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Distinct Apoptotic Signaling Characteristics of the Anti-CD40 Monoclonal Antibody Dacetuzumab and Rituximab Produce Enhanced Antitumor Activity in Non-Hodgkin Lymphoma

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Abstract

Purpose: Individually targeting B-cell antigens with monoclonal antibody therapeutics has improved the treatment of non-Hodgkin lymphoma (NHL). We examined if the antitumor activity of rituximab, CD20-specific antibody, could be improved by simultaneously targeting CD40 with the humanized monoclonal antibody dacetuzumab (SGN-40).

Experimental Design: Dacetuzumab was dosed with rituximab to determine the *in vivo* activity of this combination in a subcutaneous Ramos xenograft model of non-Hodgkin lymphoma (NHL). The effect of dacetuzumab on rituximab antibody-dependent cell mediated–cytotoxicity (ADCC), antiproliferative, and apoptotic activities were evaluated *in vitro* using NHL cell lines. Western blotting and flow cytometry were used to contrast the signaling pathways activated by dacetuzumab and rituximab in NHL cells.

Results: The dacetuzumab-rituximab combination had significantly improved antitumor activity over the equivalent dose of rituximab in the Ramos xenograft model ($P = 0.0021$). Dacetuzumab did not augment rituximab-mediated ADCC activity; however, these antibodies were additive to synergistic in cell-proliferation assays and produced increased apoptosis in combination. Rituximab signaling downregulated BCL-6 oncoprotein in a cell line–specific manner, whereas dacetuzumab strongly downregulated BCL-6 in each cell line. Dacetuzumab induced expression of the proapoptotic proteins TAp63 and Fas, whereas rituximab did not affect basal expression of either protein. Finally, rituximab partially blocked dacetuzumab-mediated upregulation of the prosurvival protein BCL-x_L.

Conclusions: Targeting CD40 with dacetuzumab enhanced the antitumor activity of rituximab in cell line and xenograft NHL models. The distinct but complementary apoptotic signal transduction profiles of dacetuzumab and rituximab are an important mechanism behind the improved activity of this combination. *Clin Cancer Res; 17(14); 4672–81. ©2011 AACR.*

Introduction

Non-Hodgkin lymphoma (NHL) is a heterogeneous group of B-cell malignancies with a considerable amount of genetic diversity, reflecting the differentiation status at the time of lymphocyte transformation (1). The majority of NHL cases are classified as aggressive diffuse large B-cell lymphoma (DLBCL), whereas follicular lymphoma (FL) is the most common subtype of indolent lymphoma. Targeting lymphomas with monoclonal antibodies has proven to be a productive therapeutic approach, and monoclonal antibodies specific for CD20, CD22, CD23, CD30, CD40, CD52, CD74, CD80, TRAIL, and VEGF are currently being tested in the clinic as unconjugated antibodies, antibody drug conjugates, or radioimmunotherapies (2, 3).

The anti-CD20 monoclonal antibody, rituximab, was the first of these to receive regulatory approval, and has significantly improved treatment of NHL. Rituximab's primary mechanism of action is effector function-based, and it is able to mediate antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and antibody dependent cellular phagocytosis (ADCP) (4). In addition to effector function activity, rituximab is a signaling antibody capable of suppressing antiapopototic pathways and chemosensitizing drug-resistant NHL cells (5–7). In the treatment of NHL, rituximab is used either as a monotherapy or in combination with chemotherapy such

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Translational Relevance

This study evaluates the potential benefit of combining the CD40-targeting monoclonal antibody dacetuzumab with rituximab in the treatment of non-Hodgkin lymphoma (NHL). Dacetuzumab mediates effector function (ADCC and ADCP), but also activates antiproliferative and proapoptotic signaling on NHL cells. Dacetuzumab is currently being evaluated in clinical trials for the treatment of NHL. In the present study, dacetuzumab was shown to enhance the antitumor activity of rituximab in NHL cell line and xenograft models. Both rituximab and dacetuzumab activated MAP kinase signaling and downregulated BCL-6 oncoprotein, which is frequently translocated and hypermutated in NHL. Dacetuzumab alone upregulated the proapoptotic p53 family member TAp63α, a suppressor of tumorigenesis. Rituximab and dacetuzumab signaling had inverse effects on the expression of Fas and BCL-xL. Our data suggest that antibody-mediated signaling through both CD20 and CD40 may be an effective strategy in the treatment of NHL.

as cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) for DLBCL treatment or cyclophosphamide, vincristine, prednisone (CVP) for follicular lymphoma (8–10).

Whereas response rates for rituximab-based therapies are high in NHL, some patients are refractory to rituximab and relapse following treatment is common, particularly in indolent lymphoma (11). Resistance to rituximab has been linked to overexpression of the complement inhibitor CD59 and the antiapoptotic proteins Bcl-X_L and Ying Yang 1 (YY1) (12). Downregulation of CD20 expression has also been reported in B-cell lymphomas following treatment with rituximab combination therapies (13). This unmet medical need warrants the development of therapeutic antibodies targeting other lymphoma cell surface antigens to supplement current NHL therapeutic regimens.

