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(54) Title: HUMANISED ANTIBODY AND METHOD OF PRODUCTION THEREOF

(57) Abstract: Described is a method of humanising antibody molecules, said method comprising using CDRs defined by a method which combines the IMGT and Rabat methods for defining CDR sequences. Specifically described is a humanised antibody with binding specificity for AREG and HBEGF and a humanised anti CD20 antibody.

Humanised Antibody and Method of Production Thereof

Field of the Invention

The application relates to the field of antibodies, in particular to humanised antibodies and methods of making such humanised antibodies. The invention also extends to the use of such humanised antibodies in therapeutic uses, such as for the treatment of medical conditions such as cancer.

Background to the Invention

The use of rodent monoclonal antibodies, such as murine monoclonal antibodies, for *in-vivo* therapeutic applications has been shown to be associated with the generation of undesirable immune responses which are generated by the subject to whom the antibodies are administered. Such immune responses can result in the production of antibodies which effectively neutralise the effectiveness of the therapeutic antibody. Such immune responses are typically referred to as human anti-mouse antibody (HAMA) responses. HAMA responses compromise the therapeutic effectiveness of the administered antibody in a number of ways, including impairing the ability of the therapeutic antibody to reach its binding target.

A number of approaches have been developed to address the issue of unwanted HAMA responses being raised against therapeutic antibodies which are administered to individuals. Typically, these approaches involve techniques which result in the replacement of certain components of the mouse antibody with equivalent portions derived from a human antibody. Such approaches can, for example, result in the production of chimeric antibodies which comprise murine variable regions joined to human-derived constant regions. Alternatively, antibodies may be humanized, using techniques such as “CDR grafting” or Composite Human Antibody technology ™, (Antitope, Cambridge, UK). Composite Human Antibodies are entirely human in origin and comprise multiple segments of human variable region sequence from different human antibodies. Such technology may thus be used to produce a fully humanized monoclonal antibody which displays a single binding specificity and which has variable

regions in which both framework and CDR regions are derived from human germline immunoglobulin sequences. This contrasts with humanised antibodies produced by "CDR grafting", wherein the complementarity determining regions (CDRs) from a murine antibody are grafted into a framework acceptor sequence, provided by regions of human antibody light and heavy chain variable domains. This results in the production of an antibody which retains the binding specificity of the murine antibody, but where the only non-human components are the grafted murine CDR regions.

With CDR grafting, the therapeutic effectiveness of the resulting humanised antibody can be impaired. For example, it has been observed that simple transplantation of the CDR regions often results in a reduced therapeutic efficacy of the antibody due to the binding affinity of the antibody being diminished. To address this problem, back mutations are often employed, back mutations being the replacement of an amino acid residue at a specific position of the framework sequence (outside of the CDR sequence) so as to improve the binding specificity of the humanised antibody.

A number of different methods of determining the complementarity determining regions of an antibody molecule are known; these include the Kabat method, (Kabat EA et al. (1991), Sequences of proteins of immunological interest, 5th edition. Bethesda:US Department of Health and Human Services), the IMGT method (Lefranc, M.-P., The Immunologist, 7, 132-136 (1999), Brochet, X. et al., Nucl. Acids Res. 36, W503-508 (2008). PMID: 18503082), and the Chothia method (Chothia, C. and Lesk, A.M., J. Mol. Biol., 196, 901-917 (1987); Chothia, C. et al., Nature, 342, 877-883 (1989); and. Al-Lazikani, B. et al., J. Mol. Biol., 273, 927-948 (1997))

In WO2009/127881, the present inventors describe cross-specific antibodies that target both HBEGF and AREG.

Summary of the Invention

As noted above, typically in CDR grafting techniques, the CDRs of a donor antibody are selected using one of the Kabat, IMGT or Chothia methods and, typically, it is necessary to back-mutate certain residues outside the CDR sequences to restore satisfactory affinity. In developing a number of humanised antibodies, the present inventors have

surprisingly shown that, when using the CDR grafting technique, humanised antibodies of high affinity may be readily obtained by using CDRs defined by a method which combines the IMGT and Kabat methods for defining CDR sequences.

Accordingly, in a first aspect of the present invention, there is provided a humanised antibody molecule having a heavy chain, wherein the complementarity determining regions (CDRs) are derived from a non-human donor antibody, wherein
(i) the first amino acid residue and the final amino acid residue of CDRH1 of the humanised antibody molecule are the first amino acid residue of CDRH1 of the donor antibody as defined by the IMGT method and the final amino acid residue as defined by the Kabat method respectively and the CDRH1 comprises the intervening amino acid residues of said donor CDRH1 between said first and final amino acid residues;
(ii) the first amino acid residue and the final amino acid residue of CDRH3 of the humanised antibody molecule are the first amino acid residue of CDRH3 of the donor antibody as defined by the IMGT method and the final amino acid residue as defined by the Kabat method respectively and the CDRH3 comprises the intervening amino acid residues of said donor CDRH3 between said first and final amino acid residues; and
(iii) CDRH2 of the humanised antibody molecule corresponds to CDRH2 of the donor antibody as defined by the Kabat method.

For the avoidance of any doubt, a humanised antibody molecule is a chimeric antibody molecule which comprises a variable domain at least part of which is derived from a human source.

Unless the context demands otherwise, the term “derived” is intended to not only encompass the source of material as being the physical source but also to define material which is structurally identical to the material but which does not originate from the reference source. Thus the CDRs derived from a non-human donor antibody need not necessarily be purified or isolated from the donor antibody or antibody framework.

Optionally, the humanised antibody molecule comprises a light chain. In one embodiment the CDRL1, CDRL2, and CDRL3 of the light chain correspond to CDRL1,

CDRL2, and CDRL3 of the donor antibody as defined by the Kabat method.

As described in the examples, it has been shown that, by employing CDRs defined in this way in a humanised antibody molecule, affinity may be maintained compared to the donor antibody without the need for as many, or indeed, in some embodiments, any additional mutations in the acceptor sequence outside of the CDRs as would be required if all CDRs were defined by the Kabat method or IMGT method alone.

Accordingly, optionally, the humanised antibody of the first aspect of the invention to maintain a predetermined affinity equivalent to the corresponding non-human donor antibody, comprises fewer back mutations in the VH sequence of the human acceptor sequence than the number of back mutations required by a corresponding humanised antibody in which the CDRs are as defined by the Kabat method only to maintain said predetermined level of affinity.

For example, optionally, the humanised antibody of the invention comprises 3 or fewer, such as 2 or fewer back mutations in the VH sequence of the human acceptor sequence. Optionally, the humanised antibody of the invention comprises no back mutations in the VH sequence of the human acceptor sequence.

As described in the Examples, the present inventors have humanised a number of distinct antibodies against different targets using the novel method for humanisation. They have demonstrated that, by using this method, antibodies with excellent binding efficacy may be readily obtained.

In one embodiment, the humanised antibody of the first aspect of the invention is an antibody which is cross-specific to two EGF molecules. For example, the humanised antibody of the invention may have binding specificity for both HBEGF and AREG. For example, in one such embodiment, the humanised antibody has binding specificity for the antigenic fragment of AREG having the amino acid sequence shown as SEQ. ID NO: 1 and the antigenic fragment of HBEGF having the amino acid sequence shown as SEQ. ID NO: 2:

SEQ. ID NO: 1:

K K N P C N A E F Q N F C I H G E C K Y I E H L E A V T C K C Q Q E Y F G E R C G E K S

SEQ. ID NO: 2:

K R D P C L R K Y K D F C I H G E C K Y V K E L R A P S C I C H P G Y H G E R C H

As described in the Examples, humanised antibody molecules according to the present invention based on a murine antibody raised against an antigenic fragment from this region were found to have specificity for both AREG and HB-EGF. In one embodiment, a humanised antibody molecule of the invention with binding specificity for both HBEGF and AREG is an antibody molecule which comprises:

a) a heavy chain having the following complementarity determining regions (CDRs):

CDRH1: SEQ ID NO: 3

CDRH2: SEQ ID NO: 4,

CDRH3: SEQ ID NO: 5,

and, optionally, b)) a light chain having the following complementarity determining regions (CDRs):

CDRL1: SEQ ID NO: 6,

CDRL2: SEQ ID NO: 7,

CDRL3: SEQ ID NO: 8.

SEQ. ID NOS: 3 to 8 are as follows:

SEQ ID NO: 3: GFNIKDSYIH

SEQ ID NO: 4 :WIDPENGNTIYDPKFQG

SEQ ID NO: 5: VSASYRYGFSY

SEQ ID NO: 6: RSNKSLLHTNGNTYLY

SEQ ID NO: 7:RMSNLAS

SEQ ID NO: 8: MQHLEYPYT

In one embodiment the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 9, SEQ. ID NO: 10, or SEQ. ID NO: 11 and/or a VL domain having an amino acid sequence shown as SEQ. ID NO: 12, SEQ. ID NO: 13, or SEQ. ID NO: 14.

SEQ. ID NOS: 9 to 14 are as follows:

SEQ. ID NO: 9:
QVQLVQSGAEVKKPGASVKVSCKASGFNIKDSYIHWVRQAPGQRLEWIGWIDPENGNTIYDPK
FQGRVTITRDTSASTAYMELSSLRSEDTAVYYCVSASYRYGFSYWGQGTLVTVSS

SEQ. ID NO: 10:
QVQLVQSGAEVKKPGASVKVSCKASGFNIKDSYIHWVRQAPGQGLEWMGWIDPENGNTIYDP
KFQGRVTVTRDTSISTAHMELSSLRSDDTAVYYCVSASYRYGFSYWGQGTAVTVSS

SEQ. ID NO: 11:
QVQLVQSGAEVKKPGASVKVSCKASGFNIKDSYIHWFQQAPTRGLEWMGWIDPENGNTIYDP
KFQGRVSITADTSTDTAYMELTSLISEDTAVYYCVSASYRYGFSYWGQGTLVTVSS

SEQ. ID NO: 12:
DIVMTQTPLSLPVTPGEPASISCRSNKSLLHTNGNTYLYWYLQKPGQSPQLLIYRMSNLASGVP
DRFSGSGSGTDFTLKISRVEAEDVGVYYCMQHLEYPYTFGQGTKLEIKR

SEQ. ID NO: 13:
DIVMTQTPLSLPVTPGEPASISCRSNKSLLHTNGNTYLYWYLQKPGQSPQLLIYRMSNLASGVP
DRFSGSGSGTDFTLRISRVEAEDVGVYYCMQHLEYPYTFGGGTKVEIK

SEQ. ID NO: 14:
DIVMTQTPLSLPVTPGEPASISCRSNKSLLHTNGNTYLYWYLQKPGQSPQLLIYRMSNLASGVP
DRFSGSGSGTAFTLKISRVEAEDVGLYYCMQHLEYPYTFGQGTKLEIKR

In a particular embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 9 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 12. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 9 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 13. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid

sequence shown as SEQ. ID NO: 9 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 14. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 10 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 12. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 10 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 13. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 10 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 14. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 11 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 12. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 11 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 13. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 11 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 14.

As described in the examples, the inventors have also applied the method of humanisation to the chimeric anti- CD-20 antibody rituximab. Accordingly, in one aspect of the invention, the humanised antibody molecule has binding specificity for CD20. In one such embodiment, the antibody molecule comprises:

a) a heavy chain having the following complementarity determining regions (CDRs):

CDRH1: SEQ ID NO: 15,

CDRH2: SEQ ID NO: 16,

CDRH3: SEQ ID NO: 17,

and optionally b) a light chain having the following complementarity determining regions (CDRs):

CDRL1: SEQ ID NO: 18,

CDRL2: SEQ ID NO: 19, and

CDRL3: SEQ ID NO: 20.

SEQ. ID NOS: 15 to 20 are as follows:

SEQ. ID NO: 15: GYTFTSYNMH

SEQ. ID NO: 16: AIYPGNGDTSYNQKFKG

SEQ. ID NO: 17: ARSTYYGGDWYFNV

SEQ. ID NO: 18: RASSSVSYIH

SEQ. ID NO: 19: ATSNLAS

SEQ. ID NO: 20: QQWTSNPPT

In one embodiment the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO:21, SEQ. ID NO: 22, or SEQ. ID NO: 23 and/or a VL domain having an amino acid sequence shown as SEQ. ID NO: 24, SEQ. ID NO: 25, or SEQ. ID NO: 26.

SEQ. ID NOS: 21 to 23 correspond to the VH sequences shown as VH1 to VH3 of Figure 9. SEQ. ID NOS: 24 to 26 correspond to the VL sequences shown as VL1 to VL3 of Figure 10.

In a particular embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO:21.

In a particular embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 21 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 24. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 21 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 25. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 21 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 26. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 22 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 24. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 22 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 25. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino

acid sequence shown as SEQ. ID NO: 22 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 26. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 23 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 24. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 23 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 25. In another embodiment, the humanised antibody molecule of the invention comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 23 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 26.

In a second aspect, the invention provides a nucleic acid molecule which encodes a humanised antibody molecule of the first aspect of the invention.

In a third aspect, the present invention provides a method of humanising an antibody molecule, said method comprising producing an antibody molecule in which the heavy chain CDRs of a non-human donor antibody are grafted onto a first human acceptor framework, and optionally the light chain CDRs of a non-human donor antibody are grafted onto a second human acceptor framework, wherein

(i) the first amino acid residue and the final amino acid residue of CDRH1 of the humanised antibody molecule are the first amino acid residue of CDRH1 of the donor antibody as defined by the IMGT method and the final amino acid residue as defined by the Kabat method and the CDRH1 comprises the intervening amino acid residues of said donor CDRH1 between said first and final amino acid residues;

(ii) the first amino acid residue and the final amino acid residue of CDRH3 of the humanised antibody molecule are the first amino acid residue of CDRH3 of the donor antibody as defined by the IMGT method and the final amino acid residue as defined by the Kabat method and the CDRH3 comprises the intervening amino acid residues of said donor CDRH3 between said first and final amino acid residues; and

(iii) CDRH2 of the humanised antibody molecule corresponds to CDRH2 of the donor antibody as defined by the Kabat method.

