

Antitumor Efficacy of a Combination of CMC-544 (Inotuzumab Ozogamicin), a CD22-Targeted Cytotoxic Immunoconjugate of Calicheamicin, and Rituximab against Non-Hodgkin's B-Cell Lymphoma

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Abstract Purpose: CMC-544 is a CD22-targeted cytotoxic immunoconjugate, currently being evaluated in B-cell non-Hodgkin's lymphoma (B-NHL) patients. Rituximab is a CD20-targeted antibody commonly used in B-NHL therapy. Here, we describe antitumor efficacy of a combination of CMC-544 and rituximab against B-cell lymphoma (BCL) in preclinical models.

Experimental Design: BCLs were cultured *in vitro* with CMC-544, rituximab, or their combination. BCLs were injected either s.c. or i.v. to establish localized s.c. BCL in nude mice or disseminated BCL in severe combined immunodeficient mice, respectively. I.p. treatment with CMC-544 or rituximab was initiated at various times either alone or in combination and its effect on s.c. BCL growth or survival of mice with disseminated BCL was monitored.

Results: *In vitro* growth-inhibitory activity of CMC-544 combined with rituximab was additive. Rituximab but not CMC-544 exhibited effector functions, such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Rituximab was less effective in inhibiting growth of established BCL xenografts than developing xenografts. In contrast, CMC-544 was equally effective against both developing and established BCL xenografts. Although CMC-544 and rituximab individually caused partial inhibition of the growth of BCL xenografts at suboptimal doses examined, their combination suppressed xenograft growth by >90%. In a disseminated BCL model, 60% of CMC-544-treated mice and 20% of rituximab-treated mice survived for 125 days. In contrast, 90% of mice treated with the combination of CMC-544 and rituximab survived for longer than 125 days.

Conclusion: The demonstration of superior antitumor activity of a combination of CMC-544 and rituximab described here provides the preclinical basis for its clinical evaluation as a treatment option for B-NHL.

CMC-544 (inotuzumab ozogamicin) is a CD22-targeted cytotoxic agent composed of a humanized IgG4 anti-CD22 antibody covalently linked to *N*-acetyl- γ -calicheamicin dimethyl hydrazide (CalichDMH) via the acid-labile 4-(4'-acetylphenoxy)butanoic acid linker (1–3). CD22 is a B-lymphoid lineage-specific differentiation antigen expressed on both normal and malignant B cells. CMC-544 binds CD22 with subnanomolar affinity, and, upon binding, is rapidly internal-

ized delivering the conjugated CalichDMH inside the cells. This preferential intracellular delivery of CalichDMH causes DNA damage resulting in B-cellular apoptosis. CalichDMH is a derivative of γ -calicheamicin, a natural product produced by *Micromonospora echinospora* and is significantly more potent than cytotoxic chemotherapeutic agents used in cancer therapy. It binds in the minor groove of DNA and causes double-strand DNA breaks in a relatively sequence-specific and thiol-dependent manner leading to apoptotic response in cells (4–6).

CMC-544 exerts potent and CD22-selective growth inhibitory activity against CD22⁺ B-cell lymphoma (BCL) cell lines *in vitro* and causes regression of developing (minimal disease), small established (palpable disease), and large BCL xenografts, with a high therapeutic index (1). In addition, CMC-544 protects severe combined immunodeficient (SCID) mice against hind limb paralysis and death caused by systemically disseminated BCL (7). In the absence of the conjugated CalichDMH, G5/44, the targeting monoclonal antibody (mAb) in CMC-544, is ineffective *in vivo* as an antitumor agent in various preclinical models (1, 7) and, thus, CMC-544 is regarded as an antibody-targeted chemotherapy agent rather than an immunotherapeutic agent like rituximab. Largely due

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Note: All authors are employed by Wyeth Research whose investigational agent, CMC-544, was studied in the present work. CMC-544 is being jointly developed by Wyeth Research and UCB Celltech (Slough, United Kingdom).

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to its tumor-targeted, drug-delivery capability, CMC-544 is likely to have a better therapeutic index than that of conventional chemotherapeutic agents. Based on its potent antitumor activity in preclinical models, CMC-544 is currently being evaluated for safety and tolerability in patients with B-cell non-Hodgkin lymphoma (B-NHL) in phase I clinical trials.

Rituximab is a chimeric human IgG1 antibody targeted to another B-lymphoid lineage-specific molecule, CD20. It represents a major therapeutic advance in B-NHL therapy (8–11). Rituximab mediates its antitumor activity by multiple mechanisms that include complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and direct induction of apoptosis in BCL (12–14). Despite the antitumor efficacy and safety shown by rituximab in B-NHL patients, only a small subset of patients achieve complete responses and the disease eventually relapses. Due to its clinically shown safety, rituximab is widely used in combination with various cytotoxic agents. Cycle treatment using a combination of rituximab with cytoreductive combination chemotherapy, such as CHOP (a combination of cyclophosphamide, doxorubicin, vincristine, and prednisone), has significantly improved clinical responses and durations of disease remission (15–18). However, such combinations also suffer from various systemic toxicities associated with the nontargeted nature of CHOP chemotherapy.

