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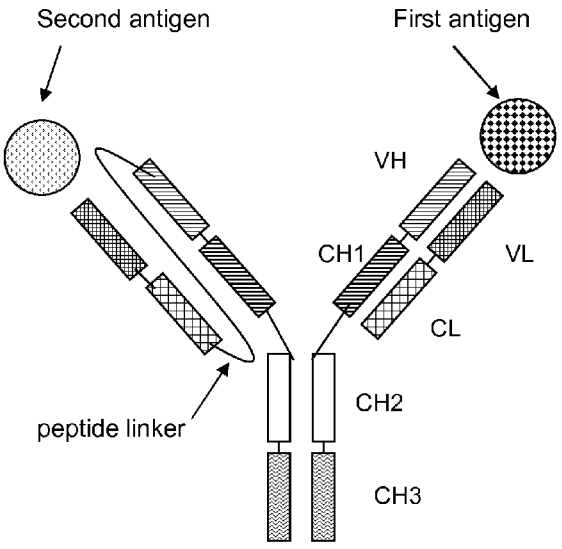
[Continued on next page]

(54) Title: BISPECIFIC ANTIBODIES

Fig. 2



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(57) **Abstract**: The present invention relates to bispecific antibodies, methods for their production, pharmaceutical compositions containing said antibodies, and uses thereof.

Bispecific antibodies

The present invention relates to bispecific antibodies, methods for their production, pharmaceutical compositions containing said antibodies, and uses thereof.

Background of the Invention

A wide variety of multispecific recombinant antibody formats have been developed in the recent past, e.g. tetravalent bispecific antibodies by fusion of, e.g., an IgG antibody format and single chain domains (see e.g. Coloma, M.J., et al., Nature Biotech 15 (1997) 159-163; WO 2001/077342; and Morrison, S.L., Nature Biotech. 25 (2007) 1233-1234).

Also several other new formats wherein the antibody core structure (IgA, IgD, IgE, IgG or IgM) is no longer retained such as dia-, tria- or tetrabodies, minibodies, several single chain formats (scFv, Bis-scFv), which are capable of binding two or more antigens, have been developed (Holliger, P., et al., Nature Biotech 23 (2005) 1126-1136; Fischer, N., Léger O., Pathobiology 74 (2007) 3-14; Shen, J., et al., Journal of Immunological Methods 318 (2007) 65-74; Wu, C., et al., Nature Biotech. 25 (2007) 1290-1297).

All such formats use linkers either to fuse the antibody core (IgA, IgD, IgE, IgG or IgM) to a further binding protein (e.g. scFv) or to fuse e.g. two Fab fragments or scFvs (Fischer N., Léger O., Pathobiology 74 (2007) 3-14). It has to be kept in mind that one may want to retain effector functions, such as e.g. complement-dependent cytotoxicity (CDC) or antibody dependent cellular cytotoxicity (ADCC), which are mediated through the Fc receptor binding, by maintaining a high degree of similarity to naturally occurring antibodies.

In WO 2007/024715 are reported dual variable domain immunoglobulins as engineered multivalent and multispecific binding proteins. A process for the preparation of biologically active antibody dimers is reported in US 6,897,044. Multivalent F_V antibody construct having at least four variable domains which are linked with each over via peptide linkers are reported in US 7,129,330. Dimeric and multimeric antigen binding structures are reported in US 2005/0079170. Tri- or tetra-valent monospecific antigen-binding protein comprising three or four Fab fragments bound to each other covalently by a connecting structure, which protein is not a natural immunoglobulin are reported in US 6,511,663. In WO 2006/020258

tetravalent bispecific antibodies are reported that can be efficiently expressed in prokaryotic and eukaryotic cells, and are useful in therapeutic and diagnostic methods. A method of separating or preferentially synthesizing dimers which are linked via at least one interchain disulfide linkage from dimers which are not linked via at least one interchain disulfide linkage from a mixture comprising the two types of polypeptide dimers is reported in US 2005/0163782. Bispecific tetravalent receptors are reported in US 5,959,083. Engineered antibodies with three or more functional antigen binding sites are reported in WO 2001/077342.

Multispecific and multivalent antigen-binding polypeptides are reported in WO 1997/001580. WO 1992/004053 reports homoconjugates, typically prepared from monoclonal antibodies of the IgG class which bind to the same antigenic determinant are covalently linked by synthetic cross-linking. Oligomeric monoclonal antibodies with high avidity for antigen are reported in WO 1991/06305 whereby the oligomers, typically of the IgG class, are secreted having two or more immunoglobulin monomers associated together to form tetravalent or hexavalent IgG molecules. Sheep-derived antibodies and engineered antibody constructs are reported in US 6,350,860, which can be used to treat diseases wherein interferon gamma activity is pathogenic. In US 2005/0100543 are reported targetable constructs that are multivalent carriers of bi-specific antibodies, i.e., each molecule of a targetable construct can serve as a carrier of two or more bi-specific antibodies.

Genetically engineered bispecific tetravalent antibodies are reported in WO 1995/009917. In WO 2007/109254 stabilized binding molecules that consist of or comprise a stabilized scFv are reported.

Bispecific antibodies against EGFR and IGF-1R are known from Lu, D., et al., Biochemical and Biophysical Research Communications 318 (2004) 507-513; Lu, D., et al., J. Biol. Chem., 279 (2004) 2856-2865; and Lu, D., et al., J. Biol Chem. 280 (2005) 19665-72.

US 2007/0274985 relates to synthetic antibody molecules comprising single chain Fab (scFab) proteins which can also be associated to dimers, including heteromeric antibodies, wherein at least two single chain antibody molecules are associated.

WO 2009/080253 relates to bispecific bivalent antibodies.

However in view of different problems and aspects of multispecific antibodies (like e.g. pharmacokinetic and biological properties, stability, aggregation, expression yield, side products) there is a need of further alternative multispecific antibody formats.

Summary of the Invention

In one aspect the invention is directed to a bispecific antibody comprising

a) the heavy chain and the light chain of a first full length antibody that specifically binds to a first antigen;

b) the heavy chain and the light chain of a second full length antibody that specifically binds to a second antigen, wherein the N-terminus of the heavy chain is connected to the C-terminus of the light chain via a peptide linker.

In another aspect of the invention the bispecific antibody according to the invention is further characterized in that

the CH3 domain of the heavy chain of the full length antibody of a) and the CH3 domain of the heavy chain of the full length antibody of b) each meet at an interface which comprises an alteration in the original interface between the antibody CH3 domains;

wherein i) in the CH3 domain of one heavy chain

an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the interface of the CH3 domain of one heavy chain which is positionable in a cavity within the interface of the CH3 domain of the other heavy chain

and wherein

ii) in the CH3 domain of the other heavy chain

an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the interface of the second CH3 domain within which a protuberance within the interface of the first CH3 domain is positionable.

In another aspect the bispecific antibody according to the invention is characterized in that

both CH3 domains are further altered by the introduction of cysteine (C) as amino acid in the corresponding positions of each CH3 domain such that a disulfide bridge between both CH3 domains can be formed.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

the antibody heavy chain variable domain (VH) and the antibody light chain variable domain (VL) of the heavy and light chain of the second full lenght antibody under b) are disulfide stabilized by introduction of a disulfide bond between the following positions:

i) heavy chain variable domain position 44 to light chain variable domain position 100,

ii) heavy chain variable domain position 105 to light chain variable domain position 43, or

iii) heavy chain variable domain position 101 to light chain variable domain position 100.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to IGF-1R and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 1, and a light chain with the amino acid sequence of SEQ ID NO: 2, and

b) the second full length antibody specifically binds to EGFR and comprises a heavy chain connected to the light chain via a peptide linker wherein said peptide connected heavy and light chain have the amino acid sequence of SEQ ID NO: 3.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to IGF-1R and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 1, and a light chain with the amino acid sequence of SEQ ID NO: 2, and

b) the second full length antibody specifically binds to EGFR and comprises a heavy chain connected to the light chain via a peptide linker wherein said peptide connected heavy and light chain have amino acid sequence of SEQ ID NO: 4.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said peptide connected heavy and light chain have amino acid sequence of SEQ ID NO: 7.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said peptide connected heavy and light chain have amino acid sequence of SEQ ID NO: 8.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that the antibody comprises a constant region of IgG1.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that the antibody is glycosylated with a sugar chain at Asn297 wherein the amount of fucose within the sugar chain is 65 % or lower.

Still further aspects of the invention are a pharmaceutical composition comprising said bispecific antibody, said composition for the treatment of cancer, the use of said bispecific antibody for the manufacture of a medicament for the treatment of cancer, a method of treatment of patient suffering from cancer by administering said bispecific antibody. to a patient in the need of such treatment.

A further aspect of the invention is a nucleic acid molecule encoding a chain of a bispecific antibody according to the invention.

The invention further provides expression vectors containing said nucleic acid according to the invention capable of expressing said nucleic acid in a prokaryotic

or eukaryotic host cell, and host cells containing such vectors for the recombinant production of a bispecific antibody according to the invention.

The invention further comprises a prokaryotic or eukaryotic host cell comprising a vector according to the invention.

The invention further comprises a method for the production of a bispecific antibody according to the invention, characterized by expressing a nucleic acid according to the invention in a prokaryotic or eukaryotic host cell and recovering said bispecific antibody from said cell or the cell culture supernatant. The invention further comprises the antibody obtained by such method for the production of a bispecific antibody.

Another aspect of the invention is a method for the preparation of a bispecific antibody according to the invention comprising the steps of

a) transforming a host cell with vectors comprising nucleic acid molecules encoding
   aa) the heavy chain and the light chain of a first full length antibody that specifically binds to a first antigen;; and
   ab) the heavy chain and the light chain of a second full length antibody that specifically binds to a second antigen, wherein the N-terminus of the heavy chain is connected to the C-terminus of light chain via a peptide linker; and
b) culturing the host cell under conditions that allow synthesis of said antibody molecule; and
c) recovering said antibody molecule from said culture.

It has now been found that the bispecific antibodies according to the invention have valuable characteristics such as good expression yields in mammalian cells (like HEK293 cells and CHO cells), stability, biological or pharmacological activity, pharmacokinetic properties. They can be used e.g. for the treatment of diseases such as cancer. These bispecific antibodies according to the invention comprising 3 poylpeptide chains especially have a valuable side product profile during expression in mammalian cells.

Description of the Figures

Figure 1 Schematic structure of a full length antibody without CH4 domain specifically binding to a first antigen 1 with two

pairs of heavy and light chain which comprise variable and constant domains in a typical order.

Figure 2 Schematic structure of the bispecific antibody according to the invention.

Figure 3a and 3b Schematic structure of the bispecific antibody according to the invention including knobs-into hole modified CH3 domains.

Figure 4 a and 4b Schematic structure of the bispecific antibody according to the invention including knobs-into hole modified CH3 domains and disulfide stabilization of the VH and VL domain of the second antibody heavy and light chain.

Figure 5 Inhibition of the HUVEC proliferation by bispecific antibody according to the invention Ang2-VEGF OA-Ava-N-scFabLC06.

Figure 6 Inhibition of the Tie2 phosphorylation by bispecific antibody according to the invention Ang2-VEGF OA-Ava-N-scFabLC06.

Figure 7 Western blot (reduced) of OA-Ak18-scFab-GA201 (Figure 7a) and OA-GA201-scFab-Ak18 (Figure 7b).

Figure 8 Growth inhibition of H322M cancer cells by the bispecific <EGFR-IGF1R> antibody OA-GA201-scFab-Ak18_WT (dose-dependently) compared to the parental monospecific antibodies <IGF-1R> HUMAB Clone 18 or <EGFR>ICR62.

Figure 9 Biacore (Surface plasmon resonance)-Sensogram: Bispecific antibody OA-GA201-scFab-Ak18_WT showed simultaneous binding of amine coupled human EGFR and human IGF1R (x-axis: response, y-axis: time).

Figure 10 Tumor growth inhibition in BxPC3 Xenograft model by OA-GA201-scFab-Ak18_WT compared to the combination of parental monospecific antibodies <IGF-1R> HUMAB Clone 18 and <EGFR>ICR62

Detailed Description of the Invention

In one aspect the invention is directed to a bispecific antibody comprising

a) the heavy chain and the light chain of a first full length antibody that specifically binds to a first antigen;
b) the heavy chain and the light chain of a second full length antibody that specifically binds to a second antigen, wherein the N-terminus of the heavy chain is connected to the C-terminus of light chain via a peptide linker.

The term "full length antibody" denotes an antibody consisting of two "full length antibody heavy chains" and two "full length antibody light chains" (see Fig. 1). A "full length antibody heavy chain" is a polypeptide consisting in N-terminal to C-terminal direction of an antibody heavy chain variable domain (VH), an antibody constant heavy chain domain 1 (CH1), an antibody hinge region (HR), an antibody heavy chain constant domain 2 (CH2), and an antibody heavy chain constant domain 3 (CH3), abbreviated as VH-CH1-HR-CH2-CH3; and optionally an antibody heavy chain constant domain 4 (CH4) in case of an antibody of the subclass IgE. Preferably the "full length antibody heavy chain" is a polypeptide consisting in N-terminal to C-terminal direction of VH, CH1, HR, CH2 and CH3. A "full length antibody light chain" is a polypeptide consisting in N-terminal to C-terminal direction of an antibody light chain variable domain (VL), and an antibody light chain constant domain (CL), abbreviated as VL-CL. The antibody light chain constant domain (CL) can be κ (kappa) or λ (lambda). The two full length antibody chains are linked together via inter-polypeptide disulfide bonds between the CL domain and the CH1 domain and between the hinge regions of the full length antibody heavy chains. Examples of typical full length antibodies are natural antibodies like IgG (e.g. IgG 1 and IgG2), IgM, IgA, IgD, and IgE. The full length antibodies according to the invention can be from a single species e.g. human, or they can be chimerized or humanized antibodies. The full lenght antibodies according to the invention comprise two antigen binding sites each formed by a pair of VH and VL, which both specifically bind to the same antigen. The C-terminus of the heavy or light chain of said full length antibody denotes the last amino acid at the C-terminus of said heavy or light chain. The N-terminus of the heavy or light chain of said full length antibody denotes the last amino acid at the N- terminus of said heavy or light chain.

The term "peptide linker" as used within the invention denotes a peptide with amino acid sequences, which is preferably of synthetic origin. These peptides according to invention are used to connect the C-terminus of the light chain to the N-terminus of heavy chain of the the second full lenght antibody (that specifically

binds to a second antigen) via a peptide linker. The peptide linker within the second full lenght antibody heavy and light chain is a peptide with an amino acid sequence with a length of at least 30 amino acids, preferably with a length of 32 to 50 amino acids. In one the peptide linker is a peptide with an amino acid sequence with a length of 32 to 40 amino acids. In one embodiment said linker is (GxS)n with G = glycine, S = serine, (x =3, n= 8, 9 or 10 and m= 0, 1, 2 or 3) or (x = 4 and n= 6, 7 or 8 and m= 0, 1, 2 or 3), preferably with x = 4, n= 6 or 7 and m= 0, 1, 2 or 3, more preferably with x = 4, n= 7 and m= 2. In one embodiment said linker is $(G_4S)_6G_2$.Preferably the CH3 domains of the bispecific antibody according to the invention can be altered by the "knob-into-holes" technology which is described in detail with several examples in e.g. WO 96/027011, Ridgway J.B., et al., Protein Eng 9 (1996) 617-621; and Merchant, A.M., et al., Nat Biotechnol 16 (1998) 677-681. In this method the interaction surfaces of the two CH3 domains are altered to increase the heterodimerisation of both heavy chains containing these two CH3 domains. Each of the two CH3 domains (of the two heavy chains) can be the "knob", while the other is the "hole". The introduction of a disulfide bridge stabilizes the heterodimers (Merchant, A.M, et al., Nature Biotech 16 (1998) 677-681; Atwell, S., et al. J. Mol. Biol. 270 (1997) 26-35) and increases the yield.

In one aspect of the invention the bispecific antibody according to the invention is further is characterized in that

the CH3 domain of one heavy chain and the CH3 domain of the other heavy chain each meet at an interface which comprises an original interface between the antibody CH3 domains;

wherein said interface is altered to promote the formation of the bispecific antibody, wherein the alteration is characterized in that:

a) the CH3 domain of one heavy chain is altered,

so that within the original interface the CH3 domain of one heavy chain that meets the original interface of the CH3 domain of the other heavy chain within the bispecific antibody,

an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the interface of the CH3 domain of one heavy chain which is positionable in a cavity within the interface of the CH3 domain of the other heavy chain

and

b) the CH3 domain of the other heavy chain is altered,

so that within the original interface of the second CH3 domain that meets the original interface of the first CH3 domain within the bispecific antibody

an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the interface of the second CH3 domain within which a protuberance within the interface of the first CH3 domain is positionable.

Thus the antibody according to invention is preferably characterized in that

the CH3 domain of the heavy chain of the full length antibody of a) and the CH3 domain of the heavy chain of the full length antibody of b) each meet at an interface which comprises an alteration in the original interface between the antibody CH3 domains;

wherein i) in the CH3 domain of one heavy chain

an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the interface of the CH3 domain of one heavy chain which is positionable in a cavity within the interface of the CH3 domain of the other heavy chain

and wherein

ii) in the CH3 domain of the other heavy chain

an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the interface of the second CH3 domain within which a protuberance within the interface of the first CH3 domain is positionable.

Preferably said amino acid residue having a larger side chain volume is selected from the group consisting of arginine (R), phenylalanine (F), tyrosine (Y), tryptophan (W).

Preferably said amino acid residue having a smaller side chain volume is selected from the group consisting of alanine (A), serine (S), threonine (T), valine (V).

In one aspect of the invention both CH3 domains are further altered by the introduction of cysteine (C) as amino acid in the corresponding positions of each CH3 domain such that a disulfide bridge between both CH3 domains can be formed.

In one embodiment, the bispecific antibody comprises a T366W mutation in the CH3 domain of the "knobs chain" and T366S, L368A, Y407V mutations in the CH3 domain of the "hole chain". An additional interchain disulfide bridge between the CH3 domains can also be used (Merchant, A.M, et al., Nature Biotech 16 (1998) 677-681) e.g. by introducing a Y349C mutation into the CH3 domain of the "knobs chain" and a E356C mutation or a S354C mutation into the CH3 domain of the "hole chain".

In another embodiment, the bispecific antibody according to the invention comprises Y349C, T366W mutations in one of the two CH3 domains and E356C, T366S, L368A, Y407V mutations in the other of the two CH3 domains. In a another preferred embodiment the bispecific antibody comprises Y349C, T366W mutations in one of the two CH3 domains and S354C, T366S, L368A, Y407V mutations in the other of the two CH3 domains (the additional Y349C mutation in one CH3 domain and the additional E356C or S354C mutation in the other CH3 domain forming a interchain disulfide bridge) (numbering always according to EU index of Kabat). But also other knobs-in-holes technologies as described by EP 1870459A1, can be used alternatively or additionally. Thus another example for the bispecific antibody are R409D; K370E mutations in the CH3 domain of the "knobs chain" and D399K; E357K mutations in the CH3 domain of the "hole chain" (numbering always according to EU index of Kabat).

In another embodiment the bispecific antibody comprises a T366W mutation in the CH3 domain of the "knobs chain" and T366S, L368A, Y407V mutations in the CH3 domain of the "hole chain" and additionally R409D; K370E mutations in the CH3 domain of the "knobs chain" and D399K; E357K mutations in the CH3 domain of the "hole chain".

In another embodiment the bispecific antibody comprises Y349C, T366W mutations in one of the two CH3 domains and S354C, T366S, L368A, Y407V mutations in the other of the two CH3 domains or said trivalent, bispecific antibody comprises Y349C, T366W mutations in one of the two CH3 domains and S354C, T366S, L368A, Y407V mutations in the other of the two CH3 domains and

additionally R409D; K370E mutations in the CH3 domain of the "knobs chain" and D399K; E357K mutations in the CH3 domain of the "hole chain".

In one embodiment the antibody heavy chain variable domain (VH) and the antibody light chain variable domain (VL) of the heavy and light chain of the second full lenght antibody (that specifically binds to a second antigen) are disulfide stabilized by introduction of a disulfide bond between the following positions:

i) heavy chain variable domain position 44 to light chain variable domain position 100,
ii) heavy chain variable domain position 105 to light chain variable domain position 43, or
iii) heavy chain variable domain position 101 to light chain variable domain position 100 (numbering always according to EU index of Kabat).

In one embodiment the antibody heavy chain variable domain (VH) and the antibody light chain variable domain (VL) of the heavy and light chain of the second full lenght antibody (that specifically binds to a second antigen) are disulfide stabilized by introduction of a disulfide bond between the following positions: heavy chain variable domain position 44 to light chain variable domain position 100.