The tumor necrosis factor (TNF) receptor superfamily member, CD40, is continually expressed on developing B-cells from the pro-B phase through differentiation into memory B-cells and plasma cells (14, 15). CD40 is also expressed on a variety of cells types other than lymphocytes, including dendritic cells, monocytes, and macrophages. The disease expression profile of CD40 is broader than that of CD20, and includes NHL, Hodgkin lymphoma, multiple myeloma, acute lymphoblastic leukemia, and numerous carcinomas (16–19). Binding of CD40 to its ligand (CD40L) produces a growth and differentiation signal in developing B lymphocytes via activation of the NF-κB, MAP kinase (MAPK), PI3K, and PLC-γ signal transduction pathways (20–22). As with other TNFR family members, activation of CD40 signaling in certain cancers such as aggressive NHL produces an apoptotic or antiproliferative response (19).

The humanized anti-CD40 monoclonal antibody, dacetuzumab (SGN-40), has antiproliferative and apoptotic activity against a panel of high-grade B-lymphoma cell lines (23). Dacetuzumab initiates signaling in lymphoma cells following binding to CD40, activating the ERK1/2, JNK, p38 MAP kinase, and NF-κB signal transduction pathways (24). Constitutive activation of these pathways downregulates B-cell lymphoma 6 (BCL-6) and c-Myc oncoproteins in lymphoma cell lines, whereas the proapoptotic p53 family member TAp63α, Fas, and Bim are upregulated (24). In addition, dacetuzumab is an IgG_1 antibody capable of mediating antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) effector function activities (23, 25). Dacetuzumab does not mediate CDC.

The mechanisms of action of rituximab and dacetuzumab have not been contrasted, and the potential to improve rituximab's activity in NHL with an antibody targeting CD40 has not been explored. In this study, we examined the signaling and effector function characteristics of dacetuzumab and rituximab alone and in combination. Combination index analysis determined that dacetuzumab is additive to synergistic with rituximab in NHL cell lines in an *in vitro* proliferation assay. Furthermore, we found that dacetuzumab improved the antitumor activity of rituximab in a xenograft model of NHL. The combined antitumor activity of these two antibodies *in vitro* was not due to enhanced effector function, rather the relative ability of these antibodies to activate apoptotic signaling pathways correlated with potency. Both dacetuzumab and rituximab were found to negatively regulate the prosurvival BCL-6 oncoprotein in a cell line–specific manner; however, only dacetuzumab upregulated the proapoptotic p53 family member TAp63α and Fas death receptor. The differential apoptotic signaling characteristics of dacetuzumab and rituximab are likely to be significant to the activity of this antibody combination in NHL.

Materials and Methods

Cell culture

Raji, Ramos, RL, SU-DHL-4, and WIL2-S lymphoma cell lines were obtained from American Type Culture Collection and cultured in RPMI 1640 media supplemented with 10% fetal bovine serum.

Non-Hodgkin lymphoma *in vivo* studies

Severe combined immunodeficient mice (SCID) mice were subcutaneously implanted with 5×10^6 Ramos cells and dosed when average tumor volume reached 100 mm^3 ($n = 10$ mice/group). Dacetuzumab and rituximab were dosed at 4.0 mg/kg and 8.0 mg/kg in the Ramos model [3 times weekly (mwf) for 3 weeks; ip] as single agents or in combination. Tumor progression rates were reported as percent of mice having a tumor volume less than 1,000 mm^3 using Kaplan–Meier

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plots generated in Prism (GraphPad Software). *P*-values were generated using the log-rank (Mantel–Cox) test.

Antibody-dependent cell mediated-cytotoxicity and apoptosis assays

ADCC was measured using a standard ^{51}Cr release assay. Target tumor cells were labeled for 1 hour with 100 μCi $Na_2{}^{51}CrO_4$, washed, and 5,000 cells/well plated in RPMI +1% FBS. Effector cells were isolated from the leukapheresis product (Lifeblood Biological Services) of a normal FcγRIII-158V donor. Leukocytes were enriched by centrifugation over a Ficoll–Paque density gradient (Amersham) and nonadherent cells were collected and enriched for $CD16^+$ cells by negative depletion of $CD4^+$, $CD8^+$, $CD20^+$, and $CD14^+$ cells using immunomagnetic beads (Dynal). The effector cells were added at an effector:target cell ratio of 10 $CD16^+$ cells per target cell. After a 4-hour incubation, the ^{51}Cr released from lysed cells was measured and the percent specific lysis calculated as (test sample cpm–spontaneous cpm)/(total cpm–spontaneous cpm) × 100.

Ramos cells (RPMI +5% FBS) were treated 72 hours with rituximab, dacetuzumab, or control hIgG crosslinked (2:1 ratio) with a Fcγ-specific goat antihuman IgG $F(ab')_2$ fragment (Jackson ImmunoResearch). Cell death was measured by flow cytometry using the annexin V-FITC apoptosis detection kit (EMD Biosciences).

In vitro combination index analysis

Crosslinked control hIgG, dacetuzumab, and rituximab were serially diluted 2-fold then Ramos, RL, and SU-DHL-4 cells were dosed in quadruplicate. In the dacetuzumab-rituximab combination treatment, each antibody was crosslinked separately then added to cells simultaneously. Cells were treated for 96 hours, then labeled 4 hours with ^3H-thymidine, and DNA incorporated radioactivity was measured with a TopCount scintillation counter (Perkin Elmer). Dose effect curves were generated and IC_{50} values calculated with standard error of the mean (sem) reported ($n = 3$). Combination indices (CI) were calculated with the software program Calcusyn (BioSoft) at the EC_{50}, EC_{75}, or EC_{90} with sem reported ($n = 3$).

