

HUMANIZED ANTI-CA IX ANTIBODIES AND METHODS OF THEIR USE

FIELD OF THE INVENTION

- 5 The present invention relates to humanized antibodies capable of specific binding to human carbonic anhydrase IX. More specifically, the present invention relates to antibodies directed to the proteoglycan domain of CA IX, comprising murine-derived complementarity determining regions and humanized heavy and light regions.

10 BACKGROUND OF THE INVENTION

CA IX is a cancer-related carbonic anhydrase identified by Zavada, Pastorekova, Pastorek (U.S. Patent 5,387,676) using the M75 monoclonal antibody first described by Pastorekova et al, *Virology*, 187:620e626, 1992. That antibody was employed in cloning of cDNA and gene encoding CA IX, in
15 assessment of CA IX expression in tumors and normal tissues, in study of CA IX regulation and in studies of CA IX relationship to cancer progression and therapy resistance. All these studies supported the assumption made in the original U.S Patent 5,387,676 that CA IX can be used diagnostically and/or prognostically as a preneoplastic/neoplastic tumor marker and therapeutically as a target, and showed that the M75 monoclonal antibody is a valuable CA IX-specific reagent useful for different
20 immunodetection methods and immunotargeting approaches.

CA IX (alternative name: MN protein) belongs to the carbonic anhydrase family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate ions and protons. There are 15 human CA isoforms out of which three are inactive and the other twelve range in activity from weak to very strong. Most of the isoenzymes are predominantly expressed in differentiated cells to fulfill
25 specialized physiological roles in various tissues and organs (Pastorekova et al, *J Enzyme Inhib Med Chem* 19, 199–229, 2004). CA IX has a unique position due to its strong association with cancer, hypoxia-related expression pattern, acidic pKa optimum and an extra proteoglycan-like domain (PG) protruding from the globular catalytic domain of the enzyme. CA IX enzyme active site in the catalytic domain (CA) is facing the extracellular space and contributes to pH regulation across the plasma
30 membrane. It is now well established that CA IX cooperates with diverse acid extruders and bicarbonate importers including sodium-dependent bicarbonate transporters NBCe1 and NBCn1 and lactate and protons-exporting monocarboxylate transporters MCT1 and MCT4. Involvement of CA IX in pH regulation has multiple consequences supporting tumor phenotype. CA IX also behaves as an adhesion molecule that contributes to the assembly and maturation of focal adhesion contacts during cell
35 attachment and spreading on solid support. On the contrary, CA IX can destabilize intercellular adhesion contacts by disconnection of E-cadherin from the cytoskeletal anchorage through the competitive

binding to beta catenin. Accumulating experimental evidence suggests that CA IX is functionally involved in diverse aspects of cancer development, including protection of cancer cell survival in conditions of hypoxia and acidosis, facilitation of cancer cell migration/invasion, contribution to metastatic dissemination, homing and growth of metastatic lesions.

- 5 CA IX is one of the best responders to low oxygenation (ranging from anoxia to moderate hypoxia), mainly because of its transcriptional regulation by hypoxia-inducible factor HIF-1 binding to hypoxia-response element (HRE) consensus sequence localized near the transcription initiation site (Wykoff et al, *Cancer Res* 60, 7075–7083, 2000). Inactivation of the pVHL (von Hippel-Lindau) tumor suppressor protein, which causes HIF degradation, results in the elevation of CA IX expression in kidney tumors
10 (Ivanov et al, *Proc Natl Acad Sci USA* 95, 12596–12601, 1998). Moreover, hypoxia regulates splicing of the CA IX mRNA and a protein kinase A (PKA)-mediated phosphorylation of the cytoplasmic tail of the CA IX protein, in both cases affecting its enzyme activity (Barathova et al, *Br J Cancer* 98, 129–136, 2008; Ditte et al, *Cancer Res* 71, 7558–7567, 2011).

- CA IX can internalize from the cell surface to the cell cytoplasm via endocytosis induced by hypoxia
15 and calcium depletion as well as by specific antibodies binding to its extracellular part (Zatovicova et al, *Curr Pharm Des* 16, 3255–3263, 2010). Ectodomain of CA IX can be cleaved by metalloproteinase ADAM17 and released to the microenvironment in response to hypoxia, acidosis and toxic insults of carbonic anhydrase inhibitors or chemotherapeutic drugs (Zatovicova et al, *Br J Cancer* 93, 1267–1276, 2005; Vidlickova et al, *BMC Cancer* 16, 239, 2016).

- 20 CA IX expression in non-cancerous tissues is rare and generally confined to epithelia of the stomach, gallbladder, pancreas and intestine.

There are more than 1000 studies of CA IX clinical value suggesting that it can serve as a biomarker and/or therapy target in diverse tumor types and settings (Pastorek and Pastorekova, *Seminars in Cancer Biology* 31, 52-64, 2015).

- 25 CA IX is expressed in high percentage of cells in more than 90% of clear cell renal cell carcinoma (ccRCC) that carry an inactivating mutation/deletion of the VHL tumor suppressor gene. In many other tumor types, CA IX is expressed regionally in areas that are hypoxic and/or acidic and usually increases with increasing tumor stage and grade. CA IX can be also detected in body fluids of cancer patients that can be clinically exploited for non-invasive screening or monitoring of cancer patients.
- 30 Meta-analysis of studies encompassing more than 24 thousand of patients with non-RCC tumors revealed strongly significant associations between CA IX expression evaluated by immunohistochemistry and all endpoints: overall survival, disease-free, locoregional control, disease-specific, metastasis-free survival, and progression-free survival (van Kuijk et al, *Front Oncol* 6, 69, 2016). Subgroup analyses showed similar associations in the majority of tumor sites and types. In
35 conclusion, these results show that patients having tumors with high CA IX expression have higher risk of disease progression, and development of metastases, independent of tumor type or site. In addition,

there are numerous studies showing correlation between CA IX positivity and resistance to chemotherapy, radiotherapy and even immunotherapies directed to other cancer-related molecular targets, such as HER-2 (human epidermal growth factor receptor 2), VEGF (vascular endothelial growth factor), and PD-1 (programmed cell death protein 1). These findings support the usefulness of clinical tests determining patient's prognosis and therapy outcome based on CA IX expression and provide a rationale for the development of new CA IX-targeted treatment strategies.

CA IX-targeting strategy based on immunotherapy exploits the tumor-related expression pattern of CA IX. This approach uses monoclonal antibodies (mAbs) and thus, ensures high specificity and selectivity toward CA IX that is currently not achievable with chemical compounds. In case of antibody-dependent cell-mediated cytotoxicity (ADCC) as the main mechanism of action, the killing effects is fast and does not support development of compensatory mechanisms. Previous clinical trials with the CA IX-specific monoclonal antibodies did not meet the primary endpoint due to lack of patients' stratification (ADCC response-inducing GcG250, Willex), or due to unacceptable toxicity (antibody-drug conjugate MMAE-BAY79-4620, Bayer). Thus, the preferred strategy of immunotherapy includes ADCC and stratification of patients based on the CA IX expression level.

Specificity is an important factor in decisions concerning whether a particular mAb can be successfully used for cancer therapy. This attribute is accomplished by unique tumor-related expression pattern of CA IX and on the other hand, only limited expression in few normal tissues. Previous clinical evidence from ccRCC studies suggests that the antibody-based immunotherapy targeted to CA IX is safe and well tolerated (Chamie et al, *JAMA Oncology* 3:913-920, 2017). Additionally, safety of the treatment is linked to the evidence from multiple studies that CA IX expression is strongly linked to tumor phenotype and confined to only few non-cancerous tissues where the basal membrane does not allow the intravenously administered antibodies to reach epithelial cells. Data from the literature on CA IX-specific chimeric antibody cG250 (having the variable regions of murine G250 and constant regions derived from human IgG, also known as RENCAREX® or GIRENTUXIMAB®) showed no grade III and IV as well as dose-limiting toxicity and, on the other hand, an excellent accumulation in RCC and increased median/overall survival rates (Steffens et al, *J Clin Oncol* 15:1529-1537, 1997; Davis et al, *Cancer Immunity* 7:14-23 2007; Bleumer et al, *Br J Cancer* 90:985-990, 2004). Moreover, the combination therapy of cG250 with low dose interferon alpha was safe, well tolerated and with clinical benefits for patients with progressive metastatic RCC (Siebels et al, *World J Urol* 29:121-126, 2011).

WO2003/100029 discloses CA IX-specific murine monoclonal antibodies generated in CA IX-deficient mice with targeted disruption of *Car9* gene. The set of antibodies, produced by specific hybridoma cells, includes VII/20 mAb and IV/18 mAb (as described in Zatovicova et al, *J Immunol Methods* 282, 117-134, 2003). The mAbs are highly selective to CA IX and do not cross-react with the human CA I, CA II and CA XII proteins that are expressed mostly in normal differentiated tissues. Thus, both monoclonal antibodies are expected to have strictly tumor-specific effect.

The antibody VII/20 binds to the conformational epitope in the catalytic (CA) domain of CA IX, induces internalization of CA IX and shows potent anti-tumor effect *in vivo* in mouse model with subcutaneous tumor xenografts (Zatovicova et al, *Curr Pharm Des* 16, 3255–3263, 2010). The antibody IV/18 binds to the linear epitope in the proteoglycan-like (PG) domain of CA IX and does not induce internalization.

5 The ability of these two mAbs to distinguish antigenic regions on two separate extracellular domains of CA IX offers an opportunity for effective targeting. The fact that both VII/20 and IV/18 monoclonal antibodies were generated in CA IX-deficient mice which are no longer available and thus, could not be prepared again only emphasize their uniqueness. All previously mentioned attributes of the monoclonal antibodies provide a rationale for their humanization with intent of their use in anticancer
10 immunotherapy.

There is a need in the art for safe and effective antibodies that target CA IX for the treatment of CA IX-associated conditions, such as cancer. The invention fulfills that need and provides other benefits.

SUMMARY OF THE INVENTION

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The present invention provides humanized antibodies specifically recognizing proteoglycan domain of human CA IX, which show specific and effective binding activity, as well as a surprising activity in inhibition of cancer cell invasion and phagocytic potency. Furthermore, they are safe and do not elicit undesirable side effects.

20

The present invention provides a humanized antibody specifically recognizing the proteoglycan domain of human CA IX, comprising:

a) a heavy chain variable region sequence comprising CDR sequences identical to or differing in 1 or 2 amino acids from the following sequences:

25 GFTFNTNAMH (SEQ ID NO. 1), and
RIRSKSNNTTYADSVKD (SEQ ID NO. 2), and
VCGSWFAY (SEQ ID NO. 3);

and

b) a light chain variable region sequence comprising the CDR sequences identical to or differing in 1 or
30 2 amino acids from the following sequences:

KSSQSLNSSNQKNYLA (SEQ ID NO. 4), and
FTSTRQS (SEQ ID NO. 5), and
QQHYSIPLT (SEQ ID NO. 6).

35 In some embodiments, the humanized antibody of the present invention contains a heavy chain variable region sequence comprising CDR sequences identical to or differing in 1 or 2 amino acids from the

sequences GFTFNTNAMH (SEQ ID NO. 1), and RIRSKSNNYTTYADSVKD (SEQ ID NO. 2), and VCGSWFAY (SEQ ID NO. 3); and a light chain variable region sequence comprising the CDR sequences identical to or differing in 1 or 2 amino acids from the following sequences: KSSQSLNSSNQKNYLA (SEQ ID NO. 4), and FTSTRQS (SEQ ID NO. 5), and QQHYSIPLT (SEQ ID NO. 6).

