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Therapeutic Discovery

Molecular Cancer Therapeutics

Targeted Mutations in the ATR Pathway Define Agent-Specific Requirements for Cancer Cell Growth and Survival

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Abstract

Many anticancer agents induce DNA strand breaks or cause the accumulation of DNA replication intermediates. The protein encoded by *ataxia-telangiectasia mutated* and *Rad 3-related* (*ATR*) generates signals in response to these altered DNA structures and activates cellular survival responses. Accordingly, ATR has drawn increased attention as a potential target for novel therapeutic strategies designed to potentiate the effects of existing drugs. In this study, we use a unique panel of genetically modified human cancer cells to unambiguously test the roles of upstream and downstream components of the ATR pathway in the responses to common therapeutic agents. Upstream, the S-phase–specific cyclin-dependent kinase (Cdk) 2 was required for robust activation of ATR in response to diverse chemotherapeutic agents. While Cdk2-mediated ATR activation promoted cell survival after treatment with many drugs, signaling from ATR directly to the checkpoint kinase Chk1 was required for survival responses to only a subset of the drugs tested. These results show that specifically inhibiting the Cdk2/ATR/Chk1 pathway via distinct regulators can differentially sensitize cancer cells to a wide range of therapeutic agents. *Mol Cancer Ther; 11(1); 98–107. ©2011 AACR.*

Introduction

The phosphatidylinositol kinase–like kinase ATR is directly activated by chemical agents that directly or indirectly cause active DNA replication forks to stall (1). Efficient DNA replication can be impaired by DNA adducts or cross-links that impede replication fork movement or by agents that prevent the synthesis or incorporation of nucleotides. Even double-strand DNA breaks, which are primarily sensed by the related protein ATM, can indirectly cause the activation of ATR via the transient generation of DNA repair intermediates (2). As a sensor of altered DNA structures, ATR is activated by many of the most commonly used antiproliferative agents used for cancer therapy. ATR signaling in turn activates downstream pathways that control cell-cycle arrest and mediate cell survival (3).

The central role played by ATR in the signaling pathways turned on by therapeutic agents has been confirmed by experimental systems in which ATR activity is limiting. In human cells, expression of a dominant-negative mutant ATR protein (4), ATR knockdown by short interfering RNA (5), or targeted alteration of endogenous *ATR* alleles (6) have each been shown to sensitize cells to a variety of DNA-damaging agents and DNA replication inhibitors. Furthermore, it has been shown that at least some of these sensitizing effects are enhanced in cancer cells that harbor loss-of-function mutations in *p53* (7, 8). Cumulatively, this growing body of work suggests a novel strategy of inhibiting the ATR pathway as a means of selectively sensitizing cancer cells to existing therapeutic agents.

Basic studies of ATR and its activation have revealed new approaches by which ATR signaling could potentially be inhibited (9). ATR is activated at regions of replication protein A (RPA)-coated single-stranded DNA that are generated by replication fork stalling (1). Activation of ATR at such structures clearly requires its binding partner, the ATR-interacting protein (ATRIP), the ring-shaped Rad9–Rad1–Hus1 complex that is loaded onto the opened DNA structure, and the adaptor molecules TopBP1 and RHINO that facilitate complex physical interactions between these proteins (10). The activation of ATR occurs only in S- to G_2 phase (11, 12). Recently, this cell-cycle phase–specific restriction was shown to be at least partially dependent on Cdk2, the cyclin-dependent kinase activated at the start of S-phase (13). Cdk2 is required for the stabilization of the DNA replication licensing protein Cdc6 (14), which in turn interacts with the active ATR

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holoenzyme complex (Fig. 1A; refs. 9, 13, 15). Cdk2 also facilitates ATR activation via direct phosphorylation of ATRIP (16). In principle, ATR inhibition could be achieved by directly blocking the ATR kinase moiety, by disrupting the critical protein–protein interactions required for full activation, or by inhibiting upstream signaling by Cdk2.

Once activated, ATR phosphorylates numerous downstream substrates (17) including the checkpoint kinase Chk1 (18). Chk1 phosphorylation on the conserved serine residue S317 is required for DNA damage responses, whereas phosphorylation at S345 occurs during the unperturbed cell cycle and is required for normal cell growth (19, 20). In proliferating cells, a pool of Chk1 is associated with chromatin, where it maintains activating marks and thereby facilitates transcription of cell growth–promoting genes (21). In response to stalled DNA replication forks, ATR phosphorylates Chk1 on S317; this modification is required for the phosphorylation of neighboring Chk1 sites, including S345, and the resulting activation of the G_2–M checkpoint (19, 20). Following phosphorylation of residues S317 and S345, Chk1 dissociates from chromatin and transmits signals to spatially distant cellular compartments (22).

While the ATR/Chk1 pathway is activated in many cell types by many different types of therapeutic agents, it has been shown that a second pathway, mediated by the stress response kinase p38MAPK and MAPKAP kinase 2 (MK2), may be particularly important for the survival of cancer cells that lack functional p53 (3). Signaling by p38MAPK/MK2 in response to cisplatin, doxorubicin, and camptothecin is dependent on ATR (7), indicating that this pathway is functionally parallel to the ATR/Chk1 pathway. While Chk1 has been a favored target for the development of novel therapeutic agents, the existence of a parallel pathway with a heightened requirement in p53-deficient cells suggests that a more broad approach might be desirable.

