Synergy in HA22 Combinations with Chemotherapeutic Agents: ALL cell line REH

![Synergy in HA22 Combinations with Chemotherapeutic Agents](image)

Title: USE OF ANTI-CD22 IMMUNOTOXINS AND PROTEIN-SYNTHESIS-INHIBITING CHEMOTHERAPEUTIC AGENTS IN TREATMENT OF B CELL CANCERS

Abstract: The invention relates to the discovery that contacting CD22+ cancer cells with both an anti-CD22 immunotoxin whose toxin portion is a protein synthesis inhibitor and a chemotherapeutic agent that inhibits protein synthesis results in greater cytotoxicity to the cells than would be expected. This synergistic effect can be exploited to inhibit the growth of CD22+ B cell cancers.
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USE OF ANTI-CD22 IMMUNOTOXINS AND PROTEIN-SYNTHESIS-INHIBITING CHEMOTHERAPEUTIC AGENTS IN TREATMENT OF B CELL CANCERS

CROSS-REFERENCES TO RELATED APPLICATIONS
[0001] This application claims the benefit of U.S. Provisional Application Nos. 60/892,819, filed March 2, 2007, and 60/893,596, filed March 7, 2007. The contents of both of these applications are hereby incorporated by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT
[0002] NOT APPLICABLE

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISK.
[0003] NOT APPLICABLE

BACKGROUND OF THE INVENTION
[0004] Hematological malignancies are a major public health problem. According to the website of the National Cancer Institute, there will be over 63,000 new cases of non-Hodgkin's lymphoma in the United States in 2007, with over 18,000 deaths, while there will be over 44,000 new cases of leukemia, with over 22,000 deaths. Many more patients live with chronic disease-related morbidity. Unfortunately, in a high percentage of patients, conventional therapies are not able to induce long term complete remissions.

[0005] In the past several years immunotoxins have been developed as an alternative therapeutic approach to treat these malignancies. Immunotoxins were originally composed of an antibody chemically conjugated to a plant or a bacterial toxin. The antibody binds to the antigen expressed on the target cell and the toxin is internalized causing cell death by arresting protein synthesis and inducing apoptosis (Brinkmann, U., Mol. Med. Today, 2:439-446 (1996)).
Hematological malignancies are an attractive target for immunotoxin therapies because tumor cells are easily accessible and the target antigens are highly expressed (Kreitman, R. J. and Pastan, L., Semin. Cancer Biol, 6:297-306 (1995)). One of these antigens is CD25. A clinical trial with immunotoxin LMB-2 (anti-Tac(Fv)-PE38) that targets CD25 showed that the agent was well tolerated and that it had substantial anti-tumor activity (Kreitman, R. J. et al., Blood, 94:3340-3348 (1999); Kreitman, R. J. et al., J. Clin. Oncol, 18:1622-1636 (2000)). A complete response was observed in one patient with Hairy Cell Leukemia ("HCL") and partial responses were observed in patients with HCL, chronic lymphocytic leukemia, cutaneous T cell lymphoma, Hodgkin's disease and adult T cell leukemia.

Another antigen that has been used as an immunotoxin target is CD22, a lineage-restricted B cell antigen expressed in 60-70% of B cell lymphomas and leukemias. CD22 is not present on the cell surface in the early stages of B cell development and is not expressed on stem cells (Tedder, T. F. et al., Annu. Rev. Immunol, 5:481-504 (1997)). Clinical trials have been conducted with an immunotoxin containing an anti-CD22 antibody, RFB4, or its Fab fragment, coupled to deglycosylated ricin A ("dgA"). In these trials, substantial clinical responses have been observed; however, severe and in certain cases fatal, vascular leak syndrome was dose limiting (Sausville, E. A. et al., Blood, 85:3457-3465 (1995); Amlot, P. L. et al., Blood, 82:2624-2633 (1993); Vitetta, E. S. et al., Cancer Res., 51:4052-4058 (1991)). RFB4-dgA was also studied in a "cocktail" with an anti-CD19-dgA immunotoxin in which the two immunotoxins were used in combination with various chemotherapeutic agents. Ghetie et al., Blood 84(3):702-707 (1994). The authors reported synergistic activity.

As an alternative approach, the RFB4 antibody was used to make a recombinant immunotoxin in which the Fv fragment in a single chain form is fused to a 38 kDa truncated form of Pseudomonas exotoxin A (PE38). PE38 contains the translocating and ADP ribosylating domains of PE but not the cell-binding portion (Hwang, J. et al., Cell, 48:129-136 (1987)). RFB4 (Fv)-PE38 is cytotoxic towards CD22-positive cells (Mansfield, E. et al., Biochem. Soc. Trans., 25:709-714 (1997)). To stabilize the single chain Fv immunotoxin and to make it more suitable for clinical development, cysteine residues were engineered into framework regions of the \( V_H \) and \( V_L \) (Mansfield, E. et al., Blood, 90:2020-2026 (1997)) generating the molecule RFB4 (dsFv)-PE38.
RFB4 (dsFv)-PE38 is able to kill leukemic cells from patients and induced complete remissions in mice bearing lymphoma xenografts (Kreitman, R. J. et al., Clin. Cancer Res., 6:1476-1487 (2000); Kreitman, R. J. et al., Int. J. Cancer, 81:148-155 (1999)). RFB4 (dsFv)-PE38 (which is also referred to as "BL22") was evaluated in a phase I clinical trial at the National Cancer Institute in patients with hematological malignancies. Sixteen patients with purine analogue resistant hairy cell leukemia were treated with BL22 and eleven (86%) achieved complete remissions. These results showed that BL22 was able to induce high complete remission rate in patients with purine analogue-resistant HCL and establish that immunotoxins could produce clinical benefit to patients with advanced malignancies (Kreitman, R.J., et al., N Engl J Med, 345(4):241-7 (2001); Kreitman, RJ., et al., J Clin Oncol, 23(27):6719-29 (2005)). A Phase I clinical trial is currently recruiting patients for studies of the safety of using BL22 to treat pediatric patients with relapsed or refractory acute lymphoblastic leukemia and non-Hodgkin's lymphoma, while a Phase II trial is studying the effectiveness of BL22 in treating patients who have hairy cell leukemia that has not responded to treatment with cladribine. A Phase I trial studying the side effects and best dose of BL22 immunotoxin in treating adult patients has completed accrual and disease specific trials for adults with Hairy Cell Leukemia, chronic lymphocytic leukemia, or non-Hodgkin's lymphoma have opened.

Given the promising results with BL22, intensive efforts have been made to improve it. The immunotoxin HA22 is an improved form of BL22. To produce this immunotoxin, the binding region of antibody RFB4 was mutated and antibody phage display was used to isolate mutant phage that bound better to CD22 because of mutations in complementarity determining region ("CDR") 3 of the variable region heavy chain ("V_H", the amino acid sequence of the RFB4 V_H.CDR3 is SEQ ID NO.:7). In HA22, residues SSY in the CDR3 of the antibody variable region heavy chain were mutated to THW (the amino acid sequence of the V_H.CDR3 with these substitutions is SEQ ID NO.:11). Compared to its parental antibody, RFB4, HA22 has a 5-10-fold increase in cytotoxic activity on various CD22-positive cell lines and is up to 50 times more cytotoxic to cells from patients with chronic lymphocytic leukemia ("CLL") and HCL (Salvatore, G., et al., Clin Cancer Res, 8(4):995-1002 (2002); see also, co-owned international application PCT/US02/303 16, published as International Publication WO 03/027135, and co-owned U.S. Patent Application Publication 2005/01 18182). Thus, HA22 is expected to improve the therapeutic effect shown by BL22.
Even the high affinity antibody HA22 proved capable of mutation to provide antibodies and antibody fragments that have increased binding affinity for cancer cells bearing the CD22 antigen compared not only to RFB4 but even when compared to HA22. Moreover, immunotoxins made with the new, higher affinity variants had even greater cytotoxicity to CD22+ cells than did immunotoxins made with HA22. These new mutants change the amino acid sequence of the residues at positions 30 and 31 of CDR1 of the VL chain of RFB4 ("L-CDR1" or "V_H CDR1"), as those positions are numbered under the "Kabat and Wu" antibody residue numbering system, from the wild type sequence Serine-Asparagine (in single letter code, "SN") to (a) Histidine-Glycine ("HG," the antibody made by combining an RFB4 light chain containing this L-CDR1 mutant with an RFB4 heavy chain containing the THW H-CDR3 mutant is designated as "B5", the amino acid sequence of the L-CDR1 with the HG substitutions is SEQ ID NO.: 15), (b) Glycine-Arginine ("GR," the antibody made by combining an RFB4 light chain containing this L-CDR1 mutant with an RFB4 heavy chain containing the THW H-CDR3 mutant is designated as "E6," the amino acid sequence of the L-CDR1 with the GR substitutions is SEQ ID NO.: 16), (c) Arginine-Glycine ("RG," the antibody made by combining an RFB4 light chain containing this L-CDR1 mutant with an RFB4 heavy chain containing the THW H-CDR3 mutant is designated as "B8," the amino acid sequence of the L-CDR1 with the RG substitutions is SEQ ID NO.: 17), or (d) Alanine-Arginine ("AR," the antibody made by combining an RFB4 light chain containing this L-CDR1 mutant with an RFB4 heavy chain containing the THW H-CDR3 mutant is designated as "D8," the amino acid sequence of the L-CDR1 with the AR substitutions is SEQ ID NO.: 18). These mutations are set forth in more detail in co-owned international application PCT/US2004/039617, published as International Publication WO 2005/052006.

It would be desirable to find ways to further inhibit the growth of B cell lymphomas and leukemias.