Such further disulfide stabilization is achieved by the introduction of a disulfide bond between the variable domains VH and VL of the second full lenght antibody heavy and light chain. Techniques to introduce unnatural disulfide bridges for stabilization for a single chain Fv are described e.g. in WO 94/029350, Rajagopal, V., et al, Prot. Engin. 10 (1997) 1453-59; Kobayashi, H., et al., Nuclear Medicine & Biology, Vol. 25, (1998) 387-393; or Schmidt, M., et al., Oncogene (1999) 18, 1711-1721. In one embodiment the optional disulfide bond between the variable domains of the second full lenght antibody heavy and light chain is between heavy chain variable domain position 44 and light chain variable domain position 100. In one embodiment the optional disulfide bond between the variable domains is between heavy chain variable domain position 105 and light chain variable domain position 43 (numbering always according to EU index of Kabat).

In one embodiment a bispecific antibody according to the invention with said optional disulfide stabilization between the variable domains VH and VL of the second full lenght antibody heavy and light chain is preferred.

In one embodiment a bispecific antibody according to the invention without said optional disulfide stabilization between the variable domains VH and VL of the second full lenght antibody heavy and light chain is preferred.

Both parts of the bispecific antibody according to the invention comprise antigen-binding sites (the first full length antibody heavy and light chain comprise one antigen binding site, and the second full length antibody heavy and light chain comprise one antigen binding site). The terms "binding site" or "antigen-binding site" as used herein denotes the region(s) of said bispecific antibody according to the invention to which the respective antigen actually binds. The antigen binding sites either in the first full length antibody heavy and light chain and the second full length antibody heavy and light chain are formed each by a pair consisting of an antibody light chain variable domain (VL) and an antibody heavy chain variable domain (VH).

The antigen-binding sites that bind to the desired antigen (e.g EGFR) can be derived a) from known antibodies to the antigen (e.g anti-EGFR antibodies) or b) from new antibodies or antibody fragments obtained by de novo immunization methods using inter alia either the antigen protein or nucleic acid or fragments thereof or by phage display.

An antigen-binding site of an antibody of the invention contains six complementarity determining regions (CDRs) which contribute in varying degrees to the affinity of the binding site for antigen. There are three heavy chain variable domain CDRs (CDRH1, CDRH2 and CDRH3) and three light chain variable domain CDRs (CDRL1, CDRL2 and CDRL3). The extent of CDR and framework regions (FRs) is determined by comparison to a compiled database of amino acid sequences in which those regions have been defined according to variability among the sequences.

Antibody specificity refers to selective recognition of the antibody for a particular epitope of an antigen. Natural antibodies, for example, are monospecific. The term "bispecific" antibody as used herein denotes an antibody that has two or more anigen-binding sites and binds to two different antigens or two different epitopes of the same antigen. "Bispecific antibodies" according to the invention are

antibodies which have two different antigen-binding specificities. In one embodiment antibodies of the present invention are bispecific for two different antigens, i.e. VEGF as first antigen and ANG-2 as second antigen or e.g. EGFR as first antigen and IGF-1R as second antigen, or vice versa.

The term "monospecific" antibody as used herein denotes an antibody that has one or more binding sites each of which bind to the same epitope of the same antigen.

The term "valent" as used within the current application denotes the presence of a specified number of binding sites in an antibody molecule. As such, the terms "trivalent", "tetravalent", "pentavalent" and "hexavalent" denote the presence of three binding sites, four binding sites, five binding sites, and six binding sites, respectively, in an antibody molecule. A natural antibody for example or a bispecific antibody according to the invention has two binding sites and is bivalent.

The term "EGFR" as used herein refers to human epidermal growth factor receptor (also known as HER-1 or Erb-B1, SEQ ID NO: 13) is a 170 kDa transmembrane receptor encoded by the c-erbB proto-oncogene, and exhibits intrinsic tyrosine kinase activity (Modjtahedi, H., et al., Br. J. Cancer 73 (1996) 228-235; Herbst, R.S., and Shin, D.M., Cancer 94 (2002) 1593-1611). SwissProt database entry P00533 provides the sequence of EGFR. There are also isoforms and variants of EGFR (e.g., alternative RNA transcripts, truncated versions, polymorphisms, etc.) including but not limited to those identified by Swissprot database entry numbers P00533-1, P00533-2, P00533-3, and P00533-4. EGFR is known to bind ligands including α),epidermal growth factor (EGF), transforming growth factor-α (TGf-amphiregulin, heparin-binding EGF (hb-EGF), betacellulin, and epiregulin (Herbst, R.S., and Shin, D.M., Cancer 94 (2002) 1593-1611; Mendelsohn, J., and Baselga, J., Oncogene 19 (2000) 6550-6565). EGFR regulates numerous cellular processes via tyrosine-kinase mediated signal transduction pathways, including, but not limited to, activation of signal transduction pathways that control cell proliferation, differentiation, cell survival, apoptosis, angiogenesis, mitogenesis, and metastasis (Atalay, G., et al., Ann. Oncology 14 (2003) 1346-1363; Tsao, A.S., and Herbst, R.S., Signal 4 (2003) 4-9; Herbst, R.S., and Shin, D.M., Cancer 94 (2002) 1593-1611; Modjtahedi, H., et al., Br. J. Cancer 73 (1996) 228-235).

The term "IGF-1R" as used herein refers to human Insulin-like growth factor I receptor (IGF-IR, CD 221 antigen; SEQ ID NO: 14) belongs to the family of transmembrane protein tyrosine kinases (LeRoith, D., et al., Endocrin. Rev. 16

(1995) 143-163; and Adams, T.E., et al., Cell. Mol. Life Sci. 57 (2000) 1050-1063). SwissProt database entry P08069 provides the sequence of IGF-1R. IGF-IR binds IGF-I with high affinity and initiates the physiological response to this ligand in vivo. IGF-IR also binds to IGF-II, however with slightly lower affinity. IGF-IR overexpression promotes the neoplastic transformation of cells and there exists evidence that IGF-IR is involved in malignant transformation of cells and is therefore a useful target for the development of therapeutic agents for the treatment of cancer (Adams, T.E., et al., Cell. Mol. Life Sci. 57 (2000) 1050-1063).

In a preferred aspect of the invention the bispecific antibody according to the invention specifically binds to human IGF-1R as well as to human EGFR (i.e. the bispecific antibody according to the invention is a bispecific anti-IGF-1R/ anti-EGFR antibody). The bispecific antibody is based on the antigen-binding sites of human <IGF-1R> HUMAB Clone 18 (DSM ACC 2587; WO 2005/005635, abbreviated as <IGF-1R>Clone18 or <IGF-1R> AK18) and humanized <EGFR>ICR62 (WO 2006/082515 abbreviated as <EGFR>ICR62). The relevant light and heavy chain amino acid sequences of these bispecific, bivalent antibodies are given in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 (for OA-Ak18-scFab-GA201); in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 (for OA-GA201-scFab-Ak18); in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4 (for OA-Ak18-scFab-GA201_WT), and in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 8 (for OA-GA201-scFab-Ak18_WT).

The bispecific <EGFR-IGF-1R> antibodies according to the invention show benefits for human patients in need of a EGFR and IGF-1R targeting therapy. The antibodies according to the invention have highly valuable properties causing a benefit for a patient suffering from such a disease, especially suffering from cancer. The bispecific <EGFR-IGF-1R> antibodies according to the invention show e.g. a reduction of the internalization of IGF-1R receptor compared to the monospecific parent <IGF-1R> antibody. Furthermore they show good targeting of tumor cells expressing both antigens EGFR and IGF-1R which represents a benefit with respect to the efficacy/toxicity ratio for patients suffering from a cancer expressing both antigens EGFR and IGF-1R.

Thus in one aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to IGF-1R and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 1, and a light chain with the amino acid sequence of SEQ ID NO: 2, and
b) the second full length antibody specifically binds to EGFR and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 3.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to IGF-1R and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 1, and a light chain with the amino acid sequence of SEQ ID NO: 2, and
b) the second full length antibody specifically binds to EGFR and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 4.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 7.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker

wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 8.

Thus in one embodiment of the invention the bispecific antibody is an anti-IGF-1R/ anti-EGFR antibody and is characterized in comprising the amino acid sequences of SEQ ID NO: 1, of SEQ ID NO: 2, and of SEQ ID NO: 3. 22. Accordingly one aspect of the invention is a bispecific antibody that specifically binds to human IGF-1R and to human EGFR, characterized in comprising the amino acid sequences of SEQ ID NO: 1, of SEQ ID NO: 2, and of SEQ ID NO: 3.

Thus in one embodiment of the invention the bispecific antibody is an anti-IGF-1R/ anti-EGFR antibody and is characterized in comprising the amino acid sequences of SEQ ID NO: 1, of SEQ ID NO: 2, and of SEQ ID NO: 4. Accordingly one aspect of the invention is a bispecific antibody that specifically binds to human IGF-1R and to human EGFR, characterized in comprising the amino acid sequences of SEQ ID NO: 1, of SEQ ID NO: 2, and of SEQ ID NO: 4.

Thus in one embodiment of the invention the bispecific antibody is an anti-IGF-1R/ anti-EGFR antibody and is characterized in comprising the amino acid sequences of SEQ ID NO: 5, of SEQ ID NO: 6, and of SEQ ID NO: 7. Accordingly one aspect of the invention is a bispecific antibody that specifically binds to human IGF-1R and to human EGFR, characterized in comprising the amino acid sequences of SEQ ID NO: 5, of SEQ ID NO: 6, and of SEQ ID NO: 7.

Thus in one embodiment of the invention the bispecific antibody is an anti-IGF-1R/ anti-EGFR antibody and is characterized in comprising the amino acid sequences of SEQ ID NO: 5, of SEQ ID NO: 6, and of SEQ ID NO: 8. Accordingly one aspect of the invention is a bispecific antibody that specifically binds to human IGF-1R and to human EGFR, characterized in comprising the amino acid sequences of SEQ ID NO: 5, of SEQ ID NO: 6, and of SEQ ID NO: 8.

In one embodiment of the invention the bispecific antibody is an anti-IGF-1R/ anti-EGFR antibody and is characterized in

a) comprising the amino acid sequences of SEQ ID NO: 1, of SEQ ID NO: 2, and of SEQ ID NO: 3.

b) comprising the amino acid sequences of SEQ ID NO: 1, of SEQ ID NO: 2, and of SEQ ID NO: 4.

c) comprising the amino acid sequences of SEQ ID NO: 5, of SEQ ID NO: 6, and of SEQ ID NO: 7, or

d) comprising the amino acid sequences of SEQ ID NO: 5, of SEQ ID NO: 6, and of SEQ ID NO: 8.

In one embodiment of the invention said bispecific antibody anti-IGF-1R/ anti-EGFR antibody is characterized in having one or more of the following properties (determined in assays as described in Example 4 and 5):

- the anti-IGF-1R/anti-EGFR antibody inhibits the phosphorylation of IGF-1R with an IC50 of 5 nM or less (preferably 2 nM or less) on H322M tumor cells;
- the bispecific anti-IGF-1R/anti-EGFR antibody inhibits the phosphorylation of EGFR with an IC50 of 5 nM or less (preferably 2n M or less) on H322M tumor cells;
- the bispecific anti-IGF-1R/ anti-EGFR antibody reduces the downregulation of IGF-1R by 50% or more compared to the anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18 (DSM ACC 2587).

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 7 with no more than 1 amino acid residue substitutions in the CDRs, and wherein the KD value of binding affinity is equal or is reduced less than 4fold when compared to the KD value of binding affinity of unmutated amino acid sequence of SEQ ID NO: 7.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 8 with no more than 1 amino acid residue substitutions in the CDRs, and wherein the KD value of binding affinity is equal or is reduced less than 4fold when compared to the KD value of binding affinity of unmutated amino acid sequence of SEQ ID NO: 8.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 7 with no more than 1 amino acid residue substitutions in the CDR3H, and wherein the KD value of binding affinity is equal or is reduced less than 4fold when compared to the KD value of binding affinity of unmutated amino acid sequence of SEQ ID NO: 7.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 8 with no more than 1 amino acid residue substitutions in the CDR3H, and wherein the KD value of binding affinity is equal or is reduced less than 4fold when compared to the KD

value of binding affinity of unmutated amino acid sequence of SEQ ID NO: 8.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 7 with no more than 1 amino acid residue substitutions in the CDRs, and wherein the KD value of binding affinity is equal or is reduced less than 4fold when compared to the KD value of binding affinity of unmutated amino acid sequence of SEQ ID NO: 7;

and having one or more of the following properties (determined in assays as described in Example 4 and 5):

- the anti-IGF-1R/ anti-EGFR antibody inhibits the phosphorylation of IGF-1R with an IC50 of 5nM or less (preferably 2nM or less) on H322M tumor cells

- the bispecific anti-IGF-1R/ anti-EGFR antibody inhibits the phosphorylation of EGFR with an IC50 of 5nM or less (preferably 2nM or less) on H322M tumor cells

- the bispecific anti-IGF-1R/ anti-EGFR antibody reduces the downregulation of IGF-1R by 50% or more compared to the anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18 (DSM ACC 2587).

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid

sequence of SEQ ID NO: 8 with no more than 1 amino acid residue substitutions in the CDRs, and wherein the KD value of binding affinity is equal or is reduced less than 4fold when compared to the KD value of binding affinity of unmutated amino acid sequence of SEQ ID NO: 8;

and having one or more of the following properties (determined in assays as described in Example 4 and 5):

- the anti-IGF-1R/ anti-EGFR antibody inhibits the phosphorylation of IGF-1R with an IC50 of 5nM or less (preferably 2nM or less) on H322M tumor cells
- the bispecific anti-IGF-1R/ anti-EGFR antibody inhibits the phosphorylation of EGFR with an IC50 of 5nM or less (preferably 2nM or less) on H322M tumor cells
- the bispecific anti-IGF-1R/ anti-EGFR antibody reduces the downregulation of IGF-1R by 50% or more compared to the anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18 (DSM ACC 2587).

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 7 with no more than 1 amino acid residue substitutions in the CDR3H, and wherein the KD value of binding affinity is equal or is reduced less than 4fold when compared to the KD value of binding affinity of unmutated amino acid sequence of SEQ ID NO: 7;

and having one or more of the following properties (determined in assays as described in Example 4 and 5):

- the anti-IGF-1R/ anti-EGFR antibody inhibits the phosphorylation of IGF-1R with an IC50 of 5nM or less (preferably 2nM or less) on H322M tumor cells

- the bispecific anti-IGF-1R/ anti-EGFR antibody inhibits the phosphorylation of EGFR with an IC50 of 5nM or less (preferably 2nM or less) on H322M tumor cells

- the bispecific anti-IGF-1R/ anti-EGFR antibody reduces the downregulation of IGF-1R by 50% or more compared to the anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18 (DSM ACC 2587).

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and
b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 8 with no more than 1 amino acid residue substitutions in the CDR3H, and wherein the KD value of binding affinity is equal or is reduced less than 4fold when compared to the KD value of binding affinity of unmutated amino acid sequence of SEQ ID NO: 8;

and having one or more of the following properties (determined in assays as described in Example 4 and 5):

- the anti-IGF-1R/ anti-EGFR antibody inhibits the phosphorylation of IGF-1R with an IC50 of 5nM or less (preferably 2nM or less) on H322M tumor cells
- the bispecific anti-IGF-1R/ anti-EGFR antibody inhibits the phosphorylation of EGFR with an IC50 of 5nM or less (preferably 2nM or less) on H322M tumor cells
- the bispecific anti-IGF-1R/ anti-EGFR antibody reduces the downregulation of IGF-1R by 50% or more compared to the anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18 (DSM ACC 2587).

Examples of amino acid residue substitutions in the CDR3H of SEQ ID NO: 7 or of SEQ ID NO: 8 wherein the KD value of binding affinity is equal or is reduced less than 4fold when compared to the KD value of binding affinity of unmutated amino acid sequence, are described e.g in EP10166860.6.

The term "VEGF" as used herein refers to human vascular endothelial growth factor (VEGF/VEGF-A) (SEQ ID No: 15) which is described in e.g. Leung, D.W., et al., Science 246 (1989) 1306-9; Keck, P.J., et al., Science 246 (1989) 1309-12 and Connolly, D.T., et al., J. Biol. Chem. 264 (1989) 20017-24. VEGF is involved in the regulation of normal and abnormal angiogenesis and neovascularization associated with tumors and intraocular disorders (Ferrara, N., et al., Endocr. Rev. 18 (1997) 4-25; Berkman, R.A., et al., J. Clin. Invest. 91 (1993) 153-159; Brown, L.F., et al., Human Pathol. 26 (1995) 86-91; Brown, L.F., et al., Cancer Res. 53 (1993) 4727-4735; Mattern, J., et al., Brit. J. Cancer. 73 (1996) 931-934; and Dvorak, H., et al., Am. J. Pathol. 146 (1995) 1029-1039). VEGF is a homodimeric glycoprotein that has been isolated from several sources. VEGF shows highly specific mitogenic activity for endothelial cells.

The term "ANG-2" as used herein refers to human angiopoietin-2 (ANG-2) (alternatively abbreviated with ANGPT2 or ANG2) (SEQ ID No: 16) which is described in Maisonpierre, P.C., et al, Science 277 (1997) 55-60 and Cheung, A.H., et al., Genomics 48 (1998) 389-91. The angiopoietins-1 and -2 and ANG-2 were discovered as ligands for the Ties, a family of tyrosine kinases that is selectively expressed within the vascular endothelium. Yancopoulos, G.D., et al., Nature 407 (2000) 242-48. There are now four definitive members of the angiopoietin family. Angiopoietin-3 and -4 (Ang-3 and Ang-4) may represent widely diverged counterparts of the same gene locus in mouse and man. Kim, I., et al., FEBS Let, 443 (1999) 353-56; Kim, I., et al., J Biol Chem 274 (1999) 26523-28. ANG-1 and ANG-2 were originally identified in tissue culture experiments as agonist and antagonist, respectively (see for ANG-1: Davis, S., et al., Cell 87 (1996) 1161-69; and for ANG-2: Maisonpierre, P.C., et al., Science 277 (1997) 55- 60) All of the known angiopoietins bind primarily to Tie2, and both Ang-1 and -2 bind to Tie2 with an affinity of 3 nM (Kd). Maisonpierre, P.C., et al., Science 277 (1997) 55-60.

In a preferred embodiment said bispecific antibody according to the invention specifically binds to human VEGF as well as to human ANG-2 (i.e. the bispecific antibody according to the invention is a bispecific anti-VEGF/anti-ANG-2 antibody). The bispecific antibody preferably based on the antigen-binding sites of

the anti-VEGF antibody bevacizumab and ANG2i-LC06 (which is decribed in the WO2010/040508 (PCT application No. PCT/EP2009/007182) and which was obtained via phage display). The relevant light and heavy chain amino acid sequences of these bispecific, bivalent antibodies are given in SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 (for Ang2-VEGF OA-Ava-N-scFabLC06SS), and in SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 12 (for Ang2-VEGF OA-Ava-N-scFabLC06).

Thus in one aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to VEGF and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 9, and a light chain with the amino acid sequence of SEQ ID NO: 10, and
b) the second full length antibody specifically binds to ANG-2 and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 11.

In another aspect of the invention the bispecific antibody according to the invention is characterized in that

a) the first full length antibody specifically binds to VEGF and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 9, and a light chain with the amino acid sequence of SEQ ID NO: 10, and
b) the second full length antibody specifically binds to ANG-2 and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 12.

Thus in one embodiment of the invention the bispecific antibody is an anti-VEGF/ anti-ANG-2 antibody and is characterized in comprising the amino acid sequences of SEQ ID NO: 9, of SEQ ID NO: 10, and of SEQ ID NO: 11.

Thus in one embodiment of the invention the bispecific antibody is an anti-VEGF/ anti-ANG-2 antibody and is characterized in comprising the amino acid sequences of SEQ ID NO: 9, of SEQ ID NO: 10, and of SEQ ID NO: 12.

The full length antibodies of the invention comprise immunoglobulin constant regions of one or more immunoglobulin classes. Immunoglobulin classes include IgG, IgM, IgA, IgD, and IgE isotypes and, in the case of IgG and IgA, their subtypes. In a preferred embodiment, an full length antibody of the invention has a constant domain structure of an IgG type antibody.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of a single amino acid composition.

The term "chimeric antibody" refers to an antibody comprising a variable region, i.e., binding region, from one source or species and at least a portion of a constant region derived from a different source or species, usually prepared by recombinant DNA techniques. Chimeric antibodies comprising a murine variable region and a human constant region are preferred. Other preferred forms of "chimeric antibodies" encompassed by the present invention are those in which the constant region has been modified or changed from that of the original antibody to generate the properties according to the invention, especially in regard to C1q binding and/or Fc receptor (FcR) binding. Such chimeric antibodies are also referred to as "class-switched antibodies.". Chimeric antibodies are the product of expressed immunoglobulin genes comprising DNA segments encoding immunoglobulin variable regions and DNA segments encoding immunoglobulin constant regions. Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques are well known in the art. See, e.g., Morrison, S.L., et al., Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855; US 5,202,238 and US 5,204,244.

