Differential Activity of ATR and WEE1 Inhibitors in a Highly Sensitive Subpopulation of DLBCL Linked to Replication Stress

Lucy A. Young1, Lenka Oplustil O’Connor1, Christelle de Renty1, Margaret H. Veldman-Jones2, Thierry Dorval3, Zena Wilson2, David R. Jones2, Deborah Lawson4, Rajesh Odedra2, Apolinar Maya-Mendoza5, Corinne Reimer4, Jiri Bartek5,6, Alan Lau1, and Mark J. O’Connor1

Abstract

DNA damage checkpoint kinases ATR and WEE1 are among key regulators of DNA damage response pathways protecting cells from replication stress, a hallmark of cancer that has potential to be exploited for therapeutic use. ATR and WEE1 inhibitors are in early clinical trials and success will require greater understanding of both their mechanism of action and biomarkers for patient selection. Here, we report selective antitumor activity of ATR and WEE1 inhibitors in a subset of non-germinal center B-cell (GCB) diffuse large B-cell lymphoma (DLBCL) cell lines, characterized by high MYC protein expression and CDKN2A/B deletion. Activity correlated with the induction of replication stress, indicated by increased origin firing and retardation of replication fork progression. However, ATR and WEE1 inhibitors caused different amounts of DNA damage and cell death in distinct phases of the cell cycle, underlying the increased potency observed with WEE1 inhibition. ATR inhibition caused DNA damage to manifest as 53BP1 nuclear bodies in daughter G1 cells leading to G1 arrest, whereas WEE1 inhibition caused DNA damage and arrest in S phase, leading to earlier onset apoptosis. In vivo xenograft DLBCL models confirmed differences in single-agent antitumor activity, but also showed potential for effective ATR inhibitor combinations. Importantly, insights into the different inhibitor mechanisms may guide differentiated clinical development strategies aimed at exploiting specific vulnerabilities of tumor cells while maximizing therapeutic index.

Significance: ATR and WEE1 inhibitors demonstrate effective antitumor activity in preclinical models of DLBCL associated with replication stress, but new mechanistic insights and biomarkers of response support a differentiated clinical development strategy.

Introduction

Replication stress (RS), broadly defined as the slowing or stalling of replication fork (RF) progression, poses a significant problem for genome stability and cell survival (1). Generation of aberrant RF structures containing single-strand DNA activates a replication stress response (RSR) mediated by ataxia telangiectasia and Rad3-related (ATR) and checkpoint kinase 1 (CHK1; ref. 2), leading to stabilization of RF structures, and delayed cell-cycle progression for DNA repair and completion of DNA synthesis before mitosis (2, 3). Regulation of replication origin firing helps prevent unscheduled DNA synthesis and rescue stalled RFs (2, 4). ATR and CHK1 negatively regulate cyclin-dependent kinase (CDK) activity through inhibition of the CDC25 family of CDK phosphatases (5). The WEE1 kinase that regulates mitotic entry through CDK1 phosphorylation, also plays an important role in regulating replication initiation through phosphorylation of CDK2 (6, 7).

Compelling evidence links oncogene-induced RS to tumor progression and its prevalence in human cancers (8). Thus, cancer-specific dependency on the RSR for survival could be exploited by pharmacologic inhibition of ATR, CHK1, or WEE1, ultimately causing cancer cell death (9–12). Accordingly, it has been demonstrated that CHK1 and WEE1 inhibitors induce strong antitumor effects in MYC-driven lymphoma mouse models and neuroblastoma (13, 14). Furthermore, hypomorphic Atr alleles in mouse models prevent the development of MYC-driven B-cell lymphomas (15), suggesting that MYC-driven tumors are dependent on ATR for survival, and that MYC-driven patient tumors could be preferentially targeted by ATR inhibitors. Diffuse large B-cell lymphoma (DLBCL), the most common form of non-Hodgkin lymphoma (16), is associated with MYC overexpression. Although a subset of patients with DLBCL are cured with chemotherapeutic...
regimens, approximately 40% have refractory disease or will relapse after an initial response (17). ATR, CHK1, and WEE1 inhibitors potentiate the genotoxic properties of cytotoxic drugs and radiotherapy, and clinical trials assessing tolerability and efficacy of this therapeutic strategy are ongoing. However, broad clinical utility, especially as single agents, will likely depend on identifying and targeting patient tumors with high RS. A lack of robust biomarkers for detecting RS in human tumors has limited clinical testing of this hypothesis. Furthermore, understanding how these agents kill cancer cells will be essential for optimizing patient treatments. In this study, we identified that the non-GBR subtype of DLBCL, as well as MYC overexpression and CDKN2A/B deletion, are associated with increased RS and in vitro and in vivo sensitivity to ATR and WEE1 inhibition. Our data also demonstrate different modes of action for ATR and WEE1 inhibition and highlights the potential for an effective combination.

Materials and Methods

Cell lines and compounds

DLBCL cell lines were purchased from DSMZ unless otherwise specified. Pfeiffer and Toledo were purchased from ATCC; HBL-1 was from Professor Masafumi Abe, under license from Tokyo Medical and Dental University Tokyo, Japan; TMD8 was from Dr. Daniel Krappmann, German Research Center for Environmental Health, Munich, Germany; OCI-LY10 was from Dr. Louis Staudt, Center for Cancer Research, NCI, Bethesda, MD. GM144680 and GM03567 were purchased from the Coriell Institute for Medical Research, Camden, NJ. All cell lines were cultured in RPMI supplemented with 10% to 15% FBS and 2 mM/l-glutamine, except OCI-LY-10, which was cultured in IMDM supplemented with 20% FBS, 2 mM/l-glutamine, and 50 µM/1 β-mercaptoethanol. Cells with ≥90% viability, determined using trypan blue dye exclusion, were used in experiments. Cell line identification was validated using the CellCheck assay (IDEXX Bioanalytics). All cell lines were validated free of virus by IMPACT tests (IDEXX Bioanalytics) and validated free of Mycoplasma contamination using the MycoSEQ assay (Thermo Fisher Scientific) or STAT-Mycop assay (IDEXX Bioanalytics). The genomics of the cell lines was acquired from the AZ Cell Line Exploration Tool, a database containing genetic characterization of cell lines from AstraZeneca, CCLE, and COSMIC. AZD6738 (18) and AZD6738 (19) were made by AstraZeneca and solubilized in DMSO.

