S Phase and G₂ Arrests Induced by Topoisomerase I Poisons Are Dependent on ATR Kinase Function*

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ATR, a human phosphatidylinositol 3-kinase-related kinase, is an important component of the cellular response to DNA damage. In the present study, we evaluated the role of ATR in modulating the response of cells to S phase-associated DNA double-stranded breaks induced by topoisomerase poisons. Prolonged exposure to low doses of the topoisomerase I poison topotecan (TPT) resulted in S phase slowing because of diminished DNA synthesis at late-firing replicons. In contrast, brief TPT exposure, as well as prolonged exposure to the topoisomerase II poison etoposide, resulted in subsequent G₂ arrest. These responses were associated with phosphorylation of the checkpoint kinase Chk1. The cell cycle responses and phosphorylation of Chk1 were markedly diminished by forced overexpression of a dominant negative, kinase-inactive allele of ATR. In contrast, deficiency of the related kinase ATM had no effect on these events. The loss of ATR-dependent checkpoint function sensitized GM847 human fibroblasts to the cytotoxic effects of the topoisomerase I poisons TPT and 7-ethyl-10-hydroxycamptothecin, as assessed by inhibition of colony formation, increased trypan blue uptake, and development of apoptotic morphological changes. Expression of kdATR also sensitized GM847 cells to the cytotoxic effects of prolonged low dose etoposide and doxorubicin, albeit to a smaller extent. Collectively, these results not only suggest that ATR is important in responding to the replication-associated DNA damage from topoisomerase poisons, but also support the view that ATM and ATR have unique roles in activating the downstream kinases that participate in cell cycle checkpoints.

ATR has been identified as one of the protein kinases that transduces signals to the cell cycle machinery during normal DNA replication (1) and after DNA damage (2–4). Like the structurally related kinases human ATM, Schizosaccharomyces pombe Rad3 (Rad3SP), and Saccharomyces cerevisiae Mec1 (Mec1SP), ATR contains a conserved C-terminal kinase domain that phosphorlotes downstream substrates (5). The nature of the DNA damage that activates ATR, the identity of its substrates, and the impact of ATR on cell cycle progression are currently the subject of extensive investigation.

Previous studies have suggested that ATR and ATM might have distinct but overlapping functions. In response to IR, ATR has been observed to phosphorylate and activate the checkpoint kinase Chk1 (4), which in turn phosphorylates Cdc25c, inactivating its phosphatase activity and contributing to the ensuing G₂ arrest (6–8). In contrast, ATM, which appears to play the more critical role in response to IR, phosphorylates Chk2 (1, 9, 10). Despite these differences, Chen et al. (11) observed that Chk1 overexpression can complement the G₂/M checkpoint defect in AT cells and restore IR resistance. These results suggest redundancy and overlap in the specific roles of ATM and ATR.

Several observations indicate that ATM and ATR are also important in the intra-S checkpoint, a series of biochemical reactions that inhibit DNA synthesis in the face of DNA damage or stalled replication forks (12–15). ATM has been shown recently to initiate signaling through Chk2 and Cdc25A to inhibit Cdk2 and prevent DNA synthesis after IR (1). The response of AT cells to other inhibitors of DNA replication, however, appears intact despite the absence of functional ATM (10). Moreover, the observation that several proteins known to be regulated by ATM can still be activated by IR in AT cells (10) indicates that at least one additional upstream regulatory protein can initiate the S phase checkpoint.

The possibility that ATR might play this role was initially suggested by the observation that ATR inhibition results in hypersensitivity to the replication inhibitors hydroxyurea and aphidicolin (2). Subsequent results indicated that ATR phosphorylates Chk1 in response to hydroxyurea (4). More recent experiments demonstrated that Xenopus ATR associates with chromatin in a replication-dependent manner, whereas ATM and DNA-PK do not (15). Interestingly, depletion of ATR in a cell-free Xenopus replication system blocked the Chk1 phosphorylation that ordinarily occurs after treatment with inhibitors of DNA replication (15). Collectively, these observations suggest that ATR is poised to respond to DNA damage occurring specifically during S phase.

