In another preferred embodiment, the humanized anti-CD22 antibody is a CDR-grafted antibody comprising a light chain having a sequence set forth in SEQ ID NO:28 and a heavy chain having a sequence set forth in SEQ ID NO:30.

In another embodiment, the humanized anti-CD22 antibody is a CDR-grafted antibody that is a variant antibody obtained by an affinity maturation protocol and has increased ~~specificity~~ affinity for human CD22.

In another aspect, the cytotoxic drug used to generate the monomeric cytotoxic drug/carrier conjugate of the present invention is either an inhibitor of tubulin polymerization, an alkylating agent that binds to and disrupts DNA, an inhibitor protein synthesis, or an inhibitor of tyrosine kinases.

In one embodiment, the cytotoxic drug is selected from calicheamicins, thiotepa, taxanes, vincristine, daunorubicin, doxorubicin, epirubicin, esperamicins, actinomycin, authramycin, azaserines, bleomycins, tamoxifen, idarubicin, dolastatins/auristatins, hemiasterlins, and maytansinoids.

In a preferred embodiment, the cytotoxic drug is calicheamicin. In a particularly preferred embodiment, the calicheamicin is gamma calicheamicin or N-acetyl gamma calicheamicin derivative.

In yet another aspect, the cytotoxic drug is functionalized with 3-mercapto-3-methyl butanoyl hydrazide and conjugated to a proteinaceous carrier via a hydrolyzable linker that is capable of releasing the cytotoxic drug from the conjugate after binding and entry into target cells. In a preferred embodiment of this aspect, the hydrolyzable linker is 4-(4-acetylphenoxy) butanoic acid (AcBut).

In yet another aspect of the invention, octanoic acid or its salt, or decanoic acid or its salt is used as an additive during the conjugation process to decrease aggregation and increase drug loading.

In yet another aspect of the invention, the conjugates of the invention are purified by a chromatographic separation process.

In one embodiment, the chromatographic separation process used to separate the monomeric drug derivative-carrier conjugate is size exclusion chromatography (SEC).

In still another embodiment, the humanized anti-CD22 antibody is a CDR-grafted antibody that is a variant antibody obtained by an affinity maturation protocol which has increased ~~specificity~~ affinity for human CD22.

In a preferred embodiment, the calicheamicin is gamma calicheamicin or N-acetyl gamma calicheamicin.

In one embodiment, the calicheamicin derivative is functionalized with 3-mercapto-3-methyl butanoyl hydrazide.

In another embodiment, the linker used to conjugate the drug to the carrier is a hydrolyzable linker that is capable of releasing the cytotoxic drug from the conjugate after binding and entry into target cells. In a preferred embodiment, the hydrolyzable linker is 4-(4-acetylphenoxy) butanoic acid (AcBut).

Another aspect of the invention is directed to a monomeric calicheamicin derivative/anti-CD22 antibody conjugate having the formula, $Pr(-X-S-S-W)_m$ wherein: Pr is an anti-CD22 antibody; X is a hydrolyzable linker that comprises a product of any reactive group that can react with an antibody; W is a calicheamicin radical; m is the average loading for a purified conjugation product such that the calicheamicin constitutes 4 - 10% of the conjugate by weight; and (-X-S-S-W)m is a calicheamicin derivative generated by the process of the invention.

In one embodiment of this aspect, the antibody is selected from a group consisting of a monoclonal antibody, a chimeric antibody, a human antibody, a humanized antibody, a single chain antibody, a Fab fragment and a $F(ab)_2$ fragment.

In a preferred embodiment, the antibody is an anti-CD22 antibody that has specificity for human CD22, and comprises a heavy chain wherein the variable domain comprises a CDR having at least one of the sequences given as H1 in Figure 1 (SEQ ID NO:1) for CDR-H1, as H2 in Figure 1 (SEQ ID NO:2) or H2' (SEQ ID NO:13) or H2" (SEQ ID NO:15) or H2"' (SEQ ID NO:16) for CDR-H2, or as H3 in Figure 1 (SEQ ID NO:3) for CDR-H3, and comprises a light chain wherein the variable domain comprises a CDR having at least one of the sequences given as L1 in Figure 1 (SEQ ID NO:4) for CDR-L1, as L2 in Figure 1 (SEQ ID NO:5) for CDR-L2, or as L3 in Figure 1 (SEQ ID NO:6) for CDR-L3.

In another preferred embodiment, the anti-CD22 antibody comprises a heavy chain wherein the variable domain comprises a CDR having at least one of the sequences given in SEQ ID NO:1 for CDR-H1, SEQ ID NO:2 or SEQ ID NO:13 or

In one embodiment, the anti-CD22 CDR-grafted antibody is a variant antibody obtained by an affinity maturation protocol and has increased ~~specificity~~ affinity for human CD22.

In another embodiment, the anti-CD22 antibody is a chimeric antibody comprising the sequences of the light and heavy chain variable domains of the monoclonal antibody set forth in SEQ ID NO:7 and SEQ ID NO:8, respectively.

In yet another embodiment, the anti-CD22 antibody comprises a hybrid CDR with a truncated donor CDR sequence wherein the missing portion of the donor CDR is replaced by a different sequence and forms a functional CDR.

In a particularly preferred embodiment, the cytotoxic drug derivative is either a gamma calicheamicin or a N-acetyl gamma calicheamicin derivative.

In another aspect, the invention is directed to a method for the preparation of a stable lyophilized composition of a monomeric cytotoxic drug derivative/carrier conjugate. In a preferred embodiment, the stable lyophilized composition of the monomeric cytotoxic drug derivative/carrier conjugate is prepared by (a) dissolving the monomeric cytotoxic drug derivative/carrier conjugate to a final concentration of 0.5 to 2 mg/ml in a solution comprising a cryoprotectant at a concentration of 1.5%-5% by weight, a polymeric bulking agent at a concentration of 0.5-1.5% by weight, electrolytes at a concentration of 0.01 M to 0.1 M, a solubility facilitating agent at a concentration of 0.005-0.05% by weight, buffering agent at a concentration of 5-50 mM such that the final pH of the solution is 7.8-8.2, and water; (b) dispensing the above solution into vials at a temperature of +5 °C to +10 °C; (c) freezing the solution at a freezing temperature of -35 °C to -50 °C; (d) subjecting the frozen solution to an initial freeze drying step at a primary drying pressure of 20 to 80 microns at a shelf temperature at -10 °C to -40 °C for 24 to 78 hours; and (e) subjecting the freeze-dried product of step (d) to a secondary drying step at a drying pressure of 20 to 80 microns at a shelf temperature of +10 °C to +35 °C for 15 to 30 hours.

In one embodiment, the cryoprotectant used in the lyophilization of the cytotoxic drug/carrier conjugate is selected from alditol, mannitol, sorbitol, inositol, polyethylene glycol, aldonic acid, uronic acid, aldaric acid, aldoses, ketoses, amino sugars, alditols, inositols, glyceraldehydes, arabinose, lyxose, pentose, ribose, xylose, galactose, glucose, hexose, idose, mannose, talose, heptose, glucose, fructose, gluconic acid, sorbitol, lactose, mannitol, methyl α-glucopyranoside,

region 5/44-gL1 (SEQ ID NO:19), and a heavy chain variable region 5/44-gH7 (SEQ ID NO:27).

In another particularly preferred embodiment, the humanized anti-CD22 antibody is a CDR-grafted antibody having specificity for human CD22 and comprises a light chain having a sequence set forth in SEQ ID NO:28 and a heavy chain having a sequence set forth in SEQ ID NO:30.

In one embodiment, the CDR-grafted antibody is a variant antibody which has increased ~~specificity~~ affinity for human CD22, and the antibody is obtained by an affinity maturation protocol.

In one embodiment, the monomeric cytotoxic drug is calicheamicin and is preferably selected from gamma calicheamicin or N-acetyl calicheamicin.

In one embodiment, the composition may optionally contain an additional bioactive agent. Such a bioactive agent may be a cytotoxic drug, a growth factor or a hormone.

Yet another aspect of the invention is directed to a method of treating a subject with a proliferative disorder by administering to the subject a therapeutically effective dose of the composition of the invention. The composition may be administered subcutaneously, intraperitoneally, intravenously, intraarterially, intramedullarly, intrathecally, transdermally, transcutaneously, intranasally, topically, entereally, intravaginally, sublingually or rectally. In a preferred embodiment, the composition of the invention is administered intravenously.

In one embodiment, the composition is administered to a human subject suffering from a proliferative disorder such as cancer. In a preferred embodiment, the cancer is a B-cell malignancy. The B-cell malignancy may be a leukemia or lymphoma that express cell surface antigen CD22.

In yet another embodiment, the cancer is a carcinoma or a sarcoma.