In vitro growth inhibition and cell viability assays

Cells in 96-well plates were compound dosed using an Echo 555 (LabCyte) and viability determined by alamarBlue assay (Life Technologies). Fluorescence intensity was measured using a SpectraMax i3 (Molecular Devices). Percentage growth was determined using fluorescence values in the equation \( \frac{T - T_0}{C - T_0} \times 100 \), where \( T \) is the drug-treated cells, \( T_0 \) the cells at time zero, and \( C \) is the control cells. Dose-response curves were plotted in GraphPad prism V6 using the nonlinear regression model, sigmoidal dose response. Fifty percent growth inhibition (GI50) and 100% total growth inhibition (TGI) values were determined using data from ≥3 independent experiments. Cell viability and apoptosis were assessed using the Guava Viacount and Nexin Kits and analyzed on the Guava easyCyte HT Flow Cytometer (Merck-Millipore).

Cell lines and compounds

Cell lines from AstraZeneca, CCLE, and COSMIC. AZD6738 (18) and AZD1775 (19) were made by AstraZeneca and solubilized in DMSO. Cells grown on 96-well poly-D-lysine coated black-walled plates were dosed with compounds (Echo 555; LabCyte) for 40 hours, then fixed and permeabilized with 4% parformaldehyde and 0.5% Triton X100, blocked in 3% BSA in TBS-Tween (0.05%) and incubated with primary antibodies at 4°C, followed by Alexa Fluor secondary antibodies and Hoechst 33342 (Supplementary Methods Table S1). A series of 48 frames (8 per well, 6 wells per condition) were acquired using a 40× objective on the Operetta (Perkin Elmer). Harmony software was used to define nuclei, cyclin A intensity, and quantify 53BPI foci in G1 phase.

Cell-cycle analysis

To measure the S-phase population, cells were treated with compound for the indicated times, then 1 hour prior to fixation labeled with 10 µmol/L EdU (pulse-harvest). To measure cell-cycle progression, cells were first labeled with 10 µmol/L EdU for 1 hour, washed, then resuspended in media containing compounds (pulse-chase). Cells were fixed and EdU detected using the

Cell-of-Origin subtype classification

Total RNA extracted from cells was applied to a NanoString code set for determination of Cell-of-Origin (COO) gene expression subtyping, as described previously (20).

Western analysis

Equal amounts of whole cell lysates, prepared in RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Sigma-Aldrich), were analyzed by standard SDS-PAGE/immunoblotting, using antibodies listed in Supplementary Methods Table S1.

DNA fiber analysis

Cells were compound treated for 1 hour before labeling with 25 µmol/L CldU for 20 minutes, then collected by centrifugation and incubated for 20 minutes in media containing compound and 250 µmol/L IdU. Labeled cells were harvested and DNA fiber spreads prepared as described previously (21). Antibodies listed in Supplementary Methods Table S1. Fibers were imaged using an LSM710 (Carl Zeiss Microscopy) confocal microscope and 63× objective with numerical aperture of 1.2. The lengths of CldU tracks (≥300 per condition and experiment) were measured using ZenLite software (Carl Zeiss Microscopy).

DNA molecular combing assay

DNA combing was performed using the Molecular Combing Platform (Genomic Vision). Briefly, cells were compound treated for 1 hour before adding 25 µmol/L IdU and incubating the cells for 20 minutes. Fifty µmol/L CldU was then added to the media and the cells were incubated for another 20 minutes. Cells were harvested and DNA was extracted in agarose plugs using FiberPrep DNA Extraction Kit (Genomic Vision). DNA molecules were then stretched onto silanized coverslips (CombiCoverslips) using FiberComb Molecular Combing System (Genomic Vision). Immunodetection of IdU, CldU, and ssDNA was performed with antibodies listed in Supplementary Methods Table S1. Slides were entirely scanned with automated FiberVision scanner and analyzed with dedicated FiberStudio software (Genomic Vision). To determine the median replication fork velocity >700 tracks per condition were measured, and >100 tracks per condition for the median inter-origin distance.

53BPI nuclear bodies assay

Cells grown on 96-well poly-D-lysine coated black-walled plates were dosed with compounds (Echo 555; LabCyte) for 40 hours, then fixed and permeabilized with 4% paraformaldehyde and 0.5% Triton X100, blocked in 3% BSA in TBS-Tween (0.05%) and incubated with primary antibodies at 4°C, followed by Alexa Fluor secondary antibodies and Hoechst 33342 (Supplementary Methods Table S1). A series of 48 frames (8 per well, 6 wells per condition) were acquired using a 40× objective on the Operetta (Perkin Elmer). Harmony software was used to define nuclei, cyclin A intensity, and quantify 53BPI foci in G1 phase.
Click-iT Plus Kits (Life Technologies). Cells were subsequently incubated with fluorescein-conjugated antibodies (Supplementary Methods Table S1) and DAPI. Data were acquired using a flow cytometer (FACS Aria II) and analyzed in FlowJo (TreeStar).