In one aspect, the humanized antibody specifically recognizing human CA IX according to the invention comprises at least one variable region selected from the group consisting of:

- a heavy chain variable region comprising or having the sequence:

10 X³²VQLVESGGGX³³VQPGX³⁴SLX³⁵LSCAASGFTFNTNAMHWVRQAX³⁶GX³⁷GLEWVX³⁸RI
RSKSNNYTTYADSVKDRFTISRDX³⁹SKX⁴⁰TX⁴¹YLQX⁴²NSLX⁴³X⁴⁴EDTAVYYCVCGSWF
AYWGQGTX⁴⁵VTVSS (SEQ ID NO. 7)

wherein

X³² = E or Q

15 X³³ = L or V

X³⁴ = G or R

X³⁵ = K or R

X³⁶ = S or P

X³⁷ = K or R

20 X³⁸ = A or G

X³⁹ = D or N

X⁴⁰ = N or S

X⁴¹ = A or L

X⁴² = M or V

25 X⁴³ = K or R

X⁴⁴ = T or A

X⁴⁵ = L or T; and

- a light chain variable region comprising or having the sequence:

30 DX⁴⁶X⁴⁷MTQSPDSLAVSLGERX⁴⁸TINCKSSQSLNSSNQKNYLAWX⁴⁹QQKPGQX⁵⁰PX⁵¹X⁵²X⁵³
IYFTSTRQSGVPDRFX⁵⁴GSGSGTDFLTIX⁵⁵SLQAEDVAVYX⁵⁶CQQHYSIPLTFGQGT
X⁵⁷X⁵⁸EIK (SEQ ID NO. 8)

X⁴⁶ = V or I

X⁴⁷ = V or Q

X⁴⁸ = V or A

35 X⁴⁹ = Y or F

X⁵⁰ = S or P

$X^{51} = \text{K or N}$

$X^{52} = \text{L or V}$

$X^{53} = \text{L or V}$

$X^{54} = \text{S or T}$

5 $X^{55} = \text{S or N}$

$X^{56} = \text{Y or F}$

$X^{57} = \text{K or Q}$

$X^{58} = \text{L or V.}$

10 In one preferred aspect, the invention provides the humanized antibody specifically recognizing human CA IX, comprising at least one variable region selected from the group consisting of:

a) a heavy chain variable region amino acid sequence comprising or having the sequence selected from the group consisting of

15 EVQLVESGGGLVQPGGSLKLSCAASGFTFNTNAMHWVRQASGKGLEWVGRIRSKSNNYTTY
YADSVKDRFTISRDDSKNTAYLQMNSLKTEDTAVYYCVCGSWFAYWGQGTLLTVSS (SEQ
ID NO. 9),

EVQLVESGGGLVQPGGSLKLSCAASGFTFNTNAMHWVRQASGKGLEWVGRIRSKSNNYTTY
YADSVKDRFTISRDDSKSTAYLQMNSLKTEDTAVYYCVCGSWFAYWGQGTLLTVSS (SEQ

20 ID NO. 10),

QVQLVESGGGVVQPGGSLRLSCAASGFTFNTNAMHWVRQAPGRGLEWVARIRSKSNNYTTY
YADSVKDRFTISRDN SKNTLYLQVNSLRAEDTAVYYCVCGSWFAYWGQGTLLTVSS (SEQ
ID NO. 11),

EVQLVESGGGVVQPRSLRLSCAASGFTFNTNAMHWVRQAPGKGLEWVARIRSKSNNYTTY

25 YADSVKDRFTISRDN SKNTLYLQVNSLRAEDTAVYYCVCGSWFAYWGQGTLLTVSS (SEQ
ID NO. 12),

EVQLVESGGGLVQPGGSLKLSCAASGFTFNTNAMHWVRQASGKGLEWVGRIRSKSNNYTTY
YADSVKDRFTISRDDSKNTAYLQMNSLKTEDTAVYYCVCGSWFAYWGQGTLLTVSS (SEQ

ID NO. 13); and

30

b) a light chain variable region amino acid sequence comprising or having the sequences selected from the group consisting of

DVVMTQSPDSLAVSLGERVTINCKSSQSLLNSSNQKNYLAWYQQKPGQSPKLLIYFTSTRQSG
VPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQHYSIPLTFGQGTKLEIK (SEQ ID NO. 14),

35 DIVMTQSPDSLAVSLGERATINCKSSQSLLNSSNQKNYLAWFQQKPGQPPNLIYFTSTRQSG
VPDRFSGSGSGTDFTLTINSLQAEDVAVYFCQQHYSIPLTFGQGTQVEIK (SEQ ID NO. 15),

DIQMTQSPDSLAVSLGERATINCKSSQSLLNSSNQKNYLAWYQQKPGQPPKLLIYFTSTRQSG
 VPDRFSGSGSGTDFTLTISSLQAEDVAVYFCQQHYSIPLTFGQGTKVEIK (SEQ ID NO. 16),
 DIVMTQSPDSLAVSLGERATINCKSSQSLLNSSNQKNYLAWFQQKPGQPPKVLIIYFTSTRQSG
 VPDRFTGSGSGTDFTLTISSLQAEDVAVYYCQQHYSIPLTFGQGTKLEIK (SEQ ID NO. 17), and
 5 DIVMTQSPDSLAVSLGERATINCKSSQSLLNSSNQKNYLAWYQQKPGQPPKLLIYFTSTRQSG
 VPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQHYSIPLTFGQGTKLEIK (SEQ ID NO. 18).

Preferably, the humanized antibody of the present invention contains a heavy chain variable region amino acid sequence comprising or having the sequence selected from a group consisting of SEQ ID
 10 NO. 11 and SEQ ID NO. 12; and a light chain variable region amino acid sequence comprising or having the sequence selected from a group consisting of SEQ ID NO. 14, SEQ ID NO. 15 and SEQ ID NO. 18.

According to a particularly preferred embodiment of the invention, the antibody of the invention comprises a heavy chain variable region amino acid sequence comprising or having the sequence of
 15 SEQ ID NO. 12 and a light chain variable region amino acid sequence comprising or having the sequence of SEQ ID NO. 18.

Preferably, the humanized antibody of the present invention has human IgG constant regions allotype G1m17,1 of the heavy chains and human kappa constant regions allotype Km3 of the light chains.

20 The present invention further provides a pharmaceutical composition comprising the humanized antibody as described above, which specifically recognizes human CA IX, and a pharmaceutically acceptable carrier, diluent or excipient.

25 The present invention encompasses also the humanized antibody or the pharmaceutical composition as described above, for use in the treatment of a disease or disorder associated with expression, activation or function of a CA IX protein. Such diseases and disorders typically include cell proliferative disease or disorder, such as a cancer selected from the group consisting of: squamous cell carcinoma, myeloma, small-cell lung cancer, non-small cell lung cancer, glioma, Hodgkin's lymphoma, non-Hodgkin's
 30 lymphoma, acute myeloid leukemia, multiple myeloma, gastrointestinal (tract) cancer, renal cancer, ovarian cancer, liver cancer, lymphoblastic leukemia, lymphocytic leukemia, colorectal cancer, endometrial cancer, kidney cancer, prostate cancer, thyroid cancer, melanoma, chondrosarcoma, neuroblastoma, pancreatic cancer, glioblastoma multiforme, cervical cancer, brain cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, mesothelioma, and head and neck
 35 cancer.

Preferably, the present invention provides the humanized antibody or the pharmaceutical composition as described above for use in the treatment of breast cancer, mesothelioma, or glioblastoma expressing CA IX.

5 In the medical use of the humanized antibodies of pharmaceutical compositions, more than one humanized antibody can be used. The humanized antibodies or pharmaceutical compositions comprising the humanized antibodies may be administered simultaneously or sequentially. Preferably, they are administered sequentially.

10 The present invention further provides a method of treating a disease or disorder associated with expression, activation or function of a CA IX protein, comprising administering to a subject in need thereof a therapeutically effective amount of the humanized antibody or of the pharmaceutical composition as described above. Such diseases or disorders typically include cell proliferative diseases or disorders, such as a cancer selected from the group consisting of: squamous cell carcinoma, myeloma,
15 small-cell lung cancer, non-small cell lung cancer, glioma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, acute myeloid leukemia, multiple myeloma, gastrointestinal (tract) cancer, renal cancer, ovarian cancer, liver cancer, lymphoblastic leukemia, lymphocytic leukemia, colorectal cancer, endometrial cancer, kidney cancer, prostate cancer, thyroid cancer, melanoma, chondrosarcoma, neuroblastoma, pancreatic cancer, glioblastoma multiforme, cervical cancer, brain cancer, stomach
20 cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, mesothelioma, and head and neck cancer.

Preferably, the present invention provides a method of treating of breast cancer, mesothelioma, or glioblastoma expressing CA IX, comprising administering to a subject in need thereof a therapeutically
25 effective amount of the humanized antibody or the pharmaceutical composition as described above.

Yet furthermore, the present invention provides a method of reducing or inhibiting invasiveness of a tumor of a subject, comprising administering to a subject in need thereof a therapeutically effective amount of the humanized antibody or the pharmaceutical composition as described above, thereby
30 reducing or inhibiting invasiveness of a tumor in the subject.

The appropriate daily or weekly dose of the humanized antibody to CA IX for administration to a patient is preferably ranging from 0.001 mg/kg to 15 mg/kg body weight.

35 The humanized antibody to CA IX may be administered in a number of possible regimens. Typically, the following regimens may be suitable:

- i) multiple, identical or different doses of the humanized antibody;
- ii) multiple escalating doses of the humanized antibody; or
- iii) administering a dose of the humanized antibody once every week, once every 2 weeks, once every 3 weeks, once every 4 weeks, or once every 5 weeks.

5

In some embodiments, the administration of the humanized antibody to CA IX or the pharmaceutical composition as described above comprises 1-10 administration cycles, each cycle comprising 2-5 infusions/doses every 1-4 weeks, with a humanized antibody, followed by a break of 1-8 weeks between each two cycles.

10

The present invention further provides a diagnostic composition comprising at least one humanized antibody as described herein above, and at least one carrier, diluent, or excipient.

Suitable diagnostic assays in which the antibody of the present invention include immunoassays, such as ELISA, affinity chromatography, immunohistochemistry and Western blotting.

15

The present invention thus provides a method for diagnosing a cancer in a subject in need thereof, the method comprising contacting a biological sample derived or obtained from said subject with the diagnostic composition as described herein, wherein a complex formation beyond a predetermined threshold is indicative of the cancer in said subject.

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In the diagnostic composition or in the method for diagnosing a cancer in a subject the humanized antibody may be linked, bound or conjugated to a paramagnetic, radioactive or fluorogenic moiety that is detectable upon imaging.

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BRIEF DESCRIPTION OF FIGURES

Figure 1: Effect of murine IV/18 monoclonal antibody on lung metastases formation. (A) Total radiant efficiency reflects the amount of HT1080-RFP cancer cells in murine lungs from either control or IV/18 mAb-treated group. (B) Representative *ex vivo* images of fluorescent lung metastases of control mice and of mice treated with IV/18 mAb.

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Figure 2: Reactivity of CA9hu-2 variants with either CA IX-positive (C-33a_CA IX) or CA IX-negative (C-33a_neo) antigen determined via ELISA. Samples containing only antibody diluent are marked as "no Ab". Parental IV/18 (A) (marked as "mouse Ab") as well as chimeric HC0LC0 (having the murine variable domains and the human Ig constant domains) antibodies were used as reference samples. Data in the graph are expressed as a fold of induction and are calculated as O.D. values of absorbance

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measured at 492 nm from CA IX-positive antigen/O.D. values of absorbance measured at 492 nm from CA IX-negative antigen.

