Trivalent, bispecific antibodies

The present invention relates to trivalent, 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., and 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., and 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.

Summary of the Invention

A first aspect of the current invention is a trivalent, bispecific antibody comprising

a) a full length antibody specifically binding to a first antigen and consisting of two antibody heavy chains and two antibody light chains;

b) a polypeptide consisting of
   ba) an antibody heavy chain variable domain (VH); or
   bb) an antibody heavy chain variable domain (VH) and an antibody constant domain 1 (CH1),
   wherein said polypeptide is fused with the N-terminus of the VH domain

via a peptide connector to the C-terminus of one of the two heavy chains of said full length antibody

c) a polypeptide consisting of
ca) an antibody light chain variable domain (VL), or
cb) an antibody light chain variable domain (VL) and an antibody light chain constant domain (CL);
wherein said polypeptide is fused with the N-terminus of the VL domain via a peptide connector to the C-terminus of the other of the two heavy chains of said full length antibody;

and wherein the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) together form an antigen-binding site specifically binding to a second antigen

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

Still further aspects of the invention are a pharmaceutical composition comprising said trivalent, bispecific antibody.

The trivalent, bispecific antibodies according to the invention one the one hand show new properties due to their binding to different antigens, and on the other hand are suitable for production and pharmaceutical formulation due to their stability, low aggregation and pharmacokinetic and biological properties. Due to their Ig core they still retain the properties of natural antibodies like ADCC and CDC.

Detailed Description of the Invention

One aspect of the invention is trivalent, bispecific antibody comprising

a) a full length antibody specifically binding to a first antigen and consisting of two antibody heavy chains and two antibody light chains;

b) a polypeptide consisting of
ba) an antibody heavy chain variable domain (VH); or
bb) an antibody heavy chain variable domain (VH) and an antibody constant domain 1 (CH1),

wherein said polypeptide is fused with the N-terminus of the VH domain via a peptide connector to the C-terminus of one of the two heavy chains of said full length antibody

c) a polypeptide consisting of

ca) an antibody light chain variable domain (VL), or

cb) an antibody light chain variable domain (VL) and an antibody light chain constant domain (CL);

wherein said polypeptide is fused with the N-terminus of the VL domain via a peptide connector to the C-terminus of the other of the two heavy chains of said full length antibody;

and wherein the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) together form an antigen-binding site specifically binding to a second antigen

Optionally the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) are linked and stabilized via a interchain disulfide bridge 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).

Techniques to introduce unnatural disulfide bridges for stabilization are described e.g. in WO 94/029350, Rajagopal, V., et al., Prot. Engin. (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 polypeptides under b) and c) 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 of the polypeptides under b) and c) 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

trivalent, bispecific antibody without said optional disulfide stabilization between the variable domains VH and VL of the single chain Fab fragments is preferred.

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 length 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 antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) denotes the last amino acid at the N- terminus of VH or VL domain.

The CH3 domains of said full length 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 further 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.

Thus in one aspect of the invention said trivalent, bispecific antibody is further is characterized in that

the CH3 domain of one heavy chain of the full length antibody and the CH3 domain of the other heavy chain of the full length antibody 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 bivalent, 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 bivalent, 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 trivalent, 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.

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 a preferred embodiment, said trivalent, bispecific 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". Thus in a another preferred embodiment, said trivalent, bispecific antibody 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 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 (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 1 870 459A1, can be used alternatively or additionally. A preferred example for said trivalent, 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 preferred embodiment said trivalent, 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 preferred embodiment 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 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".

The bispecific antibody to the invention comprises three antigen-binding sites (A) the full length antibody according comprises two identical antigen-binding sites specifically binding to a first antigen, and B) the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) form together one antigen binding site specifically binding to a second antigen). 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 specifically binds. The antigen binding sites either in the full length antibody or by the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) 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 specifically bind to the desired antigen can be derived a) from known antibodies to the antigen 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. "Bispecific antibodies" according to the invention are antibodies which have two different antigen-binding specificities. Where an antibody has more than one specificity, the recognized epitopes may be associated with a single antigen or with more than one antigen. 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. A natural antibody for example or a full length antibody

according to the invention has two binding sites and is bivalent. As such, the terms "trivalent", denote the presence of three binding sites in an antibody molecule. The bispecific antibodies according to the invention are "trivalent". The term "trivalent, bispecific" antibody as used herein denotes an antibody that has three antigen-binding sites of which two bind to the same antigen (or the same epitope of the antigen) and the third binds to a different antigen or a different epitope of the same antigen. Antibodies of the present invention have three binding sites and are bispecific.