**In vivo studies**

All in vivo studies were performed at United States and United Kingdom locations within AstraZeneca. Study protocols at the U.S. site were reviewed and approved by the Institutional Animal Care and Use Committees, whereas those performed in the United Kingdom were approved by the Home Office. All studies were performed in accordance with the Animal Scientific Procedures Act 1986 (ASPA), and AstraZeneca Global Bioethics policy. Data were reported following the Animal Research: Reporting In Vivo (ARRIVE) experiments guidelines (22). Female NOD.CB17-SCID mice were purchased from Charles River Laboratories and used between the ages of 8 and 12 weeks in accordance with institutional guidelines. Animals were randomized into vehicle and treatment groups based on mean tumor volume of approximately 0.1 to 0.2 cm$^3$. For the OCI-LY-19 study, animal treatment groups received AZD6738 at either 25 or 50 mg/kg orally or AZD1775 at either 120, 30, or 60 mg/kg orally once daily as monotherapy, or in combination where AZD1775 was dosed 1 hour following the AZD6738 dose. AZD6738 was prepared in 10% DMSO + 40% propylene glycol + 50% water and AZD1775 was prepared in 0.5% methylcellulose. For the TMD8 study, 5 × 10$^6$ cells in 50% Matrigel were inoculated subcutaneously on the left flank of female NOD.CB17-SCID mice. Treatment groups were dosed as follows: AZD6738 was dosed as monotherapy at 50 mg/kg orally daily; the combination of rituximab and bendamustine (R-Benda) was dosed for only one cycle: rituximab at 10 mg/kg i.p. on day 1 and bendamustine at 12.5 mg/kg i.v. on days 1 and 2. AZD6738 dosed in combination with R-Benda was dosed for 1 cycle (daily for 5 days). Tumor volume was measured bilaterally by caliper using the formula $\pi/6,000 \times x \times y \times z$; animal body weight and tumor condition was recorded twice weekly for the duration of the study. Growth inhibition from the start of treatment was assessed by comparison of the geometric mean of tumor volume between the ages of 8 and 12 weeks in accordance with institutional guidelines. Animals were randomized into vehicle and treatment groups based on mean tumor volume of approximately 0.1 to 0.2 cm$^3$. For the OCI-LY-19 study, animal treatment groups received AZD6738 at either 25 or 50 mg/kg orally or AZD1775 at either 120, 30, or 60 mg/kg orally once daily as monotherapy, or in combination where AZD1775 was dosed 1 hour following the AZD6738 dose. AZD6738 was prepared in 10% DMSO + 40% propylene glycol + 50% water and AZD1775 was prepared in 0.5% methylcellulose. For the TMD8 study, 5 × 10$^6$ cells in 50% Matrigel were inoculated subcutaneously on the left flank of female NOD.CB17-SCID mice. Treatment groups were dosed as follows: AZD6738 was dosed as monotherapy at 50 mg/kg orally daily; the combination of rituximab and bendamustine (R-Benda) was dosed for only one cycle: rituximab at 10 mg/kg i.p. on day 1 and bendamustine at 12.5 mg/kg i.v. on days 1 and 2. AZD6738 dosed in combination with R-Benda was dosed for 1 cycle (daily for 5 days). Tumor volume was measured bilaterally by caliper using the formula $\pi/6,000 \times x \times y \times z$; animal body weight and tumor condition was recorded twice weekly for the duration of the study. Growth inhibition from the start of treatment was assessed by comparison of the geometric mean change in tumor volume for the control and treated groups. Statistical significance was evaluated using a one-tailed, t test.

**Immunohistochemistry**

DLBCL xenograft tumors grown in CB17-SCID mice were harvested between 0.2 and 0.5 cm$^3$ and fixed in 10% neutral buffered formalin then subjected to routine vacuum processing through graded ethanol, xylene, and paraffin. Tumors embedded in paraffin blocks were sectioned at a thickness of 3 μm, air dried, and heated to 65°C for 20 minutes. IHC was run on the Ventana Discovery XT using primary antibodies listed in Supplementary Methods Table S1. Detection was performed with Vectastain biotinylated secondary reagents (antirabbit PK-6101 and antirat PK-6102), and the DABMap Kits (Ventana Medical 760-124, and UltraMap Kit Ventana 760-152 for anti-P16). Digital slide images were acquired with the Aperio Scanscope XT using a 20× objective.

**Statistical analysis**

A Student unpaired Mann–Whitney t test was used to determine statistical differences between 2 groups of data, whereas one-way ANOVA with Kruskal–Wallis multiple comparisons test was used for the DNA fiber analysis and DNA combing analysis (GraphPad Prism V6). A value of $P < 0.05$ was considered statistically significant.

**Results**

The ATR inhibitor AZD6738 reduces proliferation in non-GCB DLBCL cell lines

Tumors with RS are hypothesized to be sensitive to ATR inhibition. To identify cell line models of RS, we evaluated the antiproliferative effects of ATR inhibitor AZD6738 (18) across 12 cancer cell line panels. The concentration of AZD6738 resulting in GI$_{50}$ was lowest in hemolologic cancer cell lines (Fig. 1A). Eight of 26 DLBCL cell lines were highly sensitive to AZD6738 (GI$_{50}<0.5$ μmol/L), therefore we examined this cell panel for correlative biomarkers of response and levels of RS.

