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Additive and synergistic inhibition of mantle cell lymphoma cell growth by combining olaparib with ibrutinib

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Abstract

Mantle cell lymphoma (MCL) presents a therapeutic challenge. The B cell targeting agent, ibrutinib, is currently one of the most effective second-line therapies for MCL, but frequently leads to development of drug resistance, and short overall survival time upon relapse. Olaparib targets tumor cells with deficiencies in single-strand DNA break repair and thus may slow the development of genetic drug resistance. We found that the olaparib-ibrutinib combination significantly inhibits cell culture growth compared to either drug alone in two genetically distinct MCL cell lines. Moreover, these inhibitory effects are either additive or synergistic, depending on genetic background. Culture growth is inhibited due to increases in apoptosis, cell death, and cell cycle arrest, and the magnitude of each is cell line dependent. The additive and synergistic inhibition of this combination additionally supports a therapeutic strategy involving lower dosing of each drug to reduce potential side effects.

KEYWORDS

apoptosis, cell cycle changes, chemotherapeutic approaches, ibrutinib, lymphoma, mantle cell lymphoma

1 | INTRODUCTION

Mantle Cell Lymphoma (MCL) is a B cell malignancy that accounts for 3-6% of non-hodgkin lymphoma (NHL) in Western countries.[1] It is an aggressive disease seen predominantly in older males, with a median survival time of only 3-4 years.[1,2] MCL is usually stage III or IV at diagnosis, involving multiple lymph nodes, and other sites of the body.[2] Rapid progression is common in MCL, with only temporary responses to chemotherapy.[3] New therapies for relapsed patients is a pressing need.

The tumor microenvironment plays a significant role in the pathogenesis of MCL and thus provides a key opportunity for targeted therapy.[1] Mantle cell lymphoma derives its name from the malignant B cells that originate in the mantle zone surrounding the normal germinal center in the follicle of the lymph node.[3] MCL relies on activation of the B cell receptor (BCR) pathway by the tumor microenvironment for proliferation and survival.[4] Inhibition of the BCR signaling pathway leads to down-regulation of chemokines, particularly CCL3 and CCL4, that in turn help maintain the tumor microenvironment[4] With a disrupted tumor architecture, malignant cells stop proliferating and become prone to apoptosis, and destruction by the immune system.[4]

Ibrutinib is an inhibitor of the BCR signaling pathway, directly inhibiting the Bruton's tyrosine kinase (BTK). This B cell targeting therapy currently is the most effective single agent in the second line treatment for MCL.[5] Initial response to treatment with Ibrutinib in MCL patients is excellent, with an overall response rate of 68%, complete response of 12%, and a progression free survival of 13.9 months.[5] The prognosis for patients that relapse after treatment with

ibrutinib, however, is very poor; median overall survival is only 8 months on salvage therapy.[6] Evaluation of the efficacy of combining other drugs, such as rituximab, with ibrutinib in second line treatment is currently being studied. However other therapy options are needed, particularly to reduce the development of ibrutinib resistance.

One class of drugs that may potentially provide a longer duration of MCL response is the polyADP ribose polymerase (PARP) inhibitors (PARPi). The PARP family member, PARP-1, functions as part of the DNA repair machinery, sensing, and binding to sites of DNA damage and initiating DNA repair.[7] The anti-cancer mechanism of PARPi's involves the inactivation of salvage DNA repair pathways in cells that are already compromised for DNA repair. PARPi's force these tumor cells toward apoptosis and death due to the complete inability to repair DNA. This concept is known as "synthetic lethality."[8] In 2014, the drug olaparib became the first PARPi to receive FDA approval, in this case for second line treatment of BRCA mutated ovarian cancer.[9]

MCL cells are marked by a relatively high degree of genomic instability and thus may be prime targets for PARPi therapy[10]. MCL is characterized by chromosomal rearrangements that lead to constitutive overexpression of cyclin D1, loss of TP53, and/or loss of the DNA repair factor, ATM. PARPi's are not currently approved for MCL treatment, but preliminary studies indicate the potential for their effectiveness, particularly in cases with mutations in BRCA1, BRCA2, ATM, and TP53 genes.[7] MCL cell lines harboring a mutation in ATM were shown to be more sensitive to olaparib than those with wild type ATM.[11] In addition, MCL cells deficient for both TP53 and ATM were more sensitive to olaparib than those deficient for ATM alone.[12] One potential drawback to the use of olaparib for MCL is an increase in hematological side effects, including anemia and lymphocytopenia, which have been reported for ovarian cancer patients treated with olaparib.

