NEW POLYPEPTIDE

Field of the invention

The present disclosure relates to a class of engineered polypeptides having a binding affinity for B7-H3. The present disclosure also relates to the use of such a B7-H3 binding polypeptide as a therapeutic, prognostic, diagnostic, and/or theranostic agent.

Background

B7-H3 biology

B7-H3 (CD276), a member of the B7 family, is a type I transmembrane protein. Other members of this family include CD80 (B7-1), CD86 (B7-2) and PD-L1 (B7-H1). The overall role of the B7 family proteins and their receptors is to act as coregulators of immune responses. B7-H3 has been reported to have both costimulatory and coinhibitory functions in the immune system and to play a role in both innate and adaptive immune responses. However, most reports suggest B7-H3 as co-inhibitor with a role primarily in the innate immune response. B7-H3 has been shown to inhibit TH1 response, CD4/CD8 T cell activation and proliferation, IFNγ production and diminish transcription factor activity and thereby turn off T cell response (Kontos *et al.*, 2021, Clin Cancer Res 27, 1227-1235).

Structurally, B7-H3 (45–66 kDa) is composed of extracellular Ig-like domains, a transmembrane region and a short intracellular region. B7-H3 exists in two isoforms, denoted 4Ig-B7-H3 and 2Ig-B7-H3 based on the number of extracellular Ig-like domains. In humans, the extracellular domain consists either of a single pair of immunoglobulin variable domain (IgV) and immunoglobulin constant domain (IgC) (2IgB7-H3) or two identical pairs of the immunoglobulins (4IgB7-H3) due to exon duplication. Mice only have the 2IgB7-H3 variant. Soluble B7-H3 (sB7-H3; approximately 37 kDa) results from alternative splicing or, more commonly, metalloprotease mediated cleavage from the cell surface. The short intracellular domain has no known signaling. Further research is warranted to define and characterize B7-H3 receptor interactions and elucidate their physiologic role, which may aid in design of B7-H3 interactome inhibitors. (Kanchan *et al.*, 2022, Biochim Biophys Acta Rev Cancer 1877(5),188783).

B7-H3 has a broad mRNA expression profile while the protein expression at steady-state is limited, suggesting the presence of an important post-transcriptional control mechanism. Protein expression can be found in both immune and non-immune cells. The expression on immune cells is quite low but can be induced, especially on antigen presenting cells (APCs). Expression in normal non-immune cells has been detected in resting fibroblast, osteoblasts and endothelial cells and in adrenal gland, pancreas, liver, colon, stomach, placenta, testis and prostate tissue (Du *et al.*, 2019 Cancer Cell 35, 221-237). While the expression in normal tissue is low, tumor tissues frequently display elevated levels of B7-H3. This differential between tumor and healthy tissue is important in the context of utilizing B7-H3 binding molecules for targeted therapy or as diagnostic tools (Kontos *et al., supra* and references therein).

B7-H3 in cancer

B7-H3 has been described to contribute to tumorigenesis through several different mechanisms including pro-invasion, pro-angiogenesis, pro-proliferation, anti-apoptosis and metabolic reprogramming (reviewed in Castellanos *et al.*, 2017, Immunol 6, 66-75).

B7-H3 overexpression is found in 60-90% of patient tumor tissues, e.g. neuroblastoma, medulloblastoma, glioma, melanoma, leukemia as well as breast, prostate, ovarian, cervical, gastric, pancreatic, renal and colorectal cancer. High expression correlates to tumor progression and poor clinical outcome. The expression can be found both in the stroma, including tumor associated vasculature, as well as on tumor cells. Tissue staining has shown expression of B7-H3 in the membrane, cytoplasm and nucleus of tumor cells and tumor stroma. In colon cancer nuclear staining of B7-H3 was strongly correlated to poor clinical outcome (Ingebrigtsen *et al.*, 2012, Int J Cancer 131, 2528-2536), pointing to a potential complexity in the expression pattern of B7-H3 in tumor tissues.

In an analysis of 1342 tumor tissues and 245 normal tissues, Seaman *et al.* demonstrated expression of B7-H3 in a range of tumor tissues and importantly even higher expression in the tumor stroma. Healthy tissues showed no or only moderate B7-H3 expression, with the highest expression found in the liver (Seaman *et al.*, 2017, Cancer Cell 31, 501-515). In this study, 65% of normal tissues tested were B7-H3 negative, 33% were weakly positive and only 1% were medium positive. For tumor tissues, only 27% of

tumor cells and 6% of stromal cells were found to be B7-H3 negative, while 30% of tumor cells and 51% of stromal cells showed the highest expression scores, with the remaining percentage distributed between weak and medium expression. This underlines the overall high expression of B7-H3 on a wide variety of tumor types, as well as the pronounced expression on tumor stromal cells. Furthermore, high expression of B7-H3 has been strongly correlated with metastasis and sB7-H3 has been observed in the serum of advanced-stage cancer patients (Kanchan *el. al., supra*). Thus, based on available information on B7-H3 overexpression in many different tumor types, a potential marker of disease aggressiveness and metastasis, it appears as an attractive protein for targeted radiotherapy, *in vivo* diagnostic applications as well as use of B7-H3 as a noninvasive target for diagnosis and prognosis.

Clinical and preclinical development of B7-H3 targeting molecules

Several anti-B7-H3 monoclonal antibodies, or antibody-drug conjugates, are in clinical development for treatment of various tumors (reviewed in Kontos *et al.*, *supra* and Kasten *et al.*, 2020, Curr Med Chem 27, 4016-4038). These include enoblituzumab (an Fc optimized B7-H3 targeting monoclonal antibody using antibody-dependent cell cytotoxicity for killing of tumor cells); MGC018, DS-7300a, BAT8009 (antibody-drug conjugates carrying cytotoxic payloads to B7-H3 expressing tumors); MVC-280 / TAK280 (a bispecific antibody targeting B7-H3 and CD3); and omburtamab (8H9, a.k.a. Omblastys®; a radiolabeled monoclonal B7-H3 antibody granted priority review by the FDA for treatment of pediatric patients with CNS/leptomeningeal metastasis from neuroblastoma). In clinical trials, omburtamab has been administered intracerebroventricularly, by positive pressure infusion to tumors in the CNS, intraperitoneally for treatment of desmoplastic small round cell tumors and intrathecally for treatment of patients with refractory, recurrent or advanced CNS or leptomeningeal disease.

Orlotamab, a bispecific antibody targeting B7-H3 and CD3, was in clinical development but was discontinued due to an observed increase in liver enzymes, suggested to be a result of cytokine release triggered by the interaction of orlotamab with CD3 on T cells.

The possibility to use a B7-H3 targeting antibody for diagnostic purposes is supported by results from Burvenich *et al.* who demonstrated specific tumor localization of the zirconium-89 labeled B7-H3 targeted

humanized monoclonal antibody DS-5573a (Burvenich *et al.*, 2018, Theranostics 8, 4199-4209).

To date, no B7-H3 targeted clinically explored radiotherapy other than the monoclonal antibody Omblastys® has been identified. As described above, it is not developed for systemic delivery.

Because the tissue penetration rate of a molecule is negatively associated with its size, a relatively large antibody molecule inherently has poor tissue distribution and penetration capacity. As such, it may not be an optimal targeting moiety for delivery of radioisotopes to solid tumors in a therapeutic setting. Moreover, although antibodies are widely used in a variety of routine contexts owing to high affinity and specificity to a multitude of possible antigens, such as for analytical, purification, diagnostic and therapeutic purposes, they still suffer from drawbacks. Such drawbacks include aggregation tendencies and long systemic half-lives which, in the case of targeted radiotherapy, increase the risk of damaging healthy tissue. When used for diagnostic (e.g. imaging) purposes, monoclonal antibodies again have drawbacks due to their long half-lives that result in poor contrast unless sufficient time is allowed for levels of unbound antibody to decline. Thus, monoclonal antibodies are not always optimal for therapeutic and diagnostic uses.

Polypeptides based on the Z scaffold and binding to B7-H3 have also been reported. Using a cell-based selection system, Stern *et al.* identified a B7-H3 binding polypeptide of modest B7-H3 affinity (denoted AC2; Kd 310 nM) that could be further optimized to generate B7-H3 binders of higher affinity (Kd 0.9 – 20 nM) (Stern *et al.*, 2019, ACS Comb Sci 21, 207-222; see also WO2020041626). By conjugating AC2 to either indocyanine green (Bam *et al.*, 2019, Bioconjug Chem 30, 1677-1689) or microbubbles (Bam *et al.*, 2020, Clin Cancer Res 26, 2140-2150), the feasibility of using a B7-H3 targeting Z scaffold variant to visualize tumors could be demonstrated. Furthermore, AC12 modified to comprise a peptide-based cysteine containing chelator at the C-terminus and labeled with ^{99}mTc was recently used for preclinical imaging of B7-H3 expressing tumors (Oroujeni *et al.*, 2022, Pharmaceutics, 14(9):1780).

Pre-clinical *in vivo* assessment of AC2 and AC12, respectively, suggests that the affinity for B7-H3 is insufficient for translation into clinical use, in particular for improved sensitivity and specificity in clinical imaging

(Bam *et al.*, 2019 *supra*, Bam *et al.*, 2020 *supra* and Oroujeni *et al.*, 2022 *supra*). These polypeptides also have unsatisfactory immunogenic profiles.

From the above, it is clear that there is continued need for provision of agents with a high affinity and specificity for B7-H3 for high contrast imaging and off-target effect, as well as agents with low immunogenicity to ensure safe and well tolerated therapeutics.

Summary of the invention

It is an object of the present disclosure to provide new B7-H3 binding agents, which could for example be used for therapeutic, prognostic and diagnostic applications.

It is an object of the present disclosure to provide a molecule allowing for efficient therapy, including theranostic applications, of for example various forms of cancer, while alleviating the abovementioned and other drawbacks of current therapies.

It is furthermore an object of the present disclosure to provide a molecule suitable for prognostic and diagnostic applications, for example prognostic and diagnostic applications in relation to various forms of cancer.

These and other objects, which are evident to the skilled person from the present disclosure, are met by the different aspects as claimed in the appended claims and as generally disclosed herein.

Thus, in a first aspect of the disclosure, there is provided a B7-H3 binding polypeptide, comprising a B7-H3 binding motif *BM*, which motif consists of an amino acid sequence selected from:

i) $EKX_3X_4ALX_7EIX_{10}X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}X_{21}AFIX_{25}X_{26}LNX_{29}X_{30}$
(SEQ ID NO:567)

wherein, independently of each other,

X_3 is selected from I and V;
X_4 is selected from A, D, E, G, H, K, L, M, N, Q, S, T and Y;
X_7 is selected from A, G, H and S;
X_{10} is selected from I and V;
X_{11} is selected from N and W;
X_{16} is selected from N and T;

X_{17} is selected from H and Y;
X_{18} is selected from A, D, E, G, H, N, Q, S and T;
X_{20} is selected from I and V;
X_{21} is selected from K and R;
X_{25} is selected from A, E, F, H, L and W;
X_{26} is selected from K and S;
X_{29} is selected from A, D and E;
X_{30} is selected from A, D and H; and

ii) an amino acid sequence which has at least 93% identity to the sequence defined in i).

In one embodiment, there is provided a B7-H3 binding polypeptide, comprising a B7-H3 binding motif *BM*, which motif consists of an amino acid sequence selected from:

i) $EKX_3X_4ALX_7EIX_{10}X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}X_{21}AFIX_{25}X_{26}LNX_{29}X_{30}$
(SEQ ID NO:567)
wherein, independently of each other,

X_3 is selected from I and V;
X_4 is selected from A, D, E, G, H, K, L, M, N, Q, S, T and Y;
X_7 is selected from A, G, H and S;
X_{10} is selected from I and V;
X_{11} is selected from N and W;
X_{16} is selected from N and T;
X_{17} is selected from H and Y;
X_{18} is selected from A, D, E, G, H, N, Q, S and T;
X_{20} is selected from I and V;
X_{21} is selected from K and R;
X_{25} is selected from A, E, F, H, L and W;
X_{26} is selected from K and S;
X_{29} is selected from A, D and E;
X_{30} is selected from A, D and H; and

ii) an amino acid sequence which has at least 93% identity to the sequence defined in i) provided that if X_4 is Y or X_{18} is G, then X_{26} is K.

In one embodiment, there is provided a B7-H3 binding polypeptide, wherein, in sequence i),
X_3 is selected from I and V;
X_4 is selected from A, D, E, G, H, K, L, M, N, Q, S, T and Y;
X_7 is selected from A, G, H and S;
X_{10} is selected from I and V;
X_{11} is selected from N and W;
X_{16} is selected from N and T;
X_{17} is selected from H and Y;
X_{18} is selected from A, D, E, G, H, N, Q, S and T;
X_{20} is selected from I and V;
X_{21} is selected from K and R;
X_{25} is selected from A, E, F, H, L and W;
X_{26} is selected from K and S;
X_{29} is selected from A, D and E; and
X_{30} is selected from A, D and H.

In one embodiment, there is provided a B7-H3 binding polypeptide, wherein, in sequence i),
X_3 is selected from I and V;
X_4 is selected from D, E, G, H, K, L, M, N, Q, S, T and Y;
X_7 is selected from A, G, H and S;
X_{10} is selected from I and V;
X_{11} is selected from N and W;
X_{16} is selected from N and T;
X_{17} is selected from H and Y;
X_{18} is selected from A, D, E, G, H, N, Q, S and T;
X_{20} is selected from I and V;
X_{21} is selected from K and R;
X_{25} is selected from A, E, F, H, L and W;
X_{26} is selected from K and S;
X_{29} is selected from A, D and E; and
X_{30} is selected from A, D and H.

In one embodiment, there is provided a B7-H3 binding polypeptide, wherein, in sequence i),

X_3 is selected from I and V;
X_4 is selected from D, G, K, L, M, N, S, T and Y;
X_7 is selected from A, G, H and S;
X_{10} is I;
X_{11} is selected from N and W;
X_{16} is T;
X_{17} is Y;
X_{18} is selected from A, D, E, H, N, Q, S and T;
X_{20} is selected from I and V;
X_{21} is selected from K and R;
X_{25} is selected from A, E, H and W;
X_{26} is K;
X_{29} is selected from A, D and E; and
X_{30} is selected from A, D and H.

In yet another embodiment, there is provided a B7-H3 binding polypeptide, wherein, in sequence i),
X_3 is I;
X_4 is selected from K and S;
X_7 is selected from G and S;
X_{10} is I;
X_{11} is W;
X_{16} is T;
X_{17} is Y;
X_{18} is selected from E, N and Q;
X_{20} is I;
X_{21} is K;
X_{25} is selected from A and H;
X_{26} is K;
X_{29} is selected from A and D; and
X_{30} is selected from A and D.

The symbols "X_n" and "X_m" are used herein to indicate amino acids in positions n and m in the various amino acid sequences defined herein, wherein n and m frequently are integers which indicate the position of an amino acid within said sequence as counted from the N-terminal end of said sequence. In other amino acid sequences, n and m may be lowercase letters

which merely indicate the internal order of the "X_n" and "X_m" residues even though other residues may be interspersed between them. As an example of the former, X_4 and X_7 indicate the amino acids in position four and seven, respectively, from the N-terminal end of sequence i).

In embodiments according to the first aspect, there are provided polypeptides wherein X_n in sequence i) is independently selected from a group of possible residues according to Table 1. The skilled person will appreciate that X_n may be selected from any one of the listed groups of possible residues and that this selection is independent from the selection of amino acids in X_m, wherein $n \neq m$. Thus, any of the listed possible residues in position X_n in Table 1 may be independently combined with any of the listed possible residues in any other variable position in Table 1.

The skilled person will appreciate that Table 1 is to be read as follows: In one embodiment according to the first aspect, there is provided a polypeptide wherein amino acid residue "X_n" in sequence i) is selected from "Possible residues". Thus, Table 1 discloses several specific and individualized embodiments of the first aspect of the present disclosure. For example, in one embodiment according to the first aspect, there is provided a polypeptide wherein X_7 in sequence i) is selected from A, G, H and S, and in another embodiment according to the first aspect, there is provided a polypeptide wherein X_7 in sequence i) is selected from G and S. For avoidance of doubt, the listed embodiments may be freely combined in yet other embodiments. For example, one such combined embodiment is a polypeptide in which X_7 is selected from G and S, while X_{18} is selected from E, N and Q, and X_{25} is selected from A and H, and so on.

Table 1

X_n	**Possible Residues**
X_3	I and V
X_3	I
X_3	V
X_4	A, D, E, G, H, K, L, M, N, Q, S, T and Y
X_4	A, E, G, H, K, L, M, N, Q, S, T and Y
X_4	A, D, G, H, K, L, M, N, Q, S, T and Y
X_4	D, E, G, H, K, L, M, N, Q, S, T and Y
X_4	A, D, E G, H, K, L, M, N, Q, S and T

X_n	**Possible Residues**
X_4	A, G, H, K, L, M, N, Q, S, T and Y
X_4	D, E, G, H, K, L, M, N, S, T and Y
X_4	D, G, H, K, L, M, N, S, T and Y
X_4	A, D, G, K, L, M, N, S and T
X_4	D, G, K, L, M, N, S, T and Y
X_4	D, K, L, M, N, S, T and Y
X_4	G, K, L, M, N, S, T and Y
X_4	K, L, M, N, S, T and Y
X_4	K, L, M, S, T and Y
X_4	K, L, M, N, S and Y
X_4	K, L, M, N, S and T

X_n	Possible Residues
X_4	K, L, M, and S
X_4	K, M, and S
X_4	K and M
X_4	M and S
X_4	K and S
X_4	K
X_4	S
X_7	A, G, H and S
X_7	A, G and S
X_7	G, H and S
X_7	H and S
X_7	G and H
X_7	G and S
X_7	G
X_7	S
X_{10}	I and V
X_{10}	I
X_{10}	V
X_{11}	N and W
X_{11}	N
X_{11}	W
X_{16}	N and T
X_{16}	N
X_{16}	T
X_{17}	H and Y
X_{17}	H
X_{17}	Y
X_{18}	A, D, E, G, H, N, Q, S and T
X_{18}	A, D, E, G, H, N, Q and S
X_{18}	A, D, E, G, H, N, Q and T
X_{18}	D, E, G, H, N, Q, S, and T
X_{18}	A, D, E, H, N, Q, S and T
X_{18}	A, D, E, H, N, Q and S
X_{18}	D, E, H, N and Q;
X_{18}	D, E, N and Q;
X_{18}	E, N and Q;
X_{18}	E and Q
X_{18}	E and N

X_n	Possible Residues
X_{18}	N and Q
X_{18}	E
X_{18}	N
X_{18}	Q
X_{20}	I and V
X_{20}	I
X_{20}	V
X_{21}	K and R
X_{21}	K
X_{21}	R
X_{25}	A, E, F, H, L and W
X_{25}	A, E, H, L and W
X_{25}	A, E, F, H and W
X_{25}	A, E, F, H and L
X_{25}	A, E, H and W
X_{25}	A, E and H
X_{25}	A, H and W
X_{25}	A and W
X_{25}	A and H
X_{25}	A
X_{25}	H
X_{26}	K and S
X_{26}	K
X_{26}	S
X_{29}	A, D and E
X_{29}	A and D
X_{29}	A and E
X_{29}	D and E
X_{29}	A
X_{29}	D
X_{29}	E
X_{30}	A, D and H
X_{30}	A and D
X_{30}	A and H
X_{30}	D and H
X_{30}	A
X_{30}	D
X_{30}	H

In a more specific embodiment defining a sub-class of B7-H3 binding polypeptides, sequence i) fulfils at least five of the nine conditions I-IX:

I. X_3 is I;

II. X_{10} is I;

III. X_{11} is selected from N and W;

IV. X_{16} is T;

V. X_{17} is Y;
VI. X_{20} is I
VII. X_{21} is K
VIII. X_{25} is selected A, E and H; and
IX. X_{26} is K

In some examples of a B7-H3 binding polypeptide according to the first aspect, sequence i) fulfils at least six of the nine conditions I-IX. More specifically, sequence i) may fulfil at least seven of the nine conditions I-IX, such as at least eight of the nine conditions I-IX, such as all of the nine conditions I-IX.

In some embodiments of a B7-H3 binding polypeptide according to the first aspect, X_{10} is I, X_{17} is Y and X_{21} is K. In some embodiments, X_{10} is I, X_{17} is Y and X_{20} is I. In some embodiments, X_{11} is W, X_{17} is Y and X_{20} is I. In some embodiments, X_3 is I, X_{10} is I and X_{17} is Y. In some embodiments, X_3 is I, X_{17} is Y and X_{21} is K. In some embodiments, X_3 is I, X_{10} is I and X_{21} is K.

As described in detail in the experimental section to follow, the selection of B7-H3 binding polypeptide variants led to the identification of a number of individual B7-H3 binding motif (*BM*) sequences belonging to the class defined in the first aspect of the disclosure. These sequences constitute individual embodiments of sequence i) or ii) according to this aspect. The sequences of individual B7-H3 binding motifs correspond to amino acid positions 8-37 in SEQ ID NO:1-535. In one embodiment of the B7-H3 binding polypeptide according to this first aspect, sequence i) corresponds to the sequence from position 8 to position 37 in a sequence selected from the group consisting of SEQ ID NO:1-535. In another embodiment of the B7-H3 binding polypeptide according to this first aspect, sequence i) corresponds to the sequence from position 8 to position 37 in a sequence selected from the group consisting of SEQ ID NO:1-21 and 480-535. In one embodiment, sequence i) corresponds to the sequence from position 8 to position 37 in a sequence selected from the group consisting of SEQ ID NO:1-21. In one embodiment, sequence i) corresponds to the sequence from position 8 to position 37 in a sequence selected from the group consisting of SEQ ID NO:1-3. In one embodiment, sequence i) corresponds to the sequence from position 8 to position 37 in SEQ ID NO:1. In one embodiment, sequence i) corresponds to the sequence from position 8 to position 37 in SEQ ID NO:2.

In one embodiment, sequence i) corresponds to the sequence from position 8 to position 37 in SEQ ID NO:3.

As the skilled person will realize, the function of any polypeptide, such as the B7-H3 binding capacity of the polypeptide of the present disclosure, is dependent on the tertiary structure of the polypeptide. It is therefore possible to make minor changes to the sequence of amino acids in a polypeptide without affecting the function thereof. Thus, the disclosure encompasses modified variants of the B7-H3 binding polypeptide, which have retained B7-H3 binding characteristics.

In this way, encompassed by the present disclosure is a B7-H3 binding polypeptide comprising an amino acid sequence with 93% or greater identity, such as 96% or greater identity, to a polypeptide as defined in i). For example, it is possible that an amino acid residue belonging to a certain functional grouping of amino acid residues (e.g. hydrophobic, hydrophilic, polar etc.) could be exchanged for another amino acid residue from the same functional group.

In some embodiments, such changes may be made in any position of the sequence of the B7-H3 binding polypeptide as disclosed herein. In other embodiments, such changes may be made only in the non-variable positions, also denoted scaffold amino acid residues. In such cases, changes are not allowed in the variable positions. In other embodiments, such changes may be only in the variable positions.

According to one contemplated definition of such “variable positions”, these are positions denoted with an “X” in sequence i) as defined above.

According to another contemplated definition, “variable positions” are those positions that are randomized in a selection library of Z variants prior to selection, and may thus for example be positions 2, 3, 4, 6, 7, 10, 11, 17, 18, 20, 21, 25, 28, 29 and 30 in sequence i). This definition of “variable positions” does not include positions 16 and 26, which are scaffold positions in this context, albeit allowed to be either one of two alternatives in each position. Reference is made to Nord *et al.* (1995), Prot Eng 8:601-608, and Löfblom *et al.* (2010), FEBS Letters, 584:2670-2680. Like the B7-H3-binding Z variants of the present disclosure, the polypeptides disclosed in Nord *et al.* and Löfblom *et al.* are also based on a scaffold from the Z derivative of domain B of protein A from *Staphylococcus aureus*, although directed to other targets. As shown in Nord *et al.* (see for example Figure 4), the amino acids in

positions 23 (corresponding to position 16 in the instant B7-H3-binding motif) and 33 (corresponding to position 26 in the instant B7-H3-binding motif) are N and S, respectively. As also shown in Löfblom *et al.*, polypeptides with amino acid residues N and S in positions 23 and 33, respectively (corresponding to positions 16 and 26 in the instant B7-H3-binding motif; see Figure 2 of Löfblom *et al.*), and polypeptides with amino acid residues T and K in positions 23 and 33, respectively, all have a maintained basic structure and function. Thus, in the context of this definition of "variable positions", the amino acid residues at positions 16 and 26 form part of the common scaffold, and it is contemplated to have either N or T in scaffold position 16 and either S or K in scaffold position 26.

The term "% identity", as used throughout the specification, may for example be calculated as follows. The query sequence is aligned to the target sequence using the CLUSTAL W algorithm (Thompson *et al.*, 1994, Nucleic Acids Research, 22: 4673-4680). A comparison is made over the window corresponding to the shortest of the aligned sequences. The shortest of the aligned sequences may in some instances be the target sequence. In other instances, the shortest of the aligned sequences may be the query sequence. The amino acid residues at each position are compared and the percentage of positions in the query sequence that have identical correspondences in the target sequence is reported as % identity.

In another embodiment, there is provided a B7-H3 binding polypeptide comprising a binding motif sequence that corresponds to the sequence from position 8 to position 37 in a sequence selected from the group consisting of SEQ ID NO:1-535; or a sequence having 93% or greater identity thereto, such as 96% identity thereto.

In some embodiments, the *BM* as defined above "forms part of" a three-helix bundle protein domain. This is understood to mean that the sequence of the *BM* is "inserted" into or "grafted" onto the sequence of the original three-helix bundle domain, such that the *BM* replaces a similar structural motif in the original domain. For example, without wishing to be bound by theory, the *BM* is thought to constitute two of the three helices of a three-helix bundle and can therefore replace such a two-helix motif within any three-helix bundle. As the skilled person will realize, the replacement of two helices of the three-helix bundle domain by the two *BM* helices has to be performed so as not to affect the basic structure of the polypeptide. That is,

the overall folding of the Cα backbone of the polypeptide according to this embodiment of the invention is substantially the same as that of the three-helix bundle protein domain of which it forms a part, e.g. having the same elements of secondary structure in the same order etc. Thus, a *BM* according to the present disclosure "forms part" of a three-helix bundle domain if the polypeptide according to this embodiment has the same fold as the original domain, implying that the basic structural properties are shared, those properties e.g. resulting in similar CD spectra. The skilled person is aware of other parameters that are relevant.

In particular embodiments, the B7-H3 binding motif (*BM)* thus forms part of a three-helix bundle protein domain. For example, the *BM* may essentially constitute two alpha helices with an interconnecting loop, within said three-helix bundle protein domain. In particular embodiments, said three-helix bundle protein domain is selected from bacterial receptor domains. In some embodiments, the three-helix bundle protein domain is selected from domains of protein A from *Staphylococcus aureus* or derivatives thereof. Non-limiting examples of such domains are the five different three-helical domains of Protein A from *Staphylococcus aureus*, such as domain B, and derivatives thereof. In some embodiments, the three-helical bundle protein domain is a variant of protein Z, which is derived from domain B of staphylococcal Protein A (Wahlberg E *et al.,* 2003, PNAS 100(6):3185-3190).

In some embodiments where the B7-H3 binding polypeptide as disclosed herein forms part of a three-helix bundle protein domain, the B7-H3 binding polypeptide comprises a binding module (*BMod*), the amino acid sequence of which is selected from:

iii) K-[*BM*]-PSQSX$_a$X$_b$LLX$_c$EAKKLX$_d$X$_e$X$_f$Q (SEQ ID NO:568);

wherein [*BM*] is a B7-H3 binding motif according to any definition herein;
X$_a$ is selected from A and S;
X$_b$ is selected from E and N;
X$_c$ is selected from A, S and C;
X$_d$ is selected from E, N and S;
X$_e$ is selected from D, E and S; and
X$_f$ is selected from A and S; and

iv) an amino acid sequence which has at least 93% identity to a sequence defined in iii).

In some embodiments, said polypeptide may beneficially exhibit a high structural stability, such as resistance to chemical modifications, to changes in physical conditions and to proteolysis, during production and storage, as well as *in vivo*.

As discussed above, polypeptides comprising minor changes as compared to the above amino acid sequences, which do not largely affect the tertiary structure and the function of the polypeptide, are also within the scope of the present disclosure. Thus, in some embodiments, sequence iv) has at least 93%, such as at least 95%, such as at least 97% identity to a sequence defined by iii).

In one embodiment, X_a in sequence iii) is A.
In one embodiment, X_a in sequence iii) is S.
In one embodiment, X_b in sequence iii) is N.
In one embodiment, X_b in sequence iii) is E.
In one embodiment, X_c in sequence iii) is A.
In one embodiment, X_c in sequence iii) is S.
In one embodiment, X_c in sequence iii) is C.
In one embodiment, X_d in sequence iii) is E.
In one embodiment, X_d in sequence iii) is N.
In one embodiment, X_d in sequence iii) is S.
In one embodiment, X_e in sequence iii) is D.
In one embodiment, X_e in sequence iii) is E.
In one embodiment, X_e in sequence iii) is S.
In one embodiment, X_dX_e in sequence iii) is selected from EE, ES, SD, SE and SS.
In one embodiment, X_dX_e in sequence iii) is ES.
In one embodiment, X_dX_e in sequence iii) is SE.
In one embodiment, X_dX_e in sequence iii) is SD.
In one embodiment, X_f in sequence iii) is A.
In one embodiment, X_f in sequence iii) is S.
In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is A and X_f is A.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is A and X_f is A.
In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is C and X_f is A.

In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is S and X_f is S.
In one embodiment, in sequence iii), Xa is S; X_b is E; X_c is C and X_f is S.
In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is A; X_dX_e is ND and X_f is A.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is A; X_dX_e is ND and X_f is A.
In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is C; X_dX_e is ND and X_f is A.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is S; X_dX_e is ND and X_f is S.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is C; X_dX_e is ND and X_f is S.
In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is A; X_dX_e is SE and X_f is A.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is A; X_dX_e is SE and X_f is A.
In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is C; X_dX_e is SE and X_f is A.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is S; X_dX_e is SE and X_f is S.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is C; X_dX_e is SE and X_f is S.
In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is A; X_dX_e is SD and X_f is A.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is A; X_dX_e is SD and X_f is A.
In one embodiment, in sequence iii), X_a is A; X_b is N; X_c is C; X_dX_e is SD and X_f is A.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is S; X_dX_e is SD and X_f is S.
In one embodiment, in sequence iii), X_a is S; X_b is E; X_c is C; X_dX_e is SD and X_f is S.

In a further embodiment, sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-535. In one embodiment, sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a

sequence selected from the group consisting of SEQ ID NO:1-21 and 480-535. In another embodiment, sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-21. In one embodiment, sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-3. In yet another embodiment, sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in SEQ ID NO:1. In yet another embodiment, sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in SEQ ID NO:2. In yet another embodiment, sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in SEQ ID NO:3.

Also, in a further embodiment, there is provided a B7-H3 binding polypeptide as described herein, which comprises an amino acid sequence selected from:

v) YA-[*BMod*]-AP (SEQ ID NO:569);
wherein [*BMod*] is a B7-H3 binding molecule as defined above; and

vi) an amino acid sequence which has at least 86% identity to a sequence defined by v).

As discussed above, polypeptides comprising minor changes as compared to the above amino acid sequences without largely affecting the tertiary structure and the function thereof also fall within the scope of the present disclosure. Thus, in some embodiments, the B7-H3 binding polypeptides as defined above may for example have a sequence vi) which is at least 86%, such as at least 88%, such as at least 90%, such as at least 92%, such as at least 94%, such as at least 96%, such as at least 98% identical to a sequence defined by v).

In some embodiments, the B7-H3 binding motif may form part of a polypeptide comprising an amino acid sequence selected from:
VDAKYAK-[*BM*]-PSQSSELLSEAKKLNDSQAPK (SEQ ID NO:570);
AEAKFAK-[*BM*]-PSQSSELLSEAKKLSESQAPK (SEQ ID NO:571);

AEAKYAK-[*BM*]-PSQSSELLSEAKKLNDSQAPK (SEQ ID NO:572);
AEAKFAK-[*BM*]-PSQSSELLSEAKKLNDSQAPK (SEQ ID NO:573);
AEAKFAK-[*BM*]-PSQSSELLSEAKKLNESQAPK (SEQ ID NO:574);
AEAKYAK-[*BM*]-PSQSSELLSEAKKLSESQAPK (SEQ ID NO:575);
ADNNFNK-[*BM*]-PSQSANLLSEAKKLNESQAPK (SEQ ID NO:576);
ADNKFNK-[*BM*]-PSQSANLLAEAKKLNDAQAPK (SEQ ID NO:577);
ADNKFNK-[*BM*]-PSVSKEILAEAKKLNDAQAPK (SEQ ID NO:578);
ADAQQNNFNK-[*BM*]-PSQSTNVLGEAKKLNESQAPK (SEQ ID NO:579);
AQHDE-[*BM*]-PSQSANVLGEAQKLNDSQAPK (SEQ ID NO:580);
VDNKFNK-[*BM*]-PSQSANLLAEAKKLNDAQAPK (SEQ ID NO:581);
AEAKYAK-[*BM*]-PSESSELLSEAKKLNKSQAPK (SEQ ID NO:582);
VDAKYAK-[*BM*]-PSQSSELLAEAKKLNDAQAPK (SEQ ID NO:583);
VDAKYAK-[*BM*]-PSQSSELLAEAKKLNDSQAPK (SEQ ID NO:584);
VDAKYAK-[*BM*]-PSQSSELLSEAKKLSESQAPK (SEQ ID NO:585);
VDAKYAK-[*BM*]-PSQSSELLSEAKKLESSQAPK (SEQ ID NO:586);
VDAKYAK-[*BM*]-PSQSSELLAEAKKLNKAQAPK (SEQ ID NO:587); and
AEAKYAK-[*BM*]-PSQSSELLAEAKKLNKAQAPK (SEQ ID NO:588);

wherein [*BM*] is a B7-H3 binding motif as defined above.