Another embodiment of the current invention is a trivalent, bispecific antibody comprising:

a) a full length antibody specifically binding to a first antigen and consisting of:
aa) two antibody heavy chains 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); and
ab) two antibody light chains consisting in N-terminal to C-terminal direction of an antibody light chain variable domain (VL), and an antibody light chain constant domain (CL) ; and

b) a polypeptide consisting of
ba) an antibody heavy chain variable domain (VH); or
bb) an antibody heavy chain variable domain (VH) and an antibody constant domain 1 (CH1),
wherein said polypeptide is fused with the N-terminus of the VH domain via a peptide connector to the C-terminus of one of the two heavy chains of said full length antibody wherein said peptide connector is a peptide of at least 5 amino acids, preferably between 25 and 50 amino acids;

c) a polypeptide consisting of
ca) an antibody light chain variable domain (VL), or
cb) an antibody light chain variable domain (VL) and an antibody light chain constant domain (CL);
wherein said polypeptide is fused with the N-terminus of the VL domain via a peptide connector to the C-terminus of the other of the two heavy chains of said full length antibody;

wherein said peptide connector is identical to the peptide connector under b);

and wherein the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) together form an antigen-binding site specifically binding to a second antigen.

Within this embodiment, preferably the trivalent, bispecific antibody comprises a T366W mutation in one of the two CH3 domains of and T366S, L368A, Y407V mutations in the other of the two CH3 domains and more preferably the trivalent, bispecific antibody comprises Y349C, T366W mutations in one of the two CH3 domains of and D356C, T366S, L368A, Y407V mutations in the other of the two CH3 domains (the additional Y349C mutation in one CH3 domain and the additional D356C mutation in the other CH3 domain forming a interchain disulfide bridge).

In one embodiment of the invention the trivalent, bispecific antibody according to the invention is characterized in that

a) said full length antibody is specifically binding to ErbB-3 comprises as heavy chain variable domain the sequence of SEQ ID NO: 1 , and as light chain variable domain the sequence of SEQ ID NO: 2

b) said polypeptide under b) comprises as the heavy chain variable domain the sequence of SEQ ID NO: 3; and

c) said polypeptide under c) comprises as the light chain variable domain the sequence of SEQ ID NO: 4.

In another aspect of the current invention the trivalent, bispecific antibody according to the invention comprises

a) a full length antibody binding to a first antigen consisting of two antibody heavy chains VH-CH1-HR-CH2-CH3 and two antibody light chains VL-CL ;
(wherein preferably one of the two CH3 domains comprises Y349C, T366W mutations and the other of the two CH3 domains comprises S354C, T366S, L368A, Y407V mutations);

b) a polypeptide consisting of

ba) an antibody heavy chain variable domain (VH); or

bb) an antibody heavy chain variable domain (VH) and an antibody constant domain 1 (CH1),

wherein said polypeptide is fused with the N-terminus of the VH domain via a peptide connector to the C-terminus of one of the two heavy chains of said full length antibody

c) a polypeptide consisting of

ca) an antibody light chain variable domain (VL), or

cb) an antibody light chain variable domain (VL) and an antibody light chain constant domain (CL);

wherein said polypeptide is fused with the N-terminus of the VL domain via a peptide connector to the C-terminus of the other of the two heavy chains of said full length antibody;

and wherein the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) together form an antigen-binding site specifically binding to a second antigen.

Another embodiment of the current invention is a trivalent, bispecific antibody comprising

a) a full length antibody specifically binding to human ErbB-3 and consisting of:

aa) two antibody heavy chains 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); and

ab) two antibody light chains consisting in N-terminal to C-terminal direction of an antibody light chain variable domain (VL), and an antibody light chain constant domain (CL) (VL-CL).; and

b) one single chain Fv fragment specifically binding to human c-Met),

wherein said single chain Fv fragment under b) is fused to said full length antibody under a) via a peptide connector at the C- or N- terminus of the

heavy or light chain (preferably at the C-terminus of the heavy chain) of said full length antibody;

wherein said peptide connector is a peptide of at least 5 amino acids, preferably between 25 and 50 amino acids.

Preferably such trivalent, bispecific antibody further comprises Y349C, T366W mutations in one of the two CH3 domains of the full length antibody and S354C (or E356C), T366S, L368A, Y407V mutations in the other of the two CH3 domains of the full length antibody.

