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Cancer Research

Venetoclax Synergizes with Radiotherapy for Treatment of B-cell Lymphomas

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Abstract

Constitutive B-cell receptor signaling leads to overexpression of the antiapoptotic BCL-2 protein and is implicated in the pathogenesis of many types of B-cell non-Hodgkin lymphoma (B-NHL). The BCL-2 small-molecule inhibitor venetoclax shows promising clinical response rates in several lymphomas, but is not curative as monotherapy. Radiotherapy is a rational candidate for combining with BCL-2 inhibition, as DNA damage caused by radiotherapy increases the activity of pro-apoptotic BCL-2 pathway proteins, and lymphomas are exquisitely sensitive to radiation. We tested B-NHL responses to venetoclax combined with either external beam radiotherapy or radioimmunotherapy (RIT), which joins the selectivity of antibody targeting with the effectiveness of irradiation. We first tested cytotoxicity of cesium-137 irradiation plus venetoclax in 14 B-NHL cell lines representing five lymphoma subtypes. Combination treatment synergistically increased cell death in 10 of 14 lines. Lack of synergy was predicted by resistance to single-agent venetoclax and high BCL-XL expression. We then assessed the efficacy of external beam radiotherapy plus venetoclax in murine xenograft models of mantle cell (MCL), germinal-center diffuse large B-cell (GCB-DLBCL), and activated B-cell (ABC-DLBCL) lymphomas. In each model, external beam radiotherapy plus venetoclax synergistically increased mouse survival time, curing up to 10%. We finally combined venetoclax treatment of MCL and ABC-DLBCL xenografts with a pretargeted RIT (PRIT) system directed against the CD20 antigen. Optimal dosing of PRIT plus venetoclax cured 100% of mice with no detectable toxicity. Venetoclax combined with radiotherapy may be a promising treatment for a wide range of lymphomas. *Cancer Res; 77(14); 1–9. ©2017 AACR.*

Introduction

Non-Hodgkin lymphoma (NHL) developed in an estimated 72,580 Americans in 2016, and over 20,000 will die despite the abundance of treatment options available (1). Over 75% of these NHL cases will be of B-cell origin. Constitutive B-cell receptor (BCR) signaling has been implicated in the pathogenesis of many types of B-NHLs, including chronic lymphocytic leukemia (CLL), follicular lymphoma, the activated B-cell (ABC) subtype of diffuse large B-cell lymphoma (DLBCL), and mantle cell lymphoma (MCL). Tonic signaling downstream of the BCR ultimately upregulates antiapoptotic BCL-2 pathway proteins including BCL-2 itself (2, 3). Constitutively high levels of BCL-2 are separately produced by the t(14;18)(q32;q21) translocation, which is found in up to 85% of follicular lymphoma and 34% of the germinal center (GCB) subtype of DLBCL, and by overexpression of the unrearranged *BCL2* gene (2, 3). These findings have made BCL-2 an important therapeutic target in NHL, leading to the development of several novel drugs. The BCL-2 inhibitor venetoclax is one of the most promising new agents, as evidenced by a breakthrough status designation in 2015 followed by full FDA approval in 2016 for the treatment of CLL. Despite this promise, venetoclax is not curative as monotherapy (4), and here we examine the efficacy of combining venetoclax with radiotherapy.

Radiotherapy is among the oldest cancer treatments in the modern era and remains an effective tool both as external beam radiotherapy (5), and as radioimmunotherapy (RIT), which combines the selectivity of antibody targeting with the effectiveness of irradiation (6–11). Radiation causes DNA strand breaks that ultimately increase the activity of proapoptotic members of the BCL-2 family (12, 13), making radiotherapy a rational candidate for combination with BCL-2–inhibiting drugs. Such drugs have been shown to synergize with DNA-damaging drugs (14) and apoptosis promoters (15–17) in several cancers, yet to our knowledge, no study has combined BCL-2 inhibition with radiotherapy. Lymphomas are uniquely sensitive to radiation (18), increasing the promise of this combination. While venetoclax is most intensively studied in CLL, it promotes apoptosis in a variety of NHL subtypes (4), and we hypothesized that venetoclax would combine synergistically with either external beam radiotherapy or targeted RIT to treat a range of B-NHL diseases.

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To test this hypothesis, we studied *in vitro* cytotoxicity resulting from cesium-137 (^{137}Cs) irradiation combined with venetoclax, using 14 B-NHL cell lines representing five lymphoma subtypes (Table 1). As predicted, the combination treatment synergistically increased cell mortality in the majority of cell lines. We then performed *in vivo* studies using three murine xenograft models, Rec-1 (MCL), U2932 (ABC-DLBCL), and SU-DHL-6 (GCB-DLBCL), chosen for their divergent single-agent sensitivities to venetoclax. For these experiments, we investigated combining venetoclax with external beam radiotherapy using a ^{137}Cs irradiator, and with RIT using a two-step "pretargeted" system (PRIT) directed against the CD20 antigen. PRIT dissociates the slow, antibody distribution phase of RIT from the administration of the radionuclide, and typically delivers an order of magnitude greater tumor-to-normal organ ratio of radiotherapy than single-step RIT (11, 19, 20). In all three *in vivo* models, optimal dose combinations of venetoclax plus external beam radiotherapy, and venetoclax plus PRIT, caused synergistic reduction or eradication of lymphoma.

Materials and Methods

Cell lines

Human cell lines Ramos, Jeko-1, JVM-2, Rec-1, SU-DHL-4, and SU-DHL-8 were obtained from the ATCC between 2006 and 2014; OCI-Ly3 and OCI-Ly19 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in 2014; and HT, Pfeiffer, Ri-1, SU-DHL-6, U2932, and WSU-FSCCL were generous gifts from Gilead Sciences in 2014. Cell line characteristics are shown in Table 1. Cells were maintained in log-phase growth at >95% viability (Trypan blue exclusion) in RPMI1640 or αMEM (OCI-Ly19) media supplemented with 10%–20% FBS, 50 U/mL penicillin G, and 50 μg/mL streptomycin sulfate, and studied within 6 weeks of thawing. All cells were tested for mycoplasma and authenticated by DNA profiling (ATCC kit 135-XV).

