

Synergistic Effects of the Fenretinide (4-HPR) and Anti-CD20 Monoclonal Antibodies on Apoptosis Induction of Malignant Human B Cells¹

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ABSTRACT

Retinoids have been shown to be clinically useful in the biological therapy of certain myeloid and T-cell malignancies, whereas CD20 has proven to be an effective target in B-cell lymphoma immunotherapy. Both retinoic acid derivatives and anti-CD20 monoclonal antibodies have also been shown to induce apoptosis of malignant cells *in vitro*. Retinoid-induced apoptosis is thought to be mediated by nuclear retinoid receptor binding and transcriptional activation, whereas CD20 ligation appears to initiate transmembrane Ca²⁺ influx with resultant programmed cell death. In this report, we evaluate the *in vitro* effects of *N*-(4-hydroxyphenyl) retinamide (4-HPR) with and without anti-CD20 antibodies in B-cell lymphoma lines. We demonstrate that 4-HPR inhibits the growth of malignant B-cells beyond that of all-*trans*-retinoic acid and 13-*cis*-retinoic acid. We also show that this 4-HPR-mediated growth inhibition is attributable to apoptosis, is consistent across a variety of malignant B-cell lines (Ramos, Ramos AW, SU-DHL4, and Raji), peaks at 96 to 144 h, and is attainable with concentrations as low as 2 μM. As with CD20-mediated apoptosis, we show that the final common pathway includes caspase activation that can be blocked by 2-val-Ala-Asp-fluoromethyl ketone (z-VAD), a specific inhibitor of caspase function. Coincubation of a 2 μM concentration of 4-HPR and the anti-CD20 antibodies rituximab and tositumomab exhibited a supra-additive increase in levels of apoptosis induction of 24%

($P = 0.009$) and 42% ($P = 0.0019$) relative to expected additive levels of these same agents. These *in vitro* findings suggest that the potential *in vivo* synergy of these well-tolerated drugs may augment the previously demonstrated clinical activity of anti-CD20 monoclonal antibodies in the treatment of B-cell malignancies.

INTRODUCTION

Retinoic acid derivatives have emerged as a group of promising new therapeutic agents for the treatment and prevention of a number of malignancies, including certain myeloid, T-lymphoid, and epithelial neoplasms (1–6). The retinoids, a family of vitamin A derivatives, are thought to function via two classes of nuclear retinoid receptors: the RARs⁵ and the RXRs (7). These receptors form stable heterodimers and homodimers to function as ligand-activated transcription factors regulating the transcription activity of target genes by binding to response elements specific for RARs or RXRs located in the promoter region of these genes. In the normal cell, endogenous retinoids and their receptors mediate the critical processes of proliferation, differentiation, and apoptosis (8).

Recently, studies have demonstrated that some of these vitamin A derivatives can also induce apoptosis in certain types of cancer cells (9). One particularly promising agent, the synthetic retinoid, 4-HPR, also known as fenretinide, has been observed to induce apoptosis in malignant hematopoietic, neuroblastoma, cervical, breast, ovarian, head and neck, and lung cancer cell lines, including those exhibiting resistance to the effects of the natural vitamin A metabolite ATRA (10–14). 4-HPR was specifically designed to have low clinical toxicity yet retain the therapeutic effects of other retinoic acid derivatives. Unlike the endogenous retinoids, 4-HPR binds poorly to the traditional RARs, and several alternative mechanisms of apoptosis induction have been proposed, including an increase of reactive oxygen species, a decrease of Bcl-2 expression, and most notably transglutaminase induction sensitizing cells to Ca²⁺-mediated apoptosis (13, 15–17). TTG is an intracellular protein cross-linking enzyme that performs a critical role in assembling the intracellular protein scaffold in cells undergoing apoptotic death (17, 18). TTG has been shown to be induced by retinoic acid derivatives by a predominantly RXR-mediated process and activated by Ca²⁺ influx (reviewed in Ref. 9).

