DNA Damage Response Inhibitor Combinations Exert Synergistic Antitumor Activity in Aggressive B-Cell Lymphomas

Valentina Restelli1, Monica Lupi1, Rosaria Chià1, Micaela Vagni1, Chiara Tarantelli2, Filippo Spiriano2, Eugenio Gaudio2, Francesco Bertoni2, Giovanna Damia1, and Laura Carrassa1

Abstract

The DNA damage response (DDR) kinases ATR, Chk1, and Wee1 play vital roles in the response to replication stress and in maintaining cancer genomic stability. Inhibitors of these kinases are currently under clinical investigation. Mantle cell lymphoma (MCL) and diffuse large B-cell lymphoma (DLBCL) are aggressive lymphomas whose clinical outcome is still largely unsatisfactory. These cell lymphoma subtypes are highly dependent on both Chk1 and Wee1 for survival. We investigated the activity of the ATR inhibitor AZD6738 as single agent and in combination with either Chk1 (AZD6738) or Wee1 (AZD1775) inhibitors in several preclinical models of MCL and DLBCL. This study included preclinical in vitro activity screening on a large panel of cell lines, both as single agent and in combination, and validation experiments on in vivo models. Cellular and molecular mechanisms of the observed synergistic effect as well as pharmacodynamic analysis of in vivo samples were studied. AZD6738 exerted a strong synergistic cytotoxic effect in combination with both AZD7762 and AZD1775 in the 2 lymphoma subtypes regardless of their TP53, MYC, and ATM mutational status. These DDR inhibitor combinations, similarly to the Chk1/Wee1 inhibitor combination, caused a marked S-phase delay, with an increase in cyclin-dependent kinases (CDK) activity, increased DNA damage, and decreases in Wee1, MYC, and RRm2 protein levels. The synergistic in vitro activity translated to striking in vivo antitumor activity. DDR–DDR inhibitor combinations could potentially offer promising novel therapeutic strategies for patients with B-cell lymphoma.

Introduction

The DNA damage response (DDR) kinases ATR, Chk1, and Wee1 are required during normal S phase to avoid deleterious DNA breakage, and to maintain cancer cell survival under replication stress (RS; ref. 1). RS may be caused by improper control of replication initiation, associated with molecular features very common in cancer (e.g., constitutive activation of oncogenes such as RAS, MYC, cyclin E, cyclinD; inactivation of key onc suppressors such as TP53, RB1, CDKN2A; ref. 2). RS activates the ATR/CHK1 pathway. The ATR kinase is activated by the ssDNA generated during RS, phosphorylates CHK1, and activates the S-phase checkpoint, preventing the collapse of the replication fork, the generation of DSB, and the formation of new origin firing, allowing the maintenance of DNA damage at a tolerable level (3, 4). The inhibition of the ATR/CHK1 pathway leads to an inappropriate initiation of DNA replication, consumption of replication factors, fork stalling, and fork collapse (5). The proper timing of DNA replication is also controlled by Wee1, which inhibits cyclin-dependent kinase 2 (CDK2) responsible for the regulation of replication origin firing. As a result, ATR, CHK1, and Wee1 directly control the proper DNA replication rate during S-phase progression and appropriately respond to RS (6). Inhibitors of Chk1, Wee1, and ATR are currently under clinical investigation, mainly in combination with chemotherapy or radiotherapy (5, 7). The combined targeting of these DDR proteins with other targeted drugs (without chemotherapy) is approaching the clinical setting (e.g., the PARP inhibitor olaparib, the p38/MAPK kinase inhibitor ralimetinib; ref. 5). Recent data from the literature and our own support the hypothesis that combining inhibitors against these DDR components with non-redundant roles could be of therapeutic value in preclinical models (8–11).