In contrast to IR, which induces multiple types of DNA damage throughout the cell cycle (16), the topo I poison CPT and its derivatives produce a single type of DNA lesion that is largely replication-dependent (17–21). Early studies demonstrated that CPT is selectively toxic during S phase (22–25). Subsequent investigations demonstrated that this S phase selectivity reflects the formation of DNA ds breaks when advancing replication forks collide with drug-stabilized topo I-DNA complexes (26–29). Additional studies revealed that brief ex-
posure of exponentially growing cells to high CPT concentrations produces a subsequent G2 arrest (30) as a consequence of impaired activation of cdk2-cyclin B complexes (31). In contrast, prolonged treatment with lower, therapeutically achievable CPT concentrations causes S phase slowing (32–34). Although it has been observed that CPT-induced S phase arrest is abolished by 7-hydroxystaurosporine (33), an inhibitor of Chk1 and tac1 (35, 36), other events that lie upstream of the CPT-induced G2 and S phase arrests have remained unclear. The present study examines the role of ATR in the response to CPT derivatives. Because deletion of ATR is lethal at an early stage of development (37), studying the function of this kinase in mammalian cells has been difficult. Previous studies have demonstrated that ATR enzymatic activity and function can be abrogated by overexpression of a kinase-inactivated ATR allele (2). We have utilized cells expressing this kdATR allele in an inducible fashion to (i) evaluate the role of ATR in the G2 and S phase checkpoint activation after topo poisons, (ii) determine whether the abrogation of these checkpoints is related to altered phosphorylation of Chk1 and Chk2, and (iii) assess the effect altered ATR function on the cytotoxic affects of topo poisons. Results of these studies provide the first evidence that ATR plays a major role in checkpoint activation and cell survival with replication-associated DNA ds breaks.

MATERIALS AND METHODS

Reagents—TPT was obtained from the Pharmaceutical Resources Branch of the National Cancer Institute. SN-38 was provided by Pharmacia. Additional reagents were purchased from the following suppliers: etoposide from Biomol (Plymouth Meeting, PA), paclitaxel and doxorubicin from Sigma, nocodeone from Aldrich, and G418 from Invitrogen.

The following murine monoclonal antibodies were utilized: C-21 antitopo I (Y.-C. Cheng, Yale University Medical School, New Haven, CT), Ki-S1 anti-topo IIa (13, 14) (Dr. Udo Kellner, Kiel, Germany), anti-Chk1 (Santa Cruz Biotechnology), anti-Chk2 (Dr. Junjie Chen, Mayo Foundation, Rochester, MN), and anti-BrdUrd (Becton Dickinson). In addition, affinity-purified epitope-specific rabbit antisera that recognizes phospho-254-Ser-Chk1 (Cell Signaling Technology, Beverly, MA) and phospho-Thr68-Chk2 (Junjie Chen), as well as goat anti-Chk1 (Upstate Biotechnology, Inc.), were used. Affinity-purified peroxidase-coupled secondary antibodies were purchased from Kierkegaard and Perry (Gaithersburg, MD).

Tissue Culture—GM847 is an SV40-transformed fibroblast line. The GM847/kdATR line was previously constructed to contain a kinase-inactivated allele of ATR under the positive control of a doxycycline-responsive promoter (2). GM847/kdATR cells were cultured in Dulbecco’s minimal essential medium with 4.5 g/liter glucose, 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine (medium A) supplemented with 400 μg/ml leupeptin, 1 mM Na3VO4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, and 10 mM NaF) were reacted overnight with 15 μl of beads (packed volume) containing covalently bound Chk1 or Chk2 antibodies. After beads were washed twice with PBS, polypeptides were eluted in SDS sample buffer, separated on 12% acrylamide gels, and probed with antibodies as described in the individual figure legends.

RESULTS

Experiments described below were designed to assess the potential role of ATR in modulating the response to DNA damage from topo poisons. For these studies, we employed GM847/kdATR, a recently described cell line that contains CDNA encoding kdATR under the positive control of a doxycycline-responsive promoter (2, 40). In the presence of doxycycline, the kdATR expressed by these cells diminishes the ability of endogenous ATR to phosphorylate itself (2) and downstream substrates such as p53 and human Chk1 in response to DNA damage (3, 4).