In one embodiment, there is provided a B7-H3 binding polypeptide as described herein, which comprises an amino acid sequence selected from:

vii) VDAKYAK-[*BM*]-PSQSSELLSEAKKLNDSQAPK (SEQ ID NO:570);
wherein [*BM*] is a B7-H3 binding motif as defined herein; and
viii) an amino acid sequence which has at least 86% identity to a sequence defined by vii).

In a further embodiment, there is provided a B7-H3 binding polypeptide as described herein, which comprises an amino acid sequence selected from:

ix) AEAKFAK-[*BM*]-PSQSSELLSEAKKLSESQAPK (SEQ ID NO:571);
wherein [*BM*] is a B7-H3 binding motif as defined herein; and
x) an amino acid sequence which has at least 86% identity to a sequence defined by ix).

In a further embodiment, sequence ix) is selected from the group consisting of SEQ ID NO:15-16, 420-424, 427-428, 430-436, 438-444 and 480-535.

In another further embodiment, there is provided a B7-H3 binding polypeptide as described herein, which comprises an amino acid sequence selected from:

xi) AEAKYAK-[*BM*]-PSQSSELLSEAKKLNDSQAPK (SEQ ID NO:572);
wherein [*BM*] is a B7-H3 binding motif as defined herein; and

xii) an amino acid sequence which has at least 86% identity to a sequence defined by xi).

In another further embodiment, sequence xi) is selected from the group consisting of SEQ ID NO:1-13, 17-418 and 446-479. In another embodiment, sequence xi) is selected from the group consisting of SEQ ID NO:1-13 and SEQ ID NO:17-21. In another embodiment, sequence xi) is selected from the group consisting of SEQ ID NO:1-3. In another embodiment, sequence xi) is SEQ ID NO:1. In another embodiment, sequence xi) is SEQ ID NO:2. In another embodiment, sequence xi) is SEQ ID NO:3.

In another further embodiment, the B7-H3 binding polypeptide comprises an amino acid sequence selected from:

xiii) AEAKFAK-[*BM*]-PSQSSELLSEAKKLNESQAPK (SEQ ID NO:573);
wherein [*BM*] is a B7-H3 binding motif as defined herein; and

xiv) an amino acid sequence which has at least 86% identity to a sequence defined by xiii).

In another further embodiment, sequence xiii) is selected from SEQ ID NO:14, 425-426, 429, 437 and 445.

In one embodiment, there is provided a B7-H3 binding polypeptide of the first aspect as described herein which is capable of binding to B7-H3 such that the K_D value of the interaction with B7-H3 is at most 1×10^{-6} M, for example at most 5×10^{-7} M, for example at most 1×10^{-7} M, for example at most 5×10^{-8} M, for example at most 1×10^{-8} M.

The terms “B7-H3 binding” and ”binding affinity for B7-H3” as used in this specification refer to a property of a polypeptide which may be tested for example by ELISA, by Kinetic Exclusion Assay (KinExA®) and/or by various sensor-based technologies, such as surface plasmon resonance (SPR) technology, biolayer interferometry (BLI; e.g. Octet®) and quartz crystal microbalance (QCM) technology.

For example as described in the experimental section below, B7-H3 binding affinity may be tested in an experiment in which samples of the polypeptide are captured on antibody-coated ELISA plates and biotinylated B7-H3 is added followed by streptavidin-conjugated HRP. TMB substrate is added and the absorbance at 450 nm is measured using a multi-well plate reader. The skilled person may then interpret the results obtained by such experiments to establish at least a qualitative measure of the binding affinity of the polypeptide for B7-H3. If a quantitative measure is desired, for example to determine the EC50 value (the half maximal effective concentration) for the interaction, ELISA may also be used. The response of the polypeptide against a dilution series of biotinylated B7-H3 is measured using ELISA as described above. The skilled person may then interpret the results obtained by such experiments, and EC50 values may be calculated from the results using for example GraphPad Prism 5 and nonlinear regression.

The affinity of a binding interaction, e.g. defined as the equilibrium dissociation constant (K_D), may also be determined by different sensor-based methods as mentioned above. Importantly, as these technologies measure binding in real-time, the kinetics properties of the interaction can also be assessed, including the rate of association (k_a) and rate of dissociation (k_d). One such technology is based on surface plasmon resonance (SPR). Here, B7-H3, or a fragment thereof, is immobilized on a sensor chip and samples of the polypeptide whose affinity is to be determined are prepared by serial dilution and injected over the chip. Alternatively, the polypeptide to be tested is immobilized on a sensor chip of the instrument, and a sample containing B7-H3, or a fragment thereof, is passed over the chip. Binding values may for example be defined in a Biacore (Cytiva), Sierra (Bruker), Carterra (Carterra) or ProteOn XPR 36 (Bio-Rad) instrument. K_D values and/or kinetic constants may then be calculated from the results using for example the 1:1 Langmuir binding model of the Biacore Insight Evaluation software, or other suitable model and software, provided by the instrument manufacturer. Analogously,

B7-H3 binding kinetics and affinity may also be assessed by BLI, an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer. Measurements by BLI technology may for example be performed in an Octet® HTX system (Sartorius) and data monitored and analyzed by appropriate model and software provided by the instrument manufacturer.

A measure of B7-H3 binding affinity may also be obtained using a continuous-flow system based on QCM technology. To monitor binding interactions, one of the interacting molecules, or fragment thereof, is immobilized on the sensor surface and the sample containing the other one is injected over the sensor surface. The signal output is given in frequency (Hz) and is directly related to changes in mass on the sensor surface. Kinetic measurements by QCM technology may for example be performed in an Attana A200® (Attana) instrument. Data is collected by Attester Software and KD values may then be calculated from the results using for example a 1:1 Langmuir binding model and subsequently processed in the Evaluation Software, or other suitable software, provided by the instrument manufacturer.

Another method for determining binding affinity for B7-H3 is the Kinetic Exclusion Assay (KinExA®; Sapidyne Instruments Inc; Darling and Brault, 2004, Assay and Drug Dev Tech 2(6):647-657) for measurements of the equilibrium binding affinity and kinetics between unmodified molecules in solution. For affinity analysis, the equilibrium dissociation constant, K_D, and the rate of association, k_a, are experimentally determined, while the rate of dissociation, kd, may be calculated based on the equation $k_d = K_D * k_a$.

A KinExA® K_D analysis requires immobilization of one interaction partner (e.g. the titrated binding partner) to a solid phase, which is then used as a probe to capture the other interaction partner (e.g. the constant binding partner) free in solution once an equilibrium is reached. For each experiment, a series of solutions with a constant concentration of one binding partner and a titration of the other binding partner are equilibrated. The solutions are then briefly exposed to the solid phase and a portion of free constant binding partner is captured and labeled with a fluorescent secondary molecule. The short contact time with the solid phase is less than the time needed for dissociation of the pre-formed complex in solution, meaning that competition between the solution and the solid phase titrated binding partner is “kinetically

excluded". Because the solid phase is only used as a probe for the free constant binding partner in each sample, the solution equilibrium is not altered during measurements. A K_D value is calculated from signals generated from captured free constant binding partner, which are directly proportional to the concentration of free constant binding partner in the equilibrated sample. The data may be analyzed using the KinExA® Pro software and least squares analysis to fit the optimal solutions for the K_D and the Active Binding site Concentration (ABC) to a curve representative of a stoichiometric relevant model, for instance a 1:1 reversible bi-molecular interaction.

Determination of binding kinetics via KinExA® may be done in a similar format as the equilibrium analysis, except measurements are collected "pre equilibrium" and the binding signals are a function of time and total concentration of the titrated binding partner. There are two methods that can be used to determine the k_a. The "direct method" holds the concentrations of titrated and constant binding partners fixed, and the solution is probed over time. The amount of the free constant binding partner in the solution will decrease as the sample moves toward equilibrium. The "inject method" holds incubation time and one partner's concentration fixed, while titrating concentrations of the other partner. As the concentration of the titrated binding partner increases, the amount of free constant binding partner will decrease as more complexes are formed.

The terms "albumin binding" and "binding affinity for albumin" as used in this disclosure refer to a property of a polypeptide which may be tested for example by ELISA, SPR, BLI, QCM and/or KinExA®, in an analogous way to the examples described above for B7-H3.

The skilled person will understand that various modifications and/or additions can be made to a B7-H3 binding polypeptide according to any aspect disclosed herein in order to tailor the polypeptide to a specific application without departing from the scope of the present disclosure. For example, in one embodiment, there is provided a B7-H3 binding polypeptide as described herein, which polypeptide has been extended by and/or comprises additional amino acids at the C terminus and/or N terminus. Such a polypeptide should be understood as a polypeptide having one or more additional amino acid residues at the very first and/or the very last position in

the polypeptide chain. Thus, the B7-H3 binding polypeptide may comprise any suitable number of additional amino acid residues, for example at least one additional amino acid residue. Each additional amino acid residue may individually or collectively be added in order to, for example, improve and/or simplify production, purification, stabilization *in vivo* or *in vitro*, coupling or detection of the polypeptide. Such additional amino acid residues may comprise one or more amino acid residues added for the purpose of chemical coupling. One example of this is the addition of a cysteine residue. Additional amino acid residues may also provide a ”tag” for purification or detection of the polypeptide, such as a His_6 tag, a $(HisGlu)_3$ tag (“HEHEHE” tag) or a ”myc” (c-myc) tag or a ”FLAG” tag for interaction with antibodies specific to the tag or immobilized metal affinity chromatography (IMAC) in the case of a His_6-tag.

In one embodiment, there is provided a B7-H3 binding polypeptide as described herein which comprises additional amino acids at the C-terminal and/or N-terminal end. For example, in one embodiment of the B7-H3 binding polypeptide as disclosed herein, it consists of any one of the sequences disclosed herein, having from 0 to 30 additional C-terminal and/or N-terminal residues, such as from 0 to 25, such as from 0 to 20, such as from 0 to 15, such as from 0 to 10, such as from 0 to 7 additional C-terminal and/or N-terminal residues. In one embodiment, the B7-H3 binding polypeptide consists of any one of the sequences disclosed herein, having from 0 to 25, such as from 0 to 15, such as from 0 to 4, such as 3 additional C-terminal residues.

In one further embodiment, there is provided a B7-H3 binding polypeptide as described herein which comprises additional amino acids at the C-terminal and/or N-terminal end wherein said additional amino acid(s) improve(s) production, purification, stabilization *in vitro* or *in vivo*, coupling or detection of the polypeptide.

The further amino acids as discussed above may be coupled to the B7-H3 binding polypeptide by means of chemical conjugation (using known organic chemistry methods) or by any other means, such as expression of the B7-H3 binding polypeptide as a fusion protein or joined in any other fashion, either directly or via a linker, for example an amino acid linker.

A further polypeptide domain may moreover provide another B7-H3 binding moiety. Thus, in a further embodiment, there is provided a B7-H3

binding polypeptide in a multimeric form. Said multimer is understood to comprise at least two B7-H3 binding polypeptides as disclosed herein as monomer units, the amino acid sequences of which may be the same or different. Multimeric forms of the polypeptides may comprise a suitable number of domains, each having a B7-H3 binding motif, and each forming a monomer within the multimer. These domains may have the same amino acid sequence, but alternatively, they may have different amino acid sequences. In other words, the B7-H3 binding polypeptide of the invention may form homo- or heteromultimers, for example homo- or heterodimers. In one embodiment, there is provided a B7-H3 binding polypeptide, wherein said monomer units are covalently coupled together. In another embodiment, said B7-H3 binding polypeptide monomer units are expressed as a fusion protein. In one embodiment, there is provided B7-H3 binding polypeptide in dimeric form. In one particular embodiment, said dimeric form is a homodimeric form. In another embodiment, said dimeric form is a heterodimeric form. For the sake of clarity in this regard, throughout this disclosure, the term “B7-H3 binding polypeptide” is used to encompass B7-H3 binding polypeptides in all forms, i.e. including both monomeric and multimeric forms.

The further amino acids as discussed above may for example comprise one or more further polypeptide domain(s). A further polypeptide domain may provide the B7-H3 binding polypeptide with another function, such as for example another binding function, or an enzymatic function, or a toxic function or a fluorescent signaling function, or combinations thereof.

Furthermore, it may be beneficial that the B7-H3 binding polypeptide as defined herein is part of a fusion protein or a conjugate comprising a second or further moieties. Second and further moiety/moieties of the fusion polypeptide or conjugate in such a protein may suitably have a desired biological activity.

Thus, in a second aspect of the present disclosure, there is provided a fusion protein or conjugate comprising:

- a first moiety consisting of a B7-H3 binding polypeptide according to the first aspect; and
- a second moiety consisting of a polypeptide having a desired biological activity.

In one embodiment, said fusion protein or conjugate may additionally comprise further moieties, comprising desired biological activities that can be either the same as or different from the biological activity of the second moiety.

Non-limiting examples of a desired biological activity comprise a therapeutic activity, a binding activity and an enzymatic activity. In one embodiment, there is provided a fusion protein according to the second aspect, wherein said biological activity is a therapeutic activity. In one embodiment, there is provided a fusion protein according to the second aspect, wherein said biological activity is a binding activity. In one embodiment, there is provided a fusion protein according to the second aspect, wherein said biological activity is an enzymatic activity.

In one embodiment, the second moiety having a desired biological activity is a therapeutically active polypeptide. Non-limiting examples of therapeutically active polypeptides are biomolecules, such as molecules selected from the group consisting of human endogenous enzymes, hormones, growth factors, chemokines, cytokines and lymphokines.

In one embodiment of the first or second aspect of the disclosure, there is provided a B7-H3 binding polypeptide, fusion protein or conjugate which comprises an anti-cancer agent. In one embodiment of the second aspect, said second moiety in the fusion protein or conjugate is an anti-cancer agent. Non-limiting examples of anti-cancer agents for use in this context include agents selected from the group consisting of auristatin, anthracycline, calicheamicin, combretastatin, doxorubicin, duocarmycin, the CC-1065 anti-tumor-antibiotic, ecteinsascidin, geldanamycin, maytansinoid, methotrexate, mycotoxin, taxol, ricin, bouganin, gelonin, pseudomonas exotoxin 38 (PE38), diphtheria toxin (DT), and their analogues, and derivates thereof and combinations thereof. A skilled person would appreciate that the non-limiting examples of anti-cancer agents include all possible variants of said agents, for example the agent auristatin is intended to include for example auristatin E, auristatin F, auristatin PE, and derivatives thereof.

In one embodiment of the second aspect of the disclosure, there is provided a B7-H3 binding polypeptide, fusion protein or conjugate, wherein in said biological activity of the second moiety is a binding activity. In one embodiment, said binding activity is an albumin binding activity which increases the *in vivo* half-life of the fusion protein or conjugate and/or

changes the biodistribution properties of the fusion protein or conjugate. In one embodiment, there is provided a fusion protein as described herein, wherein said binding activity increases the *in vivo* half-life of the fusion protein or conjugate. In another embodiment, there is provided a fusion protein as described herein, wherein said binding activity changes the biodistribution properties of the fusion protein or conjugate. In one embodiment, the binding activity acts to block biological activity. Non-limiting examples of binding activities are binding activities which increase the *in vivo* half-life of the fusion protein or conjugate, changes the biodistribution properties of the fusion protein or conjugate, and/or binding activities which act to e.g. block, inhibit, activate, increase, antagonize or agonize a biological activity. One example of such a binding activity is a binding activity, which increases the *in vivo* half-life of a fusion protein or conjugate. In one embodiment of said fusion protein or conjugate, the *in vivo* half-life of said fusion protein or conjugate is longer than the *in vivo* half-life of the B7-H3 binding polypeptide *per se*. In one embodiment, said *in vivo* half-life is increased at least 10 times, such as at least 25 times, such as at least 50 times, such as at least 75 times, such as at least 100 times, compared the *in vivo* half-life of the B7-H3 binding polypeptide *per se*. Another example of such binding activity is a binding activity, which changes the biodistribution properties of a fusion protein or conjugate. In one embodiment of said fusion protein or conjugate, the biodistribution properties of said fusion protein or conjugate are changed such that a reduced amount of the fusion protein or conjugate is retained in the kidney than of the B7-H3 binding polypeptide *per se*. In one embodiment, said retention is reduced at least 2 times, such as at least 4 times, such as at least 6 times, such as at least 8 times, such as at least 10 times, such as at least 15 times, compared to the retention of the B7-H3 binding polypeptide *per se*.

As discussed, the fusion protein or conjugate may comprise at least one further moiety with a binding activity towards a target. In one particular embodiment, said target is albumin, binding to which increases the *in vivo* half-life of said fusion protein or conjugate. In one such embodiment, said albumin binding activity is provided by an albumin binding domain (ABD) of streptococcal protein G, or a derivative thereof. Thus, said fusion protein may for example comprise a B7-H3 binding polypeptide in monomeric or multimeric form (such as a homodimeric or heterodimeric form) as defined herein and an ABD of streptococcal protein G or a derivative thereof. Derivatives of ABDs of streptococcal protein G are known to persons of skill

in the art, for example from WO2009/016043, WO2012/004384 and WO2014/048977, all hereby incorporated by reference. The ABD may for example comprise an amino acid sequence selected from the group consisting of SEQ ID NO:547, 548 and 631. In one embodiment, the ABD comprises SEQ ID NO:547. In another embodiment, the ABD comprises SEQ ID NO:548. In a further embodiment, the ABD comprises SEQ ID NO:631. It will be appreciated that said albumin binding domain (ABD) may be positioned at the C-terminal end of the B7-H3 binding polypeptide and/or at the N-terminal end of the B7-H3 binding polypeptide.

In a specific embodiment of this aspect of the disclosure, using specific polypeptides of the disclosure as B7-H3 binding moieties, albumin binding moieties and linker sequences (see further below), the fusion protein or conjugate comprises (or consists of) an amino acid sequence selected from the group consisting of SEQ ID NO:550-566 and SEQ ID NO:632-634. In one embodiment, said amino acid sequence is selected from the group consisting of SEQ ID NO:550-557. In another embodiment, said amino acid sequence is selected from the group consisting of SEQ ID NO:558-560. In another embodiment, said amino acid sequence is selected from the group consisting of SEQ ID NO:561-563. In another embodiment, said amino acid sequence is selected from the group consisting of SEQ ID NO:564-566. In another embodiment, said amino acid sequence is selected from the group consisting of SEQ ID NO:632-634. In one embodiment, said amino acid sequence is selected from the group consisting of SEQ ID NO:557, 560, 563 and 566. In a specific embodiment, said amino acid sequence is SEQ ID NO:557. In another specific embodiment, said amino acid sequence is SEQ ID NO:560. In a specific embodiment, said amino acid sequence is SEQ ID NO:563. In a specific embodiment, said amino acid sequence is SEQ ID NO:566. In one embodiment, said amino acid sequence is selected from the group consisting of SEQ ID NO:555, 558, 561 and 564. In a specific embodiment, said amino acid sequence is SEQ ID NO:555. In another specific embodiment, said amino acid sequence is SEQ ID NO:558. In a specific embodiment, said amino acid sequence is SEQ ID NO:561. In a specific embodiment, said amino acid sequence is SEQ ID NO:564. In one embodiment, said amino acid sequence is selected from the group consisting of SEQ ID NO:556, 559, 562 and 565. In a specific embodiment, said amino acid sequence is SEQ ID NO:556. In another specific embodiment, said amino acid sequence is SEQ ID NO:559. In a specific embodiment, said amino acid sequence is SEQ ID

NO:562. In a specific embodiment, said amino acid sequence is SEQ ID NO:565. In one embodiment, said amino acid sequence is selected from the group consisting of SEQ ID NO:632, 633 and 634. In a specific embodiment, said amino acid sequence is SEQ ID NO:632. In another specific embodiment, said amino acid sequence is SEQ ID NO:633. In a specific embodiment, said amino acid sequence is SEQ ID NO:634.

In another embodiment, there is provided a fusion protein or a conjugate wherein said second moiety having a desired binding activity is a protein based on protein Z, derived from the B domain of protein A from *Staphylococcus aureus*, which has a binding affinity for a target other than B7-H3.

In another embodiment, there is provided a fusion protein or a conjugate as described herein, wherein said second moiety is selected from the group consisting of human endogenous enzymes, hormones, growth factors, chemokines, cytokines and lymphokines.

In another embodiment, there is provided a fusion protein or a conjugate wherein said second moiety having a desired binding activity is an antibody or antigen binding fragment thereof. As is well known, antibodies are immunoglobulin molecules capable of specific binding to a target (an antigen), such as a carbohydrate, polynucleotide, lipid, polypeptide or other, through at least one antigen recognition site on the immunoglobulin molecule. As used herein, the term “antibody or an antigen binding fragment thereof” encompasses not only full-length or intact polyclonal or monoclonal antibodies, but also antigen-binding fragments thereof, such as Fab, Fab′, F(ab′)2, Fab3, Fv and variants thereof, fusion proteins comprising one or more antibody portions, humanized antibodies, chimeric antibodies, minibodies, diabodies, triabodies, tetrabodies, linear antibodies, single chain antibodies, multispecific antibodies (e.g. bispecific antibodies) and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity, including glycosylation variants of antibodies, amino acid sequence variants of antibodies and covalently modified antibodies. Further examples of modified antibodies and antigen binding fragments thereof include nanobodies, AlbudAbs, DARTs (dual affinity re-targeting), BiTEs (bispecific T-cell engager), TandAbs (tandem diabodies), DAFs (dual acting Fab), two-in-one antibodies, SMIPs (small modular immunopharmaceuticals), FynomAbs (fynomers fused to

antibodies), DVD-Igs (dual variable domain immunoglobulin), CovX-bodies (peptide modified antibodies), duobodies and triomAbs. This listing of variants of antibodies and antigen binding fragments thereof is not to be seen as limiting, and the skilled person is aware of other suitable variants.

In one embodiment, said at least one antibody or antigen binding fragment thereof is selected from the group consisting of full-length antibodies, Fab fragments, Fab' fragments, $F(ab')_2$ fragments, single chain Fab (scFab) fragments, Fc fragments, Fv fragments, single chain Fv (scFv) fragments, $(scFv)_2$, scFv-Fc constructs and domain antibodies. In one embodiment, said at least one antibody or antigen binding fragment thereof is selected from full-length antibodies, Fab fragments and scFv fragments. In one particular embodiment, said at least one antibody or antigen binding fragment thereof is a full-length antibody.

In one embodiment, the antibody or antigen binding fragment thereof is selected from the group consisting of monoclonal antibodies, human antibodies, humanized antibodies, chimeric antibodies, and antigen-binding fragments thereof.

In one embodiment, the antibody or antigen binding fragment thereof has affinity for an antigen, for example an antigen associated with cancer.

A conjugate as disclosed herein may be produced by the conjugation of at least one B7-H3 binding polypeptide or fusion protein as described herein to at least one additional moiety. The skilled person is aware of conjugation methods, such as conventional chemical conjugation methods, for example using charged succinimidyl esters or carbodiimides.

The skilled person is aware that the construction of a fusion protein often involves the use of linkers between the functional moieties to be fused, and that there are different kinds of linkers with different properties, such as flexible amino acid linkers, rigid amino acid linkers and cleavable amino acid linkers. Linkers are useful to for example increase stability or improve folding of fusion proteins, increase expression, improve biological activity, enable targeting and alter pharmacokinetics of fusion proteins. Thus, in one embodiment, there is provided a B7-H3 binding polypeptide, fusion protein or conjugate according to any aspect disclosed herein further comprising at least one linker, such as at least one linker selected from flexible amino acid linkers, rigid amino acid linkers and cleavable amino acid linkers. In one embodiment, said linker is arranged between said B7-H3 binding polypeptide

and a further polypeptide domain. In one embodiment, a linker is arranged between two B7-H3 binding polypeptides or domains as disclosed herein; for example between a B7-H3 binding domain as disclosed herein and an albumin binding domain; or between a B7-H3 binding domain as disclosed herein and a Z variant binding a different target which is not B7-H3; or for example between a B7-H3 binding domain as disclosed herein and an antibody or antigen binding fragment thereof. Flexible linkers are often used in the art when the joined domains require a certain degree of movement or interaction, and may be particularly useful in some embodiments. Such linkers are generally composed of small, non-polar (for example G) or polar (for example S or T) amino acids. Some flexible linkers primarily consist of stretches of G and S residues, for example $(GGGGS)_p$. Adjusting the copy number "p" allows for optimization of linker in order to achieve appropriate separation between the functional moieties or to maintain necessary inter-moiety interaction. Apart from G and S linkers, other flexible linkers are known in the art, such as G and S linkers containing additional amino acid residues, such as T and A, to maintain flexibility, as well as polar amino acid residues to improve solubility. Additional non-limiting examples of linkers include AGS, ASGS (SEQ ID NO:589), GAPGGGGS (SEQ ID NO:590), GAPGGGGSGGGGSGGGGSTSA (SEQ ID NO:591), GGGGSLVPRGSGGGGS (SEQ ID NO:592), $(GS)_3$ (SEQ ID NO:593), $(GS)_4$ (SEQ ID NO:594), $(GS)_8$ (SEQ ID NO:595), GGSGGHMGSGG (SEQ ID NO:596), GGSGGSGGSGG (SEQ ID NO:597), GGSGG (SEQ ID NO:598), GGSGGGGG (SEQ ID NO:599), GGGSEGGGSEGGGSEGGG (SEQ ID NO:600), AAGAATAA (SEQ ID NO:601), GGGGG (SEQ ID NO:602), GGSSG (SEQ ID NO:603), GSGGGTGGGSG (SEQ ID NO:604), GSGSGSGSGGSG (SEQ ID NO:605), GSGGSGGSGGSGGS (SEQ ID NO:606), GSGGSGSGGSGGSG (SEQ ID NO:607), GGGGSAS (SEQ ID NO:608), respectively, and GT. The skilled person is aware of other suitable linkers.

In one embodiment, said linker is a flexible linker comprising glycine (G), serine (S) and/or threonine (T) residues. In one embodiment, said linker has a general formula selected from $(G_nS_m)_p$ and $(S_nG_m)_p$, wherein, independently, n = 1-7, m = 0-7, n + m ≤ 8 and p = 1-7. In one embodiment, n = 1-5. In one embodiment, m = 0-5. In one embodiment, p = 1-5. In a more specific embodiment, n = 4, m = 1 and p = 1-5. In one embodiment, said linker is selected from the group consisting of S_4G (SEQ ID NO:609), $(S_4G)_3$

(SEQ ID NO:610) and $(S_4G)_4$ (SEQ ID NO:611). In one embodiment, said linker is selected from the group consisting of G_4S (SEQ ID NO:612), $(G_4S)_2$ (SEQ ID NO:613), $(G_4S)_3$ (SEQ ID NO:614), $(G_4S)_4$ (SEQ ID NO:615) and $(G_4S)_5$ (SEQ ID NO:616). In one particular embodiment, said linker is G_4S. In another embodiment, said linker is $(G_4S)_2$. In another embodiment, said linker is $(G_4S)_3$. In another embodiment, said linker is $(G_4S)_5$.

In another embodiment, said linker is a rigid linker comprising alanine (A), glutamic acid (E), lysine (K), proline (P) and/or threonine (T) residues. Non-limiting examples of linkers include $A(EAAAK)_4A$, (SEQ ID NO:635), $A(EAAAK)_5A$ (SEQ ID NO:617), $(KEAAA)_4KAK$ (SEQ ID NO:636), $(KEAAA)_5KAK$ (SEQ ID NO:618) and $(TP)_8$ (SEQ ID NO:619).

With regard to the description above of fusion proteins or conjugates incorporating a B7-H3 binding polypeptide according to the disclosure, it is to be noted that the designation of first, second and further moieties is made for clarity reasons to distinguish between B7-H3 binding polypeptide or polypeptides according to the invention on the one hand, and moieties exhibiting the same or other functions on the other hand. These designations are not intended to refer to the actual order of the different domains in the polypeptide chain of the fusion protein or conjugate. Similarly, the designations first and second monomer units are made for clarity reasons to distinguish between said units. Thus, for example, said first moiety (or monomer unit) may without restriction appear at the N-terminal end, in the middle, or at the C-terminal end of a fusion protein or conjugate of the disclosure.

The above aspects furthermore encompass polypeptides in which the B7-H3 binding polypeptide according to the first aspect, or the B7-H3 binding polypeptide as comprised in a fusion protein or conjugate according to the second aspect, further comprises a label. In a further embodiment, said label is selected from the group consisting of fluorescent dyes and metals, chromophoric dyes, chemiluminescent compounds, bioluminescent proteins, enzymes, radionuclides, radioactive particles and pretargeting recognition tags. Such labels, which a person of skill in the art will be familiar with, may for example be used for detection of the polypeptide.

In some embodiments, the labeled B7-H3 binding polypeptide is present as a moiety in a fusion protein or conjugate also comprising a second or further moiety having a desired biological activity. The label may in some

instances be coupled only to the B7-H3 binding polypeptide, and in some instances both to the B7-H3 binding polypeptide and to the second moiety of the fusion protein or conjugate. Furthermore, it is also possible that the label may be coupled to a second moiety and not to the B7-H3 binding moiety. Hence, in yet another embodiment, there is provided a B7-H3 binding polypeptide comprising a second moiety, wherein said label is coupled to the second moiety only. Thus, when reference is made to a labeled polypeptide, this should be understood as a reference to all aspects of polypeptides as described herein, including B7-H3 binding polypeptides, fusion proteins and conjugates comprising a B7-H3 binding polypeptide.

Indirect labeling of a Z variant polypeptide was recently shown using pretargeting recognition tags (Westerlund *et al*, 2015, Bioconjugate Chem 26:1724-1736). Similarly, the disclosure provides a B7-H3-binding polypeptide as described herein labeled with a pretargeting moiety, which may then be used for indirect labeling with a moiety complementary to the pretargeting moiety. In one embodiment, there is provided a B7-H3 binding polypeptide, fusion protein or conjugate as described herein, which comprises a pretargeting recognition tag able to associate with a complement to form a complementary pair of pretargeting moieties, for example selected from strept(avidin)/biotin, oligonucleotide/complementary oligonucleotide such as DNA/complementary DNA, RNA/complementary RNA, phosphorothioate nucleic acid/ complementary phosphorothioate nucleic acid and peptide nucleic acid/complementary peptide nucleic acid and morpholinos/complementary morpholinos. In a further embodiment, said pretargeting recognition tag is a peptide nucleic acid tag. In a further embodiment, said pretargeting recognition tag is a 10-20-mer peptide nucleic acid sequence, such as a 15-mer peptide nucleic acid sequence. When comprising a pretargeting moiety, a B7-H3-binding agent of the present disclosure is able to associate with a complementary pretargeting moiety, and such complementary pretargeting moiety may then comprise or be attached to a suitable radionuclide. The skilled person is aware of suitable radionuclides for therapeutic, diagnostic and/or prognostic purposes. Such a radionuclide may be chelated to said complementary pretargeting moiety via a chelating environment as generally described for the B7-H3-binding agent below.

A majority of radionuclides have a metallic nature and metals are typically incapable of forming stable covalent bonds with elements presented

in proteins and peptides. For this reason, labeling of proteins and peptides with radioactive metals is performed with the use of chelators, i.e. multidentate ligands, which form non-covalent compounds, called chelates, with the metal ions. In an embodiment of the B7-H3-binding polypeptide, fusion protein or conjugate, the incorporation of a radionuclide is enabled through the provision of a chelating environment, through which the radionuclide may be coordinated, chelated or complexed to the polypeptide.

One example of a chelator is the polyaminopolycarboxylate type of chelator. Two classes of such polyaminopolycarboxylate chelators can be distinguished: macrocyclic and acyclic chelators.

In one embodiment, there is provided a B7-H3-binding polypeptide, fusion protein or conjugate comprising a chelating environment provided by a polyaminopolycarboxylate chelator conjugated to the B7-H3 binding polypeptide via a thiol group of a cysteine residue or an amine group of a lysine residue.

The most commonly used macrocyclic chelators for radioisotopes of indium, gallium, yttrium, bismuth, radioactinides and radiolanthanides are different derivatives of DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid). In one embodiment, a chelating environment of the B7-H3-binding polypeptide, B7-H3-binding polypeptide in heterodimeric form, fusion protein or conjugate is provided by DOTA or a derivative thereof. More specifically, in one embodiment, a chelating polypeptide encompassed by the present disclosure is obtained by reacting the DOTA derivative 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidoethylacetamide (maleimidomonoamide-DOTA) with said polypeptide. In one embodiment, a chelating polypeptide encompassed by the present disclosure is obtained by reacting the DOTA derivative DOTAGA (2,2',2''-(10-(2,6-dioxotetrahydro-2H20-pyran-3-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid) with said polypeptide. Additionally, 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) and derivatives thereof may be used as chelators. Hence, in one embodiment, a chelating environment of the B7-H3-binding polypeptide, B7-H3-binding polypeptide in heterodimeric form, fusion protein or conjugate is provided by NOTA or a derivative thereof. In one embodiment, a chelating polypeptide encompassed by the present disclosure is obtained by reacting the NOTA derivative NODAGA (2,2'-(7-(1-carboxy-4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxobutyl)-1,4,7-triazonane-1,4-diyl)diacetic acid) with said polypeptide.

The most commonly used acyclic polyaminopolycarboxylate chelators are different derivatives of DTPA (diethylenetriamine-pentaacetic acid). Hence, polypeptides, fusion proteins or conjugates having a chelating environment provided by diethylenetriaminepentaacetic acid or derivatives thereof are also encompassed by the present disclosure.

Another example of a chelator is an N_3S chelator, which is a type of peptide-based, tetradentate chelator. As the term N_3S indicates, the four attaching or coordinating groups of such a chelator are formed from three nitrogen atoms and one sulfur atom, suitably provided by consecutive amino acid residues in a polypeptide chain. In an N_3S chelator, the N and S atoms are spatially arranged to provide a suitable "pocket" for complexing or attachment of the radioactive metal.

In one embodiment, there is provided a B7-H3 binding polypeptide of the first aspect, the fusion protein, or conjugate of the second aspect, comprising a chelating environment provided by a peptide based chelator, wherein the peptide sequence encoding the peptide based chelator is located in the C-terminus of said B7-H3 binding polypeptide, fusion protein or conjugate.