Another aspect of the present invention is directed to a method of treating a B-cell malignancy by administering to a patient with such malignancy a therapeutically effective composition comprising a cytotoxic drug-anti-CD22-antibody conjugate of the invention. In a preferred embodiment, the B-cell malignancy is a lymphoma, particularly Non-Hodgkin's lymphoma.

In one embodiment, the cytotoxic drug used to prepare the conjugates of the present invention is selected from the group consisting of calicheamicins, thiotepa,

(etoposide, vincristine, and doxorubicin for 96 hours with bolus doses of cyclophosphamide and oral prednisone), ICE (ifosfamide, cyclophosphamide, and etoposide), CEPP(B) (cyclophosphamide, etoposide, procarbazine, prednisone, and bleomycin), CHOP-B (cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin), CEPP-B (cyclophosphamide, etoposide, procarbazine, and bleomycin), and P/DOCE (epirubicin or doxorubicin, vincristine, cyclophosphamide, and prednisone).

In one preferred embodiment, the monomeric calicheamicin derivative/anti-CD22 antibody conjugate is administered prior to the administration of one or more combinations of cytotoxic agents as a part of a treatment regimen.

In another preferred embodiment, the therapeutically effective dose of the monomeric calicheamicin derivative/anti-CD22 antibody conjugate is administered subsequent to the administration of one or more combinations of cytotoxic agents as part of a treatment regimen.

In yet another preferred embodiment, the therapeutically effective dose of the monomeric calicheamicin derivative/anti-CD22 antibody conjugate is administered together with an antibody directed against a cell surface antigen on B-cell malignancies, and optionally comprising one or more combinations of cytotoxic agents as part of a treatment regimen.

In another aspect, the invention is directed to the use of the monomeric calicheamicin derivative/anti-CD22 antibody conjugate of the present invention in the manufacture of a medicament for the treatment of a proliferative disorder. Such a medicament can be used to treat B-cell proliferative disorders either alone or in combination with other bioactive agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequence of the CDRs of mouse monoclonal antibody 5/44 (SEQ ID NOS:1 to 6).

Figure 2 shows the DNA (SEQ ID NOS:48 and 52) and protein (SEQ ID NO:7) sequence of the light chain variable (V_L) domain of mouse monoclonal antibody 5/44.

Figure 3 shows the complete DNA (SEQ ID NOS:49 and 53) and protein (SEQ ID NO: 8) sequence of the heavy chain variable domain (V_H) of mouse monoclonal antibody 5/44.

Figure 4 shows the strategy for removal of the glycosylation site and reactive lysine in CDR-H2 (SEQ ID NOS:9-12 and 14).

Figure 5 shows the graft design for the 5/44 light chain sequence (SEQ ID NO:7). DPK-9 is the human germ-line acceptor framework sequence (SEQ ID NO:17). Vertical lines indicate differences between mouse and human residues. Sequences underlined indicate donor residues which have been retained in the graft. CDRs are indicated in bold italicized letters (not shown for DPK-9). Graft gL1 (SEQ ID NO:19) has 6 donor framework residues, gL2 (SEQ ID NO:20) has 7.

Figure 6 shows the graft design for the 5/44 heavy chain sequence (SEQ ID NO:8); DP7 (SEQ ID NO: 21) is the human germ-line acceptor framework sequence. Vertical lines indicate differences between mouse and human residues. Sequences underlined indicate donor residues which have been retained in the graft. CDRs are indicated initalicized, bold letters (not shown for DP7). Grafts gH4 (SEQ ID NO:24) and gH6 (SEQ ID NO:26) have 6 donor framework residues. Grafts gH5 (SEQ ID NO:25) and gH7 (SEQ ID NO:27) have 4 donor framework residues.

Figure 7 shows the map of vector pMRR14.

Figure 8 shows the map of vector pMRR10.1.

Figure 9 shows the Biacore assay results of the chimeric 5/44 mutants.

Figure 10 shows the oligonucleotides for 5/44 gH1 and gL1 gene assemblies (SEQ ID NOS:32-47).

Figure 11 shows the plasmid map of intermediate vector pCR2.1(544gH1).

Figure 12 shows the plasmid map of intermediate vector pCR2.1(544gL1).

Figure 13 shows the oligonucleotide cassettes used to make further grafts (SEQ ID NOS: 56-63).

Figure 14 is a graph which shows a competition assay between fluorescently labeled mouse 5/44 antibody and grafted variants.

Figure 15 is a graph which shows a competition assay between fluorescently labeled mouse 5/44 antibody and grafted variants.

Figure 16 shows the full DNA (SEQ ID NOS:29, 31, 64 and 65) and protein (SEQ ID NOS:28 and 30) sequences of the grafted heavy and light chains.

Figure 17 is a schematic representation of an antibody-NAc-gamma calicheamicin DMH conjugate.

Figure 18 is a graph which shows the effect of CMC-544 on growth of RAMOS B-cell lymphoma.

Figure 19 is a graph which shows the effect of CMC-544 on large B-cell lymphomas in an in vivo xenograft model in nude mice.

Figure 20 is a graph which compares the effects of CMC-544 made with the CMA-676 conjugation process and the CMC-544 conjugation process on the growth of RL lymphoma.

Figure 21 is a graph which shows that rituximab (Rituxan™)-treated large RL lymphoma is susceptible to CMC-544 treatment.

Figure 22 is a graph which shows the effect of rituximab (Rituxan™) on the cytotoxic effect of CMC-544.

Figure 23 is a graph which shows the effect of CMC-544, rituximab (Rituxan™), and CMA-676 on the survival of SCID mice with disseminated early RAMOS B lymphoma.

Figure 24 is a graph which shows the effect of CMC-544, rituximab (Rituxan™), and CMA-676 on the survival of SCID mice with disseminated late RAMOS B lymphoma.

Figure 25 is a graph which shows the effect of CMC-544, rituximab (Rituxan™), and CMA-676 on the survival of SCID mice with disseminated late RAMOS B lymphoma.

Figure 26 is a graph which shows the effect of CMC-544, rituximab (Rituxan™), and CMA-676 on the survival of SCID mice with disseminated late RAMOS B lymphoma.

Figure 27 is a graph which shows the effect of CMC-544, rituximab (Rituxan™), and CMA-676 on the survival of SCID mice with disseminated late RAMOS B lymphoma.

Figure 28 is a graph which shows the anti-tumor activity of CMC-544 with and without rituximab (Rituxan™) on RL Non-Hodgkin's lymphoma.

Figure 29 is a graph which shows the antitumor activity of CMC-544 and CHOP on RL Non-Hodgkin's lymphoma.

DETAILED DESCRIPTION OF THE INVENTION

The conjugates of the present invention comprise a cytotoxic drug derivatized with a linker that includes any reactive group that reacts with a proteinaceous carrier to form a cytotoxic drug derivative-proteinaceous carrier conjugate. Specifically, the conjugates of the present invention comprise a cytotoxic drug derivatized with a

Exemplified herein for use as a carrier in the present invention is a CDR-grafted humanized antibody molecule directed against cell surface antigen CD22, designated G5/44. This antibody is a humanized form of a murine anti-CD22 monoclonal antibody that is directed against the cell surface antigen CD22, which is prevalent on certain human lymphomas. The term "a CDR-grafted antibody molecule" as used herein refers to an antibody molecule wherein the heavy and/or light chain contains one or more complementarity determining regions (CDRs) including, if desired, a modified CDR (hereinafter CDR) from a donor antibody (e.g., a murine monoclonal antibody) grafted into a heavy and/or light chain variable region framework of an acceptor antibody (e.g., a human antibody). Preferably, such a CDR-grafted antibody has a variable domain comprising human acceptor framework regions as well as one or more of the donor CDRs referred to above.

When the CDRs are grafted, any appropriate acceptor variable region framework sequence may be used having regard to the class/type of the donor antibody from which the CDRs are derived, including mouse, primate and human framework regions. Examples of human frameworks, which can be used in the present invention are KOL, NEWM, REI, EU, TUR, TEI, LAY and POM (~~Kabat *et al.* Seq. of Proteins of Immunol. Interest, 1:310-334 (1994)~~Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Edition, 1991, U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health). For example, KOL and NEWM can be used for the heavy chain, REI can be used for the light chain and EU, LAY and POM can be used for both the heavy chain and the light chain.

In a CDR-grafted antibody of the present invention, it is preferred to use as the acceptor antibody one having chains which are homologous to the chains of the donor antibody. The acceptor heavy and light chains do not necessarily need to be derived from the same antibody and may, if desired, comprise composite chains having framework regions derived from different chains.