ATR Is Necessary for the G2/M Arrest after Brief Exposure to TPT—To assess the effect of ATR on G2 arrest,2 cells grown in the absence or presence of doxycycline were exposed to 125 nM TPT for 2 h,3 washed, incubated in the continued absence or presence of doxycycline for 24 h, and harvested for flow cytometry. Cells grown in the absence of doxycycline underwent a TPT-induced G2 arrest, with the percentage of cells in G2/M increasing from 15% prior to TPT treatment to 42% 24 h later (Fig. 1A). In contrast, cells expressing the kdATR allele had a markedly diminished G2 arrest after TPT treatment, with the

2 Because GM847 cells lack p53 function, G2 arrest is not observed after DNA damage.
3 The concentration of 125 nM was chosen because it has minimal effect on cell survival after a 2-h exposure (Fig. 6C), yet alters cell cycle distribution.
G2/M population increasing from 15% prior to treatment to only 24% after TPT exposure (Fig. 1B). Analysis at later time points demonstrated that this TPT-induced G2 arrest was transient in the presence of the kdATR allele, with return to essentially a pretreatment cell cycle distribution by 48 h (Fig. 1B). In contrast, the G2 arrest was more persistent in the presence of normal ATR function (Fig. 1A, 48 h).

**Fig. 1.** ATR is required for G2 checkpoint function after brief exposure to TPT. A and B, cell cycle profiles of GM847/kdATR cells grown in the absence (A, wild-type ATR function) or presence (B, kdATR allele overexpressed) of doxycycline (Dox) were examined by flow cytometry. At t = 0, cells were harvested for analysis or exposed to 125 nM TPT for 2 h, incubated in fresh medium with or without doxycycline, and subsequently harvested for analysis at the indicated time points. Results are representative of three independent experiments.

**ATR Is Also Required for Normal S Phase Arrest during Prolonged TPT Treatment—Prolonged exposure to topo I poisons results in slowing of DNA replication (32–34), presumably because of activation of the S phase checkpoint by replication-induced conversion of the stabilized DNA-topo I complexes into frank DNA ds breaks (26–29). To evaluate the possibility that ATR might also function as part of this S phase checkpoint, we pretreated cells for 48 h with doxycycline (to inactivate ATR function) or diluent, then exposed them to 125 nM TPT for 8 h in the continued presence or absence of doxycycline. At this point cells were pulse-labeled with BrdUrd and returned to TPT-containing medium in the presence or absence of doxycycline for varying lengths of time. After staining with anti-BrdUrd and propidium iodide, the fate of the cells that synthesized DNA in the presence of TPT was then determined by multiparameter flow cytometry.

Control cells (no doxycycline or TPT) uniformly incorporated BrdUrd throughout S phase after a 30-min pulse (t = 0 indicates time after the BrdUrd pulse) (Fig. 2A, gated). By 8 h after completion of the pulse, the majority of the labeled cells had completed S phase in the absence of DNA damaging agents (Fig. 2A’, gated). These results provide a base line for comparison.

When cells were exposed to TPT for 8 h (no doxycycline), an increased S phase population was observed (19% of total cells in Fig. 2B versus 11% in Fig. 2A), reflecting the previously reported S phase slowing. In addition, consistent with S phase checkpoint activity, these cells incorporated decreased amounts of BrdUrd into DNA, particularly when they were in late S phase (Fig. 2B, arrowhead). Moreover, with continued TPT incubation, fewer labeled cells progressed to G2 relative to untreated cells. By 8 h after the completion of the BrdUrd pulse, almost all of the labeled cells were still in late S phase (17% of total cell population, Fig. 2B’, gated, versus 19% at start of incubation), again reflecting S phase arrest as a consequence of checkpoint activation.

Expression of the kdATR allele abrogated all of these features of the S phase checkpoint (Fig. 2, C and C’). In particular, cells grown in the presence of doxycycline appeared to incorporate label equally throughout S phase after TPT treatment (Fig. 2C, gated). Moreover, the majority of these cells had completed S phase 8 h after pulse labeling (Fig. 2C’, gated), indicating that they progressed through S phase at a rate
similar to that of untreated cells (Fig. 2A’, gated). These findings demonstrate that ATR is required for TPT-induced inhibition of DNA replication.