The term "humanized antibody" refers to antibodies in which the framework or "complementarity determining regions" (CDR) have been modified to comprise the CDR of an immunoglobulin of different specificity as compared to that of the parent immunoglobulin. In a preferred embodiment, a murine CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody." See, e.g., Riechmann, L., et al., Nature 332 (1988) 323-327; and Neuberger, M.S., et al., Nature 314 (1985) 268-270. Particularly preferred CDRs correspond to those representing sequences recognizing the antigens noted above for chimeric antibodies. Other forms of "humanized antibodies" encompassed by the present invention are those in which the constant region has been additionally modified or changed from that of the original antibody to generate the properties according to

the invention, especially in regard to C1q binding and/or Fc receptor (FcR) binding.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germ line immunoglobulin sequences. Human antibodies are well-known in the state of the art (van Dijk, M.A., and van de Winkel, J.G., Curr. Opin. Chem. Biol. 5 (2001) 368-374). Human antibodies can also be produced in transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire or a selection of human antibodies in the absence of endogenous immunoglobulin production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge (see, e.g., Jakobovits, A., et al., Proc. Natl. Acad. Sci. USA 90 (1993) 2551-2555; Jakobovits, A., et al., Nature 362 (1993) 255-258; Bruggemann, M., et al., Year Immunol. 7 (1993) 33-40). Human antibodies can also be produced in phage display libraries (Hoogenboom, H.R., and Winter, G., J. Mol. Biol. 227 (1992) 381-388; Marks, J.D., et al., J. Mol. Biol. 222 (1991) 581-597). The techniques of Cole, et al. and Boerner, et al., are also available for the preparation of human monoclonal antibodies (Cole, et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); and Boerner, P., et al., J. Immunol. 147 (1991) 86-95). As already mentioned for chimeric and humanized antibodies according to the invention the term “human antibody” as used herein also comprises such antibodies which are modified in the constant region to generate the properties according to the invention, especially in regard to C1q binding and/or FcR binding, e.g. by “class switching” i.e. change or mutation of Fc parts (e.g. from IgG1 to IgG4 and/or IgG1/IgG4 mutation).

The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from a host cell such as a NS0 or CHO cell or from an animal (e.g. a mouse) that is transgenic for human immunoglobulin genes or antibodies expressed using a recombinant expression vector transfected into a host cell. Such recombinant human antibodies have variable and constant regions in a rearranged form. The recombinant human antibodies according to the invention have been subjected to in vivo somatic hypermutation. Thus, the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germ line VH and VL sequences, may not naturally exist within the human antibody germ line repertoire in vivo.

The "variable domain" (variable domain of a light chain (VL), variable region of a heavy chain (VH) as used herein denotes each of the pair of light and heavy chains which is involved directly in binding the antibody to the antigen. The domains of variable human light and heavy chains have the same general structure and each domain comprises four framework (FR) regions whose sequences are widely conserved, connected by three "hypervariable regions" (or complementarity determining regions, CDRs). The framework regions adopt a β-sheet conformation and the CDRs may form loops connecting the β-sheet structure. The CDRs in each chain are held in their three-dimensional structure by the framework regions and form together with the CDRs from the other chain the antigen binding site. The antibody heavy and light chain CDR3 regions play a particularly important role in the binding specificity/affinity of the antibodies according to the invention and therefore provide a further object of the invention.

The terms "hypervariable region" or "antigen-binding portion of an antibody" when used herein refer to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from the "complementarity determining regions" or "CDRs". "Framework" or "FR" regions are those variable domain regions other than the hypervariable region residues as herein defined. Therefore, the light and heavy chains of an antibody comprise from N- to C-terminus the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. CDRs on each chain are separated by such framework amino acids. Especially, CDR3 of the heavy chain is the region which contributes most to antigen binding. CDR and FR regions are determined according to the standard definition of Kabat et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991).

As used herein, the terms “binding to” or “which specifically binds to” or “specifically binding to” refers to the binding of the antibody to an epitope of the antigen in an in vitro assay, preferably in an plasmon resonance assay (BIAcore, GE-Healthcare Uppsala, Sweden) with purified wild-type antigen. The affinity of the binding is defined by the terms ka (rate constant for the association of the antibody from the antibody/antigen complex), k_D (dissociation constant), and K_D (k_D/ka). In one embodiment binding or specifically binding means a binding affinity (K_D) of 10^{-8} mol/l or less, preferably 10^{-9} M to 10^{-13} mol/l. Thus, a bispecific antibody according to the invention is preferably specifically binding to each antigen for which it is specific with a binding affinity (K_D) of 10^{-8} mol/l or less, preferably 10^{-9} M to 10^{-13} mol/l.

Binding of the antibody to the FcγRIII can be investigated by a BIAcore assay (GE-Healthcare Uppsala, Sweden). The affinity of the binding is defined by the terms ka (rate constant for the association of the antibody from the antibody/antigen complex), k_D (dissociation constant), and K_D (k_D/ka).

The term "epitope" includes any polypeptide determinant capable of specific binding to an antibody. In certain embodiments, epitope determinant include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl, and, in certain embodiments, may have specific three dimensional structural characteristics, and or specific charge characteristics. An epitope is a region of an antigen that is bound by an antibody.

In certain embodiments, an antibody is said to specifically bind an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules.

The term “constant region” as used within the current applications denotes the sum of the domains of an antibody other than the variable region. The constant region is not involved directly in binding of an antigen, but exhibit various effector functions. Depending on the amino acid sequence of the constant region of their heavy chains, antibodies are divided in the classes: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses, such as IgG1, IgG2, IgG3, and IgG4, IgA1 and IgA2. The heavy chain constant regions that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The light chain constant regions (CL) which can be found in all five antibody classes are called κ (kappa) and λ (lambda).

The term “constant region derived from human origin” as used in the current application denotes a constant heavy chain region of a human antibody of the subclass IgG1, IgG2, IgG3, or IgG4 and/or a constant light chain kappa or lambda region. Such constant regions are well known in the state of the art and e.g. described by Kabat, E.A., (see e.g. Johnson, G. and Wu, T.T., Nucleic Acids Res. 28 (2000) 214-218; Kabat, E.A., et al., Proc. Natl. Acad. Sci. USA 72 (1975) 2785-2788).

The term “complement-dependent cytotoxicity (CDC)” denotes a process initiated by binding of complement factor C1q to the Fc part of most IgG antibody subclasses. Binding of C1q to an antibody is caused by defined protein-protein interactions at the so called binding site. Such Fc part binding sites are known in

the state of the art (see above). Such Fc part binding sites are, e.g., characterized by the amino acids L234, L235, D270, N297, E318, K320, K322, P331, and P329 (numbering according to EU index of Kabat). Antibodies of subclass IgG1, IgG2, and IgG3 usually show complement activation including C1q and C3 binding, whereas IgG4 does not activate the complement system and does not bind C1q and/or C3.

While antibodies of the IgG4 subclass show reduced Fc receptor (FcγRIIIa) binding, antibodies of other IgG subclasses show strong binding. However Pro238, Asp265, Asp270, Asn297 (loss of Fc carbohydrate), Pro329, Leu234, Leu235, Gly236, Gly237, Ile253, Ser254, Lys288, Thr307, Gln311, Asn434, and His435 are residues which, if altered, provide also reduced Fc receptor binding (Shields, R.L., et al., J. Biol. Chem. 276 (2001) 6591-6604; Lund, J., et al., FASEB J. 9 (1995) 115-119; Morgan, A., et al., Immunology 86 (1995) 319-324; EP 0 307 434).

In one embodiment an antibody according to the invention has a reduced FcR binding compared to an IgG1 antibody and the full lenght parent antibody is in regard to FcR binding of IgG4 subclass or of IgG1 or IgG2 subclass with a mutation in S228, L234, L235 and/or D265, and/ or contains the PVA236 mutation. In one embodiment the mutations in the full length parent antibody are S228P, L234A, L235A, L235E and/or PVA236. In another embodiment the mutations in the full length parent antibody are in IgG4 S228P and L235E and in IgG1 L234A and L235A.

In a further embodiment the bispecific antibody according to the invention is characterized in that said full length antibody is of human IgG1 subclass.

The term "antibody-dependent cellular cytotoxicity (ADCC)" refers to lysis of human target cells by an antibody according to the invention in the presence of effector cells. ADCC is measured preferably by the treatment of a preparation of EGFR and IGF-1R expressing cells with an antibody according to the invention in the presence of effector cells such as freshly isolated PBMC or purified effector cells from buffy coats, like monocytes or natural killer (NK) cells or a permanently growing NK cell line.

The term "complement-dependent cytotoxicity (CDC)" denotes a process initiated by binding of complement factor C1q to the Fc part of most IgG antibody subclasses. Binding of C1q to an antibody is caused by defined protein-protein interactions at the so called binding site. Such Fc part binding sites are known in

the state of the art (see above). Such Fc part binding sites are, e.g., characterized by the amino acids L234, L235, D270, N297, E318, K320, K322, P331, and P329 (numbering according to EU index of Kabat). Antibodies of subclass IgG1, IgG2, and IgG3 usually show complement activation including C1q and C3 binding, whereas IgG4 does not activate the complement system and does not bind C1q and/or C3.

The constant region of an antibody is directly involved in ADCC (antibody-dependent cell-mediated cytotoxicity) and CDC (complement-dependent cytotoxicity). Complement activation (CDC) is initiated by binding of complement factor C1q to the constant region of most IgG antibody subclasses. Binding of C1q to an antibody is caused by defined protein-protein interactions at the so called binding site. Such constant region binding sites are known in the state of the art and described e.g. by Lukas, T.J., et al., J. Immunol. 127 (1981) 2555-2560; Brunhouse, R., and Cebra, J.J., Mol. Immunol. 16 (1979) 907-917; Burton, D.R., et al., Nature 288 (1980) 338-344; Thommesen, J.E., et al., Mol. Immunol. 37 (2000) 995-1004; Idusogie, E.E., et al., J. Immunol. 164 (2000) 4178-4184; Hezareh, M., et al., J. Virol. 75 (2001) 12161-12168; Morgan, A., et al., Immunology 86 (1995) 319-324; and EP 0 307 434. Such constant region binding sites are, e.g., characterized by the amino acids L234, L235, D270, N297, E318, K320, K322, P331, and P329 (numbering according to EU index of Kabat).

In one embodiment the bispecific antibodies according to the invention comprise a constant region of IgG1 or IgG3 subclass (preferably of IgG1 subclass), which is preferably derived from human origin. In one embodiment the bispecific antibodies according to the invention comprise a Fc part of IgG1 or IgG3 subclass (preferably of IgG1 subclass), which is preferably derived from human origin.

Antibody-dependent cell-mediated cytotoxicity (ADCC) of monoclonal antibodies can be enhanced by engineering their oligosaccharide component as described in Umana, P., et al., Nature Biotechnol. 17 (1999) 176-180, and US 6,602,684. IgG1 type antibodies, the most commonly used therapeutic antibodies, are glycoproteins that have a conserved N-linked glycosylation site at Asn297 in each CH2 domain. The two complex biantennary oligosaccharides attached to Asn297 are buried between the CH2 domains, forming extensive contacts with the polypeptide backbone, and their presence is essential for the antibody to mediate effector functions such as antibody dependent cellular cytotoxicity (ADCC) (Lifely, M.R., et al., Glycobiology 5 (1995) 813-822; Jefferis, R., et al., Immunol. Rev. 163

(1998) 59-76; Wright, A., and Morrison, S.L., Trends Biotechnol. 15 (1997) 26-32). Umana, P., et al. Nature Biotechnol. 17 (1999) 176-180 and WO 99/154342 showed that overexpression in Chinese hamster ovary (CHO) cells of ß(1,4)-N-acetylglucosaminyltransferase III ("GnTIII"), a glycosyltransferase catalyzing the formation of bisected oligosaccharides, significantly increases the in vitro ADCC activity of antibodies. Alterations in the composition of the Asn297 carbohydrate or its elimination affect also binding to FcγR and C1q (Umana, P., et al., Nature Biotechnol. 17 (1999) 176-180; Davies, J., et al., Biotechnol. Bioeng. 74 (2001) 288-294; Mimura, Y., et al., J. Biol. Chem. 276 (2001) 45539-45547; Radaev, S., et al., J. Biol. Chem. 276 (2001) 16478-16483; Shields, R.L., et al., J. Biol. Chem. 276 (2001) 6591-6604; Shields, R.L., et al., J. Biol. Chem. 277 (2002) 26733-26740; Simmons, L.C., et al., J. Immunol. Methods 263 (2002) 133-147).

Methods to enhance cell-mediated effector functions of monoclonal antibodies by reducing the amount of fucose are described e.g. in WO 2005/018572, WO 2006/116260, WO 2006/114700, WO 2004/065540, WO 2005/011735, WO 2005/027966, WO 1997/028267, US 2006/0134709, US 2005/0054048, US 2005/0152894, WO 2003/035835, WO 2000/061739, Niwa, R., et al., J. Immunol. Methods 306 (2005) 151-160; Shinkawa, T., et al., J Biol Chem, 278 (2003) 3466-3473; WO 03/055993 or US 2005/0249722.

Glycosylation of human IgG1 or IgG3 occurs at Asn297 as core fucosylated biantennary complex oligosaccharide glycosylation terminated with up to two Gal residues. Human constant heavy chain regions of the IgG1 or IgG3 subclass are reported in detail by Kabat, E.A., et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), and by Brüggemann, M., et al., J. Exp. Med. 166 (1987) 1351-1361; Love, T.W., et al., Methods Enzymol. 178 (1989) 515-527. These structures are designated as G0, G1 (α-1,6- or α-1,3-), or G2 glycan residues, depending from the amount of terminal Gal residues (Raju, T.S., Bioprocess Int. 1 (2003) 44-53). CHO type glycosylation of antibody Fc parts is e.g. described by Routier, F.H., Glycoconjugate J. 14 (1997) 201-207. Antibodies which are recombinantly expressed in non-glycomodified CHO host cells usually are fucosylated at Asn297 in an amount of at least 85 %. The modified oligosaccharides of the full length parent antibody may be hybrid or complex. Preferably the bisected, reduced/not-fucosylated oligosaccharides are hybrid. In another embodiment, the bisected, reduced/not-fucosylated oligosaccharides are complex.

According to the invention "amount of fucose" means the amount of said sugar within the sugar chain at Asn297, related to the sum of all glycostructures attached to Asn297 (e.g. complex, hybrid and high mannose structures) measured by MALDI-TOF mass spectrometry and calculated as average value. The relative amount of fucose is the percentage of fucose-containing structures related to all glycostructures identified in an N-Glycosidase F treated sample (e.g. complex, hybrid and oligo- and high-mannose structures, resp.) by MALDI-TOF (see Example 10).

The bispecific <EGFR-IGF-1R> antibodies according to the invention show a reduction of the internalization of EGFR and IGF-1R receptor compared to their parent < EGFR > and/ or <IGF-1R> antibodies. Therefore in one preferred embodiment of the invention, the bispecific <EGFR-IGF-1R> antibody is glycosylated (IgG1 or IgG3 subclass, preferably IgG1 subclass) with a sugar chain at Asn297 whereby the amount of fucose within said sugar chain is 65 % or lower (Numbering according to Kabat). In another embodiment is the amount of fucose within said sugar chain is between 5 % and 65 %, preferably between 20 % and 40 %. "Asn297" according to the invention means amino acid asparagine located at about position 297 in the Fc region. Based on minor sequence variations of antibodies, Asn297 can also be located some amino acids (usually not more than $\pm$3 amino acids) upstream or downstream of position 297, i.e. between position 294 and 300. Such glycoengineered antibodies are also refer to as afocusylated antibodies herein.

The afucosylated bispecific antibody according to the invention can be expressed in a glycomodified host cell engineered to express at least one nucleic acid encoding a polypeptide having GnTIII activity in an amount sufficient to partially fucosylate the oligosaccharides in the Fc region. In one embodiment, the polypeptide having GnTIII activity is a fusion polypeptide. Alternatively α1,6-fucosyltransferase activity of the host cell can be decreased or eliminated according to US 6,946,292 to generate glycomodified host cells. The amount of antibody fucosylation can be predetermined e.g. either by fermentation conditions (e.g. fermentation time) or by combination of at least two antibodies with different fucosylation amount. Such afucosylated antibodies and respective glycoengineering methods are described in WO 2005/044859, WO 2004/065540, WO2007/031875, Umana, P., et al., Nature Biotechnol. 17 (1999) 176-180, WO 99/154342, WO 2005/018572, WO 2006/116260, WO 2006/114700, WO 2005/011735, WO 2005/027966, WO 97/028267, US 2006/0134709, US 2005/0054048, US 2005/0152894,

WO 2003/035835, WO 2000/061739. These glycoengineered antibodies have an increased ADCC. Other glycoengineering methods yielding afocusylated antibodies according to the invention are described e.g. in Niwa, R., et al., J. Immunol. Methods 306 (2005) 151-160; Shinkawa, T., et al., J Biol Chem, 278 (2003) 3466-3473; WO 03/055993 or US 2005/0249722.

One embodiment is a method of preparation of the bispecific antibody of IgG1 or IgG3 subclass which is glycosylated with a sugar chain at Asn297 whereby the amount of fucose within said sugar chain is 65 % or lower, using the procedure described in WO 2005/044859, WO 2004/065540, WO 2007/031875, Umana, P., et al., Nature Biotechnol. 17 (1999) 176-180, WO 99/154342, WO 2005/018572, WO 2006/116260, WO 2006/114700, WO 2005/011735, WO 2005/027966, WO 97/028267, US 2006/0134709, US 2005/0054048, US 2005/0152894, WO 2003/035835 or WO 2000/061739.

One embodiment is a method of preparation of the bispecific antibody of IgG1 or IgG3 subclass which is glycosylated with a sugar chain at Asn297 whereby the amount of fucose within said sugar chain is 65 % or lower, using the procedure described in Niwa, R., et al., J. Immunol. Methods 306 (2005) 151-160; Shinkawa, T., et al., J Biol Chem, 278 (2003) 3466-3473; WO 03/055993 or US 2005/0249722.

The antibody according to the invention is produced by recombinant means. Thus, one aspect of the current invention is a nucleic acid encoding the antibody according to the invention and a further aspect is a cell comprising said nucleic acid encoding an antibody according to the invention. Methods for recombinant production are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody and usually purification to a pharmaceutically acceptable purity. For the expression of the antibodies as aforementioned in a host cell, nucleic acids encoding the respective modified light and heavy chains are inserted into expression vectors by standard methods. Expression is performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NS0 cells, SP2/0 cells, HEK293 cells, COS cells, PER.C6 cells, yeast, or E.coli cells, and the antibody is recovered from the cells (supernatant or cells after lysis). General methods for recombinant production of antibodies are well-known in the state of the art and described, for example, in the review articles of Makrides, S.C., Protein Expr. Purif. 17 (1999) 183-202; Geisse,

S., et al., Protein Expr. Purif. 8 (1996) 271-282; Kaufman, R.J., Mol. Biotechnol. 16 (2000) 151-161; Werner, R.G., Drug Res. 48 (1998) 870-880.

The bispecific antibodies according to the invention are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. DNA and RNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures. The hybridoma cells can serve as a source of such DNA and RNA. Once isolated, the DNA may be inserted into expression vectors, which are then transfected into host cells such as HEK 293 cells, CHO cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of recombinant monoclonal antibodies in the host cells.

Amino acid sequence variants (or mutants) of the bispecific antibody are prepared by introducing appropriate nucleotide changes into the antibody DNA, or by nucleotide synthesis. Such modifications can be performed, however, only in a very limited range. For example, the modifications do not alter the above mentioned antibody characteristics such as the IgG isotype and antigen binding, but may improve the yield of the recombinant production, protein stability or facilitate the purification.

The term “host cell” as used in the current application denotes any kind of cellular system which can be engineered to generate the antibodies according to the current invention. In one embodiment HEK293 cells and CHO cells are used as host cells.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

Expression in NS0 cells is described by, e.g., Barnes, L.M., et al., Cytotechnology 32 (2000) 109-123; Barnes, L.M., et al., Biotech. Bioeng. 73 (2001) 261-270. Transient expression is described by, e.g., Durocher, Y., et al., Nucl. Acids. Res. 30 (2002) E9. Cloning of variable domains is described by Orlandi, R., et al., Proc.

Natl. Acad. Sci. USA 86 (1989) 3833-3837; Carter, P., et al., Proc. Natl. Acad. Sci. USA 89 (1992) 4285-4289; and Norderhaug, L., et al., J. Immunol. Methods 204 (1997) 77-87. A preferred transient expression system (HEK 293) is described by Schlaeger, E.-J., and Christensen, K., in Cytotechnology 30 (1999) 71-83 and by Schlaeger, E.-J., in J. Immunol. Methods 194 (1996) 191-199.