Also, in a CDR-grafted antibody of the present invention, the framework regions need not have exactly the same sequence as those of the acceptor antibody. For instance, unusual residues may be changed to more frequently occurring residues for that acceptor chain class or type. Alternatively, selected residues in the acceptor framework regions may be changed so that they correspond to the residue found at the same position in the donor antibody or to a residue that is a conservative substitution for the residue found at the same position in the donor antibody. Such changes should be kept to the minimum necessary to recover the affinity of the donor

evaluated. Analysis of these experiments demonstrated that calicheamicin input and additive concentration had the most significant effects on the level of the low conjugated fraction LCF and aggregate formation, while temperature and pH exerted smaller influences. In additional experiments, it was also shown that the concentrations of protein carrier (antibody) and cosolvent (ethanol) were similarly of lesser importance (compared to calicheamicin input and additive concentration) in controlling LCF and aggregate levels. In order to reduce the LCF to <10%, the calicheamicin derivative input was increased from 3% to 8.5% (w/w) relative to the

amount of antibody in the reaction. The additive was changed from octanoic acid or its salt at a concentration of 200 mM to decanoic acid or its salt at a concentration of 37.5 mM. The conjugation reaction proceeded better at slightly elevated temperature (30-35°C) and pH (8.2-8.7). The reaction conditions incorporating these changes reduced the LCF to below 10 percent while increasing calicheamicin loading, and is hereinafter referred to as CMC-544 Process Condition or "new" process conditions. A comparison of the results obtained with the CMA-676 and CMC-544 Process Conditions is shown in Table 1.

TABLE 1: COMPARISON OF THE CMA-676 AND CMC-544 PROCESS CONDITIONS

CONDITIONS/RESULTS	CMA-676 PROCESS CONDITIONS	CMC-544 PROCESS CONDITIONS
Calicheamicin Input	3.0% (w/w powder weight basis)	8.5% (w/w)
Additive Identity and Concentration	Octanoic acid/Sodium ~~octanoic~~ octanoate; 200 mM	Decanoic acid/Sodium decanoate; 37.5 mM
Temperature	26 °C	31-35 °C
pH	7.8	8.2.-8.7
Calicheamic Loading (percent by weight; by UV assay)	2.4-3.5	7.0-9.0
Low Conjugated Fraction (LCF) (before purification)	45-65 HPLC Area %	<10%
Aggregation (before purification)	~5%	<5%
Aggregation (after purification)	<2%	<2%

example, arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans (such as, for example, inulin), levan, fucoidan, carrageenan, galactocarolose, pectins, pectic acids, amylose, pullulan, glycogen, amylopectin, cellulose, dextran, pustulan, chitin, agarose, keratin, chondroitin, dermatan, hyaluronic acid, alginic acid, xanthin gum, or starch. Among particularly useful carbohydrates are sucrose, glucose, lactose, trehalose, and combinations thereof. Sucrose is a particularly useful cryoprotectant.

Preferably, the cryoprotectant of the present invention is a carbohydrate or "sugar" alcohol, which may be a polyhydric alcohol. Polyhydric compounds are compounds that contain more than one hydroxyl group. Preferably, the polyhydric compounds are linear. Suitable polyhydric compounds include, for example, glycols such as ethylene glycol, polyethylene glycol, and polypropylene glycol, glycerol, or pentaerythritol; or combinations thereof.

In some preferred embodiments, the cryoprotectant agent is sucrose, trehalose, mannitol, or sorbitol.

Once formulated, the compositions of the invention can be administered directly to the subject. The subjects to be treated can be animals. However, it is preferred that the compositions are adapted for administration to human subjects.

The compositions of the present invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, infra-arterial, intramedullary, intrathecal, intraventricular, transdermal, transcutaneous (see PCT Publication No. ~~WO98/20734~~WO 98/20734), subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, intravaginal or rectal routes. Hyposprays may also be used to administer the compositions of the invention. Typically, the compositions may be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared.

Direct delivery of the compositions will generally be accomplished by injection, subcutaneously, intraperitoneally, intravenously or intramuscularly, or delivered to the interstitial space of a tissue. The compositions can also be administered into a lesion. Dosage treatment may be a single dose schedule or a multiple dose schedule.

phase. Samples, standard, and controls (50µl) were injected over immobilized anti-mouse Fc followed by antibody in the soluble phase. After each cycle, the surface was regenerated with 50µl of 40mM HCl at 30 µl/min. The kinetic analysis was performed using the BIAevaluation 3.1 software (Pharmacia).

Removal of the glycosylation site in construct T57A resulted in a slightly faster on-rate and a significantly slower off-rate compared to the chimeric 5/44, giving an affinity improvement of approximately 5-fold. The N55Q mutation had no effect on affinity. This result was unexpected as it suggests that the removal of the carbohydrate itself apparently has no effect on binding (as with the N55Q change). The improved affinity was observed only with the T57A change. One possible explanation is that, regardless of the presence of carbohydrate, the threonine at position 57 exerts a negative effect on binding that is removed on conversion of threonine to alanine. The hypothesis that the small size of alanine is important, and that thenegative effect of threonine is related to its size, is supported from the result obtained using the T57V mutation: that replacement with valine at position 57 is not beneficial (results not shown).

Removal of the lysine residue by the K60R mutation had a neutral effect on affinity, i.e. the introduction of the arginine residue removes a potential reactive site without compromising affinity.

The mutations for removal of the glycosylation site and for removal of the reactive lysine were therefore both included in the humanization design.

EXAMPLE 2

CDR-GRAFTING OF 5/44

The molecular cloning of genes for the variable regions of the heavy and light chains of the 5/44 antibody and their use to produce chimeric (mouse/human) 5/44 antibodies has been described above. The nucleotide and amino acid sequences of the mouse 5/44 VL and VH domains are shown in Figures 2 and 3 (SEQ ID NOS:7 and 8), respectively. This example describes the CDR-grafting of the 5/44 antibody onto human frameworks to reduce potential immunogenicity in humans, according to the method of Adair et al., (PCT ~~application~~ Publication No. WO 91/09967).

I. CDR-GRAFTING OF 5/44 LIGHT CHAIN

Protein sequence alignment with consensus sequences from human sub-group I kappa light chain V region indicated 64% sequence identity. Consequently, for constructing the CDR-grafted light chain, the acceptor framework regions chosen corresponded to those of the human VK sub-group I germline 012,DPK9 sequence. The framework 4 acceptor sequence was derived from the human J-region germline sequence JK1(SEQ ID NO:18)..

A comparison of the amino acid sequences of the framework regions of murine 5/44 (SEQ ID NO:7) and the acceptor sequence (SEQ ID NO:17) is given in Figure 5 and shows that there are 27 differences between the donor and acceptor chains. At each position, an analysis was made of the potential of the murine residue to contribute to antigen binding, either directly or indirectly, through effects on packing or at the V_H/V_L interface. If a murine residue was considered important and sufficiently different from the human residue in terms of size, polarity or charge, then that murine residue was retained. Based on this analysis, two versions of the CDR-grafted light chain, having the sequences given in SEQ ID NO:19 and SEQ ID NO:20 (Figure 5), were constructed.

II. CDR-GRAFTING OF 5/44 HEAVY CHAIN

CDR-grafting of the 5/44 heavy chain was accomplished using the same strategy as described for the light chain. The V-domain of the 5/44 heavy chain was found to be homologous to human heavy chains belonging to sub-group I (70% sequence identity) and therefore the sequence of the human sub-group I germline framework VH1-3,DP7 was used as an acceptor framework. The framework 4 acceptor sequences were derived from human J-region germline sequence JH4 (SEQ ID NO:22).

A comparison of the 5/44 heavy chain with the framework regions is shown in Figure 6 where it can be seen that the 5/44 heavy chain (SEQ ID NO:8) differs from the acceptor sequence (SEQ ID NO:21) at 22 positions. Analysis of the contribution that any of these might make to antigen binding led to 5 versions of the CDR-grafted heavy chains being constructed, having the sequences given in SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26 and SEQ ID NO:27 (Figure 6).

III. CONSTRUCTION OF GENES FOR GRAFTED SEQUENCES

Genes were designed to encode the grafted sequences gH1 and gL1, and a series of overlapping oligonucleotides were designed and constructed (Figure 10, SEQ ID NOS:32-47). A PCR assembly technique was employed to construct the CDR-grafted V-region genes. Reaction volumes of 100 µL were set up containing 10 mM Tris-HCl pH8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 0.25 mM of dATP, dTTP, dCTP, and dGTP, 1 pmole each of the 'internal' primers (T1, T2, T3, B1, B2, B3), 10 pmole each of the 'external' primers (F1, R1), and 1 unit of Taq polymerase (AmpliTaq, Applied BioSystems, catalogue no. N808-0171). PCR cycle parameters were 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 1 minute, for 30 cycles. The reaction products were then run on a 1.5 % agarose gel, excised and recovered using QIAGEN spin columns (QIAquick gel extraction kit, cat no. 28706). The DNA was eluted in a volume of 30 µl. Aliquots (1 µl) of the gH1 and gL1 DNA were then cloned into the InVitrogen TOPO TA cloning vector pCR2.1 TOPO (catalogue no. K4500-01) according to the manufacturer's instructions. This non-expression vector served as a cloning intermediate to facilitate sequencing of a large number of clones. DNA sequencing using vector-specific primers was used to identify correct clones containing gH1 and gL1, creating plasmids pCR2.1 (544gH1) and pCR2.1(544gL1) (Figures 11 and 12).