The skilled person is aware of suitable peptide-based chelators for inclusion into the polypeptide chain of a B7-H3 binding molecule according to the disclosure. For example, in one specific embodiment the peptide sequence encoding the peptide-based chelator is selected from the group consisting of -GGGC (SEQ ID NO:620), -GSEC (SEQ ID NO:621), -GGSC (SEQ ID NO:622), -GGEC (SEQ ID NO:623), -GGKC (SEQ ID NO:624) and -KVDC (SEQ ID NO:625). In a specific embodiment, the peptide sequence encoding the peptide-based chelator is -GGGC.

In embodiments in which the polypeptide, fusion protein or conjugate is labeled, directly or indirectly (e.g. via pretargeting as described above), with an imaging agent (e.g. radioactive agent), measuring the amount of labeled polypeptide present in a tissue, such as in a tumor tissue, may be done using imaging equipment, such as through acquiring radioactivity counts or images of radiation density, or derivatives thereof such as radiation concentration. In one embodiment, there is provided a radiolabeled B7-H3 binding polypeptide, fusion protein or conjugate consisting of a radiochelate of the B7-H3 binding polypeptide, fusion protein or conjugate as described herein and a radionuclide. In a more specific embodiment, said radionuclide is suitable for

medical imaging. In another specific embodiment, said radionuclide is suitable for therapy. Non-limiting examples of radionuclides, suitable either for direct labeling of the B7-H3-binding agent according to any aspect disclosed herein or for indirect labeling by labeling of a complementary pretargeting moiety, include ^{225}Ac, ^{72}As, ^{212}Bi, ^{213}Bi, ^{76}Br, ^{55}Co, ^{61}Cu, ^{64}Cu, ^{67}Cu, ^{18}F, [^{18}F]AlF, ^{19}F, ^{66}Ga, ^{67}Ga, ^{68}Ga, ^{166}Ho, ^{110m}In, ^{111}In, ^{123}I, ^{124}I, ^{131}I, ^{177}Lu, ^{51}Mn, ^{52m}Mn, ^{52}Mn, ^{212}Pb, ^{149}Pm, ^{186}Re, ^{188}Re, ^{44}Sc, ^{153}Sm, ^{149}Tb, ^{152}Tb, ^{155}Tb, ^{161}Tb, ^{99m}Tc, ^{45}Ti, ^{227}Th, ^{86}Y, ^{90}Y and ^{89}Zr.

In one embodiment, wherein said radionuclide is suitable for medical imaging, said radionuclide is selected from the group consisting of ^{72}As, ^{76}Br, ^{55}Co, ^{61}Cu, ^{64}Cu, ^{18}F, [^{18}F]AlF, ^{19}F, ^{66}Ga, ^{67}Ga, ^{68}Ga, ^{110m}In, ^{111}In, ^{123}I, ^{124}I, ^{131}I, ^{177}Lu, ^{51}Mn, ^{52m}Mn, ^{52}Mn, ^{186}Re, ^{188}Re, ^{44}Sc, ^{149}Tb, ^{152}Tb, ^{155}Tb, ^{161}Tb, ^{99m}Tc, ^{45}Ti, ^{86}Y and ^{89}Zr.

In another embodiment, wherein said radionuclide is suitable for therapy, said radionuclide is selected from the group consisting of ^{225}Ac, ^{212}Bi, ^{213}Bi, ^{67}Cu, ^{166}Ho, ^{177}Lu, ^{212}Pb, ^{149}Pm, ^{186}Re, ^{188}Re, ^{153}Sm, ^{149}Tb, ^{161}Tb, ^{227}Th and ^{90}Y.

In one embodiment, the imaging equipment used in such measurements is positron emission tomography (PET) equipment, in which case the radionuclide is selected such that it is suitable for PET. The skilled person is aware of radionuclides suitable for use with PET. For example, a PET radionuclide is selected from the group consisting of ^{72}As, ^{76}Br, ^{55}Co, ^{61}Cu, ^{64}Cu, ^{18}F, [^{18}F]AlF, ^{66}Ga, ^{68}Ga, ^{110m}In, ^{44}Sc, ^{152}Tb, ^{45}Ti, ^{86}Y and ^{89}Zr. In one embodiment, there is provided a radiolabeled B7-H3 binding polypeptide, fusion protein or conjugate as described herein, wherein said nuclide is suitable for use with PET and is selected from the group consisting of ^{72}As, ^{76}Br, ^{55}Co, ^{61}Cu, ^{64}Cu, ^{18}F, [^{18}F]AlF, ^{66}Ga, ^{68}Ga, ^{110m}In, ^{44}Sc, ^{152}Tb, ^{45}Ti, ^{86}Y and ^{89}Zr.

In another embodiment, the imaging equipment used is single-photon emission computed tomography (SPECT) equipment, in which case the radionuclide is selected such that it is suitable for SPECT. The skilled person is aware of radionuclides suitable for use with SPECT. For example, a SPECT radionuclide is selected from the group consisting of ^{67}Ga, ^{111}In, ^{123}I, ^{131}I, ^{177}Lu, ^{155}Tb, ^{99m}Tc. In another embodiment there is provided a radiolabeled B7-H3 binding polypeptide, fusion protein or conjugate as described herein, wherein said nuclide is suitable for use with SPECT and is selected from the group consisting of ^{67}Ga, ^{111}In, ^{123}I, ^{131}I, ^{177}Lu, ^{155}Tb, ^{99m}Tc.

In another embodiment, said nuclide is suitable for medical imaging and the chelating environment is provided by a peptide-based chelator as described herein. In a more specific embodiment, the radionuclide is selected from the group consisting of ^{99m}Tc, ^{51}Mn, ^{52m}Mn, ^{52}Mn, ^{186}Re and ^{188}Re. In a particular embodiment, the radionuclide suitable for medical imaging is ^{99m}Tc.

In an alternative embodiment, said radionuclide is suitable for therapy and the chelating environment is provided by a peptide-based chelator as described herein. In a more specific embodiment, the radionuclide is selected from the group consisting of ^{186}Re and ^{188}Re. In a particular embodiment, the radionuclide suitable for therapy is ^{188}Re.

In these embodiments of the radiolabeled B7-H3 binding polypeptide, fusion protein or conjugate according to the second aspect, the radionuclide is complexed with the B7-H3-binding polypeptide, fusion protein or conjugate via the chelating environment provided by the C-terminal -GGGC sequence.

Thus, in one embodiment there is provided a B7-H3-binding polypeptide, fusion protein or conjugate as described herein, which comprises a direct or indirect radionuclide label, such as a radionuclide selected from the group consisting of ^{72}As, ^{76}Br, ^{55}Co, ^{61}Cu, ^{64}Cu, ^{18}F, [^{18}F]AlF, ^{19}F, ^{66}Ga, ^{67}Ga, ^{68}Ga, ^{110m}In, ^{111}In, ^{123}I, ^{124}I, ^{131}I, ^{177}Lu, ^{44}Sc, ^{99m}Tc, ^{45}Ti, ^{86}Y and ^{89}Zr, such as the group consisting of ^{68}Ga, ^{110m}In, ^{18}F, ^{152}Tb, ^{155}Tb, ^{45}Ti, ^{44}Sc, ^{61}Cu, ^{66}Ga, ^{64}Cu, ^{55}Co, ^{72}As, ^{86}Y, ^{89}Zr, ^{124}I and ^{76}Br, such as ^{18}F.

In some embodiments, the labeled B7-H3-binding polypeptide is present as a moiety in a fusion protein or conjugate also comprising a second moiety having a desired biological activity. The label may in some instances be coupled only to the B7-H3-binding polypeptide, and in some instances both to the B7-H3-binding polypeptide and to the second moiety of the fusion protein or conjugate. Furthermore, it is also possible that the label may be coupled to a second moiety only and not to the B7-H3-binding moiety. Hence, in yet another embodiment, there is provided a B7-H3-binding polypeptide comprising a second moiety, wherein said label is coupled to the second moiety only.

When reference is made to a labeled polypeptide, this should be understood as a reference to all aspects of polypeptides as described herein, including B7-H3-binding polypeptides, fusion proteins and conjugates comprising a B7-H3-binding polypeptide. Thus, a labeled polypeptide may contain only the B7-H3-binding polypeptide and e.g. a radionuclide, which may be chelated or covalently coupled to the B7-H3-binding polypeptide, or

contain the B7-H3-binding polypeptide, a radionuclide and a second moiety such as a small molecule having a desired biological activity, for example a therapeutic efficacy. A labeled polypeptide may contain a B7-H3-binding polypeptide in heterodimeric form and e.g. a radionuclide, which may be chelated or covalently coupled to the B7-H3-binding polypeptide, or contain the B7-H3-binding polypeptide in heterodimeric form, a therapeutic radionuclide and a second moiety such as a small molecule having a desired biological activity, for example a therapeutic efficacy. Further, the radionuclide as described herein may serve multiple purposes, i.e. said radionuclide may fulfill both the capacity of a radionuclide suitable for therapy and the capacity of a radionuclide suitable for medical imaging. Thus, the radionuclide labeled molecule is said to be suitable as a theranostic, which is a molecule suitable for both therapy and medical imaging. The term “theranostic” as used herein can be used interchangeably with the term “theragnostic”. Furthermore, the skilled person will understand that a theranostic or theragnostic molecule could also be understood as 1) the same molecule, such as a polypeptide or fusion protein, labeled with the same radionuclide for both imaging and therapy as described previously, 2) use of the same molecule, such as a polypeptide or fusion protein, but labeled with different radionuclides for imaging and therapy, and/or 3) use of different molecules, such as different polypeptides or different fusion proteins, each labeled with different radionuclides for imaging and therapy. This way, the qualities of a theranostic could also be fulfilled, wherein one radionuclide could be suitable for therapy while the other radionuclide could be suitable for medical imaging. The skilled person will be able to create many other possible variants based on the teaching herein. The labeled polypeptide or fusion protein may be suitable for both therapy, and for medical imaging for the purpose of detection and for monitoring of the treatment response. It can be further contemplated, that payloads other than radionuclides could be conjugated via a cysteine residue, such as cytotoxic payloads. Some examples of cytotoxic payloads that can be conjugated to cysteine residues are: maytansinoids, such as DM1 and DM4; auristatins, such as monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF); DNA-damaging agents such as duocarmycins, pyrrolobenzodiazepines (PBDs), and calicheamicins; camptothecins, such as SN-38; taxanes; tubulysins; STING-agonists; TLR-agonists; Topoisomerase II inhibitors; small interfering RNA; antisense oligonucleotides; and protein degrader payloads.

In further aspects of the present disclosure, there is provided a polynucleotide encoding a B7-H3 binding polypeptide or fusion protein as described herein; an expression vector comprising said polynucleotide; and a host cell comprising said expression vector. Also encompassed by this disclosure is a method of producing a B7-H3 binding polypeptide or fusion protein, as described above, comprising culturing said host cell under conditions permissive of expression of said polypeptide from its expression vector, and isolating the polypeptide.

The B7-H3 binding polypeptide or fusion protein of the present disclosure may alternatively be produced by non-biological peptide synthesis using amino acids and/or amino acid derivatives having protected reactive side-chains, the non-biological peptide synthesis comprising

- step-wise coupling of the amino acids and/or the amino acid derivatives to form a polypeptide or fusion protein as described herein having protected reactive side-chains,
- removal of the protecting groups from the reactive side-chains of the polypeptide or fusion protein, and
- folding of the polypeptide or fusion protein in aqueous solution.

In another aspect, there is provided a composition comprising a B7-H3 binding polypeptide, fusion protein or conjugate as described herein and at least one pharmaceutically acceptable excipient or carrier. In one embodiment, said composition further comprises at least one additional active agent, such as at least two additional active agents, such as at least three additional active agents. Non-limiting examples of additional active agents that may prove useful in such combination are anti-cancer agents as described herein in connection with the first and second aspects of the disclosure.

The small size and robustness of the B7-H3 binding polypeptides of the present disclosure confer several advantages over conventional monoclonal antibody-based therapies. Such advantages include advantages in formulation, modes of administration, such as alternative routes of administration, administration at higher molar doses than antibodies and absence of Fc-mediated side effects.

It should be understood that the B7-H3 binding polypeptide according to the present disclosure is typically useful as a therapeutic, diagnostic and/or prognostic agent in its own right. A therapeutic effect may for example be accomplished by antagonizing B7-H3 action.

Thus, in one aspect of the present disclosure, there is provided a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein for use, e.g. *in vivo* use, as a medicament, a diagnostic agent and/or a prognostic agent.

In one embodiment, said B7-H3 binding polypeptide, fusion protein, conjugate or composition is provided for use as a medicament. In a more specific embodiment, there is provided a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein, for use as a medicament, wherein said polypeptide, fusion protein, conjugate or composition modulates B7-H3 function *in vivo*. As used herein, the term "modulate" refers to changing the activity, such as partially inhibiting or fully inhibiting B7-H3 function.

In one embodiment, said B7-H3 binding polypeptide, fusion protein, conjugate or composition is provided for use as a diagnostic agent and/or as a prognostic agent. In another embodiment, said B7-H3 binding polypeptide, fusion protein, conjugate or composition is provided for use as a diagnostic agent *in vivo* and/or as a prognostic agent *in vivo*. In a more specific embodiment, there is provided a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein, for use as a diagnostic agent *in vivo*. In another more specific embodiment, there is provided a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein, for use as a prognostic agent *in vivo*. In another specific embodiment, there is provided a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein, for use as a diagnostic agent, wherein said use in diagnosis is carried out *in vitro*. In another specific embodiment, there is provided a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein, for use as a prognostic agent, wherein said use in prognosis is carried out *in vitro*.

In one embodiment, there is provided a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein for use in the treatment, prognosis or diagnosis of a B7-H3 related disorder or disease.

As used herein, the term “B7-H3 related disorder or disease” refers to any disorder, disease or condition in which B7-H3 action plays a role and/or wherein targeting or modulation (e.g. inhibition) of B7-H3 may be beneficial. Such a disorder, disease or condition may be selected from the group consisting of cancers. Non-limiting examples include breast cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, gastric cancer, liver cancer including hepatocellular carcinoma, lung cancer including non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, renal cell carcinoma, sarcomas including osteosarcoma, urothelial cell carcinoma, neuroblastoma, medulloblastoma, glioma including glioblastoma and diffuse intrinsic pontine glioma, melanoma, leukemia and mesothelioma.

In one embodiment, there is provided a B7-H3 binding polypeptide, fusion protein, conjugate or composition for use in the treatment, diagnosis, or prognosis of a B7-H3 related disorder or disease, wherein said B7-H3 related disorder or disease is cancer. In one embodiment, said cancer is a cancer selected from the group consisting of: breast cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, gastric cancer, liver cancer including hepatocellular carcinoma, lung cancer including non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, renal cell carcinoma, sarcomas including osteosarcoma, urothelial cell carcinoma, neuroblastoma, medulloblastoma, glioma including glioblastoma and diffuse intrinsic pontine glioma, melanoma, leukemia and mesothelioma. In a specific embodiment, said cancer is selected from the group consisting of: breast cancer, pancreatic cancer and sarcomas.

It is to be understood that said B7-H3 binding polypeptide, fusion protein, conjugate or composition may be used as the sole therapeutic, diagnostic or prognostic agent or as a companion therapeutic, companion diagnostic and/or companion prognostic agent.

As such, in one embodiment of a therapeutic use, it is beneficial to administer a therapeutically effective amount of a B7-H3-binding polypeptide, fusion protein, conjugate or composition as described herein, together with at least one second drug substance, such as an anti-cancer agent.

The agents of the present disclosure are contemplated for oral, topical, intravenous, intraperitoneal, subcutaneous, pulmonary, transdermal, intramuscular, intranasal, buccal, sublingual or suppository administration. In particular for diagnostic imaging and radiotherapeutic applications,

administration via the intravenous or subcutaneous route is preferred. Thus, in another aspect of the disclosure, there is provided a B7-H3 binding polypeptide, fusion protein, conjugate or composition for use as described herein, wherein administration is selected from the group consisting of: oral, topical, intravenous, intraperitoneal, subcutaneous, pulmonary, transdermal, intramuscular, intranasal, buccal, sublingual or suppository administration, such as subcutaneous administration, such as intravenous administration.

In another aspect of the present disclosure, there is provided a method of treatment of a B7-H3 related disorder, comprising administering to a subject in need thereof an effective amount of a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein.

In one embodiment, there is provided a method of treatment of a B7-H3 related disorder, comprising administering to a subject in need thereof an effective amount of a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein wherein said B7-H3 binding polypeptide, fusion protein or conjugate modulates B7-H3 function *in vivo*.

In a further aspect of the present disclosure, there is provided a method of detecting the presence of B7-H3 in a sample, comprising providing a sample suspected to contain B7-H3, contacting said sample with a B7-H3 binding polypeptide, fusion protein, conjugate or composition as provided for herein, and detecting the binding of the B7-H3 binding polypeptide, fusion protein, conjugate or composition to indicate the presence of B7-H3 in the sample.

In another aspect of the present disclosure, there is provided a method for determining the presence of B7-H3 in a subject, comprising the steps of:

a) contacting the subject, or a sample isolated from the subject, with a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein; and

b) obtaining a value corresponding to the amount of the B7-H3 binding polypeptide, fusion protein, conjugate or composition that has bound in said subject or to said sample.

In another aspect of the present disclosure, there is provided a method for determining the presence of B7-H3 in a subject as described herein, which is a method for medical imaging in which:

- step a) comprises the systemic administration of said B7-H3 binding polypeptide, fusion protein, conjugate or composition to a subject;
- said B7-H3 binding polypeptide, fusion protein, conjugate or composition comprises a radionuclide label suitable for medical imaging; and
- step b) comprises obtaining one or more images of at least a part of the subject's body using a medical imaging instrument, said image(s) indicating the presence of the radionuclide inside the body.

In one embodiment of said method for medical imaging, said B7-H3 binding polypeptide, fusion protein or conjugate comprises a pretargeting recognition tag as described herein, or said composition comprises such a B7-H3 binding polypeptide, fusion protein or conjugate, and step a) further comprises contacting the subject with a complementary pretargeting moiety labeled with a detectable label, such as a radionuclide label.

In a related aspect, there is provided a method of diagnosis *in vivo*, comprising the steps:

- contacting a subject with a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein;
- detecting the binding of the B7-H3 binding polypeptide, fusion protein, conjugate or composition to indicate the presence of B7-H3 in the subject; and
- using the information obtained to establish a diagnosis.

In another related aspect, there is provided a method of prognosis *in vivo*, comprising the steps:

- contacting a subject with a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein;
- detecting the binding of the B7-H3 binding polypeptide, fusion protein, conjugate or composition to indicate the presence of B7-H3 in the subject; and
- using the information obtained to establish a prognosis.

In a related aspect, there is provided a method of diagnosis *in vitro*, comprising the steps:

- providing a sample suspected to contain B7-H3;

- contacting said sample with a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein;
- detecting the binding of the B7-H3 binding polypeptide, fusion protein, conjugate or composition to indicate the presence of B7-H3 in the sample; and
- using the information obtained to establish a diagnosis.

In another related aspect, there is provided a method of prognosis *in vitro*, comprising the steps:

- providing a sample suspected to contain B7-H3;
- contacting said sample with a B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein;
- detecting the binding of the B7-H3 binding polypeptide, fusion protein, conjugate or composition to indicate the presence of B7-H3 in the sample; and
- using the information obtained to establish a prognosis.

The above mentioned aspects may comprise a further step, such that the described methods are a part of a monitoring process of the subject before, during or after treatment. Therefore, in a related embodiment, there is provided a method of diagnosis or prognosis *in vivo* or *in vitro* as described herein, further comprising the steps:

- repeating the steps of detection, wherein said detecting is performed at several time points at intervals in the subject, in the same provided sample or in a different provided sample, as part of a monitoring of the subject before, during, or after treatment.

In another embodiment, there is provided a method of diagnosis *in vitro* or prognosis *in vitro* as described herein, further comprising a step of comparing the value corresponding to the amount of bound B7-H3 binding polypeptide to a reference value.

In another embodiment, said diagnosis or prognosis is in relation to a B7-H3 related disorder or disease.

In one embodiment of each of the methods for diagnosis *in vivo* and prognosis *in vivo*, said B7-H3 binding polypeptide, fusion protein or conjugate comprises a pretargeting recognition tag as described herein, or said composition comprises such a B7-H3 binding polypeptide, fusion protein or conjugate, and the contacting step further comprises contacting the subject

with a complementary pretargeting moiety labeled with a detectable label, such as with a radionuclide label.

The skilled person will appreciate that any description in relation to the use of B7-H3 binding polypeptide, fusion protein, conjugate or composition as described herein for treatment, diagnosis or prognosis of a disease or disorder is equally relevant for the related therapeutic, diagnostic, imaging, prognostic or theranostic methods of the disclosure. For the sake of brevity, such description will not be repeated here.

While the invention has been described with reference to various exemplary aspects and embodiments, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the scope of the invention. In addition, many modifications may be made to adapt a particular situation or molecule to the teachings of the invention without departing from the essential scope thereof. Therefore, it is intended that the invention not be limited to any particular embodiment contemplated, but that the invention will include all embodiments falling within the scope of the appended claims.

Brief description of the figures

Figure 1 shows examples of sensorgrams for four His_6-Z variants binding to B7-H3(2Ig)-Fc analyzed by surface plasmon resonance in a single cycle kinetic screen as described in Example 4. ZBH480 (top solid line), ZBH482 (middle solid line) and ZBH481 (lower solid line) and reference sequence ZAC12 (broken line).

Figure 2 shows binding of the indicated Z variants to B7-H3 expressing SKOV-3 cells, evaluated by fluorescence intensity as described in Example 4. Cells were incubated with a decreasing concentration (from 500 nM to 32 pM) of (A) recombinantly produced His_6-Z variants and (B) synthesized Z variants, respectively.

Figure 3 shows examples of circular dichroism (CD) spectra collected as described in Example 4. (A) CD spectra collected before (broken line) and after (solid line) heat-induced denaturation of ZBH480 and (B) melting curve of ZBH480.

Figure 4 shows examples of sensorgrams for the Z variant ZBH538 binding to Fc-fused B7-H3 from (A) human (B7-H3(2Ig)-Fc), (B) cynomolgus monkey (cB7-H3(4Ig)-Fc), (C) mouse (mB7-H3(2Ig)-Fc) and (D) rat (rB7-

H3(2Ig)-Fc) analyzed by surface plasmon resonance as described in Example 4. The Z variant was injected at four concentrations (0.56 nM (lower broken line), 1.67 nM, 5 nM and 15 nM (top solid line)) over the Fc-fused B7-H3 protein captured on the surface of a Protein A chip.

Figure 5 shows examples of sensorgrams for six His_6-Z variants from the second maturation (gray solid lines; ZBH001, ZBH002, ZBH003, ZBH007, ZBH009 and ZBH011) binding to B7-H3(2Ig)-Fc analyzed by surface plasmon resonance in a single cycle kinetic screen as described in Example 7. The reference variant ZAC12 (black broken line) and ZBH481 from the first maturation (black solid line), analyzed in parallel, are included for comparison.

Figure 6 shows binding of six His_6-Z variants to B7-H3 expressing SKOV-3 cells evaluated by fluorescence intensity as described in Example 7. Cells were incubated with a decreasing concentration (500 nM to 21 pM) of the respective indicated His_6-Z variant. The reference variant ZAC12 (open circles) and ZBH481 from the first maturation (open squares), analyzed in parallel, are included for comparison.

Figure 7 shows examples of circular dichroism (CD) spectra collected as described in Example 7. (A) CD spectra collected before (broken line) and after (solid line) heat-induced denaturation of ZBH001 and (B) melting curve of ZBH001.

Figure 8 shows SPR analysis of binding to B7-H3 by the dimeric polypeptide ZBHD01 (broken grey line) versus the binding by the monomeric Z variant ZBH538 (solid black line), as described in Example 8. The variants were injected over B7-H3 (B7-H3(4Ig)-Fc) captured on a Protein A chip.

Figure 9 shows binding to SKOV-3 cells of the dimeric polypeptide ZBHD01 versus the monomeric Z variant ZBH538, each incubated at decreasing concentrations (555 nM to 28 pM) as described in Example 8.

Figure 10 shows *in vitro* binding specificity of (A) ^{99m}Tc-ZBH536, (B) ^{99m}Tc-ZBH538, (C) ^{99m}Tc-ZBH539 and (D) the reference ^{99m}Tc-ZAC12c for BT474 and SKOV-3 cells, studied as described in Example 10. For the pre-saturation of B7-H3, a 200-fold molar excess of a non-labeled Z variant was added before addition of the labeled conjugate. Data are normalized to the average value of cell-associated radioactivity for non-blocked cells for each cell line. The data are presented as an average value from three samples ± SD.

Figure 11 shows the Interaction Map of (A) ^{99m}Tc-ZBH536, (B) ^{99m}Tc-ZBH538, (C) ^{99m}Tc-ZBH539 and (D) the reference ^{99m}Tc-ZAC12c binding to

SKOV-3 cells. Input data were obtained from LigandTracer measurement of cell-bound activity during association of labeled conjugate to, and dissociation from, SKOV-3 cells. Binding was measured at two different concentrations, 1 and 3 nM, for ^{99m}Tc-ZBH536, ^{99m}Tc-ZBH538 and ^{99m}Tc-ZBH539 and at three different concentrations, 2, 6 and 18 nM, for ^{99m}Tc-ZAC12c. Measurements were performed in duplicates.

Figure 12 shows comparative biodistribution of the indicated ^{99m}Tc-labeled Z variants in different organs and tissues of female NMRI mice at 4 h after injection. 3 µg of labeled conjugate (60 kBq) was injected into the tail vein. Data are expressed as the percentage of administered activity (injected probe) per gram of tissue (% ID/g) and are averages from four mice ± SD.

Figure 13 shows the (A) biodistribution and (B) tumor-to-organ ration of the indicated ^{99m}Tc-labeled Z variants 4 h after injection in BALB/C nu/nu mice bearing SKOV-3 xenografts. 3 µg of labeled conjugate (60 kBq) was injected into the tail vein. Data are expressed as % ID/g and are averages from four mice ± SD.

Figure 14 shows uptake of ^{99m}Tc-ZBH538 in SKOV-3 (B7-H3-positive) and Ramos (B7-H3-negative) xenografts at 4 h after injection. Data are expressed as %ID/g and are averages from four mice ± SD. P-value was obtained in unpaired t-test.

Figure 15 shows the imaging of (A) ^{99m}Tc-ZBH536, (B) ^{99m}Tc-ZBH538, (C) ^{99m}Tc-ZBH539 and (D) the reference ^{99m}Tc-ZAC12c in BALB/C nu/nu mice bearing B7-H3-positive SKOV-3 xenografts 4 h after injection. 3 µg of labeled Z variant (6 MBq) was injected into the tail vein. Arrows point at tumors (T) and liver (L).

Figure 16 shows the imaging of (A) ^{99m}Tc-ZBH536, (B) ^{99m}Tc-ZBH538, (C) ^{99m}Tc-ZBH539 in BALB/C nu/nu mice bearing B7-H3-negative Ramos xenografts 4 h after injection. 3 µg of labeled Z variant (6 MBq) was injected into the tail vein. Arrows point at tumors (T).

Figure 17 shows the Interaction Map of binding to SKOV-3 cells by (A) ^{111}In-ZBH538 and (B) the reference ^{111}In-ZAC12c. Input data were obtained from LigandTracer measurement of cell-bound activity during association of labeled conjugate to, and dissociation from, SKOV-3 cells. Binding was measured at three different concentrations: 1, 3 and 9 nM for ^{111}In-ZBH538, and 2, 6 and 18 nM for ^{111}In-ZAC12c. Measurements were performed in duplicates.

Figure 18 shows the biodistribution of ^{111}In-ZBH538 and the reference ^{111}In-ZAC12c 4 h (A) and 24 h (B) after injection in BALB/C nu/nu mice bearing SKOV-3 xenografts.

Figure 19 shows the tumor-to-organ ration of ^{111}In-ZBH538 and the reference ^{111}In-ZAC12c 4 h (A) and 24 h (B) after injection in BALB/C nu/nu mice bearing SKOV-3 xenografts.

Figure 20 shows (A) the biodistribution and (B) the tumor-to-organ ration of a direct comparison of ^{111}In-labeled and ^{99m}Tc-labeled ZBH538 and reference ZAC12c 4 h after injection in BALB/C nu/nu mice bearing SKOV-3 xenografts.

Figure 21 shows the imaging of ^{111}In-ZBH538 and reference ^{111}In-ZAC12c 4 h after injection in BALB/C nu/nu mice bearing (A) B7-H3-positive SKOV-3 xenografts and (B) B7-H3-negative Ramos xenografts. Arrows point at tumors (T) and liver (L).

Figure 22 shows SPR analysis of binding to B7-H3 by the ABD-fused polypeptides ZBHD02-ZBHD09. The sensorgrams show injection of 45 nM of the variants over immobilized B7-H3(4Ig)-His.

Figure 23 shows the result of the SPR analysis of binding to HSA and MSA, respectively, by the ABD-fused polypeptides ZBHD02, ZBHD07, ZBHD08 and ZBHD09 binding.

Figure 24 shows binding of the ABD-fused polypeptides ZBHD02-ZBHD09 to B7-H3 expressing SKOV-3 cells, evaluated by fluorescence intensity. Cells were incubated with a decreasing concentration (500 nM to 0.16 nM) of the respective indicated polypeptide.

Figure 25 shows the biodistribution of ABD-fused Z variants in BALB/C nu/nu mice bearing SKOV3 xenografts. (A) ^{177}Lu-ZBHD02, ^{177}Lu-ZBHD07, ^{177}Lu-ZBHD08, and ^{177}Lu-ZBHD09 48 h after injection. (B) ^{177}Lu-ZBHD02, 24, 48 and 168 h after injection.

Figure 26 shows the imaging of (A) ^{177}Lu-ZBHD02, (B) ^{177}Lu-ZBHD07, (C) ^{177}Lu-ZBHD08, and (D) ^{177}Lu-ZBHD09 48 h after injection in BALB/C nu/nu mice bearing B7-H3-positive SKOV-3 xenografts.

Figure 27 shows the imaging of ^{177}Lu-ZBHD02 48 h after injection in BALB/C nu/nu mice bearing (A) B7-H3-positive SKOV-3 xenografts and (B) B7-H3-negative Ramos xenografts. Arrows point at tumors (T).

Figure 28 demonstrates *in vivo* B7-H3 specificity of Z variants. Uptake of (A) ^{68}Ga-ZBH001, (B) ^{68}Ga-ZBH002, (C) ^{68}Ga-ZBH003 and (D) the reference ^{68}Ga-ZAC12, in SKOV-3 (B7-H3-positive) and Ramos (B7-H3-

negative) xenografts, respectively, at 2 h after injection. Data are expressed as %ID/g and are averages from four mice±SD.

Figure 29 shows the (A) biodistribution and (B) tumor-to-organ ration of the indicated ^{68}Ga-labeled Z variants 2 h after injection in BALB/C nu/nu mice bearing SKOV-3 xenografts. 2 µg of labeled conjugate (400 kBq) was injected into the tail vein. Data are expressed as % ID/g and are averages from four mice ± SD.

Figure 30 shows the imaging of (A) ^{68}Ga-ZBH003, (B) ^{68}Ga-ZBH001, (C) ^{68}Ga-ZBH002, and (D) the reference ^{68}Ga-ZAC12 2 h after injection in BALB/C nu/nu mice bearing B7-H3-positive SKOV-3 xenografts.

Figure 31 shows the imaging of ^{68}Ga-ZBH003 after injection in BALB/C nu/nu mice bearing (A) B7-H3-positive SKOV-3 xenografts and (B) B7-H3-negative Ramos xenografts. Arrows point at tumors (T).

Examples

Summary

The following Examples disclose the development of novel Z variant molecules targeting B7-H3, based on phage display technology. The polypeptides selected as described herein were sequenced, and their amino acid sequences are listed in the sequence listing with the indicated sequence identifiers. The Examples further describe the characterization of these selected B7-H3 binding polypeptides, variants and derivatives thereof, as well as fusions proteins comprising them, and demonstrate their *in vitro* and *in vivo* functionality.

Various B7-H3 proteins used in the Examples are listed in Table 2.

Table 2: B7-H3 proteins used in selection, screening and characterization of B7-H3 binding polypeptides

Designation	Description	Species	Vendor	Cat. no.
B7-H3(2Ig)-His	B7-H3(2Ig), His tag	Human	R&D System	1949-B3
B7-H3(2Ig)-Fc	B7-H3(2Ig)-Fc	Human	R&D System	1027-B3
B7-H3(4Ig)-His	B7-H3(4Ig), His tag	Human	R&D	2318-B3
B7-H3(4Ig)-Fc	B7-H3(4Ig)-Fc	Human	Acro biosystems	B7B-H5258
b-B7-H3(4Ig)-His_1	B7-H3(4Ig), His tag, biotinylated	Human	Sino Biological	158-11188-H08H-B

b-B7-H3(4Ig)-His_2	B7-H3(4Ig), His tag, Avitag™,biotinylated	Human	R&D Systems	AVI2318-50
b-B7-H3(4Ig)-Fc	B7-H3(4Ig), Fc, Avitag™, biotinylated	Human	ACRO biosystems	B7B-H82F5
rB7-H3(2Ig)-Fc	B7-H3(2Ig), human Fc tag	Rat	SinoBiological	80380-R02H
cB7-H3(4Ig)-Fc	B7-H3(4Ig), human Fc tag	Cynomolgus	SinoBiological	90806-C02H
mB7-H3(2Ig)-Fc	B7-H3(2Ig), human Fc tag	Mouse	SinoBiological	50973-M02H

Example 1
Design and construction of a maturation library of B7-H3 binding Z variants

Summary

In this Example, a phage selection library was designed partly based on four B7-H3 binding variants previously identified by yeast display technology as described in Stern *et al., supra* and in WO2020041626. The maturation library contained approximately 6×10^9 individual clones.