To examine the contributions of individual components of the Cdk2/ATR/Chk1 pathway to cell survival after therapy, we systematically examined drug responses in a panel of isogenic human cancer cell lines with defined genetic alterations. Cell survival was highly dependent on genotype and on the specific drug used. In agreement with previous reports, genetic inhibition of ATR was a highly potent sensitizer to numerous agents. However, genetic inhibition of ATR signaling to Chk1 could only recapitulate some of these effects, showing the importance of other ATR substrates. Upstream inhibition of Cdk2 showed sensitization to some of the same agents that could be potentiated by ATR inhibition. Therefore, while inhibition of Cdk2 has previously been viewed as a general antiproliferative strategy (23, 24), our results indicate

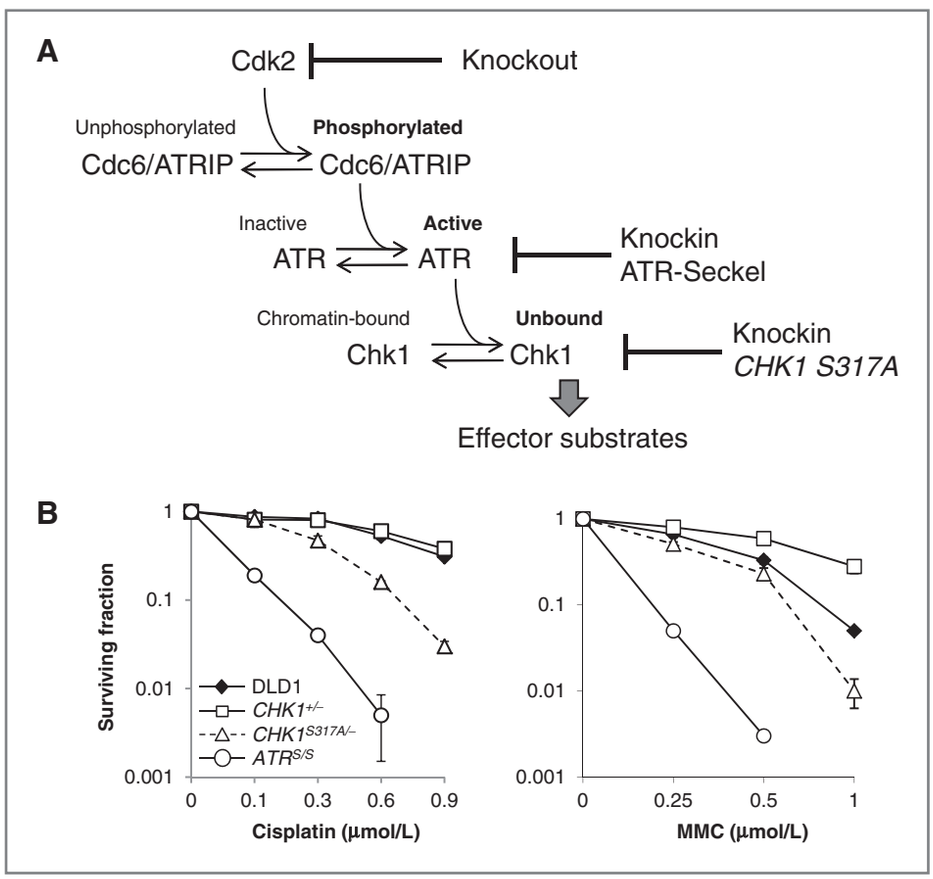

Figure 1. Evaluating drug responses in genetically modified human colorectal cancer cell lines. A, ATR can be activated during S-phase in the presence of upstream Cdk2. The contributions of individual components of the Cdk2/ATR/Chk1 pathway were assessed with the use of isogenic lines harboring knockout and knockin alleles, as indicated. See text for additional details. B, clonogenic survival of DLD-1 and $ATR^{S/S}$, $CHK1^{+/-}$ and $CHK1^{S317A/-}$ derivative cells after treatment with the indicated doses of drugs. Survival is plotted on log scale as a proportion of untreated controls.

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that Cdk2 inhibition might have highly selective effects if combined with existing therapies.

Materials and Methods

Cell culture

The human colon cancer cell lines HCT116 and DLD-1 have been genetically modified by recombinant adeno-associated virus-mediated homologous recombination (25, 26). HCT116, DLD-1, and all genetically altered derivatives were cultured at 37°C in 5% CO_2, in McCoy's 5A medium supplemented with 6% fetal calf serum and penicillin/streptomycin (Invitrogen). All cell lines were authenticated within the past 12 months by short tandem repeat fingerprinting and verification of targeted alterations by DNA sequencing.

Drugs and screening

The Approved Oncology Drug Set of 89 U.S. Food and Drug Administration (FDA)-approved drugs was obtained from the Developmental Therapeutics Program at the National Cancer Institute, Bethesda, MD. Wild-type DLD-1 cells and the derivative $ATR^{S/S}$ cell line were plated in 96-well plates (10^4 cells per well) and grown for 2 days. At approximately 80% confluence, cells were treated with drugs for 24 hours at a final concentration of 300 nmol/L for all drugs with the exception of alkylating agents (1 μmol/L) and antimetabolites (50 μmol/L). Control cells were mock treated with dimethyl sulfoxide. Following a 24-hour incubation with drug, media were removed. Cells were washed twice in Hank's buffered saline solution (Invitrogen), detached with trypsin, and resuspended. One tenth of the cells were plated to new 96-well plates in triplicate. Cells were grown for 12 days in media containing no drug. Cell viability was assessed by CellTiter-Blue Cell Viability Assay (Promega) according to manufacturer's protocol. Substrate conversion was measured with a SpectraMax M5 fluorescent plate reader (Molecular Devices). For the clonogenic survival assays, hydroxyurea, cisplatin, and mitomycin C (Sigma) were dissolved in PBS. Gemcitabine (Gemzar; Eli Lilly and Co.) was of pharmaceutical grade.