Because of the widespread therapeutic use of rituximab in B-NHL patients in the United States and Europe, initial clinical evaluation of any experimental agent intended for B-NHL is likely to be carried out in B-NHL patients that have been treated with rituximab with or without chemotherapy. Because B-NHL cells consistently express both CD20 and CD22, it is reasonable to combine rituximab and CMC-544 in an attempt to enhance the therapeutic advantage of either agent. This study evaluated antitumor activities of CMC-544, rituximab, and their combination and shows that a therapeutic combination of rituximab and CMC-544 is able to significantly inhibit the growth of s.c. human BCL xenografts and protects against systemically disseminated BCL. These results support the evaluation of a combination of CMC-544 and rituximab in patients with B-NHL with the ultimate goal of improving the therapeutic index of the combination.

Materials and Methods

Materials

A humanized anti-CD22 mAb (G5/44, an IgG4 isotype) was derived by CDR grafting from murine anti-CD22 mAb m5/44 by Celltech (Slough, United Kingdom; ref. 19) and expressed at Wyeth Biopharma (Andover, MA). G5/44 was conjugated to CalichDMH with an acid-labile 4-(4'-acetylphenoxy)butanoic acid linker and the resulting conjugate was termed CMC-544. The quantity of CalichDMH present in CMC-544 was 73 $\mu\text{g}/\text{mg}$ G5/44. Chimeric human IgG1 anti-CD20 mAb, rituximab (Rituxan, Biogen-Idex Pharmaceuticals, San Diego, CA, and Genentech, South San Francisco, CA), was purchased from Med World Pharmacy (Chestnut Ridge, NY). All conjugates were endotoxin free (<5.0 endotoxin units/mL) by a modified *Limulus* amoebocyte assay test (Biowhittaker, Walkersville, MD). Doses of calicheamicin conjugates are expressed as equivalents of CalichDMH and that of unconjugated antibody are expressed as antibody protein.

The Burkitt lymphoma cell lines Ramos (CRL-1923), Raji (CCL-86), Daudi (CCL-213), and a NHL line RL (CRL-2261) were obtained from

the American Type Culture Collection (Manassas, VA) and determined to be *Mycoplasma* free by a PCR *Mycoplasma* detection assay. Cell lines were maintained as suspension cultures in RPMI medium plus 10% FCS, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, penicillin G sodium 100 units/mL, and streptomycin sulfate 100 $\mu\text{g}/\text{mL}$. Before use, viable cells were isolated by centrifugation (30 minutes at $1,000 \times g$) using Lymphoprep (Nycomed, Oslo, Norway) density gradient.

Mice

Female, BALB/c *nu/nu* (nude) mice (18–23 g) and 6- to 8-week-old male SCID mice (CB17 SCID, body weight 20–25 g) were obtained from Charles River Laboratories (Wilmington, MA). All mice were housed in microisolator units and provided with sterile food and water *ad libitum* throughout the studies. All procedures involving mice were approved by the Wyeth Animal Care and Use Committee and carried out according to established guidelines.

Methods

Flow cytometric detection of CD22 expression. The expression of CD22 by various BCLs treated with or without rituximab was confirmed using both direct and indirect immunofluorescence analyses as described before (19). Fluorescein-conjugated murine anti-human CD22 antibody, RFB4 (Santa Cruz Biotechnology, Santa Cruz, CA) was used to assess the surface expression of CD22 on various BCL. Alternatively, murine anti-CD22 antibody, m5/44, was used during indirect immunofluorescence analysis of CD22 expression by BCL (19). RFB4 and m5/44 recognize distinct epitopes on human CD22 (19).

Cytotoxicity assays. A vital dye (MTS cell proliferation assay kit, Promega, Madison, WI) stain was used to determine the number of surviving cells following exposure to various drug treatments. BCLs were seeded in 96-well dishes at a density of either 5,000 cells per well (Ramos, Raji, and RL) or 10,000 cells per well (Daudi). Immediately after seeding, the cells were exposed to various concentrations of CMC-544 (0.0001 to 10 ng CalichDMH equivalents/mL), rituximab (20 or 200 $\mu\text{g}/\text{mL}$), or a combination of the above. The number of cells surviving 96 hours after drug exposure was determined. Percent survival of cells in these cultures was calculated using the following equation: percent survival = $100 \times (\text{no. viable cells in treated cultures} / \text{no. viable cells in control cultures})$. IC_{50} values were calculated based on a four-variable logistic model or, to obtain a better fit in some cases, a three-variable model.

The Bliss independence model was used to define the interaction of the drugs in combination (20). In the Bliss model, the expected percentage of control of a combination of two drugs is the product of the percentage of control for each drug alone at the same concentration used in the combination. The medium-alone value was used as the control and was subtracted from each well (background subtraction), and then the mean of the (background adjusted) values for cells alone wells were used to calculate the percentage of control. Statistical significance was assessed using ANOVA to calculate 95% confidence intervals on the observed mean percentage of control for each combination. If the expected percentage of control for the combination was outside of the 95% confidence interval for the observed mean, the difference between the observed and expected growth inhibition was noted as statistically significant. Separate ANOVAs were run for each cell line. In each ANOVA, CMC-544 and rituximab concentrations were the independent variables.