The skilled person will readily understand what is meant by “grafted onto a human acceptor framework” and will readily understand that the method encompasses any

method of producing an antibody having heavy chain CDRs derived from a non-human donor antibody within a human acceptor framework. The method thus encompasses any method of producing an antibody molecule according to the first aspect of the invention and will thus include methods involving expression of nucleic acid sequence(s) which encode an antibody molecule of the invention.

A fourth aspect of the invention is an antibody molecule obtained by the method of the third aspect of the invention.

A further aspect of the invention provides a humanised antibody molecule of the first or fourth aspect of the invention or a nucleic acid molecule according to the second aspect of the invention for use in medicine.

Also provided is a humanised antibody molecule of the first or fourth aspect of the invention or a nucleic acid molecule according to the second aspect of the invention for use in the treatment of neoplastic disease.

Also provided is a humanised antibody molecule of the first or fourth aspect of the invention or a nucleic acid molecule according to the second aspect of the invention for use in the treatment of a disease or disorder associated with angiogenesis.

Further provided by the invention is a method of treating neoplastic disease in a subject, said method comprising administration to said subject of an effective amount of humanised antibody molecule of the first or fourth aspect of the invention or a nucleic acid molecule of the second aspect of the invention.

Further provided by the invention is a method of treating a disease or disorder associated with angiogenesis in a subject, said method comprising administration to said subject of an effective amount of humanised antibody molecule of the first or fourth aspect of the invention or a nucleic acid molecule of the second aspect of the invention.

The invention also extends to a pharmaceutical composition comprising a humanised antibody molecule of the first or fourth aspect of the invention or a nucleic acid molecule of the second aspect of the invention. In one embodiment of this aspect of the

invention, the pharmaceutical composition further comprises a chemotherapeutic agent and/or an angiogenesis inhibitor.

Also encompassed by the invention is a kit comprising:

(i) a humanised antibody molecule of the first or fourth aspect of the invention or a nucleic acid molecule of the second aspect of the invention; and

(ii) a chemotherapeutic agent and/or an angiogenesis inhibitor for simultaneous, sequential or separate administration with said humanised antibody molecule or nucleic acid molecule.

Brief Description of the Figures

Figure 1 illustrates the VH and VL sequences of the murine Fsn1006 antibody which has binding specificity for both HBEGF and AREG; the CDRs as defined by the Kabat method, the IMGT method and the Chothia method are shown in Table 1.

Figure 2 illustrates VH acceptor frameworks with the Fsn1006 "Fusion CDR" residue sequences and VL acceptor frameworks with the Fsn1006 "Fusion CDR" residue sequences as employed in humanized Fsn 1006 variants; the CDRs are underlined.

Figure 3 illustrates a sequence alignment of the murine Fsn1006 VH sequence with three humanised VH variants.

Figure 4 illustrates a sequence alignment of the murine Fsn1006 VL sequence with three humanised VL variants.

Figure 5 illustrates the results of analysis of binding of the nine humanised antibody constructs to HB-EGF in comparison to the murine antibody (2F7) and the chimeric antibody (H0LO).

Figure 6 illustrates a Western blot of HCTt116 cells treated with HBEGF and FSN 1006 HC2LC2.

Figure 7 illustrates the results of an MTT assay performed on HCT116 cells after 72 hours incubation with three humanised antibody variants of the invention.

Figure 8 illustrates the VH and VL sequences of rituximab; the CDRs as defined by the Kabat method, the IMGT method and the Chothia method are shown in Table 2.

Figure 9 illustrates a sequence alignment of the rituximab VH sequence with three humanised VH variants.

Figure 10 illustrates a sequence alignment of the rituximab VL sequence with three humanised VL variants.

Figure 11 illustrates the results of a flow cytometry experiment in which the binding ability of humanised rituximab antibodies (panels c, d, e, f and g) to CD20 is compared with that of a negative control (panel a), and, as a positive control, rituximab (panel b).

Detailed Description of the Invention

The present invention provides humanised antibody molecules produced by a modified CDR grafting technique in which the heavy chain CDR sequences as employed in the humanised antibody molecule comprise a combination of the CDR residues as defined by the IMGT method and a combination of the CDR residues as defined by the Kabat method.

Antibody Numbering Systems

Amino acid residues in antibody domains are conventionally numbered according to a system devised by Kabat et al. (URL - www.kabatdatabase.com; see also Kabat,E.A., Wu,T.T., Perry,H., Gottesman,K. and Foeller,C. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition. NIH Publication No. 91-3242). The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain). The Kabat definition is based on sequence variability. The Kabat residue designations do not always correspond directly with the linear numbering of the amino acid residues

of the heavy and light chain variable regions of the present invention. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether a framework region or complementarity determining region (CDR), of the basic variable domain structure of the heavy or light chain. The correct Kabat numbering of residues may be determined for any given antibody by alignment of residues in the sequence of the antibody with a standard sequence to which the Kabat numbering has been applied.

Alternative numbering systems include the IMGT system and the Chothia system. The Chothia definition is based on the location of the structural loop regions. The IMGT definition is based on sequence variability, structure and evolutionary data.

Antibody Molecules

An "antibody molecule" is an immunoglobulin, whether natural or partly or wholly synthetically produced. The term also covers any polypeptide, protein or peptides having a binding domain that is, or is homologous to, the binding domain of the humanised antibody molecules of the invention.

Immunoglobulins typically have a heterotetrameric structure comprising two identical heavy chains and two identical light chains, linked together by disulphide bonds. Each heavy and light chain comprises a variable domain which confers the binding specificity of the antigen, with these domains being known as VH and VL domains for the heavy and light chains respectively. Each chain also comprises at least one constant domain, with the light chain having at a single constant domain, designated the CL domain, while the heavy domain comprises three constant domains, CH1, CH2 and CH3. Some antibody isotypes additionally include a further constant domain referred to as the CH4 domain.

In humans, there are 5 different classes of antibodies, namely; IgG, IgA, IgD IgE and IgM.

In one embodiment, the acceptor sequence of the humanised antibodies of the invention is an IgG. Although any subclass may be used, in a particular embodiment, the IgG is of subclass IgG1.

The Fc domain of an antibody typically comprises the last 2 heavy chain constant region domains of each chain. These dimerise to form the Fc domain which is responsible for mediating the effector functions of the antibody, such as ADCC (antibody-dependent cell-mediated cytotoxicity) and complement fixation. The Fc region of the antibody also has a role in the circulatory half-life of the antibody. Modifications can be made to the Fc domain to modulate antibody function.

Within each of the VH and VL domains are 3 complementarity determining regions ("CDRs"), along with 4 associated framework regions ("FRs"). A VH domain typically comprises 3 HCDRs (heavy chain complementarity determining regions), and a VL domain typically comprises 3 LCDRs (light chain complementarity determining regions). Accordingly, a binding member may comprise a VH domain comprising, in sequence, VH CDR1 (or CDRH1), CDR2 (CDRH2) and CDR3 (CDRH3) regions along with a plurality of associated framework regions. A binding member may additionally or alternatively comprise a VL domain comprising VL CDR1(or CDRL1), CDR2(CDRL2) and CDR3 (CDRL3) regions along with associated framework regions. The VH and VL domains typically each comprise four framework regions, FR1, FR2, FR3 and FR4, interspersed between the 3 complementarity determining regions in the following arrangement: FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. The complementarity determining regions which may also be known as hypervariable regions, have a role in conferring the binding specificity of the antibody, or binding fragment.

It is known that fragments of a whole antibody can perform the function of binding antigens. Examples of such binding fragments include, but are not limited to: (i) Fab fragments consisting of the VL, VH, CL and CH1 domains of a heterotetrameric antibody, (ii) F(ab')2 fragments, a bivalent fragment comprising two Fab fragments linked by a disulphide bridge at the hinge region, (iii) Fab' fragment, a Fab fragment with part of the hinge region, (iv) Fd fragments consisting of the VH and CH1 domains of a heterotetrameric antibody, (v) Fv fragments consisting of the VH and VL domains of a heterotetrameric antibody, (vi) scFv (single chain Fv molecules), wherein a VH domain

and a VL domain are linked by a peptide linker, (vii) an isolated CDR, such as CDRH3, and (viii) diabodies, these being multimers of polypeptides which may be multivalent or multispecific fragments produced by gene fusion techniques.

Accordingly, the term "antibody molecule" should be construed as extending to fragments of humanised antibodies of the invention. The invention therefore extends to a fragment of the humanized antibody molecule of the present invention, e.g. the heavy only, or, for example, the variable domain of the heavy chain.

The CDRs of the light chain associate with the CDRs of the heavy chain to confer the binding specificity of an antibody, or antibody binding fragment, in instances where both sets of CDRs are present. It is known that the contribution made by the light chain variable region to the energetics of binding is small relative to the associated heavy chain variable region. Accordingly, isolated heavy chain regions, which comprise, in sequence, the 3 complementarity determining regions (CDRH1, CDRH2, and CDRH3), are known to have an antigen binding capability and are commonly referred to as single domain antibodies. Such antibody fragments of the humanized antibody molecules of the invention are provided by the present invention.

In certain embodiments, the antibody molecule may be selected from the group comprising, but not limited to; a Fab fragment, a Fab' fragment, a scFv (single chain variable fragment), a peptidomimetic, a diabody, or a related multivalent derivative.

Techniques used for the recombinant production of Fab, Fab' and F(ab')2 fragments are well known to the person skilled in the art and include those disclosed in International PCT Patent Publication WO 92/22324, Sawai et al.,"Direct Production of the Fab Fragment Derived From the Sperm Immobilizing Antibody Using Polymerase Chain Reaction and CDNa Expression Vectors",1995, AJRI 34:26-34, Mullinax et al., "Expression of a Heterodimeric Fab Antibody Protein in One Cloning Step", 1992, BioTechniques 12(6):864-869, and Better et al., "*Escherichia coli* Secretion of an Active Chimeric Antibody Fragment" 1988, Science 240:1041-1043, the contents of each of which are hereby incorporated by reference.

Examples of techniques which can be used to produce scFv (single chain Fv fragments) are disclosed in Huston et al., "Protein Engineering of Single-Chain Fv Analogs and Fusion Proteins", *Methods in Enzymology*, vol. 203:46-88 (1991), the contents of which are hereby incorporated by reference.

In certain embodiments, antibody fragments can be derived from full length antibodies by proteolytic digestion according to the method of Morimoto (Morimoto et al., "Single-step purification of $F(ab')_2$ fragments of mouse monoclonal antibodies (immunoglobulins G1) by hydrophobic interaction high performance liquid chromatography using TSKgel Phenyl-5PW" Journal of Biochemical and Biophysical Methods 24:107-117 (1992)). Antibody fragments can also be produced directly by host cells (see Carter et al., Bio/Technology 10:163-167 (1992)).

Single domain binding members

The invention extends to single binding domains which are based on the VH region of the humanised antibodies of the antibodies of the invention.

Accordingly, in certain further embodiments of the present invention, there is provided a binding member comprising, consisting or consisting essentially of a single binding domain derived from a humanised antibody of the invention. In certain embodiments, the single binding domain is derived from the amino acid sequence of the VH (heavy chain variable domain), for example as defined in SEQ ID NO:10, of a humanized antibody molecule of the invention. Such a binding domain may be used as a targeting agent to AREG and HBEGF, as it is known that immunoglobulin VH domains are capable of binding to target antigens in a specific manner.

In certain embodiments, the amino acid sequences of the VH or VL domains may comprise at least one back mutation, said back mutation being the replacement of an amino acid residue at a specific position of the sequence so as to improve the binding specificity of the humanised antibody or fragment thereof to its target and/or to enhance the therapeutic efficacy of the humanised antibody. Typically such modification can be made to the framework residues within the light and heavy chain variable regions so as to decrease the immunogenicity of the antibody. However, as described

herein , such back mutations may not be necessary. In certain embodiments, further engineering techniques can be used to modify the antibodies of the present invention, for example by including modifications of the Fc region which can alter serum half life, complement fixation, Fc receptor binding and/or antigen dependent cellular cytotoxicity.

Further, in certain embodiments, the antibodies or antibody fragments can be produced which have altered glycosylation patterns. In certain embodiments, an antibody molecule of the invention is altered to increase or decrease the extent to which the antibody molecule is glycosylated. Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of a carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X -threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N- aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used. In certain further embodiments, the antibodies can be PEGylated by reacting the antibody with a plyethylene glycol (PEG) derivative. In certain embodiments, the antibody is defucosylated and therefore lacks fucose residues.

In certain embodiments, modifications in the biological properties of an antibody may be accomplished by selecting substitutions that affect (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Amino acids may be grouped according to similarities in of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)): (1) non-polar: Ala (A), VaI (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M); (2) uncharged polar: GIy (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gin (Q); (3) acidic: Asp (D), GIu (E); (4) basic: Lys (K), Arg (R), His(H). Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties: (1) hydrophobic: Norleucine, Met, 5 Ala, VaI, Leu, He; (2)

neutral hydrophilic: Cys, Ser, Thr, Asn, GIn; (3) acidic: Asp, GIu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: GIy, Pro; (6) aromatic: Trp, Tyr, Phe. Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Such substituted residues also may be introduced into the conservative substitution sites, or into the remaining (e.g., non-conserved) sites.

Optionally, amino acid substitutions may be made in one or more CDRs. Accordingly, the humanized antibody molecule of the invention may comprise 3 or fewer, for example 2, 1 or zero amino acid substitutions in the intervening amino acid residues between the first and the final amino acid residues of one or more of CDRH1, CDRH2, CDRH3, CDRL1, CDRL2, or CDRL3. For example, a Met may be substituted by a Leu and/or an Asn may be substituted by a Gln. Where the humanized antibody molecule of the invention comprises one or more amino acid substitutions in said CDRs, optionally the antibody molecule maintains affinity of a magnitude at least equivalent to that of the corresponding antibody molecule having no such amino acid substitutions. Optionally, the humanized antibody molecule of the invention comprises zero amino acid substitutions in the intervening amino acid residues between the first and the final amino acid residues of each of CDRH1, CDRH2, CDRH3, CDRL1, CDRL2, or CDRL3, i.e. the intervening amino acids correspond to those of the CDRs of the donor antibody as defined above.

