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ORIGINAL ARTICLE

# Preclinical anti-tumor activity of antibody-targeted chemotherapy with CMC-544 (inotuzumab ozogamicin), a CD22-specific immunoconjugate of calicheamicin, compared with non-targeted combination chemotherapy with CVP or CHOP

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**Abstract**
*Purpose* CMC-544 (inotuzumab ozogamicin) is a CD22-specific immunoconjugate of calicheamicin currently being evaluated in patients with non-Hodgkin's B-cell lymphoma (BCL). CHOP and CVP represent untargeted combination chemotherapy comprised of cyclophosphamide, vincristine and prednisone with or without doxorubicin, commonly used in the treatment of NHL. Here, we describe anti-tumor efficacy of CMC-544, CHOP or CVP against human BCL xenografts.
*Methods* In vitro, human BCLs were cultured with CMC-544 or individual constituents of CHOP for inhibition of their growth. In vivo, immunocompromised mice with established BCL xenografts were administered CHOP, CVP or CMC-544 to monitor their survival and BCL growth.
*Results* In vitro, CMC-544 was more potent in causing growth inhibition of various BCL than cyclophosphamide, doxorubicin, vincristine or dexamethasone. In vivo, treatment with CHOP or CVP inhibited growth of BCL xenografts for up to 40 days after which BCL relapsed. Tumor growth inhibition by CMC-544 (>100 days) lasted longer than that by CHOP or CVP. BCL xenografts that relapsed after the treatment with CHOP or CVP were far less responsive to CHOP or CVP re-treatment but regressed upon subsequent treatment with CMC-544. CVP could be co-administered with suboptimal doses of CMC-544, while CHOP could be administered on alternant days with CMC-544 to cause enhanced regression of established BCL xenografts.
*Conclusion* Preclinically, CMC-544 provides greater therapeutic benefit than CVP or CHOP against BCL xenografts. CMC-544 may also be co-administered with standard chemotherapeutic regimens in the treatment of B-NHL for superior anti-tumor activity.

**Keywords** CD22 · CMC-544 · CHOP · CVP · Immunoconjugate · B-cell lymphoma

**Abbreviations**

| | |
|---|---|
| BCL | B-cell lymphoma |
| CalichDMH | N-acetyl gamma calicheamicin dimethyl hydrazide |
| CHOP | Cyclophosphamide doxorubicin vincristine prednisone |
| CVP | Cyclophosphamide vincristine prednisone |
| B-NHL | Non-Hodgkin's B-cell lymphoma |

## Introduction

Combination chemotherapy using cytotoxic agents with distinct mechanisms of action is a common therapeutic strategy in the treatment of metastatic cancers. Individual components of the cytotoxic combination chemotherapy

All authors were employees of Wyeth Research during the conduct of this investigation. CMC-544 is a collaboration product being jointly developed by Wyeth Research and UCB Celltech, Slough, UK.

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are predominantly targeted to the cellular DNA, microtubular cytoskeleton and/or the enzymatic machinery that are required for DNA replication and cell division. Drugs are often administered simultaneously as a cocktail or sequentially to maximize their therapeutic impact. Although this approach is widely used in clinical practice, cumulative toxicities, narrow therapeutic windows and low therapeutic indices often hamper its use. Since these agents do not preferentially target the tumor, they often damage both normal and malignant cells indiscriminately resulting in low therapeutic indices. This realization has led to the more recent development of the strategy of antibody-targeted chemotherapy.

Antibody-targeted chemotherapy involves the use of a monoclonal antibody (mAb) with specificity for a tumor-associated antigen [1]. The mAb is covalently linked to a potent cytotoxic agent. When introduced in the systemic circulation, the mAb facilitates preferential delivery of the attached potent payload to the tumor cells and, in this process, attempts to avoid indiscriminate delivery of the cytotoxic payload to normal tissues. Such preferential tumor-targeted delivery of a cytotoxic agent not only increases its anti-tumor efficacy but also may minimize its exposure to normal tissues resulting in its improved therapeutic index. Mylotarg®, a CD33-targeted calicheamicin conjugate, is the first antibody-targeted chemotherapeutic approved for clinical use [2]. Similar applications of antibodies that deliver radiation to the tumor cells (radioimmunotherapy) have been clinically validated with tositumomab-$^{131}$I/Bexxar and ibritumomab tiuxetan/Zevalin, both of which have been licensed for clinical use in the US [3].

CMC-544 (inotuzumab ozogamicin) is a CD22-specific immunoconjugate of calicheamicin in which a humanized $IgG_4$ anti-CD22 mAb, G5/44, is covalently linked via an acid-labile AcBut linker to CalichDMH (N-acetyl gamma calicheamicin dimethylhydrazide, [4]). CalichDMH is a derivative of gamma calicheamicin, a DNA-damaging enediyne antibiotic [5], and is much more potent than traditionally used chemotherapeutic agents [6]. Gamma calicheamicin binds DNA in the minor groove and with the help of cellular thiols brings about double-strand DNA breaks [5] leading to cellular apoptosis and death. CMC-544 binds human CD22 with subnanomolar affinity and causes potent cytotoxicity against human B-lymphoma cells [4]. When administered i.p. or i.v., CMC-544 causes a dose-dependent inhibition in the growth of subcutaneous or systemically disseminated B-cell lymphoma xenografts established in immunocompromised mice [4, 7]. When combined with rituximab, CMC-544 produced a supra-additive therapeutic benefit in the above preclinical models of B-cell lymphoma [8]. Unconjugated anti-CD22 mAb, G5/44, the targeting agent in CMC-544, is ineffective as a therapeutic agent in the absence of conjugated CalichDMH [4].

