ANTIBODY-TARGETED CHEMOTHERAPY WITH CMC-544:
A CD22-TARGETED IMMUNOCONJUGATE OF CALICHEAMICIN FOR THE
TREATMENT OF B LYMPHOID MALIGNANCIES

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ABSTRACT:

Antibody-targeted chemotherapy with gemtuzumab ozogamicin (Mylotarg® or CMA-676, a CD33-targeted immunoconjugate of N-acetyl gamma calicheamicin dimethyl hydrazide (CalichDMH, a potent DNA-binding cytotoxic anti-tumor antibiotic) is a clinically validated therapeutic option for AML patients. Here, we describe the preclinical profile of another immunoconjugate of CalichDMH, CMC-544, targeted to CD22 expressed by B lymphoid malignancies. CMC-544 is comprised of a humanized IgG4 anti-CD22 mAb, G5/44, covalently linked to CalichDMH via an acid-labile AcBut linker. Both CMC-544 and unconjugated G5/44 bound human CD22 with subnanomolar affinity. CMC-544, but not unconjugated G5/44, exerted potent cytotoxicity against CD22+ B-cell lymphoma (BCL) cell lines (IC₅₀: 6 to 600 pM CalichDMH). CMC-544 caused a potent inhibition of growth of small but established BCL xenografts leading to cures (therapeutic index >10). CMC-544 prevented the establishment of BCL xenografts and also caused regression of large BCLs (>1.5 g of tumor mass). In contrast, unconjugated CalichDMH, unconjugated G5/44 and an isotype-matched control conjugate, CMA-676, were ineffective against these BCL xenografts. Thus, CD22-targeted delivery of CalichDMH is a potent and effective preclinical therapeutic strategy for BCLs. The strong anti-tumor profile of CMC-544 supports its clinical evaluation as a treatment option for B lymphoid malignancies.

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INTRODUCTION

Antibody-targeted chemotherapy is a therapeutic strategy that involves the use of a cytotoxic agent chemically linked to a monoclonal antibody (mAb) that specifically recognizes a tumor-associated antigen. The mAb specifically delivers the cytotoxic agent to tumor cells that express the tumor-associated antigen. The efficacy of antibody-targeted chemotherapy relies heavily on the specific binding of the targeting mAb-drug conjugate to the tumor antigen and on the internalization of the antigen-mAb complex to ensure focused delivery of the conjugated cytotoxic agent inside tumor cells. Such focused delivery of the cytotoxic agent to tumor cells maximizes its anti-tumor effect and minimizes its normal tissue exposure resulting in an improved therapeutic index. The concept of antibody-targeted chemotherapy is not novel and has been explored extensively with significant preclinical success. However, it had not been successfully translated into clinical benefit until the recent introduction of gemtuzumab ozogamicin (Mylotarg® or CMA-676), the first antibody-targeted chemotherapeutic. Presently, gemtuzumab ozogamicin remains the only antibody-targeted chemotherapeutic agent approved for clinical use in the US.

Gemtuzumab ozogamicin is comprised of a humanized IgG4 anti-CD33 antibody covalently linked to N-acetyl gamma calicheamicin dimethyl hydrazone (hereafter referred to as CalichDMH), a derivative of gamma calicheamicin, via an acid-hydrolysable AcBut linker. Gamma calicheamicin is a potent cytotoxic anti-tumor antibiotic that binds DNA in the minor groove and undergoes thiol-dependent structural changes in its enediyne moiety to generate a di-radical which abstracts hydrogens from the phosphodiester backbone of DNA. This causes double-strand DNA breaks resulting in apoptotic cell death. Gemtuzumab ozogamicin binds CD33 on the surface of myeloid cells and is rapidly internalized into lysosomal vesicles whose acidic pH facilitates hydrolysis of the hydrazone functional group within the AcBut linker liberating CalichDMH. Gemtuzumab ozogamicin is indicated for the treatment of elderly (≥ 60 years of age) acute myeloid leukemia (AML) patients in first relapse who are not candidates for other therapies. Its effectiveness in patients with AML can be attributed to the preferential delivery of CalichDMH to CD33+ myeloid leukemic cells.
resulting in their elimination. CD33, the molecular target of gemtuzumab ozogamicin, is a cell-surface antigen whose expression is confined to the myeloid lineage. CD33 is expressed by both normal and malignant myeloid cells but is not expressed by nonhematopoietic lineages or stem cell precursors of the myeloid lineage. The clinical effectiveness of gemtuzumab ozogamicin provides a strong rationale for applying the antibody-targeted calicheamicin strategy to other malignancies using target antigens with similar lineage-confined expression.