DLBCL can be classified into germinal center B-cell like (GCB) and activated B-cell like (ABC) subtypes, the latter associated with worse outcomes following standard chemo-immunotherapy treatments (23). Using a NanoString scoring system (20), we identified 6 cell lines of ABC subtype, 7 of GCB, and 6 unclassified (Fig. 1B and C). However, the unclassified signature closely resembles the ABC subtype (Fig. 1B). Greater AZD6738 activity was observed in the non-GCB group comprising ABC and unclassified subtypes (median GI$_{50}=0.372$ μmol/L), compared with GCB (median GI$_{50}=1.554$ μmol/L) or lymphoblastoid cell lines (Fig. 1C and D, Supplementary Table S1). AZD6738 activity was only weakly associated with doubling rates of cell lines (Supplementary Fig. S1A), and not enhanced by prolonged exposure (Supplementary Table S1; Supplementary Fig. S1B).AZD6738 sensitivity is associated with MYC overexpression and CDKN2A/B deletion

We looked for genetic correlations of increased AZD6738 activity in non-GCB DLBCL cell lines. Abrupt expression and activation of oncogenes involved in proliferation, such as RAS, CCNE1, and MYC, can induce RS by causing premature S-phase entry, transcription–replication conflicts, and nucleotide shortage (15, 24–27). Translocation and/or amplification of MYC was present in 11 of 18 of the DLBCL cell lines (Fig. 2A), consistent with higher prevalence of MYC (28) compared with RAS and CCNE1 aberrations in DLBCL (29). AZD6738 sensitivity did not correlate with MYC genomics (Supplementary Fig. S1C) but was associated with higher c-MYC protein levels (Fig. 2A and B).

Cell-cycle dysregulation contributes to RS, with reports suggesting selective toxicity of ATR and CHK1 inhibitors in TP53-defective cells (30, 31). TP53 mutations were present in 12 of 18 of DLBCL cell lines but did not enrich for AZD6738 sensitivity (Supplementary Fig. S1C). Homozygous deletion of CDKN2A/B (p16INK4a, p14ARF, and p15INK4b), a key regulator of G1 to S, occurs in 30% of ABC and 4% of GCB patients with DLBCL (32). Five of eight sensitive cell lines were homozygous CDKN2A/B deleted (Fig. 2A), which statistically enriched for AZD6738 activity (mean GI$_{50}=0.195$ μmol/L, WT = 1.561 μmol/L), whereas p16 protein levels did not (Fig. 2B). Undetectable levels of BCL-6 protein in non-GCB cell lines also enriched for AZD6738 activity, consistent with BCL-6 expression characterizing GCB DLBCL (33). In vivo protein expression of MYC, BCL6, and p16 was determined by IHC and corroborated in vitro data (Supplementary Fig. S2). Separately, each biomarker identified a cell line population enriched for AZD6738 sensitivity (Fig. 2C) as did the combination of low p16 and high c-MYC protein (Supplementary
Fig. S3A). However, combination of the non-GCB subtype with high c-MYC protein and CDKN2A/B deletion was most accurate, identifying 7 of 8 highly sensitive and 0 of 7 less sensitive cell lines.

Constitutive activation of DNA damage response (DDR) proteins is a hallmark of RS and improved response to CHK1 inhibitors in DLBCL cell lines (34). Surprisingly, we found no correlation between AZD6738 sensitivity and endogenous DDR activation, ATR/CHK1 protein levels, or ATM functionality in the DLBCL panel (Supplementary Fig. S3B–S3D). Together, these results suggest MYC protein expression and CDKN2A/B deletion could represent more reliable predictive biomarkers of ATR inhibitor response in DLBCL tumors than DDR pathway status.

AZD6738 induces RS in DLBCL cell lines

DNA lesions formed in mitosis following RS can be detected as 53BP1 nuclear bodies (NB) in daughter G1 cells (35). We therefore evaluated whether 53BP1 NB formation correlated with AZD6738 sensitivity (Fig. 3A–C). AZD6738 sensitivity was not associated with endogenous levels of 53BP1 NBs but was associated with a greater fold induction in 53BP1 NBs following AZD6738 treatment (Fig. 3C). Moderate levels of RS are tolerated by cells, with slowed RF velocity causing chromatin recruitment of ATR pathway sensors and mediators without activation of CHK1, ATM, or cell-cycle checkpoints (36). We therefore speculated that non-GCB cell lines may have higher intrinsic levels of RS that, upon exacerbation by AZD6738 treatment, would result in more 53BP1 NBs compared with GCB cell lines. To test this, we analyzed RF progression by DNA fiber analysis (Fig. 3D and E). Basal RF velocities were significantly slower in non-GCB cell lines OCI-LY-19, WILL-2, and TMD-8 compared with GCB cell lines Karpas-422 and DB (Fig. 3D; Supplementary Table S2), except for TMD-8 versus Karpas-422 (Supplementary Table S3). AZD6738 treatment reduced RF velocity, with the greatest impact in non-GCB cells (Supplementary Table S2). RF velocity in DB and
Karpas-422 cells following AZD6738 treatment (0.81–0.86 kb/min) was comparable to DMSO-treated OCI-LY-19 and WILL-2 cells (0.77–0.96 kb/min), thus from our study we propose that RF velocities of approximately 0.8 kb/min represent a moderate, but manageable level of RS requiring ATR for RF stability. AZD6738 treatment slowed RF progression below this threshold in non-GCB cells, potentially leading to underreplicated DNA, which together with abrogation of the G2–M checkpoint, increased mitotic transmission of DNA lesions. Together, these data suggest that underlying intrinsic levels of RS in DLBCL cell lines ultimately determines response to ATR inhibition.

