Inhibition of checkpoint kinase 1 following gemcitabine-mediated S phase arrest results in CDC7- and CDK2-dependent replication catastrophe

Combining DNA-damaging drugs with DNA checkpoint inhibitors is an emerging strategy to manage cancer. Checkpoint kinase 1 inhibitors (CHK1is) sensitize most cancer cell lines to DNA-damaging drugs and also elicit single-agent cytotoxicity in 15% of cell lines. Consequently, combination therapy may be effective in a broader patient population. Here, we characterized the molecular mechanism of sensitization to gemcitabine by the CHK1i MK8776. Brief gemcitabine incubation irreversibly inhibited ribonucleotide reductase, depleting dNTPs, resulting in durable S phase arrest. Addition of CHK1i 18 h after gemcitabine elicited cell division cycle 7 (CDC7)- and cyclin-dependent kinase 2 (CDK2)-dependent reactivation of the replicative helicase, but did not reinitiate DNA synthesis due to continued lack of dNTPs. Helicase reactivation generated extensive single-strand (ss)DNA that exceeded the protective capacity of the ssDNA-binding protein, replication protein A. The subsequent cleavage of unprotected ssDNA has been termed replication catastrophe. This mechanism did not occur with concurrent CHK1i plus gemcitabine treatment, providing support for delayed administration of CHK1i in patients. Alternative mechanisms of CHK1i-mediated sensitization to gemcitabine have been proposed, but their role was ruled out; these mechanisms include premature mitosis, inhibition of homologous recombination, and activation of double-strand break repair nuclease (MRE11). In contrast, single-agent activity of CHK1i was MRE11-dependent and was prevented by lower concentrations of a CDK2 inhibitor. Hence, both pathways require CDK2 but appear to depend on different CDK2 substrates. We conclude that a small-molecule inhibitor of CHK1 can elicit at least two distinct, context-dependent mechanisms of cytotoxicity in cancer cells.

DNA-damaging agents are standard-of-care therapies for many cancers, but they result in limited long-term benefit for many patients. DNA damage activates cell cycle checkpoints that arrest cell cycle progression and provide time for repair and recovery. Hence, the use of inhibitors of DNA damage checkpoints to sensitize cancer cells is a rapidly emerging therapeutic strategy (1, 2). We have previously shown that checkpoint kinase 1 inhibitors (CHK1i) can sensitize cancer cells to several DNA-damaging agents (3–6). However, it remains unclear how CHK1i increases the cytotoxic effect of each source of DNA damage. In this study, we investigated the mechanism whereby the CHK1i, MK-8776, sensitizes cancer cells to gemcitabine. Understanding the mechanism of this novel anti-cancer therapy may promote effective clinical development by aiding rational combination scheduling and provide biomarkers to predict response. In addition, CHK1i as a single agent has been shown to be acutely cytotoxic to about 15% of the cell lines tested (7). This study demonstrates that the mechanism occurring when CHK1i is combined with gemcitabine is different from the mechanism of single-agent CHK1i activity. Hence, different patients may respond better to monotherapy or combination therapies.

We previously demonstrated that CHK1i single-agent activity depends on CDK2-dependent activation of the nucleases MRE11 and MUS81 (8). However, the cause of enhanced cytotoxicity when CHK1i was combined with DNA-damaging agents remained unclear as many more cell lines are sensitive to the combination than CHK1i monotherapy (4, 5, 7). In our studies, we incubate cells with gemcitabine for a nominal 6 h and then remove the drug to mimic transient exposure that occurs in patients. Similarly, 6-h incubations with MK-8776 approximate the pharmacokinetic profile when administered to patients (9). Using this strategy, we demonstrated that a concurrent 6-h incubation of gemcitabine and MK-8776 was less cytotoxic than administering CHK1i 18–24 h after gemcitabine (5, 6). This delayed scheduling was also shown to be more effective in a mouse xenograft model (5). The enhanced sensitization with the delayed schedule suggests that cells become more dependent on CHK1 for survival over time following gemcitabine. Understanding the mechanism of action of the combination may elucidate why a delay is necessary for this treatment.

This work was supported by National Institutes of Health Grant 2R01CA117874 (to A. E.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This article contains Figs. S1–S6.

