BISPECIFIC DEATH RECEPTOR AGONISTIC ANTIBODIES

The present invention relates to bispecific antibodies comprising a first antigen binding site specific for a death receptor and a second antigen binding site specific for a second antigen, methods for their production, pharmaceutical compositions containing said antibodies, and uses thereof.

Monoclonal antibodies are proving to be powerful therapeutic agents in the treatment of cancer owing to the selective targeting of antigens which are differentially expressed on cancer cells. The therapeutic strategies of most currently developed monoclonal antibodies include the targeting of tumor-associated antigens to modify tumor-cell biology, inhibition of growth factor receptors, inhibition of angiogenesis, apoptosis induction and cytotoxicity via complement fixation or antibody-dependent cellular cytotoxicity. Some antibodies target the growth factor receptors that are crucial for cancer cell survival, such as trastuzumab (Herceptin®) and cetuximab (Erbitux®). Targeting of the TRAIL death receptors on cancer cells with agonistic monoclonal antibodies represents a new generation of monoclonal antibody therapy, as they are able to directly induce apoptosis of targeted cells. The use of an agonistic monoclonal antibody against the death receptors instead of TRAIL may be advantageous: TRAIL targets multiple receptors including both the death receptors and decoy receptors and, therefore, selectivity is a concern. In addition, TRAIL has a much shorter blood half-life compared with monoclonal antibodies, a factor which affects dose and schedule parameters. The very short blood half-life of TRAIL would require large and frequent doses compared with monoclonal antibodies. In addition recombinant TRAIL is very difficult and tedious to produce.

Michaelson J.S. et al. (mAbs, Vol 1, Issue 2, p:128 – 141; March/April 2009) describe engineered IgG like biscpecific antibodies targeting two TNF family member receptors, namely TRAIL-R2 (TNF related Apoptosis Inducing Ligand Receptor-2) and LTβR (Lymphotoxin-beta Receptor).

Herrmann T. et al. (Cancer Res 2008; 68: (4); p: 1221 – 1227) describe bispecific monovalent chemically combined Fab molecules directed to CD95/Fas/Apo-1 cell surface receptor and three target antigens on glioblastoma cells: NG2, EGFR and CD40.

The present disclosure relates to antibodies combining a death receptor targeting antigen binding site with a second antigen binding site that targets a second antigen. By that the death receptors become cross linked and apoptosis of the target cell is induced. The advantage of these

bispecific death receptor agonistic antibodies over conventional death receptor targeting antibodies is the specificity of induction of apoptosis only at the site where the second antigen is expressed.

The present disclosure provides for a bispecific antibody comprising a first antigen binding site specific for a death receptor antigen and a second antigen binding site specific for a second antigen.

In a preferred embodiment of the bispecific antibody, the death receptor is selected from death receptor 4 polypeptide (DR4), death receptor 5 polypeptide (DR5) or FAS polypeptide, preferably human DR4 polypeptide (Seq. Id. No. 1), human DR5 polypeptide (Seq. Id. No. 2) or human FAS polypeptide (Seq. Id. No. 3).

In a further preferred embodiment of the bispecific antibody, the second antigen is associated with an oncological disease or rheumatoid arthrithis.

In a further preferred embodiment of the bispecific antibody, the second antigen is selected from,carcinoembryonic antigen (CEA) polypeptide, CRIPTO protein, magic roundabout homolog 4 (ROBO4) polypeptide, melanoma-associated chondroitin sulfate proteoglycan (MCSP) polypeptide, tenascin C polypeptide and fibroblast activation protein (FAP) polypeptide, preferably human CEA polypeptide (Seq. Id. No. 4), human CRIPTO polypeptide (Seq. Id. No. 5), human ROBO4 polypeptide (Seq. Id. No. 6), human MCSP polypeptide (Seq. Id. No. 7), human tenascin C polypeptide (Seq. Id. No. 8) and human FAP polypeptide (Seq. Id. No. 9).

In a first aspect, there is provided a bispecific antibody comprising a first antigen binding site specific for DR5 and a second antigen binding site specific for FAP.

In a further preferred embodiment of the bispecific antibody, the bispecific antibody is a dimeric molecule comprising a first antibody comprising the first antigen binding site and a second antibody comprising the second antigen binding site.

In a preferred embodiment of the dimeric bispecific antibody of the present invention, the first and second antibody comprise an Fc part of an antibody heavy chain, wherein the Fc part of the first antibody comprises a first dimerization module and the Fc part of the second antibody comprises a second dimerization module allowing a heterodimerization of the two antibodies.

In a further preferred embodiment of the dimeric bispecific antibody, the first dimerization module comprises knobs and the second dimerization module comprises holes according to the knobs into holes strategy (see Carter P.; Ridgway J.B.B.; Presta L.G.: Immunotechnology, Volume 2, Number 1, February 1996 , pp. 73-73(1)).

In a further preferred embodiment of the dimeric bispecific antibody, the first antibody is an Immunoglobulin (Ig) molecule comprising a light chain and a heavy chain and the second antibody is selected from the group consisting of scFv, scFab, Fab or Fv.

In a further preferred embodiment the bispecific antibody comprises a modified Fc part having a reduced binding affinity for the Fcγ receptors compared to a wildtpye Fc part e.g. a LALA modification.

In yet a further preferred embodiment of the dimeric bispecific antibody, the Ig molecule comprises the first antigen binding site specific for the death receptor and the second antibody comprises the second antigen binding site specific for the second antigen.

In a further preferred embodiment of the bispecific antibody, the Ig molecule comprises the second antigen binding site specific for the second antigen and the second antibody comprises the antigen binding site specific for the death receptor.

In a further preferred embodiment of the dimeric bispecific antibody, the second antibody is fused to the N- or C- terminus of the heavy chain of the Ig molecule.

In a further preferred embodiment of the dimeric bispecific antibody, the second antibody is fused to the N- or C-terminus of the light chain of the Ig molecule.

In yet another preferred embodiment of the dimeric bispecific antibody, the Ig molecule is an IgG. In a further preferred embodiment of the dimeric bispecific antibody, the second molecule is fused to the Ig molecule by a peptide linker, preferably a peptide linker having a length of about 10 – 30 amino acids.

In a further preferred embodiment of the dimeric bispecific antibody, the second antibody comprises additional cysteine residues to form disulfide bonds.

The bispecific antibodies according to the invention are at least bivalent and can be trivalent or multivalent e.g. tetravalent or hexavalent.

In a second aspect the present disclosure relates to a pharmaceutical composition comprising a bispecific antibody of the present disclosure.

There is also provided a bispecific antibody of the present disclosure for the treatment of cancer or rheumatoid arthritis.

In a third aspect, there is provided the bispecific antibody as defined above for use in the treatment of cancer.

In a fourth aspect, there is provided the use of the bispecific antibody as defined above in the manufacture of a medicament for the treatment of cancer.

There is also provided a nucleic acid sequence comprising a sequence encoding a heavy chain of a bispecific antibody of the present disclosure, a nucleic acid sequence comprising a sequence encoding a light chain of a bispecific antibody of the present disclosure, an expression vector comprising a nucleic acid sequence of the present disclosure and to a prokaryotic or eukaryotic host cell comprising a vector of the present disclosure.

Detailed description of the invention

The term "polypeptide" is used herein to refer to native amino acid sequences and sequence variants of the polypeptides of the present invention i.e. DR4, DR5, FAS, CEA, CRIPTO, ROBO4, MCSP, Tenascin C and FAP from any animal, e.g. mammalian species, including humans.

"Native polypeptide" refers to a polypeptide having the same amino acid sequence as a polypeptide occurring in nature regardless of its mode of preparation. The term "native polypeptide" specifically encompasses naturally occurring truncated or secreted forms, naturally occurring variant forms (e.g. alternatively spliced forms), and naturally occurring allelic variants of the polypeptides of the present invention. The amino acid sequences in the Sequence Listing (Seq. Id. No. 1 – 9) refer to native human sequences of the proteins of the present invention.

The term "polypeptide variant" refers to amino acid sequence variants of a native sequence containing one or more amino acid substitution and/or deletion and/or insertion in the native sequence. The amino acid sequence variants generally have at least about 75%, preferably at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, most preferably at least about 95% sequence identity with the amino acid sequence of a native sequence of a polypeptide of the present invention.

The term "antibody" encompasses the various forms of antibody structures including but not being limited to whole antibodies and antibody fragments. The antibody according to the invention is preferably a fully human antibody, humanized antibody, chimeric antibody, or further genetically engineered antibody as long as the characteristic properties according to the invention are retained.

"Antibody fragments" comprise a portion of a full length antibody, preferably the variable domain thereof, or at least the antigen binding site thereof. Examples of antibody fragments include diabodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments. scFv antibodies are, e.g. described in Houston, J.S., Methods in Enzymol. 203 (1991) 46-96). In addition, antibody fragments comprise single chain polypeptides having the characteristics of a VH domain, namely being able to assemble together with a VL domain, or of a VL domain, namely being able to assemble together with a VH domain to a functional antigen binding site and thereby providing the antigen binding property of full length antibodies.