An oligonucleotide cassette replacement method was used to create the humanized grafts gH4, 5, 6 and 7, and gL2 (SEQ ID NOS:56-63). Figure 13 shows the design of the oligonucleotide cassettes. To construct each variant, the vector pCR2.1(544gH1) or pCR2.1(544gL1)) was cut with the restriction enzymes shown (XmaI/SacII for the heavy chain, XmaI/BstEII for the light chain). The large vector fragment was gel purified from agarose and was used in ligation with the oligonucleotide cassette. These cassettes are composed of 2 complementary oligonucleotides (shown in Figure 13, SEQ ID NOS:56-63), mixed at a concentration of 0.5 pmoles/µl in a volume of 200 µl 12.5 mM Tris-HCl pH 7.5, 2.5 mM $MgCl_2$, 25 mM NaCl, 0.25 mM dithioerythritol. Annealing was achieved by heating to 95 °C for 3 minutes in a water bath (volume 500 ml) then allowing the reaction to slow-cool to room temperature. The annealed oligonucleotide cassette was then diluted ten-fold in water before ligation into the appropriately cut vector. DNA sequencing was used to confirm the correct sequence, creating plasmids pCR2.1 (5/44-gH4-7) and pCR2.1 (5/44-gL2). The

verified grafted sequences were then sub-cloned into the expression vectors pMRR14 (heavy chain) and pMR10.1 (light chain).

IV. CD22 BINDING ACTIVITY OF CDR-GRAFTED SEQUENCES

The vectors encoding grafted variants were co-transfected into CHO cells in a variety of combinations, together with the original chimeric antibody chains. Binding activity was compared in a competition assay, competing the binding of the original mouse 5/44 antibody for binding to Ramos cells (obtained from ATCC, a Burkitt's lymphoma lymphoblast human cell line expressing surface CD22). This assay was considered the best way to compare the grafts in their ability to bind to cell surface CD22. The results are shown in FIGS. 14 and 15. As can be seen, there is very little difference between any of the grafts, all performed more effectively than the chimeric at competing against the murine parent. The introduction of the 3 additional human residues at the end of CDR-H2 (gH6 and gH7) did not appear to have affected binding.

The graft combination with the least number of murine residues gLlgH7 was selected. The light chain graft gL1 has 6 donor residues. Residues V2, V4, L37 and Q45 are potentially important packing residues. Residue H38 is at the V_H/V_L interface. Residue D60 is a surface residue close to the CDR-L2 and may directly contribute to antigen binding. Of these residues, V2, L37, Q45 and D60 are found in germline sequences of human kappa genes from other sub-groups. The heavy chain graft gH7 has 4 donor framework residues (Residue R28 is considered to be part of CDR-H1 under the structural definition used in CDR-grafting (see Adair et al. (1991), PCT ~~application~~ Publication No. WO_91/09967)). Residues El and A71 are surface residues close to the CDRs. Residue 148 is a potential packing residue. Residue T93 is present at the V_H/V_L interface. Of these residues, El and A71 are found in other germline genes of human sub-group I. Residue 148 is found in human germline sub-group 4, and T73 is found in human germline sub-group 3.

The full DNA and protein sequence of both the light chain and heavy chain, including approximate position of introns within the constant region genes provided by the vectors, are shown in Figure 16 and are given in SEQ ID NO:29 and SEQ ID NO:28, respectively, for the light chain and SEQ ID NO: 31 and SEQ ID NO:30, respectively, for the heavy chain.

and LCF. This pool was loaded on a Sephadex G-25 (Amersham Biosciences) desalting column for exchange to a buffer appropriate for formulation, consisting of 20 mM Tris-HCl and 100 mM sodium chloride at pH 8.0. The purified, buffer-exchanged CMC-544 preparation had the following properties: Calicheamicin Loading: 81 μg/mg; Aggregate: 0.4% (area percent by HPLC) LCF: 0.8% (area percent by HPLC).

EXAMPLE 5

BINDING ANALYSIS OF NAC-GAMMA CALICHEAMICIN DMH ACBUT-G5/44 IMMUNOCONJUGATE (CMC-544)

Immunoconjugate of humanized anti-CD22 antibody (G5/44) with calicheamicin (CMC-544) generated by the above conjugation process was analyzed in a binding study to determine whether the conjugate generated using the improved process had any adverse effect on antigen binding. Table 3 shows that the conjugation procedure does not have any impact on the antigen binding affinity of the antibody. CMC-544 immunoconjugate made by either the old or new conjugation procedure bound the target antigen with similar affinities, which did not differ from that of the unconjugated antibody G5/44.

TABLE 3: BINDING AFFINITIES OF CMC-544 MADE BY USING CMA-676 AND CMC-544 CONJUGATION PROCEDURES

Anti-CD22 Antibody	K_D (M)	K_A (1/M)	K_d (1/s)	K_a (1/Ms)	Percent LCF
Humanized G5/44	1.30×10^{-10}	7.90×10^{9}	2.80×10^{-5}	2.20×10^{5}	100
CMC-544 (21 μg/mg) (CMA-676 procedure)	1.20×10^{-10}	8.10×10^{9}	6.10×10^{-5}	4.90×10^{5}	25
CMC-544 (87 μg/mg) (CMA-544 procedure)	1.50×10^{-10}	6.60×10^{9}	6.90×10^{-5}	4.60×10^{5}	3.3

Biosensor analyses were carried out using a BIAcore 2000 (BIAcore AB, Uppsala, Sweden). CD22mFc was covalently immobilized on the N-hydroxysuccinimide-activated carboxymethyl dextran-coated biosensor chip (CM5) using a standard amine-coupling chemistry at a protein density of approximately 2000 resonance units. Samples of CMC-544 or G5/44 were diluted in the HBS buffer

III. TREATMENT OF RITUXIMAB (RITUXANTM)-RESISTANT TUMORS

The next question to be explored was whether the B-cell lymphomas grown after the discontinuation of the commercially available, anti-CD20 rituximab (Rituxan™) treatment would still responsive to the CMC-544 treatment. To this end, developing (unstaged) RL lymphomas were treated with rituximab (Rituxan™) for three weeks. As long as the rituximab (Rituxan™) therapy was continued, the growth of RL lymphoma was inhibited. Upon cessation of rituximab (Rituxan™) therapy, RL lymphomas grew rapidly to the size of ~1 g mass at which time they were treated with CMC-544 at the intraperitoneal dose of 160 μg/kg. As shown in Figures 21 and 22, these RL lymphomas were still responsive to CMC-544 with 80% of mice becoming tumor-free by day 60. Thus, CMC-544 is able to cause regression of B-cell lymphomas with three doses that could only be inhibited by the continuous dosing of rituximab (Rituxan™).

EXAMPLE 87

IN VITRO AND IN VITRO EFFECT OF CMC-544

I. BINDING AND TOXICITY STUDIES

CMC-544 was evaluated for its binding to CD22 and also for its activity in in vitro and in vivo models. CMC-544 was also compared to CMA-676, an isotype-matched control conjugate of hP67.6 (IgG4) with AcBut linked calicheamicin, and to rituximab (Rituxan™), a chimeric IgG1 anti-CD 20 mAb, (IDEC Pharmceuticals, San Diego, CA.), which is commercially available and was purchased from Medworld Pharmacy (Chestnut Ridge, NY). The following antibodies were used in the G5/44 binding domain studies: BU12 (Celltech, Slough, UK); BLCAM, HD239 (Santa Cruz Biotech, Santa Cruz, CA); RFB-4 (Ancell Corp, Bayport, MN); SHCL-1, Leu 14 (Becton Dickinson, Franklin Lakes, NJ); 4KB128 and To 15 (Dako Corp, Carpinteria, CA); M6/13 and M5/44 (Celltech, Slough, UK). Additional antibodies used in the blocking studies were SJ10 (Immunotech, Fullerton, CA) and M17.1.1, M19.1.1, M38.1.1 (Celltech, Slough, UK). Cell lines for the studies including Burkitt's lymphoma cell line Ramos (CRL-1923) and the Non-Hodgkin's lymphoma (NHL) cell line RL (CRL-2261) were all obtained from the American Type Culture Collection. The cell lines were determined to be mycoplasma free by a polymerase chain reaction mycoplasma detection assay (ATCC, Manassas, VA). The cell lines were

the murine parent of G5/44 that has the same specificity as G5/44, inhibits the binding of G5/44, and serves as a positive control. Anti-CD19 antibody BU12 serves as a negative control in these evaluations. The results are summarized in Table 7.