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2 The abbreviations used are: CHK1i, checkpoint kinase 1 inhibitor; ssDNA, single-strand DNA; TBS, Tris-buffered saline; FBS, fetal bovine serum; HRP, horseradish peroxidase; RPA, replication protein A; γH2AX, pSer-139 histone 2AX; PCNA, proliferating cell nuclear antigen; CDK, cyclin-dependent kinase; ATM, ATR, and Rad3-related; DAPI, 4′,6-diamidino-2-phenylindole.
CHK1 inhibition and replication catastrophe

The ribonucleotide reductase inhibitor gemcitabine rapidly depletes deoxyribonucleotides (dNTPs) and arrests replicating cells in S phase (10). Although low levels of dNTPs stall DNA polymerases, the DNA helicases can continue to unwind DNA. The resulting single-strand DNA (ssDNA) and subsequent regression of stalled replication forks serves as a platform to activate the DNA-damage response (11–13). Regardless, activation of CHK1 is not necessary for S phase arrest in the absence of dNTPs as cells lack the ability to synthesize new DNA. However, CHK1 promotes the integrity of partially replicated DNA resulting from incubation with gemcitabine such that CHK1i results in an increase in the DNA damage marker γH2AX, consistent with the formation of DNA breaks (4–6).

Several processes have been implicated in the origin of the DNA breaks associated with replication fork collapse. We have noted that initiation of homologous recombination occurs ~16 h after cells were incubated with gemcitabine (5); this coincides with the time of sensitivity to addition of CHK1i. Furthermore, CHK1i was found to dissociate gemcitabine-induced RAD51 foci. It has also been reported that inhibiting CHK1 in the absence of dNTPs may aberrantly activate endonucleases, such as MUS81 (14), albeit this could also be a consequence of dissociating RAD51 foci. CHK1i can also induce premature activation of cyclin-dependent kinase 1 (CDK1) in S phase-arrested cells leading to premature mitosis (15–17). Another area of investigation has been the restart of stalled replication following recovery from the genotoxic insult (18). It is possible that checkpoint inhibitors can function by preventing fork restart, thus prolonging replication arrest and slowing tumor growth. Alternatively, CHK1i-mediated DNA helicase activation and replication catastrophe has been proposed as a mechanism of cell death (19, 20). Active DNA helicases in the absence of dNTPs may generate extensive ssDNA, exhausting the available ssDNA-binding protein RPA, thereby exposing DNA to nucleases. It is important to note that most experiments describing the cellular response to dNTP depletion as well as replication catastrophe were performed with the osteosarcoma cell line U2OS (12, 13, 20–27). U2OS is one of the few cell lines hypersensitive to CHK1i as a single agent (7) and therefore may not accurately represent how the majority of cell lines respond to CHK1i in combination with replication-arresting agents. In this study, we have investigated these different mechanisms in a variety of cell lines with various phenotypes to obtain a more comprehensive analysis of the mechanism of sensitivity.

Results

Delayed CHK1i causes significantly more DNA damage than concurrent inhibition or gemcitabine alone

We previously reported that concurrent CHK1i only slightly sensitized cells to a 6-h gemcitabine treatment, while delaying addition of CHK1i until 18 h resulted in much greater cytotoxicity (5, 6). Delayed CHK1i, but not concurrent, also sensitized tumors to gemcitabine in mouse xenograft models. Gemcitabine is an irreversible inhibitor of ribonucleotide reductase, and it is administered to patients during a short infusion. Therefore, we incubated cells for 6 h with gemcitabine and then released them into fresh media to better mimic clinical applications (Fig. 1A). The concentrations of gemcitabine used result in durable arrest in early S phase for at least 24 h (Fig. S1) (5).

