POLYPEPTIDES FOR USE IN THE TREATMENT OF GLYPICAN-3-EXPRESSING TUMOURS

DESCRIPTION

Field of the present technology

The present technology relates to the field of cancer treatment. In particular, it relates to polypeptides targeting Glypican-3 (GPC3) and T cell receptor (TCR) for use in the treatment of glypican-3-positive (GPC3+) solid tumours, such as hepatocellular carcinoma (HCC) or non-small cell lung cancer (NSCLC).

Technological background

Cancer takes an enormous human toll around the world. It is nowadays the second leading cause of death globally, only preceded by heart disease and stroke. Cancer figures among the leading causes of morbidity and mortality worldwide, with approximately 19.3 million new cases and 10 million cancer related deaths in 2020. The number of new cases is expected to rise further over the next decades. Population growth, ageing and lifestyle changes have been described as contributing factors to the increasing cancer burden. In 2013, the WHO projected that by 2030 cancer will surpass ischemic heart disease and become the most common cause of death worldwide (source: WHO Cancer).

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults and the third-leading cause of cancer-related deaths worldwide. Treatment of HCC depends on the stage of the disease, the patient's ability to tolerate surgery and the availability of a liver transplant. When the cancer is early stage and limited to one or a few areas within the liver, surgically removing the tumour cells may have a curative effect. However, when the cancer has spread beyond the liver or is too advanced in the liver other treatment options have to be explored.

In 2007, sorafenib (sold under brand name Nexavar®), an oral multikinase inhibitor, was the first systemic agent approved for first-line treatment of advanced HCC. Only a modest improvement in overall survival of a little over 2-3 months was observed in trials. Additionally,

undesirable side effects were very frequently observed. Therefore, further treatment options were desired.

Additionally, in a recent phase III clinical trial the combination of atezolizumab and bevacizumab was shown to improve overall as well as progression-free survival when compared to sorafenib. Furthermore, in 2022 the FDA approved tremelimumab in combination with durvalumab for the treatment of unresectable HCC.

However, overall, the prognosis for patients with HCC is still rather poor. Only about 10-20% of hepatocellular carcinomas can be removed completely using surgery, but if the cancer is not removed entirely HCC is usually fatal to the patient within 3 to 6 months. The prognosis has slightly improved thanks to treatments like sorafenib, but there remains an urgent need for better treatment options for HCC patients.

Non-small cell lung cancer (NSCLC) is any type of epithelial lung cancer other than small cell lung cancer (SCLC). The most common types of NSCLC are squamous cell carcinoma, large cell carcinoma, and adenocarcinoma, but there are several other types that occur less frequently, and all types can occur in unusual histological variants. NSCLC accounts for about 85% of all lung cancers. As a class, NSCLCs are relatively insensitive to chemotherapy, compared to small-cell carcinoma.

The standard of care for patients with stage I and II, as well as select patients with stage IIIA, non-small-cell lung cancer (NSCLC) is surgical resection. Following resection, patients may be offered adjuvant systemic therapy. Osimertinib, a third-generation oral EGFR– tyrosine kinase inhibitor (TKI), selectively binds both EGFR driver mutations and EGFR T790M resistance mutations. It is approved for the adjuvant therapy of completely resected stage II and stage III NSCLC (Mithoowani H, Febbraro M. Non-Small-Cell Lung Cancer in 2022: A Review for General Practitioners in Oncology. Curr Oncol. 2022 Mar 9;29(3):1828-1839).

Prognosis for patients with NSCLC is also relatively poor. The 5-year relative survival rate for NSCLC in women in the United States is 33% and the 5-year relative survival rate for men is 23%. There also remains an urgent need for better treatment options for NSCLC patients.

Glypican-3 (GPC3) is a GPI anchored cell surface glycoprotein consisting of heparan sulphate GAG chains and a core protein. It has been implicated in embryogenesis and early development, controlling cell growth and differentiation. Whereas the expression of GPC3 is high during development, its expression is nearly absent in normal adult tissue, with moderate/low expression in renal proximal tubules and bronchial cells. Consistent with its role in early development, high level of GPC3 is also expressed in the placenta.

GPC3 expression is especially prevalent in tumour tissue from hepatocellular carcinoma. Soluble GPC3 can also be detected in serum of HCC patients and has been used to differentiate from liver diseases with different aetiologies.

Additionally, multiple studies have found that GPC3 is also involved in promoting cell proliferation and tumorigenesis in non-small cell lung cancer (NSCLC), particularly in squamous cell carcinoma (SCC).

GPC3 likely regulates early development via multiple signalling cascades, including the Wnt, Hh and YAP pathways. In addition, GPC3 is able to interact with basic growth factors, such as FGF2 to regulate cell growth. In an experimental setting, overexpression of GPC3 can inhibit FGF2-induced cell proliferation. By contrast, GPC3 also negatively regulates BMP7, an inhibitory growth factor. Altogether, the activity of GPC3 can be highly contextual, as it is able to not only inhibit cell proliferation, but also can promote carcinogenesis in liver and other forms of cancer, such as HCC or NSCLC.

As such, GPC3 is an attractive potential target for HCC and NSCLC treatment. Currently, no GPC3-targeting treatment for HCC or NSCLC has been approved. Several compounds are either in pre-clinical or clinical phase.

Consequently, a need remains for effective treatment of hepatocellular carcinoma and non-small cell lung cancer that can improve the prognosis for patients.

Summary of the present technology

The present inventors found that a polypeptide specifically targeting GPC3 and the T cell receptor (TCR) at the same time allows for the efficient treatment of Glypican-3-positive (GPC3+) solid tumours, such as hepatocellular carcinoma (HCC) and non-small cell lung cancer (NSCLC), with limited off-target effects. Based thereon, the inventors developed novel

treatment regimens and compositions for the treatment of Glypican-3-positive (GPC3+) solid tumours. These treatments promote efficient T cell-mediated killing of GPC3 expressing cells within GPC3+-solid tumours, resulting in tumour regression. Notably, this T-cell-mediated cytotoxic response is highly specific against Glypican-3-positive (GPC3+) solid tumours, as it only showed limited activity against cells expressing no or low levels of GPC3, thus the treatment method claimed herein exhibiting a favourable safety profile.

Specifically, the present technology provides polypeptides that comprises or consists of at least three immunoglobulin single variable domains (ISVDs), wherein at least two ISVDs specifically bind to GPC3 and one ISVD specifically binds to the constant domain of a TCR on a T cell.

In a first aspect the present technology thus provides a polypeptide for use in the treatment of GPC3+ solid tumours that comprises or consists of at least three immunoglobulin single variable domains (ISVDs), wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least three ISVDs are optionally linked via one or more peptidic linkers, and wherein:

a) a first ISVD specifically binds to the constant domain of the T cell receptor (TCR) on a T cell and comprises
   i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6;
   ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10; and
   iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14;

b) a second ISVD specifically binds to GPC3 and comprises
   iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7;
   v. a CDR2 that is the amino acid sequence of SEQ ID NO: 11; and
   vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 15; and

c) a third ISVD specifically binds to GPC3 and comprises
   vii. a CDR1 that is the amino acid sequence of SEQ ID NO: 8;
   viii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12; and
   ix. a CDR3 that is the amino acid sequence of SEQ ID NO: 16,

preferably wherein the order of the ISVDs indicates their relative position to each other considered from the *N*-terminus to the *C*-terminus of said polypeptide, wherein the first ISVDs is optionally located at the *N*-terminus of said polypeptide.

In one embodiment, the polypeptide comprising or consisting of at least three ISVDs preferably further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units. For example, the binding unit can be an ISVD that binds to a serum protein, preferably to a human serum protein such as human serum albumin. In preferred embodiments such polypeptides exhibit a half-life in the subject to be treated that is long enough such that consecutive treatments can be conveniently spaced apart.

Tumour development can be assessed by evaluating the expression of GPC3+solid tumour specific biomarkers, such as alpha-fetoprotein (AFP). Thus, in another embodiment, the polypeptide as defined above is for use in decreasing the alpha-fetoprotein (AFP) levels in a subject suffering from a Glypican-3-positive (GPC3+) solid tumour.

Moreover, the present technology provides a composition comprising the polypeptide for use in the treatment of Glypican-3-positive (GPC3+) solid tumours and/or decreasing the alpha-fetoprotein (AFP) levels in a subject suffering from a Glypican-3-positive (GPC3+) solid tumour.

In some embodiments, the polypeptide and/or a composition comprising the polypeptide is administered to a subject in need thereof intravenously or subcutaneously. In some embodiments, the polypeptide and/or composition is administered to a subject a dosage at a dosage of between 3 and 72 mg.

In some embodiments, the polypeptide can be used in monotherapy or as combination therapy with one or more additional therapeutic agents, for instance with a PD-L1 inhibitor, such as an anti-PD-L1 monoclonal antibody, preferably atezolizumab. In some embodiments, the polypeptide or composition is administered at most twice a week to a subject in need thereof, preferably once a week.

Brief description of the drawings

Figure 1. Mean A022600424 plasma concentration profiles following a single 0.5-hour intravenous infusion from 3 to 10000 µg/kg of A022600424 to female monkeys, (A) Linear-scale (B) Log-scale.

Figure 2. Mean A022600424 serum concentration-time profiles on Days 1 and 29 following twice a week IV (0.3 mg/kg/adm) administration of A022600424 to male and female monkeys for four weeks, (A) Linear-scale (B) Log-scale.

Figure 3. Mean A022600424 serum concentration-time profiles on Days 1 and 29 following twice a week IV (1 mg/kg/adm) administration of A022600424 to male and female monkeys for four weeks, (A) Linear-scale (B) Log-scale.

Figure 4. Mean A022600424 serum concentration-time profiles on Days 1 and 29 following twice a week IV (1-3-10 mg/kg/adm step up) administration of A022600424 to male and female monkeys for four weeks, (A) Linear-scale (B) Log-scale.

Figure 5. Levels (pg/mM) of IFNγ (**A**) and IL-6 (**B**) *vs*. time (Cycle 1) in blood samples from patients from the DL1 group.

Figure 6. Levels (pg/mL) of IFNγ (**A**) and IL-6 (**B**) *vs*. time (Cycle 1) in blood samples from patients from the DL1A group.

Figure 7. Study design. *Bayesian logistic regression model (BLRM) design with at least 3 participants per DL. A022600424 IV or in combination will be administered over a 21-day cycle. DL, dose level; GPC3, glypican-3; IV, intravenous; NSCLC, non-small cell lung cancer.

Figure 8. Mean PK Profiles of A022600424 at C2D1. C, cycle; D, day; DL, dose level; PK, pharmacokinetics.

Figure 9. Cytokine levels (pg/mL) in five dose levels. C, cycle; CRS, cytokine release syndrome; D, day; DL, dose level; EOI, end of infusion; h, hour; IFN, interferon; IL-6, interleukin-6; N, no; SOI, start of infusion; Y, yes.

Figure 10. AFP decrease in HCC patients with AFP decrease ≥20% from baseline. AFP, alpha fetoprotein; C, cycle; D, day; DL, dose level; HCC, hepatocellular carcinoma.

Detailed description of the present technology

1. Definitions

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.*, 1989 (Molecular Cloning: A Laboratory Manual, 2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory Press), Ausubel *et al.*, 1987 (Current protocols in molecular biology, Green Publishing and Wiley Interscience, New York), Lewin 1985 (Genes II, John Wiley & Sons, New York, N.Y.), Old *et al.,* 1981 (Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2nd Ed., University of California Press, Berkeley, CA), Roitt *et al.*, 2001 (Immunology, 6th Ed., Mosby/Elsevier, Edinburgh), Roitt *et al.*, 2001 (Roitt's Essential Immunology, 10th Ed., Blackwell Publishing, UK), and Janeway *et al.*, 2005 (Immunobiology, 6th Ed., Garland Science Publishing/Churchill Livingstone, New York), as well as to the general background art cited herein.

Unless indicated otherwise, all methods, steps, techniques and manipulations that are not specifically described in detail herein 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 and the general background art mentioned herein and to the further references cited therein; as well as to for example the following reviews: Presta 2006 (Adv. Drug Deliv. Rev., 58: 640), Levin and Weiss 2006 (Mol. Biosyst., 2: 49), Irving *et al.*, 2001 (J. Immunol. Methods, 248: 31), Schmitz *et al.,* 2000 (Placenta 21 Suppl. A: S106), Gonzales *et al.*, 2005 (Tumour Biol., 26: 31), which describe techniques for protein engineering, such as

affinity maturation and other techniques for improving the specificity and other desired properties of proteins such as immunoglobulins.

It must be noted that as used herein, the singular forms "a", "an", and "the", include plural references unless the context clearly indicates otherwise. Thus, for example, reference to "a reagent" includes one or more of such different reagents and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the technology described herein. Such equivalents are intended to be encompassed by the present technology.

The term "and/or" wherever used herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term "comprising" can be substituted with the term "containing" or "including" or sometimes when used herein with the term "having".

The term “sequence” as used herein (for example in terms like “immunoglobulin sequence”, “antibody sequence”, “variable domain sequence”, “V_{HH} sequence” or “protein sequence”), should generally be understood to include both the relevant amino acid sequence as well as nucleic acids or nucleotide sequences encoding the same, unless the context requires a more limited interpretation. Amino acid sequences are interpreted to mean a single amino acid or

an unbranched sequence of two or more amino acids, depending on the context. Nucleotide sequences are interpreted to mean an unbranched sequence of 3 or more nucleotides.

When a nucleotide sequence or amino acid sequence is said to "comprise" another nucleotide sequence or amino acid sequence, respectively, or to "essentially consist of" another nucleotide sequence or amino acid sequence, this may mean that the latter nucleotide sequence or amino acid sequence has been incorporated into the first mentioned nucleotide sequence or amino acid sequence, respectively, but more usually this generally means that the first mentioned nucleotide sequence or amino acid sequence comprises within its sequence a stretch of nucleotides or amino acid residues, respectively, that has the same nucleotide sequence or amino acid sequence, respectively, as the latter sequence, irrespective of how the first mentioned sequence has actually been generated or obtained (which may for example be by any suitable method described herein). By means of a non-limiting example, when an ISVD is said to comprise a CDR sequence, this may mean that said CDR sequence has been incorporated into the ISVD, but more usually this generally means that the ISVD contains within its sequence a stretch of amino acid residues with the same amino acid sequence as said CDR sequence, irrespective of how said ISVD has been generated or obtained. It should also be noted that when the latter amino acid sequence has a specific biological or structural function, it preferably has essentially the same, a similar or an equivalent biological or structural function in the first-mentioned amino acid sequence (in other words, the first-mentioned amino acid sequence is preferably such that the latter sequence is capable of performing essentially the same, a similar or an equivalent biological or structural function). For example, when an ISVD is said to comprise a CDR sequence or framework sequence, respectively, the CDR sequence and framework are preferably capable, in said ISVD, of functioning as a CDR sequence or framework sequence, respectively. Also, when a nucleotide sequence is said to comprise another nucleotide sequence, the first-mentioned nucleotide sequence is preferably such that, when it is expressed into an expression product (e.g., a polypeptide), the amino acid sequence encoded by the latter nucleotide sequence forms part of said expression product (in other words, that the latter nucleotide sequence is in the same reading frame as the first-mentioned, larger nucleotide sequence).

Amino acid residues will be indicated interchangeably herein according to the standard three-letter or one-letter amino acid code, as mentioned in Table 1 below.

Table 1: Common amino acids

1-Letter Code	3-Letter Code	Amino Acid Name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
X	Xaa	Unspecified
Y	Tyr	Tyrosine

When an amino acid residue is indicated as "X" or "Xaa", it means that the amino acid residue is unspecified, unless the context requires a more limited interpretation. For example, if the description provides an amino acid sequence of a CDR wherein one (or more) of the amino acid residue(s) is (are) indicated with “X”, the description may further specify which amino acid residue(s) is (can be) present at that specific position of the CDR.

Amino acids are those amino acids commonly found in naturally occurring proteins and are listed in Table 1. Any amino acid sequence that contains post-translationally modified amino acids may be described as the amino acid sequence that is initially translated using the symbols shown in the Table 1 with the modified positions, e.g., hydroxylations or glycosylations, but these modifications shall not be shown explicitly in the amino acid sequence. Any peptide or protein that can be expressed as a sequence modified linkages, cross links and end caps, non-peptidyl bonds, etc., is embraced by this definition.

The terms "protein", "peptide", and "polypeptide" are used interchangeably throughout the disclosure, and each has the same meaning for the purposes of this disclosure. By convention, the amide bond in the primary structure of polypeptides is in the order that the amino acids are written, in which the amine end (*N*-terminus) of a polypeptide is always on the left, while the acid end (*C*-terminus) is on the right.

The term "about" used in the context of the parameters or parameter ranges of the provided herein shall have the following meanings. Unless indicated otherwise, where the term "about" is applied to a particular value or to a range, the value or range is interpreted as being as accurate as the method used to measure it. If no error margins are specified in the application, the last decimal place of a numerical value indicates its degree of accuracy. Where no other error margins are given, the maximum margin is ascertained by applying the rounding-off convention to the last decimal place, e.g., for a pH value of about pH 2.7, the error margin is 2.65-2.74. In the present application, any parameter indicated with the term "about" is also contemplated as being disclosed without the term "about". In other words, embodiments referring to a parameter value using the term "about" shall also describe an embodiment directed to the numerical value of said parameter as such. For example, an embodiment specifying a pH of "about pH 2.7" shall also disclose an embodiment specifying a pH of "pH 2.7" as such; an embodiment specifying a pH range of "between about pH 2.7 and about pH 2.1" shall also describe an embodiment specifying a pH range of "between pH 2.7 and pH 2.1", etc.

For the purposes of comparing two or more nucleotide sequences, the percentage of "sequence identity" between a first nucleotide sequence and a second nucleotide sequence

may be calculated by dividing [the number of nucleotides in the first nucleotide sequence that are identical to the nucleotides at the corresponding positions in the second nucleotide sequence] by [the total number of nucleotides in the first nucleotide sequence] and multiplying by [100%], in which each deletion, insertion, substitution or addition of a nucleotide in the second nucleotide sequence – compared to the first nucleotide sequence – is considered as a difference at a single nucleotide (position). Alternatively, the degree of sequence identity between two or more nucleotide sequences may be calculated using a known computer algorithm for sequence alignment such as NCBI Blast v2.0, using standard settings. Some other techniques, computer algorithms and settings for determining the degree of sequence identity are for example described in WO 04/037999, EP 0967284, EP 1085089, WO 00/55318, WO 00/78972, WO 98/49185 and GB 2357768. Usually, for the purpose of determining the percentage of "sequence identity" between two nucleotide sequences in accordance with the calculation method outlined hereinabove, the nucleotide sequence with the greatest number of nucleotides will be taken as the "first" nucleotide sequence, and the other nucleotide sequence will be taken as the "second" nucleotide sequence.

For the purposes of comparing two or more amino acid sequences, the percentage of "sequence identity" between a first amino acid sequence and a second amino acid sequence (also referred to herein as "amino acid identity") may be calculated by dividing [the number of amino acid residues in the first amino acid sequence that are identical to the amino acid residues at the corresponding positions in the second amino acid sequence] by [the total number of amino acid residues in the first amino acid sequence] and multiplying by [100%], in which each deletion, insertion, substitution or addition of an amino acid residue in the second amino acid sequence – compared to the first amino acid sequence – is considered as a difference at a single amino acid residue (position), *i.e.,* as an "amino acid difference" as defined herein. Alternatively, the degree of sequence identity between two amino acid sequences may be calculated using a known computer algorithm, such as those mentioned above for determining the degree of sequence identity for nucleotide sequences, again using standard settings. Usually, for the purpose of determining the percentage of "sequence identity" between two amino acid sequences in accordance with the calculation method outlined hereinabove, the amino acid sequence with the greatest number of amino acid

residues will be taken as the “first” amino acid sequence, and the other amino acid sequence will be taken as the “second” amino acid sequence.

Also, in determining the degree of sequence identity between two amino acid sequences, the skilled person may take into account so-called “conservative” amino acid substitutions, which can generally be described as amino acid substitutions in which 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 3D structure, function, activity, or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example from WO 04/037999, GB 335768, WO 98/49185, WO 00/46383, and WO 01/09300; and (preferred) types and/or combinations of such substitutions may be selected on the basis of the pertinent teachings from WO 04/037999 as well as WO 98/49185 and from the further references cited therein.

Such conservative substitutions preferably are substitutions in which one amino acid within the following groups (a) – (e) is substituted by another amino acid residue within the same group: (a) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (b) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (c) polar, positively charged residues: His, Arg and Lys; (d) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (e) aromatic residues: Phe, Tyr and Trp. Particularly preferred conservative 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; and/or Phe into Val, into Ile or into Leu.