In vitro cytotoxicity of radiation combined with venetoclax

For each cell line, single-agent dose–response tests were conducted to identify an incubation period and dose range that provided approximately 0%, 20%, 40%, 60%, 80%, and 100% mortality attributable to 660 keV gamma rays from ^{137}Cs (Gammacell 1000 irradiator, MDS Nordion) or to venetoclax (donated by AbbVie). Cells ($1–2 \times 10^5$/mL) were treated with drug or irradiated, incubated 24–120 hours, and assayed for mortality using the CellTiter-Glo assay (Promega, G7571). Optimized incubation and dose parameters were subsequently used to test the efficacy of agent combinations in 6×6 dose matrices. Radiation was administered at time 0 and venetoclax added 24–48 hours later. All tests were performed in triplicate and the computed means used in further analysis. To determine synergy, additivity, or antagonism, combination indexes (CI) were calculated with CalcuSyn software (Biosoft) using the median effect equation, from mortalities in the 3×3 matrix section of each assay centered on 50% mortality (considered most accurate; refs. 21–24).

In vitro BCL-2, BCL-XL, and MCL-1 expression

BCL-2 pathway expression was characterized in B-NHL cell lines by flow cytometry. Cells were fixed in 4% formaldehyde, permeabilized in 90% methanol, and stained with PE-conjugated BCL-2, BCL-XL, MCL-1, or isotype control mAbs (Cell Signaling Technology, 26295, 13835, 65617, 6899, 5742). All cell lines were examined by flow cytometry in a single assay. Protein expression was estimated as controlled median fluorescence index (MFI), calculated for each sample by subtracting isotype control MFI from target mAb MFI. This complete assay was replicated on a different day by a different person. Assay repeatability was examined by linear regression of controlled MFI from the first versus the second assay, for each protein. Results were highly repeatable ($r^2 > 0.7$; $P < 0.01$).

Mice

NOD.*Cg-Rag1tm1Mom Il2rgtm1Wjl*/SzJ mice [(NRG), from the Jackson Laboratory or Fred Hutchinson Cancer Research Center (FHCRC)] and FoxN1Nu athymic nude mice (Envigo) were maintained under standard protocols approved by the FHCRC Institutional Animal Care and Use Committee. Individual studies used either all female mice or 50:50 sex ratios in all experimental

Table 1. B-NHL cell lines

Cell line	NHL subtype	Relevant characteristics
OCI-Ly3	ABC-DLBCL	Amplified BCL-2 (copy no. 3.8)[a], low BCL-XL and high MCL-1 expression[b]
Ri-1	ABC-DLBCL	Amplified BCL-2 (copy no. 14.7)[a], high BCL-2 expression[b]
U2932	ABC-DLBCL	Amplified BCL-2 (copy no. 14.9)[a], high BCL-2 and low BCL-XL expression[b]
HT	GCB-DLBCL	Low BCL-2[b] and high MCL-1 expression[a]
OCI-Ly19	GCB-DLBCL	t(14;18) translocation, amplified BCL-2 (copy no. 3.3)[a]
Pfieffer	GCB-DLBCL	High BCL-XL and MCL-1 expression[b], t(14;18) translocation[c]
SU-DHL-4	GCB-DLBCL	High BCL-2 expression[b], t(14;18) translocation[a]
SU-DHL-6	GCB-DLBCL	Low BCL-2 and high MCL-1 expression[b], t(14;18) translocation[a]
SU-DHL-8	GCB-DLBCL	Low BCL-2 and high BCL-XL expression[b]
Jeko-1	MCL	Overexpresses BCL-2, Bcl-1/J(H) gene rearrangement[c]
JVM2	MCL	High BCL-XL expression[b,d]
Rec-1	MCL	High BCL-XL expression [d], p53 oncogene[c]
Ramos	Burkitt's	Low BCL-2 expression[b,e]
WSU-FSCCL	Trans. follicular	t(14;18) translocation[f]

NOTE: Sources:
[a]Ref. 4.
[b]Current study, high and low expression defined as values outside 95% confidence intervals of the mean, $n = 14$ lines.
[c]ATCC.
[d]Ref. 41
[e]Ref. 51
[f]DSMZ.

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groups and controlled for gender in statistical analyses. Genders were housed separately.

Antibodies, pretargeting reagents, and radiolabeling

1F5, a murine immunoglobulin G_{2a} anti-human CD20 mAb, and HB8181, an IgG_{2a} isotype control, were produced from hybridomas using a hollow-fiber bioreactor system in the Biological Production Facility at FHCRC. The hybridoma cell line expressing 1F5 was a gift from Clay Siegall (Seattle Genetics), and the HB8181 hybridoma was purchased from ATCC. In all immunotherapy experiments, mice were coinjected with 400 μg of HB8181 to block nonspecific binding of 1F5 to Fc receptors. 1F5–streptavidin conjugates, DOTA-biotin reagents, and biotin-galactose clearing agent [(CA) N-acetyl-galactosamine; NeoRx)] were prepared as described previously (11, 25). Radiolabeling of DOTA-biotin with the pure beta-emitter yttrium-90 (^{90}Y) (Perkin Elmer, NEZ306) was performed as described previously (11, 25) with labeling efficiencies >86%.