Figure 3: Screening of humanized variants of CA9hu-2 in antibody-dependent cell-mediated cytotoxicity using either CA IX-positive (C-33a_CA IX) or CA IX-negative (C-33a_neo) cells. Chimeric HC0LC0 (having the murine variable domains and the human Ig constant domains) antibodies were used as reference samples. Data in the graph are expressed as luminescence in relative luminescence units (RLU) and represent mean $\pm$ standard deviation values.

Figure 4: 3D model of BT-20 spheroids cultivated with human PBMCs in the presence of humanized antibody variant CA9hu-2_HC4LC5. Projection of PBMC cells within BT-20 spheroids (both pre-stained using CellBrite™ Dye) from Z-stack sections after 3 days of treatment acquired across the spheroid volume (upper part of the figure). Immunohistochemical analysis of the impact of humanized antibody variant CA9hu-2_HC4LC5 on spheroid morphology. Representative sections from BT-20 spheroids co-cultivated with PBMCs and treated with humanized antibody variant for 11 days. A distinctive pattern of CA IX-staining was observed within the membranes across the BT-20 spheroids (lower part of the figure).

Figure 5: Invasion ability of hypoxia pre-incubated C-33a_CA IX cells in the presence of humanized antibody variant CA9hu-2_HC4LC5 was assessed using real-time measurement by xCELLigence device. Cells seeded in a Matrigel-coated top chamber were stimulated into invasion toward a chemoattractant in the lower chamber. C-33a_CA IX cells seeded the absence of humanized antibody are marked as "no Ab". Data in the graph shows time dependance of the cell index expressed as mean $\pm$ standard deviation values.

Figure 6: Analysis of multicellular aggregation of C-33a_CA IX with humanized antibody CA9hu-2_HC4LC5 after 24 h and 72 h on poly-HEMA coated dishes. C-33a_CA IX cells incubated in the absence of humanized antibodies are marked as "negative control".

Figure 7: Analysis of C-33a_CA IX cells by propidium iodide staining and flow cytometry after 72 h of treatment with humanized antibody CA9hu-2_HC4LC5. C-33a_CA IX cells incubated in the absence of humanized antibodies are marked as "negative control".

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations:

Throughout the detailed description and examples of the invention the following abbreviations will be used:

3D	three-dimensional
ADCC	antibody-dependent cell-mediated cytotoxicity
ADCP	antibody-dependent cell-mediated phagocytosis

	ccRCC	clear cell renal cell carcinoma
	CA IX	carbonic anhydrase IX
	CDC	complement dependent cytotoxicity
	CDR	complementarity determining regions
5	CRA	cytokine release assays
	DOX	doxorubicin
	ELISA	enzyme-linked immunosorbent assay
	FCS	fetal calf serum
	FR	framework region
10	HER-2	human epidermal growth factor receptor 2
	HIF-1	hypoxia-inducible factor 1
	HRE	hypoxia-response element
	HVR	hypervariable region
	IFN γ	interferon γ
15	IL	interleukin
	IMGT	Immunogenetics Information System
	INN	international nonproprietary names
	kDa	kilodalton
	K _D	dissociation constant
20	mAb	monoclonal antibody
	M	molar
	MCT	monocarboxylate transporter
	MHC	major histocompatibility complex
	NFAT	nuclear factor of activated T-cells
25	PBMC	peripheral blood mononuclear cells
	PBS	phosphate-buffered saline
	PCR	polymerase chain reaction
	PD-1	programmed cell death protein 1
	PD-L1	programmed cell death-ligand 1
30	PG	proteoglycan-like region
	PKA	protein kinase A
	PPA	Proteome Profiler Array
	RFP	red fluorescent protein
	SEB	staphylococcal enterotoxin B
35	TNBC	triple-negative breast cancer
	TNF α	tumor-necrosis factor α

	V _H	immunoglobulin heavy chain variable region
	V _L	immunoglobulin light chain variable region
	VEGF	vascular endothelial growth factor
	VHL	von Hippel-Lindau
5	WHO	World Health Organization

Cell lines:

	8-MG-BA	human glioblastoma cancer cells (Cellosaurus CVCL_1052)
	BT-20	human breast carcinoma cells (ATCC HTB-19)
10	C-33a	human cervical carcinoma cells (ATCC HTB-31)
	HT1080	human fibrosarcoma cancer cells (ATCC CCL-121)

Definitions:

So that the invention may be more readily understood, certain technical and scientific terms are specifically defined below. Unless specifically defined elsewhere in this document, all technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs.

The term "CA IX" is used to refer to the protein product of the CA9 gene (e.g. NP_001207.2).

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The terms "anti-CA IX antibody", "an antibody which recognizes CA IX", "an antibody against CA IX" and "an antibody to CA IX" are interchangeable, and used herein to refer to an antibody that binds to the CA IX protein (such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting CA IX).

25

The term "PG domain" is used to refer the domain homologous to proteoglycans which is localized at the N-terminal part of the CA IX protein.

The term "antigen" as used herein refers to a molecule or a portion of a molecule capable of eliciting antibody formation and being bound by an antibody. An antigen may have one or more than one epitope. The specific reaction is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies, which may be evoked by other antigens. An antigen according to the present invention is a CA IX protein or a fragment thereof.

The term "antigenic determinant" or "epitope" as used herein refers to the region of an antigen molecule that specifically reacts with a particular antibody. Peptide sequences derived from an epitope can be

used, alone or in conjunction with a carrier moiety, applying methods known in the art, to immunize animals and to produce additional, or monoclonal antibodies. Isolated peptides derived from an epitope may be used in diagnostic methods to detect antibodies and as therapeutic agents when inhibition of said antibodies is required.

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The term "antibody" or "immunoglobulin" as used herein refers to composition of two heavy chains linked together by disulfide bonds and two light chains, each light chain being linked to a respective heavy chain by disulfide bonds in a "Y" shaped configuration. Proteolytic digestion of an antibody yields Fv (Fragment variable) and Fc (Fragment crystalline) domains. The antigen binding domains, Fab, 10 include regions where the polypeptide sequence varies. The term F(ab')₂ represents two Fab' arms linked together by disulfide bonds. The central axis of the antibody is termed the Fc fragment. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains (C_H). Each light chain has a variable domain (V_L) at one end and a constant domain (C_L) at its other end, the light chain variable domain being aligned with the variable domain of the heavy chain and the light chain constant 15 domain being aligned with the first constant domain of the heavy chain. The variable domains of each pair of light and heavy chains form the antigen-binding site. The domains on the light and heavy chains have the same general structure and each domain comprises four framework regions, whose sequences are relatively conserved, joined by three hyper-variable domains known as complementarity determining regions (CDRs 1-3). These domains contribute specificity and affinity of the antigen- 20 binding site. The isotype of the heavy chain (gamma, alpha, delta, epsilon or mu) determines immunoglobulin class (IgG, IgA, IgD, IgE or IgM, respectively). The light chain is either of two isotypes (kappa, κ or lambda, λ) found in all antibody classes.

An "isolated" antibody is one, which has been separated from a component of its natural environment. 25 In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic or chromatographic.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogenous antibodies, i.e. the individual antibodies comprising the population are 30 identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigen. Furthermore, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

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The term "chimeric antibody" as used herein refers to an antibody in which a portion of the heavy and/or

light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source.

5 The term "humanized antibody" as used herein refers to an antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVR (e.g. CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A
10 "humanized form" of an antibody, e.g. a non-human antibody, refers to an antibody that has undergone humanization.

The term "acceptor human framework" for the purposes herein is a framework comprising the amino acid sequence of a heavy chain variable domain (V_H) framework or a light chain variable domain (V_L)
15 framework derived from human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments,
20 the V_L acceptor human framework is identical in sequence to the V_L human immunoglobulin framework sequence or human consensus framework sequence.

The term "hypervariable region" or "HVR" as used herein refers to each of the regions of an antibody variable domain, which is hypervariable in sequence and/or form structurally defined loops ("hypervariable loops"). Generally, native four-chain antibodies comprise six HVRs, three in the V_H ,
25 and three in the V_L . HVRs generally comprise amino acid residues from the hypervariable loops and/or from the "complementarity determining regions" (CDRs), the latter being of highest sequence variability and/or involved in antigen recognition.

30 The term "affinity" as used herein refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g. an antibody) and its binding partner (e.g. an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g. antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_D). Affinity
35 can be measured by common methods known in the art, including those described herein.

The term "effector function" as used herein refers to those biological activities attributable to the Fc-region of an antibody, which vary with the antibody class. Examples of antibody effector functions include: complement dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC).

5

The term "Fc-region" as used herein refers to a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region.

10 The term "effective amount" of an agent, e.g. a pharmaceutical formulation, as used herein refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

15 The term "pharmaceutical formulation" as used herein refers to a preparation, which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

20 The term "treatment" as used herein refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequence of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

25

30 The term "subject" or "individual" as used herein refers to mammal. Mammals include, but are not limited to, domesticated animals (e.g. cows, sheep, cats, dogs, and horses), primates (e.g. humans and non-human primates such as monkeys), rabbits, and rodents (e.g. mice and rats). In certain embodiments, the subject or individual is a human.

35 The term "cancer" and "cancerous" as used herein refers to or describes the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell carcinoma, myeloma, small-cell lung cancer, non-small cell lung cancer, glioma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, acute myeloid leukemia, multiple

myeloma, gastrointestinal (tract) cancer, renal cancer, ovarian cancer, liver cancer, lymphoblastic leukemia, lymphocytic leukemia, colorectal cancer, endometrial cancer, kidney cancer, prostate cancer, thyroid cancer, melanoma, chondrosarcoma, neuroblastoma, pancreatic cancer, glioblastoma multiforme, cervical cancer, brain cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, 5 colon carcinoma, mesothelioma, and head and neck cancer. Particularly preferred cancers that may be treated in accordance with the present invention include those characterized by elevated expression CA IX in tested tissue samples.

10 The term "anti-neoplastic composition" as used herein refers to a composition useful in treating cancer comprising at least one active therapeutic agent capable of inhibiting or preventing tumor growth or function, and/or causing destruction of tumor cells. Therapeutic agents suitable in an anti-neoplastic composition for treating cancer include, but not limited to, chemotherapeutic agents, radioactive isotopes, toxins, cytokines such as interferons, and antagonistic agents targeting cytokines, cytokine receptors or antigens associated with tumor cells.

15 The term "diagnosing" as used herein refers to determining presence or absence of a pathology, classifying a pathology or a symptom, determining a severity of the pathology, monitoring pathology progression, forecasting an outcome of a pathology and/or prospects of recovery.

20 The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein 25 are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein.

30 All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

35 Pharmaceutical formulations

The pharmaceutical composition of the invention comprises a carrier for the antibody, desirably a

pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier can be any suitable pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents, other excipients, or encapsulating substances which are suitable for administration into a human or veterinary patient (e.g. a
5 physiologically acceptable carrier or a pharmacologically acceptable carrier). The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The pharmaceutically acceptable carrier can be co-mingled with one or more of the active components, e.g. a hybrid molecule, and with each other, when more than one pharmaceutically acceptable carrier is present in the composition in a manner so as not to substantially
10 impair the desired pharmaceutical efficacy. "Pharmaceutically acceptable" materials typically are capable of administration to a patient without the production of significant undesirable physiological effects such as nausea, dizziness, rash, or gastric upset. It is, for example, desirable for a composition comprising a pharmaceutically acceptable carrier not to be immunogenic when administered to a human patient for therapeutic purposes.