Signal transduction experiments

Ramos, RL, and SU-DHL-4 cells were treated for 48 hours with 1.0 μg/mL crosslinked hIgG, rituximab, dacetuzumab, or dacetuzumab–rituximab combined. Harvested cells were lysed on ice in protein extraction buffer [50 mmol/L Tris (pH = 7.5), 1% NP-40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA (pH = 7.6), phosphatase inhibitors (EMD Biosciences), and protease inhibitors (Rocherland)], centrifuged, and protein concentrations measured. Western blotting with antibodies specific for phospho-p44/42 MAPK (Thr202/Tyr204), phospho-JNK MAPK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), BCL-xL, IκBα, BCL-2, β-actin (Cell Signaling), BCL-6 (Abcam 1:500), and TAp63α (BD Pharmingen) was executed using Super-Signal West Pico Chemiluminescent Substrate (Thermo Scientific).

Flow cytometry of fas (CD95)

Ramos, RL, and SU-DHL-4 cells were treated for 48 hours with 1.0 μg/mL crosslinked hIgG, rituximab, dacetuzumab, or dacetuzumab-rituximab combined. Cells were harvested, washed with FACs buffer (PBS + 2% FBS + 0.02% azide), and stained with PE-labeled anti-Fas (CD95) antibody (BD Biosciences). Fas expression was analyzed using a FACSCalibur flow cytometer (BD Biosciences) and fold induction over hIgG control reported.

Results

Dacetuzumab augments rituximab activity *in vivo*

Previous studies have shown *in vivo* activity of dacetuzumab in xenograft models of lymphoma (23); however, its activity has not been examined in combination with the current standard of care in NHL, rituximab. We evaluated the antitumor activity of dacetuzumab and rituximab as single agents or in combination in the subcutaneous Ramos (Burkitt's lymphoma) xenograft model (Fig. 1). As a monotherapy, dacetuzumab produced a greater tumor growth delay than rituximab (days 20–50 post dosing) at both 4.0 mg/kg and 8.0 mg/kg concentrations in the Ramos model. However, the combined activity of dacetuzumab and rituximab (4.0 mg/kg each) was significantly greater than that of dacetuzumab or rituximab alone at the 8.0 mg/kg dosing level (*P*-value of 0.0041 and 0.0021, respectively). The median time to reach the 1,000 mL tumor volume endpoint was 58.7 days for dacetuzumab (8.0 mg/kg), 34.1 days for rituximab (8.0 mg/kg), and >100 days for the dacetuzumab plus rituximab combination

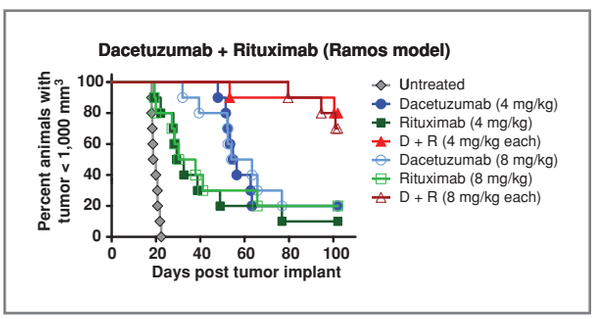

Figure 1. Dacetuzumab improves the activity of rituximab *in vivo*. SCID mice were subcutaneously implanted with 5×10^6 Ramos cells, and dosed when average tumor size reached 100 mm^3 ($n = 10$ mice/group). Dacetuzumab and rituximab were dosed as single agents or in combination (D + R) at 4.0 mg/kg or 8.0 mg/kg [q2d (mwf) × 3 weeks, 9 doses total, ip]. Tumor growth was plotted as percent of mice in each group with <1,000 mm^3 tumor volume using a Kaplan–Meier plot. Combination of dacetuzumab with rituximab (4.0 mg/kg each) significantly improved activity over dacetuzumab or rituximab alone at the 8.0 mg/kg dose level ($P = 0.0041$ or 0.0021, respectively by log-rank test).

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(4.0 mg/kg each). Furthermore, the *in vivo* activity of the dacetuzumab–rituximab combination appears to be greater than additive in this model. Dacetuzumab similarly augmented rituximab activity in the U698M lymphoma xenograft model (Supplementary Fig. S1). These studies showed that dacetuzumab is capable of improving the *in vivo* activity of rituximab, and suggests these antibodies have distinct but complementary tumor cell–killing mechanisms of action.

Apoptotic signaling, not antibody-dependent cell mediated-cytotoxicity, is enhanced in the dacetuzumab-rituximab combination

Both dacetuzumab and rituximab have well characterized ADCC effector function activity against NHL cells. To test whether the improved activity of the dacetuzumab–rituximab combination is a result of enhanced recruitment of $CD16^+$ effector cells to target lymphoma cells, we examined the *in vitro* ADCC activity of both antibodies alone and in combination. Raji and WIL2-S target cells were selected, because CD20 and CD40 expression levels are high enough to achieve comparable ADCC activity between dacetuzumab and rituximab. In the Raji target cell-line dacetuzumab and rituximab produced equivalent specific cell lysis at saturating antibody concentrations, whereas dacetuzumab showed greater activity at concentrations below 0.1 μg/mL (Fig. 2A). The dacetuzumab–rituximab combination did not produce a substantial improvement in specific cell lysis *versus* dacetuzumab alone. In the WIL2-S target cell-line rituximab produced a greater specific cell lysis relative to dacetuzumab (Fig. 2A). Combining dacetuzumab with rituximab did not improve the ADCC activity over rituximab alone, except for a modest effect at antibody concentrations below 0.01 μg/mL. Similar findings were made using Ramos and SU-DHL-4 as target cell lines (Supplementary Fig. S2). These data suggest that the improved activity of dacetuzumab–rituximab combination *in vivo* is not due to the combined ADCC activity of these antibodies. ADCP activity was also not improved with the dacetuzumab–rituximab combination (data not shown).