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

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

The term "humanized antibody" refers to antibodies in which the framework or "complementarity determining regions" (CDR) have been modified to comprise the CDR of an immunoglobulin of different specificity as compared to that of the parent immunoglobulin. In a preferred embodiment, a murine CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody." See e.g. Riechmann, L., et al., Nature 332 (1988) 323-327; and Neuberger, M.S., et al., Nature 314 (1985) 268-270. Particularly preferred CDRs correspond to those representing sequences recognizing the antigens noted above for chimeric antibodies. Other forms of "humanized antibodies" encompassed by the present invention are those in which the constant region has been additionally modified or changed from that of the original antibody to generate the properties according to the invention, especially in regard to C1q binding and/or Fc receptor (FcR) binding.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germ line immunoglobulin sequences. Human antibodies are well-known in the state of the art (van Dijk, M.A., and van de Winkel, J.G., Curr. Opin. Chem. Biol. 5 (2001) 368-374). Human antibodies can also be produced in transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire or a selection of human antibodies in the absence of endogenous immunoglobulin production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge (see, e.g., Jakobovits, A., et al., Proc. Natl. Acad. Sci. USA 90 (1993) 2551-2555; Jakobovits, A., et al., Nature 362 (1993) 255-258; Bruggemann, M., et al., Year Immunol. 7 (1993) 33-40). Human antibodies can also be produced in phage display libraries (Hoogenboom, H.R., and Winter, G., J. Mol. Biol. 227 (1992) 381-388; Marks, J.D., et al., J. Mol. Biol. 222 (1991) 581-597). The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Mono-

clonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); and Boerner, P., et al., J. Immunol. 147 (1991) 86-95). As already mentioned for chimeric and humanized antibodies according to the invention the term "human antibody" as used herein also comprises such antibodies which are modified in the constant region to generate the properties according to the invention, especially in regard to C1q binding and/or FcR binding, e.g. by "class switching" i.e. change or mutation of Fc parts (e.g. from IgG1 to IgG4 and/or IgG1/IgG4 mutation.)

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

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

The term "antigen-binding site of an antibody" when used herein refer to the amino acid residues of an antibody which are responsible for antigen-binding. The antigen-binding portion of an antibody comprises amino acid residues from the "complementary determining regions" or "CDRs". "Framework" or "FR" regions are those variable domain regions other than the hypervariable region residues as herein defined. Therefore, the light and heavy chain variable domains of an antibody comprise from N- to C-terminus the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Especially, CDR3 of the heavy chain is the region which contributes most to antigen binding and defines the antibody's properties. CDR and FR regions are determined according to the standard definition of Kabat et al., Sequences of Proteins of Immunological Inter-

est, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) and/or those residues from a "hypervariable loop".

Antibody specificity refers to selective recognition of the antibody for a particular epitope of an antigen. Natural antibodies, for example, are monospecific. "Bispecific antibodies" according to the invention are antibodies which have two different antigen-binding specificities. Antibodies of the present invention are specific for two different antigens, i.e. death receptor antigen as first antigen and a second antigen.

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

The term "bispecific" antibody as used herein denotes an antibody that has at least two binding sites each of which bind to different epitopes of the same antigen or a different antigen.

The term "valent" as used within the current application denotes the presence of a specified number of binding sites in an antibody molecule. As such, the terms "bivalent", "tetravalent", and "hexavalent" denote the presence of two binding sites, four binding sites, and six binding sites, respectively, in an antibody molecule. The bispecific antibodies according to the invention are at least "bivalent" and may be "trivalent" or "multivalent" (e.g."tetravalent" or "hexavalent").

Antibodies of the present invention have two or more binding sites and are bispecific. That is, the antibodies may be bispecific even in cases where there are more than two binding sites (i.e. that the antibody is trivalent or multivalent). Bispecific antibodies of the invention include, for example, multivalent single chain antibodies, diabodies and triabodies, as well as antibodies having the constant domain structure of full length antibodies to which further antigen-binding sites (e.g., single chain Fv, a VH domain and/or a VL domain, Fab, or (Fab)2) are linked via one or more peptide-linkers. The antibodies can be full length from a single species, or be chimerized or humanized.

A "single chain Fab fragment" is a polypeptide consisting of an antibody heavy chain variable domain (VH), an antibody constant domain 1 (CH1), an antibody light chain variable domain (VL), an antibody light chain constant domain (CL) and a linker, wherein said antibody domains and said linker have one of the following orders in N-terminal to C-terminal direction: a) VH-CH1-linker-VL-CL, b) VL-CL-linker-VH-CH1, c) VH-CL-linker-VL-CH1 or d) VL-CH1-linker-VH-CL; and wherein said linker is a polypeptide of at least 30 amino acids, preferably between 32 and 50 amino acids. Said single chain Fab fragments a) VH-CH1-linker-VL-CL, b) VL-CL-linker-VH-CH1, c) VH-CL-linker-VL-CH1 and d) VL-CH1-linker-VH-CL, are stabilized via the natural disulfide bond between the CL domain and the CH1 domain. In addition, these single chain Fab molecules might be further stabilized by generation of interchain disulfide

bonds via insertion of cysteine residues (e.g. position 44 in the variable heavy chain and positionn 100 in the variable light chain according to Kabat numbering). The term "N-terminus denotes the last amino acid of the N-terminus, The term "C-terminus denotes the last amino acid of the C-terminus.

The terms "nucleic acid" or "nucleic acid molecule", as used herein, are intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "amino acid" as used within this application denotes the group of naturally occurring carboxy α-amino acids comprising alanine (three letter code: ala, one letter code: A), arginine (arg, R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

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

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

As used herein, the term "binding" or "specifically binding" refers to the binding of the antibody to an epitope of the antigen in an in-vitro assay, preferably in a surface plasmon resonance assay (SPR, BIAcore, GE-Healthcare Uppsala, Sweden). The affinity of the binding is defined by the terms ka (rate constant for the association of the antibody from the antibody/antigen

complex), kD (dissociation constant), and KD (kD/ka). Binding or specifically binding means a binding affinity (KD) of 10-8 mol/l or less, preferably 10-9 M to 10-13 mol/l.

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

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

The "Fc part" of an antibody is not involved directly in binding of an antibody to an antigen, but exhibit various effector functions. A "Fc part of an antibody" is a term well known to the skilled artisan and defined on the basis of papain cleavage of antibodies. Depending on the amino acid sequence of the constant region of their heavy chains, antibodies or immunoglobulins are divided in the classes: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG1, IgG2, IgG3, and IgG4, IgA1, and IgA2. According to the heavy chain constant regions the different classes of immunoglobulins are called α, δ, ε, γ, and μ respectively. The Fc part of an antibody is directly involved in ADCC (antibody-dependent cell-mediated cytotoxicity) and CDC (complement-dependent cytotoxicity) based on complement activation, C1q binding and Fc receptor binding. Complement activation (CDC) is initiated by binding of complement factor C1q to the Fc part of most IgG antibody subclasses. While the influence of an antibody on the complement system is dependent on certain conditions, binding to C1q is caused by defined binding sites in the Fc part. Such binding sites are known in the state of the art and described e.g. by Boakle et al., Nature 282 (1975) 742-743, Lukas et al., J. Immunol. 127 (1981) 2555-2560, Brunhouse and Cebra, Mol. Immunol. 16 (1979) 907-917, Burton et al., Nature 288 (1980) 338-344, Thommesen et al., Mol. Immunol. 37 (2000) 995-1004, Idusogie et al., J. Immunol.164 (2000) 4178-4184, Hezareh et al., J. Virology 75 (2001) 12161-12168, Morgan et al., Immunology 86 (1995) 319-324, EP 0307434. Such binding sites are e.g. L234, L235, D270, N297, E318, K320, K322, P331 and P329 (numbering according to EU index of Kabat, see below). Antibodies of subclass IgG1, IgG2 and IgG3 usually show complement activation and C1q and C3 binding, whereas IgG4 do not activate the complement system and do not bind C1q and C3.

The antibodies according to the invention are produced by recombinant means. Thus, one aspect of the current invention is a nucleic acid encoding the antibody according to the invention

and a further aspect is a cell comprising said nucleic acid encoding an antibody according to the invention. Methods for recombinant production are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody and usually purification to a pharmaceutically acceptable purity. For the expression of the antibodies as aforementioned in a host cell, nucleic acids encoding the respective modified light and heavy chains are inserted into expression vectors by standard methods. Expression is performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NS0 cells, SP2/0 cells, HEK293 (including HEK293 EBNA) cells, COS cells, PER.C6 cells, yeast, or E.coli cells, and the antibody is recovered from the cells (supernatant or cells after lysis). General methods for recombinant production of antibodies are well-known in the state of the art and described, for example, in the review articles of Makrides, S.C., Protein Expr. Purif. 17 (1999) 183-202; Geisse, S., et al., Protein Expr. Purif. 8 (1996) 271-282; Kaufman, R.J., Mol. Biotechnol. 16 (2000) 151-161; Werner, R.G., Drug Res. 48 (1998) 870-880.

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

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

The term "host cell" as used in the current application denotes any kind of cellular system which can be engineered to generate the antibodies according to the current invention. In one embodiment HEK293 cells and CHO cells are used as host cells. As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transfectants" and "tansfected cells" include the primary subject cell and cultures derived there from without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent

mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

Expression in NS0 cells is described by, e.g., Barnes, L.M., et al., Cytotechnology 32 (2000) 109-123; Barnes, L.M., et al., Biotech. Bioeng. 73 (2001) 261-270. Transient expression is described by, e.g., Durocher, Y., et al., Nucl. Acids. Res. 30 (2002) E9. Cloning of variable domains is described by Orlandi, R., et al., Proc. Natl. Acad. Sci. USA 86 (1989) 3833-3837; Carter, P., et al., Proc. Natl. Acad. Sci. USA 89 (1992) 4285-4289; and Norderhaug, L., et al., J. Immunol. Methods 204 (1997) 77-87. A preferred transient expression system (HEK 293) is described by Schlaeger, E.-J., and Christensen, K., in Cytotechnology 30 (1999) 71-83 and by Schlaeger, E.-J., in J. Immunol. Methods 194 (1996) 191-199.

