A Synthetic Lethal Screen Reveals Enhanced Sensitivity to ATR Inhibitor Treatment in Mantle Cell Lymphoma with ATM Loss-of-Function

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Abstract

Mechanisms to maintain genomic integrity are essential for cells to remain viable. Not surprisingly, disruption of key DNA damage response pathway factors, such as ataxia telangiectasia-mutated (ATM)/ataxia telangiectasia and RAD3-related (ATR) results in loss of genomic integrity. Here, a synthetic lethal siRNA-screening approach not only confirmed ATM but identified additional replication checkpoint proteins, when ablated, enhanced ATR inhibitor (ATRI) response in a high-content γ-H2AX assay. Cancers with inactivating ATM mutations exhibit impaired DNA double-stranded break (DSB) repair and rely on compensatory repair pathways for survival. Therefore, impairing ATR activity may selectively sensitize cancer cells to killing. ATR inhibition in an ATM-deficient context results in phosphorylation of DNA-dependent protein kinase catalytic subunits (DNA-PKcs) and leads to induction of γ-H2AX. Using both in vitro and in vivo models, ATR inhibition enhanced efficacy in ATM loss-of-function mantle cell lymphoma (MCL) compared with ATM wild-type cancer cells. In summary, single-agent ATR inhibitors have therapeutic utility in the treatment of cancers, like MCL, in which ATM function has been lost.

Implications: These data suggest that single-agent ATR inhibitors have therapeutic utility and that ATR uses a complex and coordinated set of proteins to maintain genomic stability that could be further exploited. Mol Cancer Res; 13(1): 120–9. ©2014 AACR.

Introduction

Mammalian cells have evolved a complex network of surveillance mechanisms, "checkpoints," to maintain genomic integrity in the face of various genomic insults (1–3). Ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase catalytic subunits (DNA-PKcs), and ataxia telangiectasia and RAD3-related (ATR) are closely related members of the phosphatidylinositol nitse (PIKK) family and are key transducers of cell-cycle checkpoints in DNA damage response (DDR) pathways. ATM and DNA-PKcs are activated primarily in response to double-stranded DNA breaks (DSB) with accessory factors Mre11/Nbs1/Rad50 complex (4) or Ku70/80 (5). ATR is activated upon the generation of ssDNA lesions at aberrant replicative structures and resected dsDNA breaks during S phase. Replication protein A binds ssDNA and facilitates the binding of ATR and ATRIP–TopBP1, thus bringing the kinase to the lesion site (6, 7).

Inactivation of ATR is embryonic lethal (8) and leads to deleterious DNA breakage during S phase (9, 10). ATR is considered a promising target for cancer treatment because of its critical role in intra-S checkpoint in response to replication stress due to oncogenic-induced alterations in tumors or chemotherapeutics treatment (11, 12). Replication stress, in the absence of ATR can result in unrepaird stalled forks that will collapse into DSBs and eventually lead to cell death. There are several synthetic lethality-inducing agents currently targeting the ATR–CHK1 pathway with promising preclinical and/or clinical trial results (13–16). To date, two small-molecule ATR inhibitors have been reported in the literature (13–15).