In various further aspects, the present invention extends to a multivalent monospecific antigen binding protein comprising two, three, four or more of the antibody molecules as defined in the foregoing aspects of the invention, or to fragments thereof, wherein said antibody molecules are bound to each other by a connecting structure.

In certain further aspects, the invention also extends to bispecific antibodies. These may include conventional bispecific antibodies which may be prepared by chemical conjugation means, or by hybrid hybridoma cell lines. Alternatively, the bispecific antibodies may be derived from bispecific antibody fragments, such as scFv dimers or diabodies. In certain embodiments, scFv dimers may be used, rather than whole antibodies. Such diabodies can be constructed using only variable domains and

therefore are provided without an Fc region, such a structure reducing the possible occurrence of a HAMA or anti-idiotypic immune response.

Moreover, the antibody or antibody fragments of the invention may be subject to one or more techniques which may further be used to ensure that an immunogenic response is not mounted against the antibody when administered to a subject. Examples of such techniques will be well known to the person skilled in the art and include, but are not limited to, de-immunisation, gene shuffling and PEGylation. Deimmunisation techniques may involve the introduction of mutations to remove T-cell epitopes without significantly reducing the binding affinity of an antibody. Typically, such "deimmunised" antibodies are created with human constant domains.

Furthermore, in order to enhance the biologic function or therapeutic activity of an antibody or antibody fragment of the invention, further optimisation techniques may be employed. Such techniques include, but are not limited to, modifying the following attributes of the antibody: potency, affinity, binding specificity, K on (association rate constant), K off (dissociation rate constant), thermodynamic stability, solubility, serum half-life, expression, folding kinetics, protease susceptibility, Fc region effector function and drug recycling. The biological function or therapeutic activity which may be modulated includes, but is not limited to: enhancement of efficacy, improved pharmacokinetic profile, enhanced patient convenience, improved safety profile, reduced immunogenicity and extended shelf-life.

Antibody production

The antibody molecules of the invention may be produced wholly or partly by chemical synthesis. For example, the antibody molecules of the invention can be prepared by techniques which are well known to the person skilled in the art, such as standard liquid peptide synthesis, or by solid-phase peptide synthesis methods. Alternatively, the antibody molecules may be prepared in solution using liquid phase peptide synthesis techniques, or further by a combination of solid-phase, liquid phase and solution chemistry.

The present invention further extends to the production of the antibody molecules of the invention by expression in a suitable expression system of a nucleic acid which

encodes at least one amino acid sequence which, alone or in combination with one or more other amino acid sequences, comprises an antibody molecule of the invention, such that a desired peptide or polypeptide can be encoded. For example, a nucleic acid encoding the amino acid sequence of the light chain and a second nucleic acid encoding an amino acid of a heavy chain can be expressed to provide an antibody molecule of the present invention.

Accordingly, in certain further aspects of the invention, there is provided a nucleic acid encoding an amino acid sequence which forms an antibody molecule of the present invention.

Typically, nucleic acids encoding the amino acid sequences which form antibody molecules of the present invention can be provided in an isolated or purified form, or provided in a form which is substantially free of material which can be naturally associated with it, with the exception of one or more regulatory sequences. Nucleic acid which expresses an antibody molecule of the invention may be wholly or partially synthetic and may include, but is not limited to, DNA, cDNA and RNA.

Nucleic acid sequences encoding the antibody molecules of the invention can be readily prepared by the skilled person using techniques which are well known to those skilled in the art, such as those described in Sambrook et al. "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory Press, Volumes 1-3, 2001 (ISBN-0879695773), and Ausubel et al. "Short Protocols in Molecular Biology", 4th Edition, 1999, John Wiley and Sons, (ISBN – 0471250929). Said techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of nucleic acid, (ii) chemical synthesis, or (iii) preparation of cDNA sequences. DNA encoding antibody molecules of the invention may be generated and used in any suitable way known to those skilled in the art, including taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The excised portion may then be operably linked to a suitable promoter and expressed in a suitable expression system, such as a commercially available expression system. Alternatively, the relevant portions of DNA can be amplified by using suitable PCR primers. Modifications to the DNA sequences can be made by using site directed mutagenesis.

Nucleic acid sequences encoding the antibody molecules of the invention may be provided as constructs in the form of a plasmid, vector, transcription or expression cassette which comprises at least one nucleic acid as described above. Such constructs form a further aspect of the present invention. The construct may be comprised within a recombinant host cell which comprises one or more constructs as above. Expression may conveniently be achieved by culturing, under appropriate conditions, recombinant host cells containing suitable nucleic acid sequences. Following expression, the antibody or antibody fragments may be isolated and/or purified using any suitable technique, then used as appropriate.

Systems for the cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells may include bacteria, mammalian cells, yeast, insect and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney cells and NS0 mouse myeloma cells. A commonly employed bacterial host is *E. coli*. The expression of antibodies and antibody fragments in prokaryotic cells such as *E. coli* is well established in the art. Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of a binding member. The invention extends to a host cell comprising a nucleic acid or antibody molecule of the invention.

General techniques for the production of antibodies are well known to the person skilled in the field, with such methods being discussed in, for example, Kohler and Milstein (1975) Nature 256: 495-497; US Patent No. 4,376,110; and Harlow and Lane, Antibodies: A Laboratory Manual, (1988) Cold Spring Harbor. Techniques for the preparation of recombinant antibody molecules are described in the above references and also in, for example, European Patent No: 0,368,684.

In certain embodiments of the invention, a recombinant nucleic acid comprising an insert coding for a heavy chain variable domain and/or for a light chain variable domain of an antibody molecule is employed. By definition, such nucleic acids encode single stranded nucleic acids, double stranded nucleic acids consisting of said coding nucleic acids and of complementary nucleic acids thereto, or these complementary (single

stranded) nucleic acids themselves.

Furthermore, nucleic acids encoding a heavy chain variable domain and/or a light chain variable domain of antibodies can be enzymatically or chemically synthesised nucleic acids having the authentic sequence coding for a naturally-occurring heavy chain variable domain and/or for the light chain variable domain, or a mutant thereof.

An antibody of the invention may be produced by recombinant means, not only directly, but also as a fusion polypeptide with a heterologous polypeptide, for example a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected may be one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process a native antibody signal sequence, the signal sequence may be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, Lpp, or heat-stable enterotoxin II leaders.

Pharmaceutical Compositions

Antibody molecules and nucleic acids may be formulated with diluents or adjuvants and still, for practical purposes, be considered as being provided in an isolated form. For example the antibody molecules can be mixed with gelatin or other carriers if used to coat microtitre plates for use in immunoassays, or will be mixed with pharmaceutically acceptable carriers or diluents when used in diagnosis or therapy. The antibody molecules may be glycosylated, either naturally or by systems of heterologous eukaryotic cells (e.g. CHO or NSO cells), or they may be (for example if produced by expression in a prokaryotic cell) unglycosylated.

Heterogeneous preparations comprising humanised antibody molecules also form part of the invention. For example, such preparations may be mixtures of antibodies with full-length heavy chains and heavy chains lacking the C-terminal lysine, with various degrees of glycosylation and/or with derivatized amino acids, such as cyclization of an N-terminal glutamic acid to form a pyroglutamic acid residue.

Administration

The antibody molecules of the present invention may be administered alone but will preferably be administered as a pharmaceutical composition which will generally comprise a suitable pharmaceutically acceptable excipient, diluent or carrier selected depending on the intended route of administration. Examples of suitable pharmaceutical carriers include water, glycerol, ethanol and the like.

The antibody molecules of the present invention may be administered to a patient in need of treatment via any suitable route. Typically, the composition can be administered parenterally by injection or infusion. Examples of preferred routes for parenteral administration include, but are not limited to intravenous, intracardial, intraarterial, intraperitoneal, intramuscular, intracavity, subcutaneous, transmucosal, inhalation or transdermal. Routes of administration may further include topical and enteral, for example, mucosal (including pulmonary), oral, nasal, rectal.

In embodiments where the composition is delivered as an injectable composition, for example in intravenous, intradermal or subcutaneous application, the active ingredient can be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as sodium chloride injection, Ringer's injection or, Lactated Ringer's injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

The composition may also be administered via microspheres, liposomes, other microparticulate delivery systems or sustained release formulations placed in certain tissues including blood.

Examples of the techniques and protocols mentioned above and other techniques and protocols which may be used in accordance with the invention can be found in Remington's Pharmaceutical Sciences, 18th edition, Gennaro, A.R., Lippincott Williams & Wilkins; 20th edition ISBN 0-912734-04-3 and Pharmaceutical Dosage Forms and Drug Delivery Systems, Ansel, H.C. et al. 7th Edition ISBN 0-683305-72-7, the entire disclosures of which are herein incorporated by reference.

A composition of the invention may be administered to a subject in a "therapeutically effective amount", this being an amount sufficient to show benefit to the subject to whom the composition is administered. The actual dose administered, and rate and time-course of administration, will depend on, and can be determined with due reference to, the nature and severity of the condition which is being treated, as well as factors such as the age, sex and weight of the subject being treated, as well as the route of administration. Further due consideration should be given to the properties of the composition, for example, its binding activity and *in-vivo* plasma life, the concentration of the antibody or binding member in the formulation, as well as the route, site and rate of delivery.

Dosage regimens can include a single administration of the composition, or multiple administrative doses of the composition. The compositions can further be administered sequentially or separately with other therapeutics and medicaments which are used for the treatment of the condition for which the antibody or binding member of the present invention is being administered to treat.

Examples of dosage regimens which can be administered to a subject can be selected from the group comprising, but not limited to; 1µg/kg/day through to 20mg/kg/day, 1µg/kg/day through to 10mg/kg/day, and 10µg/kg/day through to 1mg/kg/day. In certain embodiments, the dosage will be such that a plasma concentration of from 1µg/ml to 100µg/ml of the antibody is obtained. However, the actual dose of the composition administered, and rate and time-course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage etc, is ultimately within the responsibility and at the discretion of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners.

In certain embodiments the formulation is a liquid formulation, a lyophilized formulation, a lyophilized formulation that is reconstituted as a liquid, or an aerosol formulation. In certain embodiments, the antibody in the formulation is at a concentration in the range of about 0.5 mg/ml to about 250 mg/ml, for example about 0.5 mg/ml to about 200 mg/ml, such as about 10 mg/ml to about 150 mg/ml, such as

about 50 mg/ml to about 100 mg/ml, or about 50 mg/ml to about 250 mg/ml, such as about 50 mg/ml to about 200 mg/ml, for example about 100 mg/ml to about 150 mg/ml. In certain embodiments, the formulation further comprises a buffer. Typically the pH of the formulation is from about pH 5.5 to about pH 6.5. In certain embodiments, the buffer may comprise from about 4 mM to about 60 mM histidine buffer, about 5 mM to about 25 mM succinate buffer, or about 5 mM to 25 mM acetate buffer. In certain embodiments, the buffer comprises sodium chloride at a concentration of from about 10mM to 300mM, for example in the range 100 to 150mM, such as about 125mM concentration and sodium citrate at a concentration of from about 5mM to 50mM, for example about 25mM. In certain embodiments the formulation can further comprise a surfactant at a concentration of about 0% to about 0.2%. In certain embodiments the surfactant is selected from the group consisting of, but not limited to: polysorbate-20, polysorbate-40, polysorbate-60, polysorbate-65, polysorbate-80, polysorbate-85, and combinations thereof. In one embodiment, the surfactant is polysorbate-20. In certain embodiments, the formulation further comprises about 0.001% to about 0.05% Tween and may further comprise sodium chloride, e.g. in the range 100 to 150mM such as at a concentration of about 125mM and sodium citrate, e.g. at a concentration in the range 5mM to 50mM, such as about 25mM.

Therapeutic Uses

An antibody molecule of the invention may be used in, for example, *in vitro*, *ex vivo*, and *in vivo* therapeutic methods. In certain further aspects, the invention extends to the use of the antibody molecules and nucleic acids of the invention for the treatment of neoplastic disease.

As used herein, the term “treatment” and associated terms such as “treat” and “treating” means the reduction of the progression, severity and/or duration of a disease or condition or at least one symptom thereof, wherein said reduction or amelioration results from the administration of a binding compound which has specificity for the target binding epitope. The term ‘treatment’ therefore refers to any regimen that can benefit a subject. The treatment may be in respect of an existing condition or may be prophylactic (preventative treatment). Treatment may include curative, alleviative or prophylactic effects. References herein to "therapeutic" and "prophylactic" treatments

are to be considered in their broadest context. The term "therapeutic" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylactic" does not necessarily mean that the subject will not eventually contract a disease condition.

In certain embodiments, antibody molecules, nucleic acids or compositions of the invention may be used in the treatment of neoplastic diseases or cancers. "Treatment of neoplastic disease or cancer" includes treatment of conditions caused by cancerous growth and/or vascularisation and includes the treatment of neoplastic growths or tumours. Examples of tumours that may be treated are, for instance, sarcomas, including osteogenic and soft tissue sarcomas, carcinomas, e.g., breast-, lung-, bladder-, thyroid-, prostate-, colon-, rectum-, pancreas-, stomach-, liver-, uterine-, prostate , kidney, cervical and ovarian carcinoma, non-small cell lung cancer, hepatocellular carcinoma, lymphomas, including Hodgkin and non-Hodgkin lymphomas, neuroblastoma, melanoma, myeloma, Wilms tumor, and leukemias, including acute lymphoblastic leukaemia and acute myeloblastic leukaemia, astrocytomas, gliomas and retinoblastomas.

Certain embodiments of the invention may be particularly useful in the treatment of existing cancer and in the prevention of the recurrence of cancer after initial treatment or surgery.