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

Purification of antibodies is performed in order to eliminate cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art. See Ausubel, F., et al., ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987). Different methods are well established and widespread used for protein purification, such as affinity chromatography with microbial proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and electrophoretical methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M.A. Appl. Biochem. Biotech. 75 (1998) 93-102).

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

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

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

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

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

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

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

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

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

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

The term "transformation" as used herein refers to process of transfer of a vectors/nucleic acid into a host cell. If cells without formidable cell wall barriers are used as host cells, transfection is carried out e.g. by the calcium phosphate precipitation method as described by Graham and Van der Eh, Virology 52 (1978) 546ff. However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used. If prokaryotic cells or cells which contain substantial cell wall constructions are used, e.g. one method of transformation is calcium treatment using calcium chloride as described by Cohen, F. N, et al, PNAS. 69 (1972) 7110ff.

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

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

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

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

Short description of the figures

Figure 1: FACS binding analysis of CEA, DR5 and FAS expression levels on different human cell lines (Lovo, OVCAR-3, AsPC-1, BxPC3, LS174T and MKN-45) using unlabelled, commercially available murine IgG1 antibodies (CEA: Abcam # 11330; DR5: R&D # MAB631; FAS: BD # 555671) and a common goat anti mouse FITC labelled IgG (Serotec Star105F) for detection. As controls samples containing only cells or cells and secondary antibody alone were used. Except Lovo cells all tested cell lines express significant amounts of DR5 and FAS on the surface. Compared to that CEA expression was rather low. When the same cells were tested with other antibodies for the three antigens also Lovo cells were positive in FACS analysis to express DR5, FAS and CEA (data not shown).

Figure 2: Analysis of apoptosis induction (DNA fragmentation assay) of different cell lines after 4 hrs incubation with commercially available antibodies that are able to induce apoptosis in solution without cross-linking (DR5: R&D # MAB631; FAS: Millipore / Upstate: CH11). For detection of apoptosis the Cell Death Detection ELISAPLUS kit for analysis of histone-associated DNA fragmentation was used. In BxPC-3, Lovo and LS174T cells apoptosis clearly can be induced via DR5 and FAS, while ASPC-1 cells do not undergo apoptosis at all. MKN-45 cells are more resistant to DR5 compared to the other cell lines.

Figure 3: Induction of apoptosis (DNA fragmentation assay) of LS174T cells after 4 hrs incubation with ApomAb (white bar), ApomAb cross-linked with an anti human Fc antibody (hatched, grey bar), ApomAb_sm3e_A (black bar) and ApomAb_sm3e_A1 (stippled grey bar)

bispecific molecules. CEA binding dependent induction of apoptosis by targeted hyper-cross-linking via the bispecific antibodies can be detected. This effect is in the same range as the apoptosis induced by cross-linking of ApomAb and could be completely abolished by pre-incubation with an excess amount of sm3e IgG. No apoptosis was observed with the controls (cells only or sm3e IgG) and also the ApomAb alone did not induce apoptosis in the used concentration (1 µg / ml).

Figure 4: Comparison of apoptosis inducing activity of different ApomAb_sm3e bispecific molecules compared to ApomAb (white bar) alone or ApomAb cross-linked with an anti human Fc antibody (hatched, grey bar), in a DNA fragmentation assay with LS174T cells incubated for 4 hrs with apoptosis inducing agents. In general, molecules where the sm3e scFv is fused to the C-terminus of the heavy chain of ApomAb (format A, black bar) seem to be more active than constructs in which the sm3e scFv is fused to the C-terminus of the light chain of ApomAb (format B, grey bar). Furthermore, disulfide stabilized scFv containing bispecific antibodies (format A1, dotted grey bar and B1, small grid bar) seem to be slightly inferior to molecules with the wild type scFv.

Figure 5: Analysis of apoptosis induction (DNA fragmentation assay) of LS174T cells after 4 hrs of incubation with either Apomab (white bars), ApomAb that was cross-linked with an anti human Fc antibody (hatched, grey bars) or ApomAb_PR1A3_A bispecific construct (black bars). In each case apoptosis induction was dependent on the concentration of the antibody used. ApomAb alone also induced low levels of apoptosis at high concentrations but this was significantly increased by cross-linking. The bispecific ApomAb_PR1A3_A molecule was even more active without secondary cross-linking agent than the cross-linked ApomAb was.

Figure 6: Analysis of apoptosis induction (DNA fragmentation assay) of Lovo cells after 4 hrs of incubation with either Apomab (white bars), ApomAb that was cross-linked with an anti human Fc antibody (grey bars) or ApomAb_PR1A3_A bispecific construct (black bars). In each case apoptosis induction was dependent on the concentration of the antibody used. ApomAb alone also induced low levels of apoptosis at high concentrations (as described) but this was significantly increased by cross-linking. The bispecific ApomAb_PR1A3_A molecule was as active on its own as the cross-linked ApomAb was.

Figure 7: Comparison of DNA fragmentation in LS174T cells after 4 hrs incubation with different apoptosis inducing bispecific antibodies. The used molecules are ApomAb_PR1A3 bispecific molecules in which the PR1A3 scFv (wt = A / B or disulfide stabilized = A1 / B1) is fused to either the C-terminus of the heavy chain (A, hatched grey bar) or the light chain (B, dotted bar). While the fusion position of the scFv in this case does not seem to make a difference in terms of apoptosis induction, the kind of used scFv is important: using the disulfide stabilized

scFv almost completely abolished induction of apoptosis compared to constructs containing the wt scFv fused to ApomAb (black and grey bar, respectively). Due to the lower affinity of PR1A3 compared to sm3e also the overall induction of apoptosis is lower with PR1A3 containing bispecific molecules.

Figure 8: FACS binding analysis of ApomAb – CEA (PR1A3) bispecific constructs on MKN-45 cells. Comparison of ApomAb_PR1A3 bispecific constructs with wild type (A) or disulfide stabilized scFv (A1). Both bispecific constructs bind in a concentration dependent manner to the target cells but the molecule containing PR1A3 in wild type scFv format binds with much higher affinity to the antigen than the disulfide stabilized PR1A3 scFv.

Figure 9: Analysis of surface expression of CRIPTO, FAS and DR5 on NCCIT and recombinant, CRIPTO expressing HEK293 cells by FACS binding experiments. NCCIT cells do not express FAS, only low amounts of CRIPTO but similar amounts of DR5 compared to recombinant HEK293 – CRIPTO cells. The latter cells show low levels of FAS, significant levels of DR5 and rather high levels of CRIPTO expression.

Figure 10: Apoptosis induction comparison (DNA fragmentation in HEK293-CRIPTO cells) using FAS (HFE7A IgG), FAS (HFE7A IgG) cross-linked via anti human Fc antibody and FAS – CRIPTO bispecific molecules (HFE7A_LC020 H3L2D1, in which the wt (A) or disulfide stabilized (A1) CRIPTO scFv is fused to the C-terminus of the heavy chain of HFE7A. FAS IgG alone, CRIPTO IgG alone and a FAS – MCSP bispecific molecule did not induce apoptosis while the cross-linked FAS and the HFE7A – CRIPTO bispecific molecules show DNA fragmentation after 4 hrs incubation which also in part could be abolished by pre-incubation with excess of anti CRIPTO IgG.

Figure 11: Apoptosis induction (DNA fragmentation assay) by HFE7A-CRIPTO bispecific molecules in recombinant HEK293-CRIPTO cells (black bars) compared to recombinant HEK293-FAP (fibroblast activation protein) cells (white bars). In both cell lines apoptosis can be induced using an apoptosis inducing commercially available antibody (CH11) and with HFE7A IgG that was cross-linked via a second, Fc specific antibody, whereas HFE7A alone did not induce apoptosis under the used conditions. Induction of apoptosis with the bispecific FAS-CRIPTO molecule was higher than with the cross-linked HFE7A IgG but could not completely be inhibited by pre-incubation with an anti CRIPTO IgG in excess. In the HEK293-FAP cells a certain low background apoptosis could be observed which also could not be out-competed by CRIPO IgG in excess. Even a negative control molecule (in which a disulfide stabilized MCSP specific scFv was fused to the C-terminus of the heavy chain of HFE7A) showed a low degree of apoptosis in HEK293-FAP cells.

Figure 12: FACS binding analysis for determination of surface expression levels of MCSP on different cell lines (MCF7, SkBr3, A431, A549, HCT-116 and U87-MG) using two different antibodies. With both antibodies the same levels of MCSP expression could be detected, indicating that U87-MG showed the highest MCSP expression, HCT-116 with a low MCSP expression whereas all other tested cell lines were MCSP negative (in the range of the negative control such as unstained cells).

Figure 13: Evaluation of apoptosis capability of U87-MG (A) and HCT-116 (B) cells using soluble and cross-linked ApomAb (black bars) and HFE7A (grey bars) and the relevant control molecules (anti FAS_CH11, anti DR5_R2 and anti Fc-IgG alone). While in HCT-116 cells apoptosis could only be induced via the DR5 receptor after four hours and not via FAS, this was different for U87-MG cells. Here, significant apoptosis only could be observed after 24 hours. In contrast to HCT-116 cells in U87-MG apoptosis induction by cross-linked HFE7A was twice as efficient as with cross-linked ApomAb. The control antibodies conferring apoptosis already in solution where even more efficient.

Figure 14: Analysis of apoptosis induction on U87-MG glioma cells after 24 hours incubation with the bispecific HFE7A-MCSP antibody (mAb 9.2.27) in which either the wild type (A format) or disulfide stabilized MCSP scFv (A1 format) is fused to the C-terminus of the heavy chain of ApomAb. In this case the construct containing the disulfide stabilized scFv demonstrated significantly higher apoptosis that the molecule containing the wild type scFv (although the amount of apoptosis measured by DNA fragmentation was relatively low). However, in both cases the induction of apoptosis could be completely abolished by pre-incubation of the cells with an excess of competing MCSP IgG.

