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(54) Title: RODENT BISPECIFIC HETERODIMERIC PROTEINS

(57) Abstract: Novel rodent bispecific heterodimeric proteins, such as rodent bispecific antibodies, in vitro method for producing such, and uses thereof.
RODENT BISPECIFIC HETERODIMERIC PROTEINS

FIELD OF THE INVENTION

The present invention relates to novel heterodimeric proteins such as bispecific antibodies of primarily rodent origin.

BACKGROUND OF THE INVENTION

Monoclonal antibodies have in recent years become successful therapeutic molecules, in particular for the treatment of cancer. Unfortunately, however, monoclonal antibodies are often unable to cure diseases when used as monotherapy. Bispecific antibodies can potentially overcome some of the limitations of monoclonal antibody therapy, e.g. they could be used as mediators to target a drug or toxic compound to target cells, as mediators to retarget effector mechanisms to disease-associated sites or as mediators to increase specificity for tumor cells, for example by binding to a combination of targets molecules that is exclusively found on tumor cells. Different formats and uses of bispecific antibodies have been reviewed by Chames and Baty ((2009) Curr Opin Drug Disc Dev 12: 276) and by Kontermann and Brinkman ((2015) Drug Discovery Today 20: 838).

An in vitro method for producing bispecific antibodies is described in WO 2008119353 (Genmab). Herein is described that a bispecific antibody is formed by "Fab-arm" or "half-molecule" exchange (swapping of a heavy chain and attached light chain) between two monospecific antibodies having IgG4-like CH3 regions upon incubation under reducing conditions. This Fab-arm exchange reaction is the result of a disulfide-bond isomerization reaction and dissociation-association of CH3 domains wherein heavy-chain disulfide bonds in the hinge regions of the parent (originally monospecific) antibodies are reduced and the resulting free cysteines form an inter-heavy-chain disulfide bond with cysteine residues of another parent antibody molecule (originally with a different specificity), and simultaneously CH3 domains of the parent antibodies dissociate and re-associate with the CH3 domains of the other parent antibody. The resulting product is a bispecific antibody having two Fab arms which potentially are composed of different sequences. WO2011131746 (Genmab) describes that by introducing asymmetrical, matching mutations in the CH3 regions of the two monospecific starting proteins, the Fab-arm exchange reaction can be forced to become directional and thereby yield highly stable heterodimeric proteins. This so-called controlled Fab-arm exchange (cFAE) involves the mixing of two separately expressed parental IgGs under controlled reducing conditions to allow the recombination of antibody half-molecules.
The recombination is driven by two matching point-mutations, such as F405L and K409R (EU-numbering index), one in either parental IgG, which weaken the non-covalent CH3-CH3 interaction in the parental Abs enough to allow dissociation of half-molecules, but at the same time, strongly favour heterodimerization, thus promoting bispecific IgG endproduct yield and post-exchange stability upon re-oxidation of the hinge disulfides.

Except for their dual specificity, it was found that bispecific antibodies generated from human IgGs using cFAE behaved as regular, monospecific human IgGs. Thus, Fc-effector functions of these bispecific human IgGs were preserved and the pharmacokinetics were similar as regular IgG (LaBriijn et al. 2013. Proc Natl Acad Sci USA 110(13):5145-50).

To predict the efficacy and safety of bispecific IgGs for further development in the clinic, extensive preclinical research is required. The use of animal models is essential for the understanding of the mechanisms of action of therapeutic bispecific human IgGs. Proof-of-concepts for therapeutic antibodies are often established in pre-clinical xenograft models using immunodeficient mice. For many therapeutic concepts, however, the use of surrogate antibodies in rodent disease models such as a mouse model is warranted. The use of mouse or other rodent antibodies enables optimal cross-talk with mouse or other rodent effector cells and proteins, it allows the bispecific antibodies to be assessed in rodent disease models and in addition limits potential immunogenicity which is likely to be a problem for human antibodies applied in rodents, thus allowing long-term treatment of the rodent.

In the early 1980s it has been shown that fusion of two mouse hybridomas (quadroma) allows for the production of murine hybrid immunoglobulin molecules containing two different binding sites (Milstein C, Cuello AC. Nature. 1983 Oct 6-12;305(5934):537-40). Because the various H and L chains expressed by the quadroma will randomly pair, the yield of the desired bispecific pair of H/L chains was very low. Therefore, another bispecific antibody format involving cross-rodent species IgGs has been developed by Lindhofer et al. (1995 J Immunol 155:219). Using this method, rat and mouse hybridomas producing different antibodies are fused, leading to enrichment of functional bispecific antibodies, because of preferential species-restricted heavy/light chain pairing. The bispecific antibodies are then purified from the mixture that also contains monospecific antibodies. Thus, half of the resulting bispecific antibody is derived from mouse IgG and the other half is derived from rat IgG. Such bispecific antibodies will most likely be immunogenic in mouse or rat as half of the molecule will be foreign.
Thus, there is a need for surrogate bispecific rodent antibodies for facilitating studies in immunocompetent mouse or other rodent models, in which antibodies will have optimal cross-talk with mouse or other rodent effector cells and proteins, which can be used in rodent disease models and will have limited immunogenicity.

It is thus an object of the present invention to provide heterodimeric proteins such as bispecific antibodies which can be used in rodent disease models as surrogate for human bispecific antibodies e.g. for investigating mechanisms of action, effector functions, toxicity, unwanted mechanisms and side effects, anti-tumor efficacy, treatment efficacy, T-cell mediated anti-tumor efficacy, immune modulation, pharmacodynamics and/or pharmacokinetics of the heterodimeric protein, which antibodies have limited immunogenicity in the rodent compared to fully human or humanized antibodies. It is a further object of the present invention to provide rodent heterodimeric proteins which can be used as research tools.

15 SUMMARY OF THE INVENTION

The present inventors surprisingly found, that the two matching point mutations of the prior art bispecifics were less optimal in driving rodent homodimeric proteins in the direction that favours heterodimerization.

The inventors found that at least one further amino acid modification on the first homodimer was needed for providing at least 85% of the desired rodent heterodimeric proteins.

Accordingly, the present invention relates in one aspect to heterodimeric proteins comprising a first polypeptide of a first homodimeric protein, said first polypeptide comprising a first variable region having a first binding specificity and a first Fc region, said first Fc region comprising a first CH3 domain; and

a second polypeptide of a second homodimeric protein, said second polypeptide comprising a second variable region having a second binding specificity and a second Fc region, said second Fc region comprising a second CH3 domain,

wherein the variable regions may originate from any species and the Fc regions originate from a rodent species, and wherein the first CH3 domain comprises an amino acid selected from Gly, Ala, Val, Leu, Ile, Ser, Lys, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr, and Met at
position 370 and a substitution of the amino acid residue at position 409 selected from Gly, Ala, Val, Me, Ser, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr and Thr and the second CH3 domain comprises a substitution of the amino acid residue at position 405 selected from Ala, Val, Leu, Ile, Ser, Lys, His, Asp, Asn, Glu, Gin, Trp, Tyr and Thr, relative to the wild type IgG isotype from said rodent species when using EU numbering index.

The invention further relates to the use of such heterodimeric proteins and to the method of production of such proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: A) Principle of controlled Fab-arm exchange for the generation of human IgG1-based bispecific antibodies. Alignment of human (Homo sapiens; hs) and rodent (Mus musculus; mm, Rattus norvegicus; rn) sequences of (B) hinge regions and (C) CH3 regions. EU-numbering convention is used to annotate amino acid residues.

Figure 2: Efficiency of controlled Fab-arm exchange (cFAE) as measured by dual-binding ELISA (solid bars) or Hydrophobic Interaction Chromatography (HIC) (open bars) of mixtures of 2F8-derived and 7D8-derived (A) hsIgG1-CH3(mmG1), (B) hsIgGl-CH3(mmG2a) or (C) hsIgGl-CH3(mmG2b) parental antibodies. The 2F8-derived and 7D8-derived parental antibodies contained the indicated mutations, respectively. Data represent mean ± SEM. nd = not done. As the presence of aggregates can influence quantification by ELISA (overestimation), the HIC values are considered more accurate.

Figure 3: Exemplary Hydrophobic Interaction Chromatography (HIC) characterization of bispecific antibodies (containing mouse-derived CH3 domains). A) Overlay of HIC profiles of 2F8 and 7D8-derived parental antibodies (black dotted and gray dashed lines, respectively) and the bispecific antibody product (black solid line) generated by controlled Fab-arm exchange. B) Exemplary HIC profiles of bispecific antibodies generated by controlled Fab-arm exchange using the indicated (above and left of panels) combination of parental antibodies. Numbers indicate percent ages of bispecific antibody product (middle peaks) and residual 2F8 and 7D8-derived parental antibodies (left and right peaks, respectively). Vertical lines correspond with the retention times of the HIC analyses of the individual parental antibodies.
Figure 4: A) Schematic representation of chimeric IgG1 molecules containing mouse-derived constant regions having the F405L and K409R matching mutations alone or in combination with additional N/R411T and V/T370K mutations, respectively. B) and C) Efficiency of controlled Fab-arm exchange as measured by dual-binding ELISA (solid bars) or HIC (open bars) of mixtures of 2F8-derived and 7D8-derived mmIgG1, mmIgG2a, mmIgG2b or mmIgG3 parental antibodies without mutations (WT) (B; left panel), with the F405L or K409R mutation, respectively, (B; right panel), with the F405L or T/V370 K-K409R mutations, respectively, (C; left panel), or with the F405L-N/R411T or T/V370 K-K409R mutations, respectively, (C; right panel). Data represent mean ± SEM. nd = not done.

Figure 5: Exemplary Hydrophobic Interaction Chromatography (HIC) characterization of bispecific antibodies (containing mouse-derived constant regions) generated by controlled Fab-arm exchange using the indicated (above and left of panels) combination of parental antibodies. Numbers indicate percent ages of bispecific antibody product (middle peaks) and residual 2F8 and 7D8-derived parental antibodies (left and right peaks, respectively). Vertical lines correspond with the retention times of the HIC analyses of the individual parental antibodies.

Figure 6: A) Isothermal titration calorimetry (ITC) analysis of the binding of mmIgG2b variants to the Z-domain. Isotherms resulting from the binding of Z-domain to mmIgG2b variants. Top panels show the isotherms representing power input over time. In the bottom panels total energy required for equilibration as a function of the molar ratio of injectant (Z-domain) to titrant (IgG). B) MAbSelect Sure separation of mmIgG2b variants and the corresponding bispecific antibody product by 3-step pH gradient elution. Chromatograms show the absorption at 280 nm (solid line; left y-axis) and the pH profile (dashed grey line; right y-axis) during the purification. C) Fractions from each elution step: pH 7.2, 4.0, and 3.4 for the mixed sample containing both parental Abs and the bispecific Ab (dotted hashed trace in panel B) were analyzed by hydrophobic interaction chromatography (HIC). Dashed lines indicate the elution volume of the two parental Abs and the bispecific Ab. Note that the elution volume of the bispecific Ab is intermediate between that of the parental Abs.

Figure 7: A) Schematic representation of chimeric IgG1 molecules containing rat-derived constant regions having the F405L and K409R matching mutations alone or in combination with additional N/S411T and S/T370K mutations, respectively. B) and C) Efficiency of controlled Fab-arm exchange as measured by dual-binding ELISA (solid bars) or HIC (open bars).
bars) of mixtures of 2F8-derived and 7D8-derived mIgGl, mIgG2a, mIgG2b or mIgG2c parent al antibodies without mutations (WT) (B; left panel), with the F405L or K409R mutation, respectively, (B; right panel), with the F405L or S/T370K-K409R mutations, respectively, (C; left panel), or with the F405L-N/S411T or S/T370K-K409R mutations, respectively, (C; right panel). Data represent mean ± SEM. nd = not done, na = not applicable.

**Figure 8**: Antibody-Dependent Cellular Phagocytosis (ADCP) of Daudi cells (target cell; T) by bone marrow-derived mouse macrophages (effect or cell; E) incubated with a fixed 1 μg/mL concentration of the indicated (A) mIgGl-7D8, (B) mIgG2a-7D8 and (C) mIgG2b-7D8 variant, with an E:T ratio of 1:1. Data represent mean ± SD of triple measurements of a representative experiment.

**Figure 9**: T-cell mediated cytoxicity of AU565 cells (target cells; T) cocultured for 3 days at 37°C with mouse splenocytes (effect or cells; E) in the presence of serial diluted (A) mIgGl-derived or (B) mIgG2a-derived bispecific antibodies and parent al control antibodies (as indicated), with a E:T ratio of 2:1. Data represents mean of a representative experiment (n=2).

**Figure 10**: Total antibody plasm a concentration over time (A) and plasma clearance rate (B) of mIgGl-derived or mIgG2a-derived bispecific antibodies in C57Bl/6J mice. Six groups of mice (3 mice per group) were injected with the indicated antibodies (100 μg/mice). Blood samples were drawn at different times and plasma concentrations were determined by antigen-specific ELISA. Data represent mean ± SD.

