs.

The sequences of the ISVDs mentioned above are summarized in Tables IIA, IIB, and IIC:

TABLE IIA: Sequences of ISVDs interfering with Wnt3a signaling:

VHH ID SEQ ID NO:	**FR1**	**CDR1**	**FR2**	**CDR2**	**FR3**	**CDR3**	**FR4**
F012909 7A08(E1 A,V23A) SEQ ID NO:11	AVQLVESG GGLVQPG GSLRLSCAA SGRTFS (SEQ ID NO:24)	TYVMG (SEQ ID NO:1)	WFRQAPG KEREFVA (SEQ ID NO:25)	AISWSGG STYYADS VKG (SEQ ID NO:2)	RFTISRDNSKNTVYL QMNSLRPEDTAVYY CAA (SEQ ID NO:26)	SRGTST PSRASG VSRYDY (SEQ ID NO:3)	WGQGTL VTVSS (SEQ ID NO:27)
F012909 3A02 SEQ ID NO:12	EVQLVESG GGLVQPG GSLRLSCAA SGLTFS (SEQ ID NO:28)	RYAVA (SEQ ID NO:4)	WFRQAPG KEREFVA (SEQ ID NO:25)	AITWSSG RIDYADS VKG (SEQ ID NO:5)	RFTISRDNSKNTVYL QMNSLRPEDTAVYY CAA (SEQ ID NO:26)	DRRPRS TGRSGT GSPSTY DY (SEQ ID NO:6)	WGQGTL VTVSSA (SEQ ID NO:29)
F012909 7A08 (SEQ ID NO:23)	EVQLVESG GGLVQPG GSLRLSCVA SGRTFS (SEQ ID NO:30)	TYVMG (SEQ ID NO:1)	WFRQAPG KEREFVA (SEQ ID NO:25)	AISWSGG STYYADS VKG (SEQ ID NO:2)	RFTISRDNSKNTVYL QMNSLRPEDTAVYY CAA (SEQ ID NO:26)	SRGTST PSRASG VSRYDY (SEQ ID NO:3)	WGQGTL VTVSS (SEQ ID NO:27)

TABLE IIB: Sequences of ISVDs interfering with Wnt1 signaling:

VHH ID SEQ ID NO:	**FR1**	**CDR1**	**FR2**	**CDR2**	**FR3**	**CDR3**	**FR4**
F012904 6C10 (E1A,N32G) SEQ ID NO:13	AVQLVES GGGLVQ PGGSLRL SCAASGSI FR (SEQ ID NO:31)	IGAMG (SEQ ID NO:7)	WYRQAPG KQRELVA (SEQ ID NO:32)	AVSSGGS TYYVDSV KG (SEQ ID NO:8)	RFTISRDNSKNT VYLQMNSLRPE DTAVYYCNR (SEQ ID NO:33)	ETGPYG PPKRDY (SEQ ID NO:9)	WGQGTL VTVSS (SEQ ID NO:27)
F012904 6C10 SEQ ID NO:14	EVQLVES GGGLVQ PGGSLRL SCAASGSI FR (SEQ ID NO:34)	INAMG (SEQ ID NO:10)	WYRQAPG KQRELVA (SEQ ID NO:32)	AVSSGGS TYYVDSV KG (SEQ ID NO:8)	RFTISRDNSKNT VYLQMNSLRPE DTAVYYCNR (SEQ ID NO:33)	ETGPYG PPKRDY (SEQ ID NO:9)	WGQGTL VTVSS (SEQ ID NO:27)

VHH ID SEQ ID NO:	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
F012904 6C10(N32G) SEQ ID NO:22	EVQLVES GGGLVQ PGGSLRL SCAASGSI FR (SEQ ID NO:34)	IGAMG (SEQ ID NO:7)	WYRQAPG KQRELVA (SEQ ID NO:32)	AVSSGGS TYYVDSV KG (SEQ ID NO:8)	RFTISRDNSKNT VYLQMNSLRPE DTAVYYCNR (SEQ ID NO:33)	ETGPYG PPKRDY (SEQ ID NO:9)	WGQGTL VTVSSA (SEQ ID NO:29)

TABLE IIC: Sequence of ISVD binding to serum albumin (Alb11 domain):

VHH ID SEQ ID NO:	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
Alb11 SEQ ID NO:21	EVQLVESG GGLVQPG NSLRLSCA ASGFTFS (SEQ ID NO:35)	SFGMS (SEQ ID NO:15)	WVRQAPG KGLEWVS (SEQ ID NO:36)	SISGSGSD TLYADSV KG (SEQ ID NO:16)	RFTISRDNAKTTLYL QMNSLRPEDTAVYY CTI (SEQ ID NO:37)	GGSLSR (SEQ ID NO:17)	SSQGTLV TVSS (SEQ ID NO:38)

In an aspect, the present invention relates to a polypeptide which binds to LRP5, comprising an ISVD which competes for binding to LRP5 with a reference ISVD, wherein the reference ISVD has the sequence identified by SEQ ID NOs: 11, 12, 13, 14, 22 or 23. Preferably the ISVD blocks a Wnt1 or Wnt3a binding site of LRP. In particular the ISVD inhibits Wnt1-driven or Wnt3a-driven target gene transcription

In another aspect, the present invention relates to a polypeptide which binds to LRP5, comprising a first ISVD which competes for binding to LRP5 with a first reference ISVD, wherein the first reference ISVD has the sequence identified by SEQ ID NOs: 11, 23 or 12, and a second ISVD which competes for binding to LRP5 with a second reference ISVD, wherein the second reference ISVD has the sequence identified by SEQ ID NOs: 13, 22 or 14. Preferably the first ISVD blocks a Wnt3a binding site of LRP5, in particular inhibits Wnt3a driven target gene transcription. Alternatively, or in addition thereto, the second ISVD may block a Wnt1 binding site of LRP5, in particular inhibit Wnt1-driven target gene transcription.

Herein, the expression “an ISVD which competes for binding to an antigen with a reference ISVD” (or similar expressions) comprises all ISVDs that bind to the same epitope as the reference ISVD or to an epitope that overlaps with the epitope of the

reference ISVD and inhibit binding of the reference ISVD to the antigen (i.e. LRP5) to a certain degree. Preferably, such ISVDs bind to the same epitope as the reference ISVD and/or essentially completely inhibit binding of the reference ISVD (such as the ones mentioned above) to the antigen (i.e. LRP5).Whether a certain ISVD is an ISVD that competes for binding with the reference ISVD can be easily tested by the skilled artisan if he has knowledge of the reference ISVD and the antigen, even without knowing the epitope of the antigen the reference ISVD binds to, by conducting any suitable competition binding assay known in the art. Such competition binding assays are well known in the art. For instance, the skilled artisan may incubate the antigen with a labelled reference ISVD and may compare the amount of antigen-bound labelled reference ISVD to the amount of labelled reference ISVD which binds to the antigen upon pre-incubation with the ISVD to be tested. If latter amount is reduced (i.e. because pre-incubation allows the ISVD to be tested to block binding sites it shares with the reference ISVD), the ISVD to be tested can be said to compete for binding to the antigen with the reference ISVD.

As set out above, the (at least two) ISVDs present in a polypeptide of the invention can be linked to each other either directly, without use of a linker, or via a linker. The linker is preferably a linker peptide and will, according to the invention, be selected so as to allow binding of the at least two different ISVDs to each of their target epitopes.

Suitable linkers will *inter alia* depend on the epitopes and, specifically, the distance between the epitopes on the target molecules to which the ISVDs shall bind. This will be clear to the skilled person based on the disclosure herein, optionally after some limited degree of routine experimentation.

Thus, suitable linkers may comprise an amino acid sequence, e.g. having a length of 9 or more amino acids, preferably at least 17 amino acids, such as about 20 to 40 amino acids. The linker sequence may be a naturally occurring sequence or a non-naturally occurring sequence. If used for therapeutic purposes, the linker is preferably non-immunogenic in the subject to which the polypeptide of the invention is administered.

One useful group of linker sequences are linkers derived from the hinge region of heavy chain antibodies as described in WO1996/34103 and WO1994/04678. Other examples are poly-alanine linker sequences such as Ala-Ala-Ala.

Further preferred examples of linker sequences are Gly/Ser linkers of different length such as $(gly_xser_y)_z$ linkers, including e.g. $(gly_4ser)_3$, $(gly_4ser)_5$, $(gly_4ser)_7$, $(gly_3ser)_3$, $(gly_3ser)_5$, $(gly_3ser)_7$, $(gly_3ser_2)_3$, $(gly_3ser_2)_5$, and $(gly_3ser_2)_7$.

Alternatively, or in addition, to a polypeptide linker, the at least two ISVDs present in a polypeptide of the invention may be linked to each other via another moiety, such as another polypeptide which, in a preferred but non-limiting embodiment, may be a further ISVD as already described above. Such moiety may either be essentially inactive or may have a biological effect such as improving the desired properties of the polypeptide or may confer one or more additional desired properties to the polypeptide. As already set out above, a preferred additional polypeptide domain will increase the half-life of the polypeptide, such as a (human) serum albumin binding domain, such as the Alb11 domain.

Thus, according to a further embodiment, the invention specifically includes polypeptides comprising any of the following sequences, wherein the exact amino acid sequences can be taken from Table III below:
F012900082, having the sequence identified by SEQ ID NO: 18,
F012900135, having the sequence identified by SEQ ID NO: 19, and
F012900141, having the sequence identified by SEQ ID NO: 20.

TABLE III: Sequences of three specific embodiments of polypeptides of the invention

ID SEQ ID NO:	**Amino Acid Sequence** (CDR sequences underlined)
F012900082 SEQ ID NO:18	EVQLVESGGGLVQPGGSLRLSCVASGRTFSTYVMGWFRQAPGKEREFVAAISWSGGSTYY ADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCAASRGTSTPSRASGVSRYDYWGQG TLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPG NSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAK TTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSSGGGGSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGSIFRINAMGWYRQAPGKQ RELVAAVSSGGSTYYVDSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCNRETGPYGPP KRDYWGQGTLVTVSS
F012900135 SEQ ID NO:19	AVQLVESGGGLVQPGGSLRLSCAASGSIFRIGAMGWYRQAPGKQRELVAAVSSGGSTYYV DSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCNRETGPYGPPKRDYWGQGTLVTVSS GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSC AASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAKTTLYLQ MNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSG GGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGLTFSRYAVAWFRQAPGKEREFVAAI TWSSGRIDYADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCAADRRPRSTGRSGTGS PSTYDYWGQGTLVTVSSA

F012900141 SEQ ID NO:20	AVQLVESGGGLVQPGGSLRLSCAASGRTFSTYVMGWFRQAPGKEREFVAAISWSGGSTYY ADSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCAASRGTSTPSRASGVSRYDYWGQG TLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPG NSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLYADSVKGRFTISRDNAK TTLYLQMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSSGGGGSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGSIFRIGAMGWYRQAPGKQ RELVAAVSSGGSTYYVDSVKGRFTISRDNSKNTVYLQMNSLRPEDTAVYYCNRETGPYGPP KRDYWGQGTLVTVSSA

As explained before, unless indicated otherwise, the polypeptides of the invention may include further moieties and/or additional polypeptide domains, as long as their binding to LRP5 will not be prevented by such additional moiety or domain.

The polypeptides of the invention can additionally contain modifications such as glycosyl residues or modified amino acid side chains, and they may be PEGylated in order to increase half-life and other properties of such molecule. Techniques and reagents useful for PEGylating ISVD constructs may be taken e.g. from WO2011/107507.

The polypeptides of the invention may have a modified N-terminal sequence, e.g. a deletion of one or more of the N-terminal amino acids, or an exchange of e.g. the first, N-terminal amino acid (e.g. glutamate to alanine), to optimize the molecule for being expressed by using certain expression systems (such as specific vectors or host cells), or for being expressed as inclusion bodies or in soluble form, or for being secreted into the medium or the periplasmic space or for being contained within the cell, or for yielding a more homogenous product. The polypeptides of the invention may have a modified C-terminal sequence, such as an additional alanine, and/or further amino acid exchanges in the C-terminal part or at other defined positions within any of the framework regions, as explained e.g. in WO2012/175741, WO2011/075861, or WO2013/024059, in order to e.g. further enhance stability or reduce immunogenicity of such polypeptides.

Furthermore, half-life of the polypeptides of the invention may be enhanced by adding an albumin domain, i.e. by converting them into albumin fusion proteins. Examples of useful albumin moieties, and how to add them to binding molecules, are e.g. provided in WO2001/079271 and WO2003/059934.

Preferably, the polypeptides of the invention are having binding (EC50) values, measured in a FACS binding assay as described in Example 7.1 below, in the range of 10^{-6} moles/liter or less, more preferably 10^{-9} moles/liter or less, and even more preferably in the range of from 10^{-10} to 10^{-13} moles/liter, or are having an IC50 value as measured in

a combined Wnt1 and Wnt3a reporter assay as set out in Example 7.3 below, of 10^{-9} moles/liter or below, and preferably in the range of from 5 x 10^{-10} moles/liter to 10^{-12} moles/liter.

The polypeptides of the invention allow a more efficient treatment of several cancer types, such as TNBC, CRC, and NSCLC. They are having improved *in vitro* characteristics (i.e. higher efficacy of Wnt pathway inhibition), cf. e.g. Examples 7 and 8 below, and significant *in vivo* tumor growth inhibition properties, leading to higher *in vivo* efficacy as compared to LRP6 binding molecules described in the art, as shown in e.g. Example 9 below. In addition, polypeptides of the invention have vastly lower gastrointestinal toxicity than LRP6 binding molecules described in the art, as shown in e.g. Example 9.