Figure 15: FACS binding analysis of two different cell lines (SW872 and GM05389) for expression levels of human fibroblast activation protein (FAP) (A). The fluorescence intensity measured with different concentrations of an anti FAP antibody is shown over a range of three magnitudes (black, grey and hatched bars). Negative control reactions as secondary antibody and cells only ate shown as stippled and white bars, respectively. While the GM05389 cells demonstrate expression of FAP over all tested antibody concentrations that was above background, with the SW872 cells FAP expression only could be detected with the highest antibody concentration used (10 µg / ml), indicating that these cells are not suitable for FAP based binding / apoptosis induction experiments. In addition it is shown that this cell line hardly undergo ApomAb mediated apoptosis (B). ApomAb alone or another, commercially available anti DR5 antibody did not induce relevant DNA fragmentation. Only when ApomAb is cross-linked with an anti human Fc antibody a detectable low level apoptosis induction can be observed.

Figure 16: Analysis of apoptosis induction of GM05389 (white bars) and MDA-MB-231 (grey bars) alone compared to apoptosis induction upon co-cultivation of both cell lines (black bars). In all cell lines ApomAb alone only had a minor effect, while cross-linking of ApomAb resulted in significant apoptosis induction in the MDA-MB-231 cells. Induction of DNA fragmentation with the death receptor agonistic bispecific constructs (ApomAb – FAP) only occurred in high levels when both cell lines are co-cultivated. Here the cross-linking of ApomAb alone did not increase apoptosis in the same range, indicating that for optimal induction of apoptosis two cell lines are necessary: one expressing the death receptor and a second one expressing the FAP antigen.

Figure 17: Results of apoptosis induction assay (24 hrs) on MKN-45 cells with tetravalent bispecific ApomAb_PR1A3_scFab molecules in which the scFab is fused to the C-terminus of the heavy chain of ApomAb (A format). Apoptosis induction is compared to ApomAb (+ / - cross-linking with 10 fold excess of anti-human-Fc- antibody) and negative controls. All constructs were used at concentrations of 0.1 and 1.0 µg / ml. Under the used assay conditions the bispecific ApomAb_PR1A3_scFab construct (black bars) clearly shows a concentration dependent induction of apoptosis which is in the same range as observed with hyper-cross-linked ApomAb (grey bars) and which is significantly higher as with ApomAb alone (hatched bars).

Figure 18: Analysis of apoptosis induction of LS174T cells by ApomAb (alone, hatched bars or hyper-cross-linked, grey bars) compared to bispecific trivalent constructs (ApomAb_sm3e_scFab; 2x1 valency, black bars) and negative controls. The assay was performed for 4 hrs using the constructs in concentrations of 0.1 and 1.0 µg / ml. The bispecific ApomAb_sm3e_scFab construct is able to induce apoptosis in a concentration dependent manner in the same range as hyper-cross-linked ApomAb does.

Figure 19: Analysis of in-vivo efficacy of ApomAb and the bispecific DR5 agonistic antibody ApomAb_sm3e_A1 compared to the vehicle control in an intrasplenic metastatic model using the human colon carcinoma cell line LS174T. Random groups of ten mice each were treated either with PBS (black line), with ApomAb (black circles) or the ApomAb-sm3e_A1 bispecific antibody (black squares). The percentage of survival is plotted against the time course of the experiment.

Examples

Example 1: Design of bispecific antibodies recognizing human death receptor 5 and human CEA

In the following tetravalent bispecific antibodies comprising a full length antibody binding to a first antigen (human death receptor, DR5) combined with two single chain Fv fragments

binding to a second antigen (human carcinoembryonic antigen, CEA) fused via a peptide linker either to the C-terminus of the heavy or light chain of the full length antibody are described. The antibody domains and the linker in said single chain Fvs have the following orientation: VH – linker – VL.

As the variable light and heavy chains of the DR5 recognizing antibody sequences of ApomAb antibody described by Adams in US2007 / 0031414 A1 were used.

For the CEA antigen binding scFvs the sequences of the variable light and heavy chains of PR1A3 (Bodmer et al., 1999; US5965710) and sm3e (Begent et al., 2003; US7232888 B2) were used.

By gene synthesis and recombinant DNA technology the VH and VL of the corresponding CEA antibodies were linked by a glycine – serine (G4S)4 linker to generate single chain Fvs which were fused by a (G4S)n connector (where n = 2 or 4) to either the C-terminus of the heavy or light chain of the ApomAb IgG1.

In addition to the 'wild type' scFvs, variants containing cysteine residues at Kabat position 44 in the variable heavy chain and Kabat position 100 in the variable light chain were produced to generate interchain disulfide bridges between VH and VL. This had the aim to stabilize the scFv molecule to minimize potential aggregation tendency.

To prevent non-specific cross-linking of the bispecific molecules, e.g. via Fc□ receptors as the human FcγRIIIa, two amino acids in the Fc region of the IgG part of the bispecific molecules were changed. By site directed mutagenesis the two leucine residues at position 234 and 235 in the Fc region were exchanged by alanine residues. This so called LALA mutation is described as to abolish Fc – FcR interaction (Hessell et al., Nature 449 (2007), 101 ff).

All these molecules were recombinantly expressed, produced and purified using standard antibody purification techniques including protein A affinity chromatography followed by size exclusion chromatography. The molecules were characterized in terms of expression yield, stability and biological activity.

A summary of the different bispecific death receptor agonistic antibody molecules consisting of ApomAb – CEA combinations is given in table 1. The description of the design of the different molecules can be concluded from the molecule names, where the first part characterizes the death receptor targeting IgG (e.g. ApomAb), the second name describes the source of the CEA targeting scFv (e.g. PR1A3 or sm3e) and the letter and number combination describes the fusion position and disulfide stabilization property of the scFv.

Table 1: Description of the different bispecific death receptor agonistic antibodies targeting human DR5 and human CEA with their relevant characteristics.

Name	IgG	scFv (CEA)	Fusion position	Linker	Connector	Disulfide stabilization
ApomAb-sm3e-A	ApomAb	sm3e	C-terminus heavy chain	$(G_4S)_4$	$(G_4S)_2$	no
ApomAb-sm3e-A1	ApomAb	sm3e	C-terminus heavy chain	$(G_4S)_4$	$(G_4S)_2$	yes
ApomAb-sm3e-B	ApomAb	sm3e	C-terminus light chain	$(G_4S)_4$	$(G_4S)_2$	no
ApomAb-sm3e-B1	ApomAb	sm3e	C-terminus light chain	$(G_4S)_4$	$(G_4S)_2$	yes
ApomAb-PR1A3-A	ApomAb	PR1A3	C-terminus heavy chain	$(G_4S)_4$	$(G_4S)_2$	no
ApomAb-PR1A3-A1	ApomAb	PR1A3	C-terminus heavy chain	$(G_4S)_4$	$(G_4S)_2$	yes
ApomAb-PR1A3-B	ApomAb	PR1A3	C-terminus light chain	$(G_4S)_4$	$(G_4S)_2$	no
ApomAb-PR1A3-B1	ApomAb	PR1A3	C-terminus light chain	$(G_4S)_4$	$(G_4S)_2$	yes

Example 2: Expression and purification of bispecific death receptor agonistic antibodies

Separate expression vectors for the light and heavy chains for each bispecific antibody were constructed. These vectors contain a prokaryotic selection marker, regulatory elements for gene expression in mammalian cells and an origin of replication, oriP, from Ebstein-Barr virus for autonomous replication of the plasmids in EBNA containing HEK293 cells. The plasmids were propagated in E. coli, amplified, purified and co-transfected into HEK293 EBNA cells us-

ing Ca-phosphate mediated precipitation for transient expression. After seven days the cell culture supernatants were harvested and the antibodies were purified by protein A and size exclusion chromatography. The purified molecules were analyzed for homogeneity, stability and integrity by analytical size exclusion chromatography (before and after one freeze-thaw step) and SDS-PAGE analysis (under non-reducing and reducing conditions).

Table 2: Summary of the purification yields and monomer content of different death receptor agonistic bispecific antibodies

Name	Yield [mg / L)	Concentration [mg / ml]	Monomer content [%]	Aggregate increase after freezing
ApomAb-sm3e-A	4.34	0.14	100.00	no
ApomAb-sm3e-A1	4.38	1.25	100.00	no
ApomAb-sm3e-B	3.18	1.27	100.00	no
ApomAb-sm3e-B1	2.19	1.10	100.00	no
ApomAb-PR1A3-A	5.83	0.22	98.48	yes
ApomAb-PR1A3-A1	5.62	0.20	100.00	no
ApomAb-PR1A3-B	5.00	0.43	98.88	yes
ApomAb-PR1A3-B1	11.46	1.25	100.00	no

All molecules could be produced and purified in sufficient amounts and with appropriate quality for further characterization and testing. The yields after purification were in the range of about 5 mg / L with some deviations for some molecules. For example the yield for ApomAb-sm3e-B1 was significantly lower (2.19 mg / L) while of the corresponding construct, ApomAb-PR1A3_B1 even more than 11 mg / L could be purified.

Determination of aggregate formation after freezing / thawing and increasing of the antibody concentration revealed that, depending on the molecule, the stabilization via interchain disulfide bridges can have beneficial effects on the tendency to form aggregates. In general the disulfide stabilization yielded in higher monomer content of the molecules at least at higher concentrations (table 3).