One way to limit the side effects of olaparib in MCL patients is by combination with a drug that synergistically, or

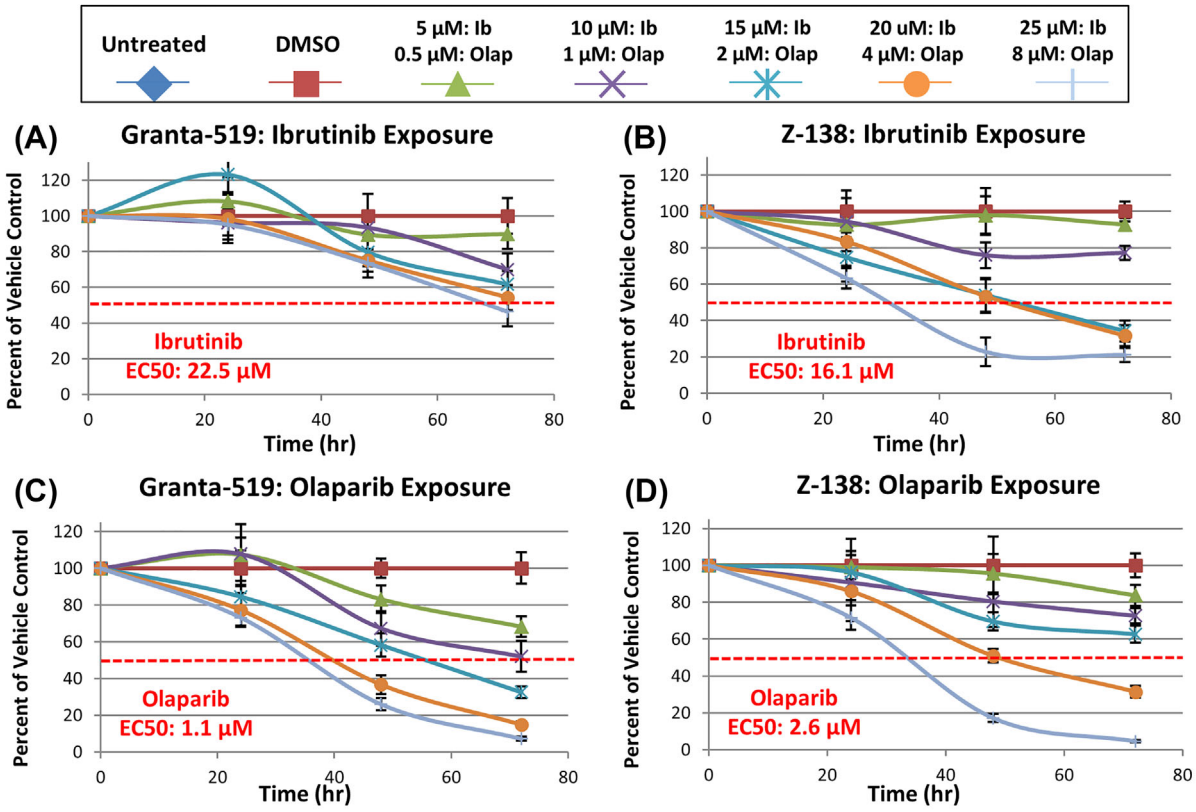

FIGURE 1 MCL cell lines are more sensitive to olaparib than ibrutinib.: 9A and C) Granta-519 and (B and D) Z-138 cells were exposed to 5, 10, 15, 20, or 25 µM ibrutinib (A and B), or 0.5. 1, 2, 4, and 8 µM olaparib (C and D), along with DMSO drug vehicle for a total of 72 hours. Cell concentrations were determined every 24 h. Cellconcentrations normalized to DMSO control are shown. Both cell lines were inhibited in a dose dependent fashion upon exposure to both drugs with both cell lines exhibiting greater sensitivity to olaparib, than ibrutinib based on molar concentration. Z-138 cells were more greatly impacted by ibrutinib exposure than Granta-519 (EC50 of 16.1 µM versus 22.5 µM, respectively). Conversely, Granta-519 cells were more greatly impacted by olaparib exposure than Z-138 (EC50 of 1.1 µM versus 2.6 µM at 72 h, respectively). 95% confidence intervals of triplicate experiments are shown