Materials and methods

Design and construction of an affinity maturation B7-H3 library: A library was designed partly based on four B7-H3 binding sequences described in Stern *et al. supra*. 13 surface exposed positions in the Z molecule scaffold were biased towards certain amino acid residues, according to a strategy mainly based on the binding motifs of the Z variants defined in SEQ ID NO:541-544. The library design is shown in Table 3, indicating the percentages of the amino acids used in each of the 13 randomized positions.

Table 3: Design of the B7-H3 maturation library

		Position in ***BM***												
		2	3	4	6	7	10	11	17	18	20	21	25	28
Amino acid		**Position in full length Z variant**												
		9	10	11	13	14	17	18	24	25	27	28	32	35
A	Ala	4.4	8.0	5.6	8.0	8.0	8.0	4.2	2.9	4.1	8.0	8.0	30.0	3.2
C	Cys	0	0	0	0	0	0	0	0	0	0	0	0	0
D	Asp	4.4	3.5	5.6	3.5	2.7	3.0	4.2	2.9	4.1	3.0	2.7	2.4	3.2
E	Glu	4.4	3.5	5.6	3.5	2.7	3.0	4.2	2.9	4.1	3.0	2.7	2.4	3.2
F	Phe	4.4	8.0	5.6	8.0	2.7	8.0	8.0	2.9	4.1	8.0	8.0	8.0	3.2
G	Gly	0	0	5.6	0	2.7	0	0	2.9	30.0	0	0	0	0
H	His	4.4	3.5	5.6	3.5	8.0	3.0	4.2	30.0	4.1	3.0	2.7	2.4	8.0
I	Ile	4.4	30.0	5.6	8.0	8.0	30.0	4.2	2.9	4.1	30.0	8.0	8.0	3.2
K	Lys	30.0	3.5	5.6	3.5	2.7	3.0	4.2	2.9	4.1	8.0	2.7	2.4	3.2
L	Leu	4.4	8.0	5.6	30.0	2.7	8.0	4.2	2.9	8.0	8.0	8.0	8.0	3.2
M	Met	0	0	5.6	0	0	0	0	2.9	0	0	30.0	0	3.2
N	Asn	4.4	3.5	5.6	3.5	2.7	3.0	4.2	8.0	4.1	3.0	2.7	2.4	30.0
P	Pro	0	0	0	0	0	0	0	0	0	0	0	0	0
Q	Gln	4.4	3.5	5.6	3.5	8.0	3.0	4.2	8.0	4.1	3.0	2.7	2.4	8.0
R	Arg	8.0	3.5	5.6	3.5	2.7	3.0	4.2	2.9	4.1	3.0	2.7	8.0	3.2
S	Ser	4.4	3.5	5.6	3.5	30.0	3.0	4.2	8.0	4.1	3.0	2.7	8.0	8.0
T	Thr	4.4	3.5	5.6	3.5	2.7	3.0	4.2	8.0	4.1	3.0	2.7	2.4	8.0
V	Val	4.4	8.0	5.6	8.0	8.0	8.0	4.2	2.9	4.1	8.0	8.0	8.0	3.2
W	Trp	4.4	3.5	5.6	3.5	2.7	3.0	30.0	2.9	4.1	3.0	2.7	2.4	3.2
Y	Tyr	4.4	3.5	5.6	3.5	2.7	8.0	8.0	2.9	4.1	3.0	2.7	2.4	3.2

Two oligonucleotides, one forward and one reverse complementary, with complementary 3'-ends were generated using TRIM technology. The oligos were ordered from Ella Biotech GmbH (Martinsried, Germany).

The construction of the library was performed essentially as described earlier (e.g. PCT publication WO2017/072280), in this case in a vector denoted pAY04242, but with the following exceptions: 1) transformation was done using approximately 270 ng per electroporation into electrocompetent *Escherichia coli* (*E. coli*) XL-1 Blue cells (Agilent technologies, cat. no. 200268); 2) cells were pooled after the electroporation, incubated in recovery medium (Lucigen) for 60 min at 37°C and thereafter cultivated for approximately 8 h in 2 L TSB-YE medium [30 g/L tryptic soy broth; 5.0 g/L yeast extract], supplemented with 10 µg/mL tetracycline and 100 µg/mL carbenicillin. The library quality and the distribution of amino acids were verified by sequencing essentially as described in WO2009/077175. In this library, an albumin binding domain (ABD, domain GA3 of protein G from *Streptococcus* strain G148; SEQ ID NO:548) is used as fusion partner to the Z variants.

Preparation of library phage stock: Cells from a glycerol stock containing the phagemid library were inoculated into 3 L TSB [30 g/L tryptic

soy broth] supplemented with 100 µg/mL carbenicillin, 10 µg/mL tetracycline and 1% glucose and cultivated at 70 rpm and 37°C until an optical density at 600 nm (OD_{600}) of 0.79 was reached. The cultivation was infected using a 50× molar excess of M13K07 helper phage (New England Biolabs) and the cells were incubated for 1.5 h at 37°C. Cells were pelleted and resuspended in 3 L TSB+YE supplemented with 100 µg/mL carbenicillin, 25 µg/mL neomycin and 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The cultivation was incubated at 30°C and 70 rpm and harvested after 20 h. The cells in the cultivation were removed by centrifugation. The phage particles were precipitated from the supernatant twice using polyethylene glycol/sodium chloride (PEG/NaCl) and filtered essentially as described in Grönwall *et al.*, 2007 J Biotechnol 128:162-183, and finally dissolved in phosphate buffered saline (PBS: Dulbecco's Gibco DPBS; 9.6 mM phosphate, 138 mM NaCl, 2.67 mM KCl, pH 7.4) and glycerol. Phage stocks were stored at −80°C until used in selection.

Results

Library construction: The library was designed based on four B7-H3 binding variants described in Stern *et al. supra*. The theoretical size of the designed library was 7.3×10^{15} Z variants. The actual size of the library, determined by titration after transformation to *E. coli.* XL-1 Blue cells, was 6×10^{9} transformants. The library quality was tested by sequencing of 96 transformants and by comparing their actual sequences with the theoretical design. Sequence analysis of individual library members verified a distribution of codons in accordance with the theoretical design. The library was designated Zlib008B7-H3.I.

Example 2

Selection and screening of B7-H3 binding Z variants

Summary

In this Example, two different recombinantly produced extracellular domains of human B7-H3 were used as targets in phage display selections using a maturation phage library of Z variants. Selected clones were cloned in bulk in an expression vector driven by a T7 promoter, DNA sequenced,

produced in *E. coli* and assayed against B7-H3 in ELISA (enzyme-linked immunosorbent assay).

Materials and methods

Phage display selection of B7-H3 binding Z variants: The phage stock of the library Zlib008B7-H3.I, designed and constructed as described in Example 1, was used in selection against two different biotinylated B7-H3 target proteins, b-B7-H3(4Ig)-His_1 and b-B7-H3(4Ig)-His_2 (see Table 2). Selection was performed using either a solid phase of streptavidin beads (SA beads, Speedbead magnetic SA, Cytiva) in track 1–2 or in solution in track 3–10 in the first cycle. In cycle 3, neutravidin beads (NA beads, Speedbead magnetic NA, Cytiva) were used. As selection proceeded, tracks 1–4 were further divided according to target concentration and number and/or time of washes.

In order to reduce the amount of background binders, pre-selection was performed in cycle 1–2 using SA beads and in cycle 3 using NA beads. During pre-selection, the phage stock was incubated with coated beads for 30–40 min at RT. Beads used in the pre-selections or selection were pre-blocked with PBS supplemented with 3% bovine serum albumin (BSA, Sigma) and 0.1% Tween20 (PBSTB) and all tubes used were of non-stick type (Preblocked 1.5 ml tubes (Protein LoBind), Eppendorf).

Selection was performed in PBSTB at 37°C. The time for selection was between 20–120 min followed by catch of target-phage particle complexes on SA beads and on NA beads in cycle 3. For solid-phase selection, the biotinylated B7-H3 was already immobilized on SA beads prior to the selection. Finally, the different beads with target-phage particle complexes were washed with PBS supplemented with 0.1% Tween-20 (PBST0.1%) during approximately 30 s per wash. Selection steps, block of beads, preselection and washing steps were made either manually in Eppendorf tubes or in a KingFisher Duo instrument (Thermo Fisher) using KingFisher Deepwell 96 Plates (Thermo Fisher) or a combination thereof. In addition to the above, phage stocks in some of the tracks were heated to 70°C before use in selection.

An overview of the selection strategy, describing an increased stringency in successive cycles with a lowered target concentration and an increased number of washes, is shown in Table 4. Elution was carried out as described in WO2009/077175.

Table 4: Overview of the selection against B7-H3 using maturation library Zlib008B7-H3.I

Cycle	Sel. track	Phage stock from library or selection track(s)	Pre-heated phage stock? (Y/N)	Target	Target conc. (nM)	HSA included? (Y/N)	Selection time (min)	Number of washes
1	1	Zlib008B7-H3.I	N	b-B7-H3(4Ig)-His_1	50	N	120	2
1	2	Zlib008B7-H3.I	N	b-B7-H3(4Ig)-His_2	50	N	120	2
1	3	Zlib008B7-H3.I	N	b-B7-H3(4Ig)-His_1	50	N	120	2
1	4	Zlib008B7-H3.I	N	b-B7-H3(4Ig)-His_2	50	N	120	2
1	5	Zlib008B7-H3.I	N	b-B7-H3(4Ig)-His_1	5	N	20	18
1	6	Zlib008B7-H3.I	N	b-B7-H3(4Ig)-His_1	5	Y	20	18
1	7	Zlib008B7-H3.I	Y	b-B7-H3(4Ig)-His_1	5	N	20	18
1	8	Zlib008B7-H3.I	N	b-B7-H3(4Ig)-His_1	5	N	20	18
1	9	Zlib008B7-H3.I	N	b-B7-H3(4Ig)-His_1	5	Y	20	18
1	10	Zlib008B7-H3.I	Y	b-B7-H3(4Ig)-His_1	5	N	20	18
2	11	1, 3	N	b-B7-H3(4Ig)-His_1	25	N	60	4
2	12	2, 4	N	b-B7-H3(4Ig)-His_2	25	N	60	4
2	13	1, 3	N	b-B7-H3(4Ig)-His_1	10	N	30	10
2	14	2, 4	N	b-B7-H3(4Ig)-His_2	10	N	30	10
2	15	1, 3	N	b-B7-H3(4Ig)-His_1	25	N	60	4
2	16	2, 4	N	b-B7-H3(4Ig)-His_2	25	N	60	4
2	17	1, 3	N	b-B7-H3(4Ig)-His_1	10	N	60	6
2	18	2, 4	N	b-B7-H3(4Ig)-His_2	10	N	60	6
3	19	11, 13, 15, 17	N	b-B7-H3(4Ig)-His_1	20	N	60	6
3	20	11, 13, 15, 17	Y	b-B7-H3(4Ig)-His_1	20	N	60	6
3	21	12, 14, 16, 18	N	b-B7-H3(4Ig)-His_2	20	N	60	6
3	22	12, 14, 16, 18	N	b-B7-H3(4Ig)-His_1	20	N	60	6
3	23	11, 13, 15, 17	N	b-B7-H3(4Ig)-His_1	20	N	75	6
3	24	11, 13, 15, 17	N	b-B7-H3(4Ig)-His_1	10	N	75	10
3	25	12, 14, 16, 18	N	b-B7-H3(4Ig)-His_2	20	N	75	6
4	26	19, 23, 24	N	b-B7-H3(4Ig)-His_1	8	N	40	10
4	27	19, 23, 24	N	b-B7-H3(4Ig)-His_1	1	N	40	24
4	28	19, 20, 23, 24	N	b-B7-H3(4Ig)-His_1	8	Y	40	10
4	29	20	Y	b-B7-H3(4Ig)-His_1	8	N	40	10
4	30	20	Y	b-B7-H3(4Ig)-His_1	1	N	40	24
4	31	21, 25	N	b-B7-H3(4Ig)-His_2	8	N	40	10
4	32	22	N	b-B7-H3(4Ig)-His_2	8	N	40	10
4	33	19, 23, 24	N	b-B7-H3(4Ig)-His_1	8	N	40	10
4	34	19, 23, 24	N	b-B7-H3(4Ig)-His_1	1	N	40	24
4	35	21, 22, 25	N	b-B7-H3(4Ig)-His_2	8	N	40	10

Amplification and preparation of phage particles: Amplification of phage particles between the different selection cycles was performed as follows. *E. coli* strain XL-1 Blue was used for phage amplification and M13K07 helper phage was used in 55–65 × excess. XL-1 Blue was cultivated in TSB medium (Tryptic Soy Broth, 30 g/L) supplemented with 1% glucose and 1 µg/mL tetracycline at 37°C to early log phase and thereafter infected with phage particles. 22–2500 × excess of bacteria was used compared to the phage

particle amount. The infection was allowed for 30 min at 37°C after which the medium volume was doubled by addition of TSB medium supplemented with 1% glucose, 1 µg/mL tetracycline and 200 µg/mL of carbenicillin. After incubation for approximately 1 h at 37°C, helper phage was added and incubated for 1.5 h at 37°C. Superinfected bacteria were pelleted by centrifugation and resuspended in 50 mL TSB+YE medium supplemented with 25 µg/mL neomycin and 0.1 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside) and incubated at 30°C overnight. The overnight cultures were pelleted and phage particles in the supernatant were precipitated twice with PEG/NaCl. Finally, the phage particles were re-suspended in selection buffer before entering the next selection cycle.

Bulk cloning: In the last selection cycle, log phase bacteria were infected with eluate and grown overnight at 37°C in TSB supplemented with 0.1 µg/mL carbenicillin. Each cultivation was pelleted, and plasmid DNA was prepared using QIAprep Spin Miniprep (Qiagen). Each plasmid preparation was used separately to subclone phage selected variants in a bulk manner using standard molecular cloning methods to a T7 promoter driven expression vector and with an N-terminal His_6-tag. Transformed *E. coli* T7E2 cells (GeneBridges) were spread onto TBAB agar plates (30 g/L tryptose blood agar base, Oxoid) supplemented with 0.2 g/L neomycin and 1% glucose. The Z gene fragments were subcloned into T7 promoter driven expression vectors, resulting in the encoded sequences MGSSHHHHHHLQ-[ZBH####] (SEQ ID NO:626). ZBH#### refers to sequences of individual, 58 amino acid residue, B7-H3 binding Z variants.

Production of Z variants for ELISA: Z variants were produced by inoculating single colonies from subcloned and transformed selected variants into 1.2 mL TSB-YE medium supplemented with 100 µg/mL ampicillin and 0.175 mM IPTG in deep-well plates (Rainin, Mettler Toledo Liquidator pyramidal well bottom 2.2 mL). The plates were incubated with rotation for 17–19 h at 37°C. Cells were pelleted by centrifugation, re-suspended in 250 µL of 2× PBST0.05% (2× PBS supplemented with 0.05%Tween20) and incubated for 7 min at 90°C. The heat-treated suspensions were filtrated using a 96-well deep filter plate (AcroPrep filter plates, Pall Laboratories). The filtrated supernatant (Heat Treated (HT) lysate), with the soluble part of the extract, contained the Z variants as fusions to His_6, expressed as MGSSHHHHHHLQ-[ZBH####] (SEQ ID NO:626). All individually picked clones were subjected to DNA sequencing.

ELISA screening of Z variants against B7-H3: 384-well ELISA plates (Greiner) were coated at 4°C overnight with 20 µL of 3 µg/mL of an anti-His_6 mouse antibody (Abcam cat.no. 18184) diluted in PBS. The wells were washed 4× with PBST0.05% and thereafter blocked with 50 µL of Blocker® casein in PBS (Thermo Scientific) for 1.5 h at RT. The wells were washed 4× with PBST0.05% and thereafter 20 µL HT lysate, diluted 1:20 in PBST was added to the respective well and incubated for 1 h and 20 min at RT. As a positive control, HT lysate prepared using ZAC12 (SEQ ID NO:543), cloned in the same way as the tested variants, was added in duplicate on each plate. As a negative control, HT lysate prepared using a Z variant binding to an irrelevant target and cloned as Z-His_6 was added. The supernatants were poured off and the wells were washed 4× with PBST0.05%. Then, 20 µL of biotinylated Fc fused B7-H3 (b-B7-H3(4Ig)-Fc) at concentrations of 5 nM, 1.7 nM, 0.56 nM and 0 nM, diluted in Blocker® casein in PBS was added to each well. The plates were incubated for 1 h and 20 min at RT followed by washes as described above. Streptavidin conjugated HRP (Thermo Scientific), diluted 1:30000 in Blocker® casein in PBS, was added to the wells and the plates were incubated for 45 min. After washing as described above, 20 µL TMB substrate (1-Step Ultra TMB-ELISA Pierce ThermoFisher Scientific) was added to the wells and the plates were treated according to the manufacturer's recommendations. The absorbance at 450 nm was measured using a multi-well plate reader (EnSpire, Perkin Elmer).

Results

Phage display selection of B7-H3 binding Z variants: Individual clones were obtained after one or four cycles of phage display selections against biotinylated B7-H3 using the maturation library Zlib008B7-H3.I.

Sequencing: Sequencing was performed for clones obtained after one or four cycles of selection. Each variant was given a unique identification number ####, and individual variants are referred to as ZBH#### herein. The amino acid sequences of selected 58 amino acid residues long Z variants are listed in the sequence listing as SEQ ID NO:480-535. The deduced B7-H3 binding motifs extend from residue 8 to residue 37 in each sequence. The amino acid sequences of the 49 amino acid residues long polypeptides predicted to constitute the complete three-helix bundle within each of these Z variants extend from residue 7 to residue 55.

ELISA screening of Z variants against B7-H3: The clones obtained after one and four cycles of selection were produced as heat treated, soluble crude samples in 96-well plates and screened for B7-H3 binding activity in ELISA. Z variants listed as (SEQ ID NO:480-535) were shown to give a response between 0.55 and 3.2 AU at a target concentration of 5 nM B7-H3, corresponding to at least 3× the blank control. The corresponding responses of binders for concentrations of biotinylated B7-H3 of 1.7 nM or 0.57 nM, respectively, were shown to give a response between 0.28 and 3.1 AU and between 0.15 and 2.0 AU, respectively. The mean responses for ZAC12 were 5 nM: 2.25 AU, 1.7 nM: 1.53 AU, and 0.57 nM: 0.76 AU, respectively.

Example 3
Production of B7-H3 binding Z variants

Summary

This Example describes the general procedure for subcloning and production of His-tagged Z variants, Z variants in fusion with a His-tag and a TEV (Tobacco Etch Virus) protease cleavage site and Z variants in fusion with ABD, which are used throughout the characterization experiments that follow. Production of Z variants using peptide synthesis is also described.

Materials and methods

Subcloning of Z variants with a His_6-tag: DNA encoding monomeric reference Z variants (SEQ ID NO:541-545) were ordered as codon optimized and synthesized genes from ATUM (Newark, California). The synthesized Z gene fragments were subcloned into T7 promoter driven expression vectors, resulting in the encoded sequences MGSSHHHHHHLQ-[ZBH####] (SEQ ID NO:626).

Subcloning of Z variant in fusion with a cleavable His_6-tag: Monomeric versions of B7-H3 binding Z variants were subcloned using standard molecular biology strategies into a T7 promoter driven vector, resulting in the encoded sequences MGSSHHHHHHSSGVDLGTENLYFQG-[ZBH###]-C (SEQ ID NO:627), respectively. After TEV protease treatment, the resulting sequences were in the format G-[ZBH###]-C (SEQ ID NO:628).

A gene encoding a dimeric version of a Z variant denoted ZBHD01 (SEQ ID NO:549) was ordered from GeneArt (Thermo Fisher Scientific, MA,

USA) as a codon optimized and synthesized gene cloned in a custom plasmid. The Z variant was cloned as a fusion protein with a His_6 tag and a TEV protease cleavage site. The synthesized dimeric Z gene fragments were subcloned into a T7 promoter driven expression vector, resulting in the encoded sequence MGSSHHHHHHSSGVDLGTENLYFQG-[ZAC12m]-GAPGGGGSGGGGSGGGGSTS-[ZAC12mc] (SEQ ID NO:629). After TEV protease treatment, resulting sequences were in the format G-[ZAC12m]-GAPGGGGSGGGGSGGGGSTS-[ZAC12mc] (SEQ ID NO:630).

Subcloning of Z variants in fusion with ABD: A set of variants with an ABD moiety (PP013; SEQ ID NO:547) incorporated at different positions within the polypeptide (at the N-terminus, at the C-terminus or in between two Z moieties) and insertion of different linkers (length and type) between moieties were ordered from GeneArt (TermoFisher Scientific GENEART GmbH, Regensburg, Germany) as cloned genes in a custom vector. All constructs contained a C-terminal cysteine to be used for subsequent conjugation. The encoded proteins were in the format ZBH481-G_4SAS-PP013-C (ZBHD02; SEQ ID NO:550), GS-PP013-$(G_4S)_2$-ZBH481-C (ZBHD03; SEQ ID NO:551), ZBH481-$(G_4S)_5$-ZBH481-G_4SAS-PP013-C (ZBHD04; SEQ ID NO:552), ZBH481-$(G_4S)_3$-ZBH481-G_4SAS-PP013-C (ZBHD05; SEQ ID NO:553), GS-PP013-$(G_4S)_2$-ZBH481-$(G_4S)_5$-ZBH481-C (ZBHD06; SEQ ID NO:554), ZBH481-$(G_4S)_3$AS-PP013-$(G_4S)_3$-ZBH481-C (ZBHD07; SEQ ID NO:555), ZBH481-A$(EAAAK)_4$AGS-PP013-$(KEAAA)_4$KAKGS-ZBH481-C (ZBHD08; SEQ ID NO:556), ZBH481-$(TP)_8$-PP013-$(TP)_8$-ZBH481-C (ZBHD09; SEQ ID NO:557).

Expression of Z variants: Generally, Z variants were expressed in autoinducing medium (Overnight Express TB, Novagen) inoculated with precultures of *E. coli* T7E2 clones carrying plasmids with sequence verified gene fragments of each B7-H3 binding Z variant. The cultures (3-200 ml) were incubated at 37°C and 150 or 300 rpm for 16 h before the cells were harvested by centrifugation.

Z variants in fusion with ABD were produced by fed-batch cultivation in 0.6 L scale. The cultivations were inoculated with precultures of *E. coli* T7E2 clones carrying plasmids with sequence verified gene fragments of each polypeptide to a final OD_{600} of 0.1 followed by cultivation at 37°C. 17.5 hours after cultivation start the temperature was lowered to 31°C followed by induction with IPTG after 18 h and expression for 9 h. After 27 h in total, the

cultivations were cooled below 18°C and cells were harvested by centrifugation.

Cell disruption: Cells were disrupted by known methods, using heat treatment, sonication or a FastPrep-24™ instrument (MP Biomedicals). Cell debris and, if applicable, residual silica spheres from FastPrep-24™ were removed by centrifugation and supernatants were either used immediately for purification or stored at −20°C until purification.

Purification of Z variants in fusion with a His_6-tag (+/- TEV cleavage site) in 96-well plate format: Lysates clarified by centrifugation were applied on a His MultiTrap HP plate (Cytiva) or a 1 mL filter plate (1.2 µm, Acroprep) prepacked in-house with 75 µL Ni Sepharose HP (Cytiva) and equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4). After washing with wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 60 mM imidazole, pH 7.4), the His_6-tag fused Z variants were eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). All wash and elution steps were performed using centrifugal force. Buffer exchange to PBS was performed using a PD MultiTrap G-25 desalting plate (Cytiva).

TEV protease cleavage followed by reverse IMAC purification in 96-well plate format: B7-H3 binding Z variants in fusion with His_6-tag and a TEV protease cleavage site were incubated overnight at 4°C with His_6-tagged TEV protease in a 25:1 molar ratio Z variant:TEV protease and DTT added to 2 mM final concentration. The incubated samples, supplemented with 20 mM imidazole were applied to a 1 mL filter plate (1.2 µm, Acroprep) prepacked with 150 µL Ni Sepharose HP (Cytiva) equilibrated with binding buffer. TEV protease cleaved Z variants were collected in the flow through using centrifugal force whereas His-tagged material bound to the IMAC resin. Buffer exchange to relevant buffer (PBS or 0.1 M HAc) was performed using a PD MultiTrap G-25 desalting plate (Cytiva) using centrifugal force.

Purification of Z variants in fusion with a His_6-tag: Lysates clarified by centrifugation were applied on a 1 mL His GraviTrap IMAC column (Cytiva). Contaminants were removed by washing with wash buffer and the Z variants were subsequently eluted with elution buffer. For constructs with purity below 95% (SDS-PAGE based) or otherwise deemed applicable, a second purification step was performed using reverse phase chromatography (RPC). Each Z variant was loaded onto a 1 or 3 mL Resource 15RPC column (Cytiva), pre-equilibrated with RPC solvent A (0.1% trifluoroacetic acid (TFA),

10% acetonitrile (ACN), 90% water). After column wash with RPC solvent A, bound proteins were eluted with a linear gradient of 0–60% RPC solvent B (0.1% TFA, 80% ACN, 20% water) for 18 column volums (CV). The buffer was then exchanged to PBS using PD-10 desalting columns (Cytiva).

Purification of Z variants in fusion with a His_6-tag and a TEV protease cleavage site: B7-H3 binding Z variants in fusion with a His_6-tag and a TEV protease cleavage site were purified as described in the previous section, with 1 mM DTT added to all buffers used for Z variants containing a C-terminal cysteine. Furthermore, a step for cleaving the tag prior to purification by RPC was included: the Z variants were buffer exchanged to PBS and incubated over night at 4°C with His-tagged TEV protease in a 25:1 or 30:1 molar ratio of Z variant:TEV protease, and DTT was added to 2 mM final concentration. The incubated samples, supplemented with 20 mM imidazole, were applied on a 1 mL His GraviTrap IMAC column (Cytiva) equilibrated with binding buffer. TEV protease cleaved Z variants were collected in the flow through whereas His-tagged material bound to the IMAC resin. The non-tagged Z variants were further purified by reverse phase chromatography (RPC) and buffer exchanged to PBS as described in the previous section or to 0.2 M NaAc pH 6.0 if subsequently to be conjugated.

Purification of Z variants in fusion with ABD: The respective cell pellet was re-suspended in purification buffer (50 mM sodium phosphate, 200 mM NaCl, 1 mM EDTA, pH 7.0), followed by addition of DENARASE® (c-Lecta; 1 µL/g pellet) and DTT (final concentration 20 mM). After cell disruption by sonication, clarification by centrifugation and filtration, the supernatant was applied on HiScale 10 column, packed with resin comprising an immobilized an anti-ABD ligand (in-house developed), connected to an ÄKTA Avant 25 system (Cytiva). After washing with purification buffer, the ABD fused Z variant was eluted with 0.1 M HAc, 1 mM EDTA, pH 3.1. 10% ACN was added to each eluate before further purification by RPC performed essentially as described above, but using a 20 ml SOURCE 30RPC column (Cytiva).

DOTA conjugation of Z variants with or without ABD: Each purified polypeptide with a unique C-terminal cysteine, was buffer exchanged to conjugation buffer (0.2 M NaAc, 2 mM EDTA, pH 6, treated with Chelex® 100 resin), using a PD-10 desalting column. Maleimido-mono-amide-DOTA (Macrocyclics, cat. No. B-272) was added at 4-fold molar excess and each sample was incubated at 22ºC and 600 rpm for 60 min, followed by buffer exchange to 0.2 M NaAc, pH 6 (treated with Chelex® 100 resin) using a PD-

10 desalting column. Samples to be used for radiolabeling were conjugated likewise but omitting EDTA in the conjugation buffer, and two additional rounds of buffer exchange were performed.

NOTA conjugation of Z variants: Z variants with a unique C-terminal cysteine was conjugated with NOTA (CheMatech, cat. no. C101) according to the methodology described above for DOTA conjugation, but incubation was performed at 24ºC and 450 rpm for 90 min.

Peptide synthesis of Z variants: A subset of Z variants (ZBH536-ZBH539, SEQ ID NO:536-539, and the reference variant ZAC12c, SEQ ID NO:540) were produced by solid phase peptide synthesis using the Fmoc/tBu strategy, performed by the contract manufacturer Bachem (St Helens, UK) as synthesized peptides. The C-terminal amino acids in positions 56-58 (originally A, P and K, respectively) of the synthesized Z variants were each mutated to the amino acid residue G. Furthermore, the amino acid C was added to the C-terminus.

General protein characterization: Protein concentrations were determined by absorbance measurements at 280 nm. The purity was analyzed by SDS-PAGE stained with Coomassie Blue and the identity of each purified Z variant was confirmed using HPLC-MS analysis.

Results

Production of B7-H3 binding Z variants: The B7-H3 binding Z variants with His_6-tag or with His_6-tag and a TEV protease cleavage site and Z variants in fusion with ABD were successfully expressed as soluble gene products in *E. coli* and purified using affinity chromatography, in some cases followed by RPC. Where applicable, the His_6-tag was removed by a TEV protease cleavage step. Monomeric Z variants comprising a C-terminal GGGC sequence were successfully produced by chemical synthesis. SDS-PAGE analysis of each final protein preparation showed that these predominantly contained the B7-H3 binding Z variant. The correct identity and molecular weight of each Z variant, ABD fused Z variant and conjugated variant, respectively, were confirmed by HPLC-MS analysis.

Example 4
Characterization of purified B7-H3 binding Z variants

Summary

In this Example, N-terminally His_6-tagged Z variants (SEQ ID NO:480-535) produced as described in Example 3, were assessed in terms of binding characteristics and a subset was also subjected to stability studies. Surface plasmon resonance (SPR) and bio-layer interferometry (BLI) were used to characterize the interactions of the Z variants with human B7-H3. *In vitro* cell binding was assessed using B7-H3 expressing MCF-7 and SKOV-3 cells. The melting temperature and secondary structure content were analyzed by circular dichroism (CD) spectroscopy. Chemically synthesized Z variants (SEQ ID NO:536-539 and the reference SEQ ID NO:540) were also used to measure the interaction with B7-H3 from other species.

Materials and methods

SPR k_{off} screen against B7-H3: The dissociation rate constants (k_{off}) were determined for the His_6-Z variants against Fc fused B7-H3 (B7-H3(2Ig)-Fc) using a Biacore 8K instrument (Cytiva). B7-H3(2Ig)-Fc was diluted to 10 nM in HBS-EP+ and captured on flow cell 2 on protein A chip (Cytiva) resulting in a capture level of approximately 430 RU. His_6-Z variants at concentrations 1 nM and 5 nM were used as analytes and injected over both flow cell 1 and 2. Association time was 120 s (30 µL/min) and dissociation time was 300 s (30 µL/min). HBS-EP+ was used as running buffer and 10 mM glycine-HCl pH 1.5 (two pulses of 30 s / 30 µL/min) was used as regeneration buffer. The assay temperature was 30°C. Reference cell (flow cell 1) and blank cycle injection (HBS-EP+) were subtracted from sensorgrams prior to evaluation using Biacore Insight Evaluation Software.

SPR single cycle kinetic screen against B7-H3: The affinities (K_D) were determined for the His_6-Z variants against Fc fused B7-H3 (B7-H3(2Ig)-Fc) in a single cycle kinetic run using a Biacore 8K instrument (Cytiva). B7-H3(2Ig)-Fc was diluted to 10 nM in HBS-EP+ and captured on flow cell 2 on protein A chip (Cytiva) resulting in a capture level of approximately 430 RU. His_6-Z variants at concentrations 0.56, 1.67, 5 and 15 nM were used as analytes and injected over both flow cell 1 and 2. Association time was 120 s (50 µL/min) and dissociation time was 300 s (50 µL/min). HBS-EP+ was used as running buffer and 10 mM glycine-HCl pH 1.5 (two pulses of 30 s / 30 µL/min) was

used as regeneration buffer. The assay temperature was 25°C. Reference cell (flow cell 1) and blank cycle injection (HBS-EP+) were subtracted from sensorgrams prior to evaluation using Biacore Insight Evaluation Software.

BLI kinetic screen: Affinities (K_D) and kinetic values (k_{on} and k_{off}) were determined for the B7-H3 binding His_6-Z variants using BLI in an Octet HTX instrument (Sartorius). In the experiment, His_6-Z variants were diluted in 1× KB buffer (10× Octet Kinetics buffer diluted in PBS) to a concentration of 0.5 µg/mL and loaded for 300 s on Anti-His biosensors (Octet® Anti-Penta-HIS (HIS1K) Biosensors). Association was performed during 400 s using 8 nM hB7-H3(2Ig)-Fc or 1× KB buffer for reference sample. Dissociation was performed during 400 s in 1× KB buffer. The anti-His biosensors were regenerated in 10 mM glycine-HCl pH 1.5 (6 × 2 s) between each cycle. A shake speed of 1000 rpm and an assay temperature of 30°C were used during the entire experiment. Reference sample (1× KB buffer) was subtracted from sensorgrams prior to evaluation using Octet Analysis Studio 12.2 Software.

Cell binding assays: In a first screening experiment, the His_6-Z variants were tested in terms of binding to B7-H3 expressing MCF-7 cells. The cells were placed in a V-bottom 96-well plate (0.2×10^6 cells/well) and incubated at 4°C for 1 h with the His_6-Z variants at a single concentration of 50 nM. Each variant was tested in duplicates. After 1× washing in PBS with 1% fetal bovine serum (FBS), binding of His_6-Z was identified by an anti-Z polyclonal antibody added at a concentration of 4 µg/mL and incubated at 4°C for 1 h followed by an Alexa488 conjugated goat-anti-rabbit IgG diluted 1:2000 and incubated at 4°C for 1 h. After 2× washing in FBS, fluorescence intensity was measured by a multimode plate reader (Enspire).

In a second experiment, a selection of the matured His_6-Z variants (n=7) was tested for binding to B7-H3 expressing SKOV-3 cells. The experiment was performed as described above for MCF-7 cells, but with His_6-Z variants incubated at decreasing concentrations from 500 nM to 32 pM. Each variant was tested in triplicates. Binding curves were plotted and EC50 values were determined using software GraphPad Prism.

In a third experiment, chemically synthesized Z variants (SEQ ID NO:536-539 and reference variant SEQ ID NO:540) were tested for binding to B7-H3 expressing SKOV-3 cells. The same method as described above for SKOV-3 cells was used. Each Z variant was tested in quadruplicates.