**TPT-induced Phosphorylation of Chk1 Is Dependent upon ATR Kinase Function, whereas Chk2 Phosphorylation Is Not—** The observation that the kdATR allele diminishes both G2 arrest (Fig. 1) and S phase slowing (Fig. 2) after TPT treatment suggested that ATR may be signaling for cell cycle arrest via Chk1 and/or Chk2, known upstream components of the G2 and S phase checkpoints. To assess these possibilities, GM847/kdATR cells grown in the absence or presence of doxycycline were exposed to 125 nM TPT for increasing lengths of time and then analyzed for the presence of phosphorylated Chk1 and Chk2. These experiments indicated that TPT treatment resulted in Chk1 phosphorylation (cf. Fig. 3A, lanes 3, 5, and 7). This phosphorylation was evident within 2 h (Fig. 3A, lane 3) and persisted for at least 24 h (Fig. 3A, lane 7). Expression of kdATR markedly diminished this Chk1 phosphorylation (Fig. 3A, lanes 4, 6, and 8). These results indicate that Chk1 phosphorylation correlates with the intra-S phase slowing (Fig. 2, B and B’), and abrogation of Chk1 phosphorylation correlates with loss of the S phase checkpoint (Fig. 2, C and C’).

In contrast, phosphorylation of Chk2 does not correlate with the effects of ATR. In particular, prolonged TPT treatment causes a detectable increase in Chk2 phosphorylation (cf. Fig. 3B, lanes 1 and 7) that occurs independent of ATR status (Fig. 3B, lane 8), suggesting that TPT-induced Chk2 phosphorylation occurs by an ATR-independent mechanism and is not responsible for the TPT-induced checkpoint activation.

**Chk1 Activation and Cell Cycle Responses Observed after Etoposide Treatment Are Dependent upon ATR Kinase Activity—** Topo I poisons are almost exclusively dependent upon DNA replication for the formation of secondary DNA strand breaks, whereas topo II poisons, such as etoposide depend somewhat more upon RNA transcription to generate these lesions (17, 18, 24, 41). Accordingly, topo II poisons are thought to generate DNA ds breaks throughout the cell cycle. In view of this dichotomy, we investigated whether functional inactivation of ATR affected the response of cells to the topo II poison etoposide.

As indicated in Fig. 4A, treatment of GM847/kdATR cells with 300 nM etoposide for 24 h resulted in a prominent G2 arrest (44% cells in G2 versus 16%, untreated). Induction of the kdATR allele for 24 h before addition of etoposide diminished the number of cells arrested in G2 (Fig. 4B, versus untreated), although the effect was smaller than that observed with TPT. In addition, there was a relative increase in the number of cells passing through M to G1 (42% G1 in its absence; Fig. 4, A and B). These results suggest that ATR also plays a role in the cell cycle response to etoposide-induced DNA damage. Consistent with this conclusion, etoposide-induced phosphorylation of Chk1 (Fig. 4C, lanes 3, 5, and 7), but not Chk2 (Fig. 4D, lanes 4 and 5), was abrogated by overexpression of the kdATR allele.

**TPT- and Etoposide-induced Phosphorylation of Chk1 Occurs in an ATM-independent Manner—** To rule out the possibility that the effects of kdATR described above result from inhibition of the related kinase ATM, we analyzed the effects of topo poisons on the ATM-deficient SV40-transformed human fibroblast line AT4BI. Prolonged exposure of these cells to 125 nM TPT resulted in marked S phase accumulation (49% with TPT versus 25% untreated, Fig. 5A). In addition, prolonged exposure to 300 nM etoposide resulted in G2 arrest (41% with etoposide versus 18% untreated, Fig. 5B). These results indicate that ATM is not required for the TPT-induced S phase arrest or the etoposide-induced G2 arrest observed in Figs. 2A and 4A, respectively.

Our earlier results suggested that these cell cycle effects correlated with ATR-dependent Chk1 phosphorylation. If so,
examined survival and the development of apoptotic morphological changes. After a 24-h exposure to 125 nM TPT, the percentage of apoptotic cells as assessed by nuclear morphology was increased nearly 5-fold (9.7% versus 2.0%) when cells expressed the kdATR allele (Fig. 6B). After an additional 24-h incubation in fresh medium, the number of cells undergoing apoptosis increased further, but more cells expressing the kdATR allele still underwent apoptosis. When trypan blue exclusion was utilized to quantitate short term survival, complementary results were obtained, with survival after 24 and 48 h being 98 and 48%, respectively, for cells grown in the absence of doxycycline and 88 and 27% for cells grown in the presence of doxycycline.

Further experiments (Fig. 6C) demonstrated that expression of the kdATR allele also increased the sensitivity of cells to a 2-h TPT exposure, although higher concentrations of TPT were required to inhibit colony formation. A similar degree of sensitization was observed in cells treated for 24 h with another topo I poison, SN-38, the active metabolite of irinotecan (Fig. 6D), confirming that the effects of kinase-inactivated ATR were not unique to TPT.

