AXL antibodies

Description

The present invention refers to antibodies, particularly to monoclonal antibodies, which bind to the extracellular domain of the AXL receptor tyrosine kinase and which at least partially inhibit AXL activity.

Background

The AXL (Ark, UFO, Tyro-7) receptor tyrosine kinase is a member of the Tyro-3 family of kinases with the other members being Mer (Eyk, Nyk, Tyro-12) and Sky (Rse, Tyro-3, Dtk, Etk, Brt, Tif). It is activated by binding of the heterophilic ligand Gas6, a 70-kDa protein homologous to the anti-coagulation factor protein S. In contrast to other receptor tyrosine kinases, AXL tyrosine phosphorylation can also be induced by homophilic binding. AXL activation leads to signalling through PI-3-kinase/Akt (Franke et al., Oncogene 22: 8983-8998, 2003) and other major pathways like Ras/Erk and β-catenin/TCF (Goruppi et al., Mol. Cell Biol. 21: 902-915, 2001).

AXL is weakly expressed in a range of normal tissues, including brain, heart, skeletal muscle, the organ capsules and connective tissues of several other organs, and in monocytes, but not lymphocytes. Akt phosphorylation induced by AXL has been described in survival of fibroblasts (Goruppi et al., Mol Cell Biol 17: 4442-4453 1997), endothelial cells (Hasanbasic et al., Am J Physiol Heart Circ Physiol, 2004), vascular smooth muscle cells (Melaragno et al., J. Mol. Cell Cardiol. 37: 881-887, 2004) and neurons (Allen et al., Mol. Endocrinol. 13: 191-201 1999). Furthermore, AXL plays a role in cell-adhesion and chemotaxis. AXL knockouts display impaired platelet aggregate stabilization and thrombus formation as a result of reduced activation of the platelet integrin IIb3.

AXL overexpression has been demonstrated in various cancer types, e.g.

breast (Meric et al., Clin. Cancer Res. 8: 361-367, 2002; Berclaz et al., Ann. Oncol. 12: 819-824, 2001), colon (Chen et al., Int. J. Cancer 83: 579-584, 1999; Craven et al., Int. J. Cancer 60: 791-797, 1995), prostate (Jacob et al., Cancer Detect. Prev. 23: 325-332, 1999), lung (Wimmel et al., Eur J Cancer 37: 2264-2274, 2001), gastric (Wu et al., Anticancer Res 22: 1071-1078, 2002), ovarian (Sun et al., Oncology 66: 450-457, 2004), endometrial (Sun et al., Ann. Oncol. 14: 898-906, 2003), renal (Chung et al., DNA Cell Biol. 22: 533-540, 2003), hepatocellular (Tsou et al., Genomics 50:331-340, 1998), thyroid (Ito et al., Thyroid 12:971-975, 2002; Ito et al., Thyroid 9: 563-567, 1999), and esophageal carcinoma (Nemoto et al., 1997), furthermore in CML (Janssen et al., A novel putative tyrosine kinase receptor with oncogenic potential. Oncogene, 6: 2113-2120, 1991; Braunger et al., Oncogene 14:2619-2631 1997; O'Bryan et al., Mol Cell Biol 11:5016-5031,1991), AML (Rochlitz et al., Leukemia 13: 1352-1358, 1999), osteosarcoma (Nakano et al., J. Biol. Chem. 270:5702-5705, 2003) melanoma (van Ginkel et al.,. Cancer Res 64:128-134, 2004) and in head and neck squamous cell carcinoma (Green et al., Br J Cancer. 2006 94:1446-5, 2006).

Moreover AXL has been identified as a metastasis-associated gene that is upregulated in aggressive breast cancer cell lines compared to non-invasive cells. In vitro, AXL activity was found to be required for migration and invasion, and this activity could be inhibited by antibody treatment (WO04008147). Similarly, abrogation of AXL activity in vivo, either via expression of a dominant negative version of AXL (Vajkoczy, P., et al., Proc. Natl. Acad. Science U.S.A. 103: 5799-5804. 2005) or by siRNA mediated downregulation of AXL (Holland et al., Cancer Res. 65: 9294-9303, 2005) prevented subcutaneous and orthotopic cell growth in murine xenograft experiments.

So far two antibodies that bind to AXL and posses biological activity have been described. One antibody is capable of reducing AXL mediated cell invasion (WO04008147) whereas the other antibody has been reported to reduce AXL/Ligand interaction. However both antibodies are polyclonal

rendering them unsuitable for therapeutic administration.

Thus in light of the therapeutic potential of AXL there is a high need for monoclonal AXL antibodies, antibody fragments or derivatives thereof that effectively and specifically block AXL mediated signal transduction and which are suitable for therapeutic treatment.

Accordingly a first aspect of the present invention relates to a monoclonal antibody including a fragment or derivative thereof that binds to the extracellular domain of AXL, particularly of human AXL, and at least partially inhibits AXL activity.

Preferably the antibody of the present invention further possesses at least one or more of the following properties: the ability to reduce or block AXL-mediated signal transduction, the ability to reduce or block AXL phosphorylation, the ability to reduce or block cell proliferation, the ability to reduce or block angiogenesis, the ability to reduce or block cell migration, the ability to reduce or block tumor metastasis, the ability to reduce or block AXL mediated PI3K signaling and the ability to reduce or block AXL mediated anti-apoptosis, thereby increasing for example the sensitivity of a cell against treatment with an antineoplastic agent. Moreover the antibodies of the present invention may exhibit high specificity for AXL, particularly human AXL and do not significantly recognize other Tyro-3 family members, e.g. MER and/or SKY and/or mammalian non-primate AXL, such as murine AXL. Antibody specificity may be determined by measurements of cross-reactivity as described in the Examples.

The term "activity" refers to the biological function of AXL, which influences the phenotype of a cell, in particular but not limited to cancer phenotypes such as evasion of apoptosis, self sufficiency in growth signals, cell proliferation, tissue invasion and/or metastasis, insensitivity to anti-growth signals (anti-apoptosis) and/or sustained angiogenesis.

The term "AXL mediated signal transduction" means the activation of second messenger pathways triggered by direct or indirect interaction of AXL with second messenger molecules.

The term "AXL phosphorylation" refers to the phosphorylation of amino acid residues, preferably tyrosine residues, either by a second AXL protein (transphosphorylation) or by another protein having protein kinase activity.

The term "cell proliferation" refers to all AXL-involving processes underlying the reproduction of human cells, in particular but not limited to human cancer cells. They contribute to or result in the replication of cellular DNA, separation of the duplicated DNA into two equally sized groups of chromosomes, and the physical division (called cytokinesis) of entire cells, and shall be stimulated or mediated by non-catalytic or catalytic activities of AXL, preferably including AXL phosphorylation and/or AXL-mediated signal transduction.

The term "angiogenesis" refers to all AXL-involving processes that contribute to the growth of new blood vessels from pre-existing vessels, in particular but not limited to new tumor supplying blood vessels. These processes include multiple cellular events such as proliferation, survival, migration and sprouting of vascular endothelial cells, attraction and migration of pericytes as well as basal membrane formation for vessel stabilization, vessel perfusion, or secretion of angiogenic factors by stromal or neoplastic cells, and shall be stimulated or mediated by non-catalytic or catalytic activities of AXL, preferably including AXL phosphorylation and/or AXL-mediated signal transduction.

The term "metastasis" refers to all AXL-involving processes that support cancer cells to disperse from a primary tumor, penetrate into lymphatic and/or blood vessels, circulate through the bloodstream, and grow in a distant focus (metastasis) in normal tissues elsewhere in the body. In particular, it refers to cellular events of tumor cells such as proliferation,

migration, anchorage independence, evasion of apoptosis, or secretion of angiogenic factors, that underly metastasis and are stimulated or mediated by non-catalytic or catalytic activities of AXL, preferably including AXL phosphorylation and/or AXL-mediated signal transduction.

The term "AXL mediated anti-apoptosis" refers to all AXL-involving processes that prevent human cells, preferably but not limited to human cancer cells from programmed cell death (apoptosis). In particular, it refers to processes that prevent human cells, preferably but not limited to human cancer cells from induction of apoptosis through growth factor withdrawal, hypoxia, exposure to chemotherapeutic agents or radiation, or initiation of the Fas/Apo-1 receptor-mediated signaling, and are stimulated or mediated by non-catalytic or catalytic activities of AXL, preferably including AXL phosphorylation and/or AXL-mediated signal transduction.

In addition, the present invention includes antibodies whose binding activities to AXL are KD = 10^{-5} M or lower, preferably KD = 10^{-7} M or lower, and most preferably KD = 10^{-9} M or lower. Whether the binding activity of an antibody of the present invention to AXL is KD = 10^{-5} M or lower can be determined by methods known to those skilled in the art. For example, the activity can be determined using surface plasmon resonance with Biacore, and/or by ELISA (enzyme-linked immunosorbent assays), EIA (enzyme immunoassays), RIA (radioimmunoassays), or fluorescent antibody techniques, e.g. FACS.

In a second aspect, the antibody may have at least one antigen binding site, e.g. one or two antigen binding sites. Further, the antibody preferably comprises at least one heavy immunoglobulin chain and at least one light immunoglobulin chain. An immunoglobulin chain comprises a variable domain and optionally a constant domain. A variable domain may comprise complementary determining regions (CDRs), e.g. a CDR1, CDR2 and/or CDR3 region, and framework regions. The term "complementary determining region" (CDR) is well-defined in the art (see, for example, Harlow and Lane, "Antibodies, a Laboratory Manual", CSH Press, Cold

Spring Harbour, 1988) and refers to the stretches of amino acids within the variable region of an antibody that primarily makes contact with the antigen.

A further aspect of the present invention relates to an antibody including a fragment or derivative thereof that binds to the extracellular domain of AXL which comprises at least one heavy chain amino acid sequence comprising at least one CDR selected from the group consisting of

(a) a CDRH1 as shown in SEQ ID NOs: 16, 22, 28, or a CDRH1 sequence differing in 1 or 2 amino acids therefrom,
(b) a CDRH2 as shown in SEQ ID NOs: 17, 23, 29, or a CDRH2 sequence differing in 1 or 2 amino acids therefrom, and
(c) a CDRH3 as shown in SEQ ID NOs: 18, 24, 30, or a CDRH3 sequence differing in 1 or 2 amino acids therefrom,

and/or at least:
one light chain amino acid sequence comprising at least one CDR selected from the group consisting of

(d) a CDRL1 as shown in SEQ ID NOs: 13, 19, 25, or a CDRL1 sequence differing in 1 or 2 amino acids therefrom,
(e) a CDRL2 as shown in SEQ ID NOs: 14, 20, 26, or a CDRL2 sequence differing in 1 or 2 amino acids therefrom, and
(f) a CDRL3 as shown in SEQ ID NOs: 15, 21, 27, or a CDRL3 sequence differing in 1 or 2 amino acids therefrom,

or a monoclonal antibody recognizing the same epitope on the extracellular domain of AXL.

In a preferred embodiment, the antibody comprises a heavy chain comprising at least one CDR selected from the group consisting of

(a) a CDRH1 as shown in SEQ ID NO: 16, or a CDRH1 sequence

differing in 1 or 2 amino acids therefrom,

(b) a CDRH2 as shown in SEQ ID NO: 17, or a CDRH2 sequence differing in 1 or 2 amino acids therefrom, and

(c) a CDRH3 as shown in SEQ 1D NO: 18, or a CDRH3 sequence differing in 1 or 2 amino acids therefrom,

and/or a light chain comprising at least one CDR selected from the group consisting of

(d) a CDRL1 as shown in SEQ 1D NO: 13, or a CDRL1 sequence differing in 1 or 2 amino acids therefrom,

(e) a CDRL2 as shown in SEQ 1D NO: 14, or a CDRL2 sequence differing in one or two amino acids therefrom, and

(f) a CDRL3 as shown in SEQ 1D NO: 15, or a CDRL3 sequence differing in 1 or 2 amino acids therefrom,

or an monoclonal antibody recognizing the same epitope on the extracellular domain of AXL.

In a further preferred embodiment, the antibody comprises a heavy chain comprising at least one CDR selected from the group consisting of

(a) a CDRH1 as shown in SEQ ID NO: 22, or a CDRH1 sequence differing in 1 or 2 amino acids therefrom,

(b) a CDRH2 as shown in SEQ ID NO: 23, or a CDRH2 sequence differing in 1 or 2 amino acids therefrom, and

(c) a CDRH3 as shown in SEQ 1D NO: 24, or a CDRH3 sequence differing in 1 or 2 amino acids therefrom,

and/or a light chain comprising at least one CDR selected from the group consisting of

(d) a CDRL1 as shown in SEQ 1D NO: 19, or a CDRL1 sequence differing in 1 or 2 amino acids therefrom,

(e) a CDRL2 as shown in SEQ 1D NO: 20, or a CDRL2 sequence differing in one or two amino acids therefrom, and

(f) a CDRL3 as shown in SEQ 1D NO: 21, or a CDRL3 sequence differing in 1 or 2 amino acids therefrom,

or an monoclonal antibody recognizing the same epitope on the extracellular domain of AXL.

In a yet further preferred embodiment, the antibody comprises a heavy chain comprising at least one CDR selected from the group consisting of

(a) a CDRH1 as shown in SEQ ID NO: 28, or a CDRH1 sequence differing in 1 or 2 amino acids therefrom,
(b) a CDRH2 as shown in SEQ ID NO: 29, or a CDRH2 sequence differing in 1 or 2 amino acids therefrom, and
(c) a CDRH3 as shown in SEQ 1D NO: 30, or a CDRH3 sequence differing in 1 or 2 amino acids therefrom,
and/or a light chain comprising at least one CDR selected from the group consisting of
(d) a CDRL1 as shown in SEQ 1D NO: 25, or a CDRL1 sequence differing in 1 or 2 amino acids therefrom,
(e) a CDRL2 as shown in SEQ 1D NO: 26, or a CDRL2 sequence differing in one or two amino acids therefrom, and
(f) a CDRL3 as shown in SEQ 1D NO: 27, or a CDRL3 sequence differing in 1 or 2 amino acids therefrom,

or an monoclonal antibody recognizing the same epitope on the extracellular domain of AXL.

In another embodiment, the present invention refers to an antibody comprising a heavy chain amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 10, 12 or at least the variable domain thereof or an amino acid sequence having a sequence identity of at least 90% thereto and/or a light chain amino acid sequence selected from the group consisting of SEQ. ID NOs: 7, 9, 11 or at least the variable domain thereof or an amino acid sequence having a sequence identity of at least 90% thereto or to an antibody recognizing the same epitope on the extracellular domain of AXL.

As used herein, "sequence identity" between two polypeptide sequences, indicates the percentage of amino acids that are identical between the sequences. Preferred polypeptide sequences of the invention have a sequence identity of at least 90%.

In a particular preferred embodiment, the antibody is selected from the group consisting of 11B7, 11D5, 10D12 or an antibody recognizing the same epitope on the extracellular domain of AXL.

The antibody may be any antibody of natural and/or synthetic origin, e.g. an antibody of mammalian origin. Preferably, the constant domain -if present- is a human constant domain. The variable domain is preferably a mammalian variable domain, e.g. a humanized or a human variable domain. More preferably, the antibody is a chimeric, humanized or human antibody.

The antibody of the invention may be of the IgA-, IgD-, IgE, IgG-or IgM-type, preferably of the IgG-or IgM-type including, but not limited to, the IgG1-, IgG2-, IgG3-, IgG4-, IgM1-and IgM2-type. In most preferred embodiments, the antibody is of the human IgG1-, IgG2-or IgG4-type.

As discussed, supra, there are a number of isotypes of antibodies. It will be appreciated that antibodies that are generated need not initially possess such an isotype but, rather the antibody as generated can possess any isotype and that the antibody can be isotype-switched by using the molecularly cloned V region genes or cloned constant region genes or cDNAs in appropriate expression vectors using conventional molecular biological techniques that are well known in the art and then expressing the antibodies in host cells using techniques known in the art

The term antibody includes "fragments" or "derivatives", which have at least one antigen binding site of the antibody. Antibody fragments include Fab fragments, Fab' fragments $F(ab')_2$ fragments as well as Fv fragments.

Derivatives of the antibody include single chain antibodies, nanobodies, and diabodies. Derivatives of the antibody shall also include scaffold proteins having an antibody-like binding activity that bind to AXL.

Within the context of the present invention, the term "scaffold protein", as used herein, means a polypeptide or protein with exposed surface areas in which amino acid insertions, substitutions or deletions are highly tolerable. Examples of scaffold proteins that can be used in accordance with the present invention are protein A from Staphylococcus aureus, the bilin binding protein from Pieris brassicae or other lipocalins, ankyrin repeat proteins, and human fibronectin (reviewed in Binz and Plückthun, Curr Opin Biotechnol,16: 459-69, 2005). Engineering of a scaffold protein can be regarded as grafting or integrating an affinity function onto or into the structural framework of a stably folded protein. Affinity function means a protein binding affinity according to the present invention. A scaffold can be structurally separable from the amino acid sequences conferring binding specificity. In general, proteins appearing suitable for the development of such artificial affinity reagents may be obtained by rational, or most commonly, combinatorial protein engineering techniques such as panning against AXL, either purified protein or protein displayed on the cell surface, for binding agents in an artificial scaffold library displayed in vitro, skills which are known in the art (Skerra, J. Mol. Recog., Biochim Biophys Acta, 1482: 337-350, 2000; Binz and Plückthun, Curr Opin Biotechnol, 16: 459-69, 2005). In addition, a scaffold protein having an antibody like binding activity can be derived from an acceptor polypeptide containing the scaffold domain, which can be grafted with binding domains of a donor polypeptide to confer the binding specificity of the donor polypeptide onto the scaffold domain containing the acceptor polypeptide. The inserted binding domains may include, for example, at least one CDR of an anti-AXL antibody, preferably at least one selected from the group of SEQ ID NOs: 13-30. Insertion can be accomplished by various methods known to those skilled in the art including, for example, polypeptide synthesis, nucleic acid synthesis of an encoding amino acid as well by various forms of recombinant methods well known to

those skilled in the art.

As has been indicated above, the specificity of the antibody, antibody fragment, or a derivative thereof lies in the amino acid sequence of the CDR. The variable domain (the heavy chain VH and light chain VL) of an antibody preferably comprises three complementary determining regions sometimes called hypervariable regions, flanked by four relatively conserved framework regions or "FRs". Often, the specificity of an antibody is determined or largely determined by a CDR, such as a CDR of the VH chain or a plurality of CDRs. The person skilled in the art will readily appreciate that the variable domain of the antibody, antibody fragment or derivative thereof having the above-described CDRs can be used for the construction of antibodies of further improved specificity and biological function. Insofar, the present invention encompasses antibodies, antibody fragments or derivatives thereof comprising at least one CDR of the above-described variable domains and which advantageously have substantially the same, similar or improved binding properties as the antibody described in the appended examples. Starting from an antibody that comprises at least one CDR as recited in the attached sequence listing and required by the embodiments of the invention, the skilled artisan can combine further CDRs from the originally identified monoclonal antibodies or different antibodies for an enhanced specificity and/or affinity. CDR grafting is well-known in the art and can also be used to fine-tune the specific affinity and other properties of the antibody, fragment or derivative thereof of the invention, as long as the original specificity is retained. It is advantageous that the antibody, fragment or derivative comprises at least two, more preferred at least three, even more preferred at least four or at least five and particularly preferred all six CDRs of the original donor antibody. In further alternatives of the invention, CDRs from different originally identified monoclonal antibodies may be combined in a new antibody entity. In these cases, it is preferred that the three CDRs of the heavy chain originate from the same antibody whereas the three CDRs of the light chain all originate from a different (but all from the same) antibody. The antibodies of the present invention or their corresponding

immunoglobulin chain(s) can be further modified using conventional techniques known in the art, for example, by using amino acid deletion(s), insertion(s), substitution(s), addition(s), and/or recombination(s) and/or any other modification(s) known in the art either alone or in combination. Methods for introducing such modifications in the DNA sequence underlying the amino acid sequence of an immunoglobulin chain are well known to the person skilled in the art; see, e.g., Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y.

The antibodies, antibody fragments or derivative thereof are optionally deimmunized for therapeutic purposes. A deimmunized antibody is a protein devoid of or reduced for epitopes that can be recognized by T helper lymphozytes. An example how to identify said epitopes is shown in Tangri et al., (J Immunol. 174: 3187-96, 2005). The manufacture of deimmunized antibody fragments or derivative thereof may be carried out as described in U.S. Pat. Nos. 6,054,297, 5,886,152 and 5,877,293.

In one embodiment the antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)). The production of chimeric antibodies is described, for example, in WO 89/09622.

Preferably, the present invention refers to a chimerized antibody comprising a heavy chain amino acid sequence selected from the group consisting of SEQ ID NOs: 38, 39, 41, 42 or at least the variable domain thereof or an amino acid sequence having a sequence identity of at least 90% thereto

and/or a light chain amino acid sequence selected from the group consisting of SEQ. ID NOs: 37, 40 or at least the variable domain thereof or an amino acid sequence having a sequence identity of at least 90% thereto or to an antibody recognizing the same epitope on the extracellular domain of AXL.

In a further embodiment the antibodies of the present invention are humanized or fully human antibodies. Humanized forms of the antibodies may be generated according to the methods known in the art such as chimerization or CDR grafting. Alternative methods for the production of humanized antibodies are well known in the art and are described in, e.g., EP-A1 0 239 400 and W090/07861. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be for example performed following the method of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)), by substituting CDRs or CDR sequences of non human origin for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in non human antibodies.

Preferably, the present invention refers to a humanized antibody comprising a heavy chain amino acid sequence selected from the group consisting of SEQ ID NOs: 44, 46 or at least the variable domain thereof or an amino acid sequence having a sequence identity of at least 90% thereto and/or a light chain amino acid sequence selected from the group consisting of SEQ. ID NOs: 43, 45 or at least the variable domain thereof or an amino acid sequence having a sequence identity of at least 90% thereto or to an

antibody recognizing the same epitope on the extracellular domain of AXL.

One method for generating fully human antibodies is through the use of XenoMouse® strains of mice that have been engineered to contain up to but less than 1000 kb sized germline configured fragments of the human heavy chain locus and kappa light chain locus. See, Mendez et al., , (Nature Genetics 15:146-156 1997), and Green and Jakobovits, ,(J. Exp. Med. 188:483-495, 1998). The XenoMouse® strains are available from AMGEN, Inc. (formerly ABGENIX, Fremont, CA).