To confirm that MK-8776 was inhibiting CHK1, we probed lysates for the ATR activation site, Ser-345, and the CHK1 autophosphorylation site, Ser-296. CHK1–pSer-345 was induced by gemcitabine alone, but further increased with delayed CHK1i (Fig. 1B). Gemcitabine alone potently induced CHK1–pSer-296 compared with control, which was reversed by CHK1i when administered either concurrently or 18 h later (Fig. 1B). We also confirmed that MK-8776 was not inhibiting CHK2 by probing for the autophosphorylation site Ser-516. Gemcitabine induced CHK2–pSer-516, and delayed MK-8776 caused a further increase (Fig. 1B). As a positive control, administering 20 μM CHK2 inhibitor II eliminated CHK2–pSer-516 in gemcitabine-treated cells. These data demonstrate that MK-8776 is selectively inhibiting CHK1, resulting in increased DNA checkpoint signaling.

To characterize the extent of DNA damage in cells, we measured phosphorylation of histone 2AX on Ser-139 (γH2AX) by Western blotting (Fig. 1B) and flow cytometry (Fig. 2). Gemcitabine treatment alone resulted in a modest induction of γH2AX in MDA–MB-231 and HT29 cells at both 6 and 24 h. Concurrent CHK1i (0–6 h) only increased γH2AX ~4-fold compared with 6 h gemcitabine alone by Western blotting in MDA–MB-231 cells (Fig. 1B and Fig. S2). CHK1i alone for 6 h elicited negligible increase in γH2AX by Western blotting and flow cytometry (Fig. 1B and Fig. S4). In contrast, delayed CHK1i increased γH2AX 19-fold at 24 h compared with gemcitabine alone (Fig. 1B and Fig. S2A). Delayed CHK1i also dramatically increased the percentage of cells positive for γH2AX by flow cytometry (Fig. 2). There were a greater number of cells in S phase 18 h following gemcitabine, which may contribute to the increased number of cells exhibiting γH2AX with the delayed addition of CHK1i. However, the γH2AX signal in each positive cell was also markedly increased when CHK1i was administered 18 h after gemcitabine (Fig. 2). Incubation with CHK1i alone had negligible impact on γH2AX. These findings are consistent with our previous publications (4–6).

Gemcitabine plus delayed CHK1i also resulted in phosphorylation of the ssDNA-binding protein replication protein A 32-kDa subunit (RPA32) (Fig. 1B). A band shift of total RPA32 suggested that a majority of the protein in cells is phosphorylated after delayed CHK1i in MDA–MB-231 cells. Serines 4 and 8 of RPA32 are reported targets of DNA-PK, a DNA-damage response protein activated by DNA double-strand breaks (28). Using a specific antibody to RPA32–pSer-4/pSer-8, we confirmed that the mobility shift of total RPA corresponds to phosphorylation at Ser-4/Ser-8 (Fig. 1B). This mobility shift was reversible by treating the whole-cell extract with λ-phosphatase (Fig. S3). Elevation of RPA32–pSer-4/Ser-8 further suggests that delayed CHK1i causes a greater amount of DNA breaks.

We further confirmed the presence of DNA damage using the alkaline single-cell gel electrophoresis assay (alkaline comet assay). This assay measures the migration of broken DNA from individual cells by electrophoresis in agarose but does not discriminate between double- or single-strand breaks (29). Neither gemcitabine treatment alone nor concurrent CHK1i
resulted in a significant increase in damaged DNA (Fig. 1C). However, gemcitabine plus delayed CHK1i resulted in a highly significant increase in the percentage of cells with DNA breaks (Fig. 1C). The lack of cells positive for DNA breaks from gemcitabine plus concurrent CHK1i suggests that accumulation of cells in S phase is not the sole driver of increased sensitivity at delayed time points. Furthermore, these breaks are not a consequence of apoptosis as there was no cleavage of the apoptotic marker poly(ADP-ribose) polymerase within 24 h (data not shown), and cell death does not occur until after 4 days with this combination (6).

Premature mitotic entry is not required for CHK1i-mediated DNA damage following gemcitabine treatment

The ability of S phase–arrested cells to undergo premature mitosis before completing DNA synthesis is well-recognized (30). More recently, it was demonstrated that CHK1i can force some gemcitabine-arrested cell lines to prematurely enter mitosis (15–17). Mitotic catastrophe is a well-characterized cytotoxic insult that could explain CHK1i-mediated sensitization to gemcitabine (31). Here, we show that HT29 cells, but not MDA–MB-231 cells, exhibit premature mitosis.

