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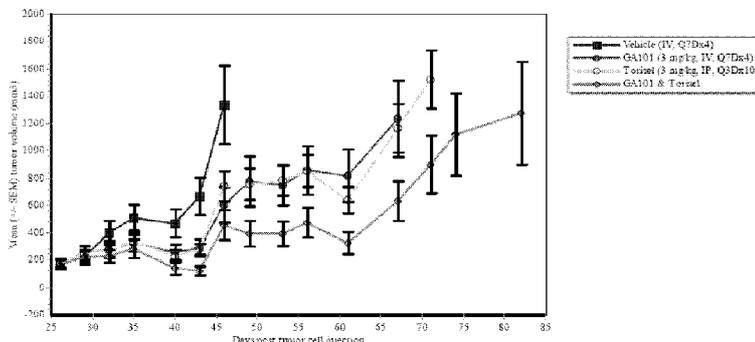
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(54) **Title:** COMBINATION THERAPY OF AN AFUCOSYLATED CD20 ANTIBODY WITH A MTOR INHIBITOR

**Figure 1**



(57) **Abstract:** The present invention is directed to the combination therapy of an afucosylated anti-CD20 antibody with a mTOR inhibitor for the treatment of cancer, especially to the combination therapy of CD20 expressing cancers with an afucosylated humanized B-Lyl antibody and a mTOR inhibitor such as Temozolimus or Everolimus.

## Combination therapy of an afucosylated CD20 antibody with a mTOR inhibitor

The present invention is directed to the combination therapy of an afucosylated CD20 antibody with a mTOR inhibitor for the treatment of cancer.

### 5 Background of the Invention

#### Afucosylated antibodies

Cell-mediated effector functions of monoclonal antibodies can be enhanced by engineering their oligosaccharide component as described in Umana, P., et al.,  
10 Nature Biotechnol. 17 (1999) 176-180; and US 6,602,684. IgG1 type antibodies, the most commonly used antibodies in cancer immunotherapy, are glycoproteins that have a conserved N-linked glycosylation site at Asn297 in each CH2 domain. The two complex biantennary oligosaccharides attached to Asn297 are buried between the CH2 domains, forming extensive contacts with the polypeptide  
15 backbone, and their presence is essential for the antibody to mediate effector functions such as antibody dependent cellular cytotoxicity (ADCC) (Lifely, M.R., et al, Glycobiology 5 (1995) 813-822; Jefferis, R., et al, Immunol. Rev. 163 (1998) 59-76; Wright, A., and Morrison, S.L., Trends Biotech. 15 (1997) 26-32). Umana, P., et al. Nature Biotechnol. 17 (1999) 176-180 and WO 99/54342 showed  
20 that overexpression in Chinese hamster ovary (CHO) cells of B(1,4)-N-acetylglucosaminyltransferase III ("GnTIII"), a glycosyltransferase catalyzing the formation of bisected oligosaccharides, significantly increases the in vitro ADCC activity of antibodies. Alterations in the composition of the N297 carbohydrate or its elimination affect also binding to Fc binding to FcγR and CI q (Umana, P., et  
25 al, Nature Biotechnol. 17 (1999) 176-180; Davies, J., et al, Biotechnol. Bioeng. 74 (2001) 288-294; Mimura, Y., et al, J. Biol. Chem. 276 (2001) 45539-45547; Radaev, S., et al, J. Biol. Chem. 276 (2001) 16478-16483; Shields, R.L., et al, J. Biol. Chem. 276 (2001) 6591-6604; Shields, R.L., et al, J. Biol. Chem. 277 (2002) 26733-26740; Simmons, L.C., et al, J. Immunol. Methods 263 (2002) 133-147).

30 Iida, S., et al, Clin. Cancer Res. 12 (2006) 2879-2887 show that efficacy of an afucosylated anti-CD20 antibody was inhibited by addition of fucosylated anti-CD20. The efficacy of a 1:9 mixture (10 microg/mL) of afucosylated and fucosylated anti-CD20s was inferior to that of a 1,000-fold dilution (0.01 microg/mL) of afucosylated anti-CD20 alone. They conclude that afucosylated

IgG1, not including fucosylated counterparts, can evade the inhibitory effect of plasma IgG on ADCC through its high FcγRIIIa binding. Natsume, A., et al., shows in *J. Immunol. Methods* 306 (2005) 93-103 that fucose removal from complex-type oligosaccharide of human IgG1-type antibody results in a great enhancement of antibody-dependent cellular cytotoxicity (ADCC). Satoh, M., et al, *Expert Opin. Biol. Ther.* 6 (2006) 1161-1173 discusses afucosylated therapeutic antibodies as next-generation therapeutic antibodies. Satoh concludes that antibodies consisting of only the afucosylated human IgG1 form are thought to be ideal. Kanda, Y., et al., *Biotechnol. Bioeng.* 94 (2006) 680-688 compared fucosylated CD20 antibody (96% fucosylation, CHO/DG44 1H5) with afucosylated CD20 antibody. Davies, J., et al, *Biotechnol. Bioeng.* 74 (2001) 288-294 reports that for a CD20 antibody increased ADCC correlates with increased binding to FcγRIII.

Methods to enhance cell-mediated effector functions of monoclonal antibodies by reducing the amount of fucose are described e.g. in WO 2005/018572, WO 2006/116260, WO 2006/114700, WO 2004/065540, WO 2005/011735, WO 2005/027966, WO 1997/028267, US 2006/0134709, US 2005/0054048, US 2005/0152894, WO 2003/035835, WO 2000/061739, Niwa, R., et al, *J. Immunol. Methods* 306 (2005) 151-160; Shinkawa, T., et al, *J. Biol. Chem.* 278 (2003) 3466-3473; WO 03/055993 or US2005/0249722.

### **CD20 and anti CD20 antibodies**

The CD20 molecule (also called human B-lymphocyte-restricted differentiation antigen or Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine, M.A., et al, *J. Biol. Chem.* 264 (1989) 11282-11287; and Einfeld, D.A., et al, *EMBO J.* 7 (1988) 711-717; Tedder, T.F., et al, *Proc. Natl. Acad. Sci. U.S.A.* 85 (1988) 208-212; Stamenkovic, I., et al, *J. Exp. Med.* 167 (1988) 1975-1980; Tedder, T.F., et al, *J. Immunol.* 142 (1989) 2560-2568). CD20 is found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs and is expressed during early pre-B cell development and remains until plasma cell differentiation. CD20 is present on both normal B cells as well as malignant B cells. In particular, CD20 is expressed on greater than 90% of B cell non-Hodgkin's lymphomas (NHL) (Anderson, K.C., et al., *Blood* 63 (1984) 1424-1433) but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells, or other normal tissues (Tedder, T.F., et al, *J. Immunol.* 135 (1985) 973-979).

The 85 amino acid carboxyl-terminal region of the CD20 protein is located within the cytoplasm. The length of this region contrasts with that of other B cell-specific surface structures such as IgM, IgD, and IgG heavy chains or histocompatibility antigens class II  $\alpha$  or  $\beta$  chains, which have relatively short intracytoplasmic regions of 3, 3, 28, 15, and 16 amino acids, respectively (Komaromy, M., et al. NAR 11 (1983) 6775-6785). Of the last 61 carboxyl-terminal amino acids, 21 are acidic residues, whereas only 2 are basic, indicating that this region has a strong net negative charge. The GenBank Accession NO. is NP-690605. It is thought that CD20 might be involved in regulating an early step(s) in the activation and differentiation process of B cells (Tedder, T.F., et al, Eur. J. Immunol. 16 (1986) 881-887) and could function as a calcium ion channel (Tedder, T.F., et al, J. Cell. Biochem. 14D (1990) 195).

There exist two different types of anti-CD20 antibodies differing significantly in their mode of CD20 binding and biological activities (Cragg, M.S., et al, Blood 103 (2004) 2738-2743; and Cragg, M.S., et al, Blood 101 (2003) 1045-1052). Type I antibodies, as e.g. rituximab, are potent in complement mediated cytotoxicity, whereas type II antibodies, as e.g. Tositumomab (B1), 11B8, AT80 or humanized B-Lyl antibodies, effectively initiate target cell death via caspase-independent apoptosis with concomitant phosphatidylserine exposure.

The sharing common features of type I and type II anti-CD20 antibodies are summarized in Table 1.

**Table 1: Properties of type I and type II anti-CD20 antibodies**

<b>type I anti-CD20 antibodies</b>	<b>type II anti-CD20 antibodies</b>
type I CD20 epitope	type II CD20 epitope
Localize CD20 to lipid rafts	Do not localize CD20 to lipid rafts
Increased CDC (if IgG1 isotype)	Decreased CDC (if IgG1 isotype)
ADCC activity (if IgG1 isotype)	ADCC activity (if IgG1 isotype)
Full binding capacity	Reduced binding capacity
Homotypic aggregation	Stronger homotypic aggregation
Apoptosis induction upon cross-linking	Strong cell death induction without cross-linking

**mTOR and mTOR inhibitors**

mTOR is the mammalian target of rapamycin (mTOR) also known as mechanistic target of rapamycin or FK506 binding protein 12-rapamycin associated protein 1

(FRAP1) is a protein which in humans is encoded by the FRAP1 gene (Brown, E.J., et al, Nature 369 (1994) 756-758; Moore, P.A., et al, Genomics 33 (1996) 331-332). mTOR is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription. (Hay, N., et al, Genes Dev. 18 (2004) 1926-1945; Beevers, C. S., et al, Int. J. Cancer 119 (2006) 757-764).

mTOR integrates the input from upstream pathways, including insulin, growth factors (such as IGF-1 and IGF-2), and mitogens. mTOR also senses cellular nutrient and energy levels and redox status. The mTOR pathway is dysregulated in human diseases, especially certain cancers. Rapamycin is a bacterial product that can inhibit mTOR by associating with its intracellular receptor FKBP12. The FKBP12-rapamycin complex binds directly to the FKBP12-Rapamycin Binding (FRB) domain of mTOR.