**BRffiF SUMMARY OF THE INVENTION**

In some embodiments, the invention provides methods of increasing inhibition of growth of a CD22+-cancer cell by contacting the cell with (a) an immunotoxin comprising (i) an anti-CD22 antibody and (ii) a protein synthesis-inhibiting toxin and then, within one week, by (b) a first chemotherapeutic agent, which first chemotherapeutic agent inhibits protein synthesis in the cell. In some embodiments, the cell is contacted with the immunotoxin and then by the first chemotherapeutic agent within a 96 hour period. In some embodiments, the
cell is contacted with the immunotoxin and then by the first chemotherapeutic agent within a 72 hour period. In some embodiments, the cell is contacted with the immunotoxin and then by the first chemotherapeutic agent within a 48 hour period. In some embodiments, the cell is contacted with the immunotoxin and then by the first chemotherapeutic agent within a 24 hour period. In some embodiments, the cell is concurrently contacted with both the immunotoxin and by the first chemotherapeutic agent. In some embodiments, the anti-CD22 antibody is a scFv or a dsFv. In some embodiments, the anti-CD22 antibody is RFB4 or RFB4(dsFv). In some embodiments, the anti-CD22 antibody has a variable light chain (VL) and a variable heavy chain (VH), wherein CDRs 1-3 of said VL chain, respectively, have the sequences of SEQ ID NOs.:8-10, the heavy chain (VH) CDRs 1 and 2, respectively, have the sequences of SEQ ID NOs.:5 and 6, respectively, and CDR3 of the VH chain has a sequence selected from any of SEQ ID NO.:11-14. In some embodiments, the VH CDR3 has the sequence of SEQ ID NO.:11. In some embodiments, the anti-CD22 antibody has a variable light chain (VL) and a variable heavy chain (VH), wherein CDRs 1-3, respectively, of said VH chain, have the sequences of SEQ ID NOs.:5-7, the VL CDRs 2 and 3 respectively, have the sequences of SEQ ID NOs.:9 and 10, respectively, and CDR1 of the VL chain has a sequence selected from any of SEQ ID NO.:15-18. In some embodiments, the anti-CD22 antibody has a variable light chain (VL) and a variable heavy chain (VH), each with three complementarity determining regions (CDRs) wherein (a) CDRs 1-2, respectively, of the VH chain have the sequences of SEQ ID NOs.:5 and 6, respectively, and CDR3 has an amino acid sequence selected from any of SEQ ID NO.:11-14, (b) VL CDRs 2 and 3 respectively, have the sequences of SEQ ID NOs.:9 and 10, respectively, and (c) VL CDR1 has a sequence selected from any of SEQ ID NO.:15-18. In some embodiments, the VH CDR3 has the sequence of SEQ ID NO.:11. In some embodiments, the anti-CD22 antibody is human. In some embodiments, the first chemotherapeutic agent is L-asparaginase. In some embodiments, the L-asparaginase is an E. coli or Erwinia carotovora L-asparaginase. In some embodiments, the cell is further contacted by a second chemotherapeutic agent. In some embodiments, the protein synthesis-inhibiting toxin is a Pseudomonas exotoxin A (PE). In some embodiments, the PE is PE38.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0014] **Figure 1.** Figure 1 sets forth the nucleotide sequence (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:2) of the variable region of the RFB4 light chain and the
nucleotide sequence (SEQ ID NO:3) and amino acid sequence (SEQ ID NO:4) of the variable region of the RFB4 heavy chain. The CDRs assigned using the IMGT program (Lefranc, *Nucl. Acids Res.* 29:207-209, 2001; also found on-line by entering http://imgt.cines.fr) are underlined (while not numbered, CDRs 1, 2 and 3 of each chain are presented within their respective chain in increasing numerical order). DNA hot spots (A/G-G-C/T-A/T and A-G-C/T) are highlighted.

[0015] **Figure 2.** Figure 2 is a graph showing the results of in vitro studies of the effect of contacting cells of the ALL cell line REH simultaneously with HA22 immunotoxin and with one of four chemotherapeutic agents. The chemotherapeutic agents are shown on the Y axis, while the X axis shows the result of subtracting from 1 the "Combination Index" calculated by the Chow-Talalay median-effect equation.

[0016] **Figure 3.** Figure 3 is a graph showing the results of in vitro studies of the effect of contacting cells of the ALL cell line KOPN-8 simultaneously with HA22 immunotoxin and with one of four chemotherapeutic agents. The chemotherapeutic agents are shown on the Y axis, while the X axis shows the result obtained by subtracting from 1 the "Combination Index" calculated by the Chow-Talalay median-effect equation.

[0017] **Figure 4.** Figure 4 is a graph showing the results of in vitro studies of the effect of contacting cells of the ALL cell line REH with either a pair of two chemotherapeutic agents or with HA22 immunotoxin and with the same pair of chemotherapeutic agents. The agents are shown on the Y axis, while the X axis shows the result obtained by subtracting from 1 the "Combination Index" calculated by the Chow-Talalay median-effect equation. Adria: adriamycin (also known as doxorubicin). Vine: vincristine. Cyta: cytarabine. L-asp: L-asparaginase.

[0018] **Figures 5A and 5B.** Figures 5A and B are graphs showing the results of in vitro studies of the effect of contacting cells of the ALL cell line REH (Fig. 5A) or the ALL cell line KOPN-8 (Fig. 5B) either first with a chemotherapeutic agent and then with HA22 immunotoxin, or vice versa. The agents were sequentially added for two day incubations. The agents are shown on the Y axis in temporal order of administration. The X axis shows the result obtained by subtracting from 1 the "Combination Index" ("CI") calculated by the Chow-Talalay median-effect equation. Each CI value is the mean ± standard deviation of three independent determinations.
DETAILED DESCRIPTION OF THE INVENTION

Introduction

[0019] As noted in the Background, many B cell lymphomas and leukemias express a
5 lineage-restricted B-cell antigen referred to as CD22, and two anti-CD22 immunotoxins,
referred to as BL22 and HA22, respectively, are currently in clinical trials for treatment of
CD22+ malignancies. These immunotoxins employ as the toxic moiety a truncated 38 kD
form of Pseudomonas exotoxin A known as PE38. As is well known, PE acts by inactivating
the ADP-ribosylation of elongation factor 2 (EF-2), thereby interfering with protein synthesis
within the cell. Studies on B cells of chronic lymphocytic leukemia (CLL) found that the
immunotoxins caused caspase-mediated apoptosis. See, Decker et al., Blood, 103(7):2718-26
(2004) ("Decker et al."). Decker et al. reported that BL22 induced caspase-9 and caspase-3
activation. Decker et al. further tested the effect of BL22 in combination with the
chemotherapeutic agents (i) vincristine, a mitotic inhibitor which inhibits assembly of
microtubules, (ii) fludarabine, a purine analog that interferes with DNA synthesis, and (iii)
doxorubicin, a drug that is believed to intercalate DNA and interfere with topoisomerase II,
thereby inhibiting DNA replication. While BL22 and vincristine by themselves each killed
approximately 40% of the CLL cells, the combination of the two agents killed almost 80% of
the cells at the doses tested.

[0020] As noted, the combinations of agents tested by Decker et al. were of agents that
work through different pathways, such as an agent that interferes with protein synthesis (e.g.,
the BL22 immunotoxin) and an agent that interferes with mitosis, such as vincristine. This is
in keeping with the general understandings in the art that (i) there is greater benefit in
attacking cancer cells from multiple directions at once and (ii) that if a particular agent has
"killed" a particular pathway or seriously disturbed it, there is little additional benefit to be
 gained by adding a second agent against the same pathway.

[0021] Surprisingly, we have now discovered that there is a synergistic effect in
administering anti-CD22 immunotoxins using as their toxin portion a cytotoxic fragment of
PE, which inhibits protein synthesis, and L-asparaginase, a chemotherapeutic agent which
also inhibits protein synthesis. This result was surprising in part because both agents have the
same final common mechanism of action. Further surprisingly, despite the fact that both the
immunotoxin and L-asparaginase inhibit protein synthesis, we found that the order of
administration also makes a difference. As shown in Figure 5, we found uniformly better
results by administering the immunotoxin before administering L-asparaginase. While
administration of the two agents may overlap, so that cells are contacted with both agents
concurrently, in preferred embodiments, the immunotoxin is administered first and then the
L-asparaginase.

[0022] Based on the results in our studies, other CD22-targeted immunotoxins, and
particularly those which have the binding specificity of the RFB4 antibody, will also show a
synergistic effect with protein synthesis-inhibiting chemotherapeutic agents in inhibiting the
growth of CD22+ B cell cancer cells, including lymphomas and leukemias. Preferred anti-
CD22 antibodies are RFB4 dsFv, HA22, other variants of RFB4 with the mutations of the
residues SSY in the CDR3 of the variable heavy chain, as taught in international application
PCT/US02/30316, published as International Publication WO 03/027135, and variants of
RFB4 in which residues 30 and 31 of the variable light chain are mutated to HG, GR, RG, or
AR, as disclosed in international application PCT/US2004/039617, published as International
Publication WO 2005/052006. Persons of skill will appreciate that RFB4 can also be
mutated at hot spot codons of CDRs 1 or 2 of the heavy chain or of CDRs 2 or 3 of the light
chain, or at combinations of these, to produce other antibodies with the binding specificity of
RFB4 and higher affinity. BL22 and HA22 are preferred, with HA22 being particularly
preferred.

[0023] One advantage of using L-asparaginase as the chemotherapeutic agent to combine
with immunotoxin therapy is that L-asparaginase has a relatively mild side effect profile. In
some embodiments, however, the L-asparaginase or other protein synthesis inhibiting
chemotherapeutic agent may be encapsulated in a liposome to reduce the patient's systemic
exposure to the agent. Encapsulating therapeutic agents in liposomes to reduce side effects is
well known in the art. For example, DOXIL®, a preparation of PEGylated liposomes
encapsulating doxorubicin, a chemotherapeutic agent, is commercially available from Ortho
Biotech Products, L.P.