We incubated HT29 and MDA–MB-231 cells with gemcitabine alone or in combination with CHK1i, and we immunostained cells with fluorescent antibodies to γH2AX and histone H3 phosphorylated on Ser-10 (pHH3), a marker of mitosis. We also quantified DNA content in cells by staining with propidium iodide to identify which phase of the cell cycle mitosis or DNA damage was occurring. In MDA–MB-231 cells, the number of pHH3-positive cells did not increase with any treatments compared with control cells, despite a dramatic increase in γH2AX with the delayed addition of CHK1i. In HT29 cells, delayed CHK1i caused an increase in S phase cells stained for pHH3, but this was still fewer than the number of γH2AX-positive cells (Fig. 2). The fact that no MDA–MB-231 cells, and only a portion of the γH2AX-positive HT29 cells, undergo a S to M transition suggests it is not the primary driver of the mechanism of CHK1i-mediated sensitization to gemcitabine.

To further elucidate the impact of aberrant mitotic entry on DNA damage in HT29 cells, we prevented mitosis by concurrently inhibiting CDK1/2 and CHK1. Although low concentrations (≤1.25 μM) of CVT-313 inhibit the CDK2-dependent mechanism of CHK1i monotherapy (7), higher concentrations (10 μM) also inhibit CDK1. Intriguingly, low concentrations of CVT-313 thought to be selective for CDK2 completely suppressed pHH3 in HT29 cells, and the higher concentration had only a marginal impact on γH2AX positivity (Fig. 2). The ability of low concentrations of CVT-313 to protect cells from the induction of pHH3, but not γH2AX, further suggests that premature induction of mitosis cannot explain the
mechanism of DNA damage induced by gemcitabine plus delayed CHK1i.

**Sensitization to gemcitabine by CHK1i is independent of homologous recombination**

We previously demonstrated that RAD51 foci appear 16 h following incubation with gemcitabine alone (5). Thus, the timing of homologous recombination coincides with the optimal window of sensitization by CHK1i. As gemcitabine-incubated cells lack dNTPs, homologous recombination is unable to proceed to completion. CHK1 stabilizes homologous recombination structures by phosphorylating BRCA2 to promote RAD51 binding to DNA (32). Additionally, CHK1i can dissociate RAD51 foci after they have formed, consistent with collapse of recombination structures (5). Here, we asked whether destabilization of homologous recombination structures is required for replication fork collapse and sensitization induced by CHK1i. The two homologous recombination-competent cell lines, MDA-MB-231 and HT29, induced RAD51 foci in a majority of cells 24 h after gemcitabine treatment (Fig. 3A). In contrast, HCC1937 cells that are deficient for BRCA1 and homologous recombination (33) exhibited very few RAD51-positive cells (Fig. 3A). To measure the sensitivity of these three cell lines to gemcitabine plus CHK1i, we incubated cells with gemcitabine alone plus concurrent or delayed CHK1i; cells then recovered for 6 days in media, and DNA content was measured. The three cell lines had differing sensitivities to gemcitabine alone, but all three were significantly more sensitive to gemcitabine plus delayed CHK1i than either gemcitabine alone or gemcitabine plus concurrent CHK1i (Fig. 3B). Sensitization of these cancer cells irrespective of their homologous recombination status suggests that collapse of stalled homologous recombination is unlikely to be the primary cause of CHK1i-mediated sensitization to gemcitabine.

The extent of sensitization observed here was only ~4-fold, but much greater sensitization was observed if incubation with CHK1i was extended from 18 to 30 or 42 h (6); however, these longer incubations would not facilitate comparison with the 6-h concurrent incubations.

**MRE11 activity is not required for delayed CHK1i-mediated sensitization to gemcitabine**

We previously reported that MRE11 nuclelease activity is required for CHK1i single-agent cytotoxicity in sensitive cell
Aberrant MRE11 activity in unperturbed S phase resulted in an increase in ssDNA and subsequent formation of MUS81-dependent double–strand breaks. As MRE11-mediated resection of DNA occurs at stalled replication forks, we hypothesized that this nuclease could also be involved in CHK1i-mediated sensitization of cancer cells to gemcitabine. We co-incubated three cell lines with the MRE11 inhibitor, mirin, and CHK1i 18 h after gemcitabine treatment (Fig. 4). Mirin failed to prevent CHK1i-mediated increases in γH2AX and phospho-RPA32 by Western blotting in all three cell lines. As a control, mirin did prevent CHK1i-mediated γH2AX and phospho-RPA32 in AsPC-1 cells, which are sensitive to CHK1i monotherapy (Fig. 4). These data suggest that MRE11 activity is not required for the CHK1i-mediated sensitization to gemcitabine.