When comparing two immunoglobulin single variable domains, the term "amino acid difference" refers to an insertion, deletion or substitution of a single amino acid residue on a position of the first sequence, compared to the second sequence; it being understood that two immunoglobulin single variable domains can contain one, two or more such amino acid differences.

For the purposes of comparing two or more immunoglobulin single variable domains or other amino acid sequences such as, e.g., the polypeptides of the present technology, etc.,

the percentage of "sequence identity" between a first amino acid sequence and a second amino acid sequence (also referred to herein as "amino acid identity") may be calculated or determined as described in paragraph f) on pages 49 and 50 of WO 08/020079 (incorporated herein by reference), such as by dividing [the number of amino acid residues in the first amino acid sequence that are identical to the amino acid residues at the corresponding positions in the second amino acid sequence] by [the total number of amino acid residues in the first amino acid sequence] and multiplying by [100%], in which each deletion, insertion, substitution or addition of an amino acid residue in the second amino acid sequence - compared to the first amino acid sequence - is considered as a difference at a single amino acid residue (position), i.e., as an "amino acid difference" as defined herein; alternatively, the degree of sequence identity between two amino acid sequences may be calculated using a known computer algorithm for sequence alignment, such as NCBI Blast v2.0, using standard settings. Some other techniques, computer algorithms and settings for determining the degree of sequence identity are for example described in WO 04/037999, EP 0 967 284, EP 1 085 089, WO 00/55318, WO 00/78972, WO 98/49185 and GB 2 357 768-A.

Usually, for the purpose of determining the percentage of “sequence identity” between two amino acid sequences in accordance with the calculation method outlined hereinabove, the amino acid sequence with the greatest number of amino acid residues will be taken as the “first” amino acid sequence, and the other amino acid sequence will be taken as the “second” amino acid sequence.

Also, in determining the degree of sequence identity between two immunoglobulin single variable domains, the skilled person may take into account so-called "conservative" amino acid substitutions, which can generally be described as amino acid substitutions in which 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 04/037999, GB-A-3 357 768, WO 98/49185, WO 00/46383 and WO 01/09300; and (preferred) types and/or combinations of such substitutions may be selected on the basis of the pertinent teachings from WO 04/037999 as well as WO 98/49185 and from the further references cited therein.

Immunoglobulin single variable domains and nucleic acid sequences are said to be "exactly the same" if they have 100% sequence identity (as defined herein) over their entire length.

The “efficacy” (of a therapeutic compound) measures the maximum strength of the effect itself, at saturating therapeutic compound concentrations. Efficacy indicates the maximum response achievable by the therapeutic compound. It refers to the ability of a therapeutic compound to produce the desired (therapeutic) effect.

The term "potency" is a measure of the biological activity of therapeutic compound. Potency of a therapeutic compound can be determined by any suitable method known in the art. Cell culture-based potency assays are often the preferred format for determining biological activity since they measure the physiological response elicited by the therapeutic compound and can generate results within a relatively short period of time. Various types of cell based assays, based on the mechanism of action of the therapeutic compound, can be used, including but not limited to proliferation assays, cytotoxicity assays, cell killing assays, reporter gene assays, cell surface receptor binding assays, and assays to measure induction/inhibition of functionally essential proteins or other signal molecules (such as phosphorylated proteins, enzymes, cytokines, cAMP and the like), Ramos B cell depletion model, T cell mediated tumour cell killing assay, all well known in the art.

2. Polypeptides

The present technology relates to multispecific-multivalent polypeptides for use in the treatment of Glypican-3-positive (GPC3+) solid tumours and/or for use in decreasing AFP levels in a subject suffering from a GPC3+ solid tumour, wherein the polypeptides comprise at least two immunoglobulin single variable domains (ISVDs) that specifically bind to a domain of GPC3 as described in detail in below, and one ISVD that specifically binds to the constant domain of a T cell receptor (TCR) on a T cell.

In the context of the present technology, the term “immunoglobulin single variable domain” (ISVD), interchangeably used with “single variable domain”, defines immunoglobulin molecules wherein the antigen binding site is present on, and formed by, a single

immunoglobulin domain. This sets ISVDs apart from "conventional" immunoglobulins (e.g., monoclonal antibodies) or their fragments (such as Fab, Fab', $F(ab')_2$, scFv, di-scFv), wherein two immunoglobulin domains, in particular two variable domains, interact to form an antigen binding site. Typically, in conventional immunoglobulins, a heavy chain variable domain (V_H) and a light chain variable domain (V_L) interact to form an antigen binding site. In this case, the complementarity determining regions (CDRs) of both V_H and V_L will contribute to the antigen binding site, i.e., a total of 6 CDRs will be involved in antigen binding site formation.

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 immunoglobulin single variable domain, 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 V_H-V_L pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen.

In contrast, ISVDs are capable of specifically binding to an epitope of the antigen without pairing with an additional immunoglobulin variable domain. The binding site of an immunoglobulin single variable domain is formed by a single V_H, a single V_{HH} or single V_L domain.

In the context of the present technology, the single variable domain may be a light chain variable domain sequence (e.g., a V_L-sequence) or a suitable fragment thereof; or a heavy chain variable domain sequence (e.g., a V_H-sequence or V_{HH} sequence) or a suitable fragment thereof; as long as it is capable of forming a single antigen binding unit (i.e., a functional antigen binding unit that essentially consists of the single variable domain, such that the single antigen binding domain does not need to interact with another variable domain to form a functional antigen binding unit).

An ISVD can for example be a heavy chain ISVD, such as a V_H, V_{HH}, including a camelized V_H or humanized V_{HH}. Preferably, it is a V_{HH}, including a camelized V_H or humanized V_{HH}. Heavy chain ISVDs can be derived from a conventional four-chain antibody or from a heavy chain antibody.

For example, the ISVD may be a single domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody), a "dAb" or dAb (or an amino acid sequence that is suitable for use as a dAb) or a NANOBODY® ISVD (as defined herein and including but not limited to a V_{HH}); other single variable domains, or any suitable fragment of any one thereof. Preferably, the ISVD is a V_H, a humanized V_H, a human V_H, a V_{HH}, a humanized V_{HH} or a camelized V_H. More preferably, the ISVD is a NANOBODY® ISVD (such as a V_{HH}, including a humanized V_{HH} or camelized V_H) or a suitable fragment thereof. *NANOBODY®* and *NANOBODIES®* are registered trademarks of Ablynx N.V.

A "humanized V_{HH}" comprises an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring V_{HH} domain, but that has been "humanized", i.e., by replacing one or more amino acid residues in the amino acid sequence of said naturally occurring V_{HH} sequence (and in particular in the framework sequences) by one or more of the amino acid residues that occur at the corresponding position(s) in a V_H domain from a conventional 4-chain antibody from a human being (e.g., indicated above). This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the further description herein and the prior art (e.g., WO 2008/020079). Again, it should be noted that such humanized V_{HH}s can be obtained in any suitable manner known per se and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring V_{HH} domain as a starting material.

A "camelized V_H" comprises an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring V_H domain, but that has been "camelized", i.e., by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring V_H domain from a conventional 4-chain antibody by one or more of the amino acid residues that occur at the corresponding position(s) in a V_{HH} domain of a heavy chain antibody. This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the further description herein and the prior art (e.g., WO 2008/020079). Such "camelizing" substitutions are preferably inserted at amino acid positions that form and/or are present at the V_H-V_L interface, and/or at the so-called Camelidae hallmark residues, as defined herein (see for example WO 94/04678 and Davies and Riechmann (1994 and 1996),

supra). Preferably, the V_H sequence that is used as a starting material or starting point for generating or designing the camelized V_H is preferably a V_H sequence from a mammal, more preferably the V_H sequence of a human being, such as a V_H3 sequence. However, it should be noted that such camelized V_H can be obtained in any suitable manner known per se and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring V_H domain as a starting material.

The structure of an ISVD sequence can be considered to be comprised of four framework regions ("FRs"), which are referred to in the art and herein as "Framework region 1" ("FR1"); as "Framework region 2" ("FR2"); as "Framework region 3" ("FR3"); and as "Framework region 4" ("FR4"), respectively; which framework regions are interrupted by three complementary determining regions ("CDRs"), which are referred to in the art and herein as "Complementarity Determining Region 1" ("CDR1"); as "Complementarity Determining Region 2" ("CDR2"); and as "Complementarity Determining Region 3" ("CDR3"), respectively.

As further described in paragraph q) on pages 58 and 59 of WO 08/020079, the amino acid residues of an immunoglobulin single variable domain can be 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 V_{HH} domains from Camelids in the article of Riechmann and Muyldermans, 1999 (J. Immunol. Methods 231(1-2):25-38; see for example Figure 2 of this publication). It should be noted that – as is well known in the art for V_H domains and for V_{HH} 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 (according to Kabat annotation or Kabat numbering) may or may not correspond to the actual numbering of the amino acid residues in the actual sequence. The total number of amino acid residues in a V_H domain and a V_{HH} 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.

In the present application, unless indicated otherwise, CDR sequences were determined according to Kabat numbering with AbM CDR annotation ("according to AbM annotation" or "AbM numbering") as described in Kontermann and Dübel (Eds. 2010, Antibody Engineering, vol 2, Springer Verlag Heidelberg Berlin, Martin, Chapter 3, pp. 33-51). According to this method, FR1 comprises the amino acid residues at positions 1-25, CDR1 comprises the amino acid residues at positions 26-35, FR2 comprises the amino acids at positions 36-49, CDR2 comprises the amino acid residues at positions 50-58, FR3 comprises the amino acid residues at positions 59-94, CDR3 comprises the amino acid residues at positions 95-102, and FR4 comprises the amino acid residues at positions 103-113.

Determination of CDR regions may also be done according to different methods. In the CDR determination according to Kabat, FR1 of an immunoglobulin single variable domain comprises the amino acid residues at positions 1-30, CDR1 of an immunoglobulin single variable domain comprises the amino acid residues at positions 31-35, FR2 of an immunoglobulin single variable domain comprises the amino acids at positions 36-49, CDR2 of an immunoglobulin single variable domain comprises the amino acid residues at positions 50-65, FR3 of an immunoglobulin single variable domain comprises the amino acid residues at positions 66-94, CDR3 of an immunoglobulin single variable domain comprises the amino acid residues at positions 95-102, and FR4 of an immunoglobulin single variable domain comprises the amino acid residues at positions 103-113.

In such an immunoglobulin sequence, the framework sequences may be any suitable framework sequences, and examples of suitable framework sequences will be clear to the skilled person, for example on the basis the standard handbooks and the further disclosure and prior art mentioned herein.

The framework sequences are preferably (a suitable combination of) immunoglobulin framework sequences or framework sequences that have been derived from immunoglobulin framework sequences (for example, by humanization or camelization). For example, the framework sequences may be framework sequences derived from a light chain variable domain (e.g., a V_L-sequence) and/or from a heavy chain variable domain (e.g., a V_H-sequence or V_{HH} sequence). In one particularly preferred aspect, the framework sequences are either framework sequences that have been derived from a V_{HH}-sequence (in which said framework

sequences may optionally have been partially or fully humanized) or are conventional V_H sequences that have been camelized (as defined herein).

For a general description of ISVDs, reference is made to the further description below, as well as to the prior art cited herein. In this respect, it should however be noted that this description and the prior art mainly described ISVDs of the so-called "V_H3 class" (i.e., ISVDs with a high degree of sequence homology to human germline sequences of the V_H3 class such as DP-47, DP-51 or DP-29). It should however be noted that the technology in its broadest sense can generally use any type of ISVD, and for example also uses the ISVDs belonging to the so-called "V_H4 class" (i.e., ISVDs with a high degree of sequence homology to human germline sequences of the V_H4 class such as DP-78), as for example described in WO 2007/118670. Preferably, the ISVD is derived from an ISVD belonging to the "V_H3 class".

Generally, ISVDs (in particular V_{HH} sequences, including (partially) humanized V_{HH} sequences and camelized V_H sequences) can be characterized by the presence of one or more "Hallmark residues" (as described herein) in one or more of the framework sequences (again as further described herein). Thus, generally, an ISVD, such as a NANOBODY® ISVD, can be defined as an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which one or more of the Hallmark residues are as further defined herein.

In particular, an ISVD can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which the framework sequences are as further defined herein.

More in particular, an ISVD can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which: one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table 2 below.

Table 2: Hallmark Residues in NANOBODY® ISVDs

Position	Human V_H3	Hallmark Residues
11	L, V; predominantly L	L, S, V, M, W, F, T, Q, E, A, R, G, K, Y, N, P, I; preferably L
37	V, I, F; usually V	$F^{(1)}$, Y, V, L, A, H, S, I, W, C, N, G, D, T, P, preferably $F^{(1)}$ or Y
$44^{(8)}$	G	$E^{(3)}$, $Q^{(3)}$, $G^{(2)}$, D, A, K, R, L, P, S, V, H, T, N, W, M, I; preferably $G^{(2)}$, $E^{(3)}$ or $Q^{(3)}$;most preferably $G^{(2)}$ or $Q^{(3)}$.
$45^{(8)}$	L	$L^{(2)}$, $R^{(3)}$, P, H, F, G, Q, S, E, T, Y, C, I, D, V; preferably $L^{(2)}$ or $R^{(3)}$
$47^{(8)}$	W, Y	$F^{(1)}$, $L^{(1)}$ or $W^{(2)}$ G, I, S, A, V, M, R, Y, E, P, T, C, H, K, Q, N, D; preferably $W^{(2)}$, $L^{(1)}$ or $F^{(1)}$
83	R or K; usually R	R, $K^{(5)}$, T, $E^{(5)}$, Q, N, S, I, V, G, M, L, A, D, Y, H; preferably K or R; most preferably K
84	A, T, D; predominantly A	$P^{(5)}$, S, H, L, A, V, I, T, F, D, R, Y, N, Q, G, E; preferably P
103	W	$W^{(4)}$, $R^{(6)}$, G, S, K, A, M, Y, L, F, T, N, V, Q, $P^{(6)}$, E, C; preferably W
104	G	G, A, S, T, D, P, N, E, C, L; preferably G
108	L, M or T; predominantly L	Q, $L^{(7)}$, R, P, E, K, S, T, M, A, H; preferably Q or $L^{(7)}$

Notes:

(1) In particular, but not exclusively, in combination with KERE or KQRE at positions 43-46.

(2) Usually as GLEW at positions 44-47.

(3) Usually as KERE or KQRE at positions 43-46, e.g. as KEREL, KEREF, KQREL, KQREF, KEREG, KQREW or KQREG at positions 43-47. Alternatively, also sequences such as TERE (for example TEREL), TQRE (for example TQREL), KECE (for example KECEL or KECER), KQCE (for example KQCEL), RERE (for example REREG), RQRE (for example RQREL, RQREF or RQREW), QERE (for example QEREG), QQRE, (for example QQREW, QQREL or QQREF), KGRE (for example KGREG), KDRE (for example KDREV) are possible. Some other possible, but less preferred sequences include for example DECKL and NVCEL.

(4) With both GLEW at positions 44-47 and KERE or KQRE at positions 43-46.

(5) Often as KP or EP at positions 83-84 of naturally occurring V_{HH} domains.

(6) In particular, but not exclusively, in combination with GLEW at positions 44-47.

(7) With the proviso that when positions 44-47 are GLEW, position 108 is always Q in (non-humanized) V_{HH} sequences that also contain a W at 103.

(8) The GLEW group also contains GLEW-like sequences at positions 44-47, such as for example GVEW, EPEW, GLER, DQEW, DLEW, GIEW, ELEW, GPEW, EWLP, GPER, GLER and ELEW

Thus, a NANOBODY® ISVD can be defined as an amino acid sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table 2.

It should be noted that, in the context of the present technology, the origin of the ISVD sequence or the origin of the nucleotide sequence used to express it is not limited, nor as to the way that the ISVD sequence or nucleotide sequence is or has been generated or obtained. Thus, the ISVD sequences may be naturally occurring sequences (from any suitable species) or synthetic or semi-synthetic sequences. In a specific but non-limiting aspect, the ISVD sequence is a naturally occurring sequence (from any suitable species) or a synthetic or semi-

synthetic sequence, including but not limited to "humanized" (as defined herein) immunoglobulin sequences (such as partially or fully humanized mouse or rabbit immunoglobulin sequences, and in particular partially or fully humanized V_{HH} sequences), "camelized" (as defined herein) immunoglobulin sequences, as well as immunoglobulin sequences that have been obtained by techniques such as affinity dematuration (for example, starting from synthetic, random or naturally occurring immunoglobulin sequences), CDR grafting, veneering, combining fragments derived from different immunoglobulin sequences, PCR assembly using overlapping primers, and similar techniques for engineering immunoglobulin sequences well known to the skilled person; or any suitable combination of any of the foregoing.

Similarly, nucleotide sequences may be naturally occurring nucleotide sequences or synthetic or semi-synthetic sequences, and may for example be sequences that are isolated by PCR from a suitable naturally occurring template (e.g., DNA or RNA isolated from a cell), nucleotide sequences that have been isolated from a library (and in particular, an expression library), nucleotide sequences that have been prepared by introducing mutations into a naturally occurring nucleotide sequence (using any suitable technique known per se, such as mismatch PCR), nucleotide sequence that have been prepared by PCR using overlapping primers, or nucleotide sequences that have been prepared using techniques for DNA synthesis known *per se.*

In one embodiment, the ISVDs comprised in the polypeptides of the present technology derive from an ISVD, such as from a heavy-chain ISVD, preferably from a NANOBODY® ISVD, which has been further engineered/modified to include mutations which prevent/remove binding of pre-existing antibodies/factors. Examples of such mutations are described, e.g., in WO 2012/175741 and WO 2015/173325. For instance, to prevent/remove binding of pre-existing antibodies/factors, the amino acid at position 11 (according to Kabat) may be Val or Leu, preferably Val; and/or the amino acid at position 89 (according to Kabat) may be preferably Val, Thr or Leu, preferably Leu; and/or the amino acid at position 110 (according to Kabat) may be preferably Thr, Lys or Gln, preferably Thr; and/or the amino acid at position 112 (according to Kabat) may be Ser, Lys or Gln, preferably Ser; and/or the ISVD-based building

block may contain a *C*-terminal extension of 1-5 amino acids chosen from any naturally occurring amino acid.

In one embodiment, the polypeptide of the present technology is at least bispecific, but can also be, e.g., trispecific, tetraspecific, pentaspecific, etc. Moreover, the polypeptide is at least trivalent, but can also be, e.g., tetravalent, pentavalent, hexavalent, etc.

The terms "bispecific", "trispecific", "tetraspecific", "pentaspecific", etc., all fall under the term "multispecific" and refer to binding to two, three, four, five, etc., different target molecules, respectively. The term "multispecific" refers to binding to multiple different target molecules.

The terms "bivalent", "trivalent", "tetravalent", "pentavalent", "hexavalent", etc. all fall under the term "multivalent" and indicate the presence of two, three, four, five, six, etc., binding units/building blocks, respectively, such as ISVDs. The term "multivalent" indicates the presence of multiple binding units/building blocks.

For example, the polypeptide of the present technology is at least bispecific-trivalent, such as a polypeptide comprising or consisting of three ISVDs, wherein two ISVD specifically bind to GPC3 and one ISVD specifically binds to the constant domain of a TCR on a T cell, wherein the GPC3 and TCR are preferably human GPC3 and human TCR. In another example, the polypeptide may be trispecific-tetravalent, such as a polypeptide comprising or consisting of four ISVDs, wherein two ISVDs specifically bind to human GPC3, one ISVD specifically binds to the constant domain of a human TCR on a T cell and one ISVD binds to human serum albumin. Such a polypeptide may at the same time be biparatopic, for example if two ISVDs bind two different epitopes on human GPC3. The term "biparatopic" refers to binding to two different parts (e.g., epitopes) of the same target molecule. A preferred trispecific-tetravalent polypeptide is, e.g., A022600424, SEQ ID NO: 1, comprising two ISVDs specifically binding to human GPC3, one ISVD specifically binding to the constant domain of a human TCR on a T cell, one ISVD binding to human serum albumin, and which is biparatopic for binding to GPC3.

As described above, the technology *inter alia* uses ISVDs that can bind to the constant domain of the TCR or GPC3.

The ISVDs used in the technology form part of a polypeptide, which comprises or consists of at least three ISVDs, such that the polypeptide can specifically bind to GPC3 and to TCR, as described herein.