In some embodiments, antibody molecules, nucleic acids and compositions of the invention may be used to treat other conditions, for example other conditions or disorders mediated by or associated with angiogenesis. Such conditions include, various inflammatory disorders, tumours, various autoimmune disorders, some hereditary disorders, and ocular disorders.

In some embodiments, the antibody molecules and methods of the invention may be used in the treatment of angiogenesis associated inflammation, including various forms of arthritis, such as rheumatoid arthritis and osteoarthritis, chronic inflammatory conditions including ulcerative colitis, Crohn's disease, bartonellosis, and atherosclerosis. Other angiogenesis-mediated disorders, for which the invention may find use include hemangioma, solid tumors, leukemia, metastasis, telangiectasia,

psoriasis, scleroderma, pyogenic granuloma, myocardial angiogenesis, Crohn's disease, plaque neovascularization, coronary collaterals, cerebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, corneal diseases, retrolental fibroplasia, arthritis, diabetic neovascularization, peptic ulcer, Helicobacter related diseases, fractures, keloids, and vasculogenesis.

Ocular Disorders mediated by angiogenesis for which some embodiments of the invention may be used include macular degeneration, ocular neovascular disease corneal graft rejection, neovascularization following injury or infection, rubeosis, diabetic retinopathy, retrolental fibroplasia and neovascular glaucoma, corneal diseases and macular degeneration, diabetic retinopathy, retinopathy of prematurity, corneal graft rejection, neovascular glaucoma and retrolental fibroplasia, diseases associated with corneal neovascularization including, but are not limited to, epidemic keratoconjunctivitis, Vitamin A deficiency, atopic keratitis, superior limbic keratitis, pterygium keratitis sicca, and periphigoid radial keratotomy, diseases associated with retinal/choroidal neovascularization including, but not limited to, macular degeneration, presumed myopia, optic pits, chronic retinal detachment, hyperviscosity syndromes, trauma and post laser complications, diseases associated with rubeosis (neovascularization of the angle) and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue including all forms of proliferative vitreoretinopathy, neovascular glaucoma, retinoblastoma, retrolental fibroplasia, rubeosis, uveitis, and corneal graft neovascularization, other eye inflammatory diseases, ocular tumors, and diseases associated with iris neovascularization.

Chemotherapeutic Agents

As described above, in certain embodiments of the invention the antibody molecules may be used in combination with chemotherapeutic agents. For example, chemotherapeutic agents which may be used include antimetabolites, including thymidylate synthase inhibitors, nucleoside analogs, platinum cytotoxic agents, topoisomerase inhibitors or antimicrotubule agents. Examples of thymidylate synthase inhibitors which may be used in the invention include 5-FU, MTA and raltitrexed (TDX). Examples of platinum cytotoxic agents which may be used include cisplatin and oxaliplatin. Other chemotherapeutic agents which may be used in the present invention,

in addition or instead of the specific agents recited above, may include alkylating agents; alkyl sulfonates; aziridines; ethylenimines; methylamelamines; nitrogen mustards; nitrosureas; anti-metabolites; folic acid analogues; purine analogs; pyrimidine analogs; androgens; anti-adrenals; folic acid replenishers; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfomithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; ionidamine; mitoguazone; and mitoxantrone.

In particular embodiments of the invention, the chemotherapeutic agent is a topoisomerase inhibitor. In a particular embodiment, the topoisomerase inhibitor is a topoisomerase I inhibitor, for example a camptothecin. A suitable topoisomerase I inhibitor, which may be used in the present invention is irinotecan (CPT-11) or its active metabolite SN-38. CPT-11 specifically acts in the S phase of the cell cycle by stabilizing a reversible covalent reaction intermediate, referred to as a cleavage or cleavage complex, and may also induce G_2-M cell cycle arrest.

In certain other embodiments of the invention, the chemotherapeutic agent is a fluoropyrimidine e.g. 5-FU.

Where reference is made to specific chemotherapeutic agents, it should be understood that analogues including biologically active derivatives and substantial equivalents thereof, which retain the antitumour activity of the specific agents, may be used.

Definitions

Unless otherwise defined, all technical and scientific terms used herein have the meaning commonly understood by a person who is skilled in the art in the field of the present invention.

Throughout the specification, unless the context demands otherwise, the terms 'comprise' or 'include', or variations such as 'comprises' or 'comprising', 'includes' or 'including' will be understood to imply the inclusion of a stated integer or group of integers, but not the exclusion of any other integer or group of integers.

As used herein, terms such as "a", "an" and "the" include singular and plural referents unless the context clearly demands otherwise. Thus, for example, reference to "an active agent" or "a pharmacologically active agent" includes a single active agent as well as two or more different active agents in combination, while references to "a carrier" includes mixtures of two or more carriers as well as a single carrier, and the like.

The terms "specifically binds", “selectively binds” or “binding specificity” refer to the ability of the antibody molecules of the invention to bind to a target epitope with a greater affinity than that which results when bound to a non-target epitope. In certain embodiments, specific binding refers to binding to a target with an affinity that is at least 10, 50, 100, 250, 500, or 1000 times greater than the affinity for a non-target epitope. In certain embodiments, this affinity is determined by an affinity ELISA assay. In certain embodiments, affinity can be determined by a BIAcore assay. In certain embodiments, affinity can be determined by a kinetic method. In certain embodiments, affinity can be determined by an equilibrium/solution method.

Examples

Example 1 Generation of Humanised Antibody with Binding Specificity for HBEGF and AREG

A Cloning of Fsn1006 humanized variant DNA sequences

Sequence analysis

The present inventors have previously developed an antibody with cross-specificity for AREG and HBEGF; this antibody is described in WO2009/127881. The VH and VL sequences of this murine antibody, designated Fsn1006, are shown in Figure 1. The CDRs as defined by the Kabat method, the IMGT method and the Chothia method are shown in Table 1 below the sequences.

Humanised versions of this antibody were prepared by CDR grafting. The inventors selected the CDR sequences by employing a combined IMGT and Kabat CDR sequence in which (i) the CDRH1 comprises the first amino acid residue of the CDRH1 of the donor antibody as defined by the IMGT method, the final amino acid residue of the CDRH1 as

defined by the Kabat method, and therebetween the intervening amino acid residues of said donor CDR between said first and final amino acid residues; (ii) the CDRH3 comprises the first amino acid residue of the CDRH3 of the donor antibody as defined by the IMGT method, the final amino acid residue of the CDRH3 as defined by the Kabat method and therebetween the intervening amino acid residues of said donor CDR between said first and final amino acid residues; and (iii) the CDRH2, CDRL1, CDRL2, and CDRL3 sequences of the humanised antibody molecule correspond to the CDRH2, CDRL1, CDRL2, and CDRL3 respectively of the donor antibody as defined by the Kabat method. For each of the VH and the VL chains, three human IgG framework sequences were selected from the NCBI BLAST database, the accession nos being (VH1) ABM67208, (VH2) ABI50723, (VH3) AAR32431, (VL1) ABC66929, (VL2) ABI74076, and (VL3) CAA51099.

Back mutations in the framework sequences were not required.

The three VH acceptor frameworks with the Fsn1006 "Fusion CDR" residue sequences and the three VL acceptor frameworks with the Fsn1006 "Fusion CDR" residue sequences are shown in Figure 2 with the CDRs underlined.

In Figure 3 the murine Fsn1006 VH sequence has been aligned with the three humanized VH variants. In the figure, Fsn1006 VH is the murine sequence and VH1-3 are the humanized variants. The CDRs are underlined. Key residues important for the VH/VL interface and canonical loop structure are asterisked (*). These have been maintained in the humanized variants but have not been mutated.

In Figure 4 the murine Fsn1006 VL sequence has been aligned with the three humanized VL variants. Fsn1006 VL is the murine sequence and VL1-3 are the humanized variants. The Fusion CDRs are underlined. Key residues important for the VH/VL interface and canonical loop structure are asterisked (*). As far as possible these have been maintained in the humanized variants and have not been mutated.

Each of the VH domains was synthesised in-frame with a human IgG1 isotype constant domain sequence. The entire heavy chain sequence was codon optimised (GeneArt, Germany) and the DNA sequence verified. The amino acid sequence of the IgG1 constant domain is

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS
LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPGK (SEQ. ID NO: 29)

Each of the VL domains was synthesised in-frame with a human IgK isotype constant domain sequence. The entire light chain sequence was codon optimised (LifeTech, Germany) and the DNA sequence verified. The amino acid sequence of the IgK constant domain is

TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ. ID NO: 30)

Each of the variant chains was verified by DNA sequencing analysis.

B: Production of humanized Fsn1006h variants

The humanised variants were expressed in CHO cells. There is one chimeric antibody (VH0-VL0), having the murine variable domains and the human IgG constant domains, and 9 humanised variants having humanised variable domains and human IgG constant domains (VH1-VL1 to VH3-VL3). N.B. VH1, VH2 etc and VL1, VL2 etc are alternative nomenclature for HC1, HC2 etc and HL1, HL2 etc respectively.

Transient Transfection

Suspension adapted CHO K1 cells (CHO-S cells) (Invitrogen, UK) were routinely cultivated at 2 - 3 X 10^5 cells/ml at 150rpm, 8% CO_2, 37°C in Pro CHO 4 serum free medium (Lonza, UK) plus 8mM L-glutamine (PAA, Analab, UK) and HT (Invitrogen, UK) in 500ml vented Erlenmeyer flasks (Corning, Netherlands). Before transfection (24hours and 48 hours) cells were passed at 3X10^5cells/ml. On the day of transfection, cells were centrifuged at 100g for 5 mins and seeded at a density of 2.0 X 10^6 cells/ml in 50 ml Pro CHO 5 medium (Lonza, UK) plus 8mM L-glutamine (PAA, Analab, UK) and plus HT. Cultures were transfected with 2.5 µg/ml of vector DNA (589 pvitro DHFR3, see Table 3 for the different vector combinations), in 150mM NaCl (5% final volume), using 15 µg/ml PEI transfection reagent (6:1 PEI: DNA transfection ratio) in 150mM

NaCl (5% final volume). The DNA-PEI complex was left standing at room temperature for 10 mins before addition to the cells. The cells were then left standing for 30 mins at 37°C and then placed on a shaker at 150 rpm, 8% CO_2 and 37°C for 4 hours. An equal volume of Pro CHO 5 medium was then added (final cell density was $1X10^6$cells/ml). After 7 days incubation at 150 rpm, 8% CO_2, 37°C, the supernatant was harvested by centrifugation at 4,000 rpm for 40 mins at 4°C for purification.

Purification method and results

Following centrifugation, all media was filtered through a 0.8µM cellulose acetate filter. Each batch was purified using an Amersham Biosciences AKTA Chromatography system. A 1ml HiTrap Protein A column was used for all purifications. All purifications were carried out using PBS pH7.4 as the wash buffer. Following overday loading, any bound antibody was eluted using a Glycine/Tris pH3.0 buffer. All eluted 1ml fractions were neutralised with 100µl Tris pH8.5 buffer. Eluted fractions were quantified by Bradford assay and fractions indicated to have high levels of antibody present were selected for overnight dialysis in PBS.

C: Selection of lead Fsn1006h drug candidate

Aim

The Murine 2F7 antibody was humanized and nine variants of this FSN1006 human antibody was produced in CHO-S cells by transient transfection and purified using in house standard operating procedures as described above. The subsequent humanized variants were sent for Biacore analysis (Biaffin, Kassel, Germany). The top three variants were then chosen based on these results according to antibody binding affinities and kinetics. Subsequent in house experiments were carried out to ascertain the best lead candidate in terms of binding affinity and activity.

Table 3 Heavy Chain to Light Chain vector combinations

Heavy Chain **1** : Light Chain **1 (HC1:LC1)**
Heavy Chain **2** : Light Chain **1 (HC2:LC1)**
Heavy Chain **3** : Light Chain **1 (HC3:LC1)**
Heavy Chain **1** : Light Chain **2 (HC1:LC2)**
Heavy Chain **2** : Light Chain **2 (HC2:LC2)**

Heavy Chain **3** : Light Chain **2 (HC3:LC2)**
Heavy Chain **1** : Light Chain **3 (HC1:LC3)**
Heavy Chain **2** : Light Chain **3 (HC2:LC3)**
Heavy Chain **3** : Light Chain **3 (HC3:LC3)**
Heavy Chain **0** : Light Chain **0** Chimeric Antibody

Results

Affinity

The affinity of the FSN1006 antibody to bind the target proteins HBEGF and AREG, was tested using Enzyme Linked Immunosorbent Assay (ELISA) and Biacore analysis. Biacore analysis was performed as a *single cycle kinetics* experiment by immobilizing the antibody to a α-human Fc on CM5 sensor chips and subsequently injecting five concentrations of the antigen without regeneration of the surface in order to monitor association and dissociation rates providing sufficiently accurate values of kinetic constants (*k*ass, *k*diss) and affinities (*K*D) for a kinetic ranking. The Biacore results are summarised in Tables 4 and 5.).