In particular, as shown *in vivo* in a Wnt driven tumor model, the LRP5 half-life extended biparatopic humanized VHH constructs could inhibit Wnt signaling and tumor growth *in vivo*, and even provided for substantial tumor shrinkage (i.e. tumor growth inhibition higher than 100%).. Tumor shrinkage (i.e. tumor regression) is the desired therapeutic effect (i.e. efficacy) for treatment of cancer patients. Furthermore, tumor regression, resulting in pathological complete response (pCR), is an acknowledged clinical endpoint, indicating significant improvement of progression free survival and overall survival.

In the same *in vivo* experiments, no significant body weight changes were observed (< 10%), and results from gastrointestinal histopathological analyses did not indicate any toxic effects of the above polypeptides of the invention. This is specifically surprising in view of the *in vivo* DKK1 expression studies discussed above (i.e. resulting in intestinal mucosa ulceration and body weight loss).

Furthermore, the examples described below demonstrate the ability of the half-life extended biparatopic LRP5 specific VHH constructs to selectively inhibit cell proliferation of cancer cells harboring mutations in *RNF43* gene which are dependent on active Wnt signaling. Specifically, it is further shown that the half-life extended biparatopic LRP5 specific VHH constructs described herein selectively inhibit Wnt signaling of cancer cells harboring mutations in *RNF43* gene.

Thus, the polypeptides of the invention are in fact providing for novel therapeutic options for the treatment of cancer diseases, and especially even for use in high unmet medical need indications such as (triple negative) breast cancer.

The above advantageous effects will further be illustrated in the Examples below and by way of the comparative data included therein.

Moreover, the polypeptides of the invention are easy to manufacture and are more soluble, which means that they may be stored and/or administered in higher concentrations compared to conventional antibodies. They are stable at room temperature and have prolonged stability even at extremes of pH, so that they may be prepared, stored and/or transported without the use of refrigeration equipment, conveying cost, time and environmental savings. Due to the above and due to their low immunogenicity, they furthermore offer a variety of options regarding administration routes other than injection and infusion, as well as regarding administration regimens and uses of specific devices.

Nucleic acids, vectors, host cells

According to further aspects, the invention relates to nucleic acid molecules and expression vectors encoding the polypeptides of the invention, as well as to host cells expressing the same. These nucleic acids, vectors, and host cells are useful for manufacturing the polypeptides of the invention, and further aspects and embodiments thereof will be described further below in connection with an outline of methods of manufacturing the polypeptides of the invention.

Therapeutic use

Due to their biological properties, the polypeptides of the invention are suitable for treating diseases characterised by excessive or abnormal cell proliferation, such as cancer and idiopathic pulmonary fibrosis (IPF).

For example, the following cancers, tumors, and other proliferative diseases may be treated with polypeptides according to the invention, without being restricted thereto:

Cancers of the head and neck; Cancers of the lung, such as e.g. non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC); Neoplasms of the mediastinum, such as e.g. neurogenic tumors and mesenchymal tumors; Cancers of the gastrointestinal (GI) tract, such as e.g. cancers of the esophagus, stomach (gastric cancer), pancreas, liver and biliary tree (including e.g. hepatocellular carcinoma (HCC)), and the small and large intestine (including e.g. colorectal cancer); Cancers of the prostate; Cancers of the testis; Gynecologic cancers, such as e.g. cancers of the ovary; Cancers of the breast, such as e.g. mammary carcinoma, hormone receptor positive

breast cancer, Her2 positive breast cancer, and triple negative breast cancer; Cancers of the endocrine system; Sarcomas of the soft tissues, such as e.g. fibrosarcoma, rhabdomyosarcoma, angiosarcoma, Kaposi´s sarcoma; Sarcomas of the bone, such as e.g. myeloma, osteosarcoma, Ewing´s tumor, fibrosarcoma, osteochondroma, osteoblastoma, and chondroblastoma; Mesotheliomas;

Cancers of the skin, such as e.g. basal cell carcinoma, squamous cell carcinoma, Merkel´s cell carcinoma, and melanoma; Neoplasms of the central nervous system and brain, such as e.g. astrocytoma, glioblastoma, gliomas neuroblastomas, and retinoblastomas; Lymphomas and leukemias such as e.g. B-cell non-Hodgkin lymphomas (NHL), T-cell non-Hodgkin lymphomas, chronic B-cell lymphocytic leukemia (B-CLL), chronic T-cell lymphocytic leukemia (T-CLL), Hodgkin´s disease (HD), large granular lymphocyte leukemia (LGL), chronic myelogenous leukemia (CML), acute myelogenous/myeloid leukemia (AML), acute lymphatic/lymphoblastic leukemia (ALL), multiple myeloma (MM), plasmacytoma, and myelodysplastic syndromes (MDS); and cancers of unknown primary site.

All cancers, tumors, neoplasms, etc., mentioned above which are characterized by their specific location/origin in the body are meant to include both the primary tumors and the metastatic tumors derived therefrom.

More specifically, the polypeptides of the invention are useful for the treatment of diseases, and esp. cancer diseases in which abnormal cell proliferation is caused by, or involves, abnormal (activated) Wnt signaling.

Thus, the polypeptides of the invention are specifically useful for the treatment of solid tumors, and more specifically for the treatment of lung, liver, colon, brain, thyroid, pancreas, breast, ovary and prostate cancers, and even more specifically for the treatment of non-small cell lung cancer (NSCLC), triple-negative breast cancer (TNBC), and colo-rectal cancer (CRC). In particular, the polypeptides of the invention may be used to treat patients with locally advanced or metastatic TNBC, patients with metastatic NSCLC or locally advanced or metastatic CRC, as single agent or in combination, to prolong progression free survival (PFS) and overall survival (OS). Furthermore, the polypeptides of the invention may be used as neoadjuvant treatment for breast cancer patients to achieve pathological complete response (pCR; defined as the absence of residual invasive and *in situ* cancer by histo-pathological evaluation of the complete

resected breast specimen and all sampled regional lymph nodes following completion of neoadjuvant systemic therapy).

The polypeptides of the invention may be used in therapeutic regimens in the context of first line, second line, or any further line treatments.

The polypeptides of the invention may be used for the prevention, short-term or long-term treatment of the above-mentioned diseases, optionally also in combination with radiotherapy and/or surgery.

Likewise, the polypeptides of the invention are specifically useful for the treatment of other diseases caused by abnormal cell proliferation involving the Wnt signaling pathway, such as idiopathic pulmonary fibrosis (IPF) (Königshoff *et al.* "Functional Wnt signaling is increased in idiopathic pulmonary fibrosis". *PLoS One* 2008;3(5):e2142; Lam *et al*. "Wnt coreceptor Lrp5 is a driver of idiopathic pulmonary fibrosis". *Am J Respir Crit Care Med*. 2014;190(2):185-95).

Furthermore, the polypeptides of the invention are specifically useful for the treatment of retinopathies, and esp. for the treatment of diabetic retinopathy, due to abnormal Wnt activation in the inner retina cells, causing the increase in abnormal new retinal vessel formation leading to development and progression of diabetic retinopathy (Chen, Y., *et al*. "Activation of the Wnt pathway plays a pathogenic role in diabetic retinopathy in humans and animal models" *The Am J Pathol.* 2009; 175(6):2676-85., Gao *et al*. "Elevated LRP6 levels correlate with vascular endothelial growth factor in the vitreous of proliferative diabetic retinopathy" *Mol Vis*. 2015;21:665-72).

Finally, as it could be shown that inhibition of Wnt1/Wnt3a signaling pathways can also have an effect on dendritic cells (DCs) and dendritic cell function, the polypeptides of the invention may also be useful in the treatment of immunologic and infectious diseases, as well as for influencing the tumor microenvironment in the various cancer diseases already set out above. Tumors actively suppress antitumor immunity, and DCs play an important role in the cancer immunoescape mechanism. In particular, studies have shown that Wnt ligands in the tumor microenvironment can also initiate paracrine signaling within the immune cells and regulate host antitumor immunity (Hong *et al.* "beta-catenin promotes regulatory T-cell responses in tumors by inducing vitamin A metabolism in dendritic cells". *Cancer Res*. 2015;75(4):656-65).

The above also includes the use of the polypeptides of the invention in various methods of treating the above diseases by administering a therapeutically effective dose to a patient in need thereof, as well as the use of these polypeptides for the manufacture of medicaments for the treatment of such diseases, as well as pharmaceutical compositions including such polypeptides of the inventions, as well as the preparation and/or manufacture of medicaments including such polypeptides of the invention, and the like.

Combinations with other active substances

The polypeptides of the invention may be used on their own or in combination with other pharmacologically active substances, such as state-of-the-art or standard-of-care compounds, such as e.g. cytostatic or cytotoxic substances, cell proliferation inhibitors, anti-angiogenic substances, steroids, immune modulators / checkpoint inhibitors, and the like.

Cytostatic and/or cytotoxic active substances which may be administered in combination with the compounds according to the invention include, without being restricted thereto, hormones, hormone analogues and antihormones, aromatase inhibitors, LHRH agonists and antagonists, inhibitors of growth factors (growth factors such as for example platelet derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insuline-like growth factors (IGF), human epidermal growth factor (HER, e.g. HER2, HER3, HER4) and hepatocyte growth factor (HGF)), inhibitors are for example (anti-)growth factor antibodies, (anti-)growth factor receptor antibodies and tyrosine kinase inhibitors, such as for example cetuximab, gefitinib, afatinib, nintedanib, imatinib, lapatinib, bosutinib and trastuzumab; antimetabolites (e.g. antifolates such as methotrexate, raltitrexed, pyrimidine analogues such as 5-fluorouracil (5-FU), capecitabine and gemcitabine, purine and adenosine analogues such as mercaptopurine, thioguanine, cladribine and pentostatin, cytarabine (ara C), fludarabine); antitumor antibiotics (e.g. anthracyclins); platinum derivatives (e.g. cisplatin, oxaliplatin, carboplatin); alkylation agents (e.g. estramustin, meclorethamine, melphalan, chlorambucil, busulphan, dacarbazin, cyclophosphamide, ifosfamide, temozolomide, nitrosoureas such as for example carmustin and lomustin, thiotepa); antimitotic agents (e.g. Vinca alkaloids such as for example vinblastine, vindesin, vinorelbin and vincristine; and taxanes such as paclitaxel, docetaxel); angiogenesis inhibitors, tubuline inhibitors; DNA synthesis inhibitors, PARP inhibitors, topoisomerase inhibitors (e.g. epipodophyllotoxins such as for example etoposide and etopophos,

teniposide, amsacrin, topotecan, irinotecan, mitoxantrone), serine/threonine kinase inhibitors (e.g. PDK1 inhibitors, Raf inhibitors, A-Raf inhibitors, B-Raf inhibitors, C-Raf inhibitors, mTOR inhibitors, mTORC1/2 inhibitors, PI3K inhibitors, PI3Kα inhibitors, dual mTOR/PI3K inhibitors, STK33 inhibitors, AKT inhibitors, PLK1 inhibitors (such as volasertib), inhibitors of CDKs, Aurora kinase inhibitors), tyrosine kinase inhibitors (e.g. PTK2/FAK inhibitors), protein protein interaction inhibitors, MEK inhibitors, ERK inhibitors, FLT3 inhibitors, BRD4 inhibitors, IGF-1R inhibitors, TRAILR2 agonists, Bcl-xL inhibitors, Bcl-2 inhibitors, Bcl-2/Bcl-xL inhibitors, ErbB receptor inhibitors, BCR-ABL inhibitors, ABL inhibitors, Src inhibitors, rapamycin analogs (e.g. everolimus, temsirolimus, ridaforolimus, sirolimus), androgen synthesis inhibitors, androgen receptor inhibitors, DNMT inhibitors, HDAC inhibitors, ANG1/2 inhibitors, CYP17 inhibitors, radiopharmaceuticals, immunotherapeutic agents such as immune checkpoint inhibitors (e.g. CTLA4, PD1, PD-L1, LAG3, and TIM3 binding molecules / immunoglobulins, such as ipilimumab, nivolumab, pembrolizumab), cancer vaccines such as traditional tumor vaccine (cell based vaccines, e.g. Sipuleucel-T for treatment of prostate cancer), personalized neoantigen vaccines, and oncolytic viruses, and various chemotherapeutic agents such as amifostin, anagrelid, clodronat, filgrastin, interferon, interferon alpha, leucovorin, rituximab, procarbazine, levamisole, mesna, mitotane, pamidronate and porfimer.

Particularly preferred are methods of treatment including the use of the polypeptides of the invention in combination with a drug selected from the group consisting of:

(i) anti-VEGF antibodies (bevacizumab and other anti-angiogenic substances) with or without chemotherapy combination (including doxorubicin / cyclophosphamide combination and/or capecitabine / docetaxel combination in neoadjuvant setting; taxane / platinum regimen for first and later line treatment) in breast cancer patients;

(ii) EGFR TKIs for EGFR mutant NSCLC or crizotinib for ALK translocated NSCLC with or without chemotherapy combination (platinum based cytotoxic combination therapy, including gemcitabine / cisplatin in first line treatment; docetaxel or pemetrexed in second line treatment in lung cancer patients;

(iii) anti-EGFR antibodies (cetuximab and panitumumab in *KRAS* wild-type tumors) with or without chemotherapy combination (including irinotecan), anti-VEGF antibody combination (bevacizumab and other anti-angiogenic substances) or regorafenib combination, e.g. for the treatment of CRC patients.