**Figure 11**: Evaluation of the in vivo efficacy of mIgG2a-derived 145-2C11xCTA99 bispecific antibodies in a syngeneic xenograft model with gp75-expressing B16/F10 tumor cells. On day 6, when average tumor size was ~200-400 mm3, mice were randomized (n = 8 or 9 per group) and treated intravenously with the indicated doses, followed by a second dose at day 8 (A) or 9 (B) (arrowheads indicate treatment days). Data represent mean tumor volumes ± SEM.
DEFINITIONS

The term "immunoglobulin" refers to a class of structurally related glycoproteins consisting of two pairs of polypeptide chains, one pair of light (L) chains and one pair of heavy (H) chains, all four inter-connected by disulfide bonds. The structure of immunoglobulins has been well characterized. See for instance Fundamental Immunology Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)). Briefly, each heavy chain typically is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain "constant region" typical ly is comprised of three domains, CH1, CH2, and CH3. The heavy chains are inter-connected via disulfide bonds in the so-called "hinge region". Each light chain typically is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region typically is comprised of one domain, CL. The VH and VL regions may be further subdivided into regions of hypervariability (or hypervariable in sequence and/or form of structurally defined loops), also termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FRs). Each VH and VL is typically composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 (see also Chothia and Lesk J. Mol. Biol. 196, 901 917 (1987)).

Herein, the numbering of amino acid residues in the constant region is performed according to the "EU numbering index" as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). The variable regions are numbered according to the IMGT numbering system as described in "IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains": Lefranc, M.-P. et al., Dev. Comp. Immunol., 27, 55-77 (2003)

When used herein, the term "Fab-arm" is used interchangeably with the term "half molecule" and refers to one heavy chain-light chain pair.

When used herein, the term "Fc region" refers to an antibody region comprising at least the hinge region, the CH2 domains and the CH3 domains. Thus, the Fc region is part of the constant region.
When used herein, the term "constant region" refers to constant regions of an immunoglobulin, i.e. the regions CH1, hinge, CH2 and CH3.

The term "antibody" (Ab) in the context of the present invention refers to an immunoglobulin molecule, a fragment of an immunoglobulin molecule, or a derivative of either thereof, which has the ability to specifically bind to an antigen under typical physiological conditions with a half-life of significant periods of time, such as at least about 30 min., at least about 45 min., at least about one hour, at least about two hours, at least about four hours, at least about 8 hours, at least about 12 hours (h), about 24 hours or more, about 48 hours or more, about 3, 4, 5, 6, 7 or more days, etc., or any other relevant functionally-defined period (such as a time sufficient to induce, promote, enhance, and/or modulate a physiological response associated with antibody binding to the antigen and/or time sufficient for the antibody to recruit an effector activity). The variable regions of the heavy and light chains of the immunoglobulin molecule contain a binding domain that interacts with an antigen. The constant regions of the antibodies (Abs) may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (such as effector cells) and components of the complement system such as C1q, the first component in the classical pathway of complement activation. An antibody may also be a bispecific antibody, diabody, or similar molecule. The term "bispecific antibody" refers to antibodies having specificities for at least two different epitopes, typically non-overlapping epitopes. As indicated above, the term antibody herein, unless otherwise stated or clearly contradicted by the context, includes fragments of an antibody that retain the ability to specifically bind to the antigen. Such fragments may be provided by any known technique, such as enzymatic cleavage, peptide synthesis and recombinant expression techniques. It has been shown that the antigen-binding function of an antibody may be performed by fragments of a full-length antibody, e.g. a F(ab')2 fragment. It also should be understood that the term antibody, unless specified otherwise, also includes polyclonal antibodies, monoclonal antibodies (mAbs), antibody-like polypeptides, such as chimeric antibodies and humanized antibodies. An antibody as generated can possess any isotype.

The term "full-length antibody" when used herein, refers to an antibody which contains all heavy and light chain constant and variable domains that are normally found in an antibody of that isotype.
As used herein, "isotype" refers to the immunoglobulin class (for humans for instance IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM) that is encoded by heavy chain constant region genes, including all allotypes. Isotypes of Mus musculus (mm) include for instance IgG1, IgG2a, IgG2b, IgG2c and IgG3. Isotypes of Rattus norvegicus (rn) include for instance IgG1, IgG2a, IgG2b, and IgG2c.

The term "allogn" when used herein, refers to variations in the amino acid sequences which are found naturally within a population.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germ line immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germ line immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germine of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "rodent" as used herein is intended to include all members of the order of Rodentia (Carleton, M. D.; Musser, G. G. (2005). "Order Rodentia", In Wilson, Don E.; Reeder, DeeAnn M. Mammal Species of the World: A Taxonomic and Geographic Reference, Volume 12. JHU Press, pp. 745-752. ISBN 978-0-8018-8221-0.)

The term "rodent antibody" or "rodent heterodimeric protein" as used herein, is intended to include antibodies or heterodimeric proteins having variable and constant regions derived from a rodent species immunoglobulin sequences, such as e.g. a mouse or a rat. The rodent antibodies or heterodimeric proteins of the invention may include amino acid residues not encoded by rodent immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). The term "rodent antibody" or "rodent heterodimeric protein", as used herein, is also intended to include antibodies or heterodimeric proteins in which CDR sequences or the variable region sequences are derived from another species such as e.g. a human or another mammalian species.

When used herein, the term "heavy chain antibody" or "heavy-chain antibody" refers to an antibody which consists only of two heavy chains and lacks the two light chains usually
found in antibodies. Heavy chain antibodies, which naturally occur in e.g. cam elids, can bind antigens despite having only VH domains.

The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. The epitope may comprise amino acid residues directly involved in the binding (also called immunodominant component of the epitope) and other amino acid residues, which are not directly involved in the binding, such as amino acid residues which are effectively blocked by the specific antigen binding peptide (in other words, the amino acid residue is within the footprint of the specific antigen binding peptide).

As used herein, the term "binding" in the context of the binding of an antibody to a predetermined antigen typically is a binding with an affinity corresponding to a KD of about $10^{-6}$ M or less, e.g. $10^{-7}$ M or less, such as about $10^{-8}$ M or less, such as about $10^{-9}$ M or less, about $10^{-10}$ M or less, or about $10^{-11}$ M or even less when determined by for instance surface plasmon resonance (SPR) technology in a BIAcore 3000 instrument using the antigen as the ligand and the antibody as the analyte, and binds to the predetermined antigen with an affinity corresponding to a KD that is at least ten-fold lower, such as at least 100-fold lower, for instance at least 1,000-fold lower, such as at least 10,000-fold lower, for instance at least 100,000-fold lower than its affinity for binding to a non-specific antigen (e.g., BSA, casein) other than the predetermined antigen or a closely related antigen. The amount with which the affinity is lower is dependent on the KD of the antibody, so that when the KD of the antibody is very low (that is, the antibody is highly specific), then the amount with which the affinity for the antigen is lower than the affinity for a non-specific antigen may be at least 10,000-fold. The term "KD" (M), as used herein, refers to the dissociation equilibrium constant of a particular antibody-antigen interaction.

When used herein the term "heterodimeric interaction between the first and second CH3 regions" refers to the interaction between the first CH3 region and the second CH3 region in a first-CH3/second-CH3 heterodimeric protein.

When used herein the term "homodimeric interactions of the first and second CH3 regions" refers to the interaction between a first CH3 region and another first CH3 region in a first-
CH3/first-CH3 homodimeric protein and the interaction between a second CH3 region and another second CH3 region in a second-CH3/second-CH3 homodimeric protein.

An "isolated antibody," as used herein, denotes that the material has been removed from its original environment (e.g., the natural environment if it is naturally occurring or the host cell if it is recombinantly expressed). It is also advantageous that the antibodies be in purified form. The term "purified" does not require absolute purity; rather, it is intended as a relative definition, indicating an increase of the antibody concentration relative to the concentration of contaminants in a composition as compared to the starting material.

The term "host cell," as used herein, is intended to refer to a cell into which an expression vector has been introduced, e.g. an expression vector encoding an antibody of the invention. Recombinant host cells include, for example, transfectom as, such as CHO cells, HEK293 cells, NS/0 cells, and lymphocytic cells.

When used herein, the term "co-expression" of two or more nucleic acid constructs, refers to expression of the two constructs in a single host cell.

The term "tumor cell protein" refers to a protein located on the cell surface of a tumor cell.

As used herein, the term "effector cell" refers to an immune cell which is involved in the effector phase of an immune response, as opposed to the cognitive and activation phases of an immune response. Exemplary immune cells include a cell of a myeloid or lymphoid origin, for instance lymphocytes (such as B cells and T cells including cytolytic T cells (CTLs)), killer cells, natural killer cells, macrophages, monocytes, eosinophils, polymorphonuclear cells, such as neutrophils, granulocytes, mast cells, and basophils. Some effector cells express specific Fc receptors and carry out specific immune functions. In some embodiments, an effector cell is capable of inducing antibody-dependent cellular cytotoxicity (ADCC), such as a natural killer cell, capable of inducing ADCC. In some embodiments, an effector cell may phagocytose a target antigen or target cell.

The term "reducing conditions" or "reducing environment" refers to a condition or an environment in which a substrate, here a cysteine residue in the hinge region of an antibody, is more likely to become reduced than oxidized.

The term "disulfide bond isomerization" refers to an exchange of disulfide bonds between different cysteines, i.e., the shuffling of disulfide bonds.
Embodiments of the invention:

In a first aspect the invention relates to a heterodimeric protein comprising:

a first polypeptide of a first homodimeric protein, said first polypeptide comprising a first variable region having a first binding specificity and a first Fc region, said first Fc region comprising a first CH3 domain; and

a second polypeptide of a second homodimeric protein, said second polypeptide comprising a second variable region having a second binding specificity and a second Fc region, said second Fc region comprising a second CH3 domain,

wherein the variable regions may originate from any species and the Fc regions originate from a rodent species, and wherein the first CH3 domain comprises an amino acid selected from Gly, Ala, Val, Leu, Me, Ser, Lys, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr and Met at position 370 and a substitution of the amino acid residue at position 409 selected from Gly, Ala, Val, Me, Ser, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr and Thr and the second CH3 domain comprises a substitution of the amino acid residue at position 405 selected from Ala, Val, Leu, Me, Ser, Lys, His, Asp, Asn, Glu, Gin, Trp, Tyr and Thr, relative to the wild type IgG isotype from said rodent species when using EU numbering index for position numbering.

In one aspect of the invention the heterodimeric protein is a bispecific antibody.

In one embodiment the Fc regions of the heterodimeric protein are from mouse, rat, hamster, cotton rat or guinea pig origin.

In one embodiment the Fc regions of the heterodimeric protein are from mouse origin.

In one embodiment the Fc regions of the heterodimeric protein are from rat origin.

In one embodiment the Fc regions of the heterodimeric protein are from hamster origin.

In one embodiment the Fc regions of the heterodimeric protein are from cotton rat origin.

In one embodiment the Fc regions of the heterodimeric protein are from guinea pig origin.
In a preferred embodiment the Fc regions of the heterodimeric protein are of mouse or rat origin. In one embodiment they are of *Mus musculus* (*mm*) origin. In another embodiment they are of *Rattus norvegicus* (*rn*) origin. The first and second Fc regions may be obtained from the same species but they may also be of two different species, so that the first Fc region is of one species and the second Fc region is of another species. It is prefer not that they are of the same species. In another embodiment the Fc regions are chimeric so that e.g. the first Fc region may be of two different species. In one embodiment the first and second Fc regions both origin from *Mus Musculus*. In another embodiment the first and second Fc regions both origin from *Rattus Norvegicus*.

In one aspect of the invention, the variable regions of the heterodimeric protein are of rodent origin. In one embodiment the variable regions are of the same origin as the Fc regions so that the heterodimeric protein is e.g. fully mouse protein, such as fully mouse bispecific antibody. In another embodiment it is a fully rat bispecific antibody. Such bispecific fully mouse or rat antibodies may be useful as surrogate antibodies for use in a mouse or rat model for predicting e.g. the efficacy and safety of human or humanized bispecific antibodies binding the same targets. These bispecific antibodies may also be useful for further preclinical research where the use of animal models is essential for the understanding of the mechanisms of action of therapeutic bispecific human IgGs. Proof-of-concepts for therapeutic antibodies are often established in pre-clinical xenograft models using immune deficient mice. For many therapeutic concepts, however, the use of surrogate antibodies in rodent disease models such as a mouse model is warranted. The use of mouse or other rodent antibodies enables optimal cross-talk with mouse or other rodent effector cells and proteins, and in addition limits potential immunogenicity which is likely to be a problem for human antibodies applied in rodents, thus allowing long-term treatment of the rodent. Thus, in certain applications it is an advantage that the bispecific antibody is fully rodent.