Accordingly, the target molecules of the ISVDs used in the technology are at least GPC3 and the constant domain of the TCR. Binding to TCR can be achieved, for example, by binding to the TCRalpha subunit and/or the TCR beta subunit. Examples are mammalian GPC3 and TCR. While human GPC3 (Uniprot accession P51654) and human TCR alpha constant domain (Uniprot accession P01848) and human TCR beta constant domain (Uniprot accession P01850) are preferred, the versions from other species are also amenable to the present technology, for example GPC3 and TCR from mice, rats, rabbits, cats, dogs, goats, sheep, horses, pigs, non-human primates, such as cynomolgus monkeys (also referred to herein as "*cyno*"), or camelids, such as llama or alpaca.

Specific examples of ISVDs specifically binding to the constant domain of a TCR on a T cell that can be used in the technology are as described in the following item A:

A. An ISVD that specifically binds to human TCR and comprises

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6

ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 and

iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14

In a preferred embodiment, the ISVD binds to the constant domain of a human TCR-α of SEQ ID NO: 135 and/or of a TCR-β of SEQ ID NO: 136, or polymorphic variants or isoforms thereof.

Preferred examples of such an ISVD that specifically binds to human TCR comprise one or more (and preferably all) framework regions as indicated for ISVD TCE01 in Table 7 (in addition to the CDRs as defined in the preceding item A), and most preferred is an ISVD that consists of the full amino acid sequence of ISVD TCE01 (SEQ ID NO: 2; see Table 6 and 7).

Also, in a preferred embodiment, the amino acid sequence of the ISVD specifically binding to human TCR may exhibit a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 2, wherein the CDRs are as defined in the preceding item A. In particular, the ISVD specifically binding to TCR most preferably is the amino acid sequence of SEQ ID NO: 2.

Specific examples of ISVDs specifically binding to GPC3 that can be used in the technology are as described in the following items B and C:

B. An ISVD that specifically binds to human GPC3 and comprises

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7;

ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 and

iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15;

C. An ISVD that specifically binds to human GPC3 and comprises

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8;

ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12; and

iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16.

In a preferred embodiment, the ISVD binds to human GPC3 of SEQ ID NO: 134.

Preferred examples of such an ISVD that specifically binds to human GPC3 comprise one or more (and preferably all) framework regions as indicated for ISVD A022600351 and A022600314, respectively, in Table 7 (in addition to the CDRs as defined in the preceding items B and C, respectively), and most preferred is a polypeptide comprising both ISVDs that consist of the full amino acid sequence of ISVD A022600351 and A022600314 (SEQ ID NOs: 3 and 4, see Tables 6 and 7).

Also, in a preferred embodiment, the amino acid sequence of the ISVDs specifically binding to human GPC3 may exhibit a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 3 or 4, respectively, wherein the CDRs are as defined in the preceding item B or C, respectively. In particular, the ISVD binding to human GPC3 most preferably is the amino acid sequence of SEQ ID NOs: 3 or 4.

Preferably, each of the ISVDs as defined under items A to C above is comprised in the polypeptide of the present technology.

Such a polypeptide comprising at least each of the ISVDs as defined under items A to C above preferably exhibits at least half the binding affinity, more preferably at least the same binding affinity, to human TCR and to human GPC3 as a polypeptide consisting of the amino acid of SEQ ID NO: 1, wherein the binding affinity is measured using the same method, such as SPR.

The SEQ ID NOs referred to in the above items A to C and item D below (see section 4 "(*In vivo)* half-life extension") are based on the CDR definition according to the AbM definition (see Table 7). It is noted that the SEQ ID NOs defining the same CDRs according to the Kabat definition (see Table 8) can likewise be used in the above items A to C and item D below (see section 4 "(*In vivo*) half-life extension").

Accordingly, the specific examples of ISVDs specifically binding to the constant domain of the TCR on a T cell or GPC3 that can be used in the technology, and are as described above using the AbM definition, can be also described using the Kabat definition as set forth in items A' to C' below:

A'. An ISVD that specifically binds to human TCR and comprises

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 31;

ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 35; and

iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14.

Preferred examples of such an ISVD that specifically binds to human TCR comprise one or more (and preferably all) framework regions as indicated for ISVD TCE01, respectively, in Table 8 (in addition to the CDRs as defined in the preceding item A'), and most preferred is an ISVD that consists of the full amino acid sequence of ISVD TCE01 (SEQ ID NO: 2; see Tables 6 and 8).

B'. An ISVD that specifically binds to human GPC3 and comprises

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 32;

ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 36 ; and

iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15;

C'. An ISVD that specifically binds to human GPC3 and comprises

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 33;

ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 37; and

iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16.

Preferred examples of such ISVDs that specifically bind to human GPC3 comprise one or more (and preferably all) framework regions as indicated for ISVD A022600351 and

A022600314, respectively, in Table 8 (in addition to the CDRs as defined in the preceding items B' and C', respectively), and most preferred is a polypeptide comprising both ISVDs that consist of the full amino acid sequence of ISVD A022600351 and A022600314 (SEQ ID NOs: 3 and 4, see Tables 6 and 8).

In one embodiment, the present technology provides a bispecific-trivalent polypeptide comprising or consisting of at least three ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least three ISVDs are optionally linked via one or more peptidic linkers, and wherein:

a) a first ISVD specifically binds the constant domain of a TCR on a T cell and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 6, a CDR2 that is the amino acid sequence of SEQ ID NO: 10, and a CDR3 that is the amino acid sequence of SEQ ID NO: 14;

b) a second ISVD specifically binds GPC3 and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 11, and a CDR3 that is the amino acid sequence of SEQ ID NO: 15; and

c) a third ISVD specifically binds GPC3 and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 16,

wherein the TCR and GPC3 bound by said polypeptide is preferably human TCR and human GPC3, respectively, and wherein the CDRs are defined according to AbM annotation.

In some embodiments such a polypeptide can be a polypeptide, wherein:

a) the amino acid sequence of said first ISVD comprised in the polypeptide exhibits a sequence identity of more than 90%, such as 95%, 97%, 99% or 100% sequence identity with SEQ ID NO: 2;

b) the amino acid sequence of said second ISVD comprised in the polypeptide exhibits a sequence identity of more than 90%, such as 95%, 97%, 99% or 100% sequence identity with SEQ ID NO: 3; and

c) the amino acid sequence of said third ISVD comprised in the polypeptide exhibits a sequence identity of more than 90%, such as 95%, 97%, 99% or 100% sequence identity with SEQ ID NO: 4.

Preferably, the multispecific-multivalent polypeptide comprises:

a) a first ISVD which consists of the amino acid sequence of SEQ ID NO: 2;

b) a second ISVD which consists of the amino acid sequence of SEQ ID NO: 3; and

c) a third ISVD which consists of the amino acid sequence of SEQ ID NO: 4.

The terms "first ISVD", "second ISVD", "third ISVD", etc., in this regard only indicate the relative position of the ISVDs to each other, wherein the numbering is started from the *N*-terminus of the polypeptide. The "first ISVD" is thus closer to the *N*-terminus than the "second ISVD", whereas the "second ISVD" is closer to the *N*-terminus than the "third ISVD". Accordingly, the ISVD arrangement is inverse when considered from the *C*-terminus. Since the numbering is not absolute and only indicates the relative position of the at least three ISVDs it is not excluded that other binding units/building blocks such as additional ISVDs binding to GPC3, or ISVDs binding to another target may be present in the polypeptide. Moreover, it does not exclude the possibility that other binding units/building blocks such as ISVDs can be placed in between. For instance, as described further below (see in particular, section 4. "(*In vivo*) half-life extension" below), the polypeptide can further comprise another ISVD binding to human serum albumin that can even be located between, e.g., the "second ISVD" and "third ISVD".

In a further aspect the present technology thus provides a trispecific-tetravalent polypeptide comprising the bispecific-trivalent polypeptides described above and a fourth ISVD binding to human serum albumin as described in detail below (section 4; "(*In vivo*) half-life extension").

In a preferred embodiment, the first ISVD binding to TCR is positioned at the *N*-terminus of the polypeptide.

The components, preferably ISVDs, of said multispecific-multivalent polypeptides described herein may be linked to each other directly or by one or more suitable linkers, such as peptidic linkers.

The use of linkers to connect two or more (poly)peptides is well known in the art. Exemplary peptidic linkers are shown in Table 3. One often used class of peptidic linker are known as the "Gly-Ser" or "GS" linkers. These are linkers that essentially consist of glycine (G) and serine (S) residues, and usually comprise one or more repeats of a peptide motif such as

the GGGGS (SEQ ID NO: 100) motif (for example, exhibiting the formula $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_n$ in which n may be 1, 2, 3, 4, 5, 6, 7 or more). Some often-used examples of such GS linkers are 9GS linkers (GGGGSGGGS, SEQ ID NO: 103) 15GS linkers (n=3) and 35GS linkers (n=7). Reference is for example made to Chen *et al.*, Adv. Drug Deliv. Rev. 2013 Oct 15; 65(10): 1357–1369; and Klein *et al.*, Protein Eng. Des. Sel. (2014) 27 (10): 325-330. In the polypeptide(s) disclosed herein, the use of 5GS and 9GS linkers to link the components of the polypeptide to each other is preferred.

Table 3: Linker sequences ("ID" refers to the SEQ ID NO as used herein)

Name	ID	Amino acid sequence
3A linker	99	AAA
5GS linker	100	GGGGS
7GS linker	101	SGGSGGS
8GS linker	102	GGGGSGGS
9GS linker	103	GGGGSGGGS
10GS linker	104	GGGGSGGGGS
15GS linker	105	GGGGSGGGGSGGGGS
18GS linker	106	GGGGSGGGGSGGGGSGGS
20GS linker	107	GGGGSGGGGSGGGGSGGGGS
25GS linker	108	GGGGSGGGGSGGGGSGGGGSGGGGS
30GS linker	109	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
35GS linker	110	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
40GS linker	111	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS

In some embodiments, the first ISVD and the second ISVD comprised in the polypeptide are linked to each other via a linker consisting of less than 10 amino acids, preferably less than 6 amino acids, wherein the linker is preferably a 5GS linker (e.g., SEQ ID NO: 100).

In one embodiment of the multispecific-multivalent polypeptide, the polypeptide comprising or consisting of at least three ISVDs, comprises the at least two ISVDs specifically binding to GPC3 and one ISVD specifically binding to TCR, wherein the ISVD binding to TCR is

linked to one of the at least two ISVDs binding to GPC3 via a 5GS linker (e.g., SEQ ID NO: 100), and the at least two ISVDs specifically binding to GPC3 are linked to each other via a 9GS linker (e.g., SEQ ID NO: 103).

In one embodiment, the multispecific-multivalent polypeptide further comprises an ISVD binding to serum albumin, which is further linked via a 9 GS linker (e.g., SEQ ID NO: 103) to the ISVD binding to GPC3 that is not linked to the ISVD binding to TCR (as described in section 4 "(*In vivo*) half-life extension" below). Such a configuration can increase the efficiency of the polypeptide in eliciting a T cell-mediated cytotoxic response.

Accordingly, it is an aspect of the technology that the polypeptide comprises or consists of the following, in the order starting from the *N*-terminus of the polypeptide: a first ISVD specifically binding to TCR, a second ISVD specifically binding to GPC3, a third ISVD specifically binding to GPC3, and an optional binding unit providing the polypeptide with increased half-life as defined herein. The binding unit providing the polypeptide with increased half-life is preferably an ISVD, that preferably binds to serum albumin, as described herein.

In one embodiment, the polypeptide comprises or consists of the following, in the order starting from the *N*-terminus of the polypeptide: an ISVD specifically binding to TCR, a linker, a second ISVD specifically binding to GPC3, a linker, a third ISVD specifically binding to GPC3, a linker, and an ISVD binding to human serum albumin, wherein the linker between the first and second ISVDs is preferably a 5GS linker (e.g., SEQ ID NO: 100), whereas the other linkers are preferably 9GS linkers (e.g., SEQ ID NO: 103).

Such configurations of the polypeptide can provide for strong potencies with regard to treating cancer as well as low binding by pre-existing antibodies.

In an embodiment, the multispecific-multivalent polypeptide exhibits reduced binding by pre-existing antibodies in human serum. To this end, in one embodiment, the polypeptide exhibits a valine (V) at amino acid position 11 and a leucine (L) at amino acid position 89 (according to Kabat numbering) in at least one ISVD (and preferably the ISVD at the *C*-terminal end of the polypeptide), but preferably in each ISVD.

In another embodiment, the polypeptide exhibits an extension of 1 to 5 (preferably naturally occurring) amino acids, such as a single alanine (A) extension, at the *C*-terminus of the *C*-terminal ISVD. The C-terminus of an ISVD is normally VTVSS (SEQ ID NO: 116). In another

embodiment, the polypeptide exhibits a lysine (K) or glutamine (Q) at position 110 (according to Kabat numbering) in at least one ISVD. In another embodiment, the ISVD exhibits a lysine (K) or glutamine (Q) at position 112 (according to Kabat numbering) in at least on ISVD. In these embodiments, the *C*-terminus of the ISVD is VKVSS (SEQ ID NO: 117), VQVSS (SEQ ID NO: 118), VTVKS (SEQ ID NO: 119), VTVQS (SEQ ID NO: 120), VKVKS (SEQ ID NO: 121), VKVQS (SEQ ID NO: 122), VQVKS (SEQ ID NO: 123), or VQVQS (SEQ ID NO: 124) such that after addition of a single alanine the *C*-terminus of the polypeptide for example exhibits the sequence VTVSSA (SEQ ID NO: 125), VKVSSA (SEQ ID NO: 126), VQVSSA (SEQ ID NO: 127), VTVKSA (SEQ ID NO: 128), VTVQSA (SEQ ID NO: 129), VKVKSA (SEQ ID NO: 130), VKVQSA (SEQ ID NO: 131), VQVKSA (SEQ ID NO: 132), or VQVQSA (SEQ ID NO: 133), preferably VTVSSA (see Table 4). In another embodiment, the polypeptide exhibits a valine (V) at amino acid position 11 and a leucine (L) at amino acid position 89 (according to Kabat numbering) in at least the C-terminal ISVD, optionally a lysine (K) or glutamine (Q) at position 110 (according to Kabat numbering) in at least one ISVD, and exhibits an extension of 1 to 5 (preferably naturally occurring) amino acids, such as a single alanine (A) extension, at the *C*-terminus of the *C*-terminal ISVD (such that the *C*-terminus of the polypeptide for example consists of the sequence VTVSSA, VKVSSA or VQVSSA, preferably VTVSSA). See e.g., WO 2012/175741 and WO2015/173325 for further information in this regard.

Table 4: *C*-termini with or without *C*-terminal extensions ("ID" refers to the given SEQ ID NO as used herein)

ID	Amino acid sequence
116	VTVSS
117	VKVSS
118	VQVSS
119	VTVKS
120	VTVQS
121	VKVKS
122	VKVQS
123	VQVKS
124	VQVQS

125	VTVSSA
126	VKVSSA
127	VQVSSA
128	VTVKSA
129	VTVQSA
130	VKVKSA
131	VKVQSA
132	VQVKSA
133	VQVQSA

In a preferred embodiment, the multispecific-multivalent polypeptide comprises or consists of an amino acid sequence exhibiting a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 1, wherein more preferably the CDRs of the four ISVDs are as defined in items A to D using the AbM definition (or A' to D' if using the Kabat definition) set forth above in this section and in section "4 (*In vivo*) half-life extension" below, respectively, wherein in particular, using the AbM annotation:

- the first ISVD specifically binding to the constant domain of a TCR on a T cell comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14;
- the second ISVD specifically binding to GPC3 comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15;
- the third ISVD specifically binding to GPC3 comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16; and
- the fourth ISVD binding to human serum albumin comprises a CDR1 being the amino acid sequence of SEQ ID NO: 9, a CDR2 being the amino acid sequence of SEQ ID NO: 13 and a CDR3 being the amino acid sequence of SEQ ID NO: 17,

or alternatively, if using the Kabat annotation:

- the first ISVD specifically binding to TCR comprises a CDR1 being the amino acid sequence of SEQ ID NO: 31, a CDR2 being the amino acid sequence of SEQ ID NO: 35 and a CDR3 being the amino acid sequence of SEQ ID NO: 14;
- the second ISVD specifically binding to GPC3 comprises a CDR1 being the amino acid sequence of SEQ ID NO: 32, a CDR2 being the amino acid sequence of SEQ ID NO: 36 and a CDR3 being the amino acid sequence of SEQ ID NO: 15;
- the third ISVD specifically binding to GPC3 comprises a CDR1 being the amino acid sequence of SEQ ID NO: 33, a CDR2 being the amino acid sequence of SEQ ID NO: 37 and a CDR3 being the amino acid sequence of SEQ ID NO: 16; and
- the fourth ISVD binding to human serum albumin comprises a CDR1 being the amino acid sequence of SEQ ID NO: 34, a CDR2 being the amino acid sequence of SEQ ID NO: 38 and a CDR3 being the amino acid sequence of SEQ ID NO: 17.

In some embodiments, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1. Preferably, the polypeptide consists of the amino acid sequence of SEQ ID NO: 1.

3. Specificity

In the context of the present technology, the terms "specificity", "binding specifically" or "specific binding" refer to the number of different target molecules, such as antigens, to which a particular binding unit can bind with sufficiently high affinity (see below). "*Specificity*", "*binding specifically*" or "*specific binding*" are used interchangeably herein with "*selectivity*", "*binding selectively*" or "*selective binding*". Generally, binding units, such as binding ISVDs, specifically bind to their designated targets.

The specificity/selectivity of a binding unit can be determined based on affinity. The affinity denotes the strength or stability of a molecular interaction. The affinity is commonly given by the K_D, or dissociation constant, which has units of mol/litre (or M). The affinity can also be expressed as an association constant, K_A, which equals $1/K_D$ and has units of $(mol/litre)^{-1}$ (or M^{-1}).

The K_D may also be expressed as the ratio of the dissociation rate constant of a complex, denoted as k_{off}, to the rate of its association, denoted k_{on} (so that $K_D = k_{off}/k_{on}$ and $K_A = k_{on}/k_{off}$). The off-rate k_{off} has units s^{-1} (where s is the SI unit notation of second). The on-rate k_{on} has

units $M^{-1}s^{-1}$. The on-rate may vary between 10^2 $M^{-1}s^{-1}$ to about 10^7 $M^{-1}s^{-1}$, approaching the diffusion-limited association rate constant for bimolecular interactions. The off-rate is related to the half-life of a given molecular interaction by the relation $t_{1/2}=\ln(2)/k_{off}$. The off-rate may vary between 10^{-6} s^{-1} (near irreversible complex with a $t_{1/2}$ of multiple days) to 1 s^{-1} ($t_{1/2}$=0.69 s).

The measured K_D may correspond to the apparent K_D if the measuring process somehow influences the intrinsic binding affinity of the implied molecules for example by artefacts related to the coating on the biosensor of one molecule. Also, an apparent K_D may be measured if one molecule contains more than one recognition sites for the other molecule or molecules. In such situation the measured affinity may be affected by the avidity of the interaction by the two molecules.

The dissociation constant (K_D) may be the actual or apparent dissociation constant, as will be clear to the skilled person. Methods for determining the K_D will be clear to the skilled person, and for example include the techniques mentioned below. In this respect, it will also be clear that it may not be possible to measure dissociation constants of more than 10^{-4} moles/litre or 10^{-3} moles/litre (e.g., of 10^{-2} moles/litre). Optionally, as will also be clear to the skilled person, the (actual or apparent) K_D may be calculated on the basis of the (actual or apparent) association constant (K_A), by means of the relationship ($K_D = 1/K_A$). $K_A = 1/K_D$ --> K_A= [AB] / [A].[B].

Typically, binding units used in the present technology (such as ISVDs) will bind to their targets with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/litre or less, and preferably 10^{-7} to 10^{-12} moles/litre or less and more preferably 10^{-8} to 10^{-12} moles/litre (i.e. with an association constant (K_A) of 10^5 to 10^{12} litre/ moles or more, and preferably 10^7 to 10^{12} litre/moles or more and more preferably 10^8 to 10^{12} litre/moles).

Any K_D value greater than 10^{-4} mol/litre (or any K_A value lower than 10^4 litres/mol) is generally considered to indicate non-specific binding.

The K_D for biological interactions, such as the binding of immunoglobulin sequences to an antigen, which are considered specific are typically in the range of 10^{-5} moles/litre (10000 nM or 10 µM) to 10^{-12} moles/litre (0.001 nM or 1 pM) or less.