Assessment of ADCC and CDC. Nude mouse peripheral blood was allowed to clot for 60 minutes at 4°C after which the tubes were centrifuged to collect serum, used as a source of murine complement. For CDC, Ramos B-lymphoma cells were used as target cells. Typically, 10,000 or 50,000 Ramos B cells were mixed in 96-well microtiter plates with increasing protein concentrations (range of 0.1–10 $\mu\text{g}/\text{mL}$) of G5/44, CMC-544, or rituximab in the presence or absence of mouse serum (1:100 final dilution). The microtiter plates were incubated at 37°C for 4 hours after which the activity of lactate dehydrogenase (LDH) in the

cell-free supernatants was assessed using the Cytotox-1 homogeneous membrane integrity kit (Promega). Ramos cells were lysed using lysis buffer to assess the maximum LDH releasable from these cells. LDH activity observed with Ramos cells maintained in RPMI 1640 in the absence of antibody or conjugate, and complement represented the background release.

The percent cytotoxicity was calculated by the following equation:

$$\%Lysis = 100 \times \frac{(\text{experimental release} - \text{background release})}{(\text{maximum release} - \text{background release})}$$

Mononuclear cells (MNC) isolated from the spleen or peripheral blood of nude mice were used as effector cells in the experiments assessing the ADCC activity of G5/44, CMC-544, and rituximab. Peripheral blood was collected in heparinized collection tubes (BD Biosciences, San Jose, CA) and MNC were separated from the heparinized blood or single-cell suspension of splenocytes using Optiprep by floatation mixture technique. MNCs were washed in TBS and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum and used as effector cells in ADCC assays.

For ADCC activity, 5,000 Ramos B cells were mixed with 250,000 MNC (effector cell to target cell ratio of 50:1) in the presence of increasing protein concentrations of G5/44, CMC-544, or rituximab (range of 0.1-10 µg/mL) and incubated at 37°C for 4 hours. The release of LDH in the cell-free culture medium was assessed using the Cytotox-1 homogeneous membrane integrity kit as described above. The negative controls included Ramos cells alone, MNC alone, a mixture of Ramos cells and MNC in the absence of antibodies, Ramos cells and antibodies without MNC, and MNC and antibodies without Ramos cells. Maximum release of LDH was derived from cells treated with the lysis buffer. Percent lysis was calculated as described above.

Subcutaneous xenografts. Female, athymic nude mice were exposed to total body irradiation (400 rad) to suppress their residual immune system and facilitate the establishment of BCL xenografts. Three days later, the irradiated mice were injected s.c. with 1×10^7 Ramos or RL cells in Matrigel (Collaborative Biomedical Products, Belford, MA, diluted 1:1 in RPMI 1640) in the dorsal, left flank. Therapeutic agents were administered i.p. at 0.2 mL/dose volume. CMC-544 (16 or 160 µg/kg), rituximab (2 or 20 mg/kg) or a combination of each was administered (1, 7). Three doses of CMC-544 were administered 4 days apart (Q4D×3), whereas rituximab was either administered according to the same schedule as CMC-544 (Q4D×3) or as six doses, twice a week for 3 weeks (Q3D×6). The initial day of drug administration was considered day 1. For the established xenograft models, therapy was initiated when tumors reached the average tumor mass of >150 mg. Doses of CMC-544 were based on the quantity of CalichDMH (calicheamicin equivalents). Tumors were measured at least weekly and their mass was calculated as follows: tumor mass (g) = $0.5 \times (\text{tumor width}^2)$ (tumor length). Mean (±SE) tumor mass for each treatment group was calculated and compared with the vehicle-

treated group for statistical significance using ANOVA and subsequent pairwise comparisons to the vehicle-treated group by a one-tailed *t* test with the error term for the *t* test based on the pooled variance across all treatment groups. Statistical significance was declared at the traditional level of 0.05.

Disseminated BCL xenografts. Male SCID mice were injected i.v. with 1×10^6 Ramos BCLs in a volume of 0.2 mL in the tail vein. Dissemination of BCL was allowed to occur for 3 days before the initiation of drug therapy (7). Mice with disseminated BCL were administered vehicle, CMC-544 (0.4 µg/kg), rituximab (1 mg/kg), or a combination of both drugs on days 3, 7, and 11 (Q4D×3, 10 mice per treatment group). Mice were monitored daily for the presence of hind limb paralysis or death. Mice exhibiting hind limb paralysis were euthanized by CO₂ asphyxiation according to institutional regulations. The average survival time (days ± SD) was calculated for each group. The percentage of mice surviving throughout the observation period was recorded. The difference in survival distributions between groups was determined by using the log-rank test. Multiple comparisons were done using the rank transformation procedure proposed by Conover and Iman (21). The rank transformation procedure consists of replacing the survival times with their ranks and performing an ANOVA test on the ranks. This approach enabled us to perform multiple comparisons using Tukey's procedure on the ranks. Statistical significance was declared at the traditional 0.05 level. The survival curves were constructed using the Kaplan-Meier method.

