Cdk Inhibition in Human Cells Compromises Chk1 Function and Activates a DNA Damage Response

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Abstract

Cyclin-dependent kinases (Cdk) promote cell proliferation, are often deregulated in human cancers, and are targets of ongoing cancer chemotherapy trials. We show here that Cdk activity is also required in human cells to maintain function of the Chk1 pathway, a key component of the response to DNA damage or stalled replication. Chk1 expression was markedly reduced in primary fibroblasts and U2OS osteogenic sarcoma cells by treatment with small molecule Cdk inhibitors or induction of a dominant-negative mutant of Cdk2. The findings of decreased Chk1 activity and accumulation of Cdc25A, a protein targeted for degradation by Chk1, confirmed that Chk1 function was impaired. Furthermore, Cdk inhibition triggered a DNA damage response, characterized by the accumulation of activated forms of ATM and Chk2 as well as nuclear foci containing phosphorylated substrates of ATM/ATR, including histone H2AX (γH2AX). Time course experiments showed that the bulk of ATM activation followed Chk1 down-regulation. Chk1 RNA interference combined with partial inhibition of DNA replication was sufficient to evoke the DNA damage response. Conversely, ectopic expression of Chk1 blunted induction of γH2AX foci by Cdk inhibitors, indicating that Chk1 down-regulation was necessary to elicit the full phenotype. Finally, both Cdk and Chk1 inhibitors enhanced the cytotoxicity of etoposide, a DNA-damaging agent. These results define a pathway through which Cdk inhibition can augment the antiproliferative effects of Cdk inhibitors, one that might be exploited in the design of cancer chemotherapy.

Introduction

Eukaryotic cells have developed complex mechanisms to ensure the precise duplication of the genome and its equal segregation to progeny. Tight regulation of the cell cycle is enforced by cyclin-dependent kinases (Cdk), in coordination with extracellular signals and checkpoint pathways (1). The latter recognize threats to genomic stability and prevent the propagation of damaged DNA (2). Two members of the phosphatidylinositol 3-kinase family, ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR), initiate the response to genotoxic insults. ATM activates the Chk1 and Chk2 kinases when DNA double-strand breaks manifest (3, 4), whereas ATR detects additional blocks to DNA replication and primarily activates Chk1 (5, 6). Chk1 and Chk2, in turn, antagonize the function of Cdc25 phosphatases, thereby constraining Cdk activity and conferring cell cycle delays (3, 7–9). During this window, checkpoint pathways may contribute to DNA repair or the induction of apoptosis if damage is extensive (2, 10).

Genomic instability, at moderate levels, is a hallmark of carcinogenesis and may result, in part, from defects in checkpoint pathways. The ATR/Chk1 pathway seems to play a critical role in genome maintenance (11–13), offering a partial explanation for Chk1 mutations and Cdc25A overexpression in some cancers (14). In virtually all human malignancies, a component of the pRb pathway is affected (15). One consequence is deregulated Cdk2-cyclin E activity, itself a potential mediator of chromosome instability (16). Whereas genomic instability is thought to contribute to tumor progression (17), most cancer chemotherapies rely on induction of sufficient DNA damage to overwhelm the repair processes in cancer cells and kill them. The enhanced proliferation and checkpoint defects of tumor cells may contribute to a therapeutic window that allows their preferential killing. Consequently, delineation of molecular determinants of checkpoint function could lead to improvements in the potency and specificity of current treatments (10).

Cdns have been targeted for drug development, primarily with the aim of blunting cell proliferation (18). This strategy has been called into question by recent targeted gene deletions in mice indicating that specific Cdns may be dispensable for normal cell proliferation (19–21). Nevertheless, these studies suggest that Cdns may perform redundant functions, which are required for cell cycle progression, and the frequent deregulation of Cdns and their regulators in tumors (22) points to key roles for these enzymes in malignant cell proliferation. Although Cdns are recognized as downstream targets of checkpoint kinases, little is known about the influence of Cdk activity on checkpoint function.