Pharmacologic inactivation of ATR and WEE1 increases RS levels

ATR, WEE1, and CHK1 are all involved in the RSR (5, 12). Consistent with this, we found a similar trend in sensitivity for
AZD6738 and the WEE1 inhibitor AZD1775 across DLBCL cell lines (Fig. 4A; Supplementary Fig. S4A–S4B), with AZD1775 showing greater reduction in cell viability (Fig. 4B; Supplementary Fig. S4C) and shared biomarkers of response (Supplementary Fig. S4D). We next compared the effects of both inhibitors on DNA replication in OCI-LY-19 and DB cell lines (the most and least sensitive cells in the panel). Distribution of cells in S-phase was determined by measuring EdU incorporation (Fig. 4C). OCI-LY-19 cells were more abundant in early S-phase, indicating slower progression of cells through S-phase, a sign of RS. ATR is critical in early S-phase to limit replication origin firing and suppress formation of ssDNA (37). Thus, OCI-LY-19 cells may be more vulnerable than DB cells to ATR or WEE1 inhibition.

We performed DNA combing with DNA counterstaining to determine the impact of AZD6738 and AZD1775 on RF progression and origin firing. In agreement with previous DNA fiber analyses, RIs in OCI-LY-19 cells progressed slower than in DB cells (Fig. 4E). RF velocity is closely correlated with origin firing (38), and both AZD6738 and AZD1775 treatments decreased RF velocity.

Figure 3.
AZD6738 treatment induces 53BP1 nuclear bodies and retardation of RF progression. A, Representative images of immunofluorescence staining for 53BP1 and cyclin A in cells treated with AZD6738 or DMSO for 40 hours acquired by wide-field microscopy (Operetta, Perkin Elmer). B, Quantification of 53BP1 nuclear bodies (NB) in G1 nuclei (Cyclin A−) of untreated DLBCL cells using Harmony software (Perkin Elmer); ≥1,000 cells were counted per condition. Error bars are calculated from ≥3 independent experiments. C, Induction of 53BP1 NBs by AZD6738 treatment. Data shown as the fold change in average number of 53BP1 NBs in AZD6738-treated cells relative to untreated cells. D, Cells were treated with 1 μmol/L AZD6738 or DMSO for 1 hour before sequentially labeling for 20 minutes with IdU, then CldU. Replication fork velocities were determined by DNA fiber assay. Dot plots show median velocity with interquartile range (IdU; n > 300 forks per cell line, n = 3 experiments; GM14680 and Karpas-422, n > 100 forks, n = 2 experiments). E, Representative fibers from each cell line (original magnification, ×63).
AZD1775 is more potent than AZD6738 in DLBCL cell lines. A, Comparison of GI50 values in DLBCL cell lines treated with AZD6738 or AZD1775 for 72 hours. B, Cell viability measured by uptake of Viacount reagent in control and drug-treated DLBCL cells at the times indicated. Graphs show the mean percentage of viable cells (±SEM) relative to controls from three independent experiments. C and D, Cells were pretreated for 1 hour with 1 μmol/L AZD6738, AZD1775, or DMSO, then EdU labeled (10 μmol/L, 40 minutes) before fixation. C, Representative cell-cycle distribution determined by flow cytometry analysis of EdU intensity and DNA content. D, EdU+ cells were gated into early-mid or mid-late S-phase populations based on DNA content. Graphs show the average ± SEM from three independent experiments. E–F, Cells were treated with 1 μmol/L AZD6738, AZD1775, or DMSO for 1 hour before sequential IdU/CldU dual labeling, then analyzed by DNA combing. E, Distribution of replication fork velocity by treatment. All distributions are statistically different from each other (P < 0.0001), except DB + ATRi vs. OCI-LY-19 + DMSO. F, Distribution of inter-origin distance in indicated conditions. A summary of DNA replication parameters and track number measurements with dot plots showing median and interquartile range from two independent experiments. **P < 0.0039; ***P < 0.0003; ****P < 0.0001. ns, not statistically significant.
velocities and increased origin firing, indicating RS (1, 39, 40). However, the overall impact was greatest in the OCI-LY-19 cell line (Fig. 4E and F), suggesting sensitivity to both inhibitors is caused by a higher capacity for excessive origin firing. Median RF velocities and inter-origin distances in AZD6738- or AZD1775-treated DB cells were equal or greater than those in untreated OCI-LY-19 cells (Fig. 4E and F). These data suggest that RS levels induced by AZD6738 and AZD1775 in OCI-LY-19 cells are likely greater than the RS threshold, pushing cells towards replication catastrophe (41). In contrast, RS levels are likely below the threshold in DB cells, providing better tolerance to AZD6738 and AZD1775.

Differences in viability and replication dynamics between AZD6738- and AZD1775-treated cell lines was not caused by differential target inhibition, as measured by phosphorylated CHK1 on serine 345 for AZD6738 (18) and phosphorylated cdc2 (CDK1) on tyrosine 15 for AZD1775 treatment (Supplementary Fig. S5A; ref. 19). Because of cdc2-pY15 antibody cross-reactivity, we cannot exclude concurrent reduction in phosphorylated CDK2 and CDK3. Re-phosphorylation of CHK1 by DNA-PK during sustained ATR inhibition (37) was not observed in AZD6738-treated DLBCL cell lines (Supplementary Fig. S5), suggesting compensatory regulators of the RSR do not affect the activity of AZD6738 or AZD1775 in DLBCL. Whether this is a general feature of DLBCL or due to the use of different ATR inhibitors remains unclear. AZD1775 treatment induced phosphorylation of CHK1 in DLBCL cell lines (Supplementary Fig. S5A–S5B), indicating WEE1 inhibition causes activation of the ATR–CHK1 pathway. Consistently, AZD1775 also decreased CHK1 and ATM protein expression between 24 and 72 hours, with earlier protein reduction correlating with increased drug sensitivity. We did not investigate the mechanism of DDR protein reduction, however proteasomal degradation of DDR proteins has been reported (42) and warrants further investigation.