Circular dichroism (CD) spectroscopy analysis: A selection of the His_6-Z variants were diluted to 0.5 mg/mL in PBS. A CD spectrum at 250–195 nm was obtained at 20°C. In addition, a variable temperature measurement (VTM) was performed to determine the melting temperature (Tm). In the VTM, absorbance was measured at 221 nm while the temperature was raised from 20°C to 90°C, with a temperature slope of 5°C/min. A new CD spectrum was obtained at 20°C after the heating procedure, in order to study the refolding ability of the Z variants. The CD measurements were performed on a Jasco J-810 spectropolarimeter (Jasco Scandinavia AB) using a cell with an optical path length of 1 mm.

SPR analysis against B7-H3 from different species: Binding of the chemically synthesized Z variants (SEQ ID NO:536-539 and reference variant SEQ ID NO:540) to Fc fused B7-H3 from human (B7-H3(2Ig)-Fc), cynomolgus monkey (cB7-H3(4Ig)-Fc), mouse (mB7-H3(2Ig)-Fc) and rat (rB7-H3(2Ig)-Fc) was analyzed using a Biacore 8K instrument (Cytiva). The Fc fused B7-H3 variants were diluted to 10 nM in HBS-EP+ and captured on flow cell 2 on protein A chip (Cytiva) resulting in a capture level of approximately 270, 250, 260 and 320 RU for human, cynomolgus monkey, mouse and rat, respectively. His_6-Z variants at concentrations 0.56 nM, 1.67 nM, 5 nM and 15 nM were used as analytes and injected over both flow cell 1 and 2. Association time was 120 s (50 µL/min) and dissociation time was 300 s (50 µL/min). HBS-EP+ was used as running buffer and 10 mM glycine-HCl pH 1.5 (two pulses of 30 s / 30 µL/min) was used as regeneration buffer. The assay temperature was 30°C. Reference cell (flow cell 1) and blank cycle injection (HBS-EP+) were subtracted from sensorgrams prior to evaluation using Biacore Insight Evaluation Software.

Results

SPR k_{off} screen against B7-H3: The interactions of His_6-Z variants with B7-H3 were analyzed in a Biacore 8K instrument by injecting two concentrations of the purified Z variants over surfaces with immobilized B7-H3(2Ig)-Fc. The dissociation rate constants (k_{off}) based on data from the 5 nM concentration of each selected B7-H3 binder, injected over B7-H3 surfaces, are given in Table 5.

SPR single cycle kinetic screen against B7-H3: A single cycle kinetic screen of a subset of purified Z variants to B7-H3 was analyzed using SPR in a Biacore 8K. B7-H3(2Ig)-Fc was captured on protein A chip and Z variants

were injected at four concentrations over a surface. The affinities (K_D) were estimated and are shown in Table 5. Examples of sensorgrams obtained for four B7-H3 binding Z variants (ZBH480-ZBH482, SEQ ID NO:480-482, and the reference variant ZAC12, SEQ ID NO:543) to B7-H3(2Ig)-Fc are displayed in Figure 1.

BLI kinetic screen: The interactions of His_6-Z variants with B7-H3 were analyzed in an Octet HTX instrument by loading His_6-Z variants on Anti-His biosensors followed by association in hB7-H3(2Ig)-Fc. The dissociation rate constants (k_{off}) are given in Table 5.

Cell binding assay: The binding of His_6-Z variants to B7-H3 expressing MCF-7 cells was evaluated by fluorescence intensity in a first screening experiment. Cells were incubated with one concentration of purified Z variants followed by an anti-Z pAb and an Alexa488-conjugated anti-rabbit IgG for detection. The fluorescence intensity varied between 488 and 6677 (arbitrary units). A summary of mean fluorescence values of selected binders (improved over ZAC12 either in the cell assay, Biacore or Octet screening assay) is given in Table 5. In a second experiment, the binding of seven matured His_6-Z variants to B7-H3 expressing SKOV-3 cells was evaluated by fluorescence intensity. Cells were incubated with decreasing concentration of purified Z variants (ranging from 500 nM to 32 pM) followed by an anti-Z pAb and a Alexa488-conjugated anti-rabbit IgG for detection. Calculated mean EC50 values are summarized in Table 6 and curves from one representative experiment are shown in Figure 2A. In a third experiment, the binding of four synthesized Z variants to B7-H3 expressing cells was evaluated by fluorescence intensity. Cells were incubated with decreasing concentration of synthesized Z variants (ranging from 500 nM to 32 pM) using an anti-Z pAb and a Alexa488-conjugated anti-rabbit IgG. Mean EC50 values are summarized in Table 7 and curves from one representative experiment are shown in Figure 2B.

Table 5: Screening data from SPR analysis, BLI analysis and cell assay

Z variant	SEQ ID NO:	Biacore k_{off} (1/s)	Biacore K_D (M)	Octet K_D (M)	Octet k_{on} (1/Ms)	Octet k_{off} (1/s)	Cell binding assay[1]	Fold increase[3] compared to ZAC12
ZBH495	495	7.91×10^{-2}	n.d.	8.02×10^{-10}	4.34×10^{5}	3.48×10^{-4}	n.d.	n.d.
ZBH483	483	4.26×10^{-2}	8.8×10^{-9}	3.22×10^{-10}	3.15×10^{5}	1.01×10^{-4}	4276	2.46
ZBH484	484	1.41×10^{-1}	1.7×10^{-8}	1.20×10^{-9}	3.00×10^{5}	3.61×10^{-4}	1945	1.12

ZBH485	485	7.72×10^{-2}	1.5×10^{-8}	7.71×10^{-10}	4.63×10^{5}	3.57×10^{-4}	1277	0.73
ZBH496	496	1.02×10^{-1}	2.4×10^{-8}	1.25×10^{-9}	4.08×10^{5}	5.12×10^{-4}	1440	0.83
ZBH497	497	1.89×10^{-1}	3.7×10^{-8}	1.31×10^{-9}	6.91×10^{5}	9.06×10^{-4}	1847	1.06
ZBH480	480	1.52×10^{-1}	2.6×10^{-9}	n.d.	9.90×10^{4}	n.d.	5257	3.02
ZBH499	499	6.43×10^{-2}	n.d.	5.66×10^{-10}	3.75×10^{5}	2.12×10^{-4}	1064	0.61
ZBH500	500	9.59×10^{-2}	n.d.	6.35×10^{-10}	3.07×10^{5}	1.95×10^{-4}	1096	0.63
ZBH501	501	1.94×10^{-2}	n.d.	1.74×10^{-10}	2.39×10^{5}	4.15×10^{-5}	1425	0.82
ZBH502	502	1.01×10^{-1}	1.8×10^{-8}	1.02×10^{-9}	5.79×10^{5}	5.89×10^{-4}	2688	1.54
ZBH503	503	3.49×10^{1}	3.5×10^{-9}	n.d.	1.30×10^{5}	n.d.	6677	3.84
ZBH486	486	3.58×10^{-2}	7.0×10^{-9}	1.22×10^{-10}	2.61×10^{5}	3.19×10^{-5}	4066	2.34
ZBH504	504	5.13×10^{-2}	n.d.	1.72×10^{-9}	2.76×10^{5}	4.75×10^{-4}	3619	2.08
ZBH505	505	5.65×10^{-2}	n.d.	7.07×10^{-10}	5.43×10^{5}	3.84×10^{-4}	n.d.	n.d.
ZBH506	506	7.27×10^{-2}	2.0×10^{-8}	1.14×10^{-9}	3.58×10^{5}	4.09×10^{-4}	2236	1.28
ZBH487	487	1.25×10^{-1}	5.9×10^{-9}	5.81×10^{-10}	3.50×10^{5}	2.03×10^{-4}	2962	1.70
ZBH507	507	5.38×10^{-2}	4.0×10^{-9}	1.72×10^{-10}	2.39×10^{5}	4.11×10^{-5}	5801	3.33
ZBH508	508	5.43×10^{-2}	n.d.	4.97×10^{-10}	3.27×10^{5}	1.63×10^{-4}	2964	1.70
ZBH509	509	5.00×10^{-2}	9.9×10^{-9}	8.75×10^{-10}	2.44×10^{5}	2.14×10^{-4}	2527	1.45
ZBH510	510	4.11×10^{-2}	8.4×10^{-9}	9.78×10^{-10}	2.29×10^{5}	2.24×10^{-4}	2822	1.62
ZBH511	511	2.39×10^{-2}	n.d.	2.09×10^{-10}	2.52×10^{5}	5.25×10^{-5}	2594	1.49
ZBH489	489	6.16×10^{-2}	1.5×10^{-8}	1.29×10^{-9}	2.83×10^{5}	3.66×10^{-4}	4511	2.59
ZBH512	512	2.30×10^{-2}	n.d.	1.03×10^{-10}	1.49×10^{5}	1.52×10^{-5}	1105	0.63
ZBH513	513	4.64×10^{-2}	7.3×10^{-9}	5.86×10^{-10}	3.68×10^{5}	2.16×10^{-4}	1517	0.87
ZBH514	514	9.12×10^{-2}	1.5×10^{-8}	9.26×10^{-10}	5.00×10^{5}	4.62×10^{-4}	2814	1.62
ZBH490	490	6.34×10^{-2}	1.2×10^{-8}	1.12×10^{-9}	2.67×10^{5}	2.98×10^{-4}	2557	1.47
ZBH515	515	7.77×10^{-2}	n.d.	1.21×10^{-9}	4.96×10^{5}	5.98×10^{-4}	2501	1.44
ZBH516	516	2.18×10^{-2}	3.6×10^{-9}	7.69×10^{-11}	1.72×10^{5}	1.32×10^{-5}	4667	2.68
ZBH517	517	1.86×10^{-1}	n.d.	1.50×10^{-9}	1.09×10^{6}	1.64×10^{-3}	2278	1.31
ZBH518	518	3.88×10^{-2}	n.d.	4.32×10^{-10}	3.40×10^{5}	1.47×10^{-4}	914	0.53
ZBH519	519	1.38×10^{-1}	2.9×10^{-8}	1.37×10^{-9}	4.99×10^{5}	6.81×10^{-4}	3360	1.93
ZBH520	520	9.45×10^{-2}	3.3×10^{-8}	1.50×10^{-9}	5.13×10^{5}	7.72×10^{-4}	2662	1.53
ZBH481	481	8.20×10^{-2}	1.6×10^{-8}	5.71×10^{-10}	2.53×10^{5}	1.45×10^{-4}	5777	3.32
ZBH491	491	5.04×10^{-2}	6.9×10^{-9}	5.09×10^{-10}	2.63×10^{5}	1.34×10^{-4}	5210	2.99
ZBH521	521	2.44×10^{-1}	n.d	7.41×10^{-10}	5.51×10^{5}	4.08×10^{-4}	n.d.	n.d.
ZBH522	522	4.96×10^{-2}	n.d.	8.62×10^{-10}	2.88×10^{5}	2.48×10^{-4}	6531	3.75
ZBH523	523	9.19×10^{-2}	n.d.	6.50×10^{-10}	2.93×10^{5}	1.91×10^{-4}	1552	0.89
ZBH524	524	6.18×10^{-2}	n.d.	6.40×10^{-10}	2.50×10^{5}	1.60×10^{-4}	939	0.54
ZBH525	525	4.27×10^{-2}	n.d.	5.24×10^{-10}	2.75×10^{5}	1.44×10^{-4}	1715	0.99
ZBH526	526	4.74×10^{-2}	n.d.	9.21×10^{-10}	2.95×10^{5}	2.71×10^{-4}	2363	1.36
ZBH527	527	4.97×10^{-2}	n.d.	5.07×10^{-10}	2.25×10^{5}	1.14×10^{-4}	3020	1.74
ZBH492	492	1.13×10^{-1}	1.5×10^{-8}	1.49×10^{-9}	2.87×10^{5}	4.27×10^{-4}	2385	1.37
ZBH482	482	6.00×10^{-2}	4.6×10^{-9}	7.09×10^{-10}	2.06×10^{5}	1.46×10^{-4}	5439	3.13
ZBH493	493	6.96×10^{-2}	1.2×10^{-8}	9.87×10^{-10}	2.88×10^{5}	2.84×10^{-4}	3030	1.74
ZBH528	528	4.63×10^{-2}	6.9×10^{-9}	5.68×10^{-10}	5.71×10^{5}	3.25×10^{-4}	3765	2.16
ZBH529	529	5.71×10^{-2}	n.d.	1.51×10^{-9}	2.22×10^{5}	3.35×10^{-4}	3694	2.12
ZBH530	530	2.82×10^{-2}	n.d.	9.53×10^{-10}	2.23×10^{5}	2.12×10^{-4}	5193	2.98
ZBH531	531	6.08×10^{-2}	2.1×10^{-8}	1.21×10^{-9}	3.56×10^{5}	4.32×10^{-4}	3623	2.08
ZBH532	532	5.99×10^{-2}	n.d.	1.04×10^{-9}	4.82×10^{5}	5.01×10^{-4}	1799	1.03
ZBH533	533	9.00×10^{-2}	1.2×10^{-8}	1.31×10^{-9}	2.90×10^{5}	3.81×10^{-4}	2930	1.68
ZBH494	494	3.08×10^{-2}	5.2×10^{-9}	4.94×10^{-10}	2.30×10^{5}	1.14×10^{-4}	3002	1.72
ZBH534	534	9.81×10^{-2}	2.0×10^{-8}	1.03×10^{-9}	4.12×10^{5}	4.24×10^{-4}	2641	1.52
ZBH535	535	3.16×10^{-2}	4.2×10^{-9}	4.74×10^{-10}	2.27×10^{5}	1.08×10^{-4}	1325	0.76
ZAC12[2]	543	7.37×10^{-2}	1.1×10^{-8}	9.20×10^{-10}	2.49×10^{5}	2.32×10^{-4}	1740	1

[1] Mean of two measurements
[2] Mean of three measurements
[3] Fold increase in the cell binding assay
n.d: not determined

Table 6: Cell binding data for His_6-Z variants binding to SKOV-3 cells

Z variant	SEQ ID NO:	EC50
ZBH480	480	2.4
ZBH481	481	18.8
ZBH482	482	7.8
ZBH486	486	23.9
ZBH487	487	51.6
ZBH491	491	14.2
ZBH494	494	6.7

Table 7: Cell binding data for synthesized Z variants binding to SKOV-3 cells

Z variant	SEQ ID NO:	EC50
ZBH536	536	5.7
ZBH537	537	4.8
ZBH538	538	7.5
ZBH539	539	7.6
ZAC12c (ref)	540	22.0

CD analysis: The CD spectra determined for nine B7-H3 binding Z variants with a His_6 tag showed that all variants have an α-helical structure at 20°C as judged from the typical minima at 208 and 222 nm. Reversible folding was seen for all Z variants when spectra measured before and after heating to 90°C were superimposed. The melting temperatures (Tm) are summarized in Table 8. Exemplary CD spectra before and after heating to 90°C is shown for His_6-ZBH480 in Figure 3A and its melting curve is shown in Figure 3B.

Table 8: Melting temperatures (Tm) determined for His_6 tagged Z variants

Z variant	SEQ ID NO:	Tm (°C)
ZBH480	480	56
ZBH481	481	56
ZBH482	482	63
ZBH486	486	61
ZBH491	491	59
ZBH516	516	70
ZBH519	519	57
ZBH531	531	50
ZBH533	533	59

SPR analysis against B7-H3 from different species: The interactions of chemically synthesized Z variants (ZBH536-ZBH539, SEQ ID NO:536-539

and reference variant ZAC12c, SEQ ID NO:540) with B7-H3 from human, cynomolgus monkey, mouse and rat were analyzed in a Biacore 8K instrument by injecting four concentrations of the purified Z variants over B7-H3(2Ig)-Fc, cB7-H3(4Ig)-Fc, mB7-H3(2Ig)-Fc and rB7-H3(2Ig)-Fc captured on the surface of a protein A chip. The Z variants showed approximately the same binding to B7-H3 from different species. Sensorgrams obtained for one B7-H3 binding polypeptide (ZBH538, SEQ ID NO:538) to human, cynomolgus monkey, mouse and rat B7-H3 are displayed in Figure 4.

Example 5
Design and construction of two maturation libraries of B7-H3 binding Z variants

Summary

In this Example, two new libraries were designed based on the B7-H3 binding variants identified in the first selection described above. The new maturation libraries contained approximately 5.2×10^8 and 5.9×10^8 individual clones, respectively.

Materials and methods

Design of two B7-H3 affinity maturation libraries: Two new libraries were designed based on the sequences of the B7-H3 binding Z variants selected, produced and characterized as described in Examples 1–4. 13 surface exposed positions in the Z molecule scaffold were biased towards certain amino acid residues, according to a strategy based on the binding motifs of the Z variants identified in the first maturation and including those defined in SEQ ID NO:480-535. The variability within the 13 varied positions was the same between the two libraries. However, they differed in three scaffold positions. One library was designed with Y5, N52 and D53 (numbering with respect to the full-length Z variant sequence), whereas the other library was designed with positions F5, S52 and E53. Furthermore, both libraries contained variability in two scaffold positions within the *BM*: X_{29} and X_{30} (numbering with respect to the *BM* defined herein). The design of the new libraries is shown in Table 9, wherein percentages of the amino acids used in each of the 15 randomized positions are indicated.

Table 9: Design of the B7-H3 maturation libraries

	Position in *BM*														
	2	3	4	6	7	10	11	17	18	20	21	25	28	29	30
Amino acid	Position in full length Z variant														
	9	10	11	13	14	17	18	24	25	27	28	32	35	36	37
A Ala	0	0	6.3	0	25	20	0	0	11.1	0	0	16.7	0	33.3	33.3
C Cys	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D Asp	0	0	6.3	0	0	0	0	0	11.1	0	0	0	0	33.3	33.3
E Glu	0	0	6.3	0	0	0	0	0	11.1	0	0	16.7	0	33.3	0
F Phe	0	0	0	0	0	0	0	0	0	0	0	16.7	0	0	0
G Gly	0	0	6.3	0	25	0	0	0	11.1	0	0	0	0	0	0
H His	0	0	6.3	0	25	0	0	33.3	11.1	0	0	16.7	0	0	33.3
I Ile	0	50	6.3	50	0	20	0	0	0	50	0	0	0	0	0
K Lys	100	0	6.3	0	0	20	0	0	0	0	16.7	0	0	0	0
L Leu	0	0	6.3	50	0	20	0	0	0	0	16.7	16.7	0	0	0
M Met	0	0	6.3	0	0	0	0	0	0	0	16.7	0	0	0	0
N Asn	0	0	6.3	0	0	0	50	0	11.1	0	0	0	100	0	0
P Pro	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Q Gln	0	0	6.3	0	0	0	0	0	11.1	0	16.7	0	0	0	0
R Arg	0	0	6.3	0	0	0	0	33.3	0	0	16.7	0	0	0	0
S Ser	0	0	6.3	0	25	0	0	0	11.1	0	0	0	0	0	0
T Thr	0	0	6.3	0	0	0	0	0	11.1	0	0	0	0	0	0
V Val	0	50	6.3	0	0	20	0	0	0	50	16.7	0	0	0	0
W Trp	0	0	0	0	0	0	50	0	0	0	0	16.7	0	0	0
Y Tyr	0	0	6.3	0	0	0	0	33.3	0	0	0	0	0	0	0

Two oligonucleotides, one forward and one reverse complementary, with complementary 3'-ends were generated using TRIM technology. These oligos were ordered from Ella Biotech GmbH (Martinsried, Germany).

The construction of the libraries was performed as described in Example 1 in vectors pAY02592 and pAY04242, but with the following exceptions: 1) library transformations were performed into electrocompetent XL-1 Blue cells; 2) transformed cells were thereafter pooled and cultivated for 6.5 h at 37°C in 1 L of TSB-YE medium, supplemented with 2% glucose, 10 g/mL tetracycline and 100 g/mL ampicillin. The library qualities and the distributions of amino acids were verified by sequencing as described in Example 1.

Preparation of phage stock: Phage stock cultivations and preparations were essentially performed as described in Example 1 in 1.5 L medium for each library. The phage particles were precipitated from the supernatant twice using PEG/NaCl and filtered as described in Example 1, and finally dissolved

in PBS and glycerol. Phage stocks were stored at −80°C until use in selection.

Results

Library construction: The two new libraries were designed based on the set of B7-H3 binding variants described in Examples 1–4. The theoretical size of the designed libraries was 4.5×10^7 Z variants. The actual size of the two libraries were approximately 5.2×10^8 and 5.9×10^8, respectively, determined by titration after transformation to *E. coli.* XL-1 Blue cells. The library quality was tested by sequencing of 192 transformants per library and by comparing their actual sequences with the theoretical design. Sequence analysis of individual library members verified a distribution of codons in accordance with the theoretical design. The libraries were designated Zlib009B7-H3.I and Zlib008B7-H3.II.

Example 6
Second selection and screening of affinity matured B7-H3 binding Z variants

Summary

In this Example, B7-H3 was used as target in phage display selections using two different B7-H3 maturation phage libraries of Z variants. Selected clones were cloned in bulk in an expression vector driven by a T7 promoter, DNA sequenced, produced in *E. coli* and assayed against different target proteins by ELISA and SPR.

Materials and methods

Phage display selection of B7-H3 binding Z variants: Phage display selection was performed using the phage stocks of the newly produced maturation libraries. Selections against biotinylated b-B7-H3(4Ig)-Fc and b-B7-H3(4Ig)-His_1 were performed in solution in all tracks and essentially as described in Example 2, using the specific conditions described in Table 10.

Washing was performed using streptavidin coated SpeedBeads as solid phase in cycle 1, 2, and 4, while neutravidin beads were used in cycle 3. Washing was performed manually in cycle 1 and manually and/or in a KingFisher Duo instrument in cycle 2–4. Selection was made at RT, 37°C or 50°C and using preblocked tubes (Protein LowBind, Eppendorf). In order to

reduce the degree of unspecific binding, pre-selection was performed in cycle 1–2 using the streptavidin coated SpeedBeads as solid phase. In some tracks, biotinylated Fc (Jackson ImmunoResearch Laboratories) was coated on the beads before use in pre-selection (tracks 15, 21 and 22). In addition, in some tracks the phage stock was preheated at 70°C for 15 min followed by centrifugation at 13000 rpm before use in selection (tracks 9, 16, 25, 32, 41 and 50).

As selection proceeded, the tracks were further divided according to target concentration and number and/or time of washes.

Table 10: Overview of the selection against B7-H3 using maturation libraries

Cycle	Selection track	Phage stock from library or selection track	Temp. during selection (RT/°C)	Target type	Target conc. (nM)	Selection time (min)	No of short washes	Temp during short wash (RT/°C)	No of long washes	Duration long washes (h)	Temp. during long wash (RT/°C)
1	1	Zlib009B7-H3.I	RT	b-B7-H3(4Ig)-His_1	50	60	3	RT			
1	2	Zlib009B7-H3.I	37	b-B7-H3(4Ig)-His_1	50	60	3	37			
1	3	Zlib009B7-H3.I	50	b-B7-H3(4Ig)-His_1	50	60	3	50			
1	4	Zlib009B7-H3.I	37	b-B7-H3(4Ig)-Fc	50	60	3	37			
1	5	Zlib008B7-H3.II	RT	b-B7-H3(4Ig)-His_1	50	60	3	RT			
1	6	Zlib008B7-H3.II	37	b-B7-H3(4Ig)-His_1	50	60	3	37			
1	7	Zlib008B7-H3.II	50	b-B7-H3(4Ig)-His_1	50	60	3	50			
1	8	Zlib008B7-H3.II	37	b-B7-H3(4Ig)-Fc	50	60	3	37			
2	9	1	RT	b-B7-H3(4Ig)-His_1	20	60	6	RT			
2	10	1	RT	b-B7-H3(4Ig)-His_1	20	60	6	RT			
2	11	1	RT	b-B7-H3(4Ig)-His_1	10	60	15	RT			
2	12	2	37	b-B7-H3(4Ig)-His_1	20	60	6	37+RT			
2	13	2	37	b-B7-H3(4Ig)-His_1	10	60	15	37+RT			
2	14	3	50	b-B7-H3(4Ig)-His_1	20	60	6	50			
2	15	4	37	b-B7-H3(4Ig)-Fc	20	60	6	37+RT			
2	16	5	RT	b-B7-H3(4Ig)-His_1	20	60	6	RT			
2	17	5	RT	b-B7-H3(4Ig)-His_1	20	60	6	RT			
2	18	5	RT	b-B7-H3(4Ig)-His_1	10	60	15	RT			
2	19	6	37	b-B7-H3(4Ig)-His_1	20	60	6	37+RT			
2	20	6	37	b-B7-H3(4Ig)-His_1	10	60	15	37+RT			
2	21	5,6,7,8	50	b-B7-H3(4Ig)-His_1	20	60	6	50			
2	22	8	37	b-B7-H3(4Ig)-Fc	20	60	6	37+RT			
2	23	1,2,3,4	RT	b-B7-H3(4Ig)-His_1	5	60	21	RT	1	1	RT
2	24	7,8	RT	b-B7-H3(4Ig)-His_1	5	60	21	RT	1	1	RT
3	25	9,10,11,12,13,14,15	RT	b-B7-H3(4Ig)-His_1	5	60	21	RT	1	1	RT
3	26	9,10,11,12,13,14,15	RT	b-B7-H3(4Ig)-His_1	10	60	15	RT			
3	27	9,10,11,12,13,14,15	RT	b-B7-H3(4Ig)-His_1	5	60	21	RT	1	1	RT
3	28	9,10,11,12,13,14,15	37	b-B7-H3(4Ig)-His_1	10	60	15	37			
3	29	9,10,11,12,13,14,15	37	b-B7-H3(4Ig)-His_1	5	60	21	RT	1	1	37
3	30	9,10,11,12,13,14,15	50	b-B7-H3(4Ig)-His_1	10	60	15	50			
3	31	9,10,11,12,13,14,15	37	b-B7-H3(4Ig)-Fc	5	60	21	RT	1	1	37
3	32	16,17,18,19,20,21,22	RT	b-B7-H3(4Ig)-His_1	5	60	21	RT	1	1	RT

3	33	16,17,18,19,20,21,22	RT	b-B7-H3(4Ig)-His_1	10	60	15	RT			
3	34	16,17,18,19,20,21,22	RT	b-B7-H3(4Ig)-His_1	5	60	21	RT	1	1	RT
3	35	16,17,18,19,20,21,22	37	b-B7-H3(4Ig)-His_1	10	60	15	37			
3	36	16,17,18,19,20,21,22	37	b-B7-H3(4Ig)-His_1	5	60	21	RT	1	1	37
3	37	16,17,18,19,20,21,22	50	b-B7-H3(4Ig)-His_1	10	60	15	50			
3	38	16,17,18,19,20,21,22	37	b-B7-H3(4Ig)-Fc	5	60	21	RT	1	1	37
3	39	23	RT	b-B7-H3(4Ig)-His_1	0.5	60	34	RT	2	~1	RT
3	40	24	RT	b-B7-H3(4Ig)-His_1	0.5	60	34	RT	2	~1	RT
4	41	25	RT	b-B7-H3(4Ig)-His_1	0.5	50	34	RT	2	~1	RT
4	42	26	RT	b-B7-H3(4Ig)-His_1	2	50	22	RT			
4	43	27	RT	b-B7-H3(4Ig)-His_1	0.5	50	34	RT	2	~1	RT
4	44	25,26,27,28,29,30,31	RT	b-B7-H3(4Ig)-His_1	0.1	50	47	RT	2	3.5 + 15. 5	RT
4	45	28	37	b-B7-H3(4Ig)-His_1	2	50	22	37			
4	46	29	37	b-B7-H3(4Ig)-His_1	0.5	50	34	37	2	~1	RT
4	47	30	50	b-B7-H3(4Ig)-His_1	2	50	22	50			
4	48	31	37	b-B7-H3(4Ig)-Fc	0.5	50	34	37	2	~1	RT
4	49	25,26,27,28,29,30,31	37	b-B7-H3(4Ig)-Fc	0.1	50	47	37	2	3.5 + 15. 5	37 + RT
4	50	32	RT	b-B7-H3(4Ig)-His_1	0.5	50	34	RT	2	~1	RT
4	51	33	RT	b-B7-H3(4Ig)-His_1	2	50	22	RT			
4	52	34	RT	b-B7-H3(4Ig)-His_1	0.5	50	34	RT	2	~1	RT
4	53	32,33,34,35,36,37,38	RT	b-B7-H3(4Ig)-His_1	0.1	50	47	RT	2	3.5 + 15. 5	RT
4	54	35	37	b-B7-H3(4Ig)-His_1	2	50	22	37			
4	55	36	37	b-B7-H3(4Ig)-His_1	0.5	50	34	37	2	~1	RT
4	56	37	50	b-B7-H3(4Ig)-His_1	2	50	22	50			
4	57	38	37	b-B7-H3(4Ig)-Fc	0.5	50	34	37	2	~1	RT
4	58	32,33,34,35,36,37,38	37	b-B7-H3(4Ig)-Fc	0.1	50	47	37	2	3.5 + 15. 5	37 + RT

Amplification and preparation of phage particles: Phage stock preparation and amplification of phage between selection cycles were performed as described in Example 1 and 2. Log phase bacteria were infected with eluates from selected tracks (cycle 2–4) and a pool of the cultivation was used to bulk clone the selected variants essentially as described in Example 2.

Production of Z variants for binding analyses: The Z variants were produced and the HT lysate of each individual variant was prepared as described in Example 2. The final filtered supernatant with the soluble part of the extract, contained the Z variants as fusions to His_6, expressed as MGSSHHHHHHLQ-[ZBH###] (SEQ ID NO:626). ZBH### refers to sequences of individual, 58 amino acid residue, B7-H3 binding Z variants.

ELISA screening of Z variants: The binding of Z variants to B7-H3 was analyzed in a set of ELISA assays. In a first assay, each Z variant was

analyzed against biotinylated B7-H3 (b-B7-H3(4Ig)-Fc) at a target concentration 0.6 nM. The ELISA was performed as described in Example 2. In a second assay, a selected subset of the binders was subjected to an ELISA against biotinylated b-B7-H3(4Ig)-Fc at concentrations 1.8, 0.6, 0.2 and 0 nM. In a third assay, another selected subset of binders was subjected to an ELISA using the target concentration 0.6 nM and using 96-well plates. In this assay the subset of binders was also analyzed against a biotinylated irrelevant target protein at a concentration of 12 nM. As a positive control, ZAC12 (SEQ ID NO:543) was used in the ELISA experiment against biotinylated b-B7-H3(4Ig)-Fc at four concentrations. The absorbance at 450 nm was measured using a multi-well plate reader, EnSpire (Perkin Elmer).

SPR k_{off} screen against captured B7-H3 using HT lysates: For ranking purposes, a screening experiment was set up to estimate the dissociation rate constants (k_{off}) of a subset of His_6-Z variants (n=1674) produced as HT lysates. The experiment was performed essentially as described in Example 4 against Fc fused B7-H3 (B7-H3(2Ig)-Fc) captured on a protein A chip and using a Biacore 8K instrument.

SPR kinetic screen of captured Z variants against B7-H3: The binding of a subset of Z variants (n=93) to B7-H3 was analyzed using SPR in a Biacore 8K. Each Z variant in HT lysates was captured on an anti-His Ab (Abcam cat. no. ab18184) immobilized on a CM5 chip surface and analyzed against B7-H3(4Ig)-Fc diluted to 30 nM in HBS-EP+. HBS-EP+ was used as running buffer and 10 mM glycine-HCl pH 2.0 (two pulses of 20 s / 30 µL/min) was used as regeneration buffer. The assay temperature was 30°C.

Sequencing: In parallel with the ELISA and SPR screening, all clones were DNA sequenced.

Results

Phage display selection of matured B7-H3 binding Z variants: Individual clones were obtained after two, three and four cycles of phage display selections against biotinylated B7-H3.

ELISA screening of Z variants: Clones obtained after two to four cycles of selection were produced in 96-well plates and screened for binding activity against B7-H3. In the first assay, binders were regarded as positive if the response was 3× the background response at 0.6 nM B7-H3 (above 0.2 AU). In the second and third assay, binders were regarded as positive if the average response was 3× the background response at 0.6 nM (above 0.2

AU) together with a sound target dependent response in the other concentrations.

SPR k_{off} screens against captured B7-H3 using HT lysates: The interactions of Z variants with B7-H3 were analyzed in an off-rate screening experiment in a Biacore 8K instrument by injection of HT lysates over captured Fc-fused B7-H3 protein. Z variants found to have an off-rate curve ranked at least two times better than the control Z variant ZAC12 were shortlisted.

SPR kinetic screen of captured Z variants against B7-H3: An affinity screen of a subset of Z variants in HT lysates to B7-H3 was analyzed using SPR in a Biacore 8K. Z variants in HT lysates were captured on an immobilized anti-His_6 antibody and B7-H3 was injected as analyte and the kinetic responses was assessed. The assay was used to rank the binders with regard to affinity. Z variants found to have an off-rate curve that ranked equal to or better than the control Z from the first maturation (ZBH481; SEQ ID NO:481) were shortlisted.

Sequencing: Sequencing was performed for clones obtained after three to four cycles of selection. Each variant was given a unique identification number ###, and individual variants are referred to as ZBH###. The amino acid sequences of the 58 amino acid residues long Z variants shortlisted after the ranking SPR screens described above are listed in in the sequence listing as SEQ ID NO:1-479. The deduced B7-H3 binding motifs extend from residue 8 to residue 37 in each sequence. The amino acid sequences of the 49 amino acid residues long polypeptides predicted to constitute the complete three-helix bundle within each of these Z variants extend from residue 7 to residue 55. It appeared that the primers used during the bulk cloning resulted in some unintentional, but yet functional, Z scaffold variants. Besides the designed variants with the amino acids Y5, N52 and D53 (SEQ ID NO:1-13, 17-418, 446-479) and amino acids F5, S52 and E53 (SEQ ID NO:15-16, 420-424, 427-428, 430-436, 438-444, 480-535), respectively, variants with F5, N52 and E53 (SEQ ID NO:14, 425-426, 429, 437, 445) and one variant with F5, N52 and D53 (SEQ ID NO:419) were identified.