The production of the XenoMouse® strains of mice is discussed and delineated in U.S. Patent Application Serial Nos. 07/466,008, filed January 12, 1990, 07/610,515, filed November 8, 1990, 07/919,297, filed July 24, 1992, 07/922,649, filed July 30, 1992, 08/031,801, filed March 15, 1993, 08/112,848, filed August 27, 1993, 08/234,145, filed April 28, 1994, 08/376,279, filed January 20, 1995, 08/430, 938, filed April 27, 1995, 08/464,584, filed June 5, 1995, 08/464,582, filed June 5, 1995, 08/463,191, filed June 5, 1995, 08/462,837, filed June 5, 1995, 08/486,853, filed June 5, 1995, 08/486,857, filed June 5, 1995, 08/486,859, filed June 5, 1995, 08/462,513, filed June 5, 1995, 08/724,752, filed October 2, 1996, 08/759,620, filed December 3, 1996, U.S. Publication 2003/0093820, filed November 30, 2001 and U.S. Patent Nos. 6,162,963, 6,150,584, 6,114,598, 6,075,181, and 5,939,598 and Japanese Patent Nos. 3 068 180 B2, 3 068 506 B2, and 3 068 507 B2. See, also European Patent No., EP 0 463 151 B1, grant published June 12, 1996, International Patent Application No., WO9402602, published February 3, 1994, International Patent Application No., WO9634096, published October 31, 1996, WO9824893, published June 11, 1998, WO0076310, published December 21, 2000. The disclosures of each of the above-cited patents, applications, and references are hereby incorporated by reference in their entirety.

In an alternative approach, others, including GenPharm International, Inc., have utilized a "minilocus" approach. In the minilocus approach, an

exogenous Ig locus is mimicked through the inclusion of pieces (individual genes) from the Ig locus. Thus, one or more VH genes, one or more DH genes, one or more JH genes, a mu constant region, and usually a second constant region (preferably a gamma constant region) are formed into a construct for insertion into an animal. This approach is described in U.S. Patent No. 5,545,807 to Surani et al. and U.S. Patent Nos. 5,545,806, 5,625,825, 5,625,126, 5,633,425, 5,661,016, 5,770,429, 5,789,650, 5,814,318, 5,877,397, 5,874,299, and 6,255,458 each to Lonberg and Kay, U.S. Patent No. 5,591,669 and 6,023.010 to Krimpenfort and Berns, U.S. Patent Nos. 5,612,205, 5,721,367, and 5,789,215 to Berns et al., and U.S. Patent No. 5,643,763 to Choi and Dunn, and GenPharm International U.S. Patent Application Serial Nos. 07/574,748, filed August 29, 1990, 07/575,962, filed August 31, 1990, 07/810,279, filed December 17, 1991, 07/853,408, filed March 18, 1992, 07/904,068, filed June 23, 1992, 07/990,860, filed December 16, 1992, 08/053,131, filed April 26, 1993, 08/096,762, filed July 22, 1993, 08/155,301, filed November 18, 1993, 08/161,739, filed December 3, 1993, 08/165,699, filed December 10, 1993, 08/209,741, filed March 9, 1994, the disclosures of which are hereby incorporated by reference. See, also European Patent No. 0 546 073 B1, International Patent Application Nos. WO9203918, WO9222645, WO9222647, WO9222670, WO9312227, WO9400569, WO9425585, WO9614436, WO9713852, and WO9824884 and U.S. Patent No. 5,981,175, the disclosures of which are hereby incorporated by reference in their entirety.

Kirin has also demonstrated the generation of human antibodies from mice in which, through microcell fusion, large pieces of chromosomes, or entire chromosomes, have been introduced. See, European Patent Application Nos. 773 288 and 843 961, the disclosures of which are hereby incorporated by reference. Additionally, KMTM mice, which are the result of cross-breeding of Kirin's Tc mice with Medarex's minilocus (Humab) mice have been generated. These mice possess the human IgH transchromosome of the Kirin mice and the kappa chain transgene of the Genpharm mice (Ishida

et al., , Cloning Stem Cells 4:91-102, 2002).

Human antibodies can also be derived by in vitro methods. Suitable examples include but are not limited to phage display (CAT, Morphosys, Dyax, Biosite/Medarex, Xoma, Symphogen, Alexion (formerly Proliferon), Affimed) ribosome display (CAT), yeast display, and the like.

For therapeutic purposes, the antibody may be conjugated with a therapeutic effector group, e.g. a radioactive group or a cytotoxic group.

For diagnostic purposes, the antibody may be labelled. Suitable labels include radioactive labels, fluorescent labels, or enzyme labels.

Further antibodies to be utilized in accordance with the present invention are so-called xenogenic antibodies. The general principle for the production of xenogenic antibodies such as human antibodies in mice is described in, e.g., WO9110741, WO 9402602, WO 9634096 and WO 9633735.

As discussed above, the antibody of the invention may exist in a variety of forms besides complete antibodies; including, for example, Fv, Fab´ and $F(ab')_2$ as well as in single chains; see e.g. W08809344.

If desired, the antibodies of the invention may be mutated in the variable domains of the heavy and/or light chains to alter a binding property of the antibody. For example, a mutation may be made in one or more of the CDR regions to increase or decrease the Kd of the antibody for AXL, or to alter the binding specificity of the antibody. Techniques in site directed mutagenesis are well-known in the art. See, e.g., Sambrook et al. and Ausubel et al., supra. Furthermore, mutations may be made at an amino acid residue that is known to be changed compared to germline in a variable region of an AXL antibody. In another aspect, mutations may be introduced into one or more of the framework regions. A mutation may be made in a framework region or constant domain to increase the half-life of the AXL

antibody. See, e.g., WO0009560. A mutation in a framework region or constant domain may also be made to alter the immunogenicity of the antibody, to provide a site for covalent or non-covalent binding to another molecule, or to alter such properties as complement fixation. Mutations may be made in each of the framework regions, the constant domain and the variable regions in a single mutated antibody. Alternatively, mutations may be made in only one of the framework regions, the variable regions or the constant domain in a single mutated antibody.

In a further aspect, the antibody may have a constant domain with effector functions, whereby AXL expressing cells which have bound the antibody, antibody fragment or derivative thereof on the cell surface may be attacked by immune system functions. For example, the antibody may be capable of fixing complement and participating in complement-dependent cytotoxicity (CDC). Moreover, the antibody may be capable of binding to Fc receptors on effector cells, such as monocytes and natural killer (NK) cells, and participate in antibody-dependent cellular cytotoxicity (ADCC).

In yet a further aspect the antibodies of the invention are applicable for therapeutic treatment, preferably for treatment of hyperproliferative diseases, cardiovascular diseases, in particular artherosclerosis and thrombosis, diabetes related diseases, in particular glomerular hypertrophy or diabetic nephropathy, and particularly of disorders associated with, accompanied by or caused by AXL expression, overexpression or hyperactivity. The hyperproliferative diseases are preferably selected from disorders associated with, accompanied by or caused by AXL expression, overexpression or hyperactivity, such as cancer, e.g. breast cancer, colon cancer, lung cancer, kidney cancer, follicular lymphoma, myeloid leukemia, skin cancer/melanoma, glioblastoma, ovarian cancer, prostate cancer, pancreatic cancer, Barrett's esophagus and esophageal cancer, stomach cancer, bladder cancer, cervical cancer, liver cancer, thyroid cancer, and head and neck cancer, or hyperplastic and neoplastic diseases or other AXL expressing or overexpressing hyperproliferative diseases.

In another aspect the antibodies of the present invention can be used for the co-administration with an antineoplastic agent for the treatment of one of the above mentioned disorders.

Co-administration as used herein includes the administration of an antibody of the present invention with an antineoplastic agent, preferably an apoptosis inducing antineoplastic agent. The term co-administration further includes the administration of the antibody of the present invention and the antineoplastic agent, preferably an apoptosis inducing antineoplastic agent, in the form of a single composition or in the form of two or more distinct compositions. Co-administration includes the administration of an antibody of the present invention with an antineoplastic agent, preferably an apoptosis inducing antineoplastic agent simultaneously (i.e. at the same time) or sequentially, (i.e. at intervals).

The invention further relates to a nucleic acid molecule encoding the antibody, antibody fragment or derivative thereof of the invention. The nucleic acid molecule of the invention encoding the above-described antibody, antibody fragment or derivative thereof may be, e.g. DNA, cDNA, RNA or synthetically produced DNA or RNA or recombinantly produced chimeric nucleic acid molecule comprising any of those nucleic acid molecules either alone or in combination. The nucleic acid molecule may also be genomic DNA corresponding to the entire gene or a substantial portion thereof or to fragments and derivatives thereof. The nucleotide sequence may correspond to the naturally occurring nucleotide sequence or may contain single or multiple nucleotide substitutions, deletions or additions. In a particular preferred embodiment of the present invention, the nucleic acid molecule is a cDNA molecule.

Preferably, the invention relates to an isolated nucleic acid molecule selected from the group consisting of:

(a) a nucleic acid sequence encoding a polypeptide of SEQ ID NOs: 7-12, 13-30, 37-42, 43-46

(b) a nucleic acid sequence as shown in SEQ ID NOs: 1-6, 31-36

(c) a nucleic acid complementary to any of the sequences in (a) or (b); and

(d) a nucleic acid sequence capable of hybridizing to (a), (b) or (c) under stringent conditions.

The term "hybridizing under stringent conditions" means that two nucleic acid fragments hybridize with one another under standardized hybridization conditions as described for example in Sambrook et al., "Expression of cloned genes in E. coli" in Molecular Cloning: A laboratory manual (1989), Cold Spring Harbor Laboratory Press, New York, USA. Such conditions are for example hybridization in 6.0xSSC at about 45° C. followed by a washing step with 2.0xSSC at 50° C, preferably 2.0xSSC at 65°C, or 0.2xSSC at 50°C, preferably 0.2xSSC at 65°C.

The invention also relates to a vector comprising a nucleic acid molecule of the invention. Said vector may be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

The nucleic acid molecules of the invention may be joined to a vector containing selectable markers for propagation in a host. Generally, a plasmid vector is introduced in a precipitate such as a calcium phosphate precipitate or rubidium chloride precipitate, or in a complex with a charged lipid or in carbon-based clusters, such as fullerens. Should the vector be a virus, it may be packaged in vitro using an appropriate packaging cell line prior to application to host cells.

Preferably, the vector of the invention is an expression vector wherein the nucleic acid molecule is operatively linked to one or more control sequences allowing the transcription and optionally expression in prokaryotic and/or

eukaryotic host cells. Expression of said nucleic acid molecule comprises transcription of the nucleic acid molecule, preferably into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They usually comprise regulatory sequences ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the lac, trp or tac promoter in E. coli, and examples for regulatory elements permitting expression in eukaryotic host cells are the AOXI or GAL1 promoter in yeast or the CMV-, SV40-, RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (Invitrogen) or pSPORTI (GIBCO BRL). Preferably, said vector is an expression vector and/ or a gene transfer or targeting vector. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the polynucleotides or vector of the invention into targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (2001, Third Edition) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1994). Alternatively, the nucleic acid molecules of the invention can be reconstituted into liposomes for delivery to target cells.

The invention further relates to a host comprising the vector of the invention.

Said host may be a prokaryotic or eukaryotic cell or a non-human transgenic animal. The polynucleotide or vector of the invention which is present in the host may either be integrated into the genome of the host or it may be maintained extrachromosomally. In this respect, it is also to be understood that the nucleic acid molecule of the invention can be used for "gene targeting" and/or "gene replacement", for restoring a mutant gene or for creating a mutant gene via homologous recombination; see for example Mouellic, Proc. Natl. Acad. Sci. USA, 87 (1990), 4712-4716; Joyner, Gene Targeting, A Practical Approach, Oxford University Press.

The host can be any prokaryotic or eukaryotic cell, such as a bacterial, insect, fungal, plant, animal, mammalian or, preferably, human cell. Preferred fungal cells are, for example, those of the genus Saccharomyces, in particular those of the species S. cerevisiae. The term "prokaryotic" is meant to include all bacteria which can be transformed or transfected with a polynucleotide for the expression of a variant polypeptide of the invention. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, E. coli, S. typhimurium, Serratia marcescens and Bacillus subtilis. A polynucleotide coding for a mutant form of variant polypeptides of the invention can be used to transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Methods for preparing fused, operably linked genes and expressing them in bacteria or animal cells are well-known in the art (Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (2001, Third Edition). The genetic constructs and methods described therein can be utilized for expression of variant antibodies, antibody fragments or derivatives thereof of the invention in, e.g., prokaryotic hosts. In general, expression vectors containing promoter sequences which facilitate the efficient transcription of the inserted nucleic acid molecule are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of the transformed cells. The transformed prokaryotic hosts can be grown in fermentors and cultured

according to techniques known in the art to achieve optimal cell growth. The antibodies, antibody fragments or derivatives thereof of the invention can then be isolated from the growth medium, cellular lysates, or cellular membrane fractions. The isolation and purification of the microbially or otherwise expressed antibodies, antibody fragments or derivatives thereof of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibodies.

In a preferred embodiment of the invention, the host is a bacterium, fungal, plant, amphibian or animal cell. Preferred animal cells include but are not limited to Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), 3T3 cells, NSO cells and a number of other cell lines including human cells, for example Per.C6. In another preferred embodiment, said animal cell is an insect cell. Preferred insect cells include but are not limited to cells of the SF9 cell lines

In a more preferred embodiment of the invention, said host is a human cell or human cell line. Said human cells include, but are not limited to Human embryonic kidney cells (HEK293, 293T, 293 freestyle). Furthermore, said human cell lines include, but are not limited to HeLa cells, human hepatocellular carcinoma cells (e. g., Hep G2), A549 cells.

The invention also provides transgenic non-human animals comprising one or more nucleic acid molecules of the invention that may be used to produce antibodies of the invention. Antibodies can be produced in and recovered from tissue or body fluids, such as milk, blood or urine, of goats, cows, horses, pigs, rats, mice, rabbits, hamsters or other mammals. See, e. g., U.S. Patent Nos. 5,827,690; 5,756,687; 5,750,172; and 5,741,957. As described above, non-human transgenic animals that comprise human immunoglobulin loci can be produced by immunizing with AXL or a portion thereof.

The invention additionally relates to a method for the preparation of an antibody, comprising culturing the host of the invention under conditions that allow synthesis of said antibody and recovering said antibody from said culture.

The transformed hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention, can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like; see, Scopes, "Protein Purification", Springer-Verlag, N.Y. (1982). The antibody or its corresponding immunoglobulin chain(s) of the invention can then be isolated from the growth medium, cellular lysates, or cellular membrane fractions. The isolation and purification of the, e.g., microbially expressed antibodies or immunoglobulin chains of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibodies directed, e.g., against the constant region of the antibody of the invention.

It will be apparent to those skilled in the art that the antibodies of the invention can be further coupled to other moieties for, e.g., drug targeting and imaging applications. Such coupling may be conducted chemically after expression of the antibody or antigen to site of attachment or the coupling product may be engineered into the antibody or antigen of the invention at the DNA level. The DNAs are then expressed in a suitable host system, and. the expressed proteins are collected and renatured, if necessary.

In a preferred embodiment of the present invention, the antibody is coupled to an effector, such as a radioisotope or a toxic chemotherapeutic agent. Preferably, these antibody conjugates are useful in targeting cells, e.g. cancer cells, expressing AXL, for elimination. The linking of

antibodies/antibody fragments of the invention to radioisotopes e.g. provides advantages to tumor treatments. Unlike chemotherapy and other forms of cancer treatment, radioimmunotherapy or the administration of a radioisotope-antibody combination directly targets the cancer cells with minimal damage to surrounding normal, healthy tissue. Preferred radioisotopes include e.g. ^{3}H, ^{14}C, ^{15}N, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I.

Furthermore, the antibodies of the invention can be used to treat cancer when being conjugated with toxic chemotherapeutic drugs such as geldanamycin (Mandler et al., J. Natl. Cancer Inst., 92(19), 1549-51 (2000» and maytansin, for example, the maytansinoid drug, DM1 (Liu et al., Proc. Natl. Acad. Sci. U.S.A. 93:8618-8623 (1996) and auristatin-E or monomethylauristatin-E (Doronina et al., Nat. Biotechnol. 21:778-784 (2003) or calicheamicit);. Different linkers that release the drugs under acidic or reducing conditions or upon exposure to specific proteases are employed with this technology. The antibodies of the invention may be conjugated as described in the art.

The invention further relates to a pharmaceutical composition comprising the antibody, the nucleic acid molecule, the vector, the host of the invention or an antibody obtained by the method of the invention.

The term "composition" as employed herein comprises at least one compound of the invention. Preferably, such a composition is a pharmaceutical or a diagnostic composition.

It is preferred that said pharmaceutical composition comprises a pharmaceutically acceptable carrier and/or diluent. The herein disclosed pharmaceutical composition may be partially useful for the treatment of disorders associated with, accompanied by or caused by AXL expression, overexpression or hyperactivity, e.g. hyperproliferative diseases, cardiovascular diseases, in particular artherosclerosis and thrombosis, diabetes related diseases, in particular glomerular hypertrophy or diabetic

nephropathy. Said disorders comprise, but are not limited to cancer, e.g. breast cancer, colon cancer, lung cancer, kidney cancer, follicular lymphoma, myeloid leukemia, skin cancer/melanoma, glioblastoma, ovarian cancer, prostate cancer, pancreatic cancer, Barrett's esophagus and esophageal cancer, stomach cancer, bladder cancer, cervical cancer, liver cancer, thyroid cancer, and head and neck cancer, or other hyperplastic or neoplastic diseases or other AXL expressing or overexpressing diseases.

The term "hyperactivity" herein refers to uncontrolled AXL signaling which may be caused by a lack and/or dysfunction of negative regulation. By way of example negative regulation comprises protein dephosphorylation, degradation and/or endocytosis. Moreover uncontrolled AXL signaling may be the result of genetic alterations, either somatic or germline, which result in changes of the AXL amino acid sequence.

Examples of suitable pharmaceutical carriers, excipients and/or diluents are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical, intradermal, intranasal or intrabronchial administration. The compositions of the invention may also be administered directly to the target site, e.g., by biolistic delivery to an external or internal target site, like the brain. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Proteinaceous pharmaceutically active matter may be present in amounts between 1 µg and 100 mg/kg body weight per

dose; however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. If the regimen is a continuous infusion, it should also be in the range of 1 pg to 100 mg per kilogram of body weight per minute.

Progress can be monitored by periodic assessment. The compositions of the invention may be administered locally or systemically. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or' fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the pharmaceutical composition of the invention may comprise further agents depending on the intended use of the pharmaceutical composition. It is particularly preferred that the pharmaceutical composition comprises further active agents like, e.g. an additional antineoplastic agent, small molecule inhibitor, anti-tumor agent or chemotherapeutic agent.

The invention also relates to a pharmaceutical composition comprising an anti-AXL-antibody, which is preferably the antibody of the invention in combination with at least one further antineoplastic agent. Said combination is effective, for example, in inhibiting abnormal cell growth.

Many antineoplastic agents are presently known in the art. In general the term includes all agents that are capable of prevention, alleviation and/or treatment of hyperproliferative disorders. In one embodiment, the

antineoplastic agent is selected from the group of therapeutic proteins including but not limited to antibodies or immunomodulatory proteins. In another embodiment the antineoplastic agent is selected from the group of small molecule inhibitors or chemotherapeutic agents consisting of mitotic inhibitors, kinase inhibitors, alkylating agents, anti-metabolites, intercalating antibiotics, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, histone deacetylase inhibitors, anti-survival agents, biological response modifiers, anti-hormones, e. g. anti-androgens, and antiangiogenesis agents.

Specific examples of antineoplastic agents which can be used in combination with the antibodies provided herein include, for example, gefitinib, lapatinib, sunitinib, pemetrexed, bevacisumab, cetuximab, imatinib, trastuzumab, alemtuzumab, rituximab, erlotinib, bortezomib and the like. Other specific antineoplastic agents to be used in the compositions as described and claimed herein include for example, chemotherapeutic agents such as capecitabine, daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide, trimetrexate, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, The Merck Manual of Diagnosis and Therapy, 15th Ed. 1987, pp. 1206-1228, Berkow et al., eds., Rahway, N.J. In particular preferred are such antineoplastic agents that induce apoptosis.

When used with the described AXL antibodies, such antineoplastic agents

may be used individually (e.g., 5-FU and an antibody), sequentially (e.g., 5-FU and an antibody for a period of time followed by MTX and an antibody), or in combination with one or more other such antineoplastic agents (e.g., 5-FU, MTX and an antibody, or 5-FU, radiotherapy and an antibody).

The term antineoplastic agent may also include therapeutic procedures, as for example irradiation or radiotherapy.

The pharmaceutical composition of the invention can be used in human medicine and can be used also for veterinary purposes.

Additionally, the invention relates to the use of the antibody of the invention, the nucleic acid molecule, the vector, the host of the invention or an antibody obtained by the method of the invention for the preparation of a pharmaceutical composition for diagnosis, prevention or treatment of hyperproliferative diseases, cardiovascular diseases, in particular artherosclerosis and thrombosis, diabetes related diseases, in particular glomerular hypertrophy or diabetic nephropathy, and particularly of disorders associated with, accompanied by or caused by AXL expression, overexpression or hyperactivity.

A hyperproliferative disease as mentioned above includes any neoplasia, i.e. any abnormal and/or uncontrolled new growth of tissue. The term "uncontrolled new growth of tissue" as used herein may depend upon a dysfunction and/or loss of growth regulation. A hyperproliferative disease includes tumor diseases and/or cancer, such as metastatic or invasive cancers.

In a preferred embodiment of the use of the invention, said hyperproliferative disease is in particular breast cancer, colon cancer, lung cancer, kidney cancer, follicular lymphoma, myeloid leukemia, skin cancer/melanoma, glioblastoma, ovarian cancer, prostate cancer, pancreatic cancer, Barrett's esophagus and esophageal cancer, stomach cancer, bladder cancer,

cervical cancer, liver cancer, thyroid cancer, and head and neck cancer, or hyperplastic or neoplastic diseases or other AXL expressing or overexpressing hyperproliferative diseases.

In yet another embodiment the present invention refers to the use of an anti-AXL-antibody, preferably the antibody of the present invention for the manufacture of a medicament for the co-administration with an antineoplastic agent for the treatment of one of the above mentioned disorders.