mTOR is the catalytic subunit of two molecular complexes. mTOR Complex 1 (mTORC1) is composed of mTOR, regulatory associated protein of mTOR (Raptor), mammalian LST8/G-protein  $\beta$ -subunit like protein (mLST8/GpL) and the recently identified partners PRAS40 and DEPTOR. This complex is characterized by the classic features of mTOR by functioning as a nutrient/energy/redox sensor and controlling protein synthesis. The activity of this complex is stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress.

mTORC1 is inhibited by low nutrient levels, growth factor deprivation, reductive stress, caffeine, rapamycin, farnesylthiosalicylic acid (FTS) and curcumin. The two best characterized targets of mTORC1 are p70-S6 Kinase 1 (S6K1) and 4E-BP1, the eukaryotic initiation factor 4E (eIF4E) binding protein 1.[3]

mTORC1 phosphorylates S6K1 on at least two residues, with the most critical modification occurring on a threonine residue (T389). This event stimulates the subsequent phosphorylation of S6K1 by PDK1. Active S6K1 can in turn stimulate the initiation of protein synthesis through activation of S6 Ribosomal protein (a component of the ribosome) and other components of the translational machinery. S6K1 can also participate in a positive feedback loop with mTORC1 by phosphorylating mTOR's negative regulatory domain at two sites; phosphorylation at these sites appears to stimulate mTOR activity.

mTORC1 has been shown to phosphorylate at least four residues of 4E-BP1 in a hierarchical manner. Non-phosphorylated 4E-BP1 binds tightly to the translation initiation factor eIF4E, preventing it from binding to 5'-capped mRNAs and recruiting them to the ribosomal initiation complex. Upon phosphorylation by mTORC1, 4E-BP1 releases eIF4E, allowing it to perform its function. The activity of mTORC1 appears to be regulated through a dynamic interaction between mTOR and Raptor, one which is mediated by GPL. Raptor and mTOR share a strong N-terminal interaction and a weaker C-terminal interaction near mTOR's kinase domain. When stimulatory signals are sensed, such as high nutrient/energy levels, the mTOR-Raptor C-terminal interaction is weakened and possibly completely lost, allowing mTOR kinase activity to be turned on. When stimulatory signals are withdrawn, such as low nutrient levels, the mTOR-Raptor C-terminal interaction is strengthened, essentially shutting off kinase function of mTOR.

mTOR Complex 2 (mTORC2) is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), GPL, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1). mTORC2 has been shown to function as an important regulator of the cytoskeleton through its stimulation of F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42, and protein kinase C  $\alpha$  (PKC $\alpha$ ). mTORC2 also appears to possess the activity of a previously elusive protein known as "PDK2." mTORC2 phosphorylates the serine/threonine protein kinase Akt/PKB at a serine residue S473. Phosphorylation of the serine stimulates Akt phosphorylation at a threonine T308 residue by PDK1 and leads to full Akt activation; curcumin inhibits both by preventing phosphorylation of the serine.

mTORC2 appears to be regulated by insulin, growth factors, serum, and nutrient levels. Originally, mTORC2 was identified as a rapamycin-insensitive entity, as acute exposure to rapamycin did not affect mTORC2 activity or Akt phosphorylation. However, subsequent studies have shown that, at least in some cell lines, chronic exposure to rapamycin, while not effecting pre-existing mTORC2s, can bind to free mTOR molecules, thus inhibiting the formation of new mTORC2.

It is hypothesized that some dietary regimes, like caloric restriction and methionine restriction, cause lifespan extension by decreasing mTor activity.

**mTOR inhibitors**

Many inhibitors of mTOR have been identified and several are in clinical trials for the treatment of cancer (e.g. RAD001 (also known as Everolimus; Novartis); CCI-779 (also known as Temsirolimus; Wyeth); AP23573 (Ariad Pharmaceuticals); and  
5 KU-0059475 (Kudus Pharmaceuticals); Mita, M.M. et al, Cancer Biology & Therapy 2, Suppl. 1 (2003) S169-S177). The potential effectiveness of combinations of such mTOR inhibitors with other anti-cancer agents has also been suggested and is being tested in clinical trials (Adjei, A. A. and Hidalgo, M., J. Clin. Oncol. 23 (2005) 5386-5403). Such combinations include combinations of  
10 mTOR inhibitors with protein-tyrosine kinase inhibitors (Sawyers, C. L., Cancer Cell 4 (2003) 343-348; Gemmill, R. M., et al, Br. J. Cancer 92 (2005) 2266-2277; Goudar, R. K., et al., Mol. Cancer Therapeutics 4 (2005) 101-112; International Patent Publication WO 2004/004644; Birle, D.C., et al, 94<sup>th</sup> Annual Meeting of the American Association for Cancer Research, Washington, D.C., Vol. 44, Second  
15 Edition, Proc. Am. Assoc. Cancer Res. (July 2003) p. 932, #R4692).

**Summary of the Invention**

The invention comprises the use of an afucosylated anti-CD20 antibody with an amount of fucose of 60% or less, for the manufacture of a medicament for the treatment of cancer in combination with a mTOR inhibitor.

20 One aspect of the invention is a method of treatment of patient suffering from cancer by administering an afucosylated anti-CD20 antibody with an amount of fucose of 60% or less in combination with a mTOR inhibitor, to a patient in the need of such treatment.

25 Another spect of the invention is an afucosylated anti-CD20 antibody with an amount of fucose of 60% or less, for the treatment of cancer in combination with a mTOR inhibitor.

Preferably the amount of fucose is between 40% and 60% of the total amount of oligosaccharides (sugars) at Asn297.

30 Preferably said afucosylated anti-CD20 antibody is humanized B-Lyl antibody, and said cancer is a CD20 expressing cancer, preferably a B-Cell Non-Hodgkin's lymphoma (NHL).

Preferably the mTOR inhibitor is a rapamycin, or a rapamycin analog or derivative.  
Preferably the mTOR inhibitor is Temsirolimus or Everolimus.

One embodiment of the invention is a composition comprising an anti-CD20  
afucosylated antibody with an amount of fucose of 60% or less, and a mTOR  
5 inhibitor for the treatment of cancer.

Surprisingly we have now found out that the combination of a mTOR inhibitor  
with an afucosylated anti-CD20 antibody showed synergistic (e.g. more than  
additive) antiproliferative effects in the treatments of cancer.

We have now found out that the combination of an afucosylated anti-CD20  
10 antibody showed synergistic (e.g. even more than additive) antiproliferative effects

### Description of the Figures

**Figure 1** In vivo antitumor activity of combined treatment of an  
afucosylated type II anti-CD20 antibody (GA101 = B-HH6-B-  
KV1 GE) with a mTOR inhibitor (Temsirolimus) in comparison  
15 in comparison with the respective monotherapies.

### Detailed Description of the Invention

The invention comprises the use of an afucosylated anti-CD20 antibody (preferably  
of IgG1 or IgG3 isotype, more preferably of IgG1 isotype) with an amount of  
20 fucose of 60%, or less of the total amount of oligosaccharides (sugars) at Asn297,  
for the manufacture of a medicament for the treatment of cancer in combination  
with a mTOR inhibitor.

Preferably the mTOR inhibitor is a rapamycin, or a rapamycin analog or derivative.  
Preferably the mTOR inhibitor is Temsirolimus or Everolimus.

25 Preferably the amount of fucose is between 40% and 60% of the total amount of  
oligosaccharides (sugars) at Asn297.

The term "antibody" encompasses the various forms of antibodies including but not  
being limited to whole antibodies, human antibodies, humanized antibodies and  
genetically engineered antibodies like monoclonal antibodies, chimeric antibodies  
30 or recombinant antibodies as well as fragments of such antibodies as long as the  
characteristic properties according to the invention are retained. The terms

"monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of a single amino acid composition. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences. In one embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic non-human animal, e.g. a transgenic mouse, having a genome comprising a human heavy chain transgene and a light human chain transgene fused to an immortalized cell.

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

The term "humanized antibody" refers to antibodies in which the framework or "complementarity determining regions" (CDR) have been modified to comprise the CDR of an immunoglobulin of different specificity as compared to that of the parent immunoglobulin. In a preferred embodiment, a murine CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody." See, e.g., Riechmann, L., et al, Nature 332 (1988) 323-327; and Neuberger, M.S., et al, Nature 314 (1985) 268-270..

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. Human antibodies are well-known in the state of the art (van Dijk, M.A., and van de Winkel, J.G., Curr. Opin. Chem. Biol. 5 (2001)

368-374). Based on such technology, human antibodies against a great variety of targets can be produced. Examples of human antibodies are for example described in Kellermann, S.A., et al, Curr Opin Biotechnol. 13 (2002) 593-597.

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

As used herein, the term "binding" or "specifically binding" refers to the binding of the antibody to an epitope of the tumor antigen in an in vitro assay, preferably in an plasmon resonance assay (BIAcore, GE-Healthcare Uppsala, Sweden) with purified wild-type antigen. The affinity of the binding is defined by the terms  $k_a$  (rate  
20 constant for the association of the antibody from the antibody/antigen complex),  $k_D$  (dissociation constant), and  $K_D$  ( $k_D/k_a$ ). Binding or specifically binding means a binding affinity ( $K_D$ ) of  $10^{-8}$  mol/l or less, preferably  $10^{-9}$  M to  $10^{-13}$  mol/l. Thus, an afucosylated antibody according to the invention is specifically binding to the tumor antigen with a binding affinity ( $K_D$ ) of  $10^{-8}$  mol/l or less, preferably  $10^{-9}$  M  
25 to  $10^{-13}$  mol/l.

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

30 The "constant domains" are not involved directly in binding the antibody to an antigen but are involved in the effector functions (ADCC, complement binding, and CDC).

The "variable region" (variable region of a light chain (VL), variable region of a heavy chain (VH)) as used herein denotes each of the pair of light and heavy chains which is involved directly in binding the antibody to the antigen. The domains of

variable human light and heavy chains have the same general structure and each domain comprises four framework (FR) regions whose sequences are widely conserved, connected by three "hypervariable regions" (or complementarity determining regions, CDRs). The framework regions adopt a b-sheet conformation and the CDRs may form loops connecting the b-sheet structure. The CDRs in each chain are held in their three-dimensional structure by the framework regions and form together with the CDRs from the other chain the antigen binding site.