Table 3: aggregate formation of bispecific death receptor agonistic antibodies in correlation with protein concentration

Construct	Concentration [mg / ml]	Monomer content [%]
ApomAb-PR1A3-A	0.22	98.48
	1.73	90.90
	3.30	81.50
ApomAb-PR1A3-A1	0.20	100.00
	1.50	100.00
	3.37	100.00
ApomAb-sm3e-A	0.14	100.00
	1.11	95.00
	2.74	94.00
ApomAb-sm3e-A1	1.25	100.00
	0.79	98.60
	2.00	97.10

The tendency to form aggregates is not only dependent on the disulfide stabilization of the scFv but also on the used antigen binding scFv. From table 3 it is obvious that bispecific ApomAb molecules containing PR1A3 scFvs undergo significant aggregation upon increase of protein concentration. At concentrations of more than 3 mg / ml only 80 % of the material appears as monomer, while after introduction of two additional cysteine residues (VH44 / VL100 according to Kabat numbering) these molecule do not form aggregates at the used concentration.

The degree of aggregate formation with bispecific ApomAb molecules containing sm3e scFvs is not as pronounced since here the monomer content still is around 94 % without and 97 % with disulfide stabilization, respectively.

Example 3: Induction of apoptosis by death receptor bispecific DR5 – CEA antibody molecules:

The human DR5 death receptor agonistic antibody ApomAb induces apoptosis of DR5 expressing tumor cells, such as the colon cancer cell lines LS180 or Colo-205. In-vitro, ApomAb on its own mediates significant apoptosis which can be dramatically increased by cross-linking of the ApomAb-bound DR5 with antibodies binding to the human Fc region of ApomAb. This induction of Apoptosis also translates into in-vivo where it could be shown for different tumor models that ApomAb exhibits significant efficacy (Jin et al., 2008; Adams et al., 2008), most probably by cross-linking events via the human Fc-receptors. To evaluate the potential of DR5 – CEA bispecific antibodies for tumor site targeted cross-linking of DR5 with subsequent induc-

tion of apoptosis the activity of ApomAb – CEA bispecific molecules in terms of apoptosis mediation was analyzed in-vitro.

In order to determine if DR5 – CEA bispecific antibody molecules are able to induce tumor antigen binding dependent apoptosis of target cells DNA fragmentation in tumor cells after incubation with death receptor agonistic bispecific antibodies as a measure of apoptosis was analyzed using a cell death detection ELISA assay.

To figure out which cell lines would be suitable to measure antigen binding dependent cross-linking of DR5 which leads to induction of apoptosis several different tumor cell lines were analyzed for surface expression of DR5, FAS and CEA.

All used target cell lines were analyzed for relative expression levels of tumor-related antigens and FAS or DR5 death receptors before apoptosis assays were performed as follows.

Number and viability of cells was determined. For this, adherently growing cells were detached with cell dissociation buffer (Gibco – Invitrogen # 13151-014). Cells were harvested by centrifugation (4 min, 400 x g), washed with FACS buffer (PBS / 0.1 % BSA) and the cell number was adjusted to 1.111 X 106 cells / ml in FACS buffer. 180 µl of this cell suspension was used per well of a 96 well round bottom plate, resulting in 2 x 105 cell per well. The cells were incubated for 30 min at 4 °C with the first antibody in appropriate dilution. Then the cells were harvested by centrifugation (4 min, 400 x g), supernatant was completely removed and cells were washed once with 150 µl of FACS buffer. The cells were resuspended in 150 µl FACS buffer and incubated with the secondary antibody (in case unlabelled first antibody was used) for 30 min at 4 °C in the dark. After two washing steps with FACS buffer cells were resuspended in 200 µl of FACS buffer and analyzed in a HTS FACSCanto II (BD, Software FACS Diva). Alternatively the cells could be fixed with of 200 µl of 2 % PFA (paraformaldehyde) in FACS buffer for 20 min at 4 °C and analyzed later. All assays were performed in triplicates.

In figure 1 the results of FACS binding analysis of different tumor cell lines with three specific antibodies recognizing CEA, DR5 or FAS are shown. Except the Lovo cells all other tested cell lines express the tested antigens at different levels. CEA expression was highest in MKN-45 cells and more or less similar in OVCAR-3, AsPC-1, BxPC-3 and LS174T. In terms of DR5 expression the AsPC-1 and BxPC.3 cells express most of the receptor compared to the other cell lines followed by OVCAR-3 and MKN-45 whereas LS174T has the lowest DR5 expression level. Regarding FAS expression the cell lines were different but all showing significant FAS expression. When the Lovo cells which were negative in this assay were analyzed later with different antibodies against CEA, DR5 and FAS they also showed significant expression of the tested antigens (data not shown).

For determination of induced apoptosis the Cell Death Detection ELISA PLUS kit from Roche was used. In short, 104 cells per well of a 96-well plate (after detaching, and determination of cell number and viability) were seeded in 200 µl appropriate medium and were incubated over night at 37 °C in a 5 % CO2 atmosphere. The next day the medium was replaced by fresh medium containing the apoptosis inducing antibodies, control antibodies and other controls in appropriate concentrations:

The bispecific antibodies were used in a final concentration of 0.01 – 10 µg / ml; control antibodies were used at 0.5 µg / ml and cross-linking antibodies were used at 100 µg / ml. Competing antibodies were used at a 100 fold excess.

The cells were incubated for 4 – 24 hrs at 37 °C, 5 % CO_2 to allow induction of apoptosis. The cells were harvested by centrifugation (10 min, 200 x g) and incubated for 1 h at room temperature in 200 µl of lysis buffer (supplied by the kit). Intact cells were sedimented by centrifugation (10 min, 200 x g) and 20 µl of the supernatant was analyzed according to the manufacturer's recommendations for induction of apoptosis.

A set of cell lines also was analyzed for the ability to undergo apoptosis by incubation with commercially available antibodies against DR5 or FAS which are known to cross-link the death receptors already in solution (figure 2).

Here significant differences among the cell lines were observed in terms of induction of apoptosis as shown in figure 2. While in MKN-45 and BxPC-3 apoptosis induction via DR5 and FAS was similar (although in MKN-45 the DNA fragmentation value reached only 50 % of that with BxPC-3), in LS174T and Lovo cells apoptosis could be induced much better with the DR5 cross-linking antibody than with the FAS binding antibody. In LS174T cells apoptosis induction via DR5 cross-linking was about two-fold as effective as apoptosis via FAS cross-linking. In Lovo cells this difference in apoptosis induction was even four-fold. ASPC-1 cells are very resistant to apoptosis induction via death receptor cross-linking. Based on these results the two cell lines Lovo and LS174T were chosen to analyze apoptosis induction by tumor antigen targeted cross-linking of DR5.

The results of apoptosis induction in LS174T cells upon treatment with bispecific DR5 – CEA molecules (ApomAb – sm3e) in comparison with the effect of ApomAb or cross-linked ApomAb is illustrated in figure 3. Under the used assay conditions (4 hrs incubation at a concentration of 1 µg / ml) ApomAb alone or sm3e in IgG1 format did not exhibit detectable DNA fragmentation (normalized to the 'cells only' value), while the bispecific ApomAb-sm3e molecules (either wild type (format A) or disulfide stabilized (format A1) scFv) showed significant induction of apoptosis which was comparable to the theoretical maximum of hyper-cross-linked ApomAb. The two bispecific molecules showed very similar activity, demonstrating that the stabili-

zation of the molecule by insertion of interchain disulfides does not affect biological activity. When the cells were pre-incubated with an excess of sm3e IgG (100-fold higher concentration compared to the bispecific constructs) no apoptosis can be induced anymore, indicating that the sm3e IgG blocks all CEA antigen on the cell surface and prevents additional binding of the bispecific death receptor agonistic molecule. This demonstrates that the induced apoptosis is specifically dependent on cross-linking of the DR5 death receptor via the tumor antigen.

In figure 4 the results of a comparison between different molecule formats of the bispecific ApomAb – sm3e constructs on apoptosis induction of LS174T cells are summarized. Induction of apoptosis was performed for 4 hrs at a concentration of 1 µg / ml. Again, the bispecific ApomAb – sm3e molecules in which the sm3e scFv is fused to the C-terminus of the heavy chain of ApomAb (A and A1 format) demonstrated significant induction of apoptosis which was, in this case, even superior to the hyper-cross-linked ApomAb. ApomAb alone did not induce detectable DNA fragmentation under the used conditions. Two additional bispecific constructs (sm3e scFv fused to the C-terminus of the light chain of ApomAb, either wild type = B format or disulfide stabilized = B1 format) also exhibited high levels of apoptosis induction which was, at least for the B format, in a similar range as with cross-linked ApomAb, indicating that both formats basically are functional. The fusion of the scFv to the C-terminus of the heavy chain of ApomAb seem to be slightly advantageous over fusion to the light chain. In comparison to the results shown in figure 4, it also might be that the disulfide-stabilized molecules exhibit a slightly reduced activity compared to molecules with wild type scFv.

The ApomAb – sm3e constructs described above worked very well in terms of antigen dependent specific induction of apoptosis as shown in figures 3 and 4. This CEA antibody, sm3e, exhibits a very high affinity towards its antigen (low picomolar range). In order to evaluate if the effect of apoptosis induction with bispecific DR5 – CEA constructs also can be mediated with molecules with lower binding affinity to the tumor antigen additional constructs, analogous to the former ones, were generated. The CEA targeting scFv was engineered using the sequence of the CEA antibody PR1A3 which has a rather low affinity to CEA which is in the micromolar range. For evaluation of this antibody bispecific constructs were generated in which the PR1A3 scFv (wild type or disulfide stabilized) was fused to either the C-terminus of the heavy or light chain of ApomAb IgG. The nomenclature of the resulting molecules is analogous to the already described: ApomAb_PR1A3_A / A1 / B / B1 where A and A1 describe fusion to the C-terminus of the heavy chain and B and B1 show fusion to the C-terminus of the light chain. A and B contain wild type scFv whereas A1 and B1 indicate disulfide stabilized scFv.