Clonogenic survival assay

Cells growing in 24-well plates were treated with drugs for 24 to 48 hours as indicated. Drug-treated cells and untreated controls were washed with Hank's buffered saline solution (Invitrogen) and harvested following detachment in 0.5-mL trypsin/EDTA. Cells were transferred to 1.5-mL medium and vortexed. Approximately, 10^4 cells were plated in triplicate to 10-cm tissue culture dishes and grown for 14 days. Colonies were stained with 0.2% crystal violet in 50% methanol. Colonies containing more than 50 cells were scored. Clonogenic survival is expressed as a proportion of the colony number on control dishes.

Immunofluorescence and cell imaging

Subconfluent cell cultures growing on chamber slides (Nunc) were fixed in 3% paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with primary antibodies overnight. DNA damage foci were visualized using a primary antibody to γH2AX (Millipore) and a secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were captured at room temperature with an AxioImager Z1 microscope equipped with an AxioCam HRm camera, Axiovision 4.6.3 software, and a Plan Neofluar 63x/1.25NA lens (Zeiss).

Antibodies and immunoblotting

Whole-cell lysates were denatured, sonicated, and fractionated on NuPAGE gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride membranes, which were then incubated with antibodies directed against Chk1, α-tubulin (Santa Cruz Biotechnology), Chk1-S317-P, Chk1-S345-P (Cell Signaling Technologies) under conditions recommended by the manufacturers. Blots were developed using enhanced chemiluminescence (Amersham).

DNA combing

To assess active DNA replication forks, cells were sequentially pulse-labeled with 25 μmol/L chlorodeoxyuridine (CldU) and then 250 μmol/L iododeoxyuridine (IdU) for 20 minutes each. Double-labeled cells were harvested and DNA fiber spreads were prepared and stained as previously described (27). Fibers were examined using a Leica SP2 confocal microscope using a ×63 (1.3 NA) glycerol immersion objective. The lengths of red- (Alexa Fluor 555) or green (Alexa Fluor 488)-labeled patches were measured using the ImageJ software (NIH, Bethesda, MD), and arbitrary length values were converted into micrometers using the scale bars created by the microscope. At least 125 replication tracks were measured per experiment.

Results

To unambiguously test the role of the ATR pathway in drug responses, we used a panel of mismatch repair (MMR)-deficient colorectal cancer cells with targeted genetic alterations that affect individual pathway components and compared them with isogenic controls (Fig. 1A). The effects of upstream signals from Cdk2 were examined with the use of a *CDK2*-knockout cell line (genotype $CDK2^{-/-}$) that exhibits reduced checkpoint activation after treatment with ionizing radiation (13, 28). ATR is essential for cell viability, but greatly reduced ATR expression is observed in cells that harbor the hypomorphic *ATR* mutant that causes the autosomal recessive disorder, ATR-Seckel syndrome (29). We used a cellular model of ATR-Seckel syndrome (6), in which the point mutation that defines the *ATR-Seckel* allele was introduced by gene

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targeting into both endogenous loci (genotype $ATR^{S/S}$). To study the role of ATR signaling downstream to Chk1, we used a cell line in which the serine residue (S317) at a conserved phosphorylation site on Chk1 was replaced with an alanine residue. This S317A point mutation has been definitively shown to abrogate ATR-dependent Chk1 phosphorylation at multiple C-terminal sites in response to DNA damage and replication stress (19, 20). Cells that exclusively express this allele (genotype $CHK1^{S317A/-}$) exhibit diminished checkpoint activation compared with cells that harbor one ($CHK1^{+/-}$) or two ($CHK1^{+/+}$) wild-type Chk1 alleles (20).

First, we tested 2 agents that have previously been shown (30) to elicit a potent requirement for ATR for clonogenic cell survival. Consistent with prior results, $ATR^{S/S}$ cells were strikingly more sensitive to both cisplatin and mitomycin C (MMC) over broad dose ranges than parental DLD-1 cells (Fig. 1B). Cells harboring a single wild-type *CHK1* allele and one knocked out allele ($CHK1^{+/-}$) exhibited resistance similar to parental cells that harbor 2 wild-type alleles. In contrast, cells that express only a single mutant allele ($CHK1^{S317A/-}$) exhibited a response that was intermediate of wild-type and ATR-deficient cells. This result shows that loss of signaling to Chk1 was only a partial cause of the dramatic sensitization caused by the $ATR^{S/S}$ genotype. It is probable that additional, Chk1-independent signaling molecules downstream of ATR play important roles in the survival responses to these alkylating agents, as first shown by Reinhardt and colleagues (7). In addition to signaling molecules, numerous proteins involved in DNA maintenance and DNA repair are also known to be important substrates of ATR (17) and are therefore potential mediators of cell survival after drug treatment.

ATR has previously been shown to be required for clonogenic survival after treatment with the most commonly used antiproliferative agents, antimetabolites, and DNA-damaging agents (4, 8, 30). To test an expanded panel of reagents, we screened a library of FDA-approved anticancer drugs. The standard clonogenic survival assay is not well suited to high-throughput applications, so we instead used a fluorometric assay that measures cell proliferation by metabolic capacity after replating (see Materials and Methods). Drug concentrations were adjusted to ranges previously established in these cell lines on the basis of drug classification (30).