CMC-544 is currently being evaluated as an antibody-targeted chemotherapy for efficacy and safety in patients with non-Hodgkin's B-cell lymphoma (B-NHL) and has shown promising clinical activity, demonstrating an overall response rate in follicular lymphoma of 80% and 47% in DLBCL [9–11]. In a dose escalation study in combination with rituximab, CMC-544 plus rituximab exhibited a similar side effect profile as was observed in previous CMC-544 monotherapy trials. The preliminary efficacy of the drug combination of CMC-544 and rituximab in recurrent/refractory follicular lymphoma and DLBCL is promising and may indicate a durable response [12]. Trials are ongoing.

The CHOP combination chemotherapy using cyclophosphamide (C), doxorubicin (initially known as hydroxydaunorubicin) (H), vincristine (V) (initially known as Oncovin®) (O) and prednisone (P) is the standard cytoreductive treatment for NHL. This regimen has become the standard treatment since various other chemotherapeutic alternatives and their combinations have not been found to be superior to CHOP in randomized cooperative group clinical trials [13]. However, newer alternatives to CHOP are continuously being evaluated by addition of new agents to the CHOP combination. The development of such combination chemotherapy was considered rational as the agents were selected based on non-overlapping toxicity and/or evidence of clinical activity as single agents. CHOP was similarly developed empirically by adding doxorubicin (hydroxydaunorubicin) to the pre-existing CVP regimen [14]. The CVP regimen is also used as a first-line treatment option for patients with follicular lymphomas. Clinical efficacy of both CHOP and CVP chemotherapeutic regimens is significantly enhanced by their use in combination with rituximab, which is used as an immunotherapeutic agent [15] so much so that R-CVP or R-CHOP has now become the first-line treatment options for almost all patients with B-cell lymphoma.

Since CMC-544 is regarded as an antibody-targeted chemotherapy and not an immunotherapy [1], it is appropriate to assess its anti-tumor efficacy in comparison with that of the current chemotherapeutic standard of care. In this preclinical study, we have evaluated the anti-tumor efficacy of CD22-targeted CMC-544 with that of untargeted CHOP or CVP combination chemotherapy against subcutaneous human B-cell lymphoma xenografts established in immunocompromised mice. Our results from this preclinical evaluation suggest that targeted chemotherapy with CMC-544 provides superior therapeutic activity against BCL compared to that of non-targeted combination chemotherapy with CVP or CHOP. Our studies also provide evidence that CMC-544 can be combined with CVP or CHOP for additional therapeutic benefit.

## Materials and methods

### Cell lines

The DLBCL lines WSU-DLCL2 (ACC 575) and SU-DHL4 (ACC 495) were obtained from DSMZ, the German Collection of Microorganisms and Cell Cultures (Bruanschweig, Germany). The Burkitt's B-lymphoma line Ramos (CRL-1923) and B-cell NHL cell line RL (CRL-2261) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell lines were determined to be mycoplasma free as determined by a polymerase chain reaction mycoplasma detection assay (ATCC, Manassas, VA). The cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 1 mM sodium pyruvate, 0.2% glucose, penicillin G sodium 100 U/ml and streptomycin sulfate 100 μg/ml. Before use, viable cells were isolated by density-gradient centrifugation (30 min at 1000×*g*) using Lymphoprep (Nycomed, Oslo, Norway).

### Mice

Female nu/nu (nude) mice (18–23 g) and male scid mice (CB17 scid, 20–25 g) were obtained from Charles River Laboratories, Wilmington, MA. All procedures using mice were approved by the Wyeth Animal Care and Use Committee according to established guidelines.

### Compounds

CMC-544 is comprised of a humanized $IgG_4$ anti-CD22 mAb, G5/44, linked to N-acetyl gamma calicheamicin dimethyl hydrazide via an acid-labile AcBut linker with an average drug loading of 73 μg of CalichDMH per mg of G5/44 protein (approximately 6 mol/mole) and has been described [4, 16]. The CD22-specific targeting antibody G5/44 used to create CMC-544 carries a serine to proline mutation (S229P) in its hinge region, which allows it to form stable interchain disulfide bonds and remove potential for any Fab exchange with natural $IgG_4$ (16). The CMC-544 conjugate preparation was confirmed as having low endotoxin (<5.0 EU/ml) as determined by the limulus amebocyte lysate test (BioWhittaker, Walkersville, MD). Doses of CMC-544 are expressed as equivalents of CalichDMH. Cyclophosphamide (Cytoxan®, Princeton, NJ), doxorubicin hydrochloride (Adriamycin, Bedford, OH), vincristine sulfate (Paramus, NJ) and prednisone (Columbus, Ohio) were obtained from Med World Pharmacy, Chestnut Ridge, NY. Dexamethasone was obtained from Sigma (St. Louis, MO).

### Growth inhibition studies

The effect of drugs on cell lines was assessed using a cellular viability indicator (MTS, Promega, Madison, WI) to determine the number of surviving cells following exposure to various drug treatments. Cells were seeded in 96-well microtiter plates at a density of 5,000 to 10,000 cells per well and exposed to various concentrations of drugs. Following determination of the number of viable cells surviving 96 h of drug exposure, the $IC_{50}$ of each treatment was calculated based on the logistic regression parameters derived from the dose–response curves. $IC_{50}$s were calculated by logistic non-linear regression and are reported as the concentration (nM) from each treatment group that causes 50% loss of cell viability.