Another embodiment of the current invention is a trivalent, bispecific antibody comprising

a) a full length antibody specifically binding to human ErbB-3 and consisting of:
aa) two antibody heavy chains 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); and
ab) two antibody light chains consisting in N-terminal to C-terminal direction of an antibody light chain variable domain (VL), and an antibody light chain constant domain (CL) ; and

b) a polypeptide consisting of
ba) an antibody heavy chain variable domain (VH); or
bb) an antibody heavy chain variable domain (VH) and an antibody constant domain 1 (CH1),
wherein said polypeptide is fused with the N-terminus of the VH domain via a peptide connector to the C-terminus of one of the two heavy chains of said full length antibody wherein said peptide connector is a peptide of at least 5 amino acids, preferably between 25 and 50 amino acids;

c) a polypeptide consisting of
ca) an antibody light chain variable domain (VL), or
cb) an antibody light chain variable domain (VL) and an antibody light chain constant domain (CL);
wherein said polypeptide is fused with the N-terminus of the VL domain

via a peptide connector to the C-terminus of the other of the two heavy chains of said full length antibody;
wherein said peptide connector is identical to the peptide connector under b);

and wherein the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) together form an antigen-binding site specifically binding to human c-Met

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; Brüggemann, 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, S.P.C., et al., and Boerner, P., et al., are also available for the preparation of human monoclonal antibodies (Cole, S.P.C., et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77-96 (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, E.A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991).

As used herein, the term "binding" or "specifically binding" 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). 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, an trivalent, bispecific antibody according to the invention is 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 "peptide connector" as used within the invention denotes a peptide with amino acid sequences, which is preferably of synthetic origin. These peptide connectors according to invention are used to fuse the polypeptides under b) and c) to the heavy chain C-termini of the full length antibody to form the trivalent, bispecific antibody according to the invention. Preferably said peptide connectors are peptides with an amino acid sequence with a length of at least 5 amino acids, preferably with a length of 10 to 100 amino acids, more preferably with a length of 25 to 50 amino acids. Preferably said peptide connector under b) and c) are

identical peptides with a length of at least 25 amino acids, preferably with a length between 25 and 50 amino acids and more preferably said peptide connector is (GxS)n or (GxS)nGm with G = glycine, S = serine, and (x = 3, n= 6, 7 or 8, and m= 0, 1, 2 or 3) or (x =4, n= 3,4, 5, 6, or 7 and m= 0, 1, 2 or 3), preferably x = 4 and n= 5, 6, or 7.

In a further embodiment the trivalent, bispecific antibody according to the invention is characterized in that said full length antibody is of human IgG1 subclass, or of human IgG1 subclass with the mutations L234A and L235A.

In a further embodiment the trivalent, bispecific antibody according to the invention is characterized in that said full length antibody is of human IgG2 subclass.

In a further embodiment the trivalent, bispecific antibody according to the invention is characterized in that said full length antibody is of human IgG3 subclass.

In a further embodiment the trivalent, bispecific antibody according to the invention is characterized in that said full length antibody is of human IgG4 subclass or, of human IgG4 subclass with the additional mutation S228P.

Preferably the trivalent, bispecific antibody according to the invention is characterized in that said full length antibody is of human IgG1 subclass, of human IgG4 subclass with the additional mutation S228P.

It has now been found that the trivalent, bispecific antibodies according to the invention have improved characteristics such as biological or pharmacological activity, pharmacokinetic properties or toxicity. They can be used e.g. for the treatment of diseases such as cancer.

In a further embodiment the trivalent, bispecific antibody according to the invention is characterized in specifically binding to ErbB3 and c-Met.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).

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 length 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 in IgG1 L234A and L235A.

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).

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 antigen 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.

Cell-mediated effector functions 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 99/54342 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 are reported 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.

Surprisingly the bispecific <ErbB3-c-Met> antibodies which are one embodiment of the invention show reduced downregulation and internalization of target antigen compared to their parent <ErbB3> and/ or <c-Met> antibodies. Therefore in one preferred embodiment of the invention, the bispecific antibody is glycosylated (if it comprises an Fc part of IgG1, IgG2, IgG3 or IgG4 subclass, preferably of IgG1 or IgG3 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 ±3 amino acids) upstream or downstream of position 297, i.e. between position 294 and 300. In one embodiment the glycosylated antibody according to the invention the IgG subclass is of human IgG1 subclass, of human IgG1 subclass with the mutations L234A and L235A or of IgG3 subclass. In a further embodiment the amount of N-glycolylneuraminic acid (NGNA) is 1 % or less and/or the amount of N-terminal alpha-1,3-galactose is 1 % or less within said sugar chain. The sugar chain show preferably the characteristics of N-linked glycans attached to Asn297 of an antibody recombinantly expressed in a CHO cell.