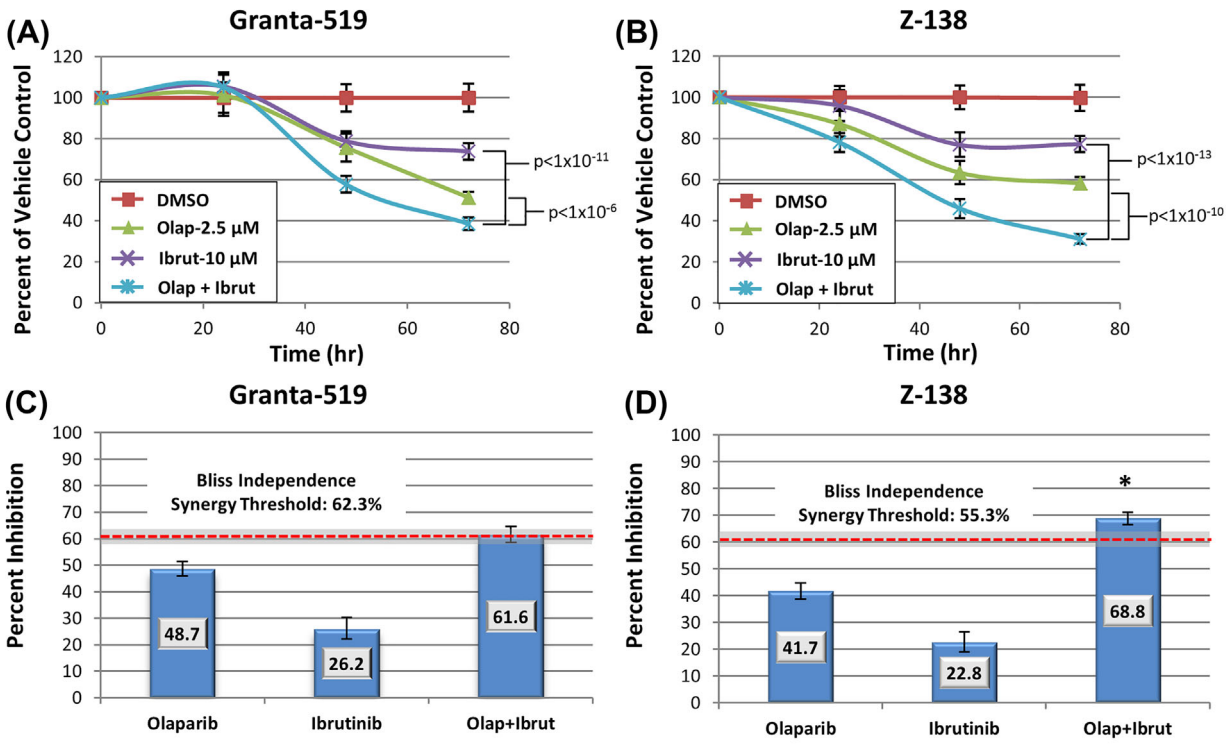

FIGURE 2 Additive and synergistic effects of ibrutinib plus olaparib treatment on MCL cell lines. (A) Granta-519 and (B) *Z*-138 cell were exposed to 2.5 μM olaparib, 10 μM Ibrutinib, or both drugs in combination for 72 h. Cell concentrations were assayed every 24 h and compared to those treated with drug vehicle (DMSO) control only. Error bars represent 95% confidence intervals of triplicate replicates from three independent experiments. *P* values derived from Student's *t*-test. (C and D)The Bliss Independence model of additivity indicates an additive effect of the drug combination for Granta-519 (C), and a synergistic effect for *Z*-138 (D). *P* value based on Student's *t*-test of the drug combination and the synergy threshold. Error bars indicate 95% confidence intervals of triplicate replicates from 3 independent experiments. Shaded area indicates 95% confidence interval of Bliss Independence threshold (64.8-59.7%, Granta-519; 57.39-53.13%, *Z*-138)

even additively, inhibits MCL growth. Combinatorial growth inhibition allows for lower dosing of each of the component drugs, particularly when one of the agents provides cell targeted therapy. Hence, we tested whether or not the addition of olaparib to the B cell targeting agent, ibrutinib, additively, or synergistically inhibits MCL cell growth. Importantly, this combination has the potential to delay ibrutinib resistance, since olaparib targets cells with high levels of DNA damage, which contributes to the development of genetic resistance. Here we report the results of the first step in evaluating this combination therapy, testing the combined effects of olaparib and ibrutinib on MCL cell lines. Our results show at least additive effects of the two drugs on MCL cell line growth, with the potential for synergistic inhibition depending on genetic background.

2 | RESULTS

To assess the combinatorial effects of olaparib and ibrutinib on MCL cells in vitro, we utilized two distinct cell lines: Granta-519 and *Z*-138. The Granta-519 line was derived from peripheral blood and has a mature B-cell phenotype. Granta-519 cells express high levels of cyclin D1 due to the chromosomal translocation of t(11;14)(q13;q32), but are deficient for ATM.[11,13–15] In contrast to Granta-519 cells, the cell line *Z*-138 was derived from the bone marrow of a blastoid mantle cell lymphoma patient[16] *Z*-138 cells express normal levels of ATM and relatively higher levels of cyclin D.[16] In terms of cell surface markers, Granta-519 and *Z*-138 cell lines both lack CD5 expression and express lambda light chain and CD19 surface proteins.[14–16] Unlike Granta-519, *Z*-138 cells do not express HLA-A2 on their surface. Thus, this marker can serve to distinguish between the two cell lines[14,16] We confirmed these differences by flow cytometry in our cultures (Data not shown).

We next generated dose response curves for olaparib and ibrutinib in Granta-519 and *Z*-138 cultures. Culture growth of both cell lines was inhibited in a dose dependent manner by exposure to ibrutinib (Figures 1A and 1B). The EC50 doses at 72 h were 22.5 μM for Granta-519 (Figure 1A) and 16.1 μM for *Z*-138 (Figure 1B). Thus *Z*-138 cultures showed greater

sensitivity to ibrutinib compared to Granta-519 cultures. Similar to ibrutinib treatment, olaparib inhibited culture growth for both cell lines in a dose dependent fashion. Both cell lines were considerably more sensitive to olaparib exposure than to ibrutinib. The EC50 doses for Granta-519 and *Z*-138 cell lines at 72 h were calculated as 1.1 μM and 2.6 μM, respectively (Figures 1C and 1D). In contrast to ibrutinib, the ATM deficient Granta-519 cultures were more sensitive to olaparib than were *Z*-138 cultures, consistent with previous reports.[11,17]