According to a further preferred embodiment the present invention is directed to the use of an anti-AXL antibody for the manufacture of a pharmaceutical composition for the treatment of drug resistant cancer. In a particularly preferred embodiment, the anti-AXL antibody is a monoclonal antibody as defined in claims 1-22.

Further the present invention relates to a diagnostic composition comprising the antibody of the invention, the nucleic acid molecule, the vector, the host of the invention or an antibody obtained by the method of the invention and optionally a pharmaceutically acceptable carrier.

The diagnostic composition of the invention is useful in the detection of an undesired expression, overexpression or hyperactivity of the mammalian AXL in different cells, tissues or another suitable sample, comprising contacting a sample with an antibody of the invention, and detecting the presence of AXL in the sample. Accordingly, the diagnostic composition of the invention may be used for assessing the onset or the disease status of a hyperproliferative disease.

Furthermore, malignant cells, such as cancer cells expressing AXL, can be targeted with the antibody of the invention. The cells which have bound the antibody of the invention might thus be attacked by immune system functions such as the complement system or by cell-mediated cytotoxicity,

thereby reducing the number of or eradicating cancer cells. These considerations equally apply to the treatment of metastases and re-current tumors.

In another aspect of the present invention, the antibody of the invention is coupled to a labelling group. Such antibodies are particularly suitable for diagnostic applications. As used herein, the term "labelling group" refers to a detectable marker, e.g. a radiolabelled amino acid or biotinyl moieties that can be detected by marked avidin. Various methods for labelling polypeptides and glycoproteins, such as antibodies, are known in the art and may be used in performing the present invention. Examples of suitable labelling groups include, but are not limited to, the following: radioisotopes or radionuclides (e.g. ^{3}H, ^{14}C, ^{15}N, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I), fluorescent groups (e.g. FITC, rhodamine, lanthanide phosphors), enzymatic groups (e.g. horseradish peroxidase, β-galactosidase, luciferase, alkaline phosphatase), chemiluminescent groups, biotinyl groups, or predetermined polypeptide epitopes recognized by a secondary reporter (e.g. leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags).

In certain aspects, it may be desirable, that the labelling groups are attached by spacer arms of various lengths to reduce potential steric hindrance.

In another embodiment the present invention relates to a method of assessing for the presence of AXL expressing cells comprising contacting the antibody of the invention with cells or a tissue suspected of carrying AXL on their/its surface. Suitable methods for detection of AXL expression in a sample may be an Enzyme-Linked Immunosorbent Assay (ELISA) or Immunohistochemistry (IHC).

An ELISA assay may be carried out in a microtiter plate format, wherein e.g. wells of a microtiter plate, are adsorbed with an AXL antibody. The wells are rinsed and treated with a blocking agent such as milk protein or albumin to

prevent nonspecific adsorption of the analyte. Subsequently the wells are treated with a test sample. After rinsing away the test sample or standard, the wells are treated with a second AXL antibody that is labelled, e.g. by conjugation with biotin. After washing away excess secondary antibody, the label is detected, e.g. with avidin-conjugated horseradish peroxidase (HRP) and a suitable chromogenic substrate. The concentration of the AXL antigen in the test samples is determined by comparison with a standard curve developed from standard samples.

For IHC, paraffin-embedded tissues may be used, wherein the tissues are, e.g. first deparaffinized in xylene and then dehydrated, e.g. with ethanol and rinsed in distilled water. Antigenic epitopes masked by formalin-fixation and paraffin-embedding may be exposed by epitope unmasking, enzymatic digestion or saponin. For epitope unmasking paraffin sections may be heated in a steamer, water bath or microwave oven for 20-40 min in an epitope retrieval solution as for example 2N HCl solution (pH 1.0). In the case of an enzyme digestion, tissue sections may be incubated at 37°C for 10-30 minutes in different enzyme solutions such as proteinase K, trypsin, pronase, pepsin etc.

After rinsing away the epitope retrieval solution or excess enzyme, tissue sections are treated with a blocking buffer to prevent unspecific interactions. The primary AXL antibody is added at appropriate concentrations. Excess primary antibody is rinsed away and sections are incubated in peroxidase blocking solution for 10 min at room temperature. After another washing step, tissue sections are incubated with a secondary labelled antibody, e.g. labelled with a group that might serve as an anchor for an enzyme. Examples therefore are biotin labelled secondary antibodies that are recognized by streptavidin coupled horseradish peroxidase. Detection of the antibody/enzyme complex is achieved by incubating with a suitable chromogenic substrate.

In an additional embodiment the present invention relates to a method of

blocking AXL function comprising contacting the antibody of the invention with cells or a tissue suspected of carrying AXL on their/its surface under conditions, wherein the antibody is capable of blocking AXL function. The contacting may be in vitro or in vivo.

The invention also relates to a method of treating a hyperproliferative disease, cardiovascular diseases, in particular artherosclerosis and thrombosis, diabetes related diseases, in particular glomerular hypertrophy or diabetic nephropathy, comprising, administering to a patient in need thereof a suitable dose of the antibody or antibody fragment or derivative thereof of the present invention. The hyperproliferative disease is preferably selected from disorders associated with, accompanied by or caused by AXL expression, overexpression or hyperactivity, such as cancer, e.g. breast cancer, colon cancer, lung cancer, kidney cancer, follicular lymphoma, myeloid leukemia, skin cancer/melanoma, glioblastoma, ovarian cancer, prostate cancer, pancreatic cancer, Barrett's esophagus and esophageal cancer, stomach cancer, bladder cancer, cervical cancer, liver cancer, thyroid cancer, and head and neck cancer, or hyperplastic and neoplastic diseases or other AXL expressing or overexpressing hyperproliferative diseases.

According to another preferred embodiment of the invention the cancer to be treated is a drug resistant cancer.

The invention further relates to a method of treating a disease wherein the antibody of the invention is administered to a mammal and wherein said disease is correlated directly or indirectly with the abnormal level of expression or activity of AXL.

Finally, the invention relates to a kit comprising an anti-AXL-antibody, preferably the antibody, antibody fragment or derivative thereof of the invention, the nucleic acid molecule encoding said components and/or the vector of the invention.

All embodiments covering the compounds disclosed herein can be used as single compounds or in combination for the preparation of a medicament.

Figure Legends

Figure 1. Flow cytometry analysis of cell surface AXL in Ratl-Mock and Ratl-AXL cl.2 fibroblasts. Polyclonal Ratl-Mock and clonal Ratl-AXL cl.2 cells, generated by infection of Ratl fibroblasts with pLXSN and pLXSN-hAXL ecotrophic virus, respectively, were collected and stained with mouse control antibody 72A1 (left panel) or mouse anti-AXL MAB154 primary antibody (right panel) at 3 µg/ml and PE-conjugated anti-mouse secondary antibody. See text for details. Staining of Ratl-AXL cl.2 cells results in a shift by three orders of magnitude and demonstrates AXL overexpression on the surface of these cells.

Figure 2. Flow cytometry analysis of cell surface AXL in NIH3T3-Mock and NIH3T3-AXL cl.7 fibroblasts. Polyclonal NIH3T3-Mock and clonal NIH3T3-AXL cl.7 cells, generated by infection of NIH3T3 fibroblasts with pLXSN and pLXSN-AXL ecotrophic virus, respectively, were collected and stained with mouse control antibody 72A1 (left panel) or mouse anti-AXL MAB154 primary antibody (right panel) at 3 µg/ml and PE-conjugated anti-mouse secondary antibody. See text for details. Staining of NIH3T3-AXL cl.7 cells results in a shift by two orders of magnitude and demonstrates AXL overexpression on the surface of these cells.

Figure 3. Flow cytometry analysis of cross-reactivity of rat-anti AXL antibodies with mouse and cynomolgus monkey AXL as well as human Mer and Sky. HEK293T fibroblasts were transiently transfected with pcDNA3, pcDNA3-hAXL, pcDNA3mAXL, pcDNA3-cyAXL, pcDNA3-hMer, or pcDNA3-hSky. Cells were collected and stained with 10 µg/ml anti-AXL 1D5, 11D5, 11B7, 10D12, 6E7, 2A1, 11D7 or 12B7 primary antibody and/or PE-conjugated donkey anti-rat secondary antibody, or PE-conjugated donkey anti-mouse secondary antibody only for control. See text for details. Except 12B7 which shows moderate cross-reactivity with mouse AXL as well as human Mer and Sky, non of the anti-AXL antibodies cross-reacted with these molecules. In contrast, all tested anti-AXL antibodies cross-reacted with

cynomolgus monkey AXL.

Figure 4. ELISA experiments to investigate the effects of rat anti-AXL antibodies on AXL receptor phosphorylation. NIH3T3-AXL cl.7 fibroblasts (A) and NCI-H292 lung cancer cells (B) were starved, pre-incubated with 10 µg/ml of mouse control antibody 72A1 as well as the rat anti-AXL antibodies 2A1, 11D7, 11D5, 11B7, 6E7, or 10D12, treated with or without 400 ng/ml mGas6, and lysed. Lysates were transferred to anti-phospho-tyrosine antibody 4G10-coated Maxi-Sorp 96 well plates, which then were washed and incubated with 0.5 µg/ml biotinylated rat anti-AXL antibody 12B7, AP-conjugated streptavidin and AttoPhos substrate solution in order to collect fluorescence intensities. See text for details. The rat anti-AXL antibodies 11B7, 11D5, 6E7, and 10D12 were able to block or reduce ligand-mediated AXL activation as indicated by decreased phosphorylation, and are thus considered antagonistic anti-AXL antibodies. In contrast, the rat anti-AXL antibodies 2A1 and 11D7 stimulate basal AXL activation as indicated by increased phosphorylation, do not significantly reduce ligand-mediated AXL activation, and are therefore considered agonistic anti-AXL antibodies.

Figure 5. ELISA experiments to investigate the effects of rat anti-AXL antibodies on p42/p44 MAP-Kinase phosphorylation. CaSki cervical cancer cells were starved, pre-incubated with 10 µg/ml of the isotypic control antibody 1D5 as well as the rat anti-AXL antibodies 11D5, 11B7, or 2A1, treated with or without 400 ng/ml mGas6, and fixed with formaldehyde. Cells were washed, quenched and incubated with anti-phospho-p44/p42 MAP Kinase (Thr202/Tyr204) primary antibody, HRP-conjugated anti-rabbit secondary antibody and Tetramethylbenzidine solution in order to measure absorbance intensities. See text for details. The rat anti-AXL antibodies 11B7 and 11D5 were able to reduce ligand-mediated p42/p44 MAP-Kinase activation as indicated by decreased phosphorylation, and are thus considered antagonistic anti-AXL antibodies. In contrast, the rat anti-AXL antibody 2A1 stimulates basal p42/p44 MAP-Kinase activation as indicated

by increased phosphorylation, does not reduce ligand-mediated p42/p44 MAP-Kinase activation, and is therefore considered an agonistic anti-AXL antibody.

Figure 6. ELISA experiments to investigate the effects of rat anti-AXL antibodies on Akt-Kinase phosphorylation. NIH3T3-AXL cl.7 fibroblasts (A) and CaLu-1 lung cancer cells (B) were starved, pre-incubated with 10 µg/ml of the isotypic control antibody 1D5 as well as the rat anti-AXL antibodies 11D5, 11B7, or 2A1, treated with or without 400 ng/ml mGas6, and fixed with formaldehyde. Cells were washed, quenched and incubated with anti-phospho-Akt (Ser473) primary antibody, HRP-conjugated anti-rabbit secondary antibody and Tetramethylbenzidine solution in order to measure absorbance intensities. See text for details. The rat anti-AXL antibodies 11B7 and 11D5 were able to block or reduce ligand-mediated Akt-Kinase activation as indicated by decreased phosphorylation, and are thus considered antagonistic anti-AXL antibodies. In contrast, the rat anti-AXL antibody 2A1 stimulates basal Akt-Kinase activation as indicated by increased phosphorylation, does not reduce ligand-mediated Akt-Kinase activation, and is therefore considered an agonistic anti-AXL antibody.

Figure 7. ELISA experiments to compare the effects of rat and chimeric anti-AXL antibodies on Akt-Kinase phosphorylation. NIH3T3-AXL cl.7 fibroblasts were starved, pre-incubated with 50 ng/ml, 100 ng/ml, 300 ng/ml, 500 ng/ml, and 1 µg/ml of rat anti-AXL antibody 11B7 or chimeric anti-AXL antibody ch11B7, as well as 50 ng/ml, 100 ng/ml, 300 ng/ml, 500 ng/ml, 1 µg/ml, 5 µg/ml, and 10 µg/ml of rat anti-AXL antibody 11D5 or chimeric anti-AXL antibody ch11D5, treated with or without 400 ng/ml mGas6, and fixed with formaldehyde. Cells were washed, quenched and incubated with anti-phospho-Akt (Ser473) primary antibody, HRP-conjugated anti-rabbit secondary antibody and Tetramethylbenzidine solution in order to measure absorbance intensities. See text for details. Rat anti-AXL antibody 11B7 and chimeric anti-AXL antibody ch11B7 as well as rat anti-AXL antibody 11D5 or chimeric anti-AXL antibody ch11D5 were able to inhibit ligand-mediated Akt-

Kinase activation to similar extent as indicated by decreased phosphorylation. Thus, as compared to their respective rat counterparts, the chimeric anti-AXL antibodies ch11B7 and ch11D5 maintained activity.

Figure 8. Competition ELISA experiments to investigate binding properties of rat anti-AXL antibodies. 96 well Maxi-Sorp plates were coated with 1 µg/ml human AXL-ECD and pre-incubated with 10 µg/ml of unbiotinylated isotypic control antibody 1D5 or rat anti-AXL antibodies 11B7, 11D5, 6E7, 10D12, 11D7, or 2A1. After incubation with 0.5 µg/ml biotinylated isotypic control antibody 1D5 or biotinylated rat anti-AXL antibodies 11B7, 11D5, 6E7, 10D12, 11D7, or 2A1, and addition of AP-conjugated Streptavidin and AttoPhos substrate solution, fluorescence was collected to measure bound biotinylated antibodies. See text for details. The control antibody 1D5 did not bind to AXL-ECD. The antagonistic anti-AXL antibodies 11B7, 11D5, 6E7, and 10D12 competed with each other for the same or structurally adjacent epitopes. The agonistic antibodies 11D7 and 2A1 recognize different epitopes and do not compete with the antagonistic antibodies for binding to the AXL-ECD.

Figure 9. Wound healing/scratch assay to investigate the effects of rat and chimeric anti-AXL antibodies on cell migration and proliferation. After grown to confluency, NCI-H292 lung cancer cells were starved and wounded with a pipette tip. In the presence of 10 µg/ml of the isotypic control antibody 1D5, the antagonistic rat anti-AXL antibodies 11D5, 11B7, 6E7, or 10D12, the chimeric anti-AXL antibodies chn11D5 IgG2 and chn11B7 IgG2, the agonistic rat anti-AXL antibodies 2A1 and 11D7, as well as 10 µg/ml of Erbitux or 5µM Sutent, cells were permitted to re-populate the area of clearing, After 24 h, cells were fixed and stained, and photos of the wounds were taken. See text for details. Compared to the isotypic control antibody 1D5, the antagonistic rat anti-AXL antibodies 11D5, 11B7, 6E7, and 10D12, as well as the chimeric anti-AXL antibodies chn11D5 IgG2 and chn11B7 IgG2 reduced the re-population of the cleared area, whereas the agonistic rat anti-AXL antibodies 2A1 and 11D7 led to complete wound closure.

Figure 10. Boyden chamber/transwell assay to investigate the effects of rat anti-AXL antibodies on directed cell migration. Serum starved NIH3T3-AXL cl.7 fibroblasts were pre-incubated with 10 µg/ml of the rat anti-AXL antibodies 4A6, 11B7 or 2A1, plated on top of collagen I-coated FluoreBlock inserts and exposed to serum-free medium with or without Gas6 in the lower compartment. After 7 h, transmigrated cells were stained with calcein-AM, and fluorescence of each well was measured. See text for details. The antagonistic anti-AXL antibody 11B7 reduced both basal and Gas6-induced migration of NIH3T3-AXL cl.7 fibroblasts, whereas the agonistic rat anti-AXL antibody 2A1 increased ligand-induced and, in particular, basal migration of NIH3T3-AXL cl.7 cells. The antibody 4A6 did not affect directed cell migration.

Figure 11. AlamarBlue™ assay to investigate the effects of rat anti-AXL antibodies on Gas6-induced cell proliferation. Serum starved NIH3T3-AXL cl.7 fibroblasts were pre-incubated with 20 µg/ml of the mouse control antibody 72A1, the rat antagonistic anti-AXL antibodies 11D5 and 11B7, as well as the agonistic anti-AXL antibody 2A1, and grown in the absence or presence of 400 ng/ml Gas6. After 4 days, AlamarBlue™ was added to the cells and absorbance was measured. See text for details. The antagonistic anti-AXL antibodies 11D5 amd 11B7 inhibited Gas6-induced proliferation of NIH3T3-AXL cl.7 fibroblasts, whereas the agonistic rat anti-AXL antibody 2A1 increased ligand-induced and, in particular, basal proliferation of NIH3T3-AXL cl.7 cells.

Figure 12. Caspase-3/7 assay to investigate the effects of rat anti-AXL antibodies on Gas6-mediated anti-apoptosis. Serum-starved NIH3T3-AXL cl.7 fibroblasts were pre-incubated with 10 µg/ml of the isotypic control antibody 1D5, the antagonistic rat anti-AXL antibodies 11B7 and 11D5, or the agonistic rat anti-AXL antibodies 11D7 and 2A1, and treated with or without Gas6. Apo-ONE substrate solution was added and flourescence was collected to measure caspase-3/7 activity. See text for details. Compared to

the isotypic control antibody, the antagonistic rat anti-AXL antibodies 11B7 and 11D5 reduced Gas6-mediated anti-apoptosis of serum-starved NIH3T3-AXL cl.7 fibroblasts, and thus induced apoptosis. In contrast, the agonistic rat anti-AXL antibodies 2A1 and 11D7 induced anti-apoptosis of serum-starved NIH3T3-AXL cl.7 cells regardless of the absence or presence of Gas6, and therefore inhibited apoptosis.

Figure 13. Spheroid-based cellular angiogenesis assay to investigate the effects of rat anti-AXL antibodies on VEGF-A-induced endothelial cell sprouting. HUVEC spheroids were embedded in a 3D collagen gel, stimulated with 25 ng/ml VEGF-A and treated with indicated concentrations of the antagonistic rat anti-AXL antibodies 11B7 (A) and 11D5 (B) for 24 h. The mean ± SEM of the cumulative sprout length of 10 randomly selected spheroids per data point was analyzed (left panel) and the relative inhibition by the antibody was determined (right panel). Fitting of IC_{50} curves and calculation of IC_{50} values was performed with GraphPad Prism 4.03. See text for details. The antagonistic rat anti-AXL antibodies 11B7 and 11D5 inhibited VEGF-A-stimulated HUVEC sprouting in the spheroid-based angiogenesis assay in a dose-dependent manner. Whereas treatment with the highest concentration of 11B7 reduced HUVEC sprouting to basal levels, inhibition with the highest concentration of 11D5 was not as effective (left panel). HUVEC sprouting was inhibited with IC_{50} values of 9.8×10^{-8} M and 7.0×10^{-7} M for 11B7 and 11D5, respectively (right panel).

Figure 14. Orthotopic xenograft model to investigate the effects of rat anti-AXL antibodies on human prostate carcinoma growth in nude mice. PC-3-LN prostate carcinoma cells were orthotopically implanted into the prostate of $NMRI^{-nu/nu}$ mice. Animals were randomized into 4 groups and received 25 mg/kg of the isotypic control antibody 1D5 or the antagonistic rat anti-AXL antibody 11B7, as well as 40 mg/kg Sutent or 12.5 mg/kg Taxotere. During the treatment period, the growth of orthotopically growing PC-3-LN tumors as well as peripheral metastases was monitored once weekly via *in vivo* bioluminescence imaging on day 15, day 23, day 29, and day 34. See

text for details. Compared to the isotypic control antibody 1D5, the antagonistic rat anti-AXL antibody 11B7 reduced the overall growth of PC-3-LN prostate tumors in nude mice.

Figure 15. Orthotopic xenograft model to investigate the effects of rat anti-AXL antibodies on human prostate carcinoma metastasis in nude mice. PC-3-LN prostate carcinoma cells were orthotopically implanted into the prostate of NMRI$^{nu/nu}$ mice. Animals were randomized into 4 groups and received 25 mg/kg of the isotypic control antibody 1D5 or the antagonistic rat anti-AXL antibody 11B7, as well as 40 mg/kg Sutent or 12.5 mg/kg Taxotere. Post necropsy, selected organs (liver, spleen, lung, femur, and a part of the lumbar spine) were collected and analyzed for the presence of metastases via bioluminescence imaging. See text for details. Compared to the isotypic control antibody 1D5, the antagonistic rat anti-AXL antibody 11B7 of the invention reduced the occurrence of spleen metastases. Noteworthy, the anti-metastatic effect of 11B7 in this experiment was stronger than that of Sutent.

Figure 16. Immunhistochemical analysis of AXL expression in different human malignancies. 17 human solid tumor types, each represented by pairs of tumor tissue and matching non-malignant tissue, were analyzed with regard to AXL expression by immunhistochemistry. See text for details. Results are summarized (A), whereby an intensity of 1 refers to weak staining in more than 25% of inspected cells. Examples of most intense staining as observed in mammary tumors and a signet ring cell carcinoma of the stomach are displayed (B).

Figure 17. ELISA experiments to compare the effects of rat and chimeric anti-Axl antibodies on Axl phosphorylation. CaSki cervical cancer cells were starved, pre-incubated with 50 ng/ml, 100 ng/ml, 300 ng/ml, 750 ng/ml, 1 µg/ml, and 10 µg/ml of rat anti-Axl antibody 11B7 (A) or chimeric anti-Axl antibody ch11B7 (B), treated with or without 400 ng/ml mGas6, and lysed. Lysates were transferred to anti-phospho-tyrosine

antibody 4G10-coated Maxi-Sorp 96 well plates. Afterwards, plates were washed and incubated with 0.5 µg/ml of biotinylated rat anti-Axl antibody 12B7, AP-conjugated streptavidin, and AttoPhos substrate solution in order to collect fluorescence intensities. See text for details. As demonstrated by concentration-dependent decrease of the relative Axl phosphorylation in the cervical cancer cell line CaSki, the rat anti-Axl antibody 11B7 (A) and the chimeric anti-Axl antibody ch11B7 (B) of the invention were able to block ligand-induced activation of the receptor tyrosine kinase Axl to similar extent.