[0024] Patients receiving therapy according to the methods of the invention will typically
have the anti-CD22 immunotoxin administered followed a day or more later by the
administration of the chemotherapeutic agent. In a preferred embodiment, the anti-CD22
immunotoxin is administered, followed by administration of chemotherapeutic agent(s), such
as L-asparaginase, at one to two day intervals. To permit observation for adverse effects,
there will usually be a space of one half hour to 168 hours between the administration of the
immunotoxin and the first administration of L-asparaginase or other protein synthesis inhibiting agent, with 1 to 96 hours being preferred, 1 to 72 hours being more preferred, 1 to 48 hours still more preferred, 1 to 36 hours even more preferred, 12 to 30 hours yet more preferred, and about 24 hours being most preferred (with "about" in this context meaning 2 hours on either side to allow the patient and staff some flexibility in scheduling and the like). Thus, a typical administration schedule might be an administration of an immunotoxin, such as BL22 or HA22, on day 1, with administration of L-asparaginase on days 2, 4, and 6.

[0025] Persons of skill will appreciate that there is a difference between the plasma half life of an agent (that is, how long it persists in the circulation) and its functional half life. In clinical trials, the plasma half life of BL22 immunotoxin has been between a few minutes and a few hours, depending on the particular patient, and the half life of HA22 immunotoxin is expected to be similar. The functional half life of the immunotoxins is, however, considerably longer, as the immunotoxin will tend to bind to CD22+-cells during its time in the circulation and be internalized, where it will exert its effect in inhibiting protein synthesis over a period of time.

[0026] Persons of skill will appreciate that bacterial toxins other than PE are known that inhibit protein synthesis and that have been used in immunotoxins. Typically, these bacterial toxins, like PE, interfere with ADP-ribosylation of Elongation Factor-2 ("EF-2"). Similarly, a number of plant toxins, such as ricin, gelonin and saporin, are known to inhibit protein synthesis and have been used to form immunotoxins. Typically, the plant toxins that inhibit protein synthesis tend to interfere with the function of ribosomes or subunits of ribosomes rather than EF-2. While PE is a particularly preferred toxin for use in the present invention, it is expected that immunotoxins using other inhibitors of protein synthesis, such as bacterial or plant toxins, as their toxic moiety will also be useful in synergizing the effect of L-asparaginase or of other chemotherapeutic agents that have as their primary mechanism of action the inhibition of protein synthesis. It should be noted that all cytotoxins can in some sense be thought of inhibiting protein synthesis in a cell since a dead cell makes no proteins. Persons of skill in constructing immunotoxins, however, are familiar with defining the activity of the and persons of skill in oncology, are famili The art The term "inhibiting protein synthesis" is used herein in a narrower sense to refer to those toxins and chemotherapeutic agents considered in the art to
Definitions

[0027] Units, prefixes, and symbols are denoted in their Systeme International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects or embodiments of the invention, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[0028] "CD22" refers to a lineage-restricted B cell antigen belonging to the Ig superfamily. It is expressed in 60-70% of B cell lymphomas and leukemias and is not present on the cell surface in early stages of B cell development or on stem cells. See, e.g. Vaickus et al., Crit. Rev. Oncol/Hematol. 11:267-297 (1991).

[0029] As used herein, the term "anti-CD22" in reference to an antibody, refers to an antibody that specifically binds CD22 and includes reference to an antibody which is generated against CD22. In preferred embodiments, the CD22 is a primate CD22 such as human CD22. In one preferred embodiment, the antibody is generated against human CD22 synthesized by a non-primate mammal after introduction into the animal of cDNA which encodes human CD22. In other preferred embodiments, the antibodies have one or more of the specific mutations discussed herein of the antibody referred to as "RFB4".

[0030] "RFB4" refers to a mouse IgGl monoclonal antibody that specifically binds to human CD22. The antibody is commercially available under the name RFB4 from several sources, such as Southern Biotechnology Associates, Inc. (Birmingham AL; Cat. No. 9360-01), Autogen Bioclear UK Ltd. (Calne, Wilts, UK; Cat. No. AB147), Axxora LLC. (San Diego, CA). RFB4 is highly specific for cells of the B lineage and has no detectable cross-reactivity with other normal cell types. Li et al., Cell. Immunol. 118:85-99 (1989). The heavy and light chains of RFB4 have been cloned. See, Mansfield et al., Blood 90:2020-2026 (1997), which is incorporated herein by reference.

[0031] Unless otherwise indicated, references herein to amino acid positions of the RFB4 heavy or light chain refer to the numbering of the amino acids under the "Kabat and Wu" system, which is the most widely used antibody numbering systems. See, Kabat, E., et al., SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST, U.S. Government Printing Office, NTH Publication No. 91-3242 (1991), which is hereby incorporated by reference. The
Chothia number scheme is identical to the Kabat scheme, but places the insertions in CDR-L1 and CDR-H1 at structurally different positions. It should be noted that the number accorded to a residue under the Kabat and Wu system does not necessarily correspond to the number that one might obtain for a residue in a given heavy or light chain by counting from the amino terminus of that chain. Thus, the position of an amino acid residue in a particular \( V_H \) or \( V_L \) sequence does not refer to the number of amino acids in a particular sequence, but rather refers to the position as designated with reference to the Kabat numbering scheme. For convenience, the "Kabat and Wu" numbering is sometimes referred to herein as "Kabat" numbering.


The term "BL22" refers to an immunotoxin in which a disulfide stabilized Fv ("dsFv") region of RFB4 is attached to a 38 kD truncated cytotoxic fragment of *Pseudomonas* exotoxin A ("PE") known as "PE38". Various mutations and truncations of PE are known in the art and are discussed in some detail in a separate section of this disclosure.

The term "HA22" refers to an antibody or fragment thereof having an RFB4 Fv region in which the native sequence SSY at positions 100, IOOA and IOOB of the CDR3 of the variable heavy chain ("H-CDR3") is mutated to THW. The term "HA22" is also used
herein to refer to an immunotoxin in which a dsFv form of an RFB4 antibody containing this mutation is fused to PE38. Which meaning is intended will be clear in context.

[0035] The terms "B5," "E6," "B8" and "D8" refer to antibodies or fragments thereof having an RFB4 Fv regions with mutations in the CDR1 of the variable light chain. These mutants change the amino acid sequence of the residues at positions 30 and 31 of CDR1 of the VL chain of RFB4 ("L-CDR1"), as those positions are numbered under the "Kabat and Wu" antibody residue numbering system, from the wild type sequence Serine-Asparagine (in single letter code, "SN") to (a) Histidine-Glycine ("HG," the antibody made by combining an RFB4 light chain containing this L-CDR1 mutant with an RFB4 heavy chain containing the THW H-CDR3 mutant is designated as "B5"), (b) Glycine-Arginine ("GR," the antibody made by combining an RFB4 light chain containing this L-CDR1 mutant with an RFB4 heavy chain containing the THW H-CDR3 mutant is designated as "E6"), (c) Arginine-Glycine ("RG," the antibody made by combining an RFB4 light chain containing this L-CDR1 mutant with an RFB4 heavy chain containing the THW H-CDR3 mutant is designated as "B8"), or (d) Alanine-Arginine ("AR," the antibody made by combining an RFB4 light chain containing this L-CDR1 mutant with an RFB4 heavy chain containing the THW H-CDR3 mutant is designated as "D8").

[0036] As used herein, "antibody" includes reference to an immunoglobulin molecule immunologically reactive with a particular antigen, and includes both polyclonal and monoclonal antibodies. The term also includes genetically engineered forms such as chimeric antibodies (e.g., humanized murine antibodies), heteroconjugate antibodies (e.g., bispecific antibodies), recombinant single chain Fv fragments (scFv), and disulfide stabilized (dsFv) Fv fragments (see, co-owned U.S. Patent No. 5,747,654, which is incorporated herein by reference). The term "antibody" also includes antigen binding forms of antibodies (e.g., Fab', F(ab')2, Fab, Fv and rlgG. See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, IL); Goldsby et al., eds., Kuby, J., Immunology, 4th Ed., W.H. Freeman & Co., New York (2000).

Typically, an immunoglobulin has a heavy and light chain. Each heavy and light chain contains a constant region and a variable region, (the regions are also known as "domains")—Light and heavy chain variable regions contain a "framework" region interrupted by three hypervariable regions, also called "complementarity-determining regions" or "CDRs". The extent of the framework region and CDRs have been defined. See, Kabat and Wu, supra. The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three dimensional space.

The CDRs are primarily responsible for binding to an epitope of an antigen. The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus, and are also typically identified by the chain in which the particular CDR is located. Thus, a V_H CDR3 is located in the variable domain of the heavy chain of the antibody in which it is found, whereas a V_L CDR1 is the CDR1 from the variable domain of the light chain of the antibody in which it is found.

References to "V_H" or a "VH" refer to the variable region of an immunoglobulin heavy chain, including an Fv, scFv, dsFv or Fab. References to "V_L" or a "VL" refer to the variable region of an immunoglobulin light chain, including of an Fv, scFv, dsFv or Fab.

The phrase "single chain Fv" or "scFv" refers to an antibody in which the variable domains of the heavy chain and of the light chain of a traditional two chain antibody have been joined to form one chain. Typically, a linker peptide is inserted between the two chains to allow for proper folding and creation of an active binding site.

The phrase "disulfide bond" or "cysteine-cysteine disulfide bond" refers to a covalent interaction between two cysteines in which the sulfur atoms of the cysteines are oxidized to form a disulfide bond. The average bond energy of a disulfide bond is about 60 kcal/mol compared to 1-2 kcal/mol for a hydrogen bond.

The phrase "disulfide stabilized Fv" or "dsFv" refer to the variable region of an immunoglobulin in which there is a disulfide bond between the light chain and the heavy chain. In the context of this invention, the cysteines which form the disulfide bond are within the framework regions of the antibody chains and serve to stabilize the conformation of the antibody. Typically, the antibody is engineered to introduce cysteines in the framework region at positions where the substitution will not interfere with antigen binding.
[0044] The term "linker peptide" includes reference to a peptide within an antibody binding fragment (e.g., Fv fragment) which serves to indirectly bond the variable domain of the heavy chain to the variable domain of the light chain.