In vivo radiotherapies combined with venetoclax

NRG or athymic nude mice were injected subcutaneously in the right flank with 1×10^7 Rec-1 or SU-DHL-6 cells or 0.5×10^7 U2932 cells 8–16 days prior to therapy to generate lymphoma xenografts, depending on growth kinetics of the individual cell line. Athymic mice were injected intraperitoneally with anti-asialoGM1 antibody according to manufacturer recommendations (Wako, 986-10001) to attenuate tumor rejection via natural killer cell activity. Injections were given one day prior to tumor implantation, five days later, and weekly thereafter. When tumors were approximately 50 mm^3, mice were randomized into groups of 8–10 with equivalent mean tumor volumes (sample size determined by power analysis). To examine interactions between external radiotherapy and venetoclax, mice were treated with either the drug diluent (60% Phosal 50PG, 30% PEG 400, 10% EtOH, oral gavage once daily for 10–28 days), venetoclax (100–200 mg/kg, same schedule), radiotherapy (single, total body dose of 6–10 Gy ^{137}Cesium from JL Shepard Mark I irradiator), or a combination of venetoclax and radiotherapy in which venetoclax treatment began one day after radiotherapy. Mice receiving >6 Gy radiotherapy underwent syngeneic bone marrow transplantation (BMT) 4 hours after radiotherapy, receiving 5×10^6 donor bone marrow cells without T-cell depletion, as described previously (26). PRIT studies used the same experimental design, but in place of external beam radiotherapy, mice were initially coinjected with 1.4 nmol (300 μg) unlabeled 1F5-SA conjugate and 2 mg/mL (400 μg) HB8181 (11). Twenty-one hours later, 5.8 nmol CA was administered, followed 3 hours later by 1.2 nmol of ^{90}Y-DOTA-biotin labeled with 400, 800, or 1,200 μCi of ^{90}Y (14.8, 29.6, or 44.4 MBq, respectively). The total amount of antibody delivered was the same for every animal, regardless of the radioactive dose of ^{90}Y-DOTA-biotin administered. In combination groups, venetoclax treatment began 2 days after ^{90}Y-DOTA-biotin administration. Tumor size and body weight were measured three times a week following treatment and continued through day 120. Mice were euthanized when they experienced excessive weight loss, hind limb paralysis, or exceeded tumor volume limits per institutional guidelines.

Statistical analyses

In vitro responses to venetoclax plus radiotherapy were analyzed using *t* tests to determine whether the mean combination index (CI, see "*In vitro* cytotoxicity" methods) of a cell line differed from 1, with CI values <1 indicating synergy, and values >1 indicating antagonism. Correlations between CI and venetoclax LD_{50}, ^{137}Cs LD_{50}, and BCL-2, BCL-XL, and MCL-1 expression were determined using simple and multiple linear regression. *In vivo* treatment effects on mouse survival were determined by log-rank comparisons of Kaplan–Meier survival functions. All analyses were performed using JMP 12.2.0 (SAS Institute).

Results

In vitro cytotoxicity of radiation combined with venetoclax

The 14 B-NHL cell lines studied showed a wide range of sensitivities to single-agent external beam radiotherapy (LD_{50} = 0.3–49 Gy) and venetoclax (LD_{50} = 0.002–13 μmol/L). Therefore, to optimally evaluate cytotoxicity of radiotherapy combined with venetoclax, each cell line was assayed using a unique 6 × 6 dose combination. The resulting cytotoxicity data (Fig. 1A) were evaluated with isobolographic analyses (Fig. 1B), which provided a CI for each dose pair (Fig. 1C), where values <1 indicate synergy, values of 1 signify additivity, and values >1 indicate antagonism. Overall, 10 of 14 NHL lines responded synergistically to combined radiotherapy and venetoclax (Fig. 1D, $P < 0.003$ for CI <1), including 4 of 6 GCB-DLBCL cell lines, 3 of 3 ABC-DLBCL cell lines, and 2 of 3 MCL lines. The remaining four lines responded antagonistically (Fig. 1D, $P < 0.027$ for CI >1). We examined two possible predictors of treatment response: single-agent sensitivity and BCL-2 pathway protein expression. Single-agent sensitivity to venetoclax strongly predicted the CI: cell lines that were more responsive to single-agent venetoclax were more likely to respond synergistically to combining the drug with radiation (Fig. 2A, $r^2 = 0.73$, $P = 0.0001$). In contrast, single-agent sensitivity to radiation had no predictive power (Fig. 2A, $r^2 = 0.03$, $P = 0.6$). Among the BCL-2 pathway proteins, lower expression of BCL-XL predicted a more synergistic response to combination treatment (Fig. 2B, $r^2 = 0.51$, $P = 0.004$), while levels of BCL-2 and MCL-1 were not predictive. Multivariate analysis, examining all possible correlations among venetoclax LD_{50}, radiotherapy LD_{50}, BCL-2, BCL-XL and MCL-1 expression, and CI, detected no further relationships among variables (multivariate, $P > 0.22$) and indicated that venetoclax LD_{50} (multivariate, $P = 0.0002$) and BCL-XL expression (multivariate, $P = 0.006$) had statistically independent influences on CI.

In vivo radiotherapies combined with venetoclax

Each of three *in vivo* lymphoma models, treated with either radiotherapy or PRIT in combination with venetoclax, responded synergistically to combination therapy without significant toxicity. These models were solid xenografts of Rec-1 (MCL), SU-DHL-6 (GCB-DLBCL), or U2932 (ABC-DLBCL), chosen to represent synergistic *in vitro* responders from different disease subtypes and with differing single-agent sensitivity to venetoclax, the primary predictor of *in vitro* synergistic response (Fig. 2).