Mantle cell lymphoma (MCL) is an incurable form of lymphoma with a median overall survival of only 4 to 5 years. Its clinical course usually involves early relapses, with no responsiveness to current standard therapies (12, 13). Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive lymphoma in Western countries, with approximately 40% of patients having refractory disease or disease that will relapse after an initial response to first-line therapy. Unfortunately, the majority of patients with relapsed DLBCL succumb to the disease (14).
Deregulation of the cell cycle is the pathogenic hallmark of MCL due to both the chromosomal translocation t(11;14), which leads to overexpression of cyclin D1, and to enhanced expression of the oncogene MYC and inactivation/genetic alteration of cell-cycle inhibitors (12, 15, 16). MYC aberrations are also present in more than 40% of DLBCL and define patients with a more aggressive phenotype and a poor outcome (14, 17). Thus, both MCL and DLBCL are characterized by having a high endogenous level of RS, due to constitutive activation of such oncogenes (e.g., MYC, cyclinD1) and oncosuppressors (e.g., TP53) that cause improper DNA replication initiation. As our recent data showed a dependence of MCL and DLBCL on Chk1 and Wee1 for survival (9, 11) and high ATR inhibitors are strongly active in experimental systems with high oncogenic replicative stress (18, 19), we investigated the cytotoxic effect of the ATR inhibitor AZD6738 as single agent and in combination with the Chk1 inhibitor AZD7762 and the Wee1 inhibitor AZD1775. AZD6738 was active at low concentrations in MCL and DLBCL cell lines. We showed that AZD6738 can act synergistically with AZD7762 and AZD1775, in vitro and in vivo. These findings indicate the therapeutic value of DDR–DDR inhibitor combinations, warranting their testing in patients with MCL and DLBCL for whom new therapeutic strategies are urgently needed.

**Materials and Methods**

**Cell culture and drugs**

Cell lines derived from mature B-cell lymphomas were used, as specified previously (9, 20). Cell lines had been authenticated by the authors in the last 6 months. The STR profiles were compared with the ATCC database or the German Collection of Microorganisms and Cell Cultures database. AZD6738, AZD1775, and AZD7762 were kindly provided by Astrazeneca, and PF-00477736 and VE-822 were commercially available (Axon Medchem). More details on cell lines and drugs are given in the Supplementary Materials and Methods.

**Quantification of the effect of the treatments**

For the initial screening 36 B-cell lymphoma cell lines (20) were seeded in 384-well plates at a density of 2,000 cells, using a VIAFLO 96/384 channel pipette (Integra Biosciences AG); compounds were distributed using a D300e Digital Dispenser (Tecan), and antiproliferative activity was calculated after 72 hours of treatment, as described previously (20). For the validation step 96-well plates were used. For the assessment of the combined treatment, each cell line was treated simultaneously with serial concentrations of the drugs. Results were examined by isobologram analysis with CalcuSyn Software (Biosoft) and combination concentrations of the drugs. Results were examined by isobologram analysis with CalcuSyn Software (Biosoft) and combination analysis of the effect of the treatments

**Flow cytometric analysis**

To analyze DNA content distribution by flow cytometry, OCIly-7 and JEKO-1 cells were fixed in 70% ethanol 8, 24 and 48 hours after treatments either singly or combined. Fixed cells were washed with PBS and stained with 25 μg/ml propidium iodide (PI; Calbiochem) in PBS plus 5 μl of 1 mg/ml RNase (Sigma) in water. After 2 hours of incubation at room temperature, at least 10,000 cells for each sample were acquired by FACScalibur (Becton Dickinson) flow cytometer. Cell-cycle percentages in the different phases were obtained by flow cytometric histograms as described previously (22). For 2-parameter flow cytometry analysis of DNA content and p-S10 histone H3 about 2 × 10^6 cells fixed in ethanol 70% were washed with PBS and permeabilized in Triton X-100 0.25% in PBS for 10 minutes on ice. Then, cells were washed and incubated with 100 μl of anti-p-S10 histone H3 (Cell Signaling Technology; #9706) diluted 1:100 in PBS plus 5 μl of 1 mg/ml RNase in water, incubated overnight, and analyzed.

**Analysis of gene expression and RT-PCR**

Gene expression profiling was done using the HumanHT-12 v4 Expression BeadChip (Illumina) as reported previously (23). Data were first extracted with the Illumina GenomeStudio software and then imported in the Partek Genomics Suite 6.4 and quantile normalized. Total RNA was extracted from cell lines and tissues using a Maxwell RSC simplyRNA Cells Kit and Maxwell RSC simplyRNA Tissue Kit with the Maxwell RSC Instrument (Promega). RNA retro-transcription and mRNA quantification was done as described previously (24). Primer specifications are given in Supplementary Table S1.