Delayed CHK1i loads the helicase co-factor CDC45 onto DNA

We next asked whether gemcitabine plus delayed CHK1i can promote DNA helicase activity, but, as the gemcitabine treatment still inhibits dNTP synthesis, this would drive the formation of excessive ssDNA. Chromatin fractionation was used to separate soluble and DNA-bound proteins. CDC7-mediated phosphorylation of the MCM2 subunit of the helicase at serine 53 is reported to promote S phase entry during normal replication (34, 35). We observed a continuous increase of DNA-bound MCM2–pSer-53 over the 24-h incubation (Fig. 5, A and B, and Fig. S2). The addition of CHK1i at 18 h did not increase the intensity of MCM2–pSer-53, but shifted all of the signal to the faster-mobility band; this band is reported to be the hyperphosphorylated form of MCM2 (34–36). The total MCM2 antibody indicated that similar proportions of both bands were present in the DNA-bound fraction following addition of CHK1i. λ-Phosphatase treatment of whole-cell extracts eliminated both bands of MCM2–pSer-53, but only the lower band when probed with the total MCM2 antibody (Fig. S3). As discussed below, both MCM2–pSer-53 bands were eliminated upon incubation with a CDC7i. The relationship of the multiple phosphorylated forms to the mechanism of helicase activation remains unclear.

Following phosphorylation of MCM2–7 during normal replication, Treslin is recruited to pre-replication complexes to facilitate loading of CDC45 and activate the helicase (37). We hypothesized that CDC45 recruitment would be stimulated by delayed CHK1i. Although there is a 2-fold increase in chromatin-bound CDC45 by 18 h after gemcitabine alone, probably because there are more cells in S phase, delayed CHK1i induced a further 3-fold increase in CDC45 loading compared with gemcitabine alone (Fig. 5, A and B, and Fig. S2). Importantly, the CHK1i-mediated increase in CDC45 preceded an increase in γH2AX and phospho-RPA32. These results suggest that CDC45 loading onto DNA may be a critical step in the mechanism by which CHK1i sensitizes cells to gemcitabine.

Figure 3. Sensitization to gemcitabine by CHK1i is independent of homologous recombination. A, cells were incubated with or without gemcitabine (Gem) for 6 h and then harvested at 24 h. DNA-bound RAD51 was immunostained, and DNA was stained with DAPI and imaged by confocal microscopy. Cells with nuclear RAD51 intensity of >2 S.D. of the mean of control cells were counted as positive. Graphs represent the mean ± S.D. of positive cells (n = 3). B, MDA–MB-231, HCC1937, and HT29 cells were incubated with gemcitabine either alone for 0–6 h, concurrently with 2 μM MK-8776 (CHK1i), or with 2 μM MK-8776 at 18–24 h. Following treatment, cells were allowed to recover in fresh media for 6 days. DNA content was stained with Hoechst 33258 and analyzed with a fluorescent plate reader. The GI50 graph represents mean ± S.D. of the concentration of gemcitabine required to inhibit growth. *, p value < 0.05; **, p value < 0.005; #, p value < 0.0001; N.S., not significant.
Soluble pools of RPA are depleted following gemcitabine plus delayed CHK1i
The most striking observation from the chromatin fractionation experiments was the rapid appearance of phosphorylated RPA32, all of which was bound to chromatin (Fig. 5, A and B). Within 4 h of addition of CHK1i, almost the entire amount of RPA32 was bound to chromatin and phosphorylated, with almost no residual RPA32 left in the soluble fraction. The 70-kDa subunit of RPA was similarly increased on chromatin and depleted from the soluble fraction (Fig. 5A). RPA depletion only occurred with delayed, not concurrent, CHK1i and correlated with an increase in γH2AX. Prior to phosphorylation on RPA32 was bound to chromatin and phosphorylated, with almost no residual RPA32 left in the soluble fraction. The 70-kDa subunit of RPA was similarly increased on chromatin and depleted from the soluble fraction (Fig. 5A). RPA depletion only occurred with delayed, not concurrent, CHK1i and correlated with an increase in γH2AX. Prior to phosphorylation on
Ser-4/Ser-8, there was an additional band of RPA with slightly retarded electrophilic mobility (Fig. 5B), which is consistent with ATR-mediated phosphorylation of RPA32 on Ser-33 that occurs in response to ssDNA (38). These results are consistent with the hypothesis that the delayed addition of CHK1i leads to excessive ssDNA that exceeds the protective capacity of RPA.