To test for combinatorial effects of olaparib and ibrutinib, we treated both cell lines with 2.5 μM olaparib plus 10 μM ibrutinib and compared the growth rates to cells exposed to each drug individually and the drug vehicle (DMSO) for 72 h (Figure 2). Inhibition of growth for the combined drugs was greater than that observed with either drug alone. This difference was statistically significant at 72 h for both cell lines ($P < 1 \times 10^{-7}$, Student's *t*-test). Determination of whether the observed combinatorial effect was additive or synergistic was achieved using the bliss independence model of additivity.[18,19] Calculation of the threshold for additive versus synergistic effects indicated that the olaparib and ibrutinib combination had an additive effect for Granta-519 cells (Figure 2C), but had a statistically significant, synergistic effect for the *Z*-138 cells ($P < 1 \times 10^{-7}$, Student's *t*-test) (Figure 2D).

Additive effects suggest that olaparib and ibrutinib are impacting mutually exclusive pathways, whereas synergistic effects indicate an enhanced impact of both drugs on one pathway or more. To determine the mechanisms through which olaparib and ibrutinib inhibit MCL growth in combination, we tested the contributions of these agents on cell survival and cell cycling. The effects of the combined drugs on apoptosis and cell death were evaluated by flow cytometry, staining with Annexin V, a marker of early apoptosis, and the vital dye 7AAD. These analyses indicated that combination drug treatments significantly increased the amount of apoptotic and dead cells in both cell lines compared to DMSO controls ($P < 0.05$, apoptotic; $P < 0.012$, dead) (Figure 3). Neither cell death nor apoptosis was increased by ibrutinib alone for either cell line ($P > 0.1$ apoptotic; $P > 0.15$ dead). In contrast, olaparib alone induced statistically significant increases in both dead and apoptotic cells compared to the drug vehicle control in both cell lines ($P < 0.012$, apoptotic; $P < 0.019$, dead) (Figure 3). There was a small increase in apoptosis at 72 h observed in the *Z*-138 cells in the drug combination compared to olaparib alone, but this increase was not statistically significant ($P > 0.2$). Therefore, the synergistic inhibition of *Z*-138 culture growth was associated with enhanced cell inhibition that did not arise from cell death or apoptosis.

Next, we tested whether or not culture growth inhibition was associated with cell cycle arrest, in addition to apoptosis

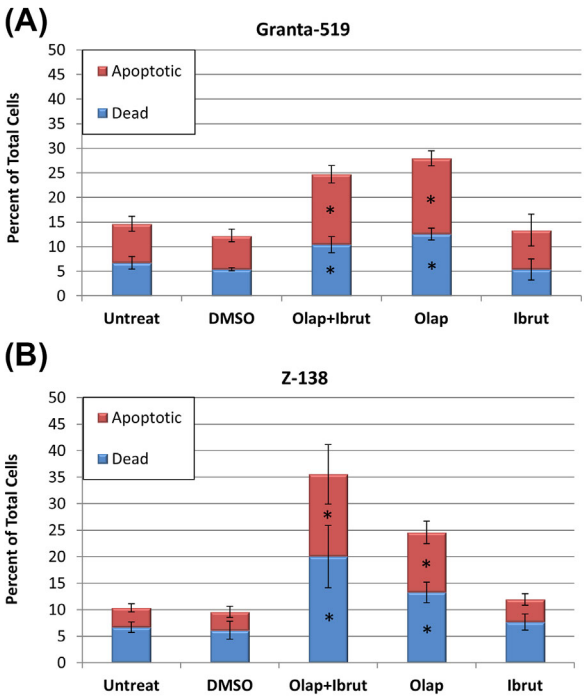

FIGURE 3 Treatments with olaparib, either alone or in combination, increased dead and apoptotic populations in both MCL cell lines. Flow cytometry with Annexin V and 7AAD was used to determine viability and apoptosis percentages at 72 h after individual drug treatments and drug combinations. Less cell death was observed in the combination of olaparib and ibrutinib in (A) Granta-519 than in (B) *Z*-138 cell cultures. Ibrutinib treatment does not result in increased cell death or apoptosis for either cell line. Error bars represent 95% confidence intervals from 3 independent experiments. Asterisks indicate significant difference from DMSO vehicle control per Student's *t*-test, $P < 0.05$

and cell death. We used Hoechst 33 342 cell staining and flow cytometry to assess G1-, S-, and G2-phase population sizes in cultures treated with single agents and the olaparib-ibrutinib combination (Figure 4). Most notably, these analyses indicated that the percentage of cells in G2-phase increased significantly for Granta-519 cultures containing olaparib (olaparib alone, olaparib plus ibrutinib, 72 h) compared to control cultures (Figures 4A and4B). Interestingly, this G2 block was not observed in *Z*-138 cells, regardless of treatment condition (Figures 4C and 4D).