The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, enhancers and polyadenylation signals.

A nucleic acid is "operably linked" when it is placed in a functional relationship with another nucleic acid sequence. For example, DNA for a pre-sequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a pre-protein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

Purification of antibodies is performed in order to eliminate cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art. See Ausubel, F., et al., ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987). Different methods are well established and widespread used for protein purification, such as affinity chromatography with microbial proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and

electrophoretical methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M.A. Appl. Biochem. Biotech. 75 (1998) 93-102).

The term "transformation" as used herein refers to process of transfer of a vectors/nucleic acid into a host cell. If cells without formidable cell wall barriers are used as host cells, transfection is carried out e.g. by the calcium phosphate precipitation method as described by Graham, F.L., and van der Eb, A.J., Virology 52 (1973) 456ff. However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used. If prokaryotic cells or cells which contain substantial cell wall constructions are used, e.g. one method of transfection is calcium treatment using calcium chloride as described by Cohen, F.N, et al, PNAS. 69 (1972) 7110ff.

As used herein, "expression" refers to the process by which a nucleic acid is transcribed into mRNA and/or to the process by which the transcribed mRNA (also referred to as transcript) is subsequently being translated into peptides, polypeptides, or proteins. The transcripts and the encoded polypeptides are collectively referred to as gene product. If the polynucleotide is derived from genomic DNA, expression in a eukaryotic cell may include splicing of the mRNA.

A "vector" is a nucleic acid molecule, in particular self-replicating, which transfers an inserted nucleic acid molecule into and/or between host cells. The term includes vectors that function primarily for insertion of DNA or RNA into a cell (e.g., chromosomal integration), replication of vectors that function primarily for the replication of DNA or RNA, and expression vectors that function for transcription and/or translation of the DNA or RNA. Also included are vectors that provide more than one of the functions as described.

An "expression vector" is a polynucleotide which, when introduced into an appropriate host cell, can be transcribed and translated into a polypeptide. An "expression system" usually refers to a suitable host cell comprised of an expression vector that can function to yield a desired expression product.

It has now been found that the bispecific antibodies according to the invention have valuable characteristics such as good expression yields in mammalian cells ,stability, biological or pharmacological activity, pharmacokinetic properties or toxicity. They can be used e.g. for the treatment of diseases such as cancer. The antibodies according to the invention, especially the bispecific < IGF-1R - EGFR > antibodies show highly valuable properties like growth inhibition of cancer cells

expressing both receptors IGF-1R and EGFR, and antitumor efficacy causing a benefit for a patient suffering from cancer. The bispecific < IGF-1R - EGFR > antibodies according to the invention show reduced internalization of both receptors IGF-1R and EGFR when compared to their parent monospecific < IGF-1R> and <EGFR > antibodies on cancer cells expressing both receptors IGF-1R and EGFR.

One aspect of the invention is a pharmaceutical composition comprising an antibody according to the invention. Another aspect of the invention is the use of an antibody according to the invention for the manufacture of a pharmaceutical composition. A further aspect of the invention is a method for the manufacture of a pharmaceutical composition comprising an antibody according to the invention. In another aspect, the present invention provides a composition, e.g. a pharmaceutical composition, containing an antibody according to the present invention, formulated together with a pharmaceutical carrier.

One embodiment of the invention is the bispecific antibody according to the invention for the treatment of cancer.

Another aspect of the invention is said pharmaceutical composition for the treatment of cancer.

Another aspect of the invention is the use of an antibody according to the invention for the manufacture of a medicament for the treatment of cancer.

Another aspect of the invention is method of treatment of patient suffering from cancer by administering an antibody according to the invention to a patient in the need of such treatment.

As used herein, "pharmaceutical carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (e.g. by injection or infusion).

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. To administer a compound of the invention by certain routes of administration, it may

be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutical carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intra-arterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intra-articular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

The term cancer as used herein refers to proliferative diseases, such as lymphomas, lymphocytic leukemias, lung cancer, non small cell lung (NSCL) cancer, bronchioloalviolar cell lung cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, gastric cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, mesothelioma, hepatocellular cancer, biliary cancer, neoplasms of the central nervous system (CNS), spinal axis tumors, brain stem glioma, glioblastoma multiforme, astrocytomas, schwanomas, ependymonas, medulloblastomas, meningiomas, squamous cell carcinomas, pituitary adenoma and Ewings sarcoma, including refractory versions of any of the above cancers, or a combination of one or more of the above cancers.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of

microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier preferably is an isotonic buffered saline solution.

Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition.

Description of the Amino acid Sequences

SEQ ID NO: 1	<IGF-1R> heavy chain, OA-Ak18-scFab-GA201 (+WT)
SEQ ID NO: 2	<IGF-1R> light chain, OA-Ak18-scFab-GA201 (+WT)
SEQ ID NO: 3	<EGFR> peptide connected heavy and light chain with disulfide stabilization VH 44 /VL100 of OA-Ak18-scFab-GA201
SEQ ID NO: 4	<EGFR> peptide connected heavy and light chain of OA-Ak18-scFab-GA201_WT
SEQ ID NO: 5	<EGFR> heavy chain, OA-GA201-scFab-Ak18 (+WT)
SEQ ID NO: 6	<EGFR> light chain, OA-GA201-scFab-Ak18 (+WT)
SEQ ID NO: 7	<IGF-1R> peptide connected heavy and light chain with disulfide stabilization VH 44 /VL100- OA-GA201-scFab-Ak18
SEQ ID NO: 8	<IGF-1R> peptide connected heavy and light chain of OA-GA201-scFab-Ak18_WT
SEQ ID NO: 9	<VEGF> heavy chain, Ang2-VEGF OA-Ava-N-scFabLC06 (+SS)
SEQ ID NO: 10	<VEGF> light chain, Ang2-VEGF OA-Ava-N-scFabLC06 (+SS)
SEQ ID NO: 11	<ANG-2> peptide connected heavy and light chain with disulfide stabilization VH 44 /VL100 of Ang2-VEGF OA-Ava-N-scFabLC06SS
SEQ ID NO: 12	<ANG-2> peptide connected heavy and light chain of Ang2-VEGF OA-Ava-N-scFabLC06
SEQ ID NO: 13	Human EGFR
SEQ ID NO: 14	Human IGF-1R
SEQ ID NO: 15	Human VEGF
SEQ ID NO: 16	Human ANG-2

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Experimental Procedure

Examples

Materials & Methods

Recombinant DNA techniques

Standard methods were used to manipulate DNA as described in Sambrook, J. et al., Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions.

DNA and protein sequence analysis and sequence data management

General information regarding the nucleotide sequences of human immunoglobulins light and heavy chains is given in: Kabat, E.A., et al., (1991) Sequences of Proteins of Immunological Interest, Fifth Ed., NIH Publication No 91-3242. Amino acids of antibody chains are numbered according to EU numbering (Edelman, G.M., et al., PNAS 63 (1969) 78-85; Kabat, E.A., et al., (1991) Sequences of Proteins of Immunological Interest, Fifth Ed., NIH Publication No 91-3242). The GCG's (Genetics Computer Group, Madison, Wisconsin) software package version 10.2 and Infomax's Vector NTI Advance suite version 8.0 was used for sequence creation, mapping, analysis, annotation and illustration.

DNA sequencing

DNA sequences were determined by double strand sequencing performed at SequiServe (Vaterstetten, Germany) and Geneart AG (Regensburg, Germany).

Gene synthesis

Desired gene segments were prepared by Geneart AG (Regensburg, Germany) from synthetic oligonucleotides and PCR products by automated gene synthesis. The gene segments encoding "knobs-into-hole" antibody heavy chains carrying S354C and T366W mutations and "knobs-into-hole" heavy chains carrying Y349C, T366S, L368A and Y407V mutations in the CH3 domain in combination with umodified VH domains or scFab antibody fragments as well as antibody light chains are flanked by singular restriction endonuclease cleavage sites (BamHI –

XbaI or BamHI – KpnI) and were cloned into pGA18 (ampR) plasmids. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The DNA sequence of the subcloned gene fragments was confirmed by DNA sequencing. All constructs were designed with a 5'-end DNA sequence coding for a leader peptide (MGWSCIILFLVATATGVHS), which targets proteins for secretion in eukaryotic cells.

Construction of the expression plasmids

A Roche expression vector was used for the construction of all "knobs-into-hole" heavy chain as well as antibody light chain encoding expression plasmids. The vector is composed of the following elements:

- a hygromycin resistance gene as a selection marker,
- an origin of replication, oriP, of Epstein-Barr virus (EBV),
- an origin of replication from the vector pUC18 which allows replication of this plasmid in E. coli
- a beta-lactamase gene which confers ampicillin resistance in E. coli,
- the immediate early enhancer and promoter from the human cytomegalovirus (HCMV),
- the human 1-immunoglobulin polyadenylation ("poly A") signal sequence, and
- unique BamHI and XbaI restriction sites.

The immunoglobulin genes comprising the "knobs-into-hole" heavy chains with unmodified VH domains or scFab fragments as well as unmodified light chains were prepared by gene synthesis and cloned into pGA18 (ampR) plasmids as described. The pG18 (ampR) plasmids carrying the synthesized DNA segments and the Roche expression vector were digested with BamHI and XbaI or BamHI and KpnI restriction enzymes (Roche Molecular Biochemicals) and subjected to agarose gel electrophoresis. Purified "knobs-into-hole" heavy and unmodified light chain encoding DNA segments were then ligated to the isolated Roche expression vector BamHI/XbaI or BamHI/KpnI fragment resulting in the final expression vectors. The final expression vectors were transformed into E. coli cells, expression plasmid DNA was isolated (Miniprep) and subjected to restriction enzyme analysis and DNA sequencing. Correct clones were grown in 150 ml LB-Amp medium, again plasmid DNA was isolated (Maxiprep) and sequence integrity confirmed by DNA sequencing.

Transient expression of bispecific antibodies in HEK293 cells

Recombinant bispecific antibodies were expressed by transient transfection of human embryonic kidney 293-F cells using the FreeStyle™ 293 Expression System according to the manufacturer's instruction (Invitrogen, USA). Briefly, suspension FreeStyle™ 293-F cells were cultivated in FreeStyle™ 293 Expression medium at 37°C/8 % CO_2 and the cells were seeded in fresh medium at a density of 1-2x10^6 viable cells/ml on the day of transfection. "Knobs-into-hole" DNA-293fectin complexes were prepared in Opti-MEM® I medium (Invitrogen, USA) using 325 µl of 293fectin™ (Invitrogen, Germany) and 250 µg of "Knobs-into-hole" heavy chain 1 and 2 and light chain plasmid DNA in a 1:1:1 or 1:2:1 molar ratio for a 250 ml final transfection volume. Antibody containing cell culture supernatants were harvested 7 days after transfection by centrifugation at 14000 g for 30 minutes and filtered through a sterile filter (0.22 µm). Supernatants were stored at -20° C until purification.

Purification of bispecific antibodies

Bispecific antibodies were purified from cell culture supernatants by affinity chromatography using Protein A-Sepharose™ (GE Healthcare, Sweden) and Superdex200 size exclusion chromatography. Briefly, sterile filtered cell culture supernatants were applied on a HiTrap ProteinA HP (5 ml) column equilibrated with PBS buffer (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.4). Unbound proteins were washed out with equilibration buffer. Antibody and antibody variants were eluted with 0.1 M citrate buffer, pH 2.8, and the protein containing fractions were neutralized with 0.1 ml 1 M Tris, pH 8.5. Then, the eluted protein fractions were pooled, concentrated with an Amicon Ultra centrifugal filter device (MWCO: 30 K, Millipore) to a volume of 3 ml and loaded on a Superdex200 HiLoad 120 ml 16/60 gel filtration column (GE Healthcare, Sweden) equilibrated with 20mM Histidin, 140 mM NaCl, pH 6.0. Fractions containing purified bispecific antibodies with less than 5 % high molecular weight aggregates were pooled and stored as 1.0 mg/ml aliquots at -80°C.

Analysis of purified proteins

The protein concentration of purified protein samples was determined by measuring the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence. Purity and molecular weight of bispecific and control antibodies were analyzed by SDS-

PAGE in the presence and absence of a reducing agent (5 mM 1,4-dithiotreitol) and staining with Coomassie brilliant blue. The NuPAGE® Pre-Cast gel system (Invitrogen, USA) was used according to the manufacturer's instruction (4-20 % Tris-Glycine gels). The aggregate content of bispecific and control antibody samples was analyzed by high-performance SEC using a Superdex 200 analytical size-exclusion column (GE Healthcare, Sweden) in 200 mM KH_2PO_4, 250 mM KCl, pH 7.0 running buffer at 25°C. 25 µg protein were injected on the column at a flow rate of 0.5 ml/min and eluted isocratic over 50 minutes. For stability analysis, concentrations of 1 mg/ml of purified proteins were incubated at 4°C and 40°C for 7 days and then evaluated by high-performance SEC The integrity of the amino acid backbone of reduced bispecific antibody light and heavy chains was verified by NanoElectrospray Q-TOF mass spectrometry after removal of N-glycans by enzymatic treatment with Peptide-N-Glycosidase F (Roche Molecular Biochemicals).

Surface Plasmon Resonance

The binding affinity is determined with a standard binding assay at 25°C, such as surface plasmon resonance technique (BIAcore®, GE-Healthcare Uppsala, Sweden). For affinity measurements, 30 µg/ml of anti Fcγ antibodies (from goat, Jackson Immuno Research) were coupled to the surface of a CM-5 sensor chip by standard amine-coupling and blocking chemistry on a SPR instrument (Biacore T100). After conjugation, mono- or bispecific Her3/cMet antibodies were injected at 25°C at a flow rate of 5 µL/min, followed by a dilution series (0 nM to 1000 nM) of human HER3 or c-Met ECD at 30 µL/min. As running buffer for the binding experiment PBS/0.1 % BSA was used. The chip was then regenerated with a 60s pulse of 10 mM glycine-HCl, pH 2.0 solution.

EGFR/IGF-1R Surface Plasmon Resonance

SPR experiments were performed using a Biacore T100 instrument (GE Healthcare Biosciences AB, Uppsala, Sweden). IGF-1R or EGFR were immobilized on the surface of a CM5 biosensorchip using standard amine-coupling chemistry. IGF-1R or EGFR were injected in sodium acetate, pH 5.0 at 1 µg/ml using the immobilization wizard procedure with an aim for 200 RU (IGF-1R) or 100 RU (EGFR). Reference control flow cells were treated in the same way but with vehicle buffer only. The antibodies were diluted in 1xPBS pH 7.4, 0.05 % Tween20 (Roche Diagnostics GmbH) and injected at increasing concentrations

between 3.125 and 50 nM with a flow rate of 30 µl/min. The contact time (association phase) was 3 min (EGFR binding) and 5 min (IGF-1R binding), the dissociation time was 10 min (EGFR) and 3 min (IGF-1R). EGFR binding was regenerated with an inject of 0.85 % phosphoric acid for 30 s at a flow rate of 5 µl/min. IGF-1R binding was regenerated with an inject of 4 M magnesium chloride for 1 min at 5 µl/min. Kinetic rate constants and equilibrium dissociation constants were calculated by using the 1:1 Langmuir binding model within the Biaevaluation software

To demonstrate simultaneous binding, the bispecific antibodies are injected onto the EGFR surface at 25 nM for 1 min, 5 µl/min flow rate. After capturing the antibody to the EGFR surface, IGF-1R is injected at increasing concentrations between 2.5 and 80 nM with a flow rate of 30 µl/min. The surface is regenerated with an inject of 0.85 % phosphoric acid for 30s at a flow rate of 5 µl/min. Kinetic rate constants and equilibrium dissociation constants are calculated by using the 1:1 Langmuir binding model within the Biaevaluation software.

ANG-2 binding Surface Plasmon Resonance (Biacore)

Binding of the antibodies to the antigen e.g. human ANG-2 is investigated by surface plasmon resonance using a BIACORE T100 instrument (GE Healthcare Biosciences AB, Uppsala, Sweden). Briefly, for affinity measurements goat<hIgG-Fcgamma> polyclonal antibodies were immobilized on a CM5 chip via amine coupling for presentation of the antibodies against human ANG-2 (Figure 6B). Binding was measured in HBS buffer (HBS-P (10 mM HEPES, 150 mM NaCl, 0.005 % Tween 20, ph 7.4), 25°C. Purified ANG-2-His (R&D systems or in house purified) was added in various concentrations between 6,25 nM and 200 nM in solution. Association was measured by an ANG-2-injection of 3 minutes; dissociation was measured by washing the chip surface with HBS buffer for 3 minutes and a KD value was estimated using a 1:1 Langmuir binding model. Due to heterogenity of the ANG-2 preparation no 1:1 binding could be observed; KD values are thus only relative estimations. Negative control data (e.g. buffer curves) were subtracted from sample curves for correction of system intrinsic baseline drift and for noise signal reduction. Biacore T100 Evaluation Software version 1.1.1 was used for analysis of sensorgrams and for calculation of affinity data. Alternatively, Ang-2 could be captured with a capture level of 2000-1700 RU via a PentaHisAntibody (PentaHis-Ab BSA-free, Qiagen No. 34660) that was immobilized on a CM5 chip via amine coupling (BSA-free) (see below).

VEGF binding Surface Plasmon Resonance (Biacore)

VEGF binding of bispecific <VEGF-Ang-2> antibodies is analyzed using surface plasmon resonance technology on a Biacore T100 instrument according to the following protocol and analyzed using the T100 software package: Briefly <VEGF> antibodies wer captured on a CM5-Chip via binding to a Goat Anti Human IgG (JIR 109-005-098). The capture antibody was immobilized by amino coupling using standard amino coupling as follows: HBS-N buffer served as running buffer, activation was done by mixture of EDC/NHS with the aim for a ligand density of 700 RU. The Capture-Antibody was diluted in coupling buffer NaAc, pH 5.0, c = 2 µg/mL, finally still activated carboxyl groups were blocked by injection of 1 M Ethanolamine. Capturing of Mabs <VEGF> antibodies was done at a flow of 5 µL/min and c(Mabs<VEGF>) = 10 nM, diluted with running buffer + 1 mg/mL BSA; a capture level of approx. 30 RU should be reached. rhVEGF (rhVEGF, R&D-Systems Cat.-No, 293-VE) was used as analyte. The kinetic characterization of VEGF binding to<VEGF> antibodies was performed at 37°C in PBS + 0.005 % (v/v) Tween20 as running buffer. The sample was injected with a flow of 50 µL/min and an association of time 80 sec. and a dissociation time of 1200 sec with a concentration series of rhVEGF from 300 - 0.29 nM. Regeneration of free capture antibody surface was performed with 10 mM Glycin pH 1.5 and a contact time of 60 sec after each analyte cycle. Kinetic constants were calculated by using the usual double referencing method (control reference: binding of rhVEGF to capture molecule Goat Anti Human IgG, blanks on the measuring flow cell, rhVEGF concentration "0", Model: Langmuir binding 1:1, (Rmax set to local because of capture molecule binding).

Generation of HEK293-Tie2 cell line

In order to determine the interference of Angiopoietin-2 antibodies with ANG2 stimulated Tie2 phosphorylation and binding of ANG2 to Tie2 on cells a recombinant HEK293-Tie cell line was generated. Briefly, a pcDNA3 based plasmid (RB22-pcDNA3 Topo hTie2) coding for full-length human Tie2 (SEQ ID 108) under control of a CMV promoter and a Neomycin resistance marker was transfected using Fugene (Roche Applied Science) as transfection reagent into HEK293 cells (ATCC) and resistant cells were selected in DMEM 10 % FCS, 500µg/ml G418. Individual clones were isolated via a cloning cylinder, and subsequently analyzed for Tie2 expression by FACS. Clone 22 was identified as clone with high and stable Tie2 expression even in the absence of G418 (HEK293-

Tie2 clone22). HEK293-Tie2 clone22 was subsequently used for cellular assays: ANG2 induced Tie2 phosphorylation and ANG2 cellular ligand binding assay.

VEGF induced HUVEC proliferation assay

VEGF induced HUVEC (Human Umbilical Vein Endothelial Cells, Promocell #C-12200) proliferation was chosen to measure the cellular function of VEGF antibodies. Briefly, 5000 HUVEC cells (low passage number, ≤5 passages) per 96 well were incubated in 100µl starvation medium (EBM-2 Endothelial basal medium 2, Promocell # C-22211, 0.5 % FCS, Penicilline/Streptomycine) in a collagen I-coated BD Biocoat Collagen I 96-well microtiter plate (BD #354407 / 35640 over night. Varying concentrations of antibody were mixed with rhVEGF (30 ng1/ml final concentration, BD # 354107) and pre-incubated for 15 minutes at room temperature. Subsequently, the mix was added to the HUVEC cells and they were incubated for 72 h at 37°C, 5 % CO2. On the day of analysis the plate was equilibrated to room temperature for 30 min and cell viability/proliferation was determined using the CellTiter-GloTM Luminescent Cell Viability Assay kit according to the manual (Promega, # G7571/2/3). Luminescence was determined in a spectrophotometer.