[0045] The term "parental antibody" means any antibody of interest which is to be mutated or varied to obtain antibodies or fragments thereof which bind to the same epitope as the parental antibody, but with higher affinity.

[0046] The term "hotspot" means a portion of a nucleotide sequence of a CDR or of a framework region of a variable domain which is a site of particularly high natural variation. Although CDRs are themselves considered to be regions of hypervariability, it has been learned that mutations are not evenly distributed throughout the CDRs. Particular sites, or hotspots, have been identified as these locations which undergo concentrated mutations. The hotspots are characterized by a number of structural features and sequences. These "hotspot motifs" can be used to identify hotspots. Two consensus sequences motifs which are especially well characterized are the tetranucleotide sequence RGYW and the serine sequence AGY, where R is A or G, Y is C or T, and W is A or T.

[0047] A "targeting moiety" is the portion of an immunoconjugate intended to target the immunoconjugate to a cell of interest. Typically, the targeting moiety is an antibody, a scFv, a dsFv, an Fab, or an F(ab')₂.

[0048] A "toxic moiety" is the portion of a immunotoxin which renders the immunotoxin cytotoxic to cells of interest.

[0049] A "therapeutic moiety" is the portion of an immunoconjugate intended to act as a therapeutic agent.

[0050] The term "therapeutic agent" includes any number of compounds currently known or later developed to act as anti-neoplasties, antiinflammatories, cytokines, anti-infectives, enzyme activators or inhibitors, allosteric modifiers, antibiotics or other agents administered to induce a desired therapeutic effect in a patient. The therapeutic agent may also be a toxin or a radioisotope, where the therapeutic effect intended is, for example, the killing of a cancer cell.

[0051] A "detectable label" means, with respect to an immunoconjugate, a portion of the immunoconjugate which has a property rendering its presence detectable. For example, the
immunoconjugate may be labeled with a radioactive isotope which permits cells in which the immunoconjugate is present to be detected in immunohistochemical assays.

[0052] The term "effector moiety" means the portion of an immunoconjugate intended to have an effect on a cell targeted by the targeting moiety or to identify the presence of the immunoconjugate. Thus, the effector moiety can be, for example, a therapeutic moiety, a toxin, a radiolabel, or a fluorescent label.

[0053] The term "immunoconjugate" includes reference to a covalent linkage of an effector molecule to an antibody. The effector molecule can be an immunotoxin.

[0054] The terms "effective amount" or "amount effective to" or "therapeutically effective amount" includes reference to a dosage of a therapeutic agent sufficient to produce a desired result, such as inhibiting cell protein synthesis by at least 50%, or killing the cell.

[0055] The term "toxin" includes reference to toxins, such as abrin, ricin, Pseudomonas exotoxin A (PE), diphtheria toxin (DT), or cytotoxic fragments or mutants thereof, which act by inhibiting protein synthesis in cells into which they are internalized. For example, PE and DT are highly toxic compounds that typically bring about death through liver toxicity. PE and DT, however, can be modified into a form for use as an immunotoxin by removing the native targeting component of the toxin (e.g., domain Ia of PE or the B chain of DT) and replacing it with a different targeting moiety, such as an antibody.

[0056] As noted in some detail below, a number of substitutions and truncations have been made to native Pseudomonas exotoxin A (PE) to reduce the non-specific toxicity of the toxin, to increase its cytotoxicity to desired targets, and to reduce the immunogenicity of the toxin. For convenience of reference, the terms "Pseudomonas exotoxin A" and "PE" are intended to encompass these truncated and mutated forms unless otherwise required by context. When specific reference to an individual form of PE is intended, it is designated by its molecular weight (e.g., the truncated form known PE38) or by the particular mutations made to produce the particular variation of the native molecule (e.g., the mutated form referred to as PE4E, as further described herein), or by a combination thereof, such as PE38KDEL.

[0057] The phrase "chemotherapeutic agent [which] inhibits protein synthesis" denotes that inhibition of protein synthesis is the primary way in which the chemotherapeutic agent is classified. It is understood that a chemotherapeutic agent that inhibits cell growth or proliferation by, for example, intercalcating with DNA, thereby interfering with DNA
replication, may also incidentally inhibit protein synthesis, but such agents are not classified in the art as protein synthesis inhibitors, and are not considered to be protein synthesis-inhibiting chemotherapeutic agents for purposes of the present invention.

[0058] "L-asparaginase" (EC 3.5.1.1) is an enzyme that degrades the amino acid asparagine. See, e.g., Goodsell, *Oncologist*, 10:238-239 (2005). Some blood cells cannot synthesize their own asparagine and the growth and proliferation of cancers deriving from such cells can be inhibited by administration of L-asparaginase into the blood. Use of L-asparaginase as a chemotherapeutic agent has been explored for over half a century. See, e.g., Capizzi et al., "L-Asparaginase", *Annual Rev of Medicine*, 21:433-444 (1970). A form of L-asparaginase, L-asparagine amidohydrolase, type EC2, derived from *E. coli*, is in clinical use as a chemotherapeutic agent under the trade name Elspar® (Merck & Co., Inc., Whitehouse Station, NJ). Another form is derived from *Erwinia carotovora* and there is also a pegylated form of the *E. coli* derived agent, both of which in commercial use. These agents are usually administered intramuscularly in a hospital setting. Detailed information on its administration and dosing are readily available in the literature and on the internet.

[0059] Non-Hodgkin's lymphoma ("NHL") refers to any of a large group of cancers of the immune system. According to the website of the National Cancer Institute, there are many different types of NHL, which can be divided into aggressive (fast-growing) and indolent (slow-growing) types and can be classified as either B-cell or T-cell NHL. B-cell NHLs include Burkitt's lymphoma, diffuse large B-cell lymphoma, follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, and mantle cell lymphoma. T-cell NHLs include mycosis fungoides, anaplastic large cell lymphoma, and precursor T-lymphoblastic lymphoma. Lymphomas related to lymphoproliferative disorders following bone marrow or stem cell transplantation are usually B-cell NHLs. Prognosis and treatment depend on the stage and type of disease. For purposes of the present disclosure, reference to non-Hodgkin's lymphoma refers to a B-cell lymphoma unless otherwise required by context.

[0060] The term "contacting" includes reference to placement in direct physical association. With respect to chemotherapeutic agents, such agents are considered to contact a CD22+ cancer cell, such as a B cell lymphoma or leukemia, in an organism if the agent is administered by a route accepted in the art for administering that agent. For example, if a particular chemotherapeutic agent is approved for treating a B cell lymphoma or leukemia by
IV administration, persons of skill would accept that the agent is contacting cells of the lymphoma or leukemia. Similarly, for purposes of the present invention, an immunotoxin is considered to "contact" a CD22+ cancer cell, such as a B cell lymphoma or leukemia if the immunotoxin is administered by routes that permit introduction of the immunotoxin into the systemic circulation or the lymphatic channels, as appropriate.

[0061] An "expression plasmid" comprises a nucleotide sequence encoding a molecule or interest, which is operably linked to a promoter.

[0062] As used herein, "polypeptide", "peptide" and "protein" are used interchangeably and include reference to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The terms also apply to polymers containing conservative amino acid substitutions such that the protein remains functional.

[0063] The term "residue" or "amino acid residue" or "amino acid" includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide (collectively "peptide"). The amino acid can be a naturally occurring amino acid and, unless otherwise limited, can encompass known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids.

[0064] As used herein, "recombinant" includes reference to a protein produced using cells that do not have, in their native state, an endogenous copy of the DNA able to express the protein. The cells produce the recombinant protein because they have been genetically altered by the introduction of the appropriate isolated nucleic acid sequence. The term also includes reference to a cell, or nucleic acid, or vector, that has been modified by the introduction of a heterologous nucleic acid or the alteration of a native nucleic acid to a form not native to that cell, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell, express mutants of genes that are found within the native form, or express native genes that are otherwise abnormally expressed, underexpressed or not expressed at all.

[0065] The term "in vivo" includes reference to inside the body of the organism in which an effect is to be studied or determined. "Ex vivo" and "in vitro" means outside the body of an organism.
The phrase "malignant cell" or "malignancy" includes reference to tumors or tumor cells that are invasive and/or able to undergo metastasis, *i.e.*, a cancerous cell.

As used herein, "mammalian cells" includes reference to cells derived from mammals including humans, rats, mice, guinea pigs, chimpanzees, or macaques. The cells may be cultured *in vivo* or *in vitro*.

The term "selectively reactive" refers, with respect to an antigen, the preferential association of an antibody, in whole or part, with a cell or tissue bearing that antigen and not to cells or tissues lacking that antigen. It is, of course, recognized that a certain degree of non-specific interaction may occur between a molecule and a non-target cell or tissue. Nevertheless, selective reactivity, may be distinguished as mediated through specific recognition of the antigen. Although selectively reactive antibodies bind antigen, they may do so with low affinity. On the other hand, specific binding results in a much stronger association between the antibody and cells bearing the antigen than between the bound antibody and cells lacking the antigen. Specific binding typically results in greater than 2-fold, preferably greater than 5-fold, more preferably greater than 10-fold and most preferably greater than 100-fold increase in amount of bound antibody (per unit time) to a cell or tissue bearing CD22 as compared to a cell or tissue lacking CD22. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats are appropriate for selecting antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow & Lane, *ANTIBODIES, A LABORATORY MANUAL*, Cold Spring Harbor Publications, New York (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

The term "immunologically reactive conditions" includes reference to conditions which allow an antibody generated to a particular epitope to bind to that epitope to a detectably greater degree than, and/or to the substantial exclusion of, binding to substantially all other epitopes. Immunologically reactive conditions are dependent upon the format of the antibody binding reaction and typically are those utilized in immunoassay protocols or those conditions encountered *in vivo*. See Harlow & Lane, *supra*, for a description of immunoassay formats and conditions. Preferably, the immunologically reactive conditions employed in the methods of the present invention are "physiological conditions" which
include reference to conditions (e.g., temperature, osmolality, pH) that are typical inside a living mammal or a mammalian cell. While it is recognized that some organs are subject to extreme conditions, the intra-organismal and intracellular environment normally lies around pH 7 (i.e., from pH 6.0 to pH 8.0, more typically pH 6.5 to 7.5), contains water as the predominant solvent, and exists at a temperature above 0°C and below 50°C. Osmolality is within the range that is supportive of cell viability and proliferation.