Cell cycle analyses throughout the entire time course of drug treatment revealed additional impacts on cell cycle dynamics. In both cell lines, temporary G1-phase blocks were detected in the ibrutinib only and drug combination samples (Figures 5A and 5B). These G1 blocks returned to control levels by 72 h. Concomitant losses in S-phase cells were observed in the ibrutinib only samples (Figures 5C and 5D). These data suggest that ibrutinib induced a small, transient G1

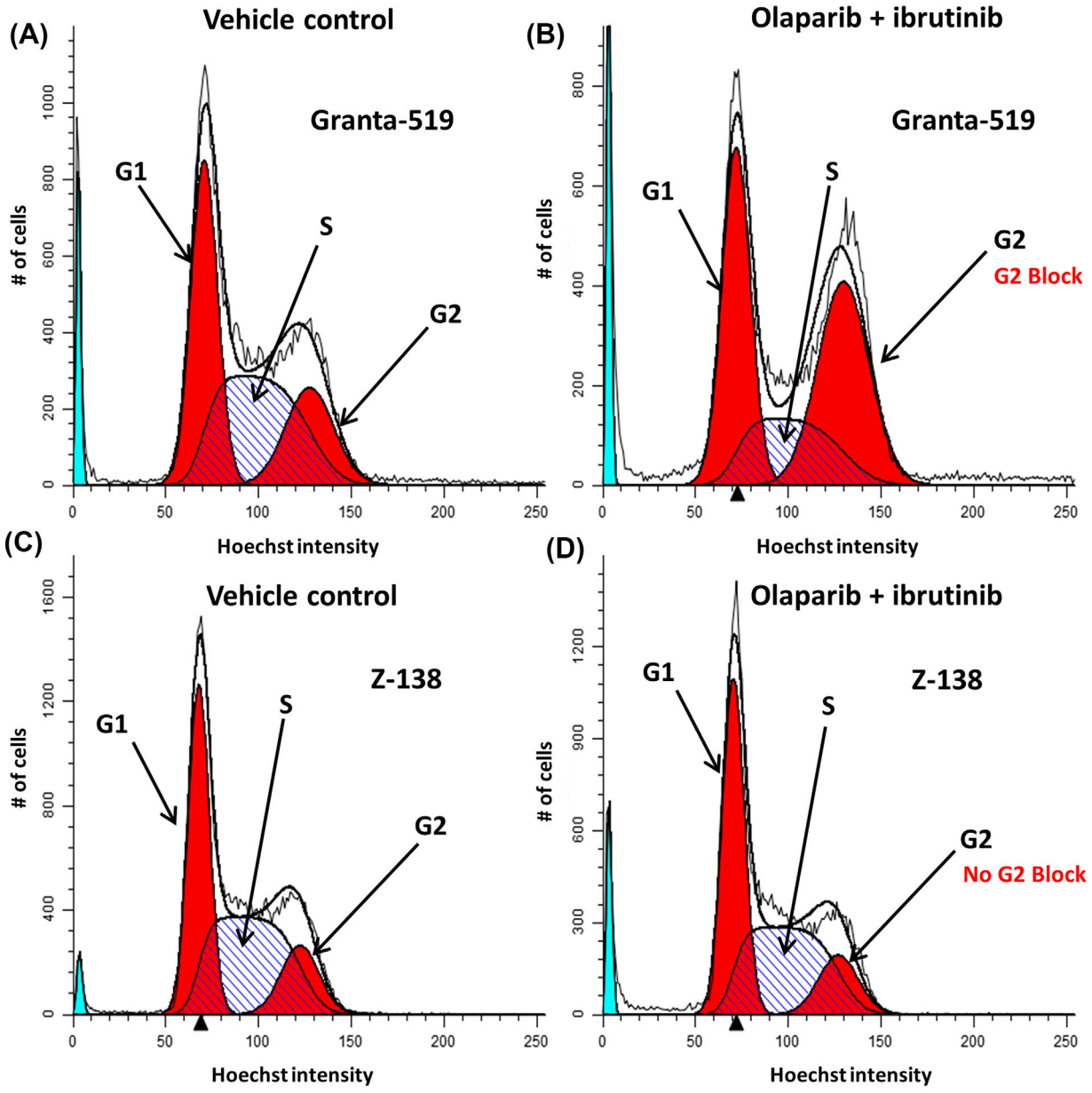

FIGURE 4 Olaparib and ibrutinib induce cell line dependent effects on cell cycle progression. (A), (B) Granta-519 and (C), (D) *Z*-138 cultures were treated with (A), (C) vehicle control or (B), (D) 2.5 μM olapariband 10 μM ibrutinib for 72 h, stained with Hoechst 33 342, and analyzed by flow cytometry and cell cycle modeling (red and hatched peaks). Granta-519, but not *Z*-138, culture showed an increase in the G2-phase cell population and a concomitant decrease in S-phase population with the combination drug treatment. Data from one representative experiment are shown

block in both cell lines that temporarily reduced the S-phase percentage.