15 The pharmaceutical composition can contain suitable buffering agents, e.g. acetic acid or a salt thereof, citric acid or a salt thereof, boric acid or a salt thereof, and phosphoric acid or a salt thereof. The pharmaceutical compositions also optionally can contain suitable preservatives, such as benzalkonium chloride, chlorobutanol, parabens, and thimerosal.

20 The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

25 The composition suitable for parenteral administration conveniently comprises a sterile aqueous preparation of the inventive composition, which preferably is isotonic with the blood of the recipient. This aqueous preparation can be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also can be a sterile injectable
30 solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed, such as synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid can be used in the
35 preparation of injectables. Carrier formulations suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in *Remington's Pharmaceutical Sciences*, Mack

Publishing Co., Easton, PA, 22nd edition, 2013.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

5

Diagnostic uses of anti-CA IX antibodies

The present invention provides a diagnostic composition comprising at least one humanized antibody, which specifically recognizes human CA IX, as described above.

10 The present invention further provides, in another aspect, a method for diagnosing a cancer in a subject in need thereof, the method comprising contacting a biological sample derived or obtained from said subject with the diagnostic composition described above, wherein a complex formation beyond a predetermined threshold is indicative of the cancer in said subject.

15 The present invention further provides, in another aspect, a method for determining the expression of CA IX, the method comprising contacting a biological sample with the antibodies thereof described above, and measuring the level of immune complex formation.

20 The present invention further provides, in another aspect, a method for diagnosing a disease or disorder associated with a CA IX protein expression, comprising the steps of incubating a biological sample with a humanized antibody as described above; detecting the bound CA IX protein using a detectable probe; comparing the amount of bound CA IX protein to a standard curve obtained from reference samples; calculating the amount of the CA IX protein in the biological sample from the standard curve; and optionally administering an appropriate treatment to the patient.

25

The present invention further provides, in an aspect, the use of humanized antibodies as described above, for preparation of a diagnostic composition for the diagnosis of a cancer-related disease or disorder.

30 The present invention further provides, in an aspect, a conjugation of the antibodies of the invention to a synthetic molecule. The synthetic molecule can be a label. Labels can be useful in diagnostic applications and can include, for example, contrast agents. A contrast agent can be a radioisotope label such as iodine (^{131}I or ^{125}I), indium (^{111}In), technetium (^{99}Tc), phosphorus (^{32}P), carbon (^{14}C), tritium (^3H), other radioisotope (e.g., a radioactive ion) or a therapeutic radioisotope listed above. Additionally, contrast agents can include radiopaque materials, magnetic resonance imaging (MRI) agents, and
35 ultrasound imaging agents, and any other contrast agents suitable for detection by a device that images a body. The synthetic molecule can also be a fluorescent label, a biologically active enzyme label, a

luminescent label, or a chromophore label.

Therapeutic uses of anti-CA IX antibodies

Any of the anti-CA IX antibodies provided herein may be used in therapeutic methods.

5

In one aspect, an anti-CA IX antibody for use as a medicament is provided. In certain embodiments, an anti-CA IX antibody for use in a method of treatment is provided.

10 In a further aspect, the invention provides for the use of an anti-CA IX antibody in the manufacture or preparation of a medicament.

In a further aspect, the invention provides pharmaceutical formulation comprising any of the anti-CA IX antibodies provided herein. In one embodiment, a pharmaceutical formulation comprises any of the anti-CA IX antibodies provided herein and a pharmaceutically acceptable carrier.

15

Antibodies of the invention can be used either alone or in combination with other agents in a therapy. For instance, an antibody of the invention may be co-administered with at least one additional therapeutic agent.

20 Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody of the invention can occur prior to, simultaneously, and/or following administration of the additional therapeutic agent and/or adjuvant. Antibodies of the invention can also be used in combination with radiation therapy.

25

The invention also provides a method of treating a subject that has a disorder associated with elevated levels of CA IX. Generally, the method includes administering a therapeutically effective amount of an isolated humanized antibody of the invention to the subject. The antibody can be any anti-CA IX antibody of the invention as described above. The antibody can be administered in combination with
30 other agents, e.g. a cytotoxic, cytostatic, anti-angiogenic, immune-checkpoint blocking agent or a therapeutic radioisotope.

Antibodies of the invention (and any additional therapeutic agent) can be administered by any suitable means, including parenteral, intrapulmonary, and intranasal, and, if desired for local treatment,
35 intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. Dosing can be by any suitable route, e.g. by injections,

such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Various dosing schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

5 Antibodies of the invention can be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody need not be, but is
10 optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosage and with administration routes as described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

15 For treatment of disease, the appropriate dosage of an antibody of the invention (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and
20 response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatment. Depending on the type and severity of the disease, about 0.001 mg/kg to 15 mg/kg of antibody can be an initial candidate dosage for administration to the patient, whether, for example by one or more separate administrations, or by continuous infusion. For repeated administration over several days or longer, depending on the condition,
25 the treatment would generally be sustained until a desired suppression of disease symptoms occurs. Such doses may be administered intermittently, e.g. every week or every three weeks. An initial higher loading dose, followed by one or more lower doses may be administered. The progress of this therapy is easily monitored by conventional techniques and assays.

30 EXAMPLES

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

35 Commercially available reagents referred to in the Examples were used according to manufacturer's instructions unless otherwise indicated.

Example 1: Humanized antibodies derived from parental IV/18 mAb

This example demonstrates the construction and characterization of humanized antibody variants
5 CA9hu-2 directed to the proteoglycan domain of CA IX.

The humanization process utilized a combination of standard CDR-grafting technologies coupled with the latest research on antibody structure and up-to-date database of mature human IgG sequences. Firstly, the VII/20 murine antibody variable domains were sequenced. The Complementarity
10 Determining Regions (CDRs) were identified using the Immunogenetics Information System® (IMGT®) or the Kabat numbering system (Lefranc et al, *Nucleic Acid Res* 27:209-212, 1999; Lefranc et al, *Dev Comp Immunol* 27:55-77, 2003; Kabat et al, Sequences of Proteins of Immunological Interest, 5th edition, 1991). For optimal retention of CDR-loop conformation, both numbering systems were used to identify CDRs within murine variable heavy (V_H) as well as variable light (V_L) domains.
15 Subsequently, a number of human framework sequences were identified and used as “acceptor” frameworks (in the text below) for the CDR sequences. Each of the V_H domains was synthesized in-frame with a human IgG isotype constant domain sequence (allotype G1m17,1). Additionally, each of the V_L domains was synthesized in-frame with a human IgK isotype constant domain sequence (allotype Km3). The entire heavy and light chain sequence was codon optimized and the DNA sequence verified.

20 The combination of five V_H and five V_L chains resulted in generation of twenty-five humanized variants having humanized variable domains [marked in the following text as heavy (HC) and light (LC) chain] and human Ig constant domains. In order to characterize twenty-five humanized antibody variants, all sequences were screened for MHC Class II binding epitopes, Fv glycosylation motifs and deamidation
25 motifs.

Murine monoclonal antibody IV/18 (isotype IgG2a) directed to the proteoglycan (PG)-like domain of CA IX was generated in the CA IX-deficient mice (WO2003/100029; Zatovicova et al, *J Immunol Methods* 282, 117-134, 2003). Pre-incubation of hypoxic tumor cells with the IV/18 mAb reduced the
30 number of lung metastases in murine lung colonization model (Figure 1A). Metastatic colonies of fluorescently tagged HT1080-red fluorescent protein (RFP) cells in PBS-perfused murine lungs were imaged *ex vivo* after 12 days using IVIS Caliper imaging system (Figure 1B). Total radiant efficiency reflects the amount of cancer cells in murine lungs. Pre-incubation of HT1080-RFP cells with IV/18 mAb, and subsequent administration of three doses of antibody (50 µg/mouse) during 12 days after the
35 initial tail vein injection (1,500,000 cells/mouse, 10 mice per group) led to a marked decrease in lung colonization by these cells as determined by the fluorescence signal by IVIS. Metastatic colonies were

evaluated shortly after tail vein inoculation, which means that reduced extravasation was a main factor behind decreased metastasis formation. These data indicate a possible benefit of anti-CA IX therapy in attenuation of tumor cell extravasation and metastasis formation.

5 Heavy chain

The murine V_H domain had the sequence below, which does not include the murine signal peptide sequence:

EVQLVETGGGLVQPKGSLKLSCAASGFTFNTNAMHWVRQAPGKGLEWVARIRSKSNNYTTY
YADSVKDRFTISRDDSQSMLYLQMNNLKTEDTAMYYCYCGSWFAYWGQGLVTVSA (SEQ

10 ID NO. 19)

The CDR residues (underlined) were identified using the IMGT numbering system or the Kabat numbering system.

15 CDR1 V_H IV/18 (SEQ ID NO. 1)

GFTFNTNAMH

CDR2 V_H IV/18 (SEQ ID NO. 2)

RIRSKSNNYTTYADSVKD

20

CDR3 V_H IV/18 (SEQ ID NO. 3)

VCGSWFAY

Online databases of Human IgG sequences were searched for comparison to the murine V_H domain
25 using BLAST search algorithms, and candidate human variable domains were selected from the top 200 BLAST results. These were reduced to five candidates (based on a combination of framework homology, maintaining key framework residues and canonical loop structure) and the CDRs were grafted in.

Five acceptor frameworks are:

30

AGP01286 (SEQ ID NO. 20)

EVQLVESGGGLVQPGGSLKLSCAASGFTFSGSAMHWVRQASGKGLEWVGRIRSKANSYATA
YAASVKGRFTISRDDSKNTAYLQMNSLKTEDTAVYYCTRLVGAIPFDYWGQGLVTVSS

35 AEX29087 (SEQ ID NO. 21)

EVQLVESGGGLVQPGGSLKLSCAASGFNFSGPAIHWVRQASGKGLEWVGRIRSKAKNFATAY

AASVKGRFTISRDDSKSTAYLQMNSLKTEDTAVYYCTTTSSSINDYWGQGLTVTVSS

ACS95862 (SEQ ID NO. 22)

QVQLVESGGGVVQPGGSLRLSCAASGFAFSSYGMHWVRQAPGRGLEWVAFIRSDGSNTYY
 5 DSVKGRFTISRDN SKNTLYLQVNSLRAEDTAVYYCAFGGDYYFGYWGQGLTVTVSS

BAC01516 (SEQ ID NO. 23)

EVQLVESGGGVVQPGSLRLSCAASSGFTFSSYGMHWVRQAPGKGLEWVAVISYDGSNKYYA
 10 DSVKGRFTISRDN SKNTLYLQVNSLRAEDTAVYYCAKGRGTGDYWGQGLTVTVSS

IGHV3-73 (SEQ ID NO. 24)

EVQLVESGGGLVQPGGSLKLSCAASGFTFSGSAMHWVRQASGKGLEWVGRIRSKANSYATA
YAASVKGRFTISRDDSKNTAYLQMNSLKTEDTAVYYCTRYYGMDVWGQGT TVTVSS

15 With the CDRs of the murine V_H grafted into these acceptor frameworks they become the humanized variants:

HC1 CA9_{hu}-2 (SEQ ID NO. 9)