Figure 18. ELISA experiments to compare the effects of rat and chimeric anti-Axl antibodies on Axl phosphorylation. CaSki cervical cancer cells were starved, pre-incubated with 50 ng/ml, 100 ng/ml, 300 ng/ml, 750 ng/ml, 1 µg/ml, and 10 µg/ml of rat anti-Axl antibody 11B7 (A) or chimeric anti-Axl antibody ch11B7 (B), treated with or without 400 ng/ml mGas6, and fixed with formaldehyde. Cells were washed, quenched and incubated with anti-phospho-p44/p42 MAP Kinase (Thr202/Tyr204) primary antibody, HRP-conjugated anti-rabbit secondary antibody and Tetramethyl-benzidine solution in order to measure absorbance intensities. See text for details. The rat anti-Axl antibody 11B7 (A) and the chimeric anti-Axl antibody ch11B7 (B) of the invention were able to block Gas6-induced activation of p42/p44 MAP-Kinase in CaSki cervical cancer cells to similar extent as indicated by concentration-dependent decrease of the relative p42/p44 MAP-Kinase phosphorylation.

Figure 19. TUNEL staining to investigate the combinatorial effect of rat anti-AXL antibodies and chemotherapeutic agents to overcome drug resistance in human ovarian cancer cells. Human NCI/ADR-RES ovarian cancer cells were pre-incubated with 10 µg/ml of control antibody or the antagonistic anti-Axl antibody 11B7 and co-incubated with doxorubicin at final concentrations of 100 µM, 150 µM, or 200 µM. Applying a commercially available kit, TUNEL staining was performed in order to visualize and determine apoptosis. See text for details. No TUNEL staining, and hence no apoptosis, was observed with NCI/ADR-RES ovarian cancer cells that were

treated with 100 µM of doxorubicin, regardless of whether cells have been co-incubated with control antibody or the antagonistic anti-Axl antibody 11B7 (top). However, at a concentration of 150 µM of doxorubicin, only very week apoptosis could be detected in cells co-treated with control antibody, whereas co-incubation with the antagonistic anti-Axl antibody 11B7 resulted in a substantial induction of apoptosis (middle). Also in the presence of 200 µM of doxorubicin, co-incubation of cells with 11B7 significantly increased apoptosis rates as compared to cells being incubated with control IgG antibody (bottom), indicating that co-treatment of even multi drug-resistant tumor cells with both chemotherapeutic agents and antagonistic anti-Axl antibodies of the invention may be suitable to overcome drug resistance.

Figure 20. Soft agar assay to investigate the combinatorial effect of rat anti-AXL antibodies and chemotherapeutic agents on anchorage-independent growth of human melanoma cells. Human C-8161 melanoma cells either remained untreated or were incubated with the rat antagonistic anti-AXL antibody 11B7 at a final concentration of 2.5 µg/ml. Combined with cisplatin at the indicated concentrations, agar-embedded cells were allowed to grow on top of a 0.7% bottom agar layer for 5 days. Stained with MTT, the area of colonies was then measured. See text for details. Absolute numbers reflecting the overall area of colonies (A) and the relative growth inhibition (B) calculated on the basis of these data are shown. As compared to untreated control cells, incubation with cisplatin led to colony growth retardation in a dose-dependent manner. In line with the inhibitory effect of 11B7 alone in the range of 30%, combination with the antagonistic anti-Axl antibody 11B7 resulted in a significantly potentiated inhibitory effect of cisplatin on soft agar growth of C-8161 melanoma cells, particularly at lower concentrations of cisplatin.

Further, the present invention shall be explained by the following examples and the accompanying drawing figures.

Examples

General comment

The following examples, including the experiments conducted and results achieved, are provided for illustrative purposes only and are not to be construed as limiting upon the present invention.

Example 1. Generation of AXL overexpressing Ratl fibroblasts as immunogen and AXL overexpressing NIH3T3 fibroblasts as experimental model system

The full length coding sequence for the human receptor tyrosine kinase AXL transcript variant 1 according to the National Center for Biotechnology Information (NCBI) reference sequence (NM_021913) was subcloned into pLXSN via flanking recognition elements for the restriction endonucleases *Eco*RI and *Bam*HI, thereby resulting in the retroviral expression vector pLXSN-hAXL.

For the generation of antibodies that specifically bind to human receptor tyrosine kinase AXL, Ratl fibroblasts stably overexpressing human AXL were generated by retroviral gene transfer. In brief, $3x10^5$ Phoenix-E cells were seeded on 60 mm culture dishes and transfected with 2 µg/ml pLXSN vector or pLXSN-hAXL using the calcium phosphate method. After 24 h, medium was replaced by fresh medium in which Phoenix-E cells were incubated for 4 h. The supernatants of Phoenix-E cells releasing pLXSN or pLXSN-hAXL ecotrophic virus were harvested and used for the incubation of subconfluent Ratl cells ($2x10^5$ cells per 6 cm dish) for 3 h in the presence of Polybrene (4 mg/ml; Aldrich). Simultaneously, Phoenix-E cells were re-incubated with fresh medium, which after another 3 h was used for a second infection of the Ratl fibroblasts in the presence of Polybrene (4 mg/ml; Aldrich). Likewise, a third infection cycle was performed. After changing the medium, selection of Ratl cells with G418 was started. Usually, stable clones were picked after selection for 21 days.

A panel of stable clones was propagated and quantified for membrane-localized human AXL expression by FACS analysis. In detail, $1x10^5$ cells were harvested with 10 mM EDTA in PBS, washed once with FACS buffer (PBS, 3% FCS, 0.4% azide) and seeded on a 96 well round bottom plate. The cells were spun for 3 min at 1,000 rpm to remove supernatant and were resuspended with mouse anti-AXL primary antibody MAB154 (R&D Systems, 3 µg/ml). Cell suspensions were incubated on ice for 1 h, washed twice with FACS buffer and resuspended in 100 µl/well of PE-conjugated donkey anti-mouse secondary antibody (Jackson) diluted 1:50 in FACS buffer. The cell suspensions were incubated on ice and in the dark for 30 min, washed twice with FACS buffer and analyzed using an Epics XL-MCL flow cytometer (Beckman Coulter).

Figure 1 shows the FACS analysis of the polyclonal Ratl-Mock population stably infected with pLXSN empty vector and Ratl-AXL cl.2 stably infected with pLXSN-hAXL, and demonstrates AXL overexpression on the cell surface of this representative clone.

Additionally, in order to generate a suitable cellular model system for experimental purposes, NIH3T3 fibroblasts stably overexpressing AXL were generated in analogy to procedures described for Ratl. In brief $3x10^5$ Phoenix-E cells were seeded on 60 mm culture dishes and transfected with 2 µg/ml pLXSN vector or pLXSN-AXL cDNA using the calcium phosphate method. After 24 h, medium was replaced by fresh medium in which Phoenix-E cells were incubated for 4 h. The supernatants of Phoenix-E cells releasing pLXSN or pLXSN-hAXL ecotrophic virus were harvested and used for the incubation of subconfluent NIH3T3 cells ($2x10^5$ cells per 6 cm dish) for 3 h in the presence of Polybrene (4 mg/ml; Aldrich). Simultaneously, Phoenix-E cells were re-incubated with fresh medium, which after another 3 h was used for a second infection of the NIH3T3 fibroblasts in the presence of Polybrene (4 mg/ml; Aldrich). Likewise, a third infection cycle was performed. After changing the medium, selection of NIH3T3 cells with G418

was started. Usually, stable clones were picked after selection for 21 days.

A panel of stable clones was propagated and quantified for membrane-localized AXL expression by FACS analysis. In detail, $1x10^5$ cells were harvested with 10 mM EDTA in PBS, washed once with FACS buffer (PBS, 3% FCS, 0.4% azide) and seeded on a 96 well round bottom plate. The cells were spun for 3 min at 1000 rpm to remove supernatant and were resuspended with mouse anti-AXL primary antibody MAB154 (R&D Systems, 3 µg/ml). Cell suspensions were incubated on ice for 1 h, washed twice with FACS buffer and resuspended in 100 µl/well of PE-conjugated donkey anti-mouse secondary antibody (Jackson) diluted 1:50 in FACS buffer. The cell suspensions were incubated on ice and in the dark for 30 min, washed twice with FACS buffer and analyzed using an Epics XL-MCL flow cytometer (Beckman Coulter).

Figure 2 shows the FACS analysis of the polyclonal NIH3T3-Mock population stably infected with pLXSN empty vector and NIH3T3-AXL cl.7 stably infected with pLXSN-hAXL, and demonstrates AXL overexpression on the cell surface of this representative clone.

Example 2. Generation of rat anti-AXL monoclonal antibodies

Monoclonal rat anti-AXL antibodies were raised by injection of approximately $10x10^6$ frozen cells of RatI-AXL cl.2 both i.p. and subcutaneously into Lou/C or Long Evans rats. After an 8-week interval, a final boost was given i.p and subcutaneously 3 d before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells was performed according to standard procedures and yielded 105 hybridomas. After 2 weeks, first supernatants from hybridomas were collected and tested in a primary FACS screen for binding to NIH3T3-AXL cl.7 fibroblasts versus NIH3T3-Mock control cells. Clones positive for AXL binding were further cultivated. From 50 ml supernatant of these clones, antibodies were purified and re-analyzed for specific binding to AXL on NIH3T3-AXL cl.7 fibroblasts versus NIH3T3-

Mock control cells. Purified antibodies specifically binding to NIH3T3-AXL cl. 7 fibroblasts but not NIH3T3-Mock control cells were furthermore tested in Akt-Kinase phosphorylation ELISAs, and ELISAs to determine the isotype were performed. For purification of rat antibodies, supernatants were spun for 20 minutes at 5,000g and subsequently sterile filtered. 500 µl of protein G sepharose FF were added and incubated at 4°C for at least 1 h on a spinning wheel. Sepharose was spun down, supernatant discarded and protein G matrix was washed twice with PBS prior to protein elution utilizing citrate buffer (100mM) pH 2.1. Elution fractions were immediately rebuffered to neutral pH by adding 1M Tris pH 8.0 and dialyzed against PBS.

Of the oligoclonal antibodies tested, 91 specifically bound to NIH3T3-AXL cl. 7 fibroblasts but not NIH3T3-Mock control cells, 9 inhibited Gas6-induced Akt phosphorylation in the same cells, whereas 71 stimulated Akt phosphorylation. Four antagonistic antibodies (I11B7, I10D12, I6E7, and III11D5, in the following examples referred to as 11B7, 10D12, 6E7 and 11D5, respectively), two agonistic antibodies (I11D7 and III2A1; in the following examples referred to as 11D7 and 2A1) and one control antibody (III1D5; in the following examples referred to as 1D5) were kryoconserved and subcloned.

Nr.	clone	subclass	FACS shift NIH3T3-pLXSN control	FACS shift NIH3T3-hAXL-Cl8
1	I 1B11	2a	0,8	53,8
2	I 1C8	IgM/2a	0,9	55,0
3	I 2F3	2a	0,8	52,4
4	I 6E7	2a	1,8	62,3
5	I 7E6	2a	0,8	47,1
6	I 7G1	G1	0,7	32,0
7	I 7G11	G1	3,5	8,8
8	I 8E5	G1	1	33,0
9	I 9H3	G1	0,5	40,4
10	I 10A10	IgM/2a	0,5	32,6
11	I 10D9	2a	0,7	47,4
12	I 10D12	G1	0,5	37,5
13	I 11B7	IgM/G1/2c	0,6	36,2

14	I 11D7	IgM/2a	0,7	9,6
15	I 12B7	2a/2c	0,8	43,6
16	II 2B8	IgM/G1	0,6	2,5
17	II 2D4	2a	0,8	46,5
18	II 6A5	G1	0,6	13,1
19	II 8A8	2a	0,6	34,6
20	III 1A10	2a	1,4	54,5
21	III 1B1	2a	7,5	24,6
22	III 1B3	IgM/2a	1,1	53,3
23	III 1B6	2b	1,1	15,3
24	III 1B11	2b	1,1	11,1
25	III 1C3	2b	1,0	24,2
26	III 1C10	-	1,1	22,1
27	III 1D2	IgM/2b	3,0	26,6
28	III 11D5	2a	1,5	8,9
29	III 1D7	2b	1,0	17,3
30	III 1D11	-	1,1	10,9
31	III 1D12	2b	1,0	7,7
32	III 1E7	-	1,1	81,4
33	III 1E11	G1/2a	1,2	44,0
34	III 1F2	G1	1,2	42,2
35	III 1F3	2b	1,1	9,0
36	III 1G2	-	1,0	30,5
37	III 1G9	2a	1,3	89,2
38	III1G11	-	1,1	54,7
39	III 1G12	-	1,1	59,4
40	III 1H4	IgM/2b	1,0	20,0
41	III 1H8	2a/2b	1,0	10,1
42	III 1H9	2b	0,9	13,6
43	III 2A1	2a	1,0	36,0
44	III 2A2	2b	1,0	10,5
45	III 2A4	2b	1,2	11,8
46	III 2B1	2b	0,9	16,0
47	III 2B6	2a/2b	1,0	39,7
48	III 2B8	2a	1,0	53,3
49	III 2B10	2b	1,1	10,6
50	III 2C12	2a/2b	1,0	11,2
51	III 2D1	2a/2b	1,0	42,0
52	III 2D3	2b	0,9	17,8
53	III 2D8	2a	1,4	109,7
54	III 2D12	2b	1,8	16,0
55	III 2E11	2b	1,0	14,8
56	III 2G4	-	1,0	8,5
57	III 2H7	-	1,0	91,2
58	III 3A1	2a	1,5	82,5
59	III 3A2	2b	1,0	7,4
60	III 3A3	IgM/G1	2,0	49,6
61	III 3B2	-	1,0	11,3
62	III 3B3	2b	0,8	12,4
63	III 3B4	IgM	1,2	98,0
64	III 3B5	IgM/2b	1,6	74,0
65	III 3B7	2b	1,8	13,4

66	III 3B10	2a	1,1	70,6
67	III 3C3	-	1,3	45,7
68	III 3C4	-	1,4	15,2
69	III 3C10	2a	15,2	83,3
70	III 3C12	2b	1,2	41,8
71	III 3D2	2b	0,9	11,8
72	III 3D3	2a	1,0	54,5
73	III 3E1	-	1,2	49,7
74	III 3E3	2a/2b	1,3	16,0
75	III 3E5	2a	1,1	56,4
76	III 3F1	2b	1,0	9,8
77	III 3G1	2a	1,2	57,8
78	III 3G3	2a	1,1	45,7
79	III 3G6	2a	1,1	55,9
80	III 3H4	2b	1,0	43,3
81	III 3H5	2b	1,2	11,8
82	III 4A4	IgM	1,3	8,5
83	III 4A5	2a	1,9	32,8
84	III 4A6	2a	2,5	10,4
85	III 4B1	2b	1,9	10,2
86	III 4B5	2b	1,6	6,4
87	III 4B6	2a	1,9	56,8
88	III 4B9	IgM/2b/2c	1,7	16,6
89	III 4B11	2a	1,2	58,1
90	III 4C2	-	1,6	7,4
91	III 4C8	2a	12,8	21,3
92	III 4D1	-	1,6	7,9
93	III 4D9	-	1,1	31,2
94	III 4D10	2a	3,8	7,5
95	III 4E11	2b	1,5	7,6
96	III 4F6	-	1,2	5,5
97	III 4F8	2a	1,2	51,3
98	III 4F11	IgM	1,2	12,9
99	III 4F12	2a	1,1	52,6
100	III 4G2	2a	1,0	52,4
101	III4G11	IgM/2b	1,1	8,9
102	III 4H4	2b	3,1	61,3
103	III 4H5	2a	2,7	20,0
104	III 4H10	IgM/2a	1,3	49,2
105	III 4H11	IgM	3,3	124,0

Example 3. Rat anti-AXL antibodies of the invention do not cross-react with mouse AXL or other members of the human AXL family, Mer and Sky

This example addressed cross-reactivity of rat anti-AXL antibodies of the

invention with mouse and cynomolgus monkey AXL as well as with the other members of the human AXL family, human Mer and human Sky. Following subcloning of the mouse and monkey AXL coding sequence as well as human Mer and Sky into pcDNA3, each expression construct was transfected into HEK293T fibroblasts. The ability of rat anti-AXL antibodies of the invention to bind these proteins was tested by FACS analysis.

3 A. Cloning of mouse AXL

In the present study, the mouse AXL expression construct pcDNA3-mAXL was generated. The full length coding sequence of mouse AXL was polymerase chain reaction (PCR) amplified using mouse heart cDNA (Ambion) as template and appropriate primers according to the National Center for Biotechnology Information (NCBI) reference sequence (NM_009465) of mouse AXL. The full length sequence coding for mouse AXL was thereby covered by two overlapping PCR fragments, a 5'-fragment and 3'-fragment. The primers for amplification of these fragments were as follows:

Forward primer MOUSE1 for the 5'-fragment carrying an *Eco*RI recognition sequence:

5'- GCG AAT TCG CCA CCA TGG GCA GGG TCC CGC TGG CCT G- 3'

Reverse primer MOUSE2 for the 5'-fragment:

5'- CAG CCG AGG TAT AGG CTG TCA CAG ACA CAG TCA G- 3'

Forward primer MOUSE3 for the 3'-fragment:

5'- GCA CCC TGT TAG GGT ACC GGC TGG CAT ATC- 3'

Reverse primer MOUSE4 for the 3'-fragment carrying a *Not*I recognition sequence:

5'- ATA AGA ATG CGG CCG CTC AGG CTC CGT CCT CCT GCC CTG- 3'

The 5'-fragment was digested with *Eco*RI and *Bst*EII, the 3'-fragment was digested with *Bst*EII and *Not*I, and pcDNA3 was cleaved with *Eco*RI and *Not*I. A three factor ligation of the isolated and purified fragments was performed and transformed into DH5α bacterial cells. A single colony was picked and grown in the presence of ampicillin. Using a commercially available plasmid purification kit (Qiagen), the mouse AXL expression vector

pcDNA3-mAXL was purified, and sequence verified for subsequent transient transfection into HEK293T cells.

3 B. Cloning of cynomolgus monkey **AXL**

In the present study, the cynomolgus monkey AXL expression constructs pcDNA3-cyAXL was generated. The full length coding sequence of cynomolgus monkey AXL was PCR amplified using cDNA prepared from cynomolgus monkey brain tissue as template. Since the nucleotide sequence of cynomolgus monkey *AXL* was not available, respective primers were designed assuming significant homology to human *AXL*. The full length sequence coding for cynomolgus monkey AXL was thereby covered by two overlapping PCR fragments, a 5'-fragment and 3'-fragment. The primers for amplification of these fragments were as follows:

Forward primer CYNO1 for the 5'-fragment carrying an *Eco*RI recognition sequence:

5'- CGG AAT TCG CCA CCA TGG CGT GGC GGT GCC CCA G- 3'

Reverse primer CYNO2 for the 5'-fragment:

5'- CTC TGA CCT CGT GCA GAT GGC AAT CTT CAT C- 3'

Forward primer CYNO3 for the 3'-fragment:

5'- GTG GCC GCT GCC TGT GTC CTC ATC- 3'

Reverse primer CYNO4 for the 3'-fragment carrying a *Not*I recognition sequence:

5'- ATA AGA ATG C GG CCG CTC AGG CAC CAT CCT CCT GCC CTG - 3'

The 5'-fragment was digested with *Eco*RI and *Dra*III, the 3'-fragment was digested with *Dra*III and *Not*I, and pcDNA3 was cleaved with *Eco*RI and *Not*I. A three factor ligation of the isolated and purified fragments was performed and transformed into DH5α bacterial cells. A single colony was picked and grown in the presence of ampicillin. Using a commercially available plasmid purification kit (Qiagen), the cynomolgus monkey AXL expression vector pcDNA3-cyAXL was purified, and sequence verified for subsequent transient transfection into HEK293T cells. The nucleotide and amino acid sequences of cynomolgus monkey are as follows:

Nucleotide sequence:

ATGGCGTGGCGGTGCCCCAGGATGGGCAGGGTCCCGCTGGCCTGGTG
CTTGGCGCTGTGCGGCTGGGTGTGCATGGCCCCCAGGGGCACACAGG
CTGAAGAAAGTCCTTTCGTGGGTAACCCAGGGAATATCACAGGTGCCC
GGGGACTCACGGGCACCCTTCGGTGTCAGCTCCAGGTTCAGGGAGAG
CCCCCCGAGGTACACTGGCTTCGGGACGGACAGATCCTGGAGCTCGC
GGACAGTACCCAGACCCAGGTGCCCCTGGGTGAAGATGAGCAGGATGA
CTGGATAGTGGTCAGCCAGCTCAGAATCGCCTCCCTACAGCTTTCCGAC
GCGGGACAGTACCAGTGTTTGGTGTTTCTGGGACATCAGAACTTCGTGT
CCCAGCCTGGCTACGTAGGGCTGGAGGGCTTACCTTACTTCCTGGAGG
AGCCTGAGGACAGGACTGTGGCCGCCAACACCCCCTTCAACCTGAGCT
GCCAAGCCCAGGGACCCCCAGAGCCCGTGGACCTACTCTGGCTCCAG
GATGCTGTCCCCCTGGCCACAGCTCCAGGTCATGGTCCCCAGCGCAAC
CTGCATGTTCCAGGGCTGAACAAGACATCCTCTTTCTCCTGCGAAGCCC
ATAACGCCAAGGGAGTCACCACATCCCGCACGGCCACCATCACAGTGC
TCCCCCAGCAGCCCCGTAACCTCCATCTGGTCTCCCGCCAACCCACGG
AGCTGGAGGTGGCTTGGACTCCAGGCCTGAGCGGCATCTACCCCCTGA
CCCACTGCACCCTGCAGGCTGTGCTGTCAGACGATGGGATGGGCATCC
AGGCGGGAGAACCAGACCCCCCAGAGGAGCCCCTCACCTTGCAAGCAT
CTGTGCCCCCCCACCAGCTTCGGCTGGGCAGCCTCCATCCTCACACCC
CTTATCACATCCGTGTGGCATGCACCAGCAGCCAGGGCCCCTCATCCT
GGACACACTGGCTTCCTGTGGAGACGCCGGAGGGAGTGCCCCTGGGC
CCCCCTGAGAACATTAGTGCCACGCGGAATGGGAGCCAGGCCTTCGTG
CATTGGCAGGAGCCCCGGGCGCCCCTGCAGGGTACCCTGTTAGGGTA
CCGGCTGGCGTATCAAGGCCAGGACACCCCAGAGGTGCTAATGGACAT
AGGGCTAAGGCAAGAGGTGACCCTGGAGCTGCAGGGGGACGGGTCTG
TGTCCAATCTGACAGTGTGTGTGGCAGCCTACACTGCTGCTGGGGATG
GACCCTGGAGCCTCCCAGTACCCCTGGAGGCCTGGCGCCCAGGGCAA
GCACAGCCAGTCCACCAGCTGGTGAAGGAAACTTCAGCTCCTGCCTTC
TCGTGGCCCTGGTGGTATATACTGCTAGGAGCAGTCGTGGCCGCTGCC
TGTGTCCTCATCTTGGCTCTCTTCCTTGTCCACCGGCGAAAGAAGGAGA
CCCGTTATGGAGAAGTGTTCGAGCCAACAGTGGAAAGAGGTGAACTGG
TAGTCAGGTACCGCGTGCGCAAGTCCTACAGTCGCCGGACCACTGAAG