AZD6738 and AZD1775 differentially affect cell-cycle progression and induce DNA damage and cell death in distinct cell-cycle phases

The differential effects of AZD6738 and AZD1775 on DNA replication in OCI-LY-19 and DB cell lines prompted further evaluation of their mechanism of action. Cells were EdU pulse-labeled then released into EdU-free medium containing DMSO (control), AZD6738, or AZD1775 and assessed by flow cytometry to measure cell-cycle progression of S-phase (EdU+ labeled) cells and DNA damage (γH2AX, Fig. 5A). Control EdU+ cells transitioned into the daughter cell cycle within 24 hours. However, OCI-LY-19 cells progressed through S-phase more slowly (compare DMSO at 8 hours, Fig. 5A), consistent with slower RF progression. AZD1775 prevented transition of EdU+ OCI-LY-19 into daughter G1 phase, indicated by 3% G1-phase EdU+ cells after 8 hours compared with 26% in control cells (Fig. 5B). Abrogation of S-phase progression correlated with γH2AX induction at 8 to 24 hours (Fig. 5A) and an increased sub-G1 population at 48 to 72 hours, indicative of cell death (Fig. 5B). Consistent with this, 53B1 NBs declined in OCI-LY-19 following AZD1775 treatment (Supplementary Fig. S6), suggesting DNA-damaged cells do not complete mitosis. In contrast, AZD1775-treated DB cells displayed normal S-phase progression, and fewer were γH2AX+ (Fig. 5A and B). Replication catastrophe, characterized by high levels of ssDNA and RPA exhaustion, causes DNA breakage and S-phase pan-nuclear γH2AX (41). Increased origin firing observed in AZD1775-treated OCI-LY-19 cells, but not in DB cells (Fig. 4F), may cause excessive ssDNA and account for the larger proportion of γH2AX+ S-phase OCI-LY-19 cells. γH2AX was mostly detected in EdU+ DB cells entering S-phase at 8 to 12 hours on AZD1775 treatment (Fig. 5A), suggesting delayed effects in DB cells.

AZD6738-treated cells showed normal S-phase progression, and accelerated progression into daughter G1 compared with DMSO control (8 hours, Fig. 5B) consistent with the regulation of intrinsic S–G2, and G2–M checkpoints by ATR (3). AZD6738 caused chronic accumulation of EdU+ OCI-LY-19 cells in the daughter G1–early S-phase, whereas DB cells continued through subsequent cell division (Fig. 5B). AZD6738 induced γH2AX in 4% to 13% of OCI-LY-19 cells predominantly in early S (6–12 hours) and daughter G1–S cells (24 hours), compared with detection only in the second S-phase in DB cells (EdU+ cells, 24 hours, Fig. 5A), consistent with increased 53B1 NBs in OCI-LY-19, but not DB cells. Increasing AZD6738 to 3 μmol/L did not exacerbate the phenotype, suggesting differences between AZD6738 and AZD1775 are mechanistic and not dose dependent (Supplementary Fig. S7A).

Cell-cycle distribution and proliferation were also evaluated by EdU pulse-labeling cells before fixation (Fig. 5C,i). AZD1775 and AZD6738 decreased EdU intensity in S-phase OCI-LY-19, but not DB cells (Fig. 5C,i), consistent with slower DNA synthesis and higher RS in OCI-LY-19 cells. AZD1775 increased the OCI-LY-19 EdU+ S-phase population, concurrent with decreased EdU+ cells (24 hours, Fig. 5C,ii), indicating S-phase arrest and inhibited proliferation. S-phase arrest occurred at 48 hours in DB cells, consistent with transition into the successive S-phase (Fig. 5A). AZD6738 reduced the population of EdU+ OCI-LY-19 cells compared with control cells at 48 to 72 hours, in agreement with G1–S arrest of daughter cells (Fig. 5A).

To assess where DNA damage occurred in the cell cycle, γH2AX+ cells were gated by DNA content (Fig. 6A). AZD1775 induced γH2AX predominantly in G1–early S-phase in OCI-LY-19 cells, and in S and G2–M phases in DB cells, indicating DNA damage was replication-associated. AZD1775 increased the population of pH3+ (mitotic) cells in both cell lines (2–8 hours, Fig. 6B,i), suggesting either unscheduled mitosis or mitotic arrest. Conversely, AZD6738 had no effect. The population of pH3+ AZD6738-treated OCI-LY-19 cells decreased by 24 hours and supports the notion that WEE1 inhibition impedes replication, whereas the mitotic population of DB cells remained higher than control cells (Fig. 6B,ii), corresponding with accumulation in G2–M. A fraction of mitotic DB cells was γH2AX+ (Fig. 6B,ii), suggesting DNA damage sustained during S-phase may have been carried into mitosis, resulting in stalling. However, most AZD1775-treated DNA damage occurred outside of mitosis (Supplementary Fig. S7B), and all pH3+ cells compromised 4N DNA (Supplementary Fig. S7C), suggesting premature mitosis is not the major source of DNA damage. Cleaved caspase 3 (CC3) was predominantly detected in γH2AX+ cells between 24 and 72 hours (Fig. 6C,i and ii), indicating DNA damage leads to apoptosis. Apoptosis and DNA damage occurred in the same cell-cycle phase, predominantly S–G2 in response to AZD1775 or the daughter G1–S phase in response to AZD6738 (Supplementary Fig. S7D).