To identify components of the replication checkpoint that play a critical role during replication stress to ATR inhibition, we adopted a focused synthetic lethal screening approach using γ-H2AX signal (17), a marker of DSBs, as a high-content readout of phenotypic enhancement. It has been established that ATR inhibition exhibits a synthetically lethal interaction in cells with ATM deficiency (13–15). Here, we identified several other genes in the replication checkpoint, in addition to ATM, that are synthetically lethal with a small molecular weight ATR inhibitor (ATRi). Of these genes, ATM is well established as being mutated in human cancer. ATM alterations have been characterized in hematologic cancers such as B-cell lymphocytic leukemia, follicular lymphoma, and mantle cell lymphoma (MCL) by high rates of inactivating mutations and/or deletion (18–21). Cancers with ATM loss-of-function are defective in repair of DSBs and rely on multiple compensatory DNA repair pathways for survival. Thus, we hypothesized that inhibiting ATR kinase activity in ATM-deficient cells would result in synthetic lethality and as such represents a therapeutic approach in ATM-deficient cancers.
To further explore the synthetic lethal relationship between ATR inhibition and ATM loss-of-function, we used a pair of MCL cell lines in vitro and further characterized the relationship in xenograft tumor models. In cells with ATM loss-of-function, phosphorylation of DNA-PKcs and γ-H2AX were identified as potential pharmacodynamics (PD) markers that tracked with single-agent ATR inhibition. Importantly, we show that ATRi in an ATM-deficient GRANTA-519 MCL xenograft model resulted in tumor regressions, whereas at similar doses having minimal antitumor effect on ATM wt JVM-2 tumors. Taken together, these data suggest that ATR inhibitors have therapeutic potential in the treatment of MCL cancers with demonstrated ATM loss-of-function.

Materials and Methods

Cell lines and cell culture

U2OS (osteosarcoma), HT144 (melanoma), JVM-2 (human MCL), and MCF10A (human normal mammary epithelial) cells were obtained from the ATCC. Cell lines were cultured in ATCC-formulated DMEM, RPMI or McCoy’s 5A supplemented with 10% FBS (Omega Scientific). For MCF10A cells, DMEM/F-12 medium was supplemented with 5% horse serum, 0.1 μg/ml cholera toxin, 20 ng/ml hEGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 2 mg/ml L-glutamine. GRANTA-519 (human MCL) cells were obtained from Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures (DSMZ) and cultured in ATCC-formulated DMEM supplemented with 10% FBS.

Drugs and compounds

ATRi was synthesized and purified as described previously (WO2010/073034). ATMi, KU55933 (WO2003/070726), DNA-PKcsi, NU7441 (WO2003/024949), mTORi, AZD8055 (WO2008/023161) were purchased from Selleckchem. Dual ATR/mTORi was synthesized and purified as described previously (WO2012/104776). Antimetabolites were obtained from Sigma (hydroxyurea, cytarabine, and cladribine) and Tocris (clofarabine). Gemcitabine (Gemzar) was obtained from Eli Lilly.

siRNA treatment

The siRNA sense sequences of genes validated are listed in Supplementary Table S2 (Dharmacon). Cells were transfected with final concentration of 50 nmol/l siRNA gene duplexes in Supplementary Table S2 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Ionizing radiation

Where indicated, tumor cells were irradiated with 2 Grays (Gy) in media plus serum using a Gammacell 40 cesium137 Irradiator (Nordion). Immediately after irradiation, cells were incubated for a 1-hour recovery and then harvested.

γ-H2AX cellular assay

Cells were fixed and permeabilized (70% ethanol) before staining with an FITC-conjugated anti-γ-H2AX monoclonal antibody (Millipore). Cells were counterstained with propidium iodide and subsequently analyzed by flow cytometry (LSR II; Becton Dickinson) at select times using FacsDIVA software. Experiments were typically done two to three times, each in duplicates and data are presented as a percentage of γ-H2AX-positive cells.

Western blot analysis of siRNA knockdowns

Cell pellets were trypsinized, washed with PBS, and lysed in L7250 lysis buffer, 50 mmol/L Tris-HCl pH 7.4, 250 mmol/L NaCl, 5 mmol/L EDTA, 0.1% NP-40, 10% glycerol, phosphatase inhibitors (phosSTOP; Roche), protease inhibitors (Complete, EDTA free; Roche), and 1 mmol/L DTT. Protein concentration was determined using Comassie Plus—The Better Bradford Assay Reagent (Thermo Fisher Scientific). Protein extracts were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Antibodies were from Cell Signaling Technology (pS345-Chk1, pT68-Chk2, pS15-TP53, pS235/236-S6RP, pS6RP, ATR, ATM, and β-actin). Genetex (pS1981-ATM), Stressgen (Chk1), Bethyl Laboratories (ATM and Msh2), Thermofisher Scientific (DNA-PKcs), and Abcam (pS2056-DNA-PKcs).