EVQLVESGGGLVQPGGSLKLSCAASGFTFNTNAMHWVRQASGKGLEWVGRIRSKSNNTTY
 20 YADSVKDRFTISRDDSKNTAYLQMNSLKTEDTAVYYCVCGSWFAYWGQGLTVTVSS

HC2 CA9_{hu}-2 (SEQ ID NO. 10)

EVQLVESGGGLVQPGGSLKLSCAASGFTFNTNAMHWVRQASGKGLEWVGRIRSKSNNTTY
YADSVKDRFTISRDDSKSTAYLQMNSLKTEDTAVYYCVCGSWFAYWGQGLTVTVSS
 25

HC3 CA9_{hu}-2 (SEQ ID NO. 11)

QVQLVESGGGVVQPGGSLRLSCAASGFTFNTNAMHWVRQAPGRGLEWVARIRSKSNNTTY
YADSVKDRFTISRDN SKNTLYLQVNSLRAEDTAVYYCVCGSWFAYWGQGLTVTVSS

30 HC4 CA9_{hu}-2 (SEQ ID NO. 12)

EVQLVESGGGVVQPGSLRLSCAASGFTFNTNAMHWVRQAPGKGLEWVARIRSKSNNTTY
YADSVKDRFTISRDN SKNTLYLQVNSLRAEDTAVYYCVCGSWFAYWGQGLTVTVSS

HC5 CA9_{hu}-2 (SEQ ID NO. 13)

35 EVQLVESGGGLVQPGGSLKLSCAASGFTFNTNAMHWVRQASGKGLEWVGRIRSKSNNTTY
YADSVKDRFTISRDDSKNTAYLQMNSLKTEDTAVYYCVCGSWFAYWGQGT TVTVSS

Table 1: Homology of humanized variants to murine V_H of IV/18 mAb. In rank order of homology the humanized variants are HC2>HC1>HC5>HC4>HC3.

	Identical amino acids	Consensus amino acids
HC1	90.6%	94.0%
HC2	91.5%	94.9%
HC3	86.3%	93.2%
HC4	88.0%	93.2%
HC5	89.7%	93.2%

5 Light chain

The murine V_L domain had the sequence below, which does not include the murine signal peptide sequence:

DIVMTQSPSSLAMSLGQKVTMSCKSSQSLNSSNQKNYLAWFQKPGQSPKLLVYFTSTRQS
GVPDRFIGSGSGTDFTLTISVQAEDLADYFCQQHYSIPLTFGAGTKLELK (SEQ ID NO. 25)

10

The CDR residues (underlined) were identified using the IMGT numbering system or the Kabat numbering system.

CDR1 VL IV/18 (SEQ ID NO. 4)

15 KSSQSLNSSNQKNYLA

CDR2 VL IV/18 (SEQ ID NO. 5)

FTSTRQS

20 CDR3 VL IV/18 (SEQ ID NO. 6)

QQHYSIPLT

Online databases of Human Ig kappa sequences were searched for comparison to the murine V_L domain using BLAST search algorithms, and candidate human variable domains were selected from the top 200

25 BLAST results. These were reduced to five candidates (based on a combination of framework homology, maintaining key framework residues and canonical loop structure) and the CDRs were grafted in.

Five acceptor frameworks are:

AAW69164 (SEQ ID NO. 26)

DVVMQTQSPDSLAVSLGERVTINCKSSQSVLNTSNNKNYLWVYQQKPGQSPKLLIYLASTREF
GVPDRFSG SGSGTDFTLTISLQAEDVAVYYCQQYHSSPHTFGQGTKLEIK

5 CAI99839 (SEQ ID NO. 27)

DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWFQQKPGQPPNLVIYWASTRES
GVPDRFSG SGSGTDFTLTINSLQAEDVAVYFCQQYYSTPLTFGQGTQVEIK

AMK70392 (SEQ ID NO. 28)

10 DIQMTQSPDSLAVSLGERATINCKASQSVLYSSKNKNYLAWYQQKPGQPPKLLIYRASTRDSG
VPDRFSG SGSGTDFTLTISLQAEDVAVYFCQQYYSTPQTFGQGTKVEIK

ALV87854 (SEQ ID NO. 29)

DIVMTQSPDSLAVSLGERATINCKSSQSVLYRSKNKNYLAWFQQKPGQPPKLLIYSTSTRASG
15 VPDRFTG SGSGTDFTLTISLQAEDVAVYYCQQYYITPYTFGQGTKLEIK

IGKV4-1 (SEQ ID NO. 30)

DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIYWASTRESG
VPDRFSGSGSGTDFTLTISLQAEDVAVYYCQQYYSTPYTFGQGTKLEIK

20

With the CDRs of the murine V_L grafted into these acceptor frameworks they become the humanized variants:

LC1 CA9_{hu}-2 (SEQ ID NO. 14)

25 DVVMQTQSPDSLAVSLGERVTINCKSSQSLLNSSNQKNYLAWYQQKPGQSPKLLIYFTSTRQSG
VPDRFSGSGSGTDFTLTISLQAEDVAVYYCQQHYSIPLTFGQGTKLEIK

LC2 CA9_{hu}-2 (SEQ ID NO. 15)

DIVMTQSPDSLAVSLGERATINCKSSQSLLNSSNQKNYLAWFQQKPGQPPNLVIYFTSTRQSG
30 VPDRFSGSGSGTDFTLTINSLQAEDVAVYFCQQHYSIPLTFGQGTQVEIK

LC3 CA9_{hu}-2 (SEQ ID NO. 16)

DIQMTQSPDSLAVSLGERATINCKSSQSLLNSSNQKNYLAWYQQKPGQPPKLLIYFTSTRQSG
VPDRFSGSGSGTDFTLTISLQAEDVAVYFCQQHYSIPLTFGQGTKVEIK

35

LC4 CA9_{hu}-2 (SEQ ID NO. 17)

DIVMTQSPDSLAVSLGERATINCKSSQSLLNSSNQKNYLAWFQQKPGQPPKVLIIYFTSTRQSG
VPDRFTGSGSGTDFTLTISSLQAEDVAVYYCQQHYSIPLTFGQGTKLEIK

LC5 CA9hu-2 (SEQ ID NO. 18)

5 DIVMTQSPDSLAVSLGERATINCKSSQSLLNSSNQKNYLAWYQQKPGQPPKLLIYFTSTRQSG
VPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQHYSIPLTFGQGTKLEIK

Table 2: Homology of humanized variants to murine V_L of IV/18 mAb. In rank order of homology the humanized variants are LC1>LC4=LC5>LC3>LC2.

	Identical amino acids	Consensus amino acids
LC1	85.8%	94.7%
LC2	82.3%	90.3%
LC3	84.1%	92.0%
LC4	85.0%	92.9%
LC5	85.0%	92.9%

10

Humanization check

The humanized variants were checked to determine whether they had been humanized in accordance with World Health Organization's (WHO) definition of humanized antibodies: The variable domain of a humanized chain has a V region amino acid sequence which, analyzed as a whole, is closer to human than to other species (assessed using the IMGT® DomainGapAlign tool) (Ehrenmann et al, *Nucleic Acids Res* 38, D301-307, 2010).

15

Table 3: WHO's assigned antibody international nonproprietary names (INN) for the murine and humanized variants. VH0/VL0 is the murine sequence of IV/18 and HC1-5/LC1-5 are the humanized variants.

20

Sequence Name	Species	Gene and Allele	Domain Label	% Identity	Overlap	WHO INN Designation
VH0	Mus musculus	IGHV10S3*01	VH	98.0	99	Mouse
HC1	Homo sapiens	IGHV3-73*01	VH	90.8	98	Humanized
HC2	Homo sapiens	IGHV3-73*01	VH	89.8	98	Humanized
HC3	Homo sapiens	IGHV3-30*02	VH	84.7	98	Humanized
HC4	Homo sapiens	IGHV3-30*01	VH	85.7	98	Humanized
HC5	Homo sapiens	IGHV3-73*01	VH	90.8	98	Humanized

VL0	Mus musculus	IGKV8-24*01	V-Kappa	95.0	101	Mouse
LC1	Homo sapiens	IGKV4-1*01	V-Kappa	89.1	101	Humanized
LC2	Homo sapiens	IGKV4-1*01	V-Kappa	87.1	101	Humanized
LC3	Homo sapiens	IGKV4-1*01	V-Kappa	90.1	101	Humanized
LC4	Homo sapiens	IGKV4-1*01	V-Kappa	89.1	101	Humanized
LC5	Homo sapiens	IGKV4-1*01	V-Kappa	92.1	101	Humanized

T-cell epitope screening

Presentation of peptide sequences in the groove of major histocompatibility complex (MHC) Class II molecules leads to activation of CD4⁺ T-cells and an immunogenic response. In order to reduce this response, therapeutic proteins can be designed to avoid the incorporation of “T-cell epitopes” that can activate T-cells by reducing the affinity of binding to the MHC Class II molecules.

The original murine antibody V_H and V_L and the humanized variant sequences were screened for MHC II binding peptides to determine that the humanization process had removed peptide sequences with high affinity using *in silico* algorithms. The following 8 alleles represent over 99% of the world's population and are the standard allele set used for prediction of MHC Class II epitopes: DRB1*01:01; DRB1*03:01; DRB1*04:01; DRB1*07:01; DRB1*08:02; DRB1*11:01; DRB1*13:02; DRB1*15:01 (Nielsen et al, *BMC Bioinformatics* 8:238, 2007; Wang et al, *BMC Bioinformatics* 11:568, 2010; Gonzalez-Galarza et al, *Nucleic Acid Research* 39, D913-D919, 2011; Greenbaum et al, *Immunogenetics* 63(6): 325-35, 2011).

For the V_H domain, all the humanized variants performed well in terms of the T-cell epitope screen with HC3 predicted to have the smallest germline T-cell epitope. Analysis by homology alone ranked HC2 and HC1 as closest to the parental murine sequence.

For the V_L domain, all the humanized variants were ranked equally from the T-cell epitope screen. By homology alone, LC1 and LC4 were ranked highest.

Screening for post-translational modifications:

Fv Glycosylation

The N-linked glycosylation motif is NXS/T where X is any amino acid except proline. This motif NYT is present in the murine CDR2 V_H variant and, as CDRs were grafted in their entirety, has been carried through to all the humanized variants.

The motif NSS is present in the murine CDR1 of the light chain. Again, this motif was carried through to all the humanized variants.

Deamidation

The amino acid motifs SNG, ENN, LNG, and LNN can be prone to deamidation of asparagines to aspartic acid (Chelius et al, *Anal Chem* 77(18): 6004-11, 2005). Asparagine within other motifs is less prone to deamidation. None of these four motifs are present in the murine or humanized variants of IV/18 mAb V_H or V_L.

The foregoing data demonstrate the generation of twenty-five humanized variants (marked in the following text as CA9hu-2_HCxLCx) having humanized variable domains and human Ig constant domains.

Example 2: Characterization of the binding capacity of the humanized antibodies

This example demonstrates the desirable binding properties of twenty-five humanized variants of CA9hu-2 for carbonic anhydrase IX.

To evaluate the antigen-binding specificity, all twenty-five humanized variants of CA9hu-2 were subjected to enzyme-linked immunosorbent assay (ELISA) using either CA IX-positive or CA IX-negative antigen. Antigens were prepared from stably transfected C-33a cell line expressing CA IX (C-33a_CA IX) and parental mock-transfected C-33a cells without CA IX expression (C-33a_neo).