In figure 5 the induction of apoptosis on LS174T cells by ApomAb, cross-linked ApomAb and ApomAb_PR1A3 bispecific antibody (wild type PR1A3 scFv fused to C-terminus of the ApomAb heavy chain) is shown over a concentration range from 0.01 to 10.0 µg / ml. ApomAb

on its own exhibits a certain degree of concentration dependent apoptosis induction which could be significantly increased by cross-linking of ApomAb with an anti human Fc antibody. The bispecific ApomAb-PR1A3 molecule also demonstrated concentration dependent induction of apoptosis, which at a concentration of 10.0 µg / ml, was even higher as with the cross-linked ApomAb at concentration at the same concentration indicating that it is not absolutely necessary to use the highest affine tumor antigen binders in this bispecific death receptor agonistic antibody format to achieve good in-vitro efficacy in terms of apoptosis induction.

To investigate, if the observed effect of induction of apoptosis upon incubation with DR5 – CEA bispecific molecules can be applied to other cell lines, a similar experiment as shown in figure 6 was performed using Lovo cells, another colon cancer cell line.

The results of apoptosis induction in Lovo cells using the death receptor agonistic bispecific molecule ApomAb_R1A3_A (DR5-CEA) compared to induction of apoptosis via ApomAb and cross-linked ApomAb are shown in figure 6. For all constructs a concentration dependent induction of apoptosis was observed. Here the ApomAb alone reached about 20 % of the activity of cross-linked ApomAb when used in concentration of 10 µg / ml. Below this concentration apoptosis induction was much lower compared to cross-linked ApomAb. The ApomAb_PR1A3 bispecific antibody, in the absence of any cross-linking molecule, showed the same induction of DNA fragmentation as the hyper-cross-linked ApomAb antibody demonstrating that the apoptosis inducing effect using death receptor agonistic antibodies is a general phenomenon that can be applied to all apoptosis competent cell lines.

In figure 7 the results of a comparison between different ApomAb – PR1A3 and ApomAb – sm3e constructs are shown. Here the induction of apoptosis in LS174T cells after 4 hrs incubation with a concentration of 1 µg / ml are summarized. From the results it becomes quite obvious that the affinity to the CEA antigen indeed might play a role in mediating apoptosis via death receptor cross-linking. There is a clear difference in apoptosis induction with constructs containing the high affinity CEA binder compared to the low affinity binder. ApomAb – PR1A3 shows only about one third of the apoptosis induction in LS174T cells compared to ApomAb – sm3e. Furthermore there seem to exist intrinsic differences in the different molecules which also are reflected in the capability of induction of apoptosis. In the cases in which the PR1A3 scFv is fused to ApomAb there is no difference in activity between molecules where the scFv is fused to either the C-terminus of the heavy or light chain. Both molecules show the same induction of apoptosis. In contrast to this, constructs containing the sm3e scFv behave different. Here the fusion of the scFv to the C-terminus of the heavy chain is superior to the fusion to the C-terminus of the light chain.

An additional difference between the two series of constructs is the fact that there is a different effect of disulfide stabilization of scFv. While disulfide stabilized sm3e scFv containing constructs are not affected regarding induction of apoptosis this is contrary for PR1A3 scFvs. These do not exhibit significant induction of apoptosis anymore if used in the disulfide stabilized form.

Example 4: Generation of bispecific death receptor agonistic antibodies targeting FAS (CD95) and CRIPTO as the tumor antigen and evaluation of these molecules in-vitro:

CRIPTO is a GPI-anchored growth factor that is reported to be over-expressed in cancer cells, but low or absent in normal cells. CRIPTO is found to be up-regulated in colon tumors and liver metastasis. As a member of the EGF family, it is considered to be an autocrine growth factor that plays a role in proliferation, metastasis, and/or survival of tumor cells. This growth factor activates a number of signaling pathways through several potential receptors or co-receptors.

To figure out if CRIPTO would be a suitable target for the death receptor agonistic bispecific antibody approach tetravalent, bispecific antibodies targeting FAS as the death receptor and CRIPTO as the tumor antigen were generated. These molecules consist of a full length IgG1 antibody (recognizing FAS) to which CRIPTO targeting scFvs are fused to the C-terminus of the heavy chain.

For the heavy and light chains of the FAS targeting IgG part of the molecule the sequences of the HFE7A antibody was used (Haruyama et al., 2002), which is a human / mouse cross-reactive antibody against CD95. The CRIPTO scFv was generated from sequences of a humanized anti-CRIPTO antibody that was generated by immunization (LC020_H3L2D1). The scFv was generated using standard recombinant DNA techniques and fused by a short peptide linker to the C-terminus of the FAS IgG1 heavy chain. The order of the single domains in the scFv is VH – (G4S)4 linker VL.

Unfortunately there are not that many suitable cell lines available that can be used for CRIPTO targeting. Therefore two cell lines were evaluated for their potential to be used as target cell line for FAS cross-linking mediated apoptosis induction via bispecific FAS / CRIPTO antibodies. In figure 9 the results of the evaluation of surface expression of FAS, DR5 and CRIPTO in NCCIT and recombinant, human CRIPTO expressing HEK cells (hereafter referred to as HEK-CRIPTO) are shown. In contrast to the HEK-CRIPTO cells NCCIT hardly express FAS on the surface and only very low levels of CRIPTO, while DR5 expression seems to be normal. In contrast to that HEK-CRIPTO cells express high levels of CRIPTO, significant levels of DR5 and suitable levels of FAS, why these cells were chosen for in-vitro analysis of apoptosis induction with FAS – CRIPTO bispecific antibodies.

Figure 10 summarizes the results of in-vitro experiments for induction of apoptosis on HEK-CRIPTO cells using either HFE7A, cross-linked HFE7A or the HFE7A – CRIPTO bispecific constructs. There is no significant apoptosis induction with HFE7A or CRIPTO (LC020) alone. Cross-linking of HFE7A with an anti human Fc antibody leads to high levels of DNA fragmentation as do the bispecific HFE7A – CRIPTO molecules. In this case bispecific molecules that contain either the wild type CRIPTO scFv (HFE7A_LC020_A) or the disulfide stabilized scFv (HFE7A_LC020_A1) fused to the C-terminus of the HFE7A heavy chain. Almost no difference in apoptosis induction between these to molecules could be observed.

In both cases, pre-incubation with excess of CRIPTO IgG significantly reduced apoptosis induction but this reduction was not complete. The reason for that is not clear and needs to be evaluated. An analogous construct in which an MCSP targeting scFv is fused to the C-terminus of the heavy chain of HFE7A (HFE7A_LC007_A1) did not induce any apoptosis of the HEK-CRIPTO cells indicating that the observed apoptosis with the bispecific HFE7A – CRIPTO molecules is tumor antigen specific.

The results from a comparison of apoptosis induction between HEK-CRIPTO and recombinant human FAP (fibroblast activating protein) expressing HEK cells (HEK-FAP) upon treatment with HFE7A – CRIPTO bispecific antibodies are shown in figure 11. Both cell lines undergo apoptosis if incubated with a positive control antibody conferring apoptosis already in solution or when treated with cross-linked HFE7A. The anti FAS antibody HFE7A on its own did not mediate apoptosis in these cell lines. The bispecific HFE7A – CRIPTO molecule induced apoptosis only in HEK-CRIPTO cells but not in the control HEK-FAP cells. There seems to be a low level of DNA fragmentation also in the HEK-FAP cells but this is non-specific basal activity since it can be observed also with a unrelated HFE7A – MCSP control molecule and even with the anti CRIPTO and anti Fc antibody alone. As observed in the experiments described in figure 10 also in this case the inhibition of apoptosis by pre-incubation with an excess of CRIPTO IgG was not complete.

Example 5: Generation of FAS – MCSP bispecific death receptor agonistic antibodies and evaluation of their apoptosis induction potential.

Among antigens that are directly expressed and displayed on the tumor cell surface also other antigens are being considered for targeted cross-linking of death receptors to induce apoptosis. In particular these are antigens from the stroma or neovasculature. One example for the latter one is the melanoma associated chondroitin sulfate proteoglycan (MCSP). MCSP is expressed on the majority of melanoma cells but also on glioma cells and on neovasculature. Several monoclonal antibodies targeting human MCSP have been described but none of them was suitable to be used in cancer therapy due to missing efficacy (e.g. lack of ADCC). Therefore

MCSP antibodies might gain value if used in a bispecific format that is able to mediate tumor site targeted apoptosis.

In order to evaluate simultaneous tumor / neovasculature targeting with respect to apoptosis induction bispecific death receptor agonistic antibodies were generated in which a MCSP specific scFv (wild type or disulfide stabilized) is fused to the C-terminus of the anti FAS antibody HFE7A. These scFvs are fused via a short peptide linker to HFE7A. The sequences of the variable light and heavy chains to generate the MCSP targeting scFv were taken from the MCSP antibody 9.2.27 (Beavers et al., 1996; US5580774).