The term "the sugar chains show characteristics of N-linked glycans attached to Asn297 of an antibody recombinantly expressed in a CHO cell" denotes that the sugar chain at Asn297 of the full length parent antibody according to the invention has the same structure and sugar residue sequence except for the fucose residue as those of the same antibody expressed in unmodified CHO cells, e.g. as those reported in WO 2006/103100.

The term "NGNA" as used within this application denotes the sugar residue N-glycolylneuraminic acid.

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.

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) 151-160; Werner, R.G., Drug Res. 48 (1998) 870-880.

The trivalent, 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 trivalent, 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, e.g. as described above. 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.

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).

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 trivalent, 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.

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.

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) 456-467. 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, S.N, et al., PNAS 69 (1972) 2110-2114.

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.

Description of the Amino acid Sequences

SEQ ID NO:1 heavy chain variable domain <ErbB3 > HER3 clone 29
SEQ ID NO:2 light chain variable domain < ErbB3> HER3 clone 29
SEQ ID NO:3 heavy chain variable domain <c-Met> Mab 5D5
SEQ ID NO:4 light chain variable domain <c-Met> Mab 5D5
SEQ ID NO:5 heavy chain <ErbB3> HER3 clone 29
SEQ ID NO:6 light chain <ErbB3> HER3 clone 29
SEQ ID NO:7 heavy chain <c-Met> Mab 5D5
SEQ ID NO:8 light chain <c-Met> Mab 5D5
SEQ ID NO:9 heavy chain <c-Met> Fab 5D5
SEQ ID NO:10 light chain <c-Met> Fab 5D5
SEQ ID NO:11 heavy chain 1 <ErbB3-c-Met> Her3/Met_KHSS
SEQ ID NO:12 heavy chain 2 <ErbB3-c-Met> Her3/Met_KHSS
SEQ ID NO:13 light chain <ErbB3-c-Met> Her3/Met_KHSS
SEQ ID NO:14 heavy chain 1 <ErbB3-c-Met> Her3/Met_SSKH
SEQ ID NO:15 heavy chain 2 <ErbB3-c-Met> Her3/Met_SSKH
SEQ ID NO:16 light chain <ErbB3-c-Met> Her3/Met_SSKH
SEQ ID NO:17 heavy chain 1 <ErbB3-c-Met> Her3/Met_SSKHSS
SEQ ID NO:18 heavy chain 2 <ErbB3-c-Met> Her3/Met_SSKHSS
SEQ ID NO:19 light chain <ErbB3-c-Met> Her3/Met_SSKHSS
SEQ ID NO:20 heavy chain 1 <ErbB3-c-Met> Her3/Met_1C
SEQ ID NO:21 heavy chain 2 <ErbB3-c-Met> Her3/Met_1C
SEQ ID NO:22 light chain <ErbB3-c-Met> Her3/Met_1C
SEQ ID NO:23 heavy chain 1 <ErbB3-c-Met> Her3/Met_6C
SEQ ID NO:24 heavy chain 2 <ErbB3-c-Met> Her3/Met_6C
SEQ ID NO:25 light chain <ErbB3-c-Met> Her3/Met_6C
SEQ ID NO:26 heavy chain constant region of human IgG1
SEQ ID NO:27 heavy chain constant region of human IgG1
SEQ ID NO:28 human light chain kappa constant region

SEQ ID NO:29 human light chain lambda constant region

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.

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 representation of a trivalent, bispecific antibody according to the invention, comprising a full length antibody specifically binding to a first antigen 1 to which a) **Fig 2a** two polypeptides VH and VL are fused (the VH and VL domains of both together forming a antigen binding site specifically binding to a second antigen 2; b) **Fig 2b** two polypeptides VH-CH1 and VL-CL are fused (the VH and VL domains of both together forming a antigen binding site specifically binding to a second antigen 2)

Figure 3 Schematic representation of a trivalent, bispecific antibody according to the invention, comprising a full length antibody specifically binding to a first antigen 1 to which two polypeptides VH and VL are fused (the VH and VL domains of both together forming a antigen binding site specifically binding to a second antigen 2) with "knobs and holes".

Figure 4 Schematic representation of a trivalent, bispecific antibody according to the invention, comprising a full length antibody specifically binding to a first antigen 1 to which two polypeptides VH and VL are fused (the VH and VL domains of both together forming a antigen binding site specifically binding to a second antigen 2, wherein these VH and VL domains comprise an interchain disulfide bridge between positions VH44 and VL100) with "knobs and holes".

Figure 5 Binding of bispecific antibodies to the cell surface of cancer cells

Figure 6 Inhibition of HGF-induced c-Met receptor phosphorylation by bispecific Her3/c-Met antibody formats

Figure 7 Inhibition of HRG-induced Her3 receptor phosphorylation by bispecific Her3/c-Met antibody formats.