B cell Lymphomas and Leukemias

[0070] Persons of skill in the art are presumed to be familiar with the various types of B cell lymphomas and leukemias, as well as with the standard regimens for treating them and the considerable literature in the field, such as Pui, ed., CHILDHOOD LEUKEMIA, Cambridge University Press, Cambridge, UK (2nd Ed., 2006), Hancock et al., eds., MALIGNANT LYMPHOMA, Oxford University Press, New York, NY (2000), and Leonard and Coleman, eds., HODGKIN'S AND NON-HODGKIN'S LYMPHOMA, Springer Science+Business Media LLC, New York, NY (2006).

[0071] The methods of the invention are expected to be useful in inhibiting the growth of CD22+ B cell lymphomas and leukemias, and in particular those for which the anti-CD22 immunotoxin called BL22, and particularly those for which the anti-CD22 immunotoxin HA22, is currently in clinical trials. These cancers include relapsed or refractory acute lymphoblastic leukemia ("ALL"), non-Hodgkin's lymphoma, hairy cell leukemia, refractory chronic lymphocytic leukemia, and prolymphocyte leukemia.

Chemotherapeutic Agents

[0072] The invention contemplates the combined use of two types of therapeutic agents: immunotoxins which are comprised of an anti-CD22 antibody attached to PE or another protein synthesis-inhibiting toxin, and a chemotherapeutic agent that inhibits protein synthesis. In preferred embodiments, the protein synthesis-inhibiting agent is L-asparaginase.

[0073] In some treatment protocols, a protein synthesis-inhibiting chemotherapeutic agent, such as L-asparaginase, may be administered in combination with one or more conventional chemotherapeutic agents. As shown in the Figures, the combination of an anti-CD22 immunotoxin, a protein synthesis-inhibiting chemotherapeutic agent, such as L-asparaginase, and optionally one or more additional conventional chemotherapeutic agents used in treating
a B cell lymphoma or leukemia other than a protein synthesis inhibitor can be synergistic in treating B cell cancers.

For example, the conventional chemotherapeutic agent other than a protein synthesis inhibiting chemotherapeutic agent can be

- a cytotoxic/antitumor antibiotic, such as daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone, valrubicin, bleomycin, hydroxyurea, or mitomycin;

- a plant alkaloid, such as docetaxel, paclitaxel, vinblastine, vincristine, vindesine, or vinorelbine;

- an alkylating agent, such as a nitrogen mustard (such as chlorambucil, chloromethine, cyclophosphamide, ifosfamide, or melphalan), a nitrosoureas: (carmustine, fotemustine, lomustine, streptozocin), a platinum drug (such as carboplatin, cisplatin, oxaliplatin, BBR3464), busulfan, dacarabazine, mechlorethamine, procarbazine, temozolomide, thioTEPA, uramustine;

- an antimetabolite, such as (a) those that interfere with the metabolism of folic acid (e.g., aminopterin, methotrexate, pemtrexed, raltitrexed), (b) purine analogs (e.g., cladribine, clofarabine, fludarabine, mercaptopurine, thioguanine, pentostatin), and (c) pyrimidine analogs (e.g., capecitabine, cytarabine, fluorouracil, and gemcitabine;

- a topoisomerase inhibitor, such as topotecan, irinotecan, podophyllum, etoposide, or teniposide;

- a therapeutic antibody, such as alemtuzumab, bevacizumab, cetuximab, gemtuzumab, panitumumab, rituximab, tositumomab, trastuzumab; or,

- a kinase inhibitor, such as imatinib, nilotinib, dasatinib, erlotinib, gefitinib, lapatinib, sorafenib, sunitinib, or vandetanib.

In some embodiments, the other conventional chemotherapeutic agent is vincristine, doxorubicin, or cytarabine.

Pseudomonas Exotoxins and Other Toxins

Toxins can be attached to anti-CD22 antibodies to yield immunotoxins for use in the methods of the present invention. Persons of skill in the art are aware of a number of toxins available for use as the toxic moiety of immunotoxins. A minireview published in 2000, for
example, listed over 30 chimeric molecules, primarily immunotoxins, which had been tested, or which were being tested, in clinical trials. See, Frankel, A., *Clin Cancer Res* 6:326-334 (2000). The toxins used in these clinical trials were: *Pseudomonas* exotoxin A, ricin, pokeweed antiviral protein, gelonin, Diphtheria toxin, and saporin. See, Frankel, *supra*, at page 327, Table 1. As demonstrated by the fact that these toxins have been used in clinical trials, they are known in the art. While persons of skill in the art are presumed to be familiar with the considerable literature on these toxins, for convenience, some aspects of these and other toxins will be discussed below.

[0084] Toxins preferred for use in the present invention are toxins that inhibit protein synthesis. Exemplary toxins include ricin, abrin, and Diphtheria toxin and subunits thereof. These toxins are readily available from commercial sources (e.g., Sigma Chemical Company, St. Louis, MO). Diphtheria toxin is isolated from *Corynebacterium diphtheriae*. Ricin is the lectin RCA60 from *Ricinus communis* (Castor bean). The term also references toxic variants thereof, as described, for example, in U.S. Patent Nos. 5,079,163 and 4,689,401. *Ricinus communis* agglutinin (RCA) occurs in two forms designated RCA60 and RCA 120 according to their molecular weights of approximately 65 and 120 kD, respectively (Nicholson & Blaustein, *J. Biochim. Biophys. Acta* 266:543 (1972)). The A chain is responsible for inactivating protein synthesis and killing cells. The B chain binds ricin to cell-surface galactose residues and facilitates transport of the A chain into the cytosol (Olsnes, et al., *Nature* 249:627-631 (1974) and U.S. Patent No. 3,060,165).

[0085] Abrin includes toxic lectins from *Abrus precatorius*. The toxic principles, abrin a, b, c, and d, have a molecular weight of from about 63 and 67 kD and are composed of two disulfide-linked polypeptide chains A and B. The A chain inhibits protein synthesis; the B-chain (abrin-b) binds to D-galactose residues (see, e.g., Funatsu, et al., *Agr. Biol. Chem.* 52:1095 (1988); and Olsnes, *Methods Enzymol.* 50:330-335 (1978)).

[0086] In particularly preferred embodiments, the toxin is a cytotoxic form of *Pseudomonas* exotoxin A ("PE"). Native PE is an extremely active monomelic protein (molecular weight 66 kD), secreted by *Pseudomonas aeruginosa*, which inhibits protein synthesis in eukaryotic cells. The native PE sequence is set forth in U.S. Patent No. 5,602,095 (the "095 patent"). The method of action is inactivation of the ADP-ribosylation of elongation factor 2 (EF-2). The exotoxin contains three structural domains that act in concert to cause cytotoxicity. Domain Ia (amino acids 1-252) mediates cell binding. Domain
II (amino acids 253-364) is responsible for translocation into the cytosol and domain III (amino acids 400-613) mediates ADP ribosylation of elongation factor 2. The function of domain Ib (amino acids 365-399) remains undefined, although a large part of it, amino acids 365-380, can be deleted without loss of cytotoxicity. See Siegall, et al, J Biol Chem 264:14256-61 (1989).

[0087] The terms "Pseudomonas exotoxin" and "PE" as used herein typically refer to a PE that has been modified from the native protein to reduce or to eliminate non-specific toxicity. Numerous such modifications are known in the art and include, but are not limited to, elimination of domain Ia, various amino acid deletions in domains Ib, II and III, single amino acid substitutions and the addition of one or more sequences at the carboxyl terminus that are known to increase toxicity. See Siegall, et al, J. Biol. Chem. 264:14256-14261 (1989). Cytotoxic fragments of PE include those which are cytotoxic with or without subsequent proteolytic or other processing in the target cell (e.g., as a protein or pre-protein). Cytotoxic fragments of PE include PE40, PE38, variants thereof such as PE38QQR and PE38KDEL, and PE35, as discussed below. In a preferred embodiment, the cytotoxic fragment of PE retains at least about 20%, preferably at least about 40%, more preferably about 50%, even more preferably 75%, more preferably at least about 90%, and still more preferably 95% of the cytotoxicity of native PE. In particularly preferred embodiments, the cytotoxic fragment has at least the cytotoxicity of native PE, and preferably has more.

[0088] In preferred embodiments, the PE has been modified to reduce or eliminate non-specific cell binding, frequently by deleting domain Ia, as taught in U.S. Patent 4,892,827, although this can also be achieved, for example, by mutating certain residues of domain Ia. U.S. Patent 5,512,658, for instance, discloses that a mutated PE in which Domain Ia is present but in which the basic residues of domain Ia at positions 57, 246, 247, and 249 are replaced with acidic residues (glutamic acid, or "E") exhibits greatly diminished non-specific cytotoxicity. This mutant form of PE is sometimes referred to as "PE4E."

In some preferred embodiments, the cytotoxic fragment PE38 is employed. PE38 contains the translocating and ADP ribosylating domains of PE but not the cell-binding portion (Hwang, J. et al., Cell, 48:129-136 (1987)). PE38 is a truncated PE pro-protein composed of amino acids 253-364 and 381-613 which is activated to its cytotoxic form upon processing within a cell (see e.g., U.S. Patent No. 5,608,039, and Pastan et al., Biochim. Biophys. Acta 1333:C1-C6 (1997)). The sequence of PE38 is therefore known in the art, but could also readily be determined by the practitioner by comparing the stated residues against the native sequence of PE as set forth, for example, in the '095 patent. Persons of skill will be aware that, due to the degeneracy of the genetic code, the amino acid sequence of PE38, of its variants, such as PE38KDEL, and of the other PE derivatives discussed herein can be encoded by a great variety of nucleic acid sequences, any of which can be expressed to result in the desired polypeptide.