CD22 is an attractive target for antibody-targeted chemotherapy and has been extensively explored as a target for immunotoxin-based therapeutic strategies. CD22 is a terminal α2,6-linked sialic acid-binding cellular lectin and a member of the Ig superfamily. CD22 is expressed on the surface of mature B-lymphocytes and their malignant counterparts but not on other non-B lineages including hematopoietic stem cells. CD22 is rapidly internalized upon binding to anti-CD22 mAb. These properties make CD22 a suitable molecular target for antibody targeted calicheamicin therapy for B lymphoid malignancies. Using a humanized anti-CD22 mAb (G5/44), we have created a CD22-targeted immunoconjugate of CalichDMH, designated as CMC-544. The present study describes the functional characterization of CMC-544 and provides evidence of its strong preclinical anti-tumor activity against human B-cell lymphoma (BCL) cell lines, both in vitro and in vivo. This study supports the evaluation of CMC-544 as an effective therapeutic option for patients with BCLs, especially non-Hodgkin’s lymphoma.
MATERIALS AND METHODS

Antibodies

Humanized anti-CD22 (G5/44, IgG4 isotype) was derived from the murine anti-CD22 mAb m5/44 by grafting the complementarity-determining regions (CDR) plus key framework residues on to human acceptor frameworks at Celltech R&D (Slough, UK) (manuscript in preparation). Antibody G5/44 was expressed in Chinese hamster ovary cells and purified at Wyeth Biopharma (Andover, MA). G5/44 was covalently linked to N-acetyl gamma calicheamicin dimethyl hydrazide (CalichDMH) via an acid-labile AcBut [4-(40-acetylphenoxy) butanoic acid] linker as described and the resulting conjugate was designated as CMC-544. Figure 1 shows a structural representation of CMC-544, which has an average loading of 65-80 µg of CalichDMH/mg of antibody protein (5 to 7 moles of CalichDMH per mole of antibody) and <10% of the antibody protein in CMC-544 is un conjugated. This conjugate preparation contains <1 µg of unconjugated CalichDMH/mg of conjugated antibody. CMA-676 (Mylotarg® or gemtuzumab ozogamicin) is a conjugate of humanized IgG4 anti-human CD33 mAb, hP67.6, covalently linked to CalichDMH via the AcBut linker. All conjugates were determined to be endotoxin free (< 5.0 EU/ml) by a modified limulus amoebocyte assay (Biowittaker, Walkersville, MD). Rituximab, a chimeric IgG1 anti-CD20 mAb (Rituxan®), was obtained from Med World Pharmacy (Chestnut Ridge, NY). Purified human IgG4 and IgG1 were obtained from Sigma Chem. Co. (St Louis, MO). A fusion protein consisting of the extracellular domain of human CD22 genetically fused to the hinge-CH2-CH3 Fc region of murine IgG1 (CD22mFc) was expressed in NS0 cells and purified at Celltech (Slough, UK). Fluorescein-conjugated affinity-purified polyclonal goat anti-human IgG (heavy and light chain specific) antibody was obtained from Zymed (South San Francisco, CA).
Cell lines

Burkitt’s lymphoma cell lines Ramos (CRL-1923), Raji (CCL-86), Daudi (CCL-213), a non-Hodgkin’s lymphoma cell line RL (CRL-2261) and a myeloid leukemic cell line HL-60 (CCL-240) were all obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell lines were determined to be mycoplasma free by a polymerase chain reaction mycoplasma detection assay (ATCC). The cell lines, Ramos, Raji, Daudi and RL were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 0.2% glucose, penicillin G sodium 100 U/ml, and streptomycin sulfate 100 µg/ml. HL-60 cells were propagated and maintained in Iscove’s DMEM plus 20% FBS, 0.02% glucose, penicillin G sodium 100 U/ml, and streptomycin sulfate 100 µg/ml.

Mice

Female, athymic BALB/c nu/nu (nude) mice (18-23 g) mice were obtained from Charles River, Wilmington, MA. All mice were provided with food and water ad libitum throughout the studies. All experimental procedures involving mice were carried out in a laminar flow hood and were approved by the Wyeth Pearl River Animal Care and Use Committee according to the established guidelines.

Biosensor analysis

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 [10 mM HEPES, pH 7.4 containing 150 mM NaCl, 3 mM EDTA and 0.005% polysorbate 20 (v/v)] and injected in the concentration range of 1 to 100 nM over the CD22mFc-coated biosensor chip surface at a flow rate of 30 µl/min for 3 min to allow binding. After the binding phase, dissociation of the bound antibody was monitored by washing the chip
with the HBS buffer over a 15 min period. The antigenic surface was regenerated by washing the biosensor chip with 15 µl of the regeneration buffer (10 mM NaOH and 200 mM NaCl) for 30 s, followed by a stabilization time of 2 min before the next cycle. Kinetic constants were calculated by nonlinear least square regression analysis using a 1:1 Langmuir binding curve fitting model and BIAevaluation program (version 3.0, BIAcore).²⁶