Figure 8 Inhibition of HGF-induced HUVEC proliferation by bispecific Her3/c-Met antibody formats

Figure 9 Inhibition of proliferation in the cancer cell line A431 by bispecific Her3/c-Met antibody formats.

Figure 10 Analysis of inhibition of HGF-induced cell-cell dissemination (scattering) in the cancer cell line A431 by bispecific Her3/c-Met antibody formats.

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 which are flanked by singular restriction endonuclease cleavage sites 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. Gene Segments coding "knobs-into-hole" Her3 (clone 29) antibody heavy chain carrying a T366W mutation in the CH3 domain with a C-terminal 5D5 VH region linked by a $(G_4S)_n$ peptide connector as well as "knobs-into-hole" Her3 (clone 29) antibody heavy chain carrying T366S, L368A and Y407V mutations with a C-terminal 5D5 VL region linked by a $(G_4S)_n$ peptide connector were synthesized with 5'-BamHI and 3'-XbaI restriction sites. In a similar manner, DNA sequences coding "knobs-into-hole" Her3 (clone 29) antibody heavy chain carrying S354C and T366W mutations in the CH3 domain with a C-terminal 5D5 VH region linked by a $(G_4S)_n$ peptide connector as well as "knobs-into-hole" Her3 (clone 29) antibody heavy chain carrying Y349C, T366S, L368A and Y407V mutations with a C-terminal 5D5 VL region linked by a $(G_4S)_n$ peptide connector were prepared by gene synthesis with flanking BamHI and XbaI restriction sites. Finally, DNA sequenes encoding unmodified heavy and light chains of the Her3 (clone 29) and 5D5 antibody were synthesized with flanking BamHI and XbaI restriction sites. 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 heavy VH /or VL fusion protein and light chain protein 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.

Aufträge CSP=erl.; PCT wird am 30.03.10 per Fahrer beim EPA eingereicht --> Termin gelöscht, mwThe immunoglobulin fusion genes comprising the heavy or light chain constucts as well as "knobs-into-hole" constructs with C-terminal VH and VL domains 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 restriction enzymes (Roche Molecular Biochemicals) and subjected to agarose gel electrophoresis. Purified heavy and light chain coding DNA segments were then ligated to the isolated Roche expression vector BamHI/XbaI 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 immunoglobulin variants in HEK293 cells

Recombinant immunoglobulin variants 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. DNA-293fectin™ complexes were prepared in Opti-MEM® I medium (Invitrogen, USA) using 325 µl of 293fectin™ (Invitrogen, Germany) and 250 µg of heavy and light chain plasmid DNA in a 1:1 molar ratio for a 250 ml final transfection volume. "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 1:1:2 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 trivalent bispecific and control antibodies

Trivalent bispecific and control 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 and control antibodies with less than 5 % high molecular weight aggregates were pooled and stored as 1.0 mg/ml aliquots at -80°C. Fab fragments were generated by a Papain digest of the purified 5D5 monoclonal antibody and subsequent removal of contaminating Fc domains by Protein A chromatography. Unbound Fab fragments were further purified on a Superdex200 HiLoad 120 ml 16/60 gel filtration column (GE Healthcare, Sweden) equilibrated with 20mM Histidin, 140 mM NaCl, pH 6.0, 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).

c-Met phosphorylation assay

5x10e5 A549 cells were seeded per well of a 6-well plate the day prior HGF stimulation in RPMI with 0.5 % FCS (fetal calf serum). The next day, growth medium was replaced for one hour with RPMI containing 0.2 % BSA (bovine serum albumine). 5 µg/mL of the bispecific antibody was then added to the medium and cells were incubated for 10 minutes upon which HGF was added for further 10 minutes in a final concentration of 50 ng/mL. Cells were washed once with ice cold PBS containing 1 mM sodium vanadate upon which they were placed on ice and lysed in the cell culture plate with 100 µL lysis buffer (50 mM Tris-Cl pH7.5, 150 mM NaCl, 1 % NP40, 0.5 % DOC, aprotinine, 0.5 mM PMSF, 1 mM sodium-vanadate). Cell lysates were transferred to eppendorf tubes and lysis was allowed to proceed for 30 minutes on ice. Protein concentration was determined using the BCA method (Pierce). 30-50 µg of the lysate was separated on a 4-12 % Bis-Tris NuPage gel (Invitrogen) and proteins on the gel were transferred to a nitrocellulose membrane. Membranes were blocked for one hour with TBS-T containing 5 % BSA and developed with a phospho-specific c-Met antibody directed against Y1230,1234,1235 (44-888, Biosource) according to the manufacturer's instructions. Immunoblots were reprobed with an antibody binding to unphosphorylated c-Met (AF276, R&D).