In addition to effector function activity, dacetuzumab and rituximab can mediate antiproliferative and apoptotic responses in cultured NHL cell lines via signaling through CD40 and CD20, respectively. We therefore evaluated if apoptotic signaling *in vitro* could be enhanced by combining dacetuzumab with rituximab. Ramos cells were treated with Fc-crosslinked antibodies at 1.0 μg/mL final concentration in media for 72 hours, then assayed for total cell death (apoptosis and necrosis) by flow cytometry (Fig. 2B). Rituximab and dacetuzumab alone produced a 2.0- and 2.7-fold increase in cell death over antibody control, respectively. In contrast, the dacetuzumab–rituximab combination produced a 3.9-fold increase in cell death over hIgG control at both 0.5 and 1.0 μg/mL mAb concentrations. These data suggest that dacetuzumab and rituximab stimulate complementary proapoptotic signaling events *in vitro*, capable of producing a stronger antitumor response when simultaneously dosed.

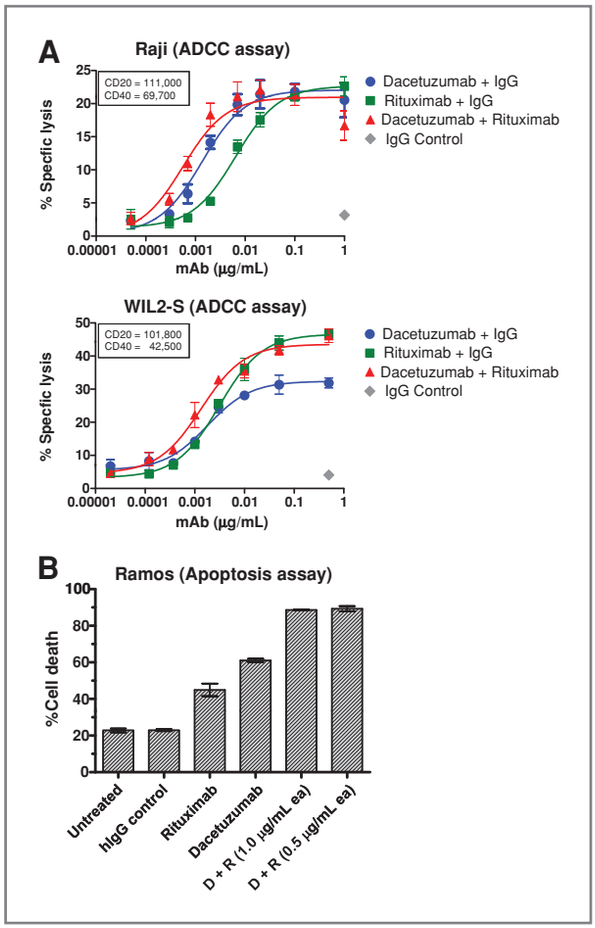

Figure 2. Dacetuzumab augments rituximab apoptotic signaling, not ADCC. A, in an ADCC assay target cells Raji and WIL2-S were labelled with ^{51}Cr, treated with dacetuzumab, rituximab, or control hIgG, then incubated with NK enriched PBMCs (effector target ratio of 10:1). After 4 hours supernatants were collected and percent cell lysis determined. CD20 and CD40 receptor densities per cell are indicated in the inset boxes. B, Ramos cells were treated with crosslinked rituximab, dacetuzumab, and hIgG control alone or in combination (D + R) for 72 hours (1.0 μg/mL). A reduced antibody concentration (0.5 μg/mL) D + R combination group was included to control for total antibody dose. Cell death (apoptotic and necrotic) was measured by flow cytometry of annexin V-FITC and propidium iodide staining, with standard error of the mean (sem) reported.

Dacetuzumab and rituximab antiproliferative activity is additive to synergistic

Next, we quantitated the relative potencies of dacetuzumab and rituximab, alone and in combination, by measuring the antiproliferative activity of these mAbs across a panel of NHL cell lines (Fig. 3A). In each of the three cell lines examined (Ramos, RL, and SU-DHL-4), dacetuzumab had substantially greater antitumor activity than