In order to define a cell line that is suitable for analysis of in-vitro apoptosis induction several cell lines were tested for MCSP expression by FACS binding analysis (figure 12). Among the tested cell lines only HCT-116 and U-87MG exhibited significant MCSP expression as detected with two anti MCSP antibodies (9.2.27 and LC007). All other cell lines tested showed only very low or no expression of MCSP. For that reason these two cell lines were analyzed if they go into apoptosis when treated with cross-linked agonistic death receptor antibodies or with control antibodies that confer apoptosis already in solution. In U-87MG cells apoptosis could be induced by both, anti FAS and anti DR5 antibodies (figure 13 A) while this was different for HCT-116 cells. Here apoptosis only could be induced with anti DR5 antibodies (figure 13 B). Therefore U-87MG cells were chosen to be used as target cells for future apoptosis induction experiments.

Figure 14 shows the results obtained from apoptosis induction experiments with the glioma cell line U-87MG after treatment with FAS agonistic bispecific antibodies (in a concentration of 1 µg / ml) consisting of FAS targeting HFE7A IgG which is combined with a MCSP binding scFv (9.2.27). Both, the wild type (A format) and the H44 / L100 disulfide stabilized scFv (A1 format) were compared to HFE7A alone or HFE7A cross-linked via a secondary anti human Fc antibody. Although, in general induction of apoptosis of these U-87MG cells is rather low (even after 24 hrs incubation) a significant DNA fragmentation can be observed when the bispecific FAS agonistic antibodies are used. In this case the construct containing the disulfide stabilized scFv seems to be superior over the one containing the wild type scFv, and both show much higher apoptosis induction capacity than the cross-linked HFE7A IgG molecule. Pre-incubation of the cells with a 100-fold excess of MCSP (9.2.27) IgG completely inhibited apoptosis induction by the bispecific constructs, indicating that the observed DNA fragmentation / apoptosis in the absence of competing antibody is specific and dependent on cross-linking of FAS via the MCSP antigen.

Example 6: A DR5 – FAP death receptor agonistic bispecific antibody is able to mediate apoptosis of one cell line via cross-linking by a second cell line.

Another approach of induction of apoptosis by cross-linking of death receptors as DR5 (apart from cross-linking via an antigen expressed by the tumor cell), is targeting the stroma surrounding the tumor. In that case the targeted antigen is not displayed directly by the tumor cells but by a second, different cell type. One example for this kind of antigen would be FAP (fibroblast activation protein). This protein is expressed on activated fibroblast as they are found in the tumor stroma.

To investigate the possibilities of tumor targeted induction of apoptosis using bispecific death receptor agonistic antibodies targeting human DR5 and an antigen from the tumor stroma, bispecific molecules were generated that consist of an IgG1 part that recognizes DR5 and a FAP binding scFv that is fused to the C-terminus of the heavy chain of the antibody. The sequence of the DR5 targeting IgG was taken from the ApomAb sequence as described in US2007 / 0031414 A1. The sequence of variable heavy and light chain of the FAP binding scFv was taken from a Fab anti FAP molecule isolated by phage display as shown in sequence # 1 and 2. The FAP scFv is fused by a (G4S)2 connector to the C-terminus of the anti DR5 IgG heavy chain.

In this kind of setting two different cell lines have to be used for the in-vitro activity assays: one cell line (the target cell line) should express human DR5, has to be apoptosis competent but does not need to express FAP. The second cell line (the effector cell line) has to be apoptosis negative (either by apoptosis resistance or by not expressing DR5) but needs to express FAP on the surface.

One possible effector cell line that fulfils the desired criteria is the human fibroblast cell line GM05389. As shown in figure 15 A this cell line expresses significant levels of FAP compared to the cell line SW872 which only showed FAP expression with the highest tested antibody concentration (10 µg / ml) but does not undergo apoptosis by non-cross-linked ApomAb as seen in figure 15 B. Therefore this cell line seems to be a potential effector cell line in an apoptosis assay where DNA fragmentation of a target cell line is induced by cross-linking via an antigen expressed on a second cell line.

As a target cell line the human breast-adenocarcinoma cell line MDA-MB-231 was used that expresses low levels of DR5 and is sensitive to DR5 mediated apoptosis induction. In figure 16 the results of induction of DNA fragmentation of GM05389 cells and MDA-MB-231 cells compared to the combination of both cell lines by tumor targeted cross-linking of DR5 via FAP is summarized. A significant apoptosis induction after incubation with death receptor agonistic antibodies only can be observed when both cell lines are co-cultivated (black bars) while apoptosis by cross-linking of DR5 with an anti human Fc targeting ApomAb can be detected to a lower degree in both cell lines separately (white and grey bars, respectively). We interpret this

result in a way that the DR5 receptors on MDA-MB-231 cells are cross-linked upon binding to the FAP antigen expressed by the fibroblast cell line GM05389.

Example 7: Fusion of CEA single chain Fab molecules (scFab) to ApomAb for the generation of DR5 – CEA bispecific agonistic antibodies.

Besides the stabilization of bispecific antibodies by defined insertion of internal cysteine residues in the variable heavy and variable light chain of scFv's to prevent aggregate formation, the use of single chain Fab's (scFab's) is another possible strategy to stabilize the entire bispecific antibody to avoid non-specific cross-linking.

To evaluate if this format (scFab fused to DR5 agonistic antibody) exhibits similar apoptosis induction activity as the corresponding scFv containing molecules, different bispecific antibodies in which a CEA scFab was fused to the C-terminus of either the heavy or light chain of ApomAb, were generated by standard recombinant DNA technology.

The orientation of the different domains of the scFab's is as follows: VL – CL – VH – CH1. The C-terminus of the constant light chain (CL) is connected to the N-terminus of the variable heavy chain (VH) via a 34mer peptide linker. Fusion of the scFab occurs by a G4S connector (either 2mer or 4mer).

Single chain Fab containing bispecific antibodies were generated in two basically different formats: in one format two scFab's are fused to the C-terminus of the heavy or light chain of ApomAb (bispecific, tetravalent homodimeric molecules). On the other hand a bispecific molecule was constructed in which only one scFab is fused to the C-terminus of only one ApomAb heavy chain (bispecific, trivalent heterodimeric molecule). This heterodimerization was achieved by using the so-called knob into holes technology which uses Fc mutations that only allow formation of heterodimeric IgG molecules.

In figure 17 the results of apoptosis induction experiments in which ApomAb_PR1A3_scFab is compared to ApomAb or hyper-cross-linked ApomAb are shown. In this assay the gastric cancer cell line MKN-45 was used and apoptosis was measured after 24 hrs using a DNA fragmentation assay. Clearly, the bispecific construct exhibits apoptosis induction activity that is in the same range as can be observed with ApomAb that was cross-linked via an anti Fc antibody, and which is significantly higher as with the ApomAb alone. However, the apoptosis induction with ApomAb on its own is rather high, which most probably is due to the elongated incubation time of 24 hrs which is necessary to demonstrate maximum apoptosis induction on the used MKN-45 cell line (in contrast to e.g. LS174T cells with which the assay is only run for four hrs).

To evaluate if bispecific, trivalent DR5 agonistic antibodies (monovalent for the tumor target, CEA, and bivalent for DR5) also are able to induce tumor targeted apoptosis, a molecule was generated in which a CEA scFab (sm3e specificity) was fused to the C-terminus of the ApomAb heavy chain (containing the knob mutation). This heavy chain was co-expressed with the corresponding ApomAb heavy chain containing the 'hole' mutations and the ApomAb light chain. The results of the 4 hrs apoptosis induction assay in which the bispecific, trivalent molecule was analyzed on LS174T cells (in concentrations of 0.1 and 1.0 µg / ml) are summarized in figure 18. From these results it is obvious that also the described trivalent format is able to induce targeted apoptosis in the same range as hyper-cross-linked ApomAb does. At a lower concentration the bispecific format even seems to be slightly more active as ApomAb upon cross-linking.

Example 8: DR5 – CEA bispecific agonistic antibody with superior in-vivo efficacy compared to ApomAb

For evaluation if the apoptotic activity of the death receptor agonistic antibodies that has been demonstrated in-vitro also translates into superior in-vivo efficacy an in-vivo experiment using the human colon carcinoma cell line LS174T as a model was set up.

In short, at day one of the experiment female SCID beige mice were treated with intrasplenic injection of 3 x 106 tumor cells. At day seven a scout animal was tested for tumor engraftment as a criterion to start with the antibody treatment one day later. The treatment consisted of a series of three injections (each 10 mg / kg, i.v. in intervals of seven days). Each day the animals were analyzed for demonstrating termination criteria.

Figure 19 summarizes the results obtained in this in-vivo experiment. Here the survival duration of three groups of mice (each consisting of initially ten animals and treated with different molecules) is compared. While the control group (PBS, black line) was completely terminated 37 days post tumor injection the group treated with ApomAb (filled circles) showed a prolonged survival (maximum of 44 days). The group treated with the bispecific antibody (ApomAb_sm3e_A1, black squares) even showed longer survival (52 days) than the group that had obtained ApomAb alone. Mathematical analysis of the obtained data demonstrated that these results are statistically significant (with p-values below 0.05) meaning that ApomAb showed in-vivo efficacy compared to the PBS control and that the bispecific ApomAb_sm3e_A1 demonstrated superior in-vivo efficacy even compared to ApomAb.

Material and Methods:

Transfection HEK293 EBNA cells

All (bispecific) antibodies used herein were transiently produced in HEK 293 EBNA cells using a Ca^{2+}-phosphate dependent co-transfection procedure for heavy and light chain vectors as described below.