Efficacy of external beam radiotherapy combined with venetoclax

In mice bearing Rec-1 tumors, venetoclax alone had no detectable effect on mouse survival (Fig. 3A, $P = 0.32$ compared with controls), and 8 Gy external-gamma radiotherapy alone, a dose necessitating BMT rescue, increased mean survival time 44% over controls ($P = 0.00002$, Kaplan–Meier log-rank test). However, 8

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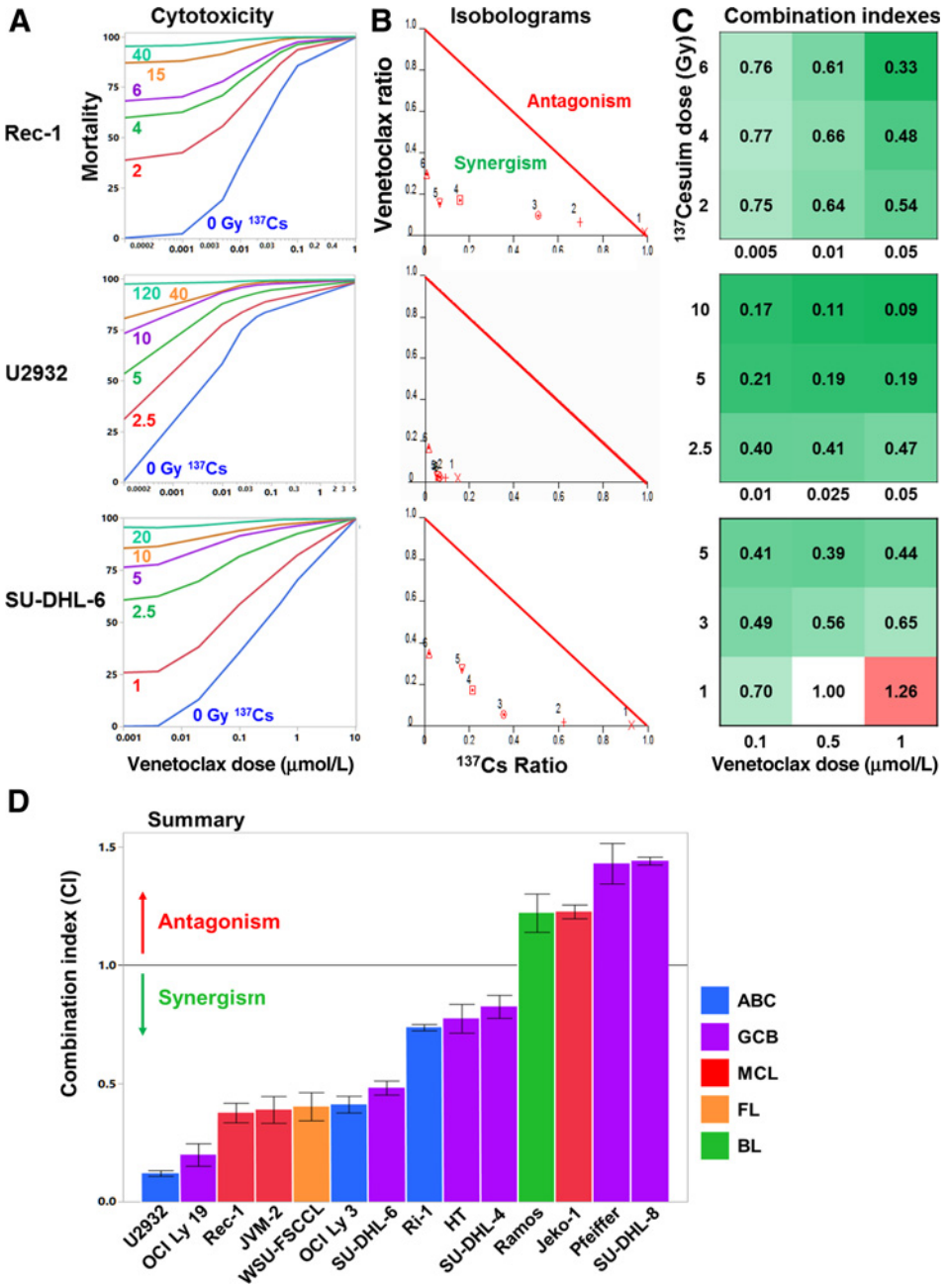

Figure 1.
Venetoclax combined synergistically with ^{137}Cs irradiation to increase mortality in the majority of B-NHL cell lines. **A,** Representative cytotoxicity profiles for cells treated with 6 levels of ^{137}Cesium irradiation combined with 6 dose levels of venetoclax. Treated cells were incubated 72–96 hours, cytotoxicity assessed using CellTiter-Glo and mortality calculated as percent of untreated control. Each assay was conducted in triplicate and the mean values used in further analyses. **B,** Normalized isobolograms were constructed from mortality data at each level of ^{137}Cs treatment. Values below the red 1:1 line of additivity indicate synergy; values above the line indicate antagonism. Isobolograms shown are for the fourth ^{137}Cs treatment level (purple lines in **A**). **C,** Matrices of combination indexes from the 3 × 3 section of the dose matrix centered on 50% mortality (considered most accurate). Combination index values < 1 indicate synergy (green), values of 1 indicate additivity (white), values > 1 indicate antagonism (red). The top row in each matrix derives from the isobologram in **B**. **D,** Summary of responses of B-NHL cell lines to ^{137}Cs irradiation combined with venetoclax. Ten of 14 lines responded synergistically (CI < 1, $P < .003$). The remaining four lines responded antagonistically (CI > 1, $P < 0.03$). $N = 9$ CIs/cell line, CIs calculated from the means of 1–2 triplicate assays; error bars, 1 SEM.

Gy radiotherapy combined synergistically with venetoclax, nearly tripling mean survival time relative to controls while curing 1 of 9 mice ($P = 0.00004$ for combination group > radiotherapy alone; cure defined as mice surviving to end of study (120 days) with no signs of relapse; synergy defined as survival of the combination group being greater than the additive survival benefits of each agent administered alone (Supplementary Table S1)]. Results for the SU-DHL-6 model were similar: venetoclax alone had no detectable benefit (Fig. 3B, $P = 0.22$), 10 Gy radiotherapy extended mean survival time 48% over controls ($P = 0.013$), while combination therapy boosted survival time 156% and cured 1 of 10 mice ($P = 0.008$ for combination group > radiotherapy alone). Using the SU-DHL-6 and U2932 models, we additionally studied combination therapy using 6 Gy radiotherapy, the highest dose not requiring BMT rescue. As a single agent, 6 Gy radiotherapy increased survival time 36% over controls in SU-DHL-6 ($P < 0.001$, Supplementary Table S1), but had no effect in U2932 ($P = 0.7$ for radiotherapy alone compared with control). However, combining 6 Gy radiotherapy with venetoclax increased mean survival time in SU-DHL-6 models an additional 18% beyond either single-agent treatment ($P < 0.001$, Supplementary Table S1), and in U2932 models the combination increased survival 58% beyond either single-agent treatment ($P < 0.004$).