To investigate the consequences of Cdk inhibition for checkpoint pathways in human cells, we used two inhibitors of Cdk1/2, olomoucine and roscovitine (23), as well as a dominant-negative mutant of Cdk2 (Cdk2 DN). We show that Cdk inhibition results in disparate effects on the Chk1 and Chk2 pathways. Chk1 expression is reduced, and this effect contributes to a broad DNA damage response marked by activation of ATM and Chk2. These results define a novel mechanism that may augment the antiproliferative effects of Cdk inhibitors, one that might be exploited in the design of cancer chemotherapeutics.

Materials and Methods

Cell Culture and Drug Treatments. Human diploid fibroblasts (HDF) from lung (a kind gift of J. Sedivy, Brown University, Providence, RI; ref 24), U2OS, HeLa, and SaOS2 cells were cultured in DMEM supplemented...
Cdk inhibition mediates Chk1 down-regulation. A, HDFs and U2OS cells treated with olomoucine (Olo) or roscovitine (Ros) displayed decreased levels of Chk1, assayed by immunoblotting. Inhibition of Cdk activity was confirmed by decreased levels of hyperphosphorylated retinoblastoma protein (ppRb). Flow cytometry (FACS) profiles (bottom) showed an accumulation of cells in G2-M. B, HDFs matched for cell cycle stage showed a reduction in Chk1 following Cdk inhibitor treatment. Serum-starved HDFs were induced to reenter the cell cycle by serum stimulation and treated with vehicle (–) or olomoucine (Olo) at 16 hours of serum stimulation. Cells were harvested at the indicated time points chosen to maintain cell cycle synchrony. C, Expression of Cdk2 DN (+) in U2OS cells decreased Chk1 levels compared with uninduced cells (–), both in asynchronous cultures (Async.) and cells synchronized by hydroxyurea treatment (G1-S) and release (Mid S), whereas Cdk2 WT did not. Immunoblotting for the HA tag on exogenous Cdk2 showed comparable induction of DN and WT proteins. D, Olomoucine (Olo) treatment of HDFs reduced Chk1 mRNA by more than 75%. Quantitative real-time PCR of reverse transcription products was normalized to the 0 hour time point. Means of three independent experiments (each done in triplicate); bars, SD.

with 10% FCS. Cells were treated 24 hours after seeding with vehicle (DMSO), 180 μmol/L olomoucine (LC Laboratories, Woburn, MA), 20 μmol/L roscovitine (LC Laboratories), and 30 μmol/L (HDF) or 50 μmol/L (U2OS) etoposide (Sigma, St. Louis, MO) for 24 hours; 500 mmol/L CEP-3891 (Cephalon, Inc., West Chester, PA) and 300 mmol/L UCN-01 (a gift from R.J. Schultz, National Cancer Institute, Bethesda, MD) for 8 hours, unless otherwise indicated. Cdk2 DN and wild-type (WT) clones were induced as described previously (8). HDFs were synchronized by serum starvation (0.2% FCS for 72 hours) and stimulated with 10% FCS. U2OS cells were synchronized with 1 mmol/L hydroxyurea (Sigma) for 24 hours, washed with PBS, and released into fresh medium.

Flow Cytometry, Preparation of Cell Extracts, and Immunoblotting. These techniques were done as described previously (8). Whole cell extracts were analyzed by immunoblotting using antibodies to pRb (XZ77; ref. 25); Chk1 (G-4); Cdk25A (F-6; Santa Cruz Biotechnology, Santa Cruz, CA); actin (clone AC-40; Sigma); HA-tag (12CA5); phospho-Chk1 (XZ77; ref. 25); Chk1 (G-4), Cdc25A (F-6; Santa Cruz Biotechnology, Santa Cruz, CA); phospho-Chk2 (Thr68, lot 2; Cell Signaling Technology, Beverly, MA); phospho-H2AX (Ser139, Upstate) and ATM/ATR phosphor-ylated substrates (Cell Signaling Technology). Image acquisition was done on a Nikon E600 microscope with a CoolSNAPcf high-resolution monochrome charge-coupled device camera (Photometrics, Tucson, AZ) on a Nikon E600 microscope with a CoolSNAPcf high-resolution monochrome charge-coupled device camera (Photometrics, Tucson, AZ) using IPLab software (Scanalytics, Fairfax, VA). In each of two to three independent experiments, ≥100 cells per treatment were scored by a blinded observer. The Cochran-Mantel-Haenszel model (a χ² test) was used for statistical analysis.