Table 4 Affinity to HB-EGF

		Kinetic global fit (Langmuir 1:1)			
Ligand		k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)	% stoichiometry
Ab FSN1006h	HC0:LC0	3.8×10^4	3.3×10^{-4}	8.9	145%
	HC1:LC1	1.4×10^4	4.0×10^{-4}	28.5	100%
	HC1:LC2	1.6×10^4	3.6×10^{-4}	23.1	100%
	HC1:LC3	**			
	HC2:LC1	5.7×10^4	2.9×10^{-4}	5.1	152%
	HC2:LC2	6.2×10^4	3.2×10^{-4}	5.2	149%
	HC2:LC3	5.4×10^4	3.8×10^{-4}	7.0	156%
	HC3:LC1	6.4×10^4	9.3×10^{-3}	145	75%
	HC3:LC2	6.2×10^4	1.5×10^{-2}	238	83%
	HC3:LC3	**			

Table 5 Affinity to AREG

		Kinetic global fit (Langmuir 1:1)			
Ligand		k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)	% stoichiometry
Ab FSN1006h	HC0:LC0	2.0×10^4	3.1×10^{-4}	15.7	145%
	HC1:LC1	**			
	HC1:LC2	**			
	HC1:LC3	**			
	HC2:LC1	2.7×10^4	3.9×10^{-4}	14.7	200%
	HC2:LC2	2.7×10^4	2.9×10^{-4}	10.8	150%
	HC2:LC3	2.2×10^4	4.8×10^{-4}	22.2	155%
	HC3:LC1	**			
	HC3:LC2	**			
	HC3:LC3	**			

The binding of the generated humanised antibodies was tested via competition ELISA. Briefly, 96-well Maxisorp plate (Nunc) were coated with 100ng/well of AREG or HB EGF antigen (1µg/ml stock) in coating buffer and incubated overnight at 4˚C. Coating solution was removed and 200 µl/well of blocking solution (3.0% w/v semi skimmed milk in 1X PBS) was added. Plates were agitated at room temperature for 2 hours. Blocking solution was removed and plates were washed 1X PBS Tween 20 (0.1%) (PBS-T). Murine or humanized Fsn1006 antibodies were diluted to a starting concentration of 1µg/ml and serial 1:1 dilutions then made down to 0.031µg/ml in PBS. Each dilution (100 µl/well) was added in triplicate with a PBS negative control. Plates were incubated with agitation at room temperature for 2 hours. Primary antibody was removed and plates were washed 4 x PBS-T. Secondary antibody (100µl/well) was added (for murine Fsn 1006 antibodies goat anti mouse HRP (Biorad) 1:5,000 in PBS and for humanized variants (goat anti human HRP (Sigma) 1:20,000 in PBS) and plates were agitated at room temperature for 1 hour. Samples were removed and plates

washed x6 in PBS-T. TMB solution was added and plates incubate for exactly 10 min at 37°C. The reaction was stopped by addition of 1 M HCL (50 µl/well) and the plates were read immediately at 450nM. Binding of humanized antibodies was shown to be of similar magnitude to that of the murine antibody.

Figure 5 illustrates the results of analysis of binding of the nine humanised antibody constructs to HB-EGF in comparison to the murine antibody (2F7) and the chimeric antibody (H0L0). The ELISA binding data confirmed that the variants H2L1(HC2:LC1), H2L2(HC2:LC2), and H2L3(HC2:LC3) had very similar binding characteristics to the murine antibody. Although binding of the variants having the H1 or H3 chains was not as strong, binding nevertheless occurred to the H1L1(HC1:LC1), H1L2(HC1:LC2), H3L1(HC3:LC1) and H3L2(HC3:LC2) antibodies. The results are summarised in Table 6 below

Table 6

Antibody	**K_{ass} (l mol^{-1} s^{-1})**	**K_{diss} (s^{-1})**	**K_D (nM)**
Chimeric H0L0	3.8×10^4	3.3×10^{-4}	8.9
HC1:LC1	1.4×10^4	4.0×10^{-4}	28.5
HC1:LC2	1.6×10^4	3.6×10^{-4}	23.1
HC1:LC3	**		
HC2:LC1	5.7×10^4	2.9×10^{-4}	5.1
HC2:LC2	6.2×10^4	3.2×10^{-4}	5.2
HC2:LC3	5.4×10^4	3.8×10^{-4}	7.0
HC3:LC1	6.4×10^4	9.3×10^{-3}	145
HC3:LC2	6.2×10^4	1.5×10^{-2}	238
HC3:LC3	**		

** not measurable

Activity

The ability of the FSN1006 antibody to inhibit phosphorylation of the Epidermal Growth Factor Receptor (EGFR) was determined using western blots. The MTT assay was used to determine the cytotoxicity and anti-proliferative activities of FSN1006.

Inhibition of EGFR phosphorylation

HBEGF (30ng/ml) and FSN 1006 antibodies (50nM-1,000nM) were pre- incubated on ice for 30 minutes before treating HCT 116 cells for 20 minutes to determine if the presence of FSN 1006 constructs could block the phosphorylation of EGFR by HBEGF. Lysates were prepared form the treated cells and separated by SDS PAGE and western blotted. The blots were then probed with anti- phospho EGFR antibody. The presence of a heavy band indicated that phopsorylated EGFR was detected and was being activated by HBEGF.

All three constructs involving HC2 were tested with HBEGF. The results are shown in Figure 6 for the HC2LC2 humanised antibody. The lanes were as follows:

Lane 1 : PBS treated HCT116 cells neg control

Lane 2 : 1,000nM HC2LC2

Lane 3 : 1,000nM HC2LC2 + 30ng/ml HBEGF

Lane 4 : 500nM HC2LC2 + 30ng/ml HBEGF

Lane 5 : 250nM HC2LC2 + 30ng/ml HBEGF

Lane 6 : 100nM HC2LC2 + 30ng/ml HBEGF

Lane 7 : 50nM HC2LC2 + 30ng/ml HBEGF

Lane 8: 30ng/ml HBEGF

At the highest concentration of 1,000nM FSN 1006 HC2LC2 in the presence of HBEGF (Lane 3) the band was significantly fainter than the control of HBEGF alone (Lane 8). As the concentration of HC2LC2 decreased (lanes 4-7) the bands become gradually heavier indicating phosphorylation of EGFR by HBEGF and a reduction of the inhibition of HBEGF by FSN 1006 HC2LC2.

In-Vitro Cytotoxic and Anti-proliferative activities

The humanised antibodies were tested for antiproliferative activities on a number of cell lines using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 5000 cells were seeded per well on 96 well plate. After 24 hours cells were treated with the humanised antibody of interest at different concentrations. After 72 hours MTT (1.0mg/ml) was added to each well and cells were incubated at 37°C for 2 hours. The culture media was removed and formazan crystals were reabsorbed in 200µl DMSO. Cell viability was determined by reading the absorbance of each well at 570nm using a microplate reader (Tecan Sunrise, Biorad, UK).

Figure 7 illustrates the results of an MTT assay using the humanised antibody variants having heavy chain 2. The HC2LC3 variant appeared to be the most effective of the three constructs with a cell kill of 30-40% observed at concentrations of 100-150µg/ml. The HC2LC2 antibody proved the next best effective with cell kill at 25-30% whilst HC2LC1 showed cell death from 15-32% observed at concentrations of 100-150µg/ml.

Similar MTT assays were performed a variety of further cancer cell lines. For example, in DLD-1 cells, each of the three humanised antibodies resulted in a cell kill effect of 25-30% at concentrations of greater than 100µg/ml. Similarly, in DKO-4 cells, each of the three humanised antibodies resulted in a cell kill effect, with the greatest effect shown by the HC2LC3 cells. In MDA-231 cells, the HC2LC2 antibodies demonstrated the greatest effect, reducing MDA-231 cell viability by 30% as compared to the PBS treated control cells.

Example 2 Generation of Humanised Antibody with Binding Specificity for CD20

Using the method as employed in Example 1, the inventors have also designed and generated a humanised anti-CD20 antibody, specifically a humanised version of rituximab.

The VH and VL sequences of rituximab are shown in Figure 8. The CDRs as defined by the Kabat method, the IMGT method and the Chothia method are shown in the Table below the sequences.

Humanised versions of this antibody were prepared by CDR grafting. The inventors selected the CDR sequences by employing a combined IMGT and Kabat CDR sequence in which (i) the CDRH1 comprises the first amino acid residue of the CDRH1 of the donor antibody as defined by the IMGT method, the final amino acid residue of the CDRH1 as defined by the Kabat method and therebetween the intervening amino acid residues of said donor CDR between said first and final amino acid residues; (ii) the CDRH3 comprises the first amino acid residue of the CDRH3 of the donor antibody as defined by the IMGT method, the final amino acid residue of the CDRH3 as defined by the Kabat method and therebetween the intervening amino acid residues of said donor CDR between said first and final amino acid residues; and (iii) the CDRH2, CDRL1, CDRL2,

and CDRL3 sequences of the humanised antibody molecule correspond to the CDRH2, CDRL1, CDRL2, and CDRL3 of the donor antibody as defined by the Kabat method. For each of the VH and the VL chains, three human IgG framework sequences were selected from the NCBI BLAST database.

Back mutations in the framework sequences were not required.

In Figure 9 the murine rituximab VH sequence has been aligned with the three humanized VH variants. In the figure, VHO is the murine sequence and VH1-3 are the humanized variants. The CDRs are marked by boxes.

In Figure 10 the murine rituximab VL sequence (VL0) has been aligned with the three humanized VL variants. Fsn1006 VL0 is the murine sequence and VL1-3 are the humanized variants. The "Fusion CDRs" are indicated by the boxes.

Each of the VH domains was synthesised in-frame with a human IgG1 isotype constant domain sequence. The entire heavy chain sequence was codon optimised (GeneArt, Germany) and the DNA sequence verified. Each of the VL domains was synthesised in-frame with a human IgK isotype constant domain sequence. The entire light chain sequence was codon optimised (LifeTech, Germany) and the DNA sequence verified.

Each of the variant chains was verified by DNA sequencing analysis, with sequence chromatograms supplied. The humanised variants were expressed in CHO cells. The humanised heavy chains and humanised light chains were recombined to 9 humanised variants.

Binding of the nine humanized rituximab variants to Raji cells (EBV-transformed lymphocytes expressing surface CD20) was assessed to compare their functional activity in binding to CD20. Raji cells were harvested in the log phase of growth and washed x3 in PBS. Cells were blocked with BSA, and then incubated with primary antibody (rituximab positive control, humanized rituximab variants, PBS control, human IgG negative control) for 1hr at room temp. Cells were washed x 3 with PBS and then incubated with secondary goat-anti-human-FITC antibody for 1hr at room temperature. Cells were washed x3 with PBS and then analysed by flow cytometry for positive binding. Sample results for the humanised variants are shown together with the

results for a negative control, and rituximab in Figure 11. The results demonstrate that, two of the humanised rituximab antibodies, AbR1 (VH1VL1) and AbR3 (VH1VL3), bind to CD20 with excellent affinity. This is particularly surprising given that only three humanised variant VH and three humanised variant VL sequences were employed to generate the humanised antibodies. By performing the equivalent CDR grafting into a small panel of 9 variants using just the Kabat or IMGT CDRs, it would be very unlikely that a humanised variant with similar affinity to the murine antibody would be identified. (Typically, it would be expected that, of humanised antibodies tested for suitable affinity, less than 10%, indeed less than 5% of those tested would be expected to demonstrate affinity and activity of a magnitude suitable for use therapeutically. For example, in a study by Hwang et al (Methods. 2005 May;36(1):35-42), of 17 CDR – grafted antibodies, only two showed no loss in avidity after CDR-grafting into human germlines; with the other antibodies requiring affinity maturation.) Moreover, in many cases it would be impossible to generate such humanised antibodies without resorting to back mutations in the non-CDR framework.

Thus, in both Examples 1 and 2, which are directed to humanisation of antibodies to different targets, the inventors, by employing the method of humanisation of the present invention, have identified humanised variants with similar affinity to the corresponding murine antibody from very small panels of variants without back mutations.

All documents referred to in this specification are herein incorporated by reference. Various modifications and variations to the described embodiments of the inventions will be apparent to those skilled in the art without departing from the scope of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes of carrying out the invention which are obvious to those skilled in the art are intended to be covered by the present invention.

Claims

1. A humanised antibody molecule comprising complementarity determining regions (CDRs) derived from a non-human donor antibody, wherein
(i) the first amino acid residue and the final amino acid residue of CDRH1 of the humanised antibody molecule are the first amino acid residue of CDRH1 of the donor antibody as defined by the IMGT method and the final amino acid residue as defined by the Kabat method respectively and the CDRH1 comprises the intervening amino acid residues of said donor CDRH1 between said first and final amino acid residues;
(ii) the first amino acid residue and the final amino acid residue of CDRH3 of the humanised antibody molecule are the first amino acid residue of CDRH3 of the donor antibody as defined by the IMGT method and the final amino acid residue as defined by the Kabat method respectively and the CDRH3 comprises the intervening amino acid residues of said donor CDRH3 between said first and final amino acid residues; and
(iii) CDRH2 of the humanised antibody molecule corresponds to CDRH2 of the donor antibody as defined by the Kabat method.

2. The humanised antibody molecule according to claim 1 wherein the humanised antibody molecule comprises a light chain.

3. The humanised antibody molecule according to claim 2 wherein the CDRL1, CDRL2, and CDRL3 of the light chain correspond to CDRL1, CDRL2, and CDRL3 of the donor antibody as defined by the Kabat method.

4. The humanised antibody molecule according to any one of claims 1 to 3 wherein the variable region sequences other than the CDRs do not comprise any backmutations to the non-CDR framework VH sequence of the non-human donor antibody.

5. The humanised antibody molecule according to any one of claims 1 to 4, wherein said antibody molecule has binding specificity for HBEGF and AREG.

6. The humanised antibody molecule according to any one of the preceding claims wherein the antibody molecule has binding specificity for an antigenic fragment of AREG having the amino acid sequence shown as SEQ. ID NO: 1 and the antigenic fragment of HBEGF having the amino acid sequence shown as SEQ. ID NO: 2.

7. The humanised antibody molecule according to any one of the preceding claims, wherein the antibody molecule comprises a heavy chain and a light chain having the following complementarity determining regions (CDRs):
CDRH1: SEQ ID NO: 3,
CDRH2: SEQ ID NO: 4,
CDRH3: SEQ ID NO: 5,
CDRL1: SEQ ID NO: 6,
CDRL2: SEQ ID NO: 7, and
CDRL3: SEQ ID NO: 8.

8. The humanised antibody molecule according to claim 7 comprising a VH domain having an amino acid sequence shown as SEQ. ID NO: 9, SEQ. ID NO: 10, or SEQ. ID NO: 11.

9. The humanised antibody molecule according to claim 7 or claim 8 comprising a VL domain having an amino acid sequence shown as SEQ. ID NO: 12, SEQ. ID NO: 13, or SEQ. ID NO: 14.

10. The humanised antibody molecule according to claim 9 wherein said antibody molecule comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 10 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 13.