ANG2 induced Tie2 phosphorylation assay

Inhibition of ANG2 induced Tie2 phosphorylation by the bispecific <ANG2-VEGF> antibodies was measured according to the following assay principle. HEK293-Tie2 clone22 was stimulated with ANG2 for 5 minutes in the absence or presence of <ANG2-VEGF> antibodies and P-Tie2 was quantified by a sandwich ELISA. Briefly, 2x105 HEK293-Tie2 clone 22 cells per well were grown over night on a Poly-D-Lysine coated 96 well- microtiter plate in 100µl DMEM, 10 % FCS, 500 µg/ml Geneticin. The next day a titration row of <ANG2-VEGF> antibodies was prepared in a microtiter plate (4-fold concentrated, 75µl final volume/well, duplicates) and mixed with 75µl of an ANGPT2 (R&D systems # 623-AN] dilution (3.2 µg/ml as 4-fold concentrated solution). Antibodies and ANG2 were pre-incubated for 15 min at room temperature. 100 µl of the mix were added to the HEK293-Tie2 clone 22 cells (pre-incubated for 5 min with 1 mM NaV3O4, Sigma #S6508) and incubated for 5 min at 37°C. Subsequently, cells were washed with 200µl ice-cold PBS + 1mM NaV3O4 per well and lysed by addition of 120µl lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1 % NP-40, 10 % glycerol, 2mM EDTA, 1 mM NaV3O4, 1 mM PMSF and 10 µg/ml

Aprotinin) per well on ice. Cells were lysed for 30 min at 4°C on a microtiter plate shaker and 100 µl lysate were transferred directly into a p-Tie2 ELISA microtiter plate (R&D Systems, R&D #DY990) without previous centrifugation and without total protein determination. P-Tie2 amounts were quantified according to the manufacturer's instructions and IC50 values for inhibition were determined using XLfit4 analysis plug-in for Excel (Dose-response one site, model 205).

Cell Titer Glow Assay

Cell viability and proliferation was quantified using the cell titer glow assay (Promega). The assay was performed according to the manufacturer's instructions. Briefly, cells were cultured in 96-well plates in a total volume of 100 µL for the desired period of time. For the proliferation assay, cells were removed from the incubator and placed at room temperature for 30 min. 100 µL of cell titer glow reagent were added and multi-well plates were placed on an orbital shaker for 2 min. Luminescence was quantified after 15 min on a microplate reader (Tecan).

Example 1a

Expression & Purification bispecific, bivalent <VEGF-ANG-2 > antibody molecules

According the procedures described in the materials and methods above, the bispecific, bivalent <VEGF-ANG-2> antibody molecules Ang2-VEGF OA-Ava-N-scFabLC06SS and Ang2-VEGF OA-Ava-N-scFabLC06, were expressed and purified. The VH and VL of <VEGF> part are based on bevacizumab. The VH and VL of <ANG2> part are based on VH and VL sequences of ANG2i-LC06 (which is decribed in the PCT application No. PCT/EP2009/007182 and which was obtained via phage display). The relevant light and heavy chain amino acid sequences of these bispecific, bivalent antibodies are given in SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 (for Ang2-VEGF OA-Ava-N-scFabLC06SS), and in SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 12 (for Ang2-VEGF OA-Ava-N-scFabLC06). Expression of Ang2-VEGF OA-Ava-N-scFabLC06SS and Ang2-VEGF OA-Ava-N-scFabLC06 was confirmed by Western blot. Purification of Ang2-VEGF OA-Ava-N-scFabLC06SS and Ang2-VEGF OA-Ava-N-scFabLC06 led to the following yields.

Clone	Supernatant	Protein A		SEC	
		Yield	Mono.	Yield	Mono.
OA-Ava-N-scFabLC06	0.5 L	42.5 mg	77 %	27.0 mg	94.8 %
OA-Ava-N-scFabLC06SS	1.5 L	160 mg	85 %	93 mg	> 99 %

* MassSpec: no homomeric heavy chain dimers were detected

Binding and other properties were determined as described .

Example 1b

Expression & Purification bispecific, bivalent <IGF-1R - EGFR> antibody molecules

Table: Overview of bispecific, bivalent <IGF-1R - EGFR> antibody molecules

Construct	Sequence	scFab VH44-VL100 Disulfide
OA-Ak18-scFab-GA201 WT	SEQ ID NO: 4 (Heavy chain 1) SEQ ID NO: 1 (Heavy chain 2) SEQ ID NO: 2 (Light chain)	-
OA-Ak18-scFab-GA201	SEQ ID NO: 3 (Heavy chain 1) SEQ ID NO: 1 (Heavy chain 2) SEQ ID NO: 2 (Light chain)	+
OA-GA201-scFab-Ak18 WT	SEQ ID NO: 8 (Heavy chain 1) SEQ ID NO: 5 (Heavy chain 2) SEQ ID NO: 6 (Light chain)	-
OA-GA201-scFab-Ak18	SEQ ID NO: 7 (Heavy chain 1) SEQ ID NO: 5 (Heavy	+

Construct	Sequence	scFab VH44-VL100 Disulfide
	chain 2) SEQ ID NO: 6 (Light chain)	

According the procedures described in the materials and methods above, the bispecific, bivalent <IGF-1R - EGFR> antibody molecules OA-Ak18-scFab-GA201 and OA-GA201-scFab-Ak18, were expressed with a 1:1:1 plasmid ratio and purified. The bispecific antibody is based on the antigen-binding sites of <IGF-1R> HUMAB Clone 18 (DSM ACC 2587; WO 2005/005635, abbreviated as <IGF-1R>Clone18 or <IGF-1R> AK18) and humanized <EGFR>ICR62 (WO 2006/082515 abbreviated as <EGFR>ICR62). The relevant light and heavy chain amino acid sequences of these bispecific, bivalent antibodies are given in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 (for OA-Ak18-scFab-GA201), and in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 (for OA-GA201-scFab-Ak18). Expression of OA-Ak18-scFab-GA201 and OA-GA201-scFab-Ak18 was confirmed by Western blot.After Protein A purification of cell culture supernatants both constructs showed between 50 and 58 % of bispecific antibody with the expected molecular weight of approximately 148 kDa as detected by analytical SEC and a high amount of half antibody with a molecular weight of 100 kDa. After SEC purification both constructs showed between 86 and 90 % homogenous monomer with a molecular weight of 148 kDa and a residual side product of 100 kDa. The OA-GA201-scFab-Ak18 was also expressed with a 1:2:1 plasmid ratio and subjected to Prot A and SEC purification. In comparison to the 1:1:1 expression ratio, the amount of half antibody after Prot A was reduced from 30 % to 6 % with a 1:2:1 expression level as detected by a Bioanalyzer (Caliper analysis). Mass Spec analysis of the SEC purified proteins confirmed the efficient removal of the half heavy chain 1 humanized <EGFR>ICR62 antibody by change of the plasmid ratio upon transfection. The OA-GA201-scFab-Ak18 purification yield was increased by 40 % with a 1:2:1 plasmid ratio upon expression in HEK293 cells.

The bispecific, bivalent <IGF-1R - EGFR> antibody molecule OA-GA201-scFab-Ak18_WT, (with the relevant light and heavy chain amino acid sequences given in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 8) was expressed with a 1:1:1 and 1:2:1 plasmid ratio and purified analogously.

Results for the 1:2:1 plasmid ratio:

Purification Construct	Protein A		SEC	
	Yield	Monomer	Yield	Monomer
OA-GA201-scFab-Ak18_WT (9.9 L)	283.5 mg	85.0 % (Analyt. SEC) 86.0 % (BioAnalyzer)	204.1 mg	98.0 % (Analyt. SEC) 95.0 % (BioAnalyzer)

The OA-scFab constructs with only 3 plasmids has the advantage of a valuable side product profile over similar heterodimeric approaches using the knobs-into-hole technology to generate bispecific molecules with 4 expression plasmids (see e.g. WO 2009/080253. The antibodies accorsign to the invention show a complete absence of wrongly paired light chains or antibodies lacking the light chain (data not shown).

The described 1:2:1 method was shown to yield bispecific molecules with high purity and a clear reduction of half antibodies and a complete absence of wrongly paired light chains or antibodies lacking the light chain (data not shown).

Binding and other properties were determined as described.

The bispecific, bivalent <IGF-1R - EGFR> antibody molecule OA-Ak18-scFab-GA201_WT, (with the relevant light and heavy chain amino acid sequences given in in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4 (for OA-Ak18-scFab-GA201_WT), can be expressed and purified analogously.

Example 2

(Simultaneous) binding of bispecific antibodies to both antigens

The binding of the different bispecific antibody formats were compared to the binding of the 'wildtype' IgGs from which the binding modules and bispecific antibodies were derived. These analyses were carried out by applying Surface Plasmon Resonance (Biacore) as described above. Simultaneous binding of bispecific antibody OA-GA201-scFab-Ak18_WT to IGF-1R and EGFR could be detected.

Instrument: Biacore T100 (GE Healthcare),T200 sensitivity enhanced
Software: T200 Control, Version 1.0
Software: T200 Evaluation, Version 1.0

Chip:CM5-Chip

Assay

Standard amine coupling on flow cells 1 to 4 according to the manufacturer's instructions: running buffer: HBS-N buffer, activation by mixture of EDC/NHS, aim for ligand density. EGFR was diluted in coupling buffer NaAc, pH 4.5, c = 15 µg/mL; finally remaining activated carboxyl groups were blocked by injection of 1 M Ethanolamine.

Amine coupled EGFR on flow cell 1 was used as reference control surface for correction of possible buffer-effects or non specific binding.

The simultaneuos binding was measured at a flow rate of 30 µL/min at 25°C. Bispecific Ab was injected for 2 minutes at a concentration of c = 10 nM followed immediately by a consecutive injection of either human or cyno IGF1R (association time: 2 minute, dissociation time: 3 minutes, c = 150 nM).

All samples were diluted with running buffer + 1 mg/mL BSA.

After each cycle the regeneration was performed using 15 mM NaOH, contact time 1 minute, flow rate 30 µL/min. Negative control: Instead of IGF1R dilution buffer was injected as negative control.

Results

Bispecific Abs: OA-GA201-scFab-Ak18_WT showed simultaneous binding of amine coupled human EGFR and human IGF1R (see sensogram Figure 9).

Example 3a

ANG2-VEGF-Mab Tie2 phoshorylation inhibition

Inhibition of VEGF induced HUVEC proliferation by the bispecific <ANG2-VEGF> antibodies was measured according to the assay principle described above the Results are shown in Figure 5.

Example 3b

ANG2-VEGF-Mab Tie2 phoshorylation inhibition

Inhibition of ANG2 induced Tie2 phosphorylation by the bispecific <ANG2-VEGF> antibodies was measured according to the assay principle. Described above the Results are shown in Figure 6.

Example 4

Internalization/Downregulation of IGF-1R by bispecific <EGFR-IGF1R> antibodies

The human anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18 (DSM ACC 2587) inhibits IGF-1R signaling and induces internalization and subsequent downregulation of IGF-1R. To evaluate the potential inhibitory activity of bispecific <EGFR-IGF1R> antibodies, the degree of downregulation of IGF-1R was analyzed.

In order to detect effects of the antibody of the invention on the amount of IGF-1 receptor (IGF-1R) in tumor cells, time-course experiments and subsequent ELISA analysis with IGF-1R and EGFR specific antibodies were performed.

Human H322M tumor cells (obtained from NCI) were cultivated in 96 well plates ($1x10^4$ cells/well) over night at 37°C and 5 % CO_2 in RPMI medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine and 1 % PenStrep.

The medium was carefully removed and replaced by bispecific <EGFR-IGF1R> antibody solution diluted in RPMI medium in a total volume of 100 µl. Cells were incubated at 37°C and 5 % CO_2 for at least 3 but no more than 24 hours.

The medium was carefully removed by aspiration and cells were lysed with 120 µl/well of cold MES-lysis buffer (25 mM MES pH 6.5, 2 % Triton X-100, 60 nM Octylglucoside, 150 mM NaCl, 10 mM Na_3VO_4, and Complete® protease inhibitor). Plates were stored at -20°C until further analysis.

For IGF-1R detection

A 1:200 dilution of antibody AK1a-Biotinylated (<IGF-1R> HUMAB Clone 1a (DSM ACC 2586) described in WO2004/087756, Roche, Germany) in PBS, 3 % BSA and 0.2 % Tween®20 at a final concentration of 2.4 µg/ml was added to each well of a streptavidin-coated MTP (Roche ID. No.: 11965891001). The

streptavidin-MTP was agitated for 1 hour at RT and then washed three times with 200 µl per well of PBS containing 0.1 % Tween®20.

After removal of the PBS/Tween solution, 100 µl cell lysate was added to each well of the antibody coated streptavidin-MTP.

The MTPs were then incubated for another hour at RT with agitation and washed 3 times with PBS containing 0.1 % Tween®20 subsequently.

IGF-1Rβ rabbit antibody (200 µg/ml, Santa Cruz Biotechnology, Cat. No. sc-713) diluted 1:750 in PBS, 3 %BSA and 0.2 % Tween®20 was used to detect IGF-1R bound by the capture antibody AK1a. 100 µl was added per well and incubated for 1 hour at RT with constant agitation. The solution was removed subsequently and the wells were washed three times with 200 µl of PBS containing 0.1 % Tween®20. The peroxidase labeled anti-rabbit IgG-HRP (Cell signaling Cat. No. 7074) was used as secondary detection antibody in a dilution of 1:4000 in PBS, 3 % BSA and 0.2 % Tween®20. 100 µl of it was added to each well and incubated for 1 hour at RT with agitation. The plate was then washed six times with PBS containing 0.1 % Tween®20 solution. 100 µl per well of the peroxidase substrate 3,3'-5,5'-Tetramethylbenzidin (Roche, BM-Blue ID-No.: 11484581) were added and incubated for 20 minutes at RT with agitation. The colourigenic reaction was stopped by adding 25 µl per well of 1M H_2SO_4 and incubating for another 5 minutes at RT. The absorbance was measured at 450nm.

The bispecific <EGFR-IGF1R> antibody OA-GA201-scFab-Ak18_WT induces less downregulation than the human anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18. Downregulation by OA-GA201-scFab-Ak18_WT was reduced by > 50% compared to <IGF-1R> HUMAB Clone 18.

Example 5

Inhibition of EGFR- as well as IGF-1R- signaling pathways by bispecific <EGFR-IGF1R> antibodies

The human anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18 (DSM ACC 2587) inhibits IGF-1R-signaling and the humanized rat anti-EGFR antibody ICR62 inhibits the signaling by EGFR. To evaluate the potential inhibitory activity of bispecific <EGFR-IGF1R> antibodies, the degree of inhibition of signaling towards both pathways was analyzed.

Human tumor cells (H322M, 3 x 10^4 cells/well) in RPMI medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine and 1 % PenStrep were seeded in 96-well microtiter plates and cultivated over night at 37°C and 5 % CO_2.

The medium was carefully removed and replaced by 100 µl serum-free DMEM medium (supplemented with 1 mg/ml RSA, 10 mM Hepes, 1 % PenStrep) and incubated for at least 2.5 hours at 37°C and 5 % CO_2.

The medium was again carefully removed and replaced by a dilution of bispecific antibodies, and control antibodies (<IGF-1R> HUMAB Clone 18 and <EGFR>ICR62 final concentration 0.01 mg/ml) in serum-free DMEM medium at a total volume of 50 µl followed by an incubation for 30 min at 37°C and 5 % CO_2. Cells were stimulated by the addition of 50 µl IGF-1 (10 nM) or EGF (20 ng/ml) (diluted in serum-free DMEM medium) and incubated for 10 min at 37°C and 5 % CO_2.

The medium was carefully removed and cells were washed once with 100 µl/well of ice cold PBS. Cells were lysed by the addition of 100 µl/well BioRad Cell Lysis buffer (BioRad Cell Lysis Kit (BioRad Cat #171-304012) Plates were stored at -20°C until further analysis.

Cell debris was removed by filtering cell lysates through MultiScreen HTS-filter plates by centrifugation at 500 g for 5 min. EGFR and IGF-1R phosphorylation in filtered cell lysates was analysed with a Luminex system using the P-EGFR (Tyr) bead kit (Millipore Cat. # 46-603) for the analysis of EGFR phosphorylation and the P-IGF-1R (Tyr1131) bead kit (BioRad Cat. # 171V27343) for the analysis of IGF-1R phosphorylation. The Luminex assays were performed as described in the BioPlex Phosphoprotein Detection manual (BioRad Bulletin # 2903) using Phosphoprotein Detection Reagent Kits (BioRad Cat. # 171-304004).

The bispecific <EGFR-IGF1R> antibody OA-GA201-scFab-Ak18_WT effectively inhibits the phosphorylation of IGF-1R (IC50: 1 nM, maximal inhibition: > 70%) and the phosphorylation of EGFR (IC50: 1 nM, maximal inhibition: > 90%) on H322M tumor cells.

Example 6

Growth inhibition of NCI-H322M tumor cells in 3D culture by bispecific <EGFR-IGF1R> antibodies

The human anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18 (DSM ACC 2587) inhibits the growth of tumor cell lines that express IGF-1R (WO 2005/005635). In a similar manner, the humanized rat anti-EGFR antibody <EGFR>ICR62 has been shown to inhibit the growth of tumor cell lines that express EGFR (WO 2006/082515). To evaluate the potential inhibitory activity of bispecific <EGFR-IGF1R> antibodies in growth assays of tumor cell lines, the degree of inhibition in H322M cells which express EGFR as well as IGF-1R was analyzed.

H322M lung carcinoma cells (NCI) were cultured in RPMI 1640 medium (PAA, Pasching, Austria) supplemented with 10 % FBS (PAA), 1mM sodium pyruvate (Gibco, Darmstadt, Germany), non-essential amino acids (Gibco) and 2mM L-glutamine (Sigma, Steinheim, Germany). 25000 cells/well were seeded in 96-well poly-HEMA (poly(2-hydroxyethylmethacrylate) (Polysciences, Warrington, PA, USA)) coated plates containing the culture medium. Concomitantly, different concentrations of bispecific antibodies were added and incubated for 7 days. The CellTiterGlo® (Promega , Madison, WI, USA) assay was used to detect cell viability by measuring the ATP-content of the cells according to the manufacturer's instructions.

Results were shown in Figure 8. The bispecific <EGFR-IGF1R> antibody OA-GA201-scFab-Ak18_WT dose-dependently inhibits proliferation of H322M cells. At a dose of 1000 nM the bispecific <EGFR-IGF1R> antibody OA-GA201-scFab-Ak18_WT showed improved inhibition of proliferation when compared to the parental monospecific antibodies <IGF-1R> HUMAB Clone 18 or <EGFR>ICR62.

Example 7

In vivo efficacy of bispecific <EGFR-IGF1R> antibodies in a subcutaneous xenograft model with EGFR and IGF-1R expression

The human anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18 (DSM ACC 2587) inhibits the growth of tumor cell lines that express IGF-1R (WO 2005/005635). In a similar manner, the humanized rat anti-EGFR antibody <EGFR>ICR62 has been shown to inhibit the growth of tumor cell lines that express EGFR (WO 2006/082515). To evaluate the potential inhibitory activity of bispecific <EGFR-IGF1R> antibodies on in vivo tumor growth the subcutaneous xenograft

model BxPC-3 that was characterized by the expression of EGFR as well as IGF-1R was used.

Cells of the human pancreatic carcinoma cell line BxPC-3 (obtained from ATCC) were cultured in RPMI 1640 medium (PAN^{TM} Biotech GmbH), supplemented with 10 % fetal bovine serum (Sera Plus; PAN^{TM} Biotech GmbH) and 2 mM L-glutamine (PAN^{TM} Biotech GmbH) at 37°C in a water saturated atmosphere at 5 % CO_2. At the day of inoculation, BxPC-3 tumor cells were harvested (1x trypsin-EDTA, Roche Diagnostics) from culture flasks and transferred into culture medium, centrifuged, washed once and re-suspended in PBS. For injection of cells, the final titer was adjusted to 1×10^8 cells/ml. Subsequently 100 µl of this suspension (corresponding to 1×10^7 cells) were injected subcutaneously into the right flank of female SCID beige mice. Treatment with vehicle, <EGFR-IGF1R> antibodies and control antibodies (<IGF-1R> HUMAB Clone 18 and <EGFR>ICR62) started after tumors were established and have reached an average size of 150-250 mm^3. Tumor volume was measured twice a week and animal weights were monitored in parallel. Single treatments and combination of the single antibodies were compared to the therapy with bispecific antibody.