The terms "hypervariable region" or "antigen-binding portion of an antibody" when used herein refer to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from the "complementarity determining regions" or "CDRs". "Framework" or "FR" regions are those variable domain regions other than the hypervariable region residues as herein defined. Therefore, the light and heavy chains of an antibody comprise from N- to C-terminus the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Especially, CDR3 of the heavy chain is the region which contributes most to antigen binding. CDR and FR regions are determined according to the standard definition of Kabat et al, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) and/or those residues from a "hypervariable loop".

A mTOR inhibitor according to the invention can be any mTOR inhibitor that is currently known in the art or that will be identified in the future, and includes any chemical entity that, upon administration to a patient, results in inhibition of mTOR in the patient. A mTOR inhibitor can inhibit mTOR by any biochemical mechanism, including competition at the ATP binding site, competition elsewhere at the catalytic site of mTOR kinase, non-competitive inhibition, irreversible inhibition (e.g. covalent protein modification), or modulation of the interactions of other protein subunits or binding proteins with mTOR kinase in a way that results in inhibition of mTOR kinase activity (e.g. modulation of the interaction of mTOR with FKBP12, GpL, (mLST8), RAPTOR (mKOG1), or RICTOR (mAV03)). Specific examples of mTOR inhibitors include: rapamycin; other rapamycin macrolides, or rapamycin analogues, derivatives or prodrugs; Everolimus (also known as RADOOL, Everolimus/RADOOL is an alkylated rapamycin (40-O-(2-hydroxyethyl)-rapamycin), disclosed in U.S. Pat. No. 5,665,772; Novartis); Temsirolimus (also known as CCI-779, Temsirolimus/CCI-779 is an ester of rapamycin (42-ester with 3-hydroxy-2-hydroxymethyl-2-methylpropionic acid), disclosed in U.S. Pat. No. 5,362,718; Wyeth); AP23573 or AP23841 (Ariad

Pharmaceuticals); ABT-578 (40-epi-(tetrazolyl)-rapamycin; Abbott Laboratories); KU-0059475 (Kudus Pharmaceuticals); and TAFA-93 (a rapamycin prodrug; Isotechnika). Examples of rapamycin analogs and derivatives known in the art include those compounds described in U.S. Pat. Nos. 6,329,386; 6,200,985; 5 6,117,863; 6,015,815; 6,015,809; 6,004,973; 5,985,890; 5,955,457; 5,922,730; 5,912,253; 5,780,462; 5,665,772; 5,637,590; 5,567,709; 5,563,145; 5,559,122; 5,559,120; 5,559,119; 5,559,112; 5,550,133; 5,541,192; 5,541,191; 5,532,355; 5,530,121; 5,530,007; 5,525,610; 5,521,194; 5,519,031; 5,516,780; 5,508,399; 5,508,290; 5,508,286; 5,508,285; 5,504,291; 5,504,204; 5,491,231; 5,489,680; 10 5,489,595; 5,488,054; 5,486,524; 5,486,523; 5,486,522; 5,484,791; 5,484,790; 5,480,989; 5,480,988; 5,463,048; 5,446,048; 5,434,260; 5,411,967; 5,391,730; 5,389,639; 5,385,910; 5,385,909; 5,385,908; 5,378,836; 5,378,696; 5,373,014; 5,362,718; 5,358,944; 5,346,893; 5,344,833; 5,302,584; 5,262,424; 5,262,423; 5,260,300; 5,260,299; 5,233,036; 5,221,740; 5,221,670; 5,202,332; 5,194,447; 15 5,177,203; 5,169,851; 5,164,399; 5,162,333; 5,151,413; 5,138,051; 5,130,307; 5,120,842; 5,120,727; 5,120,726; 5,120,725; 5,118,678; 5,118,677; 5,100,883; 5,023,264; 5,023,263; and 5,023,262; all of which are incorporated herein by reference. Rapamycin derivatives are also disclosed for example in WO 94/09010, WO 95/16691, WO 96/41807, or WO 99/15530, which are incorporated herein by 20 reference. Such analogs and derivatives include 32-deoxorapamycin, 16-pent-2-ynyloxy-32-deoxorapamycin, 16-pent-2-ynyloxy-32 (S or R)-dihydro-rapamycin, 16-pent-2-ynyloxy-32 (S or R)-dihydro-40-O-(2-hydroxyethyl)-rapamycin, 40-O-(2-hydroxyethyl)-rapamycin, 32-deoxorapamycin and 16-pent-2-ynyloxy-32(S)-dihydro-rapamycin. Rapamycin derivatives may also include the so-called rapalogs, e.g. as disclosed in WO 98/02441 and WO 01/14387 (e.g. AP23573, AP23464, AP23675 or AP23841). Further examples of a rapamycin derivative are those disclosed under the name biolimus-7 or biolimus-9 (BIOLIMUS A9™) (Biosensors International, Singapore). Any of the above rapamycin analogs or derivatives may be readily prepared by procedures as described in the above 25 references. Additional examples of mTOR inhibitors useful in the invention described herein include those disclosed and claimed in U.S. patent application Ser. No. 11/599,663.

Preferably the mTOR inhibitors are rapamycin or rapamycin analogs or derivatives, more preferably rapamycin analogs or derivatives. Preferred rapamycin analogs or derivatives are eg. Temsirolimus or Everolimus. 35

The term "afucosylated antibody" refers to an antibody of IgG1 or IgG3 isotype (preferably of IgG1 isotype) with an altered pattern of glycosylation in the Fc region at Asn297 having a reduced level of fucose residues. Glycosylation of human IgG1 or IgG3 occurs at Asn297 as core fucosylated biantennary complex oligosaccharide glycosylation terminated with up to 2 Gal residues. These structures are designated as G0, G1 (α1,6 or α1,3) or G2 glycan residues, depending from the amount of terminal Gal residues (Raju, T.S., BioProcess Int. 1 (2003) 44-53). CHO type glycosylation of antibody Fc parts is e.g. described by Routier, F.H., Glycoconjugate J. 14 (1997) 201-207. Antibodies which are recombinantly expressed in non glycomodified CHO host cells usually are fucosylated at Asn297 in an amount of at least 85%.

Thus an afucosylated antibody according to the invention means an antibody of IgG1 or IgG3 isotype (preferably of IgG1 isotype) wherein the amount of fucose is 60%, or less of the total amount of oligosaccharides (sugars) at Asn297 (which means that at least 40% or more of the oligosaccharides of the Fc region at Asn297 are afucosylated). In one embodiment the amount of fucose is between 40% and 60% of the oligosaccharides of the Fc region at Asn297. In another embodiment the amount of fucose is 50% or less, and in still another embodiment the amount of fucose is 30%, or less of the oligosaccharides of the Fc region at Asn297. According to the invention "amount of fucose" means the amount of said oligosaccharide (fucose) within the oligosaccharide (sugar) chain at Asn297, related to the sum of all oligosaccharides (sugars) attached to Asn 297 (e. g. complex, hybrid and high mannose structures) measured by MALDI-TOF mass spectrometry and calculated as average value (for a detailed procedure to determine the amount of fucose, is described e.g. in WO 2008/077546). Furthermore the oligosaccharides of the Fc region are preferably bisected. The afucosylated antibody according to the invention can be expressed in a glycomodified host cell engineered to express at least one nucleic acid encoding a polypeptide having GnTIII activity in an amount sufficient to partially fucosylate the oligosaccharides in the Fc region. In one embodiment, the polypeptide having GnTIII activity is a fusion polypeptide. Alternatively α1,6-fucosyltransferase activity of the host cell can be decreased or eliminated according to US 6,946,292 to generate glycomodified host cells. The amount of antibody fucosylation can be predetermined e.g. either by fermentation conditions (e.g. fermentation time) or by combination of at least two antibodies with different fucosylation amount. Such afucosylated antibodies and respective glycoengineering methods are described in

WO 2005/044859, WO 2004/065540, WO 2007/031875, Umana, P., et al, Nature Biotechnol. 17 (1999) 176-180, WO 99/154342, WO 2005/018572, WO 2006/1 16260, WO 2006/1 14700, WO 2005/01 1735, WO 2005/027966, WO 97/028267, US 2006/0134709, US 2005/0054048, US 2005/0152894, 5 WO 2003/035835, WO 2000/061739. These glycoengineered antibodies have an increased ADCC. Other glycoengineering methods yielding afucosylated antibodies according to the invention are described e.g. in Niwa, R., et al, J. Immunol. Methods 306 (2005) 151-160; Shinkawa, T., et al, J. Biol. Chem. 278 (2003) 3466-3473; WO 03/055993 or US 2005/0249722.

10 Thus one aspect of the invention is the use of an afucosylated anti-CD20 antibody of IgG1 or IgG3 isotype (preferably of IgG1 isotype) specifically binding to a tumor antigen with an amount of fucose of 60% or less of the total amount of oligosaccharides (sugars) at Asn297, for the manufacture of a medicament for the treatment of cancer in combination with a mTOR inhibitor. Preferably the amount 15 of fucose is between 40% and 60% of the total amount of oligosaccharides (sugars) at Asn297.

CD20 ( also known as B-lymphocyte antigen CD20, B-lymphocyte surface antigen Bl, Leu-16, Bp35, BM5, and LF5; the sequence is characterized by the SwissProt database entry PI1836) is is a hydrophobic transmembrane protein with a 20 molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes. (Valentine, M.A., et al., J. Biol. Chem. 264 (1989) 11282-11287; Tedder, T.F., et al, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 208-212; Stamenkovic, I., et al, J. Exp. Med. 167 (1988) 1975-1980; Einfeld, D.A., et al, EMBO J. 7 (1988) 711-717; Tedder, T.F., et al, J. Immunol. 142 (1989) 2560-2568). The 25 corresponding human gene is Membrane-spanning 4-domains, subfamily A, member 1, also known as MS4A1. This gene encodes a member of the membrane-spanning 4A gene family. Members of this nascent protein family are characterized by common structural features and similar intron/exon splice boundaries and display unique expression patterns among hematopoietic cells and nonlymphoid 30 tissues. This gene encodes the B-lymphocyte surface molecule which plays a role in the development and differentiation of B-cells into plasma cells. This family member is localized to 11q12, among a cluster of family members. Alternative splicing of this gene results in two transcript variants which encode the same protein.