As noted above, some or all of domain Ib may be deleted, and the remaining portions joined by a linker or directly by a peptide bond. Some of the amino portion of domain II may be deleted. And, the C-terminal end may contain the native sequence of residues 609-613, or may contain a variation found to maintain the ability of the construct to translocate into the cytosol, and repeats of these sequences. See, e.g., U.S. Patents 5,854,044; 5,821,238; and 5,602,095 and International Publication WO 99/51643. While in preferred embodiments, the PE is PE4E, PE40, or PE38, any form of PE in which non-specific cytotoxicity has been eliminated or reduced to levels in which significant toxicity to non-targeted cells does not occur can be used in the immunotoxins of the present invention so long as it remains capable of translocation and EF-2 ribosylation in a targeted cell. In some preferred embodiments, the arginine present at position 490 of the native PE sequence is replaced with a glycine, alanine, valine, leucine or isoleucine, with alanine being particularly preferred, to increase the cytotoxicity of PE. See, International Publication WO 2005/052006.

In some preferred embodiments, the PE molecules are further modified to reduce immunogenicity by having a substitution of alanine, glycine, serine or glutamine in place of the amino acid residue normally present at one or more of designated positions of the PE molecule. The particularly preferred substitutions to reduce immunogenicity are described in International Application PCT/US2006/028986, published as International Publication WO 2007/016150.
A. Conservatively Modified Variants of PE

[0093] It is understood that the sequence of native PE and the variants discussed above can have conservative substitutions and retain cytotoxic capability and, desirably, reduced antigenicity compared to the native sequence of PE. The preferred embodiments, modified variants of PE or cytotoxic fragments thereof have at least 85% sequence similarity, preferably at least 90% sequence similarity, more preferably at least 95% sequence similarity, and most preferably at least 98% sequence similarity at the amino acid level, with the PE of interest, such as PE38.

[0094] The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refer to those nucleic acid sequences which encode identical or essentially identical amino acid sequences, or if the nucleic acid does not encode an amino acid sequence, to essentially identical nucleic acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0095] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid.

B. Assaying for Cytotoxicity or Antigenicity of PE

[0096] Pseudomonas exotoxins employed in the invention can be assayed for the desired level of cytotoxicity by assays well known to those of skill in the art. Thus, cytotoxic fragments of PE and conservatively modified variants of such fragments can be readily
assayed for cytotoxicity. A large number of candidate PE molecules can be assayed simultaneously for cytotoxicity by methods well known in the art. For example, subgroups of the candidate molecules can be assayed for cytotoxicity. Positively reacting subgroups of the candidate molecules can be continually subdivided and reassayed until the desired cytotoxic fragment(s) is identified. Such methods allow rapid screening of large numbers of cytotoxic fragments or conservative variants of PE. Antigenicity can be assayed by, for example, the methods taught in the Examples herein.

**Conjugation to the Antibody**

[0097] In a non-recombinant embodiment of the invention, an anti-CD22 antibody is linked to a therapeutic agent, such as a toxin, using any number of means known to those of skill in the art. Both covalent and noncovalent attachment means may be used.

[0098] Antibodies typically contain a variety of functional groups; *e.g.*, carboxylic acid (COOH), free amine (-NH2) or sulfhydryl (-SH) groups to result in the binding of the therapeutic agent. Alternatively, the antibody is derivatized to expose or to attach additional reactive functional groups. The derivatization may involve attachment of any of a number of linker molecules, such as those available from Pierce Chemical Company, Rockford Illinois.

[0099] A "linker", as used herein, is a molecule that is used to join the antibody to the toxin or other therapeutic agent. The linker is capable of forming covalent bonds to both the antibody and to the agent. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. Where the therapeutic agent is a polypeptide, the linkers may be joined to the constituent amino acids through their side groups (*e.g.*, through a disulfide linkage to cysteine). However, in a preferred embodiment, the linkers will be joined to the alpha carbon amino and carboxyl groups of the terminal amino acids.

[0100] In some circumstances, it is desirable to free the therapeutic agent from the antibody when the immunoconjugate has reached its target site. Therefore, in these circumstances, immunoconjugates will comprise linkages which are cleavable in the vicinity of the target site. Cleavage of the linker to release the therapeutic agent from the antibody may be prompted by enzymatic activity or conditions to which the immunoconjugate is subjected either inside the target cell or in the vicinity of the target site. When the target site is a tumor, a linker which is cleavable under conditions present at the tumor site (*e.g.* when exposed to tumor-associated enzymes or acidic pH) may be used.
Production of Immunoconjugates

Immunoconjugates include, but are not limited to, molecules in which there is a covalent linkage of a therapeutic agent to an anti-CD22 antibody. Where the therapeutic agent is a polypeptide, such as PE, it can be encoded by a variety of clones containing functionally equivalent nucleic acids, such as nucleic acids which differ in sequence but which encode the the same antibody and therapeutic agent. Methods of expressing such immunoconjugates is disclosed in, for example, International Application PCT/US2006/028986, published as International Publication WO 2007/016150.

Pharmaceutical Compositions and Administration

It is contemplated that the anti-CD22 immunotoxin will be administered by parenteral means, such as by intravenous administration or administration into a body cavity, while the conventional chemotherapeutic agents will be administered by the routes currently used in the art for their administration in clinical settings.

As of this writing, the listing of clinical trials maintained by the National Institutes of Health website (found on the internet by entering "clinical trials." followed by "gov") lists six open clinical trials on the treatment of CD22+ cancers with the anti-CD22 immunotoxin referred to as "CAT-8015" (which designates a chimeric molecule of HA22 fused to PE38). For example, a clinical trial listed under identifier no. NCT00587015 is a phase I multicenter dose escalation study of CAT-8015 in patients with relapsed or refractory non-Hodgkin's lymphoma. The website further shows one completed phase I trial and one currently recruiting trial using the earlier generation immunotoxin referred to as BL22.

It is expected that immunotoxins administered in the methods of the present invention will be administered under protocols similar to the protocols developed for the clinical trials already underway with respect to BL22 and HA22. For example, in NCT00462189, a safety study of CAT-8015 immunotoxin in patients with hairy cell leukemia with advanced disease, it is contemplated that patients will receive CAT-8015 intravenously over 30 minutes on days 1, 3, and 5, followed by rest. The treatment is repeated every 4 weeks for up to a total of 10 courses in the absence of dose limiting toxicity, complete response or disease progression. Patients are followed at 1, 3, 6, 12, 15, 18, 21, 24 months following the start of the last treatment cycle. Cohorts of 3-6 patients each will receive escalating doses of recombinant CAT-8015 until the maximum tolerated dose (MTD)
is determined. The MTD is defined as the dose proceeding that at which 2 of 3 or 2 of 6 patients experience dose-limiting toxicity.

[0105] It should be noted that, under the exclusion criteria set forth in the six currently open clinical trials of CAT-8015, patients are excluded if they have not had at least a two to six week period since the administration of any cytotoxic chemotherapy. The trials on BL22 similarly require two to six weeks since such administration, depending on the particular trial.

[0106] The compositions for administration will commonly comprise a solution of the antibody and/or immunoconjugate dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of antibody or immunoconjugate in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

[0107] Thus, a typical composition for intravenous administration in the methods of the present invention would be about 0.1 to 10 mg per patient per day. Dosages from 0.1 up to about 100 mg per patient per day may be used. Actual methods for preparing administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as REMINGTON'S PHARMACEUTICAL SCIENCE, 19TH ED., Mack Publishing Company, Easton, Pennsylvania (1995).

[0108] In therapeutic applications, compositions are administered to a patient suffering from a CD22+ cancer in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health. An effective amount of the compound is that which provides either subjective relief of a symptom(s) or an objectively identifiable improvement as noted by the clinician or other qualified observer.
Single or multiple administrations of the compositions are administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the antibody or immunocojugates to effectively treat the patient. As noted, in clinical trials of BL22 and HA22, the immunotoxin is administered periodically. Generally, the dose is chosen to be sufficient to treat or ameliorate symptoms or signs of disease without producing unacceptable toxicity to the patient.

Controlled release parenteral formulations of the antibody or immunoconjugate can be made as implants, oily injections, or as particulate systems. For a broad overview of protein delivery systems see, Banga, AJ., THERAPEUTIC PEPTIDES AND PROTEINS: FORMULATION, PROCESSING, AND DELIVERY SYSTEMS, Technomic Publishing Company, Inc., Lancaster, PA, (1995) incorporated herein by reference. Particulate systems include microspheres, microparticles, microcapsules, nanocapsules, nanospheres, and nanoparticles. Microcapsules contain the therapeutic protein as a central core. In microspheres the therapeutic is dispersed throughout the particle. Particles, microspheres, and microcapsules smaller than about 100 nm are generally referred to as nanoparticles, nanospheres, and nanocapsules, respectively. Capillaries have a diameter of approximately 5-10 micrometers, so that only nanoparticles are administered intravenously. Microparticles are typically around 100 micrometers in diameter and are administered subcutaneously or intramuscularly.