Cytotoxicity assays

Various BCL cells were cultured in the presence of various concentrations of unconjugated CalichDMH, unconjugated antibody (G5/44), CMC-544 or CMA-676 for 96 hr after which cell viability was measured by propidium iodide exclusion and analyzed by flow cytometry using a Becton Dickinson FACSort. Red fluorescent intensity (emission at 617 nm in the FL2 channel) of the cells excited at 488 nm was measured. The regions for viable cells were also set using both the forward light scatter and right-angle light scatter properties of the cells. The loss of viability was determined by the loss of cells from within the gated region defining viable cells. The average number of viable cells per 6 replicate cultures was calculated. The percent survival of BCL cells in these cultures was calculated using the following equation: Percent Survival = 100 x (# of viable cells in treated cultures/# of viable cells in control cultures). IC₅₀s (with 95% confidence intervals) were calculated by logistic non-linear regression and are reported as the amount of CalichDMH equivalents from each treatment group that causes 50% loss of cell viability.

Subcutaneous xenografts

Female, athymic nude mice were exposed to total body irradiation (400 rads) to further suppress their residual immune system and facilitate the establishment of BCL xenografts. Three days later, the irradiated mice were injected subcutaneously (SC) with 1x10⁷ Ramos or RL cells in Matrigel (Collaborative Biomedical Products, Belford, MA, diluted 1:1 in RPMI 1640 medium) in the dorsal, left flank. When the tumors reached the mass of 0.3 to 0.4 g, the tumors were staged to ensure uniformity of the
tumor mass between various treatment groups (n=7-9 mice/group) prior to the administration of therapy. Conjugated CalichDMH (CMC-544 or CMA-676), unconjugated CalichDMH or unconjugated antibody (G5/44) was administered intraperitoneally (IP) in sterile saline (0.2 ml/mouse) on Day 1 and the same treatment was repeated twice four days apart (Q4Dx3). Dosages of CMC-544 and CMA-676 were based on the quantity of CalichDMH. In additional studies, the effect of CMC-544 or G5/44 on the development of the BCL was also examined. In these studies, treatments were administered 3 days after the BCL were injected SC. Tumors were measured at least once a week and their mass was defined as tumor mass (g) = 0.5 x (tumor width^2)(tumor length). Mean tumor mass (± SEM) for each treatment group was calculated and compared to the vehicle-treated group for statistical significance using a one-sided t-test, with the error term for the t-test based on the pooled variance across all treatment groups. Tumor mass values for each treatment group were recorded up to 100 days after the initiation of treatment or until either tumor-bearing mice died or the tumors grew to 15% of the body weight at which time these mice were euthanized according to institutional regulations. The number of tumor-free mice at the end of each study for each treatment group was also recorded. The minimum efficacious dose (MED) of CMC-544 was the lowest dose of CMC-544 that caused a statistically significant inhibition of tumor growth (p<0.05) at any given time point compared to the vehicle-treated group.

In order to determine the maximum nonlethal dose (MND) of CMC-544, non-irradiated, non-tumor-bearing mice (n=10) were administered IP Q4Dx3 with increasing doses of CMC-544 and monitored for 50 days. The MND was the highest dose of CMC-544 at which 100% of the treated mice survived the observation period. The therapeutic index (TI) was calculated as the ratio of the MND/MED. The minimum curative dose (MCD) was the lowest dose of CMC-544 at which no residual tumor mass could be detected and the mice remained tumor-free for at least 100 days after initiation of the treatment. The curative index was calculated as the ratio of MND/ MCD. ED_{90} is the dose of CMC-544 (Q4Dx3) at which 90% inhibition of average tumor growth is observed relative to the vehicle-treated average tumor growth (with 95 % confidence
intervals) and was estimated using inverse prediction after fitting a logistic model to relative tumor growth data from three independent experiments using RL BCL xenografts.

Pharmacokinetics of CMC-544 in tumor-bearing and non-tumor-bearing mice

RL BCL-bearing nude mice (SC tumors) and non-tumor bearing nude mice (n=52/group) were administered a single IP dose of CMC-544 (160 µg of conjugated CalichDMH/kg) at time 0. Blood samples were collected terminally by cardiac puncture under CO₂ anesthesia from these mice (n=4 mice/time point) using a non-serial bleeding design at 0.1, 0.5, 1, 4, 8, 24, 48, 72, 96, 120, 168, 240 and 336 hr post-dosing. Serum from these blood samples was collected and stored frozen at -70°C. Serum concentrations of CMC-544 were quantified using a modification of an enzyme-linked immunosorbent assay (ELISA) originally developed for the pharmacokinetic measurements of CMA-676. A soluble form of the CD22 protein, CD22mFc, was immobilized on microtiter plates to capture the G5/44 antibody portion within CMC-544. The CalichDMH portion of the molecule was then recognized with a rabbit anti-calicheamicin antibody. A goat anti-rabbit antibody conjugated to horseradish peroxidase was used to detect the bound rabbit antibody using 3,3',5,5' tetramethylbenzidine (TMB) as a substrate for colorimetric readout. CMC-544 diluted in nude mouse serum was used as a standard in these determinations. The pharmacokinetic parameters for each dose group were estimated using the pharmacokinetic and statistical analysis software, IBIS (version 1.5.0) that runs under SAS (version 6.12). Calculations were performed using a model-independent approach as described by Gibaldi and Perrier. Briefly, C_max and t_max values were taken directly from the observed mean data. The area under the serum concentration versus time curve (AUC) over the sampling period of 336 hours and its standard error were calculated using a method described by French and Powers that is based upon a rearrangement of the linear trapezoidal rule.
RESULTS