For other applications it may be an advantage that the variable regions originate from a human antibody. Accordingly, in one embodiment the variable regions of the heterodimeric protein are of human origin. In another embodiment they are humanized rodent variable regions. In yet another embodiment the first and second polypeptides of the heterodimeric protein comprise human variable regions and rodent Fc regions. In yet another embodiment the first and second polypeptides of the heterodimeric protein comprise human variable regions and mouse Fc regions, such as *Mus musculus* derived Fc regions.
Hereby chimeric heterodimeric proteins are provided which at least recognize human targets but which preferably have no or very limited immunogenicity in rodents. In this way, mechanisms of human heterodimeric protein clinical candidates, such as bispecific antibody clinical candidates may be tested in a mouse or other rodent model. Accordingly, the present invention provides chimeric bispecific antibodies that may comprise human variable regions and rodent constant regions. Using human variable regions which are identical to variable regions of a lead clinical candidate and the rest of the molecule being of rodent origin, the present invention provides a format by which e.g. mechanisms of action, effector functions, toxicity, unwanted mechanisms and/or side effects, anti-tumor efficacy, treatment efficacy, T-cell mediated anti-tumor efficacy, immune modulation, pharmacodynamics and/or pharmacokinetics of said heterodimeric protein may be investigated in a rodent model. This information may be very useful and predictive of the corresponding functions and effects in humans of the fully human antibody counterpart having identical variable regions but human constant regions.

In some embodiments, the stable heterodimeric protein can be obtained at high yield using the method of the invention on the basis of two homodimeric starting proteins containing only a few, fairly conservative, asymmetrical mutations in the CH3 regions.

Thus, in one embodiment, the sequences of said first and second CH3 regions contain amino acid substitutions at non-identical positions.

In one embodiment, said first homodimeric protein has no more than two amino acid substitutions in the CH3 region, and the second homodimeric protein has no more than one amino acid substitution in the CH3 region relative to the relevant wild-type CH3 regions.

In certain embodiments of the invention the first CH3 domain of the heterodimeric protein comprise a Lysine (K) at amino acid position 370 and a substitution of the amino acid residue at position 409 selected from Gly, Ala, Val, Me, Ser, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr, and Thr and the second CH3 domain comprises a substitution of the amino acid residue at position 405 selected from Ala, Val, Leu, Me, Ser, Lys, His, Asp, Asn, Glu, Gin, Trp, Tyr and Thr, relative to wild type mm1G1 when using EU numbering index. In a preferred embodiment the first CH3 domain of the heterodimeric protein comprise a Lysine (K) at amino acid position 370 and a substitution of the amino acid residue at position 409 with an Arginine (R) and the second CH3 domain comprises a substitution of the amino acid
residue at position 405 selected from Ala, Val, Leu, Me, Ser, Lys, His, Asp, Asn, Glu, Gin, Trp, Tyr and Thr.

In a more preferred embodiment the first CH3 domain of the heterodimeric protein comprises a Lysine (K) at amino acid position 370 and a substitution of the amino acid residue at position 409 with an Arginine (R) and the second CH3 domain comprises a substitution of the amino acid residue at position 405 with a Leucine (L). In one embodiment these mutations are the only mutations compared to the relevant wild type CH3 domain.

In another embodiment the second CH3 domain further comprises an amino acid selected from the group comprising: Gly, Ala, Val, Ile, Ser, His, Asp, Glu, Gin, Trp, Phe, Tyr, Met and Thr at position 411.

In certain embodiments the second CH3 domain further comprises a Threonine (T) at position 411 so that the second CH3 domain contains a Leucine at position 405 and a Threonine at position 411.

According to the present invention, the amino acid sequences of the first and second CH3 regions of the homodimeric starting proteins are different from each other and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions.

In one embodiment, the increased strength of the heterodimeric interaction as compared to each of the homodimeric interactions is due to CH3 modifications other than the introduction of covalent bonds, cysteine residues or charged residues.

In certain embodiments of the invention the Fc regions of the first and second polypeptides are from the same species. In other embodiments they are of the same species and isotype. In another embodiment they are of different species and/or different isotypes.

In an embodiment of the invention the Fc regions of the first and second polypeptides are both of mouse IgG1 isotype, preferably *Mus musculus* IgG1.

In another embodiment of the invention the Fc regions of the first and second polypeptides are both of mouse IgG2a isotype, preferably *Mus musculus* IgG2a.

In another embodiment of the invention the Fc regions of the first and second polypeptides are both of mouse IgG2b isotype, preferably *Mus musculus* IgG2b.
In another embodiment of the invention the Fc regions of the first and second polypeptides are both of mouse IgG2c isotype, preferably *Mus musculus* IgG2c.

In another embodiment of the invention the Fc regions of the first and second polypeptides are both of mouse IgG3 isotype, preferably *Mus musculus* IgG3.

In an embodiment of the invention the Fc regions of the first and second polypeptides are both of rat IgG1 isotype, preferably *Rattus norvegicus* IgG1.

In another embodiment of the invention the Fc regions of the first and second polypeptides are both of rat IgG2a isotype, preferably *Rattus norvegicus* IgG2a.

In another embodiment of the invention the Fc regions of the first and second polypeptides are both of rat IgG2b isotype, preferably *Rattus norvegicus* IgG2b.

In another embodiment of the invention the Fc regions of the first and second polypeptides are both of rat IgG2c isotype, preferably *Rattus norvegicus* IgG2c.

In further embodiments, the invention relates to heterodimeric protein wherein the first homodimeric protein and the second homodimeric protein are selected from the group consisting of (i) an antibody (ii) a fusion protein comprising an Fc region, such as an Fc region fused to a receptor, cytokine or hormone, (iii) an antibody conjugated to a produg, peptide, drug or a toxin, and (iv) a fusion protein comprising an Fc region conjugated to a produg, peptide, drug or a toxin.

In one embodiment the invention relates to heterodimeric protein wherein the first homodimeric protein and the second homodimeric protein are fusion proteins each comprising an Fc region which further comprises an antigen binding region, such as a Fragment antigen-binding (Fab) region or a single-chain variable fragment (scFv).

In some embodiments, said first and/or second homodimeric protein comprise, in addition to the Fc region, one or more or all of the other regions of an antibody, i.e. a CH1 region, a VH region, a CL region and/or a VL region. Thus, in one embodiment, said first homodimeric protein is a full-length antibody. In another embodiment, said second homodimeric protein is a full-length antibody.
In other embodiments, however, only one of the homodimorphic proteins is a full-length antibody and the other homodimeric protein is not a full-length antibody, e.g. an Fc region without a variable region, expressed in conjunction to another protein or peptide sequence like a receptor, cytokine or hormone, or conjugated to a prodrg, peptide, a drug or a toxin. In a further embodiment, neither of the homodimeric proteins is a full-length antibody. For example, both homodimeric proteins may be Fc regions that are fused to another protein or peptide sequence like a receptor, cytokine or hormone, or conjugated to a prodrg, peptide, a drug or a toxin.

In a further embodiment, both the first and second homodimeric proteins are antibodies, preferably full-length antibodies. Preferably the first and second homodimeric proteins bind different epitopes. The epitopes may be on the same or different antigens. Preferably the epitopes are not overlapping so that the heterodimeric protein may bind both epitopes at the same time. In such embodiments, the heterodimeric proteins that are generated are bispecific antibodies. Thus, the bispecific format may be used in many ways to generate desired combinations of bispecific antibodies. In addition to being able of combining antibodies targeting different antigens in a very selective way it can be used to change a desired property, e.g. to increase CDC, by combining two different antibodies targeting the same antigen. Furthermore, it can be used to remove partial agonistic activity of an agonistic antibody or convert an agonistic antibody into an antagonistic antibody by making a bispecific antibody thereof with an irrelevant (inactive) antibody.

In a further embodiment, one or both of the homodimeric proteins is glyco-engineered to reduce fucose and thus enhance ADCC, e.g. by addition of compounds to the culture media during antibody production as described in US2009317869 or as described in van Berkel et al. (2010) Biotechnol. Bioeng. 105:350 or by using FUT8 knockout cells, e.g. as described in Yamane-Ohnuki et al. (2004) Biotechnol. Bioeng. 87:614. ADCC may alternatively be optimized using the method described by Umaha et al. (1999) Nature Biotechnol. 17:176.

In a further embodiment, one or both of the homodimeric proteins has been engineered to enhance complement activation, e.g. as described in Natsume et al. (2009) Cancer Sci. 100:2411 or Diebolder et al. (2014) Science 343:1260.

In a further embodiment, one or both of the homodimeric proteins has been engineered to reduce or increase the binding to the neonatal Fc receptor (FcRn) in order to manipulate the serum half-life of the heterodimeric protein. In one embodiment, the homodimer starting
proteins are engineered by substitutions at positions corresponding to P257A or L225Q, T256N and P257A or P307H and M309Q in mmIgG1 which substitution decreases FcRn binding of those homodimers. In another embodiment, the homodimer starting proteins are engineered by substitutions at positions corresponding to T252L, T254S and T256F in mmIgG1 which substitution increases FcRn binding and serum half-life of those homodimers.

In a further embodiment, one of the homodimeric starting proteins has been engineered to not bind Protein A, thus allowing the separation of the heterodimeric protein from said homodimeric starting protein by passing the product over a protein A column. This may in particular be useful for embodiments wherein an excess of one homodimeric protein is used relative to the other homodimeric protein as starting material. In such embodiments, it may be useful to engineer the homodimeric protein that is in excess so that it loses its ability to bind protein A. Following the heterodimerization reaction, the heterodimeric protein may then be separated from a surplus of unexchanged homodimeric protein by passage over a protein A column. In one embodiment, the one homodimer starting protein is engineered by a substitution at a position corresponding to I253D in mmIgG2b which substitution decreases Protein A binding of that homodimer. In another embodiment the other homodimer is engineered by substituting the amino acid positions corresponding to P307T and Q309L of mmIgG2b which substitutions increases Protein A binding of that other homodimer. In a preferred embodiment, the first homodimer comprises the substitution I253D and the second homodimer comprises the substitutions P307T and Q309L. These substitutions may be made in any homodimeric starting protein such as for example mmIgG2a or mmIgG2b at corresponding amino acid positions. In another embodiment the other homodimer is engineered by substituting the amino acid positions corresponding to T252M and/or T254S of mmIgG1 which substitutions increases Protein A binding of those homodimers.

In a further embodiment, one of the homodimeric proteins is an Fc region or a full-length antibody recognizing a non-relevant epitope or a full-length antibody containing germ line-derived sequences that have not undergone somatic hyper mutation and do not bind self-antigens. In such an embodiment the heterodimeric protein functions as a monovalent antibody. In another embodiment, both homodimeric proteins comprises the same heavy chain, but only one of the homodimeric proteins contains a light chain which forms a functional antigen-binding site with said heavy chain, whereas the other homodimeric protein contains a non-functional light chain, which does not bind any antigen in combination with said heavy chain. In such an embodiment, the heterodimeric protein
functions as a monovalent antibody. Such a non-functional light chain can e.g. be a germ line-derived sequence that has not undergone somatic hypermutation and does not bind self-antigens.

In a further embodiment, one or both of the homodimeric proteins has been engineered to inhibit or abolish the interaction of the proteins according to the invention with Fc Receptors (FcRs) present on a wide range of effector cells, such as monocytes, or with C1q to activate the complement pathway in order to reduce unwanted effects. In one embodiment the homodimer starting proteins are engineered by substitutions at positions corresponding to D265A in mmlG1, mmlG2a or mmlG2b which substitution decrease FcR and C1q binding of those homodimers. In another embodiment, the homodimer starting proteins are engineered by a substitution at a position corresponding to L235E in mmlG2a which substitution decreases FcR binding of those homodimers. In a further embodiment, the homodimer starting proteins are engineered by substitutions at positions corresponding to L234A and L235A in mmlG2a which substitutions decrease FcR binding in those homodimers. In another embodiment, the homodimer starting proteins are engineered by a substitution at a position corresponding to N324D in mmlG2a which substitution decreases C1q binding of those homodimers. In a further embodiment, the homodimer starting proteins are engineered by substitutions at positions corresponding to N297A or E318A in mmlG2b which substitutions decrease FcR binding and C1q binding in those homodimers.

In another embodiment, the homodimer starting proteins are engineered by substitutions at positions corresponding to E318V, K320A, K320Q, K322A, K322Q or E318A in mmlG2b which substitutions decrease C1q binding in those homodimers. In a further embodiment, the homodimer starting proteins are engineered by deletion of amino acids at positions corresponding to 233, 234 and 235 in mmlG2b which deletion decreases FcR binding and C1q binding in those homodimers.

In one embodiment, the first and/or second homodimeric protein is conjugated to a drug, a prodrug or a toxin or contains an acceptor group for the same. Such acceptor group may e.g. be an unnatural amino acid.

In another aspect, the invention relates to the use of a heterodimeric protein according to the invention as a research tool for exploring functions of corresponding fully human heterodimeric proteins having similar or identical binding specificity in a rodent model. Thus, the invention in one aspect relates to use of rodent, such as mouse or rat, bispecific
antibodies for use in a rodent model wherein the binding part (the variable region or the
CDR regions) of the molecule is obtained from a human antibody such as a human bispecific antibody for which further characterization in an animal model is of interest. In other embodiments, the binding part of the molecule may be obtained from another species or may be humanized rodent variable regions or CDR regions.