Anti-CD20 antibodies have recently been reported to in-

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⁵ The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; 4-HPR, *N*-(4-hydroxyphenyl) retinamide; ATRA, all-*trans*-retinoic acid; TTG, tissue transglutaminase; mAb, monoclonal antibody; 13-*cis*-RA, 13-*cis*-retinoic acid; PARP, poly(ADP-ribose) polymerase; z-VAD, 2-val-Ala-Asp-fluoromethyl ketone.

duce apoptosis in malignant B-cells by several investigators (19–21). CD20 is B cell-specific regulator of transmembrane Ca^{2+} conductance and plays an important functional role in B-cell activation, proliferation, and differentiation (22). Our group has demonstrated that CD20 ligation induces a cascade of events including caspase activation and intracellular calcium influx with resultant typical programmed cell death, as evidenced by DNA fragmentation assays (20, 21). Not unexpectedly, these same anti-CD20 antibodies have also been shown to have clinical activity against CD20-expressing B-cell malignancies (23–26).

On the basis of the demonstrated mechanisms of apoptotic induction of 4-HPR and anti-CD20 antibody ligation, *i.e.*, the induction of TTG and the promotion of Ca^{2+} influx, respectively, we hypothesized that using these two agents in conjunction may result in synergistic levels of apoptosis. In this report we detail the dose-response growth inhibition of 4-HPR in relation to other commonly used retinoids and show that this inhibition correlates with apoptotic induction. Furthermore, we elaborate the time course of 4-HPR-induced apoptosis and demonstrate its consistent activity across a variety of previously unevaluated B-cell lymphoma lines. Most importantly, we illustrate the synergistic effects of anti-CD20 antibodies and 4-HPR with regard to apoptotic induction with the ultimate goal of eventually enhancing the clinical efficacy of these agents.

MATERIALS AND METHODS

Cells. The CD20-expressing human Burkitt's lymphoma cell lines Ramos, Daudi and Raji were obtained from the American Type Culture Collection (Bethesda, MD). The Ramos AWR cell line was from Dr. George Klein (Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden). The B lymphoma cell line SU-DHL4 was from Dr. David Maloney (Fred Hutchinson Cancer Research Center, Seattle, WA). All cell lines were maintained in log phase growth in RPMI 1640 supplemented with 12% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin.

Antibodies and Reagents. The IgG2a anti-CD20 mAbs 1F5 and 2H7 were produced and purified as described previously (27–29). The IgG2a anti-CD20 mAb anti-B1 (Tositumomab) was a gift from Dr. George Tidmarsh (Coulter Pharmaceuticals, South San Francisco, CA). The chimeric anti-CD20 mAb C2B8 (Rituximab) was purchased from Genentech (San Francisco, CA). ATRA and 13-*cis*-RA were purchased from Sigma Chemical Co. (St. Louis, MO). 4-HPR was a kind gift from Dr. Ronald Lubet (National Cancer Institute Prevention Branch, Frederick, MD). FITC-Annexin V was from PharMingen (San Diego, CA). z-VAD was from Enzyme Systems Products, Inc. (Dublin, CA). A rabbit anti-poly PARP antibody was from Upstate Biotechnology (Lake Placid, NY).

***In vitro* Cell Proliferation Assay.** The effects of retinoids on malignant B-cell growth *in vitro* were determined by assessing [^3H]thymidine incorporation in Ramos cells (30). Briefly, 10^4 cells were resuspended in 200- μl culture medium and plated in 96-well, flat-bottomed microtiter plates. After incubating cells at 37°C for 48 h with retinoids, 1 μCi of [^3H]thymidine/well was added, and cells were cultured for an

additional 6 h. Cells were then harvested onto glass fiber filters with an automated harvesting system from Skatron, Inc. (Sterling, VA), and [^3H]thymidine uptake was assayed with a 4000 series liquid scintillation counter (Downers Grove, IL).