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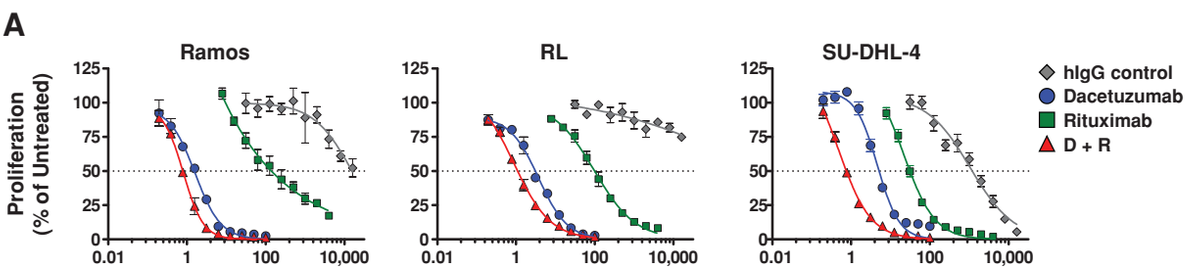
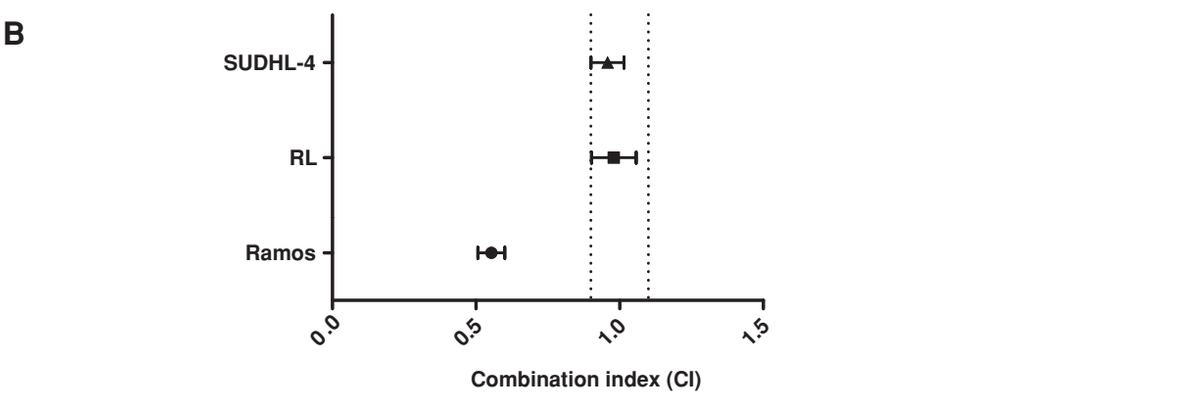


Cell line	CD20 receptor number	CD40 receptor number	Lymphoma subtype	Dacetuzumab (IC_{50} ng/mL)	Rituximab (IC_{50} ng/mL)	D + R (IC_{50} ng/mL)
Ramos	51,406	13,566	Burkitt's	1.45 ± 0.09	> 500	0.86 ± 0.11
RL	17,986	81,502	DLBCL	3.29 ± 0.06	> 500	1.92 ± 0.45
SU-DHL-4	98,273	26,396	DLBCL	5.64 ± 0.75	77.6 ± 34.3	1.86 ± 0.49



Cell line	Combination index (EC_{50})	Combination index (EC_{75})	Combination index (EC_{90})	Combined activity
Ramos	0.47 ± 0.07	0.50 ± 0.03	0.55 ± 0.05	Synergism
RL	1.02 ± 0.13	0.99 ± 0.09	0.98 ± 0.08	Additive
SU-DHL-4	1.15 ± 0.01	1.04 ± 0.04	0.96 ± 0.06	Additive

Figure 3. Dacetuzumab is additive to synergistic with rituximab *in vitro*. A, Ramos, RL, and SU-DHL-4 cell lines were treated with a 2-fold dilution series of dacetuzumab ± rituximab crosslinked with goat antihuman Fcγ chain F(ab')2 fragment (1:4 ratio). Cells were incubated for 96 hours, then labeled with ^{3}H-thymidine to measure antiproliferative effect. Dose response curves were produced and IC_{50} values determined from quadruplicate data points. B, dose effect curves were used to derive combination index (CI) values with the Calcusyn software package. CI values derived at the EC_{90} of dose effect curves are plotted for each cell line ($n = 3$). CI < 0.9 is synergistic, CI > 1.1 is antagonistic, and CI 0.9–1.1 is additive.

rituximab, which showed the greatest potency in the germinal center-derived BCL-6$^+$ cell line SU-DHL-4. The dacetuzumab–rituximab treatment group had greater activity than either antibody alone in all cell lines. To determine if dacetuzumab is additive or synergistic with rituximab, combination index analysis was carried out

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using the Chou–Talalay method (26). Dacetuzumab was found to be synergistic with rituximab in the Ramos cell line and additive in the RL and SU-DHL-4 cell lines (Fig. 3B). Thus, the combinatorial activity of dacetuzumab and rituximab observed *in vivo* is likely due to the cell-signaling events activated at the cell surface following antibody binding.

Dacetuzumab and rituximab have cell line–specific differences in signaling potential

The antiproliferative and apoptotic activities of dacetuzumab and rituximab are initiated following antibody binding to CD40 and CD20, respectively, on the lymphoma cell surface, which initiates intracellular signal transduction events. We have previously shown that activation of the NF-κB and MAP kinase pathways is important to the cytotoxicity of dacetuzumab in cultured NHL cell lines (24). Therefore, we contrasted the relative ability of dacetuzumab to activate signal transduction through these signaling cascades with that of rituximab (Fig. 4). In the Ramos cell line, dacetuzumab strongly activated the ERK1/2, JNK, and p38 MAP kinase pathways 48-hours posttreatment (Fig. 4, lane 3) in addition to the NF-κB pathway, as indicated by the degradation of IκBα inhibitory protein. In contrast, rituximab did not activate the ERK1/2 MAPK or NF-κB pathways in Ramos cells, and only weakly activated the JNK and p38 MAPK pathways (Fig. 4, lane 4). Dacetuzumab and rituximab had similar signaling profiles in the DLBCL cell lines RL and SU-DHL-4, with both antibodies activating the three MAPK cascades and the NF-κB pathway. Dacetuzumab activated the ERK1/2 MAPK, JNK MAPK, and NF-κB pathways noticeably more than rituximab in these two cell lines. Lymphoma cells treated with both dacetuzumab and rituximab had a signaling pattern nearly identical to dacetuzumab alone in terms of pathways activated and signaling intensity (Fig. 4, lane 5). These data indicate that dacetuzumab and rituximab are able to activate overlapping signal transduction pathways, but differ in the intensity of signaling produced in a cell line–specific manner. Interestingly, dacetuzumab and rituximab were synergistic in the Ramos cell line where their signaling patterns were distinct and additive in the RL and SU-DHL-4 cell lines where their signaling pathways overlapped.