Reverse Transcription and Real-Time PCR. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was prepared from 1 μg of total RNA with the SuperScript First-Strand Synthesis System for reverse transcription–PCR (Invitrogen); 0.75% to 1.5% of the reverse transcription reaction was amplified and analyzed by real-time PCR on the ABI Prism 7000 sequence detection system and software v1.0 (Applied Biosystems, Foster City, CA). Dilutions of reverse transcription product from untreated cells were amplified to create a standard curve. Glyceraldehyde-3-phosphate dehydrogenase real-time PCR was done in parallel to normalize for input. Primer Express Software v2.0 (Applied Biosystems) was used to select the following primers spanning a Chk1 exon junction: forward, 5′-TTGAAATCCTAGCAAGAATTACC-3′; reverse, 5′-ATCCACTGGGA-GACTCTGACA-3′.

Immunofluorescence. Cells were fixed with 1% paraformaldehyde and immunofluorescence was done as described (26) using mouse and rabbit antibodies specific for γH2AX (Ser139, Upstate) and ATM/ATR phosphorylated substrates (Cell Signaling Technology). Image acquisition was done on a Nikon E600 microscope with a CoolSNAPcf high-resolution monochrome charge-coupled device camera (Photometrics, Tucson, AZ) using IPLab software (Scanalytics, Fairfax, VA). In each of two to three independent experiments, ≥100 cells per treatment were scored by a blinded observer. The Cochran-Mantel-Haenszel model (a χ² test) was used for statistical analysis.

Plasmid and Small Interfering RNA Transfection. U2OS cells were transfected with 450 ng/6-cm dish Myc-tagged wild-type Chk1 (WT) and Chk1 D130A mutant (KD) plasmids (kindly provided by H. Piwnica-Worms, Washington University, St. Louis, MO; ref. 6) using Lipofectin Reagent (Invitrogen), and transfection media was replaced with DMEM after 6 hours. At 24 hours, cells were incubated with vehicle (DMSO) or roscovitine for an additional 24 hours. A Chk1 small interfering RNA (siRNA) duplex (Dharmacon, Lafayette, CO; ref. 7) or a 2-bp mismatch of
an unrelated sequence was transfected with Oligofectamine reagent (Invitrogen) into U2OS cells at 20 μmol/L. The transfection media was supplemented with DMEM containing 30% FCS after 4 hours, replaced with fresh DMEM with or without 0.1 μmol/L aphidicolin (Sigma) at 24 hours, and incubated another 16 hours.

**Viability Assays.** Cells were seeded in 96-well plates at 10^4 cells per well, and 24 hours later, vehicle (DMSO), roscovitine, olomoucine, CEP-3891, UCN-01, or 50 μmol/L (SaOS2: 25 μmol/L) etoposide was added to triplicate wells. After 24 hours, cells were washed once with PBS, and fresh DMEM was added, with or without a second drug treatment, from which cells were released after an additional 24 hours. At the start of treatment (day 0) and 72 hours later, 10 μL WST-1 reagent (Roche Applied Science, Indianapolis, IN) was added to 100 μL media. The absorbance was read at 450 nm with the EL-312 microplate reader (Bio-Tek Instruments, Winooski, VT) after 1 to 2 hours incubation. Analysis of covariance models or the Student’s t test was used for statistical analysis.