The terms "CD20" and "CD20 antigen" are used interchangeably herein, and include any variants, isoforms and species homologs of human CD20 which are naturally expressed by cells or are expressed on cells transfected with the CD20 gene. Binding of an antibody of the invention to the CD20 antigen mediate the killing of cells expressing CD20 (e.g., a tumor cell) by inactivating CD20. The killing of the cells expressing CD20 may occur by one or more of the following mechanisms: Cell death/apoptosis induction, ADCC and CDC.

Synonyms of CD20, as recognized in the art, include B-lymphocyte antigen CD20, B-lymphocyte surface antigen Bl, Leu-16, Bp35, BM5, and LF5.

The term "anti-CD20 antibody" according to the invention is an antibody that binds specifically to CD20 antigen. Depending on binding properties and biological activities of anti-CD20 antibodies to the CD20 antigen, two types of anti-CD20 antibodies (type I and type II anti-CD20 antibodies) can be distinguished according to Cragg, M.S., et al, Blood 103 (2004) 2738-2743; and Cragg, M.S., et al, Blood 101 (2003) 1045-1052, see Table 2.

**Table 2: Properties of type I and type II anti-CD20 antibodies**

<b>type I anti-CD20 antibodies</b>	<b>type II anti-CD20 antibodies</b>
type I CD20 epitope	type II CD20 epitope
Localize CD20 to lipid rafts	Do not localize CD20 to lipid rafts
Increased CDC (if IgG1 isotype)	Decreased CDC (if IgG1 isotype)
ADCC activity (if IgG1 isotype)	ADCC activity (if IgG1 isotype)
Full binding capacity	Reduced binding capacity
Homotypic aggregation	Stronger homotypic aggregation
Apoptosis induction upon cross-linking	Strong cell death induction without cross-linking

Examples of type II anti-CD20 antibodies include e.g. humanized B-Lyl antibody IgG1 (a chimeric humanized IgG1 antibody as disclosed in WO 2005/044859), 11B8 IgG1 (as disclosed in WO 2004/035607), and AT80 IgG1. Typically type II anti-CD20 antibodies of the IgG1 isotype show characteristic CDC properties. Type II anti-CD20 antibodies have a decreased CDC (if IgG1 isotype) compared to type I antibodies of the IgG1 isotype.

Examples of type I anti-CD20 antibodies include e.g. rituximab, HI47 IgG3 (ECACC, hybridoma), 2C6 IgG1 (as disclosed in WO 2005/103081), 2F2 IgG1 (as

disclosed and WO 2004/035607 and WO 2005/103081) and 2H7 IgG1 (as disclosed in WO 2004/056312).

5 The afucosylated anti-CD20 antibodies according to the invention is preferably a type II anti-CD20 antibody, more preferably an afucosylated humanized B-Lyl antibody.

The afucosylated anti-CD20 antibodies according to the invention have an increased antibody dependent cellular cytotoxicity (ADCC).

10 By "afucosylated anti-CD20 antibody with increased antibody dependent cellular cytotoxicity (ADCC)" is meant an afucosylated anti-CD20 antibody, as that term is defined herein, having increased ADCC as determined by any suitable method known to those of ordinary skill in the art. One accepted in vitro ADCC assay is as follows:

- 1) the assay uses target cells that are known to express the target antigen recognized by the antigen-binding region of the antibody;
- 15 2) the assay uses human peripheral blood mononuclear cells (PBMCs), isolated from blood of a randomly chosen healthy donor, as effector cells;
- 3) the assay is carried out according to following protocol:
  - 20 i) the PBMCs are isolated using standard density centrifugation procedures and are suspended at  $5 \times 10^6$  cells/ml in RPMI cell culture medium;
  - ii) the target cells are grown by standard tissue culture methods, harvested from the exponential growth phase with a viability higher than 90%, washed in RPMI cell culture medium, labeled with 100 micro-Curies of  $^{51}\text{Cr}$ , washed twice with cell culture medium, and resuspended in cell culture medium at a density of  $10^5$  cells/ml;
  - 25 iii) 100 microliters of the final target cell suspension above are transferred to each well of a 96-well microtiter plate;
  - iv) the antibody is serially-diluted from 4000 ng/ml to 0.04 ng/ml in cell culture medium and 50 microliters of the resulting antibody solutions are added to the target cells in the 96-well microtiter plate, testing in triplicate various antibody concentrations covering the whole concentration range above;
  - 30 v) for the maximum release (MR) controls, 3 additional wells in the plate containing the labeled target cells, receive 50 microliters of a 2% (VN)

- 16 -

aqueous solution of non-ionic detergent (Nonidet, Sigma, St. Louis), instead of the antibody solution (point iv above);

- 5
- vi) for the spontaneous release (SR) controls, 3 additional wells in the plate containing the labeled target cells, receive 50 microliters of RPMI cell culture medium instead of the antibody solution (point iv above);
- 10
- vii) the 96-well microtiter plate is then centrifuged at 50 x g for 1 minute and incubated for 1 hour at 4°C;
- viii) 50 microliters of the PBMC suspension (point i above) are added to each well to yield an effector:target cell ratio of 25: 1 and the plates are placed in an incubator under 5% CO<sub>2</sub> atmosphere at 37 C for 4 hours;
- 15
- ix) the cell-free supernatant from each well is harvested and the experimentally released radioactivity (ER) is quantified using a gamma counter;
- x) the percentage of specific lysis is calculated for each antibody concentration according to the formula  $(ER-MR)/(MR-SR) \times 100$ , where ER is the average radioactivity quantified (see point ix above) for that antibody concentration, MR is the average radioactivity quantified (see point ix above) for the MR controls (see point V above), and SR is the average radioactivity quantified (see point ix above) for the SR controls (see point vi above);
- 20
- 4) "increased ADCC" is defined as either an increase in the maximum percentage of specific lysis observed within the antibody concentration range tested above, and/or a reduction in the concentration of antibody required to achieve one half of the maximum percentage of specific lysis observed within the antibody concentration range tested above. The increase in ADCC is relative to the ADCC, measured with the above assay, mediated by the same antibody, produced by the same type of host cells, using the same standard production, purification, formulation and storage methods, which are known to those skilled in the art, but that has not been produced by host cells engineered to overexpress GnTIII.
- 25
- 30

Said "increased ADCC" can be obtained by glycoengineering of said antibodies, that means enhance said natural, cell-mediated effector functions of monoclonal antibodies by engineering their oligosaccharide component as described in Umana, P., et al, Nature Biotechnol. 17 (1999) 176-180 and US 6,602,684.

The term "complement-dependent cytotoxicity (CDC)" refers to lysis of human tumor target cells by the antibody according to the invention in the presence of complement. CDC is measured preferably by the treatment of a preparation of CD20 expressing cells with an anti-CD20 antibody according to the invention in the presence of complement. CDC is found if the antibody induces at a concentration of 100 nM the lysis (cell death) of 20% or more of the tumor cells after 4 hours. The assay is performed preferably with <sup>51</sup>Cr or Eu labeled tumor cells and measurement of released <sup>51</sup>Cr or Eu. Controls include the incubation of the tumor target cells with complement but without the antibody.

The "rituximab" antibody (reference antibody; example of a type I anti-CD20 antibody) is a genetically engineered chimeric human gamma 1 murine constant domain containing monoclonal antibody directed against the human CD20 antigen. Rituximab is not afucosylated with an amount of fucose of approximately 85% or more of the total amount of oligosaccharides (sugars) at Asn297. This chimeric antibody contains human gamma 1 constant domains and is identified by the name "C2B8" in US 5,736,137 (Andersen et. al.) issued on April 17, 1998, assigned to IDEC Pharmaceuticals Corporation. Rituximab is approved for the treatment of patients with relapsed or refracting low-grade or follicular, CD20 positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have shown that rituximab exhibits human complement—dependent cytotoxicity (CDC) (Reff, M.E., et. al, Blood 83 (1994) 435-445). Additionally, it exhibits significant activity in assays that measure antibody-dependent cellular cytotoxicity (ADCC). Rituximab is not afucosylated.

<b>Antibody</b>	<b>Amount of fucose</b>
Rituximab (non-afucosylated)	>85%
Wild type afucosylated glyco-engineered humanized B-Ly1 (B-HH6-B-KV1) (non-afucosylated)	>85%
afucosylated glyco-engineered humanized B-Ly1 (B-HH6-B-KV1 GE)	45-50%

The term "humanized B-Ly1 antibody" refers to humanized B-Ly1 antibody as disclosed in WO 2005/044859 and WO 2007/031875, which were obtained from

the murine monoclonal anti-CD20 antibody B-Lyl (variable region of the murine heavy chain (VH): SEQ ID NO: 1; variable region of the murine light chain (VL): SEQ ID NO: 2- see Poppema, S., and Visser, L., Biotest Bulletin 3 (1987) 131-139) by chimerization with a human constant domain from IgG1 and following humanization (see WO 2005/044859 and WO 2007/031875). These "humanized B-Lyl antibodies" are disclosed in detail in WO 2005/044859 and WO 2007/031875.