Some AZD6738-treated OCI-LY-19 cells undergoing apoptosis had DNA content consistent with early S-phase.
Supplementary Fig. S7D, suggesting that cells carrying DNA lesions into S-phase, in the absence of functional ATR, are predisposed to higher levels of RS, replication catastrophe, and cell death. Interestingly, AZD1775-induced apoptosis occurred later in the DB cell line, possibly due to differences in cell-cycle distribution of the DNA-damaged cells (Fig. 6A). However, we have not tested whether differential apoptotic regulation contributes to the kinetics of cell death.

AZD1775 and AZD6738 demonstrate in vivo activity in DLBCL xenograft models

Consistent with in vitro data, once daily oral dosing of AZD6738 or AZD1775 induced significant tumor growth inhibition (TGI) in the OCI-LY-19 xenograft model. The maximum-tolerated dose (MTD) of 120 mg/kg/day for AZD1775, or 50 mg/kg/day for AZD6738 caused complete TGI at day 8 (Fig. 7A; Supplementary Fig. S8A,i; Supplementary Table S4), without significant body...
However, body weight loss was observed by day 17 in some animals, leading to termination of dosing and reduced numbers of animals used to calculate end-of-study tumor volume (Supplementary Table S4). These data suggest that at high doses, either inhibitor would need to be administered using shorter cycles to allow for recovery. At the end of study (21 days), the average tumor volume in mice treated with AZD6738 (50 mg/kg/day) or AZD1775 (120 mg/kg/day), was 0.35 or 0.1 cm³, respectively. These in vivo data suggest AZD1775 has greater single-agent antitumor activity than AZD6738 at their respective MTDs.

Given the differential mechanisms of action observed in the in vitro experiments, we tested whether combination of AZD6738 and AZD1775 in vivo would improve efficacy. A less dose-intensive 5 days on, 9 days off schedule, using AZD1775 (60 mg/kg/day) and AZD6738 (25 mg/kg/day), resulted in tumor regressions comparable to the AZD1775 single-agent MTD (120 mg/kg/day; Fig. 7B; Supplementary Fig. S8B,i;
AZD6738 demonstrated lower potency compared with AZD1775, suggesting that chemotherapy combination approaches may be required in the clinic. R-Benda is a common chemotherapy regimen for DLBCL, however the ABC-subtype is frequently refractory, or patients soon relapse (43). We evaluated AZD6738 in combination with R-Benda in the TMD-8 model (Fig. 7C,i). Single-agent AZD6738 (50 mg/kg/day continuous) resulted in 82% TGI after 15 days and was superior to R-Benda (53% TGI; Fig. 7C,ii; Supplementary Fig. S8C,i). Strikingly, the combination of R-Benda with 5 days of AZD6738, resulted in tumor regression with complete responses in 8 of 8 animals, no relapse within 100 days (Fig. 7C,ii; Supplementary Table S6), and good tolerability (Supplementary Fig. S8C,ii). AZD1775 in combination with R-Benda would also be expected to demonstrate efficacy. However, only limited (2.5 days) exposure of AZD1775 has been tolerated with chemotherapy in the clinic (44), and therefore we did not test this combination in vivo.
Discussion

Preclinical activity of ATR, WEE1, and CHK1 inhibitors has been reported in numerous cancer subtypes, including hematologic cancers (13, 30). However, the genetic and molecular determinants of sensitivity and how this could be exploited in the clinic is still being defined. In this study, we identified a group of non-GCB DLBCL cell lines sensitive to both ATR (AZD6738) and WEE1 (AZD1775) inhibitors and provided preclinical evidence that RS and specific genetic features of DLBCL represent key predictive biomarkers of response. Furthermore, we have provided some of the first head-to-head mechanistic comparisons for ATR and WEE1 inhibitors in DLBCL.

Although constitutive activation of the DDR in DLBCL cell lines was previously associated with CHK1 response (34), we found no correlation with ATR inhibitor activity, consistent with other studies profiling CHK1 in hematologic cell lines (45). These results suggest endogenous DDR activation is not a reliable predictive biomarker of sensitivity to RSR inhibitors, at least in nonsolid tumors. However, we showed the non-GCB subtype, together with high expression of c-MYC and/or deletion of the CDKN2A/B locus in DLBCL cell lines, enriches for AZD6738 sensitivity, similar to that reported for CHK1 inhibition in MYC-driven lymphomas (13, 15) and CDKN2A/p16 deleted head and neck cancer cells (46). The DLBCL COO classification has profound prognostic implications, with the ABC subtype associated with an inferior outcome compared with GCB (43). Poor prognosis is also associated with CDKN2A deletion, found in 30% of ABC DLBCL (32, 47) and high MYC expression, reported in 30% of newly diagnosed patients with DLBCL (28). Furthermore, a recent comprehensive genetic analysis of primary DLBCL revealed new robust DLBCL subsets based on gene signatures, including an ABC/GCB-independent group with biallelic inactivation of TP53, CDKN2A loss, genomic instability, and increased levels of E2F targets (48). Together, our data suggest that ATR and WEE1 inhibitors could provide therapeutic benefit for patients with DLBCL subgroups characterized by CDKN2A/B deletion and/or c-MYC overexpression.