Flow Cytometric Analysis of Apoptosis Using FITC-labeled Annexin V Staining. Cells were cultured with 4-HPR and/or anti-CD20 mAbs using a concentration of 1 $\mu\text{g}/\text{ml}$ at 37°C for 1–6 days in the time-course experiments and for 4 days in the synergy experiments. Apoptosis was assessed by staining with FITC-labeled Annexin V and propidium iodide and performing flow cytometric analysis (Becton Dickinson FACScan; Becton Dickinson, Mountain View, CA) according to the manufacturer's instruction manual. Positive staining with FITC-Annexin V reflects a shift of phosphatidyl serine from the inner to the outer layer of the cytoplasmic membrane, which occurs early in apoptosis. Because positive staining of FITC-Annexin V may also occur in cells undergoing necrosis, propidium iodide was used to bind the cellular DNA of necrotic cells, but not the DNA of apoptotic cells, with intact cell membranes. This method effectively distinguishes Annexin V-positive apoptotic cells from necrotic cells (31). Annexin V-positive and propidium iodide-negative cells were scored as apoptotic cells. The paired *t* test was used to determine the statistical significance of differences in apoptosis between groups.

Immunoblotting. Ten million cells were lysed for 30 min at 4°C in 50 μl of lysis buffer containing 1% NP40, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), and 1 mM phenylmethylsulfonyl fluoride. Debris was sedimented by centrifugation for 15 min at 12,000 $\times g$. Thirty μl of supernatants were mixed with 30 μl of 2 \times Laemmli's SDS-PAGE sample buffer (Bio-Rad, Hercules, CA), and heated for 5 min at 100°C. Proteins were resolved at 150 V on 10–12% gels and electrophoretically transferred to nitrocellulose membranes for 1 h at 100 V. Membranes were blocked for 1 h in a PBS (pH 7.4) plus 0.5% Tween 20 buffer containing 5% nonfat dried milk. Blots were then probed for 1 h at room temperature with specific antibodies. Blots were developed using a goat anti-mouse or rabbit secondary antibody labeled with peroxidase (Amersham Corp., Arlington Heights, IL). Immunoreactive material was then visualized by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

RESULTS

Effects of Retinoic Acid Derivatives on B Cell Lymphoma Growth. To evaluate the dose-response relationship of the growth inhibition of malignant B lymphocytes by 4-HPR in relation to more traditional retinoids, Ramos cells were incubated with escalating doses of ATRA, 13-*cis*-RA, and 4-HPR. The effects of retinoids on the growth of Ramos cells were monitored by the [^3H]thymidine incorporation of cell DNA. 4-HPR induced a >20% and >80% growth inhibition at 1.0 and 10.0 μM concentrations, respectively (Fig. 1). In contrast, ATRA and 13-*cis*-RA induced only moderate growth inhibition at the highest dose level, considerably lower than that observed with 4-HPR. On the basis of these results, we selected 4-HPR for additional studies.

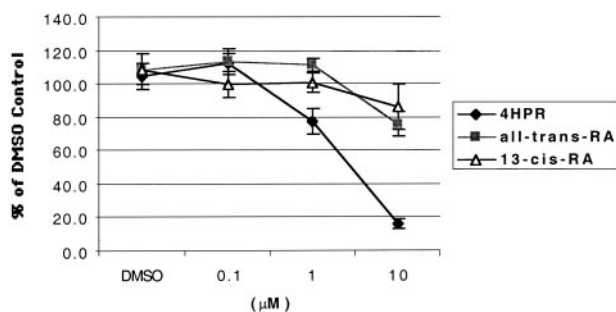


Fig. 1 Growth inhibitory effect of retinoids on Ramos cells measured by tritiated thymidine incorporation. Cells (10^4) in 200 μl /well were incubated in medium containing 0, 0.1, 1, or 10 μM retinoids at 37°C for 48 h, with the subsequent addition of 1 μCi [^3H]thymidine/well and incubating for another 6 h. Cells incubated in medium without retinoids (0 μM) were used as control. Data are representative of two concordant experiments.