Preferably the "humanized B-Lyl antibody" has variable region of the heavy chain (VH) selected from group of SEQ ID NO:3 to SEQ ID NO:20 (B-HH2 to B-HH9 and B-HL8 to B-HL17 of WO 2005/044859 and WO 2007/031875). Especially preferred are Seq. ID NO: 3, 4, 7, 9, 11, 13 and 15 (B-HH2, BHH-3, B-HH6, B-HH8, B-HL8, B-HL11 and B-HL13 of WO 2005/044859 and WO 2007/031875). Preferably the "humanized B-Lyl antibody" has variable region of the light chain (VL) of SEQ ID NO: 20 (B-KV1 of WO 2005/044859 and WO 2007/031875). Preferably the "humanized B-Lyl antibody" has a variable region of the heavy chain (VH) of SEQ ID NO:7 (B-HH6 of WO 2005/044859 and WO 2007/031875) and a variable region of the light chain (VL) of SEQ ID NO: 20 (B-KV1 of WO 2005/044859 and WO 2007/031875). Furthermore the humanized B-Lyl antibody is preferably an IgG1 antibody. According to the invention such afucosylated humanized B-Lyl antibodies are glycoengineered (GE) in the Fc region according to the procedures described in WO 2005/044859, WO 2004/065540, WO 2007/031875, Umana, P., et al, Nature Biotechnol. 17 (1999) 176-180 and WO 99/154342. The afucosylated glyco-engineered humanized B-Lyl (B-HH6-B-KV1 GE) is preferred in one embodiment of the invention. Such glycoengineered humanized B-Lyl antibodies have an altered pattern of glycosylation in the Fc region, preferably having a reduced level of fucose residues. Preferably the amount of fucose is 60% or less of the total amount of oligosaccharides at Asn297 (in one embodiment the amount of fucose is between 40% and 60%, in another embodiment the amount of fucose is 50% or less, and in still another embodiment the amount of fucose is 30% or less). Furthermore the oligosaccharides of the Fc region are preferably bisected. These glycoengineered humanized B-Lyl antibodies have an increased ADCC.

The oligosaccharide component can significantly affect properties relevant to the efficacy of a therapeutic glycoprotein, including physical stability, resistance to protease attack, interactions with the immune system, pharmacokinetics, and specific biological activity. Such properties may depend not only on the presence

or absence, but also on the specific structures, of oligosaccharides. Some generalizations between oligosaccharide structure and glycoprotein function can be made. For example, certain oligosaccharide structures mediate rapid clearance of the glycoprotein from the bloodstream through interactions with specific carbohydrate binding proteins, while others can be bound by antibodies and trigger undesired immune reactions. (Jenkins, N., et al, Nature Biotechnol. 14 (1996) 975-981).

Mammalian cells are the preferred hosts for production of therapeutic glycoproteins, due to their capability to glycosylate proteins in the most compatible form for human application. (Cumming, D.A., et al., Glycobiology 1 (1991) 115-30; Jenkins, N., et al, Nature Biotechnol. 14 (1996) 975-981). Bacteria very rarely glycosylate proteins, and like other types of common hosts, such as yeasts, filamentous fungi, insect and plant cells, yield glycosylation patterns associated with rapid clearance from the blood stream, undesirable immune interactions, and in some specific cases, reduced biological activity. Among mammalian cells, Chinese hamster ovary (CHO) cells have been most commonly used during the last two decades. In addition to giving suitable glycosylation patterns, these cells allow consistent generation of genetically stable, highly productive clonal cell lines. They can be cultured to high densities in simple bioreactors using serum free media, and permit the development of safe and reproducible bioprocesses. Other commonly used animal cells include baby hamster kidney (BHK) cells, NSO- and SP2/0-mouse myeloma cells. More recently, production from transgenic animals has also been tested. (Jenkins, N., et al, Nature Biotechnol. 14 (1996) 975-981).

All antibodies contain carbohydrate structures at conserved positions in the heavy chain constant regions, with each isotype possessing a distinct array of N-linked carbohydrate structures, which variably affect protein assembly, secretion or functional activity. (Wright, A., and Morrison, S.L., Trends Biotech. 15 (1997) 26-32). The structure of the attached N-linked carbohydrate varies considerably, depending on the degree of processing, and can include high-mannose, multiply-branched as well as biantennary complex oligosaccharides. (Wright, A., and Morrison, S.L., Trends Biotech. 15 (1997) 26-32). Typically, there is heterogeneous processing of the core oligosaccharide structures attached at a particular glycosylation site such that even monoclonal antibodies exist as multiple glycoforms. Likewise, it has been shown that major differences in antibody glycosylation occur between cell lines, and even minor differences are seen for a

given cell line grown under different culture conditions. (Lifely, M.R., et al, *Glycobiology* 5 (1995) 813-822.

One way to obtain large increases in potency, while maintaining a simple production process and potentially avoiding significant, undesirable side effects, is to enhance the natural, cell-mediated effector functions of monoclonal antibodies by engineering their oligosaccharide component as described in Umana, P., et al., *Nature Biotechnol.* 17 (1999) 176-180 and US 6,602,684. IgG1 type antibodies, the most commonly used antibodies in cancer immunotherapy, are glycoproteins that have a conserved N-linked glycosylation site at Asn297 in each CH2 domain. The two complex biantennary oligosaccharides attached to Asn297 are buried between the CH2 domains, forming extensive contacts with the polypeptide backbone, and their presence is essential for the antibody to mediate effector functions such as antibody dependent cellular cytotoxicity (ADCC) (Lifely, M.R., et al, *Glycobiology* 5 (1995) 813-822; Jefferis, R., et al, *Immunol. Rev.* 163 (1998) 59-76; Wright, A., and Morrison, S.L., *Trends Biotech.* 15 (1997) 26-32).

It was previously shown that overexpression in Chinese hamster ovary (CHO) cells of B(1,4)-N-acetylglucosaminyltransferase 111 ("GnTIII17y), a glycosyltransferase catalyzing the formation of bisected oligosaccharides, significantly increases the in vitro ADCC activity of an antineuroblastoma chimeric monoclonal antibody (chCE7) produced by the engineered CHO cells. (See Umana, P., et al, *Nature Biotechnol.* 17 (1999) 176-180; and WO 99/154342, the entire contents of which are hereby incorporated by reference). The antibody chCE7 belongs to a large class of unconjugated monoclonal antibodies which have high tumor affinity and specificity, but have too little potency to be clinically useful when produced in standard industrial cell lines lacking the GnTIII enzyme (Umana, P., et al, *Nature Biotechnol.* 17 (1999) 176-180). That study was the first to show that large increases of ADCC activity could be obtained by engineering the antibody producing cells to express GnTIII, which also led to an increase in the proportion of constant region (Fc)-associated, bisected oligosaccharides, including bisected, non-fucosylated oligosaccharides, above the levels found in naturally-occurring antibodies.

The term "cancer" as used herein includes lymphomas, lymphocytic leukemias, lung cancer, non small cell lung (NSCL) cancer, bronchioloalviolar cell lung cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer,

cancer of the anal region, stomach cancer, gastric cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, mesothelioma, hepatocellular cancer, biliary cancer, neoplasms of the central nervous system (CNS), spinal axis tumors, brain stem glioma, glioblastoma multiforme, astrocytomas, schwannomas, ependymomas, medulloblastomas, meningiomas, squamous cell carcinomas, pituitary adenoma, including refractory versions of any of the above cancers, or a combination of one or more of the above cancers. Preferably the term cancer refers to a CD20 expressing cancer.

The term "expression of the CD20" antigen is intended to indicate an significant level of expression of the CD20 antigen in a cell, preferably on the cell surface of a T- or B- Cell, more preferably a B-cell, from a tumor or cancer, respectively, preferably a non-solid tumor. Patients having a "CD20 expressing cancer" can be determined by standard assays known in the art. E.g. CD20 antigen expression is measured using immunohistochemical (IHC) detection, FACS or via PCR-based detection of the corresponding mRNA.

The term "CD20 expressing cancer" as used herein refers to all cancers in which the cancer cells show an expression of the CD20 antigen. Preferably CD20 expressing cancer as used herein refers to lymphomas (preferably B-Cell Non-Hodgkin's lymphomas (NHL)) and lymphocytic leukemias. Such lymphomas and lymphocytic leukemias include e.g. a) follicular lymphomas, b) Small Non-Cleaved Cell Lymphomas/ Burkitt's lymphoma (including endemic Burkitt's lymphoma, sporadic Burkitt's lymphoma and Non-Burkitt's lymphoma) c) marginal zone lymphomas (including extranodal marginal zone B cell lymphoma (Mucosa-associated lymphatic tissue lymphomas, MALT), nodal marginal zone B cell lymphoma and splenic marginal zone lymphoma), d) Mantle cell lymphoma (MCL), e) Large Cell Lymphoma (including B-cell diffuse large cell lymphoma (DLCL), Diffuse Mixed Cell Lymphoma, Immunoblastic Lymphoma, Primary Mediastinal B-Cell Lymphoma, Angiocentric Lymphoma-Pulmonary B-Cell Lymphoma) f) hairy cell leukemia, g ) lymphocytic lymphoma, Waldenstrom's macroglobulinemia, h) acute lymphocytic leukemia (ALL), chronic lymphocytic

leukemia (CLL)/ small lymphocytic lymphoma (SLL), B-cell prolymphocytic leukemia, i) plasma cell neoplasms, plasma cell myeloma, multiple myeloma, plasmacytoma j) Hodgkin's disease.

5 More preferably the CD20 expressing cancer is a B-Cell Non-Hodgkin's lymphomas (NHL). Especially the CD20 expressing cancer is a Mantle cell lymphoma (MCL), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), B-cell diffuse large cell lymphoma (DLCL), Burkitt's lymphoma, hairy cell leukemia, follicular lymphoma, multiple myeloma, marginal zone lymphoma, post transplant lymphoproliferative disorder (PTLD), HIV associated  
10 lymphoma, Waldenstrom's macroglobulinemia, or primary CNS lymphoma.

The term "a method of treating" or its equivalent, when applied to, for example, cancer refers to a procedure or course of action that is designed to reduce or eliminate the number of cancer cells in a patient, or to alleviate the symptoms of a cancer. "A method of treating" cancer or another proliferative disorder does not  
15 necessarily mean that the cancer cells or other disorder will, in fact, be eliminated, that the number of cells or disorder will, in fact, be reduced, or that the symptoms of a cancer or other disorder will, in fact, be alleviated. Often, a method of treating cancer will be performed even with a low likelihood of success, but which, given the medical history and estimated survival expectancy of a patient, is nevertheless  
20 deemed to induce an overall beneficial course of action.