Cell cycle dynamics for the combination treated cultures reflected the combined effects of ibrutinib and olaparib alone. For example, the decrease in S-phase percentage extended for the full time course in the drug combination sample in Granta-519. These dynamics reflect both the G1 block caused by ibrutinib that peaks at 24 h and the induction of an olaparib mediated G2 block beginning at 24 h (Figure 5E). An S-phase decrease was not sustained in the drug combination sample in *Z*-138 cells, however, because a G2 block was not induced and G1 percentages returned to control levels by 48 h (Figure 5B-F).

Although the G1 block observed in both cell lines was transient and of substantially lower magnitude than the G2 block observed in Granta-519, it could potentially contribute significantly to overall growth inhibition by slowing down the first cell division in the treated cultures. This would result in

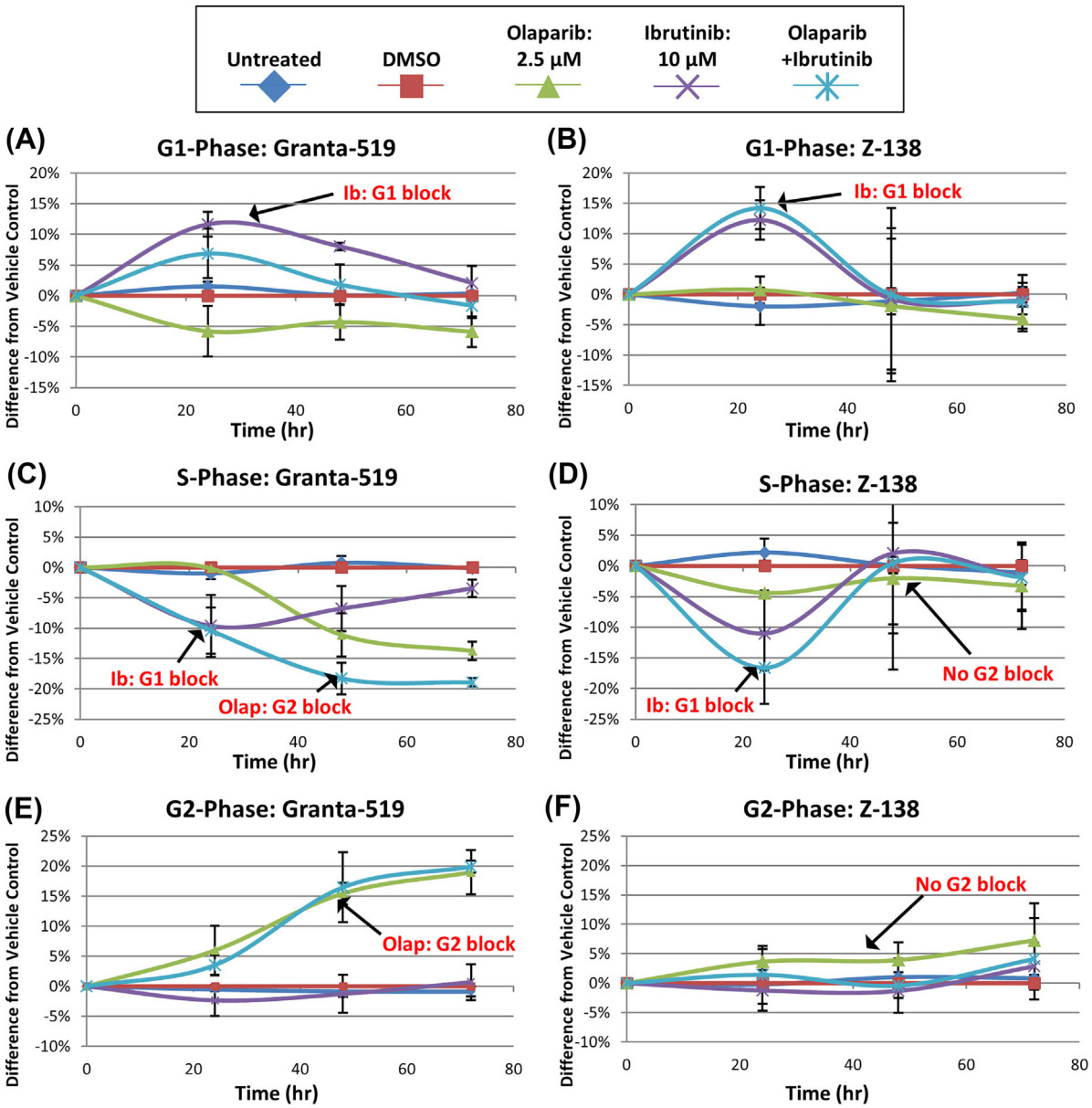

FIGURE 5 Time course experiments reveal dynamics in G1-, S-, and G2-phase cell populations caused by ibrutinib and olaparib. (A), (C), (E), Granta-519 and (B), (D), (F) *Z*-138 cells were treated as indicated (top) and analyzed by Hoechst staining and flow cytometry every day for 3 days. (A), (B) Cultures exhibited a transient G1-phase block after 24 h treatment with ibrutinib alone or in combination. C, The S-phase population was reduced transiently in Granta-519 at 24 h in the ibrutinib only sample. S-phase reduction also was seen in the olaparib only sample but not until 48 h. S-phase populations remain reduced after 24 h in cultures treated with both drugs. The dynamics of S-phase reduction are consistent with increases in G1- and G2-phase populations at different time points. D, Ibrutinib induced a temporary reduction in S-phase percentage in *Z*-138 cells, but this returned to control levels by 48 h. Olaparib had little effect on Sphase percentage in *Z*-138 cells. E, Samples exposed to olaparib alone or in combination with ibrutinib showed a G2 block in Granta-519 cells, as indicated by the increase in the percentage of cells accumulating in G2 over the time course. F, Olaparib did not induce a G2 block in *Z*-138 cells. Error bars are 95% confidence intervals from 3 independent experiments

the delayed proliferation of ibrutinib containing samples for the entire time course. In addition, *Z*-138 cultures had a more substantial increase in apoptosis and cell death (Figure 3) that, in combination with the G1 block, actually resulted in greater overall inhibition than observed in Granta-519. These findings are summarized in Table 1.

2.1 | Table 1: Summary of ibrutinib and olaparib effects on MCL cells

No significant differences compared to vehicle control = −($P > 0.05$, Student's *t*-test). Significant differences with $5 \times 10^{-3} < P \leq 0.05$ = +, significant

TABLE 1 Summary of ibrutinib and olaparib effects on MCL cells

	Ibrutinib		Olaparib		Drug Combination	
	Granta-519	Z-138	Granta-519	Z-138	Granta-519	*Z*-138
Cell death	−	+	+	+	+	++
Apoptosis	−	−	+	+	+	+
G1 block	+	+	−	−	+	+
S phase reduction	+	+	++	−	++	+
G2 block	−	−	+	−	++	−
Overall inhibition	+++	+++	+++	+++	++++	++++