In another aspect, the invention relates to the use of a heterodimeric protein according to the invention for exploring functions such as mechanisms of action, effector functions, toxicity, unwanted mechanisms and side effects, anti-tumor efficacy, treatment efficacy, T-cell mediated anti-tumor efficacy, immune modulation, pharmacodynamics and/or pharmacokinetics of said heterodimeric protein. When the heterodimeric protein is a rodent model of a human bispecific antibody this information may be valuable for the characterization of the antibodies and for selection of clinical candidates.

In yet another aspect, the invention relates to an in vitro method for generating a heterodimeric protein of the invention, said method comprising the following steps:

a) providing a first homodimeric protein comprising a first variable region having a first binding specificity, a hinge region, and a first Fc region, said first Fc region comprising a first CH3 domain; and

b) providing a second homodimeric protein comprising a second variable region having a second binding specificity, a hinge region, and a second Fc region, said second Fc region comprising a second CH3 domain,

wherein the variable regions may originate from any species and the Fc regions originate from a rodent species, and wherein the first CH3 domain comprises an amino acid selected from Gly, Ala, Val, Leu, Ile, Ser, Lys, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr and Met at position 370 and a substitution of the amino acid residue at position 409 selected from Gly, Ala, Val, Ile, Ser, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr and Thr, and the second CH3 domain comprises a substitution of the amino acid residue at position 405 selected from Ala, Val, Leu, Me, Ser, Lys, His, Asp, Asn, Glu, Gin, Trp, Tyr and Thr, relative to the wild type IgG1 isotype from said rodent when using EU-index numbering and wherein the sequences of said first and second CH3 regions are different and are such that a heterodimeric interaction between said first
and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions,

c) incubating said first homodimeric protein together with said second homodimeric protein under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide-bond isomerization, and

d) obtaining said heterodimeric protein.

The sequence of the hinge region of the homodimeric starting proteins may vary. In one embodiment both hinge regions are wild type hinge regions of the same isotype and species as the rest of the Fc regions. Thus, where the Fc region is obtained from mmlG1, the hinge region is likewise obtained from mmlG1.

In many embodiments wherein the first and said second homodimeric proteins are antibodies, said antibodies further comprise light chains. Said light chains may be different, i.e. differ in sequence and each form a functional antigen-binding domain with only one of the heavy chains. In another embodiment, however, said first and second homodimeric proteins are heavy-chain antibodies, which do not need a light chain for antigen binding, see e.g. Hamers-Casterman (1993) Nature 363:446.

As described above, step c) of the method of the invention comprises incubating said first protein together with said second protein under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide-bond isomerisation. Examples of suitable conditions are given herein. The minimal requirements for the cysteines in the hinge region for undergoing disulfide-bond isomerisation may differ depending on the homodimeric starting proteins, in particular depending on the exact sequence in the hinge region. It is important that the respective homodimeric interactions of said first and second CH3 regions are sufficiently weak to allow cysteines in the hinge region to undergo disulfide-bond isomerisation under the given conditions.

In one embodiment, the reducing conditions in step c) comprise the addition of a reducing agent, e.g. a reducing agent selected from the group consisting of: 2-mercaptooctyl hylanine (2-MEA), dithiothreitol (DTT), dithioerythritol (DTE), glutathione (GSH), tris(2-carboxyethyl)phosphine (TCEP), L-cysteine and beta-mercapto-ethanol, preferably a
reducing agent selected from the group consisting of: 2-mercaptoethylamine, dithiothreitol and tris(2-carboxyethyl)phosphine.

In a further embodiment, step c) comprises incubation for at least 90 min at a temperature of at least 20°C in the presence of at least 25 mM 2-mercaptoethylamine or in the presence of at least 0.5 mM dithiothreitol. The incubation may be performed at a pH of from 5 to 8, such as at pH 7.0 or at pH 7.4.

In a further embodiment, step d) comprises restoring the conditions to become non-reducing or less reducing, for example by removal of a reducing agent, e.g. by desalting.

In some embodiments, the method of the invention yields an antibody product wherein more than 80%, such as more than 90%, e.g. more than 95%, such as more than 99% of the antibody molecules are the desired bispecific antibodies, when measured by HIC analysis. HIC analysis may be performed as described in example 8.

**EXAMPLES**

**Table 1: Sequences**

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<th>SEQ ID</th>
<th>SEQ Desc.</th>
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<tr>
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<td>Human IgG1 (Accession number P01857) CH1</td>
<td>ASIKGPSVFLAPSSKSISGGITAALCLVYTFEPVTVSWSN SGALTSGLHVPAVLQLSGLYSLSVTVPSSSLGTQTVICN VNHPSNNTKVR</td>
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<td>RTVAAPSFVFIFIFPSDEQLKSGTASVVCNNFYPFRAKVQVWK VDNALSGNSQESVEQDSDKSTYSLSSTLTLSKADYEKHKV YACEVTHQGLSPVTSHFRC</td>
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| SEQ ID NO: 6 | Mouse IgG2b (Accession number     | AKTPPSVPYPAFCGTGGSSVTGLCLVYTFEPVTVWN SGSLSSGVHTFAPALLQSGLYTMSSSVTSTPSTWPVITC 
AHPASSTKVDDKL |
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<td>7</td>
<td>Mouse IgG3 (Accession number X00915) CHI</td>
<td>ATTTAPSVYPLVPCGSDTSSVLTGLCVKYGFPPTVTVKN YGALSGLGTVSSTVLSQSFYSLSLSTVPSSTWPSQTVCINW AHPKSTELIKRI</td>
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<td>Mouse IgG1 CH2</td>
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<td>Mouse IgG2a CH2</td>
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<td>RADAAPTVSIFPPSEQLTSGGASVCLMNNFYPRD I SVKWK</td>
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**Note:** The sequences listed are not provided in the image, but the table format is maintained for clarity.
SEQ ID NO: 22 Mouse IGHG1

KTTPSVYPLAPGSAATNSMTLGLCVKGYPFPETVVTWNS
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LTTILTPKVCVTVVDI SKDDPVEQFSEWFDVDVEVHTAQTPQR
EEQFNSTRFVSELP IMHQDWEINGKFCVRNSAFAFPPEIK
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SEQ ID NO: 23 Mouse IGHG2A

AKTTAPSVYPLAPVCGDCDGGTSSVTGLCVKGYPFPETVLTWN
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PPKIDVPLMISLSPIVTVDVSEDPDQVQSIWVDVWNEVH
TAPQTHoireNTSRLVSVAP IQHQDWSGKEFCKMNK
KLAP IEITI SKPKGSVRAQPQYVVLTPPEEMTMDQVLTMV
TDFMPEI YVEWTTNGKTELNYKNTEPVLDSGDSYFMYSSKL
VERKKNVNSYSCVSVHEGLNHKHTKFRSFRPGK

SEQ ID NO: 24 Mouse IGHG2B

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SEQ ID NO: 26 Mouse IGHG3

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KLAP IEITI SKPKGSVRAQPQYVVLTPPEEMTMDQVLTMV
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VERKKNVNSYSCVSVHEGLNHKHTKFRSFRPGK

SEQ ID NO: 27 Mouse IGKC

ADAAPTVS IFPSSESQLTSGASVSVCFLNNFYPDKINDVKWI
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SEQ ID NO: 28 Rat IGHG1

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SEQ ID NO: 29  Rat IGHG2A
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SEQ ID NO: 30  Rat IGHG2B
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SEQ ID NO: 31  Rat IGHG2C
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SEQ ID NO: 32  Rat IGKC
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SEQ ID NO: 33  Mouse IGHG1 CHI
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SEQ ID NO: 34  Mouse IGHG2A CHI
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SEQ ID NO: 35  Mouse IGHG2B CHI
AKTTPSVYPLAPGCDDTTGSSTSGCLGVKYFPEEPVTWN SGSLSSGVHHTPAVEDLYTTSSTWPSQSTRPSETVTCNV AHPASSTKVDKKI

SEQ ID NO: 36  Mouse IGHG2c CHI
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<td>53</td>
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Example 1: Expression vectors for the expression of human IgG1-2F8, IgG1-7D8 and variants thereof
The VH and VL coding regions of HuMab 2F8 (WO 02/100348) and HuMab 7D8 (WO 04/035607) were cloned in the expression vector pConGI (containing the genomic sequence of the human IgGI allotype constant region (Lonza Biologies)) for the production of the human IgGI heavy chain and pConKappa (containing the human kappa light chain constant region, Lonza Biologies) for the production of the kappa light chain. Alternatively, in follow-up constructs, vectors were used containing the fully codon-optimized coding regions of the heavy chain in the pcDNA3.3 vector (Invitrogen) or the human kappa light chain of HuMab 2F8 or HuMab 7D8 in the pcDNA3.3 vector. The heavy chain constant region amino acid sequences as used were the following:

Human IgGI (Accession number P01857) CH1, SEQ ID NO: 1:
ASTKGPSSVFPLAPSSKTSGTALGCLVKDYFEPVPVTGSWNSGALTSGVHTFPAVLQSSGLYSLSSV
TVPSSSLGTQTYICNVHKPSNTKVDKRV

Human IgGI Hinge sequence is described in Table 1 (SEQ ID NO: 61) and Figure 1B.

Human IgGI CH2, SEQ ID NO: 2:
ELLGGPSVFLPPKPDMLI SRTPETCVVVDVSHEDPEVKFNWYVEDGEVKHNAKTPREEQYNSTYR
VVSVLTVLHODWHLNGKEYKCKVSNKALPAPI EKTI SKAK

Human IgGI CH3 sequences are described in Figure 1C.

Human kappa (Accession number J00241) CL, SEQ ID NO: 3
RTVAAPSVFI FPPSD EQLKSGTASVVCLNHYFPRKVKQWKVDNALSQNSQESVTEQDSDKSTYSLS
STLTLSDKAYEKHKVYACEVTHQGLSVPVTCSFNRGEC

To introduce mutations in the CH3 regions of the antibody heavy chains, i.e., F405L or K409R (EU numbering convention as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991) is used throughout), Quickchange site-directed mutagenesis kit (Stratagen e, La Jolla, CA) was used according to the manufacturer’s recommendations. Alternatively, the constructs were fully synthesized or VH regions were cloned in a vector already containing the specific amino acid encoding substitutions.
Example 2: Expression vectors for the expression of chimeric IgG1-2F8-CH3(mmG1), IgG1-2F8-CH3(mmG2a), IgG1-2F8-CH3(mmG2b), IgG1-2F8-CH3(mmG3), IgG1-7D8-CH3(mmG1), IgG1-7D8-CH3(mmG2a), IgG1-7D8-CH3(mmG2b) and IgG1-7D8-CH3(mmG3) constructs and variants thereof

Vectors containing the coding regions for the human IgG1 CH1-hinge-CH2 regions, mouse CH3 (IgG1, IgG2a, IgG2b or IgG3) regions and human kappa light chains constant regions and the human VH and VL regions of Humab 2F8 and 7D8 were synthesized, fully codon-optimized and inserted in separate (heavy chain and light chain) pcDNA3.3 vectors. The heavy chain constant region sequences as used were the following:

Human IgG1 CH1-Hinge-CH2 sequences (SEQ ID Nos: 1, 61 and 2).

Mouse IgG1, IgG2a, IgG2b and IgG3 CH3 sequences (SEQ ID Nos: 44, 45, 46 and 48).

To introduce mutations in the CH3 regions of the antibody heavy chains, i.e. F405L, K409R, V370K, T370K, N411T or R411T, Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer's recommendations. Alternatively the constructs were fully synthesized or VH regions were cloned in a vector already containing the specific amino acid encoding substitutions.

Example 3: Expression vectors for the expression of chimeric mm1Gg1-2F8, mm1Gg2a-2F8 mm1Gg2b-2F8, mm1G3-2F8, mm1Gg1-7D8, mm1Gg2a-7D8 mm1Gg2b-7D8 and mm1G3-7D8 constructs and variants thereof

Vectors containing the coding regions for the mouse (Mus musculus) IgG1, mouse IgG2a, mouse IgG2b, mouse IgG3 heavy and kappa light chains constant regions and the human VH and VL regions of Humab 2F8 and 7D8 were synthesized, fully codon-optimized and inserted in separate (heavy chain and light chain) pcDNA3.3 vectors. The heavy chain constant region amino acid sequences as used were the following:

Mouse IgG1 (Accession number J00453) CH1, SEQ IDNO: 4
AKTTPPSVYPLAPGSAAQTNSMVTLGCVLKGFPEPVTWNSGSLSSGVHTFPAPVLESDLYTLSSSVTV
PSSRPSETVTCNAHHPASSTKVDKKI

29
Mouse IgG2 a (Accession number V0082 5) CH1, SEQ ID NO: 5
AKTTPSVYPLAPVCGDTTGGSSVLGCLVKGYFPEPVTLTWNSGSLLSSSVHTFPALQLQGLYTMSSSVTV
TSSTWPSQSI TCNVAH PASSTKVDDKI
Mouse IgG2 b (Accession number V00 763) CH1, SEQ ID NO: 6
AKTTPPSVYPLAPCG DTDTVSTSGLCLVKGIFPEPVTVWNSSGSSVHTFPAALLQSLGTYTMSSSVTV
VPSSTWPSQTVCTSAVH PASSTTVDKKL
Mouse IgG3 (Accession number X009 15) CH1, SEQ ID NO: 7
ATTTAPSVYPLAPGCDTSGSSVTLGCLVKGYFPEPVTVKWNYGALSSGVRTVSSVLQSGFYSLSSLVTV
PSSTWPSQTVI CNVAH PASKTEI KR

Mouse IgG1, IgG2a, IgG2 b and IgG3 Hinge sequences are described in Table 1 and Figure 1 B.