Her3 (ErbB3) phosphorylation assay

2x10e5 MCF7 cells were seeded per well of a 12-well plate in complete growth medium (RPMI 1640, 10 % FCS). Cells were allowed to grow to 90 % confluency within two days. Medium was then replaced with starvation medium containing 0.5 % FCS. The next day the respective antibodies were supplemented at the indicated concentrations 1 hour prior addition of 500 ng/mL Heregulin (R&D). Upon addition of Heregulin cells were cultivated further 10 minutes before the cells were harvested and lysed. Protein concentration was determined using the BCA method (Pierce). 30-50 µg of the lysate was separated on a 4-12 % Bis-Tris

NuPage gel (Invitrogen) and proteins on the gel were transferred to a nitrocellulose membrane. Membranes were blocked for one hour with TBS-T containing 5 % BSA and developed with a phospho-specific Her3/ErbB3 antibody specifically recognizing Tyr1289 (4791, Cell Signaling).

Scatter assay

A549 (4000 cells per well) or A431 (8000 cells per well) were seeded the day prior compound treatment in a total volume of 200 µL in 96-well E-Plates (Roche, 05232368001) in RPMI with 0.5 % FCS. Adhesion and cell growth was monitored over night with the Real Time Cell Analyzer machine with sweeps every 15 min monitoring the impedance. The next day, cells were pre-incubated with 5 µL of the respective antibody dilutions in PBS with sweeps every five minutes. After 30 minutes 2,5 µL of a HGF solution yielding a final concentration of 20 ng/mL were added and the experiment was allowed to proceed for further 72 hours. Immediate changes were monitored with sweeps every minute for 180 minutes followed by sweeps every 15 minutes for the remainder of the time.

FACS

a) Binding Assay

A431 were detached and counted. 1.5x10e5 cells were seeded per well of a conical 96-well plate. Cells were spun down (1500 rpm, 4°C, 5 min) and incubated for 30 min on ice in 50 µL of a dilution series of the respective bispecific antibody in PBS with 2 % FCS (fetal calf serum). Cells were again spun down and washed once with 200 µL PBS containing 2 % FCS followed by a second incubation of 30 min with a phycoerythrin-coupled antibody directed against human Fc which was diluted in PBS containing 2 % FCS (Jackson Immunoresearch, 109116098). Cells were spun down washed twice with 200 µL PBS containing 2 % FCS, resuspended in BD CellFix solution (BD Biosciences) and incubated for at least 10 min on ice. Mean fluorescence intensity (mfi) of the cells was determined by flow cytometry (FACS Canto, BD). Mfi was determined at least in duplicates of two independent stainings. Flow cytometry spectra were further processed using the FlowJo software (TreeStar). Half-maximal binding was determined using XLFit 4.0 (IDBS) and the dose response one site model 205.

b) Internalization Assay

Cells were detached and counted. 5x10e5 cells were placed in 50 µL complete medium in an eppendorf tube and incubated with 5 µg/mL of the respective

bispecific antibody at 37°C. After the indicated time points cells were stored on ice until the time course was completed. Afterwards, cells were transferred to FACS tubes, spun down (1500 rpm, 4°C, 5min), washed with PBS + 2 % FCS and incubated for 30 minutes in 50 µL phycoerythrin-coupled secondary antibody directed against human Fc which was diluted in PBS containing 2 % FCS (Jackson Immunoresearch, 109116098). Cells were again spun down, washed with PBS + 2 % FCS and fluorescence intensity was determined by flow cytometry (FACS Canto, BD).

c) Crosslinking Experiment

HT29 cells were detached counted and split in two populations which were individually stained with PKH26 and PKH67 (Sigma) according to the manufacturer's instructions. Of each of the stained populations 5x10e5 cells were taken, combined and incubated for 30 and 60 minutes with 10 µg/mL of the respective bispecific antibody in complete medium. After the indicated time points cells were stored on ice until the time course was completed. Cells were spun down (1500 rpm, 4°C, 5min), washed with PBS + 2 % FCS and fluorescence intensity was determined by flow cytometry (FACS Canto, BD).

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).

Wst-1 Assay

A Wst-1 viability and cell proliferation assay was performed as endpoint analysis, detecting the number of metabolic active cells. Briefly, 20 µL of Wst-1 reagent (Roche, 11644807001) were added to 200 µL of culture medium. 96-well plates were further incubated for 30 min to 1 h until robust development of the dye. Staining intensity was quantified on a microplate reader (Tecan) at a wavelength of 450 nm.