**Xenograft models**

Five-week-old female NCr-nu/nu mice were obtained from Envigo s.r.l., Italy, and maintained under specific pathogen-free conditions. Malignant cells were loaded and separated on SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (PROTRAN, Schleicher and Shull). Immunoblotting was done using antibodies, as detailed in Supplementary Materials and Methods and visualized using Odyssey FC Imaging System (LI-COB).
conditions. Procedures involving animals and their care were conducted in conformity with institutional guidelines, in compliance with national and international laws and policies and with guidelines for the welfare and use of animals in cancer research (25). Details of in vivo procedures and pharmacodynamic studies are given in the Supplementary Materials and Methods.

Statistical analysis and correlations
Statistical significance was determined with GraphPad Prism 7.02 (GraphPad Software). The legends to the figures specify which tests were done. The Pearson test was used to establish the correlations between different variables. A P value below 0.05 was taken as significant. Groups of functionally related genes expressed differently between classes were identified as described previously (20), using the gene expression profiles of the untreated cell lines from the GSE94669 dataset and the Gene Set Enrichment Analysis (GSEA) tool and MSigDB 5.2 gene sets (26), with a threshold based on FDR < 0.1. Raw data are available at the National Center for Biotechnology Information Gene Expression Omnibus https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64820 database.

Results

In vitro cytotoxic activity of the ATR inhibitor AZD6738 in B-cell lymphoma cell lines
We investigated the cytotoxic effect of AZD6738 in a large panel of lymphoma cell lines: 36 mature B-cell lymphoma cell lines comprising 10 MCL and 26 DLBCL cell lines [7 activated B-cell (ABC)-DLBCL and 19 germinal center B (GC-B)-DLBCL], treating them with increasing doses of the ATR inhibitor AZD6738, for 72 hours. Figure 1A reports the AZD6738 IC50 values in all the cell lines tested. AZD6738 antiproliferative activity weakly correlated with that of another ATR inhibitor, VE-822 (R = 0.541, P = 0.00063) and of the Chk1 inhibitor AZD7762 (R = 0.49, P = 0.002378), but not with the Wee1 inhibitor AZD1775 (Supplementary Fig. S1A-S1C). We then validated the results of the initial screening in 21 of these cell lines in 96-well plates (Fig. 1B) with similar results. Specifically, we rescreened all the MCL cell lines and we compared them with an equal number of DLBCL cell lines, chosen mainly on the basis of the IC50 values obtained by the first screening and selecting 5 DLBCL-ABC and 6 DLBCL-GCB with IC50 spanning from the lowest to the highest values, with the idea to catch representative cell lines. The JEKO-1R cell line with acquired resistance to the Chk1 inhibitor PF-00477736 (27) was cross resistant to the ATR inhibitor (Supplementary Fig. S1D).

Molecular determinants of the sensitivity to AZD6738 in B-cell lymphoma cell lines
Considering the wide range (100-fold) of sensitivity to AZD6738 (from 143 to 10,400 nmol/L) in this panel of cell lines, and to gain some idea of the molecular determinants associated with the response, we compared the baseline gene expression profiles of the 11 most sensitive (IC50 < 600 nmol/L) with the 11 most resistant (IC50 > 2500 nmol/L) B-cell lymphoma cell lines (Supplementary Fig. S2A). Sensitive cell lines showed enrichment of the genes related to chromosome segregation, cell-cycle checkpoint, damaged DNA binding, and cyclin genes; less sensitivity to the drug was related to enrichment in oxidative phosphorylation and reactive species genes, hydrogen transport, fatty acid metabolism, and PI3K/AKT/mTOR signaling (Fig. 1C; Supplementary Tables S2 and S3).