TABLE 7: BINDING OF MURINE ANTI-CD22 M/AB WITH DEFINED SPECIFICITIES TO G544-PRETREATED FC-CD22. BINDING RESPONSE EXPRESSED AS SURFACE PLASMON RESONANCE ~~UNITES~~ UNITS (RU)

Antibody	Epitope Ig Domain of CD22	Response 1 with G544	Response 2 with G544 after 2nd Anti-CD22 mAb	Response 3 (Response 2-1)	Response 3 adjusted for "OFF" rate of G544	Binding of 2nd mAb without G544 adjusted for background	Inhibition by G544 (%)
Anti-CD22 Leu 14	A 1,2	654.3	579.8	-74.5	9	29.3	69
Anti-CD22 HD239	A 1	710.5	628.7	-81.8	1.7	19.3	91
Anti-CD22 M 6/13	? ?	710.0	652.7	-57.3	26.2	152.4	83
Anti-CD22 RFB-4	B 3	703.5	1108.5	405	488.5	534	9
Anti-CD22 4KB128	? 2	691.0	1343.5	652.5	736.0	738.8	0
Anti-CD22 To 15	C 4	676.9	1163.6	486.7	570.2	614.6	7
Anti-CD22 M5/44	Positive Control	725.1	679.3	-45.8	37.7	613.9	94
Anti-CD22 BU12	Negative Control	686.2	602.7	-83.5	0	0	0

Using murine mAbs of known binding specificities for individual domains to CD22, the ability of G5/44 to block the binding of these antibodies to B cells was investigated. Additionally, the ability of the mAbs to block the binding of G5/44 to B cells was also investigated. In these studies, 1 x 10^5 Ramos cells were first exposed to murine anti-CD22 antibody (10 μg/ml humanized G5/44 or mouse monoclonal anti-

mouse host effector mechanisms. These mechanisms include complement-mediated cytotoxicity and/ or antibody dependent cellular cytotoxicity through recruitment of natural killer cells that are present in SCID mice. The injected Ramos tumor cells are probably more susceptible early on to the host immune mechanisms that are activated by rituximab. (Rituxan™), before the cells have an opportunity to infiltrate into the affected organs. The unconjugated G5/44 antibody (the targeting molecule in CMC-544) had not yet been tested in the disseminated tumor model in SCID mice, but it had no effect when administered in subcutaneous xenografts. G5/44, being of the IgG4 isotype, would not be expected to activate the host effector mechanisms and, therefore, would not produce anti-tumor activity.

Calicheamicin conjugated G5/44 (CMC-544) behaved in the opposite fashion than rituximab (Rituxan™), producing better results when administered in the established phase of the disease. The reason for CMC-544 performing better in the established phase is not clear, but the established phase more closely represents the linical situation. CMA-676, the isotype matched, nonbonding control conjugate, did, no have any significant effects on the average survival times. The results in the disseminated SCID model clearly suggest that the doses of CMC-544 need to be reduced to determine the maximum efficacious dose (MED). The 160 μg/kg dose was less active than the lower doses of 80 and 40 μg/kg. It is not clear why this is so but the 160 μg/kg dose may be well over the MED. Further studies are planned to address this issue.

Mohammad et al., used CHOP therapy (Cyclophosphamide (C) 40 mg/kg IV, doxorubicin (H) 3.3 mg/kg IV, vincristine (O) 0.5 mg/kg IV, and prednisone (P) 0.2 mg/kg PO) in a model of subcutaneous xenografts with a diffuse large cell lymphoma cell line, DLCL. The doses used for the CHOP therapy were determined to be their maximum tolerated dose. Therapy, CHO given once IV and P, given daily for 5 days, was rated 'active', producing a T/C of 25.8%. No tumor cures were recorded. The results in the model described by Mohammad et al., appear similar to those observed with CHOP therapy (administered IP, Q4Dx3) in the RL model described herein. In neither study did CHOP produce long-term cures, unlike CMC-544.

TABLE 10: DESCRIPTIVE MEASURES OF SURVIVAL TIME FOR COMBINATION STUDIES

Treatment	Average Survival Time	Median Survival Time	Standard Deviation	Minimum Survival Time	Maximum Survival Time	Number of Animals
CMC-544 40 μg/kg	118.90	125.00	19.29	64.00	125.00	10
CMC LD + Ritux LD	125.00	125.00	0.00	125.00	125.00	10
CMC-544 80 μg/kg	118.22	125.00	17.86	71.00	125.00	9
CMC MD + Ritux MD	125.00	125.00	0.00	125.00	125.00	10
CMC-544 160 μg/kg	85.22	82.00	40.37	35.00	125.00	9
CMC HD + Ritux HD	91.30	100.00	36.31	44.00	125.00	10
Rituximab 5 mg/kg	40.70	36.50	9.57	34.00	64.00	10
Rituximab 10 mg/kg	33.80	34.00	3.26	29.00	41.00	10
Rituximab 20 mg/kg	40.50	34.00	15.45	31.00	82.00	10
Vehicle	25.80	25.00	3.12	22.00	34.00	10

CMC LD = CMC-544 low dose, 40 μg/kg
CMC MD = CMC-544 medium dose, 80 μg/kg
CMC HD = CMC-544 high dose, 160 μg/kg
Ritux LD = Rituximab low dose, 5 mg/kg
Ritux MD = Rituximab medium dose, 10 mg/kg
Ritux HD = Rituximab high dose, 20 mg/kg

EXAMPLE 98

STABLE FORMULATIONS OF CMC-544

Stable formulations of CMC-544 for in vivo administration were prepared by adding diluents, excipients, carriers and stabilizers. Following HIC chromatography, the chromatographic fractions are assayed by SEC-HPLC and multiwavelength UV analysis. Appropriate fractions were selected for pooling on the basis of the above analysis, which provided information on aggregate content, protein concentration, and calicheamicin loading. Excipients, stabilizers, bulking agents and buffering agents were added to stabilize the solution. Since CMC-544 can undergo degradation via a number of degradation pathways, physical instabilities need to be considered in the development of formulations. One of the main considerations in the development of formulations is that the rate of hydrolysis of calicheamicin from

the antibody must be minimized while the physical and chemical integrity of the anti-CD-22 antibody must be maintained. In addition, precipitation of the calicheamicin-antibody conjugate, which can occur under certain pH and concentration conditions, needs to be minimized.

In developing a formulation of a monomeric calicheamicin derivative-antibody conjugate, the pH of the formulation is critical, as this minimizes degradation and physical instability. A pH of 8.0 was selected to minimize hydrolysis of calicheamicin and maintain adequate solubility of the conjugate. Additional data, obtained using SDS-PAGE and antigen binding ELISA, indicated that the significant structural integrity and specificity of the antibody are maintained at a pH of 8.0. Consequently, tromethamine was chosen as a buffering agent to maintain a pH of 8.0. An alternative buffer could include dibasic sodium or potassium phosphate. The range of buffer concentration can be 5 to 50 mM. A preferred pH range of 7.5 to 8.5 is suggested for optimum stability/solubility. The current pH specification for the finished product is 7.0-9.0.

Although the stability of the buffered conjugate solutions is adequate for the short time, long-term stability is poor. Lyophilization is used to improve the shelf life of the conjugates. The problems associated with lyophilization of a protein solution are well documented, and the loss of secondary, tertiary and quaternary structure can occur during freezing and drying processes. Sucrose is included in the formulation to act as an amorphous stabilizer of the conjugate and maintain the structural integrity of the antibody during freezing and drying. Concentrations of 1.5-5.0% w/v sucrose have been used. In addition, a polymeric bulking agent, such as Dextran 40 or hydroxyethyl starch can be incorporated to enhance the appearance and physical rigidity of the lyophilized cakes at a concentration of 0.5-1.5% by weight. These materials form lyophilized cakes at relatively low concentrations and can be used to minimize the overall solids content of the lyophilized formula, thus permitting more rapid freeze drying. Formulation studies have used a Dextran 40 concentration of 0.9% by weight.

Polysorbate 80 is included in the formulation to enhance the solubility of the conjugate. A preferred concentration of 0.01% is used with a potential range of 0.005-0.05%. Tween is also added to the formulation at a concentration of 0.01 ~~0.91~~ 0.05% by volume.