The terms "co-administration" or "co-administering" refer to the administration of said afucosylated anti-CD20, and said mTOR inhibitor as one single formulation or as two separate formulations. The co-administration can be simultaneous or sequential in either order, wherein preferably there is a time period while both (or  
25 all) active agents simultaneously exert their biological activities. Said anti-CD20 afucosylated antibody and said mTOR inhibitor are co-administered either simultaneously or sequentially (e.g. via an intravenous (i.v.) through a continuous infusion (one for the anti-CD20 antibody and eventually one for said mTOR inhibitor. When both therapeutic agents are co-administered sequentially the dose  
30 is administered either on the same day in two separate administrations, or one of the agents is administered on day 1 and the second is co-administered on day 2 to day 7, preferably on day 2 to 4. Thus the term "sequentially" means within 7 days after the dose of the first component ( anti-CD20 antibody or mTOR inhibitor), preferably within 4 days after the dose of the first component; and the term  
35 "simultaneously" means at the same time. The terms "co-administration" with

respect to the maintenance doses of said afucosylated anti-CD20 antibody and said mTOR inhibitor mean that the maintenance doses can be either co-administered simultaneously, if the treatment cycle is appropriate for both drugs, e.g. every week. Or said mTOR inhibitor is e.g. administered e.g. every first to third day and said afucosylated antibody is administered every week. Or the maintenance doses are co-administered sequentially, either within one or within several days.

It is self-evident that the antibodies are administered to the patient in a "therapeutically effective amount" (or simply "effective amount") which is the amount of the respective compound or combination that will elicit the biological or medical response of a tissue, system, animal or human that is being sought by the researcher, veterinarian, medical doctor or other clinician.

The amount of co-administration of said anti-CD20 afucosylated antibody and said mTOR inhibitor and the timing of co-administration will depend on the type (species, gender, age, weight, etc.) and condition of the patient being treated and the severity of the disease or condition being treated. Said afucosylated anti-CD20 antibody and said mTOR inhibitor are suitably co-administered to the patient at one time or over a series of treatments.

If the administration is intravenous the initial infusion time for said afucosylated anti-CD20 antibody or said mTOR inhibitor may be longer than subsequent infusion times, for instance approximately 90 minutes for the initial infusion, and approximately 30 minutes for subsequent infusions (if the initial infusion is well tolerated).

Depending on the type and severity of the disease, about 1 µg/kg to 50 mg/kg (e.g. 0.1-20 mg/kg) of said afucosylated anti-CD20 antibody and 1 µg/kg to 50 mg/kg (e.g. 0.1-20 mg/kg) of said mTOR inhibitor is an initial candidate dosage for co-administration of both drugs to the patient. In one embodiment the preferred dosage of said afucosylated anti-CD20 antibody (preferably the afucosylated humanized B-Ly1 antibody) will be in the range from about 0.05mg/kg to about 30mg/kg. Thus, one or more doses of about 0.5mg/kg, 2.0mg/kg, 4.0mg/kg, 10mg/kg or 30mg/kg (or any combination thereof) may be co-administered to the patient. The preferred dosage of said mTOR inhibitor will be in the range from 0.01 mg/kg to about 30 mg/kg, e.g. 0.1 mg/kg to 10.0 mg/kg. Depending on the on the type (species, gender, age, weight, etc.) and condition of the patient and on the type of afucosylated anti-CD20 antibody, the dosage and the administration

5 schedule of said afucosylated antibody can differ for said mTOR inhibitor. E.g. the said afucosylated anti-CD20 antibody may be administered e.g. every one to three weeks and said mTOR inhibitor may be administered daily or every 2 to 10 days. An initial higher loading dose, followed by one or more lower doses may also be administered.

10 Preferably said humanized B-Lyl antibody is administered in a dosage of 800 to 1200 mg on day 1, 8, 15 of a 6-weeks-dosage-cycle and then in a dosage of 800 to 1200 mg on day 1 of up to five 4-weeks-dosage-cycles. Alternatively the preferred dosage of said afucosylated anti-CD20 antibody can be 800 to 1200 mg (preferably 1000 mg) on day 1 up to eight 3-weeks-dosage-cycles

15 Preferably said mTOR inhibitor is administered in a dosage of 25 mg/weekly to 175 mg/weekly depending on the type of lymphoma disease. E.g. said mTOR inhibitor is administered in mantle cell lymphoma (MCL) in a dosage of 75 mg/weekly. (For relapsed or refractory MCL further dosage once-weekly of 175 mg for 3 weeks followed by 75 mg once weekly (175/75) is possible. For follicular NHL, DLBCL und CLL e.g. a dosage of 25 mg/weekly is administered.

20 In a preferred embodiment, the medicament is useful for preventing or reducing metastasis or further dissemination in such a patient suffering from cancer, preferably CD20 expressing cancer. The medicament is useful for increasing the duration of survival of such a patient, increasing the progression free survival of such a patient, increasing the duration of response, resulting in a statistically significant and clinically meaningful improvement of the treated patient as measured by the duration of survival, progression free survival, response rate or duration of response. In a preferred embodiment, the medicament is useful for increasing the response rate in a group of patients.

30 In the context of this invention, additional other cytotoxic, chemotherapeutic or anti-cancer agents, or compounds that enhance the effects of such agents (e.g. cytokines) may be used in the afucosylated anti-CD20 antibody and said mTOR inhibitor combination treatment of cancer. Such molecules are suitably present in combination in amounts that are effective for the purpose intended. Preferably the said afucosylated anti-CD20 antibody and said mTOR inhibitor combination treatment is used without such additional cytotoxic, chemotherapeutic or anti-cancer agents, or compounds that enhance the effects of such agents.

Such agents include, for example: alkylating agents or agents with an alkylating action, such as cyclophosphamide (CTX; e.g. Cytosan®), chlorambucil (CHL; e.g. leukeran®), cisplatin (CisP; e.g. platinol®), busulfan (e.g. myleran®), melphalan, carmustine (BCNU), streptozotocin, triethylenemelamine (TEM), mitomycin C, and the like; anti-metabolites, such as methotrexate (MTX), etoposide (VP 16; e.g. vepesid®), 6-mercaptopurine (6MP), 6-thioguanine (6TG), cytarabine (Ara-C), 5-fluorouracil (5-FU), capecitabine (e.g. Xeloda®), dacarbazine (DTIC), and the like; antibiotics, such as actinomycin D, doxorubicin (DXR; e.g. adriamycin®), daunorubicin (daunomycin), bleomycin, mithramycin and the like; alkaloids, such as vinca alkaloids such as vincristine (VCR), vinblastine, and the like; and other antitumor agents, such as paclitaxel (e.g. taxol®) and paclitaxel derivatives, the cytostatic agents, glucocorticoids such as dexamethasone (DEX; e.g. decadron®) and corticosteroids such as prednisone, nucleoside enzyme inhibitors such as hydroxyurea, amino acid depleting enzymes such as asparaginase, leucovorin and other folic acid derivatives, and similar, diverse antitumor agents. The following agents may also be used as additional agents: arnifostine (e.g. ethyol®), dactinomycin, mechlorethamine (nitrogen mustard), streptozocin, cyclophosphamide, lomustine (CCNU), doxorubicin lipo (e.g. doxil®), gemcitabine (e.g. gemzar®), daunorubicin lipo (e.g. daunoxome®), procarbazine, mitomycin, docetaxel (e.g. taxotere®), aldesleukin, carboplatin, oxaliplatin, cladribine, camptothecin, CPT 11 (irinotecan), 10-hydroxy 7-ethyl-camptothecin (SN38), floxuridine, fludarabine, ifosfamide, idarubicin, mesna, interferon beta, interferon alpha, mitoxantrone, topotecan, leuprolide, megestrol, melphalan, mercaptopurine, plicamycin, mitotane, pegaspargase, pentostatin, pipobroman, plicamycin, tamoxifen, teniposide, testolactone, thioguanine, thiotepa, uracil mustard, vinorelbine, chlorambucil. Preferably the afucosylated anti-CD20 antibody and said mTOR inhibitor combination treatment is used without such additional agents.

The use of the cytotoxic and anticancer agents described above as well as antiproliferative target-specific anticancer drugs like protein kinase inhibitors in chemotherapeutic regimens is generally well characterized in the cancer therapy arts, and their use herein falls under the same considerations for monitoring tolerance and effectiveness and for controlling administration routes and dosages, with some adjustments. For example, the actual dosages of the cytotoxic agents may vary depending upon the patient's cultured cell response determined by using

histoculture methods. Generally, the dosage will be reduced compared to the amount used in the absence of additional other agents.

5 Typical dosages of an effective cytotoxic agent can be in the ranges recommended by the manufacturer, and where indicated by in vitro responses or responses in animal models, can be reduced by up to about one order of magnitude concentration or amount. Thus, the actual dosage will depend upon the judgment of the physician, the condition of the patient, and the effectiveness of the therapeutic method based on the in vitro responsiveness of the primary cultured malignant cells or histocultured tissue sample, or the responses observed in the appropriate animal  
10 models.

In the context of this invention, an effective amount of ionizing radiation may be carried out and/or a radiopharmaceutical may be used in addition to the afucosylated anti-CD20 antibody and said mTOR inhibitor combination treatment of CD20 expressing cancer. The source of radiation can be either external or  
15 internal to the patient being treated. When the source is external to the patient, the therapy is known as external beam radiation therapy (EBRT). When the source of radiation is internal to the patient, the treatment is called brachytherapy (BT). Radioactive atoms for use in the context of this invention can be selected from the group including, but not limited to, radium, cesium-137, iridium-192,  
20 americium-241, gold-198, cobalt-57, copper-67, technetium-99, iodine-123, iodine-131, and indium-111. It is also possible to label the antibody with such radioactive isotopes. Preferably the afucosylated anti-CD20 antibody and said mTOR inhibitor combination treatment is used without such ionizing radiation.