Cell proliferation assays

For long-term proliferation assays, following siRNA knockdown in the presence or absence of hydroxyurea treatment, cells were replated in 6-well plates with fresh media at 10,000 cells per well in duplicates, and were allowed to proliferate for 7 days. Attached cells were fixed with methanol/aceton (1:1) for 20 minutes and stained with crystal violet solution [0.2% crystal violet (w/v), 2% ethanol] for 20 minutes.

Cell proliferation was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Following 24 hours exposure of ATRi cells were washed, replated in fresh media, and allowed to grow in the absence of drug until cell proliferation was assessed at 120 hours.

Immunohistochemistry

Samples were fixed in 10% neutral-buffered formalin for 24 hours and were processed in Leica ASP300 tissue processor. Tissues were dehydrated in ascending ethanol concentrations, cleared in xylene, and penetrated in heated paraffin. The tissues were embedded in paraffin, sectioned into 3- to 4-μm thickness, placed onto slides and loaded onto a Ventana Discovery XT (Ventana Medical Systems). Briefly, the IHC protocol included heat and standard exposure to Ventana cell Conditioning #1 antigen retrieval reagent. The primary antibody, phospho-Histone H2AX S139 (clone 20E3; Cell Signaling Technology) was diluted to a working concentration of 1:250 and incubated for 60 minutes at room temperature. Subsequently, incubation with a biotinylated goat anti-rabbit secondary antibody (The Jackson Laboratory) used at working concentration of 1:200 was performed. Detection was performed using the Ventana DAB Map Kit (Ventana Medical Systems). Stained slides were digitized with an Aperio ScanScope XT whole-slide scanner and qualitative assessment of the percentage of immunopositive nuclei were performed.

Human tumor xenograft studies

Human MCL GRANTA-519 or JVM-2 cell lines (5 × 10⁶ cells) were implanted subcutaneously with 50% Matrigel (Becton Dickinson) in SCID-Bg mice (Harlan). Mice were randomized into cohorts (5–7 mice/group for efficacy; 3–4 mice/group for PD studies) when tumors reached 200 to 500 mm³. ATRi was administered via oral gavage in a vehicle of 20% Captisol, as a single daily dose or every 2 hours for three doses per day. Tumor volumes, body weights, and clinical observations were noted to establish efficacy and tolerability of test agents. Tumor caliper measurements were converted into tumor volumes using the formula: 1/2 [length × (width)²]. Relative tumor inhibition was calculated as a percentage of ΔT/C = 100 × ΔT/C, where ΔT or
\[ \Delta C = \text{difference of mean tumor volume of drug treatment (T) or vehicle (C) on the treatment day of the study and the initial randomization volume (on day 0).} \]

Statistical comparisons were conducted using a one-way ANOVA, followed by a Tukey post hoc test (SigmaStat). Differences were considered statistically significant at \( P < 0.05 \). For PD studies, tumors were collected at specified times after dose, and phosphorylation of DNA-PKcs, S6RP, ATM, and \( \gamma\)-H2AX was evaluated by Western blot analysis or immunohistochemistry as described previously. The bioanalysis for ATRi in plasma was done by quantitative LC-MS.

**Results**

Identification of DNA replication checkpoint genes whose loss is synthetically lethal with ATR inhibition