### Subcutaneous BCL 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 xenografts. Three days later, mice were injected s.c. with $5 \times 10^6$ Ramos or RL cells suspended in Matrigel (Collaborative Biomedical Products, Belford, MA, diluted 1:1 in RPMI 1640 medium) in the right flank. Mice with staged tumors, approximately 0.1 to 0.2 g ($n = 6$ to 8 mice/treatment group), were administered i.p. with normal saline (vehicle), CMC-544 or the components of CHOP administered at their MTD (cyclophosphamide 40 mg/kg i.p., doxorubicin 3.3 mg/kg i.p., vincristine 0.5 mg/kg i.p., and prednisone 0.2 mg/kg po. [17]) unless otherwise indicated. Tumors were measured at least once a week, and their mass (±SEM) was calculated as described before [4].Calculated tumor mass for each calicheamicin treatment group was compared to that from the vehicle-treated group for statistical significance using ANOVA and subsequent pair wise comparisons to the vehicle-treated group using a one-tailed *t*-test analysis of variance and subsequent pairwise comparisons to vehicle with the error term for the *t*-test based on the pooled variance across all treatment groups.

### Assessment of anti-tumor efficacy against disseminated BCL

Male scid mice were injected i.v. with $3 \times 10^6$ Ramos cells in the tail vein, and the injected Ramos cells were allowed to disseminate for 9 days. Mice with disseminated Ramos were administered i.p. with vehicle (PBS, $n = 9$ mice/treatment group), or CMC-544 ($n = 10$) or CHOP ($n = 10$), and these treatments were repeated twice, 4 days apart (Q4D×3). Mice with disseminated ALL were monitored daily for the presence of hind-limb paralysis or death. The

difference in survival between groups was determined by using non-parametric methods for comparing the survival distribution of the mice using Proc Lifetest in SAS version 8.2. Multiple comparisons were performed based on the rank transformation procedure. The rank transformation procedure consists of replacing the survival times with their ranks and applying the usual parametric *F*-test to the ranks. Multiple comparisons were performed using Tukey's method on the ranks. Tukey's method indicates the difference in survival times among mice with significance reported at the 0.05 level. The survival curves were constructed using the Kaplan–Meier method.

## Results

### In vitro effect of cytotoxic chemotherapy against B-lymphoma cell lines

The effect of cyclophosphamide (C), doxorubicin (H), vincristine (V), dexamethasone (D), a combination of all 4 drugs (CHOD), CalichDMH or CMC-544 on the in vitro growth of 4 different human BCL lines was examined (Table 1). BCL cells were cultured in the presence of increasing concentrations of drugs and, after 96 h, the number of surviving live cells in culture was enumerated using the MTS assay. Vincristine was the most potent of the individual drugs that comprised CHOD and exhibited growth inhibitory activity similar to that of CalichDMH and at least fivefold more potent than that of doxorubicin. Vincristine's cytotoxic activity was most likely responsible for the cytotoxic activity of CHOD since its $IC_{50}$ approximated the $IC_{50}$ of the drug combination. Two BCL lines (Ramos and SU-DHL4) exhibited greater sensitivity to CalichDMH than to C, H or O, while WSU-DLCL2 and RL were more potently inhibited by vincristine. Cyclophosphamide requires hepatic activation, so it was not surprising that it was inactive in these in vitro studies. Prednisone also requires hepatic activation [18], which prompted us to use dexamethasone in its place. D was either poorly (RL, Ramos) or completely (WSU-DLCL2, SU-DHL4) inactive at concentrations up to 1 μM. The growth inhibitory effect of the targeted chemotherapeutic CMC-544 was greater than that of unconjugated CalichDMH or any of the components of CHOP in each of the cell lines.

### In vivo anti-tumor efficacy of cytotoxic chemotherapy against B-lymphoma xenografts

Subcutaneous Ramos and RL BCL xenografts were used in the evaluation of the anti-tumor activity of combination chemotherapy with CHOP or CVP. In clinical practice, individual cytotoxic components of the CHOP or CVP combination are administered i.v. at their respective maximum tolerated doses, whereas prednisone (P) is administered orally (p.o.), and this cycle is often repeated every 3 weeks. In our preclinical evaluation, cyclophosphamide (C), doxorubicin (H) and vincristine (O or V), used at their individual MTD, were administered either i.v. or i.p., whereas P was always administered p.o. in nude mice bearing BCL xenografts. The individual MTDs were determined separately during preliminary studies prior to their use in combination and are in agreement with Mohammad et al., [17]. No significant difference was observed in the anti-tumor activity of CHOP chemotherapy regardless of whether C, H and O were administered iv or i.p. (DiJoseph and Evans, unpublished observation). Hence, in subsequent studies, the i.p. route was used for the ease of administration.