Results

In vitro effect of a combination of CMC-544 and rituximab on BCL growth. To determine the effect of combining CMC-544 and rituximab on the growth of CD20⁺ CD22⁺ Daudi, Raji, RL, and Ramos BCL, the sensitivity of these cell lines to rituximab was first assessed. Rituximab at 20 µg/mL caused a modest inhibition (≈20%) in growth of these BCL. Increasing the rituximab concentration up to 200 µg/mL did not further increase the inhibition of BCL growth. Consistent with our previous observations, CMC-544 caused a potent and dose-dependent inhibition of growth of each BCL with IC₅₀ values ranging between 12 pmol/L and 2.2 nmol/L of conjugated CalichDMH (Table 1). Addition of rituximab at either 20 or 200 µg/mL to CMC-544 caused reduction in the IC₅₀ values of CMC-544 for each of the cell lines. The results of the Bliss analysis of the drug interactions suggested that the interaction between CMC-544 and rituximab *in vitro* was additive in each of the BCLs studied. Human IgG1 used as an isotype-matched control for rituximab did not alter the IC₅₀ values of CMC-544 against these BCLs (data not shown). Thus, rituximab can cause

Table 1. Effect of rituximab on the sensitivity of BCLs to CMC-544

Treatment	CMC-544 IC ₅₀ [CalichDMH (pmol/L), 95% confidence interval]				
	Ramos*	Ramos*	Daudi	RL	Raji
CMC-544	100 (60-160)	73 (40-147)	40 (33-60)	12 (6-24)	2,200 (1,660-2,860)
CMC-544 + rituximab 20 µg/mL	66 (40-100)	53 (33-80)	20 (13-33)	5 (2-10)	600 (413-893)
CMC-544 + rituximab 200 µg/mL	40 (20-73)	40 (20-87)			

NOTE: Ramos, Raji, RL, or Daudi BCLs were cultured for 4 days with increasing concentrations of CMC-544 in the presence of either 20 or 200 µg/mL of rituximab, after which the number of viable cells in each culture was quantified. IC₅₀ values (with 95% confidence intervals) were calculated using a three- or four-variable logistic model with the lower asymptote fixed at 0. Rituximab, when used at either 20 or 200 µg/mL concentration in the absence of CMC-544, caused ≤20% inhibition of BCL growth. Addition of nonbinding human IgG1 at 20 or 200 µg/mL to CMC-544 did not change IC₅₀ of CMC-544.

*Two independent experiments.

a modest increase in the potency of the *in vitro* anti-BCL activity of CMC-544.

Rituximab has been shown *in vitro* to slightly increase the surface expression of CD22 on BCL (22). We therefore examined whether the increased sensitivity of rituximab-treated BCL was related to their enhanced expression of CD22. Individual BCLs were cultured for 18, 44, or 66 hours with 10 $\mu\text{g}/\text{mL}$ of either rituximab or human IgG1 (isotype-matched control) after which the binding of fluorescein-conjugated murine anti-human CD22 mAb (RFB4) to treated BCL was examined by flow cytometry. Rituximab failed to cause meaningful changes in the expression of CD22 on the BCLs at any of the time frames examined (data not shown). Occasionally, there was an incremental increase in the expression of CD22 of these cultured BCLs but these changes were not consistently observed.

Effect of CMC-544 or rituximab on the growth of developing and established BCL xenografts. The effect of CMC-544 and rituximab on the growth of both the developing and established Ramos and RL BCL xenografts was evaluated. Rituximab was able to prevent the establishment of both Ramos and RL BCL developing xenografts ($P < 0.05$ versus vehicle; Fig. 1A and B). However, upon discontinuation of the rituximab treatment (days 13 and 21 in the developing Ramos and RL models, respectively), tumors began to grow, suggesting