Our screen detected numerous compounds that preferentially affected the proliferation of the $ATR^{S/S}$ cells over their parental ($ATR^{+/+}$) controls (Fig. 2). The most striking degree of genotype-specific growth suppression was observed in cells treated with dactinomycin and bortezomib. These two drugs broadly target cell growth by distinct mechanisms of action. Bortezomib is a proteasome inhibitor that alters protein stability and thereby disrupts many growth and survival pathways, whereas dactinomycin is an antiproliferative agent that inhibits RNA synthesis. 5-Fluorouracil (5-FU), a compound previously shown to preferentially inhibit clonogenic

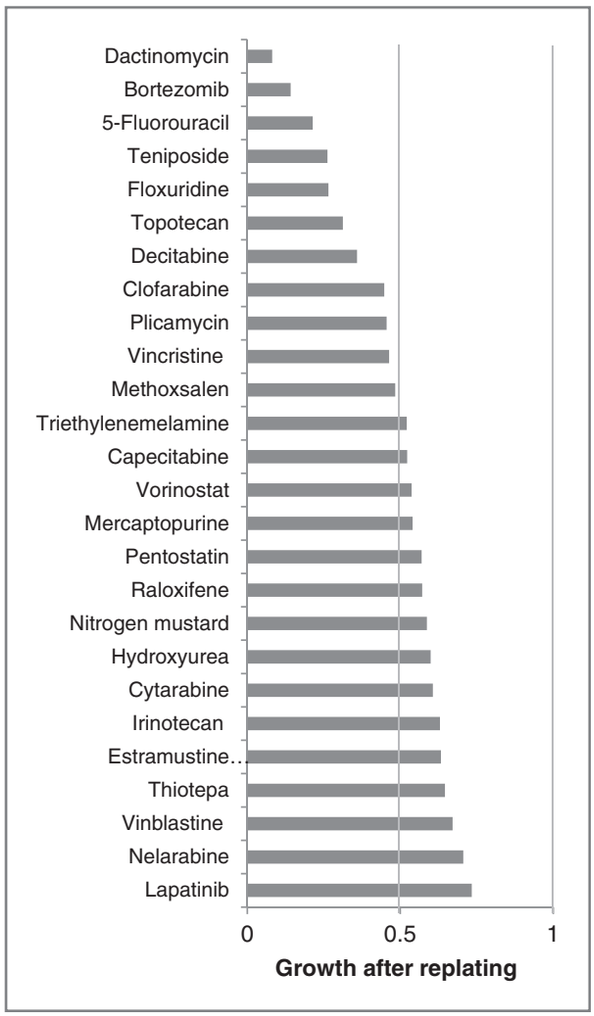

Figure 2. Inhibition of cell growth after treatment with anticancer agents. DLD-1 cells were compared with their $ATR^{S/S}$ derivative in a high-throughput growth assay (see Materials and Methods). The growth of $ATR^{S/S}$ cells was plotted as the proportional growth of wild-type DLD-1 cells treated with the same drugs.

survival of ATR-deficient cells (30), also elicited a strong genotype-dependent effect in this growth assay. Interestingly, other compounds that specifically inhibited the clonogenic survival of $ATR^{S/S}$ cells, such as cisplatin and MMC (Fig. 1B), did not significantly reduce $ATR^{S/S}$ cell growth as measured by this growth assay. Conversely, the top drugs identified in the growth assay (Fig. 2) elicited more modest genotype-specific effects when clonogenic survival was assessed (Fig. 3). These results show that different methods of assessing cell growth and long-term clonogenic survival are not directly comparable.

Next, we examined how downstream signaling to Chk1 contributed to the growth effects observed in the ATR-deficient cells after drug treatment. For a subset of the agents initially tested, we compared wild-type cells with isogenic $ATR^{S/S}$, $CHK^{+/-}$, and $CHK1^{S317A/-}$ derivatives by clonogenic survival assay (Fig. 3). In the case of

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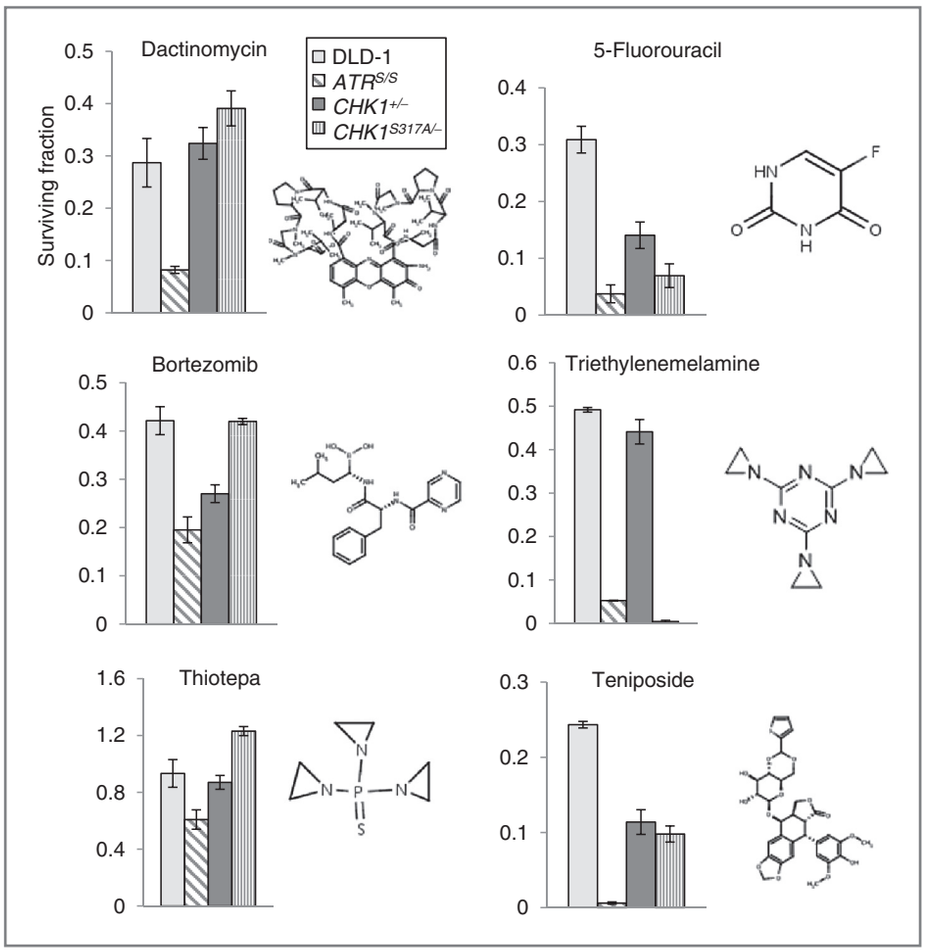