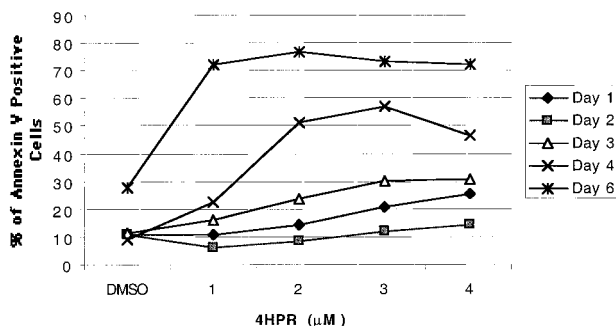


Fig. 2 4-HPR-induced apoptosis of Ramos cells demonstrated by FITC-Annexin V staining. Ramos cells (5×10^5)/ml were incubated with 0, 1, 2, 3, and 4 μM 4-HPR (X axis) at 37°C for 1, 2, 3, 4, and 6 days (curves 1–6). After the incubation, cells were stained with FITC-Annexin V and propidium iodide and analyzed by flow cytometry. Annexin V-positive and propidium iodide-negative cells were quantified (Y axis). Data are representative of three concordant experiments.

Apoptosis of B-Cell Lymphoma Lines Induced by 4-HPR. The growth inhibition observed in our initial experiments could result from multiple processes, including cell-cycle arrest, apoptosis, or cell necrosis. To assess the contribution of apoptosis to this growth inhibition induced by 4-HPR, we incubated Ramos cells with increasing concentrations of 4-HPR ranging from 0 to 4 μM (spanning the range that can be obtained *in vivo* by oral administration; Ref. 32) over time intervals of 1–6 days. Rates of apoptosis induction were enhanced by increasing the concentration of 4 HPR and by prolonging the exposure time of malignant B cells to 4-HPR. Concentrations of 4 HPR as low as 1 μM were effective at inducing apoptosis (Fig. 2). A dose-response-related induction of apoptosis was also demonstrated in Ramos AW, Raji, Daudi, and SU-DHL4 cell lines when incubated with increasing concentrations of 4-HPR for 4 days (data not shown). On the basis of these results, we selected a concentration of 3 μM of 4-HPR and a 4-day incubation period as optimal for additional studies. More importantly, the presence of the antiapoptotic factor, *bcl-2*, did not appear to abrogate this effect in the *bcl-2*-overexpressing Ramos

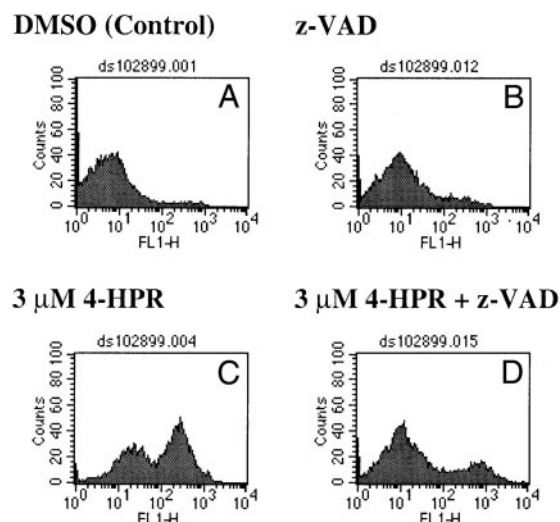


Fig. 3 z-VAD inhibits 4-HPR-induced apoptosis. Ramos cells (5×10^5)/ml were incubated with 3 μM 4-HPR at 37°C for 4 days in the absence and presence of 80 μM z-VAD (an inhibitor of apoptosis) and then stained with FITC-Annexin V and propidium iodide and analyzed by flow cytometry. Data are representative of two concordant experiments. No Annexin V positivity (apoptosis) was noted with control- (A) or z-VAD- (B) treated cells. Apoptosis induced by 4-HPR (C) is inhibited by z-VAD (D).

AW (EBV-infected Ramos cells), Raji, and SU-DHL4 cell lines. These findings suggest that 4-HPR may be able to overcome *in vivo* the effects of *bcl-2* overexpression, one of the major hypothesized mechanisms of malignant cellular immortalization.