Radiation therapy is a standard treatment for controlling unresectable or inoperable  
25 tumors and/or tumor metastases. Improved results have been seen when radiation therapy has been combined with chemotherapy. Radiation therapy is based on the principle that high-dose radiation delivered to a target area will result in the death of reproductive cells in both tumor and normal tissues. The radiation dosage regimen is generally defined in terms of radiation absorbed dose (Gy), time and  
30 fractionation, and must be carefully defined by the oncologist. The amount of radiation a patient receives will depend on various considerations, but the two most important are the location of the tumor in relation to other critical structures or organs of the body, and the extent to which the tumor has spread. A typical course of treatment for a patient undergoing radiation therapy will be a treatment schedule  
35 over a 1 to 6 week period, with a total dose of between 10 and 80 Gy administered

to the patient in a single daily fraction of about 1.8 to 2.0 Gy, 5 days a week. In a preferred embodiment of this invention there is synergy when tumors in human patients are treated with the combination treatment of the invention and radiation. In other words, the inhibition of tumor growth by means of the agents comprising the combination of the invention is enhanced when combined with radiation, optionally with additional chemotherapeutic or anticancer agents. Parameters of adjuvant radiation therapies are, for example, contained in WO 99/60023.

The afucosylated anti-CD20 antibodies are administered to a patient according to known methods, by intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, or intrathecal routes. Intravenous or subcutaneous administration of the antibodies is preferred.

The mTOR inhibitor is administered to a patient according to known methods, e.g. by intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, or peroral routes. Intravenous or intraperitoneal administration is preferred.

As used herein, a "pharmaceutically acceptable carrier" is intended to include any and all material compatible with pharmaceutical administration including solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and other materials and compounds compatible with pharmaceutical administration. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

#### **Pharmaceutical Compositions:**

Pharmaceutical compositions can be obtained by processing the anti-CD20 antibody and/or the mTOR inhibitor according to this invention with pharmaceutically acceptable, inorganic or organic carriers. Lactose, corn starch or derivatives thereof, talc, stearic acids or its salts and the like can be used, for example, as such carriers for tablets, coated tablets, dragees and hard gelatine capsules. Suitable carriers for soft gelatine capsules are, for example, vegetable oils, waxes, fats, semi-solid and liquid polyols and the like. Depending on the nature of the active substance no carriers are, however, usually required in the case

of soft gelatine capsules. Suitable carriers for the production of solutions and syrups are, for example, water, polyols, glycerol, vegetable oil and the like. Suitable carriers for suppositories are, for example, natural or hardened oils, waxes, fats, semi-liquid or liquid polyols and the like.

5 The pharmaceutical compositions can, moreover, contain preservatives, solubilizers, stabilizers, wetting agents, emulsifiers, sweeteners, colorants, flavorants, salts for varying the osmotic pressure, buffers, masking agents or antioxidants. They can also contain still other therapeutically valuable substances.

10 One embodiment of the invention is composition comprising both said afucosylated anti-CD20 antibody with an amount of fucose is 60% or less (preferably said afucosylated humanized B-Lyl antibody) and said mTOR inhibitor for use in the treatment of cancer, in particular of CD20 expressing cancer.

Said pharmaceutical composition may further comprise one or more pharmaceutically acceptable carriers.

15 The present invention further provides a pharmaceutical composition, in particular for use in cancer, comprising (i) an effective first amount of an afucosylated anti-CD20 antibody with an amount of fucose is 60% or less (preferably an afucosylated humanized B-Lyl antibody), and (ii) an effective second amount of a mTOR inhibitor. Such composition optionally comprises pharmaceutically  
20 acceptable carriers and / or excipients.

Pharmaceutical compositions of the afucosylated anti-CD20 antibody alone used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences  
25 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol,  
30 butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids

such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal  
5 complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

Pharmaceutical compositions of the mTOR inhibitor can be similar to those describe above for the afucosylated anti-CD20 antibody.

10 In one further embodiment of the invention the pharmaceutical compositions according to the invention are preferably two separate formulations for said afucosylated anti-CD20 antibody and said mTOR inhibitor.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interracial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly- (methylmethacrylate)  
15 microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano- particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

20 Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (US 3,773,919), copolymers of L-glutamic acid  
25 and gamma-ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

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

The present invention further provides a method for the treatment of cancer, comprising administering to a patient in need of such treatment (i) an effective first amount of an afucosylated anti-CD20 antibody with an amount of fucose is 60% or

less, (preferably an afucosylated humanized B-Lyl antibody); and (ii) an effective second amount of a mTOR inhibitor.

Preferably the amount of fucose of is between 40% and 60%.

Preferably said cancer is a CD20 expressing cancer.

5 Preferably said CD20 expressing cancer is a B-Cell Non-Hodgkin's lymphoma (NHL).

Preferably said afucosylated anti-CD20 antibody is a type II anti-CD20 antibody.

Preferably said antibody is a humanized B-Lyl antibody.

Preferably the mTOR inhibitor is a rapamycin, or a rapamycin analog or derivative.

10 Preferably the mTOR inhibitor is Temsirolimus or Everolimus.

Preferably said humanized B-Lyl antibody is administered in a dosage of 800 to 1200 mg on day 1, 8, 15 of a 6-weeks-dosage-cycle and then in a dosage of 800 to 1200 mg on day 1 of up to five 4-weeks-dosage-cycles. Preferably said mTOR inhibitor is administered in a dosage of 25 mg/weekly to 175 mg/weekly depending  
15 on the type of lymphoma disease. E.g. said mTOR inhibitor is administered in mantle cell lymphoma (MCL) in a dosage of 75 mg/weekly. (For relapsed or refractory MCL further dosage once-weekly of 175 mg for 3 weeks followed by 75 mg once weekly (175/75) is possible. For follicular NHL, DLBCL und CLL e.g. a dosage of 25 mg/weekly is administered.

20 As used herein, the term "patient" preferably refers to a human in need of treatment with an afucosylated anti-CD20 antibody (e.g. a patient suffering from CD20 expressing cancer) for any purpose, and more preferably a human in need of such a treatment to treat cancer, or a precancerous condition or lesion. However, the term "patient" can also refer to non-human animals, preferably mammals such as dogs,  
25 cats, horses, cows, pigs, sheep and non-human primates, among others.

The invention further comprises an afucosylated anti-CD20 antibody with an amount of fucose is 60% or less, for the treatment of cancer in combination with a mTOR inhibitor.

The invention further comprises an afucosylated anti-CD20 antibody with an amount of fucose is 60% or less, and a mTOR inhibitor for use in the treatment of cancer.

Preferably said afucosylated anti-CD20 antibody is a humanized B-Lyl antibody.

5 Preferably the mTOR inhibitor is a rapamycin, or a rapamycin analog or derivative. Preferably the mTOR inhibitor is Temsirolimus or Everolimus.

Preferably the cancer is a CD20 expressing cancer, more preferably a B-Cell Non-Hodgkin's lymphoma (NHL).

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

### Sequence Listing

15 **SEQ ID NO: 1** amino acid sequence of variable region of the heavy chain (VH) of murine monoclonal anti-CD20 antibody B-Lyl.

**SEQ ID NO: 2** amino acid sequence of variable region of the light chain (VL) of murine monoclonal anti-CD20 antibody B-Lyl.

**SEQ ID NO: 3 -19** amino acid sequences of variable region of the heavy chain (VH) of humanized B-Lyl antibodies (B-HH2 to B-HH9, B-HL8, and B-HL10 to B-HL17)

20 **SEQ ID NO: 20** amino acid sequences of variable region of the light chain (VL) of humanized B-Lyl antibody B-KV1

### Experimental Procedures

25

**Antitumor activity of combined treatment of a type II anti-CD20 antibody (B-HH6-B-KV1 GE) with Temsirolimus (Torisel®).**

#### **Test agents**

30 Type II anti-CD20 antibody B-HH6-B-KV1 GE (= humanized B-Lyl, glycoengineered B-HH6-B-KV1 = GA101; see WO 2005/044859 and WO 2007/031875) was provided from GlycArt, Schlieren, Switzerland. Antibody buffer included histidine, trehalose and polysorbate 20. Antibody solution was diluted

appropriately in PBS from stock for prior injections. Temsirolimus (Torisel®, 25 mg/ml, Wyeth Pharmaceuticals) was supplied by Oncodesign and stored at +4°C protected from light according to the supplier's instructions.

### Cell lines and culture conditions

5 SU-DHL-4 human lymphoma cells (DSMZ NO.: ACC 495) were grown as a suspension at 37°C in a humidified atmosphere (5% CO<sub>2</sub>, 95% air). The culture medium was RPMI 1640 containing 2 mM L-glutamine (Ref BE12-702F, Batch N° 8MB0056, Lonza, Venders, Belgium) and supplemented with 10% fetal bovine serum (Ref 3302, Batch N° P282005, Lonza). The cells were counted in a  
10 hemocytometer and their viability was assessed by 0.25% trypan blue exclusion.

### Animals

Female CB17 SCID beige mice, 5-6 week-old and weighing 16-20 g, were obtained from Charles River (L'Arbresle, France). Animals were observed for 7 days in our specific-pathogen-free (SPF) animal care before treatment.

15 The animal care unit is authorized by the French ministries of Agriculture and Research (agreement No A2123101 1). Animal experiments were performed according to ethical guidelines of animal experimentation (1) and the English guidelines for welfare of animals in experimental neoplasia (2).

### Induction of SC SU-DHL-4 tumors in SCID beige mice

20 Ten millions (10<sup>7</sup>) SU-DHL-4 tumor cells in 100 µl of PBS with matrigel (50:50, BD Biosciences, France) were subcutaneous ly (SC) injected into the right flank of 52 female SCID beige mice.

### Monitoring

25 All study data, including animal body weight measurements, tumor volume, clinical and mortality records, and drug treatment management were managed using Vivo Manager® software (Biosystemes, Dijon, France).

30 Isoflurane Forene (Minerve, Bondoufle, France) was used to anaesthetize the animals before SC inoculation of tumor cells, IV injection of compounds and sacrifice. Mortality, clinical signs and behaviour were recorded every day. Animal body weights and tumor volumes were monitored and recorded twice a week.