Accordingly, specific/selective binding may mean that - using the same measurement method, e.g., surface plasmon resonance SPR - a binding unit (or polypeptide comprising the

same) binds to TCR and/or GPC3 with a K_D value of 10^{-5} to 10^{-12} moles/litre or less and binds to related targets with a K_D value greater than 10^{-4} moles/litre. Examples of related targets for GPC3 are GPC1, GPC2, GPC4, GPC5 or GPC6. Thus, in an embodiment of the technology, the ISVDs comprised in the polypeptide that bind to GPC3, bind to GPC3 with a K_D value of 10^{-5} to 10^{-12} moles/litre or less and bind to GPC1, GPC2, GPC4, GPC5 and GPC6 of the same species with a K_D value greater than 10^{-4} moles/litre.

Specific binding to a certain target from a certain species does not exclude that the binding unit can also specifically bind to the analogous target from a different species. For example, specific binding to human TCR does not exclude that the binding unit (or a polypeptide comprising the same) can also specifically bind to TCR from cynomolgus monkeys. Likewise, for example, specific binding to human GPC3 does not exclude that the binding unit (or a polypeptide comprising the same) can also specifically bind to GPC3 from cynomolgus monkeys ("cyno").

The ISVD with SEQ ID NO: 2 that binds to human TCR and that is comprised in the polypeptides of the current technology, exhibits improved binding characteristics compared to ISVD T0170056G05, which is described in WO 2016/180969 A1. More specifically, the ISVD with SEQ ID NO:2 is derived from T017056G05 and comprises specific mutations in CDR1 and CDR3 which result in improved cross-reactivity for binding to human and non-human primate (such as cynomolgus monkey) TCR compared to ISVD T0170056G05.

When an ISVD is said to exhibit "improved cross-reactivity for binding to human and non-human primate TCR" compared to another ISVD, it means that for said ISVD the ratio of the binding activity (such as expressed in terms of K_D or k_{off}) for human TCR and for non-human primate TCR is higher than that same ratio calculated for the other ISVD in the same assay.

Good cross-reactivity for binding to human and non-human primate TCR allows for the assessment of toxicity of a multispecific T cell engaging polypeptide in preclinical studies conducted on non-human primates.

Specific binding of a binding unit to its designated target can be determined in any suitable manner known per se, including, for example, 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; as well as the other techniques mentioned herein.

The dissociation constant may be the actual or apparent dissociation constant, as will be clear to the skilled person. Methods for determining the dissociation constant will be clear to the skilled person, and for example include the techniques mentioned below. In this respect, it will also be clear that it may not be possible to measure dissociation constants of more than 10^{-4} moles/litre or 10^{-3} moles/litre (e.g., of 10^{-2} moles/litre). Optionally, as will also be clear to the skilled person, the (actual or apparent) dissociation constant may be calculated on the basis of the (actual or apparent) association constant, by means of the relationship [$K_D = 1/K_A$].

The affinity of a molecular interaction between two molecules can be measured via different techniques known *per se*, such as the well-known surface plasmon resonance (SPR) biosensor technique (see for example Ober *et al.,* 2001, Intern. Immunology 13: 1551-1559). The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, where one molecule is immobilized on the biosensor chip and the other molecule is passed over the immobilized molecule under flow conditions yielding k_{on}, k_{off} measurements and hence K_D (or K_A) values. This can for example be performed using the well-known BIAcore® system (BIAcore International AB, a GE Healthcare company, Uppsala, Sweden and Piscataway, NJ). For further descriptions, see Jonsson *et al.* (1993, Ann. Biol. Clin. 51: 19-26), Jonsson *et al.* (1991 Biotechniques 11: 620-627), Johnsson *et al.* (1995, J. Mol. Recognit. 8: 125-131), and Johnnson *et al.* (1991, Anal. Biochem. 198: 268-277).

Another well-known biosensor technique to determine affinities of biomolecular interactions is bio-layer interferometry (BLI) (see for example Abdiche *et al.* 2008, Anal. Biochem. 377: 209-217). The term “bio-layer Interferometry” or “BLI”, as used herein, refers to a label-free optical technique that analyses the interference pattern of light reflected from two surfaces: an internal reference layer (reference beam) and a layer of immobilized protein on the biosensor tip (signal beam). A change in the number of molecules bound to the tip of the biosensor causes a shift in the interference pattern, reported as a wavelength shift (nm),

the magnitude of which is a direct measure of the number of molecules bound to the biosensor tip surface. Since the interactions can be measured in real-time, association and dissociation rates and affinities can be determined. BLI can for example be performed using the well-known Octet® Systems (ForteBio, a division of Pall Life Sciences, Menlo Park, USA).

Alternatively, affinities can be measured in Kinetic Exclusion Assay (KinExA) (see for example Drake *et al.,* 2004, Anal. Biochem., 328: 35-43), using the KinExA® platform (Sapidyne Instruments Inc, Boise, USA). The term "KinExA", as used herein, refers to a solution-based method to measure true equilibrium binding affinity and kinetics of unmodified molecules. Equilibrated solutions of an antibody/antigen complex are passed over a column with beads precoated with antigen (or antibody), allowing the free antibody (or antigen) to bind to the coated molecule. Detection of the antibody (or antigen) thus captured is accomplished with a fluorescently labelled protein binding the antibody (or antigen).

The GYROLAB® immunoassay system provides a platform for automated bioanalysis and rapid sample turnaround (Fraley *et al*. 2013, Bioanalysis 5: 1765-74).

4. (*In vivo*) half-life extension

In some embodiments, the polypeptide further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units.

The term “half-life” as used here can generally be defined as described in paragraph o) on page 57 of WO 2008/020079 and as mentioned therein refers to the time taken for the serum concentration of the compound or polypeptide to be reduced by 50%, *in vivo*, for example due to degradation of the sequence or compound and/or clearance or sequestration of the sequence or compound by natural mechanisms. The *in vivo* half-life of the polypeptide of the technology can be determined in any manner known *per se*, such as by pharmacokinetic analysis. Suitable techniques will be clear to the person skilled in the art and may for example generally be as described in paragraph o) on page 57 of WO 2008/020079. As also mentioned in paragraph o) on page 57 of WO 2008/020079, the half-life can be expressed using

parameters such as the $t_{1/2}$-alpha, $t_{1/2}$-beta and the area under the curve (AUC). In this respect it should be noted that the term "half-life" as used herein in particular refers to the $t_{1/2}$-beta or terminal half-life (in which the $t_{1/2}$-alpha and/or the AUC or both may be kept out of considerations). Reference is for example made to the standard handbooks, such as Kenneth, A *et al*: Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists and Peters *et al*, Pharmacokinetic analysis: A Practical Approach (1996).

Reference is also made to "Pharmacokinetics", M Gibaldi & D Perron, published by Marcel Dekker, 2nd Rev. edition (1982). Similarly, the terms "increase in half-life" or "increased half-life" are also as defined in paragraph o) on page 57 of WO 2008/020079 and in particular refer to an increase in the $t_{1/2}$-beta, either with or without an increase in the $t_{1/2}$-alpha and/or the AUC or both. *In vivo* half-life extension means, for example, that the polypeptide exhibits an increased half-life in a mammal, such as a human subject, after administration.

The type of groups, residues, moieties or binding units is not generally restricted and may for example be chosen from the group consisting of a polyethylene glycol molecule, serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

In some embodiments, said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life can be chosen from the group consisting of binding units that can bind to serum proteins, such as human serum albumin, or a serum immunoglobulin, such as IgG, and preferably is a binding unit that can bind to human serum albumin. The binding unit is preferably an ISVD, preferably an ISVD which binds serum albumin.

For example, WO 04/041865 describes NANOBODY® molecules binding to serum albumin (and in particular against human serum albumin) that can be linked to other proteins (such as one or more other NANOBODY® molecules binding to a desired target) in order to increase the half-life of said protein.

The international application WO 06/122787 describes a number of NANOBODY® molecules against (human) serum albumin. These NANOBODY® molecules include the NANOBODY® molecule called Alb-1 (SEQ ID NO: 52 in WO 06/122787) and humanized variants

thereof, such as Alb-8 (SEQ ID NO: 62 in WO 06/122787). Again, these can be used to extend the half-life of therapeutic proteins and polypeptide and other therapeutic entities or moieties.

Moreover, WO 2012/175400 describes a further improved version of Alb-1, called Alb-23.

In an embodiment, the polypeptide comprises a serum albumin binding moiety selected from Alb-1, Alb-3, Alb-4, Alb-5, Alb-6, Alb-7, Alb-8, Alb-9, Alb-10 and Alb-23, preferably Alb-8 or Alb-23 or its variants, as shown in pages 7-9 of WO 2012/175400 and the albumin binders described in WO 2012/175741, WO 2015/173325, WO 2017/080850, WO 2017/085172, WO 2018/104444, WO 2018/134235, WO2 018/134234. Some preferred serum albumin binders are also shown in Table 5.

A further component of the polypeptide of the present technology is as described in item D:

D. An ISVD that binds to human serum albumin and comprises

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 9;

ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13; and

iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17;

wherein the CDRs are defined according to AbM annotation,

Preferred examples of such an ISVD that binds to human serum albumin comprise one or more (and preferably all) framework regions as indicated for ISVD ALB23002 in Table 7 below (in addition to the CDRs as defined in the preceding item D), and most preferred is an ISVD that consists of the full amino acid sequence of ISVD ALB23002 (SEQ ID NO: 5, see Table 6 below).

Item D can be also described using the Kabat definition as:

D'. An ISVD that binds to human serum albumin and comprises

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 34;

ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 38; and

iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17.

Preferred examples of such an ISVD that binds to human serum albumin comprise one or more (and preferably all) framework regions as indicated for ISVD ALB23002 in Table 8 (in addition to the CDRs as defined in the preceding item D')

Most preferred is an ISVD which comprises or consists of the full amino acid sequence of ISVD ALB23002 (SEQ ID NO: 5, see Tables 6 and 8 below).

Also, in a preferred embodiment, the amino acid sequence of an ISVD binding to human serum albumin may exhibit a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 5, wherein the CDRs are as defined in the preceding item D or D'. In particular, the ISVD binding to human serum albumin preferably is the amino acid sequence of SEQ ID NO: 5.

When such an ISVD binding to human serum albumin is at a C-terminal position it can exhibit a C-terminal alanine (A) or glycine (G) extension (preferably A) and is preferably selected from SEQ ID NOs: 83, 85, 87, 89, 91, 93, 95, 96 and 98, most preferably SEQ ID NO: 96, which represents SEQ ID NO: 5 with a single alanine extension (see Table 5 below). In some embodiments, the ISVD binding to human serum albumin is at another position than the C-terminal position (i.e., is not the C-terminal ISVD of the polypeptide) and is selected from SEQ ID NOs: 5, 82, 84, 86, 88, 90, 92, 94 and 97 (see Table 5 below).

Table 5: Serum albumin binding ISVD sequences ("ID" refers to the SEQ ID NO as used herein)

Name	ID	Amino acid sequence
Alb8	82	EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSI SGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVTVSS
Alb8-A	83	EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSI SGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVTVSSA
Alb23	84	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSI SGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVTVSS
Alb23-A	85	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSI SGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVTVSSA

Alb83	86	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTATYYCTIGGSLSR SSQGTLVTVSS
Alb83-A	87	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTATYYCTIGGSLSR SSQGTLVTVSSA
Alb132	88	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTATYYCTIGGSLSR SSQGTLVTVSS
Alb132-A	89	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTATYYCTIGGSLSR SSQGTLVTVSSA
Alb73	90	EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSI SGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVKVSS
Alb73-A	91	EVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSI SGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVKVSSA
Alb82	92	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVTVSS
Alb82-A	93	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVTVSSA
Alb199	94	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVKVSS
Alb199-A	95	EVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVKVSSA

Alb23002	5	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVTVSS
Alb223	96	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVTVSSA
Alb216	97	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVKVSS
Alb216-A	98	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVKVSSA

5. Preparation of the polypeptide

The polypeptide of the present technology can be prepared according to the methods described in WO2021/198260 and WO 2022/129560 (incorporated by reference). For instance, a method for producing the polypeptide as disclosed herein may comprise transforming/transfecting a host cell or (non-human) host organism with a nucleic acid encoding the polypeptide, expressing the polypeptide in the host, optionally followed by one or more isolation and/or purification steps. Specifically, the method may comprise:

a) expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid sequence encoding the polypeptide; optionally followed by:

b) isolating and/or purifying the polypeptide.

Suitable host cells or host organisms for production purposes will be clear to the skilled person, and may for example be any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or eukaryotic organism. Specific examples include HEK293 cells, CHO cells, *Escherichia coli* or *Pichia pastoris*. The most preferred host is *Pichia pastoris*.

6. Compositions

The technology also provides a composition comprising at least one polypeptide for use as disclosed herein, at least one nucleic acid molecule encoding a polypeptide for use as disclosed herein or at least one vector comprising such a nucleic acid molecule. The composition may be a pharmaceutical composition.

In some embodiments, the composition further comprises pharmaceutically acceptable excipients.

The composition may further comprise at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally comprise one or more further pharmaceutically active polypeptides and/or compounds.

Generally, the polypeptides of present technology can be formulated using techniques and procedures well known in the art and administered in any suitable manner known *per se*. For instance, the ISVDs can be in from of a sterile lyophilized powder. Reference is for example made to the general background art cited above (and in particular to WO 04/041862, WO 04/041863, WO 04/041865, WO 04/041867 and WO 08/020079) as well as to the standard handbooks, such as Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Company, USA (1990), Remington, the Science and Practice of Pharmacy, 21st Ed., Lippincott Williams and Wilkins (2005); or the Handbook of Therapeutic Antibodies (S. Dubel, Ed.), Wiley, Weinheim, 2007 (see for example pages 252-255).

Preferably, the composition is provided as a liquid formulation.

7. Uses of the polypeptide

The present technology relates to applications and uses of the polypeptides, and compositions for the prevention and/or treatment of Glypican-3-positive (GPC3+) solid tumours. As described above in section 2 "Polypeptides", in a preferred embodiment, the polypeptide used in the present technology (and comprised in the compositions of the present technology) comprises or consists of at least three ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least three ISVDs are optionally linked via one or more peptidic linkers, and wherein:

a) a first ISVD specifically binds the constant domain of a TCR on a T cell and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 6, a CDR2 that is the amino acid sequence of SEQ ID NO: 10, and a CDR3 that is the amino acid sequence of SEQ ID NO: 14;

b) a second ISVD specifically binds GPC3 and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 11, and a CDR3 that is the amino acid sequence of SEQ ID NO: 15; and

c) a third ISVD specifically binds GPC3 and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 16,

wherein the TCR and GPC3 bound by said polypeptide is preferably human TCR and human GPC3, respectively, and wherein the CDRs are defined according to AbM annotation.

In a preferred embodiment, the polypeptide comprises

a) a first ISVD which consists of the amino acid sequence of SEQ ID NO: 2;

b) a second ISVD which consists of the amino acid sequence of SEQ ID NO: 3; and

c) a third ISVD which consists of the amino acid sequence of SEQ ID NO: 4.

More preferably, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1.

The multispecific-multivalent polypeptides and compositions of the present technology as defined in section 2 “Polypeptides” can generally be used to activate T cells via binding to its TCRαβ constant region. Due to the simultaneous binding of TCR on T cells and an GPC3 on target cells by the multispecific-multivalent polypeptides of the present technology, the activation of the T-cells is only induced at the site of the GPC3-expressing target cancer cells, which results in the lysis (killing) of the GPC3-expressing target cells. Consequently, GPC3+ solid tumour growth can be reduced and, preferably, the tumour can be eliminated.

"T cell activation" as used herein refers to one or more cellular response(s) of a T cell, e.g., a cytotoxic T cell, such as selected from: proliferation, differentiation, cytokine secretion, cytotoxic effector molecule release, cytotoxic activity, expression of activation markers, and redirected target cell lysis. The multispecific-multivalent polypeptides of the current technology are capable of inducing T cell activation. Suitable assays to measure T cell

activation are known in the art described herein, for instance as described in WO 99/54440 or by Schlereth *et al*. 2005 (Cancer Immunol. Immunother. 20: 1-12).

The redirected lysis of target cells via the recruitment of T cells by the polypeptides of the present technology involves cytolytic synapse formation and delivery of perforin and granzymes. Cell lysis by T cells has been described, e.g., by Atkinson and Bleackley, 1995 (Crit. Rev. Immunol 15(3-4): 359-384). Preferably, the engaged T cells are capable of serial target cell lysis and are not affected by immune escape mechanisms interfering with peptide antigen processing and presentation, or clonal T cell differentiation (see, for example, WO 2007/042261). *In vitro*, redirected lysis is seen at low picomolar concentrations, suggesting that very low numbers of the polypeptides of the present technology need to be bound to target cells for triggering T cells. Accordingly, the present technology relates to potent polypeptides. Preferably, the multispecific-multivalent polypeptide of the current technology mediates killing of target cells, e.g., cancer cells, such as stimulating T cells in pore formation and delivering pro-apoptotic components of cytotoxic T cell granules. Preferably, the polypeptide of the present technology induces T cell activation only when said second and/or further ISVD is bound to an antigen (such as GPC3) on a target cell. Thus, when not bound to the antigen (such as GPC3) expressing target cells, the polypeptides of the present technology show hardly any T cell activation. As such, target-independent lysis (i.e., lysis of cells without antigen expression) by the polypeptides of the present technology is minimal. Consequently, the polypeptide of the present application has a low cytotoxicity.

Cancer

The cancer may be any type of Glypican-3-positive (GPC3+) solid tumour but is preferably HCC or NSCLC.

In a non-limiting example cancerous glypican-3-positive (GPC3+) solid tumours can be carcinomas (such as hepatocellular carcinoma and pulmonary squamous cell carcinoma), sarcomas, adenocarcinomas, and malignant melanoma.

In certain embodiments, the Glypican-3-positive (GPC3+) solid tumour is liver cancer or lung cancer, preferably liver cancer.

In a specific embodiment the liver cancer is HCC. For example, the HCC may be metastatic and/or unresectable HCC.

In another specific embodiment, the lung cancer is NSCLC, more preferably metastatic NSCLC.

In another specific embodiment, the lung cancer is lung squamous cell carcinoma (LSCC).

Preferably, the GPC3 expressing cancer cells express (on average) at least half the amount of GPC3 protein, preferably at least the same amount of GPC3 protein as Huh7 cells (on average). Huh7 cells are publicly available from, e.g., the National Institutes of Biomedical Innovation, Health and Nutrition, JCRB Cell Bank, under accession number JCRB0403.

The expressed GPC3 preferably refers to cell surface exposed GPC3. The expression of (cell surface exposed) GPC3 on a cell (and the amount thereof) can be readily determined by routine methods commonly known in the art, such as flow cytometry, or immunohistochemistry.

Alpha-fetoprotein (AFP)

Alpha-fetoprotein (AFP) is known to correlate with the incidence of hepatic cancer and is thus a commonly used serum marker to aid the diagnosis and screening of high risk hepatic cancer patients and certain lung cancers and can thus be used as a biomarker to assess tumour progression. Accordingly, a decrease of the AFP levels in a subject suffering said tumour may indicate effective treatment of a GPC3+ solid tumour expressing AFP with the polypeptide of the present technology.

In some embodiments, the administration of the polypeptide or composition of the present technology, as defined in section 2 “Polypeptides” above, leads to a decrease in AFP level of at least 20% from baseline, preferably in a decrease in AFP level of at least 30% from baseline, more preferably in a decrease in AFP level of at least 40% from baseline, such as a decrease in AFP level of at least 50%, or at least 55%, or at least 59% from baseline.

In some embodiments, the administration of the polypeptide or composition of the present technology, as defined in section 2 “Polypeptides” above, leads to a decrease in AFP level of at least 20% from baseline, preferably in a decrease in AFP level of at least 30% from baseline, more preferably in a decrease in AFP level of at least 40% from baseline, such as a

decrease in AFP level of at least 50%, or at least 55%, or at least 59%, or at least 60%, or at least 70% from baseline.

The baseline level is the level of AFP in the subject before the administration of the polypeptide or composition. Alternatively, baseline level is the level of AFP in the placebo treated subjects.

Therefore, in particular embodiments, the present technology provides a method wherein the polypeptide or the composition of the present technology can be used for use in decreasing AFP levels in a subject suffering from a Glypican-3-positive (GPC3+) solid tumour.

As described above, the decrease in AFP levels can be of at least 20% from baseline, preferably at least 30% from baseline, more preferably of at least 40% from baseline, such as a decrease of at least 50%, or at least 55%, or at least 59%, or at least 60%, or at least 70% from baseline. Preferably, the decrease in AFP levels is maintained for at least two days, three days, four days, five days, six days, one week or more, such as for at least two weeks, three weeks, four weeks or more.