Proteins were extracted from the cell monolayer with RIPA lysis buffer (0.1% deoxycholate, 1% Triton X-100 and protease inhibitor cocktail in PBS). Protein concentrations were determined by bicinchoninic acid assay (ThermoFisher Scientific, Waltham, MA USA) according to the manufacturer's instructions. Protein extracts were diluted to final concentration 0.2 mg/ml in PBS. Protein concentration of antigen samples used in screening of antigen-antibody specific interaction tested by ELISA meets the requirements for low detergent content that could otherwise interfere throughout the analysis. 50 µl of either CA IX-positive or CA IX-negative antigen was coated on the surface of microplate wells

overnight at 37°C. After washing with PBS-T 0.05% Tween-20 in PBS pH7.2, 50 µl of all humanized variants of CA9hu-2 (diluted to concentration 5 µg/ml in 10% FCS in PBS-T) were added and incubated for 2 h at room temperature. Peroxidase-labeled swine anti-human IgG (diluted 1:5000 in 10% FCS in PBS-T; Sigma-Aldrich, St. Louis, MO USA) was used as detector. Parental IV/18 antibody (marked as "mouse Ab") as well as chimeric HC0LC0 antibody (having the murine variable domains and the human Ig constant domains) were used as reference samples. Results are expressed as a fold of induction and are calculated as O.D. values of absorbance measured at 492 nm from CA IX-positive antigen/O.D. values of absorbance measured at 492 nm from CA IX-negative antigen.

- Figure 2 demonstrates specific and effective binding of twenty-five humanized variants of anti-CA IX antibodies. A majority of CA9hu-2 antibody variants from showed even higher specificity than the chimeric variant.

The foregoing results demonstrate that humanized antibody variants CA9hu-2 retain desirable specificity for their antigen and can be used to specifically distinguish tumor cells expressing CA IX.

Example 3: ADCC (antibody-dependent cell-mediated cytotoxicity) and CDC (complement-dependent cytotoxicity) effects of the humanized antibodies

- This example demonstrates the desirable participation of humanized variants of anti-CA IX antibodies in antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).

ADCC

- To evaluate the ability of humanized antibody variants to mediate the cytotoxic effect, ADCC Reporter Bioassay System (Promega, Madison WI, USA) was applied. ADCC Reporter Bioassay System represents a bioluminescence reporter array for quantifying biological activity on pathway activation by therapeutic antibody drugs in an ADCC mechanism of action (Chung et al., *Monoclonal Antibodies* 4:326–40, 2012). It uses engineered Jurkat cells stably expressing the FcγRIIIa receptor, V158 high affinity variant, and NFAT (nuclear factor of activated T-cells) response element driving expression of firefly luciferase as effector cells. Thus, ADCC mechanism of action is quantified through the luciferase production as a result of NFAT activation.

- ADCC reporter assay was performed according to the manufacturer's instructions using C-33a_CA IX as well as C-33a_neo cells. Both cell types (12,500 cells/well) were plated onto sterile 96-well plate and incubated in culture medium overnight at 37°C. Humanized antibody variants CA9hu-2 were diluted to

1 $\mu\text{g}/\text{ml}$ in PBS and 75,000 of effector cells (according to the recommended effector:target ratio 6:1) were used per well. After 6 hours of incubation, detection of firefly luciferase was performed using Bio-Glo™ Luciferase Assay Reagent (Promega). Mixture of sample with ADCC assay buffer and effector cells without adding the humanized antibody is marked as "no Ab". Mixture of sample without antibody and effector cells is marked as "no Ab, no EC", and serves as "plate background". Results are expressed as luminescence in relative luminescence units (RLU) and are calculated as a fold of induction (RLU induced by humanized antibody variant/RLU no Ab).

As shown in Figure 3, CA9hu-2 variants exhibited high luminescence signal and thus, high cytotoxicity against C-33a_CA IX expressing cells. According to the fact that the RLU of samples were 100 times higher than the plate background RLU (marked as "no Ab, no EC cells"), there was no need to subtract plate background from sample RLU.

Evaluation of the effector functions of humanized antibody variants of the invention (via ADCC reporter assay), as well as their antigen-binding specificity (via ELISA) resulted in the selection of the following antibody variants: HC3LC1, HC3LC2, HC4LC1, HC4LC2 and HC4LC5 and the last one was selected as the best candidate.

To prove antibody-dependent cell-mediated cytotoxicity on cancer cell naturally expressing CA IX, we analyzed triple-negative breast cancer (TNBC) cell line BT-20 as well as glioblastoma cell line 8-MG-BA. One day before analysis, cancer cell lines were pre-incubated in hypoxia to ensure the highest CA IX expression. After hypoxic pre-incubation, 12,500 cells/well were plated onto sterile 96-well plate and incubated in culture medium overnight at 37°C. Similarly as in case of ADCC screening, ADCC reporter assay was performed according to the manufacturer's instructions. Humanized antibody variant CA9hu-2_HC4LC5 was diluted to 1 $\mu\text{g}/\text{ml}$ in PBS and 75,000 of effector cells (according to the recommended effector:target ratio 6:1) were used per well. After 6 hours of incubation, detection of firefly luciferase was performed using Bio-Glo™ Luciferase Assay Reagent.

Table 4: ADCC activity of humanized antibody variant CA9hu-2_HC4LC5 analyzed using cancer cell lines derived either from glioblastoma (8-MG-BA), or breast cancer (BT-20). Cancer cells incubated in the absence of humanized antibody are marked as "no Ab". Data in the table are expressed as luminescence in relative luminescence units (RLU) and represent mean $\pm$ standard deviation values.

	no Ab		CA9hu-2_HC4LC5	
	mean	stdev	mean	stdev
8-MG-BA	6504.8 (100%)	3061.6	27920.3 (429.2%)	2722.4

BT-20	1628.0 (100%)	171.0	3296.3 (202.5%)	780.9
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The ADCC reporter assay enables us to analyze an earlier point in ADCC pathway through the NFAT-mediated activation of gene transcription in the effector cells. Table 4 clearly shows that the humanized antibody variant CA9hu-2_HC4LC5 retains the ability to activate ADCC pathway and to mediate cytotoxic effect on target cells expressing CA IX. In comparison with no Ab treatment, ADCC reporter activity was elevated after incubation of both cancer cells with CA9hu-2_HC4LC5. The highest induction (> 4-fold) was observed in glioblastoma 8-MG-BA cells.

CDC

To evaluate the ability of humanized antibody variants to participate on CDC, Cell Titer Blue Viability Assay Kit (Promega) was applied. Cell Titer Blue Viability Assay provides a homogenous fluorometric method for estimating the number of viable cells via indicator dye resazurin and thus, measurement of metabolic capacity of cells as an indicator of their viability. In viable cell, resazurin is reduced into highly fluorescent resorufin generating a fluorescent signal, which can be measured (530_{Ex}/590_{Em}). Thus, the fluorescent signal from the Cell Titer Blue Reagent is proportional to the number of viable cells.

Cell Titer Blue assay was performed according to the manufacturer's instructions using C-33a_CA IX as well as C-33a_neo cells. Both cell types (200,000 cells/well) were plated onto sterile 96-well plate and incubated in culture medium overnight at 37°C. Humanized antibody variant CA9hu-2_HC4LC5 diluted to 5 µg/ml was added to both cell lines. Rabbit complement serum (10% from the total volume, BAG Health Care, Lich, Germany) was added to each well, mixed and incubated. Cell viability was quantified and analyzed after 24 h. Results are expressed as fluorescence measured at 530_{Ex}/590_{Em}.

Table 5: Effect of humanized antibody CA9hu-2_HC4LC5 on the viability of analyzed cells +/- expressing CA IX (C-33a_CA IX versus C-33a_neo) in the presence of complement determined via Cell Titer Blue Viability Assay. Cancer cells incubated in the absence of humanized antibodies are marked as "no Ab". Data in the table are expressed as fluorescence and represent mean ± standard deviation values.

	C-33a_CA IX		C-33a_neo	
	mean	stdev	mean	stdev
no Ab	3393.5 (100%)	389.6	3096.5 (100%)	289.2
CA9hu-2_HC4LC5	2159 (63.6%)	240.4	2807 (90.7%)	193.7

Table 5 depicts the ability of humanized antibody variant CA9hu-2_HC4LC5 to affect the viability of CAIX-expressing cells in the presence of complement. After 24 h incubation with CA9hu-2_HC4LC5

in the presence of complement, C-33a_CA IX cells showed only 64% viability. The viability of C-33a_neo cells was almost not affected.

The foregoing results demonstrate that humanized antibody variants CA9hu-2 can be used to specifically distinguish and consequently mediate the cytotoxic response via ADCC or CDC on tumor cells expressing CA IX.

Example 4: ADCC effects of the humanized antibodies mediated via peripheral blood mononuclear cells in three-dimensional spheroids

This example demonstrates the desirable property of humanized antibody variant to mediate the ADCC activity in three-dimensional cultures.

Three-dimensional (3D) cultures like multicellular spheroids are increasingly used in basic research to study cell biology and physiology under more realistic conditions. To validate the efficiency of humanized antibodies in mediating the cytotoxic effect on cancer cells in 3D system, we performed co-cultivation of TNBC BT-20 cells and peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from human peripheral blood (healthy donor) by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). In order to visualize the PBMCs within spheroids, isolated cells were stained with CellBrite™ Orange Cytoplasmic membrane Labelling Dye (Biotium, Hayward CA, USA). BT-20 cells were firstly stained with CellBrite™ Green Cytoplasmic Membrane Labelling Dye and subsequently, BT-20 spheroids were pre-formed from 10,000 cells per 25 µl of culture medium in drops hanging on the lid of tissue culture dish for 7 days at 37°C. After 10 days of cultivation, pre-stained PBMC/Orange cells (2,000,000) were added together with forty BT-20 spheroids into Petri dishes and mixed with humanized antibody CA9hu-2_HC4LC5 (25 µg/ml). Spheroids cultivated without humanized antibodies were treated with PBS (negative control). The distribution of pre-stained PBMC cells within spheroids was analyzed after 3 days of treatment by confocal laser scanning microscope Zeiss LSM510 Meta.

To validate the effect of prolonged (11 days) co-cultivation with PBMC cells, BT-20 spheroids were collected and fixed in Carnoy's fixative solution for 2 hours and embedded into paraffin according to the standard histological procedures. Spheroid blocks were sliced into 4 µm thin sections and subjected to immunohistochemical staining using DAKO Cytomation EnVision+ System-HRP (DAB; DAKO, Glostrup, Denmark) according to the manufacturer's recommendation. Primary antibody specific for CA IX mouse monoclonal M75 was diluted (1 µg/ml) and incubated for 60 min at RT. Staining was visualized with DAB solution. Finally, the sections were counterstained with Mayer's hematoxylin. The

stained sections were examined with LeicaDM4500B microscope and photographed with Leica DFC480 camera.

Figure 4 clearly shows the incorporation of pre-stained PBMCs within BT-20 spheroids. Visualization by confocal microscopy revealed more intensive incorporation of PBMCs after the treatment with humanized antibody CA9hu-2_HC4LC5. The percentage of positive signal from PBMCs was evaluated for the entire BT-20 spheroid by ImageJ 1.38x software (Rasband, W.S., ImageJ, NIH, Bethesda MD, USA). The proportion of PBMCs-stained pixels in the entire spheroid was 6.624% after the treatment with CA9hu-2_HC4LC5. In case of untreated spheroids, only 2.781% of PBMC-positive pixels were revealed. The effect of prolonged treatment of BT-20 spheroids with PBMCs and humanized antibody was examined after 11 days. As shown in Figure 4, significant morphological changes were observed after the co-cultivation of BT-20 spheroids with human PBMCs and treatment with humanized variant CA9hu-2_HC4LC5. The immunohistochemical analysis was employed to visualize the CA IX expression across the BT-20 spheroids.