Figure 3. Inhibition of cell survival after treatment with selected anticancer agents. DLD-1 cells were compared with their isogenic derivatives in a clonogenic survival assay. Drug concentrations used were bortezomib (30 nmol/L), thiotepa (1 μmol/L), triethylenemelamine (1 μmol/L), dactinomycin (30 nmol/L), teniposide (300 nmol/L), and 5-fluorouracil (50 μmol/L). Cell lines were treated with drugs for 24 hours before replating.

dactinomycin, bortezomib, and the alkylating agent thiotepa, the signaling-defective $CHK1^{S317A/-}$ cells exhibited clonogenic survival that was similar to that observed in wild-type cells. In contrast, 5-FU, triethylenemelamine, and teniposide elicited a strong requirement for signaling to Chk1, with gene dosage having a more modest effect. These results underscore the diverse signaling responses and downstream survival pathways triggered by different anticancer agents. While ATR is important for clonogenic survival in response to many types of cell stress and DNA damage, the role of Chk1 appears to be highly stimulus-specific.

To examine in additional detail the molecular mechanisms that might underlie the varying requirement for Chk1, we examined signaling to Chk1 in wild-type cells after drug treatment. Bortezomib and triethylenemelamine both induced phosphorylation on Chk1 S317 (Fig. 4A). Paclitaxel and temozolomide, which did not elicit ATR-specific growth suppression, did not cause Chk1 phosphorylation under the conditions used.

In contrast to the other agents, dactinomycin caused a dramatic reduction in Chk1 protein expression, consistent with its known role as an inhibitor of transcription (Fig. 4A). It is possible that limiting intracellular pools of Chk1 after dactinomycin treatment might have caused the observed requirement for ATR. Chk1 phosphorylation–deficient cells were not more growth sensitive to dactinomycin than wild-type cells (Fig. 3). Treatment with dactinomycin did not cause an observable increase in DNA damage, as measured by immunostaining with the DNA damage marker γH2AX. Consistent with the increased levels of Chk1 phosphorylation after bortezomib treatment (Fig. 4A), bortezomib-treated DLD-1 cells did express γH2AX (Fig. 4B); approximately 35% of bortezomib-treated cells had more than 10 γH2AX foci. The increase in phospho-Chk1 and γH2AX may have been due to the stabilization of these phosphoproteins, which are normally turned over by proteasomal degradation (31, 32). It is also possible that altered protein turnover could trigger the activation of DNA damage signaling pathways. Bortezomib has been reported to induce γH2AX expression and apoptosis through multiple mechanisms including a p38MAPK-dependent mechanism (33, 34).

We next looked upstream of ATR and tested the cellular effects of targeting Cdk2. Cdk2 is activated during the transition from G_1 to S-phase and subsequently

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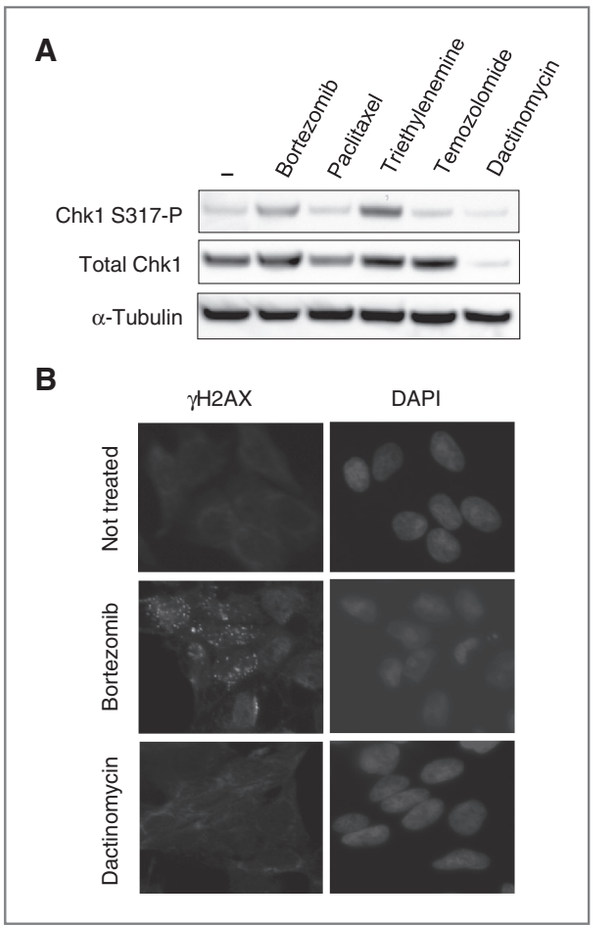