Example 7
Characterization of 2nd generation of matured B7-H3 binding Z variants

Summary

In this Example, a subset (n=21) from the matured Z variants selected as described in Example 6 were further characterized. N-terminally His_6-tagged Z variants (SEQ ID NO:1-21), produced in analogy to Example 3, were assessed in terms of binding to B7-H3 using SPR, and further subsets were tested in a cell binding assay and/or subjected to stability and secondary structure evaluation using CD spectroscopy.

Materials and methods

SPR binding kinetics of Z variants against B7-H3: A single cycle kinetic screen of a subset of Z variants (n=21) to B7-H3 was run using SPR in a Biacore 8K instrument (Cytiva). ZBH481 (SEQ ID NO:481) from the first maturation and the reference variant ZAC12 (SEQ ID NO:543) were included in the assay for comparison. B7-H3(4Ig)-His was immobilized through amine coupling onto the carboxylated dextran layer of the surface of a CM5 chip (Cytiva) according to the manufacturer's recommendations. Surface 1 was activated and then deactivated and used as a reference. Each Z variant was injected over chip surfaces for 165 sec at concentrations of 0.33, 1, 3, 9 and 27 nM followed by dissociation for 720 sec (flow rate 50 µl/min). The chip surfaces were regenerated using 50 mM NaOH (two pulses of 20 s / 30 µL/min). HBS-EP+ was used as running and dilution buffer. The assay temperature was 25°C. The reference surface and a blank cycle injection (HBS-EP+) were substracted and curves were fitted to a kinetic 1:1 binding model in the Biacore Insight Evaluation software (Cytiva) to estimate the binding properties (k_{on}, k_{off} and KD).

Cell assay: The experiment was performed as described in Example 4 for SKOV-3 cells, but with His_6-Z variants incubated at decreasing concentrations from 500 nM to 21 pM. Each variant was tested in triplicates. ZBH481 (SEQ ID NO:481) from the first maturation and the reference variant ZAC12 (SEQ ID NO:543) were included in the assay for comparison. Binding curves were plotted and EC50 values were determined using software GraphPad Prism.

CD spectroscopy analysis: For a subset of His_6-Z variants (n=9), a VTM was performed for each Z variant to determine the Tm, and a CD

spectrum at 250–195 nm was collected at 20°C before and after heating to 90°C, as described in Example 4.

Results

Biacore binding kinetics of Z variants against B7-H3: A single cycle kinetic screen of a subset of Z variants to B7-H3 was analyzed using SPR in a Biacore 8K. The Z variants were injected at five concentrations over immobilized B7-H3 and the binding properties (k_{on}, k_{off} and KD) were estimated. The results are summarized in Table 11. Examples of sensorgrams obtained for six B7-H3 binding polypeptides to B7-H3(4Ig)-His are displayed in Figure 5. All analyzed Z variants showed improved binding compared to the reference variant ZAC12 as well as to the previously matured variant ZBH481.

Cell assay: The binding of six His_6-Z variants to B7-H3 expressing SKOV-3 cells was evaluated by fluorescence intensity. Cells were incubated with decreasing concentrations of purified Z variants (ranging from 500 nM to 21 pM), followed by an anti-Z pAb and a Alexa488-conjugated anti-rabbit IgG for detection. Calculated mean EC50 values are summarized in Table 11 and binding curves are shown in Figure 6. All new matured Z variants showed improved cell binding compared to the reference variant ZAC12 as well as to the previously matured variant ZBH481.

Table 11: Summary of kinetic values determined by SPR and EC50 values calculated from SKOV-3 cell binding assay

Z variant	SEQ ID NO	Biacore approx. K_D (M)	Biacore approx. k_{on} (1/Ms)	Biacore k_{off} (1/s)	EC50 (nM)
ZBH001	1	6.0×10^{-10}	5.8×10^{6}	3.5×10^{-3}	10.0
ZBH002	2	7.4×10^{-10}	8.3×10^{6}	6.2×10^{-3}	9.1
ZBH003	3	8.2×10^{-10}	6.8×10^{6}	5.6×10^{-3}	6.5
ZBH004	4	2.6×10^{-9}	2.8×10^{7}	7.1×10^{-2}	n.d.
ZBH005	5	7.7×10^{-10}	6.7×10^{6}	5.2×10^{-3}	n.d.
ZBH006	6	1.6×10^{-9}	7.2×10^{6}	1.2×10^{-2}	n.d.
ZBH007	7	2.8×10^{-10}	9.2×10^{6}	2.5×10^{-3}	5.2
ZBH008	8	2.4×10^{-9}	4.2×10^{6}	1.0×10^{-2}	n.d.
ZBH009	9	3.0×10^{-10}	7.1×10^{6}	2.2×10^{-3}	n.d.
ZBH010	10	3.7×10^{-9}	4.1×10^{6}	1.5×10^{-2}	n.d.
ZBH011	11	3.9×10^{-10}	1.1×10^{7}	4.2×10^{-3}	10.8
ZBH012	12	4.2×10^{-10}	8.8×10^{6}	3.7×10^{-3}	8.9
ZBH013	13	1.3×10^{-9}	8.5×10^{6}	1.1×10^{-2}	n.d.
ZBH014	14	1.1×10^{-9}	4.4×10^{6}	4.9×10^{-3}	n.d.
ZBH015	15	8.9×10^{-10}	1.8×10^{7}	1.6×10^{-2}	n.d.

ZBH016	16	5.9×10^{-10}	1.1×10^{7}	6.6×10^{-3}	n.d.
ZBH017	17	3.2×10^{-9}	6.8×10^{6}	2.2×10^{-2}	n.d.
ZBH018	18	1.8×10^{-9}	7.4×10^{6}	1.4×10^{-2}	n.d.
ZBH019	19	1.3×10^{-9}	4.7×10^{6}	6.3×10^{-3}	n.d.
ZBH020	20	3.3×10^{-9}	4.8×10^{6}	1.6×10^{-2}	n.d.
ZBH021	21	1.2×10^{-9}	7.0×10^{6}	8.7×10^{-3}	n.d.
ZBH481	481	3.5×10^{-9}	3.3×10^{6}	1.2×10^{-2}	18.0
ZAC12	543	1.2×10^{-8}	3.1×10^{6}	3.7×10^{-2}	62.77

n.d: not determined

CD analysis: The CD spectra determined for nine B7-H3 binding Z variants with a His_6 tag showed that all variants have an α-helical structure at 20°C as judged from the typical minima at 208 and 222 nm. After heating to 90°C, five of the Z variants (ZBH001-ZBH004 and ZBH008), showed completely reversible folding, two of the Z variants (ZBH007 and ZBH009) showed nearly reversible folding, whereas two Z variants (ZBH011 and ZBH012) appeared sensitive to the harsh heating to 90°C. The melting temperatures (Tm) are summarized in Table 12. Exemplary CD spectra before and after heating of ZBH001 to 90°C are shown in Figure 7A. The corresponding melting curve for ZBH001 is shown in Figure 7B.

Table 12: Melting temperatures (Tm) determined for His_6 tagged Z variants

Z variant	**SEQ ID NO**	**Tm (°C)**
ZBH001	1	54
ZBH002	2	58
ZBH003	3	68
ZBH004	4	70
ZBH007	7	48
ZBH008	8	59
ZBH009	9	54
ZBH011	11	50
ZBH012	12	58

Example 8

In vitro assessment of a polypeptide in dimeric format

Summary

To test if improved efficacy can be achieved by incorporating additional B7-H3 binding moieties, a dimeric polypeptide ZBHD01 (SEQ ID NO:549) was constructed and produced as described in Example 3 and

studied in binding assays by means of SPR analysis and binding to SKOV-3 cells.

Materials and methods

SPR analysis: Binding of the dimeric polypeptide ZBHD01 and the monomeric Z variant ZBH538 (SED ID NO:538; included for comparison) to B7-H3(4Ig)-Fc was studied using a Biacore 8K instrument (Cytiva). B7-H3(4Ig)-Fc was diluted to 2 nM in HBS-EP+ and captured on flow cell 2 on a Protein A chip (Cytiva) resulting in a capture level of approximately 45 RU. Each of the dimeric polypeptide and the monomeric Z variant was used as analyte at a concentration of 40 nM, injected over both flow cell 1 and 2. Association time was 120 s (50 µL/min) and dissociation time was 600 s (50 µL/min). HBS-EP+ was used as running buffer and 10 mM glycine-HCl pH 1.5 (two pulses of 30 s / 30 µL/min) was used as regeneration buffer. The assay temperature was 25°C. Reference cell (flow cell 1) and blank cycle injection (HBS-EP+) were subtracted from sensorgrams prior to evaluation using Biacore Insight Evaluation Software.

Cell assay: The experiment was performed as described in Example 4 for SKOV-3 cells, but the polypeptide ZBHD01 or ZBH538 was incubated at decreasing concentrations from 555 nM to 28 pM. Each variant was tested in duplicates. Binding curves were plotted and EC50 values were determined using software GraphPad Prism.

Results

SPR analysis: The binding of the dimeric polypeptide ZBHD01 and the monomeric Z variant ZBH538 to B7-H3 was analyzed qualitatively using SPR in a Biacore 8K instrument. The variants were injected over B7-H3 captured on a Protein A chip and the binding curves were compared. The sensorgrams obtained for ZBHD01 and ZBH538, respectively, binding to B7-H3(4Ig)-Fc are displayed in Figure 8 and show a significantly slower off-rate for the dimeric polypeptide.

Cell assay: The binding of ZBHD01 and ZBH538 to B7-H3 expressing SKOV-3 cells was evaluated by fluorescence intensity. Cells were incubated with a decreasing concentration of dimeric or monomeric variant (ranging from 555 nM to 28 pM) followed by an anti-Z pAb and a Alexa488-conjugated anti-rabbit IgG for detection. Calculated mean EC50 values are summarized in Table 13 and the binding curves are shown in Figure 9, exhibiting an

approximately 3.8 times improvement in the EC50 value for the dimeric format.

Table 13: Cell binding data of a dimeric polypeptide to SKOV-3 cells and comparison with a monomeric Z variant

Z variant	SEQ ID NO	EC50
ZBHD01 (dimer)	549	1.8
ZBH538 (monomer)	538	6.8

Example 9
Radiolabeling of B7-H3 binding Z variants

Summary

This Example describes radiolabeling of B7-H3 binding Z variants subsequently used in the experiments described in Example 10-12 and 14-15.

Materials and methods

Labeling with ^{99m}Tc: The Z variants ZBH536 (SEQ ID NO:536), ZBH538 (SEQ ID NO:538), ZBH539 (SEQ ID NO:539) and the reference Z variant ZAC12c (SEQ ID NO:540), all with a unique C-terminal cysteine were site-specifically labeled with ^{99m}Tc. A freeze-dried labelling kit containing 75 µg of tin (II) chloride dihydrate (Fluka Chemika), 5 mg of gluconic acid sodium salt (Celsus Laboratories) and 100 µg of ethylenediaminetetraacetic acid tetra sodium salt ($EDTANa_4$) (Sigma-Aldrich) was prepared as described previously (Ahlgren *et al.*, 2010, Nucl Med Biol 37:539-546). ^{99m}Tc was obtained as pertechnetate by elution of Ultra TechneKow generator (Mallinckrodt) with sterile 0.9 % sodium chloride (Mallinckrodt). Radiolabeling of each Z variant was performed by adding the contents of the freeze-dried kit, dissolved in 120 µL degassed PBS, to 100 µg of Z variant. 80 µL (200–300 MBq) of ^{99m}Tc-pertechnetate was added to the reaction mixture, and the vial was degassed to protect the mixture from oxidation. The reaction vial was thoroughly vortexed and incubated at 90°C for 1 h.

Labeling with ^{111}In: The Z variant ZBH538 (SEQ ID NO:538) and the reference Z variant ZAC12c (SEQ ID NO:540) were DOTA conjugated as described in Example 3 and site-specifically labeled with ^{111}In. 30 µL of 0.2 M

NH_4OAc (pH 5.5) was added to ZBH538-DOTA and ZAC12c-DOTA (40 µg/22 µL, dissolved in 0.2 M NH_4OAc, pH 5.5). The mixture was incubated with [^{111}In]$InCl_3$ (Curium Pharma; 20-40 MBq) at 90°C for 1 h. To remove loosely bound ^{111}In, $EDTANa_4$ (5 mg/mL in Milli-Q water) was added at a 500-fold molar excess to the reaction mixture and incubated at 90°C for 10 min. Purification was performed using NAP-5 columns, pre-equilibrated and eluted with 1% BSA in PBS.

Labeling with ^{68}Ga: Gallium-68 was obtained by fractionated elution of the ^{68}Ge/^{68}Ga generator (Eckert and Ziegler AG) with 0.1 M HCl. The eluate with the highest radioactivity concentration was used for labeling. 40 µg of each NOTA conjugated Z variant ZBH001 (SEQ ID NO:1), ZBH002 (SEQ ID NO:2), ZBH003 (SEQ ID NO:3) and the reference Z variant ZAC12 (SEQ ID NO:543), all in the format G-[ZBH###]-C (SEQ ID NO:628), in 0.2 M NaAc pH 6 was mixed with 100 µL of 1.25 M of NaAc, pH 3.6. Generator eluate (100 µl, 80 MBq), was added and the mixtures were incubated for 10 min at 60°C. Purification was performed using NAP-5 columns, pre-equilibrated and eluted with 1% BSA in PBS.

Labeling with ^{177}Lu: 50 µg of a each DOTA conjugated ABD-fused Z variant ZBHD02 (SEQ ID NO: 550), ZBHD07 (SEQ ID NO: 555), ZBHD08 (SEQ ID NO: 556) and ZBHD09 (SEQ ID NO: 557) in 0.2 M NaAc pH 6 was mixed with 70 µL of 0.2 M sodium acetate, pH 6.25 (metal-free). 50 MBq of $^{177}LuCl_3$ (PerkinElmer) was added and the mixtures were incubated at 75°C for 1 h. To remove loosely bound radionuclide, a 1000-fold excess of $EDTANa_4$ was added and the mixture was incubated at 75°C for 10 min. Purification was performed using NAP-5 columns, pre-equilibrated and eluted with 1% BSA in PBS.

Evaluation of radiolabeling: The radiochemical yield of each Z variant was analyzed by instant thin layer chromatography (ITLC-SG) (Agilent Technologies).

To cross-validate radio-ITLC data, RP-HPLC was conducted on a LaChrom Elite® system (VWR Hitachi) equipped with an L-2130 pump, a UV detector (L-2400), and a radiation flow detector (Bioscan) coupled in series. Purity analysis of labeled compounds was performed using an analytical column (Vydac RP C18 column, 300 Å; 3 × 150 mm; 5 µm). The RP-HPLC conditions were as follows: Solvent A = 10 mM TFA in H_2O, Solvent B = 10 mM TFA in acetonitrile; UV-detection at 220 nm; gradient elution: 0–15 min at

5% to 70% B, 15–18 min at 70% to 95% B, 19–20 min at 5% B; and the flow rate was 1.0 mL/min.

To evaluate the *in vitro* stability of the ^{99m}Tc labeled conjugates, fractions of each of the freshly labeled Z variants (10 µL, 4 µg) were incubated with an excess amount of PBS (40 µL) for 1 h at 37°C. To evaluate the *in vitro* stability of the ^{68}Ga and ^{177}Lu labeled conjugates, fractions of each of the freshly labeled compound were incubated with an excess of EDTA: ^{68}Ga labeled conjugates were incubated at a 1000 molar excess of EDTA for 2 h at 37°C, whereas ^{177}Lu labeled conjugates were incubated at a 500 molar excess of EDTA for 1 h at RT. Incubation was also performed in PBS as a control. The tests were run in triplicates. The release of radionuclide was monitored by ITLC-SG as described above.

Results

Labeling with ^{99m}Tc: B7-H3 binding Z variants were successfully labeled with ^{99m}Tc with a radiochemical yield exceeding 95%, and the RHT was less than 5%.

The specific activity of ^{99m}Tc-labeled conjugates was 3 MBq/µg (molar activity 18.9 GBq/µmol). All ^{99m}Tc-labeled conjugates were stable during incubation at 37°C for 1 h in the presence of excess PBS. Less than 5% release of ^{99m}Tc was observed.

The results of radiolabeling and *in vitro* stability tests are summarized in Table 14A. In the RP-HPLC analysis, the retention time of 11-12 min of labeled Z variant (measured by radioactivity detector) was the same as for non-labeled polypeptide (measured by UV detector). This confirms the authenticity of the labeled conjugates.

Table 14A: Results of ^{99m}Tc radiolabeling and in vitro stability

Z variant	SEQ ID NO	Radiochemical yield (%)	Stability in PBS 37 °C, 1 h (%)
ZBH536	536	96 ± 2	98 ± 1
ZBH538	538	95 ± 4	97 ± 1
ZBH539	539	95 ± 3	98 ± 1
ZAC12c	540	96 ± 2	96 ± 1

Labeling with ^{111}In: The DOTA conjugated ZBH538 and reference Z variant ZAC12c were successfully labeled with ^{111}In with a radiochemical yield exceeding 91%. The radiochemical purities were over 98% following

purification on NAP-5 columns.

Labeling with ^{68}Ga: The NOTA conjugated B7-H3 binding Z variants were successfully labeled with ^{68}Ga with a radiochemical yield exceeding about 88%. The radiochemical purities were over 99% following purification on NAP-5 columns. The specific activity of all ^{68}Ga-labeled conjugates was 1.2 MBq/µg. The Z variants demonstrated high stability during incubation for up to 2 h with a 1000-fold molar excess of EDTA. The results of radiolabeling and *in vitro* stability tests are summarized in Table 14B. In the RP-HPLC analysis, only one major peak was observed for each Z variant at a retention time of 10-12 min.

Table 14B: Results of ^{68}Ga radiolabeling and in vitro stability

Z variant	SEQ ID NO	Radiochemical yield (%)	Stability (%) 37 °C, 2 h	
			PBS	1000×EDTA
ZBH001	1	88 ± 2	96 ± 1	96 ± 1
ZBH002	2	90 ± 2	94 ± 1	94 ± 1
ZBH003	3	90 ± 2	97 ± 1	96 ± 1
ZAC12	543	90 ± 2	96 ± 1	95 ± 1

Labeling with ^{177}Lu: The DOTA conjugated ABD-fused Z variants were successfully labeled with ^{177}Lu with a radiochemical yield exceeding about 86% after EDTA treatment at 75°C for 10 min. The radiochemical purities were over 98% following purification on NAP-5 columns. The specific activity of all ^{177}Lu-labeled conjugates was 1 MBq/µg. The Z variants demonstrated high stability during incubation for up to 1 h with a 500-fold molar excess of EDTA. The results of radiolabeling and *in vitro* stability tests are summarized in Table 14C. In the RP-HPLC analysis, only one major peak was observed for each ABD-fused Z variant at a retention time of around 12 min.

Table 14C: Results of ^{177}Lu radiolabeling and in vitro stability

ABD fusion variant	SEQ ID NO	Radiochemical yield (%)		Stability (%) RT, 1 h	
		Before EDTA	After EDTA	PBS	500×EDTA
ZBHD02	550	98 ± 1	86 ± 2	99 ± 1	97 ± 1
ZBHD07	555	97 ± 1	88 ± 2	96 ± 1	96 ± 1
ZBHD08	556	98 ± 2	89 ± 3	100 ± 1	99 ± 1
ZBHD09	557	96 ± 1	86 ± 1	98 ± 2	97 ± 2

Example 10
In vitro assessment of ^{99m}Tc-labeled B7-H3 binding Z variants

Summary

This Example describes *in vitro* cell based assays for evaluating the binding and specificity of ^{99m}Tc-labeled conjugates before the variants were used in the animal studies described in Example 11.

Materials and methods

Cell cultures: Ovarian carcinoma SKOV-3 and breast carcinoma BT-474 cell lines, both obtained from the American Type Culture Collection (ATCC), were used as B7-H3 expressing positive cell lines. Ramos lymphoma cell line (ATCC) was used as a non-B7-H3 expressing negative control cell line. A B7-H3 specific antibody (mouse IgG1; R&D Systems, cat. no. MAB1027) was used to rank receptor expression levels of different cell lines by titration and quantification of expression using BD Quantibrite beads (BD Biosciences). The B7-H3 expression levels were estimated to be 68000 (high), 45000 (intermediate) and 250 (low) receptors per cell for SKOV-3, BT-474 and Ramos, respectively (Oroujeni *el al.*, 2022, Pharmaceutics 14:1780). Cells were cultured in RPMI medium (Flow Laboratories) supplemented with 10% fetal calf serum (20% fetal calf serum for BT-474), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin. Cells were seeded in cell-culture dishes (35 mm in diameter) with a density of 10^6 cells/dish. A set of three dishes was used for *in vitro* binding specificity assessment.

In vitro binding specificity: In three control dishes each, SKOV-3 and BT-474 cells were pre-saturated with a 200-fold excess of each non-labeled Z variant for 15 min before addition of labeled conjugate. The cells in both pre-saturated and non-saturated dishes were incubated with a labeled conjugate (10 nM) in a humidified incubator (5% CO_2, 37°C) for 1 h. The medium was discarded, and the cells were washed with cold serum-free medium before trypsin–EDTA solution (0.5 mL per dish) was added and cells incubated for an additional 10 min. Detached cells were diluted with 0.5 mL complete medium, re-suspended and transferred to fraction tubes. The radioactivity of cells was measured using an automated gamma-spectrometer equipped with a 3-inch NaI (Tl) well detector (2480 Wizard, Wallac) and the cell-bound radioactivity was calculated. Data was analyzed by unpaired 2-tailed t-test.

In vitro binding affinity: The binding kinetics of the ^{99m}Tc-labeled Z variants to B7-H3 receptors on cells were measured using a LigandTracer Yellow instrument (Ridgeview Instruments) as previously described (Björke *et al.*, 2006 Appl. Radiat. Isot., 64:901–905). SKOV-3 cells were seeded on a local area of a cell culture dish (89 mm in diameter, Nunclon™, NUNC A/S). The measurements were performed at RT to prevent internalization. Uptake curves were recorded at 1 and 3 nM for ^{99m}Tc-ZBH536, ^{99m}Tc-ZBH538 and ^{99m}Tc-ZBH539 and at 2, 6 and 18 nM for ^{99m}Tc-ZAC12c, after which the radioactive medium was withdrawn and replaced with fresh non-radioactive medium, and the dissociation curve was recorded. The data was analyzed using the Interaction Map software (Ridgeview Diagnostics) to calculate association rate, dissociation rate and dissociation constant at equilibrium (KD). Analysis was performed in duplicates.

Results

In vitro binding specificity: The B7-H3-binding specificity of ^{99m}Tc-labeled conjugates was tested using a saturation experiment. The binding was significantly ($p < 5 \times 10^{-5}$) decreased when the cells were pre-saturated with an excess amount of non-labeled anti-B7-H3 Z variant (Figure 10), demonstrating that the binding was B7-H3-mediated.

In vitro binding affinity: The binding kinetics of ^{99m}Tc-labeled conjugates to SKOV-3 cells measured in real-time using a LigandTracer Yellow instrument are presented in Figure 11 and Table 15. The best fit of the binding of the radiolabeled conjugates to SKOV-3 cell line was achieved using a 1:2 model, suggesting that there were two types of interactions with B7-H3. All variants showed K_{D2} values in the low nanomolar range. ^{99m}Tc-ZBH538 showed a K_{D1} value in the low picomolar range (K_{D1}=28 ± 1.3 pM, % weight=12), i.e. the variant with the apparent highest affinity.

Table 15: Apparent kinetic parameters of ^{99m}Tc-labeled Z variants interacting with B7-H3-expressing SKOV-3 cells

Z variant	SEQ ID NO	k_{a1} ($M^{-1}s^{-1}$) $\times10^{5}$	k_{d1} (s^{-1}) $\times10^{-5}$	K_{D1} (M) $\times10^{-9}$	Weight$_1$ (%)	k_{a2} ($M^{-1}s^{-1}$) $\times10^{5}$	k_{d2} (s^{-1}) $\times10^{-3}$	K_{D2} (M) $\times10^{-9}$	Weight$_2$ (%)
ZBH536	536	0.77 ±0.31	0.45 ±2.8	0.121 ±0.0062	27	2.5±1.2	2.9±0.6	13±3.4	73
ZBH538	538	1.2 ±0.5	0.33 ±0.03	0.028 ±0.001	12	3.5±0.5	2.8±0.2	8.2±0.5	88
ZBH539	539	1.1 ±1	1.5 ±1	0.138 ±0.004	21	2.6±0.5	2.1±0.3	8.2±0.4	79

ZAC12c	540	0.17 ±0.5	3.0 ±0.4	1.9 ±0.8	23	1.2±0.1	8.1±0.5	69±7.4	77

Example 11

In vivo biodistribution and imaging studies of ^{99m}Tc labeled B7-H3 binding Z variants

Summary

The use of radionuclide molecular imaging for monitoring B7-H3 expression could replace biopsy sampling, providing a non-invasive, repeatable alternative also allowing for simultaneous detection of B7-H3 in metastases. This Example describes *in vivo* biodistribution studies in both non-tumor and tumor bearing mice performed with the B7-H3 binding Z variants ZBH536 (SEQ ID NO:536), ZBH538 (SEQ ID NO:538), ZBH539 (SEQ ID NO:539) and the reference Z variant ZAC12c (SEQ ID NO:540), all labeled with ^{99m}Tc.

Materials and methods

Animal handling: Animal experiments were performed in accordance with the national legislation for work with laboratory animals. Approval was granted by the Ethical Committee for Animal Research in Uppsala.

Biodistribution in non-tumor-bearing mice: Female NMRI mice (average weight 36.5 ± 6.4 g) were intravenously injected with 3 µg of ^{99m}Tc-labeled Z variant (60 kBq, 100 µL in PBS) into the tail vein. After 4 h, mice were euthanized by overdosing of anaesthetic solution (20 µL of solution per gram of body weight: ketamine, 10 mg/mL; xylazine, 1 mg/mL). This was followed by a heart puncture, and blood samples were collected. Organs and tissue samples were collected and weighed. The organ radioactivity was measured using a gamma spectrometer with a NaI(Tl) detector (2480 Wizard, Wallac), along with three standards and empty syringes for each animal. Uptake values for organs were calculated as the percentage-injected dose per gram tissue (%ID/g). Data was analyzed by unpaired 2-tailed t-test and ANOVA using GraphPad Prism (version 6 for Windows; GraphPad Software) to determine significant differences ($p < 0.05$).

Biodistribution in tumor-bearing mice: Biodistribution and targeting properties were evaluated in BALB/C nu/nu mice bearing B7-H3-positive SKOV-3 xenografts. To establish xenografts, SKOV-3 cells (10^7 cells/mouse)

were subcutaneously injected into the right hind leg of female BALB/c nu/nu mice. As a specificity control, B7-H3-negative Ramos cells (5×10^6 cells/mouse) were subcutaneously implanted on the left hind leg of female BALB/c nu/nu mice. The experiments were performed three weeks after cell implantation. The average animal weight was 19.1 ± 1.9 g. The average tumor weight was 0.10 ± 0.08 g and 0.09 ± 0.07 g for SKOV-3 and Ramos xenografts, respectively. Groups of four tumor bearing mice were injected with ^{99m}Tc-labeled Z variant (3 µg, 60 kBq, 100 µL in PBS) into the tail vein. To test B7-H3-specific accumulation, one group of animals bearing B7-H3-negative Ramos xenografts was injected with the same peptide and activity doses for each conjugate. The biodistribution was measured 4 h after injection and was performed and analyzed as described above for NMRI mice.

In vivo imaging: To confirm biodistribution results, a small animal SPECT/CT imaging was performed. One SKOV-3 xenograft mouse and one Ramos xenograft mouse was intravenously injected with 6 MBq/3 µg of ^{99m}Tc-labeled Z variant. The mice were imaged at 4 h after injection using a nanoScan SPECT/CT scanner (Mediso Medical Imaging Systems). The mice were euthanized by CO_2 asphyxiation immediately before being placed in the camera. The computed tomography (CT) acquisition was carried out at the following parameters: energy peak of 50 kV, 670 µA, 480 projections, and 2.29 min acquisition time. SPECT acquisition was performed at the following parameters: ^{99m}Tc energy peak of 140 keV, window width of 20%, matrix of 256 × 256, and acquisition time of 1 h. CT images were reconstructed in real-time using Nucline 2.03 Software (Mediso Medical Imaging Systems). SPECT raw data were reconstructed using TeraTomo™ 3D SPECT reconstruction technology.

Results

Biodistribution in non-tumor-bearing mice: The results of biodistribution of ^{99m}Tc-labeled Z variants in NMRI mice 4 h after injection are presented in Figure 12 and Table 16. Biodistribution data demonstrated that there was no significant difference in blood concentration. Less uptake in almost all organs and tissues was observed for ^{99m}Tc-ZBH538. The hepatic uptake as well as uptake in spleen was significantly lower for ^{99m}Tc-ZBH536, ^{99m}Tc-ZBH538 and ^{99m}Tc-ZBH539 compared to the reference variant ^{99m}Tc-ZAC12c; the renal uptake was at the same level for all radioconjugates.

Table 16. Biodistribution of ^{99m}Tc labeled Z variants in female NMRI mice at 4 h after injection. Data expressed as %ID/g; averages from 4 mice ± SD

Organ	ZBH536	ZBH538	ZBH539	ZAC12c (ref)
Blood	0.11 ± 0.04	0.06 ± 0.03	0.09 ± 0.01	0.10 ± 0.01
Salivary	0.11 ± 0.02	0.04 ± 0.01	0.12 ± 0.02	0.07 ± 0.01
Lung	0.28 ± 0.01	0.17 ± 0.09	0.43 ± 0.09	0.14 ± 0.02
Liver	0.55 ± 0.04	0.25 ± 0.10	0.49 ± 0.08	1.94 ± 0.16
Spleen	0.27 ± 0.04	0.13 ± 0.06	0.29 ± 0.10	0.68 ± 0.09
Pancreas	0.11 ± 0.01	0.14 ± 0.07	0.13 ± 0.03	0.06 ± 0.01
Stomach	0.22 ± 0.01	0.09 ± 0.01	0.21 ± 0.02	0.10 ± 0.02
Small int	0.20 ± 0.06	0.15 ± 0.04	0.19 ± 0.07	0.15 ± 0.04
Kidney	4.65 ± 0.72	5.23 ± 0.60	3.36 ± 0.42	5.91 ± 0.32
Muscle	0.04 ± 0.01	0.10 ± 0.08	0.04 ± 0.01	0.03 ± 0.01
Bone	0.06 ± 0.01	0.03 ± 0.01	0.07 ± 0.01	0.09 ± 0.03
GI*	1.72 ± 0.04	1.61 ± 0.42	3.81 ± 0.35	1.50 ± 0.08
Carcass*	1.79 ± 0.13	1.97 ± 1.18	1.73 ± 0.38	1.44 ± 0.06

* *Data for gastrointestinal (GI) tract with content and carcass are presented as % of injected dose per whole sample.*

Biodistribution in tumor-bearing mice: The results of biodistribution of ^{99m}Tc-labeled Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts 4 h after injection are presented in Figure 13A and Table 17. Tumor-to-organ ratios are presented in Figure 13B and Table 18. The tumor uptake of ^{99m}Tc-ZBH536 (2.15±1.01 %ID/g), ^{99m}Tc-ZBH538 (1.54±0.19 %ID/g) and ^{99m}Tc-ZBH539 (3.19±0.21 %ID/g) was higher than for the reference variant ^{99m}Tc-ZAC12c (1.04±0.08 %ID/g). ^{99m}Tc-ZBH538 showed the lowest blood concentration (0.06 ± 0.01 %ID/g) and significantly ($p<0.05$) less uptake in liver (0.26 ± 0.02 %ID/g) and bone (0.006 ± 0.001 %ID/g) compared to the other radioconjugates. ^{99m}Tc-ZBH539 showed significantly ($p<0.05$) less renal uptake (5.85 ± 0.28 %ID/g) compared to ^{99m}Tc-ZBH538 (10.37 ± 1.34 %ID/g) and ^{99m}Tc-ZAC12c (10.30 ± 1.13 %ID/g). The biodistribution profile resulted in significantly ($p<0.05$) higher tumor-to-blood ratio for ^{99m}Tc-ZBH538 (25.7 ± 2.5) compared to ^{99m}Tc-ZBH536 (11.3 ± 4.2) and ^{99m}Tc-ZAC12c (11.0 ± 0.5). The tumor-to-liver ratio for ^{99m}Tc-ZBH538 (5.9 ± 0.8) was significantly ($p<0.05$) higher than for the other radioconjugates.

Furthermore, the uptake of ^{99m}Tc-ZBH538 in B7-H3-positive SKOV-3 xenografts was significantly ($p < 5 \times 10^{-5}$) higher than in B7-H3-negative Ramos xenografts 4 h after injection (Figure 14), which supports that the tumor accumulation was B7-H3-specific *in vivo*.