BIBLIOGRAPHY

1. G. Kohler and Milstein, C., Nature, 256:495 (1975).

2. T. G. Hose and Blair, A.H., CRC Critical Rev. Drug Carrier Systems, 3:263 (1987).

3. U.S. Patent No. 5,877,296

4. U.S. Patent No. 5,773,001

5. U.S. Patent No. 5,714,586

6. U.S. Patent No. 5,712,374

7. U.S. Patent No. 5,053,394

8. J. Tramontano, et al., J. Mol. Recognit., 7:9 (1994).

9. H. McConnell and Hoess, J., J. Mol. Biol., 250:460 (1995).

10. Nord et al., Nat Biotechnol., 15:772 (1997).

11. Nord et al., Protein Eng., 8:601 (1995).

12. Ku and Schultz, Proc. Natl. Acad. Sci., USA 92:6552 (1995).

13. Markand et al., Biochemistry, 35:8045 (1996).

14. Markand et al., Biochemistry, 35:8098 (1996).

15. Rottgen and Collins, Gene, 164:243 (1995).

16. Wang et al, J. Biol. Chem., 270:12250 (1995).

17. I.D. Bernstein et al., J. Clin. Invest., 79:1153 (1987).

18. I.D. Bernstein et al., J. Immunol., 128:867-881 (1992).

19. ~~Kabat et al. Seqencing. of Proteins of Immunological Interest, 1:310-334 (1994).~~ Kabat et al., Sequences of Proteins of Immunological Interest, 5th Edition, 1991, U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health).

20. PCT ~~publication~~ Publication No. WO 91/09967.

21. Yang et al., J. Mol. Biol., 254, :392-403 (1995).

22. Low et al., J. Mol. Biol., 260:359-368 (1996).

23. Patten et al., Curr. Opin. Biotechnol., 8, :724-733, (1997).

24. Thompson et al., J. Mol. Biol., 256, :77-88, (1996).

25. Crameri et al., Nature, 391, :288-291, (1998).

26. U.S. Patent No. 4,671,958

27. U.S. Patent No. 4,970,198

28. U.S. Patent No. 5,037,651

29. U.S. Patent No. 5,079,233

30. U.S. Patent No. 5,877,296

31. PCT ~~publication~~ Publication No. WO 98/20734

32. Trail P and Bianchi A., Current Opin. Immunol., 11:584-588~~,~~ (1999).

33. Dubowchik G. and Walker M., Pharmacol. & Therapeutics, 83:67-123 (1999).

34. Bross P.F., Beitz J., Chen G., Chen X.H., Duffy E., Keiffer-Bross P., Beitz J., Chen G., Chen X., Duffy E., Kieffer L., Roy S., Sridhara R., Rahman A., Williams G., Pazdur R., Clin. Cancer Res., 7:1490-1496 (2001).

35. Berger M., Leopold L., Dowell J., Korth-Bradley J., Sherman M., Invest. New Drugs~~;~~ ~~,~~ 20: 395-406 (2002).

36. Sievers E., Larson R., Stadmauer E., Estey E., Lowenberg B., Dombret H., Karanes C., Theobald M., Bennet J., Sherman M., et al., J. Clin. Oncol~~;~~ ~~,~~ 19:3244-3254 (2001).

37. Larson R., Boogaerts M., Estey E., Karanes C., Stadtmauer E., Sievers E., Mineur P., Bennett J., Berger M., Eten C. et al. Leukemia~~;~~ ~~,~~ 16:1627-1636 (2002).

38. Hamann P., Hinman L., Beyer C., Kindh D., Upeslacis J., Flowers D., Bernstein I., Choice of Linker. Bioconj. Chem~~;~~ ~~,~~ 13:40-46 (2002).

39. Hamann P., Hinman L., Hollander I., Beyer C., Lindh D., Holcomb R., Hallet W., Tsou H., Upeslacis J., Shochat D., et al., Bioconj. Chem~~;~~ ~~,~~ 13:47-58 (2002).

40. Lee M., Dunne T., Chang C., Siegal M., Morton G., Ellestad G., McGahren W., Borders D.., J. Am. Chem. Soc. 1992~~;~~ ~~,~~ 114:985-987 (1992).

41. Zein N., Sinha A., McGahren W., Ellestad G., Science~~;~~ ~~,~~ 240:1198-1201 (1988).

42. Thorson J., Sievers E., Ahlert J., Shepard E., Whitwam R., Onwueme K., Ruppen M., Current Pharmaceut. Design~~;~~ ~~,~~ 6:1841-1879 (2000).

43. Andrews R., Singer J., Bernstein I., J. Exp. Med.; 169:1721-1731 (1989).

44. Kreitman R.J., Current Pharmaceut. Biotech~~;~~ ~~,~~ 2:313-325 (2001).

45. Pastan I., Kreitman R.J., Current Opin. Investig. Drugs, 3(7):1089-1091 (2002).

46. Kreitman R.J., Curr. Opin. Mol. Ther. ~~;~~ ~~,~~ 5:44-551 (2003).

47. Crocker P.R. and Varki A. Siglecs, Trends in Immunol~~;~~ ~~,~~ 22:337-342 (2001).

48. Hursey M., Newton D.L., Hansen H.J., Ruby D., Goldenberg D.M., Rybak S.M., Leukemia and Lymphoma~~;~~ ~~,~~ 43:953-959 (2002).

49. Nitschke L., Floyd H., and Crocker P.R., Scand. J. Immunol., 53:227-234 (2001).
50. Moyron-Quiroz J.E., Partida-Sanchez S., Donis-Hernandez R., Sandoval-Montes C. and Santos-Argumedo L., Scand. J. Immunol., 55:343-351 (2002).
51. Tedder T.F., Tuscano J., Sato S., Kehrl J.H., Ann. Rev. Immunol., 15:481-504 (1997).
52. Hanna R., Ong G.L., Mattes M.J., Cancer Res., 56:3062-3068 (1996).
53. Shan D. and Press O.W., J. Immunol., 1995; 154:4466-4475 (1995).
54. Dowell J.A., Korth-Bradley J., Liu H., King S.P., Berger M.S., J. Clin. Pharmacol., 41:1206-1214 (2001).
55. Gibaldi M., Perrier D., Pharmacokinetics, 2nd ed., Marcel-Dekker Inc., NY (1982).
56. Van Horssen P.J., Preijers, F.W., Van Oosterhout, Y.V., Eling W.M. and De Witte, T., Leukemia & Lymphoma, 39(5-6):591-599 (2000).
57. Hinman L.M., Hamann P.R., Wallace R., Menendez A.T., Durr F.E., Upeslacis J., Cancer Res., 53:3336-3342 (1993).
58. Kreitman R.J., Wilson W.H., Bergeron K., Raggio M., Stetler-Stevenson M., Fitzgerald D.J., Pastan, Engl. J. Med., 345:241-247 (2001).
59. Leonard J.P. and Link B.K., Sem. Oncol., 29:81-86 (2002).
60. Schindler J., Sausville E., Messmann R., Uhr J.W. & Vitetta, E.S., Clin.Cancer Res., 7;:255-258 (2001).
61. Vincent T. DeVita, Samuelo Hellman, Steven A. Rosenberg, Eds., Cancer Principles and Practice of Oncology, 6th Edition, Publishers: Lippincott, Williams and Wilkins (2001).
62. Edward Chu and Vincent T. DeVita, Physician's Cancer Chemotherapy Drug Manual, Publishers: Jones and Bartlett (2002).

CLAIMS:

1. A monomeric calicheamicin derivative/anti-CD22 antibody conjugate having the formula, Pr(-X-W)m wherein:

Pr is an anti-CD22 antibody;

X is a hydrolyzable linker having the formula (CO - Alk^1 - Sp^1 - Ar - Sp^2 - Alk^2 - $C(Z^1)$ = Q - Sp), wherein

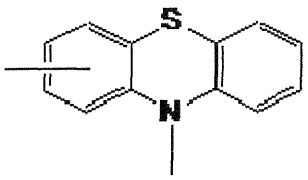
Alk^1 and Alk^2 are independently a bond or branched or unbranched (C_1-C_{10}) alkylene chain;

Sp^1 is a bond, -S-, -O-, -CONH-, -NHCO-, -NR'-, $-N(CH_2CH_2)_2N-$, or -X-Ar'-Y-$(CH_2)_n$-Z wherein X, Y, and Z are independently a bond, -NR'-, -S-, or -O-, with the proviso that when n = 0, then at least one of Y and Z must be a bond and Ar' is 1,2-, 1,3-, or 1,4-phenylene optionally substituted with one, two, or three groups of (C_1-C_5) alkyl, (C_1-C_4) alkoxy, (C_1-C_4) thioalkoxy, halogen, nitro, -COOR', -CONHR', $-(CH_2)_nCOOR'$, $-S(CH_2)_nCOOR'$, $-O(CH_2)_nCONHR'$, or $-S(CH_2)_nCONHR'$, with the proviso that when Alk^1 is a bond, Sp^1 is a bond;

n is an integer from 0 to 5;