(iv) immunotherapeutic agents, including anti-PD-1 agents, such as pembrolizumab and nivolumab, anti-PD-L1 agents, anti-CTLA4 agents, anti-BTLA agents, anti-LAG3 agents, and anti-TIM3 agents, such as anti-PDL1 antibodies etc., e.g. for treatment of breast cancer, lung cancer and CRC patients

(v) chemotherapeutic agents, such as platinum-based antineoplastic agents, or in a combination with a FOLFOX chemotherapy regimen, including folinic acid, 5'-fluorouracil, and oxaliplatin, or in a combination with a FOLFOXIRI chemotherapy regimen, including folinic acid, 5'-fluorouracil, oxaliplatin, and irinotecan, e.g. for treatment of breast cancer or CRC patients.

When two or more substances or principles are to be used as part of a combined treatment regimen, they can be administered via the same route of administration or via different routes of administration, at essentially the same time (i.e. simultaneously, concurrently) or at different times (e.g. sequentially, successively, alternately, consecutively, or according to any other sort of alternating regime).

When the substances or principles are to be administered simultaneously via the same route of administration, they may be administered as different pharmaceutical formulations or compositions or as part of a combined pharmaceutical formulation or composition. Also, when two or more active substances or principles are to be used as part of a combined treatment regimen, each of the substances or principles may be administered in the same amount and according to the same regimen as used when the compound or principle is used on its own, and such combined use may or may not lead to a synergistic effect. However, when the combined use of the two or more active substances or principles leads to a synergistic effect, it may also be possible to reduce the amount of one, more or all of the substances or principles to be administered, while still achieving the desired therapeutic action. This may for example be useful for avoiding, limiting or reducing any unwanted side-effects that are associated with the use of one or more of the substances or principles when they are used in their usual amounts, while still obtaining the desired pharmacological or therapeutic effect.

The above includes the preparation, and methods of preparing, the polypeptides of the invention for the combined use with the above combination partners. Also included are the preparation, and methods of preparing, the above-mentioned combination partners for the combined use with the polypeptides of the invention. Thus, the invention hereby e.g. provides methods of using, or preparing for use, an immune modulator / checkpoint

inhibitor, such as an anti-PD1 antibody, such as pembrolizumab or nivolumab, for the administration in combination with a polypeptide of the invention, and more specifically for the administration in a combination therapy regimen with a polypeptide of the invention.

Furthermore, the invention also encompasses kits comprising at least one polypeptide of the invention and one or more other components selected from the group consisting of other drugs used for the treatment of the diseases and disorders as described above, and devices as described below.

Pharmaceutical compositions, methods of administration, dosages

It will be clear to the skilled person that the above methods of treatment of a disease include the preparation of a medicament for the treatment of said disease. Thus, the invention further relates to pharmaceutical compositions for the treatment of the diseases mentioned hereinabove, wherein such compositions comprise at least one polypeptide of the invention.

The polypeptides of the invention and/or the compositions comprising the same can be administered to a patient in need thereof in any suitable manner, depending on the specific pharmaceutical formulation or composition to be used. Thus, the polypeptides of the invention and/or the compositions comprising the same can for example be administered intravenously (i.v.), subcutaneously (s.c.), intramuscularly (i.m.), intraperitoneally (i.p.), transdermally, orally, sublingually (e.g. in the form of a sublingual tablet, spray or drop placed under the tongue and adsorbed through the mucus membranes into the capillary network under the tongue), (intra-)nasally (e.g. in the form of a nasal spray and/or as an aerosol), topically, by means of a suppository, by inhalation, or any other suitable manner in an effective amount or dose.

The polypeptides of the invention and/or the compositions comprising the same are administered according to a regimen of treatment that is suitable for treating and/or alleviating the disease, disorder or condition to be treated or alleviated. The clinician will generally be able to determine a suitable treatment regimen, depending on factors such as the disease, disorder or condition to be treated or alleviated, the severity of the disease, the severity of the symptoms thereof, the specific polypeptide of the invention to be used, the specific route of administration and pharmaceutical formulation or composition to be used, the age, gender, weight, diet, general condition of the patient, and similar factors well known to the clinician. Generally, the treatment regimen will

comprise the administration of one or more polypeptides of the invention, or of one or more compositions comprising the same, in therapeutically effective amounts or doses.

Generally, for the treatment and/or alleviation of the diseases, disorders and conditions mentioned herein and depending on the specific disease, disorder or condition to be treated, the potency of the specific polypeptide of the invention to be used, the specific route of administration and the specific pharmaceutical formulation or composition used, the polypeptides of the invention will generally be administered in an amount between 0.005 and 20.0 mg per kilogram of body weight and dose, preferably between 0.05 and 10.0 mg/kg/dose, and more preferably between 0.5 and 10 mg/kg/dose, either continuously (e.g. by infusion) or more preferably as single doses (such as e.g. twice a week, weekly, or monthly doses; cf. below), but can vary, especially, depending on the before-mentioned parameters. Thus, in some cases it may be sufficient to use less than the minimum dose given above, whereas in other cases the upper limit may have to be exceeded. When administering large amounts it may be advisable to divide them up into a number of smaller doses spread over the day.

Depending on the specific polypeptide of the invention and its specific pharmacokinetic and other properties, it may be administered daily, every second, third, fourth, fifth or sixth day, weekly, monthly, and the like. An administration regimen could include long-term, weekly treatment. By "long-term" is meant at least two weeks and preferably months, or years of duration.

The efficacy of the polypeptides of the invention, and of compositions comprising the same, can be tested using any suitable *in vitro* assay, cell-based assay, *in vivo* assay and/or animal model known *per se*, or any combination thereof, depending on the specific disease involved. Suitable assays and animal models will be clear to the skilled person, and for example include the assays and animal models used in the Examples below.

Preferably, the polypeptides of the invention are having better characteristics than conventional antibodies known in the art in at least one of these assays or models, and preferably in one or more of the *in vivo* models.

Formulations

For pharmaceutical use, the polypeptides of the invention may be formulated as a pharmaceutical preparation comprising (i) at least one polypeptide of the invention and (ii) at least one pharmaceutically acceptable carrier, diluent, excipient, adjuvant, and/or stabilizer, and (iii) optionally one or more further pharmacologically active polypeptides and/or compounds. By "pharmaceutically acceptable" is meant that the respective material does not show any biological or otherwise undesirable effects when administered to an individual and does not interact in a deleterious manner with any of the other components of the pharmaceutical composition (such as e.g. the pharmaceutically active ingredient) in which it is contained. Specific examples can be found in standard handbooks, such as e.g. Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Company, USA (1990). For example, the polypeptides of the invention may be formulated and administered in any manner known *per se* for conventional antibodies and antibody fragments and other pharmaceutically active proteins. Thus, according to a further embodiment, the invention relates to a pharmaceutical composition or preparation that contains at least one polypeptide of the invention and at least one pharmaceutically acceptable carrier, diluent, excipient, adjuvant and/or stabilizer, and optionally one or more further pharmacologically active substances.

Pharmaceutical preparations for parenteral administration, such as intravenous, intramuscular, subcutaneous injection or intravenous infusion may for example be sterile solutions, suspensions, dispersions, emulsions, or powders which comprise the active ingredient and which are suitable, optionally after a further dissolution or dilution step, for infusion or injection. Suitable carriers or diluents for such preparations for example include, without limitation, sterile water and pharmaceutically acceptable aqueous buffers and solutions such as physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution; water oils; glycerol; ethanol; glycols such as propylene glycol, as well as mineral oils, animal oils and vegetable oils, for example peanut oil, soybean oil, as well as suitable mixtures thereof.

Solutions of the polypeptides of the invention may also contain a preservative to prevent the growth of microorganisms, such as antibacterial and antifungal agents, for example, p-hydroxybenzoates, parabens, chlorobutanol, phenol, sorbic acid, thiomersal, (alkali metal salts of) ethylenediamine tetraacetic acid, and the like. In many cases, it will be

preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Optionally, emulsifiers and/or dispersants may be used. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. Other agents delaying absorption, for example, aluminum monostearate and gelatin, may also be added. The solutions may be filled into injection vials, ampoules, infusion bottles, and the like.

In all cases, the ultimate dosage form must be sterile, fluid and stable under the conditions of manufacture and storage. Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

Usually, aqueous solutions or suspensions will be preferred. Generally, suitable formulations for therapeutic proteins such as the polypeptides of the invention are buffered protein solutions, such as solutions including the protein in a suitable concentration (such as from 0.001 to 400 mg/ml, preferably from 0.005 to 200 mg/ml, more preferably 0.01 to 200 mg/ml, more preferably 1.0 - 100 mg/ml, such as 1.0 mg/ml (i.v. administration) or 100 mg/ml (s.c. administration) and an aqueous buffer such as:

- phosphate buffered saline, pH 7.4,
- other phosphate buffers, pH 6.2 to 8.2,
- acetate buffers, pH 3.2 to 7.5, preferably pH 4.8 to 5.5
- histidine buffers, pH 5.5 to 7.0,
- succinate buffers, pH 3.2 to 6.6, and
- citrate buffers, pH 2.1 to 6.2,

and, optionally, salts (e.g. NaCl) and/or sugars (such as e.g. sucrose and trehalose) and/or other polyalcohols (such as e.g. mannitol and glycerol) for providing isotonicity of the solution.

Preferred buffered protein solutions are solutions including about 0.05 mg/ml of the polypeptide of the invention dissolved in 25 mM phosphate buffer, pH 6.5, adjusted to isotonicity by adding 220 mM trehalose. In addition, other agents such as a detergent, e.g. 0.02 % Tween-20 or Tween-80, may be included in such solutions. Formulations for subcutaneous application may include higher concentrations of the polypeptide of the invention, such as up to 100 mg/ml or even above 100 mg/ml. However, it will be clear to the person skilled in the art that the ingredients and the amounts thereof as given above do only represent one, preferred option. Alternatives and variations thereof will be immediately apparent to the skilled person, or can easily be conceived starting from the above disclosure.

Also, compared to conventional antibodies or antibody fragments, one major advantage of the use of the polypeptides of the invention is that they can also be easily administered via routes other than parenteral administration and can be easily formulated for such administration. For example, as described in international patent application WO2004/041867, such polypeptides may be formulated for oral, intranasal, intrapulmonary and transdermal administration.

According to a further aspect of the invention, a polypeptide of the invention may be used in combination with a device useful for the administration of the polypeptide, such as a syringe, injector pen, micropump, or other device.

Methods of manufacture and purification

The invention further provides methods of manufacturing a polypeptide of the invention, such methods generally comprising the steps of:

- culturing host cells comprising a nucleic acid encoding a polypeptide of the invention (hereinafter: "nucleic acid of the invention") under conditions that allow expression of the polypeptide of the invention; and,

- recovering or isolating the polypeptide expressed by the host cells from the culture; and

- optionally further purifying and/or modifying and/or formulating the polypeptide of the invention.

A nucleic acid of the invention can e.g. be a DNA molecule comprising coding sequences as well as regulatory sequences and optionally natural or artificial introns, or can be a cDNA molecule. It may have its original codons or may have an optimized codon usage

that has been specifically adapted for expression in the intended host cell or host organism. According to one embodiment of the invention, the nucleic acid of the invention is in essentially isolated form, as defined above.

The nucleic acid of the invention may also be in the form of, be present in and/or be part of a vector, such as for example a plasmid, cosmid or YAC, which again may be in essentially isolated form. The vector may especially be an expression vector, i.e. a vector that can provide for expression of the polypeptide *in vitro* and/or *in vivo* (e.g. in a suitable host cell, host organism and/or expression system). Such expression vector generally comprises at least one nucleic acid of the invention that is operably linked to one or more suitable regulatory element(s), such as promoter(s), enhancer(s), terminator(s), and the like. Specific examples of such regulatory elements and other elements, such as integration factor(s), selection marker(s), signal or leader sequence(s), reporter gene(s), and the like, useful or necessary for expressing polypeptides of the invention, are disclosed e.g. on pp. 131 to 133 of WO2006/040153.

The nucleic acids of the invention can be prepared or obtained in a manner known *per se* (e.g. by automated DNA synthesis and/or recombinant DNA technology), based on the information on the amino acid sequences for the polypeptides of the invention given herein.

According to another embodiment, the invention relates to a host or host cell that expresses or is capable of expressing a polypeptide of the invention; and/or that contains a nucleic acid encoding a polypeptide of the invention. According to a particularly preferred embodiment, said host cells are bacterial cells, yeast cells, fungal cells or mammalian cells.

For production on industrial scale, preferred heterologous hosts for the (industrial) production of ISVD polypeptides and protein therapeutics containing them include strains of *E. coli*, *Pichia pastoris*, and *S. cerevisiae* that are suitable for large scale expression, production and fermentation, and in particular for large scale (bio-)pharmaceutical expression, production and fermentation.

Polypeptides of the invention produced in a cell as set out above can be produced either intracellularly (e.g. in the cytosol, in the periplasma or in inclusion bodies) and can then be isolated from the host cells and optionally be further purified; or they can be produced extracellularly (secreted into the medium in which the host cells are cultured) and then isolated from the culture medium and optionally further purified.

Further methods and reagents used for the recombinant production of polypeptides, such as suitable expression vectors, transformation or transfection methods, selection markers, methods of induction of protein expression, culture conditions, and the like, are known in the art. Similarly, protein isolation and purification techniques useful in a method of manufacture of a polypeptide of the invention are well known to the skilled person.

Production of the polypeptides of the invention through fermentation in convenient recombinant host organisms such as *E. coli* and yeast is cost-effective, as compared to conventional antibodies which usually require expensive mammalian cell culture facilities. Furthermore, achievable levels of expression are high and yields of the polypeptides of the invention are in the range of 1 to 10 g/l (E. coli) and up to 10 g/l (yeast) and more.