ATR plays an essential role in DDR to DNA replication stress (i.e., factors that contribute to replication fork stalling) to maintain genomic integrity (11, 22, 23). ATR, and not ATM or DNA-PKcs, is required in preventing the collapse of stalled replication forks (11), and as a consequence, we hypothesized that an inhibitor of ATR would enhance DNA damage and ultimately lead to cell death in the presence of a replication stress agent such as hydroxyurea. To identify whether ATR was effective at conferring this effect, we used a flow cytometry assay to measure accumulation of \( \gamma\)-H2AX (17), a marker of DNA DSBs (24, 25) in U2OS cells following hydroxyurea treatment. Hydroxyurea depletes dNTP pools inhibiting DNA replication and promoting stalled replication forks that cannot be repaired when ATR is inhibited. This leads to fork collapse causing an increase in \( \gamma\)-H2AX signal (Fig. 1B–D). In contrast, under similar conditions, inhibition of ATM or DNA-PKcs kinase with siRNA shows little or no \( \gamma\)-H2AX signal compared with luciferase siRNA controls (Fig. 1A–C); thus, this assay can be used to evaluate whether compounds are effectively inhibiting ATR.

**Figure 1.** Assessment of \( \gamma\)-H2AX following inhibition of ATR with siRNA or inhibitor in U2OS cells. A, following 32 hours of siRNA transfection of the luciferase control, ATR, ATM, and DNA-PKcs siRNA duplexes, cells (in presence of siRNA) were then incubated with 1 mmol/L hydroxyurea (HU) for an additional 16 hours, and then cells were harvested. Western blots were immunoblotted as indicated. B, DNA damage was analyzed for \( \gamma\)-H2AX by flow cytometry. C, graphical representation of data, and includes siRNA knockdown of ATM and DNA-PKcs. Data, three independent experiments performed in duplicates \( \pm \) SD. D, cells were treated with 1 mmol/L hydroxyurea for 16 hours. ATRi was dose titrated onto cells for 2 hours (in the presence or absence of hydroxyurea). Graphical representation of \( \gamma\)-H2AX (DNA damage) assessed by flow cytometry. Structure of ATRi, and cellular \( \gamma\)-H2AX EC\(_{50}\) generated from two independent experiments.
We used the functional γ-H2AX assay (17) to discriminate a small-molecule ATRi that could be used in a synthetic lethal screen (Fig. 1D and Supplementary Fig. S2). A small-molecule inhibitor for ATR (ATRi) phenocopied the siRNA data (Fig. 1D), demonstrating its suitability for our screen (WO2010/073034). The selectivity of ATRi was demonstrated using biochemical (kinase) and cellular-based assays (Supplementary Fig. S1B and Supplementary Materials and Methods) and in binding assays at a screening concentration of 1 μmol/L using the DiscoveRx KINOMescan panel (Supplementary Fig. S1A and Supplementary Table S1). ATRi demonstrated specificity to ATR kinase and not ATM or DNAPK kinases, with low level of inhibition of mTOR, another member of PIKK family. In agreement with siRNA data, small-molecule inhibitors of ATM, DNA-PKcs, or mTOR had little or no effect on γ-H2AX signal (Supplementary Fig. S2).

This selective ATRi was then taken forward in a focused siRNA screen using 19 genes with established roles in DNA replication and checkpoint responses. To determine the optimal exposure time for ATRi, we tested whether loss of these genes in combination with 1 μmol/L ATRi for 2 hours enhanced accumulation of DNA damage as measured by γ-H2AX phosphorylation (Supplementary Fig. S4). The 2-hour time point was selected because upon exposure to ATRi for longer periods such as 24 hours, U2OS cells accumulate double-stranded DNA breaks as monitored by γ-H2AX phosphorylation and display reduced cell proliferation (Supplementary Fig. S4); whereas at 2 hours, little or no γ-H2AX phosphorylation is detectable and there is no effect on cell proliferation compared with luciferase control.