Binding of CMC-544 to CD22

The binding of CMC-544 to human CD22 was evaluated by surface plasmon resonance analysis using CD22mFc covalently immobilized on a biosensor chip. The results of kinetic analyses of the binding of CMC-544 and G5/44 to CD22mFc are shown in Figure 2. The data were fitted globally to a 1:1 Langmuir binding model with compensation for mass transfer. Both CMC-544 and unconjugated G5/44 bound CD22 with a similar affinity (CMC-544:CD22 $K_D = 200$ pM; G5/44:CD22 $K_D = 235$ pM). Conjugation to CalichDMH did not impact the ability of G5/44 to bind CD22mFc effectively. The binding of CMC-544 and G5/44 to CD22 expressed on the surface of human B lymphoma cells was further confirmed by flow cytometry. CMC-544 specifically recognizes CD22 on human B cells but not on murine, rat, canine, porcine or primate (cynomolgus and rhesus) B cells (data not shown).

In Vitro effects of CMC-544 against human B lymphoma cells

The effect of CMC-544 on the in vitro growth of various CD22$^+$ B lymphoma cell lines was determined. CD33-targeted CMA-676 was used as an isotype-matched non-binding control conjugate to explore antigen-nonspecific effects of the conjugates. CalichDMH was also included for comparison in these studies. The use of unconjugated CalichDMH indicated sensitivity of each cell line to this drug. Table 1 shows results of the cytotoxic effect ($IC_{50}$ based on the CalichDMH equivalents defined as the concentration of conjugated CalichDMH that causes 50% reduction in the cell number compared to untreated controls) of CMC-544 and CMA-676. CMC-544 was more potent (1.5 to 39 fold) against CD22$^+$ B lymphoma cells (Ramos, Raji, and RL) than unconjugated CalichDMH. The cytotoxic effect of the isotype-matched control conjugate (CMA-676) against CD22$^+$ B lymphoma cells was less than that of unconjugated CalichDMH. Unconjugated G5/44 mAb up to 1 $\mu$M (antibody protein) had no effect on the in vitro growth of any of the B lymphoma cell lines studied. Against the CD22$^-$ CD33$^+$ leukemic cell line HL-60, the relative potencies of CD22-targeted CMC-
544 and CD33-targeted CMA-676 were reversed. CMA-676 was at least 12 fold more potent than CMC-544 against HL-60 cells. The antigen-nonspecific effects of CMC-544 and CMA-676 can be explained by the hydrolytic release of CalichDMH from its conjugated state by the acidification of the culture medium of actively cycling cells. These results demonstrate that CMC-544 exerts CD22-specific cytotoxic effect with subnanomolar potency against CD22⁺ B lymphoma cells. Furthermore, the observed greater cytotoxic potency of CMC-544 over unconjugated CalichDMH is indicative of the efficiency of CD22-mediated intracellular delivery of CalichDMH over its untargeted transport through the cell membrane.

Anti-tumor efficacy of CMC-544 against established B lymphoma xenografts

CMC-544 was evaluated for its anti-tumor activity against established SC xenografts of CD22⁺ Ramos or RL human BCL. The tumor-bearing mice were staged to an average tumor mass of 300 mg (established, small SC tumors). CMC-544 was administered at 10 to 320 µg of conjugated CalichDMH/kg of body weight (0.15 to 5 mg/kg of conjugated antibody protein) IP on Days 1, 5, and 9 (Q4Dx3). CMA-676 was similarly administered as a negative control conjugate. This dosing schedule has been shown to be effective for immunoconjugates of CalichDMH in a number of preclinical tumor models.⁷,⁸,³⁰ CMC-544 inhibited the SC growth of Ramos BCL in a dose-dependent manner (Figure 3). The lowest dose of CMC-544 causing significant (p<0.05) growth inhibition (MED) of Ramos BCL xenografts was 10 µg conjugated CalichDMH/kg. CMC-544, administered IP Q4Dx3 at 160 µg of conjugated CalichDMH/kg, caused complete regression of Ramos BCL xenografts and 6 out of 7 mice in this treatment group remained tumor-free up to 100 days and were considered cured. In a separate experiment (data not shown), CMC-544 at the dose of 120 µg conjugated CalichDMH/kg (MCD) also cured Ramos xenografted mice. In contrast, CMA-676 (isotype-matched non-binding control conjugate), at 160 µg/kg (administered IP Q4Dx3) had no significant effect on the growth of Ramos BCL xenografts.