Previous studies have suggested that pretreatment levels of topo I can affect sensitivity to topo I poisons (42, 43). More recently, CPT has also been shown to induce proteasome-mediated topo I degradation in resistant cell lines but not sensitive cell lines, raising the possibility that CPT-induced proteasome-mediated degradation might play a role in resistance to topo I poisons (44). To evaluate the possibility that ATR affected either pretreatment topo I levels or drug-induced topo I degradation, GM847/kdATR cells were grown in absence or presence of doxycycline for 48 h and harvested before or after treatment with TPT. These studies demonstrated that expression of the kdATR allele had no effect on basal topo I polypeptide levels (Fig. 6E). Moreover, expression of the kdATR allele did not affect the degradation of topo I in response to TPT. Whether the kdATR allele was expressed or not, topo I levels in these transformed fibroblasts remained constant during the course of topo treatment (Fig. 6F). Likewise, expression of the kdATR allele did not affect basal levels of topo IIα (Fig. 6E).

During the course of TPT or etoposide treatment, however, topo IIα levels increased (Fig. 6F), possibly reflecting the higher expression of topo II in S and G2 phase cells (45, 46). Once again, expression of the kdATR allele did not affect this change.

In a final series of experiments, the effect of the kdATR allele on the response to topo II poisons was examined. Previous reports have suggested that a brief exposure to high concentrations of etoposide kills cells in a manner that does not require ongoing DNA synthesis (41, 47). Consistent with this conclusion, expression of kdATR had no effect on the antiproliferative effects observed after exposure to high concentrations of etoposide for 2 h (data not shown). A different picture emerged after more prolonged etoposide treatment. Although the effects were somewhat smaller than those observed with TPT, presumably because only part of the toxic DNA strand breaks are being generated during S phase (24), expression of kdATR enhanced the antiproliferative effects during a 24-h exposure to lower etoposide concentrations (Fig. 7A). Similar effects were observed when etoposide was replaced doxorubicin (Fig. 7B), another agent that stabilizes topo II-DNA cleavage complexes (48). In contrast, the kdATR allele had little effect on the cytotoxicity of the microtubule stabilizing agent paclitaxel (Fig. 7C).

**DISCUSSION**

Results of the present study have demonstrated that ATR kinase function is necessary for both the G2 and S phase arrests induced by topo I poisons. In particular, forced overexpression

![Fig. 5. TPT-induced checkpoint activation and Chk1 phosphorylation are independent of ATM function.](image)

A and B, cell cycle profiles of the AT-deficient AT4BI fibroblast cell line were examined by flow cytometry. At t = 0, cells were harvested for analysis or exposed to 125 nM TPT (A) or 300 nM etoposide (B) and subsequently harvested at 24 h. C, Chk1 phosphorylation in response to topo poisons in AT-deficient fibroblasts. AT4BI cells were treated with diluent (0 h) or the indicated concentration of TPT or etoposide for 8 h. After completion of incubations, samples were immunoprecipitated with anti-Chk1 antibody covalently linked to protein A-Sepharose. Immunoprecipitates were probed with anti-Chk1 or epitope-specific anti-phospho-Ser345-Chk1 antisera.

then the effects of these drugs on Chk1 phosphorylation would be predicted to occur normally in ATM-deficient cells. Consistent with this prediction, we observed that Chk1 phosphorylation at Ser345 was readily detectable when AT4BI cells were treated with TPT or etoposide (Fig. 5C, lanes 2 and 3). These results provide further support for the view that ATR is responsible for the activating phosphorylations of Chk1 after treatment with these drugs.

The kdATR Allele Enhances the Antiproliferative Effects of Topo I and II Poisons—To determine whether abrogation of the ATR-induced signaling events altered the sensitivity of cells to the antiproliferative effects of topoisomerase poisons, GM847/kdATR cells grown in the absence or presence of doxycycline for 48 h were exposed to increasing concentrations of topo I or topo II poisons. As illustrated in Fig. 6A (open circles), a 24-h exposure to increasing concentrations of TPT in the absence of doxycycline decreased subsequent colony formation of GM847/kdATR cells with an IC50 of 75 nM and an IC90 of >200 nM. Treatment with doxycycline (Fig. 6A, closed circles) resulted in a 2–3-fold decrease in the IC50 (30 nM) and IC90 (100 nM), suggesting that ATR normally modulates the antiproliferative effects of TPT. Control experiments revealed that doxycycline had no effect on TPT sensitivity in parental GM847 cells (data not shown), indicating that the sensitization observed in GM847/kdATR cells was caused by overexpression of the kdATR allele.