To ensure the effectiveness of the screen, several positive controls were included. First, inhibition of DNA polymerase α (PolA) is known to be synergistic with inhibition of ATR (26). In addition, we included RRM1 and RRM2, subunits of ribonucleotide reductase (RNR), the clinically validated target of hydroxyurea and gemcitabine, as we predicted these were synergistic based on our combinatorial data with other inhibitors of RNR, including gemcitabine, cytarabine, clofarabine, and cladribine (Supplementary Fig. S3A–S3E). RNR inhibition leads to replication fork stall as a result of dNTP pool depletion, which requires the ATR–Chk1 pathway to stabilize the stalled forks (11). As expected, depletion of DNA replication components such as PolA (47.4% ± 3.1%), RRM1 (39.4% ± 0.4%), or RRM2 (77% ± 0.1%) showed increased levels of γ-H2AX with ATRi compared with luciferase control (3.5% ± 0.9%; Fig. 2 and Table 1). In addition to our positive controls,
we identified ATM (19% ± 4.1%), Chk1 (23% ± 0.4%), Wee1 (31.6% ± 0.9%), and Clspn (24.2% ± 1.1%) as enhancers of γ-H2AX signal (Fig. 2 and Table 1).

Of the genes we explored (Table 1), ATM is the best established as being mutated in human cancer. ATM alterations have been described in a range of solid tumors and perhaps even more extensively in hematopoietic and lymphoid tissues (19). Thus, we further explored the underlying biology of this interaction and whether ATM mutation/deletion could provide therapeutic opportunity for an ATRi as monotherapy.

To verify that inhibition of ATM in the presence of ATRi affects cell proliferation, U2OS cells were replated and allowed to grow further for 7 days following siRNA knockdown of ATM in the absence or presence of ATRi. In addition to luciferase, DNA-PKcs was used as a negative control because depletion of DNA-PKcs showed no effect on the γ-H2AX signal (Fig. 3A). Figure 3B shows growth inhibitory effect following ATM inhibition in the presence of ATRi and not with DNA-PKcs or luciferase control. siRNA knockdown of ATM and DNA-PKcs were confirmed by Western blot analysis (Fig. 3C).

An accumulation of γ-H2AX-positive cells in S phase was observed following ATM knockdown and ATRi treatment (Fig. 3A), indicating a specific intra-S phase arrest (i.e., stalled replication forks). Collectively, these data suggest a role for ATM at stalled replication forks following ATR inhibition (17, 24, 25).

When ATR is inhibited in ATM proficient cells, the activity of the inhibitor can be monitored by ATM S1981 phosphorylation, and...
this can serve as a useful biomarker in both in vitro and in vivo settings. However, in cells deficient in ATM, an alternative biomarker is required, and we hypothesized that the ensuing induction of γ-H2AX might be a consequence of DNA-PKcs activation. To test this hypothesis, we evaluated induction of ATM and DNA-PKcs phosphorylation in U2OS cells. Transfected luciferase-negative control cells treated with ATRi induce both phosphorylation of DNA-PKcs S2056 and ATM S1981 (Fig. 3C). As predicted by our hypothesis, levels of DNA-PKcs phospho-S2056 were significantly increased in cells with specific depletion of ATM in the presence of ATRi compared with luciferase control (Fig. 3C), suggesting that DNA-PKcs is required for DDR to ATR inhibition in ATM-deficient cells and that phosphorylation of DNA-PKcs can be used as a marker of pathway response.

Mechanistic and functional effects of ATR inhibition in MCL cell lines

ATM mutations have been identified in lymphoma, leukemia, breast, ovarian, and lung cancer cell lines (19). In solid tumors, mutations are often heterozygous and not always functional. In contrast, the loss of functional ATM has been well documented in MCL and provided a model system to examine the synthetic lethal relationship with ATR. Deletion of 11q22-23 encompassing ATM locus is a common genetic event in MCL tumors that leads to ATM inactivation or deficiency (18, 20). In addition, MCL has the highest frequency of ATM mutations of any non-Hodgkin lymphoma subtype (18). To enable in vitro and in vivo modeling, we selected a pair of MCL lines, ATM-mutant GRANTA-519 and ATM-wt JVM-2.