EXAMPLES

Example 1: Immunization of llamas with LRP5 for the induction of humoral immune responses

Several protocols for the immunization of llamas needed to be worked out and implemented for identification of LRP5 binding VHH domains: Llamas were initially immunized with recombinant extracellular domains of LRP5 protein (mouse). However, functional characterization of the above mentioned LRP5 recombinant protein revealed that the recombinant protein was not properly folded. Thus, further work was required to develop suitable antigens for immunization. As a work-around, llamas were immunized with HEK293 cells stably transfected with human LRP5 or cynomolgus (cyno) LRP5. However, also then, only very low expression of human or cyno LRP5 could be achieved, by transient or by stable transfection, and using different cell lines (HEK293 and CHO). Thus, even further work was required to achieve sufficient expression of LRP5. In the end, and after some unsuccessful trial and error, this could be achieved by developing a protocol involving stable co-transfection of HEK293 cells with MesDC-2, a chaperone intended to increase exogenous LRP5 expression. Even then, i.e. upon co-expression of MesDC-2 during the generation of the LRP5 stable transfected cell line, instability of protein expression was repeatedly observed. In contrast, significantly higher and stable expression levels were achieved upon co-expression of human LRP6 with MesDC-2 in HEK293 cells. These findings are in line with published data showing the inherent challenge of human LRP5 as very poorly expressed protein (Fleury et al, Protein Expr

Purif. 2010 Mar;70(1):39-47). This resulted in the problem that LRP5 expression could be lost during immunization and selection. To solve this further problem, passaging of the cells expressing LRP5 was limited as much as possible and additional cell sorting was performed to enrich for cells expressing LRP5.

Llamas were additionally immunized with LRP5-coding DNA with and without the hMesDC-2 chaperone in opposite flanks. Additional boosts were delivered to several llamas in an attempt to enhance the species cross-reactive immune response, with the objective to increase chances of identifying anti-human-LRP5 VHH domains cross-reactive with mouse and cyno LRP5.

Immune blood (PBL) samples were taken at regular intervals, serum responses were determined, and total RNA was prepared from the isolated PBL. Low serum responses to LRP5 upon immunization with recombinant protein; in contrast, medium LRP5 immune response was observed for llamas immunized with DNA. Very low immune response was observed for the cell immunizations. Additionally, synthetic libraries were also explored. Nevertheless, finally, sufficient diversity of the repertoire could be achieved for continuing with the next steps, as outlined in Example 2.

Example 2: Isolation of LRP5 binding monovalent VHH domains (VHHs)

Library construction:

Total RNA was extracted immediately following collection of the immune tissues, and RNA integrity and concentration was verified. cDNA samples were made from these RNA preparations. Nucleotide sequences encoding VHHs were amplified from the cDNA samples in a one-step RT-PCR reaction. The 700bp amplicons specifically amplified from the IgG2 and IgG3 cDNAs in the sample were isolated from agarose gel and subsequently used as template in a nested PCR reaction. The PCR products were subsequently digested with *Sfi*I and *BstE*II and ligated into the corresponding restriction sites of phagemid vector pAX50. The ligation mixtures were electroporated into *Escherichia coli* TG-1. The resulting pool of transformants constituted the genetic diversity of the phage display library.

pAX50 is an expression vector derived from pUC119, which contains a resistance gene for ampicillin and the *lac* promoter followed by the coding sequence of the pIII protein signal peptide in frame with a downstream VHH domain cloning site. In frame with the VHH domain coding sequence, the vector codes for a C-terminal Myc and hexa-histidine

tag and a coli phage pIII protein. After infection of the *E. coli* TG-1 library clones with helper phage, the presence of pAX50 allows for production of phage particles from these clones, displaying the individual VHH domains as a fusion protein with the pIII protein.

Selection:

The VHH domain-phagemid libraries were constructed and used for selections. Given the very high species homology across species (between llama and human LRP5), it was uncertain whether the immune response raised in the llamas would raise sufficient diversity of the VHH domains. Therefore, two synthetic libraries were used in parallel with the immune libraries during selections.

Different strategies were used during selections as follows:

- Alternation of species source (i.e. human and mouse LRP5 derived tools) to enhance chances of identifying human/mouse LRP5 species cross-reactive VHH domains, e.g. selections on libraries from human LRP5 immunized llamas with HEK293 stably expressing mouse LRP5 or the use of both mouse and human LRP5 expressing HEK293 cell lines during selection on synthetic libraries (mouse cross-reactivity of such LRP5 antagonist allowing to evaluate efficacy, i.e. tumor growth inhibition, and safety profiles, required for assessment of the therapeutic window, in the same pre-clinical models (i.e. in xenograft tumor-mouse models)).

- “In solution” selections with LRP5 recombinant protein to keep the epitopes in their native conformation: As an additional obstacle, LRP5 recombinant protein was found to lose proper folding if directly coated on ELISA binding plates. Therefore, the recombinant protein was biotinylated and, after confirming proper folding in functional assays, was used for selection “in solution”.

- Selections using cells overexpressing LRP5, to have native conformation of the receptors. This surprisingly turned out to be an important ruse, especially needed to improve selection of binders to the Wnt3a–class binding domain of LRP5, since functional data of recombinant protein showed lack of proper folding of the Wnt3a binding epitope.

- Negative selection using cells overexpressing LRP6 to identify LRP5-selective binders.

Example 3: Screening of the monovalent VHHs

After selection, clones were grown in 96 deep well plates (1 mL volume), and VHH expression was induced by adding IPTG. Periplasmic extracts of single clones were prepared according to standard methods, as e.g. reported in WO2011/107507, and screened for binding to human LRP5. Initially, the periplasmic extracts were screened in binding ELISA assays using recombinant LRP5, which represent sensitive, robust and high-throughput assays, when compared to FACS-based binding assays. After purification, VHHs identified in ELISA assays were further characterized using binding FACS assay to confirm binding of purified VHHs to the LRP5 receptor in their native conformation.

Usually, a good correlation between ELISA and FACS binding assays is expected. However, in the present case, the best LRP5 binding VHHs in ELISA assays (i.e. with high affinity to recombinant LRP5 ectodomain) did not show any binding or very weak binding to human LRP5 in a FACS binding assay. The use of different coating buffers (dPBS vs. bicarbonate buffer) and blocking solutions in the ELISA set-up (Marvel vs. BSA) did not resolve the observed discrepancy. Instead, it was found out that very weak binders in ELISA showed high affinity to LRP5 in binding FACS assays using LRP5 expressing HEK293 cells. These further data and experiments thus allowed for selection of high affinity binders recognizing the native conformation of the two receptors. Additionally, confirmation was thereby obtained that these high affinity binders are recognizing a conformation dependent epitope, and not a linear epitope in LRP5 protein. These further non-routine data and experiments thus allowed for selection of therapeutically relevant LRP5 binders, which should have high affinity towards LRP5 expressed on the plasma membrane in their native conformation.

Thus, despite (i) the low throughput of FACS binding assays, (ii) the less robust assay set-up, and (iii) the above-described difficulties encountered due to the loss of recombinant protein expression upon passaging of the cells over-expressing LRP5, these assays were subsequently used for further selection and characterization of high affinity VHH binders. Briefly, cells were incubated with purified VHH dilutions (1:5 serial dilutions from 1μM to 1pM, final concentration) for 1.5 hours at 4 °C on a plate shaker. After washing the cells 5 x with FACS buffer, consisting of 1x phosphate buffered saline (PBS) + 10% fetal bovine serum (FBS) + 0,05% sodium azide, they were incubated for 30 minute up to 1 hour at 4°C with a polyclonal mouse antibody which binds to the

framework regions of the VHHs and, therefore, binds to all the LRP5 binders tested. After washing the cells 3X with FACS buffer, the cells were incubated for 30 minutes up to 1 hour at 4 °C with the labeled secondary antibody (anti-mouse PE), followed by 3X washing step with FACS buffer. Fluorescence was measured using FACS Array (BD).

A total of around fifty LRP5-selective VHHs families/clusters were identified from immune libraries and from synthetic origin, based on binding FACS data and sequence analysis. Representative examples thereof are shown, and defined by their sequence, further below. The VHHs were expressed in *E.coli* and purified. In case expression in *E.coli* proved insufficient, VHHs were produced in *Pichia pastoris*. A brief description of expression and purification of VHHs is reported further below.

Generic expression of VHHs in *E.coli:*

The encoding sequences were cloned into the pAX100 expression vector and expressed in *E.coli* as c-Myc hexa-histidine-tagged proteins. *E.coli* TG-1 cells containing the VHH constructs of interest were grown (37 °C, 250 rpm) in shake flasks in TB medium supplemented with kanamycin and induced by addition of 1mM IPTG for expression. After spinning the cell cultures, periplasmic extracts were prepared by freeze-thawing the pellets and resuspending in dPBS.

Generic expression of VHHs in *Pichia (P.) pastoris:*

The encoding sequences were cloned into the pAX159 expression vector and expressed in *P. pastoris* as c-Myc hexa-histidine-tagged proteins. *P. pastoris* X-33 cells containing VHH constructs of interest were grown (30 °C, 250 rpm) in BGCM (Buffered Glycerol-Complex Medium; Invitrogen). On the third day, the medium was switched to BMCM (Buffered Methanol-Complex Medium; Invitrogen) and the culture was grown further and was regularly induced by addition of 0.5 vol% methanol (100%). After spinning the cell culture, supernatant (containing the secreted VHH) was collected.

VHH purification:

Hexa-histidine-tagged VHHs were purified on the Tecan EVO150 by immobilised metal affinity chromatography (RoboColumns 100ul Nickel Sepharose™ 6 FF, Atoll), eluted from the column with 250mM imidazole and subsequently desalted towards dPBS. The purity and integrity of VHHs was verified by SDS-PAGE and/or western blot using anti-Myc and anti-VHH detection.

Example 4: *In vitro* characterization of purified monovalent VHHs

After VHH screening, the purified VHHs having high affinity to cells expressing LRP5 were characterized by using several functional and biophysical assays as described below:

4.1 LRP5 selective binding potency and cross-reactivity: FACS-based DKK1 competition assay

During characterization of LRP5 selective monovalent VHHs, it was observed that data obtained in binding FACS assay did not always correlate with the potency observed in Wnt1 and Wnt3a reporter assays, most likely due to fast off-rate of some of the VHHs. Therefore, this additional assay needed to be established (i.e. a DKK1 competition FACS), which proved to be more reliable for selectivity and binding potency determination including LRP5 binding and lack of LRP6 binding. The aim was to select functional VHHs which bind selectively to LRP5 with no binding to LRP6 detected at concentration up to 1μM. The identified Wnt1 and Wnt3a functional VHHs were thus characterized in DKK-1 competition FACS as follows:

For FACS-based DKK1 competition assay, HEK293 cells with stable overexpression of human LRP5 or human LRP6 were used. Human recombinant DKK1 (rhDKK1 - R&D Systems, Cat 5439-DK/CF) was added to the cells at the constant final concentration of 1nM. Cells were incubated with rhDKK1 and LRP5 binder dilutions (1:5 serial dilution of the purified VHHs) for 1.5 hours at 4 °C on a plate shaker. After washing the cells three times with FACS buffer, they were incubated with biotinylated goat anti-human DKK1 (R&D Systems, Cat BAF1096) for 30 minutes at 4 °C on a plate shaker. After washing the cells three times with FACS buffer, they were incubated with Streptavidin PE (BD Biosciences, Cat 554061) for 30 minute to 1 hour at 4 °C on a plate shaker in the dark. Cells were washed twice with FACS buffer and fluorescence was measured using FACS Array (BD) and mean channel fluorescence (MCF) values were reported.

LRP5 selective VHHs are expected to compete with human DKK1 for binding to HEK293 cells overexpressing human LRP5 but not, or with very low potency (>1μM) for binding to HEK293 cells overexpressing human LRP6 (and the same would apply, vice versa, to LRP6 specific VHHs). In contrast, LRP5/LRP6 cross-reactive VHHs would compete with human DKK1 for binding to HEK293 overexpressing human LRP5, as well as for binding to HEK293 overexpressing human LRP6. As a result of this experiment, it could be shown that the present LRP5 selective VHHs competed with human DKK1 for binding to

HEK293 cells overexpressing human LRP5 (i.e. decrease in MCF value with increasing concentration of the binder with complete inhibition of DKK1 binding corresponded to ≤ 60 MCF values at the highest concentration tested).

4.2 Species cross-reactivity: mouse and cynomolgus monkey

To determine whether the selected panel of LRP5 selective VHHs was able to bind to LRP5 of mouse and of cynomolgus monkey origin, DKK1 competition FACS was performed as follows:

Serial dilutions of VHHs were incubated with HEK293 cells stably expressing mouse LRP5 or cyno LRP5 in the presence of 1 and 0.3 nM hDKK1 (concentration below EC50 value of mouse and cyno, respectively). Binding of DKK1 to the cells was detected using a biotinylated anti-DKK1 antibody with Streptavidin-PE as secondary detection as described above. As a result, such cross-reactivity could be demonstrated.

4.3 Epitope binning

Binning experiments were performed for the most potent Wnt1 signaling blocking LRP5 selective VHHs, to identify different epitope bins. In particular, individual VHHs were analyzed for their ability to compete with other biotinylated VHHs (called reference VHHs) for LRP5 receptor binding using FACS based assays. Serial dilutions of the individual VHHs were incubated on HEK293 stably expressing human LRP5 together with 200pM or 500pM biotinylated reference VHH (concentrations below EC50 value). The binding of biotinylated reference VHH to the cells was detected using streptavidin-PE. A VHH competing with the reference VHHs for binding to LRP5 shows a decrease in the fluorescence measured using FACS Array.

As a result of these experiments, the Wnt1 blockers and Wnt3a blockers could be categorized into two and six bins, respectively.