The bispecific <EGFR-IGF1R> antibody OA-GA201-scFab-Ak18_WT (20 mg/kg; i.p., once weekly (q7d)) showed strong anti-tumor efficacy in the s.c. BxPC3 xenograft model see Fig.10 and Table below) and inhbited tumor growth slightly better than the combination of monospecific antibodies <IGF-1R> HUMAB Clone 18 (10 mg/kg; i.p., once weekly (q7d)) and <EGFR>ICR62 (10 mg/kg; i.p., once weekly (q7d))

	TGI	TCR
OA-GA201-scFab-Ak18_WT (20 mg/kg; i.p. q7d)	93,5%	0,26
HUMAB Clone 18 (10 mg/kg; i.p. q7d) + ICR62 (10 mg/kg; i.p. q7d)	91,5%	0,27

Example 8

Preparation of the glycoengineered derivatives of bispecific <EGFR-IGF1R> antibodies

Glycoengineered derivatives of bispecific <EGFR-IGF1R> antibodies were produced by co-transfecting HEK293-EBNA cells with the mammalian antibody heavy and light chain expression vectors using a calcium phosphate-transfection approach. Exponentially growing HEK293-EBNA cells were transfected by the calcium phosphate method. For the production of unmodified antibody, the cells were transfected only with antibody heavy and light chain expression vectors in a 1:1 ratio. For the production of the glycoengineered antibody, the cells are co-transfected with four plasmids, two for antibody expression, one for a fusion GnTIII polypeptide expression, and one for mannosidase II expression at a ratio of 4:4:1:1, respectively. Cells were grown as adherent monolayer cultures in T flasks using DMEM culture medium supplemented with 10 % FCS, and were transfected when they were between 50 and 80 % confluent. For the transfection of a T75 flask, 7.5 (to 8) million cells were seeded 24 hours before transfection in ca 14 ml DMEM culture medium supplemented with FCS (at 10 % V/V final), (eventually 250 µg/ml neomycin,) and cells were placed at 37°C in an incubator with a 5 % CO2 atmosphere overnight. For each T75 flask to be transfected, a solution of DNA, CaCl2 and water was prepared by mixing 47 µg total plasmid vector DNA divided equally between the light and heavy chain expression vectors, 235 µl of a 1M CaCl2 solution, and adding water to a final volume of 469 µl. To this solution, 469 µl of a 50mM HEPES, 280 mM NaCl, 1.5 mM Na2HPO4 solution at pH 7.05 were added, mixed immediately for 10 sec and left to stand at room temperature for 20 sec. The suspension was diluted with ca. 12 ml of DMEM supplemented with 2 % FCS, and added to the T75 in place of the existing medium. The cells were incubated at 37°C, 5 % CO2 for about 17 to 20 hours, then medium was replaced with ca. 12 ml DMEM, 10 % FCS. The conditioned culture medium was harvested 5 to 7 days post-transfection centrifuged for 5 min at 210 – 300 *g , sterile filtered through a 0.22 µm filter (or alternatively centrifuged for 5 min at 1200 rpm, followed by a second centrifugation for 10 min at 4000 rpm) and kept at 4°C.

The secreted antibodies were purified by Protein A affinity chromatography, and a final size exclusion chromatographic step on a Superdex 200 column (Amersham Pharmacia) exchanging the buffer to phosphate buffer saline and collecting the pure monomeric IgG1 antibodies. Antibody concentration ia estimated using a

spectrophotometer from the absorbance at 280 nm. The antibodies are formulated in a 25 mM potassium phosphate, 125 mM sodium chloride, 100 mM glycine solution of pH 6.7.

Glycoengineered variants of the bispecific antibodies were produced by co-transfection of the antibody expression vectors together with a GnT-III glycosyltransferase expression vector, or together with a GnT-III expression vector plus a Golgi mannosidase II expression vector. Glycoengineered antibodies were purified and formulated as described above for the non-glycoengineered antibodies. The oligosaccharides attached to the Fc region of the antibodies were analysed by MALDI/TOF-MS as described below to determine the amount of fucose.

Purification Glycoengineered Construct	**Protein A**		**SEC**	
	Yield	**Monomer**	**Yield**	**Monomer**
OA-GA201-scFab-Ak18_WT GE GE (4.2 L)	87.3 mg	92.0 % (Analyt. SEC)	65.1 mg	100.0 % (Analyt. SEC)

Oligosaccharides are enzymatically released from the antibodies by PNGaseF digestion, with the antibodies being either immobilized on a PVDF membrane or in solution.

The resulting digest solution containing the released oligosaccharides is either prepared directly for MALDI/TOF-MS analysis or is further digested with EndoH glycosidase prior to sample preparation for MALDI/TOF-MS analysis. For all bispecific antibodies according to the invention, GE means glycoengineered.

Example 9

Binding to FcgRIIIa and ADCC-competence of bispecific <EGFR-IGF1R> antibodies

The degree of ADCC mediation by a given antibody depends not only on the antigen that is bound, but is also dependent on affinities of constant regions to the FcgRIIIa, which is known as the Fc receptor that triggers the ADCC reaction. For the analysis of binding of the bispecific <EGFR-IGF1R> antibodies to the FcgRIIIa, a Biacore technology is applied. By this technology, binding of

bispecific <EGFR-IGF1R> antibodies to recombinantly produced FcgRIIIa domains is assessed.

All surface plasmon resonance measurements are performed on a BIAcore 3000 instrument (GE Healthcare Biosciences AB, Sweden) at 25°C. The running and dilution buffer was PBS (1 mM KH2PO4, 10mM Na2HPO4, 137 mM NaCl, 2.7mM KCl), pH6.0, 0.005 % (v/v) Tween20. The soluble human FcgRIIIa was diluted in 10 mM sodium-acetate, pH 5.0 and immobilized on a CM5 biosensor chip using the standard amine coupling kit (GE Healthcare Biosciences AB, Sweden) to obtain FcgRIIIa surface densities of approximately 1000 RU. HBS-P (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005 % Surfactant P20; GE Healthcare Biosciences AB, Sweden) is used as running buffer during immobilization. XGFR bispecific antibodies are diluted with PBS, 0.005 %(v/v) Tween20, pH6.0 to a concentration of 450 nM and injected over 3 minutes at a flow rate of 30µl/minute. Then, the sensor chip is regenerated for 1 minute with PBS, pH8.0, 0.005 % (v/v) Tween20. Data analysis is performed with the BIAevaluation software (BIAcore, Sweden).

To analyze to what degree the binding competency of bispecific <EGFR-IGF1R> antibodies to FcgRIIIa translates also into in-vitro ADCC activity towards tumor cells, ADCC competency is determined in cellular assays. For these assays, glycomodified derivatives bispecific <EGFR-IGF1R> antibodies are prepared (see above) and tested in a BIAcore ADCC-competence assay format and also an in-vitro ADCC assay as described below.

Human peripheral blood mononuclear cells (PBMC) are used as effector cells and were prepared using Histopaque-1077 (Greiner Leucosep # 227288) following essentially the manufacturer's instructions. In brief, venous blood is taken with heparinized syringes from healthy volunteers. The blood is diluted 1:0.75-1.3 with PBS (not containing Ca^{++} or Mg^{++}) and layered on Histopaque-1077. The gradient was centrifuged at 800 x g for 30 min at room temperature (RT) without breaks. The interphase containing the PBMC is collected and washed with PBS (50 ml per cells from two gradients) and harvested by centrifugation at 400 x g for 10 minutes at RT. After resuspension of the pellet with PBS, the PBMC are counted and washed a second time by centrifugation at 400 x g for 10 minutes at RT. The cells are then resuspended in the appropriate medium for the subsequent procedures.

The effector to target ratio used for the ADCC assays is 25:1 for PBMC. The effector cells are prepared in AIM-V medium at the appropriate concentration in

order to add 50 µl per well of round bottom 96 well plates. Target cells were human EGFR/IGFR expressing cells (e.g., H322M, A549, or MCF-7) grown in DMEM containing 10 % FCS.

Target cells are washed in PBS, counted and adjusted at 1x10E6 cells/ml. Cells are labeled with Calcein AM (10µM) for 30min at 37°/5%CO2. After labeling cells are washed twice in PBS and seeded at 5000 cells/well in 50µl (AIM-V medium) in 96-well round bottom plates. Antibodies are diluted in AIM-V, added in 50 µl to the pre-plated target cells. Then the effector cells are added and the plate is incubated for 4 hours at 37°C in a humidified atmosphere containing 5 % CO2. After the incubation period plates are centrifuged at 200g for 10min and 80µl supernatant is transferred to a black fluorescent plate/transparent bottom and fluorescence (Ex 485nm/Em 535nm) is measured with a Tecan Infinite reader.

Following controls are included in the assay:

- background: 50µl supernatant-aliquot after labeling of cells + 100µl medium
- spontaneous lysis: 50µl target cell suspension + 100µl medium
- maximum lysis: 50µl target cell suspension + 100µl medium/1,5% Triton X-100
- lysis control w/o antibody: 50µl target cell suspension + 50µl medium + 50µl PBL´s

% antibody dependent cytotoxicity is calculated as follows:
% ADCC = x - spontaneous release/ max. lysis – spontaneous lysis x 100

Example 10

Analysis of glycostructure of bispecific <EGFR-IGF1R> antibodies

For determination of the relative ratios of fucose- and non-fucose (a-fucose) containing oligosaccharide structures, released glycans of purified antibody material are analyzed by MALDI-Tof-mass spectrometry. For this, the antibody sample (about 50µg) is incubated over night at 37°C with 5mU N-Glycosidase F (Prozyme# GKE-5010B) in 0.1M sodium phosphate buffer, pH 6.0, in order to release the oligosaccharide from the protein backbone. Subsequently, the glycan structures released are isolated and desalted using NuTip-Carbon pipet tips (obtained from Glygen: NuTip1-10 µl, Cat.Nr#NT1CAR). As a first step, the NuTip-Carbon pipet tips are prepared for binding of the oligosaccharides by

washing them with 3 µL 1M NaOH followed by 20 µL pure water (e.g. HPLC-gradient grade from Baker, # 4218), 3 µL 30 % v/v acetic acid and again 20 µl pure water. For this, the respective solutions are loaded onto the top of the chromatography material in the NuTip-Carbon pipet tip and pressed through it. Afterwards, the glycan structures corresponding to 10 µg antibody are bound to the material in the NuTip-Carbon pipet tips by pulling up and down the N-Glycosidase F digest described above four to five times. The glycans bound to the material in the NuTip-Carbon pipet tip are washed with 20 µL pure water in the way as described above and are eluted stepwise with 0.5 µL 10 % and 2.0 µL 20 % acetonitrile, respectively. For this step, the elution solutions are filled in a 0.5 mL reaction vails and are pulled up and down four to five times each. For the analysis by MALDI-Tof mass spectrometry, both eluates are combined. For this measurement, 0.4 µL of the combined eluates are mixed on the MALDI target with 1.6 µL SDHB matrix solution (2.5-Dihydroxybenzoic acid/2-Hydorxy-5-Methoxybenzoic acid [Bruker Daltonics #209813] dissolved in 20 % ethanol/5mM NaCl at 5 mg/ml) and analysed with a suitably tuned Bruker Ultraflex TOF/TOF instrument. Routinely, 50-300 shots are recorded and sumed up to a single experiment. The spectra obtained are evaluated by the flex analysis software (Bruker Daltonics) and masses are determined for the each of the peaks detected. Subsequently, the peaks are assigned to fucose or a-fucose (non-fucose) containing glycol structures by comparing the masses calculated and the masses theoretically expected for the respective structures (e.g. complex, hybride and oligo-or high-mannose, respectively, with and without fucose).

For determination of the ratio of hybride structures, the antibody sample are digested with N-Glycosidase F and Endo-Glycosidase H concommitantlyN-glycosidase F releases all N-linked glycan structures (complex, hybride and oligo- and high mannose structures) from the protein backbone and the Endo-Glycosidase H cleaves all the hybride type glycans additionally between the two GlcNAc-residue at the reducing end of the glycan. This digest is subsequently treated and analysed by MALDI-Tof mass spectrometry in the same way as described above for the N-Glycosidase F digested sample. By comparing the pattern from the N-Glycosidase F digest and the combined N-glycosidase F / Endo H digest, the degree of reduction of the signals of a specific glyco structure is used to estimate the relative content of hybride structures.

The relative amount of each glycostructure is calculated from the ratio of the peak height of an individual glycol structure and the sum of the peak heights of all glyco

structures detected. The amount of fucose is the percentage of fucose-containing structures related to all glyco structures identified in the N-Glycosidase F treated sample (e.g. complex, hybride and oligo- and high-mannose structures, resp.). The amount of afucosylation is the percentage of fucose-lacking structures related to all glyco structures identified in the N-Glycosidase F treated sample (e.g. complex, hybride and oligo- and high-mannose structures, resp.).

The amount of fucose determined for OA-GA201-scFab-Ak18_WT was between 25% and 40%.

Example 11

Binding of bispecific <EGFR-IGF1R> antibodies to cells with different EGFR and IGF-1R expression

The human anti-IGF-1R antibody <IGF-1R> HUMAB Clone 18 (DSM ACC 2587) binds to cells expressing IGF-1R and the humanized rat anti-EGFR antibody ICR62 binds to cells that express EGFR on their surface. To evaluate the binding properties of bispecific <EGFR-IGF1R> antibodies compared to monospecific bivalent antibodies towards EGFR and IGF-1R, competitive binding assays were performed on cells with different IGF-1R / EGFR expression ratio.

Human tumor cells (e.g. A549, TC-71, MDA-MB-231, 2 x 105 cells/well) diluted in ice-cold buffer (PBS + 2 % FCS, Gibco) were added to a mixture of labeled monospecific antibodies (HUMAB Clone 18 or humanized rat anti-EGFR antibody ICR62) (final concentration of 1 µg/ml) and different concentrations of unlabeled <EGFR-IGF1R> antibodies or unlabeled monospecific antibodies or Fab fragments as controls (final titration range of 100 to 0.002 µg/ml) in a 96-well microtiter plate. The mixture was incubated on ice for 45 minutes. Cells were washed 2 times by addition of 150-200 µl buffer (PBS + 2% FCS) and subsequent centrifugation (300g; 5 min, 4°C). The cells were then resuspended in 200 µl fixation buffer (1x CellFix, BD #340181) containing 6.25 µl/ml 7-AAD (BD #559925) and incubated for 10-20 min on ice to allow for fixation and penetration of 7-AAD in dead cells. Fluorescent signal of the samples was analysed by FACS and IC50 values were calculated.

The results of the competitive binding analysis versus <IGF-1R> HUMAB Clone 18 (IC50 values) are shown in Table X. On A549 tumor cells, expressing both IGF-1R and EGFR, binding of the bispecific <EGFR-IGF1R> antibody OA-GA201-scFab-Ak18_WT was superior to <IGF-1R> HUMAB Clone 18 (~ 3x) and

superior to the <IGF-1R> HUMAB Clone 18 Fab fragment (~ 30x) likely due to the ability of the bispecific antibody to bind both IGF-1R and EGFR simultaneously (avidity effects). On TC-71 tumor cells, expressing IGF-1R but not EGFR binding of <EGFR-IGF1R> antibody OA-GA201-scFab-Ak18_WT was comparable to the IGF-1R> HUMAB Clone 18 Fab fragment. In this setting, were only IGF-1R but not EGFR is expressed OA-GA201-scFab-Ak18_WT can only bind to IGF-1R with one binding arm.

This capacity of bispecific <EGFR-IGF1R> antibodies to bind stronger to cells expressing IGF-1R and EGFR can be exploited to achieve superior targeting to tumor tissue and potentially resulting in favorable safety profiles and PK properties compared to monospecific IGF-1R and EGFR targeting antibodies.

Cell line	**Test compound (unlabeled)**	**Competing compound (labeled)**	**IC50 [nM] Exp. #1**	**IC50 [nM] Exp. #2**
A549	<IGF-1R> HUMAB Clone 18	<IGF-1R> HUMAB Clone 18	1.6	1.5
A549	<IGF-1R> HUMAB Clone 18 Fab fragment	<IGF-1R> HUMAB Clone 18	21.1	19.3
A549	<EGFR-IGF1R> antibody OA-GA201-scFab-Ak18_WT	<IGF-1R> HUMAB Clone 18	0.6	0.6
TC-71	<IGF-1R> HUMAB Clone 18	<IGF-1R> HUMAB Clone 18	1.4	1.5
TC-71	<IGF-1R> HUMAB Clone 18 Fab fragment	<IGF-1R> HUMAB Clone 18	24.6	26.2
TC-71	<EGFR-IGF1R> antibody OA-GA201-scFab-Ak18_WT	<IGF-1R> HUMAB Clone 18	24.3	24.9

Patent Claims

1. A bispecific antibody comprising

a) the heavy chain and the light chain of a first full length antibody that specifically binds to a first antigen;

b) the heavy chain and the light chain of a second full length antibody that specifically binds to a second antigen, wherein the N-terminus of the heavy chain is connected to the C-terminus of the light chain via a peptide linker.

2. The antibody according to claim 1, characterized in that

the CH3 domain of the heavy chain of the full length antibody of a) and the CH3 domain of the heavy chain of the full length antibody of b) each meet at an interface which comprises an alteration in the original interface between the antibody CH3 domains;

wherein i) in the CH3 domain of one heavy chain

an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the interface of the CH3 domain of one heavy chain which is positionable in a cavity within the interface of the CH3 domain of the other heavy chain

and wherein

ii) in the CH3 domain of the other heavy chain

an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the interface of the second CH3 domain within which a protuberance within the interface of the first CH3 domain is positionable.

3. The antibody according to claim 2, characterized in that

the said amino acid residue having a larger side chain volume is selected from the group consisting of arginine (R), phenylalanine (F), tyrosine (Y), tryptophan (W) and
said amino acid residue having a smaller side chain volume is selected from the group consisting of alanine (A), serine (S), threonine (T), valine (V).

4. The antibody according to claims 2 to 3, characterized in that

both CH3 domains are further altered by the introduction of cysteine (C) as amino acid in the corresponding positions of each CH3 domain such that a disulfide bridge between both CH3 domains can be formed.

5. The antibody according to claims 1 to 4, characterized in that

the antibody heavy chain variable domain (VH) and the antibody light chain variable domain (VL) of the heavy and light chain of the second full lenght antibody under b) are disulfide stabilized by introduction of a disulfide bond between the following positions:
i) heavy chain variable domain position 44 to light chain variable domain position 100,
ii) heavy chain variable domain position 105 to light chain variable domain position 43, or
iii) heavy chain variable domain position 101 to light chain variable domain position 100.

6. The bispecific antibody according to claims 1 to 5, wherein the antibody comprises a constant region of IgG1.

7. The bispecific antibody according to claim 1, characterized in that

a) the first full length antibody specifically binds to IGF-1R and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 1, and a light chain with the amino acid sequence of SEQ ID NO: 2, and
b) the second full length antibody specifically binds to EGFR and comprises a heavy chain connected to the light chain via a peptide linker wherein said peptide connected heavy and light chain have the amino acid sequence of SEQ ID NO: 3.

8. The bispecific antibody according to claim 1, characterized in that

a) the first full length antibody specifically binds to IGF-1R and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 1, and a light chain with the amino acid sequence of SEQ ID NO: 2, and

b) the second full length antibody specifically binds to EGFR and comprises a heavy chain connected to the light chain via a peptide linker wherein said peptide connected heavy and light chain have the amino acid sequence of SEQ ID NO: 4.

9. The bispecific antibody according to claim 1, characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and

b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said peptide connected heavy and light chain have the amino acid sequence of SEQ ID NO: 7.

10. The bispecific antibody according to claim 1, characterized in that

a) the first full length antibody specifically binds to EGFR and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 5, and a light chain with the amino acid sequence of SEQ ID NO: 6, and

b) the second full length antibody specifically binds to IGF-1R and comprises a heavy chain connected to the light chain via a peptide linker wherein said peptide connected heavy and light chain have the amino acid sequence of SEQ ID NO: 8.

11. The bispecific antibody according to claim 1, characterized in that

a) the first full length antibody specifically binds to VEGF and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 9, and a light chain with the amino acid sequence of SEQ ID NO: 10, and

b) the second full length antibody specifically binds to ANG-2 and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 11.

12. The bispecific antibody according to claim 1, characterized in that

a) the first full length antibody specifically binds to VEGF and comprises a heavy chain with the amino acid sequence of SEQ ID NO: 9, and a light chain with the amino acid sequence of SEQ ID NO: 10, and

b) the second full length antibody specifically binds to ANG-2 and comprises a heavy chain connected to the light chain via a peptide linker wherein said connected heavy and light chain have the amino acid sequence of SEQ ID NO: 12.

13. The bispecific antibody according to claims 6 to 10, wherein the antibody is glycosylated with a sugar chain at Asn297 wherein the amount of fucose within the sugar chain is 65 % or lower.