Treatment regimes

A “subject” as referred to in the context of the technology can be any animal, preferably a mammal. Among mammals, a distinction can be made between humans and non-human mammals. Non-human animals may be for example companion animals (e.g., dogs, cats), livestock (e.g., bovine, equine, ovine, caprine, or porcine animals), or animals used generally for research purposes and/or for producing antibodies (e.g., mice, rats, rabbits, cats, dogs, goats, sheep, horses, pigs, non-human primates, such as cynomolgus monkeys, or camelids, such as llama or alpaca).

In the context of prophylactic and/or therapeutic purposes, the subject can be any animal, and more specifically any mammal, but preferably is a human subject.

As used herein, the terms "treat", "treatment" and "treating" in the context of administering a therapy/therapies to a subject refers to the reduction or amelioration of the progression, severity, and/or duration of a disorder associated with a GPC3+ solid tumour, such as an

increase in progression-free survival time, or increase in survival time of the subject with a GPC3+ cancer.

In specific embodiments, the terms "treat", "treatment" and "treating" in the context of administering a therapy/therapies to a subject refers to the reduction or amelioration of the progression and/or severity of a GPC3+ solid tumour. For instance, in some embodiments, treatment may lead to inhibition of an activity of GPC3+ cancer cells in a GPC3+ solid tumour, such as to delay or minimize the spread of the GPC3+ cancer cells, to inhibit or delay growth and/or proliferation of the GPC3+ cancer cells, and/or to kill the GPC3+ cancer cells (e.g., cause regression of the disorder and/or symptoms), by more than about 10%, such as 20%, 30%, or 40%, or even more than 50%, such as more than 60%, such as 70%, 80% or even more than 90%, such as 100%.

In specific embodiments, treatment refers to a reduction in cancer cells by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% relative to a control (e.g., a negative control such as phosphate buffered saline).

In other embodiments, the terms "treat", "treatment" and "treating" in the context of administering a therapy/therapies to a subject refers to the reduction or amelioration of the progression and/or severity of a GPC3+ solid tumour, in particular they refer to no change in cancer cell number, a reduction in hospitalization time, a reduction in mortality, or an increase in progression-free survival time, or increase in survival time of the subject with a GPC3+ cancer.

Administration

In a preferred embodiment, the polypeptide and/or composition of the present technology is administered to a subject in need thereof intravenously or subcutaneously.

Intravenous administration can be, for example, by infusion. This type of injection is most typically performed in the arm or leg muscles.

An effective amount of a polypeptide or a composition comprising the polypeptide can be administered to a subject in order to provide the intended treatment results.

Dosage

As described above, the polypeptides or compositions comprising the same may be administered in various dosage forms, for example solutions, suspensions or emulsions, preferably solutions.

In some embodiments, the polypeptide or composition is administered at a dosage of between 1 and 200 mg of the polypeptide, preferably between 1 and 72 mg, and more preferably at a dosage of between 3 and 72 mg. In some other embodiments, the polypeptide or composition is administered to a subject in need thereof at a dosage of between 1 and 200 mg, preferably between 1 and 70 mg.

In some embodiments, the polypeptide or composition is administered at a dosage of between 1 to 200 mg of the polypeptide, such as between 2 and 200 mg, such as between 10 and 200 mg, between 50 and 100 mg, between 60 and 80 mg or between 65 and 75 mg, preferably between 3 and 72 mg, e.g., between 5 and 50 mg, between 10 and 30 mg, or between 15 and 25 mg.

In some embodiments, the polypeptide or composition is administered at a dosage of between 15 and 3500 μg/kg of the polypeptide, such as between 15 and 3000 μg/kg, or between 45 and 3000 μg/kg, such as between 15 and 2500 μg/kg, or between 40 and 2000 μg/kg, preferably between 15 and 1100 μg/kg, more preferably 15 and 1000 μg/kg, even more preferably between 40 and 1100 μg/kg, such as between 40 and 400 μg/kg, or at a dosage of between 150 and 360 μg/kg.

One or more doses can be administered. If more than one dose is administered, the doses can be administered in suitable intervals in order to maximize the effect of the polypeptide, and composition.

In some embodiments, the polypeptide or composition is administered at most twice a week to a subject in need thereof, preferably once a week.

In some embodiments, an effective amount of the polypeptide or composition is administered to a subject in need thereof once every week, once every two weeks, once every three weeks, once every 4 weeks. For instance, an effective amount of the polypeptide or

composition is administered to a subject in need thereof once a week, or twice a week, or three times a week, preferably once or twice a week, even more preferably once a week.

In some embodiments, the polypeptide or composition according to the present technology is administered once every 3, 4, 5, 6, or 7 days. In other embodiments, the polypeptide or composition according to the present technology is administered twice every 3, 4, 5, 6, or 7 days.

For example, one dose of the polypeptide or composition according to the present technology may be administered at day 0 and, after 7 days, another dose is administered. For instance, a dose of between 1 and 200 mg, such as between 2 and 200 mg, 10 and 200 mg, between 50 and 100 mg, between 60 and 80 mg or between 65 and 75 mg, preferably between 3 and 72 mg, e.g., between 5 and 50 mg, or between 10 and 30 mg respectively, of the polypeptide according to the present technology, may be administrated to the same subject after 7 days.

Hence, the administration regimen may be the administration of the polypeptide or composition according to the present technology of between 1 and 200 mg, such as between 2 and 200 mg, between 50 and 100 mg, between 60 and 80 mg or between 65 and 75 mg, preferably between 3 and 72 mg, e.g., between 5 and 50 mg, between 10 and 30 mg, or between 15 and 25 mg of the polypeptide according to the present technology at day 0 and the administration of the same amount of the polypeptide according to the present technology one week later, for at least two weeks, such as for three weeks, or four weeks, or five weeks, or six weeks, or longer, as long as the treatment is effective and well tolerated.

For example, a dose of about 300 µg/kg of the polypeptide or composition according to the present technology may be administered at day 0 and another dose of 300 µg/kg of the polypeptide or composition according to the present technology may be administrated to the same subject after 7 days. This can be repeated as many times as necessary, i.e., as long as the treatment is effective and well tolerated.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, e.g., into a number of discrete loosely spaced administrations. In some embodiments, the polypeptide or composition is infused over about

30-240 minutes. In some embodiments, polypeptide or composition is infused over about 30-90 minutes.

The effective dosage of the polypeptide or composition of the present application can be determined by measuring the decrease in AFP level.

In some embodiments, the effective dosage of the polypeptide or composition (corresponding to effective amount of polypeptide or composition) is equivalent to a decrease in AFP level of at least 20% from baseline, preferably a decrease in AFP level of at least 30% from baseline, more preferably a decrease in AFP level of at least 40% from baseline, such as a decrease in AFP level of at least 50%, or at least 55%, or at least 59%, or at least 60%, or at least 70% from baseline. The term "baseline" has been described above in the present description.

Consequently, in some embodiments, the dosage of the polypeptide or composition of the present technology will be adjusted as the effective dosage that achieves a decrease in AFP level of at least 20% from baseline, preferably a decrease in AFP level of at least 30% from baseline, more preferably a decrease in AFP level of at least 40% from baseline, such as a decrease in AFP level of at least 50%, or at least 55%, or at least 59%, or at least 60%, or at least 70% from baseline.

Accordingly, in the present technology, the effective dosage of a polypeptide or composition may be determined by (i): administering to said human a dose of between 1 and 200 mg of the polypeptide, preferably between 2 and 200 mg, more preferably between 1 and 72 mg, and (even more) preferably at a dosage of between 3 and 72 mg, or in an amount of between 1 to 70 mg, or in an amount of between 1 and 36 mg, (ii) measuring the AFP levels of said patient; and if said decrease in AFP level is less than 20% from baseline, then increasing the dose of said step (i) until a decrease in AFP level of at least 20% from baseline, preferably a decrease in AFP level of at least 30% from baseline, more preferably a decrease in AFP level of at least 40% from baseline, such as a decrease in AFP level of at least 50%, or at least 55%, or at least 59%, or at least 60%, or at least 70% from baseline is measured.

In other embodiments, the effective dosage of a polypeptide or composition may be determined by (i): administering to said human a dose of between 1 and 200 mg, such as between 2 and 200 mg, e.g., between 1 and 70 mg, or between 1 and 72 mg, or between 3

and 72 mg, or between 5 and 50 mg, between 10 and 30 mg, or between 15 and 25 mg, or between 1 and 36 mg (ii) measuring the AFP levels of said patient; and if said decrease in AFP level is less than 20% from baseline, then increasing the dose of said step (i) until a decrease in AFP level of at least 20% from baseline, preferably a decrease in AFP level of at least 30% from baseline, more preferably a decrease in AFP level of at least 40% from baseline, such as a decrease in AFP level of at least 50%, or at least 55%, or at least 59%, or at least 60%, or at least 70% from baseline is measured.

In other embodiments, the effective dosage of a polypeptide or composition may be determined by (i): administering to said human a dose of between 10 and 200 mg, such as between 50 and 100 mg, such as between 60 and 80 mg, or between 65 and 75 mg, (ii) measuring the AFP levels of said patient; and if said decrease in AFP level is less than 20% from baseline, then increasing the dose of said step (i) until a decrease in AFP level of at least 20% from baseline, preferably a decrease in AFP level of at least 30% from baseline, more preferably a decrease in AFP level of at least 40% from baseline, such as a decrease in AFP level of at least 50%, or at least 55%, or at least 59%, or at least 60%, or at least 70% from baseline is measured.

In addition, the polypeptide according to the present technology is administered to a human subject in need thereof in an amount which is effective to maintain the decrease the AFP levels for at least two days, three days, four days, five days, six days, one week or more, such as for at least two weeks, three weeks, four weeks or more, such as five, six, seven, eight, nine, ten, eleven or twelve weeks or more.

For instance, the polypeptide according to the present technology is administered to a human subject in need thereof in an amount which is effective to maintain a decrease in AFP levels of at least 20% from baseline for at least two days, three days, four days, five days, six days, one week or more, such as for at least two weeks, three weeks, four weeks or more.

Thus, the present technology also provides a polypeptide or composition as described herein for use in decreasing AFP levels in a subject suffering from a Glypican-3-positive (GPC3+) solid tumour with at least 20% from baseline for at least one week, such as at least two weeks, wherein the polypeptide or composition is administered at a dosage between 1 and 200 mg,

preferably between 1 and 72 mg, and more preferably at a dosage of between 3 and 72 mg. In another embodiment, the present technology provides a polypeptide or composition as described herein for use in decreasing AFP levels in a subject suffering from a Glypican-3-positive (GPC3+) solid tumour with at least 20% from baseline for at least one week, such as at least two weeks, wherein the polypeptide or composition is administered at a dosage between 1 and 200 mg, preferably between 1 and 70 mg, and more preferably at a dosage of between 1 and 36 mg.

In some embodiments, the AFP levels are decreased with at least 30% from baseline for at least one week, such as at least two weeks, at least three weeks, at least four weeks, at least five weeks, at least six weeks, or more; such as a decrease of at least 40%, at least 50%, at least 55%, or at least 59%, or at least 60%, or at least 70% from baseline for at least one week, such as at least two weeks, at least three weeks, at least four weeks, at least five weeks, at least six weeks, or more.

In some embodiments, the subject is administered the polypeptide or composition at a dosage between 10 and 200 mg, such as between 50 and 100 mg, such as between 60 and 80 mg, or between 65 and 75 mg.

In an embodiment the subject is administered the polypeptide or composition at a dosage between 3 to 72 mg, such as from 5 to 50 mg, from 10 to 30 mg, or from 15 to 25 mg.

In another embodiment the subject is administered the polypeptide or composition at a dosage between 1 to 70 mg, such as from 5 to 50 mg, from 10 to 30 mg, or from 15 to 25 mg, or from 1 to 36 mg.

The amount of the polypeptide according to the present technology is administered to a human subject in need thereof in an amount which is effective to decrease the AFP levels in the human subject by at least 20%, preferably at least 30% from baseline, more preferably by at least 40% from baseline, such by at least 50%, or at least 55%, or at least 59% from baseline on the 2nd week of treatment, or on the 3rd week of treatment, or on the 4th week of treatment, or on the 5th week of treatment, or on the 6th week of treatment, or on the 7th week of treatment, or on the 8th week of treatment, or on the 9th week of treatment, or on the 10th week of treatment, or on the 11th week of treatment, or on the 12th week of treatment, or on the 13th week of treatment, or on the 14th week of treatment, or on the 15th week of treatment,

or on the 16th week of treatment, or on the 17th week of treatment, or on the 18th week of treatment, or on the 19th week of treatment, or on the 20th week of treatment, or on the 21st week of treatment, or later.

Thus, the present technology also provides a polypeptide or composition as described herein for use in decreasing AFP levels in a subject suffering from a Glypican-3-positive (GPC3+) solid tumour with at least 20% from baseline on the 2nd week of treatment, such as on the 3rd week of treatment, wherein the polypeptide or composition is administered at a dosage between 1 and 200 mg, preferably between 1 and 72 mg, and more preferably at a dosage of between 3 and 72 mg.

In some embodiments, the AFP levels are decreased with at least 20%, such as at least 30%, from baseline on the 2nd week of treatment, or on the 3rd week of treatment, or on the 4th week of treatment, or on the 5th week of treatment, or on the 6th week of treatment, or on the 7th week of treatment, or on the 8th week of treatment, or on the 9th week of treatment, or on the 10th week of treatment, or on the 11th week of treatment, or on the 12th week of treatment, or on the 13th week of treatment, or on the 14th week of treatment, or on the 15th week of treatment, or on the 16th week of treatment, or on the 17th week of treatment, or on the 18th week of treatment, or on the 19th week of treatment, or on the 20th week of treatment, or on the 21st week of treatment, or later; such as a decrease of at least 40%, at least 50%, at least 55%, or at least 59% from baseline on the 2nd week of treatment, or on the 3rd week of treatment, or on the 4th week of treatment, or on the 5th week of treatment, or on the 6th week of treatment, or on the 7th week of treatment, or on the 8th week of treatment, or on the 9th week of treatment, or on the 10th week of treatment, or on the 11th week of treatment, or on the 12th week of treatment, or on the 13th week of treatment, or on the 14th week of treatment, or on the 15th week of treatment, or on the 16th week of treatment, or on the 17th week of treatment, or on the 18th week of treatment, or on the 19th week of treatment, or on the 20th week of treatment, or on the 21st week of treatment, or later.

In some embodiments, the subject is administered the polypeptide or composition at a dosage between 10 and 200 mg, such as between 50 and 100 mg, such as between 60 and 80 mg, or between 65 and 75 mg.

In an embodiment the subject is administered the polypeptide or composition at a dosage between 3 to 72 mg, such as from 5 to 50 mg, from 10 to 30 mg, or from 15 to 25 mg.

In an embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", or composition thereof, is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 3 to 72 mg, preferably in an amount of about 3 mg.

In an embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", or composition thereof, is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 36 mg, such as in an amount of 1 mg, 2,5 mg, 3 mg, 4,5 mg, 18 mg or 36 mg, preferably in an amount of about 18 mg.

In one embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, preferably in an amount of about 1 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by at least 20% from baseline, such as at least 40%, or at least 50%, preferably by at least 56%.

In one embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 3 to 72 mg, preferably in an amount of about 3 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by at least 20% from baseline, such as at least 40%, at least 50%, or at least 55%, preferably by at least 59%.

In one embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 36 mg, preferably in an amount of about 18 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject

are decreased by at least 20% from baseline, such as at least 40%, at least 50%, or at least 55%, or at least 60%, preferably by at least 70%.

In one embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 36 mg, preferably in an amount of about 36 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by at least 20% from baseline, such as at least 25%, preferably at least 30%.

In another embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 36 mg, preferably in an amount of about 36 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by at least 20% from baseline, such as at least 40%, preferably at least 50% or at least 55%.

In an embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, such as about 3 to 72 mg, or about 3 mg, preferably in an amount of about 3-36 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by at least 20% from baseline, such as at least 40%, at least 50%, or at least 55%, preferably by at least 59% on the 2nd week of treatment, on the 3rd week of treatment, on the 4th week of treatment, on the 5th week of treatment, on the 6th week of treatment, on the 7th week of treatment, on the 8th week of treatment, on the 9th week of treatment, on the 10th week of treatment, on the 11th week of treatment, on the 12th week of treatment, on the 13th week of treatment, on the 14th week of treatment, on the 15th week of treatment, on the 16th week of treatment, on the 17th week of treatment, on the 18th week of treatment, on the 19th week of treatment, on the 20th week of treatment, on the 21st week of treatment, or later.

In an embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-

positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, preferably in an amount of about 1 mg, wherein the AFP levels in the human subject decrease by at least 20%, such as at least 50%, or at least 55%, preferably at least 56%, on the third week of treatment.

In another embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 3 to 72 mg, preferably in an amount of about 3 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by at least 20% from baseline, such as at least 40%, at least 50%, or at least 55%, preferably by at least 59%, wherein a decrease in AFP levels of at least 40% from baseline is maintained for at least two weeks, such as for at least three weeks, at least four weeks, at least five weeks, at least six weeks, or more.

In an embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, preferably in an amount of about 3 mg, wherein the AFP levels in the human subject decrease by at least 20%, such as at least 50%, or at least 55%, preferably at least 59%, on the third week of treatment.

In other embodiments, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, preferably in an amount of about 18 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by least 20%, such as at least 30%, at least 45%,or at least 60%, preferably at least 70%, wherein the decrease in AFP levels of at least 20% from baseline is maintained for at least two weeks, such as for at least three weeks, at least four weeks, at least five weeks, at least six weeks, or more.

In an embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, preferably in an amount of about 18 mg, wherein the AFP levels in the human subject decrease by at least 20%, such as at least 50%, or at least 60%, preferably at least 70%, on the third week of treatment.

In other embodiments, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a

Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, preferably in an amount of about 36 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by least 20%, such as at least 25%, preferably at least 30%, wherein the decrease in AFP levels of at least 20% from baseline is maintained for at least two weeks, such as for at least three weeks, at least four weeks, at least five weeks, at least six weeks, or more.

In an embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, preferably in an amount of about 36 mg, wherein the AFP levels in the human subject decrease by at least 20%, such as at least 25%, preferably at least 30%, on the third week of treatment.

In one embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 36 mg, preferably in an amount of about 18 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by at least 20% from baseline, such as at least 40%, at least 50%, or at least 55%.

In one embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 36 mg, preferably in an amount of about 36 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by at least 20% from baseline, such as at least 40%, at least 50%, or at least 55%.

In an embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 3 to 72 mg, preferably in an amount of about 3 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by at least 20% from baseline, such as at least 40%, at least 50%, or at least 55%, preferably by at least 59% on the 2nd week of treatment, or on the 3rd week of treatment or on the 4th week of treatment, or on the 5th week of treatment, or on the 6th week of treatment, or on the 7th week of treatment, or on the 8th week of treatment, or on the 9th week of treatment, or on the 10th week of treatment, or on the 11th week of treatment, or on the 12th week of treatment, or on the 13th week of treatment, or on the 14th week of treatment, or on

the 15th week of treatment, or on the 16th week of treatment, or on the 17th week of treatment, or on the 18th week of treatment, or on the 19th week of treatment, or on the 20th week of treatment, or on the 21st week of treatment, or later.

In one embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, preferably in an amount of about 1 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by at least 20%, such as at least 40%, or at least 41%.

In an embodiment, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, preferably in an amount of about 1 mg, wherein the AFP levels in the human subject decrease by at least 20%, such as at least 50%, or at least 55%, preferably at least 56%, on the third week of treatment.

In other embodiments, the polypeptide according to the present technology, as defined in section 2 "Polypeptides", is administered to a human subject suffering from a Glypican-3-positive (GPC3+) solid tumour in an amount of about 1 to 72 mg, preferably in an amount of about 1 mg, for use in decreasing AFP levels, wherein the AFP levels in the human subject are decreased by least 20%, such as at least 50%, or at least 55%, preferably at least 56%, wherein the decrease in AFP levels of at least 20% from baseline is maintained for at least two weeks, such as for at least three weeks, at least four weeks, at least five weeks, at least six weeks, or more.

Combination therapy

The polypeptides of the present technology may also be used in combination with one or more further pharmaceutically active compounds or principles, i.e., as a combined treatment regimen, which may or may not lead to a synergistic effect against GPC3+ solid tumours and/or the amelioration of one or more symptoms thereof resulting from the administration of the polypeptide or composition. Any suitable combination of the polypeptides according to the technology with one or more of compounds, is within the scope of the present technology. The clinician will be able to select such further compounds or principles, as well as a suitable combined treatment regimen, based on the factors cited above and his expert judgement.