Since both TP53 and MYC have already been found to be associated with a strong response to ATR inhibitors (19, 28, 29), we looked for a putative association of these molecular features with the degree of sensitivity to AZD6738 in 26 of the 36 B-cell lymphoma cell lines tested (with known functional status for TP53 and MYC), as specified in Supplementary Fig. S2B. No association could be found with inactivation of TP53 and/or genetic alterations of MYC (translocation, amplification; refs. 11, 20, 30–32), as these were observed in both sensitive and resistant cell lines (Supplementary Fig. S2B). In addition, no association was detected between sensitivity to AZD6738 and ATM deficiency (gene deletion/lack of expression, where data where available) in these cell lines in contrast with previous data (28, 29, 33). Indeed, 2 cell lines with no protein expression of ATM (among those shown in Supplementary Fig. S3) (Z-138 and SP-S3) displayed at least a 4-fold difference in sensitivity to AZD6738 (IC50 143.6 and 756.2 nmol/L, respectively). Moreover, 2 MCL cell lines with known deletion of ATM (Granta-519 and MAVER1; refs. 34, 35) were not the most sensitive.

We then investigated whether drug sensitivity was associated with endogenous DNA damage (γH2AX and p-S317Chk1 levels), the protein levels of the oncoprogenic protein MYC, of the DDR protein ATM (both phosphorylated form and total protein), of Wee1 and of the cell-cycle inhibitors p16, p18, and p27 in the 21 cell lines used in the validation (Supplementary Fig. S3). The majority of the MCL and DLBCL cell lines had high levels of constitutive DNA damage (high levels of γH2AX and pChk1), as already reported (11, 36). There was a negative correlation between γH2AX levels and sensitivity to AZD6738 (R = 0.44; P = 0.044; Supplementary Fig. S4A). The correlation was even stronger (R = 0.70; P = 0.022) in the MCL subgroup and was observed not only with the sensitivity to AZD6738, but also with that of the Chk1 inhibitor AZD7762 (R = 0.85; P = 0.001; Supplementary Fig. S4B), and with another Chk1 inhibitor, PF-00477736 (R = 0.758; P = 0.0109; data not shown). No correlation was also found between AZD6738 sensitivity and ATM and ATR expression at mRNA levels (Supplementary Fig. S4C and S4D). Fig. 1B (lower part) reports the CI at the IC50 of each drug combination were extrapolated (Fig. 2A). Both drug combinations were strikingly synergistic in all the cell lines investigated, regardless of the lymphoma subgroups and of MYC, TP53 and ATM status. Interestingly, the synergistic effect was also observed in the AZD6738 insensitive cell lines (SUDHL2, MINO). There was a negative correlation between CI at the IC50 of the AZD6738 synergizes with both the Chk1 inhibitor AZD7762 and the Wee1 inhibitor AZD1775
Based on previous data showing the high susceptibility of B-cell lymphomas to the DDR inhibitors against Chk1 and Wee1, we investigated the effects of combined AZD6738 treatments with either the Chk1 inhibitor AZD7762 or the Wee1 inhibitor AZD1775 in 11 cell lines out of the 21 used in the rescreening panel (5 MCL cell lines and 6 DLBCL cell lines), based on their sensitivities to the 3 inhibitors. Combination indexes at the IC50 of each drug combination were extrapolated (Fig. 2A). Both drug combinations were strikingly synergistic in all the cell lines investigated, regardless of the lymphoma subgroups and of MYC, TP53 and ATM status. Interestingly, the synergistic effect was also observed in the AZD6738 insensitive cell lines (SUDHL2, MINO). There was a negative correlation between CI at the IC50 of the
Wee1 + ATR inhibitor combination and Wee1 protein levels ($R = -0.66, P = 0.026$), similarly to what has been reported with Chk1/Wee1 dual inhibition in DLBCL cell lines (11). The negative correlation was even stronger when only DLBCL cell lines were considered both at protein ($R = -0.929, P = 0.0073$) and mRNA levels ($R = -0.887, P = 0.0184$; Fig. 2B).