Figure 4. Phosphorylation of Chk1 in response to genotype-selective chemotherapeutics. A, levels of total Chk1 protein and Chk1 S317-P phosphoprotein in drug-treated DLD-1 cells were determined by immunoblotting. Drug concentrations used were bortezomib (300 nmol/L), paclitaxel (300 nmol/L), temozolomide (1 μmol/L), triethylenemelamine (1 μmol/L), and dactinomycin (300 nmol/L). Lysates were harvested after 24 hours of drug treatment. B, DLD-1 cells were treated with 300 nmol/L bortezomib or 300 nmol/L dactinomycin for 24 hours. The localization and distribution of γH2AX were assessed by immunofluorescence. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

phosphorylates downstream targets, including ATRIP and Cdc6 (14, 35, 36), that promote DNA replication. *CDK2*-nullizygous mice and human cells are fully viable (13, 37, 38), as the essential Cdk1 is able to compensate for Cdk2 loss by forming alternative cyclin–Cdk complexes during S-phase (13, 39). While genetic inhibition of Cdk2 is compatible with cell viability, it is not known whether DNA replication is equally efficient in the absence of Cdk2 expression. To quantitatively measure the dynamics of DNA replication, we used a DNA fiber labeling technique (27) to measure the speed of DNA replication forks. Interestingly, we observed an increased distribution of fast forks and a higher average fork speed in $CDK2^{-/-}$ cells, compared with isogenic cells that express normal levels of Cdk2 (see Supplementary Data). The proportion of labeled fibers derived from bidirectional origins or terminations were similar in both cell lines. These results suggest that the atypical cyclin–Cdk1 complexes that form in the absence of Cdk2 expression can support highly efficient DNA replication. On the basis of these genetic data, we speculate that highly specific inhibitors of Cdk2 might be well tolerated if they do not also prevent the activity of compensatory Cdk1 complexes.

Cdk2 cooperates with p53 to activate the G_2–M checkpoint after exposure to γ-radiation; cells lacking both proteins exhibit reduced phosphorylation of Chk1 and are profoundly checkpoint defective (13). We tested whether the chemotherapeutic drugs that most robustly suppressed the clonogenic survival of ATR-deficient cells over wild-type cells similarly elicited a requirement for upstream Cdk2 for Chk1 activation. In wild-type cells, Chk1 was robustly phosphorylated on S317 and S345 after treatment with the nucleoside analogue gemcitabine, the DNA replication inhibitor hydroxyurea, and the alkylating agents cisplatin and MMC (Fig. 5A). The extent of Chk1 phosphorylation in response to each of these agents was reduced in isogenic $CDK2^{-/-}$ cells. This reduced signaling to Chk1 was associated with diminished clonogenic survival (Fig. 5B). To further examine the mechanism by which Cdk2 deficiency caused chemosensitization, we tested the effects of gemcitabine and MMC in combination with UCN-01, a nonspecific inhibitor of Chk1 (40, 41). At the dose tested, UCN-01 alone did not reduce clonogenic survival (Fig. 6A and B). Combined treatment with UCN-01 potentiated the effects of gemcitabine so that the survival of wild-type cells was similar to that of $CDK2^{-/-}$ cells. In contrast, survival after MMC appeared to be unaffected by UCN-01treatment, suggesting that deficient phosphorylation of Chk1 was not the primary contributor to the observed survival defect.

Our recent studies (D. Wilsker and colleagues, manuscript in preparation) have shown that Chk1 haploinsufficiency in cells with one disrupted *CHK1* allele ($CHK1^{+/-}$) caused an elevated rate of tetraploidization. As an aberrant chromosome complement is a cardinal characteristic of many cancer cells, we wondered whether tetraploidy might alter some responses to therapeutic agents. To test this idea, we isolated a subclone in which every cell was tetraploid and examined its responses to MMC, an alkylating agent that elicited a strong ATR-specific survival response (Fig. 1A) and nitrogen mustard, an alkylating agent which caused a modest ATR-specific growth defect (Fig. 2). These tetraploid cells were compared with isogenic cells that remained primarily diploid. As observed previously (Fig. 1B), cells with a reduced *CHK1* gene dose were more resistant to MMC, and this resistance was further enhanced in tetraploid cells (Fig. 6B and C). In the case of nitrogen mustard, there was no effect of *CHK1* gene dosage, but the resistance of the tetraploid cells was elevated. No ploidy-specific increase in resistance to daunorubicin, mitotane, docetaxel, or paclitaxel was observed (data not shown).