Figure 1 shows the anti-tumor efficacy of CHOP or CVP combination therapy against either Ramos or RL BCL sc xenografts and its comparison with that of CMC-544, administered at 80 μg/kg in the Ramos model and 160 μg/kg in the RL model. These doses of CMC-544 were threefold and 1.5-fold lower, respectively, than its MTD established previously [4]. Both the CHOP and CVP

**Table 1** In vitro activity

| Treatment | $IC_{50}$ (nM with 95% CI) | | | |
|---|---|---|---|---|
| | Cell line | | | |
| | WSU-DLCL2 | RL | Ramos | SU-DHL4 |
| CMC-544 | 0.2 (0.03–1.4) | 0.1 (0.002, 8.3) | 0.02 (0.008–0.03) | 0.5 (0.003–91) |
| CalichDMH | 2.3 (1.3–4.2) | 3.3 (1.3, 8.6) | 0.31 (0.13–0.73) | 1.2 (0.3–4.1) |
| CHOD[a] | 1.3 (0.6–3.2) | 2.3 (1.3, 4.2) | 3.0 (2.9–3.0) | 9.2 (2.6–32.2) |
| Doxorubicin (H) | 21.6 (11.1–42.1) | 12.1 (4.3, 34.2) | 10.0 (3.9–25.9) | 19.4 (10.3–36.4) |
| Vincristine (O) | 1.2 (1.1–1.2) | 1.8 (1.3, 2.6) | 2.3 (2.2–2.3) | 4.1 (2.9–5.8) |
| Dexamethasone (D) | NA[b] | 43.5 (16.5, 114.8) | 30[c] | NA |
| Cyclophosphamide (C) | NA | NA | NA | NA |
| Prednisone | NA | NA | NA | NA |

[a] CHOD = cyclophosphamide, doxorubicin, vincristine, dexamethasone

[b] NA = not active up to 1 uM

[c] Approximate $IC_{50}$

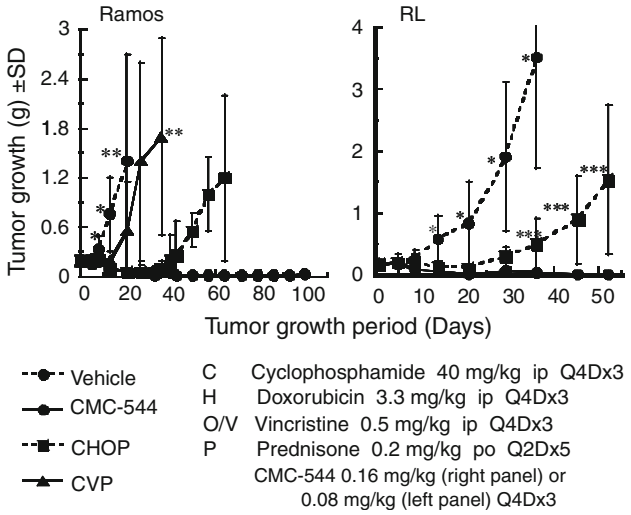


**Fig. 1** Effect of CMC-544, CHOP or CVP on tumor growth in Ramos and RL BCL sc xenografts. All drugs or vehicle was administered by the i.p. route of administration beginning on day 1 and continuing on days 5 and 9 except prednisone, which was given orally on days 1, 3, 5, 7 and 9. *$P < 0.05$ vs. all treatment groups, **$P < 0.05$ vs. CMC-544 and CHOP, ***$P < 0.05$ vs. CMC-544

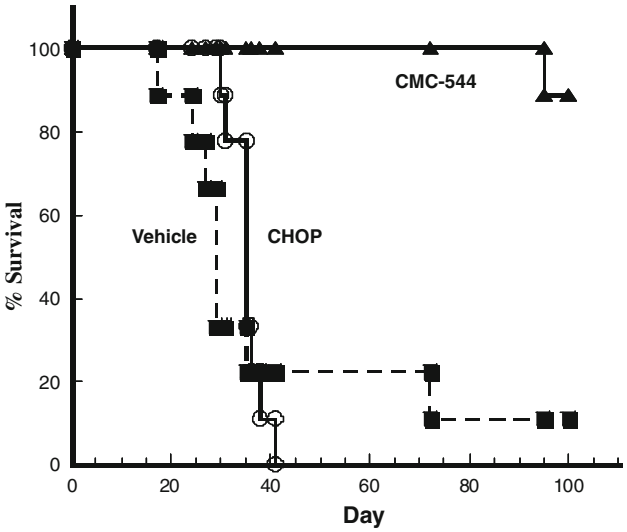


**Fig. 2** Effect of CMC-544 and CHOP on the survival of scid mice with disseminated Ramos BCL. The Ramos BCL cells were injected iv in mice to facilitate their dissemination. Nine days later, vehicle, CMC-544 (80 μg/kg) or CHOP (cyclophosphamide 20 mg/kg i.p., doxorubicin 1.65 mg/kg i.p., vincristine 0.25 mg/kg i.p., and prednisone 0.1 mg/kg p.o.) were given on days 9, 13 and 17 ($n = 9$/treatment group). Mice were monitored daily for hind-limb paralysis or death for up to 100 days

combinations were able to suppress the growth of established Ramos BCL xenografts for a short period after which tumors relapsed. CHOP was able to suppress the tumor growth for a longer duration than CVP. Similarly, CHOP combination was able to suppress the growth of RL BCL xenografts for up to 30 days after which the tumor relapsed. In contrast, CMC-544 was able to cause the complete regression of both Ramos and RL BCL xenografts, and the tumors remained suppressed for a period of >100 days. These results suggest that unlike non-targeted combination therapy with CVP or CHOP, targeted chemotherapy with CMC-544 can provide long-lasting suppression of established BCL.