Molecular characterization of the DDR inhibitor drug combinations

We further investigated the 2 drug combinations (ATR/Chk1 and ATR/Wee1 inhibition) to clarify the cellular and molecular mechanisms of the observed synergistic effect, and compared them with the previously characterized Chk1/Wee1 dual inhibition (9, 11). We selected the OCILY-7 cell line (DLBCL) and the JEKO-1 cell line (MCL). Figure 3A shows the cell growth of the 2 cell lines every 24 hours to 96 hours when treated with drug concentrations, exerting a strong synergistic effect when combined (concentrations specified in the Figure legend). There was no effect on cell growth after a single treatment, but starting from 24 hours, the 3 combinations strongly reduced cell growth up to 96 hours in both cell lines (more than 90% of growth inhibition). The combined treatments induced clear cell-cycle perturbation in both cell lines (Fig. 3B; Supplementary Fig. S5). OCILY-7 cells treated with the combinations were able to exit G1, but accumulated in early S phase. This effect was already detectable at 8 hours of treatment (still detectable at 8 hours) were able to divide or undergo cell death and only a small amount of p-S10 Histone H3-positive cells (less than 1%) had a DNA content between 2N and 4N, excluding the hypothesis of a massive premature entrance
The presence of sub-G1 events, detected at 48 hours (Fig. 3B; Supplementary Fig. S5), together with the increased caspase-3 activity at 24 and 48 hours with all the combinations (Supplementary Fig. S6B) confirmed that cell death was occurring. Single drug treated JEKO-1 cells were almost unaffected by the inhibitors, whereas cells treated with the combinations stopped growing and DNA distributions highlighted the S-phase delay. The effect and its kinetics were similar for all the combinations and the accumulation of cells became evident at 24 hours, clearly increasing at 48 hours in cells treated with the Chk1/Wee1 inhibitor combination. Between 24 and 48 hours cell death occurred, as demonstrated by the slight increase in sub-G1 events and the high caspase-3 activity detected from 8 hours and further increasing at the subsequent time points (Supplementary Fig. S5).

The 3 drug combinations induced a strong decrease in MYC, Wee1, and RRM2 protein levels and an increased DNA damage, measured by upregulation of γH2AX protein levels in both cell lines (Fig. 3C). MYC and Wee1 mRNA levels did not decrease after the drug combinations, suggesting that this expression is modulated at the protein levels. There was a slight decrease of RRM2 expression at mRNA levels in OCILY-7 cells at 48 hours after treatment with AZD6738 combined with AZD1775 and AZD7762 (Supplementary Fig. S6C).

DNA Damage Response Inhibitor Combinations

**Figure 2.** Synergistic combinations with the ATR inhibitor AZD6738. A, Normalized IC50 isobolograms showing the synergistic effects of the combination of the ATR inhibitor AZD6738 with the Wee1 inhibitor AZD1775 (left) and with the Chk1 inhibitor AZD7762 (right) in 5 MCL cell lines and in 6 DLBCL cell lines. B, Graphs showing the negative correlation between Wee1 protein levels (left) or Wee1 mRNA levels (right) and the CI at IC50 of the Wee1/ATR inhibitor combination in the 6 DLBCL cell lines tested.