Table 17. Biodistribution 4 h after injection of ^{99m}Tc labeled B7-H3 targeted Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts. Data expressed as %ID/g; averages from 4 mice ± SD

Organ	ZBH536	ZBH538	ZBH539	ZAC12c (ref)
Blood	0.18±0.04	0.06±0.01	0.13±0.03	0.10±0.01
Salivary	0.96±0.23	0.08±0.01	0.42±0.04	0.15±0.03
Lung	0.33±0.03	0.12±0.01	0.30±0.07	0.14±0.03
Liver	2.82±0.56	0.26±0.02	1.27±0.31	2.57±0.42
Spleen	0.62±0.20	0.11±0.01	0.59±0.11	0.63±0.11
Pancreas	0.14±0.03	0.05±0.01	0.13±0.01	0.06±0.01
Stomach	0.99±0.21	0.13±0.02	0.28±0.01	0.20±0.05
Small int	0.27±0.06	0.17±0.01	0.22±0.07	0.16±0.03
Kidney	7.90±1.42	10.37±1.34	5.85±0.28	10.30±1.13
Tumor	2.15±1.01	1.54±0.19	3.19±0.21	1.04±0.08
Muscle	0.03±0.01	0.02±0.01	0.02±0.01	0.014±0.004
Bone	0.02±0.01	0.006±0.001	0.018±0.00	0.024±0.002
GI tract*	1.95±0.28	1.47±0.08	4.18±0.53	1.37±0.29
Carcass*	2.57±1.22	0.61±0.04	1.98±0.84	1.39±0.28

* *Data for gastrointestinal (GI) tract with content and carcass are presented as % of injected dose per whole sample.*

Table 18. Tumor-to-organ ratios 4 h after injection of ^{99m}Tc labeled B7-H3 targeted Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts. Data expressed as %ID/g; averages from 4 mice ± SD

Organ	ZBH536	ZBH538	ZBH539	ZAC12c (ref)
Blood	11.3±4.2	25.7±2.5	23.5±6.2	11.0±0.5
Salivary	2.3±1.0	19.1±4.0	7.4±0.4	7.1±1.8
Lung	6.2±2.7	12.5±1.1	10.4±1.9	7.6±1.8
Liver	0.8±0.4	5.9±0.8	2.6±0.5	0.4±0.1
Spleen	3.5±1.4	14.3±2.8	5.5±0.7	1.7±0.3
Pancreas	14.4±5.4	29.9±7.0	24.0±1.9	17.3±3.4
Stomach	2.1±0.9	12.0±2.0	11.1±0.7	5.9±1.6
Small int	7.8±3.5	9.0±2.1	14.3±3.6	6.5±1.1
Kidney	0.3±0.1	0.15±0.01	0.55±0.04	0.10±0.01
Muscle	46.2 ± 14.6	152.9 ± 85.8	203.1 ± 92.0	72.2 ± 20.8
Bone	131.8 ± 56.4	280.5 ± 50.8	183.5 ± 39.7	43.6 ± 5.7

In vivo imaging: Results of the nanoSPECT/CT imaging (Figure 15) demonstrated a high-contrast visualization of B7-H3 expression in B7-H3 expressing SKOV-3 tumor 4 h post injection of ^{99m}Tc-labeled Z variants. Less accumulation of activity in the liver was visualized in particular for ^{99m}Tc-ZBH538. Activity uptake in B7-H3 negative Ramos Xenografts was

considerably lower than in the SKOV-3 xenograft (Figure 16), which confirmed B7-H3-mediated binding of these tracers *in vivo*.

Example 12

In vitro and *in vivo* assessment of an ^{111}In-labeled B7-H3 binding Z variant

Summary

This Example describes *in vitro* characterization as well as *in vivo* biodistribution studies in tumor bearing mice, performed with the B7-H3 binding Z variant ZBH538 (SEQ ID NO:538) and the reference Z variant ZAC12c (SEQ ID NO:540), each labeled with ^{111}In. The experiments were performed essentially as described in Example 10 and 11 for ^{99m}Tc-labeled conjugates. A side-by-side evaluation with the same variants labeled with ^{99m}Tc was performed *in vivo* for a more reliable comparison of differences relating to the radiolabel as such, independently of mice batch-to-batch variability of the animal physiology that may otherwise influence biodistribution.

Materials and methods

In vitro assessment: *In vitro* cell based assays for evaluating the binding specificity and the binding affinity of ^{111}In-labeled conjugates were performed essentially as described in Example 10. For determination of the binding affinity by LigandTracer measurements, the uptake curves were recorded at 1, 3 and 9 nM for ^{111}In-ZBH538 and 2, 6 and 18 nM for ^{111}In-ZAC12c.

Biodistribution in tumor-bearing mice: BALB/C nu/nu mice bearing SKOV-3 and Ramos xenograft, respectively, were prepared as described in Example 11. The average animal weight was 17.8±1.3 g. The average tumor weights were 0.10±0.06 g and 0.7±0.4 g for SKOV-3 and Ramos xenografts, respectively. Groups of four tumor bearing mice were injected with ^{111}In-labeled Z variant (3 µg, 20 kBq, 100 µL in PBS) into the tail vein. To test B7-H3-specific accumulation, one group of animals bearing B7-H3-negative Ramos xenografts was injected with the same peptide and activity doses for each conjugate. The biodistribution was measured 4 and 24 h after injection in mice bearing SKOV-3 xenografts and at 4 h after injection in mice bearing Ramos xenografts. For comparison, two groups of mice were injected with

^{99m}Tc-ZBH538 and ^{99m}Tc- ZAC12c (3 µg, 60 kBq, 100 µL in PBS) into the tail and the biodistribution measurements were performed at 4 h after injection.

In vivo imaging: Two SKOV-3 bearing xenograft mice were intravenously injected with 1-2 MBq/3 µg of ^{111}In-labeled ZBH538 and ZAC12c, respectively. To confirm *in vivo* specificity, two mice bearing Ramos xenograft were intravenously injected with the same activity dose of each radiolabeled Z variant. The mice were imaged at 4 h after injection using a nanoScan SPECT/CT scanner as described in Example 11.

Results

In vitro binding specificity: The B7-H3-binding specificity of ^{111}In-labeled conjugates was tested using a saturation experiment. The binding was significantly ($p < 5 \times 10^{-5}$) decreased when the cells were pre-saturated with an excess amount of non-labeled anti-B7-H3 Z variant, demonstrating that the binding was B7-H3-mediated.

In vitro binding affinity: The binding kinetics of ^{111}In-labeled conjugates to SKOV-3 cells measured in real-time using a LigandTracer Yellow instrument are presented in Figure 17 and Table 19. The best fit of the binding of the ^{111}In-labeled conjugates to SKOV-3 cell line was achieved using a 1:1 model, resulting in a K_D in the subnanomolar range.

Table 19: Apparent kinetic parameters of ^{111}In-labeled Z variants interacting with B7-H3-expressing SKOV-3 cells

Z variant	SEQ ID NO	k_a ($M^{-1}s^{-1}$) $\times 10^4$	k_d (s^{-1}) $\times 10^{-6}$	K_D (M) $\times 10^{-9}$
ZBH538	538	14.2±0.2	7.0±4.9	0.049±0.034
ZAC12c	540	7.3±1.7	17.7±5.0	0.259±0.131

Biodistribution in tumor-bearing mice: The results of biodistribution of ^{111}In-labeled Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts 4 h and 24 h after injection are presented in Figure 18A-B and Table 20. Tumor-to-organ ratios are presented in Figure 19A-B and Table 21. The tumor uptake of ^{111}In-ZBH538 was 3.63±0.31 and 0.78±0.18 %ID/g at 4 and 24 h after injection, respectively, significantly ($p<0.05$) higher than the corresponding tumor uptake of ^{111}In-ZAC12c (1.80±0.49 and 0.37±0.13 %ID/g at 4 and 24 h after injection, respectively). ^{111}In-ZBH538 showed significantly ($p<0.05$) lower hepatic uptake at both time points of the study (1.07±0.08 and 0.80±0.02 %ID/g, respectively) than ^{111}In- ZAC12c (6.43±1.05 and 4.07±0.21

%ID/g, respectively). A quick washout of activity from blood and almost all organs and tissues over time was observed for both radioconjugates.

Table 20. Biodistribution 4 h and 24 h after injection of ^{111}In labeled B7-H3 targeted Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts. Data expressed as %ID/g; averages from 4 mice ± SD

Organ	ZBH538		ZAC12c (ref)	
	4 h	24 h	4 h	24 h
Blood	0.12±0.01	0.019±0.002	0.09±0.02	0.018±0.001
Salivary glands	0.28±0.05	0.23±0.04	0.20±0.07	0.11±0.01
Lung	0.36±0.02	0.19±0.02	0.25±0.05	0.09±0.03
Liver	1.07±0.08	0.80±0.02	6.43±1.05	4.07±0.21
Spleen	0.38±0.04	0.37±0.06	1.49±0.24	1.14±0.14
Pancreas	0.21±0.04	0.12±0.0	0.15±0.05	0.06±0.02
Stomach	0.26±0.03	0.16±0.04	0.21±0.02	0.10±0.03
Small intestine	0.29±0.03	0.21±0.04	0.24±0.03	0.11±0.04
Kidney	317.06±8.16	270.72±8.43	308.27±30.0	225.57±58.89
Tumor	3.63±0.31	0.78±0.18	1.80±0.49	0.37±0.13
Muscle	0.04±0.01	0.03±0.0	0.03±0.01	0.02±0.0
Bone	0.02±0.01	0.014±0.001	0.018±0.001	0.008±0.005
GI tract*	0.54±0.15	0.27±0.03	0.84±0.24	0.18±0.04
Carcass*	3.22±0.07	2.40±0.27	2.59±0.39	1.86±0.67

** Data for gastrointestinal (GI) tract with content and carcass are presented as % of injected dose per whole sample.*

The measured tumor-to-organ ratios showed a significantly ($p<0.05$) higher tumor-to-blood ratio for ^{111}In-ZBH538 at both 4 and 24 h after injection (31.09±2.9 and 43.18±13.82, respectively) compared to ^{111}In- ZAC12c (20.60±7.10 and 20.41±6.75, respectively). Due to the reduction in uptake in almost all organs and tissues over time, higher tumor-to-organ ratios were observed at 4 h compared to at 24 h for both radioconjugates. ^{111}In-ZBH538 generally showed a higher tumor-to-organ ratio 4 h after injection compared to ^{111}In- ZAC12c. For example, the tumor-to-liver ratio (3.40±0.49) and the tumor-to-bone ratio (168.57±29.09) of ^{111}In-ZBH538 was significantly higher than for ^{111}In-ZAC12c (0.28±0.05 and 100.00±29.43, respectively) making ^{111}In-ZBH538 more favorable for imaging of bone and liver metastases at an early time point of imaging.

Table 21. Tumor-to-organ ratios 4 h and 24 h after injection of ^{111}In labeled B7-H3 targeted Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts. Data expressed as %ID/g; averages from 4 mice ± SD

Organ	ZBH538		ZAC12c (ref)	
	4 h	24 h	4 h	24 h
Blood	31.09±2.9	43.18±13.82	20.60±7.10	20.41±6.75
Salivary glands	13.40±2.87	3.45±0.89	9.34±2.53	3.40±1.13
Lung	10.16±0.60	4.01±0.87	7.38±1.50	4.05±0.66
Liver	3.40±0.49	0.97±0.23	0.28±0.05	0.22±0.24
Spleen	9.65±1.56	2.18±0.68	1.24±0.43	0.36±0.09
Pancreas	17.95±3.97	6.77±1.77	12.53±2.98	6.26±1.48
Stomach	13.92±1.89	5.00±1.48	8.45±2.06	3.97±1.30
Small intestine	12.59±1.49	3.81±0.89	7.51±2.14	3.29±0.54
Kidney	0.011±0.001	0.003±0.001	0.006±0.002	0.002±0.000
Muscle	101.78±46.3	26.40±10.03	60.94±15.80	26.94±5.28
Bone	168.57±29.0	57.74±15.24	100.00±29.4	40.58±7.87

The results of a head-to-head comparison of the biodistribution of ^{111}In-labeled and ^{99m}Tc-labeled Z variants in tumor-bearing mice 4 h after injection are presented in Figure 20 and Table 22-23. The data show that the uptake in tumor was significantly ($p<0.05$) higher for ^{111}In-ZBH538 (3.63±0.31 %ID/g) compared with both ^{99m}Tc-labeled conjugates (^{99m}Tc-ZBH538:1.59±0.19 %ID/g and ^{99m}Tc-ZAC12c: 0.84±0.18 %ID/g). The hepatic uptake was significantly ($p<0.05$) lower for ^{111}In-ZBH538 (1.07±0.08 %ID/g) compared to ^{111}In-ZAC12c (6.43±1.05 %ID/g) and also ^{99m}Tc-ZAC12c (2.70±0.30 %ID/g). ^{99m}Tc-ZBH538 showed the lowest uptake in almost all organs and tissues including tumor uptake. The difference in renal uptake for ^{111}In-labels and ^{99m}Tc-labels is due to difference between residualizing and non-residualizing properties of the radiolabels.

Table 22. Comparative biodistribution 4 h after injection of ^{111}In- and ^{99m}Tc-labeled B7-H3 targeted Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts. Data expressed as %ID/g; averages from 4 mice ± SD

Organ	^{111}In-ZBH538	^{111}In-ZAC12c (ref)	^{99m}Tc-ZBH538	^{99m}Tc-ZAC12c (ref)
Blood	0.12±0.01	0.09±0.02	0.11±0.02	0.21±0.03
Salivary	0.28±0.05	0.20±0.07	0.21±0.03	0.62±0.19
Lung	0.36±0.02	0.25±0.05	0.19±0.04	0.29±0.04
Liver	1.07±0.08	6.43±1.05	0.39±0.04	2.70±0.30
Spleen	0.38±0.04	1.49±0.24	0.15±0.07	0.80±0.47
Pancreas	0.21±0.04	0.15±0.05	0.09±0.06	0.15±0.05
Stomach	0.26±0.03	0.21±0.02	0.28±0.07	0.56±0.18
Small int	0.29±0.03	0.24±0.03	0.22±0.06	0.31±0.05
Kidney	317.06±8.16	308.27±30.02	10.89±3.03	15.99±3.59
Tumor	3.63±0.31	1.80±0.49	1.59±0.19	0.84±0.18
Muscle	0.04±0.01	0.03±0.01	0.011±0.002	0.06±0.05
Bone	0.02±0.01	0.018±0.001	0.008±0.003	0.03±0.01
GI tract*	0.54±0.15	0.84±0.24	2.18±0.90	2.19±0.37
Carcass*	3.22±0.07	2.59±0.39	1.56±0.68	4.17±0.70

** Data for gastrointestinal (GI) tract with content and carcass are presented as % of injected dose per whole sample.*

Such a biodistribution profile resulted in significantly ($p<0.05$) higher tumor-to-blood ratio for ^{111}In-ZBH538 (31.09±2.91) compared to both ^{99m}Tc-labeled conjugates(^{99m}Tc-ZBH538:15.74±4.13 and ^{99m}Tc-ZAC12c: 4.11±0.87). There was no significant difference in tumor-to-liver ratio for ^{111}In-ZBH538 (3.40±0.49) and ^{99m}Tc-ZBH538 (4.17±0.60).

Table 23. Tumor-to-organ ratios 4 h after injection of ^{111}In- and ^{99m}Tc-labeled B7-H3 targeted Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts.

Data expressed as %ID/g; averages from 4 mice ± SD

Organ	^{111}In-ZBH538	^{111}In-ZAC12c (ref)	^{99m}Tc-ZBH538	^{99m}Tc-ZAC12c (ref)
Blood	31.09±2.91	20.60±7.10	15.74±4.13	4.11±0.87
Salivary	13.40±2.87	9.34±2.53	7.61±1.36	1.39±0.22
Lung	10.16±0.60	7.38±1.50	8.63±1.95	2.90±0.32
Liver	3.40±0.49	0.28±0.05	4.17±0.60	0.34±0.03
Spleen	9.65±1.56	1.24±0.43	13.11±7.79	0.90±0.14
Pancreas	17.95±3.97	12.53±2.98	20.11±7.33	5.83±0.62
Stomach	13.92±1.89	8.45±2.06	6.04±1.89	1.53±0.23
Small int	12.59±1.49	7.51±2.14	7.41±1.49	2.67±0.38
Kidney	0.011±0.001	0.006±0.002	0.156±0.047	0.05±0.01
Muscle	101.78±46.38	60.94±15.80	156.22±38.60,f	22.78±3.83
Bone	168.57±29.09	100.00±29.43	208.04±69.05	35.01±12.77

In vivo imaging: Results of the nanoSPECT/CT imaging (Figure 21A) of ^{111}In-ZBH538 and ^{111}In-ZAC12c in BALB/C nu/nu mice bearing B7-H3-positive SKOV-3 xenografts 4 h after injection confirmed *ex vivo* biodistribution data. Less accumulation of activity in the liver and higher accumulation in tumor for ^{111}In-ZBH538 compared to ^{111}In-ZAC12c were observed. Furthermore, activity uptake in B7-H3-negative Ramos xenografts was considerably lower than in the SKOV-3 xenograft (Figure 21B), which confirmed B7-H3-mediated binding of these ^{111}In-labeled conjugates *in vivo*.

Example 13

In vitro characterization of ABD fused B7-H3 binding Z variants

Summary

For *in vivo* use, extended half-life of a polypeptide may be desirable and one mean of achieving this is by fusion to an ABD moiety. This example describes *in vitro* characterization of eight DOTA conjugated ABD fused Z variants (ZBHD02-ZBHD09; SEQ ID NO: 550-557) constructed in different formats with regards to 1) placement of the ABD moiety; 2) number of B7-H3 binding Z moieties and 3) design of linkers between these moieties. The polypeptides were cloned and produced as described in Example 3 and studied in binding assays by means of SPR analysis and binding to SKOV-3 cells.

Materials and methods

SPR analysis - binding to B7-H3: A multi cycle kinetic (MCK) screen of the polypeptides binding to B7-H3 was run using SPR in a Biacore 8K. B7-H3(4Ig)-His was immobilized through amine coupling onto the carboxylated dextran layer of the surface of a CM5 chip (Cytiva) according to the manufacturer's recommendations. Surface 1 was activated and then deactivated and used as a reference. Each polypeptide was injected over chip surfaces for 150 s at concentrations of 15, 45 and 135 nM followed by dissociation for 720 s (flow rate 50 µl/min). The chip surfaces were regenerated using 50 mM NaOH (two pulses of 20 s / 30 µL/min). HBS-EP+ was used as running and dilution buffer. The assay temperature was 25°C. Reference cell (flow cell 1) and blank cycle injection (HBS-EP+) were subtracted from sensorgrams prior to evaluation using Biacore Insight Evaluation Software.

SPR analysis - binding to albumin: A similar MCK analysis as described in the previous section was used. HSA (Albumedix, cat. no. 205-005) and MSA (Sigma Aldrich, cat. no. A3559), respectively, was immobilized on the CM5 chip. The polypeptides ZBHD02 (SEQ ID NO:550), ZBHD07 (SEQ ID NO:555), ZBHD08 (SEQ ID NO:556) and ZBHD09 (SEQ ID NO:557), was injected over chip surfaces for 400 s at concentrations of 1.6, 8, 40, 200 and 1000 nM followed by dissociation for 4 h (flow rate 30 µl/min).

Cell assay: The binding of the polypeptides to B7-H3 expressing SKOV-3 cells was evaluated by fluorescence intensity. The experiment was performed as described in Example 4 for SKOV-3 cells, but the polypeptides were incubated at decreasing concentrations from 500 nM to 0.16 nM and in the presence of 1.5 µM HSA.

Results

SPR analysis - binding to B7-H3: A multi cycle kinetic screen of polypeptides binding to B7-H3 was analyzed using SPR in a Biacore 8K. The polypeptides were injected at three concentrations over immobilized B7-H3. An example of sensorgrams obtained for the eight analyzed B7-H3 binding polypeptides ZBHD02-ZBHD09 (SEQ ID NO: 550-557) to B7-H3(4Ig)-His is displayed in Figure 22.

SPR analysis - binding to albumin: All four tested polypeptides showed retained and similar binding kinetic values towards HSA as well as similar

binding kinetic values towards MSA (Figure 23). As expected, a faster off-rate and a lower K_D was seen for binding to MSA compared to binding to HSA.

Cell assay: Calculated EC50 values for binding of the ABD-fused polypeptides to B7-H3 expressing SKOV-3 cells are summarized in Table 24 and binding curves are shown in Figure 24.

Table 24: Cell binding data for ABD fused Z variants binding to SKOV-3 cells

Z variant	SEQ ID NO:	EC50 (nM)
ZBHD02	550	16.3
ZBHD03	551	106.1
ZBHD04	552	2.8
ZBHD05	553	4.6
ZBHD06	554	4.2
ZBHD07	555	2.9
ZBHD08	556	1.7
ZBHD09	557	1.3

In line with the observations described in Example 8, both the SPR and the cell assay analysis show that the ABD fusion polypeptides that are dimeric with regards to the Z moiety (ZBHD04-ZBHD09) show a stronger binding to B7-H3 compared to the ABD fusion polypeptides containing one Z moiety (ZBHD02 and ZBHD03).

Example 14

In vitro and *in vivo* assessment of ^{177}Lu-labeled ABD-fused B7-H3 binding Z variants

Summary

This Example describes *in vitro* characterization as well as *in vivo* biodistribution studies in tumor bearing mice, performed with a subset of the ABD fused B7-H3 binding Z variants assessed in Example 13: ZBHD02 (SEQ ID NO:550), ZBHD07 (SEQ ID NO:555), ZBHD08 (SEQ ID NO:556) and ZBHD09 (SEQ ID NO:557), each labeled with ^{177}Lu as described in Example 9.

Materials and methods

In vitro assessment: *In vitro* cell based assays for evaluating the binding specificity and the binding affinity of ^{177}Lu-labeled ABD-fused Z variants were performed essentially as described in Example 10. For

determination of the binding affinity by LigandTracer measurements, the uptake curves were recorded at 1 and 3 nM both in the presence and absence of 100 nM HSA.

Biodistribution in tumor-bearing mice: BALB/C nu/nu mice bearing SKOV-3 and Ramos xenograft, respectively, were prepared, and biodistribution measurements performed two weeks after cell implantation, essentially as described in Example 11. The average animal weight was 18.5±0.7 g. The average tumor weight was 0.4±0.2. Groups of four SKOV-3 xenograft mice were injected with ^{177}Lu-labeled ABD-fused Z variant (468 pmol, 260 kBq, 100 µL in PBS) into the tail vein. The biodistribution was measured 48 h after injection.

Additional biodistribution studies were performed on ^{177}Lu-ZBHD02 (SEQ ID NO:550) which was also assessed at 24 and 168 h. Two groups of four mice bearing SKOV3 xenograft were injected with ^{177}Lu- ZBHD02 (468 pmol, 260 kBq, 100 µL in PBS).

In vivo imaging: One group of four SKOV-3 xenograft mice were intravenously injected with 3 MBq of ^{177}Lu-ZBHD02, ^{177}Lu-ZBHD07, ^{177}Lu-ZBHD08 and ^{177}Lu-ZBHD09, respectively. To confirm the *in vivo* specificity, one Ramos xenograft mouse and one SKOV-3 xenografts mouse were intravenously injected with 3 MBq of ^{177}Lu-ZBHD02. In both experiments, nanoSPECT/CT images were collected 48 h after injection using a NanoScan SC. CT acquisitions were carried out using an X-ray energy of 50 keV; 20-min SPECT helical scans were acquired using energy windows 50–62, 103–124, and 188–230 keV. The data were reconstructed using Tera-Tomo™ 3D SPECT Software.

Results

In vitro binding specificity: The B7-H3-binding specificity of ^{177}Lu-labeled ABD-fused Z variants was tested using a saturation experiment. The binding was significantly ($p < 0.05$) decreased when the cells were pre-saturated with 200-fold excess of non-labeled anti-B7-H3 Z variant, demonstrating that the binding was B7-H3-mediated.

In vitro binding affinity: The binding kinetics of ^{177}Lu-labeled ABD-fused Z variants to SKOV-3 cells measured in real-time using a LigandTracer Yellow instrument are presented in Table 25. The best fit of the binding was achieved using a 1:2 model. Higher affinity binding (lower K_D) were observed for the variants comprising two Z moieties (ZBHD07, ZBHD08 and ZBHD09)

compared to the varaints cointaining a single Z moiety (ZBHD02), which is in line with the results presented in Exampe 13 for corresponding unlabeled variants.

Table 25: Apparent equilibrium dissociation constants (K_D) of ^{177}Lu-labeled ABD-fused Z variants interacting with B7-H3-expressing SKOV-3 cells

ABD fusion variant	SEQ ID NO	K_{D1} (M) $\times 10^{-9}$	Weight (%)	K_{D2} (M) $\times 10^{-9}$	Weight (%)
ZBHD02	550	0.245±0.01	11	31±4	89
ZBHD02 + HSA	550	0.484±0.194	19	41±6	81
ZBHD07	555	0.012±0.005	34	3±1	66
ZBHD07 + HSA	555	0.428±0.110	82	17±8	18
ZBHD08	556	0.017±0.003	88	9±3	12
ZBHD08 + HSA	556	0.032±0.003	92	8±1	8
ZBHD09	557	0.009±0.0002	75	10±2	25
ZBHD09 + HSA	557	0.058±0.022	88	9±1	12

Biodistribution in tumor-bearing mice: The results of a head-to-head comparison of the biodistribution of four ^{177}Lu-labeled ABD-fused Z variants in SKOV-3 tumor-bearing mice 48 h after injection are presented in Figure 25A and Table 26. The data show that the tumor uptake of ^{177}Lu-ZBHD02 (19.64±0.77 %ID/g) was significantly ($p<0.05$) higher than that of ^{177}Lu-ZBHD08 (13.13±1.15 %ID/g) and ^{177}Lu-ZBHD09 (13.95±0.52 %ID/g). The renal uptake of ^{177}Lu-ZBHD02 (16.44±1.46 %ID/g) was significantly ($p<0.05$) lower than for ^{177}Lu-ZBHD08 (19.77±2.07 %ID/g) and ^{177}Lu-ZBHD09 (23.99±2.30 %ID/g). No significant difference was observed in the hepatic

uptake. A significantly ($p<0.05$) higher blood concentration was seen for ^{177}Lu-ZBHD02 and ^{177}Lu-ZBHD07 than for ^{177}Lu-ZBHD08.

Table 26: Comparative biodistribution 48 h after injection of ^{177}Lu-labeled B7-H3 targeted ABD-fused Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts. Data expressed as %ID/g; averages from 4 mice ± SD

Organ	ZBHD02	ZBHD07	ZBHD08	ZBHD09
Blood	6.52±0.49	7.39±0.54	5.06±0.66	6.36±0.43
Lung	4.34±0.42	4.22±0.35	3.20±0.32	3.79±0.36
Liver	5.97±1.07	4.02±0.35	4.23±0.55	4.41±0.69
Spleen	5.00±0.48	4.89±0.56	5.42±0.63	5.08±0.35
Stomach	1.34±0.16	1.13±0.14	1.04±0.11	1.14±0.10
Small intestine	2.45±0.44	1.57±0.4	2.01±0.20	2.07±1.04
Kidney	16.44±1.46	27.85±3.55	19.77±2.07	23.99±2.30
Tumor	19.64±0.77	16.33±2.36	13.13±1.15	13.95±0.52
Muscle	0.96±0.11	0.90±0.08	0.81±0.09	0.88±0.11
Bone	1.87±0.32	1.52±0.17	1.61±0.18	1.58±0.09
GI tract*	2.58±0.35	1.87±0.39	2.22±0.17	1.96±0.06
Carcass*	29.77±4.64	23.31±4.22	26.99±0.87	26.33±1.99

** Data for gastrointestinal (GI) tract with content and carcass are presented as % of injected dose per whole sample.*

The biodistribution of ^{177}Lu-ZBHD02 at additional time points, 24 and 168 h after injection, are presented in Figure 25B and Table 27. The clearance from blood and normal organs and tissues such as, lung, stomach, muscle and bone was rapid over time. The tumor uptake was 20.29±3.14 %ID/g 24 h after injection with a retention up to 48 h (19.64±0.77 %ID/g) followed by a decrease to 12.61±2.46 %ID/g at 168 h after injection. No significant difference was observed in the renal uptake between 24 h (14.84±0.61 %ID/g) and 48 h (16.44±1.46 %ID/g) after injection, but the activity in the kidney had dropped significantly ($p<0.05$) at 168 h (5.97±1.03 %ID/g). Based on the area under the curve for blood, kidney, tumour and

bone, the tumour-to-blood, tumour-to-kidney and tumour-to-bone ratios were 3.5, 1.4 and 10.6, respectively.

Table 27: Biodistribution at, 24, 48 and 168 h after injection of ^{177}Lu-ZBHD02 *in BALB/C nu/nu mice bearing SKOV-3 xenografts. Data expressed as %ID/g; averages from 4 mice ± SD*

Organ	24 h	48 h	168 h
Blood	10.22±0.26	6.52±0.49	0.74±0.15
Lung	5.53±0.18	4.34±0.42	1.18±0.16
Liver	6.90±2.26	5.97±1.07	2.80±0.25
Spleen	4.85±0.56	5.00±0.48	3.48±0.43
Stomach	2.27±0.41	1.34±0.16	0.42±0.12
Small intestine	4.43±0.79	2.45±0.44	0.93±0.17
Kidney	14.84±0.61	16.44±1.46	5.97±1.03
Tumor	20.29±3.14	19.64±0.77	12.61±2.46
Muscle	1.75±0.39	0.96±0.11	0.54±0.15
Bone	2.83±0.19	1.87±0.32	0.96±0.11
GI tract*	4.64±0.27	2.58±0.35	0.91±0.23
Carcass*	38.75±2.91	29.77±4.64	11.96±1.71

* *Data for gastrointestinal (GI) tract with content and carcass are presented as % of injected dose per whole sample.*

In vivo imaging: Results of nanoSPECT/CT imaging (Figure 26) in BALB/C nu/nu mice bearing B7-H3-positive SKOV-3 xenografts 48 h after injection confirmed *ex vivo* biodistribution data. I.e.a higher accumulation in tumor and a lower accumulation in kidney was observed for ^{177}Lu-ZBHD02 compared to the other variants. Furthermore, activity uptake in the B7-H3-positive SKOV-3 xenograft (Figure 27A) was considerably higher than in the B7-H3 negative Ramos xenograft (Figure 27B), which confirmed B7-H3-mediated binding *in vivo*.

To summarize, ^{177}Lu-ZBHD02 with a single Z moiety showed a better biodistribution profile compared to ^{177}Lu-ZBHD07, ^{177}Lu-ZBHD08 and ^{177}Lu-ZBHD09, comprising two Z moieties, despite binding to B7-H3 with a lower affinity.

Example 15

In vitro and *in vivo* assessment of 2nd generation of matured B7-H3 binding Z variants

Summary

This Example describes *in vitro* cell binding specificity as well as *in vivo* biodistribution studies in tumor bearing mice, performed with the B7-H3 binding Z variant ZBH001 (SEQ ID NO:1), ZBH002 (SEQ ID NO:2), ZBH003 (SEQ ID NO:3) and the reference Z variant ZAC12 (SEQ ID NO:543), each in the format G-[ZBH###]-C (SEQ ID NO:628), NOTA conjugated as described in Example 3 and labeled with ^{68}Ga as described in Example 9.

Materials and methods

In vitro binding specificity: The *in vitro* cell based assay for evaluating the binding specificity of ^{68}Ga-labeled Z variants were performed essentially as described in Example 10.

Biodistribution in tumor-bearing mice: BALB/C nu/nu mice bearing SKOV-3 and Ramos xenograft, respectively, were prepared, and biodistribution measurements performed three weeks after cell implantation, essentially as described in Example 11. The average animal weight was 18.2±1.2 g. The average tumor weight was 0.11±0.04 and 0.41±0.17 g for SKOV-3 and Ramos xenografts, respectively. Groups of four SKOV-3 xenograft mice were injected with ^{68}Ga-labeled Z variant (2 µg, 400 kBq, 100 µL in PBS) into the tail vein. To test B7-H3-specific accumulation, one group of mice bearing B7-H3-negative Ramos xenografts was injected with the same peptides and dose. The biodistribution was measured 2 h after injection.

In vivo imaging: To confirm biodistribution results, a small animal PET/CT imaging was performed. One SKOV-3 xenograft mouse was intravenously injected with 2.5 MBq of the respective Z variant. In addition, one Ramos xenograft mouse was injected with 2.5 MBq ^{68}Ga ZBH003. The mice were imaged at 2 h after injection using a PET/CT scanner (Mediso Medical Imaging Systems). The mice were euthanized by CO_2 asphyxiation immediately before being placed in the camera. CT acquisition was performed using a nanoScan SPECT/CT (Mediso Medical Imaging Systems Ltd) immediately after PET acquisition using the same bed position. The PET scans were performed for 30 min, followed by CT examination at the following parameters: CT-energy peak of 50 keV, 670 A, 480 projections, 2.29 min acquisition time. The PET data were reconstructed into a static image using Tera-Tomo™ 3D reconstruction engine. CT raw files were reconstructed in real time using Filter Back Projection in Nucline 2.03 Software (Mediso

Medical Imaging Systems). PET and CT files were fused and presented as maximum intensity projections (MIP).

Results

In vitro binding specificity: *In vitro* B7-H3-binding specificity of ^{68}Ga-labeled Z variants was tested using a saturation experiment. The binding was significantly ($p < 0.05$) decreased when the cells were pre-saturated with a 200-fold excess of non-labeled anti-B7-H3 Z variant demonstrating that the binding was B7-H3-specific.

Biodistribution in tumor-bearing mice: Biodistribution in nude mice bearing human cancer xenografts showed that the tumor uptake of ^{68}Ga-labeled Z variants in B7-H3-positive SKOV-3 xenografts was significantly ($p <0.05$) higher than in B7-H3-negative Ramos xenografts 2 h after injection (Figure 28), demonstrating that the tumour accumulation was B7-H3-specific *in vivo*.

The results of biodistribution in mice bearing SKOV-3 xenografts 2 h after injection are presented in Figure 29A and in Table 28. The tumour uptake of all three new variants, ^{68}Ga-ZBH003 (8.96±1.16 %ID/g), ^{68}Ga-ZBH001 (6.61±0.41 %ID/g) and ^{68}Ga-ZBH002 (7.25±1.56 %ID/g) was significantly ($p<0.05$) higher than of the reference variant, ^{68}Ga-ZAC12 (3.24±0.77 %ID/g). The reference variant ^{68}Ga-ZAC12 showed a significantly

($p<0.05$) lower hepatic uptake compared to new variants. The tumour-to-organ ratios are presented in Figure 29B and in Table 29.