## Effect of CHOP and CMC-544 on systemically disseminated BCL

When injected i.v. into scid mice, Ramos BCL disseminates systemically and invades various organs, including the CNS, resulting in hind-limb paralysis and/or death [19]. Administration of CMC-544 to mice with disseminated BCL protects them from both the hind-limb paralysis and death [7]. The effect of non-targeted CHOP combination chemotherapy on the survival of scid mice with disseminated Ramos BCL was further examined. The MTD of CHOP in scid mice was half of that in nude mice (unpublished observation). CHOP chemotherapy at the scid mouse MTD was administered 9 days post-systemic dissemination of Ramos BCL, and the treated mice were monitored for hind-limb paralysis or death. As shown in Fig. 2, untargeted combination chemotherapy with CHOP was unable to significantly ($P > 0.05$ vs. vehicle-treated mice) protect scid mice with disseminated BCL from hind-limb paralysis and/or death. In contrast, CMC-544 treatment almost completely protected ($P < 0.05$ vs. vehicle- or CHOP-treated mice) these mice against the disseminated BCL. These results further suggest that tumor-targeted chemotherapy with CMC-544 may provide greater therapeutic benefit than the non-targeted combination chemotherapy with CHOP.

## Combination of untargeted chemotherapy and targeted chemotherapy with CMC-544

Whether CMC-544 can be therapeutically effective in combination with CVP or CHOP was further examined. This issue was approached experimentally by either concurrent or sequential administration of CHOP or CVP with CMC-544 to tumor-bearing mice in order to assess not only the anti-tumor efficacy of the combination but also its tolerability.

The effect of sequential administration of CHOP and CMC-544 on the growth of sc BCL xenografts was evaluated. CHOP, administered at its MTD, was given with CMC-544 (80 μg/kg) on an alternating dosing schedule. Compounds were either administered once a week for 4 weeks beginning with CHOP and then alternating with CMC-544 or given once every 4 days (final dose was administered 5 days apart) with the same sequence as above

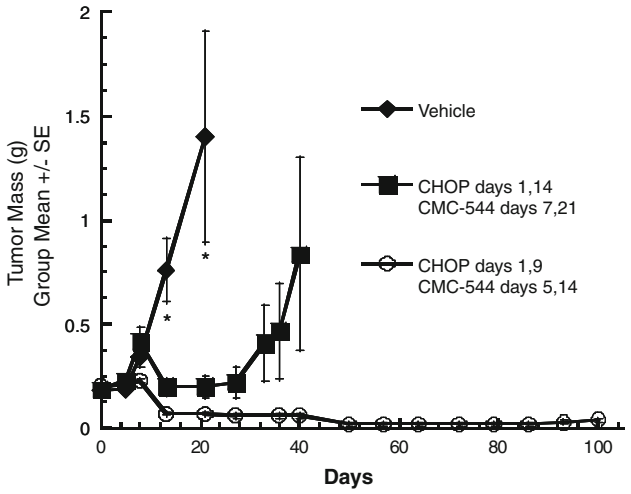


**Fig. 3** Effect of CMC-544 and CHOP administered as alternating doses on tumor growth in Ramos BCL sc xenografts. Drugs or vehicle was administered as indicated by the i.p. route of administration except prednisone which was given orally. * $P < 0.05$ vs. all treatment groups

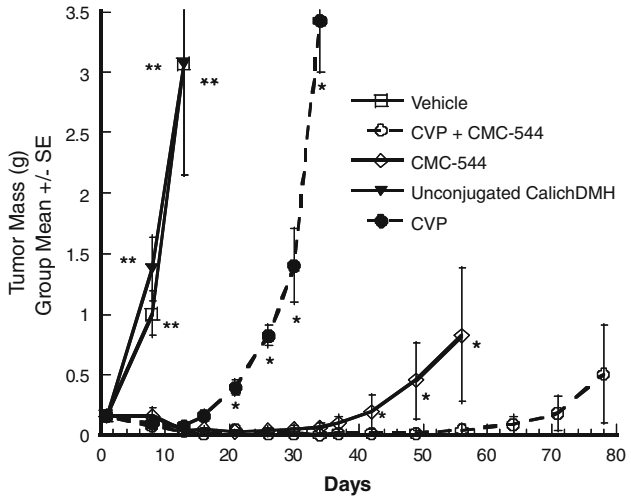


**Fig. 4** Effect of vehicle, CMC-544, unconjugated calichDMH, CVP or CMC-544 + CVP on tumor growth in Ramos sc xenografts. All drugs or vehicle were administered by the i.p. route of administration beginning on day 1 and continuing on days 5 and 9 except prednisone, which was given orally on days 1, 3, 5, 7 and 9. * $P < 0.05$ vs. all treatment groups, ** $P < 0.05$ vs. CMC-544, CVP and CMC-544 + CVP

for a total of 2 dosages of CHOP and 2 dosages of CMC-544 (Fig. 3). Administration once weekly was not sufficiently dose-dense to induce sustained tumor regression. Administering the treatments on an alternating schedule once every 4 days caused complete tumor regression, which was maintained for greater than 100 days. Two dosages of CMC-544 have been shown previously to produce significant tumor regression which, however, was not sustainable [7].