differences with $5 \times 10^{-3} < P \leq 5 \times 10^{-4} = ++$, significant differences with $5 \times 10^{-4} < P \leq 5 \times 10^{-10} = +++$, significant differences with $P < 5 \times 10^{-10} = ++++$.

3 | DISCUSSION

Our findings suggest that combining a PARP inhibitor, such as olaparib, with ibrutinib therapy has the potential to be an effective therapeutic approach and may result in greater efficacy in treating MCL than either drug used alone. The differential response to the drug combination demonstrated in Granta-519 and *Z*-138 cell lines suggests that this therapy may have differential effects depending on the genetic profile of the malignant cells, and may act synergistically on certain genotypes. Since the greatest obstacle in mantle cell lymphoma treatment is the difficulty to treat after recurrence, the addition of a PARPi to ibrutinib therapy also may help to delay the development of ibrutinib resistance by targeting cells with the highest levels of DNA damage.

Olaparib previously has been reported as an effective agent for the inhibition of the growth of MCL cultures in vitro,[11] and we have confirmed this finding with olaparib alone and in combination with ibrutinib. Further, we found that the combination of ibrutinib with olaparib results in synergistic inhibition of *Z*-138 culture growth and additive inhibition of Granta-519 cultures. The additive inhibition of Granta-519 cells can be attributed largely to G2 arrest and cell death through apoptosis. The G2 arrest is likely due to the actions of olaparib in these ATM deficient cells, which eventually leads to apoptosis and cell death. In contrast, the synergistic inhibition of *Z*-138 cultures was associated primarily with increased apoptosis and cell death rather than G2 arrest. The mechanisms for this synergy are unclear, but must result from some genetic difference between *Z*-138 and Granta-519 cells.[7]

It is interesting to note that the cell lines have inverse sensitivities to the single agent drugs, with olaparib having a greater effect on Granta-519 cells compared to *Z*-138, and ibrutinib having a greater effect on *Z*-138 compared to Granta-519 cells. The lack of ATM expression may sensitize Granta-519 to olaparib, as evidenced by the induction of the G2 block. Nearly all of the increased cell death and apoptosis could be attributed to olaparib based on comparison to single agent olaparib exposures. This suggests that ibrutinib primarily inhibits cell growth rather than killing cells outright. This finding is in agreement with the previously described role of BTK inhibition in vivo, to disrupt pro-growth signals from the B cell receptor and also to disrupt the tumor microenvironment by disrupting the cells response to chemokine signaling, but not directly to induce apoptosis.[4] It is unclear what the mechanism is for the greater ibrutinib sensitivity displayed by *Z*-138, but the faster constitutive growth rate of *Z*-138 may amplify this effect. *Z*-138 cultures were found to have a doubling time of 24.1 h versus 30.8 h for Granta-519 (Figures 2A and 2B). Although ibrutinib is not a direct cell cycle regulator, it does disrupt pro-growth signalling.[4,5]