To validate ATM functionality in MCL cell lines, we exposed cells to ionizing radiation (IR) and extracts were prepared for Western blotting with P-S1981 ATM antibody. P-S1981 ATM is a well-established radiation-induced autophosphorylation site used as a readout of ATM pathway activation (27). Autophosphorylation of ATM on S1981 was detectable in JVM-2 but not in GRANTA-519 (Fig. 4A). For additional confirmation, we assessed IR-induced autophosphorylation of ATM in HT144 (ATM homozygous null, ref. 28) and MCF10A (ATM wt). As expected, P-S1981 ATM is detectable in MCF10A but not in HT144 cells (Fig. 4A). These data verify that the ATM heterozygous mutation in GRANTA-519 cells results in loss of function as previously described (27).

Next, in-cell activity of ATRi was assessed in paired MCL cell lines using mechanistic (γ-H2AX and P-S2056 DNA-PKcs) and functional readouts (CellTiter-Glo). Cells were exposed to a dose titration of ATRi for 24 hours in GRANTA-519 (ATM deficient) and JVM-2 (ATM wt) cells. GRANTA-519 showed approximately
18% γ-H2AX–positive cells and a 100% growth inhibition at 1 μmol/L. In comparison, JVM-2 cells showed 1.5% γ-H2AX–positive cells and only 7.7% growth inhibition (Fig. 4B and C). Thus, GRANTA-519 is highly sensitive to ATR inhibition compared with JVM-2.

In analyzing the downstream consequences of ATR inhibition in U2OS cells with ATM ablation, we detected phosphorylation of DNA-PKcs, suggesting a useful PD marker for pathway readout (Fig. 3C). To verify that DNA-PKcs activation tracks with single-agent ATR inhibition in ATM-deficient GRANTA-519 MCL cell lines, cells were treated with a fixed concentration of 1 μmol/L ATRi for various times and analyzed by Western blot analysis. JVM-2 cells showed little or no phosphorylation of DNA-PKcs S2056 (Fig. 4D). In contrast, in GRANTA-519 cells treated with ATRi, significant levels of pDNA-PKcs S2056 were induced as compared with JVM-2 cells (Fig. 4D). Consistent with our results in U2OS cells (Fig. 3D), autophosphorylation of DNA-PKcs occurs as a consequence of ATR inhibition and is enhanced in ATM-deficient cancers.

In vivo PD and efficacy of ATR inhibition in GRANTA-519 (ATM mut) and JVM-2 (ATM wt) MCL xenograft models

To evaluate the relationship between pharmacokinetics and PD (PK–PD) effects for ATRi, we evaluated phosphorylation of DNA-PKcs S2056, γ-H2AX, S6RP S235/236, ATM S1981 in the GRANTA-519 and JVM-2 tumor xenograft models after ATRi dosing (Fig. 5A–C). Dose and schedule were modeled from the mouse PK parameters to achieve plasma exposure of free ATRi exceeding the cellular EC50 (0.5 μmol/L γ-H2AX, Fig. 5A and B), for >6 hours. In GRANTA-519 tumors, administration of ATRi resulted in a dose-proportional induction of pDNA-PKcs that appeared to saturate at doses >5 mg/kg (Fig. 5A), and paralleled the plasma levels exceeding the cellular EC50 (Fig. 5F). Activation of pDNA-PKcs in GRANTA-519 tumors was accompanied by induction of γ-H2AX foci (Fig. 5C). Pronounced levels of γ-H2AX were detectable in GRANTA-519 tumors from mice treated with the 25 mg/kg dose, whereas only slight changes in pDNA-PKcs or γ-H2AX were observed in JVM-2 (wt) tumors at this dose (Fig. 5A–C). In the GRANTA-519 model, ATM levels are low, and tumor pATM S1981 was largely undetectable after drug treatment (not shown). As expected, in JVM-2 tumors phosphorylation of ATM S1981 was induced following ATR inhibition, demonstrating a response to ATR-induced replication stress (Fig. 5B).

To evaluate the antitumor activity of ATRi, we established that the administration of ATRi at 40 mg/kg every 2 hours for three doses per day achieved sustained ATRi levels and was associated with the PD response described above (Supplementary Fig. S5). In the GRANTA-519 xenograft model, we then administered ATRi at 10, 25, and 40 mg/kg (Fig. 6). Antitumor activity was observed in mice treated with 10 mg/kg ATRi, and at higher doses, which resulted in tumor regressions.