4.4 Wnt1 and Wnt3a reporter assays

The ability of the LRP5 selective VHHs to inhibit Wnt signaling was tested in functional Wnt1 and Wnt3a assays. Also in this regard, no established protocol could be used, but several attempts needed to be tried to establish biochemical functional assays, such as Wnt1/Wnt3a – LRP5 blocking assays: In addition to the difficulties encountered with recombinant LRP5 proteins (see Example 1), a functional recombinant Wnt1 ligand is not available, let alone commercially available. Wnt proteins contain many conserved cysteines and are modified by mono-unsaturated fatty acid (palmitoleic acid), attached to

a conserved serine. These post-translational modifications are required for efficient signaling and for Wnt secretion. Structural analyses show that one of the domains, containing the palmitoleic acid lipid, is required for binding to the Frizzled receptors, leading to a conformational change that allows the interaction of the Wnt ligands with LRP5 receptor on the cell surface. Thus, it turned out that such post-translational modification is required for functional studies involving this protein, but at the same time such lipid-based post-translational modifications render these proteins very difficult to express and purify (low solubility). Thus, this turned out to be a major hurdle for biochemical assays.

Thus, a cell based functional assay was developed for characterization of the purified VHHs: A Wnt beta-lactamase reporter gene assay. In particular for the Wnt1 pathway inhibition, CellSensor LEF/TCF-bla FreeStyle 293F cells (Invitrogen, Cat. K1677) were transfected with human Wnt1, and clones with stable overexpression of human Wnt1 were selected. For testing the Wnt3a pathway inhibition, CellSensor LEF/TCF-bla FreeStyle 293F cells with stable overexpression of human Wnt3a were generated and, two days before treatment with LRP5 selective VHHs, transient knockdown of human LRP6 was performed via siRNA (SMARTpool, ON-TARGETplus siRNA, Dharmacon, Cat L-003845-0010) according to the manufacturer's instructions.

The CellSensor® LEF/TCF - bla FreeStyle™ 293 cell line contains a beta-lactamase reporter gene under control of the Wnt inducible LEF/TCF promoter, which is stably integrated into FreeStyle™ 293 cells (Invitrogen). The expression of Wnt1 or Wnt3a in these cells thus results in the constitutive expression and, therefore, enzymatic activity of the beta-lactamase. The treatment with LRP5 selective functional VHHs is thus expected to lead to inhibition of Wnt1 or Wnt3a pathway leading to inhibition of beta-lactamase enzymatic activity.

For the assay, 1E06 / ml cells with overexpression of Wnt1 or Wnt3a were seeded into a 384 well tissue culture plate and incubated overnight at 37 °C. The following day, serial dilutions of various LRP5 selective VHH solutions were prepared and added to the cells in the presence of LiCl at the final concentration of 10nM. DKK1, as positive control, was added to the cells at the final concentration of 200nM. DKK1 treatment resulted in a complete inhibition of Wnt1 and Wnt3a pathway, and therefore a complete inhibition of beta-lactamase enzymatic activity. The cells were incubated overnight at 37 °C. The following day, beta-lactamase enzymatic activity was measured according to the

manufacturer's instructions (Invitrogen, Cat K1085). For fluorescence emission, values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 nm emission ratios plotted for the indicated treatment. Efficacy was calculated against the positive control (DKK1; 200nM final concentration).

Two Wnt1 blockers were identified. A total of eight Wnt3a blockers showed good potency (IC_{50} below 100nM) and full efficacy.

4.5 Wnt1 and Wnt3a phosphorylation assays

The most potent and efficacious leads from each bin for the Wnt1 blockers and the most potent and efficacious Wnt3a blockers were subsequently tested in Wnt1 and Wnt3a dependent LRP5 phosphorylation assays. Cellsensor LEF/TCF 293F cells from Invitrogen (cat K1677), co-transfected with expression vectors coding for either Wnt1 or Wnt3a, were used in the phosphorylation assays. Since Wnt-Frizzled-LRP5 complex formation results in LRP5 phosphorylation and subsequent downstream signaling, quantification of phosphorylation can be used to measure such signaling. To obtain a LRP5 specific read-out, the cells were lysed and immuno-precipitation was performed with a LRP5 selective antibody (directed against the intracellular domain of the receptor). In Western blot, phosphorylated LRP5 was detected using a polyclonal anti-phospho-LRP6 (Ser1490) antibody (Cell Signaling Technology), which cross-reacts with LRP5 phosphorylated protein. A selected panel of purified Wnt1 and Wnt3a blocking VHHs, containing at least one representative VHH from each bin, were tested at final concentrations of 10 to 100nM. In particular, cells were incubated overnight in the presence of the blocking VHHs prior cell lysis and LRP5 immuno-precipitation. Efficacy of the Wnt1 orWnt3a blocking VHHs in blocking LRP5 phosphorylation was calculated via quantification of the Western blot bands against the positive control (DKK1, final concentration of 1 µM).

4.6 Biophysical characterization

The LRP5 selective VHHs were further characterized for expression and purification in *E. coli* and in *Pichia pastoris,* as reported in Example 3. In particular, expression yields for the monovalent lead panel VHHs was considered acceptable if they were above 0.1 mg/L. The selected LRP5 selective VHHs showed expression ranging between 0.3 and 22.6 mg/L in *E.coli* and higher in *Pichia pastoris* (> 1mg/L). The expression was evaluated by SDS-PAGE analysis.

Thermal stability of the monovalent LRP5 selective VHHs was determined in a fluorescence-based thermal shift assay (TSA) using the Lightcycler (Roche). VHHs were incubated at different pH values in the presence of Sypro Orange and a temperature gradient was applied. Upon heat-induced unfolding, hydrophobic patches of the proteins are exposed to which the Sypro Orange binds resulting in an increase in fluorescence intensity (Ex/Em = 465/580nm). The inflection point of the first derivative of the fluorescence intensity curve serves as a measure of the melting temperature (Tm). For all VHHs, Tm increased with increasing pH and levels off at pH 6, a typical Tm pattern seen for VHHs. An average of more than 68 °C at pH 7 was obtained for the LRP5 selective Wnt1 blocker VHHs and Wnt3a blocker VHHs.

Potential occurrence of aggregation and multimerisation for LRP5 selective VHHs was investigated by analytical size exclusion chromatography (SEC). To this end, 8ug of purified VHH sample at 0,5 mg/mL were injected via the Dionex Ultimate 3000 equipment on an Agilent SEC-3 column. L-arginine buffer (10mM phosphate, 300mM Arg-HCl, pH 6.0) was used as mobile phase and a flow rate of 1mL/min was applied. None of the LRP5 selective VHHs showed major aggregation issues during SEC analysis: profiles indicated more than 95% monomer for most samples.

Example 5: Generation and characterization of half-life extended biparatopic constructs

The LRP5 selective Wnt1 and Wnt3a VHHs were used as building blocks to generate a biparatopic construct as depicted in Figure 1. A genetic fusion to a serum albumin binding VHH was used as a half-life extension methodology. The three building blocks (Wnt1 blocker, Wnt3a blocker, and the albumin binder) were linked via a flexible linker. VHHs were produced in *P. pastoris* and purified as described in Example 3. The resulting constructs, i.e. biparatopic, half-life extended LRP5 selective VHH constructs, were cloned in *P. pastoris* expression vector pAX159, in the form of C-terminally cMyc-hexa-histidine tagged VHH constructs, according to standard procedures as e.g. reported in WO2012/131078. Different orientation of the building blocks and different linkers, esp. GS-linkers, were explored. A relatively long GS-linker was selected, based on homology modeling data reflecting an extended surface area between the potential Wnt1 and Wnt3a binding sites in LRP5. Best results regarding potency in the combined Wnt1 and Wnt3a reporter assay were obtained by putting the human serum albumin/HSA binding

VHH in the middle. A 35 GS linker was used, and Wnt1 and Wnt3a VHH blockers arranged in a preferred order.

For selection of optimal VHH binders and binder combinations, a library was generated where the human serum albumin (HSA) binding VHH was placed in between the LRP5 selective Wnt1-Wnt3a blockers. In particular, the panel of high affinity binders with high potency and efficacy in Wnt1 or Wnt3a assays (reporter and phosphorylation assays) were used in the library to generate half-life extended biparatopic constructs designed as depicted in Figure 1. After expression in *Pichia pastoris* (as reported in Example 3) followed by purification, the half-life extended biparatopic constructs were subsequently screened in the Wnt1 and Wnt3a reporter assays (described in Example 4) in the presence of 30uM HSA at three dilutions (1/100, 1/1000, 1/7000), to assess efficacy and relative potency. In general, a good correlation was observed between the data in the Wnt1 and Wnt3a reporter assays and high efficacies were measured for numerous formatted binders. A total of ten half-life extended biparatopic LRP5 selective constructs were selected for further characterization, taking into account the efficacy in both reporter assays and diversity of the Wnt1 and Wnt3a blockers. This further characterization assays are described below.

Wnt1 / Wnt3a reporter assays:

Wnt1 and Wnt3a reporter assays were performed as described in Example 4.4, in the presence of 30uM HSA final concentration. The purified biparatopic LRP5 selective constructs were tested at 12 dilutions, starting from 2.5 µM.

The majority of the constructs showed high potency - with IC_{50} values below 1nM in Wnt3a and below 5.7nM in Wnt1 reporter assays, respectively- and full efficacy in both reporter assays for LRP5 dependent Wnt signaling.

Example 6: Sequence optimization of VHHs and VHH constructs

Sequence optimization is a process in which the parental sequence is mutated to make it more identical to the human IGHV3-IGHJ germline consensus sequence. For example, specific amino acids in the framework regions (with the exception of the so-called hallmark residues) are exchanged for their human counterparts, in a way that the protein structure, activity and stability shall be preserved.

These mutations may be categorized as follows:

1. Standard: Sequence optimization of these positions is not expected to dramatically change the stability or activity or affinity of the VHH and they are therefore altered all at once, yielding a basic variant.
2. Unique: It is not known if sequence optimization of these positions affects the stability or activity or affinity of the VHH and therefore they are investigated on an individual basis on top of the basic variant.

Hallmark residues are known to be critical for the stability, activity and affinity of the VHH and are therefore not mutated.

In addition, the amino acids present in the CDRs for which there is experimental evidence that they are sensitive to post-translational modifications (PTM) were altered in such a way that the PTM site is inactivated while the protein structure, activity and stability shall remain intact. The most common post-translational modifications described for antibodies and VHHs are listed in Table IV below. The sensitivity of the VHHs for post-translational modifications was analysed in accelerated stress studies, applying several standard conditions including H_2O_2 treatment to analyze methionine oxidation, high temperature, high pH and long storage to study asparagine deamidation and aspartate isomerization. The percentage of oxidation, deamidation and isomerization was measured according to standard procedures and compared to the reference samples (VHHs stored at -20°C). Whole protein analysis in Reverse Phase Chromatography (RPC) and peptide mapping using Mass Spectrometry (MS) were performed to identify potentially sensitive residues. In case of post-translational modifications observed in the VHHs after stress test, the corresponding amino acid(s) were mutated.

TABLE IV: Potential post-translational modifications, and motifs potentially triggering them

Motif	Modification
M	Met oxidation
N-S/G/H/N/A	Asn deamidation
D-S/G/H	Asp isomerisation
N-X-S/T-X (X ≠ P)	Asn glycosylation
Q/E	pyroglutamate

As a result, several mutations were introduced in the constructs set out above, resulting i.a. in the three constructs shown in Table III above, which were chosen for further *in vitro* and *in vivo* characterization, as set out in the Examples further below.

Example 7: *In vitro* characterization of three half-life extended biparatopic LRP5 specific VHH constructs; Comparison to LRP6 binding molecules

After VHH sequence optimization, three half-life extended biparatopic LRP5 selective VHH constructs (cf. Table III above) were recombinantly expressed and purified, and were characterized by using several functional and biophysical assays as described below.

7.1 FACS binding assays

Binding to human LRP5 and LRP6 was determined on cells by FACS analysis, as reported in Figures 2A and 2B, respectively. In particular, binding to human LRP5 was tested on HEK293 cells with stable overexpression of human LRP5. For human LRP6 binding, HEK293 cells with stable overexpression of human LRP6 were used. The cells were incubated with the LRP5 binder dilutions (1:5 serial dilution of the binders corresponding to the final concentrations indicated in Figure 2A and Figure 2B) for 1.5 hours at 4°C on a plate shaker. After washing the cells 5 times with FACS buffer (1x PBS (Invitrogen cat.no.141190-094) + 10% FBS (Sigma cat.no. F7524) + 0,05% sodium azide), they were incubated for 1 hour at 4°C with a polyclonal mouse antibody which binds to the framework regions of the VHHs. After washing the cells 3 times with FACS

buffer, the cells were incubated for 1 hour at 4°C with the labelled secondary antibody (anti-mouse PE (115-116-071), followed by 3 times washing step with FACS buffer. Fluorescence was measured using FACS Array (BD). Binding to human LRP5 and LRP6 corresponds to ≥ 3000 MCF values at the highest concentration tested. Negative control consisted of a non-targeting binder (VHH construct that binds to a bacterial protein which is not expressed in HEK293 cells). As shown in Figure 2A, binding to human LRP5 corresponds to ≥ 3000 MCF values at the highest tested concentrations of the three half-life extended biparatopic LRP5 selective VHH constructs. In contrast, no binding to human LRP6 was detected (MCF values ≤ 300) as shown Figure 2B. These data confirm that the formatted, biparatopic, and sequence-optimized binding molecules selectively bind to human LRP5 receptors in their native conformation in a cellular assay system. EC50 values of binding to hLRP5 are reported in Table V below.