CTACCTTGAACAGCCTGGGCATCAGTGAAGAGCTGAAGGAGAAGCTGC
GGGATGTGATGGTGGACCGGCACAAGGTGGCCCTGGGGAAGACTCTG
GGAGAAGGAGAGTTTGGAGCCGTGATGGAAGGCCAGCTCAACCAGGA
CGACTCCATCCTCAAGGTGGCTGTGAAGACAATGAAGATTGCCATCTGC
ACAAGGTCAGAGCTGGAGGATTTCCTGAGTGAAGCAGTCTGCATGAAG
GAATTCGACCATCCCAATGTCATGAGGCTCATCGGTGTCTGTTTCCAGG
GTTCTGAACGAGAGAGCTTTCCAGCACCTGTGGTCATCTTACCTTTCAT
GAAGCATGGAGACCTACACAGCTTCCTCCTCTATTCCCGGCTTGGGGA
CCAGCCAGTGTACCTGCCCACTCAGATGCTAGTGAAGTTCATGGCGGA
CATCGCCAGTGGCATGGAATATCTGAGTACCAAGAGATTCATACACCGG
GACCTGGCGGCCAGGAACTGCATGCTGAATGAGAACATGTCCGTGTGT
GTGGCGGACTTCGGGCTCTCCAAGAAGATCTACAACGGGGACTACTAC
CGCCAGGGACGTATCGCCAAGATGCCAGTCAAGTGGATTGCCATTGAG
AGTCTAGCTGACCGTGTCTACACGAGCAAGAGTGATGTGTGGTCCTTC
GGGGTGACAATGTGGGAGATTGCCACAAGAGGCCAAACCCCATATCCA
GGCGTGGAGAACAGCGAGATTTATGACTATCTGCGCCAGGGAAATCGC
CTGAAGCAGCCTGCGGACTGTCTGGATGGACTGTATGCCTTGATGTCG
CGGTGCTGGGAGCTAAATCCCCAGGACCGGCCAAGTTTTACAGAGCTG
CGGGAAGATTTGGAGAACACACTGAAGGCCTTGCCTCCTGCCCAGGAG
CCTGACGAAATCCTCTATGTCAACATGGATGAAGGTGGAGGTTATCCTG
AACCTCCCGGCGCTGCTGGAGGAGCTGACCCCCCAACCCAGCTAGACC
CTAAGGATTCCTGTAGCTGCCTCACTTCGGCTGAGGTCCATCCTGCTGG
ACGCTATGTCCTCTGCCCTTCCACAGCCCCTAGCCCCGCTCAGCCTGC
TGATAGGGGCTCCCCAGCAGCCCCAGGGCAGGAGGATGGTGCC

Amino acid sequence:

MAWRCPRMGRVPLAWCLALCGWVCMAPRGTQAEESPFVGNPGNITGAR
GLTGTLRCQLQVQGEPPEVHWLRDGQILELADSTQTQVPLGEDEQDDWIV
VSQLRIASLQLSDAGQYQCLVFLGHQNFVSQPGYVGLEGLPYFLEEPEDRT
VAANTPFNLSCQAQGPPEPVDLLWLQDAVPLATAPGHGPQRNLHVPGLNK
TSSFSCEAHNAKGVTTSRTATITVLPQQPRNLHLVSRQPTELEVAWTPGLS
GIYPLTHCTLQAVLSDDGMGIQAGEPDPPEEPLTLQASVPPHQLRLGSLHP
HTPYHIRVACTSSQGPSSWTHWLPVETPEGVPLGPPENISATRNGSQAFV
HWQEPRAPLQGTLLGYRLAYQGQDTPEVLMDIGLRQEVTLELQGDGSVSN

LTVCVAAYTAAGDGPWSLPVPLEAWRPGQAQPVHQLVKETSAPAFSWPW
WYILLGAVVAAACVLILALFLVHRRKKETRYGEVFEPTVERGELVVRYRVRK
SYSRRTTEATLNSLGISEELKEKLRDVMVDRHKVALGKTLGEGEFGAVMEG
QLNQDDSILKVAVKTMKIAICTRSELEDFLSEAVCMKEFDHPNVMRLIGVCF
QGSERESFPAPVVILPFMKHGDLHSFLLYSRLGDQPVYLPTQMLVKFMADI
ASGMEYLSTKRFIHRDLAARNCMLNENMSVCVADFGLSKKIYNGDYYRQG
RIAKMPVKWIAIESLADRVYTSKSDVWSFGVTMWEIATRGQTPYPGVENSEI
YDYLRQGNRLKQPADCLDGLYALMSRCWELNPQDRPSFTELREDLENTLK
ALPPAQEPDEILYVNMDEGGGYPEPPGAAGGADPPTQLDPKDSCSCLTSA
EVHPAGRYVLCPSTAPSPAQPADRGSPAAPGQEDGA

3 C. Cloning of human Mer

In the present study, the human Mer expression construct pcDNA3-hMer was generated. The full length coding sequence of human Mer was obtained through cleavage of the vector pCMV6-XL4-human Mer (Origene #TC116132) with *Eco*RI and and *Xba*I. After digestion of pcDNA3 with the same restriction endonucleases, both fragments were ligated to generate pcDNA3-hMer. In order to introduce a Kozak consensus sequence, the 5'-region of the human Mer coding sequence in pcDNA3-hMer was PCR amplified using appropriate primers according to the NCBI reference sequence (NM_006343) of human Mer. The primers for amplification of this fragment were as follows:

Forward primer MER1 carrying an *Eco*RI recognition sequence and the Kozak consensus sequence:

5'-CGG AAT TCG CCA CCA TGG GGC CGG CCC CGC TGC CGC-3'

Reverse primer MER2 for the 5'-fragment:

5'-TCG GCT GCC ATT CTG GCC AAC TTC C-3'

The PCR product and pcDNA3-hMer were digested with *Eco*RI and *Eco*RV and ligated to generate pcDNA3-Kozak-hMer, in which the full length human Mer coding sequence is preceded by a Kozak consensus sequence. Transformed into DH5α bacterial cells, a single colony was picked and grown in the presence of ampicillin. Using a commercially available plasmid purification kit (Qiagen), the pcDNA3-Kozak-hMer expression vector was

purified, and sequence verified for subsequent transient transfection into HEK293T cells.

3 D. Cloning of human Sky

In the present study, the human Sky expression construct pcDNA3-hSky was generated. The full length coding sequence of human Sky was PCR amplified using the vector pCMV6-XL4-human Sky (Origene #MG1044_A02) as template and appropriate primers according to the NCBI reference sequence (NM_006293) of human Sky. The primers for amplification were as follows:

Forward primer SKY1 carrying an *Eco*RI recognition sequence:

5'-CGG AAT TCG CCA CCA TGG CGC TGA GGC GGA GC-3'

Reverse primer SKY2 carrying a *Xho*I recognition sequence:

5'-GCC CTC GAG CTA ACA GCT ACT GTG TGG CAG TAG-3'

The PCR product and pcDNA3 were digested with *Eco*RI and *Xho*I and ligated to generate the pcDNA3-hSky expression vector. Transformed into DH5α bacterial cells, a single colony was picked and grown in the presence of ampicillin. Using a commercially available plasmid purification kit (Qiagen), the pcDNA3-hSky expression vector was purified, and sequence verified for subsequent transient transfection into HEK293T cells.

3 E. Transfection and expression of mouse AXL, cynomolgus monkey AXL, human Mer, and human Sky

For transient expression of mouse AXL, cynomolgus monkey AXL, human Mer or human Sky, HEK293T cells were transiently transfected with either pcDNA3 empty vector, pcDNA3-hAXL, pcDNA3mAXL, pcDNA3-cyAXL, pcDNA3-hMer, or pcDNA3-hSky applying the calcium phosphate method. In brief, prior to transfection, $3x10^6$ HEK293T cells in 16 ml medium were seeded on a 15 cm cell tissue culture dish and grown at 7% CO_2 and 37°C for 30 h. 32 µg DNA of the respective expression construct or empty vector in 720 µl of ddH_2O were mixed with 2.5 M $CaCl_2$ and 2xBBS (pH 6.96) and kept at room temperature for 10 min. The solutions were gently added to the

cell cultures and incubated at 3% CO_2 and 37°C for 8 h. The medium then was replaced with fresh growth medium and cells were cultured at 7% CO_2 and 37°C for 24 h.

3 F. FACS analysis to test rat anti-AXL antibodies for cross-reactivity

For FACS analysis, $2x10^5$ cells were harvested with 10 mM EDTA in PBS, washed once with FACS buffer (PBS, 3% FCS, 0.4% azide) and seeded on a 96 well round bottom plate. To remove the supernatant, plates were spun for 3 min at 1000 rpm and cells were resuspended in 10 µg/ml isotypic control antibody 1D5 as well as anti-AXL 11D5, 11B7, 10D12, 6E7, 2A1, 11D7 and 12B7 primary antibody solutions (100 µl/well). After incubation on ice for 1 h, cells were washed twice with chilled FACS buffer and resuspended with PE-conjugated donkey anti-rat (Jackson) secondary antibody diluted 1:50 in FACS buffer (100 µl/well) or PE-conjugated donkey anti-mouse secondary antibody for control. Protected from light, cells were incubated on ice for 30 min, washed twice with FACS buffer and analyzed using an Epics XL-MCL flow cytometer (Beckman Coulter).

Figure 3 shows representative results of this experiment. With exception of 12B7 which shows moderate cross-reactivity with mouse AXL as well as human Mer and Sky, non of the other anti-AXL antibodies of the invention cross-reacted with these molecules. In contrast, all of the tested rat anti-AXL antibodies of the invention cross-reacted with cynomolgus monkey AXL.

Example 4. Rat anti-AXL antibodies of the invention inhibit ligand-induced AXL phosphorylation *in vitro*

ELISA experiments were performed in order to investigate whether the rat anti-AXL antibodies of the invention are able to block ligand Gas6-mediated activation of AXL. Gas6-mediated AXL activation was detected by increased receptor tyrosine phosphorylation. In brief, on day 1, $3x10^4$ cells per well were seeded in normal growth medium in flat-bottom 96 well plates. The next day, growth medium was replaced by serum-free medium to starve cells

over night for 24 h. Also over night, black Maxi-Sorp 96 well plates (Nunc) were coated with mouse anti-phospho-tyrosine antibody 4G10 at 2 µg/ml PBS and 4°C. On day 3, the 4G10 antibody solution was removed and Maxi-Sorp wells were blocked with PBS, 0.5% BSA for at lest 4 h at room temperature. In parallel, cells were pre-incubated with 10 µg/ml of the mouse control antibody 72A1 as well as the rat anti-AXL antibodies 2A1, 11D7, 11D5, 11B7, 6E7, and 10D12 for 1 h at 37°C and then treated with or without 400 ng/ml Gas6 (R&D Systems) for 10 min at 37°C. Medium was then flicked out and cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerine, and 1% Triton X-100) supplemented with phosphatase and protease inhibitors (10 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 1 mM NaF, and 0,5% aprotinin) for 30 min on ice. Meanwhile, blocking buffer was removed and Maxi-Sorp plates were washed 6x with wash buffer (PBS, 0.05% Tween 20), before lysates were transferred and incubated over night at 4°C. After plates were washed 6x with wash buffer on day 4, wells were incubated with biotinylated rat anti-AXL antibody 12B7 at 0.5 µg/ml PBS for 2 h at room temperature. Plates were washed 6x with wash buffer and AP-conjugated streptavidin (Chemicon #SA110) diluted 1:4,000 in PBS was added to each well and incubated for 30 min at room temperature. Afterwards, wells were washed 6x with wash buffer and AttoPhos substrate solution (Roche #11681982) was added. Using a Victor plate reader (Perkin Elmer), the fluorescence of each well was collected at an excitation wavelength of 430 nm and an emission wavelength of 580nm.

Figure 4 shows representative results of this experiment for NIH3T3-AXL cl.7 fibroblasts (A) and NCI-H292 lung cancer cells (B). The rat anti-AXL antibodies 11B7, 11D5, 6E7, and 10D12 of the invention were able to block or reduce ligand-mediated AXL activation as indicated by decreased phosphorylation, and are thus considered antagonistic anti-AXL antibodies. In contrast, the rat anti-AXL antibodies 2A1 and 11D7 of the invention stimulate basal AXL activation as indicated by increased phosphorylation, do not significantly reduce ligand-mediated AXL activation, and are therefore

considered agonistic anti-AXL antibodies. Similar effects with the same panel of antibodies were observed in the lung cancer cell lines CaLu-1 and CaLu-6, the breast cancer cell lines Hs578T and MDA-MB-231, the prostate cancer cell line PC-3, the pancreas cancer cell line PANC-1, the melanoma cell line C-8161, the ovarian cancer cell lines SkOV-3 and SkOV-8, the glioblastoma cell line SF-126, the cervical cancer cell line CaSki, as well as the gastric cancer cell lines Hs746T and MKN-1.

Example 5. Rat anti-AXL antibodies of the invention inhibit ligand-induced p42/p44 MAP-Kinase phosphorylation *in vitro*

Next, ELISA experiments were performed in order to investigate whether the rat anti-AXL antibodies of the invention are able to block ligand Gas6-mediated activation of p42/p44 MAP-Kinase. Gas6-mediated p42/p44 MAP-Kinase activation was detected by increased protein (Thr202/Tyr204) phosphorylation. In brief, on the first day, $2x10^4$ cells per well were seeded in flat-bottom 96 well plates. The next day, normal growth medium was replaced by serum-free medium to starve cells for 36 h. Thereafter, cells were pre-incubated with 10 µg/ml of the isotypic control antibody 1D5 as well as the rat anti-AXL antibodies 11D5, 11B7, and 2A1 for 1 hr at 37°C and then treated with or without 400 ng/ml Gas6 (R&D Systems) for 10 min at 37°C . Medium was flicked out and cells were fixed with 4% formaldehyde in PBS (pH 7.5) for 30 min at room temperature. Formaldehyde solution was removed and cells were washed twice with wash buffer (PBS, 0.1% Tween 20). Cells were quenched with 1% H_2O_2, 0.1% NaN_3 in wash buffer and incubated for 20 min at room temperature. Afterwards, the quenching solution was removed, and cells were washed twice with wash buffer and blocked with PBS, 0.5% BSA for 4 h at 4°C. Anti-phospho-p42/p44 MAP Kinase (Thr202/Tyr204) primary antibody (polyclonal rabbit; Cell Signaling #9101) diluted 1:500 in PBS, 0.5% BSA, 5 mM EDTA was added over night at 4°C. On day 4, the antibody solution was removed and the plate was washed 3x with wash buffer. HRP-conjugated anti-rabbit secondary antibody (Dianova #111-036-045) diluted 1:2,500 in PBS, 0.5% BSA was then added

to each well and incubated for 1.5 h at room temperature. The plate was washed 3x with wash buffer and twice with PBS for 5 min each. Tetramethylbenzidine (TMB, Calbiochem) was added and monitored at 620 nm. The reaction was stopped by addition of 100 µl of 250 nM HCl and the absorbance was read at 450 nm with a reference wavelength of 620 nm using a Vmax plate reader (Thermo Lab Systems).

Figure 5 shows representative results of this experiment for the cervical cancer cell line CaSki. The rat anti-AXL antibodies 11B7 and 11D5 of the invention were able to reduce ligand-mediated p42/p44 MAP-Kinase activation as indicated by decreased phosphorylation, and are thus considered antagonistic anti-AXL antibodies. In contrast, the rat anti-AXL antibody 2A1 of the invention stimulates basal p42/p44 MAP-Kinase activation as indicated by increased phosphorylation, does not reduce ligand-mediated p42/p44 MAP-Kinase activation, and is therefore considered an agonistic anti-AXL antibody. Similar effects with the same panel of antibodies were observed in the breast cancer cell line Hs578T and the lung cancer cell line NCI-H292.

Example 6. Rat anti-AXL antibodies of the invention inhibit ligand-induced Akt phosphorylation *in vitro*

Furthermore, ELISA experiments were performed in order to investigate whether the rat anti-AXL antibodies of the invention are able to block ligand Gas6-mediated activation of Akt-Kinase. Gas6-mediated Akt-Kinase activation was detected by increased protein (Ser473) phosphorylation. In brief, on day 1. $2x10^4$ cells per well were seeded in flat-bottom 96 well plates. The next day, normal growth medium was replaced by serum-reduced (DMEM, 0.5% FCS for NIH3T3-AXL cl.7 fibroblasts) or serum-free (for cancer cell lines) medium to starve cells for 36 h. Thereafter, cells were pre-incubated with 10 µg/ml of the isotypic control antibody 1D5 as well as the rat anti-AXL antibodies 11D5, 11B7, and 2A1 for 1 h at 37°C and then treated with or withour 400 ng/ml Gas6 (R&D Systems) for 10 min at 37°C.

Medium was flicked out and cells were fixed with 4% formaldehyde in PBS (pH 7.5) for 30 min at room temperature. Formaldehyde solution was removed and cells were washed twice with wash buffer (PBS, 0.1% Tween 20). Cells were quenched with 1% H_2O_2, 0.1% NaN_3 in wash buffer and incubated for 20 min at room temperature. Afterwards, the quenching solution was removed, and cells were washed twice with wash buffer and blocked with PBS, 0.5% BSA for 4 h at 4°C. Anti-phospho-Akt (Ser473) primary antibody (polyclonal rabbit; Cell Signaling #9271) diluted 1:500 in PBS, 0.5% BSA, 5 mM EDTA was added over night at 4°C. On day 4, the antibody solution was removed and the plate was washed 3x with wash buffer. HRP-conjugated anti-rabbit secondary antibody (Dianova #111-036-045) diluted 1:2,500 in PBS, 0.5% BSA was then added to each well and incubated for 1.5 h at room temperature. The plate was washed 3x with wash buffer and twice with PBS for 5 min each. Tetramethylbenzidine (TMB, Calbiochem) was added and monitored at 620 nm. The reaction was stopped by addition of 100 µl of 250 nM HCl and the absorbance was read at 450 nm with a reference wavelength of 620 nm using a Vmax plate reader (Thermo Lab Systems).

Figure 6 shows representative results of this experiment for NIH3T3-AXL cl.7 fibroblasts (A) and CaLa-1 lung cancer cells (B). The rat anti-AXL antibodies 11B7 and 11D5 of the invention were able to block or reduce ligand-mediated Akt-Kinase activation as indicated by decreased phosphorylation, and are thus considered antagonistic anti-AXL antibodies. In contrast, the rat anti-AXL antibody 2A1 of the invention stimulates basal Akt-Kinase activation as indicated by increased phosphorylation, does not reduce ligand-mediated Akt-Kinase activation, and is therefore considered an agonistic anti-AXL antibody. Similar effects with the same panel of antibodies were observed in the lung cancer cell line NCI-H292, the breast cancer cell lines Hs578T and MDA-MB-231, the prostate cancer cell line PC-3, the pancreas cancer cell line PANC-1, the melanoma cell line C-8161, the ovarian cancer cell lines SkOV-3 and SkOV-8, the bladder cancer cell line TCC-Sup, as well as the fibrosarcoma cell line HT1080.

Example 7. Rat and chimeric anti-AXL antibodies of the invention inhibit ligand-induced Akt phosphorylation *in vitro* to similar extent

Chimeric derivatives of the rat anti-AXL antibodies 11B7 and 11D5 were generated as part of this invention (see below). In order to investigate whether the rat anti-AXL antibodies of the invention and the corresponding chimeric anti-AXL antibodies of the invention were able to block ligand Gas6-mediated activation of the Akt-Kinase in NIH3T3-AXL cl.7 fibroblasts to similar extent, ELISA experiments were performed. Antibody-mediated Akt-Kinase inhibition was detected by decreased protein (Ser473) phosphorylation. In brief, on day 1. $2x10^4$ cells per well were seeded in flat-bottom 96 well plates. The next day, normal growth medium was replaced by serum-reduced medium (DMEM, 0.5% FCS) to starve cells for 36 h. Thereafter, cells were pre-incubated with 50 ng/ml, 100 ng/ml, 300 ng/ml, 500 ng/ml, and 1 µg/ml of rat anti-AXL antibody 11B7 or chimeric anti-AXL antibody ch11B7, as well as 50 ng/ml, 100 ng/ml, 300 ng/ml, 500 ng/ml, 1 µg/ml, 5 µg/ml, and 10 µg/ml of rat anti-AXL antibody 11D5 or chimeric anti-AXL antibody ch11D5 for 1 h at 37°C and then treated with or without 400 ng/ml Gas6 (R&D Systems) for 10 min at 37°C. Medium was flicked out and cells were fixed with 4% formaldehyde in PBS (pH 7.5) for 30 min at room temperature. Formaldehyde solution was removed and cells were washed twice with wash buffer (PBS, 0.1% Tween 20). Cells were quenched with 1% H_2O_2, 0.1% NaN_3 in wash buffer and incubated for 20 min at room temperature. Afterwards, the quenching solution was removed, and cells were washed twice with wash buffer and blocked with PBS, 0.5% BSA for 4 h at 4°C. Anti-phospho-Akt (Ser473) primary antibody (polyclonal rabbit; Cell Signaling #9271) diluted 1:500 in PBS, 0.5% BSA, 5 mM EDTA was added over night at 4°C. On day 4, the antibody solution was removed and the plate was washed 3x with wash buffer. HRP-conjugated anti-rabbit secondary antibody (Dianova #111-036-045) diluted 1:2,500 in PBS, 0.5% BSA was then added to each well and incubated for 1.5 h at room temperature. The plate was washed 3x with wash buffer and twice with PBS for 5 min each. Tetramethylbenzidine (TMB, Calbiochem) was added and

monitored at 620 nm. The reaction was stopped by addition of 100 µl of 250 nM HCl and the absorbance was read at 450 nm with a reference wavelength of 620 nm using a Vmax plate reader (Thermo Lab Systems).