**Results**

**Cdk Inhibition Compromises Chk1 Expression and Function.** Several lines of evidence suggest that inhibition of Cdk2 complexes might repress Chk1 expression. The p53 tumor sup-

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**Figure 2.** Cdk inhibition impairs Chk1 activity. A. Roscovitine (Ros) and olomoucine (Olo) reduced levels of active Chk1 in U2OS cells and HDFs, respectively, as did Cdk2 DN induction in U2OS cells. Levels of Chk1 phosphorylated on Ser345 (pChk1) were monitored by immunoblotting in the absence and presence of hydroxyurea (HU; bottom left load, Coomassie blue stain). Roscovitine decreased Chk1 activity (Act.) in U2OS cells treated with HU. Whole cell lysates were immunoprecipitated with normal sheep immunoglobulin G (IgG) or a Chk1 antibody, and activity was determined by kinase assay, using a synthetic peptide derived from Cdc25C as substrate (bottom right). B. Cdc25A protein levels accumulated with Cdk2 DN induction in U2OS cells. Whole cell lysates were prepared from asynchronous cultures (Async.) and from cells synchronized by HU and released into mid S phase (HU 6h rel.) and immunoblotted for Cdc25A.

pressor decreases Chk1 transcription through induction of the Cdk (and proliferating cell nuclear antigen) inhibitor, p21, and dephosphorylation of pRb (27, 28). Consistent with regulation by the Rb-E2F pathway, Chk1 expression is limited to cycling cells (29), its promoter is bound by E2F4 in quiescent cells, and its expression is deregulated in cells made deficient for the Rb family members, p107 and p130 (30). We found that Chk1 was indeed down-regulated by Cdk inhibitor treatment in both primary human diploid fibroblasts (HDFs) and a human osteosarcoma cell line (U2OS; Fig. 1A). This effect was independent of cell cycle position (Fig. 1B and C) and occurred at the transcriptional level (Fig. 1D). Expression of Cdk2 DN in U2OS cells (8) similarly decreased Chk1 protein levels in both asynchronous cells and cells matched for cell cycle stage (Fig. 1C). In contrast, induction of wild-type Cdk2 to comparable levels was without effect (Fig. 1C).

To assess the functional consequences of Chk1 down-regulation, we determined the impact of Cdk inhibition on Chk1 activity. Effects on Chk1 function were most readily asayed under conditions of checkpoint activation, following treatment with the ribonucleotide reductase inhibitor hydroxyurea. Cdk inhibitors and Cdk2 DN induction reduced levels of an active, Ser345-phosphorylated form of Chk1 (6) and Chk1 kinase activity (Fig. 2A). Cdc25A, which is targeted for degradation by active Chk1 (4), accumulated in cells expressing Cdk2 DN (Fig. 2B). Together, these results indicate that Chk1 function is compromised by inhibition of Cdk activity.

**Cdk Inhibitors Elicit a Broad DNA Damage Response.** In yeast and avian cells, mutations in functional homologues of Chk1 enhance activation of Chk2 homologues in the setting of replication blocks (31, 32). We reasoned that Cdk inhibition would lead to DNA damage and subsequent activation of the ATM/Chk2 pathway through compromised replication and Chk1 down-regulation. In preliminary studies, we identified olomoucine as the stronger inhibitor of pRb phosphorylation in HDFs and roscovitine as the more potent inhibitor in U2OS cells (data not shown). Immunofluorescence staining revealed that these Cdk inhibitors induced nuclear foci containing the phosphorylated form of histone H2AX (γH2AX; Fig. 3A and B), an early event in the ATM-dependent response to double-strand breaks (33–35). Consistent with the notion that Cdk inhibition may lead to activation of ATM, an antibody recognizing phosphorylated epitopes on ATM and ATR substrates (pATM/ATR) detected increased nuclear foci upon Cdk inhibitor treatment of U2OS cells and HDFs (Fig. 3A). Cdc25A, which is targeted for degradation by active Chk1 (4), accumulated in cells expressing Cdk2 DN (Fig. 2B). Together, these results indicate that Chk1 function is compromised by inhibition of Cdk activity.
Chk1 Down-regulation Augments DNA Damage. To assess the involvement of Chk1 down-regulation in the DNA damage phenotype mediated by Cdk inhibition, we dissected the time course of these two events. Chk1 protein levels were substantially reduced by 8 hours of Cdk inhibitor treatment in HDFs and by 12 hours in U2OS cells (Fig. 4A). Although there was a mild, transient increase in pATM at 4 hours of olomoucine treatment, a strong, sustained activation of ATM followed Chk1 down-regulation in both cell types. This correlation suggested that decreased Chk1 levels might contribute to activation of an ATM-dependent checkpoint pathway.