DDR–DDR inhibitor combinations exert antitumor activity in vivo

The 3 drug combinations were tested in vivo in nude mice bearing OCILY-7 xenografts. Doses and schedules were based on previous data (8–10, 37, 38). No significant effect on tumor growth was observed in mice treated with AZD6738, AZD7762, and AZD1775 in monotherapy, except for 1 mouse in the AZD7762-treated group, whose tumor regressed after treatment and the mouse was sacrificed at day 150 without tumor. On the contrary, all the 3 combinations were quite active, significantly delaying tumor growth, including tumor regressions, and increasing overall survival (Fig. 4A). Single treatments were well tolerated, as suggested by the fact that there were almost no body weight changes, although 2 toxic deaths were recorded in the AZD7762 group. The combinations caused body weight loss, which was rapidly regained upon drug withdrawal (Supplementary Fig. S7A), and all the combined treatments resulted in very low T/Cs, indicative of high antitumor activity (Supplementary Fig. S7B). Interestingly, tumor responses were clearly observed also at the third treatment cycle for the AZD7762 þ AZD1775 and AZD6738 þ AZD1775 combinations. In addition, in these latter groups, long-term survivals (no tumor regrowth 5 months after transplantation) were recorded (3 and 2 mice out of 7 respectively in the AZD7762 þ AZD1775 and AZD6738 þ AZD1775 combinations; Fig. 4A). The AZD6738 þ AZD1775 combination was also active when tested in a late-stage setting, in which treatment started at tumor weights of 580 mg (as opposed to 150 mg in the previous experiment), causing tumor regression or stabilization (Supplementary Fig. S7C). The drugs were able in vivo to inhibit their respective targets: ATR (decreased pS317 of Chk1), Chk1 (increased p-S317 of Chk1 and decreased pY15 CDK1), and Wee1 (decreased pY15 CDK1; Supplementary Fig. S8A). MYC, Wee1, RRM2, and pCDK2 protein levels clearly decreased after all 3 combinations, although it was more marked after AZD6738 þ AZD1775 and AZD1775 þ AZD7762, which exerted higher antitumor activity. No clear-cut increase in γH2AX was detected after the combinations as compared with single drugs in vivo (Fig. 4B).
Figure 3.
Cellular and molecular effects induced by the DDR–DDR inhibitor combinations. A, Cell growth curve of OCILY-7 (left) and JEKO-1 (right) untreated or treated with the drugs either singly or combined at the following concentrations: OCILY-7: AZD7762 at 50 nmol/L; AZD1775 at 200 nmol/L; AZD6738 at 500 nmol/L. JEKO-1: AZD7762 at 17.5 nmol/L; AZD1775 at 60 nmol/L; AZD6738 at 400 nmol/L. These concentrations were used for single and combined treatments. The data are representative of 3 independent experiments and are expressed as the mean cell number/mL ± SD of 3 replicates (error bars are not visible when smaller than symbols). ANOVA with GraphPad Prism Software was used for statistical analysis. Statistically significant differences are as follows: from 48 to 96 hours in OCILY-7 and in JEKO-1 cells combined treatment groups vs. ctrl and vs. corresponding single agents: ****, P < 0.0001; from 48 hours to 96 hours in OCILY-7 ATR inh vs. ctrl: ***, P < 0.001. B, Flow cytometric analysis of DNA content 8, 24, and 48 hours after treatment with ATR, Chk1, and Wee1 inhibitors either singly or combined at the above concentrations. C, Western blot analysis showing MYC, Wee1, RRM2, γH2AX, and actin protein levels in protein extracts from OCILY-7 and JEKO-1 cells treated with the 3 drugs, as above.
AZD1775 + AZD6738 was also tested in JEKO-1 MCL xenografts (Fig. 5A). Again, using the same drug schedules as in the previous experiment, there was no significant body weight loss (Supplementary Fig. S8B), maximum loss being 5.7%. In this model, tumor growth inhibition was significant in animals treated with the drugs as monotherapy, but the antitumor effect was greater in mice treated with the combination, as suggested by the lower T/C% (Supplementary Fig. S8D). In addition, tumor regressions were observed in all the animals treated with the combination (Supplementary Fig. S8D). Again, tumors were still responsive to the third drug combination cycle. The AZD6738 and AZD1775 targets were inhibited in vivo (Fig. 5B). The increased Chk1 activation (increased pChk1) exerted by AZD1775 was partly neutralized by AZD6738. MYC, Wee1, and RRM2 protein levels decreased clearly after the drug combination, whereas the decreases in pCDK2 and the increases in γH2AX were less clear (Fig. 5B).
Here, we investigated the role of the DDR kinase ATR as a potential therapeutic target in B-cell lymphomas, both MCL and DLBCL. AZD6738 is a novel oral selective inhibitor of ATR under early clinical trial development both in monotherapy in hematologic malignancies with reported ATM deficiencies (NCT01955668) and in combination with chemotherapy (e.g., carboplatin, paclitaxel), DNA damage repair agents (olaparib), and new anticancer agents (MEDI4736) in advanced solid tumors (for recent reviews, see refs. 5–7). We studied the cytotoxic activity of AZD6738 in a wide panel of MCL and DLBCL cell lines, both as single agent and in combination with other DDR (Chk1 and Wee1) inhibitors. MCL and DLBCL displayed a wide range of sensitivity to AZD6738, with IC50 ranging from 143 to 10,400 nmol/L. AZD6738 sensitivity was unrelated to the genetic and/or mutational status of the known oncogenes and/or onco-suppressors previously found in synthetic lethality with ATR (such as TP53, ATM, MYC; refs. 19, 28, 29, 33), and not even associated with the combination of alteration/lack of expression of more than one molecular feature (MYC, p53, p16, p27) related with the increased G1–S transition.