The ability of CMC-544 to inhibit the establishment of developing Ramos BCL xenografts was examined. Ramos cells were injected SC and 3 days later IP treatment
(Q4Dx3) with CMC-544 was initiated. CMC-544 completely suppressed the establishment of the developing Ramos BCL (Figure 4A). The effect of CMC-544 was also studied with large (almost 10% of the body weight) Ramos BCL xenografts. Ramos xenografts were staged at 2 g of tumor mass after which treatment with CMC-544 was initiated. CMA-676 was again used as a negative control conjugate. In the CMA-676 treatment group, the tumors grew rapidly and within less than 2-weeks post-initiation of therapy, these mice had to be euthanized. In contrast, similar treatment with CMC-544 caused almost complete regression of lymphoma xenografts (Figure 4B). Monitoring these tumor-freed mice for up to 50 days did not demonstrate any re-growth of the Ramos BCL. Thus, CMC-544 not only inhibited the establishment of BCL xenografts but also caused precipitous regression of large BCL xenografts.

A similar evaluation of the anti-tumor effect of CMC-544 was carried out using the RL BCL xenograft model. CMC-544, in a dose-dependent manner, caused RL xenografts to regress within 3 weeks. In contrast, neither unconjugated CalichDMH nor a mixture of unconjugated CalichDMH and unconjugated G5/44 had any effect on the growth of RL BCL xenografts. The MED of CMC-544 in the RL lymphoma model was 20 μg of conjugated CalichDMH/kg (Figure 5). Regression analysis of the dose response of CMC-544 from this and additional studies not shown in Figure 6 indicated the ED90 to be 53 μg of conjugated CalichDMH/kg (95% CI: 19.6 –137.6). The MCD of CMC-544 in the RL BCL model was 160 μg/kg. CMC-544, administered Q4Dx3 via either the IP or IV route had almost identical anti-tumor activity irrespective of the dose of the conjugate (data not shown). Taken together, these results obtained with two distinct BCL xenograft models clearly demonstrate the ability of CMC-544 to cause regression of developing as well as established BCL tumors.

The effect of unconjugated anti-CD22 mAb G5/44 on the growth of developing or established RL BCL was also investigated. Administration of G5/44 at a dose as high as 20 mg/kg IP did not influence the growth of developing (Figure 6A) or established (Figure 6B) RL BCL xenografts. The dose of 20 mg/kg of G5/44 was selected based on the ability of anti-CD20 rituximab to inhibit the establishment of RL BCL at this dose (data not shown). In contrast, CMC-544, administered IP at a suboptimal dose of 80 μg
of conjugated CalichDMH/kg Q4Dx3, suppressed the growth of small RL xenografts. Large RL BCL xenografts, which had grown in spite of the high dose treatment with unconjugated G5/44, were further treated with CMC-544 and regressed in response to the CMC-544 treatment (Figure 6B). Thus in the absence of CalichDMH, the unconjugated anti-CD22 mAb G5/44 had no impact on the growth of BCL xenografts and these G5/44-treated BCLs were still susceptible to CMC 544.

Pharmacokinetics of CMC-544 in nude mice

To determine the serum levels of CMC-544 that were associated with the anti-tumor effects described above, tumor-bearing (SC RL B lymphoma) or non-tumor-bearing nude mice received a single dose of CMC-544 IP at 160 µg of conjugated CalichDMH/kg and serum samples from these mice were assayed for the presence of CMC-544 using an enzyme-linked immunoassay. The immunoassay was specifically designed and validated to detect antibody conjugates of CalichDMH.27 As shown in Figure 7, the mean (± SE) Cmax values for non-tumor-bearing and RL BCL-bearing nude mice, respectively, were 2.6 ± 0.1 and 2.4 ± 0.2 µg/ml (CalichDMH equivalents of CMC-544), the t1/2 were 34.2 and 35 h, and the corresponding mean AUC0-∞ values were 145 and 93 µg·hr/ml in non-tumor-bearing and RL BCL-bearing mice, respectively. The difference between the AUC0-∞ values for non-tumor-bearing mice and RL BCL-bearing mice was statistically significant (p<0.05). The single dose of CMC-544 administered in this study prevented the growth of the existing RL BCL mass for 9 days after which tumor measurements were not taken. The lower systemic exposure (AUC0-∞) of CMC-544 in tumor-bearing mice than that in non-tumor-bearing mice is consistent with the uptake of CMC-544 by the tumor.