In particular, the polypeptides of the present technology may be used in combination with other pharmaceutically active compounds or principles that are or can be used for the prevention and/or treatment of the hyperproliferative cell disorders, e.g., cancer, diseases and/or disorders cited herein, as a result of which a synergistic effect may or may not be obtained. Examples of such compounds and principles, as well as routes, methods and pharmaceutical formulations or compositions for administering them will be clear to the clinician. In a non-limiting example, the polypeptide and/or composition is administered in combination with other anti-cancer agents.

In some embodiments, the polypeptide and/or composition is administered to a subject in need thereof together with a PD-L1 inhibitor.

In some embodiments, the PD-L1 inhibitor is an anti-PD-L1 monoclonal antibody, preferably Atezolizumab.

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 or at different times (e.g., essentially simultaneously, consecutively, or according to an 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 part of a combined pharmaceutical formulation or composition, as will be clear to the skilled person.

8. Sequences

Table 6: Amino acid sequences of the multivalent polypeptide A022600424 and its different monovalent ISVD building blocks

Name	ID	Amino acid sequence
A022600424	1	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTLVTVSSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGF TFSSFAMTWVRRPPGKGLEWVATITNKGVTSYADSVKGRFTISRDNAKNT LYLQMNSLRPEDTALYICANARRTGPRAPTDIGSYRGQGTLVTVSSGGGG SGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFRSVFSSSTMEWYRQAP GKKRELVARIAPGEGTYYGALYADSVKGRFTISRDNAKNTVYLQMNSLRPE DTALYYCASGVAWGQGTLVTVSSGGGGSGGGSEVQLVESGGGVVQPGG SLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKG RFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA
TCE01	2	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTLVTVSS
A022600351*	3	EVQLVESGGGVVQPGGSLRLSCAASGFTFSSFAMTWVRRPPGKGLEWVA TITNKGVTSYADSVKGRFTISRDNAKNTLYLQMNSLRPEDTALYICANARR TGPRAPTDIGSYRGQGTLVTVSS
A022600314°	4	EVQLVESGGGVVQPGGSLRLSCAASGSIFRSVFSSSTMEWYRQAPGKKRE LVARIAPGEGTYYGALYADSVKGRFTISRDNAKNTVYLQMNSLRPEDTALY YCASGVAWGQGTLVTVSS
ALB23002	5	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWV SSISGSGSDTLYADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTIGG SLSRSSQGTLVTVSS

* Sequence optimized variant of A02260018C08 (SEQ ID NO: 48,

EVQLVESGGGLVQPGGSLRLSCVASGFTFSSFAMTWVRRPPGKGLEWVATITNGGVTSYRDSVKGRFTIS RDNAKNTLYLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTLVTVSS)

° Sequence optimized variant of A02260015A08 (SEQ ID NO: 47,
EVQLVESGGGLVQAGGSLRLSCVASGSIFRSVFSSSTMEWYRQPPGKKRELVARIAPGDGTNYGALYADS
VKGRFTISRDDAKKTVDLQMNSLKPEDTGVYFCASGVAWGQGTLVTVSS)

Table 7: Sequences for CDRs and frameworks according to AbM numbering ("ID" refers to the given SEQ ID NO)

ID	ISVD	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
2	TCE01	18	DVQLVESGGGVVQPGGSLRLSCVAS	6	GYVHKINFYG	20	WYRQAPGKEREKVA	10	HISIGDQTD	24	YADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRA	14	LSRIWPYDY	28	WGQGTLVTVSS
3	A022600351*	19	EVQLVESGGGVVQPGGSLRLSCAAS	7	GFTFSSFAMT	21	WVRRPPGKGLEWVA	11	TITNKGVTS	25	YADSVKGRFTISRDNAKNTLYLQMNSLRPEDTALYICAN	15	ARRTGPRAPTDIGSY	29	RGQGTLVTVSS
4	A022600314°	19	EVQLVESGGGVVQPGGSLRLSCAAS	8	GSIFRSVFSSSTME	22	WYRQAPGKKRELVA	12	RIAPGEGTYYGAL	26	YADSVKGRFTISRDNAKNTVYLQMNSLRPEDTALYYCAS	16	GVA	28	WGQGTLVTVSS
5	ALB23002	19	EVQLVESGGGVVQPGGSLRLSCAAS	9	GFTFRSFGMS	23	WVRQAPGKGPEWVS	13	SISGSGSDTL	27	YADSVKGRFTISRDNSKNTLYLQMNSLRPEDTALYYCTI	17	GGSLSR	30	SSQGTLVTVSS

* Sequence optimized variant of A02260018C08 (SEQ ID NO: 48)

° Sequence optimized variant of A02260015A08 (SEQ ID NO: 47)

Table 8: Sequences for CDRs and frameworks according to Kabat numbering ("ID" refers to the given SEQ ID NO)

ID	ISVD	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
2	TCE01	39	DVQLVESGGG VVQPGGSLRL SCVASGYVHK	31	INFYG	20	WYRQAPG KEREKVA	35	HISIGD QTDYA DSAKG	43	RFTISRDESKNTVYLQM NSLRPEDTAAYYCRA	14	LSRIWPYDY	28	WGQGT LVTVSS
3	A022600 351*	40	EVQLVESGGG VVQPGGSLRL SCAASGFTFS	32	SFAMT	21	WVRRPPGK GLEWVA	36	TITNKG VTSYA DSVKG	44	RFTISRDNAKNTLYLQM NSLRPEDTALYICAN	15	ARRTGPRAP TDIGSY	29	RGQGTL VTVSS
4	A022600 314°	41	EVQLVESGGG VVQPGGSLRL SCAASGSIFR	33	SVFSSST ME	22	WYRQAPG KKRELVA	37	RIAPGE GTYYG ALYADS VKG	45	RFTISRDNAKNTVYLQM NSLRPEDTALYYCAS	16	GVA	28	WGQGT LVTVSS
5	ALB23002	42	EVQLVESGGG VVQPGGSLRL SCAASGFTFR	34	SFGMS	23	WVRQAPG KGPEWVS	38	SISGSG SDTLYA DSVKG	46	RFTISRDNSKNTLYLQM NSLRPEDTALYYCTI	17	GGSLSR	30	SSQGTL VTVSS

* Sequence optimized variant of A02260018C08 (SEQ ID NO: 48)

° Sequence optimized variant of A02260015A08 (SEQ ID NO: 47)

Table 9: Amino acid sequences related to GPC3 and TCR

ID	description	Amino acid sequence
134	Human GPC3 (P51654)	MAGTVRTACLVVAMLLSLDFPGQAQPPPPPPDATCHQVRSFFQRLQPGL KWVPETPVPGSDLQVCLPKGPTCCSRKMEEKYQLTARLNMEQLLQSASM ELKFLIIQNAAVFQEAFEIVVRHAKNYTNAMFKNNYPSLTPQAFEFVGEFFT DVSLYILGSDINVDDMVNELFDSLFPVIYTQLMNPGLPDSALDINECLRGA RRDLKVFGNFPKLIMTQVSKSLQVTRIFLQALNLGIEVINTTDHLKFSKDCG RMLTRMWYCSYCQGLMMVKPCGGYCNVVMQGCMAGVVEIDKYWRE YILSLEELVNGMYRIYDMENVLLGLFSTIHDSIQYVQKNAGKLTTTIGKLCA HSQQRQYRSAYYPEDLFIDKKVLKVAHVEHEETLSSRRRELIQKLKSFISFYS ALPGYICSHSPVAENDTLCWNGQELVERYSQKAARNGMKNQFNLHELK MKGPEPVVSQIIDKLKHINQLLRTMSMPKGRVLDKNLDEEGFESGDCGDD EDECIGGSGDGMIKVKNQLRFLAELAYDLDVDDAPGNSQQATPKDNEIST FHNLGNVHSPLKLLTSMAISVVCFFFLVH
135	Human TCR alpha constant domain (derived from P01848)	PNIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKDSDVYITDKTVL DMRSMDFKSNSAVAWSNKSDFACANAFNNSIIPEDTFFPSPESSC
136	Human TCR beta constant domain (derived from P01850)	EDLNKVFPPEVAVFEPSEAEISHTQKATLVCLATGFFPDHVELSWWVNGK EVHSGVSTDPQPLKEQPALNDSRYALSSRLRVSATFWQNPRNHFRCQVQ FYGLSENDEWTQDRAKPVTQIVSAEAWGRADC

Examples

Example 1: Single dose pharmacokinetics study of A022600424 in cynomolgus monkeys

Objectives

The objective of this study was to determine the potential toxicity of A022600424 (SEQ ID NO: 1) when administered as a single dose to Cynomolgus monkey by intravenous (IV) infusion followed by a 21-day observation period. In addition, the systemic exposure of A022600424 was determined.

This study was performed at Sanofi-Aventis recherche & développement, Montpellier, France.

Materials and methods

This study and the laboratory procedures comply with the Directive 2010/63/EU of the European parliament and the related French transposition texts.

The animals enrolled, *Macaca fascicularis*, were housed in stainless steel pens and fed a controlled pelleted primate diet (re. Primaten, SSNIFF, Germany), 200 g daily per animal, with unlimited access to water.

A022600424 is described in WO 2022/129560 (SEQ ID NO: 1). A022600424 was provided as a liquid stock solution at 4.97 mg/mL in a histidine sucrose buffer.

Protocol

Dosing

A total of 15 female animals of the species *Macaca fascicularis* were enrolled in this study and divided over 7 treatment groups. The dose levels of the different groups are shown below in Table 10.

Table 10: dose levels of the 5 treatment groups

Group	Number of animals	Dose level (µg/kg)	Concentration (µg/mL)
1	1 female	3	1.2
2	3 female	10	4
3	1 female	30	12
4	3 female	100	40
5	1 female	300	120
6	1 female	1000	400
7	3 female	1000	400
8	1 female	3000	1200
9	1 female	10000	4000

A starting dose of 3 µg/kg was chosen based on the analysis of available preclinical data. Additional doses were tested based on the results of the previous doses.
A022600424 was administered as a single intravenous (IV) infusion over a period of 30 minutes. Except for Group 7, where the administration was a single subcutaneous (SC) infusion over a period of 30 minutes.

Dosing volume was kept constant at 2.5 mL/kg and the site of administration was in the right or left saphenous veins unless administration was subcutaneous.

The lowest dose level (group 1) was administered A022600424 first. When no adverse clinical signs or mortality were observed, the second dose group (group 2) was administered the following dosing day, which continued for each following dose group as long as no adverse clinical signs or mortality were observed.

Four additional dose levels (group 6-9) were selected based on the safety data obtained from dose groups 1-5.

Mortality and adverse clinical signs

Mortality and adverse clinical signs were evaluated before treatment and 1, 2 and 6 hours after the start of the infusion. For the 21-day observation period, mortality and clinical signs were evaluated at least twice daily on workdays and once daily on weekend and public holidays.

Pharmacokinetic and toxicokinetic analysis

Specimens, i.e., blood samples, from the living animals were collected for toxicokinetic analysis at the end of the infusion and 2, 6, 24, 48, 72, 96, 168, 336 and 504 hours after the start of the infusion.

Specimens were analysed using an MSD (Meso Scale Discovery) based ligand binding assay.

The following parameters were assessed:

- Observed maximum concentration (C_{max})
- Time of maximum concentration (T_{max})
- Time corresponding to the last observed concentration above the limit of quantification (T_{last})
- Area under the serum concentration-time curve calculated by the linear trapezoidal method from the 0 to last timepoint post-dose with a concentration measured (AUC_{last})
- Area under the concentration time curve calculated by the linear-log trapezoidal rule extrapolated to infinity (AUC_{inf})
- Percentage of extrapolation to infinity of the AUC (AUC_E_{xt})
- Apparent terminal elimination of half-life ($t_{1/2}$)
- Coefficient of Variation (CV)
- Volume of distribution at steady state (V_{ss})
- Volume of distribution at elimination (V_z)
- Clearance (CL)

Results

All animals remained alive until after the end of the observation period. Results of the measurements performed are visualized below in Table 11 and in Figures 1A and 1B.

Table 11: Mean serum pharmacokinetic parameters

Group	C_{max} (ng/mL)	T_{max} (hr)	AUC_{last} (hr*ng/mL)	T_{last} (hr)	AUC_{inf} (hr*ng/mL)	CL (mL/hr/kg)	$t_{1/2}$ (hr)	V_{ss} (mL/kg)	V_z (mL/kg)
1	71.6	0.5	1590	168	1590	1.88	22.3	46.6	60.5
2	267	0.5	6250	168	6290	1.59	25.7	46.1	59.2
3	817	0.5	19400	168	19500	1.54	22.1	41.9	49.1
4	2620	0.5	74100	448	74100	1.35	48.5	46.0	95.4
5	7320	0.5	228000	504	228000	1.31	35.6	52.9	67.6
6	18100	0.5	644000	504	644000	1.55	36	67.8	80.7
7	5800	24	612000	336	613000	N/A	29.5	N/A	N/A
8	61500	0.5	2850000	336	2860000	1.05	36.4	60.8	55.2
9	175000	0.5	13600000	504	13600000	0.734	55.2	74.3	58.4

Following a single 0.5-hour intravenous infusion from 3 to 10000 µg/kg, the C_{max} of A022600424 was typically observed at the end of infusion and increased in a mostly dose proportional manner from 3 to 1000 µg/kg and greater than dose proportional increase from 1000 to 10000 µg/kg.

The terminal half-lives ranged from 22.1 to 55.2 hours after a single intravenous infusion and 29.5 hours after a single subcutaneous administration.

The total clearance ranged from 0.734 to 1.88 mL/h/kg after single intravenous infusion.

The maximum drug concentrations were observed at 24 hours after single subcutaneous (SC) administration. The mean SC bioavailability was calculated to be 95%.

From Table 11 and Figure 1 it can be concluded that A022600424 showed higher clearance and a shorter half-life than a typical humanized antibody administered to a cynomolgus monkey, whereas it had a high bioavailability after a single SC administration.

Example 2: Repeat-dose toxicokinetic study of A022600424 in cynomolgus monkeys

Objectives

The objective of this study phase was to determine the toxicokinetic parameters of A022600424 after twice a week intravenous administration to cynomolgus monkeys for a total of nine doses.

This study was performed at Sanofi-Aventis recherche & développement, Montpellier, France.

Materials and methods

This study and the laboratory procedures comply with the Directive 2010/63/EU of the European parliament and the related French transposition texts.

The animals enrolled, *Macaca fascicularis,* were housed in stainless steel pens and fed a controlled pelleted primate diet (re. Primaten, SSNIFF, Germany), 200 g daily per animal, with unlimited access to water.

A022600424 was provided as a liquid stock solution at 4.97 mg/mL in a histidine sucrose buffer.

Protocol

Dosing

The selection of the dose level and dosing frequency was based on the data obtained from the study described in Example 1 here above. The dose levels for this study have been selected aiming to identify toxicities and a no observed adverse effect level (NOAEL).

A total of 16 cynomolgus monkeys (8 male, 8 female) was enrolled in the study. The animals were divided over 4 groups with 2 of each sex per group.

The dosing regimen per group can be found below in Table 12.

Table 12: dose levels and number of animals per group

Group	Number and sex of animals	Dosage (mg/kg)	Concentration
1[1]	2 male, 2 female	0	0
2	2 male, 2 female	0.3	0.12

3	2 male, 2 female	1	0.4
4	2 male, 2 female	1-3-10^2	0.4-1.2-4
Notes 1. Control group 2. The seven following administrations			

Group 1 was selected as control group and was only administered a histidine sucrose buffer. The 4th group was an intra-animal escalating dose group, where the final dosage of 10 mg/kg was administered a total of 7 times.

A022600424 was administered as a single intravenous (IV) infusion over a period of 30 minutes.

Dosing volume was kept constant at 2.5 mL/kg and the site of administration was in the right or left saphenous veins.

The treatment duration was 29 days with an IV administration of A022600424 twice a week.

Mortality and adverse clinical signs

Mortality and adverse clinical signs were evaluated before administration, after administration to the last animal and 6 hours after start of the treatment of the last animal on days of administration. On other days, evaluation took place twice daily on workdays and once daily on weekends and public holidays.

Pharmacokinetic and toxicokinetic analysis

Specimens, i.e., blood samples, from the living animals were collected for toxicokinetic analysis on Days 1 and 29 before the infusion, at the end of the infusion and 2, 4, 6 and 24 hours after the infusion.

Specimens were analysed using an MSD (Meso Scale Discovery) based ligand binding assay.

The Pharsight WinNonLin (version 8.1) was used for toxicokinetic evaluation and for statistical evaluation of the determined concentrations.

Mean (±SD) serum concentrations of A022600424 were calculated at each sampling time for each treatment group. Sample concentrations less than the lower limit of quantitation (LLOQ) (154.0 pg/mL in serum) were taken as equal to zero for calculations.

The following toxicokinetic parameters were calculated from individual concentrations at each time point using a non-compartmental approach:

- Observed maximum concentration (C_{max})
- Time of maximum concentration (T_{max})
- AUC_{0-24}: area under the serum concentration time curve calculated by the linear trapezoidal method from time 0 to 24h post-dose
- R_{ac}: accumulation ratio (AUC_{0-24} on Day 29 divided by AUC_{0-24} on Day 1).

Results

All animals remained alive until after the end of the study.

Table 13 shows the mean serum concentrations of A022600424 on first day and last day of administration (Day 1 and 29 respectively). The mean serum concentrations per dosage group are also illustrated in Figures 2-4.

Table 14 shows the mean serum toxicokinetic parameters of A022600424 on first day and last day of administration (Day 1 and 29 respectively).

Table 15 shows the Day effect (Day 29/Day 1) for the C_{max} and AUC_{last} observed for male and female monkeys.

Table 13: Mean serum concentrations of A022600424 on Days 1 and 29 per dosage group

	Time after last dose (hr)					
	0	**0.5**	**2**	**4**	**6**	**24**
Group - day	**Concentration (ng/mL)**					
2 – Day 1	0	6340	4950	4910	4370	2520
2 – Day 29	0.0701	878	322	146	93	0.945
3 – Day 1	0	22000	20100	18200	16200	10500

3 – Day 29	3160	19100	15200	13600	11700	9000
4 – Day 1	0	20900	19600	18100	16600	11600
4 – Day 29	79200	249000	238000	217000	204000	186000

Table 14: Mean serum toxicokinetic parameters of A022600424 on Days 1 and 29 per dosage group

Group - day	C_{max} (ng/mL)	T_{max} (hr)	AUC_{last} (hr*ng/mL)	T_{last} (hr)	AUC_{0-24} (hr*ng/mL)
2 – Day 1	6340	0.5	91200	24	91200
2 – Day 29	878	0.5	2650	8.1	2670
3 – Day 1	22000	0.5	350000	24	350000
3 – Day 29	19100	0.5	272000	24	272000
4 – Day 1	20900	0.9	362000	24	362000
4 – Day 29	253000	0.9	4840000	24	4840000

Table 15: Day effect for C_{max} and AUC_{0-24} in male and female monkeys

Dose (mg/kg)	R_{ax} C_{Max}		R_{ac} AUC_{0-24}	
	Male	**Female**	**Male**	**Female**
0.3	0.001	0.303	0.000	0.058
1	1.15	0.620	1.09	0.486

As can be seen from Tables 13-15 and Figures 2-4, after twice per week repeated IV infusion for four weeks at 0.3 mg/kg dose, exposure decreased over time, while at 1 mg/kg dose exposure remained comparable to Day 1. In the intra-animal dose escalation group at both 3 and 10 mg/kg the dose exposure increased in a proportional manner.

Example 3: A phase I/II open label, first-in-human, dose escalation and expansion study of A022600424 in GPC3-positive tumours

Protocol

This is a Phase 1/Phase 2 open-label, multicentre, single agent or in combination with other anti-cancer agent study to evaluate the safety, pharmacokinetics (PK), pharmacodynamics (PD), and signs of clinical activity of A022600424 administered intravenously (IV) in participants with GPC3 positive (GPC3+) solid tumours (with GPC3 expression on tumour tissue). This first-in-human study consists of several parts/cohorts (see Figure 7):

- Part 1A: An intravenous (IV) monotherapy dose escalation part for safety and dose findings, conducted in patients with GPC3+ solid tumours, and who have exhausted all standard of care therapy.
- Part 2A: An IV monotherapy expansion part for the assessment of safety and efficacy at the recommended phase 2 dose (RP2D) in GPC3+ non-small cell lung cancer (NSCLC) cohorts.
- Part 1B: A dose escalation part for safety and dose findings of A022600424 in combination with atezolizumab in patients with GPC3+ solid tumours.