We extended our investigation to 12 different cell lines from different tumor types to characterize the level of RPA32 phosphorylation following inhibition of ribonucleotide reductase and CHK1. Hydroxyurea (2 mM), an alternative ribonucleotide reductase inhibitor, was used because it efficiently arrests all the cell lines tested in early S phase (Fig. S1),3 whereas the required concentration of gemicitabine differs for each cell line. The levels of RPA32 in each cell line varied, perhaps as a consequence of differing levels of DNA, but almost all cell lines demonstrated extensive phosphorylation with concomitant depletion of the unphosphorylated form (Fig. 6). The notable exception was the nontransformed fibroblast ATLD1 cells, which are deficient for MRE11 nuclease protein (8, 39). In addition, ATLD1 was the only cell line that failed to induce phosphorylation of MCM2–pSer-53 following hydroxyurea plus CHK1i. Failure to activate the DNA helicase may explain the lack of RPA phosphorylation in this cell line.

Delayed CHK1i drives formation of ssDNA, which co-localizes with PCNA

We used BrdU staining to confirm that RPA depletion was due to formation of excessive ssDNA. BrdU was incorporated into genomic DNA and, following various treatments, stained with an antibody that only detects BrdU in ssDNA (20). Gemcitabine alone caused an initial increase in ssDNA that decreased again by 24 h perhaps as a consequence of processing of stalled replication forks (Fig. 7A). Gemcitabine plus concurrent CHK1i did not increase ssDNA staining, whereas gemcitabine plus delayed CHK1i resulted in a dramatic increase of BrdU signal. Concurrently, we assessed PCNA binding to chromatin and observed that almost all the cells were negative 24 h after gemcitabine even though the majority were in S phase (Fig. 7A). This is consistent with a report that the DNA replication machinery dissociates from stalled replication forks over time (40). Addition of CHK1i caused a significant increase in cells positive for PCNA, concurrent with an increase in BrdU; the majority of BrdU-positive cells was also positive for PCNA. CHK1i alone did not significantly affect either PCNA or BrdU staining. Furthermore, BrdU and PCNA staining exhibited significant co-localization in cells treated with gemicitabine plus delayed CHK1i (Fig. 7B). These results suggest that aberrant activation of replication was the cause of ssDNA.

DNA replicative helicase is regulated by CDK2 following gemicitabine plus delayed CHK1i

Activation of CHK1 inhibits CDC25A, which in turn prevents activation of CDK2. Active CDK2 phosphorylates Treslin to facilitate CDC45 loading onto DNA during normal replication (37). We hypothesized that CDK2i would prevent CDC45 loading and DNA damage caused by CHK1i. Checkpoint-mediated inhibition of CDK2 following gemicitabine was confirmed by accumulation of cyclin E, whose degradation is mediated by active CDK2. Conversely, the addition of CHK1i rapidly depleted cyclin E, suggesting activation of CDK2 (Fig. 8A). However, low concentrations of CVT-313 thought selective for CDK2 only partially rescued cyclin E. Furthermore, high levels of phosphorylation of H2AX, RPA32, and MCM2–Ser-53 were still observed upon addition of up to 10 μM CVT-313 in MDA–MB-231, HCC1937, and HT29 cells (Figs. 8, A and B, and Fig. S5). As discussed above, this concentration of CVT-313 was enough to eliminate pHH3 staining (Fig. 2), although even lower concentrations protect from CHK1i as a single agent in sensitive cell lines (7). Therefore, 10 μM CVT-313 was thought to fully inhibit CDK1 and CDK2. However, we found that higher concentrations of CVT-313 (20 – 80 μM) were required to fully prevent cyclin E degradation (Fig. 8A). In addition, these higher concentrations inhibited CDC45 loading (Fig. 8B), BrdU staining (Fig. 9A), depletion of soluble RPA (Fig. 8B), phosphorylation of RPA32 and H2AX (Figs. 8, A and B, and Figs. S4 and S5), and DNA breaks (Fig. 10A). Additionally, 20 μM CVT-313 protected cells from growth inhibition induced by CHK1i plus gemicitabine (Fig. 10B). The experiment in Fig. 9B also confirmed co-localization of RPA32 with BrdU staining consistent with RPA32 binding to regions of ssDNA.