Mouse IgG1 CH2, SEQ ID NO: 8
VPEVSSVFIPFPKPDVLTI TLTPKTVCVVVD ISKD DPEVQFSWFV DVEVHTAQTQPREEQFNSTFRSV
SELPI MHQDWLNGKEFKCRVNSAFFAPI EKTI SKTK
Mouse IgG2 a CH2, SEQ ID NO: 9
APNLLLLGQSFPKI KDVLMI ISLSPI VTCVVDVSED DPDVQI SWFVNNVEVHTAQQTTH REDYNSTL
RVSALPI QHSDMSGKEFKCKVNNKDLAPI ERTI SKPK

Mouse IgG2 b CH2, SEQ ID NO: 10
APNLEGGSVPFI FPPN KDVLMI SLTPKTCVVVDVSED DPDVQI SWFVNNVEVHTAQQTTH REDYNSTL
RVSTLPI QHSDMSGKEFKCKVNNKDLPSPI ERTI SKK

Mouse IgG3 CH2, SEQ ID NO: 11
PGNLLGGSVPFI FPPLPKDLALMI SLTPKTCVVVDVSED DPDVQI SWFVNNVEVHTAWTFAQYNNST
FRVVSALPI QHSDWNGREKKEFKCVNNKALAPI ERTI SKPK

Mouse IgG1, IgG2 a, IgG2 b and IgG3 CH3 sequences are described in Figure 1 C.

Mouse kappa (Accession number V00807) CL, SEQ ID NO: 12
RADAAPTVSI FPZPSQELTSGAGSCVFLNN FYPKDI NKWKI DGSEQONGVLSWTDQDSKSTDYAM
SSLTLTLDK EYERHNSYTCEATHKETSPI VKSFN RNEC

To introduce mutations in the CH3 regions of the antibody heavy chains, i.e. F405 L, K409 R, V370 K, T370 K, N411 T or R411 T, Quickch ange site-directed mutagenesis kit (Stratagen e, La

Example 4: Expression vectors for the expression of chimeric rnlG1-2F8, rnlG2a-2F8, rnlG2b-2F8, rnlG2c-2F8, rnlG1-7D8, rnlG2a-7D8, rnlG2b-7D8 and rnlG2c-7D8 constructs and variants thereof

Vectors containing the coding regions for the rat (Rattus norvegicus) IgG1, rat IgG2a, rat IgG2b, rat IgG2c heavy and kappa light chains constant regions and the VH and VL regions of Humab 2F8 and 7D8 were synthesized, fully codon-optimized and inserted in separate (heavy chain and light chain) pcDNA3.3 vectors. The heavy chain constant region amino acid sequences as used were the following:

Rat IgG1 (Accession number AABR0304890 5) CH1, SEQ ID NO: 13
AETTAPSVYPLAPGTALKSNSMVTLGLVKGYFPEPVTWTNSGALSSGVHFTPAVLQSGLYTLTSSVT
PSSTWPSQTVCNVAHPASSTKVDKKI

Rat IgG2a (Accession number AABR030495 60) CH1, SEQ ID NO: 14
AETTAPSVYPLAPGTALKSNSMVTLGLVKGYFPEPVTWTNSGALSSGVHFTPAVLQSGLYTLTSSVT
PSSTWSSQAVTNVHAHPASSTKVDKKI

Rat IgG2b (Accession number AABR030489 05) CH1, SEQ ID NO: 15
AQTTAPSVYPLAPGCGDTTSTTVTGLVKGYFPEPVTWTNSGALSSDVHTFPAVLQSGLYTLTSSVTS
STWPSQTVCNVAHPASSTKVDKKV

Rat IgG2c (Accession number AABR030499 12) CH1, SEQ ID NO: 16
ARTTAPSVYPLVPGCGSTGSLVTTLGLVKGYFPEPVTWKNSGALSSGVHFTPAVLQSGLYTLTSSVTV
PSSTWSSQVTCSVAHPATKSNLJKRI

Rat IgG1, IgG2a, IgG2b and IgG2c Hinge sequences are described in Table 1 and Figure 1B.
Rat IgG1 CH2, SEQ ID NO: 17
GSEVSSVFIPPPKPKDVLTLTPKVTCCVVDISQDDPEVHFSWFVDVDEVHTAQTRPPEEQFNSTFRSV
SELPI LHQDWLNGRTFRCKVTSAAFPSPI EKTI SKPE

5 Rat IgG2a CH2, SEQ ID NO: 17
GSEVSSVFIPPPKTDVLTLTPKVTCCVVDISQNDPEVRFPSWFDVDEVHTAQTHAPEKQNSNTRLRSV
SELPI VHRDWLNGKTFLCKVNSGAPPSI EKSI SKPE

10 VPELLGGSFVI FPPKPDII SQAQVTCCVVDVSEEPDQFSWVFVNNVEVHTAQTPREEQYNSTF
RVSALPI QHODWSMGEFCKCVMNKLPSPI EKTI SKPK

15 Rat IgG2c CH2, SEQ ID NO: 20
CDDNLGRPSFVI FPPKPDILTLTPKVTCCVVDVSEEPDQFSWFDVNVRVTAQTOPHEEQLNGTF
RVSSTLHIQHODWSMGEFCKCVMNKDLPSPI EKTI SKPR

1 Rat IgG1, IgG2a, IgG2b and IgG2c CH3 sequences are described in Table 1 (SEQ ID NOs 57-60).

20 Rat kappa (Accession number V01241) CL, SEQ ID NO: 21
RADAAPTVSI FPPSTEQLATGGASVVCLMNNFPRDI SVKWKI DGTERRDGVLDSVDQDSDKYSTSYM

To introduce mutations in the CH3 regions of the antibody heavy chains, i.e. F405L, K409R,
S370K, T370K, N411T or S411T, Quickchange site-directed mutagenesis kit (Stratagene, La
Joa, CA) was used according to the manufacturer’s recommendations. Alternatively, the
constructs were fully synthesized or VH regions were cloned in a vector already containing
the specific amino acid encoding substitutions.

Example 5: Antibody production by transient expression in Freestyle™ 293-F or
Exp293F™ cells

Antibodies were produced, under serum-free conditions, by co-transfecting relevant heavy
and light chain expression vectors in Freestyle™ 293-F cells (LifeTech nologies), using
293fect in™ (LifeTech nologies), according to the manufacturer’s instructions. Alternatively,
antibodies were produced, under serum-free conditions, by co-transfecting relevant heavy
and light chain expression vectors in Expi293 F™ cells (LifeTechnologies), using ExpiFectamine™ 293 (LifeTechnologies), according to the manufacturer's instructions.

Example 6: Purification of antibodies
Antibodies were purified by protein A affinity chromatography. In short, culture supernatant was filtered over 0.2 μm dead-end filters, loaded on 5 mL MabSelect SuRe columns (GE Health Care) and eluted with 0.1 M sodium citrate- NaOH, pH 3. The eluate was immediately neutralized with 2 M Tris- HCl, pH 9 and dialyzed overnight to 12.6 mM NaH₂PO₄, 140 mM NaCl, pH 7.4 (B.Braun). Alternatively, subsequent to purification, the eluate was loaded on a HiPrep Desalting column and the antibody was exchanged into 12.6 mM NaH₂PO₄, 140 mM NaCl, pH 7.4 (B.Braun) buffer. After dialysis or exchange of buffer, samples were sterile filtered over 0.2 μm dead-end filters. Alternatively, antibodies were purified by protein G affinity chromatography.

Purity was determined by SDS-PAGE/CE-SDS and concentration was measured by absorbance at 280 nm. Batches of purified antibody were tested by high-performance size-exclusion chromatography (HP-SEC) for aggregates or degradation products. Purified antibodies were stored at 2-8°C.

Example 7: Dual-binding ELISA to determine efficiency of bispecific antibodies generation
The presence of bispecific antibodies was tested by determination of bispecific antigen binding using a sandwich enzyme-linked immunosorbent assay (ELISA). ELISA plates (Greiner bio-one, Frickenhausen, Germa n) were coated overnight with 2 μg/100 μL/well (100 μL/well) of recombinant extracellular domain of EGFR in PBS at 4°C. The plates were washed once with PBST. Dilution series of the antibody samples (0.1 μg/100 μL/well in 3-fold dilutions) in PBST/0.2% BSA (PBSTB) were transferred to the coated ELISA plates (100 μL/well) and incubated on a plate shaker (300 rpm) for 60 min at room temperature (RT). Samples were discarded and the plates were washed once with PBS/0.05% Tween 20 (PBST). Next, the plates were incubated on a plate shaker (300 rpm) with 2 μg/mL mouse anti-idiotypic monoclonal antibody 2F2 (directed against 7DB; Genmab) in PBSTB (100 ML/well) for 60 min. The plates were washed once with PBS/0.05% Tween 20 (PBST). Next, the plates were incubated on a plate shaker (300 rpm) with an HRP-conjugated goat anti-mouse IgG (15G; Jackson Immuno Research Laboratories, Westgrove, PA, USA;
1:5,000) in PBSTB (100 µL/well) for 60 min at RT. The plates were washed once with PBS/0.05% Tween 20 (PBST). ABTS (50 mg/mL; Roche Diagnostics GmbH, Mannheim, Germany) was added (100 µL/well) and incubated protected from light for 30 min at RT. The reaction was stopped with 2% oxalic acid (100 µL/well; Riedel de Haen, Seelze, Germany). After 10 min at RT, absorbance at 405 nm was measured in an ELISA plate reader. Percentage (%) Bispecifics is defined as ((absorbance at 405 nm of the sample) - (absorbance at 405 nm of assay diluent only) / (absorbance at 405 nm of reference bslgGl - 7D8x2 F8) - (absorbance at 405 nm of assay diluent only)) x 100.

**Example 8: Hydrophobic interaction chromatography to determine efficiency of bispecific antibody generation**

Hydrophobic interaction chromatography (HIC) can be used to separate both parent antibodies from the bispecific antibody product, based on the inherent hydrophobic properties of the different proteins. Here, High Pressure Liquid Chromatography (HPLC)-HIC was applied to quantify the efficiency of the cFAE reaction. For this, samples of the parent antibodies and the bispecific antibody product, generated by cFAE, were diluted twofold with HIC eluent A (15.4 mM K2HPO4, 9.6 mM KH2PO4, 1.5 M (NH4)2S04; pH 7.0) to a final concentration of 1 mg/mL for injection into the HPLC. The IgG molecules with different hydrophobic properties were separated by using a Butyl-NPR, 2.5 µm, 4.6 x 35 mm HIC-HPLC column (Tosoh Bioscience) with a flow rate of 1 mL/min. 50 µL was injected and elution was performed with a 12-min gradient of HIC eluent A to HIC eluent B (15.4 mM K2HPO4, 9.6 mM KH2PO4; pH 7.0) and detection occurred at 280 nm. Empower 3 software (Waters) was used to assign and integrate peak areas. Chromatograms of the parental antibodies were used as reference to identify their position in the end-product. The relative peak areas of the bispecific antibody and residual parent antibodies were used to calculate the efficiency of the cFAE reaction.

**Example 9: Generation of bispecific chimeric antibodies, containing mouse-derived CH3 domains that include matching mutations F405L and K409R in combination with additional CH3 mutations by controlled Fab-arm exchange**

Bispecific antibodies were generated in vitro using the DuoBody® platform technology, i.e. controlled Fab-arm exchange (cFAE) or 2-MEA-induced Fab-arm exchange as described in WO 2011/131746; Labrijn et al., PNAS 2013, 110: 5145-50; Gramer et al., MAbs 2013, 5:
962-973 and Labrijn et al., Nat Biotechnol 2014, 9: 2450-63 (summarized in Figure 1A). Mixtures of constructs were subjected to cont rolled Fab-arm exchange (cFAE):

To generate bispecific antibodies, 100 μg of each parental antibody was mixed and incubated with 75 mM 2-mercaptoethylamidine-HCl (2-MEA) in a total volume of 400 μL PBS (B.Braun, product #3623 140) at 31°C for 5 hours. The reduction reaction was stopped when the reducing agent 2-MEA was removed by using Amicon Ultra 0.5 ml centrifugal units (10 kD MWCO, Millipore, product # UFC501096) and washing 4× with 400 μL PBS by centrifuging 10 min at 13000xg. Samples were collected in a new tube by inverting the filter and centrifuging 2 min at 10000xg. Volumes were adjusted to 200 μL (when needed) with PBS. The absorbance of 280 nm (A280) of bispecific products was measured to determine the final concentration. Samples were stored at 2-8 °C until further analysis. Dual-binding ELISA (as described in Example 7) and HIC analysis (as described in Example 8) was performed to determine the amount of bispecific product.