Figure 7 demonstrated that rat anti-AXL antibody 11B7 and chimeric anti-AXL antibody ch11B7 of the invention as well as rat anti-AXL antibody 11D5 and chimeric anti-AXL antibody ch11D5 of the invention were able to inhibit ligand-mediated Akt-Kinase activation to similar extent as indicated by decreased phosphorylation. Thus, as compared to their respective rat counterparts, the chimeric anti-AXL antibodies ch11B7 and ch11D5 maintained activity.

Example 8. Antagonistic rat-anti AXL antibodies of the invention compete with each other for the same or structurally related epitopes and do not share binding sites with agonistic rat-anti AXL antibodies of the invention

Anti-AXL antibodies of the invention were examined whether they compete with each other for similar binding epitopes on the AXL-ECD domain. Therefore binding of biotinylated anti-AXL antibodies to AXL-ECD domain-coated plates preincubated with anti-AXL antibodies was determined in a competition ELISA. In brief, 30 µg of isotypic control antibody 1D5 as well as rat anti-AXL antibodies 11B7, 11D5, 6E7, 10D12, 11D7, and 2A1 were biotinylated with Sulfo-NHS-Biotin (Pierce #21217) according to the manufacturers´ instructions and purified utilizing Micro-BioSpin P6 columns SSC (BIO-RAD #732-6200). On day 1, black 96 well Maxi-Sorp plates (Nunc) were coated with 100 µl/well of 1 µg/ml human AXL-ECD (R&D Systems #154-AL) in PBS over night at 4°C. On day 2, coated Maxi-Sorp plates were blocked with blocking buffer (PBS, 1% BSA, 0.05% TWEEN-20) for 2 h at room temperature (250 µl/well), and subsequently incubated with PBS or unbiotinylated isotypic control antibody 1D5 as well as unbiotinylated rat anti-AXL antibodies 11B7, 11D5, 6E7, 10D12, 11D7, or 2A1 at 10 µg/ml in blocking buffer (100 µl/well) for 1 h at room temperature. Antibody

solutions were flicked out without washing and 100 µl/well PBS or biotinylated isotypic control antibody 1D5 as well as biotinylated rat anti-AXL antibodies 11B7, 11D5, 6E7, 10D12, 11D7, or 2A1 at 0.5 µg/ml in blocking buffer were added and incubated for 15 min at room temperature. After washing 6x with wash buffer (PBS, 0,1% TWEEN-20), 80 µl/well AP-conjugated Streptavidin (Chemicon #SA110) diluted 1:4,000 in blocking buffer were added, incubated for 20 min at room temperature washed again 6x with wash buffer and finally washed once with PBS. For detection, 100 µl/well Attophos substrate solution (Roche # 11681982) were added. Using a Victor plate reader (Perkin Elmer), the fluorescence of each well was collected at an excitation wavelength of 430 nm an emission wavelength of 580 nm.

Figure 8 shows representative results of this analysis. The antagonistic anti-AXL antibodies 11B7, 11D5, 6E7, and 10D12 of the invention compete with each other for the same or structurally adjacent epitopes. The two agonistic antibodies 11D7 and 2A1 of the invention recognize individually different epitopes and therefore are not mutually exclusive. Moreover, 11D7 and 2A1 do not compete with the antagonistic antibodies for binding to the AXL-ECD. The control antibody 1D5 did not bind to AXL-ECD.

Example 9. Rat and chimeric anti-AXL antibodies of the invention inhibit lung cancer cell migration and proliferation *in vitro*

To examine the migration and proliferation rates of different cells and culture conditions, *in vitro* wound healing/scratch assays are being employed for many years. These assays generally involve growing a confluent cell monolayer first. A small area is then disrupted and a group of cells are being destroyed or displaced by scratching a line through the layer with, for example, a pipette tip. The open gap is then inspected microscopically over time as the cells move in and fill the damaged area ("healing"). In brief, $1.5x10^6$ NCI-H292 lung cancer cells were seeded per well of a 12 well dish and cultured in normal growth medium (RPMI, 10% FCS). After 8 h, cells

were rinsed with PBS and starved in low serum medium (RPMI, 0.5% FCS) over night for 24 h. Using a sterile 200 µl pipette tip, three separate uniform wounds per well were scratched through the confluent NCI-H292 cell monolayers. Cells were gently rinsed with PBS and incubated with low serum medium (RPMI, 0.5% FCS) containing no additive, 10 µg/ml of the isotypic control antibody 1D5, the antagonistic rat anti-AXL antibodies 11D5, 11B7, 6E7, or 10D12, the chimeric anti-AXL antibodies chn11D5 IgG2 and chn11B7 IgG2, the agonistic rat anti-AXL antibodies 2A1 and 11D7 as well as 10 µg/ml of Erbitux or 5µM Sutent for comparison. Cells were permitted to migrate into the area of clearing for 24 h, washed once with PBS and fixed with ice cold Methanol (100%) at -20°C. After cells were stained with crystal violet (0.5% in 20% Methanol), rinsed with water and dried over night, photos of the wounds were taken.

Figure 9 shows representative results of this experiment for NCI-H292 lung caner cells. Compared to the isotypic control antibody, the antagonistic rat anti-AXL antibodies 11D5, 11B7, 6E7, and 10D12 of the invention, as well as the chimeric anti-AXL antibodies chn11D5 IgG2 and chn11B7 IgG2 of the invention reduced the re-population of the cleared area, whereas the agonistic rat anti-AXL antibodies 2A1 and 11D7 of the invention led to a complete closure of the wound. Similar results with the same panel of antibodies were observed with the ovarian cancer cell line SkOv-3 or the gastric cancer cell line MKN-1.

Example 10. Rat anti-AXL antibodies of the invention inhibit ligand-induced migration of NIH3T3-AXL cl.7 fibroblasts *in vitro*

Transmigration experiments were performed in order to investigate whether the antibodies of the invention block cell migration. For this purpose, in the morning of day 1, NIH3T3-AXL cl.7 cells were seeded on 15 cm dished in normal growth medium, which in the evening was replaced by serum-reduced medium (DMEM, 0.5% FCS) in order to starve cells for 36 h. The next day, a FluoroBlock 96 well plate (Becton Dickinson #351164, 8 µm pore

size) was coated with 10 µg collagen I/ml 0.1 M acetic acid over night at 37°C. On day 3, the serum-reduced medium (DMEM, 0.5% FCS) was replaced by serum-free medium (DMEM, 0% FCS, 0.1% BSA) for another 4 h. Cells were harvested with 10 mM EDTA in PBS and pre-incubated with rat anti-AXL antibodies 4A6, 11B7 or 2A1 at a cell density of $4x10^5$ cells/ml and an antibody concentration of 10 µg/ml for 45 min. 50 µl cell suspension (20,000 cells) per well were then placed in the top chamber of the FluoroBlock 96-well plate, 225 µl medium (DMEM, 0%FCS, 0.1%BSA) with or without 400 ng/ml mouse Gas6 (R&D Systems) were used per well in the bottom chamber. Cells were left to migrate for 7 h at 37°C and stained afterwards with 4.2 µM calcein-AM (Molecular Probes #C3099) in PBS, 1mM $CaCl_2$, 1 mM $MgCl_2$ for 1 h at 37°C. Using a Victor plate reader (Perkin Elmer), the fluorescence of each well was measured at a wavelength of 530 nm.

Figure 10 shows that the antagonistic anti-AXL antibody 11B7 of the invention reduced both basal and Gas6-induced migration of NIH3T3-AXL cl. 7 fibroblasts, whereas the agonistic rat anti-AXL antibody 2A1 of the invention increased ligand-induced and, in particular, basal migration of NIH3T3-AXL cl.7 cells. The antibody 4A6 did not affect cell migration.

Example 11. Rat anti-AXL antibodies of the invention inhibit ligand-induced proliferation of NIH3T3-AXL cl.7 fibroblasts *in vitro*

In vitro experiments were conducted in order to determine the ability of the rat anti-AXL antibodies of the invention to inhibit Gas6-induced cell proliferation. For this purpose, 2,500 NIH3T3-AXL cl.7 fibroblasts per well were seeded in FCS-containing medium on 96 well plates over night. The next day, cells were starved in serum-reduced medium (DMEM, 0.5% FCS) for 10 h and subsequently pre-incubated with 20 µg/ml of the mouse control antibody 72A1, the antagonistic rat anti-AXL antibodies 11D5 and 11B7, as well as the agonistic antibody 2A1 in DMEM, 0.5% FCS for 1 h at 37°C. Cells were treated with or without 400 ng/ml mouse Gas6 (R&D Systems) by adding ligand directly to the antibody solution, and were then left to grow for

96 h. AlamarBlue™ (BIOSOURCE #DAL1100) was added and incubated at 37°C in the dark. Absorbance was measured at 590 nm every 30 min. The data were taken 4 h after addition of AlamarBlue™.

Figure 11 shows representative results of this experiment. The antagonistic anti-AXL antibodies 11D5 amd 11B7 of the invention blocked Gas6-induced proliferation of NIH3T3-AXL cl.7 fibroblasts, whereas the agonistic rat anti-AXL antibody 2A1 of the invention increased ligand-induced and, in particular, basal proliferation of NIH3T3-AXL cl.7 cells.

Example 12. Rat anti-AXL antibodies of the invention inhibit ligand-mediated anti-apoptosis of serum-starved NIH3T3-AXL cl.7 fibroblasts *in vitro*

Induction of apoptosis and activation of caspases can result from a variety of stimuli including growth factor withdrawal, exposure to chemotherapeutic agents or radiation, or initiation of the Fas/Apo-1 receptor-mediated cell death process. Gas6-AXL interaction has been shown to be implicated in the protection of a range of cell types from apoptosis, including serum-starved NIH3T3 fibroblasts (Goruppi et al., 1996, Oncogene 12, 471-480) or pulmonary endothelial cells (Healy et al., 2001, Am. J. Physiol., 280, 1273-1281). In the present example we examined whether rat anti-AXL antibodies of the invention interfere with Gas6-mediated anti-apoptosis of serum-starved NIH3T3-AXL cl.7 fibroblasts, and thus induce apoptosis. Apoptosis rates were thereby determined by measurement of the cellular caspase-3/7 activity. For this purpose, NIH3T3-AXL cl.7 cells were seeded at a density of $1.5x10^3$ cells per well in black clear-bottom 96 well plates (100 µl/well). The day after, normal growth medium was replaced by serum-reduced medium (DMEM, 0.5% FCS) to starve cells over night for 24 h. The next day, antibody solutions of the isotypic control antibody 1D5, the antagonistic rat anti-AXL antibodies 11B7 and 11D5, as well as the agonistic rat anti-AXL antibodies 11D7 and 2A1 at 80 µg/ml in DMEM, 0% FCS, 0.01% BSA were prepared. The cells were washed with PBS, covered with 60 µl of

DMEM, 0% FCS, 0.01% BSA, and 10µl of the respective antibody solution were added. After 1 h incubation at 37°C, 10 µl of DMEM, 0% FCS, 0.01% BSA with or without 3.2 µg/ml mouse Gas6 (R&D Systems) were added (the final concentrations of antibody and Gas6 were 10 µg/ml and 400 ng/ml, respectively), and cells were incubated at 37°C for another 5 h. The following steps refer to the technical bulletin to the Apo-ONE Homogenwous Caspase-3/7 Assay (Promega, G7791). In brief, culture plates were removed from the incubator and allowed to equilibrate at room temperature for 20 min. 60 µl of Apo-ONE substrate and 6 ml buffer were thawed, combined, and added to the samples (75 µl/well). The contents of wells was gently shaken for 30 sec, and, protected from light, incubated at room temperature for 1 h. Using a Victor plate reader (Perkin Elmer), the fluorescence of each well was measured at an excitation wavelength of 485 nm and an emission wavelength of 530nm.

Figure 12 shows representative results of this experiment. Compared to the isotypic control antibody, the antagonistic rat anti-AXL antibodies 11B7 and 11D5 of the invention reduced Gas6-mediated anti-apoptosis of serum-starved NIH3T3-AXL cl.7 fibroblasts, and thus induced apoptosis. In contrast, the agonistic rat anti-AXL antibodies 2A1 and 11D7 of the invention strongly induced anti-apoptosis of serum-starved NIH3T3-AXL cl.7 cells regardless of the absence or presence of Gas6, and therefore inhibited apoptosis.

Example 13. Rat anti-AXL antibodies of the invention inhibit spheroid-based cellular angiogenesis *in vitro*

AXL is a key regulator of multiple angiogenic behaviors including endothelial cell migration, proliferation, and tube formation *in vitro* (Holland et al., Cancer Res: 65, 9294-9303, 2005). Therefore, the rat anti-AXL monoclonal antibodies 11B7 and 11D5 of the invention were tested for inhibitory effects on VEGF-A–induced vessel sprouting of HUVEC-spheroids. The experiments were pursued in modification of the originally published protocol

(Korff and Augustin: J Cell Sci 112: 3249-58, 1999). In brief, spheroids were prepared as described (Korff and Augustin: J Cell Biol 143: 1341-52, 1998) by pipetting 500 human umbilical vein endothelial cells (HUVEC) in a hanging drop on plastic dishes to allow over night spheroid aggregation. 50 HUVEC spheroids were then seeded in 0.9 ml of a collagen solution (2 mg/ml) and pipetted into individual wells of a 24 well plate to allow polymerization. Decreasing concentrations of the rat anti-AXL antibodies 11B7 and 11D5 ($1x10^{-6}$ M, $1x10^{-7}$ M, $1x10^{-8}$ M, 1x10-9 M, $1x10^{-10}$ M) were directly mixed in the collagen solution before polymerization, whereas the growth factor VEGF-A (final concentration 25 ng/ml) was added after 30 min by pipetting 100 µl of a 10-fold concentrated working dilution on top of the polymerized gel. Plates were incubated at 37°C for 24 hours and fixed by adding 4% paraformaldehyde. Sprouting intensity of HUVEC spheroids was quantified by an image analysis system that determines the cumulative sprout length per spheroid using an inverted microscope and the digital imaging software Analysis 3.2 (Soft imaging system, Münster, Germany). The mean of the cumulative sprout length of 10 randomly selected spheroids was analyzed as an individual data point.

Figure 13 shows the results of this experiment. The antagonistic rat anti-AXL antibodies 11B7 (A) and 11D5 (B) of the invention inhibited VEGF-A-stimulated HUVEC sprouting in the spheroid-based angiogenesis assay in a dose-dependent manner. Whereas treatment with the highest concentration of 11B7 reduced HUVEC sprouting to basal levels, inhibition with the highest concentration of 11D5 was not as effective (left panel). HUVEC sprouting was inhibited with IC_{50} values of 9.8×10^{-8} M and 7.0×10^{-7} M for 11B7 and 11D5, respectively (right panel).

Example 14. Rat anti-AXL antibodies of the invention reduce human prostate carcinoma growth in nude mice

The anti-tumor efficacy of therapeutic antibodies is often evaluated in human xenograft tumor studies. In these model systems, human tumors grow as xenografts in immunocompromised mice and therapeutic efficacy is

measured by the degree of tumor growth inhibition. The aim of this study was to evaluate whether the antagonistic rat anti-AXL antibody 11B7 of the invention interferes with tumor growth of human prostate cancer cells in nude mice. In brief, on day 0, 7-8 weeks old male NMRI$^{-nu/nu}$ mice (approximate weight: 30 g after acclimatization) were anesthesized with 1.5-2.0 volume percent isoflurane at an oxygen flow rate of 2 l/min, and $1x10^6$ PC-3-LN cells in 25 µl PBS were orthotopically implanted into the prostate. PC-3-LN cells are derived from the PC-3 prostate carcinoma cell line which was infected with a retrovirus coding for a luciferase-neomycin fusion protein. The onset of tumor growth and tumor growth progression was therefore measurable via *in vivo* bioluminescence imaging. For this purpose, luciferin was injected intraperitoneally (i.p.) into the mice and light emission was measured 10 min post injection using a NightOWL LB 981 bioluminescence imaging system (Berthold Technologies, Germany). Prior to first treatment, mice were randomized and statistical tests performed to assure uniformity in starting tumor volumes (mean, median and standard deviation) across the treatment groups of 10 animals each. On day 8, all treatments started and were continued until day 34, followed by necropsy on day 35. 25 mg/kg of the isotypic control antibody 1D5 and the antagonistic rat anti-AXL antibody 11B7 were intraperitoneally (i.p.) administered 3x a week (Mo, Wed, Fr) into animals of group 1 and 2, respectively. Animals of group 3 orally (p.o.) received 40 mg/kg of Sutent once a day. Animals of Group 4 received three intraveneous (i.v.) injections with 12.5 mg/kg of Taxotere 4 days apart of each other. An overview of the treatment groups is given below.

Group		Treatment	Application		Animal Number
			Route	Scheme	
1	1D5	25 mg/kg	i.p.	3 times per week (Mo, Wed, Fr) starting one day after randomization[2)]	10
2	11B7	25 mg/kg	i.p.	3 times per week (Mo, Wed, Fr) starting one day after randomization[2)]	10
5	Sutent	40 mg/kg	p.o.	**daily** starting one day after randomization[2)]	10
6	Taxotere	12.5 mg/kg	i.v.	**3 doses 4 days apart** starting one day after randomization	10

Figure 14 shows the results of this experiment. Compared to the isotypic control antibody 1D5, the antagonistic rat anti-AXL antibody 11B7 of the invention reduced the overall growth of PC-3-LN prostate tumors in nude mice.

Example 15. Rat anti-AXL antibodies of the invention inhibit metastasis of human prostate carcinoma

In the same experiment as described under "Rat anti-AXL antibodies of the invention reduce human prostate carcinoma growth in nude mice", relocalization of PC-3-LN tumor cells into other organs (metastasis) was analyzed post necropsy to evaluate anti-metastatic effects of the antagonistic rat anti-AXL antibody 11B7 of the invention..For this purpose, selected organs (liver, spleen, lungs, femur, part of the lumbar spine) were collected post necropsy, homogenized, and supplemented with luciferin. Subsequently, light emission was measured using a NightOWL LB 981 bioluminescence imaging system (Berthold Technologies, Germany).

Figure 15 shows the results of this experiment for the analysis of spleens. Compared to the isotypic control antibody 1D5, the antagonistic rat anti-AXL antibody 11B7 of the invention reduced the occurrence of spleen metastases. Noteworthy, the anti-metastatic effect of 11B7 in this experiment was stronger than that of Sutent. Similar observations were obtained for liver, lung, femur, and lumbar spine metastasis.

Example 16. AXL is predominantly expressed in tumor rather than adjacent normal tissue

In this study, the AXL expression in 17 different human malignancies was immunohistochemically analysed on formalin-fixed paraffin-embedded tissues in tissue multiarray format. For each tumor type, pairs of tumor tissue and matching non-malignant tissue were examined. In brief, tissue was fixed for 16 to 20 h in 4% neutral buffered formalin and embedded in paraffin. For

construction of a 60-core tissue microarray (TMA), one punch of healthy tissue and one punch of corresponding tumor tissue of each case was chosen by a pathologist. A 96-core TMA with normal control tissue punches (three of each tissue type) was generated regarding to FDA guidelines. Each punch was 1.5 mm in diameter.

With a microtome, 2-4 µm sections of selected tissue blocks were cut, mounted on silanized glass slides (Sigma) and dried at 60°C for 30 min and 38°C over night. Sections were deparaffinized by incubation in a xylene bath for 5 min twice, in acetone for 5 min twice and finally in distilled water for 5 min. Heat pre-treatment of the sections was performed in 10 mM citrate buffer, pH 6.0 in a steamer for 30 min, followed by washing in distilled water. Endogenous peroxidase was blocked by incubation with a freshly prepared solution of 0.3% H_2O_2 in methanol for 20 min at room temperature, followed by washing with distilled water and PBS for 5 min each. Sections were incubated with polyclonal goat anti-human AXL antibody (Santa Cruz SC-1096) for 60 min (1:20 dilution in TBST) at room temperature. After three washes in TBST, the sections were incubated with biotinylated rabbit anti-goat secondary antibody (Dianova, 1:200 dilution in TBST) for 45 min at room temperature. After washing as before, the sections were incubated with Streptavidin/HRP (DAKO, 1:300 dilution in TBST) for 30 min at room temperature, followed by washing as before. Staining was achieved with DAB solution (DAKO; 1:50 dilution in substrate buffer) for 10 min at room temperature. Finally, the slides were rinsed with water, counterstained with Harris' hematoxylin, and covered with a glass slide. Control sections were incubated with goat IgG control antibody (R&D) instead of anti-AXL primary antibody.

Figure 16 summarizes the results of this analysis on AXL expression in 17 different human solid tumors and corresponding non-malignant tissue (A). Among all cases screened for each indication, no marked expression was detected in follicular lymphoma, prostate cancer (except for single cells), and in kidney cancer. Melanoma and Merkel cell tumors showed very low

expression of AXL. A weak expression was observed in a few tumors of the lung, mainly adenocarcinomas. Esophagus and Barrett tumors, ovarian, colon and pancreatic tumors as well as liver tumors (hepatocellular carcinoma) showed weak staining in about 30% of the cases. Head and neck tumors showed weak to moderate staining in about 40% of the tumors. Weak to moderate staining was detected in 60% to 100% of the analyzed tumors of the breast, cervix, bladder, thyroid and the stomach. Most intense staining was seen in mammary tumors and in a signet ring cell carcinoma of the stomach (B). Non-malignant tissues mainly showed no specific staining except from tubuli of the kidney which sometimes showed weak staining over background.

Example 17: Structure and characteristics of anti-AXL antibodies

17 A. Nucleotide sequences of rat antibody variable domains

Rat anti-AXL antibody variable domains were cloned from hybridoma cells. RNA was prepared utilizing the RNA-Extraction kit RNeasy (RNeasy midi-kit, Qiagen). cDNA encoding for the antibody genes was prepared using the 5 ´RACE kit (Invitrogen) according to manufacturer's instructions.