Efficacy of PRIT combined with venetoclax

Combination therapy using PRIT was also effective across models, and produced more cures than combinations using external beam radiotherapy. The PRIT studies examined Rec-1

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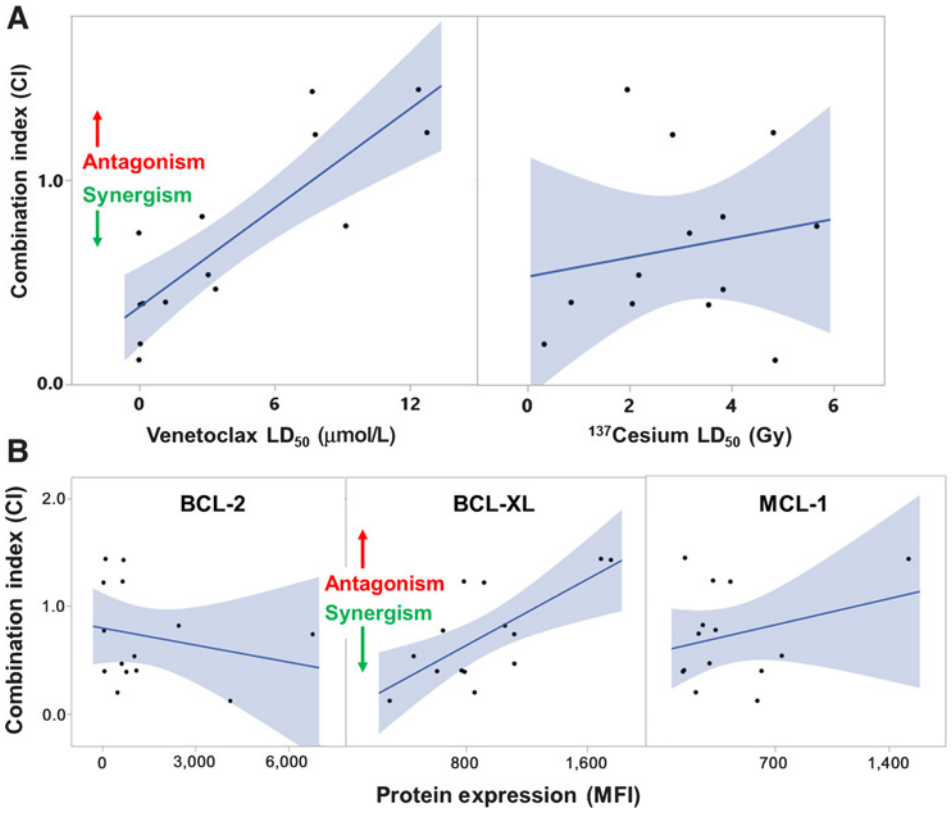

Figure 2.

Predictors of *in vitro* efficacy of radiotherapy plus venetoclax combination treatments in B-NHL. **A,** Single-agent cytotoxicities (LD_{50}) were examined for ability to predict CI (data from Fig. 1). Regression analysis detected a significant positive correlation between venetoclax LD_{50} and CI ($r^2 = 0.73$, $P = 0.0001$, $n = 14$ cell lines), thus greater sensitivity to single-agent venetoclax (lower LD_{50}) predicted a more synergistic response (CI < 1) to combination treatment. Sensitivity to single-agent ^{137}Cesium irradiation did not predict CI ($r^2 = 0.03$, $P = 0.6$). **B,** Protein levels of BCL-2, BCL-XL, and MCL-1 were assayed by flow cytometry in independent duplicate assays. Median fluorescence index (MFI) was highly correlated (repeatable) across assays ($r^2 > 0.7$, $P < 0.01$). Regression analyses detected a positive correlation between BCL-XL protein levels and CI ($r^2 = 0.51$, $P = 0.004$, $n = 14$), while BCL-2 and MCL-1 did not correlate with CI ($r^2 < 0.08$, $P > 0.3$). Thus greater BCL-XL expression predicted a more antagonistic response to combination treatment. Multivariate analysis indicated that BCL-XL and venetoclax LD_{50} had independent influences on CI (see Results).

and U2932 models, assaying two levels of PRIT activity in each. In Rec-1, venetoclax alone only marginally increased mean survival time (Fig. 4A, $P = 0.05$ compared with controls, Kaplan–Meier log-rank test), internal beta radiation from suboptimal doses (800 μCi) of PRIT increased survival 111% beyond controls ($P = 0.0001$), while the combination synergistically extended survival 483% and included 25% cures ($P = 0.001$ for combination group > PRIT alone). In the same study, 400 μCi PRIT alone increased survival time 46% over controls ($P = 0.02$, Supplementary Table S1) while combining PRIT and venetoclax increased survival by 106% over controls ($P = 0.0001$ for combination group > PRIT alone). The U2932 model proved more sensitive to all treatments (Fig. 4B). In this model, venetoclax alone doubled mean survival time but with no cures, while 800 μCi and 1,200 μCi PRIT alone cured 10% and 30% of mice, respectively ($P < 0.0001$ for any single-agent group > controls). At both PRIT doses, combination with venetoclax cured 100% of mice bearing U2932 tumors (Fig. 4B, $P < 0.0006$ for any combination group > any single agent group).