Involvement of Caspase Pathways in 4-HPR-induced Apoptosis. Because programmed cell death initiated by most apoptosis-inducing factors involves the activation of caspase pathways, we tested the involvement of caspases in 4-HPR-induced apoptosis using the broad caspase inhibitor z-VAD. When Ramos cells were incubated with 3 μM 4-HPR for 4 days in the presence of 80 μM z-VAD, the fraction of apoptotic cells dropped from 60 to 20% (Fig. 3), indicating that activation of caspases was critical in 4-HPR-induced apoptosis. The involvement of caspase pathways was also demonstrated by the inhibition of the cleavage of PARP, one of caspase substrates, by z-VAD in 4-HPR-induced apoptosis. As shown in Fig. 4, cleavage of PARP to the M_r 85,000 fragment was observed in cells treated with different concentrations of 4-HPR. In contrast, the M_r 85,000 fragment of PARP did not appear in cells treated with 4-HPR in the presence of z-VAD.

Synergistic Effect of Anti-CD20 Antibodies and 4-HPR in Inducing Apoptosis of B Cell Lymphoma Lines. We and others have previously demonstrated that anti-CD20 mAbs can directly induce apoptosis of B lymphocytes via induction of Ca^{2+} influx (19, 20). Thus, we hypothesized that the Ca^{2+} -dependent TTG induced by 4-HPR (demonstrated by other groups; Ref. 16) could be activated by the CD20-mediated Ca^{2+} influx and, thus, further augment apoptosis. To test this premise, we coinubated various anti-CD20 mAbs with escalating doses of 4-HPR within the clinically attainable range. As depicted in Fig. 5, the combination of the antibodies that poorly initiate

Fig. 4 z-VAD inhibits 4-HPR-induced PARP cleavage in Ramos cells. Ramos cells were cultured for 4 days with 0, 1, 2, and 3 μM 4-HPR in the absence (Lanes 1–4) or presence (Lanes 5–8) of 80 μM z-VAD. The expression of PARP and its cleavage product (arrow) were determined by immunoblotting with an anti-PARP antibody. Data are representative of three experiments.

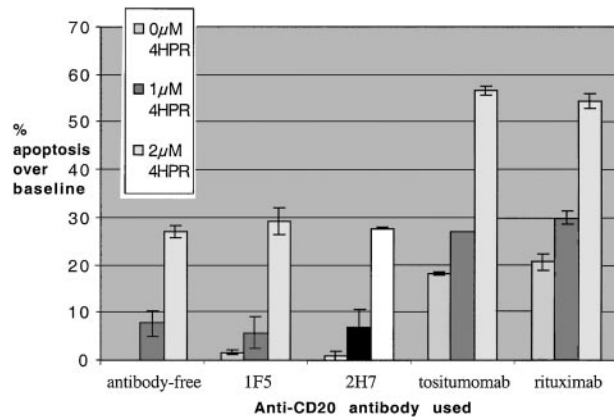
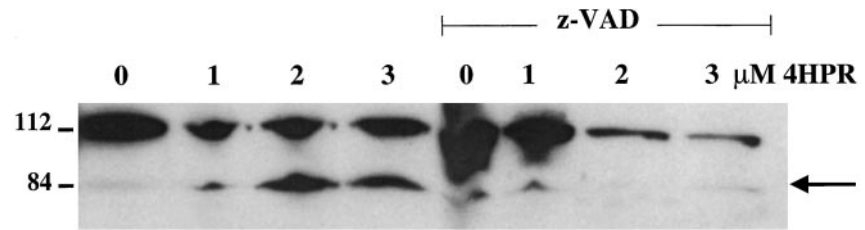


Fig. 5 Apoptosis of Ramos cells induced by 4-HPR and/or anti-CD20 mAbs. Ramos cells (5×10^5)/ml were incubated with 4-HPR and/or anti-CD20 mAbs 1F5, 2H7, B1, and C2B8 at 37°C for 4 days. After the incubation, cells were stained with FITC-Annexin V and propidium iodide and analyzed by flow cytometry. Annexin V-positive and propidium iodide-negative cells were quantified. Data are representative of three concordant experiments.

apoptosis on their own, *i.e.*, 1F5 and 2H7, showed, at best, additive effects with 4-HPR. In contrast, tositumomab and rituximab, both antibodies with the ability to induce low levels of apoptosis as a single agent, demonstrated significant supraadditive levels of apoptosis at the 2 μM concentration of 4-HPR. These findings are more clearly depicted in Fig. 6, where the expected levels of apoptosis from an additive effect are compared with the observed synergistic levels of apoptosis at the 2 μM 4-HPR concentration. Rituximab and tositumomab increased the relative fraction of apoptotic cells of over expected additive levels by 24% ($P = 0.009$) and 42% ($P = 0.0019$), respectively. Together, these findings suggest that the initiating events of apoptosis by tositumomab and rituximab are different from that of 4-HPR, yet may share a final common pathway that can be mutually enhanced by the presence of the other agent.