11. The humanised antibody molecule according to claim 9 wherein said antibody molecule comprises a VH domain having an amino acid sequence shown as SEQ. ID NO: 10 and a VL domain having an amino acid sequence shown as SEQ. ID NO: 14

12. The humanised antibody molecule according to any one of claims 1 to 4, wherein said antibody molecule has binding specificity for CD20.

13. The humanised antibody molecule according to claim 12 wherein the antibody molecule comprises a heavy chain and a light chain having the following complementarity determining regions (CDRs):
CDRH1: SEQ ID NO: 15,
CDRH2: SEQ ID NO: 16,
CDRH3: SEQ ID NO: 17,
CDRL1: SEQ ID NO: 18,
CDRL2: SEQ ID NO: 19, and
CDRL3: SEQ ID NO: 20.

14. The humanised antibody molecule according to claim 12 or claim 13 comprising a VH domain having an amino acid sequence shown as SEQ. ID NO: 21, SEQ. ID NO: 22, or SEQ. ID NO: 23.

15. The humanised antibody molecule according to any one claims 12 to 14 comprising a VL domain having an amino acid sequence shown as SEQ. ID NO: 24, SEQ. ID NO: 25, or SEQ. ID NO: 26.

16. A nucleic acid molecule which encodes a humanised antibody molecule according to any one of claims 1 to 4.

17. The nucleic acid molecule according to claim 16, wherein the nucleic acid molecule encodes a humanised antibody molecule according to any one of claims 5 to 15.

18. A humanised antibody molecule according to any one of claims 1 to 15 or a nucleic acid molecule according to claim 16 or claim 17 for use in medicine.

19. A humanised antibody molecule according to any one of claims 1 to 15 or a nucleic acid molecule according to claim 16 or 17 for use in the treatment of neoplastic disease.

20. A humanised antibody molecule according to any one of claims 1 to 15 or a nucleic acid molecule according to claim 16 or 17 for use in the treatment of a disease or disorder associated with angiogenesis.

21. A method of treating neoplastic disease in a subject, said method comprising administration to said subject of an effective amount of humanised antibody molecule according to any one of claims 1 to 15 or a nucleic acid molecule according to claim 16 or claim 17.

22. A method of treating a disease or disorder associated with angiogenesis in a subject, said method comprising administration to said subject of an effective amount of humanised antibody molecule according to any one of claims 1 to 15 or a nucleic acid molecule according to claim 16 or claim 17.

23. A pharmaceutical composition comprising a humanised antibody molecule according to any one of claims 1 to 15 or a nucleic acid molecule according to claim 16 or claim 17.

24. The pharmaceutical composition according to claim 23, wherein the humanised antibody molecule is a humanised antibody molecule according to any one of claims 5 to 15 or the nucleic acid molecule is a nucleic acid molecule according to claim 17 and said pharmaceutical composition further comprises a chemotherapeutic agent and/or an angiogenesis inhibitor.

25. A kit comprising:

(i) a humanised antibody molecule according to any one of claims 1 to 15 or a nucleic acid molecule according to claim 16 or claim 17; and

(ii) a chemotherapeutic agent and/or an angiogenesis inhibitor for simultaneous, sequential or separate administration.

26. A method of humanising an antibody, said method comprising producing an antibody molecule in which the heavy chain CDRs of a non-human donor antibody are grafted onto a human acceptor framework, and optionally the light chain CDRs of a non-human donor antibody are grafted onto a human acceptor framework, wherein

(i) the first amino acid residue and the final amino acid residue of CDRH1 of the humanised antibody molecule are the first amino acid residue of CDRH1 of the donor antibody as defined by the IMGT method and the final amino acid residue as defined by the Kabat method respectively and the CDRH1 comprises the intervening amino acid residues of said donor CDRH1 between said first and final amino acid residues;

(ii) the first amino acid residue and the final amino acid residue of CDRH3 of the humanised antibody molecule are the first amino acid residue of CDRH3 of the donor antibody as defined by the IMGT method and the final amino acid residue as defined by the Kabat method respectively and the CDRH3 comprises the intervening amino acid residues of said donor CDRH3 between said first and final amino acid residues; and

(iii) CDRH2 of the humanised antibody molecule corresponds to CDRH2 of the donor antibody as defined by the Kabat method.

27. The method according to claim 26 wherein the humanised antibody generated by the method is a humanised antibody according to any one of claims 1 to 13.

Figure 1

Murine VH domain: (SEQ. ID NO: 31)

EVQLQQSGAELVRPGALVKLSCKASGFNIKDSYIHWVNQRPEQGLEWIGWI
DPENGNTIYDPKFQGKASITADTSSNTAYLQLSSLTSEDTAVYYCVSASYRY
GFSYWGQGTLVTVSA (SEQ. ID NO: 32)

Murine VL domain: (SEQ. ID NO: 33)

DIVMTQAAPSVPVTPGESVSISCRSNKSLLHTNGNTYLYWFLQRPGQSPQLLIYRMSNL
ASGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPYTFGGGTKLEIKR

Table 1

	Kabat	IMGT	Chothia
CDR H1	DSYIH (SEQ. ID NO: 33)	GFNIKDSY (SEQ. ID NO:34)	GFNIKD (SEQ. ID NO:35)
CDR H2	WIDPENGNTIYDPKFQG (SEQ. ID NO:36)	IDPENGNT (SEQ. ID NO:37)	WIDPENGNTI (SEQ. ID NO:38)
CDR H3	ASYRYGFSY (SEQ. ID NO:39)	VSASYRYGFSY (SEQ. ID NO:40)	ASYRYGFSY (SEQ. ID NO:41)
CDR L1	RSNKSLLHTNGNTYLY (SEQ. ID NO:42)	KSLLHTNGNTY (SEQ. ID NO:43)	RSNKSLLHTNGNTYLY (SEQ. ID NO:44)
CDR L2	RMSNLAS (SEQ. ID NO:45)	RMS (SEQ. ID NO:46)	RMSNLAS (SEQ. ID NO:47)
CDR L3	MQHLEYPYT (SEQ. ID NO:48)	MQHLEYPYT (SEQ. ID NO:49)	MQHLEYPYT (SEQ. ID NO:50)

Figure 2

>VH1

QVQLVQSGAEVKKPGASVKVSCKASGFNIKDSYIHWVRQAPGQRLEWIGWI
DPENGNTIYDPKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYCVSASYRYG
FSYWGQGTLVTVSS

> VH2

QVQLVQSGAEVKKPGASVKVSCKASGFNIKDSYIHWVRQAPGQGLEWMGW
IDPENGNTIYDPKFQGRVTVTRDTSISTAHMELSSLRSDDTAVYYCVSASYRY
GFSYWGQGTAVTVSS

> VH3

QVQLVQSGAEVKKPGASVKVSCKASGFNIKDSYIHWFQQAPTRGLEWMGWI
DPENGNTIYDPKFQGRVSITADTSTDTAYMELTSLISEDTAVYYCVSASYRYG
FSYWGQGTLVTVSS

>VL1

DIVMTQTPLSLPVTPGEPASISCRSNKSLLHTNGNTYLYWYLQKPGQSPQLLIY
RMSNLASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQHLEYPYTFGQG
TKLEIKR

>VL2

DIVMTQTPLSLPVTPGEPASISCRSNKSLLHTNGNTYLYWYLQKPGQSPQLLIY
RMSNLASGVPDRFSGSGSGTDFTLRISRVEAEDVGVYYCMQHLEYPYTFGGG
TKVEIK

>VL3

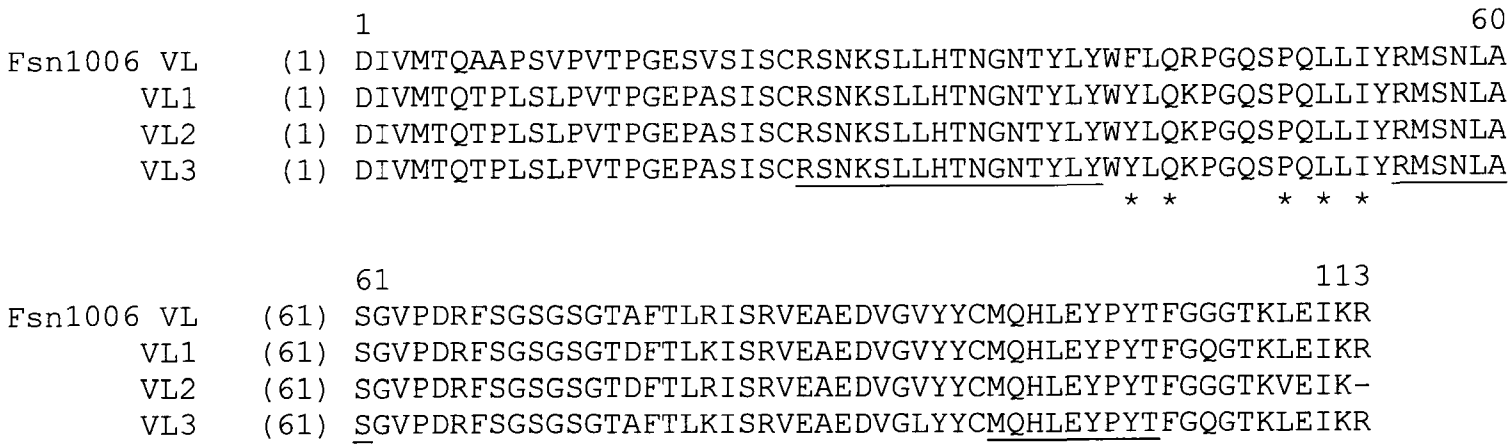
DIVMTQTPLSLPVTPGEPASISCRSNKSLLHTNGNTYLYWYLQKPGQSPQLLIY
RMSNLASGVPDRFSGSGSGTAFTLKISRVEAEDVGLYYCMQHLEYPYTFGQG
TKLEIKR

Figure 3

```
                    1                                                          60
Fsn1006 VH    (1)  EVQLQQSGAELVRPGALVKLSCKASGFNIKDSYIHWVNQRPEQGLEWIGWIDPENGNTIY
       VH1    (1)  QVQLVQSGAEVKKPGASVKVSCKASGFNIKDSYIHWVRQAPGQRLEWIGWIDPENGNTIY
       VH2    (1)  QVQLVQSGAEVKKPGASVKVSCKASGFNIKDSYIHWVRQAPGQGLEWMGWIDPENGNTIY
       VH3    (1)  QVQLVQSGAEVKKPGASVKVSCKASGFNIKDSYIHWFQQAPTRGLEWMGWIDPENGNTIY
                                           *            * *      * *

                    61                                                      118
Fsn1006 VH   (61)  DPKFQGKASITADTSSNTAYLQLSSLTSEDTAVYYCVSASYRYGFSYWGQGTLVTVSA
       VH1   (61)  DPKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYCVSASYRYGFSYWGQGTLVTVSS
       VH2   (61)  DPKFQGRVTVTRDTSISTAHMELSSLRSDDTAVYYCVSASYRYGFSYWGQGTAVTVSS
       VH3   (61)  DPKFQGRVSITADTSTDTAYMELTSLISEDTAVYYCVSASYRYGFSYWGQGTLVTVSS
                            *                       *           *
```

Figure 4



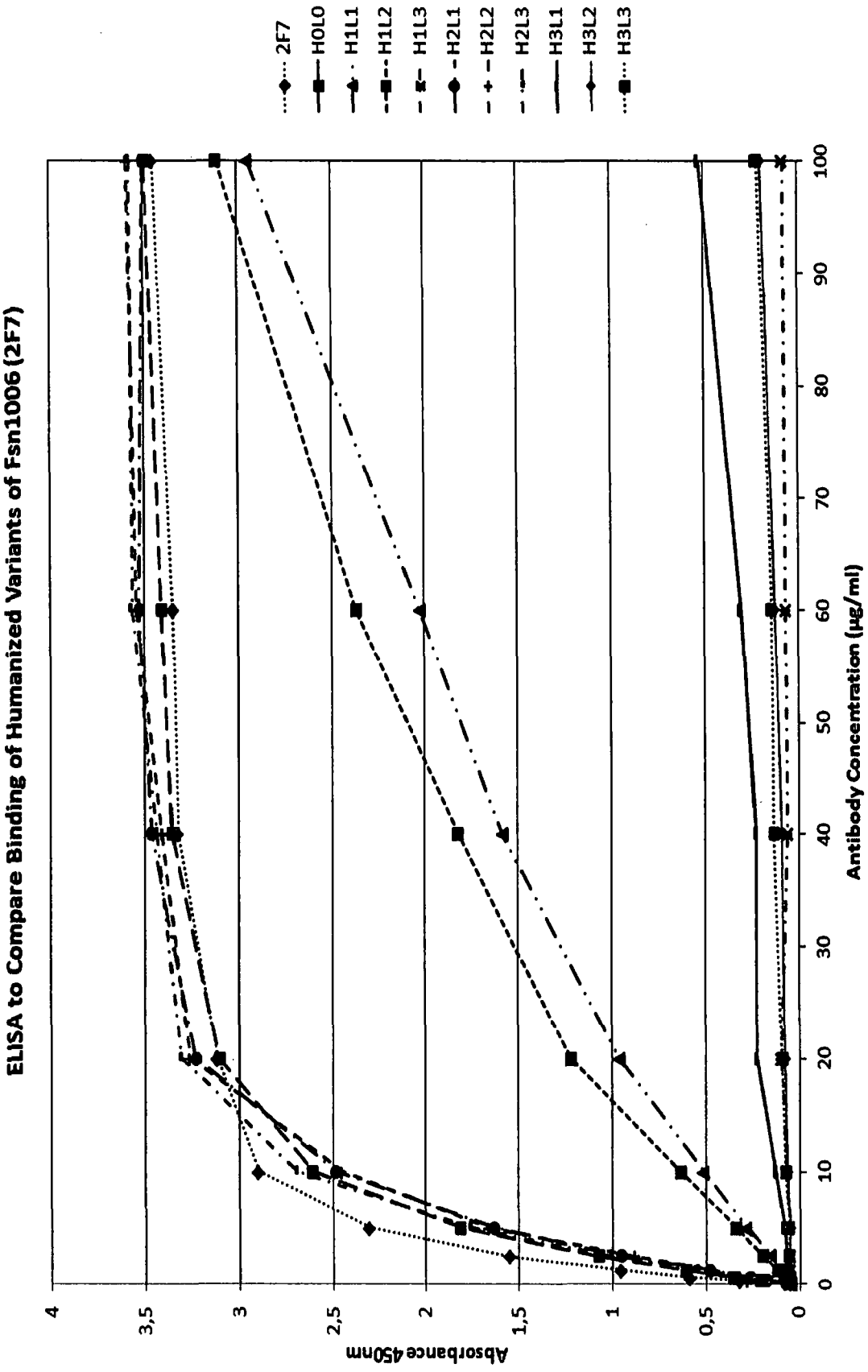

ELISA to Compare Binding of Humanized Variants of Fsn1006 (2F7)
2F7
H0L0
H1L1
H1L2
H1L3
H2L1
H2L2
H2L3
H3L1
H3L2
H3L3
Absorbance 450nm
Antibody Concentration (µg/ml)
0
0,5
1
1,5
2
2,5
3
3,5
4
0
10
20
30
40
50
60
70
80
90
100