Treatment toxicities

Single-agent venetoclax treatment caused no detectable weight loss nor nontumor-related mortality in mice in any study at any dose tested. Single-agent radiotherapy treatments of 6, 8, and 10 Gy caused maximum weight loss (averaged over all mice/treatment) of 6%, 12%, and 13%, respectively, at four days after treatment, with full recovery to baseline weight within 12 days. Combining radiotherapy with venetoclax had no additional effects on weight. Single-agent PRIT treatments of 400, 800, and 1,200 μCi caused maximum weight loss of 3%, 6%, and 4%, respectively, with full recovery within 14 days, and with no additional weight loss from combining PRIT with venetoclax. Nontumor-related mortality, prior to study day 105, was limited to 2 of 39 mice (5%) for all single-agent radiotherapy treatments, 3 of 47 (6%) for radiotherapy plus venetoclax treatments, 1 of 50 (2%) for single-agent PRIT treatments, and 0 of 76 mice for PRIT plus venetoclax treatments. After study day 105, 3 single-agent PRIT mice (Fig. 4B), and 1 PRIT plus venetoclax-treated mouse (Fig. 4A) died without apparent cause (body weights equivalent to healthy study mates and no tumor evidence on necropsy). In age-matched controls for the Fig. 4 studies, 2 of 7 of these immunodeficient mice died without apparent cause in this same time frame, suggesting that late deaths among study mice may have been nonstudy related.

Discussion

Finding cures for inoperable cancers will usually require identifying a combination of treatments that effectively targets multiple oncogenic mechanisms (27, 28). Ideally targeted combinations will both minimize toxicity to healthy tissues and eradicate cancer quickly, as treatments that do not cure quickly can promote the evolution of resistant subclones. Our results demonstrate that the combination of radiotherapy and venetoclax may achieve these goals to treat B-NHL. Ten of 14 *in vitro* cell lines and 3 of 3 *in vivo* xenograft models (GCB-DLBCL, ABC-DLBCL, and MCL) demonstrated synergistic responses to

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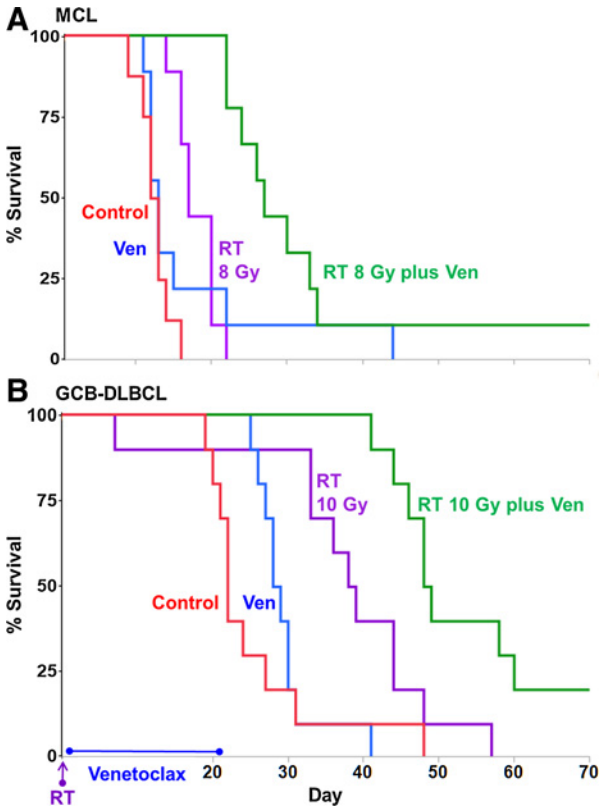

Figure 3.

External beam radiotherapy (RT) synergizes with venetoclax to lengthen survival of mice bearing B-NHL xenografts. Mice implanted with subcutaneous xenografts of Rec-1 (MCL; **A**) or SU-DHL-6 (GCB-DLBCL; **B**) were treated with either drug diluent only (control), 8 or 10 Gy external beam ^{137}Cs irradiation (RT), venetoclax (daily for 21 days), or radiotherapy plus venetoclax, when tumors were approximately 50 mm^3. Mouse survival was plotted on Kaplan–Meier curves. In both xenograft models, single-agent venetoclax (blue) did not significantly affect survival time ($P > 0.2$ compared with controls), but combining venetoclax with radiotherapy (green) extended mean survival times significantly beyond that provided by radiotherapy alone (purple) and cured at least 10% of mice ($P < 0.007$, combination groups > radiotherapy groups). Cure defined as survival to 120 days with no sign of relapse; synergy defined as survival of the combination group being greater than the additive survival benefits of each agent administered alone (Supplementary Table S1). $N = 9$–10 mice/group, additional statistics in the text.

combination therapy. In the mouse models, combining venetoclax with radiotherapy or PRIT added no toxicity, and optimal dosing cured 100% of mice.

These results suggest a way to improve on the already notable clinical success of small-molecule inhibitors. Venetoclax is a highly promising drug and the subject of at least 22 NIH-sponsored clinical trials. Nonetheless, the most impressive results using venetoclax as monotherapy are from a phase I CLL study showing 79% overall (ORR) and 20% complete (CR) response rates (29). In relapsed/refractory (R/R) NHL, a phase I trial of venetoclax monotherapy showed an ORR of 30% and a CR rate of 10% (30). These rates are typical of other promising drugs used as single agents: auspicious ORRs, but few if any durable cures. Another small molecule, the Bruton tyrosine kinase inhibitor ibrutinib, is heralded as a top new MCL therapy (31), yet the best monotherapy results in R/R MCL show a CR of only 21% with a continuous pattern of relapse (32, 33). Prognosis is dire when resistant disease evolves; median survival was only 2.9 months in MCL patients following ibrutinib failure (34, 35). While preliminary data suggest a less grim prognosis when venetoclax resistance evolves (36), the above studies highlight the importance of identifying combination treatments capable of rapid disease eradication.