Polymers can be used for ion-controlled release of antibodies or immunoconjugates. Various degradable and nondegradable polymeric matrices for use in controlled drug delivery are known in the art (Langer, R., Accounts Chem. Res. 26:537-542 (1993)). For example, the block copolymer, polaxamer 407 exists as a viscous yet mobile liquid at low temperatures but forms a semisolid gel at body temperature. It has shown to be an effective vehicle for formulation and sustained delivery of recombinant interleukin-2 and urease (Johnston, et al, Pharm. Res. 9:425-434 (1992); and Pec, et al, J. Parent. Sci. Tech. 44(2):58-65 (1990)). Alternatively, hydroxyapatite has been used as a microcarrier for controlled release of proteins (Ijntema, et al, Int. J. Pharm. 112:215-224 (1994)). In yet another aspect, liposomes are used for controlled release as well as drug targeting of the lipid-capsulated drug (Betageri,
et al., LIPOSOME DRUG DELIVERY SYSTEMS, Technomic Publishing Co., Inc., Lancaster, PA (1993)). Numerous additional systems for controlled delivery of therapeutic proteins are known. See, e.g., U.S. Pat. No. 5,055,303, 5,188,837, 4,235,871, 4,501,728, 4,837,028, 4,957,735 and 5,019,369, 5,055,303; 5,514,670; 5,413,797; 5,268,164; 5,004,697; 4,902,505; 5,506,206, 5,271,961; 5,254,342 and 5,534,496, each of which is incorporated herein by reference.

EXAMPLES

Example 1

Materials and Methods

The effects of combining HA22 with the chemotherapeutic agents doxorubicin, taxol, cisplatin, and etoposide were investigated using CA46, a human Burkitt lymphoma cell line. Various drug combinations and ratios were used to evaluate the cytotoxicity of combinations of the agents to the target cells. Drugs were added either (a) simultaneously, for 48-hour and 72-hour assays, or (b) in sequence for 72-hour assays. Sequential additions were performed according to the scheme of adding Drug 1 at 0 hours after plating of cells, Drug 2 at 24 hours, and performing an analysis of cell viability at 72 hours. Cell cytotoxicity efficacy was assessed for the individual drugs and the combinations of drugs using colorimetric viability assays (WST-8).

The combination effect of the drugs was analyzed using a mathematical model based on the median-effect equation of Chou-Talalay (Chou TC, Talalay P, "Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors," Adv Enzyme Regul 22:27-55 (1984)). Median-effect results were expressed as combination index ("CI") values, which are a quantitative measure of the degree of drug interaction. CI values that are < 0.7 indicate drug synergy, values of 1 ± 0.3 indicate additivity, and values > 1.3 indicate antagonism.

Example 2

CA46 cells were sensitive to each of the individual tested cytotoxic drugs. As shown by the drug concentrations required to inhibit cell proliferation by 50% (the "IC₅₀"), the cytotoxic activity of HA22 was at least a thousand-fold higher compared to that of the tested chemotherapeutic agents (Table 1, below).

Table 2 shows the results of assays in which CA46 cells were contacted with a combination of (a) one of several chemotherapeutic agents and (b) HA22 immunotoxin. The
drugs were administered at several combination ratios and concentration levels. The combination index (CI) was calculated for three different drug-effect levels: 50%, 75%, and 90% killing (shown on Table 2 as ED50, ED75, and ED90). CI values < 0.7 indicate drug synergy.

HA22 in combination with each of doxorubicin, taxol, cisplatin, and etoposide demonstrated a synergistic cytotoxicity at various combination ratios in three different administration regimens.

Table 3 shows the results of assays in which (a) HA22 immunotoxin was added to culture medium of CA46 cells at time 0, one of several chemotherapeutic drugs was added to the medium at 24 hours, and cell cytotoxicity was evaluated at 72 hours. CI values were calculated at the 50% drug-effect level. Nearly all the CI values were < 0.7, indicating that the drug combinations were synergistic regardless of whether the HA22 immunotoxin was added before the drug or the drug was added before the immunotoxin.

<table>
<thead>
<tr>
<th>Drug</th>
<th>48-hour Incubation IC50</th>
<th>72-hour Incubation IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA22</td>
<td>3.44 x 10^11</td>
<td>3.16 x 10^12</td>
</tr>
<tr>
<td>Taxol</td>
<td>1.13 x 10^8</td>
<td>1.73 x 10^9</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>9.66 x 10^7</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>3.16 x 10^8</td>
<td>9.89 x 10^7</td>
</tr>
<tr>
<td>Etoposide</td>
<td>1.65 x 10^8</td>
<td>4.04 x 10^7</td>
</tr>
</tbody>
</table>
Table 2  Chou and Talalay's Combination Index (CI) at three effect levels (50%, 75%, and 90% killing) based on WST-8 cell viability assay for coincubations of chemotherapeutic drugs with HA22 on CA46.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Molar ratio HA22:Drug</th>
<th>Incubation Time (hrs)</th>
<th>CI50 (M)</th>
<th>m&lt;sup&gt;a&lt;/sup&gt;</th>
<th>r&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA22 + Taxol</td>
<td>1:2000</td>
<td>48</td>
<td>0.16</td>
<td>0.14</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>1:700</td>
<td>48</td>
<td>0.24</td>
<td>0.16</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>1:2000</td>
<td>72</td>
<td>0.48</td>
<td>0.37</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>1:700</td>
<td>72</td>
<td>0.38</td>
<td>0.30</td>
<td>0.23</td>
</tr>
<tr>
<td>HA22 + Doxorubicin</td>
<td>1:217200</td>
<td>48</td>
<td>0.66</td>
<td>0.92</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>1:81500</td>
<td>48</td>
<td>0.27</td>
<td>0.86</td>
<td>2.77</td>
</tr>
<tr>
<td></td>
<td>1:36200</td>
<td>48</td>
<td>0.41</td>
<td>1.17</td>
<td>3.38</td>
</tr>
<tr>
<td></td>
<td>1:13600</td>
<td>48</td>
<td>0.64</td>
<td>0.96</td>
<td>1.46</td>
</tr>
<tr>
<td>HA22 + Cisplatin</td>
<td>1:1049700</td>
<td>48</td>
<td>0.38</td>
<td>0.24</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>1:209900</td>
<td>48</td>
<td>0.05</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>1:1049700</td>
<td>72</td>
<td>1.44</td>
<td>1.29</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>1:209900</td>
<td>72</td>
<td>0.70</td>
<td>0.45</td>
<td>0.31</td>
</tr>
<tr>
<td>HA22 + Etoposide</td>
<td>1:214100</td>
<td>48</td>
<td>2.08</td>
<td>0.56</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>1:53500</td>
<td>48</td>
<td>2.01</td>
<td>0.26</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>1:214100</td>
<td>72</td>
<td>0.94</td>
<td>0.68</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>1:53500</td>
<td>72</td>
<td>0.80</td>
<td>0.47</td>
<td>0.32</td>
</tr>
</tbody>
</table>

<sup>a</sup> CI < 0.7 indicates drug synergism (bold), CI of 1±0.3 indicates additivity, and CI > 1.3 indicates antagonism.

<sup>b</sup> m is an exponent signifying the sigmoidicity (shape) of the dose-effect curve.

<sup>c</sup> r is the linear correlation coefficient of the median effect plot

Table 3  Combination Index at 50% killing (CI50) for sequential addition of HA22, and then chemotherapeutic drug to CA46 cells:<sup>*</sup>

<table>
<thead>
<tr>
<th>Combination</th>
<th>Molar ratio HA22:drug</th>
<th>HA22, then drug CI50&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Drug, then HA22 CI50&lt;sup&gt;**&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA22 + Taxol</td>
<td>1:2000</td>
<td>0.30</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>1:700</td>
<td>0.47</td>
<td>0.52</td>
</tr>
<tr>
<td>HA22 + Cisplatin</td>
<td>1:1049700</td>
<td>0.62</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>1:209900</td>
<td>0.67</td>
<td>0.20</td>
</tr>
<tr>
<td>HA22 + Etoposide</td>
<td>1:214100</td>
<td>0.58</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>1:53500</td>
<td>0.75</td>
<td>0.35</td>
</tr>
</tbody>
</table>

<sup>*</sup> CI < 0.7 indicates drug synergism (bold), CI of 1±0.3 indicates additivity, and CI > 1.3 indicates antagonism.

<sup>**</sup> Scheme of drug addition: Drug 1 at 0 hours, Drug 2 at 24 hours, and WST-8 analysis at 72 hours.

Example 3

[0118] CA46 cells were injected into severely immunodeficient (SCID) mice. After the tumor cells had been given time to establish themselves, the animals were injected with vehicle (as a control), the chemotherapeutic agent doxorubicin, the anti-CD22 immunotoxin...
HA22, or with HA22 and doxorubicin. Animals administered both agents received doxorubicin on day 1 and received HA22 on days 2, 4, and 6.

**Example 4**

Cells of CA46, a Burkitt’s lymphoma cell line, of two ALL cell lines and of two ALL patient samples, were tested for inhibition of cell proliferation by a number of agents. Table 4 sets forth the drug concentrations required to inhibit cell proliferation by 50% (the "IC<sub>50</sub>") of each of the agents tested. The IC<sub>50</sub> values listed are the mean of multiple replicates, with the standard deviation shown.

A separate but similar series of studies was conducted using the same two ALL cell lines and four ALL patient samples. Table 5 sets forth the drug concentrations required to inhibit cell proliferation by 50% (the "IC<sub>50</sub>") of each of the agents tested. The IC<sub>50</sub> values listed are the mean of multiple replicates, with the standard deviation shown.