The cells were grown in standard DMEM medium (Invitrogen) containing 10 % FCS (Gibco, # 16000) at 37 °C in humidified incubators with 5 % CO2 atmosphere. 48 hrs prior to transfection 3 x 107 cells were inoculated in 200 ml DMEM / 10 % FCS in roller bottles (Falcon # 353069, 1400 cm^2) and were incubated at 37 °C in a roller bottle incubator (0.3 rpm). For transfection 880 µg total DNA (440 µg for each, heavy and light chain vector) + 4.4 ml $CaCl_2$ were filled up with H_2O to a total volume of 8.8 ml. The solution was mixed shortly. After mixing 8.8 ml of 1.5 mM phosphate buffer (50 mM Hepes, 280 mM NaCl, 1.5 mM NaH_2PO_4; pH7.05) were added for DNA precipitation. After additional mixing for ten seconds and short incubation at room temperature (20 seconds) 200 ml of DMEM / 2 % FCS was added to the DNA solution. The medium / DNA solution was used to replace the original medium in the roller bottle to transfect the cells. After 48 hrs incubation at 37 °C the transfection medium was replaced by 200 ml DMEM / 10 % FCS and antibody production was continued for 7 days.

After production the supernatants were harvested and the antibody containing supernatants were filtered through 0.22 µm sterile filters and stored at 4 °C until purification.

Purification

The proteins were produced by transient expression in HEK293 EBNA cells. All bispecific molecules described here were purified in two steps using standard procedures, such as protein A affinity purification (Äkta Explorer) and size exclusion chromatography.

The supernatant was adjusted to pH 8.0 (using 2 M TRIS pH 8.0) and applied to Mabselect Sure resin (GE Healthcare) packed in a TricornTM 5/50 column (GE Healthcare, column volume (cv) = 1 ml) equilibrated with buffer A (50 mM sodiumphosphate, pH 7.0, 250 mM NaCl). After washing with 10 column volumes (cv) of buffer A, 20 cv of buffer B (50 mM sodiumphosphate, pH 7.0, 1 M NaCl) and again 10 cv of buffer A, the protein was eluted using a pH-step gradient to buffer B (50 mM sodiumphosphate, 50 mM sodiumcitrate pH 3.0, 250 mM NaCl) over 20 cv. Fractions containing the protein were pooled and the pH of the solution was gently adjusted to pH 6.0 (using 2 M TRIS pH 8.0). Samples were concentrated to 2 ml using ultra-concentrators (Vivaspin 15R 30.000 MWCO HY, Sartorius) and subsequently applied to a HiLoadTM 16/60 SuperdexTM 200 preparative grade (GE Healthcare) equilibrated with 20 mM Histidine, pH 6.0, 150 mM NaCl. The aggregate content of eluted fractions was analyzed by analytical size exclusion chromatography. Therefore 50 µl of each fraction was load to a SuperdexTM200 10/300 GL column (GE Healthcare) equilibrated with 2 mM MOPS, pH 7.4, 150 mM NaCl, 0.02% w/v NaN_3. Fractions containing less than 2 % oligomers were pooled and concentrated to final con-

centration of 1 - 1.5 mg/ml using ultra concentrators (Vivaspin 15R 30.000 MWCO HY, Sartorius). Purified proteins were frozen in liquid N_2 and stored at -80 °C.

FACS binding analysis

All used target cell lines were analyzed for relative expression levels of tumor-related antigens and FAS or DR5 death receptors before apoptosis assays were performed.

Number and viability of cells was determined. For this, adherently growing cells were detached with cell dissociation buffer (Gibco – Invitrogen # 13151-014). Cells were harvested by centrifugation (4 min, 400 x g), washed with FACS buffer (PBS / 0.1 % BSA) and the cell number was adjusted to 1.111 X 106 cells / ml in FACS buffer. 180 µl of this cell suspension was used per well of a 96 well round bottom plate, resulting in 2 x 105 cell per well. The cells were incubated for 30 min at 4 °C with the first antibody in appropriate dilution. Then the cells were harvested by centrifugation (4 min, 400 x g), supernatant was completely removed and cells were washed once with 150 µl of FACS buffer. The cells were resuspended in 150 µl FACS buffer and incubated with the secondary antibody (in case unlabelled first antibody was used) for 30 min at 4 °C in the dark. After two washing steps with FACS buffer cells were resuspended in 200 µl of FACS buffer and analyzed in a HTS FACSCanto II (BD, Software FACS Diva). Alternatively the cells could be fixed with of 200 µl of 2 % PFA (paraformaldehyde) in FACS buffer for 20 min at 4 °C and analyzed later. All assays were performed in triplicates.

Used antibodies and concentrations:

Antibody	Source	Description	Conc. [mg / ml]	Conc. in test [µg / ml]
1. First antibodies				
anti hu CD95 (FAS)	BD #555671	mu IgG1, kappa	0.5	5 - 10
anti hu DR5 (TRAIL R2)	R&D #MAB631	mu IgG1, clone 71903	0.5	5 - 10
anti hu CEA	Abcam #ab11330	mu IgG1, clone C6G9	9.0	30
Isotype control	BD #554121	mu IgG1 clone MOPC1		
anti hu MCSP	in house (e.g. M9.2.27, LC007)	human(ized) / chimeric IgG1	diff.	30.
anti hu CRIPTO	in house (e.g. LC020, H3L2D1)	human(ized) / chimeric IgG1	diff.	30.
anti hu ROBO4-PE	R&D #FAB2454P	mu IgG2a	0.05	0.005

Isotype control	BD #555574	mu IgG2a-PE	0.05	0.005
2. Secondary antibodies:				
goat anti mouse IgG-PE	Serotec# STAR105PE		0.1	
$(Fab')_2$ goat anti huManFc-PE	Jackson Immunoresearch# 109-116-170			

Biacore analysis (Surface Plasmon Resonance, SPR)

SPR experiments were performed on a Biacore T100 with HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005 % Surfactant P20, GE Healthcare) as running buffer. Direct coupling of 1220, 740 and 300 resonance units (RU), respectively of biotinylated antigen was performed on a Streptavidin chip using the standard method (GE Healthcare). Different concentrations of the bispecific death receptor agonistic antibodies were passed with a flow of 40 µl/min through the flow cells at 278 K for 90 s to record the association phase. The dissociation phase was monitored for 300 s and triggered by switching from the sample solution to HBS-EP. Bulk refractive index differences were corrected for by subtracting the response obtained from a empty Streptavidin surface. Kinetic constants were derived using the Biacore T100 Evaluation Software (vAA, Biacore, Freiburg/Germany), to fit rate equations for 1:1 Langmuir binding by numerical integration. Since the antigen was immobilized the obtained kinetic constants using the 1:1 Langmuir binding by numerical integration are merely given the apparent KD-value or the avidity.

Induction of apoptosis

For determination of induced apoptosis the Cell Death Detection ELISA PLUS kit from Roche was used. In short, 104 cells per well of a 96-well plate (after detaching, and determination of cell number and viability) were seeded in 200 µl appropriate medium and were incubated over night at 37 °C in a 5 % CO_2 atmosphere. The next day the medium was replaced by fresh medium containing the apoptosis inducing antibodies, control antibodies and other controls in appropriate concentrations:

The bispecific antibodies were used in a final concentration of 0.01 – 10 µg / ml; control antibodies were used at 0.5 µg / ml and cross-linking antibodies were used at 100 µg / ml. Competing antibodies were used at a 100 fold excess.

The cells were incubated for 4 – 24 hrs at 37 °C, 5 % CO_2 to allow induction of apoptosis. The cells were harvested by centrifugation (10 min, 200 x g) and incubated for 1 h at room temperature in 200 µl of lysis buffer (supplied by the kit). Intact cells were sedimented by centrifugation (10 min, 200 x g) and 20 µl of the supernatant was analyzed according to the manufacturer's recommendations for induction of apoptosis.

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

Claims

1. A bispecific antibody comprising a first antigen binding site specific for DR5 and a second antigen binding site specific for FAP.

2. The bispecific antibody of claim 1, wherein the bispecific antibody is a dimeric molecule comprising a first antibody comprising the first antigen binding site specific for DR5 and a second antibody comprising the second antigen binding site specific for FAP.

3. The bispecific antibody of claim 2, wherein the first and second antibody comprise an Fc part of an antibody heavy chain, wherein the Fc part of the first antibody comprises a first dimerization module and the Fc part of the second antibody comprises a second dimerization module allowing a heterodimerization of the two antibodies.

4. The bispecific antibody of claim 3, wherein the first dimerization module comprises knobs and the second dimerization module comprises holes according to the knobs into holes strategy.

5. The bispecific antibody of claim 2, wherein the first antibody is an Immunoglobulin (Ig) molecule comprising a light chain and a heavy chain and the second antibody is selected from the group consisting of scFv, scFab, Fab or Fv.

6. The bispecific antibody of claim 5, wherein the second antibody is fused to the N- or C-terminus of the heavy chain of the Ig molecule.

7. The bispecific antibody of claim 5, wherein the second antibody is fused to the N- or C-terminus of the light chain of the Ig molecule.

8. The bispecific antibody of any of claims 5 – 7, wherein the Ig molecule is a IgG.

9. The bispecific antibody of any of claims 5 - 8, wherein the second molecule is fused to the Ig molecule by a peptide linker, preferably a peptide linker having a length of about 10 – 30 amino acids.

10. The bispecific antibody of any of claims 5- 9, wherein the second molecule comprises additional cystein residues to form disulfide bonds.

11. The bispecific antibody of any of claims 5- 10, wherein the Ig molecule comprises a Fc variant having a reduced affinity to Fcγ receptors compared to a wildtype Fc region.

12. A pharmaceutical composition comprising a bispecific antibody of any of claims 1 to 11.

13. The bispecific antibody of any of claims 1 to 11 for use in the treatment of cancer.

14. Use of the bispecific antibody of any of claims 1 to 11 in the manufacture of a medicament for the treatment of cancer.