3 N. J. H. Warren and A. Eastman, unpublished observations.
CHK1 inhibition and replication catastrophe

To investigate whether concentrations of CVT-313 >10 μM were still targeting CDK1/2, we used an antibody that detects the CDK1/2 substrate consensus sequence, pTPXK. Addition of CHK1i to gemcitabine-arrested cells increased phosphorylation of a number of substrates. Different substrates were inhibited at different concentrations of CVT-313 with 50% inhibitory concentrations varying from 1.2 to >80 μM (Figs. S4A and Fig. S6A). Proteins nonresponsive to CVT-313 may be the result of alternative kinases phosphorylating this consensus sequence, but the proteins that are responsive are likely phosphorylated by either CDK1 or CDK2.

To confirm that CDK1 and/or CDK2 were activated upon addition of CHK1i, we assessed the inhibitory tyrosine phosphorylation of each kinase. Phospho-specific antibodies have previously been unable to resolve these two kinases as the proteins that are responsive are likely phosphorylated by either CDK1 or CDK2.

CDC7 is required for replication catastrophe

In addition to CDK2’s involvement in the mechanism of gemcitabine plus CHK1i, we confirmed that CDC7 kinase is also required for DNA helicase activation. Phosphorylation of MCM2–Ser-53 is mediated by the replication-associated kinase, CDC7/DBF4 (35). We hypothesized that inhibiting this phosphorylation would prevent DNA loading of CDC45 and DNA damage caused by gemcitabine plus CHK1i. Addition of CDC7i (XL413) at the same time as CHK1i reduced MCM2–Ser-53 in MDA–MB-231, HCC1937, and HT29 cells and decreased DNA-bound CDC45 (Figs. 8B and Fig. S5). Concur-
rently, CDC7i prevented markers of replication catastrophe, including DNA-bound RPA32 and BrdU staining (Fig. 9A), phosphorylation of RPA32 and H2AX (Fig. 8, A and B, and Figs. S4 and S5), DNA breaks (Fig. 10A), and restored soluble levels of RPA32 (Fig. 7B). Additionally, CDC7i protected cells from CHK1i-mediated growth inhibition in combination with gemcitabine (Fig. 10B). The data demonstrate that CDC7-mediated helicase activity is required, in concert with CDK2, for the cytotoxic mechanism of gemcitabine plus delayed CHK1i.

Discussion

There are multiple mechanisms proposed to describe how DNA checkpoint inhibitors function. We previously published that CHK1i as a single agent exhibits acute cytotoxicity in about
15% of cancer cell lines by activating CDK2 in S phase to aberrantly initiate MRE11 and MUS81 nuclease-mediated DNA breaks (7, 8). Other proposed mechanisms of cytotoxicity by CHK1i, particularly in combination with DNA damage, include premature entry into mitosis with incompletely replicated DNA, inhibition of homologous recombination, inability to restart replication following a genotoxic insult, and firing of replication machinery in the absence of dNTPs. Here, we ruled out most of these mechanisms as being critical for the cytotoxicity of gemcitabine plus CHK1i, while providing data in support of the hypothesis that aberrant activation of DNA helicases in the absence of dNTPs leads to replication catastrophe (Fig. 11). Importantly, this differs from the cytotoxic mechanism described for the single-agent action of CHK1i.