The AZD6738 IC50 in the most sensitive cell lines were lower than those recently reported in the chronic lymphocytic leukemia cell lines (29) and gastric cancer cells with ATM or TP53 dysfunction (37), corroborating the evidence that at least in this experimental setting the highest sensitivity to AZD6738 is not strictly and exclusively associated with ATM mutational status/expression. Hocke and colleagues (39) identified POLD1 and other DNA repair genes, other than ATM, as synthetically lethal with ATR. In a recent paper Menezes and colleagues (40) demonstrated that the ATM-deficient cancer cell line GRANTA-519 is more sensitive to the ATR inhibitor WO2010/073034 than the ATM proficient JVM-2 cell line, but these are not identical experimental systems and thus the different sensitivity may be due to other molecular features. Our data indeed demonstrated that very sensitive lymphoma cell lines (e.g., UPN1, OCILY-10) display ATM expression and that 2 cell lines lacking ATM expression (Z-138 and SP-53) differ in sensitivity to AZD6738 (of at least 4-fold; Fig. 1B). Others, not yet identified molecular features, are responsible for the sensitivity to ATR inhibitor in these lymphoma cell lines. Unexpectedly, we found that the most sensitive cell lines to both ATR and Chk1 inhibitors were those with lower γH2AX levels. One might hypothesize that in cell lines with low constitutive γH2AX levels, the proteins responsible for this phosphorylation (e.g., DNAPK and/or ATM activity; ref. 41) have lower activity, and this might explain the greater susceptibility to the ATR inhibitor, but this remains to be demonstrated. The baseline gene expression profiles indicated that cell-cycle–related gene sets and genes related to DDR were associated with higher sensitivity to AZD6738 (of at least 4-fold; Fig. 1B). Others, not yet identified molecular features, are responsible for the sensitivity to ATR inhibitor in these lymphoma cell lines. Unexpectedly, we found that the most sensitive cell lines to both ATR and Chk1 inhibitors were those with lower γH2AX levels. One might hypothesize that in cell lines with low constitutive γH2AX levels, the proteins responsible for this phosphorylation (e.g., DNAPK and/or ATM activity; ref. 41) have lower activity, and this might explain the greater susceptibility to the ATR inhibitor, but this remains to be demonstrated. The baseline gene expression profiles indicated that cell-cycle–related gene sets and genes related to DDR were associated with higher sensitivity to AZD6738 (of at least 4-fold; Fig. 1B). Others, not yet identified molecular features, are responsible for the sensitivity to ATR inhibitor in these lymphoma cell lines. Unexpectedly, we found that the most sensitive cell lines to both ATR and Chk1 inhibitors were those with lower γH2AX levels. One might hypothesize that in cell lines with low constitutive γH2AX levels, the proteins responsible for this phosphorylation (e.g., DNAPK and/or ATM activity; ref. 41) have lower activity, and this might explain the greater susceptibility to the ATR inhibitor, but this remains to be demonstrated. The baseline gene expression profiles indicated that cell-cycle–related gene sets and genes related to DDR were associated with higher sensitivity to AZD6738 (of at least 4-fold; Fig. 1B). Others, not yet identified molecular features, are responsible for the sensitivity to ATR inhibitor in these lymphoma cell lines. Unexpectedly, we found that the most sensitive cell lines to both ATR and Chk1 inhibitors were those with lower γH2AX levels. One might hypothesize that in cell lines with low constitutive γH2AX levels, the proteins responsible for this phosphorylation (e.g., DNAPK and/or ATM activity; ref. 41) have lower activity, and this might explain the greater susceptibility to the ATR inhibitor, but this remains to be demonstrated. The baseline gene expression profiles indicated that cell-cycle–related gene sets and genes related to DDR were associated with higher sensitivity to AZD6738 (of at least 4-fold; Fig. 1B). Others, not yet identified molecular features, are responsible for the sensitivity to ATR inhibitor in these lymphoma cell lines. Unexpectedly, we found that the most sensitive cell lines to both ATR and Chk1 inhibitors were those with lower γH2AX levels. One might hypothesize that in cell lines with low constitutive γH2AX levels, the proteins responsible for this phosphorylation (e.g., DNAPK and/or ATM activity; ref. 41) have lower activity, and this might explain the greater susceptibility to the ATR inhibitor, but this remains to be demonstrated. The baseline gene expression profiles indicated that cell-cycle–related gene sets and genes related to DDR were associated with higher sensitivity to AZD6738 (of at least 4-fold; Fig. 1B). Others, not yet identified molecular features, are responsible for the sensitivity to ATR inhibitor in these lymphoma cell lines.
In contrast, we found no correlation between the sensitivity to ATR and Wee1 inhibitors in these cell lines. Taken together these data reinforce the fact that, although the 3 proteins belong to the DDR pathway and share similar functions, they also act independently in the DDR pathway. Strong synergistic activity was observed with AZD6738/AZD1775 and AZD6738/AZD7762 combinations in all the cell lines (DLBCL and MCL); in addition even in tumors less sensitive to the inhibitors as single agents, the combinations were synergistic. Our findings not only corroborated recent data on the synergistic effect with Chk1 and ATR inhibitors (10), but also clearly indicate a therapeutic synergism of the combinations with Wee1 and ATR inhibitors, in support of very recent data that showed this synergism in triple negative breast cancer (42). We investigated the molecular mechanism at the basis of the synergy of the investigated drug combinations and compared it with the Chk1+Wee1 inhibitor combination previously characterized (9). All the 3 drug combinations blocked cell growth, caused general mis-coordination of the cell cycle mainly due to substantial S-phase delay, induced DNA damage, and activated apoptosis. In the search for biomarkers of response, we found that all the DDR–DDR inhibitor combinations downregulated MYC, Wee1, and RRM2 protein levels both in vitro and in vivo. The decrease in Wee1 protein levels is not transcriptionally dependent and could be due to an ubiquitin ligase activity associated with the increased CDK activity induced by the drug combination, as recently suggested (43, 44). We have already reported that MYC protein levels were destabilized after Chk1/Wee1 dual inhibition (11). This effect was confirmed after the other DDR–DDR inhibitor combinations as well since to be clarified are the molecular mechanisms. The effect on MYC protein further stresses the therapeutic potential of these treatments in MYC-dependent lymphoma subtypes. We also observed a significant decrease of RRM2 protein, likely to be due to increased CDKs activity caused by the drug combination, as recently reported (45, 46) and observed in pancreatic cancer cells after Chk1 inhibitor in combination with gemcitabine (47). The decreased RRM2 levels leading to dNTP exhaustion can partially explain the appreciable S-phase delay after the drug combinations, which consequently induced DNA damage and cell death. The in vitro synergism translated into striking in vivo antitumor activities in 2 different xenograft models. Indeed, in both models, the combinations were very active, regardless the activity as single agent. Our data provide the first evidence that the combination of ATR and Wee1 inhibitors is effective and well tolerated. This new drug combination exerts significant antitumor activity even in a late stage of disease.