The therapeutic impact of concurrent administration of CVP and CMC-544 was evaluated on the s.c. xenografts in nude mice. CVP combination therapy (administered at the MTD of its individual components) was co-administered i.p. Q4D×3 with CMC-544 (40 μg/kg of conjugated CalichDMH) to sc BCL-bearing nude mice, and the BCL growth was monitored for up to 100 days. As shown in Fig. 4, CMC-544 monotherapy at this suboptimal dose inhibited BCL growth for a longer duration than non-targeted CVP combination chemotherapy used at its MTD. Non-targeted, unconjugated CalichDMH used at a dose of 160 μg/kg was ineffective against established BCL xenografts. Concurrent administration of the combination of CVP at MTD and CMC-544 at the above suboptimal dose caused regression of established BCL xenografts and significantly ($P < 0.05$) improved the anti-tumor activity of the combination therapy. In a similar evaluation using CHOP and CMC-544 combination, there was evidence of lethality in the mice, which was observed along with the anti-tumor activity of this drug combination. These results suggest that CMC-544 can be combined with CVP or administered with CHOP as alternating dosages to obtain greater anti-tumor therapeutic activity.

## Effect of CMC-544 on the BCL xenografts relapsed after the CVP or CHOP chemotherapy

The anti-tumor effect of CVP and CHOP on the sc BCL xenografts was of shorter duration compared to that of CMC-544. We further examined whether the BCL that had relapsed upon the initial Q4D×3 treatment with CVP or CHOP was still responsive to initial treatment with CMC-544. Nude mice with sc Ramos BCL xenografts were first treated Q4D×3 with either CHOP or CVP at their MTD. When the tumors began to re-grow, the mice with the relapsed BCL were randomized into groups to ensure that each group would have mice with both small and large tumors for subsequent treatment with vehicle, CVP, CHOP (each administered at the MTD) or CMC-544 (160 μg/kg). As shown in Fig. 5, mice with sc Ramos BCL xenografts initially responded to CVP or CHOP therapy. and then the tumors relapsed within approximately 20 days after the initiation of the combination chemotherapy. The relapsed Ramos BCL xenografts were poorly responsive to re-treatment with CVP or CHOP but regressed upon further treatment with CMC-544. Previous studies have demonstrated that CHOP therapy can cause the regression of tumors of the size (approximately 750 mg) encountered in this relapsed study (unpublished observation). These results suggest that targeted chemotherapy with CMC-544 is effective against BCLs that are refractory to the CVP or CHOP combination chemotherapy.

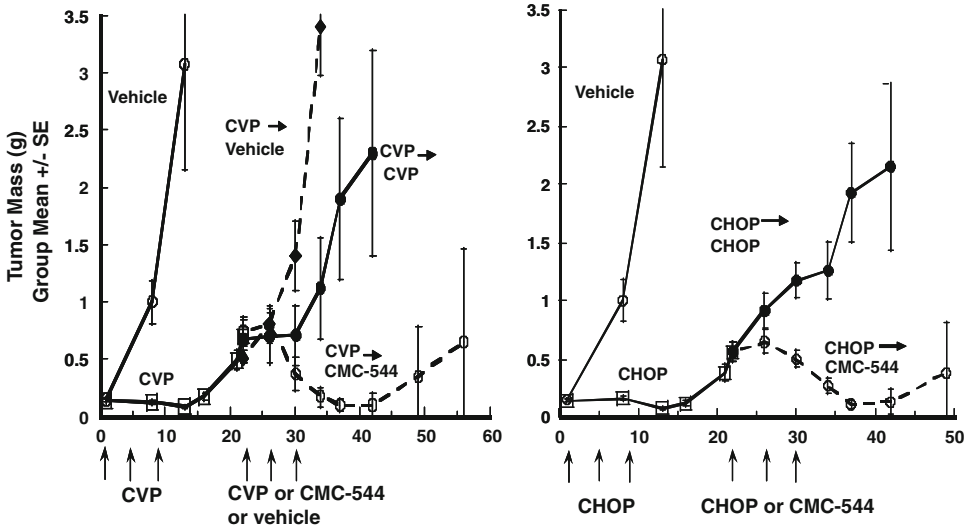


**Fig. 5** Effect of CMC-544 on BCL xenografts relapsed after CVP or CHOP chemotherapy. CVP (*left panel*) or CHOP (*right panel*) was administered on days 1, 5 and 9 to mice with Ramos sc xenografts. When tumors began to relapse, a second course of therapy with either vehicle, CVP or CMC-544 (*left panel*) or CHOP or CMC-544 (*right panel*) was initiated. All drugs or vehicle was administered by the i.p. route of administration except prednisone, which was given orally

## Discussion

The present study compared the anti-B-cell lymphoma preclinical efficacy of CMC-544 (inotuzumab ozogamicin), a CD22-targeted chemotherapeutic agent, with non-targeted CHOP or CVP combination chemotherapy. Our results demonstrate the superior anti-lymphoma activity of CMC-544 over that of CHOP and CVP.