The roles of CDKN2A/B genes and MYC in regulating cell-cycle progression and replication (26) prompted us to assess whether non-GCB DLBCL cell lines exhibited higher levels of RS. We observed increased origin firing, slower RF velocity, and increased 53BP1 NB formation following ATR inhibition, specifically in the AZD6738 sensitive non-GCB cell lines, which correlated with subsequent G1 arrest and cell death. Based on these findings, we propose that AZD6738 sensitivity in DLBCL cell lines is due to RS-associated ATR dependency. Consistent with this, CDKN2A/p16 deleted HNSCC cell lines were hypersensitive to CHK1 inhibition and reduced RF velocity was mitigated by reexpression of p16 (46). MYC depletion is lethal across both ABC and GCB cell lines (29), thus we were unable to confirm a causal link between MYC and RS levels and cannot exclude the possibility that other genetic drivers may be involved. Our results are also consistent with recent data showing sensitivity to ATR inhibition is prevented by inactivation of the CDC25A phosphatase, which delays entry into mitosis, allowing increased time for completion of DNA replication (49). Accordingly, upon AZD6738 treatment, we observed a modest increase in Cdk activity and cell-cycle transition in sensitive, but not insensitive, cell lines.

Interestingly, although ATR inhibition caused increased 53BP1 NBs, inhibition of WEE1 did not, suggesting a differential mode of action of the two drugs. Cytotoxicity induced by WEE1 inhibitors is attributed to aberrant CDK1 and CDK2 activation leading to abrogation of the DNA-damage G2–M checkpoint (5) and RS (7, 50), respectively. Therefore, it has been proposed that WEE1 inhibitors would behave similarly to ATR inhibitors, preferentially targeting cancer cells with high RS and promoting mitotic catastrophe by forcing cells with under-replicated DNA to prematurely enter mitosis (51). Although we did observe an increase in mitotic cells at early time points following WEE1 inhibition, these cells had a full complement of DNA and only a fraction were positive for DNA damage. In contrast, the majority of DNA damage occurred in S-phase cells, which, in the highly sensitive OCI-LY-19 model, underwent cell death before entering mitosis, presumably due to replication catastrophe (41).

Through both in vitro and in vivo studies, we have shown that AZD1775 and AZD6738 are effective at killing cancer cells with higher levels of endogenous RS. However, AZD1775 appears to be a more potent option for a monotherapy approach, whereas AZD6738 may have utility in combination therapy. Our in vitro data demonstrated enhanced AZD6738 efficacy in combination with AZD1775 or chemotherapy. In the latter example, combination with R-Benda induced complete tumor regression in an ABC-DLBCL CDKN2A/B-/+ xenograft model. This finding is important when considering the high-unmet need for this patient population (47). Given the tolerability challenges of AZD1775 in combination with chemotherapy (51), our data suggest development of a WEE1 inhibitor as a monotherapy option in non-GCB DLBCL with CDKN2A/B deletion and/or MYC overexpression, whereas ATR inhibitor development in this same population may be more likely to succeed using a combination approach.

Finding functional biomarkers or assays relevant for the many genetic drivers of RS is a major challenge for effective deployment of RSR inhibitors in the clinic (12). Our results show drug-induced 53BP1 NBs are an indication of intrinsic RS and predict AZD6738 sensitivity in DLBCL cancer cells, whereas endogenous levels were not predictive, potentially limiting the application of such an assay as a patient selection biomarker. In addition, we showed potential to predict cancer cell sensitivity to ATR inhibitors using RF velocity measurements, thus warranting the investigation of this approach in more relevant in vivo settings. Of note, CDKN2A/B deletion and MYC overexpression are both prevalent in other tumor types such as T-ALL, TNBC, and squamous NSCLC. Additional studies providing functional and prognostic links between these biomarkers, as well as high levels of RS and ATR and WEE1 inhibitor sensitivity, has the potential to expand the use of these DDR targeted agents to additional tumor indications.

Disclosure of Potential Conflicts of Interest

R. Odera has ownership interest (including stock, patents, etc.) in AstraZeneca. C. Reimer has ownership interest (including stock, patents, etc.) in AstraZeneca. M.J. O’Connor has ownership interest (including stock, patents, etc.) in AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: L.A. Young, C. Reimer, J. Bartek, A. Lau, M.J. O’Connor

Development of methodology: L.A. Young, A. Maya-Mendoza

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.A. Young, L.O. O’Connor, C. de Renty, M.H. Veldman-Jones, T. Dorval, D.R. Jones, R. Odedra, C. Reimer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.A. Young, L.O. O’Connor, C. de Renty
Young et al.


Writing, review, and/or revision of the manuscript: L.A. Young, L.O. O’Connor, C. de Renty, C. Reimer, J. Bartek, A. Lau, M.J. O’Connor

Administrative, technical, or material support: J. Bartek, A. Lau, M.J. O’Connor

Study supervision: J. Bartek, A. Lau, M.J. O’Connor

Acknowledgments

We thank staff in Laboratory Animal Sciences at AstraZeneca for technical support and Mark Wappett for bioinformatic support. A. Maya-Mendoza and J. Bartek are funded by the Danish Cancer Society (R1123-A7785-15-S2) and the Swedish Research Council (VR-MH 2014-46602-117891-30).

References


Data and materials availability: Researchers may obtain AZD6738, AZD1775, and AZD7762 with a material transfer agreement from AstraZeneca. All reasonable requests for collaboration involving materials used in the research will be fulfilled provided that a written agreement is executed in advance between AstraZeneca and the requester (and his or her affiliated institution). Such inquiries or requests should be directed to the corresponding author.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 11, 2018; revised December 23, 2018; accepted May 20, 2019, published first May 23, 2019.
Differential Activity of ATR and WEE1 Inhibitors in a Highly Sensitive Subpopulation of DLBCL Linked to Replication Stress

Lucy A. Young, Lenka Oplustil O'Connor, Christelle de Renty, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-18-2480

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2019/05/23/0008-5472.CAN-18-2480.DC1

Cited articles
This article cites 51 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/79/14/3762.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/79/14/3762.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.