Briefly, first strand cDNA was synthesized from total or RNA using the gene-specific GSP1-primers and SuperScript™ II Reverse Transcriptase. After first strand cDNA synthesis, the original mRNA template is removed by treatment with the RNase Mix. A homopolymeric tail is then added to the 3'-end of the cDNA. PCR amplification is accomplished using *Taq* DNA polymerase, a nested, gene-specific primer (GSP2) that anneals to a site located within the cDNA molecule and an anchor primer provided with the kit. Following amplification 5' RACE products were cloned into the pLXSN-ESK vector for sequencing. To facilitate cloning the Anchor Primer (AP) included a recognition sequence for *Sal* I, GSP2 primers contained a XhoI site.

GSP1 primer:

kappa_GSP1: GATGGATGCATTGGTGCAGC
new_kappa_GSP1: ATAGATACAGTTGGTGCAGC
heavy_GSP1: CAGGGTCACCATGGAGTTA

GSP2 primer:

XhoI-hGSP2: CCGCTCGAGCGGGCCAGTGGATAGACAGATGG
XhoI-kGSP2: CCGCTCGAGCGGCCGTTTCAGCTCCAGCTTGG

Utilization of GSP primers for rat anti-AXL Mab cloning:

11B7: kappa GSP1; XhoI-kGSP2
heavy GSP1; XhoI-hGSP2

10D12: kappa_GSP1, new_kappa_GSP1; XhoI-kGSP2
heavy GSP1; XhoI-hGSP2

11D5: new_kappa_GSP1; XhoI-kGSP2
heavy GSP1; XhoI-hGSP2

17 B. Aminoacid sequence rat anti-AXL antibody variable domains

Rat antibody variable domain sequences were translated from sequenced genes cloned into the pLXSN-ESK vectors. The given amino acid sequences start at position one of the variable domain. The complementarity determining regions (CDRs) required for the specific binding of the antibody to its target are defined according to Kabat (Kabat et al. Sequences of Proteins of Immunological Interest, Fifth Edition. NIH Publication No. 91-3242, 1991). The Kabat definition is based on the sequence variability

within the variable domains. Anti-AXL specific CDR regions of the antibodies are listed in SEQ ID NO: 13-30. The individual CDRs include the following positions:

CDR-L1: 24-34
CDR-L2: 50-56
CDR-L3: 89-97

CDR-H1: 31-35b
CDR-H2: 50-65
CDR-H3: 95-102

17 C Rat antibody expression and purification:

Hybridomas were cultured in Celline CL 1000 bioreactors (Integra Biosciences) at 37°C, 5-7% CO_2 using DMEM including 4.5g/L glucose; 1% Glutamine, 1% Pyruvate 1% Pen/Strep. FCS supplementation is 1 % FCS for the nutrient compartment and 5 % low IgG FCS for the cell compartment. Harvest and media exchange is performed twice a week. Cell splitting 1/1 ->1/3 depending on cell growth. Productivity is tested once a week via SDS-PAGE analysis. Supernatants are stored at –20°C until purification. Mycoplasma test of running cultures is done once a week.

Antibodies are purified using Protein A or G Sepharose FF (GE-Healthcare) via an Äkta Explorer 100 System (GE-Healthcare). Columns are individually packed for each purification. The column size is adjusted to the expected productivity and size of each batch (usually 50 – 500 mg). Protein containing solutions are kept on ice or at 4°C wherever possible. Sterile buffers and double destilled water are used for the entire process.

Supernatants are thawed, buffered with 50 mM TRIS pH 8.5, centrifuged, filtered through a 0.22 µm membrane and loaded onto the column. After

washing with 8 column volumes (CV) 50 mM PO_4, pH8.5 the antibody is eluted within 10 CV 100 mM Glycin, pH 3.3. Eluate fractions are rebuffered immediately to neutral pH by adding 1/5 1M Tris pH 8.0 (1 ml Tris per 4ml eluate fraction) and analysed by rSDS-PAGE subsequently. Fractions containing pure antibody are pooled, dialysed against PBS at 4°C and sterile filtered.

Buffer system requirements are adjusted according to the individual properties of each antibody. In particular, rat IgG2a antibody 11D5 was bound to ProteinG 4 FF matrix (GE-Healthcare) and washed under high salt conditions (2M NaCl). Rat antibody IgG1 11B7 was purified via rProteinA (GE-Healthcare) under high salt conditions according to 11D5. Antibody elution was performed at pH 5.5. Flow rate for rat antibody purification has to be kept low for increased binding efficiency.

As a second purification step either ion exchange chromatography (under individual, suitable conditions) or preparative size exclusion chromatography (PBS, pH 7.4) can be implemented.

The standard protocol for quality control of the purified antibodies includes:

- rSDS-PAGE gel analysis; Coommassie or silver stained
- BCA test (Pierce #23227 BCA Protein Assay Kit; rat IgG standard #31233)
- Analytical size exclusion (Superdex 200 Tricorn 10/300 GL,~250 mg in 250 µl; 0.5 ml/min, Äkta Explorer 100)
- Endotoxin test (LAL, Cambrex QCL-1000® Chromogenic LAL Endpoint Assay # US50-648U)
- Cell based activity assays (FACS binding; pAkt; pAXL)

Purified antibodies are stored in PBS, pH 7.4 under steril conditions at 4°C or –20°C depending on their stability.

17 D. Antibody Affinity determination by FACS scatchard

Human AXL overexpression NIH3T3 cells were harvested by incubation with 10 mM EDTA in PBS and resuspended at 6 million cells per ml in FACS buffer (PBS pH 7.4, 3% FCS, 0.1% NaN_3). In a round-bottom microtiter plate, 100 µl of cell suspension were added to 100µl of antibody solution containing antibodies 11B7, 11D5, ch11B7-IgG2 or ch11D5-IgG2 at concentrations between 40 and 0.002 µg/ml (266 and 0.01 nM) in FACS buffer. Antibody binding was allowed to proceed for 2 hours on ice. Then, cells were washed twice with 250 µl FACS buffer per well, and resuspended in 200µl of secondary antibody (anti-rat-PE; Jackson) diluted 1:50 in FACS buffer. After 45 minutes of incubation, cells were again washed twice in FACS buffer and resuspended in 500ml PBS for FACS analysis. Analysis was carried out on a Beckman-Coulter FACS FC500. To determine the apparent affinity constant K_{Dapp}, mean fluorescence values were plotted against the ratio of mean fluorescence and the corresponding antibody concentration ([M]). The calculated K_{Dapp} resulted from the inverse slope of the straight line are listed below:

Clone	K_D value (nM)
11B7	0.38
ch11B7-IgG2	0.6
11D5	0.81
Ch11D5-IgG2	0.9

18. Chimerization of rat anti-AXL antibodies:

Human kappa light chain and heavy chain IgG1/2 genes were cloned from peripheral blood mononuclear cells (PBMC) of a human volunteer as described below:

PBMCs were prepared from whole blood. Blood was diluted 1/2,5 in

PBS/ 2 mM EDTA with 10 U/ml heparin at RT, layered over 15 ml Biocoll solution covered by a diaphragm (35 ml/ tube) [Biocoll from Biochrom # L6115]. Samples were centrifuged at RT for 30 min at 400xg and serum (~ 15 ml) was discarded. Interface containing PBMCs was carefully recovered using a Pasteur pipette. PBMCs were washed 2x in PBS/2 mM EDTA (first wash 100ml, second wash 50 ml) and spun down at 300xg for 10 min. Cell pellet was resuspended in RPMI/ 10% FCS (25 ml) and yielded $5.5x10^7$ PBMCs.

RNA was prepared from PBMCs using RNeasy kit from Qiagen (# 75142) according to manufacturer's instructions. Purified RNA (30 µg) was stored in aliquots at –80°C.

cDNA for antibody IgG gamma 1 and 2 as well as kappa chains were prepared from isolated RNA by RT-PCR using Superskript III Reverse Transkriptase (invitrogen # 18080 – 93) according to manufacturers instructions using the following primers:

1) RT-gamma: GCG TGT AGT GGT TGT GCA GAG

2) RT-gamma2: GGG CTT GCC GGC CGT G

3) RT-kappa: TGG AAC TGA GGA GCA GGT GG

4) 5´Blp: AGA TAA GCT TTG CTC AGC GTC CAC CAA GGG CCC ATC GGT

5) 3´Bam(GAG): AGA TGG ATC CTC ATT TAC CCG GAG ACA GGG AGA G

6) 5´Bsi: AGA TAA GCT TCG TAC GGT GGC TGC ACC ATC TGT CTT CAT

7) 3´Bam(CTT): AGA TGG ATC CCT AAC ACT CTC CCC TGT TGA AGC TCT

Primers were dissolved at 100µM. RT-PCR reactions were performed using 2pmol oligo RTγand RTκ respectively, adding 1 µg RNA, 10 mM dNTP mix and heat for 5 min to 65 °C. 4µl first strand buffer, 1µl 0.1M DTT, 1 µl RNase inhibitor (40 U/µl Fermentas # EO0311) and 2 µl Superscript III RT were added, mixed and incubated at 50°C for 1h followed by a heat inactivation step for 15 min at 70 °C.

2µl of first strand reaction were used for second step PCR using Taq polymerase (Eurochrom # EME010001) to yield double stranded DNA of antibody constant domains. The primer 5´Blp and 3´Bam(GAG) were used to amplify γ–chain, and 5´Bsi and 3´Bam(CTT) were used to amplify κ-chain constant regions using the following PCR settings:.

κ-chain amplification:

94°C	120 sec
94°C	30 sec
55°C	30 sec
72°C	45 sec cycle 35 times
72°C	10 min

γ–chain amplification:

94°C	120 sec
94°C	30 sec
45°C	30 sec
72°C	60 sec cycle 5 times

94°C 30 sec
50°C 30 sec
72°C 60 sec cycle 35 times

72°C 10 min

The PCR products were analysed on a TAE buffered 2% agarose gel. A single band of ~350 bp for kappa light chain and a single band of ~ 1000 bp for the heavy chains γ1 and γ2 were found. The PCR products were purified by Qiagen gel extraction kit, (QIAGEN, #28784) according to the manufacturer's instructions. To clone the PCR fragments into the multiple cloning site of the pcDNA3 vector (Invitrogen), pcDNA3 vector and PCR fragments were digested with HindIII (5´) and BamHI (3´) restriction endonucleases. Restriction sites were encoded within the PCR primers. Digested fragments were purified using the Qiagen PCR purification kit (QIAGEN, 28104), and DNA encoding the γ1, γ2 and κ chains were ligated into the pcDNA3 vector facilitating T4 DNA ligase at 16°C overnight. Ligase was inactivated for 10 min. at 65°C. Ligated DNA plasmids were directly transformed into $CaCl_2$ competent *E.coli* using standard protocol and plated onto Ampicillin containing LB-plates. After incubation at 37°C overnight single colonies were picked, suspended in 10 µl H_2O and proofed for containing the respective antibody chain carrying plasmid by PCR (5 µl suspended cells, Taq polymerase, primer 5Blp and 3Bam(GAG) γ1/γ2 and 5Bsi and 3Bam(CTT) for κ colonies:

94°C 120 sec

94°C 30 sec
55°C 30 sec
72°C 60 sec cycle 35 times

72°C 10 min

Samples were analysed on 1.5% agarose gel for PCR products. Antibody gene containing colonies were selected to inoculate 5 ml LB/Ampicillin medium. After incubation at 37 °C overnight *E.coli* were harvested and DNA was prepared using Qiagen miniprep kit (QIAGEN, # 12123). A control digest (HindIII, BamHI) showed all κ and γ chain gene inserts at the expected size; sequences were verified by DNA sequencing at Medigenomix.

Rat variable domains were amplified by PCR from pLXSN-ESK vector and cloned into g1/g2 and k pcDNA3 vectors to yield the chimeric full length antibodies. Variable VL domains were amplified with the following primers, containing a HindIII and BsmI site at the 5´end and a BsiWI site at the 3´end:

VL-11B7-5´: AGA TAA GCT TGT GCA TTC CGA CAT CCA GAT GAC CCA GGC TCC

VL-11B7-3´: AGA TCG TAC GTT TCA GCT CCA GCT TGG TGC CTC

VL-11D5-5´: AGA TAA GCT TGT GCA TTC CGA CAT CCA GAT GAC CCA GTC TCC ATC

VL-11D5-3´: AGA TCG TAC GTT TCA GCT TGG TCC CAG

Variable VH domains were amplified with the following primers, containing a HindIII and BsmI site at the 5´end and a BlpI site at the 3´end:

VH-11B7/11D5-5´: AGA TAA GCT TGT GCA TTC CGA GGT GCA GCT TCA GGA GTC AGG

VH-11B7/11D5-3´: AGA TGC TGA GCT GAC AGT GAC CAT GAC

TCC TTG GCC

BsiWI for the light chain and the BlpI for the heavy chain are single sites at the 5´end of the constant regions to enable the direct fusion with the 3 ´end of the variable domain genes.

Fused to the leader sequence SEQ ID No.: 69 derived from pLNOH2 vector (Norderhaug et. al. J. Immunol. Methods 204, 1997; Neuberger EMBO J. 1983; 2 (8): 1373-8,1983) genes encoding the chimeric antibody chains were cloned into pCEP vector system for recombinant expression. Light chain genes were cloned NheI (5´) and XhoI (3`) into pCEP4 (Invitrogen) heavy chain genes KpnI (5´) and XhoI (3`) into pCEP-Pu (Kohfeld FEBS Vol 414; (3) 557ff, 1997).

HEK 293 cells seeded on 20x20 cm plates were co-transfected with 1µg/ml of each plasmid coding for light and heavy chain genes using standard $CaPO_4$ transfection method for transient expression. Culture conditions were 37°C, 5% CO_2 in DMEM/F12 high glucose medium containing 5% low IgG FCS, 1% pyrovate, 1% glutamine, 1% penicillin/streptomycin. 24h after transfection medium was exchanged by fresh medium. Supernatants were collected every 2-3 days for approximately 3 weeks. Chimeric antibodies were purified from approximatelly 600ml supernatant utilizing 1 ml Hitrap rProtein A columns (GE-Healthcare) under standard buffer conditions (loading: 50 mM Tris; pH=8.5, wash: 50mM PO_4; pH= 8.5, elution: 100mM Glycin; pH 3,3) as described for rat antibody purification.

Example 19. Humanization of rat anti-AXL antibody variable domains

The rat variable regions of the chimeric antibodies were compared to human antibody germline sequences at the protein level via a BLAST search for immunoglobulin domains The closest human counterpart within the V-genes, which in addition had identical CDR loop lengths was identified. The associated D and J segments were selected from the V-

BASE database (http://vbase.mrc-cpe.cam.ac.uk/) according to their homology to the rat sequences in an analogous approach.

For the rat variable domains of the 11B7 and 11D5 antibodies the following bestfitting human germline sequences (V, D and J segments) were found and defined as human framework:

VL11B7hum: Vκ1-O12 + Jk1
VH11B7hum: VH4-59 + D4-4 (reading frame 3) + JH4

VL11D5hum: Vκ1-L1 + Jκ4
VH11D5hum: VH4-59 + D4-4 (reading frame 3) + JH4

Leader sequences for humanized variable domains were adopted from the associated germline V-gene sequences as selected. CDR residues of rat anti-AXL antibodies defined according to Kabat (Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition. NIH Publication No. 91-3242, 1991) were grafted into human germline frameworks for anti-AXL specificity to obtain the final humanized version of anti-AXL antibodies hum11B7 and hum11D5.

The protein sequences of the humanized anti-AXL antibodies hum11B7 and hum11D5 are as follows:

Protein sequences were back translated into DNA sequences. DNA sequences were CODON optimized for recombinant expression in mammalian cells using the Kazusa-Codon-Usage Database. The resulting DNA sequences for the humanized anti-AXL antibodies are as follows:

The optimized DNA sequences encoding for the humanized anti-AXL antibodies were synthesized by a PCR-method based on overlapping oligonucleotides.

VL-genes were cloned into pCEP4 vector utilizing the plasmid of the chimeric antibody construct pCEP4_ch11B7k1. Cloning sites are NheI (5´) and BsiWI (3´) which were already included in the synthesized genes of the humanized antibodies. VH genes were cloned into the corresponding chimeric heavy chain vector pCEP-PU_ch11B7g1 utilizing KpnI (5´) and BlpI (3´) as restriction sites. DNA optimization, gene synthesis, cloning and sequence verification was conducted at Eurofins Medigenomix GmbH, Martinsried, Germany.

Example 20. Rat and chimeric anti-Axl antibodies of the invention inhibit ligand-induced Axl phosphorylation *in vitro* to similar extent

Chimeric derivatives of the rat anti-Axl antibodies 11B7 and 11D5 were generated as part of this invention (see below). In order to investigate whether the rat anti-Axl antibodies of the invention and the corresponding chimeric anti-Axl antibodies of the invention were able to inhibit ligand Gas6-mediated Axl activation *in vitro* to similar extent, ELISA experiments on CaSki cervical cancer cells were performed. Gas6-mediated Axl activation was thereby detected by increased receptor tyrosine phosphorylation. In brief, on day 1, $3x10^4$ cells per well were seeded in normal growth medium in flat-bottom 96 well plates. The next day, growth medium was replaced by serum-free medium to starve cells over night for 24 h. Also over night, black Maxi-Sorp 96 well plates (Nunc) were coated with mouse anti-phospho-tyrosine antibody 4G10 at 2 µg/ml PBS and 4°C. On day 3, the 4G10 antibody solution was removed and Maxi-Sorp wells were blocked with PBS, 0.5% BSA for at lest 4 h at room temperature. In parallel, cells were pre-incubated with 50 ng/ml, 100 ng/ml, 300 ng/ml, 750 ng/ml, 1 µg/ml, and 10 µg/ml of rat anti-Axl antibody 11B7 or chimeric anti-Axl antibody ch11B7 for 1 h at 37°C and subsequently treated with or without 400 ng/ml Gas6 (R&D Systems) for 10 min at 37°C. Medium was then flicked out and cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerine, and 1% Triton X-100) supplemented with phosphatase and protease inhibitors (10 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride, 1

mM orthovanadate, 1 mM NaF, and 0,5% aprotinin) for 30 min on ice. Meanwhile, blocking buffer was removed and Maxi-Sorp plates were washed 6x with wash buffer (PBS, 0.05% Tween 20), before lysates were transferred and incubated over night at 4°C. After plates were washed 6x with wash buffer on day 4, wells were incubated with biotinylated rat anti-Axl antibody 12B7 at 0.5 µg/ml PBS for 2 h at room temperature. Plates were washed 6x with wash buffer and AP-conjugated streptavidin (Chemicon #SA110) diluted 1:4,000 in PBS was added to each well and incubated for 30 min at room temperature. Afterwards, wells were washed 6x with wash buffer and AttoPhos substrate solution (Roche #11681982) was added. Using a Victor plate reader (Perkin Elmer), the fluorescence of each well was collected at an excitation wavelength of 430 nm and an emission wavelength of 580nm.

Figure 17 shows representative results of this experiment for the cervical cancer cell line CaSki. As demonstrated by concentration-dependent decrease of the relative Axl phosphorylation, the rat anti-Axl antibody 11B7 (A) and the chimeric anti-Axl antibody ch11B7 (B) of the invention were able to block ligand-induced activation of the receptor tyrosine kinase Axl to similar extent. Comparable effects applying the same experimental settings were observed with the melanoma cell line C-8161.

Example 21. Rat and chimeric anti-Axl antibodies of the invention inhibit ligand-induced p42/p44 MAP-Kinase phosphorylation *in vitro* to similar extent

To additionally verify whether the rat anti-Axl antibodies of the invention and the corresponding chimeric anti-Axl antibodies of the invention were also able to inhibit Gas6-induced activation of p42/p44 MAP-Kinase in CaSki cervical cancer cells to similar extent, ELISA experiments were performed. Here, Gas6-induced p42/p44 MAP-Kinase activation was detected by increased protein (Thr202/Tyr204) phosphorylation. In brief, on the first day, $2x10^4$ cells per well were seeded in flat-bottom 96 well plates. The next day, normal growth medium was replaced by serum-free medium to starve cells

for 24 h. Thereafter, cells were pre-incubated with 50 ng/ml, 100 ng/ml, 300 ng/ml, 750 ng/ml, 1 µg/ml, and 10 µg/ml of rat anti-Axl antibody 11B7 or chimeric anti-Axl antibody ch11B7 for 1 h at 37°C and then treated with or without 400 ng/ml Gas6 (R&D Systems) for 10 min at 37°C. Medium was flicked out and cells were fixed with 4% formaldehyde in PBS (pH 7.5) for 30 min at room temperature. Formaldehyde solution was removed and cells were washed twice with wash buffer (PBS, 0.1% Tween 20). Cells were quenched with 1% H_2O_2, 0.1% NaN_3 in wash buffer and incubated for 20 min at room temperature. Afterwards, the quenching solution was removed, and cells were washed twice with wash buffer and blocked with PBS, 0.5% BSA for 4 h at room temperature. Anti-phospho-p42/p44 MAP Kinase (Thr202/Tyr204) primary antibody (polyclonal rabbit; Cell Signaling #9101) diluted 1:1,000 in PBS, 0.5% BSA, 0.05% Tween 20, 5 mM EDTA was added over night at 4°C. On day 4, the antibody solution was removed and the plate was washed 3x with wash buffer. HRP-conjugated anti-rabbit secondary antibody (Dianova #111-036-045) diluted 1:2,500 in PBS, 0.5% BSA, 0.05% Tween 20, 5 mM EDTA was then added to each well and incubated for 1.5 h at room temperature. The plate was washed 3x with wash buffer for 5 min each. Tetramethylbenzidine (TMB, Calbiochem) was added and monitored at 620 nm. The reaction was stopped by addition of 100 µl of 250 nM HCl and the absorbance was read at 450 nm with a reference wavelength of 620 nm using a Vmax plate reader (Thermo Lab Systems).

Figure 18 shows representative results of this experiment. The rat anti-Axl antibody 11B7 (A) and the chimeric anti-Axl antibody ch11B7 (B) of the invention were able to block Gas6-induced activation of p42/p44 MAP-Kinase in CaSki cervical cancer cells to similar extent as indicated by concentration-dependent decrease of the relative p42/p44 MAP-Kinase phosphorylation.