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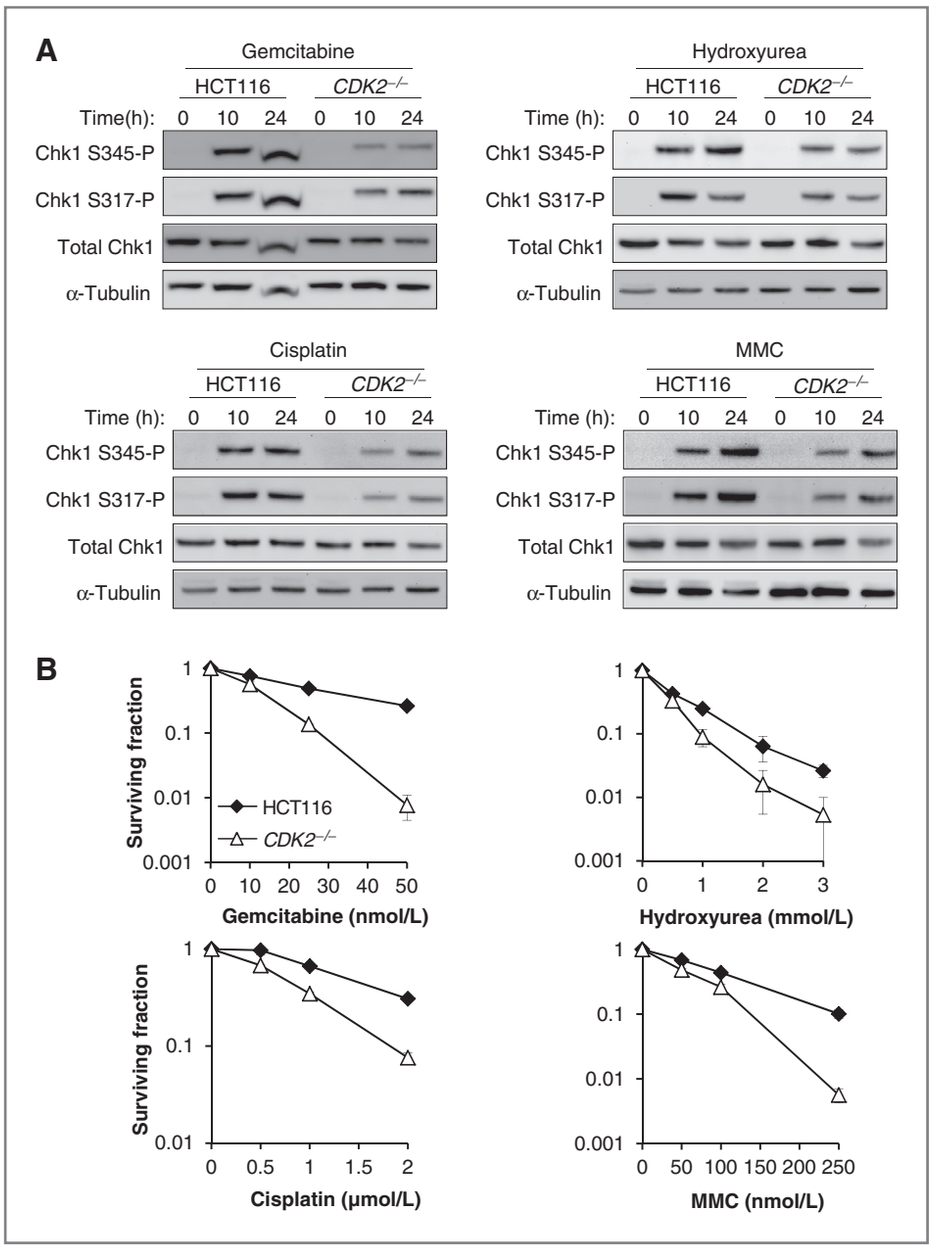

Figure 5. The role of Cdk2 in the cellular responses to ATR genotype–selective agents. A, HCT116 and isogenic *CDK2*$^{-/-}$ cells were treated with the indicated chemotherapeutic agents and harvested at 10 or 24 hours. Time "0" indicates no treatment. Levels of the Chk1 phosphoproteins Chk1S345-P and Chk1S317-P and total Chk1 were determined by immunoblotting. α-Tubulin was assessed as a loading control. B, clonogenic survival was measured in wild-type HCT116 and *CDK2*$^{-/-}$ cells treated with gemcitabine, hydroxyurea, or MMC for 24 hours or cisplatin for 48 hours, as indicated.

Discussion

Recent efforts to understand the biochemical basis for ATR activation at stalled DNA replication forks have led to new insight into how ATR is regulated and how ATR in turn regulates a network of downstream signaling molecules (9). The seminal observation by Reinhardt and colleagues that loss of p53 function, which occurs in approximately 50% of all cancers, functionally "rewires" the pathways downstream of ATR underscores the complexity of these survival pathways (7). A detailed understanding of how these pathways are activated by specific therapeutic agents is needed to formulate combinatorial strategies that can successfully create and exploit survival defects. Our study shows, from a genetic standpoint, that not all prospective molecular targets are equivalent, even though they may function in a common pathway. Genetic inhibition of ATR and Cdk2 showed distinct effects from the genetic or pharmacologic inhibition of Chk1 (Figs. 1B, 5B, and 6A).

The HCT116 and DLD-1 cell lines harbor inactivating mutations in MMR genes; all genetically modified derivatives used here are therefore also MMR-deficient. The MMR system has been shown to participate in double-strand break responses (42) and in the activation of ATR following treatment with certain alkylating agents, such

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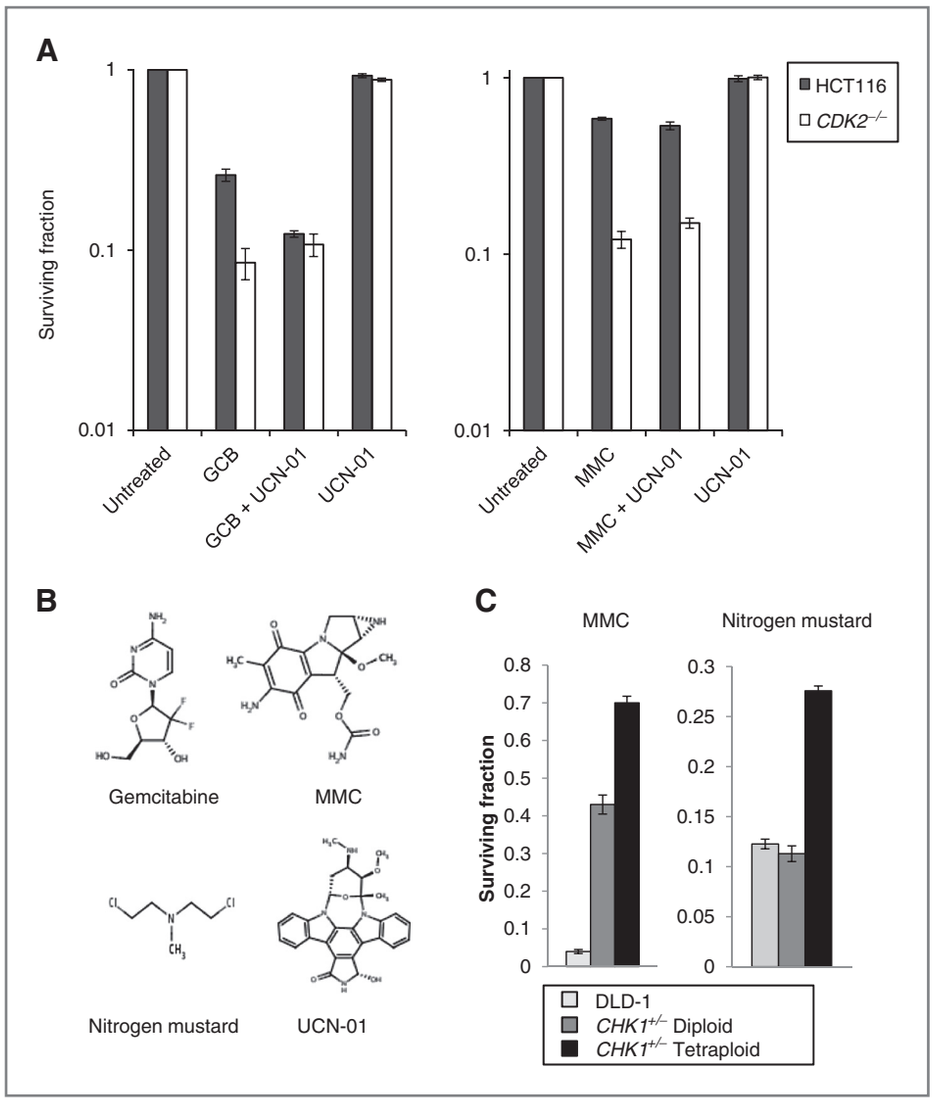