Figure 5

Figure 6

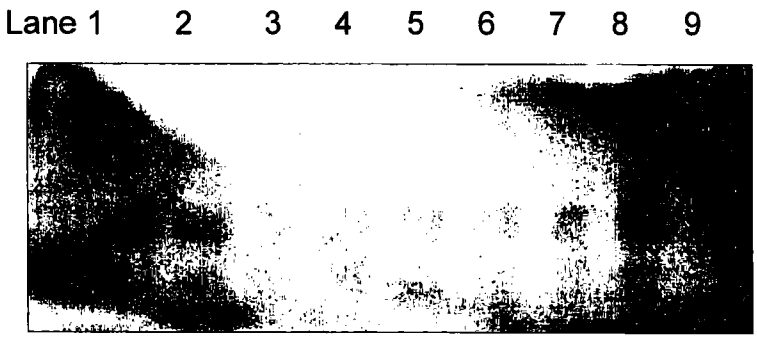


Figure 7

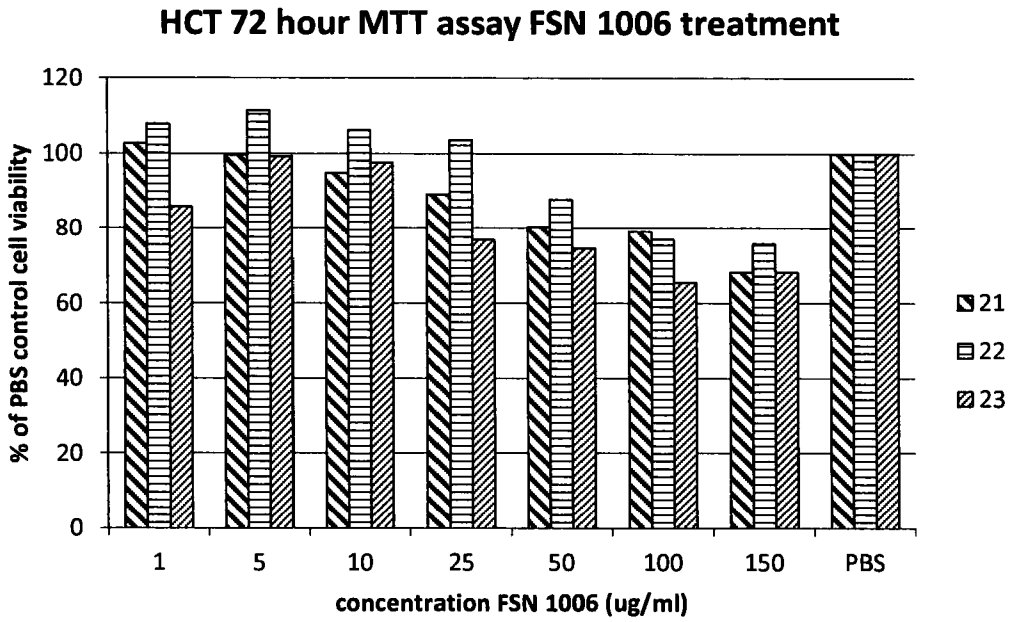

HCT 72 hour MTT assay FSN 1006 treatment
% of PBS control cell viability
120
100
80
60
40
20
0
1
5
10
25
50
100
150
PBS
concentration FSN 1006 (ug/ml)
21
22
23

Figure 8

Rituximab

Original Murine VH (SEQ. ID NO: 27)

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNG
DTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGA
GTTVTVSA

Original Murine VL (SEQ. ID NO: 28)

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVR
FSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIK

Table 2

	Kabat	IMGT	Chothia
CDR H1	SYNMH (SEQ. ID NO:51)	GYTFTSYN (SEQ. ID NO:52)	GYTFTS (SEQ. ID NO:53)
CDR H2	AIYPGNGDTSYNQKFKG (SEQ. ID NO:54)	IYPGNGDT (SEQ. ID NO:55)	AIYPGNGDTS (SEQ. ID NO:56)
CDR H3	STYYGGDWYFNV (SEQ. ID NO:57)	ARSTYYGGDWYFNV (SEQ. ID NO:58)	STYYGGDWYFNV (SEQ. ID NO:59)
CDR L1	RASSSVSYIH (SEQ. ID NO:60)	SSVSY (SEQ. ID NO:61)	RASSSVSYIH (SEQ. ID NO:62)
CDR L2	ATSNLAS (SEQ. ID NO:63)	ATS (SEQ. ID NO:64)	ATSNLAS (SEQ. ID NO:65)
CDR L3	QQWTSNPPT (SEQ. ID NO:66)	QQWTSNPPT (SEQ. ID NO:67)	QQWTSNPPT (SEQ. ID NO:68)

Figure 9

Heavy chain variable domain

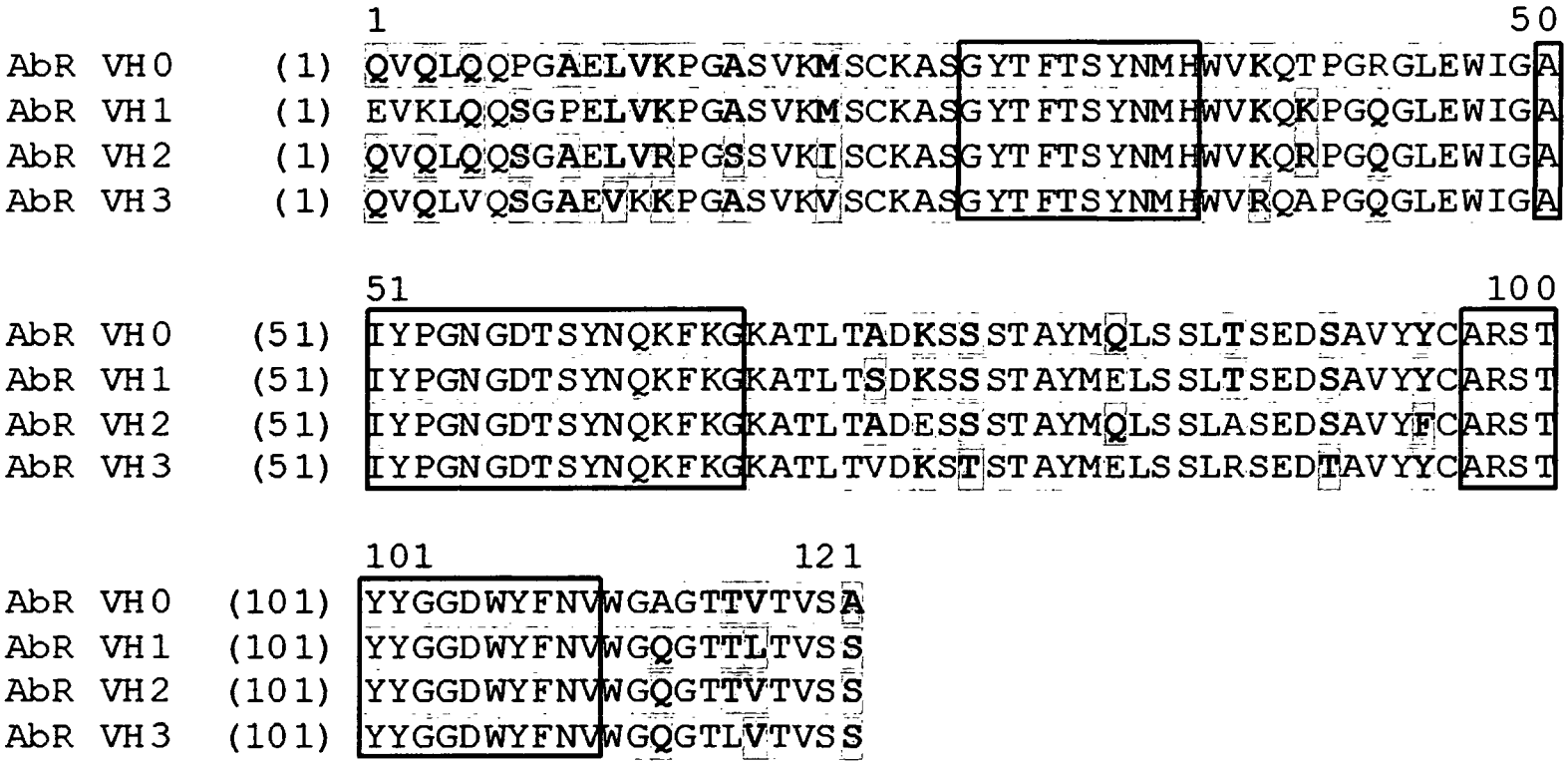


Figure 10

Light chain variable domain

```
                          1                                                  50
AbR VL0     (1)  QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYAT
AbR VL1     (1)  QFVLTQSPAFLSASPGEKVTMTCRASSSVSYIHWYQQKSGTSPKKWIYAT
AbR VL2     (1)  DIQLTQSPAIMSASPGEKVTMTCRASSSVSYIHWYQQKSGTSPKRWIYAT
AbR VL3     (1)  DIQLTQSPAIMSASPGEKVTMTCRASSSVSYIHWYQQKSGASPKLWIYAT

                         51                                                 100
AbR VL0    (51)  SNLASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGG
AbR VL1    (51)  SNLASGVPVRFSGSGSGTSYSLTISSMETEDAATYYCQQWTSNPPTFGAG
AbR VL2    (51)  SNLASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWTSNPPTFGAG
AbR VL3    (51)  SNLASGVPARFSGSGSGTSYSLTISSVEAEDAATYYCQQWTSNPPTFGGG

                         101
AbR VL0   (101)  TKLEIK
AbR VL1   (101)  TRVELK
AbR VL2   (101)  TKLELK
AbR VL3   (101)  TKLEIK
```