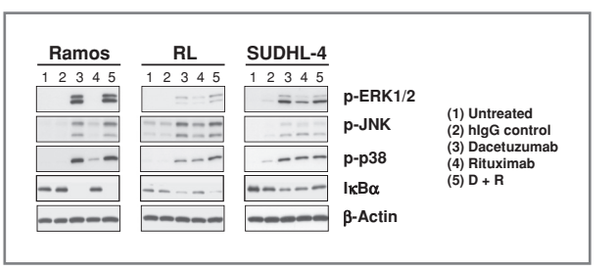

Figure 4. Dacetuzumab and rituximab show cell line-specific differences in MAPK and NF-κB signaling. Ramos, RL, and SU-DHL-4 cell lines were treated with crosslinked dacetuzumab ± rituximab for 48 hours (1.0 μg/mL). Activation of MAP Kinase pathways was analyzed by western blotting with phospho-specific antibodies recognizing phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), and phospho-p38 (Thr180/Tyr182). NF-κB pathway activation was monitored by detecting degradation of the negative regulatory protein IκBα. β-actin was included as a protein load control.

Dacetuzumab and rituximab activate distinct apoptotic signaling pathways in non-Hodgkin lymphoma cells

Our group has previously reported that dacetuzumab causes a sustained downregulation of the BCL-6 oncoprotein and upregulates the proapoptotic p53 family member TAp63α in NHL cell lines (24). Because rituximab and dacetuzumab activate similar signaling pathways in 2/3 cell lines, we evaluated the ability of rituximab to affect BCL-6 and TAp63α expression (Fig. 5A). Dacetuzumab strongly upregulated TAp63α protein in Ramos cells and weakly upregulated it in the RL and SU-DHL-4 cell lines (Fig. 5A, lane 3), whereas rituximab did not affect expression of this transcriptional regulator (Fig. 5A, lane 4). In contrast, both rituximab and dacetuzumab were able to downregulate BCL-6 protein in a cell line–specific manner correlating with their ability to activate MAPK pathways. Rituximab did not affect BCL-6 expression in the Ramos cell line where it does not substantially activate MAPK signaling, whereas BCL-6 was downregulated following rituximab treatment in the RL and SU-DHL-4 cell lines where MAPK signaling was activated (Fig. 5A, lane 4). Dacetuzumab downregulated BCL-6 protein in all three lymphoma lines, mirroring its ability to activate all MAPK cascades in each cell line (Fig. 5A, lane 3).

One mechanism by which rituximab reportedly sensitizes lymphoma cells to apoptosis is by downregulation of the prosurvival protein BCL-xL (5, 7). In our hands, rituximab did not affect basal levels of BCL-xL; however, dacetuzumab upregulated this antiapoptotic BCL-2 family member in the RL and SU-DHL-4 cell lines (Fig. 5A, lane 3). Surprisingly, rituximab partially blocked the dacetuzumab-mediated upregulation of BCL-xL in the combination treatment group (Fig. 5A**,** lane 5) in both of these cell lines. In contrast, BCL-2 expression was unaffected by both antibodies in the RL and SU-DHL-4 cell lines. The ability of rituximab to minimize the upregulation of BCL-xL likely potentiates the apoptotic activity of dacetuzumab in these two DLBCL cell lines.

Next, we compared the ability of dacetuzumab and rituximab to upregulate the proapoptotic death receptor Fas (CD95) on lymphoma cells. Basal expression levels of Fas were not detectable in Ramos or SU-DHL-4 cell lines by flow cytometry; however, the RL cell line has low levels of Fas on the cell surface (Fig. 5B). Dacetuzumab treatment produced an elevation in Fas expression above basal levels of 39-fold, 2.4-fold, and 6.6-fold in the Ramos, RL, and SU-DHL-4 cell lines, respectively.