Figure 6. Effects of a Chk1 inhibitor and cell ploidy. A, wild-type HCT116 and *CDK2*$^{-/-}$ cells were treated for 24 hours with 100 nmol/L UCN-01 alone or in combination with 20 nmol/L gemcitabine (GCB; left) or 150 nmol/L MMC (right). B, chemical structures of gemcitabine, MMC, UCN-01, and nitrogen mustard. C, *CHK1*$^{+/-}$ cells that were primarily diploid were compared with wild-type DLD-1 and an isolated subclone of *CHK1*$^{+/-}$ tetraploid cells. All cells were treated with the alkylating agents MMC (1 μmol/L) or nitrogen mustard (1 μmol/L) for 24 hours. Clonogenic survival is expressed as proportional survival compared with untreated controls of the same genotype.

as temozolomide (43, 44). The strategy applied here would not be expected to identify drugs that preferentially induce MMR-dependent signaling pathways. As a result, it is possible that the agents identified by our screens may in fact be an underrepresentation of the types of anticancer drugs that might be potentiated by inhibiting the ATR pathway.

Pharmacologic inhibition of Cdk2 was originally conceived as a strategy for preventing cell proliferation. The discovery that *CDK2*-null cells are viable and proliferate normally has forced a rethinking of this idea (23). The requirement for Cdk2 for robust activation of ATR after DNA damage provides a new framework for considering how best to use Cdk2 inhibitors in the clinic. On the basis of our genetic model, highly specific Cdk2 inhibitors might be predicted to have minimal effects on DNA replication and cell growth when used alone but might be considered for use in combination with currently approved anticancer agents. It is important to note that some level of ATR activation persists in Cdk2-nullizygous cells (Fig. 5A) and that the requirement for Cdk2 upstream of ATR is therefore not absolute. Nonetheless, pharmacologic inhibition of Cdk2 might sufficiently downregulate ATR activation to an extent that improves efficacy. Our study suggests that this idea merits thorough testing in preclinical models.

Of the molecular targets evaluated in this study, perhaps the most intensively studied has been Chk1 (45). Chk1 is an essential protein, required for cellular viability even in the absence of exogenous DNA damage. The roles of Chk1 during normal cell division remain incompletely understood, but recent insights into Chk1 functions during DNA replication (46) and mitosis (47) and the requirement for Chk1 for chromosomal stability (48)—all underscore the need for caution when considering Chk1 inhibition as a therapeutic strategy. The forms of

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chromosomal instability caused by genetic manipulation of Chk1 have been shown to alter cellular sensitivity to therapeutic agents (49); our isogenic system offers further support for this effect (Fig. 6C). While the basis for the drug resistance of the tetraploid cells we studied remains obscure, it is tempting to speculate that the evolutionary bottleneck successfully bypassed by surviving tetraploid clones might confer broad survival advantages. It is also possible that additional copies of DNA repair genes in cells with increased ploidy could contribute to survival. Ongoing studies of the mechanisms of tetraploidization and the evolution of tetraploid clones will likely provide new insights into the basis of drug resistance. Whether the inhibition of Chk1 may contribute to the clonal evolution of multidrug-resistant tetraploid clones *in vivo* is a consideration for future clinical applications of Chk1 inhibitors.

ATR is known to be activated by a broad range of agents that directly or indirectly inhibit DNA replication. Our study of a large panel of approved anticancer agents shows just how broad this range can be and how seemingly disconnected pathways can affect DNA metabolism. The finding that dactinomycin and bortezomib induce a similar dependence on ATR pathways for cell growth after treatment despite having very dissimilar modes of action suggests that there may be many avenues by which ATR inhibitors might provide enhanced therapeutic effects. From a practical standpoint, our study highlights the differences between different methods to assess drug responses *in vitro*. Comparisons between studies that use different methodologies should be conducted with appropriate caution.

A number of ATR inhibitors have been recently developed and are now being tested preclinically (50). Our studies suggest that if these inhibitors prove to be sufficiently specific, they may facilitate many potential combinatorial strategies that may be broadly applicable to many types of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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