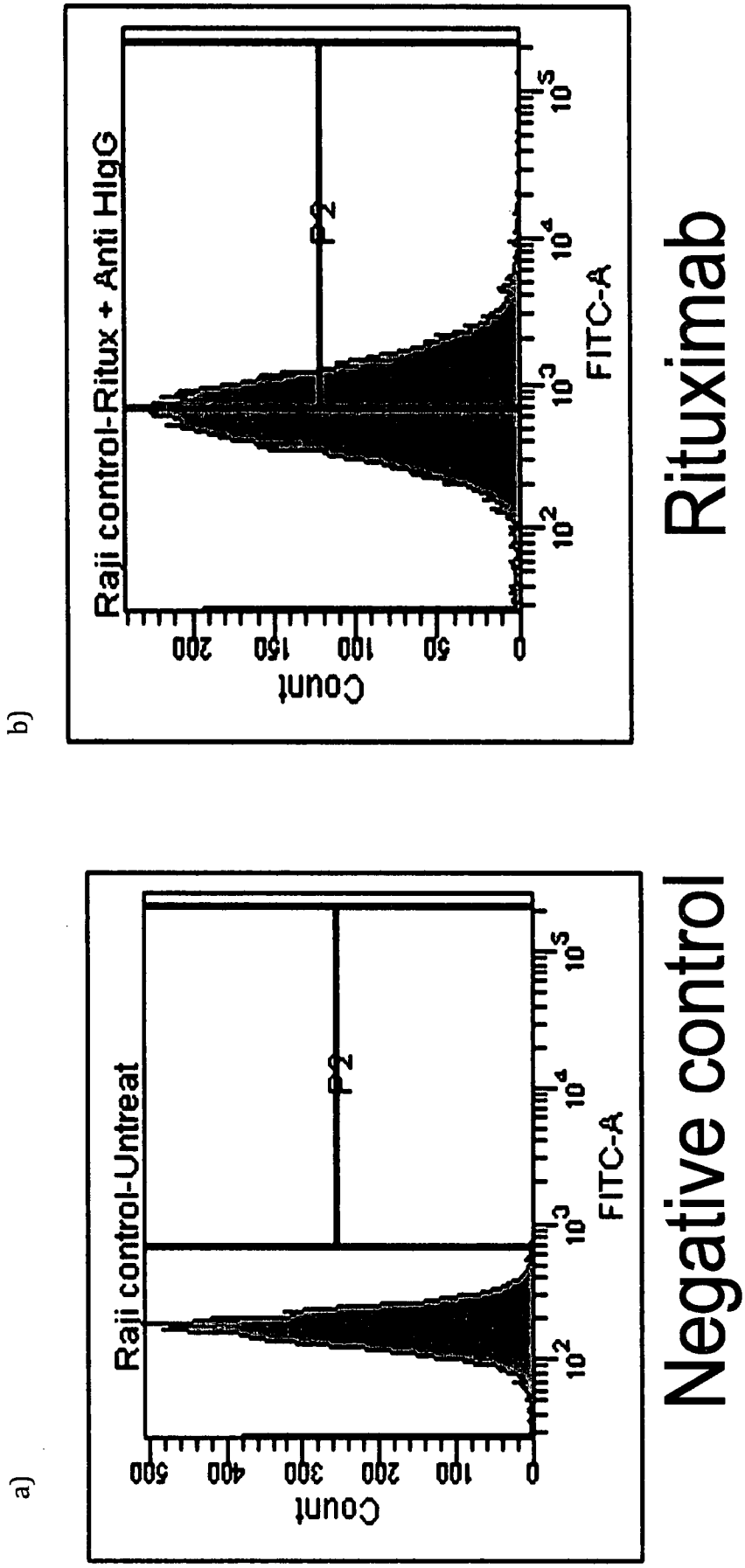
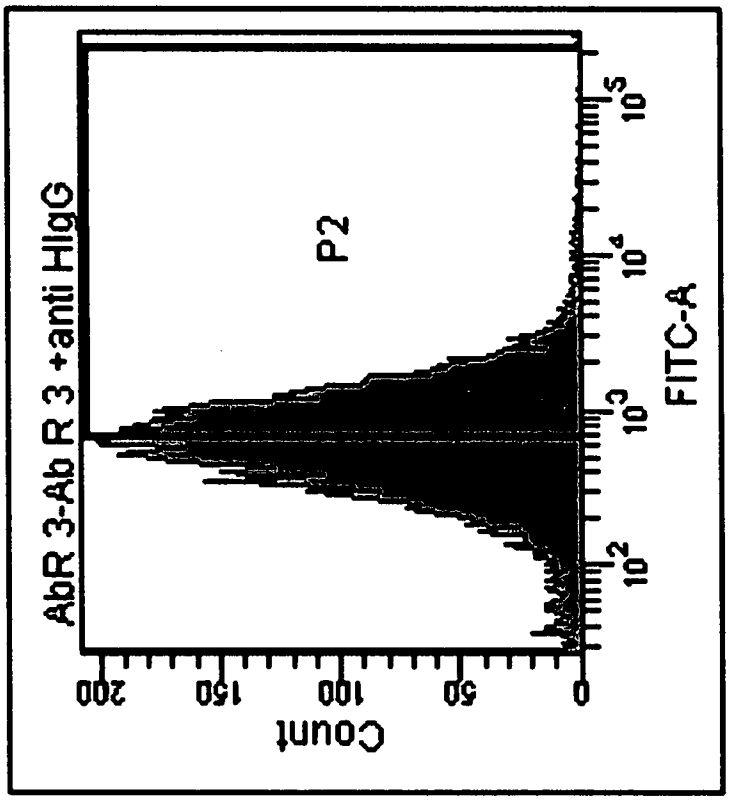
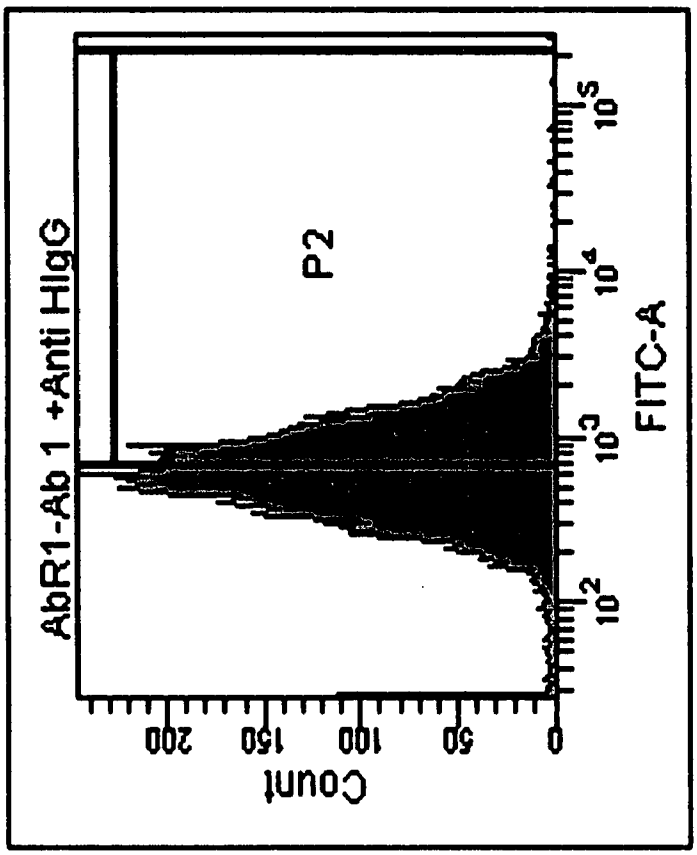

a)
Raji control-Untreat
P2
Count
FITC-A
Negative control
b)
Raji control-Ritux + Anti HIgG
P2
Count
FITC-A
Rituximab

Figure 11

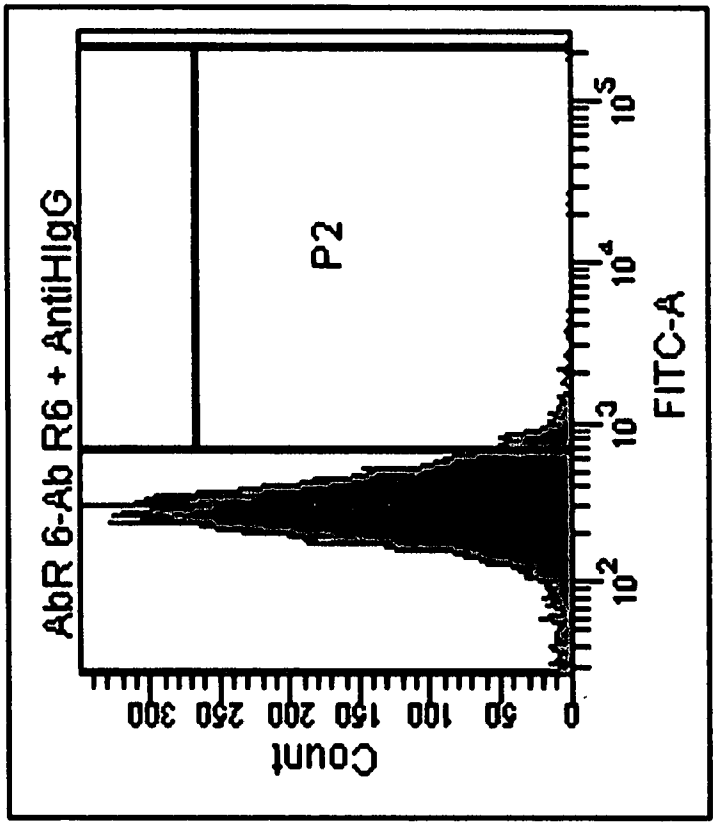
d)


AbR 3-Ab R 3 +anti HIgG
P2
FITC-A
Count
0
50
100
150
200
10^2
10^3
10^4
10^5

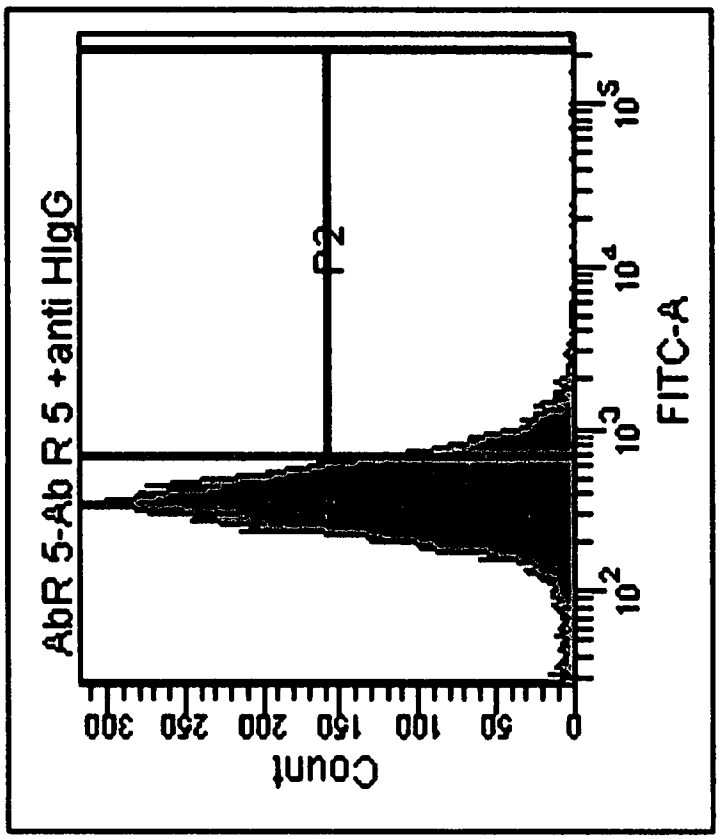
c)


AbR1-Ab 1 +Anti HIgG
P2
FITC-A
Count
0
50
100
150
200
10^2
10^3
10^4
10^5

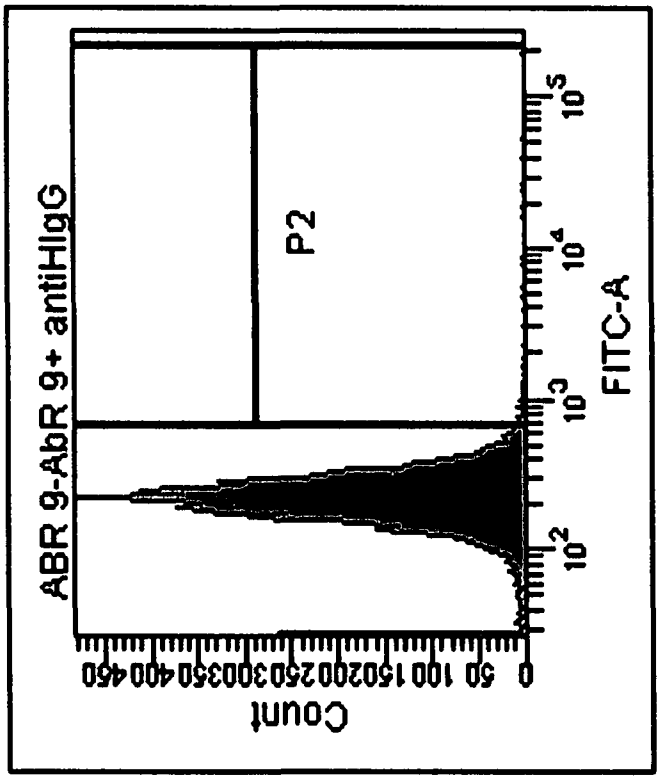
f)


AbR 6-Ab R6 + AntiHIgG
P2
Count
FITC-A
0
50
100
150
200
250
300
10^2
10^3
10^4
10^5

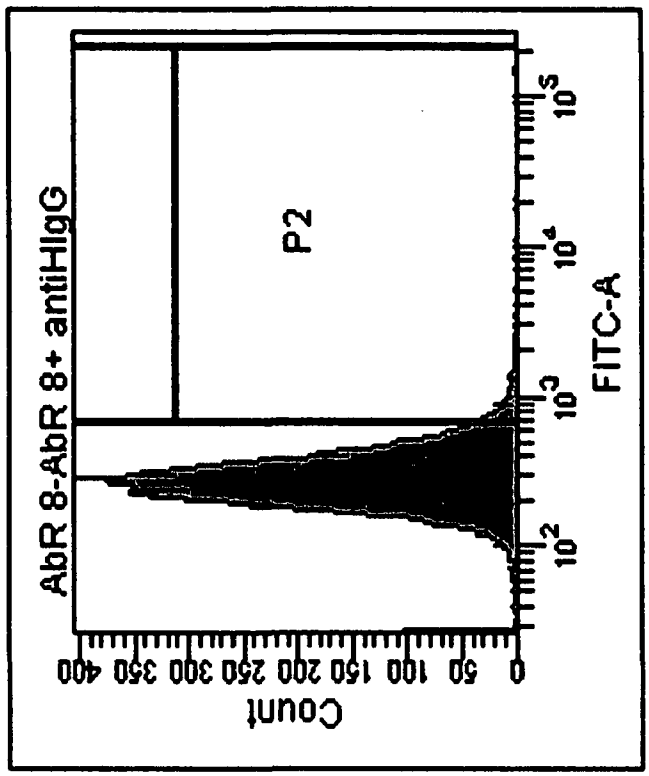
e)


AbR 5-Ab R 5 +anti HIgG
P2
Count
FITC-A
0
50
100
150
200
250
300
10^2
10^3
10^4
10^5

h)


ABR 9-AbR 9+ antiHIgG
P2
FITC-A
Count
0 50 100 150 200 250 300 350 400 450

g)


AbR 8-AbR 8+ antiHIgG
P2
FITC-A
Count
0 50 100 150 200 250 300 350 400

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/052965

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

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 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, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2009/103113 A1 (G2 INFLAMMATION PTY LTD [AU]; WHITFELD PETER [AU]; ZAHRA DAVID [AU]; M) 27 August 2009 (2009-08-27) page 2, line 10 - page 3, line 6; example 1	1-3,16, 18,23, 26,27 5-15,17, 19-22, 24,25
X Y	WO 2008/141391 A1 (CRC FOR ASHTMA AND AIRWAYS LTD [AU]; MACKAY CHARLES [AU]; ZAHRA DAVID) 27 November 2008 (2008-11-27) page 12, line 20 - page 13, line 12; example 3	1-4,16, 18,23, 26,27 5-15,17, 19-22, 24,25
Y	WO 2009/127881 A1 (FUSION ANTIBODIES LTD [GB]; CASWELL JILL [GB]; OLWILL SHANE [GB]; JOHN) 22 October 2009 (2009-10-22) claims 1-36	5-11,17, 19-22, 24,25

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 2014	03/02/2014

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2013/052965

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/052965

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2011/137362 A1 (ROTHER RUSSELL P [US]; TAMBURINI PAUL P [US]) 3 November 2011 (2011-11-03) page 68, line 9 - page 69, line 2 -----	12-15, 17,24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2013/052965

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2011044996 A1	24-02-2011
		WO 2008141391 A1	27-11-2008
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		JP 2013531476 A	08-08-2013
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