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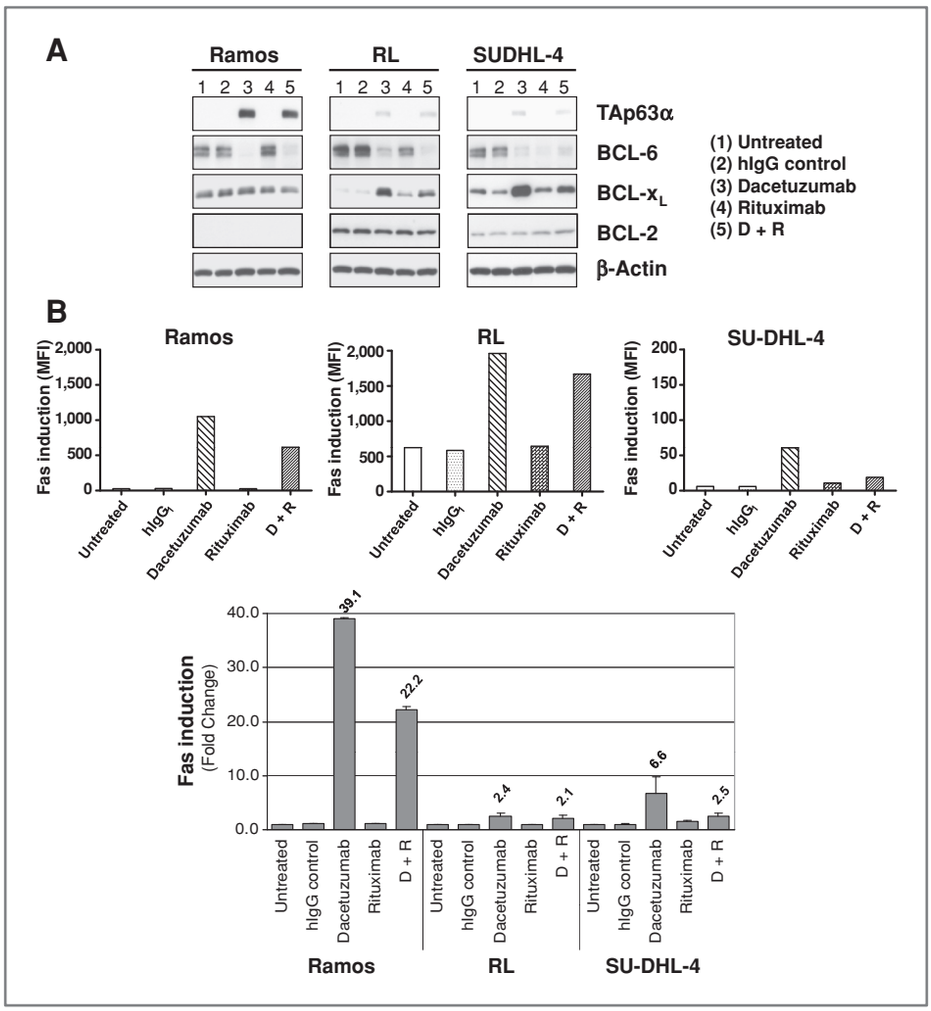

Figure 5. Dacetuzumab and rituximab differentially regulate apoptosis and prosurvival proteins. A, NHL cell lines were treated with crosslinked dacetuzumab ± rituximab for 48 hours (1.0 μg/mL), and cell extracts analyzed by western blotting for expression of the proapoptotic p53 family member TAp63α and the prosurvival proteins BCL-6, BCL-xL, and BCL-2. B, Ramos, RL, and SU-DHL-4 cell lines were treated with crosslinked dacetuzumab or rituximab (1.0 μg/mL) alone or in combination for 24 hours. Cells were stained with anti-Fas(CD95)-PE or mIgG$_1$ isotype control and analyzed by flow cytometry. Fas expression is plotted as mean fluorescence intensity (mfi) units (upper panels) from one representative experiment for each cell line. The fold increase in Fas expression over untreated cells is reported with standard deviation from $n = 2$ independent experiments (bottom).

Rituximab did not alter endogenous Fas expression levels, but it did partially attenuate dacetuzumab-mediated Fas induction in each cell line examined. Therefore, dacetuzumab and rituximab signaling may have an opposing effect on the regulation of the extrinsic apoptotic pathway through this death receptor.

Discussion

Given the genetic diversity of B-cell lymphomas and differential antigen expression patterns across lymphoma subtypes, it is unlikely that a single small molecule or antibody-based therapeutic will effectively treat all categories of NHL. Therefore, the use of therapeutic antibody combinations targeting different tumor antigens is expected to produce a more robust antitumor response. Simultaneously targeting CD20 and the TNFR family member CD40 may be productive, because both are expressed on the majority of B-cell lymphomas and mediate differential signaling events through their cytoplasmic domains. We evaluated the potential of improving rituximab-based therapies in NHL by targeting CD40 with dacetuzumab. *In vivo* analysis of the dacetuzumab–rituximab combination in the Ramos NHL xenograft model showed the capacity of dacetuzumab to augment rituximab activity. Potential mechanisms of action behind the ability of dacetuzumab to enhance rituximab activity *in vivo* include improved recruitment of effector cells to the tumor site and amplified cytotoxic signaling. Our data suggest that apoptotic signal transduction mechanisms are a greater contributor than effector function to the improved activity of the dacetuzumab–rituximab combination.

Rituximab has clearly established ADCC, CDC, and ADCP effector function activities on NHL cell lines and patient lymphoma B cells (27–30). The importance of ADCC for the *in vivo* and clinical activity of rituximab is highlighted by studies showing loss of efficacy in FcγR knock-out mice and the observation that NHL patients expressing the FcγRIIIa 158 valine/valine polymorphism have a greater response rate to rituximab (31, 32). Dacetuzumab also has well characterized ADCC and ADCP

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effector activity (23, 25), but our data showed that the effector function activity is not significantly improved when these antibodies are combined. This suggested an alternative mechanism is behind the observed *in vivo* combination activity. A previous study comparing the activity of rituximab and dacetuzumab in a Ramos xenograft model found that rituximab lost activity in the NK cell-deficient SCID-beige mouse strain whereas dacetuzumab maintained potency in this background (23). This finding indicated that antiproliferative or apoptotic signaling, not just effector function, is an important mechanism of action for dacetuzumab *in vivo*. We found that combining dacetuzumab with rituximab *in vitro* increased apoptosis and that dacetuzumab and rituximab are additive to synergistic. This supports the hypothesis that dacetuzumab and rituximab activate complementary proapoptotic and antiproliferative signaling pathways to produce an enhanced antitumor response.