CHOP combination chemotherapy represents a front-line therapy in the treatment of patients with either follicular or DLBCL. Additionally, the CVP combination (CHOP minus doxorubicin) is also used as a first-line therapy for patients with follicular B-cell lymphoma. CMC-544 is a targeted chemotherapeutic agent currently in clinical trials [9–12]. In the present study, both CHOP and CVP therapies were used at the MTD of each component of the combination. These drug cocktails were initially effective in suppressing BCL growth, but tumors relapsed relatively quickly. In contrast, treatment with CMC-544 at doses well below its MTD of 240 μg calichDMH/kg [4] caused regression of the established B-cell lymphomas leading to long-term tumor-free survival. These in vivo results were consistent with the in vitro studies in which CMC-544 as monotherapy was more potent than the CHOP(D) combination. The concentrations of CMC-544 used in the in vitro cytotoxicity studies were well below the $C_{max}$ values reported for CMC-544 in pharmacokinetic studies conducted in mice [4]. Similar therapeutic superiority of CMC-544 over the CHOP chemotherapy was also clearly apparent in SCID mice with systemically disseminated B-cell lymphoma. In the s.c. xenograft studies, equivalent dosages of unconjugated, non-targeted calicheamicin were completely inactive (Fig. 4). These results demonstrate the therapeutic advantage of the tumor-targeted delivery of a potent cytotoxic agent such as calicheamicin over its own non-targeted delivery as well as that of non-targeted CHOP combination. The improved half-life of antibody-targeted chemotherapeutics [4] coupled with targeting to the tumors could account for much of the differences in the anti-tumor response observed with the non-targeted therapies.

Since both CVP and CHOP chemotherapies in combination with rituximab represent first-line treatments for FL and DLBCL, respectively, any experimental anti-lymphoma therapeutic would be required to demonstrate antitumor activity in patients that had failed the above first-line chemotherapy. CD20-specific rituximab represents an immunotherapeutic agent with a very distinct mechanism of action from that of CHOP chemotherapy. Our previous preclinical study [8] has demonstrated that CMC-544, as a CD22-targeted chemotherapeutic agent, can provide supra-additive therapeutic activity when used in combination with rituximab. We examined whether the tumors that had initially responded to the CHOP or CVP chemotherapy and subsequently relapsed would still respond to CMC-544. The present study demonstrates that BCL xenografts that had relapsed in response to the CHOP or CVP therapy remained refractory to subsequent CHOP or CVP therapy. However, both CHOP- and CVP-refractory tumors retained their susceptibility to CMC-544. This preclinical evidence of the efficacy of CMC-544 against these chemotherapy refractory B-cell lymphoma xenografts is consistent with the clinical activity of CMC-544 observed in phase I studies in patients with B-NHL who had failed multiple chemotherapies [9–12].

In spite of the fact that the treatment with CMC-544 caused regression of CHOP- and CVP-refractory tumors, the duration CMC-544's effect was not as sustained as that previously observed in the treatment of naïve BCL xenografts [4]. This observation suggests that even if treatment with CMC-544 is effective in causing regression of the

CHOP- and-CVP-refractory tumors, some BCL cells can escape the full anti-tumor activity of the above treatment and re-establish themselves.

The preclinical demonstration of superior antitumor activity of CMC-544 over either CVP or CHOP raises an interesting possibility of using CMC-544 in combination with either of these chemotherapies. We further evaluated preclinically whether CMC-544, at suboptimal dosages, can be added to CVP or CHOP chemotherapy to gain greater antitumor efficacy. The preliminary assessment suggested that the addition of CMC-544 to CHOP but not CVP was toxic in nude mice (data not shown). This effect could be due to the combination of the two potent DNA-damaging agents, doxorubicin and calicheamicin in the mouse. Hence, combination studies were only carried out with the CVP + CMC-544 combination. In this evaluation, the CVP treatment at MTD was not as effective as suboptimal dosages of CMC-544 (Fig. 4). However, when used together, the CVP + CMC-544 combination resulted in stronger growth inhibition of BCL xenografts and significantly prolonged relapse-free survival of tumor-bearing mice. These results suggest that CMC-544 can be used in combination with the CVP chemotherapy and support clinical evaluation of this combination in patients with FL.

CHOP chemotherapy represents the first-line therapy for patients with DLBCL [13]. CMC-544, therefore, might be used in combination with CHOP in DLBCL patients. However, as noted above, our preliminary studies suggested that the simultaneous use of CHOP and CMC-544 in mice can lead to toxicities. In order to derive the therapeutic benefit of both CHOP and CMC-544 without associated toxicities, we assessed the antitumor efficacy of CHOP and CMC-544 by alternating 4 days apart (dose-dense) or weekly treatment with either CHOP or CMC-544. The dose-dense sequential treatment with 2 dosages of CHOP and CMC-544 was more therapeutically effective than the similar treatment with weekly administration of each agent. There was no evidence of toxicities with either dosing schedule based on the gain in mouse body weights. Mylotarg, the CD33-targeted calicheamicin conjugate [20], has been added to existing chemotherapeutic therapies in AML in a sequential manner demonstrating the feasibility of this approach [21, 22]. The experiments presented here suggest that CMC-544 can be used in combination with the CHOP chemotherapy as alternating treatments.

CMC-544 is currently in expanded clinical trials as monotherapy and in combination with rituximab for follicular and DLBCL. In the phase I reported results, CMC-544, administered at 1.8 mg G544 Ab/m$^2$ every 4 weeks as monotherapy to relapsed/refractory follicular and DLBCL patients, produced an objective response rate of 69 and 33%, respectively [10]. The overall safety profile was manageable, with thrombocytopenia and neutropenia as the most severe toxicities observed with a frequency >10%. In combination with rituximab, where rituximab was administered on day 1 and CMC-544 given 24 h later, the 6-month progression-free survival of patients who had relapsed after 1 or 2 prior therapies was 100% for follicular and 66% for DLBCL [12]. The main toxicity was self-limiting thrombocytopenia. As demonstrated by these clinical studies, CMC-544, as a tumor-targeted agent, may have a better safety/tolerability profile than the currently used chemotherapeutic agents (e.g. no anthracycline-induced cardiomyopathy, cytoxan-induced hemorrhagic cystitis, or vincristine-induced peripheral neuropathy or alopecia, etc.). Certainly, the side effect profile of CMC-544 differs from the above cited chemotherapeutic agents and would allow its combination with agents with non-overlapping toxicities. The efficacy of CMC-544 in follicular and DLBCL is promising and suggests durable responses in these hard-to-treat refractory patients. Clinical studies pursuing the use of CMC-544 as monotherapy and in combination with rituximab are continuing. The preclinical results provided here suggest that CMC-544 can be combined with the standard chemotherapy regimens for use in for follicular and DLBCL.