14. A pharmaceutical composition comprising an antibody according to claims 1 to 13.

15. The bispecific antibody according to any one of claims 1 to 13 for the treatment of cancer.

16. Use of the the bispecific antibody according to any one of claims 1 to 13 for the preparation of a medicament for the treatment of cancer.

17. A method of treatment of patient suffering from cancer by administering an antibody according to any one of claims 1 to 13 to a patient in the need of such treatment.

18. A nucleic acid molecule encoding a chain of a bispecific antibody according to any one of claims 1 to 13.

19. Expression vector containing said nucleic acid according claim 18 capable of expressing said nucleic acid in a prokaryotic or eukaryotic host cell.

20. A prokaryotic or eukaryotic host cell comprising a vector according to claim 19.

21. A method for the preparation of a bispecific antibody according to claims 1 to 13

comprising the steps of

a) transforming a host cell with vectors comprising nucleic acid molecules encoding

aa) the heavy chain and the light chain of a first full length antibody that specifically binds to a first antigen; and

ab) the heavy chain and the light chain of a second full length antibody that specifically binds to a second antigen, wherein the N-terminus of the heavy chain is connected to the C-terminus of light chain via a peptide linker; and

b) culturing the host cell under conditions that allow synthesis of said antibody molecule; and

c) recovering said antibody molecule from said culture.

22. A bispecific antibody that specifically binds to human IGF-1R and to human EGFR, characterized in comprising the amino acid sequences of SEQ ID NO: 1, of SEQ ID NO: 2, and of SEQ ID NO: 3.

23. A bispecific antibody that specifically binds to human IGF-1R and to human EGFR, characterized in comprising the amino acid sequences of SEQ ID NO: 1, of SEQ ID NO: 2, and of SEQ ID NO: 4.

24. A bispecific antibody that specifically binds to human IGF-1R and to human EGFR, characterized in comprising the amino acid sequences of SEQ ID NO: 5, of SEQ ID NO: 6, and of SEQ ID NO: 7.

25. A bispecific antibody that specifically binds to human IGF-1R and to human EGFR, characterized in comprising the amino acid sequences of SEQ ID NO: 5, of SEQ ID NO: 6, and of SEQ ID NO: 8.

26. The bispecific antibody according to claims 22 to 25, wherein the antibody is glycosylated with a sugar chain at Asn297 wherein the amount of fucose within the sugar chain is 65 % or lower.

27. A bispecific antibody that specifically binds to human VEGF and to human ANG-2, characterized in comprising the amino acid sequences of SEQ ID NO: 9, of SEQ ID NO: 10, and of SEQ ID NO: 11.

28. A bispecific antibody that specifically binds to human VEGF and to human ANG-2, characterized in comprising the amino acid sequences of SEQ ID NO: 9, of SEQ ID NO: 10, and of SEQ ID NO: 12.

Fig. 1

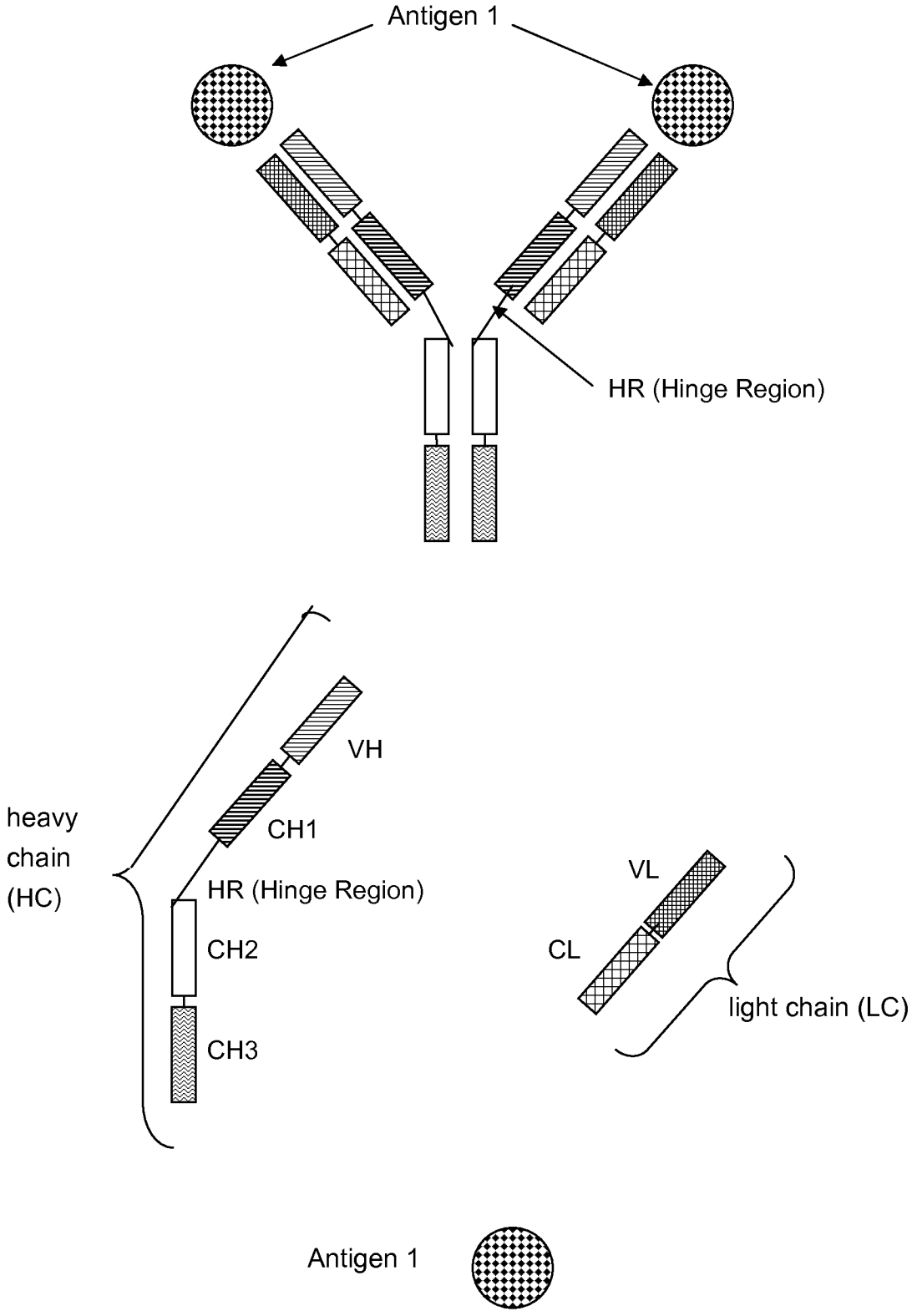

Antigen 1
HR (Hinge Region)
heavy
chain
(HC)
VH
CH1
HR (Hinge Region)
CH2
CH3
VL
CL
light chain (LC)
Antigen 1

Fig. 2

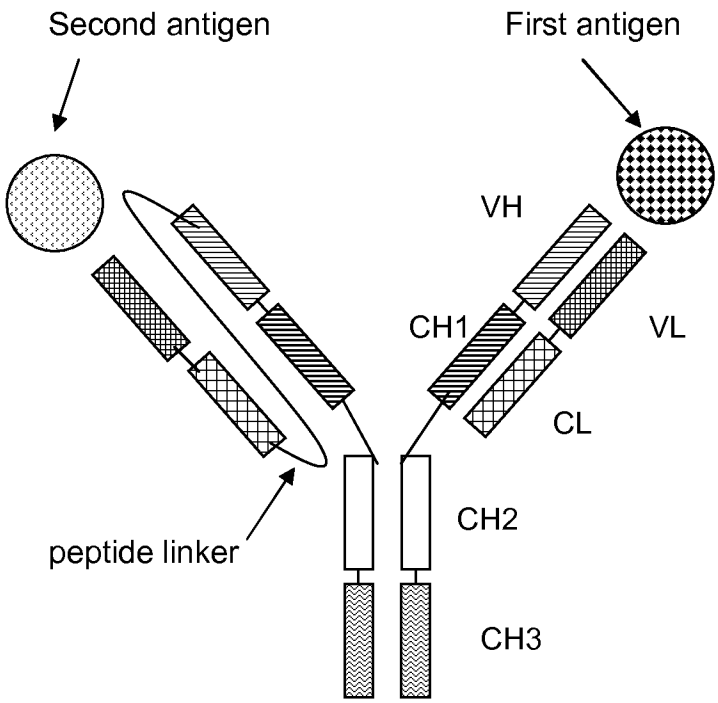

Second antigen
First antigen
VH
CH1
VL
CL
CH2
peptide linker
CH3

Fig. 3a

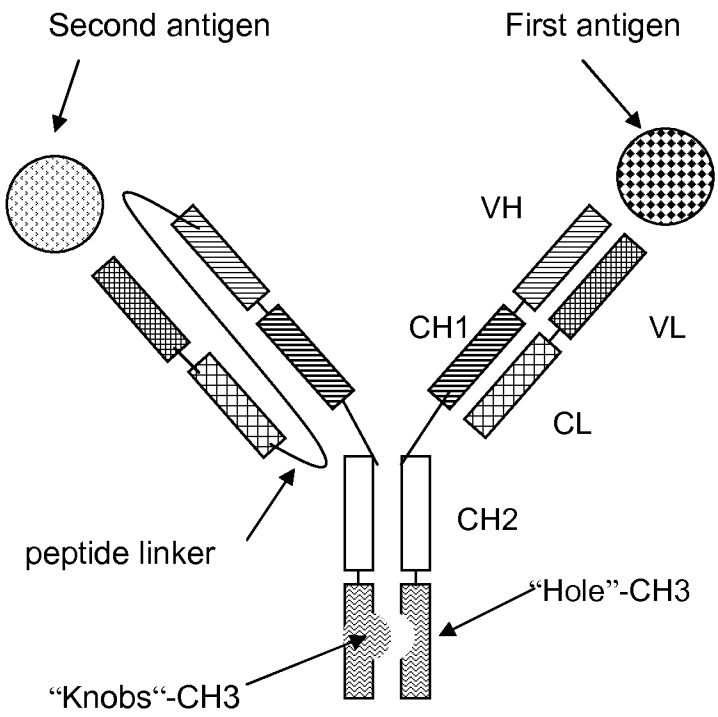

Second antigen
First antigen
VH
CH1
VL
CL
CH2
peptide linker
"Hole"-CH3
"Knobs"-CH3

Fig. 3b

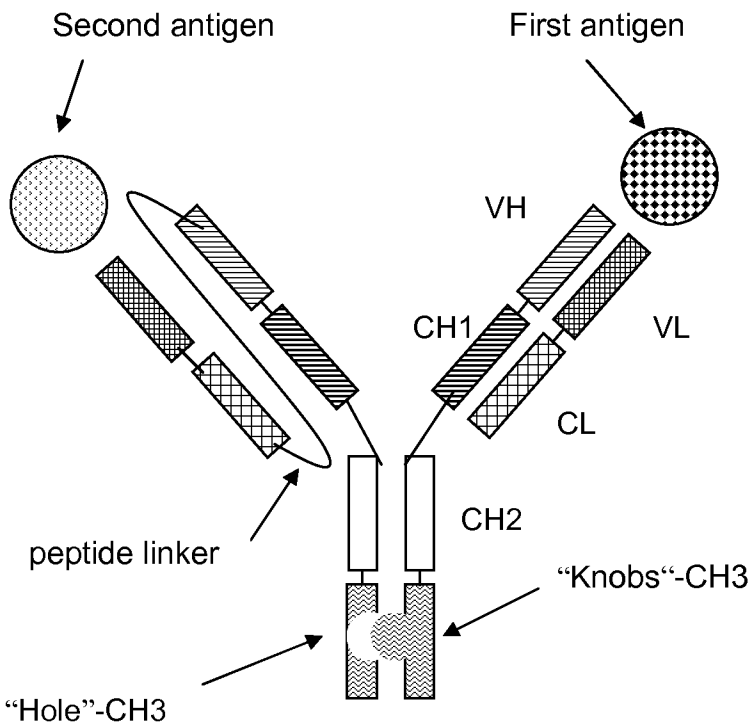

Second antigen
First antigen
VH
CH1
VL
CL
CH2
peptide linker
"Knobs"-CH3
"Hole"-CH3

Fig. 4a

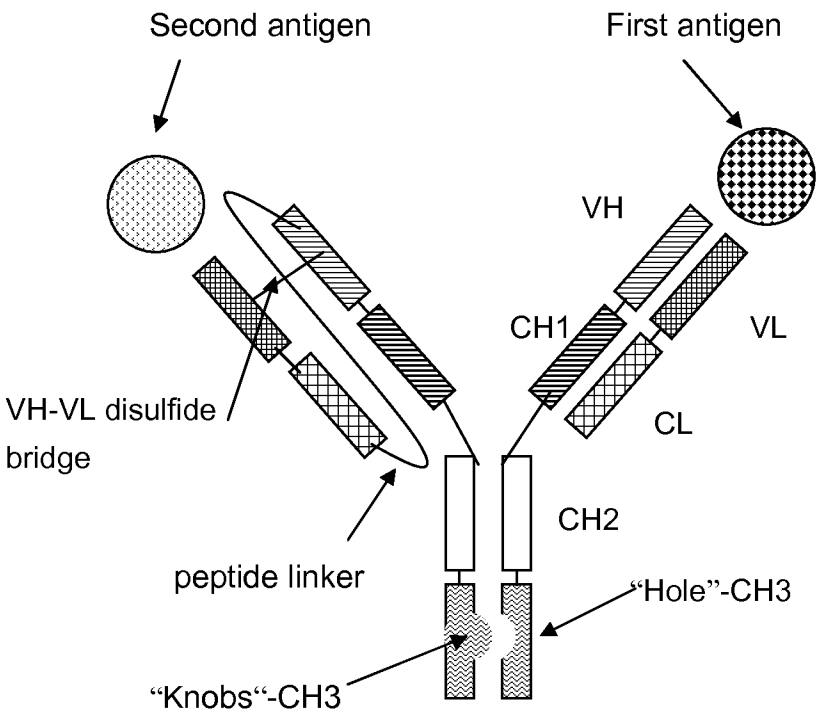

Second antigen
First antigen
VH
CH1
VL
CL
VH-VL disulfide bridge
CH2
peptide linker
"Hole"-CH3
"Knobs"-CH3

Fig. 4b

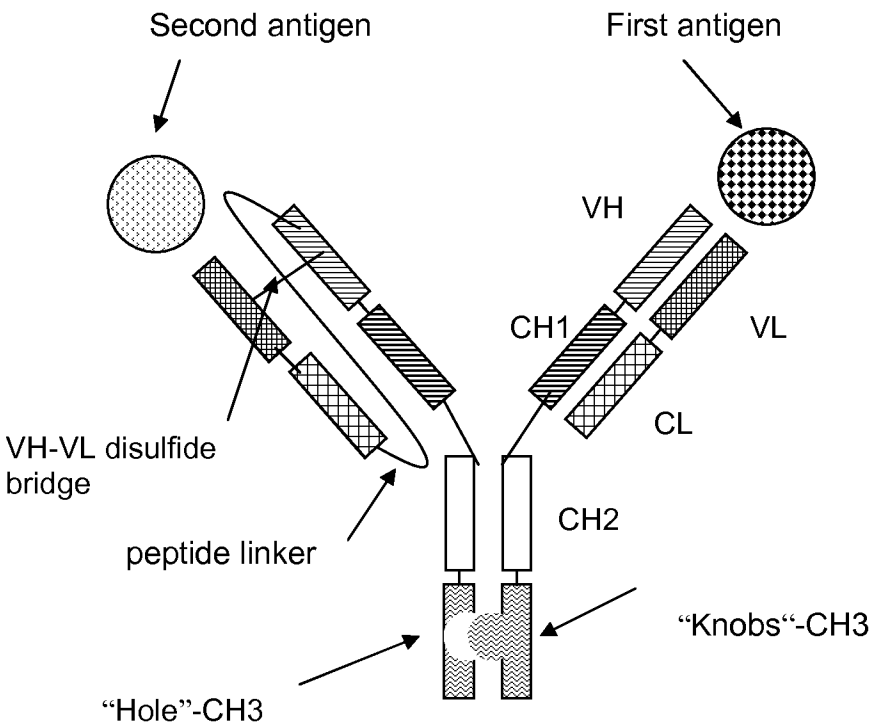

Second antigen
First antigen
VH
CH1
VL
CL
VH-VL disulfide bridge
peptide linker
CH2
"Knobs"-CH3
"Hole"-CH3

Fig. 5

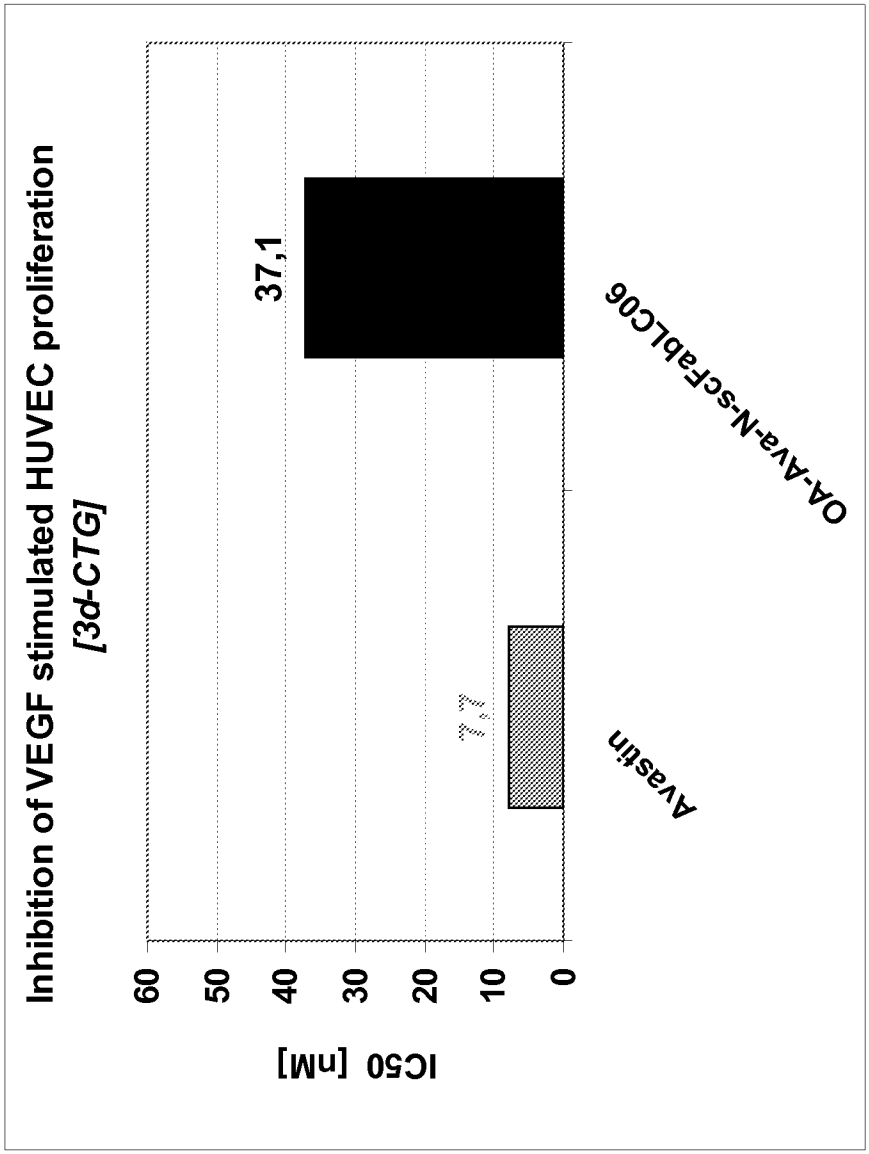

Inhibition of VEGF stimulated HUVEC proliferation
[3d-CTG]
IC50 [nM]
60
50
40
30
20
10
0
37,1
7,7
OA-Ava-N-scFabLC06
Avastin