Figure 2 shows that introduction of the matching mutations F405L and K409R in the chimeric constructs containing mouse IgG1 and mouse IgG2a CH3 regions, i.e. hslG1-CH3(mmG1) and hslG1-CH3(mmG2a), had no effect, whereas their introduction in chimeric constructs containing the mouse IgG2b CH3 region, i.e. hslG1-CH3(mmG2b) enabled cFAE. To find additional CH3 domain mutations for increasing the efficiency of heterodimerization of parental rodent homodimers into the bispecific product, the following constructs were generated:

<table>
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<th>Table 2: Chimeric constructs containing mouse-derived CH3 domains</th>
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<td>hslG1-CH3(mmG1)-7D8-K409R</td>
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<td>hslG1-CH3(mmG1)-7D8-M368L-K409R</td>
</tr>
<tr>
<td>hslG1-CH3(mmG1)-7D8-T370K-K409R</td>
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<tr>
<td>hslG1-CH3(mmG1)-7D8-K409R-N411T</td>
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<tr>
<td>hslG1-CH3(mmG1)-7D8-M368L-T370K-K409R</td>
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<td>hslG1-CH3(mmG1)-7D8-M368L-K409R</td>
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</table>
Mixtures of constructs were subjected to cFAE (as described above) and dual-binding ELISA (as described in Example 7) and HIC analysis (as described in Example 8) was performed to determine the amount of bispecific product.

Figure 2A and 2B show that, whereas introduction of the M368L mutation alone had no significant effect, introducing mutations T370K and N411T, either alone or together, in both parental antibodies, increased the cFAE efficiency in IgG1-CH3 (mmG1) (A) and IgG1-CH3 (mmG2a) (B) backbones as measured by Dual-binding ELISA and HIC. Introducing
M368L together with T370K or N/R411T had no additional effect compared to the introduction of T370K or N/R411T alone. The T370K-F405L x T370K-K409R, T370K-F405L-N/R411T x T370K-K409R-N/R411T, M368L-T370K-F405L x M368L-T370K-K409R and M368L-F405L-N/R411T x M368L-K409R-N/R411T mixtures in the IgG1-CH3 (mmG1) (A) and IgG1-CH3 (mmG2a) (B) backbones reached higher cFAE efficiencies. Surprisingly, the highest cFAE efficiencies in IgG1-CH3 (mmG1) and IgG1-CH3 (mmG2a) backbones, as measured by both dual-binding ELISA and HIC (see also Figure 3 for exemplary chromatograms), were obtained when the T370K mutation alone was introduced in the K409R parental antibody only, and mixed with parental antibodies containing the F405L mutation only or the combination of F405L with the N/R411T mutation, i.e. F405L x T370K-K409R or F405L-N/R411T x T370K-K409R.

Figure 2C and Figure 3B show that although the combination of F405L x K409R in a IgG1-CH3 (mmG2b) backbone was already able to undergo cFAE, the introduction of mutations in the equivalent positions could still increase the cFAE efficiency. F405L x V370K-K409R or F405L-N-411T x T370K-K409R were the most efficient combinations.

Example 10: Generation of bispecific mouse antibodies, containing mouse-derived constant regions that include matching mutations F405L and K409R alone or in combination with additional CH3 mutations, N/R411T and V370K, respectively, by controlled Fab-arm exchange

To verify that the identified combinations also supported efficient cFAE in the context of an entire mouse constant domain, they were introduced in chimeric constructs containing mouse-derived constant regions (Figure 4A). This resulted in the following constructs:

<table>
<thead>
<tr>
<th>Table 3: Chimeric constructs containing mouse-derived constant regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmIgG1-7D8</td>
</tr>
<tr>
<td>mmIgG1-7D8-K409R</td>
</tr>
<tr>
<td>mmIgG1-7D8-T370K-K409R</td>
</tr>
<tr>
<td>mmIgG2a-7D8</td>
</tr>
<tr>
<td>mmIgG2a-7D8-K409 R</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>mmIgG2a-7D8-T370K-K409 R</td>
</tr>
<tr>
<td>mmIgG2b-7D8</td>
</tr>
<tr>
<td>mmIgG2b-7D8-K409 R</td>
</tr>
<tr>
<td>mmIgG2b-7D8-T370K-K409 R</td>
</tr>
<tr>
<td>mmIgG3-7D8</td>
</tr>
<tr>
<td>mmIgG3-7D8-K409 R</td>
</tr>
</tbody>
</table>

Mixtures of constructs were subjected to cFAE (as described in Example 9) and dual-binding ELISA (as described in Example 7) and HIC analysis (as described in Example 8) was performed to determine the amount of bispecific product.

Figure 4 shows that, like for the chimeric constructs containing mouse CH3 domains, introduction of the matching mutations F405L and K409R in the chimeric constructs containing the entire mouse constant regions, only resulted in sub-efficient cFAE (approx. 80%) in a mmIgG2b backbone (Figure 4B) and almost no cFAE in mmIgG1 and mmIgG2a backbones. Surprisingly, the constructs containing the entire constant region of mmIgG3 enabled sub-efficient cFAE (approx. 80%) (Figure 4A).

High cFAE efficiencies such as 95-100% and 86-95%, as measured by both dual-binding ELISA and HIC, respectively, were observed in the chimeric constructs containing the entire mouse constant regions when the T/V370K mutation was introduced in the K409R parental antibody (mmIgG1-T370K-K409R, mmIgG2a-T370K-K409R and mmIgG2b-V370K-K409R), and mixed with parental antibodies containing the F405L mutation only (mmIgG1-F405L, mmIgG2a-F405L and mmIgG2b-F405L) or the combination of F405L with the N/R411T mutation (mmIgG1-F405L-N411T, mmIgG2a-F405L-R411T and mmIgG2b-F405L-N411T) (Figure 4C). Figure 5 shows exemplary HIC chromatograms.

Example 11: differential protein A purification to polish bispecific mouse antibodies
In order to facilitate differential protein A purification for cases where the presence of residual parental antibodies are undesired, a set of mutations in the CH2 domain were designed that disrupt (I253D) or enhance (P307T, Q309L) binding of mmlG2b to protein A. These mutations were introduced into a set of parental mmlG2b antibodies to yield mmlG2b-I253D-F405L and mmlG2b-P307T-Q309L-K409R and purified proteins were assessed for their ability to bind the Z-domain (an engineered analogue if the IgG-binding domain of protein A) by isothermal titration calorimetry. Figure 6A shows that compared to mmlG2b-F405L, the mmlG2b-I253D-F405L construct does not bind the Z-domain and mmlG2b-P307T-Q309L-K409R binds the Z-domain 5-fold tighter.

Mixtures of constructs were subjected to cFAE (as described in Example 9) and purified by protein A affinity chromatography, basically as described in Example 6, with the exception that a 3-step pH gradient was used for elution (pH 7.2, pH 4.0, pH 3.4). The flow-through and elution fractions were subsequently analyzed by HIC. Figure 6B shows that the mmlG2b-I253D-F405L parental antibody does not bind to protein A and can only be detected in the flow through. In contrast, mmlG2b-P307T-Q309L-K409R parental antibody binds tightly to protein A and is mainly detected after the pH 3.4 elution. The bispecific mouse antibody mixture was resolved into three fractions (flowthrough, pH elution 4.0 and pH elution 3.4) which contained, respectively, the residual mmlG2b-I253D-F405L parental antibody, the bs-mmlG2b-I253D-F405LxP307T-Q309L-K409R, and the residual mmlG2b-P307T-Q309L-K409R parental antibody (with a trace of bispecific mouse antibody) (Figure 6B and C). Thus highly pure bispecific molecules could be obtained using differential protein A purification from a mixture of parental and bispecific mmlG2b antibodies. Similar results are expected for mmlG2a antibodies with corresponding mutations.

Example 12: Generation of bispecific rat antibodies, containing rat-derived constant regions that include mutations F405L and K409R alone or in combination with additional CH3 mutations, N/S411T and S/I T370K, respectively, by controlled Fab-arm exchange.

To assess whether these identified combinations of mutations were also relevant for other rodent species, like rats (Rattus norvegicus), they were introduced in chimeric constructs containing rat-derived constant regions (Figure 7A). This resulted in the following constructs:
**Table 4: Chimeric constructs containing rat-derived constant regions**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>rnlG1-7D8</td>
<td>rnlG1-2F8</td>
</tr>
<tr>
<td>rnlG1-7D8-K409 R</td>
<td>rnlG1-2F8-F405 L</td>
</tr>
<tr>
<td>rnlG2a-7D8</td>
<td>rnlG1-2F8-F405 L-N411T</td>
</tr>
<tr>
<td>rnlG2a-7D8-K409 R</td>
<td>rnlG2a-2F8</td>
</tr>
<tr>
<td>rnlG2b-7D8</td>
<td>rnlG2a-2F8-F405 L</td>
</tr>
<tr>
<td>rnlG2b-7D8-K409 R</td>
<td>rnlG2a-2F8-F405 L-N411T</td>
</tr>
<tr>
<td>rnlG2b-7D8-S370 K-K409 R</td>
<td>rnlG2b-2F8</td>
</tr>
<tr>
<td>rnlG2c-7D8</td>
<td>rnlG2b-2F8-F405 L</td>
</tr>
<tr>
<td>rnlG2c-7D8-K409 R</td>
<td>rnlG2b-2F8-F405 L-N411T</td>
</tr>
<tr>
<td>rnlG2c-7D8-T370 K-K409 R</td>
<td>rnlG2c-2F8</td>
</tr>
<tr>
<td>rnlG2c-2F8-F405 L</td>
<td>rnlG2c-2F8-F405 L-S411T</td>
</tr>
</tbody>
</table>

Mixtures of constructs were subjected to cFAE (as described in Example 9) and dual-binding ELISA (as described in Example 7) and HIC analysis (as described in Example 8) was performed to determine the amount of bispecific product.

**Figure 7** shows that, introduction of the matching mutations F405L and K409R in the chimeric constructs containing the entire rat constant regions, resulted in highly efficient cFAE (such as 95-100% and 86-95%, as measured by both dual-binding ELISA and HIC, respectively) in the rnlG1, rnlG2a and rnlG2c backbones (**Figure 7B**) and almost no cFAE in rnlG2b backbones. High cFAE efficiencies were observed in the chimeric constructs containing the entire rat constant regions when the K409R parental antibody naturally contained the K370 (rnlG1-K409 R and rnlG2a-K409 R) or when the S/T370 K mutation was introduced in the K409 R parental antibody (rnlG2b-S370 K-K409 R and mnlG2c-T370 K-K409 R), and mixed with parental antibodies containing the F405L mutation only.
(rnlG1-F405L, rnlG2a-F405L, rnlG2b-F405L and rnlG2c-F405L) or the combination of F405L with the N411T mutation (rnlG1-F405L-N411T, rnlG2a-F405L-N411T, rnlG2b-F405L-N411T and rnlG2c-F405L-S411T) (Figure 7C).

Example 13: Culturing bone marrow-derived macrophages
Bone marrow was isolated from the hind legs of BALB/c mice (Charles Rivers Laboratories) by flushing the femoral and tibial bones using an insulin syringe filled with bone marrow medium (DMEM (Cambrex, product # BE12-709F) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin (Cambrex, product # DE17-603E), 10% (v/v) heat-inactivated donor bovine serum (Gibco, product # 10371-029) and 2 mM L-glutamine (Cambrex, product # US17-905C)) until the bones turned white. The cells were collected in a 50 ml tube and the volume was adjusted to 30 ml with bone marrow medium. After passing through a cell strainer, the cells were seeded in petri dishes at a cell concentration of 1.25x10^5 cells/mL in 10 mL. Cells were cultured for 7-8 days at 37°C/5% CO_2 in the presence of 50 U/mL M-CSF (PeproTech Inc., product # 315-02). After 3-4 days of incubation, 5 ml/dish fresh bone marrow medium with M-CSF was added.

Example 14: Antibody-dependent Cellular Phagocytosis (ADCP)
On day 7-8, bone-marrow derived macrophages were washed with PBS and harvested by incubating the cells for 10 min at 37°C with 2 mL Versene (Gibco, product # 15040-033). Detached cells were collected in a 50 ml tube and Versene was inactivated by adding bone marrow medium. Cells were washed twice and resuspended in 5 mL ADCP working medium (DMEM (Cambrex, product # BE12-917F) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin, 10% (v/v) donor bovine serum, 2 mM L-glutamine) and 2.5% HEPES (Sigma Aldrich, product # H0887)). Cells were counted and the concentration was adjusted to 0.5 x 10^6 cells/mL with ADCP working medium. The bone-marrow derived macrophages were seeded into 96-well culture plates (200 µL/well) and allowed to adhere overnight at 37°C, 5% CO_2.