TABLE V: EC_{50} values of binding to human LRP5 determined by FACS binding assays

FACS based binding assay	F012900082	F01290135	F012900141
hLRP5, EC_{50} (nM)	0.21	0.042	0.2

Specificity of the LRP5 selective formatted, biparatopic, and sequence-optimized binding molecules was compared to previously disclosed LRP6 binding molecules reported in WO2011/138391:

In WO2011/138391, multivalent antibodies binding to LRP6 and inhibiting both Wnt1 and Wnt 3 ligand interaction were disclosed. These multivalent LRP6 binding antibodies are biparatopic LRP6 binding molecules consisting of an IgG antibody as a first receptor binding domain and of an scFv fragment as a second receptor binding domain, where the IgG antibody and scFv fragment are linked together by a linker. In WO2011/138391 it is reported that all the LRP6 binding molecules have roughly the same potency in Wnt1 and Wnt3a reporter assay (Fig. 18 of WO2011/138391). Therefore, any of those multivalent LRP6 binding molecules could be chosen for comparative expriments. Thus, it was decided to use the “901” construct (referred to as MOR08168IgG1LALA 6475 scfv; also shown in Fig. 27 of WO2011/138391) as a first comparative compound.

Derivatives of this "901" construct are shown in WO2013/067355. Specifically, compounds named 801T and 802T are disclosed (cf. disclosure on p.132 of the specification), both having two LRP6 binding scFv domains, plus a half-life extending moiety. As 801T and 802T appear to have the same in vitro potency and biophysical characteristics, only one of them - variant 802T - was included in the experiments described in the following.

Binding affinities of the half-life extended biparatopic LRP5 specific VHH constructs were compared to MOR08168IgG1LALA 6475 scfv biparatopic LRP6 binding molecule using FACS binding assays described in Example 3. As was found in the course of the present invention and shown in Figure 2A and Figure 2B, MOR08168IgG1LALA 6475 scfv biparatopic LRP6 binding molecule binds to both human LRP5 and human LRP6 corresponding to ≥ 3000 MCF values at the highest concentration tested; in contrast, the present half-life extended biparatopic LRP5 specific VHH constructs only bind to human LRP5 with no binding detected on human LRP6 (MCF values ≤ 300). These data show that the half-life extended biparatopic LRP5 specific VHH constructs have distinct binding specificity (LRP5 selective binders) when compared to previously disclosed LRP6 binding molecules reported in WO2011/138391 which turned out to be LRP5/LRP6 cross-reactive binders when tested in the course of the present invention.

7.2 FACS - DKK1 competition assay

Potency and efficacy of LRP5 selective VHH constructs were further analysed using the FACS-based DKK1 competition assay, as described in Example 4.1. HEK293 cells with stable overexpression of human LRP5 or mouse LRP5 were incubated with serial dilution of the LRP5 selective VHH constructs (1:5 serial dilution corresponding to the final concentrations indicated in Figure 3A and Figure 3B). The LRP5 selective VHHs competed with human DKK1 for binding to HEK293 cells overexpressing human LRP5, as well as for binding to HEK293 cells overexpressing mouse LRP5, as shown in Figures 3A and 3B, respectively. Complete inhibition of DKK1 binding was achieved at the highest tested concentrations (≥ 1 µM) and corresponded to MCF values ≤ 100. As a result of this experiment, it could be shown that the present LRP5 selective VHHs competed with human DKK1 for binding to HEK293 cells overexpressing human LRP5, as well as to those overexpressing mouse LRP5 (i.e. decrease in MCF value with increasing concentration of the binder with complete inhibition of DKK1 binding

corresponded to ≤ 100 MCF values at the highest concentration tested). Therefore, the present LRP5 selective VHHs are mouse cross-reactive. The data strengthen the notion that the formatted, biparatopic, and sequence-optimized binding molecules are binding to human/mouse LRP5 receptors in their native conformation.

7.3 Combined Wnt1 and Wnt3a reporter assay

Potency and efficacy of the formatted, biparatopic, and sequence-optimized LRP5 binding molecules were analysed using a Wnt1 and Wnt3a reporter assay as described in Example 4.4. In Figure 4A, the value of the ratio of fluorescence [460/535nm] reported as "Wnt1" corresponds to activation of Wnt1 pathway, determined from the Wnt1 overexpressing cells; complete inhibition of Wnt1 pathway corresponds to a ratio of fluorescence [460/535nm] ≤ 0.8. In Figure 4B, the value of the ratio of fluorescence [460/535nm] reported as "Wnt3a" corresponds to activation of Wnt3a pathway, determined from the Wnt3a overexpressing cells; the value of the ratio of fluorescence [460/535nm] reported as "baseline" in Figure 4B corresponds to complete inhibition of Wnt3a pathway, determined by the treatment with the positive control (DKK1; 200nM final concentration). As shown in Figure 4A and 4B, complete inhibition of both Wnt1 and Wnt3a pathways is achieved by treatment with the three LRP5 selective, formatted, biparatopic, and sequence-optimized binding molecules. Furthermore, high potency is also reported, as shown in Table VI below by the IC50 values.

TABLE VI: IC_{50} values of Wnt1 and Wnt3a pathway inhibition

Wnt1 and Wnt3a reported assay	F012900082	F01290135	F012900141
IC_{50} (nM) Wnt1 IC_{50} (nM) Wnt3a	0.37 < 0.1	0.37 < 0.1	0.3 0.05

Example 8: Effects of three half-life extended biparatopic LRP5 selective VHH constructs on Wnt Signaling and viability in cancer cell lines

The ability of the half-life extended biparatopic LRP5 selective VHH constructs to inhibit active Wnt signaling was further characterized using cancer cell lines with active Wnt signaling, as previously described (Bafico *et al*. "An autocrine mechanism for constitutive

Wnt pathway activation in human cancer cells". *Cancer Cell* 2004;6(5):497-506; DeAlmeida *et al*. "The soluble wnt receptor Frizzled8CRD-hFc inhibits the growth of teratocarcinomas in vivo". *Cancer Res*. 2007;67(11):5371-9); Akiri *et al*. "Wnt pathway aberrations including autocrine Wnt activation occur at high frequency in human non-small-cell lung carcinoma". *Oncogene*. 2009; 28(21):2163-72). Briefly, a cancer cell line with active Wnt signaling, PA-TU-8988S, was seeded in 12-well plates and treated overnight with the LRP5 selective VHH constructs at the final concentration of 1 µM. The ability of inhibiting Wnt signaling was detected by inhibition of mRNA expression of Axin2, the endogenous Wnt target gene. qPCR expression analysis was performed using standard RNA techniques: RNA isolation was performed using the QIAGEN RNeasy Mini Kit according to the protocol provided by QIAGEN; cDNA synthesis using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Cat. No. 11754050) and qPCR using TaqMan Gene Expression Assay with Axin2 TaqMan primers/probe (Hs00610344_m1 AXIN2 FAM, Life Technologies) and with eukaryotic 18s endogenous control VIC-MGB (4319413E-1307061, Applied Biosystems).

As shown in Figure 5 (right panel), PA-TU8988S cancer cells treated with a half-life extended biparatopic LRP5 selective VHH construct showed significantly reduced Axin2 relative mRNA levels (i.e. normalized to the endogenous control) when compared to untreated cells or cells treated with a not targeting VHH construct (negative control). These data demonstrated the ability of the half-life extended biparatopic LRP5 selective VHH construct to inhibit Wnt signaling in cancer cell lines with active Wnt signaling. Furthermore, the effect of Wnt signaling blockade on cell viability was investigated in PA-TU8988S cancer cell line, whose proliferation was previously reported to be dependent on active Wnt signaling (Jiang *et al.* "Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma*". Proc Natl Acad Sci U S A*. 2013; 110(31):12649-54). Cell viability was measured by performing an Alamar Blue assay (Invitrogen, Cat.# DAL1100) after ten days of treatment with a half-life extended biparatopic LRP5 specific VHH constructs (final concentration of 1 µM) or with a not targeting VHH construct (negative control) or with doxorubicin (final concentration of 1 µM; positive control). As shown in Figure 5 (left panel), PA-TU8988S cancer cells treated half-life extended biparatopic LRP5 selective VHH construct showed significantly reduced percentage of viable cells cell (≥ 75% reduction) when compared to untreated cells or cells treated with the negative control which had no effect on cell viability. The maximum reduction on cell viability corresponds to treatment with a chemotherapy agent

such as doxorubicin (≥ 80% reduction). These data demonstrate the ability of the half-life extended biparatopic LRP5 selective VHH constructs to inhibit cell proliferation of cancer cells which are dependent on active Wnt signaling.

Example 9: *In vivo* Efficacy

The LRP5 selective biparatopic half-life extended VHH constructs / binding molecules were further characterized *in vivo* in a Wnt driven tumor model. Experiments were conducted to determine if these binding molecules inhibit tumor growth *in vivo*. Transgenic expression of the Wnt ligands using a mouse mammary tumor virus LTR enhancer (MMTV promoter) leads in mice to extensive ductal hyperplasia followed by mammary adenocarcinomas in transgenic (TG) mice by 6 months of age. These mammary tumors are driven by glucocorticoid induced overexpression of Wnt ligands and have characteristics similar to TNBC tumors, including expression of epithelial and mesenchymal markers (basal-like phenotype) and active Wnt signaling as assessed by intracellular beta-catenin localization. In particular, mammary tumors derived from MMTV-Wnt-1 transgenic mice are Wnt1 dependent. Blocking of Wnt activity using a soluble Wnt receptor comprising the Frizzled8 cysteine-rich domain (CRD) fused to the human Fc domain (F8CRDhFc) (DeAlmeida *et al*. "The soluble wnt receptor Frizzled8CRD-hFc inhibits the growth of teratocarcinomas in vivo". *Cancer Res*. 2007;67(11):5371-9) was reported to inhibit tumor growth *in vivo*. Therefore, tumors isolated from MMTV-Wnt1 transgenic mice were passaged subcutaneously as tumor pieces in nude mice for 2 to 5 passages prior to initiation of the efficacy experiment. Between 14 to 21 days post-implant, when tumors reached a mean volume of approximately 150 to 300 mm^3, mice were randomized into groups with 7 mice per group and dosed *i.v.* with the compounds. The LRP5 selective biparatopic half-life extended VHH constructs were administered to the mice *i.v.* three times (F012900082) or twice per week (F012900141), with the dosages shown in Figure 6A for F012900082 and in Figure 6B for F012900141. Tumor volume (left panels) and body weight (right panels) were monitored during the efficacy experiment and the median tumor volumes are reported in Figures 6A and 6B. Tumor growth inhibition (TGI) was determined at the end of the efficacy experiment. In particular, TGI was determined for each treatment group as compared to the control group (treatment of mice with histidine buffer - 20mM Histidine pH6.5 buffer). Furthermore, gastro-intestinal (GI) histo-pathological analysis (via H&E staining of sections of the GI tract from the duodenum to the rectum) was performed at the end of the efficacy experiment to evaluate potential toxicity of the LRP5 antagonists.

Tumor growth inhibition (TGI), outcome of the GI histo-pathological analysis at the end of the *in vivo* efficacy study, mortality which corresponds to the number of mice that needed to be sacrified due to significant loss of body weight (>18% loss of body weight compared to start of the efficacy experiment) and number of tumor regressions (tumor volume at the end of the experiment smaller than tumor volume measure at the start of the treatment) are reported for each treatment group in Tables VIIA and VIIB, and , relating to the experiments and data shown also in Figures 6A and 6B, respectively.

Table VIIA: *In vivo* efficacy of F012900082 administered *i.v.* three times per week. Results of this experiment are also shown in Figure 6A

	Dose [mg/kg]	TGI [%]	Regressions [x/7]	Mortality [x/7]	GI Histopathological evaluation
Control	Histidine buffer	-	-	-	-
F012900082	50	128	6	0	No findings

Table VIIB: *In vivo* efficacy of F012900141 administered *i.v.* twice per week. Results of this experiment are also shown in Figure 6B

	Dose [mg/kg]	TGI [%]	Regressions [x/7]	Mortality [x/7]	GI Histopathological evaluation
Control	Histidine buffer	-	-	-	-
F012900141	50	118	7	0	No findings
	15	115	7	0	No findings
	5	98	0	0	n.d.

As shown Figures 6A to 6B and Tables VIIA and VIIB above, treatment with the LRP5 selective half-life extended biparatopic VHH constructs (F012900082 at 50 mg/kg in a 3x/week schedule and F012900141 at 15 and 50mg/kg in a 2x/week schedule) resulted

in tumor regression (i.e. tumor growth inhibition (TGI) > 100% which corresponds to tumor shrinkage; decrease of the tumor volume at the end of the efficacy experiment compared to the tumor volume at the start of the experiment), with no significant body weight changes (< 10%) and no findings reported after GI histopathological analysis, suggesting a well-tolerated treatment.

Tumor shrinkage (i.e. tumor regression) is a desired therapeutic effect (i.e. efficacy) for treatment of cancer patients. In clinical studies treatments that induce tumor regression resulting in pathological complete response (pCR) positively lead to significant improvement of progression free survival and overall survival in high unmet medical need indications such as in breast cancer. Furthermore, it is of clinical relevance that the treatment is well tolerated with no off tumor – on target adverse effects at efficacious dose levels.

As comparison, it was investigated whether MOR08168IgG1LALA 6475 scfv biparatopic LRP6 binding molecule may provide for a similar advantageous effect. For this purpose an in vivo tolerability study was performed in mice as follows: MOR08168IgG1LALA 6475 scfv compound was administered *i.v.* at 3 mg/kg, twice per week (2qw); the same dose/regimen at which in vivo efficacy was detected in a xenograft tumor model in WO2011/138391, as described in Fig. 22 therein. First treatment with MOR08168IgG1LALA 6475 scfv was performed at day 1 and starting from day 6 significant loss of body weight was detected in mice. At day 10, some of the mice treated with MOR08168IgG1LALA 6475 scfv compound showed significant body weight loss (>10%). At day 11 mice were sacrificed and gastro-intestinal (GI) histopathological analysis revealed inflammation with erosion in the colon and in the cecum of the mice. These data suggest that MOR08168IgG1LALA 6475 scfv is not tolerated at the efficacious dose/regimen. Therefore, LRP5 selective biparatopic half-life extended VHH constructs are superior with regard to therapeutic window; i.e. they induce tumor regression with no significant body weight changes (< 10%) and no findings reported after GI histopathological analysis.