To this end, 19 of the aforementioned 22 venetoclax clinical trials, and multiple preclinical studies, combine venetoclax with other agents, but none to our knowledge has combined the drug with radiotherapy. We predicted this combination would synergize due to the complementary molecular mechanisms that underlie radiotherapy and venetoclax activity. Radiation damage to DNA creates a signaling cascade that interacts with BCL-2 family proteins via at least two pathways. In one, DNA damage activates the ATM/ATR kinases, which activate checkpoint kinase 2 (CHK2), phosphorylating tumor suppressor p53 that subsequently activates transcription of proapoptotic BCL-2 family proteins Bax, Noxa, and PUMA (13). Increased levels of Bax directly promote apoptosis, while Noxa and PUMA inhibit multiple antiapoptotic BCL-2 proteins including BCL-XL and MCL-1, important because BCL-XL and MCL-1 overexpression is implicated in venetoclax resistance (37, 38). In a second pathway, DNA damage acts independently of p53 by activating the checkpoint protein RAD9, which inhibits antiapoptotic BCL-XL (39). Hence, radiotherapy both directly promotes apoptosis and inhibits antiapoptotic alternatives to BCL-2, thus complementing the selective BCL-2 inhibition of venetoclax (4, 12, 40) and suggesting that combining radiotherapy with venetoclax may be valuable even in venetoclax-resistant disease.

This multiplicity of mechanisms joining radiotherapy and venetoclax activities suggests that their combination might be effective across lymphomas with different molecular profiles. The cell line Rec-1 overexpresses BCL-XL (41) and is venetoclax resistant (Figs. 3A, 4A; ref. 41), yet consistent with the idea that radiotherapy plus venetoclax may be valuable in venetoclax-resistant disease, Rec-1 responded synergistically to combination therapy (Figs. 1D, 3A, and 4A). U2932 has an opposite profile, with extremely high BCL-2 expression, low BCL-XL expression (Table 1; ref. 4) and a predictably extreme sensitivity to venetoclax (Fig. 4B). Yet venetoclax alone was not curative, and U2932 showed the most synergistic responses to combination therapy in our study (Figs. 1D and 4B; Supplementary Table S1). Our final *in vivo* cell line, SU-DHL-6, had a yet different BCL-2 profile (Table 1) and a synergistic response to combination therapy (Figs. 1D, 3B). These data support the possibility that radiotherapy plus venetoclax may be effective in a broad range of NHL subtypes.

These results also suggest that treatment efficacy might result from different mechanisms in different diseases, posing a challenge for identification of biomarkers for responsiveness to radiotherapy plus venetoclax. Exhaustive studies identifying biomarkers were beyond the scope of this investigation, but may be important for clinical translation of our findings, as 4 of 14 cell lines tested *in vitro* responded antagonistically to radiotherapy plus venetoclax (Fig. 1D). The BCL-2 family may be the most promising source of biomarkers. Cell sensitivity to single-agent ^{137}Cs irradiation was not correlated with the antagonistic response to radiotherapy plus venetoclax (Fig. 2A), perhaps because irradiation influences cell survival via multiple complex pathways, not all of which intersect with the BCL-2 family

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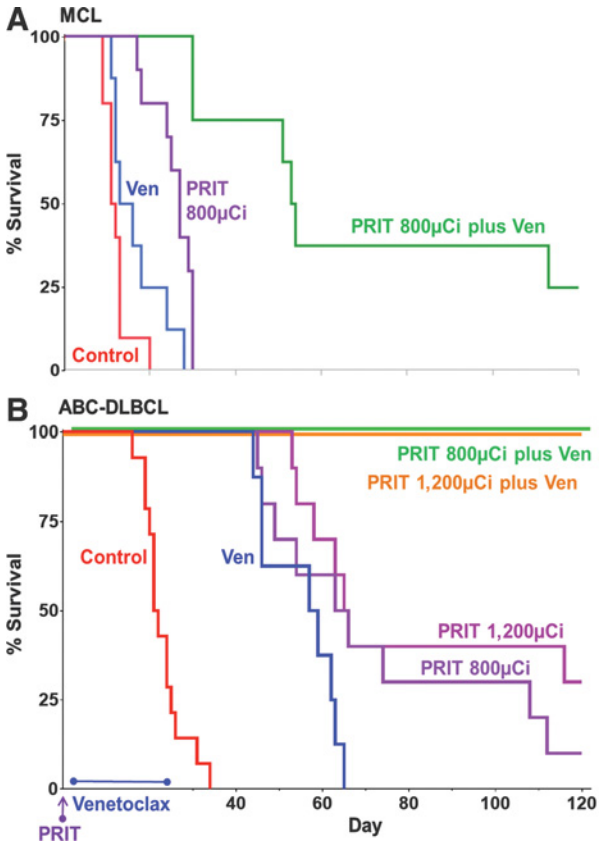

Figure 4.

PRIT synergizes with venetoclax to cure up to 100% of mice bearing B-NHL xenografts. Mice implanted with subcutaneous xenografts of Rec-1 (MCL; **A**) or U2932 (ABC-DLBCL; **B**) were treated with drug diluent only (control), PRIT (CD20-pretargeted RIT using ^{90}Y), venetoclax (daily for 21 days), or PRIT plus venetoclax, when tumors were approximately 50 mm^3. **A,** In Rec-1, our most drug and radiotherapy-resistant model, single-agent venetoclax (blue) had only marginal effects ($P = 0.05$ compared with control, Kaplan–Meier log-rank test), and single-agent 800 μCi PRIT (purple) induced some remission but all mice died from tumor burden by day 30. Yet combining venetoclax with PRIT (green) produced 75% disease-free survival through day 45 and 38% through day 100 ($P = 0.001$ for combination group > PRIT group). **B,** In U2932, single-agent venetoclax (blue) caused complete remission during treatment but no cures, and single-agent 800 and 1,200 μCi PRIT (purples) cured 10% and 30%, respectively. However, combinations of venetoclax with 800 or 1,200 μCi of PRIT (orange and green, respectively, offset for visual clarity) each cured 100% of mice ($P < 0.0006$ for either combination group > any single agent group). Cure defined as survival to 120 days with no sign of relapse; synergy defined as survival of the combination group being greater than the additive survival benefits of each agent administered alone (Supplementary Table S1). $N = 8$–10/group, additional statistics in text.