The following day, Daudi cells (ATCC; CCL-213)(cultured in RPMI 1640 (Lonza) supplemented with 10% (v/v) heat-inactivated donor bovine serum, 2 mM L-glutamine (Lonza), 1 mM sodium pyruvate (Lonza), 50 IU/mL penicillin, and 50 µg/mL streptomycin) were harvested and labeled for 25 min at 37°C with 0.01 nM Calcein-AM (Molecular probes, product # C-3100), according to the manufacturer's instructions. Labeled Daudi cells were
washed twice with ADCP working medium, counted and the concentration was adjusted to 1x10⁶ cells/ml. Supernatant from the bone-marrow derived macrophages culture plates was removed and Daudi target cells (T) were added to the macrophage effector cells (E) at an E:T ratio of 1:1 in the presence of a fixed antibody concentration of 1 µg/ml. After a 4 h incubation at 37°C/5% CO₂, target cells were washed away with PBS and macrophages were detached with Trypsin-EDTA (Gibco, product # 15400-054) and stained with F4/80-PE (AbD Serotec) and CD19-APC (DAKO, Glostrup, Denmark). ADCP was evaluated on a FACSCanto II flow cytometer (BD Biosciences) and defined as percent age of macrophages that had phagocytized. Percentage of phagocytosis was calculated using the following gate settings: the percent age of calcein-AM-positive and CD19-negative cells within the F4/80-positive cells.

**Figure 8** shows that the K409R mutation alone or in combination with T370K (in mmIgGl and mmIgGl2a) or V370K (in mmIgGl2b) had no effect on the phagocytic activity of the mouse antibody backbones. Introduction of point-mutation described in literature to affect effector functions did, in some cases, influence the phagocytic activity. For example, introducing D265A diminished the phagocytic activity in both mmIgGl-T370K-K409R and mmIgGl2a-T370K-K409R backbones, and completely inactivated the phagocytic activity in a mmIgGl2b-V370K-K409R backbone. Introduction of the L235E and N324D point-mutations, either alone or combined, had no effect on the phagocytic activity in mmIgGl2a-T370K-K409R, whereas introducing L235E in combination with D265A completely inactivated the phagocytic activity. The mutation-combinations L234A-L235A, L234A-L235A-D265A or L234A-L235A-N324D also completely inactivated the phagocytic activity in mmIgGl2a-T370K-K409R. Introduction of the L234F and K322A point-mutations alone had no effect on the phagocytic activity in mmIgGl2b-V370K-K409R. The mutation-combinations L234F-D265A or L234F-D265A-K322A also completely inactivated the phagocytic activity in mmIgGl2b-V370K-K409R.

**Example 15: Isolation of mouse splenocytes**

Splenocytes were extracted from freshly removed spleens of untreated BALB/cJ mice (Charles River Laboratories) by using the mouse spleen dissociation kit (Miltenyi Biotec, product # 130-095-926) and the GentleMACS dissociator (Miltenyi Biotec, product # 130-093-235) as per the manufacturer's instructions. In short, spleens were added to GentleMACS C tubes (Miltenyi Biotec, product # 130-093-237) each containing 2.4 ml 1x Buffer S, 50 µL Enzyme D and 15 µL Enzyme A and placed on the GentleMACS dissociator.
GentleMACS programs m_spleen_02 was run, followed by incubation for 15 minutes at 37°C in a water bath. Subsequently GentleMACS programs m_spleen_03 was run, cells were collected and a single cell suspension was made by passing the cells through a cellstrainer. The cell strainer was washed with 2.5 ml 1x buffer S and cells were pelleted at 300 g for 10 min and resuspended in 10 ml PEB buffer (PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA). Splenocytes were counted using Burker turk solution and counting chamber.
**Example 16 In vitro cytotoxicity induced by HER2 xmmCD3 bispecific mouse antibodies using mouse splenocytes as effect or cells**

CD3 is a protein complex that is associated with the T cell receptor α and β chain expressed on mature T cells. Combination of a CD3 specific antibody Fab-arm with a tumor antigen specific antibody Fab-arm in a bispecific antibody would result in the specific retargeting of T cells to tumor cells, leading to T cell mediated tumor cell lysis. Likewise, CD3 positive T cells could be targeted to other derailed cells in the body, to infected cells or directly to pathogens. To assess whether mouse T-cells could be retargeted to tumor cell lines *in vitro*, various bispecific antibodies were generated using anti-human HER2 (HER2-169) and anti-mouse CD3s (145-2C11) antibody heavy and light chain variable region sequences.


15 VH HER2-169, SEQ ID NO: 62:
QVQLVQSGAEVKPGASVKVSCKASGYTFTNYGISWVRQAPGQGLEWMGWLASAYSGNTIYAAKLLQRGRVTMTTDTSTTTAYMLRDSELQPSLALGLVQLSRRSLRSDTAVYYCARDRIVVRPDYFDYWGQGTTLVT1SSLEPEDFAVYYCQORSNWPTFGQGTKVEIK

VL HER2-169, SEQ ID NO: 63:
ELVLTQSPATLSPGERATLSCRASQSVSSYLAWSVQKPGQAPRLLV/DASN/RATGIPARFSGS GSGTDFTLTISSLEPEDFAVYYCQORSNWPTFGQGTKVEIK


25 VH 145-2C11, SEQ ID NO: 64:
EVQLVESGGGLVQPGKSLKLSCESAGFTFSGYGMHWVRQAPGRGLESVAYFTSSSNIKYADAVKGRFTVSRDNAKNLFLQMNILKEDTAMYYCARFDWKNYWQGTMVT1SSLEPEDFAVYYCQORSNWPTFGQGTKVEIK

30 VL 145-2C11, SEQ ID NO: 65:
DIQMTQSPSSLAPLSGDRVTICNQASQDINSNYLNWYQQKPGKAPKLIIYTYNKLAGVSFGSGSR DSSFTISSLSEDIGSYYCQQYNYWPWFGPGTKLEIK
The VH and VL sequences were introduced into chimeric constructs containing mouse-derived constant regions that contained F405L, T370K-K409R, D265A-F405L-N411T, or D265A-T370K-K409R mutations. This resulted in the following constructs:

**Table 5: HER2 and mmCD3 chimeric constructs**

<p>| | |</p>
<table>
<thead>
<tr>
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</tr>
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<tbody>
<tr>
<td>mmIgG1-HER2-169-T370K-K409R</td>
<td>mmIgG1-145-2C11-F405L</td>
</tr>
<tr>
<td>mmIgG1-HER2-169-D265A-T370K-K409R</td>
<td>mmIgG1-145-2C11-D265A-F405L-N411T</td>
</tr>
<tr>
<td>mmIgG2a-HER2-169-T370K-K409R</td>
<td>mmIgG2a-145-2C11-F405L</td>
</tr>
</tbody>
</table>

Bispecific antibodies using these constructs (Table 5) and mmIgG1-7D8-T370K-K409R (Table 3) were generated (as described in Example 9) and HIC analysis (as described in Example 8) was performed to determine the amount of bispecific product.

The HER2x mmCD3 bispecific antibodies (and CD20x mmCD3 included as control) were then tested in an *in vitro* cytotoxicity assay using AU565 cells as target cells (T) and mouse splenocytes as effector cells (E). For this, AU565 cells (ATCC; CRL-2351) were cultured to near confluence. Cells were washed twice with PBS, and trypsinized for 5 minutes at 37°C. 12 mL culture medium was added to inactivate trypsin. After being collected and washed, the cells were resuspended in 10 mL culture medium and a single cell suspension was made by passing the cells through a cellstrainer. 100 μL of a 4x10⁵ cells/mL suspension was added to each well of a 96-well culture plate, and cells were incubated at least 3 hrs at 37°C, 5% CO₂ to allow adherence to the plate. Mouse splenocytes were isolated as described in Example 15. Isolated splenocytes were resuspended in culture medium to a final concentration of 8x10⁶ cells/mL. Culture medium was removed from the adhered AU565 cells, and replaced with 50 μL/well 2x concentrated antibody dilution and 50 μL/well splenocyte suspension (final ratio E:T = 10:1). Plates were incubated for 3 days at 37°C, 5% CO₂. Supernatants were removed and plates were washed twice with PBS. To each well 150 μL culture medium and 15 μL Alamar blue was added. Plates were incubated for 4 hours at 37°C, 5% CO₂, and absorbance (OD 590 nm) was measured (Envision, Perkin Elmer).

Figure 9A and B show that all bispecific HER2x mmCD3 antibodies induced dose-dependent T-cell mediated killing of AU565 cells in an *in vitro* cytotoxicity assay with splenocytes. Killing
was critically dependent on the presence of a tumor-targeting Fab-arm (bs-mmIgG1-145-2C11- F405LxHER2-169-T370 K-K409 R, bs-mmIgG1-145-2C11-D265A-F405L-N411TxHER2-169-D265A-T370 K-K409 R, bs-mmIgG2a-145-2C11-1-F405LxHER2-169-T370K-K409 R), whereas control antibodies (CD3 monospecific (mmIgGl -145-2C11-F405L, mmIgG1-145-2C11-D265A-F405L-N411T, mmIgG2a-145-2C11-F405L), HER2 monospecific (mmIgGl -HER2-169-T370 K-K409 R, m mIgG1-HER2-169-D265A-T370 K-K409 R, mmIgG2a-HER2-169-T370 K-K409 R), irrelevant monospecific (mmIgG1-7D8-T370 K-K409 R) and irrelevant bispecific (bs-mmIgG1-145-2C11-F405Lx7D8-T370K-K409 R)) did not induce T cell cytotoxicity. As shown in Figure 9A, the presence of the D265A mutation in the mmIgGl backbone, and thus the inability to interact with Fc-receptors, did not impact the potential to induce dose-dependent cytotoxicity of AU565 cells using splenocytes.

Example 17: Pharmacokinetic (PK) analysis of bispecific mouse antibody variants

The mice in this study were housed in a barrier unit of the Central Laboratory Animal Facility (Utrecht, The Netherlands) and kept in filter-top cages with water and food provided ad libitum. All experiments were approved by the Utrecht University animal ethics committee. 8-11 weeks old, female C57Bl/6J mice (Charles River Laboratories) were injected intravenously with 100 μg wild type antibody (mmIgG1-2F8 or mmIgG2a-b12), mmIgGl-based bispecific antibody (F405L-N411TxT370 K-K409 R), mmIgG2a-based bispecific antibody (F405L-R411TxT370 K-K409 R), mmIgGl-based bispecific antibody with additional D265A mutations or mmIgG2a-based bispecific antibody with additional L234A-L235A mutations, using 3 mice per group. 50 μL blood samples were collected from the saphenous vein at 10 minutes, 4 hours, 1 day, 2 days, 7 days, 14 days and 21 days after antibody administration. Blood was collected into heparin containing vials and centrifuged for 5 minutes at 10,000 x g. Plasma was stored at -20 °C until determination of antibody concentrations.

Antibody concentrations were determined using an EGFR ELSA. For this assay, EGFR-ECDHis (Genmab), coated to 96-well Microlon ELSA plates (Greiner, Germany) at a concentration of 2 μg/mL was used as capturing antigen. Alternatively, antibody concentrations were determined using a gp120 ELISA, for which, gp120-JRFL (Progenies), coated to 96-well Microlon ELSA plates at a concentration of 0.5 μg/mL was used as capturing antigen. After blocking plates with PBS supplemented with 0.2% bovine serum albumin, the plates were washed and samples were added, serially diluted with ELSA buffer (PBS supplemented with 0.05% Tween 20 and 0.2% bovine serum albumin), and incubated
on a plate shaker for 1 h at room temperature (RT). Plates were subsequently washed and incubated with HRP-labeled goat anti-mouse IgG immunoglobulin (# 115-036-072, Jackson, West Grace, PA) and developed with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Roche, Mannheim, Germany). The reaction was stopped after 30 min by adding 2% oxalic acid to the wells. Absorbance was measured in a microplate reader (Biotek, Winooski, VT) at 405 nm.

Plasma clearance rates (mL/day/kg) were calculated based on the area under the curve (AUC), according to the following equation:

Plasma clearance = Dose (g/kg) / AUC (g/mL/day)

Data analysis was performed using Graphpad prism software.

Figure 10 A shows total antibody plasma concentrations over time. The shape of the PK profiles is identical in all groups. Figure 10B shows plasma clearance rates. No statistically significant differences in the clearance rates were observed between all groups. These data indicate that introduction of the matching mutations F405L-N411T and T370K-K409R in mmIgGl or F405L-R411T and T370K-K409R in mmIgG2a, nor the D265A mutation in mmIgGl or the L234A-L235A mutations in mmIgG2a had any effect on the pharmacokinetic properties.

Example 18: in vivo anti-tumor efficacy of bispecific mouse antibody variants in T-cell redirection model

To assess whether mouse T-cells could be redirected to tumor cells in vivo, various bispecific antibodies were generated using anti-gp75 (CTA99; tumor-specific arm), anti-gp120 (b12; irrelevant arm) and anti-mouse CD3s (145-2C11) antibody heavy and light chain variable region sequences.