Because of their ability to induce synthetic lethality in cells with compromised DNA repair mechanisms, PARPi's are attractive for use in combination therapy with an established treatment regimen. Many combinations of chemo, immune, and radiation therapy have been tried with varying success. Among the more promising combination partners is the chemotherapy Temozolomide (TMZ), which takes advantage of the "PARP trapping" properties of olaparib to produce a synergistic therapeutic effect, not dependent on homologous recombination deficiencies.[20] Combining a PARPi with taxanes, such as paclitaxel and docetaxel, also has shown improvement in overall survival in recurrent or metastatic gastric cancer.[21] PARPi combination with platinum based chemotherapy has shown to be synergistic in breast cancer mouse xenografts and in ovarian cancer cell lines. However, significant myelosuppression and neutropenia were seen in phase I clinical trials and further study is needed[22,23] PARP inhibitors work very well to sensitize tumors for radiation therapy in many different tissue types.[24,25] It is currently being determined whether concurrent or neoadjuvant treatment with PARPi's work best with radiation therapy.[7] PARP inhibitors also show synergistic anti-tumor effects when combined with targeted therapies such as EGFR and VEGFR inhibitors.[26,27] Based on our

findings, combining a PARP inhibitor with ibrutinib therapy for mantle cell lymphoma has the potential reduce recurrence and increase efficacy in mantle cell lymphoma. Given the synergistic, or at least additive, effects of the drug combination, there is also the potential of dose reduction and concurrent side effect reduction with this therapeutic approach.

4 | MATERIALS AND METHODS

4.1 | Cell lines and culture

Granta-519 cells were a gift from Dr. David Weinstock (Dana Farber Cancer Institute, Boston, MA). *Z*-138 cells were obtained from ATCC. Control primary CLL cells from peripheral blood were obtained from the Eastern Maine Medical Center (EMMC) BioBank, which collected blood from patients with informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the Institutional Review Board (IRB) of EMMC (Bangor, ME). Cell lines were cultured in RPMI-1640 (Fisher Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (Rocky Mountain Biologicals, Missoula, MT), L-glutamine (Fisher Scientific), and penicillin-streptomycin (Fisher Scientific). Cells were maintained in T-75 flasks at 37C with a 5% atmospheric CO_2 concentration.

4.2 | Drug exposure growth curves

For drug exposure treatments, cells were seeded at 5×10^5 cells/mL in 125 μL of complete media (see above) in 48-well culture plates (Fisher Scientific). Cells were exposed to DMSO (Sigma-Aldrich, St. Louis, MO), ibrutinib (Selleck Chemical, Houston, TX), olaparib (Sigma-Aldrich), or a combination of ibrutinib and olaparib. Drugs were initially dissolved in DMSO, and then diluted to a working stock in PBS with 10% DMSO. Working stocks were further diluted in RPMI complete media described above for drug exposure experiments. Cells were cultured for 72 h and counted every 24 h using a hemocytometer and Trypan Blue viability dye (Fisher Scientific) to distinguish viable cells.

Student's *t*-tests were used to compare treated and untreated cell concentrations at a given time point, using $P < 0.05$ as the threshold for significant differences. The Bliss Independence Model was applied to differentiate between synergistic, additive, and sub-additive effects of drug combinations. This model assumes that drugs act independently in such a manner that neither of them interferes with the other, but each contributes to a common result.[18,19] The expected additive effect was calculated by the common formula for probabilistic independence: $(E_A + E_B)$-(E_AE_B), where E_A = the effect of drug A alone, and E_B = the effect of drug B alone.[19] This value established a threshold value for synergistic effect and was then compared to the effect of drug A and drug B in combination. Combinatory effects below this threshold were considered to be sub-additive, those that were equal to the threshold were additive, and those that exceeded the threshold were considered to be synergistic.[19]

4.3 | Cell line characterization

5×10^5 cells were labelled with 1/22 dilutions of CD19-APC, CD5-APC-Vio770, HLA-A2, anti-Kappa, and anti-Lambda antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 min at room temperature, washed twice and analyzed on the Beckman Coulter Gallios flow cytometer. Control primary CLL cells were labeled with 1/22 dilutions of anti-CD10 Vioblue (isotype control) or anti-CD5-APC-Vio770 (Miltenyi Biotec) following this same protocol. Flow cytometry data files were analyzed using WinList 7 analysis software (Verity Software House).

4.4 | Viability and apoptosis

For viability and apoptosis analysis cells were seeded at 5×10^5 cells/mL in 48-well plates, treated with drug or vehicle for 72 h, and analyzed at 24 h intervals. Cells were labelled with Annexin-V-FITC (BD Biosciences, San Jose, CA) and 7AAD (Fisher Scientific) for 10 min at room temperature, washed twice, and analyzed on the Beckman Coulter Gallios flow cytometer. Flow cytometry data files were analyzed using WinList 7 analysis software (Verity Software House, Topsham, ME).

4.5 | Cell cycle

Cells were seeded at 5×10^5 cells/mL in 48-well plates, treated with drug or vehicle for 72 h, and analyzed at 24 h intervals by flow cytometry for cell cycle. Cells were labelled with 500 μg/mL Hoechst-33342 (BD Biosciences) for 15 min at 37C, washed twice, and analyzed on the Beckman Coulter Gallios flow cytometer. Flow cytometry data files were analyzed using ModFitLT cell cycle analysis software (Verity Software House).

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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