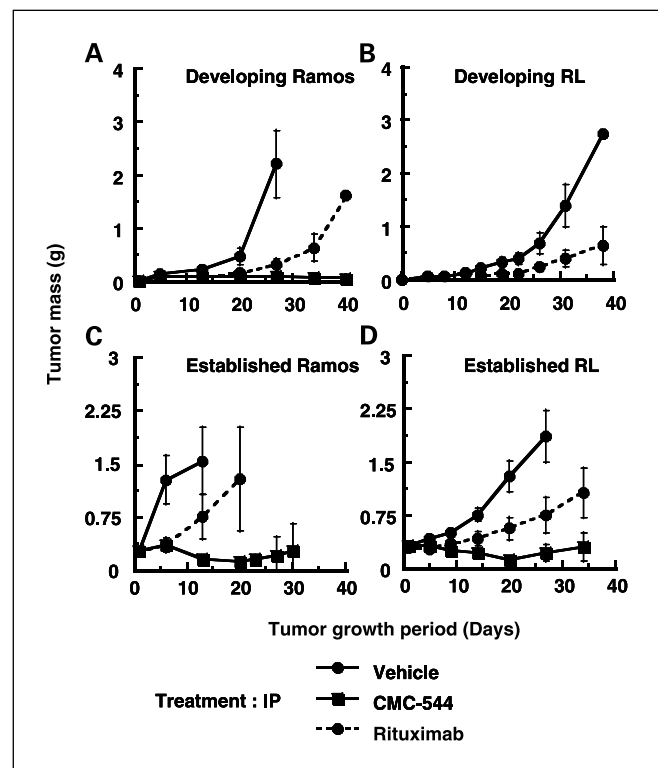


Fig. 1. Effect of rituximab and CMC-544 on the growth of developing (A and B) or established (C and D) B-lymphoma xenografts. Ramos or RL B-lymphoma cells were injected s.c. in nude mice to establish s.c. xenografts. Treatment with vehicle ($n = 8$ to 10 per treatment group), CMC-544 (160 $\mu\text{g}/\text{kg}$; A, $n = 12$), or 80 $\mu\text{g}/\text{kg}$ (C, $n = 8$ and D, $n = 9$; Q4D \times 3), or rituximab Q3D \times 3 (20 mg/kg; A, $n = 11$; C, $n = 8$; and D, $n = 7$) or Q3D \times 6 (B, $n = 8$) was initiated i.p. either 4 to 5 days after the s.c. injection of lymphoma cells (developing tumor) or after the establishment of B lymphomas to an average mass of 300 mg. Tumor growth was monitored for up to 40 days postinitiation of the treatment. Error bars, average tumor mass \pm SE.

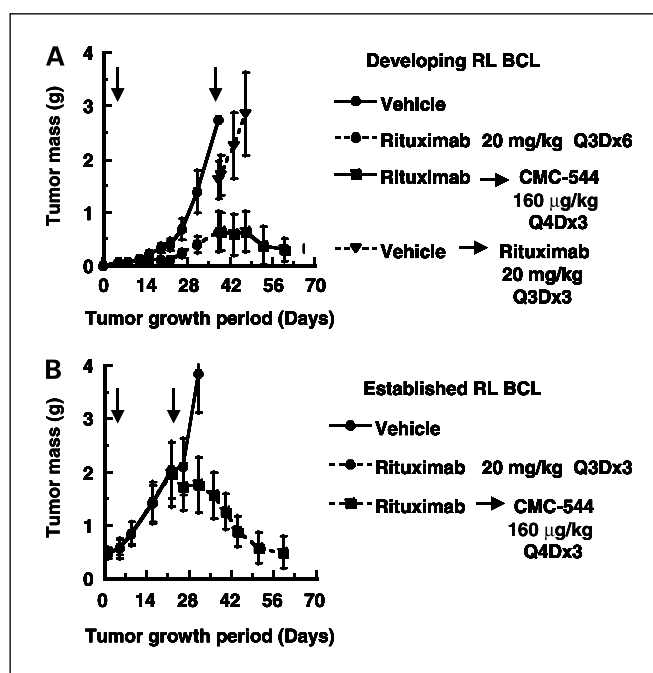


Fig. 2. BCL relapsed after rituximab treatment retain their sensitivity to CMC-544. RL BCLs were injected s.c. to initiate development of xenografts. Rituximab (20 mg/kg, $n = 8$) or vehicle (A, $n = 8$ and B, $n = 7$) was administered i.p. Q3D \times 6 either 5 days after the s.c. injections of RL cells to assess its effect on the establishment of RL xenografts (developing tumor; A) or after the RL xenografts were allowed to grow to an average mass of 400 mg (established tumor; B). Rituximab-treated RL xenografts were further treated with CMC-544 (160 $\mu\text{g}/\text{kg}$) i.p. Q4D \times 3 beginning on day 39 (developing tumor, $n = 5$) or day 22 (established tumor, $n = 6$). Vehicle-treated developing tumors were also treated with rituximab (20 mg/kg Q4D \times 3, $n = 4$) beginning on day 39. Subsequent tumor growth in these various treatment groups was monitored until 62 days. Arrows, initiation of the treatments.

that the inhibitory effect of rituximab was principally manifested during the period of drug administration. In contrast, the inhibitory effect of rituximab on the growth of established BCL xenografts was inconsistent, either moderate antitumor activity (Fig. 1C and D) or, in a number of experiments, no antitumor activity (see below Fig. 2). In Fig. 1C and D, rituximab showed modest but statistically significant activity. In contrast, CMC-544 at the doses evaluated caused potent inhibition of growth ($P < 0.05$ versus vehicle) of both developing and established BCL xenografts (Fig. 1A, C, D).

The influence of prior exposure of BCL to rituximab on the subsequent antitumor efficacy of CMC-544 was also examined. The developing RL BCL xenografts were treated with rituximab (20 mg/kg Q3D \times 6) resulting in the inhibition of RL BCL growth ($P < 0.05$ rituximab versus vehicle on days 19-38; Fig. 2A). However, discontinuation of rituximab treatment allowed regrowth of the RL xenografts. When these RL BCL regrew to an average tumor mass of 750 mg, they were further treated with CMC-544. CMC-544 caused regression of RL BCL that had regrown after the discontinuation of rituximab treatment. In contrast, rituximab had no effect on vehicle-treated and now established RL BCL xenografts (Fig. 2A). Similarly, rituximab failed to inhibit growth of established RL BCL xenografts (Fig. 2B). However, CMC-544 treatment caused regression of the same rituximab-refractory RL xenografts ($P < 0.05$ CMC-544 treated on days 51 and 59 versus rituximab treated on

day 22). These results indicate that BCL that are refractory to the rituximab treatment can still be sensitive to the antitumor activity of CMC-544.