(13, 40). Insensitivity to single-agent venetoclax did predict a more antagonistic response to combination therapy (Fig. 2A), as did higher BCL-XL expression levels (Fig. 2B). While high BCL-XL expression is a documented venetoclax escape mechanism, resistance to venetoclax correlates with different BCL-2 proteins in different diseases, and these correlations are not fully predictive (4, 37, 38, 41–43). Similarly, in our study, neither BCL-XL levels nor venetoclax sensitivity fully predicted response to combination therapy (Fig. 2). Identifying biomarkers of response to venetoclax plus radiotherapy merits further study, potentially including transcript and protein level examination of BCL-2 pathway, BCR signaling, and DNA damage cascades, across a range of B-NHL subtypes.

Recent, comprehensive reviews confirm the ongoing importance of radiotherapy for treating NHL (20, 44–46). Two types of radiotherapy are currently available for clinical use, RIT, and external beam radiotherapy. While RIT generally provides a superior therapeutic index due to superior targeting, RIT is not recommended for all patients, and external beam modalities remain effective in many settings (5, 47). In a study of DLBCL patients over the age of 60, radiotherapy consolidation after R-CHOP improved OS from 67 to 89% and PFS from 49 to 79% at five years, with no reported adverse effects (47). A review of NHL studies from 2004 through 2015 concluded that radiotherapy use in NHL has declined in the rituximab era, but that excluding radiotherapy decreases response rates and worsens toxicity in many disease subtypes (5). Radiotherapy efficacy has improved with modified dosing and more nuanced approaches to identifying patients most likely to benefit (5, 47). Our findings suggest that adding venetoclax might further improve the therapeutic index of radiotherapy, even at reduced radiotherapy doses, and that this combination deserves consideration.

In most situations however, radiolabeled antibodies will be therapeutically preferable to external beam radiotherapy, and our results support the benefits of this approach. In xenograft models, combinations of venetoclax with PRIT showed greater synergism and produced more cures than combinations of venetoclax with external beam radiotherapy (Fig. 3 vs. 4; Supplementary Table S1). PRITs' superiority is likely the result of multiple factors that differentiate these delivery methodologies, including differences in both the dose rate and the total dose delivered to tumor. Prior dosimetry and biodistribution studies published by our group demonstrate that the enhanced therapeutic ratios of ^{90}Y-SA PRIT allow delivery of the highest total radiation dose to lymphoma xenografts of any radiotherapy method (11, 25, 48–50). In the current study, we purposefully included lower, suboptimal doses of radiotherapy to examine the range of synergistic responses, and demonstrate in both external beam radiotherapy and PRIT experiments that higher radiation doses produce greater synergistic responses in combinations with venetoclax (Supplementary Table S1). These results suggest that higher total radiation doses absorbed by tumor tissue with PRIT vs. external beam radiotherapy contribute, at least in part, to the greater efficacy and synergy of PRIT plus venetoclax.

We elected to study two-step PRIT rather than single-step RIT as our group and others have previously established the superior therapeutic index of PRIT. Our current findings are consistent with this prior experience, here demonstrating that adding venotoclax to PRIT greatly increases cure rates without adding detectable toxicity. Anti-CD20 PRIT is currently being studied in a phase I/II clinical trial for high-risk B-NHL (Trial NCT02483000, ClinicalTrials.gov), raising the possibility of future clinical translation of our combination approach. Furthermore, our results suggest that combinations of venetoclax and conventional, single-step RIT may also lead to improved responses among patients with B-cell lymphoma. Single-step RIT is commercially available and effective against several hematologic malignancies (20, 44–46). While we did not directly test conventional RIT, the synergy observed between

venetoclax and both external beam radiotherapy and PRIT strongly suggests venetoclax would also synergize with single-step RIT. Reasons for RIT efficacy include the presence of surface antigens largely restricted to specific hematologic tissues, the availability of mAbs that efficiently target these antigens, and the extreme radiosensitivity of leukemias and lymphomas. In studies of follicular lymphoma and DLBCL, conventional CD20-targeted RITs produced ORRs of 80%–100% and CRs of 72%–96% when used as consolidation after frontline treatments, and significantly improved treatment responses in R/R disease (20, 45). In MCL, CD20 RIT contributed to ORRs of 88%–100% and CRs of 67%–100% in first-line treatments, and improved R/R treatment outcomes (45). In both DLBCL and MCL, RIT was additionally effective as a conditioning agent prior to hematopoietic cell transplant (HCT), improving response rates while reducing toxicity (20, 44). Moreover, RIT may be more cost effective and simpler to administer than many treatment alternatives including prolonged, continuous drug therapies (46). Despite these benefits, conventional RIT is underutilized in current practice, and when used, is most effective when combined with toxic agents or HCT, making it important to identify safer treatment regimens. In our mouse models, combining venetoclax with radiotherapies ranging from low-dose, minimally toxic PRIT to high-dose, myeloablative external beam radiotherapy consistently improved survival with no added toxicity (see "Treatment toxicities" in Results). We conclude that venetoclax plus PRIT, and likely venetoclax plus conventional single-step RIT, represent safe and promising therapeutic combinations. Our immunocompromised mouse model, and our use of single-dose rather than fractionated external beam radiotherapy, differ from clinical settings and should prompt caution when considering translation of this research. Well-designed clinical trials can address these issues, however, and we believe the combination of radiotherapy and venetoclax may offer a valuable treatment option in the large range of diseases constituting B-NHL.

Disclosure of Potential Conflicts of Interest

B.G. Till reports receiving other commercial research support from Genentech. O.W. Press has ownership interest (including patents) in Emergent Biosolutions. No potential conflicts of interest were disclosed by the other authors.

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Venetoclax Synergizes with Radiotherapy for Treatment of B-cell Lymphomas

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