To determine whether the effects of the clonogenic assays reflected altered survival rather than prolonged arrest, we
of the kdATR allele diminishes both the G₂ arrest observed after a brief TPT exposure (Fig. 1) and the S phase arrest observed after prolonged treatment (Fig. 2). Abrogation of these TPT-induced checkpoints is accompanied by decreased Chk1 phosphorylation (Fig. 3A) but not by any change in Chk2 phosphorylation (Fig. 3B). Moreover, inhibition of ATR function, Chk1 phosphorylation and the ensuing arrests is associated with increased sensitivity to brief (Fig. 6C) or prolonged (Fig. 6, A, B, and D) exposures to the topo I poisons TPT and SN-38. Additional experiments have demonstrated that inhibition of ATR function also abrogates the Chk1 activation and cell cycle response observed after prolonged treatment with
tolerance to damage (52). Interestingly, yeast lacking Mec1Sc
function, thereby allowing adequate time for repair or for more
Mec1Sc-dependent checkpoint involves slowing of DNA replica-
tors of Chk1 phosphorylation have been described recently (6–8).
These agents stabilize covalent intermediates between topo I
and its DNA substrate, providing complexes that can be con-
verted into frank DNA ds breaks through interactions with
advancing replication forks (17, 20, 42, 49). In contrast to
ionizing radiation, which produces a variety of DNA lesions,
topo I poisons are currently thought to produce only a single
type of cytotoxic DNA damage (17, 18). As a result, these agents
have been recognized as unique tools for dissecting the bio-
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chemistry of checkpoint activation (31, 32).

The present results indicate that ATR plays a role in both of
the cell cycle responses observed after exposure to topo I poions.
Brief exposure results in a subsequent G2 arrest, which is
markedly attenuated by expression of the kdATR allele (Fig.
1B). The attenuation of this TPT-induced G2 arrest by kdATR
is similar to the effect observed after ionizing radiation (9).
The previously reported phosphorylation of Chk1 by ATR (12)
provides a potential mechanism by which ATR may activate the G2
checkpoint after DNA damage. Consistent with this hypothe-
sis, we observed that TPT treatment for as little as 2 h resulted
in Chk1 phosphorylation that was inhibited by expression of the
kdATR allele (Fig. 3A).

Prolonged exposure to topo I poisons leads to S phase slowing
(32, 33). The observation that ATR is associated with DNA
during replication (15) raised the possibility that ATR might
also be involved in this response to topo I poisons. As reviewed
by Naegeli (50), replication of damaged DNA is a primary
mechanism of genetic instability because the rate of DNA syn-
thesis determines the frequency of conversion to permanent
changes in the DNA. The S phase checkpoint presumably func-
tions to prevent the consequences of replication in the face of
significant DNA damage (51). In budding yeast, the ATR ho-
omologue Mec1Sc is required for the S phase slowing seen after
several forms of DNA damage; and interruption of Mec1Sc is
associated with decreased survival after this damage (51). This
Mec1Sc-dependent checkpoint involves slowing of DNA replica-
tion, thereby allowing adequate time for repair or for more
tolerance to damage (52).

Interestingly, yeast lacking Mec1Sc are able to progress through S phase despite significant DNA
damage (52). This replication in the face of DNA damage cor-
relates with late origin firing, suggesting that the unregulated
DNA synthesis in Mec1Sc-deficient cells might result from a
failure to prevent initiation at late origins (52). The parallels of
the Mec1Sc-deficient yeast to cells expressing the kdATR allele
are striking. In GM847 cells expressing wild-type ATR, the
TPT-induced S phase slowing reflects two related changes:
decreased synthesis of DNA, particularly during late S phase
(arrowhead, Fig. 2B), and a decreased rate of S phase transit
(Fig. 2B’). Both of these changes are abrogated by overexpression
of the dominant negative kdATR protein (Fig. 2, C and C’).
These results suggest that ATR, like Mec1Sc, slows S phase
transit in response to DNA damage, possibly by inhibiting late
firing DNA replicons.