Effector functional capabilities of CMC-544 and rituximab. Rituximab inhibited the growth of developing BCL xenografts but was poorly active against established BCL xenografts in nude mice. The *in vivo* antitumor efficacy of rituximab (IgG1) is thought to be largely dependent on its capabilities to mediate ADCC and CDC against BCL targets (12, 13). The ability of CMC-544 and unconjugated G5/44 (both human IgG4 isotype) and rituximab (human IgG1) to mediate ADCC and CDC against CD20⁺CD22⁺ Ramos B-lymphoma cells was explored using murine effector cells and complement. MNCs isolated from spleens of nude mice were used as a source of effector cells in the ADCC assays and freshly prepared murine serum from nude mice was used as a source of complement.

Rituximab mediated potent, dose-dependent ADCC against Ramos BCL targets using murine effector cells from nude mice (Fig. 3A). In contrast, neither CMC-544 nor G5/44 was capable of mediating ADCC in the same model system. Similarly, rituximab but not CMC-544 or G5/44 mediated dose-dependent CDC activity against Ramos BCL targets in the presence of murine complement (Fig. 3B). Similar results were obtained using either human serum or SCID mouse serum as a source of complement and either human peripheral blood MNCs or splenic MNCs from SCID mice as effector cells and Raji BCL as target cells (data not shown). Both CMC-544 and G5/44 are human IgG4 isotype that is unable to effectively engage FcRs

and fix complement to mediate ADCC and CDC, respectively. These results suggest that conjugation to calicheamicin does not alter the inherent inability of human mAbs of IgG4 isotype to mediate Fc-dependent effector functions. This study further establishes that the immunocompromized mice (nude and SCID) possess both the effector MNCs capable of mediating ADCC activity and the complement to mediate CDC.

Effect of a combination of CMC-544 and rituximab on developing BCL xenografts. Whether a combination of CMC-544 and rituximab would be therapeutically more effective than either agent alone was examined using the developing Ramos BCL xenograft model (a minimal disease state). Because both CMC-544 and rituximab are each effective in inhibiting the establishment of developing Ramos BCL xenografts, these two agents were administered at suboptimal doses in the combination study. Rituximab (2 mg/kg Q4D \times 3) and CMC-544 (16 μ g/kg Q4D \times 3) individually caused significant inhibition of growth ($P < 0.05$ versus vehicle) of developing Ramos xenografts (Fig. 4). In spite of the growth delay caused by these agents, tumors still continued to grow. In contrast, a combination of CMC-544 and rituximab at the same suboptimal doses produced greater inhibition of growth of Ramos xenografts ($P < 0.05$ a combination of CMC-544 and rituximab versus CMC-544 beyond day 40 or rituximab beyond day 29) with eight of nine xenografted mice tumor-free when assessed at day 71 (Fig. 4). However, the same combination failed to show enhanced antitumor activity against established Ramos or RL xenografts than that of CMC-544 alone (data not shown), most likely due to the poor activity of rituximab against established tumors. These results suggest that in preclinical BCL models where rituximab shows activity, CMC-544 and rituximab can be used in combination to derive superior therapeutic effect.

Effect of a combination of CMC-544 and rituximab on the disseminated BCL in SCID mice. We have previously shown that both CMC-544 and rituximab individually can prolong survival of SCID mice with systemically disseminated developing BCL (7). Whether a combination of suboptimal doses of CMC-544 and rituximab would confer protection against systemically disseminated minimal BCL was investigated. Ramos BCLs were injected *i.v.*, and 3 days later, rituximab (1 mg/kg Q4D \times 3) or CMC-544 (4 μ g/kg Q4D \times 3) were administered *i.p.* either alone or in combination. The dose of rituximab and CMC-544 used represents one tenth the protective dose of either agent in the SCID mouse model (7). By day 35, all of the vehicle-treated mice succumbed to the disseminated BCL (Fig. 5). Only 20% of the rituximab-treated diseased mice survived for 125 days, whereas 60% of CMC-544-treated diseased mice survived for 125 days. In contrast, 90% of the diseased mice treated with the combination of CMC-544 and rituximab survived the entire observation period of 125 days. These results suggest that CMC-544 and rituximab can be administered together as a combination to derive greater therapeutic advantage.