CTA99 (WO 2009114585 A1) hereby incorporated by reference in its entirety, including sequence disclosures.

VH CTA99, SEQ ID NO: 66:
EVQLQSQGELVRPGALVKLSCKTSGFNKDYFLHLVRQRPDQGLESIWI NPDINGNTVD PKFGTASLTSADTSSNTYLSLGTSEDATAYFCTTRDYTYEKAALDYWGQASWSS

VL CTA99, SEQ ID NO: 67:
b12 (Barbas, CF. J Mol Biol. 1993 Apr 5;230 (3):812-23.) hereby incorporated by reference in its entirety, including sequence disclosure.

 VH b12, SEQ ID NO: 68:
QVQLVQGGAEVKKPGASVKVSCQASGRYFSNQVHKVRQAPGQRFQEWFGW ISPYGKPEKFDQD RVTFTADTSANTAYMLRSLRSDATAVYYCARVPYSWQDPSQDNYYMDILVWKGTTTVI VSS
VL b12, SEQ ID NO: 69:
ELTQSPGTLSSPGGERATFCSRSHSIRSRVAVYHQKPGQAPRLVIHGVSNNRASGIDRFSSGSIG TFDTLTTIRVEPEDFALYYCQVYGASSYTQGQGTVKLERK

 The VH and VL sequences were introduced into chimeric constructs containing mouse IgG2a-derived constant regions that contained L234A-L235A-T370 K-K409 R or L234A-L235A-F405L-R411T mutations. This resulted in the following constructs:

<table>
<thead>
<tr>
<th>Table 6: gp75 and mmCD3 chimeric constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmIgG2a-b12-L234A-L235A-T370K-K409R</td>
</tr>
</tbody>
</table>

 Bispecific antibodies using these constructs (Table 6) and mmIgG1-7D8-T370K-K409 R (Table 3) were generated (as described in Example 9) and HIC analysis (as described in Example 8) was performed to determine the amount of bispecific product.

 The mice in this study were housed in a barrier unit of the Central Laboratory Animal Facility (Utrecht, The Netherlands) and kept in filter-top cages with water and food provided ad libitum. All experiments were approved by the Utrecht University animal ethics committee.

 8-11 weeks old, female C57Bl/6J mice (Charles River Laboratories) were injected subcutaneously into the right flank with 1 x 10^5 B16/F10 cells (ATCC; CRL-6475) in PBS. The B16/F10 cells were previously cultured to 70% confluency in IMDM medium.
supplemented with HEPES, L-Glutamine (Lonza, cat #: BE12-722F), 10% Donor Bovine Serum with Iron (Life Technologies, 10371-029) and Pen/Strep (Lonza, cat DE17-603E).

The mice received intravenous injections of mmIgG2a-derived 145-2C11xCTA99 bispecific antibodies (bs-mmIgG2a-L234A-L235A-F405L-R411TxlL234A-L235A-T370K-K409R) or their monovalent counterparts (145-2C11xbl2 and b12xCTA99) at 6 and 8 (or 9) days after tumor cell injection. The antibodies were dosed at concentrations ranging from 0.005 mg/kg to 5 mg/kg. Tumor-size was subsequently followed over time.

Figure 11 shows that whereas doses of 0.005 mg/kg and 0.05 mg/kg mmIgG2a-derived 145-2C11xCTA99 bispecific antibody did not inhibit B16/F10 tumor growth, doses of 0.5 mg/kg or 5 mg/kg mmIgG2a-derived 145-2C11xCTA99 bispecific antibody could inhibit tumor growth, compared to equal doses of their monovalent counterparts (145-2C11xbl2 and b12xCTA99).
Claim s

1. A heterodimeric protein comprising:

a first polypeptide of a first homodimeric protein, said first polypeptide comprising a first
variable region having a first binding specificity and a first Fc region, said first Fc region
comprising a first CH3 domain; and

a second polypeptide of a second homodimeric protein, said second polypeptide comprising
a second variable region having a second binding specificity and a second Fc region, said
second Fc region comprising a second CH3 domain,

wherein the variable regions may originate from any species and the Fc regions originate from
a rodent species, and wherein the first CH3 domain comprises an amino acid selected from
Gly, Ala, Val, Leu, Me, Ser, Lys, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr and Met at
position 370 and a substitution of the amino acid residue at position 409 selected from Gly,
Ala, Val, Me, Ser, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr, and Thr, and the second CH3
domain comprises a substitution of the amino acid residue at position 405 selected from Ala,
Val, Leu, Me, Ser, Lys, His, Asp, Asn, Glu, Gin, Trp, Tyr, and Thr, relative to the wild type
IgG isotype from said rodent species when using EU numbering index.

2. The heterodimeric protein according to claim 1, wherein the Fc regions are of mouse
or rat origin, preferably Mus musculus or Rattus norvegicus.

3. The heterodimeric protein according to any of the above claims wherein the variable
regions are of human origin or are humanized rodent variable regions.

4. The heterodimeric protein according to any of the above claims, wherein the first and
second polypeptides comprise human variable regions and rodent Fc regions.

5. The heterodimeric protein according to claim 4, wherein the rodent Fc regions are
Mus musculus or Rattus norvegicus Fc regions.

6. The heterodimeric protein according to claim 5, wherein the rodent Fc regions are
Mus musculus Fc regions.
7. The heterodimeric protein according to any of the above claims, wherein the amino acid residue at position 370 of the first CH3 domain is Lys (K).

8. The heterodimeric protein according to any of the above claims, wherein the amino acid residue at position 409 of the first CH3 domain is substituted with an Arg (R).

9. The heterodimeric protein according to any of the above claims, wherein the amino acid residue at position 405 of the second CH3 domain is substituted with a Leu (L).

10. The heterodimeric protein according to any of the above claims, wherein the first CH3 domain comprises a Lys at position 370 and an Arg at position 409 and the second CH3 domain comprises a Leu at position 405.

11. The heterodimeric protein according to any of the above claims, wherein the second CH3 domain further comprises a Thr at position 411.

12. The heterodimeric protein according to any of the above claims wherein the amino acid residue at position 253 of the first homodimer is substituted with Asp (D).

13. The heterodimeric protein according to any of the above claims wherein the amino acid residues at positions 307 and/or 309 of the second homodimer are substituted with Thr (T) and Leu (L), respectively.

14. The heterodimeric protein according to any of the above claims, wherein said first homodimeric protein and said second homodimeric protein are selected from the group consisting of (i) an antibody (ii) a fusion protein comprising an Fc region, such as an Fc region fused to a receptor, cytokine or hormone, (iii) an antibody conjugated to a prodrug, peptide, drug or a toxin, and (iv) a fusion protein comprising an Fc region conjugated to a prodrug, peptide, drug or a toxin.

15. The heterodimeric protein according to claim 14, wherein the fusion protein comprising an Fc region further comprises an antigen binding region, such as a Fragment antigen-binding (Fab) or a single-chain variable fragment (scFv).

16. The heterodimeric protein according to any of the above claims, wherein the first and second polypeptides both comprise a CH1 region, a hinge region, a CH2 region and a CH3 region.
17. The heterodimeric protein according to any of the above claims, wherein said first homodimeric protein is a full-length antibody.

18. The heterodimeric protein according to any of the above claims, wherein said second homodimeric protein is a full-length antibody.

19. The heterodimeric protein according to any of the above claims, wherein said first and second homodimeric proteins are both antibodies and bind different epitopes.

20. The heterodimeric protein according to any of the above claims, wherein the Fc region of the first polypeptide is of an isotype and species selected from the group consisting of mmIgG1, mmIgG2a, mmIgG2b, mmIgG3, mmIgG1, mmIgG2a, mmIgG2b and mmIgG2c and the Fc region of the second polypeptide is of an isotype selected from the group consisting of mmIgG1, mmIgG2a, mmIgG2b, mmIgG2c, mmIgG3, mmIgG1, mmIgG2a, mmIgG2b and mmIgG2c.

21. The heterodimeric protein according to any of the above claims, wherein the Fc regions of both said first and said second polypeptides are of the same isotype and species.

22. The heterodimeric protein according to any of the above claims, wherein the Fc regions of both said first and said second polypeptides are of Mus musculus origin and are both of the same isotype, such as IgG1, IgG2a or IgG2b isotype.

23. Use of a heterodimeric protein according to any of the above claims for exploring functions of corresponding fully human heterodimeric proteins having similar binding specificities in a rodent model.

24. Use of a heterodimeric protein according to any of the above claims for exploring functions of corresponding fully human heterodimeric proteins having identical binding specificities in a rodent model.

25. Use according to claims 19 or 20 wherein the explored function are selected form the list comprising; mechanisms of action, effector functions, toxicity, unwanted mechanisms and side effects, anti-tumor efficacy, treatment efficacy, T-cell mediated anti-tumor efficacy, immune modulation, pharmacodynamics and/or pharmacokinetics of said heterodimeric protein.
26. An in vitro method for generating a heterodimeric protein according to any of claims 1-20, said method comprising the following steps:

a) providing a first homodimeric protein comprising a first variable region having a first binding specificity, a hinge region, and a first Fc region comprising a first CH3 domain; and

b) providing a second homodimeric protein comprising a second variable region having a second binding specificity, a hinge region, and a second Fc region comprising a second CH3 domain, wherein the variable regions may originate from any species and the Fc regions originate from a rodent species, and wherein the first CH3 domain comprises an amino acid selected from Gly, Ala, Val, Leu, Ile, Ser, Lys, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr and Met at position 370 and a substitution of the amino acid residue at position 409 selected from Gly, Ala, Val, Me, Ser, Arg, His, Asp, Asn, Glu, Gin, Trp, Phe, Tyr and Thr, and the second CH3 domain comprises a substitution of the amino acid residue at position 405 selected from Ala, Val, Leu, Me, Ser, Lys, His, Asp, Asn, Glu, Gin, Trp, Tyr and Thr, relative to the wild type IgG isotype from said rodent species when using EU-index numbering and wherein the sequences of said first and second CH3 regions are different and are such that a heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions,

c) incubating said first homodimeric protein together with said second homodimeric protein under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide bond isomerization, and

d) obtaining said heterodimeric protein.
FIG. 4

A

hsVH + hsVL
mmlG1 +/- F405L(-N411T)/T370K-K409R
mmlG2a +/- F405L(-R411T)/T370K-K409R
mmlG2b +/- F405L(-N411T)/V370K-K409R
mmlG3

B

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FIG. 7

A

hsVH + hsVL
rnlG1 +/- F405L(-N411T)/K409R
rnlG2a +/- F405L(-N411T)/K409R
rnlG2b +/- F405L(-N411T)/S370K-K409R
rnlG2c +/- F405L(-S411T)/T370K-K409R

B

WT x WT

F405L x K409R

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C

F405L x S/T370K-K409R

F405L-N/S411T x S/T370K-K409R

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<td>rnlG2c</td>
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**FIG. 10**

### A

Plasma concentration (µg/ml)

```
-○- mmilG1-2F8
-■- bs-mmilG1-2F8-F405L-N411T x7D8-T370K-K409R
-□- bs-mmilG1-2F8-D265A-F405L-N411T x7D8-D265A-T370K-K409R
-○- mmilG2a-b12
-■- bs-mmilG2a-2F8-F405L-R411T x7D8-T370K-K409R
```

Time (days)

### B

Clearance F0 -> d14

```
D'/1000/AUC (ml/day/kg)
```

```
-○- mmilG1
-■- bs-mmilG1-2F8-F405L-N411T x7D8-T370K-K409R
-□- bs-mmilG1-D265A-F405L-N411T x7D8-T370K-K409R
-○- mmilG2a
-■- bs-mmilG2a-L234A-L235A-F405L-R411T x7D8-T370K-K409R
```
FIG. 11

A

- ▼ CTA99x145-2C11-mmlG2a-LALA 5mg/kg
- ◇ CTA99x145-2C11-mmlG2a-LALA 0.5mg/kg
- ▲ CTA99x145-2C11-mmlG2a-LALA 0.05mg/kg
- □ CTA99x145-2C11-mmlG2a-LALA 0.005mg/kg
- ○ PBS
- ▼ Treatment

Days after tumor inoculation

B

- ○ PBS
- ▼ b12x145-2C11-mmlG2a-LALA 5mg/kg
- ◇ b12x145-2C11-mmlG2a-LALA 0.5mg/kg
- ▲ CTA99xb12-mmlG2a-LALA 5mg/kg
- □ CTA99xb12-mmlG2a-LALA 0.5mg/kg
- ▼ CTA99x145-2C11-mmlG2a-LALA 5mg/kg
- □ CTA99x145-2C11-mmlG2a-LALA 0.5mg/kg
- ▼ Treatment

Days after tumor inoculation
According to International Patent Classification (IPC) and to both national classification and IPC

**B. FIELDS SEARCHED**

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

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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**Further documents are listed in the continuation of Box C.**

**See patent family annex.**

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  - "Z" document member of the same patent family

**Date of the actual completion of the international search:** 18 March 2016

**Date of mailing of the international search report:** 30/03/2016

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

**Authorized officer:** Lonnoy, Olivier
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