This conclusion is consistent with other emerging informa-
tion on the biological functions of ATR. Tibbetts et al. (53)
recently demonstrated that ATR phosphorylates the tumor
suppressor protein BRCA1 and colocalizes with this substrate
at sites of stalled replication forks after treatment with aphidi-
colin or hydroxyurea. Coupled with the present results, these
observations suggest that ATR is a key member of an intra-S
phase checkpoint pathway.

Results of the present study also demonstrated quantitative
and qualitative differences between the effects of the kdATR
allele on sensitivities to topo I and topo II poisons. The kdATR
allele affected sensitivity to topo I poisons more than topo II
poisons (Fig. 6 (A and D) versus Fig. 7 (A and D)). Moreover, the
kdATR allele sensitized cells to topo I poisons during exposures
as brief as 2 h (Fig. 6C), whereas it had little effect on sensitiv-
ity to topo II poisons when they were applied for these brief
time periods. These differences might be related to the fact that
drug-stabilized topo I-DNA covalent complexes are particularly
efficiently converted into cytotoxic lesions by advancing repli-
cation forks (24, 27, 47), whereas drug-stabilized topo II cleav-
age complexes can also be converted into cytotoxic lesions in a
transcription-dependent manner, especially when cells are
affected with high concentrations of these agents for brief periods
(24, 41). The inability of kdATR to affect the cytotoxicity
after brief exposure to these higher levels of topo II poions
raises the possibility that the resulting lesions are different from
the DNA ds breaks that result from brief exposure to topo I poions.

It is unclear at present how ATR and related kinases are
activated by DNA damage. Current understanding suggests
that DNA damage sensors, possibly Rad proteins, are recruited
to sites of stalled replication forks and serve as scaffolds for the
assembly and/or activation of these upstream signaling kinases
(54, 55). Studies in yeast also suggest that Rad proteins, par-
icularly Rad1, Rad9, Rad17, and Hus1, might be upstream of
ATR and ATM homologs in DNA repair pathways (6). Consist-
ent with this model, recent results have demonstrated that
CPT and etoposide alter the association between the Rad9-
Rad1-Hus1 complex and chromatin in K562 human leukemia
cells (56). Further studies are required to assess whether these
putative DNA damage sensors are upstream of ATR and to
determine how topo I poions and other types of DNA damage
activate this pathway.

The present demonstration that ATR plays an important role
in the cell cycle responses and survival after treatment of cells
with topo I poions does not rule out the possibility that other
related kinases are also activated by these drugs. Recent ex-
periments suggest that DNA-PK is activated after treatment
with topo I poions and, in turn, phosphorylates replication
protein A (57). ATM also appears to play a role in the response
to topo I poions. A variety of studies (reviewed in Refs. 18 and
58) have indicated that fibroblasts from patients with AT are

![Fig. 8. Proposed pathway for S and G2 arrests after treatment with topoisoenerase poisons.](image-url)
particularly sensitive to the antiproliferative effects of topo I-directed agents. More recently, Chk2 phosphorylation has been shown to occur in an ATM-dependent manner after exposure of mammalian cells to TPT (10).

Despite evidence that DNA-PK and ATM are activated in response to topo I poisons, the respective roles of these kinases in topo I poison-induced S phase checkpoint function has not been clear. On the one hand, the results of Chaturvedi et al. (10) have implicated ATM and Chk2 in the response to topo I poisons. On the other hand, Shao et al. reported that the S phase slowing induced by SN-38 can be abrogated by treatment with 7-hydroxystaurosporine (33). Because this agent does not inhibit Chk1 (35, 36), the observation of Shao et al. implicates kinases upstream of Chk1, which include ATR (4), in activation of the S phase checkpoint by topo I poisons. Our results (Figs. 2 and 3) likewise implicate ATR and Chk1 in the S phase checkpoint but do not rule out the possibility that other related kinases might also play a role.

In summary, the present results indicate that ATR plays an important role in the regulation of DNA replication after replication-associated DNA damage and, as such, may be a key component of a mammalian S phase DNA damage response pathway. As a result, cell survival after treatment with topo-isomerase poisons, particularly topo I poisons, is enhanced by normal ATR function. These observations raise the possibility that alterations in ATR-dependent pathways might affect sensitivity to topo I poisons in the clinical setting. In addition, these observations suggest that ATR might be a good candidate for future development of biochemical modulating agents designed to enhance sensitivity of tumor cells to chemotherapeutic agents that target topo-isomerases.

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