Determination of maximum non-lethal dose of CMC-544 in BALB/c nude mice

In order to determine the maximum non-lethal dose (MND) of CMC-544, non-tumor-bearing nude mice (10 animals/group) were administered CMC-544 (80-640 µg of conjugated CalichDMH/kg IP Q4Dx3) and their survival was monitored daily for up to 50 days. All mice receiving CMC-544 at doses ≤ 240 µg of conjugated CalichDMH/kg
survived the entire period of evaluation (Table 2). However, at the next higher dose of 320 µg/kg of CMC-544, only 4 out of 10 mice were alive by Day 50. Thus, the dose of 240 µg/kg of CMC-544 was regarded as the MND of CMC-544. It is interesting to note that the dose of 320 µg/kg, which was the LD50 in non-tumor-bearing mice, was curative and non-lethal in RL B lymphoma-bearing mice. This observation is consistent with the reduced systemic exposure of tumor-bearing mice versus non-tumor-bearing mice as noted above. CMC-544 demonstrates strong anti-tumor activity against BCL xenografts with therapeutic indices (MND/MED) of 24 and 12 in Ramos and RL xenograft models, respectively, and curative indices (MND/MCD) of 2 and 1.5 in Ramos and RL models, respectively.

Collectively, these results suggest that CMC-544 not only prevents the establishment of subcutaneous BCL xenografts but also causes regression of established BCL xenografts at doses lower than its maximum nonlethal dose.
DISCUSSION

Antibody-targeted chemotherapy is an emerging therapeutic strategy that preferentially delivers the cytotoxic agent to tumor cells and minimizes the exposure of normal tissues resulting in an improved therapeutic index. CMC-544 represents a new therapeutic opportunity for CD22-targeted delivery of calicheamicin to various B-cell malignancies. CD22 was chosen as a target for conjugate delivery for several reasons. CD22 is preferentially expressed on both normal and malignant cells of the mature B lymphocyte lineage. It is not expressed on non-B lymphoid cells, myeloid cells, hematopoietic stem cells, hematopoietic precursors of B lymphocytes or any other non-hematopoietic lineage. CD22-targeted chemotherapy is not expected to affect specifically any tissue not expressing CD22 and it should not inhibit the generation of new B cells from their hematopoietic progenitors. Moreover, CD22 is one of the better internalizing molecules expressed on the surface of mature and malignant B cells and is not routinely shed into the extracellular environment. The preclinical anti-tumor activity of a number of CD22-targeted immunotoxins prepared with various xenotoxins has been reviewed. Of interest in this context is a recent clinical study by Kreitman et al. in which a recombinant immunotoxin (BL22 targeted to CD22) was shown to cause complete remission of 68% of patients with chemorefractory hairy-cell leukemia, thus further validating the choice of CD22 as a targeting antigen.

CMC-544 bound CD22 with subnanomolar affinity and exhibited a potent, dose-dependent cytotoxicity against CD22+ B lymphoma cells. The CD22-mediated intracellular delivery of CalichDMH was more effective in exerting cytotoxic activity than the untargeted uptake of CalichDMH. This enhanced potency can be attributed to the efficient internalization and subsequent intracellular delivery of the CD22-bound CMC-544. In contrast, unconjugated G5/44 antibody had no effect on the growth of various BCLs, both in vitro and in vivo. CMC-544 caused a dose-dependent regression of BCL xenografts grown as subcutaneous solid tumors in athymic nude mice. It prevented the development of BCL growth and was also effective in causing regression of pre-existing small and large established BCLs. The growth of established BCLs was inhibited by CMC-544 at a dose less than 1/10th the MND (240 µg/kg) and cured tumor-bearing mice.
of the tumor at half the MND. The anti-tumor effect of CMC-544 requires that both the targeting antibody and CalichDMH are chemically linked to each other. Neither unconjugated G5/44, unconjugated CalichDMH nor their admixture had any effect on the BCL growth in vivo. Not surprisingly, in the absence of the targeting moiety, unconjugated CalichDMH lacked anti-tumor activity at the dose evaluated, further illustrating the advantage of tumor-targeted therapy. Consequently, we conclude that the effectiveness of CMC-544 observed in vivo in this study was a direct result of the specific and targeted delivery of CalichDMH to tumor cells.