Prolonged inhibition of ATR for most of the dosing interval was required for efficacy, as ATRi dosed three times per day provided a significantly greater therapeutic benefit compared with the daily doses of 25 mg/kg (Fig. 6A). All dose levels and schedules were well tolerated with <10% body weight loss observed, indicating that mice can tolerate ATR inhibition for at least 4 days, and suggesting that a therapeutic window with ATRi may be achieved.

Finally, we evaluated whether there was a differential antitumor effect of ATR inhibition based on ATM status, using the paired MCL models. To enable this comparison, GRANTA-519 and JVM-2 tumors were randomized at similar tumor volumes, and treated with the same doses and schedules (10 and 25 mg/kg). In mice treated at 10 mg/kg, 8% tumor regressions were observed in the GRANTA-519 model, whereas, minimal activity was observed in the JVM-2 model (∆T/C of 86%; Fig. 6). At the 25 mg/kg dose level, in both GRANTA-519 and JVM-2 tumor models, partial inhibition of pS6RP (a PD marker for mTOR) was observed, suggesting at doses ≥25 mg/kg that antitumor activity could be partially attributable to inhibition of mTOR (Fig. 5A and B). Collectively, these data suggest a therapeutic benefit of ATRi in MCL tumors with demonstrated ATM loss-of-function (Figs. 5 and 6).

Discussion

In this study, we explore genetic settings in which ATR inhibition will be most effective and provide a therapeutic window in the clinic. Using a γ-H2AX high-content screen, we demonstrate that loss of ATM is synthetic lethal with an ATRi. Our data support a functional relationship between ATR and ATM as a result of unresolved single strand breaks (SSBs) into DSBs at replication forks (11). It follows that in wild-type cells, a collapsed replication fork will activate ATM and DNA-PKcs. In ATM-deficient cells, repair of DSBs using homologous recombination is impaired (31, 32), suggesting that DSBs associated with fork collapse could potentially be repaired by non-homologous end joining (NHEJ; refs. 31, 32). Consistent with this, we observed phosphorylation of DNA-PKcs in ATM-deficient cells following ATR inhibition.

We further investigated the synthetic lethal relationship between ATR and ATM using ATRi in MCL cell lines, as functional loss of ATM has been well documented in this indication (18, 19). More specifically, deletion of 11q22–23 encompassing ATM locus is a common genetic event in MCL that leads to ATM inactivation or deficiency (18, 20) and could potentially provide a patient population to evaluate the synthetic lethality relationship with ATR inhibitors. We evaluated in the cellular activity of ATRi in the MCL cells lines, JVM-2 (ATM wt) and GRANTA-519 (ATM deficient) in addition to immortalized epithelial cells (data not shown). We showed that GRANTA-519 cells were sensitive to ATRi compared with JVM-2 cells. These data suggest that cells with impaired ATR activity appear to enhance a preexisting defect in repair of DSBs in MCL cells with ATM loss-of-function, leading to an accumulation of unrepaired DSBs and reduced cell proliferation (Figs. 2 and 3). Similar results were seen with two additional cell lines, HT144 and MCF10A (data not shown). These data support the synthetic lethality for ATM loss-of-function with ATR inhibition shown previously (Figs. 2 and 3; refs. 13–15).