Example 22. Rat anti-Axl antibodies of the invention synergize with chemotherapeutic agents to overcome drug resistance *in vitro*

As rat anti-Axl antibodies of the invention turned out to interfere with Gas6-mediated anti-apoptosis of serum-starved NIH3T3-Axl cl.7 fibroblasts, the question arose, whether antagonistic anti-Axl antibodies would synergize with chemotherapeutic agents in inducing apoptosis, thereby contributing to overcome drug resistance. In this example, NCI/ADR-RES (originally named MCF-7/AdrR) cells - a ovarian cancer cell line (Liscovitch and Ravid, 2007, Cancer Letters, 245, 350-352) displaying a high level of resistance to several agents including doxorubicin (Fairchild et al., 1987, Cancer Research, 47, 5141-5148; Xu et al., 2002, The Journal of Pharmacology and Experimental Therapeutics, 302, 963-971) – were incubated with the antagonistic anti-Axl antibody 11B7 and/or doxorubicin, and apoptosis rates were determined by TUNEL staining. In brief, $3x10^4$ NCI/ADR-RES cells in normal growth medium were seeded per well of an 8-chamber culture slide (BD Falcon, cat# 354118) which were pre-incubated with the same medium for 1 h at 37°C. The next morning, normal growth medium was removed and cells were washed with and cultured in serum-reduced (0.5% FCS) medium. In the evening, isotypic control antibody 1D5 or the antagonistic anti-Axl antibody 11B7 were added at final concentrations of 10 µg/ml each. In the morning of day 3, doxorubicin at final concentrations of 100 µM, 150 µM, or 200 µM was added, and cells were incubated at 37°C. After 24 h, cells were rinsed once with PBS, fixed with 4% formaldehyde in PBS (pH 7.5) for 20 min at room temperature, air-dried for 5 min, and stored at -20°C. Using the commercially available Fluorescein-FragEL™ kit (Oncogene, cat# QIA39, presently being distributed through Merck-Calbiochem), TUNEL staining was performed according to the supplier's manual instructions (chapter 'Fluorescein-FragEL™ of cell preparations fixed on slides', page 10). Applying fluorescence microscopy, cells were analyzed and photos were taken.

Figure 19 shows representative results of this experiment. No TUNEL staining, and hence no apoptosis, was observed with NCI/ADR-RES ovarian cancer cells that were treated with 100 µM of doxorubicin, regardless of

whether cells have been co-incubated with control antibody or the antagonistic anti-Axl antibody 11B7 (top). However, at a concentration of 150 µM of doxorubicin, only very week apoptosis could be detected in cells co-treated with control antibody, whereas co-incubation with the antagonistic anti-Axl antibody 11B7 resulted in a substantial induction of apoptosis (middle). Also in the presence of 200 µM of doxorubicin, co-incubation of cells with 11B7 significantly increased apoptosis rates as compared to cells being incubated with control IgG antibody (bottom), indicating that co-treatment of even multi drug-resistant cells with both chemotherapeutic agents and antagonistic anti-Axl antibodies of the invention may be suitable to overcome drug resistance.

Example 23. Rat anti-Axl antibodies of the invention synergize with chemotherapeutic agents in reducing anchorage-independent colony growth *in vitro*

Soft agar assays were conducted in order to investigate the ability of anti-Axl antibodies of the invention to inhibit anchorage-independent cell growth either alone or in combination with chemotherapeutic agents. The soft agar colony formation assay is a standard *in vitro* assay to test for transformed cells, as only transformed cells are able to grow in soft agar.

In brief, 750 C-8161 melanoma cells either remained untreated or were pre-incubated with the antagonistic rat anti-Axl antibody 11B7 at 15 µg/ml in IMDM medium (Gibco) for 30 min at 37°C. Subsequently, cells were combined with Difco noble agar solution resulting in 50 µl of top agar cell suspension at concentrations of Agar, FCS, and 11B7 of 0.35%, 0.2%, and 7.5 µg/ml, respectively. This cell suspension was plated on top of 50 µl of a 0.7% agarose bottom layer containing 20% FCS, and was finally covered with another 50 µl of a feeding layer solution that contains 0.2% FCS as well as cisplatin in according concentrations. In the whole of 150 µl per sample, the final concentrations of 11B7 and cisplatin were 2.5 µg/ml and 1.5 µM, 1.0 µM, 0.75 µM, 0.5 µM, or 0.25 µM, respectively. Colonies were allowed to form for 5 days and were then stained with 50 µl MTT (Sigma, 1 mg/ml in

PBS) for 3 hours at 37°C. Using a Scanalyzer HTS camera system in conjunction with the HTS Bonit coloniy formation software (Lemnatec, Wuerselen), the effect of the antagonistic rat anti-Axl antibody 11B7 in the absence or presence of cisplatin were analyzed in triplicates.

Figure 20 shows representative results of this experiment. The presented data refer to the overall area of colonies and reflect both the absolute numbers being measured (A) and the relative growth inhibition (B) exerted by cisplatin and/or the antagonistic rat anti-Axl antibodies 11B7. As compared to untreated control cells, incubation with cisplatin led to colony growth retardation in a dose-dependent manner. In line with the inhibitory effect of 11B7 alone in the range of 30%, combination with the antagonistic anti-Axl antibody 11B7 resulted in a significantly potentiated inhibitory effect of cisplatin on soft agar growth of C-8161 melanoma cells, particularly at lower concentrations of cisplatin.

Example 24. Rat anti-Axl antibodies of the invention synergize with anti-neoplastic agents in reducing tumor-related phenomena

In the previous examples, synergistic effects of antagonistic anti-Axl antibodies of the invention co-administered with doxorubicin have been observed with regard to inducing apoptosis and overcoming drug resistance in multi drug-resistant cancer cells such as the ovarian cancer cell line NCI/ADR-RES. Moreover, combination effects of antagonistic anti-Axl antibodies of the invention and cisplatin in reducing anchorage-independent colony growth were detected with the melanoma cell line C-8161. Therefore, synergistic effects in inducing apoptosis in and/or overcoming drug resistance of tumor cells, suppressing tumor cell survival, inhibiting tumor cell growth and/or proliferation, reducing tumor cell migration, spreading and metastasis, or impairing tumor angiogenesis are to be expected when cancer cells or patients suffering from cancer diseases are treated with antagonistic anti-Axl antibodies in combination with irradiation and/or one or more further anti-neoplastic agent. In particular, synergistic effects in

inducing apoptosis in and/or overcoming drug resistance of tumor cells, suppressing tumor cell survival, inhibiting tumor cell growth and/or proliferation, reducing tumor cell migration, spreading and metastasis, or impairing tumor angiogenesis are to be expected when melanoma cells or patients suffering from melanoma are treated with antagonistic anti-Axl antibodies in combination with irradiation and/or any further anti-neoplastic agent which is preferably but not limited to cisplatin, dacarbazine, temozolomide/temodal, muphoran/fotemustine, paclitaxel, or docetaxel. Furthermore, synergistic effects in inducing apoptosis in and/or overcoming drug resistance of tumor cells, suppressing tumor cell survival, inhibiting tumor cell growth and/or proliferation, reducing tumor cell migration, spreading and metastasis, or impairing tumor angiogenesis are to be expected when ovarian cancer cells or patients suffering from ovarian cancer are treated with antagonistic anti-Axl antibodies in combination with irradiation and/or any further anti-neoplastic agent which is preferably but not limited to doxorubicin, cisplatin, carboplatin, paclitaxel, docetaxel, melphalan, altretamine, topotecan, ifosfamide, etoposide, or 5-fluorouracil. Additionally, synergistic effects in inducing apoptosis in and/or overcoming drug resistance of tumor cells, suppressing tumor cell survival, inhibiting tumor cell growth and/or proliferation, reducing tumor cell migration, spreading and metastasis, or impairing tumor angiogenesis are to be expected when prostate cancer cells or patients suffering from prostate cancer are treated with antagonistic anti-Axl antibodies in combination with irradiation and/or any further anti-neoplastic agent which is preferably but not limited to mitozantrone, doxorubicin, paclitaxel, docetaxel, or vinblastine. Moreover, synergistic effects in inducing apoptosis in and/or overcoming drug resistance of tumor cells, suppressing tumor cell survival, inhibiting tumor cell growth and/or proliferation, reducing tumor cell migration, spreading and metastasis, or impairing tumor angiogenesis are to be expected when gastric/stomach cancer cells or patients suffering from gastric/stomach cancer are treated with antagonistic anti-Axl antibodies in combination with irradiation and/or any further anti-neoplastic agent which is preferably but not limited to 5-fluorouracil, mitomycin C, cisplatin, doxorubicin, methotrexate,

etoposide, leucovorin, epirubicin, paclitaxel, docetaxel, or irinotecan. Also, synergistic effects in inducing apoptosis in and/or overcoming drug resistance of tumor cells, suppressing tumor cell survival, inhibiting tumor cell growth and/or proliferation, reducing tumor cell migration, spreading and metastasis, or impairing tumor angiogenesis are to be expected when breast cancer cells or patients suffering from breast cancer are treated with antagonistic anti-Axl antibodies in combination with irradiation and/or any further anti-neoplastic agent which is preferably but not limited to doxorubicin, epirubicin, paclitaxel, docetaxel, cyclophosphamide, 5-fluorouracil, gemcitabine, capecitabine, vinorelbine, or trastuzumab. Furthermore, synergistic effects in inducing apoptosis in and/or overcoming drug resistance of tumor cells, suppressing tumor cell survival, inhibiting tumor cell growth and/or proliferation, reducing tumor cell migration, spreading and metastasis, or impairing tumor angiogenesis are to be expected when cervical cancer cells or patients suffering from cervical cancer are treated with antagonistic anti-Axl antibodies in combination with irradiation and/or any further anti-neoplastic agent which is preferably but not limited to cisplatin, ifosfamide, irinotecan, 5-fluorouracil, paclitaxel, docetaxel, gemcitabine, or topotecan. Moreover, synergistic effects in inducing apoptosis in and/or overcoming drug resistance of tumor cells, suppressing tumor cell survival, inhibiting tumor cell growth and/or proliferation, reducing tumor cell migration, spreading and metastasis, or impairing tumor angiogenesis are to be expected when pancreatic cancer cells or patients suffering from pancreatic cancer are treated with antagonistic anti-Axl antibodies in combination with irradiation and/or any further anti-neoplastic agent which is preferably but not limited to gemcitabine, capecitabine, or 5-fluorouracil. Finally, but not excluding other cancer types, synergistic effects in inducing apoptosis in and/or overcoming drug resistance of tumor cells, suppressing tumor cell survival, inhibiting tumor cell growth and/or proliferation, reducing tumor cell migration, spreading and metastasis, or impairing tumor angiogenesis are to be expected when lung cancer cells or patients suffering from lung cancer are treated with antagonistic anti-Axl antibodies in combination with irradiation and/or any further anti-neoplastic

agent which is preferably but not limited to cisplatin, carboplatin, doxorubicin, paclitaxel, docetaxel, etoposide, vinorelbine, vincristine, ifosfamide, gemcitabine, methotrexate, cyclophosphamide, lomustine, or topotecan.

Claims

1. A monoclonal antibody that binds to the extracellular domain of AXL and at least partially inhibits AXL activity.

2. The monoclonal antibody of claim 1, which reduces and/or blocks AXL-mediated signal transduction.

3. The monoclonal antibody according to anyone of claims 1-2, which reduces and/or blocks AXL phosphorylation.

4. The monoclonal antibody according to anyone of claims 1-3, which reduces and/or blocks cell proliferation.

5. The monoclonal antibody according to anyone of claims 1-4, which reduces and/or blocks angiogenesis.

6. The monoclonal antibody according to anyone of claims 1-5, which reduces and/or blocks cell migration.

7. The monoclonal antibody according to anyone of claims 1-6, which reduces and/or blocks tumor metastasis.

8. The monoclonal antibody according to anyone of claims 1-7, which reduces and/or blocks the AXL mediated anti-apoptosis.

9. The monoclonal antibody according to anyone of claims 1-8 , which reduces and/or blocks AXL mediated PI3K signaling.

10. The monoclonal antibody according to anyone of claims 1-9, which is a recombinant antibody, a humanized antibody, a chimeric antibody, a

multispecific antibody, or a fragment thereof.

11. The monoclonal antibody of claim 10, which is a chimeric antibody and comprises a heavy chain amino acid sequence selected from the group consisting of SEQ ID NOs: 38, 39, 41, 42, or at least the variable domain thereof or an amino acid sequence having a sequence identity of at least 90% thereto and/or a light chain amino acid sequence selected from the group consisting of SEQ ID NOs 37, 40, or at least the variable domain thereof or an amino acid having a sequence identity of at least 90% thereto or an antibody recognizing the same epitope on the extracellular domain of AXL.

12. The monoclonal antibody of claim 10, which is a humanized antibody and comprises a heavy chain amino acid sequence selected from the group consisting of SEQ ID NOs: 44, 46 or at least the variable domain thereof or an amino acid sequence having a sequence identity of at least 90% thereto and/or a light chain amino acid sequence selected from the group consisting of SEQ ID NOs 43, 45, or at least the variable domain thereof or an amino acid sequence having a sequence identity of at least 90% thereto or an antibody recognizing the same epitope on the extracellular domain of AXL.

13. The monoclonal antibody according to anyone of claims 1-12, which is a Fab fragment, a Fab' fragment, a F(ab'), fragment, a Fv fragment, a diabody, or a single chain antibody molecule.

14. The monoclonal antibody according to anyone of claims 1-13, which is of the IgG1-, IgG2-, IgG3-or IgG4-type.

15. The monoclonal antibody according to anyone of claims 1-14, which is coupled to a labelling group.

16. The monoclonal antibody according to anyone of claims 1-15, which is

coupled to an effector-group.

17. The monoclonal antibody according to anyone of claims 1-16, which is a scaffold protein.

18. The monoclonal antibody according to anyone of claims 1-17 which comprises at least:
one heavy chain amino acid sequence comprising at least one CDR selected from the group consisting of
(a) a CDRH1 as shown in SEQ ID NOs: 16, 22, 28, or a CDRH1 sequence differing in 1 or 2 amino acids therefrom,
(b) a CDRH2 as shown in SEQ ID NOs: 17, 23, 29 or a CDRH2 sequence differing in 1 or 2 amino acids therefrom, and
(c) a CDRH3 as shown in SEQ ID NOs: 18, 24, 30, or a CDRH3 sequence differing in 1 or 2 amino acids therefrom,
and/or at least:
one light chain amino acid sequence comprising at least one CDR selected from the group consisting of
(d) a CDRL1 as shown in SEQ ID NOs: 13, 19, 25, or a CDRL1 sequence differing in 1 or 2 amino acids therefrom,
(e) a CDRL2 as shown in SEQ ID NOs: 14, 20, 26, or a CDRL2 sequence differing in 1 or 2 amino acids therefrom, and
(f) a CDRL3 as shown in SEQ ID NOs: 15, 21, 27, or a CDRL3 sequence differing in 1 or 2 amino acids therefrom,
or a monoclonal antibody recognizing the same epitope on the extracellular domain of AXL.

19. The monoclonal antibody according to anyone of claims 1-17 which comprises a heavy chain comprising at least one CDR selected from the group consisting of
(a) a CDRH1 as shown in SEQ ID NO: 16, or a CDRH1 sequence differing in 1 or 2 amino acids therefrom,
(b) a CDRH2 as shown in SEQ ID NO: 17, or a CDRH2 sequence

differing in 1 or 2 amino acids therefrom, and

(c) a CDRH3 as shown in SEQ ID NO: 18, or a CDRH3 sequence differing in 1 or 2 amino acids therefrom,

and/or a light chain comprising at least one CDR selected from the group consisting of

(d) a CDRL1 as shown in SEQ ID NO: 13, or a CDRL1 sequence differing in 1 or 2 amino acids therefrom,

(e) a CDRL2 as shown in SEQ ID NO: 14, or a CDRL2 sequence differing in one or two amino acids therefrom, and

(f) a CDRL3 as shown in SEQ ID NO: 15, or a CDRL3 sequence differing in 1 or 2 amino acids therefrom, or an monoclonal antibody recognizing the same epitope on the extracellular domain of AXL.

20. The monoclonal antibody according to any one of claims 1-17 which comprises a heavy chain comprising at least one CDR selected from the group consisting of

(a) a CDRH1 as shown in SEQ ID NO: 22, or a CDRH1 sequence differing in 1 or 2 amino acids therefrom,

(b) a CDRH2 as shown in SEQ ID NO: 23, or a CDRH2 sequence differing in 1 or 2 amino acids therefrom, and

(c) a CDRH3 as shown in SEQ ID NO: 24, or a CDRH3 sequence differing in 1 or 2 amino acids therefrom,

and/or a light chain comprising at least one CDR selected from the group consisting of

(d) a CDRL1 as shown in SEQ ID NO: 19, or a CDRL1 sequence differing in 1 or 2 amino acids therefrom,

(e) a CDRL2 as shown in SEQ ID NO: 20, or a CDRL2 sequence differing in one or two amino acids therefrom, and

(f) a CDRL3 as shown in SEQ ID NO: 21, or a CDRL3 sequence differing in 1 or 2 amino acids therefrom, or an monoclonal antibody recognizing the same epitope on the extracellular domain of AXL.

21. The monoclonal antibody according to anyone of claims 1-17 which

comprises a heavy chain comprising at least one CDR selected from the group consisting of

(a) a CDRH1 as shown in SEQ ID NO: 28, or a CDRH1 sequence differing in 1 or 2 amino acids therefrom,

(b) a CDRH2 as shown in SEQ ID NO: 29, or a CDRH2 sequence differing in 1 or 2 amino acids therefrom, and

(c) a CDRH3 as shown in SEQ ID NO: 30, or a CDRH3 sequence differing in 1 or 2 amino acids therefrom,

and/or a light chain comprising at least one CDR selected from the group consisting of

(d) a CDRL1 as shown in SEQ ID NO: 25, or a CDRL1 sequence differing in 1 or 2 amino acids therefrom,

(e) a CDRL2 as shown in SEQ ID NO: 26, or a CDRL2 sequence differing in one or two amino acids therefrom, and

(f) a CDRL3 as shown in SEQ ID NO: 27, or a CDRL3 sequence differing in 1 or 2 amino acids therefrom, or an monoclonal antibody recognizing the same epitope on the extracellular domain of AXL.

22. The monoclonal antibody according to anyone of claims 1-17, which comprises a heavy chain amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 10, 12, or at least the variable domain thereof or an amino acid having a sequence identity of at least 90% thereto and/or a light chain amino acid sequence selected from the group consisting of SEQ ID NOs 7, 9, 11, or at least the variable domain thereof or an amino acid sequence having a sequence identity of at least 90% thereto or an antibody recognizing the same epitope on the extracellular domain of AXL.

23. An isolated nucleic acid molecule selected from the group consisting of:

(a) a nucleic acid sequence encoding an monoclonal antibody, antibody fragment or a derivative thereof of any of claims 1-22,

(b) a nucleic acid sequence as shown in SEQ ID NOs: : 1-6, 31-36,

(c) a nucleic acid complementary to any of the sequences in (a) or (b); and

(d) a nucleic acid sequence capable of hybridizing to (a), (b) or (c) under stringent conditions.

24. A vector comprising a nucleic acid sequence of claim 23.

25. The vector according to claim 24, which is an expression vector and the nucleic acid sequence is operably linked to a control sequence.

26. A host comprising the vector of claim 24 or 25.

27. The host of claim 26 which is a human, bacteria, animal, fungal, amphibian or plant cell.

28. The host of claim 26 which is a non-human transgenic animal.

29. A process of manufacturing a monoclonal antibody according to anyone of claims 1-22 comprising the step of obtaining said polypeptide from the host of claim 26, 27 or 28.

30. A pharmaceutical composition comprising an anti-AXL-antibody, preferably the monoclonal antibody of anyone of claims 1-22, the nucleic acid molecule of claim 23 the vector of claim 24 or 25, the host of claim 26, 27 or 28, or a polypeptide generated by the process of claim 29.

31. The pharmaceutical composition of claim 30 comprising pharmaceutically acceptable carriers, diluents and/or adjuvants.

32. The pharmaceutical composition according to claim 30 or 31, comprising a further active agent.

33. The pharmaceutical composition according to anyone of claims 30-32,

for the diagnosis, prevention or treatment of a hyperproliferative disease.

34. The pharmaceutical composition according to anyone of claims 30-33, wherein said hyperproliferative disease is associated with AXL expression, overexpression and/or hyperactivity.

35. The pharmaceutical composition of claim 33, wherein said hyperproliferative disease is selected from the group consisting of breast cancer, lung cancer and other AXL expressing or overexpressing cancers, and formation of tumor metastases.

36. The monoclonal antibody according to anyone of claims 1-22, for the diagnosis, prevention or treatment of a hyperproliferative disease.

37. Use of the monoclonal antibody according to anyone of claims 1-22 for the manufacture of a pharmaceutical composition for the diagnosis, prevention or treatment of a hyperproliferative disease.

38. The use according to claim 36 or 37, wherein said hyperproliferative disease is a hyperproliferative disease as defined in anyone of claims 34 or 35.

39. A method for diagnosing a condition associated with the expression of AXL, comprising contacting a sample with an monoclonal antibody according to anyone of claims 1-22, and detecting the presence of AXL.

40. The method according to claim 39, wherein the condition is a hyperproliferative disease as defined in anyone of claims 34 or 35.

41. A method for preventing or treating a condition associated with the expression of AXL in a patient, comprising administering to a patient in need thereof an effective amount of at least the monoclonal antibody according to anyone of claims 1-22.

42. The method according to claim 41, wherein the condition is a hyperproliferative disease as defined in anyone of claims 34 or 35.

43. The method according to claim 41 or 42, wherein the patient is a mammalian patient, particularly a human patient.

44. A kit comprising an anti-AXL-antibody, preferably a monoclonal antibody according to anyone of claims 1-22, a nucleic acid sequence according to claim 23 or a vector according to claim 24 or 25.

45. The kit according to claim 44, further comprising a further antineoplastic agent.

46. Use of an anti-AXL antibody for the manufacture of a pharmaceutical composition for the treatment of drug resistant cancer.

47. Use according to claim 46, wherein the anti-AXL antibody is a monoclonal antibody according to anyone of claims 1-22.

48. Use of an anti-AXL antibody for the manufacture of a medicament for the co-administration with an antineoplastic agent for the treatment of a hyperproliferative disease.

49. Use according to claim 48, wherein the anti-AXL antibody is a monoclonal antibody according to any one of claims 1-22.