Unconjugated G5/44, an IgG4 isotype anti-CD22 antibody, did not inhibit the establishment of BCL xenografts nor their subsequent growth. This lack of effect of G5/44 in vivo is consistent with its inability to inhibit the growth of BCL in vitro. Human IgG4 antibodies fix complement poorly, are poor binders to Fc receptors and, thus cannot support complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). The lack of anti-tumor effect of G5/44 in the BCL xenograft models was not due to the inability of murine FcR to mediate ADCC or murine complement to mediate CDC with human(ized) IgG antibodies nor was the lack of activity by G5/44 due to the resistance of the B lymphoma cells toward ADCC or CDC since rituximab, an anti-CD20 chimeric mAb, at a dose of 20 mg/kg inhibited the establishment of RL BCL xenografts in nude mice (data not shown). Rituximab is a chimeric mAb with human IgG1 constant region that can mediate both ADCC and CDC and the anti-tumor activity of Rituximab is consistent with these capabilities. Another CD22-targeted humanized IgG1 antibody, epratuzumab, has also been shown to mediate both ADCC and CDC. The clinical activity of both rituximab and epratuzumab is consistent with their ability to mediate both these activities. Since the targeting antibody in CMC-544 lacks these effector capabilities, CMC-544 should not be regarded as an immunotherapeutic agent but rather a CD22-targeted chemotherapeutic agent.

When serum CMC-544 levels were analyzed, the $C_{\text{max}}$ of CMC-544 was approximately 2.5 µg of CalichDMH/ml of serum (1.67 µM) in both tumor- and non-tumor bearing mice. The IC$_{50}$s calculated in vitro for CMC-544’s cytotoxic activity
against a series of B lymphoma cells ranged from 0.009 to 0.9 ng of CalichDMH equivalents/ml (6 to 600 pM). Therefore, the maximum serum level of CMC-544 (after a single IP dose) was substantially higher than that needed to produce cytotoxic effects in vitro. Given the systemic t1/2 of 35 h for CMC-544, the dosing regimen of Q4Dx3 ensures that a constant and efficacious systemic exposure of CMC-544 is maintained over a 2-week period resulting in tumor-regression. The AUC values of CMC-544 in tumor-bearing mice were 37% lower (p<0.05) than in non-tumor bearing mice suggesting that the conjugate can be targeted to and absorbed by the tumor, thereby decreasing serum levels of CMC-544 in tumor-bearing mice. The t1/2 of CMC-544 in tumor-bearing and non-tumor-bearing nude mice was similar.

A number of B-cell - targeted immunotoxins have been evaluated as a therapy for BCL, both preclinically and clinically and, while objective clinical responses were observed, dose-limiting toxicities of these agents such as vascular leak syndrome have precluded their broader clinical use. An additional limiting feature of the immunotoxins is their immunogenicity in man. Patients treated with these agents developed antibodies that negatively impacted the pharmacokinetics of these agents and may have neutralized their anti-tumor activity. Clinical experience with gemtuzumab ozogamicin has demonstrated that it is relatively nonimmunogenic in humans. No antibody response was detected to either the targeting antibody nor the CalichDMH-linker component of gemtuzumab ozogamicin in phase II clinical trials involving >200 patients. Based on the clinical experience with gemtuzumab ozogamicin, CMC-544 is likely to be poorly immunogenic in man.

In summary, CMC-544, as a potent antibody-targeted chemotherapeutic agent, shows promising activity in preclinical models of BCL and may provide a strong anti-tumor therapeutic advantage in man. CMC-544 should be regarded as a targeted chemotherapeutic agent and not an immunotherapeutic agent, and its safety profile should be compared with that of chemotherapeutic agents currently used in the treatment of B-cell malignancies. While some toxicity may be observed with CMC-544 due to the potency of CalichDMH, its targeted delivery is expected to result in less severe toxicity than the non-targeted systemic delivery of currently used cytotoxic
combination chemotherapy. How extensively this agent can be used in a clinical setting will be guided largely by its clinical safety and efficacy profile. CMC-544 is currently being evaluated as a targeted chemotherapeutic for non-Hodgkin’s B-cell lymphoma patients in a multicenter phase I clinical trial.
References


26) BIAevaluation version 3.0. Software handbook. 1997; BIAcore AB.


Table 1. Cytotoxic activity of calicheamicin immunoconjugates against human B lymphoma cell lines in vitro a

<table>
<thead>
<tr>
<th>Human tumor cell line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; = pM (CalichDMH equivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conjugated CalichDMH</td>
</tr>
<tr>
<td></td>
<td>CD22-targeted</td>
</tr>
<tr>
<td></td>
<td>CD33-targeted</td>
</tr>
<tr>
<td></td>
<td>Unconjugated</td>
</tr>
<tr>
<td></td>
<td>CMC-544</td>
</tr>
<tr>
<td></td>
<td>CMA-676</td>
</tr>
<tr>
<td></td>
<td>untargeted CalichDMH</td>
</tr>
<tr>
<td>CD22&lt;sup&gt;+&lt;/sup&gt; RL</td>
<td>6</td>
</tr>
<tr>
<td>CD22&lt;sup&gt;+&lt;/sup&gt; Daudi</td>
<td>21</td>
</tr>
<tr>
<td>CD22&lt;sup&gt;+&lt;/sup&gt; Raji</td>
<td>300</td>
</tr>
<tr>
<td>CD22&lt;sup&gt;+&lt;/sup&gt; Ramos</td>
<td>200</td>
</tr>
<tr>
<td>CD33&lt;sup&gt;+&lt;/sup&gt; HL-60</td>
<td>2500</td>
</tr>
</tbody>
</table>