The unique signal transduction characteristics of CD20 and CD40 provide insight into the combination activity of dacetuzumab and rituximab. CD20 is a membrane-spanning 4 domain, subfamily A (MS4A) protein capable of associating with the Src family tyrosine kinases Lyn, Lck, and Fyn (33). Type I anti-CD20 antibodies including rituximab are able to stimulate calcium release, localization of CD20 to lipid raft domains, and PLCγ2 phosphorylation producing apoptosis in some B-lymphoma cell lines (34–37). In contrast, signaling through CD40 is mediated by TNFR-associated factors (TRAFs) 1–6 to activate signal transduction through the NF-κB, MAP kinase (ERK1/2, JNK, and p38), PI_3K, and PLC-γ pathways. We recently reported that dacetuzumab signals through the NF-κB and MAPK pathways in a sustained manner, producing antiproliferative and apoptotic responses in NHL cells (24). In the present study we found that dacetuzumab consistently activated NF-κB and MAP kinase signaling pathways in each NHL cell line examined, whereas the ability of rituximab to activate these pathways was cell line–specific. Significantly, the cytotoxic activity of rituximab was the greatest in cell lines where it activated the strongest signaling response (SU-DHL-4 and RL) and the weakest in the cell line where minimal signaling was observed (Ramos). The dual signaling inputs triggered by simultaneously binding both CD20 and CD40 with rituximab and dacetuzumab, respectively, may therefore explain the observed stronger antiproliferative and apoptotic activity of this combination.

Antibody-mediated signaling affected expression of key proteins involved in proliferation and survival in lymphoma. Both rituximab and dacetuzumab downregulated expression of BCL-6 oncoprotein, but in a cell line–specific manner. BCL-6 is a transcriptional repressor of the poxvirus and zinc finger (POZ–ZF) protein family, which regulates germinal center formation, plasma cell fate, and germ cell apoptosis in normal B-cell differentiation (38). In germinal center derived B cell (GCB) lymphomas BCL-6 expression is inappropriately upregulated by chromosomal translocation into Ig as well as non-Ig-encoding loci (39), and *in vivo* studies have shown that BCL-6 overexpression drives lymphomagenesis (40, 41). BCL-6 is a target of interest in lymphoma therapy and inhibitors of its transcriptional repressor activity are in development (42). We previously showed that dacetuzumab stimulation of ERK1/2 MAP kinase signaling causes the sustained degradation of BCL-6 protein through a proteasomal pathway, followed by growth arrest and apoptosis (24). Our study is the first report that rituximab can also downregulate this oncoprotein, suggesting that BCL-6 downregulation may be a common mechanism of action for antilymphoma antibodies capable of activating ERK1/2 MAPK signaling. This finding is of particular importance for the germinal center B-cell-like (GCB) subtype of NHL.

In addition to BCL-6, other regulators of cell survival were affected by the rituximab–dacetuzumab combination. TAp63α, a proapoptotic p53 family member capable of inducing senescence, mediating chemosensitization, and suppressing tumorigenesis and metastasis (43–47), is induced by dacetuzumab in NHL cell lines (24). Upregulation of TAp63α is likely to sensitize NHL cells to apoptotic stimuli by partially restoring p53 checkpoint activity. In this study only dacetuzumab upregulated TAp63α, whereas rituximab did not affect TAp63α protein levels in any of the NHL cell lines examined. Therefore, the ability of dacetuzumab to induce TAp63α expression is a major distinguishing characteristic and a likely determinant of additive or synergistic activity observed in combination studies.

Rituximab partially blocked the upregulation of the prosurvival Bcl-2 family member Bcl-xL by dacetuzumab in the RL and SU-DHL-4 cell lines. Rituximab has been reported to downregulate endogenous levels of Bcl-xL (5, 7); however, we observed only an attenuation of its upregulation by dacetuzumab. The ability of rituximab to minimize Bcl-xL induction by dacetuzumab may further sensitize cells to proapoptotic signaling, and provides another mechanism for the improved combination activity of these antibodies. In another example, illustrating their distinct signaling differences, the death receptor Fas was strongly upregulated by dacetuzumab, whereas rituximab did not affect basal levels of Fas expression. Interestingly, rituximab did partially attenuate dacetuzumab-mediated Fas induction. Both upregulation of Fas and decrease in Bcl-xL are likely to further contribute to lymphoma cell apoptosis *in vivo* with a dacetuzumab-rituximab combination therapy, provided that FAS ligand expressing cells are present.

Our data suggest that dacetuzumab may potentiate rituximab-based therapies in NHL patients through enhanced activation of apoptotic signaling. In the clinic dacetuzumab is well tolerated and has been dosed up to 8.0 mg/kg weekly in NHL patients with the most common adverse events being fatigue, pyrexia, and headache (48). Furthermore dacetuzumab monotherapy is active, producing objective responses in 6 (12%) of 50 patients with refractory or recurrent B-cell NHL.

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Disclosure of Potential Conflicts of Interest

All authors are employees and shareholders of Seattle Genetics, Inc.

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