### Treatment of animals

When the tumors reached a mean volume of  $173 \pm 95 \text{ mm}^3$ , 40 out of 52 tumor bearing nude mice were distributed in 4 groups of 10 mice. The treatment schedule was chosen as following:

- 5           • Mice from group 1 received once weekly IV bolus injection of vehicle for 4 consecutive weeks (Q7Dx4).
- Mice from group 2 received once weekly IV bolus injection of anti-CD20 antibody GAI01 (B-HH6-B-KV1 GE) at 3 mg/kg/inj for 4 consecutive weeks (Q7Dx4).
- 10          • Mice from group 3 received once daily IP injection of Torisel® at 3 mg/kg/inj with a 3-day interval between each dosing and a total of 10 injections (Q3Dxl0).
- Mice from group 4 received once weekly IV bolus injection of anti-CD20 antibody GAI01 (B-HH6-B-KV1 GE) at 3 mg/kg/inj for 4 consecutive weeks (Q7Dx4) in combination with once daily IP injection of Torisel® at 3 mg/kg/inj with a 3-day interval between each dosing and a total of 10 injections (Q3Dxl0)].
- 15

### Tumor growth inhibition study in vivo

20           The antitumor efficacy of anti-CD20 antibody GAI01 (B-HH6-B-KV1 GE) alone and in combination with Torisel® was examined. Torisel® injected as a single agent was used as the reference compound. In comparison with tumors from vehicle-treated animals, SU-DHL-4 tumors growth was partially delayed in mice treated with 3 mg/kg anti-CD20 antibody GAI01 (B-HH6-B-KV1 GE) or Torisel® alone. Combined treatment of anti-CD20 antibody GAI01 (B-HH6-B-KV1 GE) plus Torisel® improved antitumor activity in vivo compared with either agent alone. For combination, the tumor growth delay value was higher than that for the corresponding doses of anti-CD20 antibody GAI01 (B-HH6-B-KV1 GE) or Torisel® alone. Tumor growth was consistently slower in combination treatment group than in the single-agent groups.

30           On day 46 after tumor cell injection, the T/C (%) values of the treatment groups compared to the vehicle group were 51, 60 and 35 with anti-CD20 antibody GAI01 (B-HH6-B-KV1 GE), Torisel and the combination of both, respectively.

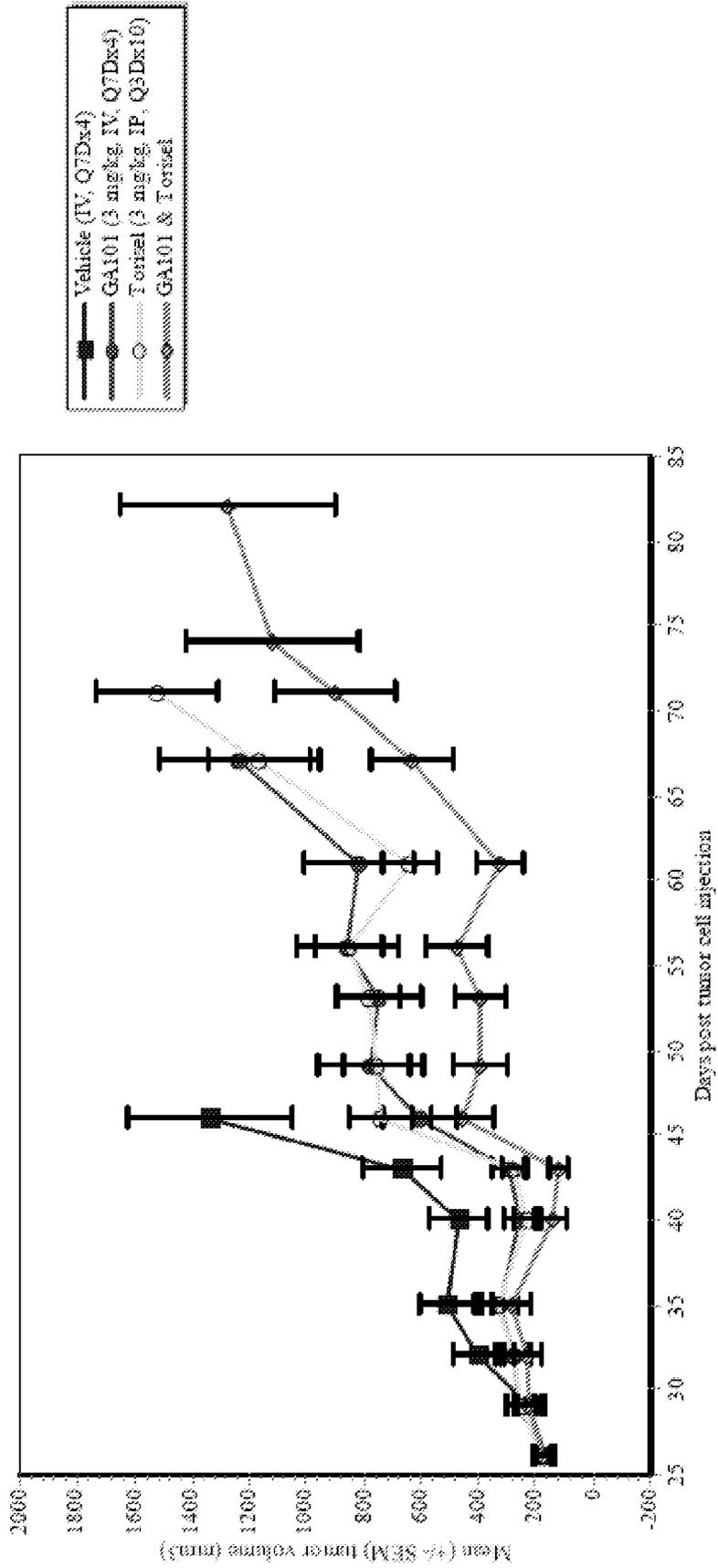
Results of the Tumor volume development during therapy are shown in Figure 1.

**Patent Claims**

1. Use of an afucosylated anti-CD20 antibody with an amount of fucose of 60% or less, for the manufacture of a medicament for the treatment of cancer in combination with a mTOR inhibitor.
- 5 2. Use according to claim 1 characterized in that the amount of fucose of is between 40% and 60%.
3. Use according to claim 1 characterized in that the amount of fucose of is 50% or less.
4. Use according to any one of claims 1 to 3 characterized in that said cancer  
10 is a CD20 expressing cancer.
5. Use according to claim 4 characterized in that said CD20 expressing cancer is a B-Cell Non-Hodgkin's lymphoma (NHL).
6. Use according to any one of claims 1 to 5, characterized in that said afucosylated anti-CD20 antibody is a type II anti-CD20 antibody.
- 15 7. Use according to claims 1 or 6, characterized in that said antibody is a humanized B-Lyl antibody.
8. Use according to claim 7, characterized in that said mTOR inhibitor is rapamycin, or a rapamycin analog or derivative.
9. Use according to claim 7, characterized in that said mTOR inhibitor is is  
20 Temsirolimus or Everolimus.
10. Use according to any one of claims 1 to 9, characterized in that one or more additional other cytotoxic, chemotherapeutic or anti-cancer agents, or compounds or ionizing radiation that enhance the effects of such agents are administered.
- 25 11. A composition comprising an afucosylated anti-CD20 antibody with an amount of fucose of 60% or less and a mTOR inhibitor for the treatment of cancer.
12. The composition according to claim 11, characterized in that said anti-afucosylated CD20 antibody is a humanized B-Lyl antibody.

13. A method of treatment of a patient suffering from cancer by administering an afucosylated anti-CD20 antibody with an amount of fucose of 60% or less in combination with a mTOR inhibitor, to a patient in the need of such treatment.
- 5
14. The method of treatment according to claim 13 characterized in that the amount of fucose of is between 40% and 60%>.
  15. The method of treatment according to claims 13 to 14 characterized in that said cancer is a B-Cell Non-Hodgkin's lymphoma (NHL).

**Figure 1**



# INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2011/056461

**A. CLASSIFICATION OF SUBJECT MATTER**  
INV. A61K39/395 A61K31/0Q  
ADD.

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal , BIOSIS, EMBASE, PAJ, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GOY A: "New directions in the treatment of mantle cell lymphoma : an overview" , CLINICAL LYMPHOMA & MYELOMA, CANCER INFORMATION GROUP, US, vol . 7, no. Suppl .1, 1 October 2006 (2006-10-01) , pages S24-S32, XP008085786, ISSN : 1557-9190 the whole document -----	1-15
Y	Wo 2008/121876 A2 (BIOGEN IDEC INC [US] ; REFF MITCHELL [US] ; HARIHARAN KANDASAMY [US] ; MA) 9 October 2008 (2008-10-09) the whole document -----	1-15
A	US 2009/060921 AI (DICKEY LYNN F [US] ET AL) 5 March 2009 (2009-03-05) the whole document -----	1-15
- / - -		

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

27 June 2011

Date of mailing of the international search report

04/07/2011

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## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2011/056461

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>FANALE MICHELLE A ET AL: "Monoclonal antibodies in the treatment of non-Hodgkin's lymphoma", DRUGS, ADIS INTERNATIONAL LTD, NZ LNKD- DOI : 10.2165/00003495-200767030-00002 , vol . 67, no. 3, 1 January 2007 (2007-01-01) , pages 333-350, XP009101539 , ISSN : 0012-6667 the whole document</p> <p style="text-align: center;">-----</p>	1-15
A	<p>ZHOU YUHONG ET AL: "Immunotherapy in mantle cell lymphoma: Anti-CD20-based therapy and beyond", AMERICAN JOURNAL OF HEMATOLOGY, NEW YORK, NY, us LNKD- DOI : 10.1002/AJH .21036, vol . 83, no. 2, 1 August 2007 (2007-08-01) , pages 144-149, XP002519364, ISSN : 0361-8609 [retrieved on 2007-08-23] the whole document</p> <p style="text-align: center;">-----</p>	1-15

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2 011/056461

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

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

a. (means)

on paper

in electronic form

b. (time)

in the international application as filed

together with the international application in electronic form

subsequently to this Authority for the purpose of search

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

3. Additional comments:

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2011/056461

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008121876 A2	09-10-2008	CA 2682382 A1 EP 2142569 A2	09-10-2008 13-01-2010
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US 2009060921 A1	05-03-2009	NONE	
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