Using several methods, we tested whether Chk1 inhibition was sufficient to produce a DNA damage response. Two Chk1 kinase inhibitors, CEP-3891 (4) and UCN-01 (39, 40), increased pATM in a dose-dependent manner, but γH2AX and pATM/ATR foci were not detected (Fig. 4B, data not shown). Transfection of a Chk1-specific siRNA oligonucleotide (7) into U2OS cells strongly reduced Chk1 protein levels, mimicking the effect of Cdk inhibitors (Fig. 4A and C). pATM was modestly and variably increased (Fig. 4C), but the proportion of cells with foci containing γH2AX or pATM/ATR substrates was not (Fig. 4D and E). We then supplemented Chk1 RNA interference with doses of aphidicolin sufficient to slow DNA replication, another consequence of Cdk inhibition (8). This combination markedly increased ATM activation (Fig. 4C) as well as the percentage of cells manifesting γH2AX and pATM/ATR foci (Fig. 4D and E). Thus, the combination of diminished Chk1 function and impaired DNA synthesis, both of which follow sustained Cdk inhibition, is sufficient to evoke a broad DNA damage response.

Chk1 Suppresses the DNA Damage Response. To determine whether Chk1 down-regulation is necessary for the DNA damage phenotype observed with Cdk inhibition, we transfected U2OS cells with either wild-type Chk1 (WT) or a kinase-defective (KD) mutant of Chk1 (6). Reconstitution of Chk1 expression could be achieved without a distinct change in cell cycle profile (Fig. 5A). The transfection procedure itself resulted in more variability in γH2AX focus formation, but roscovitine still conferred a strong effect in mock-transfected cells (Fig. 5B and C; P < 0.01). Chk1 WT reduced the percentage of roscovitine-treated cells with γH2AX foci to near-baseline levels (Fig. 5B and C). In contrast, cells transfected with Chk1 KD retained γH2AX foci (Fig. 5B and C), indicating that loss of Chk1 activity contributes to the roscovitine-dependent DNA damage response.

Cdk Inhibitors Increase Cell Killing. Given the effects of Cdk inhibitors on checkpoint pathways, we asked whether these drugs would kill cells either alone or in combination with etoposide. Our laboratory previously observed an increasing amount of cell death after several days of Cdk2 DN induction in U2OS cells (ref. 8; data not shown). Consistent with preclinical data showing antiproliferative and antitumoral effects (41, 42), roscovitine and olomoucine treatment produced a net loss of viable SaOS2 osteosarcoma cells, and olomoucine blunted proliferation of normal HDFs, U2OS cells, and HeLa cervical carcinoma cells (Fig. 6A). Notably, Cdk inhibitors significantly improved the efficacy of an established DNA-damaging agent. The strongest effects occurred when etoposide treatment preceded Cdk inhibition, possibly because prior cell cycle arrest was partially protective. Roscovitine yielded a net cell loss in only the three cancer cell lines. Olomoucine increased net cell killing in all four cell types, although the effect was most dramatic in HeLa and SaOS2 cells (Fig. 6A). UCN-01, a Chk1 kinase inhibitor with some activity toward Cdkks (18), augmented cell loss following etoposide treatment in all cell types tested (Fig. 6B). The more