Design of expressed and purified trivalent, bispecific <ErbB3-c-Met> antibodies

In Table 1: Trivalent, bispecific <ErbB3-c-Met> antibodies based on a full length ErbB-3 antibody (HER3 clone29) and the VH and VL domain from a C-met antibody (c-Met 5D5) with the respective features shown in Table1 one were expressed and purified according to the general methods described above. The corresponding VH and VL of HER3 clone29 and c-Met 5D5 are given in the sequence listing.

Table 1: Trivalent, bispecific antibody < ErbB3-c-Met> with the VHVL-Ab-nomenclature in Table 1 were expressed and purified (see also in the Examples below and Fig 3c)

Molecule Name VHVL-Ab-nomenclature for bispecific antibodies	**Her3/Met_KHSS**	**Her3/Met_SSKH**	**Her3/Met_SSKHSS**	**Her3/Met_1C**	**Her3/Met_6C**
Features:					
Knobs-in-hole mutations	S354C:T366W/ Y349'C:T366'S: L368'A:Y407'V	T366W/ T366'S:L368'A :Y407'V	S354C:T366W/ Y349'C:T366'S: L368'A:Y407'V	S354C:T366W/ Y349'C:T366'S: L368'A:Y407'V	S354C:T366W/ Y349'C:T366'S: L368'A:Y407'V
Full length antibody backbone derived from	Her3 clone 29 (chimeric)	Her3 clone 29 (chimeric)	Her3 clone 29 (chimeric)	Her3 clone 29 (chimeric)	Her3 clone 29 (chimeric)
VHVL fragment derived from	cMet 5D5 (humanized)	cMet 5D5 (humanized)	cMet 5D5 (humanized)	cMet 5D5 (humanized)	cMet 5D5 (humanized)
Position of VH attached to antibody	C-terminus knob heavy chain	C-terminus knob heavy chain	C-terminus knob heavy chain	C-terminus knob heavy chain	C-terminus knob heavy chain
Position of VL attached to antibody	C-terminus hole heavy chain	C-terminus hole heavy chain	C-terminus hole heavy chain	C-terminus hole heavy chain	C-terminus hole heavy chain
Peptide connector	$(G_4S)_3$	$(G_4S)_3$	$(G_4S)_3$	$(G_4S)_1$	$(G_4S)_6$
VHVL disulfide VH44/ VL100 stabilized	-	+	+	-	-

Example 1 (Figure 5):

Binding of bispecific antibodies to the cell surface of cancer cells.

The binding properties of the bispecific antibodies to their respective receptor on the cell surface was analyzed on A431 cancer cells in a flow cytometry based assay. Cells were incubated with the mono- or bispecific primary antibodies and binding of these antibodies to their cognate receptors was detected with a secondary antibody coupled to a fluorophore binding specifically to the Fc of the primary antibody. The mean fluorescence intensity of a dilution series of the primary antibodies was plotted against the concentration of the antibody to obtain a sigmoidal binding curve. Cell surface expression of c-Met and Her3 was validated by incubation with the bivalent 5D5 and Her3 clone 29 antibody only. The Her3/c-Met_KHSS antibody readily binds to the cell surface of A431. Under these experimental settings, the antibody can only bind via its Her3 part and consequently the mean fluorescence intensity does not exceed the staining for Her3 clone 29 alone.

Example 2 (Figure 6):

Inhibition of HGF-induced c-Met receptor phosphorylation by bispecific Her3/c-Met antibody formats

To confirm functionality of the c-Met part in the bispecific antibodies a c-Met phosphorylation assay was performed. In this experiment A549 lung cancer cells or HT29 colorectal cancer cells were treated with the bispecific antibodies or control antibodies prior exposure to HGF. Cells were then lysed and phosphorylation of the c-Met receptor was examined. Both cell lines can be stimulated with HGF as can be observed by the occurrence of a phospho-c-Met specific band in the immunoblot.

Example 3 (Figure 6):

Inhibition of HRG-induced Her3 receptor phosphorylation by bispecific Her3/c-Met antibody formats

To confirm functionality of the Her3 part in the bispecific antibodies a Her3 phosphorylation assay was performed. In this experiment MCF7 cells were treated with the bispecific antibodies or control antibodies prior exposure to HRG (Heregulin). Cells were then lysed and phosphorylation of the Her3 receptor was examined. Her3/c-Met_KHSS inhibit Her3 receptor phosphorylation to the same

extent as the parental Her3 clone29 indicating that Her3 binding and functionality of the antibody are not compromised by the trivalent antibody format.