Dose determination

A022600424 is designed to simultaneously bind TCRαβ and GPC3 to co-engage T-cells with GPC3-expressing tumour cells, resulting in T cell activation, T-cell dependent cellular cytotoxicity (TDCC), and specific killing of tumour cells. Due to its immune agonistic properties, an approach based on the minimal anticipated biological effect level (MABEL) was proposed to define the starting dose (i.e., the first lead-in dose). The MABEL was derived from the concentration corresponding to 80% pharmacological activity among a large panel of human cell killing assays and cytokine release cell assays and *in vivo* monkey biomarker response.

Based on this approach, 0.035 mg (35 µg) was selected as the maximum recommended starting dose for the clinical study, and it was rounded down to 0.03 mg (30 µg).

The human active dose was calculated using a mechanistic physiologically based pharmacokinetic model.

In this model-based approach, a mechanistic model was developed based on *in vitro* TDCC for multiple cell-lines. This model was then incorporated into a physiologically-based pharmacokinetic model where a tumour compartment had been incorporated. As the lower end of the human active dose range, synapse formation and cell killing was assumed to be as efficient and effective in the tumour as in the *in vitro* experiments. For the upper end of the human active dose range, TDCC in the tumour was assumed to require the same free A022600424 concentration as required *in vitro*. This is a conservative estimate as the target concentration in the tumour is much greater compared to *in vitro*, which means that the target-bound A022600424 concentration (which drives the TDCC) is also much greater in the tumour relative to the free A022600424 concentration compared to the *in vitro* situation.

In addition to that, a fast internalization of A022600424 is anticipated, which further reduces the predicted free A022600424 concentrations in the tumour. The uncertainty brings the predicted human active dose range to 2 – 200 mg. Thus, the planned target doses in the dose escalation part were selected to cover this therapeutic dose range.

In addition, in the primate study described in Example 1, a single dose highest non-severely toxic dose (HNSTD) was established at 8 mg/kg. Therefore, an initial lead-in dose lower than one-sixth of the human equivalent dose (HED) of the HNSTD, 0.5 mg/kg will not be exceeded. Furthermore, in the primate study described in Example 2, a dose of 10 mg/kg was well tolerated in cynomolgus monkeys. The HED (corrected for body surface area) of this dose level would correspond to a dose of 3.3 mg/kg. This information supports the current possible higher end of the active dose of 200 mg for a 60 kg patient. As the main safety concern is cytokine release syndrome for this class of therapeutics and cytokine release may be mitigated by lead-in dosing and the use of premedication, the proposed maximum dose to be tested is 450 mg.

During the clinical trial the maximum dose may be adjusted since the predicted maximum tolerable dose of A022600424 is based on the clinical data from the initial dose levels tested in the patients with GPC3 immunohistochemistry positive tumours.

A022600424 theoretically only activates T cells and subsequently induces cytokine release syndrome (CRS) when both GPC3 and T cell receptor are bound to the GPC3-binding and TCR-binding ISVDs making up A022600424.

As the preclinical GPC3 testing models don't always exactly reproduce the environment of patients with GPC3 positive tumours, the predicted dose is updated based on the clinical safety data comprised of incidence and intensity of the low-grade CRS observed in the initial patients.

Monotherapy dose escalation

Monotherapy dose escalation (Part 1A) evaluates the safety, including potential dose-limiting toxicities (DLTs) and the maximum tolerated doses (MTDs) of A022600424, and determines the recommended phase 2 dose (RP2D) for IV administration.

The recommended starting dose, 0.03 mg IV (approximately 0.5 µg/kg, assuming average body weight is 65 kg), was selected based on a minimal anticipated biological effect level (MABEL) approach, while an average therapeutic dose to obtain tumour killing is currently predicted to be around 19.5 mg (19 500 µg, approximately 300 µg/kg).

The duration of each monotherapy treatment cycle is 21 days, and the monotherapy DLT observation period consists of the first 21 and 28 days of treatment for the 2-week and the 3-week lead-in dose schedule sub cohorts, respectively. To minimize the risk of cytokine release syndrome (CRS), lead-in doses are used on Cycle 1 Day 1, Day 4, Day 8, and on Day 11. On Day 15 either lead-in or target dose of A022600424 will be administered and starting from Cycle 2 Day 1, the target dose will be administered weekly. This is to allow a gradually increasing dose within a dose level (DL) to minimize the risk of CRS. In the dose escalation part, different lead-in and target doses are tested for each dose level (DL1 to DL7A). Cycle 1 is completed after the target dose (the maximum weekly dose achieved in Cycle 1) on Cycle 1 Day 15 (2 weeks lead-in dose) or Cycle 2 Day 1 (3 weeks lead-in dose) is reached. After Cycle 1, each participant receives a weekly target dose until the end of treatment (EOT), Cycle 2 and beyond, unless the dose needs to be modified for safety reasons. Cycles will be continued until there is a reason to stop treatment, e.g. disease progression or intolerance to treatment. The dose levels are defined in Table 16.

During Cycle 1 of each dose level, each participant receives multiple increasing lead-in doses (until the target dose is reached) and 1 target dose. Each participant receives target doses from Cycle 2 and beyond until permanent discontinuation criteria are met.

Table 16: Dosing schedule

Cycle	**Cycle 1**					**Cycle 2+**		
	Lead-in dose (mg)				**Target dose (mg)**			
Week	**W1**		**W2**		**W3**	**W1**	**W2**	**W3**
Day	**D1**	**D4**	**D8**	**D11**	**D15**	**D1**	**D8**	**D15**
DL1	0.03	0.1	0.3	1	3	3	3	3
DL1A	0.03	0.06	0.1	0.3	1	1	1	1
DL2A	0.06	0.12	0.25	0.75	2.5	2.5	2.5	2.5
DL3A	0.12	0.25	0.5	1.5	4.5	4.5	4.5	4.5
	Lead-in dose (mg)					**Target dose (mg)**		
	W1		**W2**		**W3**	**W1**	**W2**	**W3**
	D1	**D4**	**D8**	**D11**	**D15**	**D1**	**D8**	**D15**
DL4A	0.25	0.5	1	2	6	18	18	18
DL5A	0.5	1	2	4	12	36	36	36
DL6A	1.00	2	4	8	24	70	70	70
DL7A	0.50	1	3	9	24	70	70	70

Up to 4 participants are enrolled per DL. Once at least 3 participants in a cohort are evaluable for dose-limiting toxicities in Cycle 1, the next dose level, which is escalation, maintaining or de-escalation of the dose, is recommended using the Bayesian logistic regression model (BLRM).

All treatment emergent adverse events (TEAEs) are included in the safety data. Adverse events (AEs) are graded according to NCI CTCAE version 5.0.

The DLT observation period for monotherapy (Part 1A) consists of the first 21 and 28 days of therapy for the 2-week and the 3-week lead-in dose schedule sub cohorts, respectively.

Combination therapy dose escalation

The combination therapy dose escalation (Part 1B) follows the rationale and procedures as described for monotherapy dose escalation (Part 1A). Combination therapy of A022600424 and atezolizumab dose escalation (Part 1B) begins after biological evidence of T-cell activation is observed. The starting dose of Part 1B is at a dose level below the cleared dose level from Part 1A, and one in which it is considered to be safe.

Dose escalation proceeds via the dose levels for A022600424 listed in Table 16. Part 1B will begin after biological evidence of T cell activation is observed in Part 1A. The starting dose of Part 1B will be at a dose level below the cleared dose level from Part 1A that demonstrate biological activation of T cell.

The DLT observation period for combination therapy (Part 1B) consists of the first 21 days of combination therapy (i.e., from Cycle 2 Day 1 [the first dose of atezolizumab] to 21 days thereafter).

Monotherapy dose expansion

The recommended phase 2 dose (R2PD) is used for the dose expansion start of the study (2A). The RP2D is determined based on the totality of the information available at all dose levels obtained in dose escalation (based on totality of tolerability, PD, PK, and preliminary anti-tumour activity) and should not exceed the maximum tolerable dose (MTD) or maximum administered dose (MAD).

Study population

Inclusion criteria

1. Participants are at least 18 years of age, or the legal age of consent in the jurisdiction in which the study takes place.
2. Cancer diagnosis for participants for parts 1A and 1B:
    - metastatic and/or unresectable HCC diagnosed by histology and/or cytology, or diagnosed clinically by the American Association for the Study of Liver Diseases

(AASLD) criteria for participants with liver cirrhosis (participants without liver cirrhosis must be diagnosed histologically); or

- other histologically/cytologically proven advanced and/or metastatic non-HCC solid tumours; and
- participants must have experienced disease progression on/after standard of care, or no acceptable standard curative or palliative treatments exist (or are no longer effective).

3. Cancer diagnosis for participants for part 2A:
   - metastatic NSCLC with no actionable driver gene mutants (such as epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK)), diagnosed by histology and/or cytology not amenable to available standard of care and must have progressed on/after therapy that included an anti-PD(L)-1 agent with or without platinum-based chemotherapy; and
   - progressive disease should be observed during the course of anti-PD(L)-1 therapy or within 12 weeks from the last dose of anti-PD(L)-1 therapy.
4. For all participants: a positive GPC3 expression (defined by GPC3 IHC membranous H score ≥ 1) on tumour tissue as determined locally or centrally (GPC3 expression is determined centrally when a local reading is not available). For the participants in Part 2A, GPC3 expression should be confirmed by central reading.
5. Provision of tumour tissue for which GPC3 expression has been determined histologically with a minimum of 5 slides with 4-5-micron thickness (ideally 9 slides if possible). Provision of tumour tissue is optional for participants with GPC3 expression level from tumour tissue determined locally, except for part 2A.
6. For participants in Part 2A: At least 1 measurable lesion per RECIST 1.1 criteria. Target lesions may be located in a previously irradiated field or previously treated with local therapy (e.g., radiofrequency ablation, percutaneous ethanol or acetic acid injection, cryoablation, high-intensity focused ultrasound, transarterial chemoembolization, transarterial embolization) if there is documented radiographic disease progression in that site.

7. Body weight within 45 - 120 kg (inclusive).

Exclusion criteria

1. Eastern Cooperative Oncology Group (ECOG) performance status of ≥2.
2. Predicted life expectancy ≤3 months.
3. For participants with HCC: Child Pugh Class B or C liver score within 14 days of initiation of A022600424. Participants with Child Pugh Class B-7 score are allowed for Part 1A.
4. Known active brain metastases or leptomeningeal metastases:
    - For Parts 1A and 1B: participants with new or progressive active brain metastases or leptomeningeal metastases are eligible if the Investigator determines that immediate CNS specific treatment is not required and is unlikely to be required during the DLT observation period.
    - For Part 2A: participants with previously treated brain metastases may participate provided they are clinically stable for at least 2 weeks without evidence of progression (clinically stable, which is defined as neurologic symptoms have returned to baseline or resolved, and the participant is not using steroids for at least 2 weeks prior to study treatment). Participants with asymptomatic brain metastases (i.e., no neurological symptoms, with no requirements for corticosteroids) may participate but require regular imaging of the brain as a site of disease.
5. History of allogenic or solid organ transplant (participants with prior corneal transplant may be allowed to enrol).
6. Treatment-related immune-mediated (or immune-related) AEs from immune-modulatory agents (including but not limited to anti-PD-1/PD-L1 agents and anti-cytotoxic T lymphocyte associated protein 4 monoclonal antibodies) that caused permanent discontinuation of the agent, or that were Grade 4 in severity.
7. Significant cardiovascular disease (such as New York Heart Association Class III or IV, myocardial infarction, or cerebrovascular accident) within 3 months prior to initiation of IMP, uncontrolled arrhythmia requiring medication, or unstable angina.

8. Ongoing AEs caused by any prior anti-cancer therapy >Grade 2 (NCI CTCAE version 5.0)
9. Known uncontrolled human immunodeficiency virus (HIV). Controlled HIV is defined as undetectable viral load and CD4+ T cells >350 cells/mm^3.
10. Known uncontrolled hepatitis B infection or known untreated current hepatitis C infection.
11. Known second malignancy either progressing or requiring active treatment within the last year. Participants with basal cell carcinoma of the skin, squamous cell carcinoma of the skin, or carcinoma in situ (e.g., breast carcinoma, cervical cancer in situ) that have undergone potentially curative therapy are not excluded.
12. Known hypersensitivity to or contraindication for the use of any study intervention including premedication to be administered in this study.
13. Participants with baseline SpO2 ≤92% (without oxygen therapy)
14. Participants with hypotension defined as BP <90/60 mmHg.
15. For combination therapy: Ongoing or recent (within 5 years) evidence of significant autoimmune disease that required treatment with systemic immunosuppressive treatments, which may suggest risk for immune-related AEs.
16. Last administration of prior antitumor therapy (chemotherapy, targeted agents, and immunotherapy) within 21 days or less than 5 times the half-life, whichever is shorter; major surgery or local therapy within 21 days prior to first dose of A022600424.
17. For Part 2A, uncontrolled pleural effusion, pericardial effusion, or ascites requiring recurrent drainage procedures (twice monthly or more frequently). Participants with indwelling catheters are allowed.
18. Participants who have received prior radiotherapy within 2 weeks of start of study intervention. Participants must have recovered from all radiation-related toxicities, and not require corticosteroids. A 1-week washout is permitted for palliative radiation (≤2 weeks of radiotherapy) to non-CNS disease.
19. Receipt of a live-virus vaccination within 28 days of planned treatment start
20. For Part 2A, participants that received prior GPC3 targeted anticancer treatment.
21. Is unable or unwilling to take premedication.

22. Current enrolment or past participation in a study of an investigational treatment or has used an investigational device within 21 days prior to the first dose of study treatment.
23. Absolute neutrophil count < 1500/µL (1.5×10^9/L).
24. Platelets $<100 \times 10^3$ µ/L (after at least 3 days without platelet transfusion) for participants with non-HCC solid tumour (including NSCLC), or $<75 \times 10^3$ µ/L (after at least 3 days without platelet transfusion) for participants with HCC.
25. Haemoglobin <9 g/dL.
26. Total bilirubin >1.5 × upper limit of normal (ULN) unless direct bilirubin ≤ULN (participants with known Gilbert disease who have serum bilirubin level ≤3 × ULN may be enrolled), or >3 × ULN for participants with HCC.
27. Aspartate aminotransferase and/or alanine aminotransferase >3 × ULN (or >5 × ULN for participants with HCC or liver metastases).
28. Estimated glomerular filtration rate (eGFR) <50 mL/min/1.73 m^2 (Modification of Diet in Renal Disease [MDRD] Formula).
29. For participants with non-HCC solid tumour (including NSCLC), International Normalized Ratio (INR) or prothrombin time (or activated partial thromboplastin time [aPTT]) >1.5 × ULN unless the participant is receiving anticoagulant therapy as long as the prothrombin time or aPTT is within therapeutic range of intended use of anticoagulants. For participants with HCC, INR >2 × ULN.
30. For participants with HCC, albumin <2.8 g/dL.
31. A baseline QTcF interval of >480 ms. In the case of a right bundle branch block (RBBB), participants may be eligible for study participation after a cardiology consult and discussion with the Medical Monitor.
32. Individuals accommodated in an institution because of regulatory or legal order; prisoners or participants who are legally institutionalized.
33. Any country-related specific regulation that would prevent the participant from entering the study.

34. Participants not suitable for participation, whatever the reason, as judged by the Investigator, including medical or clinical conditions, or participants potentially at risk of noncompliance to study procedures.
35. Participants are employees of the clinical study site or other individuals directly involved in the conduct of the study, or immediate family members of such individuals (in conjunction with section 1.61 of the ICH-GCP Ordinance E6).
36. Current pneumonitis or interstitial lung disease, or history of interstitial lung disease or pneumonitis that required oral or IV glucocorticoids to assist with management.

Materials and methods

Investigational medicinal products

For IV administration, A022600424, as a liquid formulation in a histidine sucrose buffer and water, is diluted once in 0.9% sodium chloride solution bags pre-coated with polysorbate 80 to reach the desired concentrations as required according to the clinical protocol dosing levels.

Atezolizumab (Texentriq®) is a concentrate for solution for infusion supplied in a single-dose vial 1200 mg/20 mL (60 mg/mL), clear, colourless to slightly yellowish liquid. The formulation contains excipients: histidine, glacial acetic acid, sucrose, Polysorbate 20 and water for injection.

Atezolizumab is to be administered within approximately 30 minutes after the end of the A022600424 infusion on combination therapy co-administration days (Day 1 of each cycle beginning with Cycle 2). The atezolizumab IV infusion duration is 1 hour at the first administration and 30 minutes on the subsequent cycles.

Efficacy assessments

Imaging

The initial CT/MRI tumour imaging is performed within 21 days prior to Cycle 1 Day 1. Scans performed as part of routine clinical management are acceptable for use as the screening scan if they are of diagnostic quality and performed within 21 days prior to the date of enrolment. On-study imaging is performed every 9 weeks (63 ±7 days) after the date of first treatment

administration and if clinically indicated. Imaging studies should follow calendar days and should not be adjusted for delays in cycle starts or extension. After Week 45, tumour imaging should be performed every 12 weeks (84 ±7 days). Per the primary tumour assessment criteria, after the first documentation of response or the first documentation of progression (if the participant is clinically stable), confirmatory imaging may be performed no fewer than 28 days later. Alternately, the scan performed at the next scheduled time point (e.g., every 63 ±7 days) may be used as confirmation.

For all participants, including those with no previous history of brain metastases, screening brain imaging needs to be obtained. MRI is the preferred imaging modality however CT is acceptable if an MRI is clinically contraindicated. Participants with asymptomatic brain metastases (i.e., no neurological symptoms, no requirements for corticosteroids) are eligible but require regular imaging of the brain as a site of disease. Participants with previously treated brain metastases may participate provided they are clinically stable, which is defined as neurologic symptoms have returned to baseline or resolved, and the participant is not using steroids for at least 2 weeks prior to study treatment.

During the follow-up period, tumour imaging (CT/MRI scans) should be performed every 12 weeks (84 ±7 days) until disease progression, the start of new anticancer therapy, death, or withdrawal of participant's consent, whichever comes first. Efficacy follow-up (CT/MRI scans and AFP assessments) stop at the primary analysis cut-off date.

Patient-reported outcomes

All participants enrolled in the trial are expected to fill out the Patient's Qualitative Assessment of Treatment version 2 (PQATv2), which is a 6-item patient-reported outcome (PRO) measure intended for the collection of participant perceived benefits and disadvantages of treatment received within a clinical trial. Participants are expected to fill out the PQATv2 during the dose expansion phase according to a pre-defined schedule. The PQATv2 is filled out through an electronic clinical outcome assessment (eCOA) using a site-based tablet device.

The PQATv2 includes: two 11-point numeric rating scales assessing participant-perceived treatment benefits and disadvantages (0 = not at all beneficial/disadvantageous to 10 =

extremely beneficial/disadvantageous); an item assessing participants' willingness to continue treatment after the trial (yes/no dichotomous response scale); a 7-point Likert-type item assessing participants' overall benefit/risk ratio perceptions (-3 = 'disadvantages of the drug I received significantly outweighs the benefits' to 3 = 'the benefits of the drug I received significantly outweigh the disadvantages'); and 3 open-ended, free-text items assessing participant-perceived treatment benefits, treatment disadvantages, and reasons why the participant would be willing or unwilling to continue the treatment after the trial.

Alpha-Fetoprotein (AFP)

An AFP test is conducted at the same time points as the imaging of the tumour. This test is conducted locally at the clinical site and follows standard AFP test procedures.

A commercially available AFP kit, such as the AFP Human ELISA Kit (ThermoFisher Scientific), is used at the clinical site to obtain the AFP data. The protocol provided with the kit is followed.

In addition, participants with HCC who discontinued treatment for a reason other than disease progression have AFP assessments (as an efficacy marker for HCC) at the same timepoints as tumour imaging during the follow-up period.

AFP levels before administration of A022600424 (baseline AFP level) were compared to the AFP levels measured during the different treatment cycles.

A decrease in AFP level of at least 20% from baseline is considered as positive and an indication of possible tumour regression. A decrease in AFP level that is less than 20%, but is still a decrease, is considered not positive or negative. It could be an indication of stable disease. An increase in AFP level of at least 20% is considered negative and could be an indication of disease progression.

Pharmacokinetic (PK) assay

An analytical MSD-based method for the quantification of A022600424 concentrations in human plasma is conducted. Binding of A022600424 to both targets, GPC3 and TCR, simultaneously is assessed. Samples from patients enrolled in the clinical trial are analysed in this assay.