Figure 3. Cdk inhibitors evoke markers of DNA damage. A, Immunofluorescence analysis revealed accumulation of γH2AX in nuclear foci (red) in HDFs treated with olomoucine or U2OS cells treated with roscovitine for 24 hours [blue, 4',6-diamidino-2-phenylindole (DAPI)]. B, An antibody to phosphorylated epitopes on ATM and ATR substrates (pATM/ATR) recognized nuclear foci (red) in U2OS cells treated with roscovitine. C and D, Quantification showed increased cells exhibiting ≥5 foci. Columns, means of two to three independent experiments; bars, SD. In each experiment, ≥100 cells per treatment were scored by a blinded observer. E, Olomoucine (Olo) or roscovitine (Ros) treatment of HDFs and U2OS cells, respectively, increased levels of ATM phosphorylated at Ser1981 (pATM) compared with vehicle (−). Cells treated with vehicle or Cdk inhibitor, either alone or in combination with etoposide (Etop), were immunoblotted for pATM and Chk2 phosphorylated at Thr34 (pChk2). A long exposure of the pChk2 blot is shown for the first two HDF lanes to show the Olo-dependent increase in pChk2 observed in the absence of Etop. Loading controls are actin (pATM blots) and Chk2 (pChk2 blots).
selective Chk1 inhibitor, CEP-3891 (4), potentiated the etoposide-dependent decrease in cell viability specifically in the three cancer cell lines (Fig. 6B). These findings suggest that Cdk and Chk1 inhibitors enhance the cytotoxicity of DNA-damaging chemotherapies.

**Discussion**

Checkpoint pathways provide a surveillance mechanism to ensure genomic stability. One important checkpoint outcome, cell cycle blockade, is achieved by inhibition of Cdk activity. Evidence for Cdk-dependent expression of Chk1 (refs. 27, 28, this study) points to a reciprocal relationship. Chk1 down-regulation upon Cdk inhibition may constitute a negative feedback loop that allows cell cycle recovery following physiologic conditions of checkpoint activation (4, 7). Consistent with this model, down-regulation of p21, an inhibitor of Cdk2 complexes and the replicative clamp proliferating cell nuclear antigen, is needed to efficiently resume DNA synthesis in human cells released from a hydroxyurea block (43). After a prolonged replication block by aphidicolin, *Xenopus* egg extracts undergo adaptation and enter mitosis with regions of unreplicated DNA. Under these circumstances, termination of cell cycle arrest requires the Polo-like kinase-mediated inhibition of Claspin (44), an adaptor protein necessary for Chk1 activation in both *Xenopus* and humans (45, 46). Although an analogous mechanism has yet to be characterized in human cells, these observations suggest that prolonged replicational stress may lead to Chk1 inactivation and, potentially, DNA damage. Accordingly, our findings indicate that a pharmacologically imposed, sustained period of Cdk inhibition leads to a broad DNA damage response, including formation of DNA damage foci and activation of ATM/Chk2. In this setting, Chk1 down-regulation is a key mechanism contributing to this phenotype.

![Figure 4](image-url). Chk1 down-regulation elicits a DNA damage response in the setting of stalled replication. A. pATM accumulation mediated by Cdk inhibition largely followed the reduction in Chk1 levels. HDFs and U2OS cells treated with vehicle (−), roscovitine (Ros), or olomoucine (Olo) were harvested for immunoblotting at the indicated times. B. Chk1 inhibition by CEP-3891 and UCN-01 increased pATM levels. HDFs and U2OS cells were treated with vehicle (−) or the indicated concentrations of drug for 8 hours and prepared for immunoblotting. C. Chk1 down-regulation by RNA interference combined with aphidicolin treatment augmented pATM levels. U2OS cells were transfected with control (Con) or Chk1 siRNA for 40 hours and treated with aphidicolin (aph) for the final 16 hours. D and E. Immunofluorescence for γH2AX (green) and pATM/ATR (red) showed increased foci formation with Chk1 RNA interference + aphidicolin (aph) but not Chk1 RNA interference alone (blue, DAPI). U2OS cells were transfected with siRNA as in (C). In each of three independent experiments, >100 cells per condition were scored by a blinded observer. Columns, means; bars, SD.
During preparation of this manuscript, Zhu et al. (47) presented evidence for checkpoint activation by Cdk2 inhibition that is in large part consistent with our findings. Treatment of an ovarian carcinoma cell line with the Cdk2 inhibitor aminothiazole compound 25 elicited phosphorylation of ATM and other biochemical features of DNA damage (47). Although Chk1 levels were not affected, that study focused on the very early response, when we also observed a mild, transient activation of ATM before Chk1 levels decrease (Fig. 4A). During the longer periods of Cdk inhibition examined here, the preponderance of checkpoint activation followed and was largely dependent on Chk1 down-regulation.