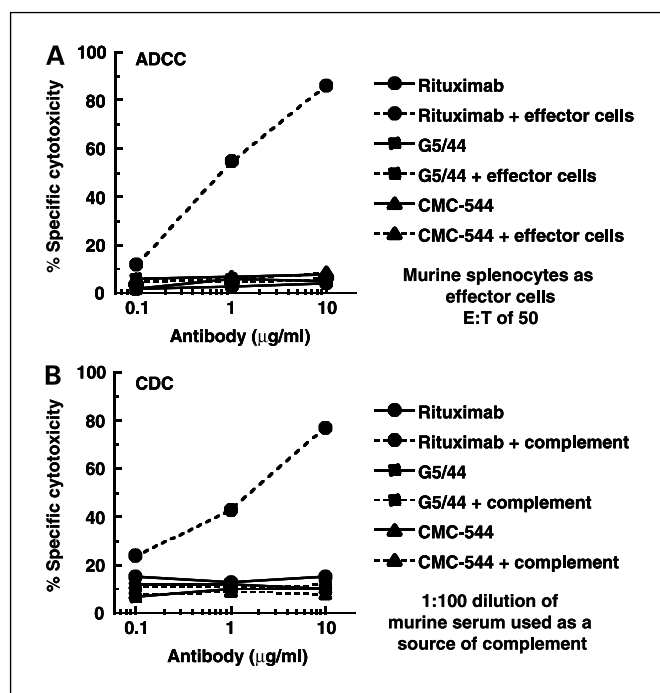


Fig. 3. *In vitro* ADCC (A) and CDC (B) of rituximab, CMC-544, or unconjugated G5/44 anti-CD22 antibody against Ramos B-lymphoma cells. Ramos B cells were incubated at 37 °C with increasing concentrations of indicated antibody or conjugate in the presence or absence of either mononuclear splenocytes from nude mice as effector cells (effector-to-target cell ratio = 50) or 1:100 dilution of fresh nude mouse serum as a source of complement. Four hours later, the cell-free supernatants were assayed for the released LDH activity as an indication of cell lysis.

Discussion

This preclinical study shows that a combination of a targeted cytotoxic chemotherapeutic agent, CMC-544, and an immunotherapeutic agent, rituximab, can provide superior antitumor activity than either agent administered alone against xenografts

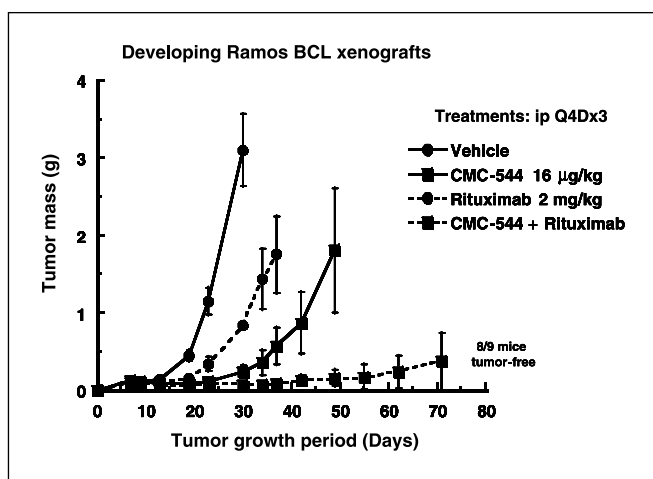


Fig. 4. Effect of a combination of CMC-544 and rituximab on s.c. growth of developing Ramos BCL xenografts. Ramos BCLs were injected s.c. in nude mice ($n = 9$ per group) and 5 days later, i.p. treatment with CMC-544 (16 µg/kg), rituximab (2 mg/kg), or a combination of CMC-544 and rituximab at the above doses was initiated and repeated twice 4 days apart (Q4D \times 3). Tumor growth was monitored for up to 72 days. There were no tumor-free mice in the rituximab treatment group, whereas four of nine mice were tumor-free in the CMC-544 treatment group when assessed at day 50. Error bars, average tumor mass \pm SE.

of human BCL. CMC-544 is a CD22-targeted cytotoxic chemotherapeutic agent devoid of any effector capability, whereas rituximab is a CD20-targeted immunotherapeutic agent capable of mediating both ADCC and CDC (12, 13). This combination makes use of the CD22-specific cytoreductive capability of CMC-544 in addition to the effector functionality of rituximab as well as its direct antiproliferative and apoptotic activity (23, 24). Because both CMC-544 and rituximab exert antitumor effects by distinct mechanisms, it is reasonable to anticipate that CMC-544 could be combined with rituximab to derive greater therapeutic advantage.

In vitro, rituximab caused $\sim 20\%$ inhibition of growth of four distinct BCLs. Because the *in vitro* conditions lacked various cellular and humoral components required to mediate ADCC and CDC, the observed growth inhibitory effect of rituximab was most likely due to its direct but weak antiproliferative activity against these BCLs (25, 26). In contrast, CMC-544 exerted a potent growth-inhibitory effect against the same BCLs attributed to the potent cytotoxic activity of CalichDMH conjugated to anti-CD22 mAb. Combining rituximab with CMC-544 produced an "additive" growth-inhibitory effect as analyzed by the bliss independence model.

When evaluated *in vivo*, rituximab was able to significantly inhibit growth of developing BCL xenografts. However, upon discontinuation of the rituximab treatment, these tumors relapsed again. Unlike its potent inhibitory effect against developing s.c. BCL xenografts, the activity of rituximab against established s.c. BCL xenografts was modest. In contrast, CMC-544 was able to mediate potent antitumor activity against both the developing and established BCL xenografts in nude mice (ref. 1 and this study). More importantly, CMC-544 was able to cause regression of BCL that were refractory to prior rituximab treatment (Fig. 2). Similarly, rituximab was able to inhibit growth of disseminated BCLs when administered early during the disseminated phase of the disease but not when the disseminated BCL disease was already established (7). The

preclinical demonstration of antitumor efficacy of CMC-544 against rituximab-refractory BCL is relevant to the ongoing clinical trials of CMC-544 as most patients being treated with CMC-544 are likely to have had rituximab-refractory BCL.