DISCUSSION

Both mAbs and retinoic acid derivatives have emerged as two of the most encouraging groups of biological agents for the treatment of malignancies. Unfortunately, despite the promise of the clinical use of the retinoids for certain myeloid and T-lymphoid disorders, many details still remain unclear with regard to the direct mechanisms of action, *in vitro* efficacy, and optimal clinical utility of this diverse group of compounds. The synthetically developed retinoid 4-HPR has been evaluated in a

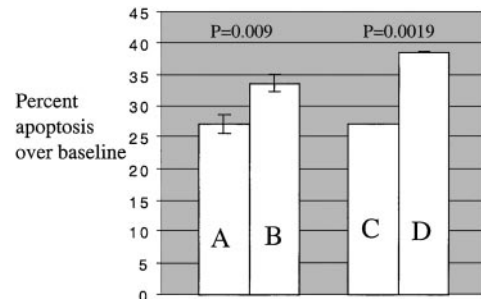


Fig. 6 Synergy of rituximab and tositumomab with 2 μM 4-HPR measured by FITC-Annexin V and propidium iodide staining and analyzed by flow cytometry. A, summation of levels of apoptosis induction when rituximab and 2 μM 4-HPR were incubated separately. B, level of apoptosis when rituximab and 2 μM 4-HPR were coincubated ($P = 0.0009$). C, summation of levels of apoptosis induction when tositumomab and 2 μM 4-HPR were incubated separately. D, level of apoptosis when tositumomab and 2 μM 4-HPR were coincubated ($P = 0.0019$).

number of *in vitro* and human settings, though the data regarding its effects on B-cell malignancies remains somewhat limited (3, 4, 10, 12, 13, 32).

Our studies presented here suggest that a variety of retinoic acid derivatives can successfully inhibit malignant B-cell growth *in vitro*, demonstrating a clear dose-dependent inhibitory effect. These results also confirm and expand further the findings of other investigators evaluating the effects of retinoids on malignant B cells by exhibiting the improved efficacy of 4-HPR-mediated B-cell growth inhibition as compared with ATRA and 13-*cis*-RA across the range of clinically attainable drug concentrations. We additionally demonstrated that 4-HPR induces apoptosis in a time- and dose-dependent fashion. Notably, this activity peaks at 96–144 h in these predominantly *bcl-2*-containing cells, paralleling the observations by other investigators that a delay, but not an abrogation of apoptosis, may be influenced by the overexpression of this antiapoptotic factor (13). *bcl-2* overexpression is thought to play a major role in immortalizing malignant B cells in patients with indolent lymphomas; thus, overcoming this effect with 4-HPR could result in a novel mechanism of inducing or enhancing clinically meaningful responses. The observed time course of programmed cell death also parallels the proposed mechanism of action of 4-HPR, *i.e.*, via mRNA induction and protein synthesis, which has been previously shown to not be mediated by classical RARs (9, 13, 16). Importantly, we were able to demonstrate that the level of apoptosis rises within the range of the clinically attainable single-dose peak serum concentrations of

1–2 μM (32). Our findings also strengthen the argument that this 4-HPR-mediated outcome may not be tumor-specific and is conserved across multiple cell lines, suggesting that these results may be more readily extrapolated to the myriad of *in vivo* and clinical settings of B cell malignancies. To confirm the pathway of 4-HPR-mediated apoptosis induction, we demonstrated that a specific inhibitor of caspase function could abrogate the effects of 4-HPR. Similarly, these findings were substantiated further by showing that the caspase substrate PARP was cleaved into its component parts with 4-HPR but not in the presence of specific caspase inhibitors.