**Table 4. Single agent IC<sub>50</sub> values (ng/ml) ± SD of 3 or more determinations. Values for asparaginase are IU/ml.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>CA46</th>
<th>REH</th>
<th>KOPN-8</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT-8015 (HA22)</td>
<td>0.27 ± 0.05</td>
<td>0.35 ± 0.09</td>
<td>0.12 ± 0.02</td>
<td>11 ± 1</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Asparaginase</td>
<td>0.69 ± 0.08</td>
<td>0.14 ± 0.05</td>
<td>0.12 ± 0.04</td>
<td>0.25 ± 0.07</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>&gt;1000</td>
<td>99 ± 15</td>
<td>110 ± 30</td>
<td>345 ± 7</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>77 ± 11</td>
<td>6.5 ± 0.5</td>
<td>6.2 ± 1.6</td>
<td>160 ± 30</td>
<td>250 ± 40</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>10.2 ± 0.7</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.6 ± 0.1</td>
<td>0.20 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>2.8 ± 1.2</td>
<td>0.65 ± 0.07</td>
</tr>
</tbody>
</table>

**Table 5. Single Agent IC<sub>50</sub> values (ng/ml) for CD22+ ALL Cell Lines and Patient Samples**

<table>
<thead>
<tr>
<th></th>
<th>REH</th>
<th>KOPN</th>
<th>Patient 1</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT-8015 (HA22)</td>
<td>0.3</td>
<td>0.07</td>
<td>11</td>
<td>12</td>
<td>30</td>
<td>75</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>11</td>
<td>10</td>
<td>150</td>
<td>600</td>
<td>10.5</td>
<td>300</td>
</tr>
<tr>
<td>CAT-3888 (BL22)</td>
<td>-</td>
<td>-</td>
<td>45</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>110</td>
<td>150</td>
<td>340</td>
<td>&gt;1000</td>
<td>80</td>
<td>900</td>
</tr>
<tr>
<td>L-Asparaginase</td>
<td>2</td>
<td>3</td>
<td>0.0008</td>
<td>0.5</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>&gt;1000</td>
<td>10</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>-</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.2</td>
<td>0.15</td>
<td>1.5</td>
<td>0.7</td>
<td>0.1</td>
<td>-</td>
</tr>
</tbody>
</table>

- = drug not tested or the result / cytotoxicity curve was inconclusive
Example 5

[0121] Figures 2 and 3 show the results of a series of studies contacting cells of the ALL cell line REH (Figure 2) or KOPN-8 (Figure 3) with simultaneous addition of HA22 immunotoxin and one of four chemotherapeutic agents (Figure 2) or one of five chemotherapeutic agents (Figure 3). For ALL cell lines, cells were seeded at 30,000 cells/well and incubated for two days. As shown in Figure 2, surprisingly, the combination of immunotoxin and L-asparaginase not only showed a synergistic effect, but showed a degree of synergy greater than that of two of the other chemotherapeutic agents tested. Similarly, a synergistic effect was observed with the combination of HA22 immunotoxin and L-asparaginase on KOPN-8 cells, as shown in Figure 3. For both Figures, the Combination Index was calculated according to the Chou Talalay mean-effect equation, as described in Example 1, above, and the result obtained was subtracted from 1, which is an art-standard method for determining whether two agents are antagonistic, additive, or synergistic effects.

Example 6

[0122] Figure 4 shows the results of studies in which cells of the ALL cell line REH were contacted with either a pair of chemotherapeutic agents or with both the pair of chemotherapeutic agents and with HA22 immunotoxin. The REH cells were seeded at 30,000 cells/well and incubated for two days prior to testing. As shown in Figure 4, each of the pairs of chemotherapeutic agents including L-asparaginase showed a synergistic effect when used in combination with HA22. The Combination Index was calculated according to the Chou Talalay mean-effect equation, as described in Example 1, above, and the result obtained was subtracted from 1, which is an art-standard method for determining whether administration of two agents results in antagonistic, additive, or synergistic effects. It should be noted that the bars set forth in Figure 4 reflect the calculation (of 1 minus the Combination Index) following this method; they are not merely a graphic depiction of the addition of the immunotoxin to the two chemotherapeutic agents.

Example 7

[0123] A series of studies was conducted on ALL cell lines to observe the effect, if any, of the temporal order in which the immunotoxin and the chemotherapeutic agent were administered. Figure 5A shows the results of studies using the ALL cell line REH, which Figure 5B shows the same studies but using the ALL cell line KOPN-8. In all but the case of the administration of cytarabine to KOPN-8 cells (Figure 5B), better results were obtained when the immunotoxin was administered before the chemotherapeutic agent. For ALL cell
lines, cells were seeded at 30,000 cells/well and incubated for two days. The Y axis of each Figure shows the agents administered, and the order in which they were administered in each pair of experiments, while the X axis shows the result obtained by subtracting from 1 the Combination Index calculated according to the Chou Talalay mean-effect equation, as described in Example 1, above.

[0124] While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

[0125] All publications and patent documents cited herein are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were individually denoted to be incorporated. Citation of various references in this document is not an admission that any particular reference is considered to be "prior art" to the invention.
WHAT IS CLAIMED IS:

1. A method of increasing inhibition of growth of a CD22+-cancer cell, said method comprising contacting said cell with (a) an immunotoxin comprising (i) an anti-CD22 antibody and (ii) a protein synthesis-inhibiting toxin, and then within one week, by (b) a first chemotherapeutic agent, which first chemotherapeutic agent inhibits protein synthesis in said cell.

2. A method of claim 1, wherein said cell is contacted with said immunotoxin and then by said first chemotherapeutic agent within a 96 hour period.

3. A method of claim 1, wherein said cell is contacted with said immunotoxin and then by said first chemotherapeutic agent within a 72 hour period.

4. A method of claim 1, wherein said cell is contacted with said immunotoxin and then by said first chemotherapeutic agent within a 48 hour period.

5. A method of claim 1, wherein said cell is contacted with said immunotoxin and then by said first chemotherapeutic agent within a 24 hour period.

6. A method of claim 1, wherein said cell is concurrently contacted with both said immunotoxin and by said first chemotherapeutic agent.

7. A method of claim 1, wherein said anti-CD22 antibody is a scFv or a dsFv.

8. A method of claim 1, wherein said anti-CD22 antibody is RFB4 or RFB4(dsFv).

9. A method of claim 1, wherein said anti-CD22 antibody has a variable light chain (VL) and a variable heavy chain (VH), wherein CDRs 1-3 of said VL chain, respectively, have the sequences of SEQ ID NOs.: 8-10, the heavy chain (VH) CDRs 1 and 2, respectively, have the sequences of SEQ ID NOs.: 5 and 6, respectively, and CDR3 of the VH chain has a sequence selected from any of SEQ ID NO.: 11-14.

10. A method of claim 9, wherein said VH CDR3 has the sequence of SEQ ID NO.: 11.
11. A method of claim 1, wherein wherein said anti-CD22 antibody has a variable light chain (VL) and a variable heavy chain (VH), wherein CDRs 1-3, respectively, of said VH chain, have the sequences of SEQ ID NOs.:5-7, the VL CDRs 2 and 3 respectively, have the sequences of SEQ ID NOs.:9 and 10, respectively, and CDR1 of the VL chain has a sequence selected from any of SEQ ID NO.:15-18.

12. A method of claim 1, wherein said anti-CD22 antibody has a variable light chain (VL) and a variable heavy chain (VH), each with three complementarity determining regions (CDRs) wherein

(a) CDRs 1-2, respectively, of said VH chain have the sequences of SEQ ID NOs.:5 and 6, respectively, and CDR3 has an amino acid sequence selected from any of SEQ ID NO.:11-14,

(b) VL CDRs 2 and 3 respectively, have the sequences of SEQ ID NOs.:9 and 10, respectively, and

(c) VL CDR1 has a sequence selected from any of SEQ ID NO.:15-18.

13. A method of claim 12, wherein said VH CDR3 has the sequence of SEQ ID NO.:11.

14. A method of claim 1, wherein said anti-CD22 antibody is humanized.

15. A method of claim 1, wherein said anti-CD22 antibody is human.

16. A method of claim 1, wherein said chemotherapeutic agent is L-asparaginase.

17. A method of claim 17, wherein said L-asparaginase is an E. coli or Erwinia carotovora L-asparaginase.

18. A method of claim 1, wherein said cell is further contacted by a second chemotherapeutic agent.

19. A method of claim 1, wherein said protein synthesis-inhibiting toxin is a Pseudomonas exotoxin A (PE).

20. A method of claim 19, wherein said PE is PE38.
VL
DIQMTQTTSSLASLGDRTVT

gatatcagagacagagcacatataccactcctcgctctggacagcagactcacc

ISCRASQDISNYLNWYYQK

P

attagttgcaggccagctcggacattttataatatattaactgtatctcagcagaacca.

DGTVKLLILYIYTSILHSGVPS

gatgggacgtttaaacctctgtatctatctaacatacatcaatattgcaactcctggagctactca

RFSGSGGTGTDYSLTISNLQE

aggttcagtggcagtgggtctctggaactattatctctcacattagcaacacttgtgaggacaa

EDFATYFCQQGNTLTPHTFGG

gaagatttttgcaccatctattttgcccacagctctacgtctccggtgacgttggtgga

GTKLEIK

ggcaccaagcgtggaatcaca

VH
EVLVESGGGLVKPGGSGLKL

gaaagtcacgtgtggagctgtgtggaggtgctcttgacacagtctgaggtcctctgaacctc

SCAAASGFASISLYDMSWVRQT

tctgtgcagcctgctggtctctgtatgattgtcttctgtcttggttcctgcagact

FEKRLEWVAYISSGTYY

cggagagagagaggtggtcgcctcaacattattattattattattggttgctacacacctat

PDTVKGRFTISRNKNTLY

ccoagcacttgtgagggcgattttcttcagagacaattgcaacactggtactgtac

LQMSSLKSEDTAMYYCARH

ctgcaatggagacagtctgtgattttctttcttgacacagtattgtaaaaagactagtgt

GYGSYGYVLFAYWQGTLVT

FIG. 1
Synergy in HA22 Combinations with Chemotherapeutic Agents: ALL cell line REH

FIG. 2
Synergy and/or Additivity in HA22 Combinations Against ALL cell line KOPN-8

FIG. 3
Addition of HA22 to Paired Chemo Drugs Further Enhances Synergy in REH cells

FIG. 4
Sequence-Specific Synergy in HA22 Combinations against ALL cell lines

Figure 5. Comparison of combination index values for two schemes of sequential drug addition in REH (A) and KOPN-8 (B). Drugs were sequentially added for two day incubations. The value of [1-CI] is shown for each combination, such that [1-CI] < -0.3 indicates drug antagonism, [1-CI] between -0.3 and 0.3 indicates drug additivity, and [1-CI] > 0.3 indicates drug synergism. Each CI value is the mean ± standard deviation of three independent determinations.

FIG. 5