R' is a branched or unbranched (C_1-C_5) chain optionally substituted by one or two groups of -OH, (C_1-C_4) alkoxy, (C_1-C_4) thioalkoxy, halogen, nitro, (C_1-C_3) dialkylamino, or (C_1-C_3) trialkylammonium $-A^-$ where A^- is a pharmaceutically acceptable anion completing a salt;

Ar is 1,2-, 1,3-, or 1,4-phenylene optionally substituted with one, two, or three groups of (C_1-C_6) alkyl, (C_1-C_5) alkoxy, (C_1-C_4) thioalkoxy, halogen, nitro, -COOR', -CONHR', $-O(CH_2)_nCOOR'$, $-S(CH_2)_nCOOR'$, $-O(CH_2)_nCONHR'$, or $-S(CH_2)_nCONHR'$ wherein n and R' are as hereinbefore defined or a 1,2-, 1,3-, 1,4-, 1,5-, 1,6-, 1,7-, 1,8-, 2,3-, 2,6-, or 2,7-naphthylidene or



with each naphthylidene or phenothiazine optionally substituted with one, two, three, or four groups of (C_1-C_6) alkyl, (C_1-C_5) alkoxy, (C_1-C_4) thioalkoxy, halogen, nitro, -COOR', -CONHR', $-O(CH_2)_nCOOR'$, $-S(CH_2)_nCOOR'$, or $-S(CH_2)_nCONHR'$ wherein n and R' are as defined above, with the proviso that when Ar is phenothiazine, Sp^1 is a bond only connected to nitrogen;

Sp^2 is a bond, -S-, or -O-, with the proviso that when Alk^2 is a bond, Sp^2 is a bond;

Z^1 is H, (C_1-C_5) alkyl, or phenyl optionally substituted with one, two, or three groups of (C_1-C_5) alkyl, (C_1-C_5) alkoxy, (C_1-C_4) thioalkoxy, halogen, nitro, -COOR', -ONHR', -O$(CH_2)_n$COOR', -S$(CH_2)_n$COOR', -O$(CH_2)_n$CONHR', or -S$(CH_2)_n$CONHR' wherein n and R' are as defined above;

Sp is a straight or branched-chain divalent or trivalent (C_1-C_{18}) radical, divalent or trivalent aryl or heteroaryl radical, divalent or trivalent (C_3-C_{18}) cycloalkyl or heterocycloalkyl radical, divalent or trivalent aryl- or heteroaryl-aryl (C_1-C_{18}) radical, divalent or trivalent cycloalkyl- or heterocycloalkyl-alkyl (C_1-C_{18}) radical or divalent or trivalent (C_2-C_{18}) unsaturated alkyl radical, wherein heteroaryl is preferably furyl, thienyl, N-methylpyrrolyl, pyridinyl, N-methylimidazolyl, oxazolyl, pyrimidinyl, quinolyl, isoquinolyl, N-methylcarbazoyl, aminocourmarinyl, or phenazinyl and wherein if Sp is a trivalent radical, Sp can be additionally substituted by lower (C_1-C_5) dialkylamino, lower (C_1-C_5) alkoxy, hydroxy, or lower (C_1-C_5) alkylthio groups; and

Q is =NHNCO-, =NHNCS-, =NHNCONH-, =NHNCSNH-, or =NHO-;

W is a calicheamicin;

m is the average loading for a purified conjugation product such that the calicheamicin constitutes 4 – 10% of the conjugate by weight; and

(-X-W)m is a calicheamicin derivative, wherein the antibody molecule comprises a heavy chain variable domain comprising

(a) SEQ ID NO: 1 for CDR-H1,

(b) SEQ ID NO: 2 or SEQ ID NO: 13 or SEQ ID NO: 15 or SEQ ID NO: 16 or residues 50-66 of SEQ ID NO: 23 or residues 50-66 of SEQ ID NO: 27 for CDR-H2, and

(c) SEQ ID NO: 3 for CDR-H3;

and a light chain variable domain comprising

(d) SEQ ID NO: 4 for CDR-L1,

(e) SEQ ID NO: 5 for CDR-L2, and

(f) SEQ ID NO: 6 for CDR-L3.

2. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 1, wherein the antibody is a CDR-grafted anti-CD22 antibody.

3. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 2, wherein the antibody comprises a variable domain comprising human acceptor framework regions and non-human donor CDRs.

4. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 3, wherein the human acceptor framework regions of the variable domain of the heavy chain of the antibody are based on SEQ ID NOs: 21 and 22 and comprise donor residues at positions 1, 28, 48, 72 and 97 in SEQ ID NO: 8.

5. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 4, wherein the antibody further comprises non-human donor residues at positions 68 and 70 in SEQ ID NO: 8.

6. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 2, wherein the CDR-grafted antibody comprises a variable domain of the light chain comprising a human acceptor framework region based on SEQ ID NOs: 17 and 18 and further comprising donor residues at positions 2, 4, 42, 43, 50 and 65 in SEQ ID NO: 7.

7. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 6, wherein the CDR-grafted antibody further comprises a donor residue at position 3 in SEQ ID NO: 7.

8. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 2, wherein the CDR-grafted antibody comprises a light chain variable region comprising SEQ ID NO: 19 and a heavy chain variable region comprising SEQ ID NO: 27.

9. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 2, wherein the CDR-grafted antibody comprises a light chain having the sequence as set forth in SEQ ID NO: 28 and a heavy chain having the sequence as set forth in SEQ ID NO: 30.

10. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 2, wherein the CDR-grafted antibody is a variant antibody obtained by an affinity maturation protocol.

11. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 2, wherein the antibody comprises a light chain consisting of an amino acid sequence resulting from the expression of SEQ ID NO: 29 in a mammalian cell and a heavy chain consisting of an amino acid sequence resulting from the expression of SEQ ID NO: 31 in a mammalian cell.

12. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of any one of the preceding claims, wherein the calicheamicin is a gamma calicheamicin or a N-acetyl gamma calicheamicin.

13. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 12, wherein the calicheamicin is functionalized with 3-mercapto-3-methyl butanoyl hydrazide.

14. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 13, wherein the linker comprises ~~is~~ 4-(4-acetylphenoxy) butanoic acid (AcBut).

15. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate according to any one of claims 1 to 14 for use in the treatment of a proliferative disorder.

16. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 15, wherein the proliferative disorder is a B-cell malignancy.

17. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 16, wherein the B-cell malignancy is leukemia.

18. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 17 wherein the leukemia expresses cell surface antigen CD22.

19. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 17 or 18, wherein the leukemia is acute lymphocytic leukemia.

20. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 16, wherein the B-cell malignancy is lymphoma.

21. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 20, wherein the lymphoma expresses cell surface antigen CD22.

22. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 20 or 21, wherein the lymphoma is a Non-Hodgkin's lymphoma.

23. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to any one of claims 15-22, wherein the conjugate is administered ~~administrable~~ with one or more bioactive agents.

24. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 23, wherein the bioactive agents are selected from a group consisting of antibodies, growth factors, hormones, cytokines, anti-hormones, xanthines, interleukins, interferons, and cytotoxic agents.

25. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 24, wherein the bioactive agent is an antibody.

26. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 25, wherein the antibody is directed against a cell surface antigen expressed on B-cell malignancies.

27. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 26, wherein the antibody directed against cell surface antigens expressed on B-cell malignancies is selected from a group consisting of anti-CD19, anti-CD20 and anti-CD33 antibodies.

28. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 27, wherein the anti-CD20 antibody is rituximab.

29. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 24, wherein the cytotoxic agent is selected from the group consisting of doxorubicin, daunorubicin, idarubicin, aclarubicin, zorubicin, mitoxantrone, epirubicin, carubicin, nogalamycin, menogaril, pitarubicin, valrubicin, cytarabine, gemcitabine, trifluridine, ancitabine, enocitabine, azacitidine, doxifluridine, pentostatin, broxuridine, capecitabine, cladribine, decitabine, floxuridine, fludarabine, gougerotin, puromycin, tegafur, tiazofurin, adriamycin, cisplatin, carboplatin, cyclophosphamide, dacarbazine, vinblastine, vincristine, mitoxantrone, bleomycin, mechlorethamine, prednisone, procarbazine methotrexate, flurouracils, etoposide, taxol, taxol analogs, and mitomycin.

30. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 24, wherein the conjugate is administered ~~administrable~~ together with, prior to, or subsequent to the administration of one or more combinations of cytotoxic agents as part of a treatment regimen, wherein the combination of cytotoxic agents is selected from:

A. CHOPP (cyclophosphamide, doxorubicin, vincristine, prednisone, and procarbazine);

B. CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone);

C. COP (cyclophosphamide, vincristine, and prednisone);

D. CAP–BOP (cyclophosphamide, doxorubicin, procarbazine, bleomycin, vincristine, and prednisone);

E. m–BACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone, and leucovorin);

F. ProMACE–MOPP (prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, leucovorin, mechloethamine, vincristine, prednisone, and procarbazine);

G. ProMACE–CytaBOM (prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, leucovorin, cytarabine, bleomycin, and vincristine);

H. MACOP–B (methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin, and leucovorin);

I. MOPP (mechloethamine, vincristine, prednisone, and procarbazine);

J. ABVD (adriamycin/doxorubicin, bleomycin, vinblastine, and dacarbazine);

K. MOPP (mechloethamine, vincristine, prednisone, and procarbazine) alternating with ABV (adriamycin/doxorubicin, bleomycin, and vinblastine);

L. MOPP (mechloethamine, vincristine, prednisone, and procarbazine) alternating with ABVD (adriamycin/doxorubicin, bleomycin, vinblastine, and dacarbazine);

M. ChlVPP (chlorambucil, vinblastine, procarbazine, and prednisone);

N. IMVP–16 (ifosfamide, methotrexate, and etoposide);

O. MIME (methyl–gag, ifosfamide, methotrexate, and etoposide);

P. DHAP (dexamethasone, high–dose cytarabine, and cisplatin);

Q. ESHAP (etoposide, methylpredisolone, high–dose cytarabine, and cisplatin);

R. CEPP(B) (cyclophosphamide, etoposide, procarbazine, prednisone, and bleomycin);

S. CAMP (lomustine, mitoxantrone, cytarabine, and prednisone);

T. CVP–1 (cyclophosphamide, vincristine, and prednisone);

U. ESHOP (etoposide, methylpredisolone, high–dose cytarabine, vincristine and cisplatin);

V. EPOCH (etoposide, vincristine, and doxorubicin for 96 hours with bolus doses of cyclophosphamide and oral prednisone);

W. ICE (ifosfamide, cyclophosphamide, and etoposide);

X. CHOP–B (cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin); and

Y. P/DOCE (epirubicin or doxorubicin, vincristine, cyclophosphamide, and prednisone).

31. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 24, wherein the conjugate is administered ~~administrable~~ together with an antibody directed against a cell surface antigen on B-cell malignancies, and optionally comprising one or more combinations of cytotoxic agents as part of a treatment regimen, wherein the combination of cytotoxic agents is selected from:

A. CHOPP (cyclophosphamide, doxorubicin, vincristine, prednisone, and procarbazine);

B. CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone);

C. COP (cyclophosphamide, vincristine, and prednisone);

D. CAP–BOP (cyclophosphamide, doxorubicin, procarbazine, bleomycin, vincristine, and prednisone);

E. m–BACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone, and leucovorin);

F. ProMACE–MOPP (prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, leucovorin, mechloethamine, vincristine, prednisone, and procarbazine);

G. ProMACE–CytaBOM (prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, leucovorin, cytarabine, bleomycin, and vincristine);

H. MACOP–B (methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin, and leucovorin);

I. MOPP (mechloethamine, vincristine, prednisone, and procarbazine);

J. ABVD (adriamycin/doxorubicin, bleomycin, vinblastine, and dacarbazine);

K. MOPP (mechloethamine, vincristine, prednisone, and procarbazine) alternating with ABV (adriamycin/doxorubicin, bleomycin, and vinblastine);

L. MOPP (mechloethamine, vincristine, prednisone, and procarbazine) alternating with ABVD (adriamycin/doxorubicin, bleomycin, vinblastine, and dacarbazine);

M. ChlVPP (chlorambucil, vinblastine, procarbazine, and prednisone);

N. IMVP–16 (ifosfamide, methotrexate, and etoposide);

O. MIME (methyl–gag, ifosfamide, methotrexate, and etoposide);

P. DHAP (dexamethasone, high–dose cytarabine, and cisplatin);

Q. ESHAP (etoposide, methylpredisolone, high–dose cytarabine, and cisplatin);

R. CEPP(B) (cyclophosphamide, etoposide, procarbazine, prednisone, and bleomycin);

S. CAMP (lomustine, mitoxantrone, cytarabine, and prednisone);

T. CVP–1 (cyclophosphamide, vincristine, and prednisone);

U. ESHOP (etoposide, methylpredisolone, high–dose cytarabine, vincristine and cisplatin);

V. EPOCH (etoposide, vincristine, and doxorubicin for 96 hours with bolus doses of cyclophosphamide and oral prednisone);

W. ICE (ifosfamide, cyclophosphamide, and etoposide);

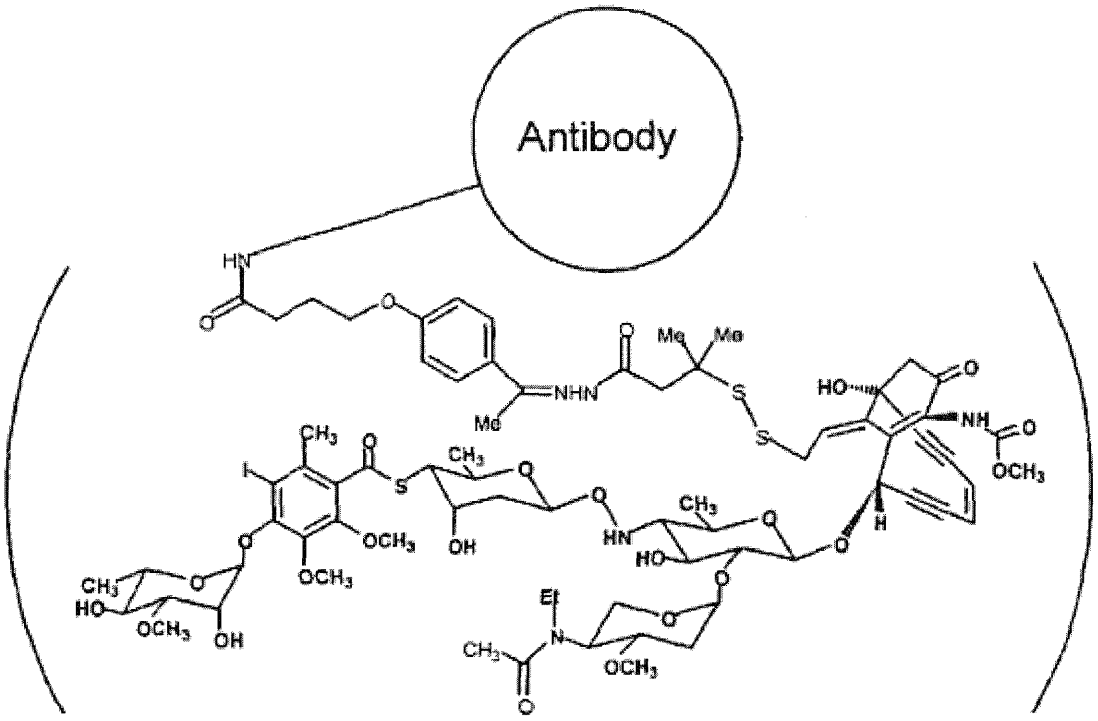
X. CHOP–B (cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin); and

Y. P/DOCE (epirubicin or doxorubicin, vincristine, cyclophosphamide, and prednisone).

32. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to any one of claims 15-31, wherein the conjugate is ~~administrable~~ administered at an effective dose of from 0.1 mg/m^2 to 50 mg/m^2.

33. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate for use according to claim 32, wherein the conjugate is administered~~administrable~~ at an effective dose of from 0.4 mg/m^2 to 30 mg/m^2.

34. A monomeric calicheamicin derivative/anti-CD22 antibody conjugate having the formula:



wherein the antibody comprises SEQ ID NO: 1 for CDR-H1, residues 50-66 of SEQ ID NO: 27 for CDR-H2, SEQ ID NO: 3 for CDR-H3, SEQ ID NO: 4 for CDR-L1, SEQ ID NO: 5 for CDR-L2 and SEQ ID NO: 6 for CDR-L3; and

wherein n is 3 to 9.

35. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 34, wherein the antibody comprises a light chain variable region as set forth in SEQ ID NO: 19 and a heavy chain variable region as set forth in SEQ ID NO: 27.

36. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 34, wherein the antibody is expressed in a mammalian cell and comprises a light chain consisting of residues 21 to 239 of SEQ ID NO: 28 and a heavy chain consisting of residues 20 to 466 of SEQ ID NO: 30.

37. The monomeric calicheamicin derivative/anti-CD22 antibody conjugate of claim 34, wherein the antibody comprises a light chain consisting of an amino acid sequence resulting from the expression of SEQ ID NO: 29 in a mammalian cell and a heavy chain consisting of an amino acid sequence resulting from the expression of SEQ ID NO: 31 in a mammalian cell.

38. A pharmaceutical composition comprising the monomeric calicheamicin derivative/anti-CD22 antibody conjugate of any one of claims 34 to 37 and a pharmaceutically acceptable excipient, diluent or carrier.