## References


1. Damle NK (2004) Tumor-targeted chemotherapy with immunoconjugates of calicheamicin. Expert Opin Biol Ther 4:1445–1452
2. Bross PF, Beitz J, Chen G et al (2001) Gemtuzumab ozogamicin: approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. Clin Cancer Res 7:1490–1496
3. Ghobrial I, Witzig T (2004) Radioimmunotherapy: a new treatment modality for B-cell non-Hodgkin's lymphoma. Oncology 18:623–630
4. DiJoseph JF, Armellino DC, Boghaert E et al (2004) Antibody-targeted chemotherapy with CMC-544: a CD22-targeted immunoconjugate of calicheamicin for the treatment of B lymphoid malignancies. Blood 103:1807–1814
5. Zein N, Sinha A, McGahren W, Ellestad G (1988) Calicheamicin γI: an antitumor antibiotic that cleaves double-stranded DNA site specifically. Science 240:1198–1201
6. Hamann PR, Hinman LM, Beyer CF et al (2002) An anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Choice of linker. Bioconj Chem 13:40–46
7. DiJoseph JF, Goad ME, Dougher MM et al (2004) Potent and specific anti-tumor efficacy of CMC-544, a CD22-targeted immunoconjugate of calicheamicin, against systemically disseminated B-cell lymphoma. Clin Cancer Res 10:8620–8629
8. DiJoseph JF, Dougher MM, Kalyandrug LB et al (2006) 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. Clin Cancer Res 12:242–249
9. Advani A, Gisselbrecht EGC, Rohatiner A et al (2005) Preliminary report of a phase 1 study of CMC-544, an antibody-targeted chemotherapy agent, in patients with B-cell non Hodgkin's lymphoma (NHL). Blood 106:230a
10. Fayad L, Patel H, Verhoef G et al (2006) Clinical activity of the immunoconjugate CMC-544 in B-cell malignancies: Preliminary

report of the expanded maximum tolerated dose (MTD) cohort of a phase 1 study. Blood 108:766a
11. Tobinai K, Ogura M, Hatake K et al (2008) Phase I pharmacokinetic study of inotuzumab ozogamicin (CMC-544) as a single agent in Japanese patients with follicular lymphoma pretreated with rituximab. Blood 112:1565
12. Fayad L, Patel H, Verhoef G et al (2008) Safety, clinical activity of the anti-CD22 immunoconjugate inotuzumab ozogamicin (CMC-544) in combination with rituximab in follicular lymphoma or diffuse large B-cell lymphoma: preliminary report of a phase 1/2 study. Blood 112:266
13. Fisher RI, Gaynor ER, Dalhberg S et al (1993) Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. N Engl J Med 328:1002–1006
14. McKelevet EM, Gottlieb JA, Wilson HE et al (1976) Hydroxyldaunomycin (adriamycin) combination chemotherapy in malignant lymphoma. Cancer 38:1484–1493
15. Coiffier B, Lepage E, Briere J et al (2002) CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med 346:235–242
16. DiJoseph JF, Popplewell A, Tickle S et al (2005) Antibody-targeted chemotherapy of B-cell lymphoma using calicheamicin conjugated to murine or humanized antibody against CD22. Cancer Immunol Immunother 54:11–24
17. Mohammad RM, Wall NR, Dutcher JA, Al-Katib AM (2000) The addition of bryostatin 1 to cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) chemotherapy improves response in a CHOP-resistant human diffuse large cell lymphoma xenografts model. Clin Cancer Res 6:4950–4956
18. Jenkins JS, Sampson PA (1967) Conversion of cortisone to cortisol and prednisone to prednisolone. Br Med J 2:205–207
19. Flavell DJ, Boehm DA, Emery L, Noss A, Ramsey A, Flavell SU (1995) Therapy of human B-cell lymphoma bearing SCID mice is more effective with anti-CD19-and anti-CD38-saporin immunotoxins used alone in combination than with either immunotoxin alone. Int J Cancer 62:337–344
20. Hamann P, Hinman L, Hollander I et al (2002) Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Bioconj Chem 13:47–58
21. Clavio M, Albarello A, Vignolo L (2007) Adding low-dose gemtuzumab ozogamicin to fludarabine, Aca-C and idarubicin (MY-FLAI) may improve disease-free and overall survival in elderly patients with non-M3 acute myeloid leukemia: results of a prospective, pilot, multi-centre trial and comparison with a historical cohort of patients. Br J Haematol 138:186–195
22. Burnett AK, Kell WJ, Goldstone AH et al (2006) The addition of gemtuzumab ozogamicin to induction chemotherapy for AML improves disease free survival without extra toxicity. Preliminary analysis of 1115 patients in the MRC AML15 trial. Blood 13:8a