Firstly, 150 µl of Superblock T20™ (Thermo Scientific) is added per well of Streptavidin coated MSD GOLD 96-well SMALLSPOT® plates (Meso Scale Discovery) as a blocking agent. The plates are incubated on an incubating shaker at 600 rpm for at least 30 minutes at room temperature (RT).

Next, a wash cycle of 3 washes with 300 µL per well wash buffer (PBS/0.05% Tween-20) using an automated plate washer is performed.

Next, 2.0 µg/mL of biotinylated Human soluble T cell receptor (sTCR) (2XN9)-Zipper-TwinStrep (Sanofi) in PBS/0.1% Casein as capture reagent is added at 50 µL per well and incubated on an incubating shaker at 600 rpm for 1 hour at RT, followed by another wash cycle as described here above.

After this, calibration standards (STDs), quality control samples (QCs) in PBS/0.1% Casein and the patient samples containing A022600424 are diluted 1:20 in PBS/0.1% Casein and added to separate wells at 50 µL per well. The plates are incubated on an incubating shaker at 600 rpm for 2 hours at RT. This is followed by another wash cycle as described here above.

The STDs are prepared by spiking A022600424 in neat blank Pooled Human Plasma (PHuP; BioIVT) and have the concentrations as listed below in Table 17 to prepare a 12-point calibration curve and regression model (5 PL – 1/Y2 weighted).

The QCs are prepared by spiking A022600424 in neat PHuP and have the concentrations as listed below in Table 18.

Complexation with the target is performed by adding 1.0 µg/mL human soluble Glypican 3 (sGPC3; R&D Systems) in PBS/0.1% Casein at 50 µL per well. This is then incubated on an incubating shaker at 600 rpm for 1 hour at RT, followed by another wash cycle as described above.

As primary detection, 2.0 µg/mL mouse anti-human sGPC3 monoclonal antibody (R&D Systems) in PBS/0.1% Casein at 50 µL per well is added and incubated on an incubating shaker at 600 rpm for 1 hour at RT, followed by another wash cycle as described above.

As secondary detection, 2.0 µg/mL of sulfo-labelled goat anti-mouse IgG polyclonal antibody (R&D Systems) in PBS/0.1% Casein at 50 µL per well is added and incubated on an

incubating shaker at 600 rpm for 30 minutes at RT in the dark. A final wash cycle as described here above is then performed.

Results are read on a Sector Imager Quickplex SQ 120 (Meso Scale Discovery) by adding MSD Gold Read buffer A (Meso Scale Discovery) at 150 µL per well and placing the plates in the reader within 10 minutes of adding the buffer.

Table 17: concentration of calibration standards (STDs)

STD1	1000.0 ng/mL (anchor)	STD7	3.24 ng/mL
STD2	384.62 ng/mL (anchor)	STD8	1.25 ng/mL
STD3	147.93 ng/mL (ULOQ)	STD9	0.48 ng/mL (LLOQ)
STD4	56.90 ng/mL	STD10	0.18 ng/mL (anchor)
STD5	21.88 ng/mL	STD11	0.07 ng/mL (anchor)
STD6	8.42 ng/mL	STD12	0.0 ng/mL (blank)

Table 18: Concentrations of quality control samples

ULOQ (upper limit of quantification)	147.93 ng/mL
HiQC (high quality control)	120.00 ng/mL
MeQC (medium quality control)	12.00 ng/mL
LoQC (low quality control)	1.20 ng/mL
LLOQ (lower limit of quantification)	0.48 ng/mL
DilQC (dilution quality control)	100000.0 ng/mL

Cytokine analysis

Blood samples are collected throughout the trial 3 hours and 24 hours after administration of A022600424 and processed to plasma to monitor concentrations of pro-inflammatory cytokines and soluble factors.

Cytokine analysis is done in a central laboratory using a validated multiplex ELISA-based assay. Results are correlated with participants' clinical parameters and are used as pharmacodynamic biomarkers of T-cell engagement as well as for evaluation of possible cytokine release syndrome (CRS). Cytokines that are monitored include, but are not limited to, IL-6, IL-8, TNF-α, and IFN-γ).

Adverse events (AEs)

Adverse events are reported by the participant. The Investigator and any qualified designees are responsible for detecting, documenting, and recording events that meet the definition of an AE or SAE (serious adverse event) and remain responsible for following up AEs that are serious, considered related to the study intervention or study procedures, or that caused the participant to discontinue the study intervention.

The Investigator will make an assessment of intensity for each AE and SAE reported during the study and assign it to 1 of the following categories, as per NCI CTCAE version 5.0 definitions:

- Grade 1 Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
- Grade 2 Moderate; minimal, local or non-invasive intervention indicated; limiting age-appropriate instrumental activities of daily living (ADL). Instrumental ADLs refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.
- Grade 3 Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care ADL. Self-care ADLs refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.
- Grade 4 Life-threatening consequences; urgent intervention indicated.
- Grade 5 Death related to AE.

Any AEs that are found to be directly related to the treatment with A022600424 are reported and followed-up.

Immunogenicity assessments

Antibodies to A022600424 and atezolizumab (anti-drug antibodies, ADAs) are evaluated in plasma and serum samples, respectively, collected from all participants throughout the clinical trial. Additionally, plasma or serum samples are also collected at the final visit from participants who discontinued study intervention or were withdrawn from the study.

A 3-tiered approach is employed to assess the immunogenicity of A022600424 and atezolizumab when applicable. Samples are screened and then confirmed for antibodies binding to A022600424 and atezolizumab, and the titer of confirmed positive samples are reported.

Results

As of January 19, 2024, a total of 24 patients (DL1: n=4, DL1A: n=4, DL2A: n=4, DL3A: n=4, DL4A: n=4, DL5A: n=4) with GPC3+ tumours received A022600424 in Part 1A dose escalation (Table 19). Patients were heavily pre-treated; median number of prior lines of therapy was 4 (2.0–7.0). Most of the patients (n=17, 71%) had hepatocellular carcinoma. See Table 19, describing the patient baseline characteristics.

No dose-limiting toxicities were observed.

Preliminary efficacy based on tumour imaging

4 patients from the DL1 group (see Table 16) at a dosage of 3 mg and 4 patients from the DL1A group (see Table 16) at a dosage of 1 mg were assessed for preliminary efficacy of A022600424 through CT imaging.

All patients have already completed at least 1 cycle of treatment.

From the tumour imaging it was observed that 1 patient had stable disease in group DL1 and 2 patients had stable disease in group DL1A.

Safety

Twenty-two patients (92%) reported treatment-related adverse events (TRAEs) of any grade, including three patients with a serious TRAE (2 events with hospitalization prolongation for a Grade 1 and 2 cytokine release syndrome [CRS] that recovered completely without Tocilizumab, 1 event with pneumonitis Grade 3. All CRS (19 patients, 79%) and infusion-related reaction (7 patients, 29%) were Grade 1 or 2. C_{max} was observed at the end or shortly after completion of infusion, then concentrations appeared to decline with mean half-life value (CV%) close to 26 hours (83%) at DL5A, see Figure 8. The maximum anti-drug antibody titer, though high, stabilized despite the target dose increased from Cycle 2.

Some TRAEs are to be expected, but the number of serious TRAEs was at an acceptable level. This suggests that A022600424 is tolerable at the tested dose levels.

Cytokine analysis

In total, patients from 5 dose levels were tested for cytokine levels (DL1, DL1A, DL2A, DL3A and DL4A).

8 patients (4 patients from the DL1 group and 4 patients from the DL1A group, see Table 16) were analysed during Cycle 1 for presence of the inflammatory cytokines TNFα, IL-8, IFNγ and IL-6. The results are shown in Figures 5 and 6.

Biomarker analysis showed an increase of interleukin-6 (IL-6) and interferon gamma (IFNG/ IFNγ) during lead-in doses, supporting CRS, see Figure 9. Cytokine levels declined after Cycle 1.

Based on this data the maximum dose to be tested has been reduced to 72 mg.

AFP

Several patients from different dosage groups were analysed for AFP level changes from baseline.

Among 14 HCC patients with baseline alpha-fatal protein (AFP) higher than 20 ng/ml, 4 (29%) had at least a 20% AFP decrease on treatment, see Figure 10. Two patients (both with HCC) have been on A022600424 for more than 6 months.

One patient in the DL1 group had a decrease in AFP level from baseline of 59.59% when the AFP level was measured on the first day of the 2nd treatment cycle. When this patient reached the 4th cycle the decrease in AFP level measured was still 41.51%.

A patient from the DL1A group had a decrease in AFP level from baseline of 56.51% when it was measured on the first day of the 2nd treatment cycle.

One patient from the DL4A group had a decrease in AFP level from baseline of 70.93% when it was measured on the first day of the 2nd treatment cycle, which on the first day of the 3rd treatment cycle was still 38.28%, and on the first day of the 4th treatment cycle it was 23.05%.

A patient in the DL5A group had a decrease in AFP level from baseline of 30.63% when it was measured on the first day of the 2nd treatment cycle. When this patient reached day 1 of the 3rd treatment cycle, the decrease in AFP level was still at 28.75%.

This indicates that, in these four patients, at least during the mentioned treatment cycles, the tumour may have been in regression.

A different patient in the DL1A group had a decrease in AFP level from baseline of 12.21% on the first day of the 2nd treatment cycle and a decrease from baseline of 18.26% on the first day of the 3rd treatment cycle.

A patient in the DL2A group had a decrease in AFP level of 17.55% from baseline on the first day of the 2nd treatment cycle.

A different patient in the DL1 group had a decrease in AFP level of 6.77% from baseline on the first day of the 2nd treatment cycle.

Although these decreases in AFP level do not reach 20% from baseline, they are still an indication that disease is potentially at least stable.

In conclusion, A022600424 is tolerated at the tested dose levels in patients with GPC3+ advanced solid tumors. AFP decreases and disease stability in a subset of patients are indicative of preliminary anti-tumor efficacy.

Table 19: Patient baseline characteristics

Patient baseline characteristics	DL1 3 mg (n=4)	DL1A 1 mg (n=4)	DL2A 2.5 mg (n=4)	DL3A 4.5 mg (n=4)	DL4A 18 mg (n=4)	DL5A 36 mg (n=4)	All (N=24)
Age, median (range), years	56.0 (29.0-76.0)	55.0 (51.0-69.0)	56.5 (47.0-72.0)	53.5 (30.0-61.0)	61.5 (40.0-66.0)	65.5 (55.0-76.0)	59 (29.0-76.0)
Male, n (%)	3 (75.0)	3 (75.0)	3 (75.0)	3 (75.0)	2 (50.0)	4 (100)	18 (75.0)
Race, n (%)							
White	1 (25.0)	1 (25.0)	2 (50.0)	2 (50.0)	1 (25.0)	1 (25.0)	8 (33.3)
Asian	3 (75.0)	3 (75.0)	2 (50.0)	2 (50.0)	3 (75.0)	3 (75.0)	16 (66.7)
GPC3 expression membranous H-score, median (range)	16 (7-70)	14 (3-20)	102 (2-300)	95 (0-190)	30 (5-280)	155 (40-160)	22 (0-300)
Number of prior regimens, median (range)	4.5 (2.0-6.0)	3.5 (2.0-6.0)	4.5 (3.0-5.0)	4 (4.0-5.0)	3.5 (2.0-7.0)	3 (3.0-4.0)	4 (2.0-7.0)
Disease location at diagnosis, n (%)*							
Liver	3 (75.00)	4 (100.00)	2 (50.00)	1 (25.00)	4 (100.00)	3 (75.00)	17 (70.83)
Cecum	0	0	0	1 (25.00)	0	0	1 (4.17)
Colon	0	0	1	0	0	0	1 (4.17)
Esophagus	0	0	0	1 (25.00)	0	0	1 (4.17)
Gall bladder	1 (25.00)	0	0	0	0	0	1 (4.17)
GI tract	0	0	0	1 (25.00)	0	0	1 (4.17)
Lung	0	0	0	0	0	1 (25.00)	1 (4.17)
Stomach	0	0	1 (25.00)	0	0	0	1 (4.17)
Notes: * cut-off date for the disease location was April 2024 DL, dose level; GPC3, glypican-3; n/N; number of patients							

CLAIMS

1. A polypeptide for use in the treatment of Glypican-3-positive (GPC3+) solid tumours wherein the polypeptide comprises or consists of at least three immunoglobulin single variable domains (ISVDs), wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), and wherein:

a) a first ISVD specifically binds to the constant domain of the T cell receptor (TCR) on a T cell and comprises

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6;

ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10; and

iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14;

b) a second ISVD specifically binds to GPC3 and comprises

iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7;

v. a CDR2 that is the amino acid sequence of SEQ ID NO: 11; and

vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 15; and

c) a third ISVD specifically binds to GPC3 and comprises

vii. a CDR1 that is the amino acid sequence of SEQ ID NO: 8;

viii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12; and

ix. a CDR3 that is the amino acid sequence of SEQ ID NO: 16,

wherein the CDRs are defined according to AbM annotation, and

wherein the order of the ISVDs indicates their relative position to each other considered from the *N*-terminus to the *C*-terminus of said polypeptide.

2. The polypeptide for use according to claim 1, wherein the first ISVDs is located at the N-terminus of said polypeptide.

3. The polypeptide for use according to claim 1 or 2, wherein

a) the amino acid sequence of said first ISVD comprised in the polypeptide exhibits a sequence identity of more than 90% with SEQ ID NO: 2;

b) the amino acid sequence of said second ISVD comprised in the polypeptide exhibits a sequence identity of more than 90% with SEQ ID NO: 3; and

c) the amino acid sequence of said third ISVD comprised in the polypeptide exhibits a sequence identity of more than 90% identity with SEQ ID NO: 4,

wherein the CDRs are defined according to AbM annotation.

4. The polypeptide for use according to any one of claims 1-3, wherein

a) said first ISVD comprised in the polypeptide consists of the amino acid sequence of SEQ ID NO: 2;

b) said second ISVD comprised in the polypeptide consists of the amino acid sequence of SEQ ID NO: 3; and

c) said third ISVD comprised in the polypeptide consists of the amino acid sequence of SEQ ID NO: 4,

wherein the CDRs are defined according to AbM annotation.

5. The polypeptide for use according to any one of claims 1-4, wherein the at least three ISVDs are linked via one or more peptidic linkers.

6. The polypeptide for use according to claim 5, wherein the first ISVD and the second ISVD comprised in the polypeptide are linked to each other via a linker consisting of less than 10 amino acids, preferably less than 6 amino acids, wherein the linker most preferably is a 5GS linker.

7. The polypeptide for use according to any one of claims 1-6, wherein the polypeptide further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units.

8. The polypeptide for use according to claim 7, wherein said one or more other groups, residues, moieties, or binding units that provide the polypeptide with increased half-life is chosen from the group consisting of a polyethylene glycol molecule, serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

9. The polypeptide for use according to claim 7 or 8, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of binding units that can bind to serum albumin or a serum immunoglobulin.

10. The polypeptide for use according to claim 9, in which said binding unit that provides the polypeptide with increased half-life is an ISVD that binds to human serum albumin.

11. The polypeptide for use according to claim 10, wherein the ISVD binding to human serum albumin comprises:

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 9;

ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13; and

iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17,

wherein the CDRs are defined according to AbM annotation.

12. The polypeptide for use according to claim 10 or 11, wherein the amino acid sequence of said ISVD binding to human serum albumin exhibits a sequence identity of more than 90% with SEQ ID NO: 5.

13. The polypeptide for use according to any one of claims 10-12, wherein said ISVD binding to human serum albumin consists of the amino acid sequence of SEQ ID NO: 5.

14. The polypeptide for use according to any one of claims 1-13, wherein the polypeptide comprises or consists of an amino acid sequence exhibiting a sequence identity of more than 90% with SEQ ID NO: 1.

15. The polypeptide for use according to any one of claims 1-14, wherein the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1.

16. Composition for use in the treatment of GPC3+ solid tumours, wherein the composition comprises the polypeptide as defined in any one of claims 1-15.

17. Composition for use according to claim 16, further comprising pharmaceutically acceptable excipients.

18. Composition according to claim 16 or 17, wherein the composition is provided as a liquid formulation.

19. The polypeptide or composition for use according to any one of claims 1-18, wherein the polypeptide or composition is administered to a subject in need thereof together with a PD-L1 inhibitor.

20. The polypeptide or composition for use according to claim 19, wherein the PD-L1 inhibitor is an anti-PD-L1 monoclonal antibody, preferably Atezolizumab.

21. The polypeptide or composition for use according to any one of claims 1-20, wherein the GPC3+ solid tumour is selected from hepatocellular carcinoma (HCC) and non-small cell lung cancer (NSCLC), preferably wherein NSCLC is metastatic NSCLC.

22. The polypeptide or composition for use according to any one of claims 1-21, wherein the polypeptide and/or composition is administered to a subject in need thereof intravenously or subcutaneously.

23. The polypeptide or composition for use according to any one of claims 1-22, wherein the polypeptide and/or composition is administered to a subject in need thereof at a dosage of between 1 and 200 mg, preferably between 1 and 70 mg.

24. The polypeptide or composition for use according to any one of claims 1-23, wherein the polypeptide and/or composition is administered to a subject in need thereof at a dosage of between 15 and 3500 µg/kg, preferably between 45 and 1100 µg/kg.

25. The polypeptide or composition for use according to any one of claims 1-24, wherein the polypeptide or composition is administered at most twice a week to a subject in need thereof, preferably once a week.

26. The polypeptide or composition for use according to any one of claims 1-25, wherein the administration of the polypeptide to a subject in need thereof results in a decrease in alpha-fetoprotein (AFP) level of at least 20% from baseline, preferably in a decrease in AFP level of at least 30% from baseline, more preferably in a decrease in AFP level of at least 40% from baseline, such as a decrease in AFP level of at least 50%, at least 55%, at least 59%, at least 60%, or at least 70% from baseline.

27. Polypeptide for use in decreasing AFP levels in a subject suffering from a Glypican-3-positive (GPC3+) solid tumour, wherein the polypeptide comprises or consists of at least three immunoglobulin single variable domains (ISVDs), wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least three ISVDs are optionally linked via one or more peptidic linkers, and wherein:

a) a first ISVD specifically binds to the constant domain of the T cell receptor (TCR) on a T cell and comprises
a CDR1 that is the amino acid sequence of SEQ ID NO: 6;
a CDR2 that is the amino acid sequence of SEQ ID NO: 10; and
a CDR3 that is the amino acid sequence of SEQ ID NO: 14;
b) a second ISVD specifically binds to GPC3 and comprises
a CDR1 that is the amino acid sequence of SEQ ID NO: 7;
a CDR2 that is the amino acid sequence of SEQ ID NO: 11; and
a CDR3 that is the amino acid sequence of SEQ ID NO: 15; and
c) a third ISVD specifically binds to GPC3 and comprises
a CDR1 that is the amino acid sequence of SEQ ID NO: 8;
a CDR2 that is the amino acid sequence of SEQ ID NO: 12; and
a CDR3 that is the amino acid sequence of SEQ ID NO: 16,
wherein the order of the ISVDs indicates their relative position to each other considered from the *N*-terminus to the *C*-terminus of said polypeptide, wherein the first ISVDs is optionally located at the *N*-terminus of said polypeptide.

28. The polypeptide for use according to claim 27, wherein the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1.

29. Polypeptide for use in the treatment of Glypican-3-positive (GPC3+) solid tumours as defined in any one of claims 1-26, or for use in decreasing AFP levels in a subject suffering from a Glypican-3-positive (GPC3+) solid tumour as defined in any one of claims 27 to 28, wherein the polypeptide is administered to the human subject in an amount of 1 to 70 mg, or 1 to 72 mg, or about 1 mg, or about 3 mg, preferably in an amount of between 1 and 36 mg.

30. Polypeptide for use according to claim 29, wherein the amount of polypeptide administered is effective to decrease the AFP levels in the subject by at least 20%, or by at least

40%, preferably by at least 50%, or by at least 55%, more preferably by at least 60%, most preferably by at least 70% from baseline.

31. Polypeptide for use according to any one of claims 29 or 30, wherein a decrease in AFP levels of at least 20% from baseline is maintained for at least two, preferably for at least three, more preferably for at least six weeks or more of treatment.

32. Method for treating Glypican-3-positive (GPC3+) solid tumours, wherein the method comprises the administration to a subject in need thereof the peptide or composition as defined in any one of claims 1-26.

33. Use of a peptide or composition as defined in any one of claims 1-26 for the manufacture of a medicament for the treatment of Glypican-3-positive (GPC3+) solid tumours.