Example 10: *In vivo* Wnt pathway inhibition

To further characterize the effect of the LRP5 selective biparatopic half-life extended VHH constructs / binding molecules on Wnt signaling, tumors were isolated at the end of the efficacy experiment described in Example 9. In particular, tumors were isolated 24 hours after the last injection with the compounds (F012900082 at 50 mg/kg or

F012900141 at 15 mg/kg) or with the control treatment. Wnt signaling inhibition was determined by reduction of mRNA expression of Axin2, as well as additional Wnt target genes: RNF43 and Notum in tumors treated with F012900082 and with F012900141, respectively, analysed as described in Example 8. TaqMan primers/probes were used for analyses of Notum and RNF43, (Mm01253278_m1 Notum FAM and Mm00552558_m1 RNF43 FAM Life Technologies). The fold change of Axin2, RNF43 or Notum mRNA expression relative to the control group is reported in Figure 7 (left-hand side) for the *in vivo* efficacy with F012900082 (cf. Figure 6A) and in Figure 7 (right-hand side) for the *in vivo* efficacy with F012900141 (cf. Figure 6B).

As can be seen from Figure 7, a significant reduction of Axin2, RNF43 and Notum mRNA expression was observed in tumors treated with the LRP5 selective binding molecules, compared to the control group. These results suggest that the LRP5 selective binding molecules are able to inhibit tumor growth by suppressing Wnt signaling in the tumor cells.

Example 11: Industrial manufacturing process

11.1 Fermentation: Any of the polypeptides set out in Table III above can be expressed in the cytoplasm of different E. coli strains like W3110, TG1, BL21, BL21(DE3), HMS174, HMS174(DE3), MM294 under control of an inducible promoter. This promoter can be chosen from lacUV5, tac, T7, trp, T5, araB. The cultivation media are preferably fully defined according to Wilms *et al.*, 2001 (Wilms, B., Hauck, A., Reuss, M., Syldatk, C., Mattes, R., Siemann, M., and Altenbuchner, J.: High-Cell-Density Fermentation for Production of L-N-Carbamoylase Using an Expression System Based on the Escherichia coli rhaBAD Promoter. Biotechnology and Bioengineering, 73: 95-103 (2001)), DeLisa *et al.*, 1999 (DeLisa, M. P., Li, J. C., Rao, G., Weigand, W. A., and Bentley, W. E.: Monitoring GFP-operon fusion protein expression during high cell density cultivation of Escherichia coli using an on-line optical sensor. Biotechnology and Bioengineering, 65: 54–64.(1999)) or equivalent. However, supplementation of the medium with amino acids like isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valin or complex media components such as soy peptone or yeast extract may be beneficial. The process for fermentation is performed in a fed-batch mode. Conditions: Temperature 30 – 40 °C, pH 6 – 7.5, dissolved oxygen is kept above 20%. After consumption of the initial C-source the culture is fed with the feed media stated above (or equivalent). When a dry cell weight in the fermenter of 40 to 90 g/L is reached the culture

is induced with an appropriate inducer corresponding to the used promoter system (e.g. IPTG, lactose, arabinose). The induction can either be performed as a pulsed full induction or as a partial induction by feeding the respective inducer into the fermenter over a prolonged time. The production phase should last 4 hours at least. The cells are recovered by centrifugation in bowl centrifuges, tubular bowl centrifuges or disc stack centrifuges, the culture supernatant is discarded.

11.2 Purification: The E. coli cell mass is resuspended in 6- to 8-fold amount of lysis buffer (phosphate or Tris buffer, pH 7-8.5). Cell lysis is preferably performed by high pressure homogenization followed by removing of the cell debris by centrifugation in bowl, tubular bowl or disc stack centrifuges. Supernatant containing the target protein is optionally filtrated using a 0.22-10 µm filter and separated via cation exchange chromatography (e.g. Toyopearl MegaCap® II SP-550EC, Toyopearl GigaCap S-650M, SP Sepharose BB, SP Sepharose FF or S HyperCel™) at pH 7-8.5. Elution is performed by a linear increasing NaCl gradient at pH 7-8.5. Fractions containing the target protein are pooled and subsequently incubated with 5-10 mM DTT in order to prevent dimerization or aggregation mediated by free cysteine residues. After further addition of 0.8-1 M ammonium sulfate or 2-3 M NaCl, solution is separated via hydrophilic interaction chromatography (e.g. Phenyl Sepharose HP, Phenyl Sepharose FF, Butyl Sepharose HP, Butyl Sephrose FF, Butyl Toyopearl 650 (S,M,C), Phenyl Toyopearl 650 (S,M,C)) at pH 7-8.5. Elution is carried out at pH 7-8.5 by a linear decreasing ammonium sulfate or NaCl gradient in presence of 5 mM DTT. Fractions containing the target protein with a purity level of minimally 90% are pooled and desalted by diafiltration in presence of 5 mM DTT followed by concentration to approximately 5 mg/ml. Subsequent refolding is performed by diluting the protein solution 1:5-1:20 with 50 mM Tris, 150 mM NaCl, 4 mM Cystamin, 10 mM CHAPS at pH 8.5 to a final protein concentration of 0.25-1 mg/ml. Refolding solution is incubated under stirring for 12-36 h at room temperature and then separated by cation exchange chromatography (e.g. SP Sepharose FF, SP Sepharose HP, Toyopearl SP-650 (S, M, C)) at pH 7-8.5. Elution is performed by a linear increasing NaCl gradient at pH 7-8.5. Fractions containing monomeric target protein are pooled and formulated in 25 mM Na-phosphate, 220 mM endotoxin free trehalose, pH 7.5 via diafiltration. The solution is sterilized by filtration and stored at 2 to 8 °C.

Example 12: Pharmaceutical formulation for s.c. administration

Any of the above biparatopic polypeptide constructs of the invention can be selected for the manufacture of a pharmaceutical formulation for subcutaneous application having a composition as follows:

Drug substance:	100 mg/ml (1 to 3 nmol/ml)
Acetate buffer:	25 mM
Trehalose:	220 mM
Tween-20:	0.02 %

Drug substance is formulated in a solution having the above composition, sterilized and stored at 2 to 8 °C.

Example 13: Pharmaceutical use in humans

The solution as prepared in Example 11.2 above is applied to a patient in need thereof, such as a human being suffering from a cancer sensitive to Wnt signaling inhibitors, by intravenous infusion (dosage of 100 to 200 mg) every two to four weeks.

Example 14: Effect of Wnt3a-signaling inhibition on pro-inflammatory cytokine release by dendritic cells in an *ex-vivo* assay

PBMCs were obtained from healthy donors with informed consent. Human monocyte-derived dendritic cells (Mo-DCs) were generated as follows: PBMCs were cultured in X-VIVO medium supplemented with 50 ng/mL GM-CSF and 50 ng/mL IL-4. After 24 h of culture, the supernatant was carefully removed and replaced with X-VIVO medium supplemented with the same GM-CSF and IL-4. On the fourth day, an aliquot of cells was removed for FACS analysis of LRP5 and LRP6 expression, and the supernatant of the remaining cells was carefully removed and replaced with X-VIVO medium in the presence of LPS only or in combination with human Wnt3a or with human Wnt3a and LRP5 selective binding molecules. On the following day, the supernatants were collected and subjected to analysis of TNF-alpha through ELISA according to the manufacturer's instructions.

FACS analysis of differentiated dendritic cells (DCs) was performed as described in Example 7.1 using the following antibodies:

- LRP5 specific monoclonal antibody: mouse IgG1 anti-human LRP5 - clone 1E9 (Sigma #WH0004041M1) at serial dilutions of 1:100 from 1mg/ml to 10 µg/ml

- LRP6 specific monoclonal antibody: mouse IgG2a anti-human LRP6 (R&D #Mab1505); at serial dilutions of 1:50 from 500µg/ml to10 µg/ml)

- Control: mouse IgG2a isotype control (Dako #X0943); at serial dilutions of 1:10

- Secondary labelled polyclonal antibody: goat anti-mouse IgG/Fc - PE (Dianova #115-115-164); 1:100 dilution.

As reported in Figure 8 (left panel), high expression of LRP5 (high PE-A values) was detected by FACS analysis of differentiated DCs when compared to the control. LRP6 expression was also detected but to a lower extent when compared to LRP5 (LRP6 PE-A values < LRP5 PE-A values).

As previously reported (Oderup et al." Canonical and noncanonical Wnt proteins program dendritic cell responses for tolerance". *J Immunol.* 2013;190(12): 6126-34), and as shown in Figure 8, Wnt3a directly inhibits pro-inflammatory cytokine secretion (i.e. TNF-alpha release) by differentiated DCs. Wnt3a driven suppression of TNF-alpha release from DCs was restored by addition of LRP5 selective binding molecules.

These data show that the formatted, biparatopic, and sequence-optimized binding molecules are able to restore TNFalpha secretion by Wnt3a treated dendritic cells, thereby suppressing the Wnt inhibitory effect on dendritic cells.

It is important to notice that blocking the Wnt pathway in dendritic cells in the tumor microenviroment represents a potential therapeutic approach towards breaking tumor-mediated immune suppression and augmenting anti-tumor immunity.

To investigate the effects of DCs on T cells (effector T cells), DCs pre-treated with Wnt3a with or without LRP5/ selective binding molecules were co-cultured with T cells, isolated from PBMCs, as previously described (Oderup et al. "Canonical and noncanonical Wnt proteins program dendritic cell responses for tolerance". J Immunol. 2013;190(12): 6126-34). After 3 days of DC/T cells co-culture, the supernatants were collected and subjected to analysis of IFN gamma through ELISA, according to the manufacturer's instructions.

IFNgamma secretion is a marker of T cell activation. As shown in Figure 8 (center and right panels), Wnt3a mediated DC inhibition leads to reduced IFNgamma secretion by T

cells (inhibition of T cell function), which is restored by treatment with LRP5 selective binding molecules.

In summary, these data show that LRP5 selective binding molecules suppress the Wnt inhibitory effect on dendritic cells, leading to restoration of T cell function.

It is known that continuous activation/stimulation of T cells induces terminal differentiation, resulting in an exhausted T cell phenotype, a progressive loss of T-cell function. Therefore, it is envisaged that the effect of LRP5 selective binding molecules on T cells, mediated by activation of DCs, can be limited by T cell exhaustion. Combination treatments, combining the administration of LRP5 selective binding molecules with the administration of an immune checkpoint inhibitor which blocks T cell exhaustion, are therefore expected to help to activate and maintain T cell function, thereby changing the tumor microenvironment, and thereby supporting the therapeutic effect of the molecules of the invention.

Example 15: Effects of three half-life extended biparatopic LRP5 selective VHH constructs on Wnt Signaling and viability in *RNF43* mutant CRC organoids

The ability of the half-life extended biparatopic LRP5 selective VHH constructs to selectively inhibit proliferation (detected by inhibition of cell viability), and active Wnt signaling (detected by reduction of Axin2 mRNA level) was characterized using *RNF43* mutant colorectal cancer (CRC) organoids. CRC organoids were established as previously described (van de Wetering *et* al. “Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell* 2015;161(4):933-45). Briefly, to perform the cell viability assay, each of the three CRC patient derived organoids (*RNF43* mut1, *RNF43* mut2 and *RNF43* WT) were filtered through a 40 µm filter and were seeded in 384 well plates (coated with Basement Membrane Matrix -BME). In particular, ~500 organoids were resuspended in a total volume of 40 µl of medium (with 5% BME) per well. The culture medium did not contain Wnt ligands. Compounds were distributed using a Tecan D300 Digital Dispenser. Organoids were exposed to an 8-point dilution series of the LRP5 selective VHH construct or control compound and one fixed concentration of staurosporin (2 µM). Fresh compounds were added at day 0 and day 3 after seeding of the organoids. After 5 days, the CellTiter-Glo 3D Cell Viability Assay (Promega) was used to determine cell viability of the CRC patient derived organoids. The CellTiter-Glo 3D Cell Viability Assay is a homogeneous method to determine the number of viable cells in 3D cell culture based on quantitation of the ATP present, which is a marker for

the presence of metabolically active cells. In each experiment, treatment with a half-life extended biparatopic LRP5 selective VHH construct and control compounds were run in triplicate per organoid line. As internal controls, the three CRC patient derived organoids were also treated with DMSO and Staurosporin and the corresponding cell viability data were used as reference: DMSO values as 100% survival and Staurosporin values as 0% survival. DMSO was added to all wells to normalize to the highest concentration (0.1% for DMSO in Staurosporin).

To analyze Axin2 mRNA levels using qRT-PCR, each of the three CRC patient derived organoids (*RNF43* mut1, *RNF43* mut2 and *RNF43* WT) were filtered through a 40 µm filter and were seeded in 384 well plates (coated with BME). In particular, ~500 organoids were resuspended in a total volume of 40 µl of medium (with 5% BME) per well. The culture medium did not contain Wnt ligands. Compounds were distributed using a Tecan D300 Digital Dispenser. Organoids were exposed to a final concentration of 62 nM of a half-life extended biparatopic LRP5 sleective VHH construct or 1µM of control compound). The compounds were added at day 0 and after 24 hours of treatment organoids were harvested to perform qRT-PCR analysis and determine Axin2 mRNA levels as biomarker of active Wnt signaling inhibition.