Our data in GRANTA-519 and JVM-2 xenograft models showed that PD markers for ATR inhibition pDNA-PKcs and γ-H2AX tracked with ATM functional status (Fig. 5). We also demonstrated that pDNA-PKcs provided a functional, sensitive PD readout for ATR inhibition that was proportional to drug exposure up to 10 mg/kg and associated with maximal antitumor activity. To date, two small-molecule ATR inhibitors have been reported in the literature (13–15); however, little is known about the requirements of ATR target engagement and PD-efﬁcacy relationships. Using the GRANTA-519 model, the following parameters were considered: the extent and duration of the pDNA-PKcs PD...
response, and ATRi PK. Our in vitro data suggested that at least 6 hours of exposure to ATRi was required to activate the DNA-PKcs response, leading to increased induction of γ-H2AX (Fig. 4) and subsequent growth inhibition. Given the requirement of long duration of target inhibition, we used the single-dose PK parameters to predict drug exposures required to inhibit ATR for greater than 6 hours. One approach to achieve this was with a thrice daily dose schedule, as the ATRi PK in mice has a relatively short plasma half-life and high clearance. In the GRANTA-519 model, tumor xenograft regression was observed following dosing at 10 mg/kg with the thrice daily administrations for 4 days, which coincided with the maximal PD effect in tumors. Short exposure to ATRi (with daily 25 mg/kg doses) was unable to induce substantial tumor growth inhibition, suggesting insufficient pathway inhibition and/or that the longer intervals between doses may allow tumor cells to use alternative repair pathways for survival.

A challenge in evaluating ATRi antitumor efficacy was the ability to discriminate ATR effects from mTOR activity due to cross-reactivity of the inhibitor with mTOR (Supplementary Table S1). Although mTOR inhibition has been shown to spare replicative-stress in vitro (Supplementary Fig. S2; ref. 12),
inhibiting the pathway can affect tumor cell growth. Hence, in our studies, efficacy was compared at 10 mg/kg, where putative mTOR inhibition, as measured by modulation of pS6RP was minimal (Fig. 5). Although this is not a perfect ATRi, a more highly selective inhibitor without cross-reactivity with mTOR would provide a better understanding of ATR efficacy.

Given the essential role of ATR in DNA replication and checkpoint (33), it was presumed that cancer and normal tissues could be equally sensitive to ATR inhibitors with chronic dosing. It has been shown that partial ATR knockdown in genetically engineered mouse models had minimal impact on normal tissue homeostasis (34). Although we did not directly measure the extent of ATR kinase inhibition, our results suggest that mice can similarly tolerate chronic ATR inhibition eluding to a potential therapeutic index for ATR inhibitors as single agents if tumors have the appropriate genetic background that will enhance their sensitivity to the inhibitor.

In summary, our data support chronic single-agent ATR inhibition to induce replicative stress, and reliance on the synthetic lethal interaction with ATM to induce tumor regression in vivo. For this therapeutic approach, ATM functionality needs to be assessed for patient selection. In addition, we identified other components of the ATR–CHK1 pathway that were synthetically lethal with ATRi (Fig. 2 and Table 1). Interestingly, RRM1 and RRM2, subunits of RNR, the targets of antimetabolites (hydroxyurea and gemcitabine) were synthetically lethal with ATRi, providing mechanistic understanding for ATRi combinations with antimetabolites (Fig. 2, Table 1; Supplementary Fig. S3).

Depletion of Wee1, CHK1, or Claspin with ATRi leads to an enhanced accumulation of γ-H2AX, suggesting that activation of ATR in this context is essential for suppression of DNA damage. CHK1 was a synthetically lethal hit with ATR, suggesting that ATR and CHK1 are not functionally epistatic, and may have discrete roles in maintenance of genomic integrity following replication stress (35). Additional genes, like Wee1, and adaptor protein Claspin may collaborate with ATR for activation of the intra-S checkpoint response to replicative stress, through mechanisms that may be independent of the CHK1–Cdc25–CDK pathway (36–40). These data suggest that ATR can use a complex, and coordinated set of proteins involved in intra-S checkpoint function to maintain genomic integrity. In this scenario, we hypothesize that these approaches may be expanded to other cancer types, in which high levels of replicative stress, like oncogene-induced, genomic instability or repair deficiencies may further expand the therapeutic window for treatment activity (12, 34, 41).

Disclosure of Potential Conflicts of Interest
All authors are employed at Novartis.

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