The foregoing results demonstrate that the humanized antibody CA9hu-2_HC4LC5 of the invention facilitates the ADCC response in 3D cultures-spheroids. This is the first demonstration of an aforementioned effect described using PG-specific humanized antibodies.

Example 5: ADCP (antibody-dependent cell-mediated phagocytosis) effect of the humanized antibodies

This example demonstrates the desirable participation of humanized antibody in antibody-dependent cell-mediated phagocytosis (ADCP).

To evaluate the ability of humanized antibody variant to mediate phagocytosis, ADCP Reporter Bioassay System (Promega) was applied. The FcγRIIa-H ADCP Reporter Bioassay is a bioluminescent cell-based assay to measure the potency and stability of antibodies and other biologics with Fc domains that specifically bind and activate FcγRIIa. It uses engineered Jurkat T cells expressing the FcγRIIa receptor, H131 high affinity variant, and NFAT response element driving expression of firefly luciferase as effector cells. Thus, ADCP mechanism of action is quantified through the luciferase production as a result of NFAT activation.

ADCP reporter assay was performed according to the manufacturer's instructions using BT-20, C-33a_CA IX as well as C-33a_neo cells. BT-20 cells were pre-incubated in hypoxia for 48 h to ensure the highest CA IX expression. One day before analysis, all three cell lines (12,500 cells/well) were plated onto sterile 96-well plate and incubated in culture medium overnight at 37°C. Humanized

antibody variant CA9hu-2_HC4LC5 was diluted to 2 µg/ml in PBS and 75,000 of effector cells (according to the recommended effector:target ratio 6:1) were used per well. Chimeric antibody HC0LC0 (having the murine variable domains and Ig constant domains) diluted to 2 µg/ml was used as reference sample. After 6 hours of incubation, detection of firefly luciferase was performed using Bio-Glo™ Luciferase Assay Reagent (Promega). Mixture of sample with ADCP assay buffer and effector cells without adding the humanized antibody is marked as "no Ab". Mixture of sample without antibody and effector cells is marked as "no Ab, no EC", and serves as "plate background". Results are expressed as luminescence in relative luminescence units (RLU). According to the fact that the RLU of samples were 100 times higher than the plate background RLU (marked as "no Ab, no EC cells"), there was no need to subtract plate background from sample RLU.

Table 6: Phagocytic potency of humanized antibody variant CA9hu-2_HC4LC5 analyzed on CA IX-expressing (C-33a_CA IX and hypoxia pre-incubated BT-20) cells in comparison with CA IX-negative (C-33a_neo) cells. Chimeric antibody HC0LC0 (having the murine variable domains and the human Ig constant domains) was used as reference samples. Cancer cells incubated in the absence of antibodies are marked as "no Ab". Data in the table are expressed as luminescence in relative luminescence units (RLU) and represent mean ± standard deviation values.

	C-33a_neo		C-33a_CA IX		BT-20	
	mean	stdev	mean	stdev	mean	stdev
no Ab	376.7	20.4	435.3	52.5	594.3	19.2
	(100%)		(100%)		(100%)	
CA9hu-2_HC4LC5	410.3	36.1	808.7	42.8	1029	85.2
	(108.9%)		(185.8%)		(173.1%)	
HC0LC0	379	9.8	682.7	78.9	940.3	51.2
	(100.6%)		(156.8%)		(158.2%)	

As shown in Table 6, almost no phagocytic activity was observed when CA IX-negative C-33a_neo cell were used as target cells. These results are independent whether C-33a cells were incubated in the presence or absence of CA IX-specific antibody. The highest phagocytic potency was acquired after the incubation of CA IX-expressing cancer cells in the presence of humanized antibody variant CA9hu-2_HC4LC5. In comparison with no Ab treatment, the highest luminescence signal was observed in C-33a_CA IX cells as well as hypoxia pre-incubated BT-20 cells. ADCP reported assay also revealed that the phagocytic potency of humanized antibody was even higher than chimeric antibody HC0LC0.

The foregoing results demonstrate that humanized antibody variant CA9hu-2_HC4LC5 can be used to specifically recognize and consequently mediate phagocytosis of cancer cells expressing CA IX.

Considering the fact that ADCP is an important mechanism of action of therapeutic antibodies, the phagocytic potency of the humanized antibody of the invention represents an extraordinary beneficial property. This the first demonstration of an aforementioned effect described using PG-specific humanized antibodies.

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Example 6: Effect of humanized antibodies on invasion of cancer cells

This example demonstrates the extraordinary property of humanized antibody to inhibit an invasion of cancer cells.

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The metastatic cascade can be divided into three main processes: invasion, intravasation and extravasation. Murine lung colonization model enabled us to reveal a possible benefit of anti-CA IX therapy in attenuation of cancer cell extravasation and metastasis formation (Figure 1). Reduced number of lung metastases which were observed with hypoxic tumor cells pre-incubated with parental IV/18 mAb *in vivo* prompted us to investigate the effect of humanized antibodies on cancer cells invasion *in vitro*. For this purpose, we performed the xCELLigence cell index impedance measurements using CIM-Plate16 and RTCA DP station according to the instructions of the supplier (Roche, Basel, Switzerland). C-33a_CA IX cells were resuspended at the density of 40,000 cell/ml in serum-free medium in the presence or absence of humanized antibody variant CA9hu-2_HC4LC5 diluted to 25 µg/ml. After addition to the Matrigel-coated top chamber of the CIM-Plate, C-33a_CA IX cells were allowed to migrate towards bottom chamber containing medium with 10% FCS as a chemoattractant. The CIM-Plate was placed in the RTCA DP station and invasion was monitored under hypoxic conditions every 15 min for 60 h.

Figure 5 demonstrates the ability of humanized antibody to inhibit invasion of cancer cells in comparison with no Ab treatment. Invasion ability of C-33a cells expressing CA IX was significantly reduces after the treatment with humanized antibody variant CA9hu-2_HC4LC5.

The foregoing results demonstrate the ability the humanized antibody variant CA9hu-2_HC4LC5 of the invention to inhibit invasion of CA IX-expressing C-33a cells. Considering the fact that inhibition of cancer cell invasion could lead to limited tumor progression, and consequently, to reduced mortality of cancer patients, this mechanism of action represents an extraordinary beneficial property. Additionally, this the first demonstration of an aforementioned effect described using PG-specific humanized antibodies.

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Example 7: Effect of humanized antibodies on cell viability

This example demonstrates the desirable property of humanized antibody variant not to affect cell viability.

- 5 To estimate the effect of humanized antibodies on cell viability, Cell Titer Blue Viability assay was performed similarly as in Example 3 (in the absence of complement), and according to the manufacturer's instructions using C-33a_CA IX as well as C-33a_neo cells. Both cell types (200,000 cells/well) were plated onto sterile 96-well plate and incubated in culture medium overnight at 37°C. Humanized antibody variant CA9hu-2_HC4LC5 diluted to 5 µg/ml was added to both cell lines. Cell viability was measured after 24 h. The fluorescent signal from the Cell Titer Blue Reagent is proportional to the number of viable cells.

Table 7: Effect of humanized antibody CA9hu-2_HC4LC5 on the viability of analyzed cells +/- expressing CA IX (C-33a_CA IX versus C-33a_neo) determined via Cell Titer Blue Viability Assay.

- 15 Cancer cells incubated in the absence of humanized antibodies are marked as "no Ab". Data in the table are expressed as fluorescence and represent mean ± standard deviation values.

	C-33a_CA IX		C-33a_neo	
	mean	stdev	mean	stdev
no Ab	7398 (100%)	192.3	7594.5 (100%)	34.6
CA9hu-2_HC4LC5	7842 (106%)	291.3	8263 (108.8%)	718.4

As shown in Table 7, Cell Titer Blue Viability Assay revealed that the viability of treated C-33a cells, neither CA IX-positive nor CA IX-negative, was not affected after 24 h.

- 20 The foregoing data demonstrate that the humanized antibody variant of the invention, CA9hu-2_HC4LC5, does not exert toxic effect on treated cancer cells.

Example 8: Prediction of the humanized antibody safety via cytokine release assay ("cytokine storm")

- 25 This example demonstrates the desirable property of selected humanized antibody variant in an *in vitro* cytokine release assay.

- 30 Cytokine release assays (CRAs) are best used for hazard identification but not risk quantification, and can help to understand potential risk and inform risk mitigation strategies (Vidal et al, *Cytokine* 51: 213-215, 2010). CRAs could be used to rank therapies by predicted safety and may provide additional data on the potential mechanisms for cytokine release in humans. Drugs targeting membrane-bound antigens

or receptors carry a greater risk of inducing cytokine release than those targeting soluble molecules. Measurement of cytokine release is performed in comparison to drug compounds known to cause high and low responses as controls. The assay results can inform hazard identification and relative risk estimation. The following cytokines are measured: interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10, interferon γ (IFN γ), and tumor-necrosis factor α (TNF α) for a complete cytokine response profile (Suntharalingam et al, *N Engl J Med* 355(10): 1018-1028, 2006).

To evaluate cytokine release associated with humanized antibodies of the invention, CRA (ProImmune Ltd., Oxford, UK) was performed and analyzed using fresh whole blood samples from 20 healthy donors. Undiluted whole blood samples were incubated in the presence of tested antibodies at various concentrations (100, 10, 1, and 0.1 μ g/ml) at 37°C for 24 h. Measurement of cytokine release was performed by ProArray Ultra® microarray assay. All cytokines were quantified against a standard curve of known concentrations. Two control antibodies (Erbix®/Cetuximab as a low response control and Campath®/Alemtuzumab as a high response control) were also included in CRA. PBS was used as an assay negative control. The assay positive control staphylococcal enterotoxin B (SEB) was used to elicit elevated cytokine release for all donors and thus, to confirm that the assay is performed within expectations.

Table 8 demonstrates results from cytokine release assay with the median values (pg/ml) for each drug/dose combination. The median response to SEB for all cytokines was greater than zero, demonstrating that donor cells have the functional capacity to produce cytokines. While Erbix® elicited low levels of cytokine release overall, application of Campath® led to elevated levels of IL-6, IL-8 and IFN γ release in the majority of donors (clinically this drug is associated with cytokine release syndrome). Similarly as in case of Erbix®, humanized antibody variant CA9hu-2_HC4LC5 had no effect on the release of tested cytokines, which indicates a beneficial property of this particular humanized variant.

Table 8: Reactivity of CA9hu-2_HC4LC5 in Cytokine Release Assay. Two control antibodies (Erbix® as a low response control and Campath® as a high response control) were also included and analyzed. PBS was used as an assay negative control and staphylococcal enterotoxin B (SEB) as positive control. Results are expressed as median cytokine levels (pg/ml) for each drug/dose combination.