As with retinoids, the clinical use of anti-CD20 antibodies has outpaced the clear understanding of their mechanisms of action. We and others, however, have performed studies in malignant human B cells, investigating the pathways of cell-growth inhibition by anti-CD20 antibodies (20, 21, 33). These experiments revealed that CD20 ligation with or without Fc-hypercross-linking induces rapid calcium influx, caspase activation, and programmed cell death (20, 21, 33). Interestingly, unlike the proposed mechanism of action of the apoptotic induction of 4-HPR, these events were not abrogated by inhibitors of specific protein tyrosine kinases nor significantly influenced by *bcl-2* expression. With apparently alternative initiating events of apoptosis and a common final pathway, we hypothesized that the combination of anti-CD20 antibodies could result in a synergistic effect. Our studies were able to clearly demonstrate that the combination of either tositumomab or rituximab with low levels of 4-HPR could augment the degree of apoptosis in a supra-additive fashion. We hypothesize that the mechanism of this synergy could be related to 4-HPR's 96-h induction of TTG that is then activated further by the CD20-mediated Ca^{2+} influx with a resultant increased apoptosis, though additional studies are required to confirm this speculation. Interestingly, 1F5 and 2H7 were not as effective at synergizing with 4-HPR. This observation may be related to the observed individual relative efficacy of apoptosis induction of the various antibodies. Nonetheless, we were able to show that a combination of clinically attainable levels of specific anti-CD20 antibodies and 4-HPR did result in the augmented programmed cell death of malignant B cells.

The results of the initial studies that we present here could have a significant clinical impact. Non-Hodgkin's lymphoma afflicts over 55,000 new individuals each year in the United States, with a majority of these cases having a B-cell immunophenotype (34). One of the major recent breakthroughs in this field of lymphoma treatment has been the therapeutic application of mAbs. mAbs appear to have a variety of potential mechanisms of cytotoxic action including antibody dependent cellular cytotoxicity, complement fixation, direct induction of apoptosis, and as carriers for radionuclides or toxins (35). Although it is unclear which of these mechanisms predominate *in vivo*, anti-CD20 antibodies have clearly demonstrated clinical responses in patients suffering from CD20-bearing B-cell lymphomas with little associated toxicity when compared with traditional chemotherapy regimens (24–26, 29).

Despite the promise of these anti-CD20 monoclonals, the majority of patients treated with this approach alone do not attain complete or long-lasting responses. To improve the efficacy of this therapy, investigators have evaluated *in vitro* syn-

ergy between traditional chemotherapeutic drugs and anti-CD20 mAbs (36, 37). This approach has also been tested in the clinic with resultant improved responses over antibody alone, but with similar toxicities to traditional chemotherapy regimens (38). Ideally, one would hope to optimize the efficacy of CD20-targeted therapy without incurring the added expected toxicities of standard chemotherapeutic agents. Fenretinide has been evaluated in a number of large therapeutic and chemopreventive clinical trials having long treatment periods and large numbers of patients with a variety of cancers, all demonstrating minimal significant toxicity (3–5). Importantly, peak serum concentrations subsequent to one administration of 4-HPR in this oral dose range (300 mg) have been shown to be $>1 \mu\text{M}$, with some studies suggesting that even higher levels may be observed within malignant tissues (3, 32, 39). Unfortunately, these studies have not demonstrated clinical efficacy for 4HPR, possibly because these serum levels are inadequate to induce meaningful apoptosis in humans on their own. On the basis of the *in vitro* synergism of apoptosis induction with 4-HPR and specific anti-CD20 antibodies suggested by our studies, we postulate that the combination of these two minimally toxic compounds may improve the clinical efficacy of these agents without incurring additional toxicity. Because the predominant mechanisms of *in vivo* cytotoxicity of anti-CD20 antibodies likely includes both antibody-dependent cellular cytotoxicity and complement fixation, these results regarding enhancing apoptotic induction by the addition of 4HPR will ultimately require additional testing to determine whether the *in vitro* data will translate into improved responses and survival in patients with B-cell non-Hodgkin's lymphoma.

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