The detailed mechanism by which Cdk inhibition might cause DNA damage warrants further investigation. One model is that fork stalling in regions that are difficult to replicate is exacerbated by loss of Cdk2 activity, perhaps due to a decreased number of active replication forks. Without the fork stabilizing activity thought to be provided by the ATR/Chk1 pathway (32, 48, 49), slow or stalled replication forks can lead to double-strand breaks. Notably, the budding yeast homologue of ATR (Mec1), prevents breaks resulting from fork collapse in replication slow zones (50). Furthermore, mammalian cells deficient in ATR develop chromosome breaks after treatment with replication inhibitors (11–13). The present study confirms

Figure 5. The DNA damage phenotype associated with Cdk inhibition requires Chk1 down-regulation. A, U2OS cells were mock transfected or transfected with Chk1 WT or Chk1 KD for 48 hours and treated with vehicle (−) or roscovitine (Ros) for the final 24 hours. Immunoblotting with a Chk1 antibody showed that the (myc-tagged) exogenous (exo) Chk1 proteins were expressed at comparable levels to endogenous (endo) Chk1 in untreated cells. Flow cytometry (bottom) confirmed that the transfection did not affect cell cycle profiles. B and C, Chk1 WT expression markedly reduced roscovitine-induced γH2AX foci (red; blue, DAPI), whereas Chk1 KD did not. In each of three independent experiments, >100 cells per condition were scored by a blinded observer. Columns, means; bars, SD.

Figure 6. Cdk and Chk1 inhibitors enhance net cell loss following etoposide treatment. A, Roscovitine (Ros) and olomoucine (Olo) treatment yielded a statistically significant net loss in viable SaOS2 cells (*, P < 0.01) and enhanced cell killing by etoposide (**, P < 0.01 compared with Etop). For the single agent assays, cells were treated with vehicle (Veh), Cdk inhibitors, or etoposide (Etop) for 24 hours and released into drug-free media for an additional 48 hours. In the combination experiments, cells were treated sequentially with Etop for 24 hours followed by Cdk inhibitors for 24 hours. Viable cell content was assessed by the WST-1 assay and normalized to day 0. Values below the dashed line, treatments that resulted in a net loss of viable cells. Columns, means of triplicate wells in two to four independent experiments; bars, SD. B, The Chk1 inhibitors, UCN-01 and CEP-3891, similarly augmented cell loss following Etop treatment (*, P < 0.01 compared with Etop). Cells were treated with inhibitors and assayed for viability as in A.
and extends these findings by implicating human Chk1 in the prevention of double-strand breaks under conditions of replicational stress, associated with either decreased polymerase activity or Cdk inhibition.

It has been proposed that inhibition of Chk1 in cancer cells (which often harbor other checkpoint defects) may potentiate the effects of DNA-damaging agents (10, 51). Consistent with this notion, we observed that both Cdk and Chk1 inhibitors enhanced net cell loss following etoposide treatment. Although we cannot rule out the contribution of off-target effects, the observation that four different drugs, each of which antagonize Chk1 function, sensitize cells to etoposide suggests that Chk1 inhibition plays a role. Because both roscovitine and UCN-01 are currently in clinical trials, insight into the mechanism and timing of Cdk inhibitor effects on checkpoint function may assist in the rational design of improved chemotherapy regimens.

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