	IL-2	IL-4	IL-6	IL-8	IL-10	IFN γ	TNF α
	(pg/ml)	(pg/ml)	(pg/ml)	(pg/ml)	(pg/ml)	(pg/ml)	(pg/ml)
PBS	0	0	0	0	0	0	0
SEB	38106.5	59.7	26016.9	4632.5	442.0	37354.4	3609.9

CA9hu- 2_HC4LC5 ($\mu\text{g/ml}$)	100	0	0	4594.3	51.5	4.8	0	0
	10	0	0	508.6	0	0	0	0
	1	0	0	0	0	0	0	0
	0.1	0	0	0	0	0	0	0
Erbitux® ($\mu\text{g/ml}$)	100	0	0	0	0	0	0	0
	10	0	0	4.6	2.9	0	0	0
	1	0	0	0	0	0	0	0
	0.1	0	0	0	0	0	0	0
Campath® ($\mu\text{g/ml}$)	100	0	0	3307.4	93.1	0	753.3	0
	10	0	0	1728.9	38.8	0	1642.9	0
	1	0	0	1733.5	82.3	0	2806.9	36.8
	0.1	0	0	851.1	37.4	0	1800.1	0

The foregoing results demonstrate the desirable property of humanized antibody variant CA9hu-2_HC4LC5 not to induce cytokine response.

5 Example 9: Effects of humanized antibodies on multicellular aggregation

This example demonstrates the extraordinary property of humanized antibody variant to inhibit a multicellular aggregation during detached conditions.

- 10 To validate the effect of humanized antibody of the invention on the ability of treated cells to form multicellular aggregates, we performed multicellular aggregation analysis. The non-ionic acid poly(2-hydroxyethyl methacrylate) (poly-HEMA; Sigma-Aldrich) which inhibits matrix deposition and cell attachment was dissolved in 99% ethanol at 10 mg/ml. 6-well tissue culture plates were coated with 0.5 ml of poly-HEMA solution, allowed to dry, washed with PBS and stored at 4 °C. C-33a_CA IX cells
- 15 (400,000 cells/well) were added to poly-HEMA-coated wells and cultivated in the presence or absence of humanized antibody variant CA9hu-2_HC4LC5 (30 $\mu\text{g/ml}$) for 24 and 72 h. To evaluate the ability of C-33a_CA IX cells to form multicellular aggregates, images from either treated and untreated cells were acquired and the accumulated pixel density was measured using the ImageJ software. At the end of the longer treatment (72 h), C-33a_CA IX cells were recovered, centrifuged, and subsequently
- 20 analyzed via flow cytometry using propidium iodide to stain dead cells.

Multicellular aggregation of cancer cells during extracellular matrix (ECM)-detachment represents an efficient mechanism for anoikis inhibition. Figure 6 clearly shows that the humanized antibody variant of the invention inhibits the ability of C-33a_CA IX cells to form multicellular aggregates during

detached condition on poly-HEMA coated dishes.

To validate the enhanced sensitivity of C-33a_CA IX cells to anoikis after the treatment with humanized antibody, we performed flow cytometry and propidium iodide staining.

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Figure 7 shows that humanized antibody variant CA9hu-2_HC4LC5 affects the viability of C-33a_CA IX-treated cells after 72 h grown in detached conditions. The percentage of dead cells treated with CA9hu-2_HC4LC5 was 35.1%. Only 15.7% of dead cells were observed in case of C-33a_CA IX cells without antibody treatment ("negative control").

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The foregoing data demonstrate the ability of humanized antibody of the invention to inhibit multicellular aggregation of CA IX-expressing C-33a cancer cells (during detached conditions) and subsequently, to enhance their sensitivity to anoikis. This mechanism of action represents an extraordinary beneficial property. Moreover, CA9hu-2_HC4LC5 reduced the viability of treated cells and the percentage of dead cells cultivated in the presence of humanized antibody was higher when comparing with control cells.

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Example 10: Effect of the humanized antibody on proteome/secretome as well as transcriptome of the affected cells

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This example demonstrates the unexpected properties of humanized antibody to affect the cytokine pattern as well as the expression of proteins responsible for an evasion of antitumor immunity.

The impact of the humanized antibody variants on the cytokine pattern *in vitro* was analyzed using Proteome Profiler Cytokine Array (PPA; R&D Systems, Inc.). PPA is a rapid, sensitive, and economic tool to simultaneously detect cytokine differences between samples on nitrocellulose membranes. PPA Cytokine Array was performed using TNBC cell line BT-20 incubated in hypoxia for 72 h according to the manufacturer's instructions. BT-20 cells were seeded onto 12-well plate (200,000 cell/well) and incubated in the presence or absence of humanized antibody CA9hu-2_HC4LC5 (50 µg/ml). Cell lysates (BT-20_proteome) as well as cell culture supernatants (BT-20_secretome) were subsequently prepared and analyzed. Diluted samples were incubated with PPA membranes overnight, washed (to remove unbound material) and incubated with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were then applied and developed. Signal is produced at each capture spot corresponding to the amount of protein bound. Pixel densities on developed X-ray films were collected and analyzed by ImageJ 1.38x software. The average signal (pixel density) of the pair of duplicate spots representing each sample was determined and subsequently, an averaged background

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signal was subtracted from each spot. Results are expressed as a fold change after antibody treatment (Table 9).

Table 9: Proteome Profiler Cytokine analysis of VEGF and IL-8 in cell lysates from BT-20 cells (BT-20_proteome) as well as culture medium from BT-20 cells (BT-20_secretome) after the treatment with humanized antibody CA9hu-2_HC4LC5 for 72 h in hypoxic conditions. Cancer cells incubated in the absence of humanized antibodies are marked as "no Ab". Results are expressed as a fold change after antibody treatment.

	BT-20_proteome		BT-20_secretome	
	VEGF	IL-8	VEGF	IL-8
no Ab	1	1	1	1
CA9hu-2_HC4LC5	0.83	0.59	0.86	0.68

- As expected, Proteome Profiler Cytokine Array revealed several differently affected cytokines (up- or down-regulated), either expressed (BT-20_proteome) or released (BT-20_secretome). The expression of IL-8 and VEGF was consistently down-regulated in case of secretome, as well as proteome after the incubation of BT-20 cells in the presence of humanized antibody variant CA9hu-2_HC4LC5 (Table 9).
- To validate the effect of humanized antibody of the invention on the transcriptional profile of treated cells, we employed reverse transcription-quantitative real-time PCR (RT-qPCR). The expression of genes coding for proteins responsible for an evasion of antitumor immunity was quantified and analyzed using TNBC cell line BT-20. BT-20 cells pre-formed in spheroids were firstly exposed to a chemotherapeutic drug, doxorubicin (DOX; Sigma-Aldrich) at concentration 1 μ M for 4 days, following the 3 days cultivation without DOX. Treatment with humanized antibody variant CA9hu-2_HC4LC5 (25 μ g/ml) was performed for the whole time period (7 days). At the same time, BT-20 cells without pre-treatment with DOX were exposed to humanized antibody for 7 days. Total RNA was extracted using TRIzol (ThermoFisher Scientific) and subsequently transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City CA, USA). Quantitative-PCR was performed on StepOne Real-Time PCR System (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers for CD47 and programmed cell death-ligand 1 (PD-L1), as well as primers for β -actin that served as an internal control. The primers were as follows: CD47 sense: 5'-AGAAGGTGAAACGATCATCGAGC-3' (SEQ ID NO. 31) and CD47 antisense: 5'-CTCATCCATACCAACCGATCT-3' (SEQ ID NO. 32); PD-L1 sense: 5'-TGGCATTTGCTGAACGCATTT-3' (SEQ ID NO. 33) and PD-L1 antisense: 5'-AGTGCAGCCAGGTCTAATTGT-3' (SEQ ID NO. 34); β -actin sense: 5'-CCAACCGCGAGAAGATGACC-3' (SEQ ID NO. 35) and β -actin antisense: 5'-

GATCTTCATGAGGTAGTCAGT-3' (SEQ ID NO. 36). Results are expressed as a fold change after antibody treatment (Table 10).

Table 10: RT-qPCR analysis of the expression levels of CD47 and PD-L1 after the treatment with humanized antibody CA9hu-2_HC4LC5 for 7 days. Doxorubicin as a chemotherapeutic drug was either added (DOX+) or not added (DOX-) for the first four days of the treatment. Cancer cells incubated in the absence of humanized antibodies are marked as "no Ab". Results are expressed as a fold change after antibody treatment.

	CD47		PD-L1	
	average	stdev	average	stdev
DOX-/no Ab	1 (100%)	0.426	1 (100%)	0.179
DOX-/CA9hu-2_HC4LC5	1.042 (104.2%)	0.037	0.891 (89.1%)	0.042
DOX+/no Ab	2.249 (100%)	0.033	4.940 (100%)	0.071
DOX+/CA9hu-2_HC4LC5	1.533 (68.2%)	0.237	3.011 (60.9%)	0.026

As shown in Table 10, RT-qPCR analysis of total RNA isolated from BT-20 cells treated with humanized antibody variant CA9hu-2_HC4LC5 for 7 days revealed decreased expression of CD47 as well as PD-L1. The down-regulation of both mRNAs was more evident in doxorubicin-exposed (DOX+) BT-20 cells and resulted into almost 40% and more than 30% reduction of PD-L1 and CD47 expression after the treatment with CA9hu-2_HC4LC5 humanized antibody, respectively.

The foregoing data demonstrate the ability of humanized antibody CA9hu-2_HC4LC5 to affect the cytokine profile of cancer cells. VEGF and IL-8 are two potent angiogenic factors secreted by breast cancer cells, which contribute to the establishment and expansion of tumor neovasculature. Angiogenesis is a crucial for tumor progression, and pro-angiogenic molecules such as VEGF and IL-8 have been investigated as potential targets for cancer therapy. Considering the fact that the treatment of TNBC cells with humanized antibody of the invention possesses the ability to induce some indirect effects, e.g. down-regulation of the expression of VEGF and IL-8, we assume that the CA IX-targeted therapy could bring additional therapeutic benefits for the patients.

The ability of cancer cells to evade immune system (both the innate as well as adaptive responses) plays a crucial role in cancer relapse and metastasis. CD47 is a cell-surface protein that interacts with signal regulatory protein α on macrophages to block phagocytosis. Its expression represents a major mechanism mediating evasion of innate immunity by cancer cells. PD-L1, also known as CD274, is a transmembrane protein commonly expressed on the surface of antigen presenting cells and tumor cells. PD-L1 specifically binds to its receptor PD-1, which is expressed on the surface of immune-related

- lymphocytes. Breakdown of the PD-L1/PD-1 interaction leads to T cells activation, proliferation, cytokine generation and cancer cell elimination. Therefore, the down-regulation of tumor PD-L1 and CD47 expression in chemotherapy-exposed cancer cells treated with humanized antibody of the invention could result in inhibition of cancer cell growth and moreover, present an unexpected property
- 5 of humanized antibodies of the invention. In addition, the coordinate inhibition of PD-L1 and CD47 expression in response to humanized antibody treatment of doxorubicin-exposed BT-20 cells provides a rationale for combining chemotherapy and anti-CA IX antibodies of the invention to improve the outcome of cancer patients.
- 10 In conclusion, the humanized antibodies of the invention were demonstrated to retain antigen-binding specificity and to possess effector functions (ADCC, CDC, ADCP). Furthermore, desirable safety of the use of the humanized antibodies was determined by cytokine release assay using fresh whole blood samples from 20 donors. An extraordinary beneficial property, e.g. inhibition of cancer cell invasion which represents an important mechanism of action for the therapy of cancer patients, was proved. More
- 15 importantly, unexpected and extraordinary properties of humanized antibodies were revealed in multicellular aggregation assay during detached conditions and in the analysis of proteome, secretome, and transcriptome of treated cancer cells using Proteome Profiler Array and RT-qPCR. Finally, this is the first description and demonstration of unexpected beneficial effects of humanized antibodies directed against PG domain of CA IX and thus, the humanized antibodies of the invention are novel and
- 20 inventive over the cited prior art.