Table 28: Comparative biodistribution 2 h after injection of ^{68}Ga-labeled B7-H3 targeted Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts. Data expressed as %ID/g; averages from 4 mice ± SD

Organ	ZBH001	ZBH002	ZBH003	ZAC12 (ref)
Blood	0.27±0.02	0.21±0.04	0.24±0.03	0.11±0.01
Lung	0.96±0.11	0.90±0.11	0.78±0.07	0.28±0.04
Liver	1.69±0.14	1.63±0.21	1.19±0.07	0.41±0.09
Spleen	0.64±0.05	0.78±0.04	0.59±0.07	0.25±0.04
Stomach	0.87±0.04	1.09±0.12	0.90±0.19	0.26±0.07
Small intestine	0.80±0.12	0.96±0.30	0.71±0.15	0.31±0.13
Kidney	207.45±73.64	282.93±27.34	296.93±26.8	315.76±35.56
Tumor	6.61±0.41	7.25±1.56	8.96±1.16	3.24±0.77
Muscle	0.26±0.03	0.41±0.23	0.27±0.04	0.08±0.02
Bone	0.83±0.09	0.96±0.05	1.07±0.32	0.30±0.06
GI tract*	1.28±0.44	1.33±0.31	1.07±0.33	0.53±0.24
Carcass*	8.30±0.55	9.39±1.49	9.46±1.65	2.41±0.73

** Data for gastrointestinal (GI) tract with content and carcass are presented as % of injected dose per whole sample.*

Table 29: Tumor-to-organ ratios 2 h after injection of ^{68}Ga-labeled B7-H3 targeted Z variants in BALB/C nu/nu mice bearing SKOV-3 xenografts. Data expressed as %ID/g; averages from 4 mice ± SD

Organ	ZBH001	ZBH002	ZBH003	ZAC12 (ref)
Blood	24.7±1.4	35.3±8.5	37.3±5.6	28.0±4.8
Lung	6.9±0.7	8.0±1.5	11.6±1.2	11.6±1.3
Liver	3.9±0.2	4.4±0.8	7.5±0.7	7.9±1.0
Spleen	10.4±1.0	9.2±1.8	15.3±1.2	13.2±2.1
Stomach	7.6±0.7	6.6±1.2	10.2±1.6	13.2±4.1
Small intestine	8.4±1.1	8.0±2.3	13.1±3.5	11.5±3.2
Kidney	0.036±0.015	0.025±0.004	0.030±0.003	0.010±0.002
Muscle	25.5±3.7	25.2±9.5	34.5±7.8	40.7±8.4
Bone	8.1±1.3	7.6±1.7	8.7±1.8	11.1±2.0

In vivo imaging: Results of nanoPET/CT imaging (Figure 30) of ^{68}Ga-labeled Z variants in BALB/C nu/nu mice bearing B7-H3-positive SKOV-3 xenografts 2 h after injection confirmed *ex vivo* biodistribution data, i.e all three new variants showed a higher tumor uptake than the reference variant ZAC12. A pronouncedly higher accumulation of activity in tumour for ^{68}Ga ZBH003 compared to other variants was observed. Furthermore, activity uptake of ^{68}Ga ZBH003 in the B7-H3-positive SKOV-3 xenograft (Figure 31A)

was considerably higher than in the B7-H3 negative Ramos xenograft (Figure 31B), which confirmed B7-H3-mediated binding *in vivo*.

ITEMIZED LIST OF EMBODIMENTS

1. B7-H3 binding polypeptide, comprising a B7-H3 binding motif *BM*, which motif consists of an amino acid sequence selected from:

i) $EKX_3X_4ALX_7EIX_{10}X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}X_{21}AFIX_{25}X_{26}LNX_{29}X_{30}$ (SEQ ID NO:567)

wherein, independently of each other,

X_3 is selected from I and V;

X_4 is selected from A, D, E, G, H, K, L, M, N, Q, S, T and Y;

X_7 is selected from A, G, H and S;

X_{10} is selected from I and V;

X_{11} is selected from N and W;

X_{16} is selected from N and T;

X_{17} is selected from H and Y;

X_{18} is selected from A, D, E, G, H, N, Q, S and T;

X_{20} is selected from I and V;

X_{21} is selected from K and R;

X_{25} is selected from A, E, F, H, L and W;

X_{26} is selected from K and S;

X_{29} is selected from A, D and E;

X_{30} is selected from A, D and H; and

ii) an amino acid sequence which has at least 93% identity to the sequence defined in i).

2. B7-H3 binding polypeptide according to item 1, wherein in sequence i),

X_3 is selected from I and V;

X_4 is selected from D, E, G, H, K, L, M, N, Q, S, T and Y;

X_7 is selected from A, G, H and S;

X_{10} is selected from I and V;

X_{11} is selected from N and W;

X_{16} is selected from N and T;

X_{17} is selected from H and Y;

X_{18} is selected from A, D, E, G, H, N, Q, S and T;

X_{20} is selected from I and V;

X_{21} is selected from K and R;

X_{25} is selected from A, E, F, H, L and W;
X_{26} is selected from K and S;
X_{29} is selected from A, D and E; and
X_{30} is selected from A, D and H.

3. B7-H3 binding polypeptide according to any preceding item, wherein in sequence i),
X_3 is selected from I and V;
X_4 is selected from D, G, K, L, M, N, S, T and Y;
X_7 is selected from A, G, H and S;
X_{10} is I;
X_{11} is selected from N and W;
X_{16} is T;
X_{17} is Y;
X_{18} is selected from A, D, E, H, N, Q, S and T;
X_{20} is selected from I and V;
X_{21} is selected from K and R;
X_{25} is selected from A, E, H and W;
X_{26} is K;
X_{29} is selected from A, D and E; and
X_{30} is selected from A, D and H.

4. B7-H3 binding polypeptide according to any preceding item, wherein in sequence i),
X_3 is I;
X_4 is selected from K and S;
X_7 is selected from G and S;
X_{10} is I;
X_{11} is W;
X_{16} is T;
X_{17} is Y;
X_{18} is selected from E, N and Q;
X_{20} is I;
X_{21} is K;
X_{25} is selected from A and H;

X_{26} is K;
X_{29} is selected from A and D; and
X_{30} is selected from A and D.

5. B7-H3 binding polypeptide according to item 1, wherein sequence i) corresponds to the sequence from position 8 to position 37 in a sequence selected from the group consisting of SEQ ID NO:1-535.
6. B7-H3 binding polypeptide according to item 5, wherein sequence i) corresponds to the sequence from position 8 to position 37 in a sequence selected from the group consisting of SEQ ID NO:1-21 and 480-535.
7. B7-H3 binding polypeptide according to item 6, wherein sequence i) corresponds to the sequence from position 8 to position 37 in a sequence selected from the group consisting of SEQ ID NO:1-21.
8. B7-H3 binding polypeptide according to item 7, wherein sequence i) corresponds to the sequence from position 8 to position 37 in a sequence selected from the group consisting of SEQ ID NO:1-3.
9. B7-H3 binding polypeptide according to item 8, wherein sequence i) corresponds to the sequence from position 8 to position 37 in SEQ ID NO:1.
10. B7-H3 binding polypeptide according to item 8, wherein sequence i) corresponds to the sequence from position 8 to position 37 in SEQ ID NO:2.
11. B7-H3 binding polypeptide according to item 8, wherein sequence i) corresponds to the sequence from position 8 to position 37 in SEQ ID NO:3.
12. B7-H3 binding polypeptide according to any preceding item, wherein said binding motif forms part of a three-helix bundle protein domain.
13. B7-H3 binding polypeptide according to item 12, wherein said binding motif essentially constitutes two alpha helices with an interconnecting loop, within said three-helix bundle protein domain.
14. B7-H3 binding polypeptide according to item 12 or 13, wherein said three-helix bundle protein domain is selected from bacterial receptor domains.

15. B7-H3 binding polypeptide according to any one of items 12-14, wherein said three-helix bundle protein domain is selected from domains of protein A from *Staphylococcus aureus* or derivatives thereof.

16. B7-H3 binding polypeptide according to any preceding item, which comprises a binding module (*BMod*), the amino acid sequence of which is selected from:

iii) K-[*BM*]-PSQSX$_a$X$_b$LLX$_c$EAKKLX$_d$X$_e$X$_f$Q (SEQ ID NO:568); wherein [*BM*] is a B7-H3 binding motif as defined in any one of items 1-11;

X$_a$ is selected from A and S;

X$_b$ is selected from E and N;

X$_c$ is selected from A, S and C;

X$_d$ is selected from E, N and S;

X$_e$ is selected from D, E and S; and

X$_f$ is selected from A and S; and

iv) an amino acid sequence which has at least 93% identity to a sequence defined in iii).

17. B7-H3 binding polypeptide according to item 16, wherein sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-535.

18. B7-H3 binding polypeptide according to item 17, wherein sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-21 and 480-535.

19. B7-H3 binding polypeptide according to item 18, wherein sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-21.

20. B7-H3 binding polypeptide according to item 19, wherein sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in a sequence selected from the group consisting of SEQ ID NO:1-3.

21. B7-H3 binding polypeptide according to item 20, wherein sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in sequence SEQ ID NO:1.

22. B7-H3 binding polypeptide according to item 20, wherein sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in sequence SEQ ID NO:2.

23. B7-H3 binding polypeptide according to item 20, wherein sequence iii) corresponds to the amino acid sequence from position 7 to position 55 in sequence SEQ ID NO:3.

24. B7-H3 binding polypeptide according to any preceding item, which comprises an amino acid sequence selected from:

v) YA-[*BMod*]-AP (SEQ ID NO:569);
wherein [*BMod*] is as defined according to any one of items 16-23; and

vi) an amino acid sequence which has at least 86% identity to a sequence defined by v).

25. B7-H3 binding polypeptide according to any preceding item, which comprises an amino acid sequence selected from:

vii) VDAKYAK-*[BM]*-PSQSSELLSEAKKLNDSQAPK (SEQ ID NO:570);
wherein [*BM*] is as defined in any one of items 1-11; and

viii) an amino acid sequence which has at least 86% identity to a sequence defined by vii).

26. B7-H3 binding polypeptide according to any one of items 1-23, which comprises an amino acid sequence selected from:

ix) AEAKFAK-[*BM*]-PSQSSELLSEAKKLSESQAPK (SEQ ID NO:571); wherein [*BM*] is as defined in any one of items 1-11; and

x) an amino acid sequence which has at least 86% identity to a sequence defined by ix).

27. B7-H3 binding polypeptide according to item 26, wherein sequence ix) is selected from the group consisting of SEQ ID NO:15-16, 420-424, 427-428, 430-436, 438-444 and 480-535.

28. B7-H3 binding polypeptide according to any one of items 1-23, which comprises an amino acid sequence selected from:

xi) AEAKYAK-[*BM*]-PSQSSELLSEAKKLNDSQAPK (SEQ ID NO:572); wherein [*BM*] is as defined in any one of items 1-11; and

xii) an amino acid sequence which has at least 86% identity to a sequence defined by xi).

29. B7-H3 binding polypeptide according to item 28, wherein sequence xi) is selected from the group consisting of SEQ ID NO:1-13, 17-418 and 446-479.
30. B7-H3 binding polypeptide according to item 29, wherein sequence xi) is selected from the group consisting of SEQ ID NO:1-13 and 17-21.
31. B7-H3 binding polypeptide according to item 30, wherein sequence xi) is selected from the group consisting of SEQ ID NO:1-3.
32. B7-H3 binding polypeptide according to item 31, wherein sequence xi) is SEQ ID NO:1.
33. B7-H3 binding polypeptide according to item 31, wherein sequence xi) is SEQ ID NO:2.
34. B7-H3 binding polypeptide according to item 31, wherein sequence xi) is SEQ ID NO:3.
35. B7-H3 binding polypeptide according to any one of items 1-23, which comprises an amino acid sequence selected from:
    xiii) AEAKFAK-[*BM*]-PSQSSELLSEAKKLNESQAPK (SEQ ID NO:574); wherein [*BM*] is as defined in any one of items 1-11; and
    xiv) an amino acid sequence which has at least 86% identity to a sequence defined by xiii).
36. B7-H3 binding polypeptide according to item 35, wherein sequence xiii) is SEQ ID NO:14, 425-426, 429, 437 and 445.
37. B7-H3 binding polypeptide according to any preceding item, which is capable of binding to B7-H3 such that the K_D value of the interaction with B7-H3 is at most 1×10^{-6} M, for example as at most 5×10^{-7} M, for example at most 1×10^{-7} M, for example at most 5×10^{-8} M, for example at most 1×10^{-8} M.
38. B7-H3 binding polypeptide according to any preceding item, which comprises additional amino acids at the C-terminal and/or N-terminal end.
39. B7-H3 binding polypeptide according to item 38, wherein said additional amino acid(s) improve(s) production, purification, stabilization *in vitro* or *in vivo*, coupling or detection of the polypeptide.

40. B7-H3 binding polypeptide according to any preceding item in multimeric form, comprising at least two B7-H3 binding polypeptide monomer units, whose amino acids may be the same or different.
41. B7-H3 binding polypeptide according to item 40, wherein said B7-H3 binding polypeptide monomer units are covalently coupled together.
42. B7-H3 binding polypeptide according to item 40, wherein said B7-H3 binding polypeptide monomer units are expressed as a fusion protein.
43. B7-H3 binding polypeptide according to any one of items 40-42, in dimeric form.
44. Fusion protein or conjugate comprising:
    - a first moiety consisting of a B7-H3 binding polypeptide according to any preceding item; and
    - a second moiety consisting of a polypeptide having a desired biological activity.
45. Fusion protein or conjugate according to item 44, wherein said desired biological activity is a therapeutic activity.
46. Fusion protein or conjugate according to item 44, wherein said desired biological activity is a binding activity.
47. Fusion protein or conjugate according to item 44, wherein said desired biological activity is an enzymatic activity.
48. Fusion protein or conjugate according to item 46, wherein said binding activity is an albumin binding activity which increases the *in vivo* half-life of the fusion protein or conjugate and/or changes the biodistribution properties of the fusion protein or conjugate.
49. Fusion protein or conjugate according to item 48, wherein said second moiety comprises the albumin binding domain of streptococcal protein G or a derivative thereof.
50. Fusion protein or conjugate according to item 49, wherein said albumin domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO:547, 548 and 631.
51. Fusion protein or conjugate according to item 50, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:550-566 and SEQ ID NO:632-634.

52. Fusion protein or conjugate according to item 51, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:550-557.

53. Fusion protein or conjugate according to item 51, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:558-560.

54. Fusion protein or conjugate according to item 51, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:561-563.

55. Fusion protein or conjugate according to item 51, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:564-566.

56. Fusion protein or conjugate according to item 51, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:632-634.

57. Fusion protein or conjugate according to item 51, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:557, 560, 563 and 566.

58. Fusion protein or conjugate according to item 51, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:555, 558, 561 and 564.

59. Fusion protein or conjugate according to item 51, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:556, 559, 562 and 565.

60. Fusion protein or conjugate according to item 51, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:632, 633 and 634.

61. Fusion protein or conjugate according to item 46, wherein said binding activity acts to block biological activity.

62. Fusion protein or conjugate according to item 45, wherein the second moiety is a therapeutically active polypeptide.

63. Fusion protein or conjugate according to item 62, wherein the second moiety is an anti-cancer agent.

64. Fusion protein or conjugate according to any one of items 44-47 and 61-63, wherein the second moiety is selected from the group consisting of human endogenous enzymes, hormones, growth factors, chemokines, cytokines and lymphokines.

65. Fusion protein or conjugate according to any one of items 44-47, wherein the second moiety is selected from the group consisting of an antibody and an antigen binding fragment thereof.

66. Fusion protein or conjugate according to item 65, wherein said antibody or antigen binding fragment thereof is selected from the group consisting of full-length antibodies, Fab fragments, Fab' fragments, $F(ab')_2$ fragments, single chain Fab (scFab) fragments, Fc fragments, Fv fragments, single chain Fv (scFv) fragments, $(scFv)_2$, scFv-Fc constructs and domain antibodies.

67. Fusion protein or conjugate according to item 66, wherein said at least one antibody or antigen binding fragment thereof is selected from the group consisting of full-length antibodies, Fab fragments and scFv fragments.

68. Fusion protein or conjugate according to item 67, wherein said at least one antibody or antigen binding fragment thereof is a full-length antibody.

69. Fusion protein or conjugate according to any one of items 65-68, wherein said antibody or antigen binding fragment thereof has affinity for an antigen, for example an antigen associated with cancer.

70. B7-H3 binding polypeptide, fusion protein or conjugate according to any preceding item, further comprising at least one linker, such as at least one linker selected from flexible amino acid linkers, rigid amino acid linkers and cleavable amino acid linkers.

71. B7-H3 binding polypeptide, fusion protein or conjugate according to any preceding item, further comprising a label.

72. B7-H3 binding polypeptide, fusion protein or conjugate according to item 71, wherein said label is selected from the group consisting of fluorescent dyes and metals, chromophoric dyes, chemiluminescent

compounds and bioluminescent proteins, enzymes, radionuclides, radioactive particles and pretargeting recognition tags.

73. B7-H3 binding polypeptide, fusion protein or conjugate according to any preceding item, comprising a chelating environment provided by a polyaminopolycarboxylate chelator conjugated to the B7-H3 binding polypeptide via a thiol group of a cysteine residue or an amine group of a lysine residue.

74. B7-H3 binding polypeptide, fusion protein or conjugate according to item 71, wherein the polyaminopolycarboxylate chelator is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, 1,4,7-triazacyclononane-1,4,7-triacetic acid, diethylenetriaminepentaacetic acid or a derivative thereof.

75. B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 1-72, comprising a chelating environment provided by a peptide based chelator, wherein the peptide sequence representing the peptide based chelator is located in the C-terminus of said B7-H3 binding polypeptide, fusion protein or conjugate.

76. B7-H3 binding polypeptide, fusion protein or conjugate according to item 75, wherein said C-terminal peptide sequence representing the peptide based chelator is selected from the group consisting of -GGGC (SEQ ID NO:620), -GSEC (SEQ ID NO:621), -GGSC (SEQ ID NO:622), -GGEC (SEQ ID NO:623), -GGKC (SEQ ID NO:624) and -KVDC (SEQ ID NO:625).

77. B7-H3 binding polypeptide, fusion protein or conjugate according to item 76, wherein said C-terminal peptide sequence representing the peptide based chelator is -GGGC (SEQ ID NO:620).

78. B7-H3 binding polypeptide, fusion protein or conjugate according to item 72, which comprises a pretargeting recognition tag able to associate with a complement to form a complementary pair of pretargeting moieties, said pair being for example selected from strept(avidin)/biotin, oligonucleotide/complementary oligonucleotide such as DNA/complementary DNA, RNA/complementary RNA, phosphorothioate nucleic acid/ complementary phosphorothioate

nucleic acid and peptide nucleic acid/complementary peptide nucleic acid and morpholinos/complementary morpholinos.

79. B7-H3 binding polypeptide, fusion protein or conjugate according to item 78, wherein said pretargeting recognition tag is a peptide nucleic acid tag.

80. B7-H3 binding polypeptide, fusion protein or conjugate according to item 79, wherein said pretargeting recognition tag is a 10-20-mer peptide nucleic acid sequence, such as a 15-mer peptide nucleic acid sequence.

81. A radiolabeled B7-H3 binding polypeptide, fusion protein or conjugate consisting of a radiochelate of the B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 73-77 and a radionuclide.

82. A radiolabeled B7-H3 binding polypeptide, fusion protein or conjugate according to item 81, wherein said radionuclide is suitable for medical imaging.

83. A radiolabeled B7-H3 binding polypeptide, fusion protein or conjugate according to item 82, wherein said radionuclide is selected from the group consisting of ^{72}As, ^{76}Br, ^{55}Co, ^{61}Cu, ^{64}Cu, ^{18}F, $[^{18}F]AlF$, ^{19}F, ^{66}Ga, ^{67}Ga, ^{68}Ga, ^{110m}In, ^{111}In, ^{123}I, ^{124}I, ^{131}I, ^{177}Lu, ^{51}Mn, ^{52m}Mn, ^{52}Mn, ^{186}Re, ^{188}Re, ^{44}Sc, ^{149}Tb, ^{152}Tb, ^{155}Tb, ^{161}Tb, ^{99m}Tc, ^{45}Ti, ^{86}Y, and ^{89}Zr.

84. A radiolabeled B7-H3 binding polypeptide, fusion protein or conjugate according to item 81, wherein said radionuclide is suitable for therapy.

85. A radiolabeled B7-H3 binding polypeptide, fusion protein or conjugate according to item 84, wherein said radionuclide is selected from the group consisting of ^{225}Ac, ^{212}Bi, ^{213}Bi, ^{67}Cu, ^{166}Ho, ^{177}Lu, ^{212}Pb, ^{149}Pm, ^{186}Re, ^{188}Re, ^{153}Sm, ^{149}Tb, ^{161}Tb, ^{227}Th and ^{90}Y.

86. A polynucleotide encoding a B7-H3 binding polypeptide or fusion protein according to any preceding item.

87. Expression vector comprising a polynucleotide according to item 86.

88. Host cell comprising an expression vector according to item 87.

89. Method of producing a B7-H3 binding polypeptide or fusion protein according to any one of items 1-80, comprising

- culturing a host cell according to item 88 under conditions permissive of expression of said polypeptide from said expression vector, and
- isolating said polypeptide.

90. Method of producing a B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 1-85 by non-biological peptide synthesis using amino acids and/or amino acid derivatives having protected reactive side-chains, the non-biological peptide synthesis comprising
- step-wise coupling of the amino acids and/or the amino acid derivatives to form a polypeptide or fusion protein as described herein having protected reactive side-chains,
- removal of the protecting groups from the reactive side-chains of the polypeptide or fusion protein, and
- folding of the polypeptide, fusion protein or conjugate in aqueous solution.

91. Composition comprising a B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 1-85 and at least one pharmaceutically acceptable excipient or carrier.

92. Composition according to item 91, further comprising at least one additional active agent, such as at least two additional active ingredients, such as at least three additional active ingredients.

93. Composition according to item 92, wherein said at least one additional active agent is an anti-cancer agent.

94. B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 1-85 or a composition according to any one of items 91-93 for use as a medicament, a diagnostic agent, a theranostic agent and/or a prognostic agent.

95. B7-H3 binding polypeptide, fusion protein, conjugate or composition for use according to item 94 as a medicament.

96. B7-H3 binding polypeptide, fusion protein, conjugate or composition for use according to item 95, wherein said polypeptide, fusion protein, conjugate or composition modulates B7-H3 function *in vivo*.

97. B7-H3 binding polypeptide, fusion protein, conjugate or composition for use according to item 94 as a diagnostic agent *in vivo* and/or as a prognostic agent *in vivo*.

98. B7-H3 binding polypeptide, fusion protein, conjugate or composition for use according to item 97 as a diagnostic agent *in vivo*.

99. B7-H3 binding polypeptide, fusion protein, conjugate or composition for use according to item 97 as a prognostic agent *in vivo*.

100. B7-H3 binding polypeptide, fusion protein, conjugate or composition for use according to any one of items 94-99, in the treatment, prognosis or diagnosis of a B7-H3 related disorder or disease.

101. B7-H3 binding polypeptide, fusion protein, conjugate or composition for use according to item 100, wherein said B7-H3 related disorder or disease is cancer.

102. B7-H3 binding polypeptide, fusion protein, conjugate or composition for use according to item 101, wherein said cancer is selected from the group consisting of:
breast cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, gastric cancer, liver cancer including hepatocellular carcinoma, lung cancer including non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, renal cell carcinoma, sarcomas including osteosarcoma, urothelial cell carcinoma, neuroblastoma, medulloblastoma, glioma including glioblastoma and diffuse intrinsic pontine glioma, melanoma, leukemia and mesothelioma.

103. B7-H3 binding polypeptide, fusion protein, conjugate or composition for use according to item 102, wherein said cancer is selected from the group consisting of breast cancer, pancreatic cancer and sarcomas.

104. B7-H3 binding polypeptide, fusion protein, conjugate or composition for use according to any one of items 94-103 wherein administration is selected from the group consisting of:
oral, topical, intravenous, intraperitoneal, subcutaneous, pulmonary, transdermal, intramuscular, intranasal, buccal, sublingual or

suppository administration, such as subcutaneous administration, such as intravenous administration.

105. Method of diagnosis *in vitro*, comprising the steps:
- providing a sample suspected to contain B7-H3;
- contacting said sample with a B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 1-83 or a composition according to any one of items 91-93;
- detecting the binding of the B7-H3 binding polypeptide, fusion protein, conjugate or composition to indicate the presence of B7-H3 in the sample; and
- using the information obtained to establish a diagnosis.

106. Method of prognosis *in vitro*, comprising the steps:
- providing a sample suspected to contain B7-H3;
- contacting said sample with a B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 1-83 or a composition according to any one of items 91-93;
- detecting the binding of the B7-H3 binding polypeptide, fusion protein, conjugate or composition to indicate the presence of B7-H3 in the sample; and
- using the information obtained to establish a prognosis.

107. Method of diagnosis *in vitro* or prognosis *in vitro* according to any one of items 105-106, further comprising the steps:
- repeating the steps of detection, wherein said detecting is performed at several time points at intervals in the same provided sample or a different provided sample, as part of a monitoring of the subject before, during, or after treatment.

108. Method of diagnosis *in vitro* or prognosis *in vitro* according to any one of items 106-107, further comprising a step of obtaining a value corresponding to the amount of the B7-H3 binding polypeptide, fusion protein, conjugate or composition that has bound in or to said sample.

109. Method of diagnosis *in vitro* or prognosis *in vitro* according to item 108, further comprising a step of comparing said value to a reference.

110. Method of diagnosis *in vitro* or prognosis *in vitro* according to any one of items 105-109, wherein said diagnosis or prognosis is in relation to a B7-H3 related disorder or disease.

111. Method of diagnosis *in vitro* or prognosis *in vitro* according to item 110, wherein said B7-H3 related disorder or disease is cancer.

112. Method of diagnosis *in vitro* or prognosis *in vitro* according to item 111, wherein said cancer is selected from the group consisting of: breast cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, gastric cancer, liver cancer including hepatocellular carcinoma, lung cancer including non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, renal cell carcinoma, sarcomas including osteosarcoma, urothelial cell carcinoma, neuroblastoma, medulloblastoma, glioma including glioblastoma and diffuse intrinsic pontine glioma, melanoma, leukemia and mesothelioma.

113. Method of diagnosis *in vitro* or prognosis *in vitro* according to item 112, wherein said cancer is selected from the group consisting of breast cancer, pancreatic cancer and sarcomas.

114. Method of detecting the presence of B7-H3 in a sample, comprising providing a sample suspected to contain B7-H3, contacting said sample with a B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 1-85 or a composition according to any one of items 91-93, and detecting the binding of the B7-H3 binding polypeptide, fusion protein, conjugate or composition to indicate the presence of B7-H3 in the sample.

115. Method for determining the presence of B7-H3 in a subject, comprising the steps of:

a) contacting the subject, or a sample isolated from the subject, with a B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 1-85 or a composition according to any one of items 91-93; and

b) obtaining a value corresponding to the amount of the B7-H3 binding polypeptide, fusion protein, conjugate or composition that has bound in said subject or to said sample.

116. Method according to item 115, in which said B7-H3 binding polypeptide, fusion protein or conjugate is according to any one of items 78-80, or said composition comprises such a B7-H3 binding polypeptide, fusion protein or conjugate, and step a) further comprises contacting the subject with a complementary pretargeting moiety labeled with a detectable label, such as a radionuclide label.

117. Method according to any one of items 114-116, further comprising a step of comparing said value to a reference.

118. Method of treatment of a B7-H3 related disorder, comprising administering to a subject in need thereof an effective amount of a B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 1-85 or a composition according to any one of items 91-93.

119. Method according to item 118, wherein said B7-H3 binding polypeptide, fusion protein or conjugate modulates B7-H3 function *in vivo*.

120. Method according to any one of items 115-119, wherein said method is in relation to a B7-H3 related disorder or disease.

121. Method according to item 120, wherein said B7-H3 related disorder or disease is cancer.

122. Method according to item 121, wherein said cancer is selected from the group consisting of:
Breast cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, gastric cancer, liver cancer including hepatocellular carcinoma, lung cancer including non-small cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, renal cell carcinoma, sarcomas including osteosarcoma, urothelial cell carcinoma, neuroblastoma, medulloblastoma, glioma including glioblastoma and diffuse intrinsic pontine glioma, melanoma, leukemia and mesothelioma.

123. Method according to item 122, wherein said cancer is selected from the group consisting of breast cancer, pancreatic cancer and sarcomas.

124. Method according to item 123, further comprising the steps of:

- contacting the subject with a B7-H3 binding polypeptide, fusion protein or conjugate according to any one of items 78-80 comprising a pretargeting recognition tag, or with a composition comprising such a B7-H3 binding polypeptide, fusion protein or conjugate, and
- contacting the subject with a complementary pretargeting moiety comprising a radionuclide.

125. Method according to any one of items 115-124, wherein said subject is a mammalian subject, such as a human subject.

126. Method according to any one of items 115-125, wherein the method is performed *in vivo*.

127. Method according to item 115, which is a method for medical imaging in which:

- step a) comprises the systemic administration of said B7-H3 binding polypeptide, fusion protein, conjugate or composition to a subject;
- said B7-H3 binding polypeptide, fusion protein, conjugate or composition comprises a radionuclide label suitable for medical imaging; and
- step b) comprises obtaining one or more images of at least a part of the subject's body using a medical imaging instrument, said image(s) indicating the presence of the radionuclide inside the body.

128. Method according to item 116, which is a method for medical imaging in which:

step a) comprises the systemic administration of said B7-H3 binding polypeptide, fusion protein, conjugate or composition to a subject;

- said B7-H3 binding polypeptide, fusion protein, conjugate, composition or pretargeting moiety comprises a radionuclide label suitable for medical imaging; and
- step b) comprises obtaining one or more images of at least a part of the subject's body using a medical imaging instrument, said image(s) indicating the presence of the radionuclide inside the body.

CLAIMS

1. B7-H3 binding polypeptide, comprising a B7-H3 binding motif *BM*, which motif consists of an amino acid sequence selected from:

i) $EKX_3X_4ALX_7EIX_{10}X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}X_{21}AFIX_{25}X_{26}LNX_{29}X_{30}$ (SEQ ID NO:567)

wherein, independently of each other,

X_3 is selected from I and V;

X_4 is selected from A, D, E, G, H, K, L, M, N, Q, S, T and Y;

X_7 is selected from A, G, H and S;

X_{10} is selected from I and V;

X_{11} is selected from N and W;

X_{16} is selected from N and T;

X_{17} is selected from H and Y;

X_{18} is selected from A, D, E, G, H, N, Q, S and T;

X_{20} is selected from I and V;

X_{21} is selected from K and R;

X_{25} is selected from A, E, F, H, L and W;

X_{26} is selected from K and S;

X_{29} is selected from A, D and E;

X_{30} is selected from A, D and H; and

ii) an amino acid sequence which has at least 93% identity to the sequence defined in i).

2. B7-H3 binding polypeptide according to claim 1, wherein sequence i) corresponds to the sequence from position 8 to position 37 in a sequence selected from the group consisting of SEQ ID NO:1-535.

3. B7-H3 binding polypeptide according to any preceding claim, wherein said binding motif forms part of a three-helix bundle protein domain, for example selected from bacterial receptor domains, for example

selected from domains of protein A from *Staphylococcus aureus* or derivatives thereof.

4. B7-H3 binding polypeptide according to any one of claims 1-3, which comprises an amino acid sequence selected from:
   xi) AEAKYAK-[*BM*]-PSQSSELLSEAKKLNDSQAPK (SEQ ID NO:572); wherein [*BM*] is as defined in any one of claims 1-2; and
   xii) an amino acid sequence which has at least 86% identity to a sequence defined by xi).

5. B7-H3 binding polypeptide according to claim 4, wherein sequence xi) is selected from the group consisting of SEQ ID NO:1-13, 17-418 and 446-479.

6. B7-H3 binding polypeptide according to any preceding claim, which is capable of binding to B7-H3 such that the K_D value of the interaction with B7-H3 is at most 1 x 10^{-6} M, for example as at most 5 x 10^{-7} M, for example at most 1 x 10^{-7} M, for example at most 5 x 10^{-8} M, for example at most 1 x 10^{-8} M.

7. Fusion protein or conjugate comprising:
   - a first moiety consisting of a B7-H3 binding polypeptide according to any preceding claim; and
   - a second moiety consisting of a polypeptide having a desired biological activity.

8. Fusion protein or conjugate according to claim 7, wherein said desired biological activity is a binding activity, for example an albumin binding activity which increases the *in vivo* half-life of the fusion protein or conjugate and/or changes the biodistribution properties of the fusion protein or conjugate.

9. Fusion protein or conjugate according to claim 8, wherein said second moiety comprises the albumin binding domain of streptococcal protein G or a derivative thereof, for example comprising an amino acid sequence selected from the group consisting of SEQ ID NO:547, 548 and 631.

10. Fusion protein or conjugate according to claim 9, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:550-566 and 632-634.

11. B7-H3 binding polypeptide, fusion protein or conjugate according to any preceding claim, further comprising a label, for example selected from the group consisting of fluorescent dyes and metals, chromophoric dyes, chemiluminescent compounds and bioluminescent proteins, enzymes, radionuclides, radioactive particles and pretargeting recognition tags.

12. B7-H3 binding polypeptide, fusion protein or conjugate according to any preceding claim, comprising a chelating environment provided by
- a polyaminopolycarboxylate chelator conjugated to the B7-H3 binding polypeptide via a thiol group of a cysteine residue or an amine group of a lysine residue, or by
- a peptide based chelator, wherein the peptide sequence representing the peptide based chelator is located in the C-terminus of said B7-H3 binding polypeptide, fusion protein or conjugate.

13. A radiolabeled B7-H3 binding polypeptide, fusion protein or conjugate consisting of a radiochelate of the B7-H3 binding polypeptide, fusion protein or conjugate according to claim 12 and a radionuclide.

14. A polynucleotide encoding a B7-H3 binding polypeptide or fusion protein according to any preceding claim.

15. Composition comprising a B7-H3 binding polypeptide, fusion protein or conjugate according to any one of claims 1-13 and at least one pharmaceutically acceptable excipient or carrier.

16. B7-H3 binding polypeptide, fusion protein or conjugate according to any one of claims 1-13 or a composition according to claim 15 for use as a medicament, a diagnostic agent *in vivo*, a theranostic agent *in vivo* and/or a prognostic agent *in vivo*.