Fig. 6

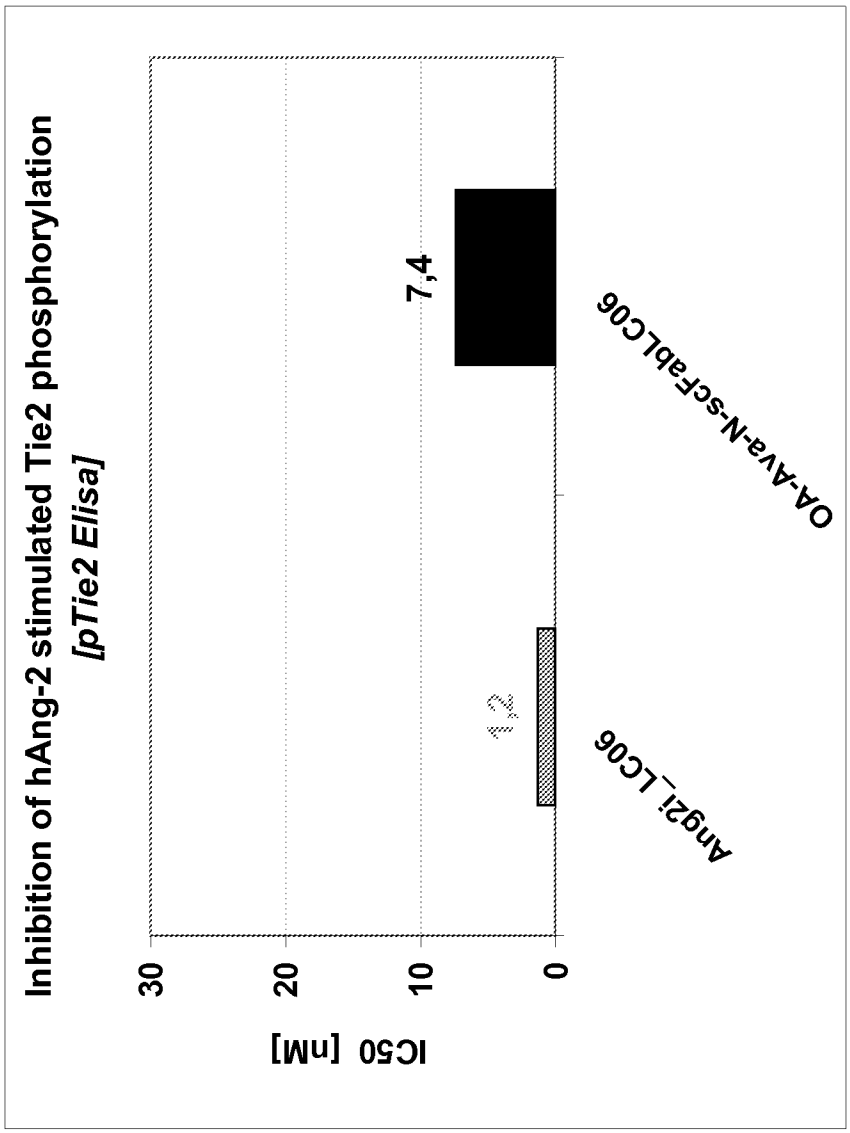

Inhibition of hAng-2 stimulated Tie2 phosphorylation
[pTie2 Elisa]
IC50 [nM]
30
20
10
0
7,4
1,2
OA-AVa-N-scFabLC06
Ang2i_LC06

Fig. 7a

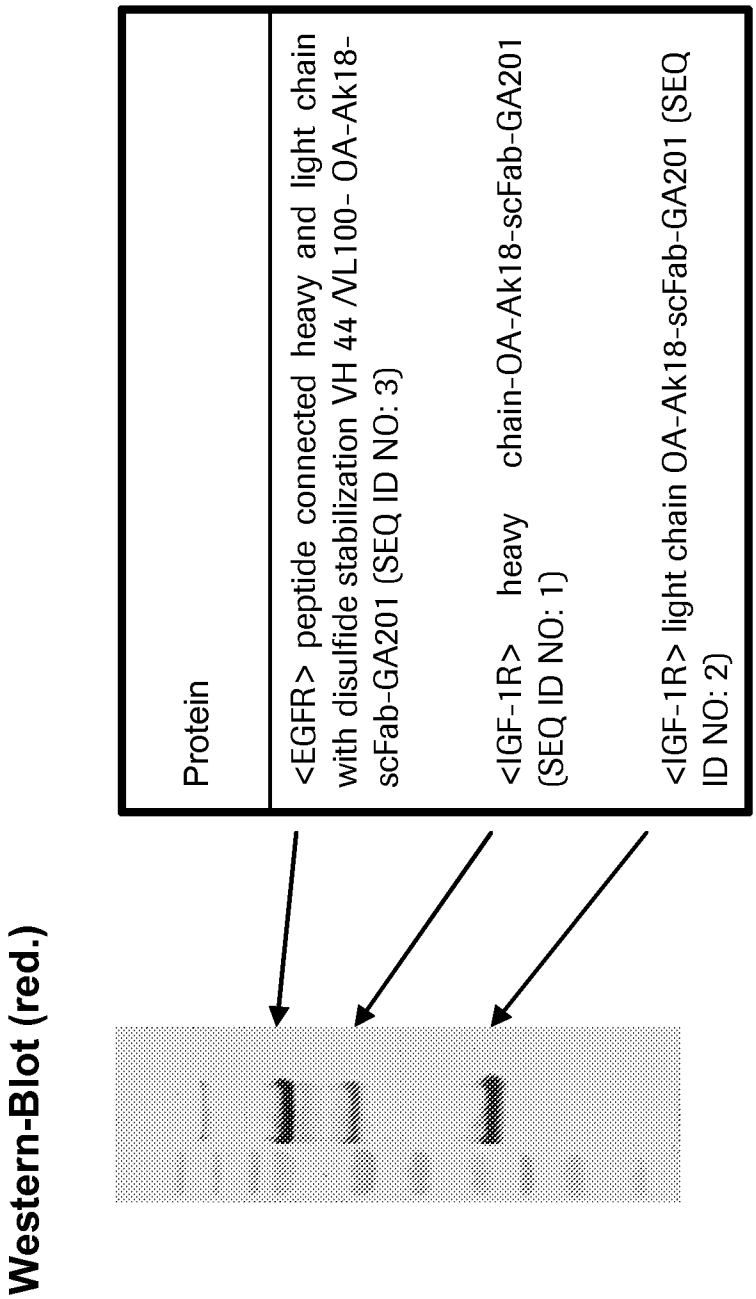

Western-Blot (red.)
Protein
<EGFR> peptide connected heavy and light chain with disulfide stabilization VH 44 /VL100- OA-Ak18-scFab-GA201 (SEQ ID NO: 3)
<IGF-1R> heavy chain-OA-Ak18-scFab-GA201 (SEQ ID NO: 1)
<IGF-1R> light chain OA-Ak18-scFab-GA201 (SEQ ID NO: 2)

Fig. 7b

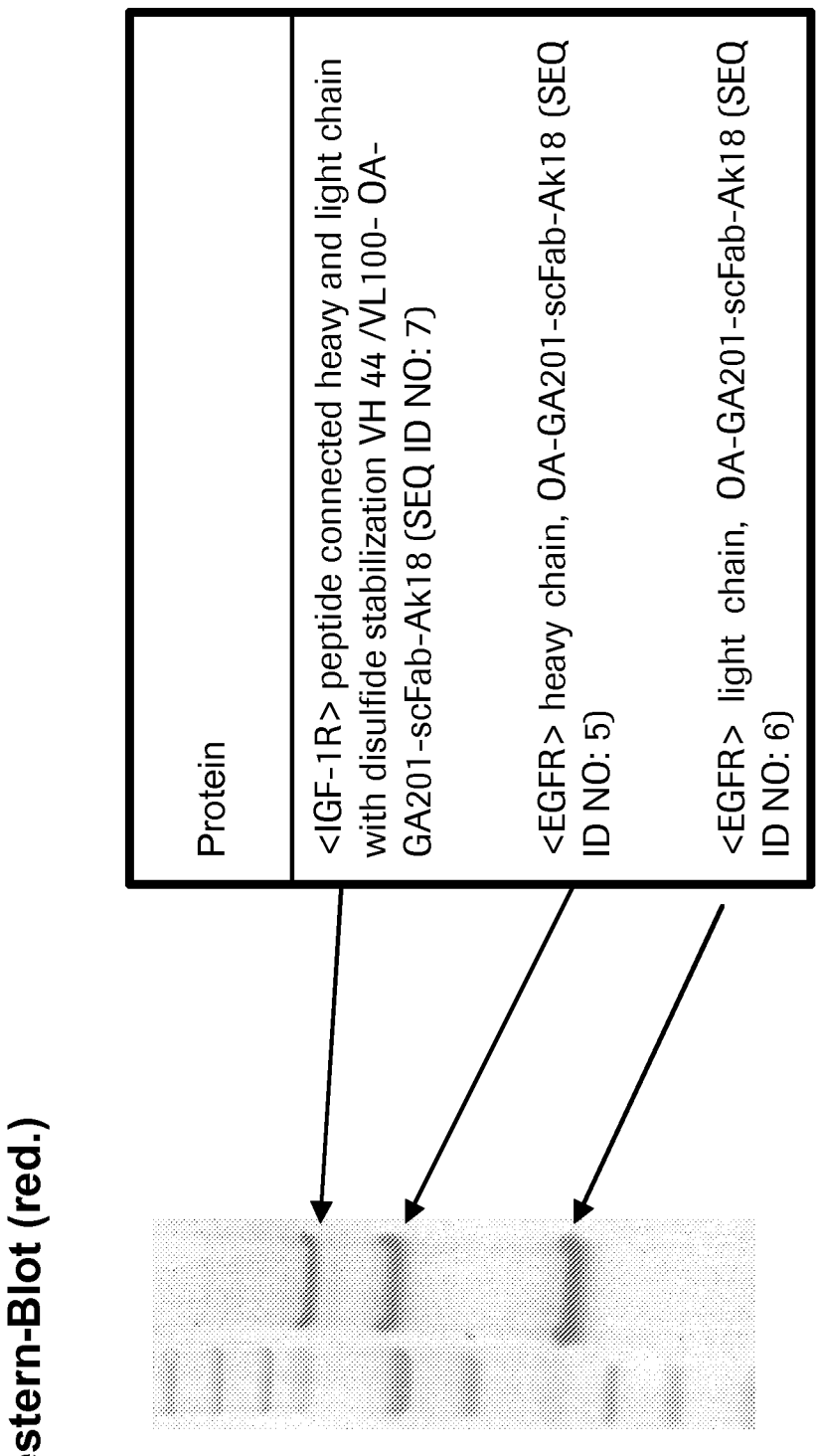

Western-Blot (red.)
Protein
<IGF-1R> peptide connected heavy and light chain with disulfide stabilization VH 44 /VL100- OA-GA201-scFab-Ak18 (SEQ ID NO: 7)
<EGFR> heavy chain, OA-GA201-scFab-Ak18 (SEQ ID NO: 5)
<EGFR> light chain, OA-GA201-scFab-Ak18 (SEQ ID NO: 6)

Fig. 8

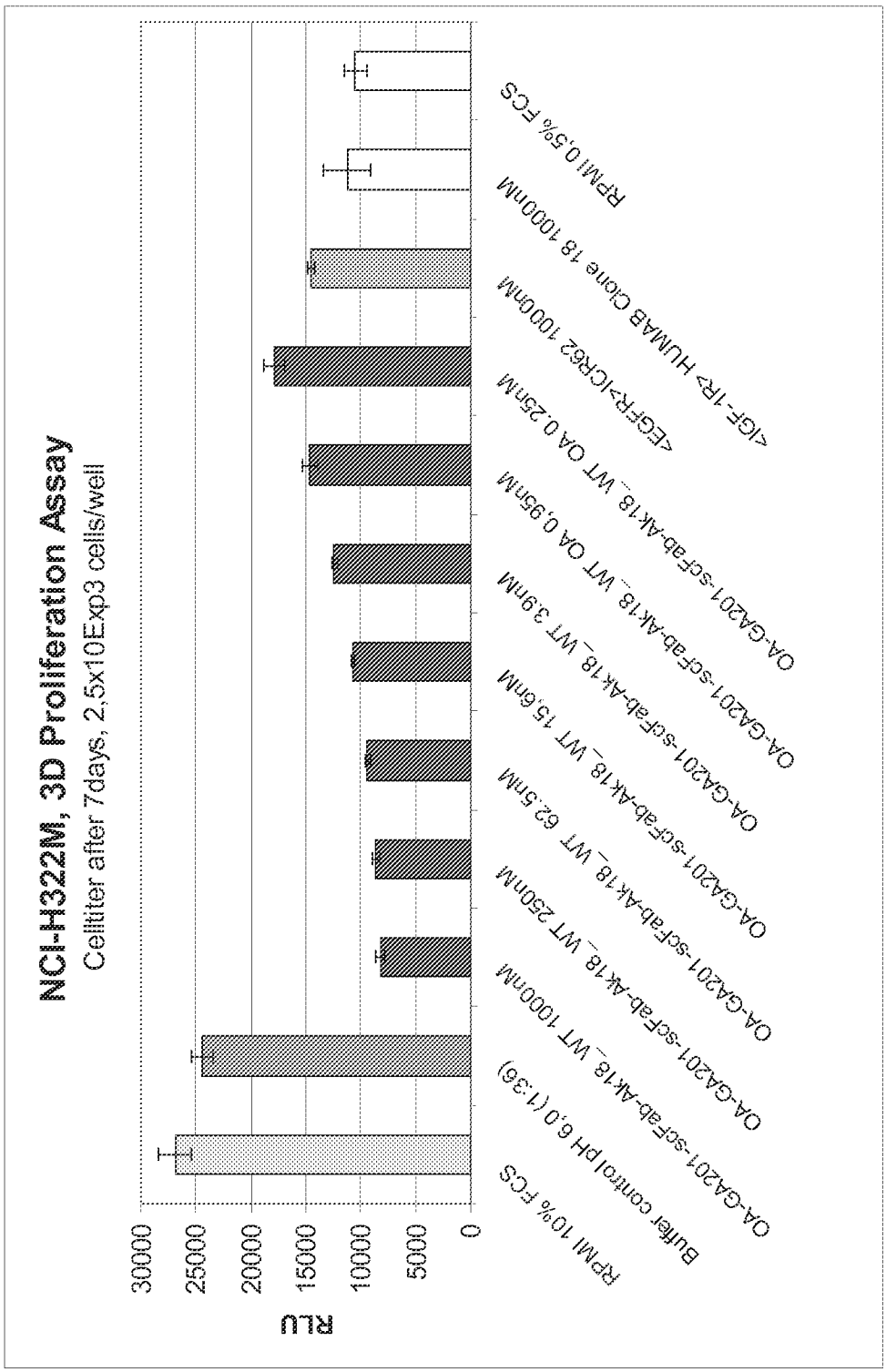

NCI-H322M, 3D Proliferation Assay
Celltiter after 7days, 2,5x10Exp3 cells/well
RLU
30000
25000
20000
15000
10000
5000
0
RPMI 10% FCS
Buffer control pH 6,0 (1:36)
OA-GA201-scFab-Ak18_WT 1000nM
OA-GA201-scFab-Ak18_WT 250nM
OA-GA201-scFab-Ak18_WT 62,5nM
OA-GA201-scFab-Ak18_WT 15,6nM
OA-GA201-scFab-Ak18_WT 3,9nM
OA-GA201-scFab-Ak18_WT OA 0,95nM
OA-GA201-scFab-Ak18_WT OA 0,25nM
<EGFR>ICR62 100nM
<IGF-1R> HUMAB Clone 18 100nM
RPMI 0,5% FCS

Fig. 9

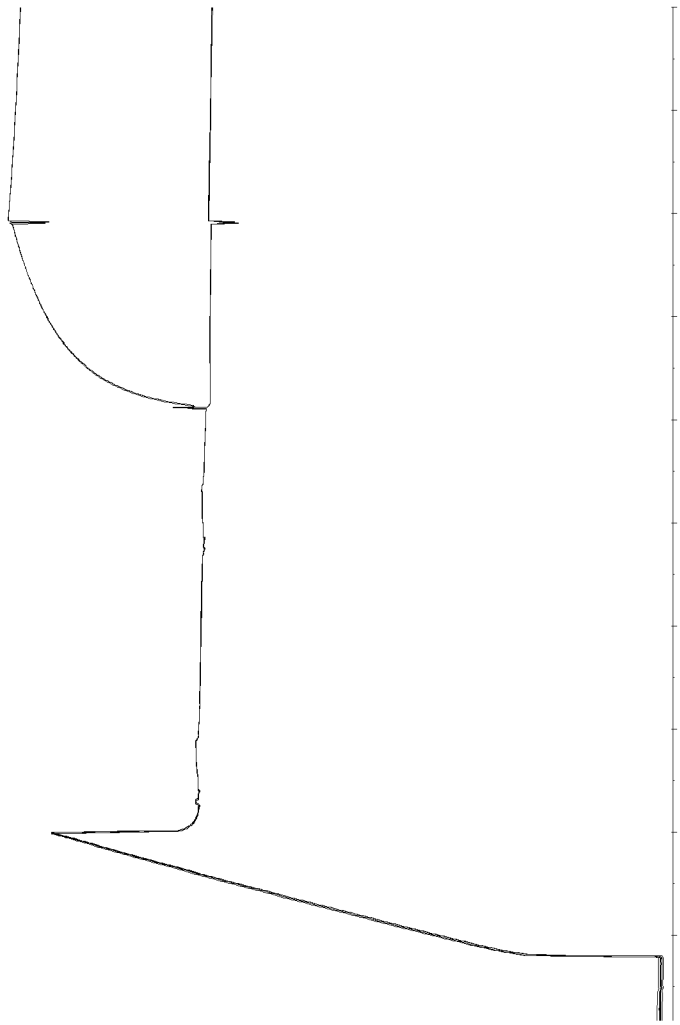
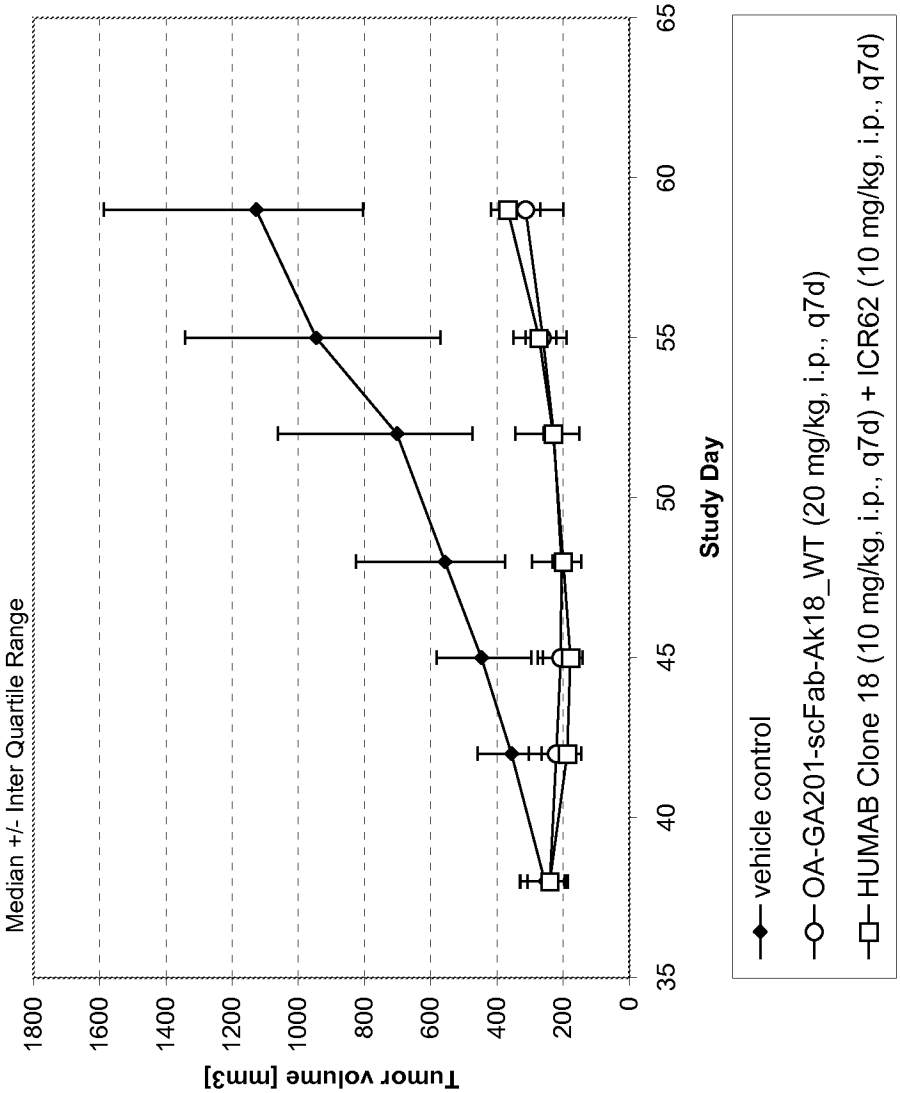


Fig. 10


Median +/- Inter Quartile Range
Tumor volume [mm3]
1800
1600
1400
1200
1000
800
600
400
200
0
35
40
45
50
55
60
65
Study Day
vehicle control
OA-GA201-scFab-Ak18_WT (20 mg/kg, i.p., q7d)
HUMAB Clone 18 (10 mg/kg, i.p., q7d) + ICR62 (10 mg/kg, i.p., q7d)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/054505

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 A61P35/00 C07K16/22 C07K16/28 C07K16/46
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 June 2011	28/06/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cilensek, Zoran

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Form PCT/ISA/210 (second sheet) (April 2005)

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International application No
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