aHuman B lymphoma cells were cultured for 96 hr in the presence of various concentrations of CMC-544, CMA-676 or unconjugated CalichDMH after which the viable cell number in each culture was enumerated by their exclusion of propidium iodide and detected by flow cytometry. Unconjugated anti-CD22 mAb G5/44 up to 1 µM had no effect on the growth of these B cell lines.
Table 2. Maximum tolerated non-lethal dose of CMC-544 in nude mice\(^a\)

<table>
<thead>
<tr>
<th>Dose of CMC-544 (µg of conjugated CalichDMH/kg)</th>
<th>% Survival of nude mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>160</td>
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<td>320</td>
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<tr>
<td>400</td>
<td>30</td>
</tr>
<tr>
<td>480</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)CMC-544 was administered IP Q4Dx3 at various doses to non-tumor-bearing nude mice (10 mice/group) after which their survival was monitored for up to 50 days. Maximum non-lethal dose (MND) of CMC-544 was the highest dose at which all CMC-544-treated mice survived after 50 days.
Figure Legends:

**Figure 1:** Structure of CMC-544, a CD22-targeted immunoconjugate of CalichDMH.

**Figure 2:** Sensograms from Biosensor analysis of the interaction between CMC-544 or G5/44 and immobilized CD22mFc. CMC-544 or G5/44 at indicated protein concentrations was injected over CD22Fc immobilized onto the Biosensor chip surface. Curves were fitted to a 1:1 Langmuir binding model with allowance for mass transfer effects.

**Figure 3.** Effect of CMC-544 on the growth of small but established Ramos BCL xenografts (A). Effect of an isotype-matched control conjugate, CMA-676, was also evaluated in the same tumor model (B). G5/44 dose is shown in antibody protein equivalents and CMC-544 dose is shown in CalichDMH equivalents. The dose of 7.5 mg/kg of G5/44 is equivalent to the dose of antibody protein in 160 µg/kg of CMC-544.

**Figure 4.** Effect of CMC-544 on developing (A) or large established Ramos BCL xenografts (B). CMA-676 was used as a nonbinding control conjugate. Doses are shown in CalichDMH equivalents.

**Figure 5.** Effect of CMC-544, unconjugated CalichDMH or an admixture unconjugated anti-CD22 mAb G5/44 and CalichDMH on the growth of small but established RL BCL xenografts. CMC-544 dose is shown in CalichDMH equivalents. Unconjugated G5/44 dose is in antibody protein equivalents.
Figure 6. Effect of unconjugated anti-CD22 mAb G5/44 on developing (A) or small but established (B) RL BCL xenografts. In addition, CMC-544 was also administered Q4Dx3 starting Day 23 to established RL BCL xenografts-bearing mice that had been pre-treated with unconjugated G5/44 over a period of 21 days (B). CMC-544 was also administered on Day 1 Q4Dx3 as a positive control. G5/44 dose is shown in antibody protein equivalents and CMC-544 dose is shown in CalichDMH equivalents.

Figure 7: Pharmacokinetics of CMC-544 in nude mice. CMC-544 was administered IP at 160 µg/kg to tumor-bearing (RL BCL) and non-tumor-bearing nude mice. Serum was collected from these mice at different time intervals (n=3 mice/time point) and assessed for the presence of CMC-544 as described in the Methods section. CMC-544 is expressed as CalichDMH equivalents.
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Figure 2: Sensograms from Biosensor analysis of the interaction between CMC-544 or G5/44 and immobilized CD22mFc. CMC-544 or G5/44 at indicated protein concentrations was injected over CD22mFc immobilized onto the Biosensor chip surface. Curves were fitted to a 1:1 Langmuir binding model with allowance for mass transfer effects.

<table>
<thead>
<tr>
<th></th>
<th>$K_d$ (1/s)</th>
<th>$K_a$ (1/M/s)</th>
<th>$K_D$ (M)</th>
<th>$K_A$ (1/M)</th>
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</thead>
<tbody>
<tr>
<td>G5/44</td>
<td>$1.73 \times 10^{-4}$</td>
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<td>$4.26 \times 10^9$</td>
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<td>CMC-544</td>
<td>$1.72 \times 10^{-4}$</td>
<td>$8.61 \times 10^5$</td>
<td>$2.00 \times 10^{-10}$</td>
<td>$5.01 \times 10^9$</td>
</tr>
</tbody>
</table>
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