We investigated whether the weak activity of rituximab against established tumors was related to the lack of host-derived effector activity in immunocompromized mice. Splenocytes from both nude and SCID mice were able to mediate rituximab-mediated ADCC (refs. 27, 28 and present study) and murine complement was able to mediate CDC against rituximab-treated ramos BCL targets. In contrast, CMC-544 was unable to mediate either of these activities. The lack of ADCC and CDC activities by CMC-544 is consistent with its human IgG4 isotype that is a poor mediator of either activity. Rituximab-mediated ADCC and CDC activities against the BCL may contribute toward its *in vivo* antitumor activity observed in both the s.c. xenograft and disseminated BCL models. If the therapeutic effect of rituximab in these models is primarily dependent on the participation of effector cells, then it is likely that, with increasing BCL burden in the established disease, the available effector cells are unable to effectively eliminate all BCL and, as a result, allow progression of the established disease (7). Consistent with this hypothesis is the study reported by Bertolini et al. (29), wherein rituximab was effective in inhibiting the development of BCL but was inactive against established or "bulky" disease. Because the antitumor activity of rituximab could only be consistently shown against the developing BCL xenografts, we evaluated the activity of a combination of CMC-544 and rituximab against developing s.c. or disseminated ramos BCL xenografts. The results of these studies show enhanced antitumor activity when the compounds were used in combination.

Both the *in vitro* and *in vivo* results described above support the combined use of CMC-544 with rituximab in B-NHL. There was no indication of antagonism between CMC-544 and rituximab in any of the studies. Both the antiproliferative effect

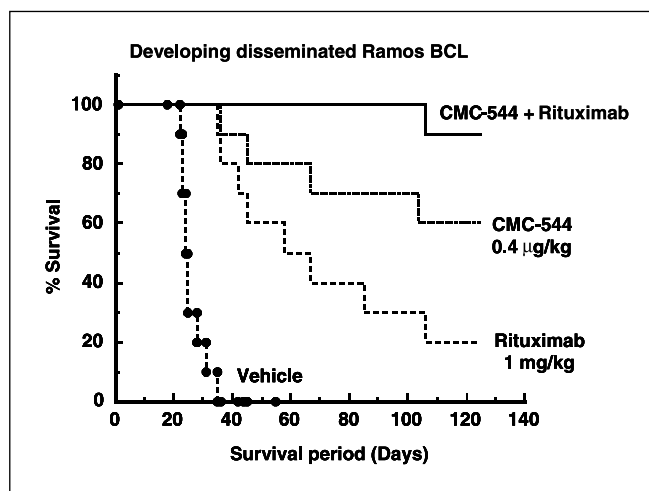


Fig. 5. Effect of a combination of CMC-544 and rituximab at suboptimal doses on the survival of SCID mice with developing disseminated Ramos BCL. SCID mice ($n = 10$ per group) were injected i.v. with Ramos BCL to cause their dissemination and 3 days later, CMC-544 (0.4 µg/kg), rituximab (1 mg/kg), or a combination of CMC-544 and rituximab at the above doses was administered. The same treatments were repeated twice 4 days apart (Q4D \times 3). Mice in each group were monitored up to 125 days for hind limb paralysis or death due to the disseminated disease.

observed *in vitro* as well as the antitumor activity exerted in both of the tumor models suggest that combining these two agents may produce, at minimum, an additive inhibitory effect on BCL growth. The dose-limiting adverse event profile of CMC-544 is currently being determined in ongoing phase I clinical trials. Combining subtherapeutic doses of CMC-544 with rituximab may derive the same antilymphoma effect achievable with higher doses of CMC-544 without precipitating dose-limiting toxicity. How rituximab enhances the activity of CMC-544 *in vivo* is not known. Rituximab binds CD20 on BCL and causes its rapid segregation into lipid rafts (30). What effect rituximab-mediated CD20 segregation into lipid rafts has on the internalization of CMC-544 by BCL has not been studied. If rituximab binding to BCL resulted in the increased expression of CD22, it may allow quantitatively greater accumulation of CMC-544 on the surface of BCL leading to its internalization. Such an effect could conceivably result in the increased intracellular delivery of the conjugated calicheamicin, leading to higher degree of cytotoxicity. However, we were unable to reproducibly show *in vitro* any meaningful increase in the surface expression of CD22 on rituximab-treated BCL.⁴

Several studies have shown that rituximab can be combined with cytotoxic agents and induce a synergistic antiproliferative effect *in vitro*. Rituximab sensitized B-NHL lines to various lympholytic agents, such as paclitaxel, dexamethasone, fludarabine, and other cytotoxic agents, causing either synergistic or additive growth inhibitory activity (10, 26, 31–38). The ability of rituximab to sensitize BCL to cytotoxic agents has been partly attributed to the nullification of the apoptosis-resisting molecular components of the Bcl₂/Bcl-X_l family of molecules (31). In addition, rituximab is also used in radioimmunotherapy with radiolabeled anti-CD20 ibritumomab (⁹⁰Y)ibritumomab tiuxetan/Zevalin; ref. 39). Given its unique mechanism(s) of antitumor activity, rituximab can be combined with various agents to improve their combined therapeutic potential. The antitumor activity of a combination of CMC-544 and rituximab as shown in the current study is consistent with this rationale. Of interest in this context is the recent demonstration of significant clinical activity of a combination of a CD22-targeted antibody, epratuzumab, and rituximab in B-NHL patients (40).

⁴ D. Armellino, unpublished observation.

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