In conclusion, these findings suggest that DDR–DDR inhibitor combinations are feasible and strongly effective in MCL and DLBCL. Despite the limitations due to the lack of data on primary lymphoma samples and/or patient derived xenografts, these data do suggest these combinations warrant clinic investigation as new and effective therapeutic strategies in aggressive patients with B-cell lymphoma.

 Disclosure of Potential Conflicts of Interest
Francisco Bertoni reports receiving Commercial Research Grant from Acerta, Bayer AG, Celestia, CITI Life Sciences, Helsinn, Menarini Ricerche, NEOQED Therapeutics 1, and PIQUR Therapeutics AG; and is a consultant/advisory board member of Helsinn and Menarini; has provided expert testimony for HTG, Amgen, Astra Zeneca, Jazz Pharmaceuticals, and Helsinn. No potential conflicts of interest were disclosed by the other authors.

Availability of Data and Materials

Authors’ Contributions
Conception and design: G. Damia, L. Carrassa
Development of methodology: R. Chilà, M. Vagni, C. Tarantelli, L. Carrassa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Restelli, M. Lupi, F. Spriamo, E. Gaudio
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Restelli, M. Lupi, M. Vagni, F. Bertoni, G. Damia, L. Carrassa
Writing, review, and/or revision of the manuscript: V. Restelli, M. Lupi, C. Tarantelli, F. Bertoni, G. Damia, L. Carrassa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Tarantelli, F. Bertoni
Study supervision: G. Damia, L. Carrassa

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References
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