Example 4 (Figure 8):

Inhibition of HGF-induced HUVEC proliferation by bispecific Her3/c-Met antibody formats

HUVEC proliferation assays were performed to demonstrate the mitogenic effect of HGF. Addition of HGF to HUVEC leads to a twofold increase in proliferation. Addition of human IgG control antibody in the same concentration range as the bispecific antibodies has no impact on cellular proliferation while the 5D5 Fab fragment inhibits HGF-induced proliferation. Titration of Her3/c-Met_KHSS demonstrate a weak inhibitory effect of the antibody (Fig8). The effect is more pronounced for the Her3/Met-6C antibody indicating that a longer connector improves efficacy of the antibody. This demonstrates the functionality of the c-Met component in the trivalent antibody format.

Example 5 (Figure 9):

Inhibition of proliferation in the cancer cell line A431 by bispecific Her3/c-Met antibody formats

If A431 were seeded in serum reduced medium, addition of HGF induces apart from scattering a weak mitogenic effect. This was exploited to analyze the impact of Her3/c-Met_KHSS on HGF treated A431 proliferation. Indeed, the bispecific antibodies can largely inhibit the HGF-induced increase of proliferation (15 %). A control human IgG1 antibody has no influence on HGF promoted A431 cell growth.

Example 6 (Figure 10):

Analysis of inhibition of HGF-induced cell-cell dissemination (scattering) in the cancer cell line A431 by bispecific Her3/c-Met antibody formats

HGF-induced scattering includes morphological changes of the cell, resulting in rounding of the cells, filopodia-like protrusions, spindle-like structures and a certain motility of the cells. The Real Time Cell Analyzer (Roche) measures the impedance of a given cell culture well and can therefore indirectly monitor changes in cellular morphology and proliferation. Addition of HGF to A431 and A549 cells resulted in changes of the impedance which was monitored as function of time. Her3/c-Met_KHSS and Her3/Met-6C inhibited HGF-induced scattering with

Her3/Met-6C being more efficacious (20,7 % and 43,7 % scatter inhibition) (Fig. 10).

Patent Claims

1. A trivalent, bispecific antibody comprising

a) a full length antibody specifically binding to a first antigen and consisting of two antibody heavy chains and two antibody light chains;

b) a polypeptide consisting of
ba) an antibody heavy chain variable domain (VH); or
bb) an antibody heavy chain variable domain (VH) and an antibody constant domain 1 (CH1),
wherein said polypeptide is fused with the N-terminus of the VH domain via a peptide connector to the C-terminus of one of the two heavy chains of said full length antibody

c) a polypeptide consisting of
ca) an antibody light chain variable domain (VL), or
cb) an antibody light chain variable domain (VL) and an antibody light chain constant domain (CL);
wherein said polypeptide is fused with the N-terminus of the VL domain via a peptide connector to the C-terminus of the other of the two heavy chains of said full length antibody;

and wherein the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) together form an antigen-binding site specifically binding to a second antigen

2. The trivalent, bispecific antibody according to claim 1, 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 bivalent, bispecific antibody, wherein the alteration is characterized in that:

i) 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 bivalent, 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

ii) 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 trivalent, 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.

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

i) 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

ii) 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 trivalent, bispecific antibody according to claims 2 or 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 trivalent, bispecific antibody according to any one of claims 2 to 4, characterized in that

the CH3 domain under i) comprises a T366W mutation; and

the CH3 domain under ii) comprises T366S, L368A, Y407V mutations.

6. The trivalent, bispecific antibody according to claim 4, characterized in that

the CH3 domain under i) comprises Y349C, T366W mutations; and

the CH3 domain under ii) comprises S354C, T366S, L368A, Y407V mutations.

7. The trivalent, bispecific antibody according to any one of claims 1 to 6, characterized in that

the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) are linked and stabilized via a interchain disulfide bridge 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.

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

the antibody heavy chain variable domain (VH) of the polypeptide under b) and the antibody light chain variable domain (VL) of the polypeptide under c) are linked and stabilized via a interchain disulfide bridge by introduction of a disulfide bond between the following positions:
i) heavy chain variable domain position 44 to light chain variable domain position 100.

9. The trivalent, bispecific antibody according to claim 6, characterized in that the peptide connectors under b) and c) are identical peptides with a length between 25 and 50 amino acids.

10. A pharmaceutical composition comprising a trivalent, bispecific antibody according to claims 1 to 9

11. A nucleic acid encoding a trivalent, bispecific antibody according to claim 1 to 9.

12. The trivalent, bispecific antibody according to claim 1 to 9 for the treatment of cancer.

13. The use of the trivalent, bispecific antibody according to claim 1 to 9 for the manufacture of a medicament for the treatment of cancer.