As shown in Figure 9, *RNF43* mutant CRC organoids (*RNF43* mut1, *RNF43* mut2) treated with half-life extended biparatopic LRP5 selective VHH construct showed significantly reduced percentage of viable cells cell ($\geq$ 50% reduction) when compared to cells treated with a not targeting VHH construct (control), which had no effect on cell viability. RNF43 wild type CRC organoid (*RNF43* WT) treated with half-life extended biparatopic LRP5 selective VHH construct showed no effect on cell viability when compared to cells treated with the control compound. These data demonstrate the ability of the half-life extended biparatopic LRP5 selective VHH constructs to selectively inhibit cell proliferation of cancer cells harboring mutations in *RNF43* gene which are dependent on active Wnt signaling.

As shown in Figure 10, *RNF43* mutant CRC organoids (*RNF43* mut1, *RNF43* mut2) treated with half-life extended biparatopic LRP5 selective VHH construct (final concentration of 62nM) showed significantly reduced Axin2 relative mRNA levels (i.e. normalized to the endogenous control) when compared to cells treated with the control (final concentration of 1 µM). RNF43 wild type CRC organoid (*RNF43* WT) treated with half-life extended biparatopic LRP5 selective VHH construct (final concentration of 62

nM) showed no significant effect on Axin2 mRNA level, when compared to cells treated with the control compound (final concentration of 1 µM). These data demonstrated the ability of the half-life extended biparatopic LRP5 selective VHH construct to selectively inhibit Wnt signaling of cancer cells harboring mutations in *RNF43* gene.

5

Claims:

1. A polypeptide which binds to low-density lipoprotein receptor-like protein 5 (LRP5), the polypeptide comprising an immunoglobulin single variable domain (ISVD) selected from the group consisting of ISVDs (i) to (iv) :

(i) an ISVD comprising the following complementarity-determining region (CDR) sequences:

CDR1: TYVMG (SEQ ID NO:1)

CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)

CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3),

(ii) an ISVD comprising the following CDR sequences:

CDR1: RYAVA (SEQ ID NO:4)

CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)

CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6),

(iii) an ISVD comprising the following CDR sequences:

CDR1: IGAMG (SEQ ID NO:7)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9), and

(iv) an ISVD comprising the following CDR sequences:

CDR1: INAMG (SEQ ID NO:10)

CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)

CDR3: ETGPYGPPKRDY (SEQ ID NO:9).

2. The polypeptide of claim 1, comprising a first ISVD selected from ISVDs (i) and (ii) and a second ISVD selected from ISVDs (iii) and (iv).
3. The polypeptide of claim 2, wherein the first ISVD is ISVD (i) and the second ISVD is ISVD (iii).
4. The polypeptide of claim 2, wherein the first ISVD is ISVD (i) and the second ISVD is ISVD (iv).
5. The polypeptide of claim 2, wherein the first ISVD is ISVD (ii) and the second ISVD is ISVD (iii).
6. The polypeptide of claim 2, wherein the first ISVD is ISVD (ii) and the second ISVD is ISVD (iv).
7. The polypeptide of any one of claims 1 to 6, wherein said ISVDs are VHH domains, preferably humanized VHH domains.

8. The polypeptide of claim 1, wherein
ISVD (i) comprises the sequence of SEQ ID NO:11 or the sequence of SEQ ID NO:23,
ISVD (ii) comprises the sequence of SEQ ID NO:12
ISVD (iii) comprises the sequence of SEQ ID NO:13 or the sequence of SEQ ID NO:22, and/or
ISVD (iv) comprises the sequence of SEQ ID NO:14.

9. The polypeptide of claim 8, comprising a first ISVD and a second ISVD, wherein said first ISVD comprises the sequence of SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:23, and said second ISVD comprises the sequence of SEQ ID NO:13, SEQ ID NO:14 or SEQ ID NO:22.

10. The polypeptide of claim 9, wherein said first ISVD comprises the sequence of SEQ ID NO: 11 and said second ISVD comprises the sequence of SEQ ID NO:22.

11. The polypeptide of claim 9, wherein said first ISVG comprises the sequence of SEQ ID NO:12 and said second ISVD comprises the sequence of SEQ ID NO:13.

12. The polypeptide of claim 9, wherein said first ISVD comprises the sequence of SEQ ID NO:23, and said second ISVD comprises the sequence of SEQ ID NO:14.

13. The polypeptide of any one of claims 2 to 12, wherein said first ISVD and said second ISVD are covalently linked by a linker peptide, wherein said linker peptide optionally comprises or consists of a third ISVD.

14. The polypeptide of any one of claims 2 to 13, wherein said polypeptide further comprises a half-life extending moiety, wherein said half-life extending moiety is covalently linked to said polypeptide and is optionally selected from the group consisting of an albumin binding moiety, such as an albumin binding peptide or an albumin binding immunoglobulin domain, a transferrin binding moiety, such as an anti-transferrin immunoglobulin domain, a polyethylene glycol molecule, human serum albumin, and a fragment of human serum albumin.

15. The polypeptide of to claim 14, wherein said half-life extending moiety is an albumin binding moiety, preferably an albumin binding ISVD, more preferably the Alb11 domain comprising the sequence of SEQ ID NO:21.

16. A polypeptide comprising a first and a second LRP5 binding ISVD and one albumin binding ISVD,
- said first LRP5 binding ISVD selected from the group consisting of ISVDs (i) and (ii):
(i) an ISVD comprising the following CDR sequences:
CDR1: TYVMG (SEQ ID NO:1)
CDR2: AISWSGGSTYYADSVKG (SEQ ID NO:2)
CDR3: SRGTSTPSRASGVSRYDY (SEQ ID NO:3)
(ii) an ISVD comprising the following CDR sequences:
CDR1: RYAVA (SEQ ID NO:4)
CDR2: AITWSSGRIDYADSVKG (SEQ ID NO:5)
CDR3: DRRPRSTGRSGTGSPSTYDY (SEQ ID NO:6),
-said second ISVD selected from the group consisting of ISVDs (iii) and (iv):
(iii) an ISVD comprising the following CDR sequences:
CDR1: IGAMG (SEQ ID NO:7)
CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3: ETGPYGPPKRDY (SEQ ID NO:9), and
(iv) an ISVD comprising the following CDR sequences:
CDR1: INAMG (SEQ ID NO:10)
CDR2: AVSSGGSTYYVDSVKG (SEQ ID NO:8)
CDR3: ETGPYGPPKRDY (SEQ ID NO:9),
-said albumin binding ISVD defined by comprising the following CDR sequences:
CDR1: SFGMS (SEQ ID NO:15)
CDR2: SISGSGSDTLYADSVKG (SEQ ID NO:16)
CDR3: GGSLSR (SEQ ID NO:17)

17. The polypeptide of claim 16, wherein the first ISVD is ISVD (i) and the second ISVD is ISVD (iii).
18. The polypeptide of claim 16, wherein the first ISVD is ISVD (i) and the second ISVD is ISVD (iv).
19. The polypeptide of claim 16, wherein the first ISVD is ISVD (ii) and the second ISVD is ISVD (iii).
20. The polypeptide of claim 16, wherein the first ISVD is ISVD (ii) and the second ISVD is ISVD (iv).

21. A polypeptide which binds to LRP5, comprising or consisting of a sequence selected from SEQ ID NOs: 18, 19 and 20.

22. A polypeptide which binds to LRP5, comprising an ISVD which competes for binding to LRP5 with a reference ISVD, wherein the reference ISVD has the sequence identified by SEQ ID NOs: 11, 12, 13, 14, 22 or 23; preferably wherein the ISVD blocks a Wnt1 or Wnt3a binding site of LRP5, in particular wherein the ISVD inhibits Wnt1-driven or Wnt3a-driven target gene transcription.

23. A polypeptide of claim 22, comprising a first ISVD which competes for binding to LRP5 with a first reference ISVD, wherein the first reference ISVD has the sequence identified by SEQ ID NOs: 11, 23 or 12, and a second ISVD which competes for binding to LRP5 with a second reference ISVD, wherein the second reference ISVD has the sequence identified by SEQ ID NOs: 13, 22 or 14; preferably wherein the first ISVD blocks a Wnt3a binding site of LRP5, in particular inhibits Wnt3a driven target gene transcription, and/or preferably wherein the second ISVD blocks a Wnt1 binding site of LRP5, in particular inhibits Wnt1-driven target gene transcription.

24. The polypeptide of claim 23, wherein a linker peptide covalently links said first ISVD to said second ISVD, wherein said linker peptide optionally comprises or consists of a further ISVD, preferably an albumin-binding ISVD, especially the albumin-binding ISVD as set forth in claim 16.

25. The polypeptide of any one of claims 23 to 24, wherein said first and/or said second ISVD is a VHH domain, preferably a humanized VHH domain.

26. A polypeptide which binds to LRP5, comprising a first LRP5-binding domain, preferably an ISVD, and a second LRP5-binding domain, preferably an ISVD, wherein the first LRP5-binding domain binds a region of LRP5 that is distinct from the LRP5 region which the second LRP5-binding domain binds to.

27. The polypeptide of claim 26, wherein the first domain blocks a Wnt3a binding site of LRP5, and preferably inhibits Wnt3a-driven target gene transcription, and/or wherein the second domain blocks a Wnt1 binding site of LRP5, and preferably inhibits Wnt1-driven target gene transcription.

28. The polypeptide of any one of claims 22 to 27, wherein the polypeptide further comprises a half-life extending moiety, wherein said half-life extending moiety is covalently linked to said polypeptide and is preferably selected from the

group consisting of an albumin binding moiety, such as an albumin binding peptide or an albumin binding immunoglobulin domain (e.g. an albumin binding ISVD), a transferrin binding moiety, such as an anti-transferrin immunoglobulin domain, a polyethylene glycol molecule, human serum albumin, and a fragment of human serum albumin.

29. The polypeptide according to any one of claims 1 to 28, wherein the polypeptide has an affinity and/or avidity for LRP5, especially human LRP5, that is at least 10 times, preferably at least 100 times, more preferably at least 1000 times, even more preferably at least 10000 times, yet even more preferably at least 100000 times stronger than their affinity and/or avidity for LRP6, in particular human LRP6.

30. The polypeptide according to any one of claims 1 to 28, wherein the polypeptide is not cross-reactive to LRP6, in particular human LRP6.

31. A nucleic acid molecule, preferably in isolated form, encoding the polypeptide according to any one of claims 1 to 30.

32. An expression vector comprising the nucleic acid of claim 31.

33. A host cell carrying an expression vector according to claim 32.

34. A method of manufacturing a polypeptide according to any one of claims 1 to 30, comprising the steps of

- culturing a host cell according to claim 33 under conditions that allow expression of a polypeptide according to any one of claims 1 to 30; and
- recovering said polypeptide.

35. The method of claim 34, additionally comprising the step of

- purifying said polypeptide.

36. A pharmaceutical composition comprising (i) as the active ingredient, the polypeptide according to any one of claims 1 to 30, and (ii) a pharmaceutically acceptable carrier, and optionally (iii) a diluent, excipient, adjuvant and/or stabilizer.

37. A polypeptide according to any one of claims 1 to 30 for use as a medicament in a method for the treatment, prevention or alleviation of a disease, disorder or condition in a human being or an animal.

38. A polypeptide according to any one of claims 1 to 30 for use in the treatment of cancer, preferably breast cancer, lung cancer, specifically non-small-cell lung carcinoma (NSCLC), pancreatic cancer, colorectal cancer, sarcomas, ovarian cancer or hepatocellular carcinoma, or for use in the treatment of idiopathic pulmonary disease, or for use in the treatment of a retinopathy caused by abnormal Wnt signaling.

39. A polypeptide according to any one of claims 1 to 30 for use in the treatment of triple negative breast cancer (TNBC).

40. A polypeptide according to any one of claims 1 to 30 for use in combination with a chemotherapeutic agent, a therapeutically active compound that inhibits angiogenesis, a signal transduction pathway inhibitor, an EGFR inhibitor, an immune modulator, an immune checkpoint inhibitor, or a hormonal therapy agent.

41. A therapeutic agent selected from the group consisting of chemotherapeutic agents, therapeutically active compounds that inhibit angiogenesis, signal transduction pathway inhibitors, EGFR inhibitors, immune modulators, immune checkpoint inhibitors, and hormonal therapy agents for use in combination with the polypeptide according to any one of claims 1 to 30.

42. A method of treatment of cancer or idiopathic pulmonary disease or a retinopathy in a patient in a need thereof comprising administering to said patient an effective amount of the polypeptide of any one of claims 1 to 30, or the pharmaceutical composition of claim 36.

43. Use of a polypeptide of any one of claims 1 to 30 to modify a tumor microenvironment by inhibiting Wnt1- and Wnt3a-driven target gene transcription in dendritic cells, preferably wherein said use is an *in vitro* use.

44. A polypeptide of any one of claims 1 to 30 for use in the treatment of cancer in combination with an immune checkpoint inhibitor selected from the group consisting of anti-PD1 antibodies, anti-PDL1 antibodies, anti-CTLA4 antibodies, anti-BTLA antibodies, anti-LAG3 antibodies, and anti-TIM3 antibodies, or in combination with a cancer vaccine.

45. Use of a polypeptide of any one of claims 1 to 30 in the manufacture of a medicament in the treatment of cancer.

46. Use of a polypeptide of any one of claims 1 to 30 in the manufacture of a medicament in the treatment of cancer in combination with an immune

checkpoint inhibitor selected from the group consisting of anti-PD1 antibodies, anti-PDL1 antibodies, anti-CTLA4 antibodies, anti-BTLA antibodies, anti-LAG3 antibodies, and anti-TIM3 antibodies, or in combination with a cancer vaccine.

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