Following incubation with gemcitabine, cells arrested in early S phase. Addition of CHK1i at 18 h, but not concurrent incubation, caused rapid chromatin loading of CDC45 and PCNA, followed by the appearance of ssDNA that appeared to exceed the protective capacity of RPA resulting in DNA breaks. These events are consistent with the process of replication catastrophe (20). The ability of CDC7i to protect cells from cytotoxicity further supports the necessity of aberrant helicase activity in this mechanism. However, it was initially surprising that the CDK1/2i, CVT-313, did not protect cells. The concentrations of CVT-313 used had been established in a prior study, wherein 0.1 μM prevented γH2AX induced by CHK1i monotherapy, whereas 10 μM prevented mitosis (7). The 10-fold difference in these concentrations is consistent with the differential inhibition of CDK2 and CDK1 in vitro kinase assays (44).

Many substrates for CDK2 can also be phosphorylated by CDK1, albeit often at different phases of the cell cycle. One substrate presumed to be unique to CDK2 is cyclin E; active CDK2 phosphorylates cyclin E which, in concert with GSK3, leads to its degradation (45). Cyclin E accumulated dramatically

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**Figure 8. DNA replicative helicase is regulated by both CDC7 and CDK2 following gemcitabine plus delayed CHK1i.** A, MDA–MB-231 cells were incubated with gemcitabine (Gem) 0–6 h, MK-8776 (CHK1i) 18–24 h, and CVT-313 (CDK1/2i) 18–24 h. Lysates were analyzed by Western blotting. See Fig. S4 for densitometric quantification of pTPXK bands; values represent mean ± S.D. of the concentration of CVT-313, which reduced the band intensity by 50% compared with gemcitabine plus CHK1i, n = 3. B, MDA–MB-231 cells were incubated with gemcitabine 0–6 h, MK-8776 (CHK1i) 18–24 h, CVT-313 (CDK1/2i) 18–24 h, or XL413 (CDC7i) 18–24 h. DNA-bound proteins were separated from soluble proteins by chromatin fractionation and analyzed by Western blotting. C, MDA–MB-231 and HT29 cells were incubated with gemcitabine 0–6 h and then MK-8776 (CHK1i) and harvested from 18 to 24 h. Western blottings were probed for pTyr-15–CDK1/2 with fluorescent-tagged secondary antibodies. Values indicate the mean band intensity of each phosphorylated band relative to gemcitabine alone at 18 h (n = 4). See Fig. S5 for full densitometric analysis of the band intensities.
Figure 9. CDK1/2i and CDC7i reduce ssDNA and DNA-bound RPA32. A, MDA–MB-231 cells were incubated with gemcitabine (Gem) and MK-8776 (CHK1i) as indicated, with the addition of 40 μM CVT-313 (CDKi) or 20 μM XL413 (CDC7i) administered at 18–24 h. Cells were immunostained for DNA-bound RPA32, pMCM2, and BrdU. Cells exhibiting nuclear signal of pMCM2 and BrdU > 2 S.D. of the mean of the control sample were counted as positive; a threshold of > 1 S.D. of the mean of the control sample was used for RPA32 positivity. *, p value < 0.05; **, p value < 0.005; #, p value < 0.0001, n = 3. B, zoomed in view of a cell from A, treated with gemcitabine plus CHK1i. Pearson’s correlation Coefficient represents three replicates, mean ± S.D.

Figure 10. CDC7i, CDK1/2i, and CDC25i prevent CHK1i-mediated sensitization to gemcitabine. A, MDA–MB-231 cells were incubated with gemcitabine (Gem) and MK-8776 (CHK1i) as indicated, with the addition of 40 μM CVT-313 (CDKi) or 20 μM XL413 (CDC7i) administered at 18–24 h. Cells were analyzed by alkaline single-cell gel electrophoresis. Inverse images are shown. Cells with a tail moment > 1 S.D. of the mean tail moment of control cells were counted as positive. Graphs represent the mean ± S.D. of the percentage of positive cells. #, p value < 0.0001. B, MDA–MB-231 cells were incubated with gemcitabine 0–6 h, 2 μM MK-8776 (CHK1i) 18–24 h, 20 μM CVT-313 (CDKi) 18–24 h, and 20 μM XL413 (CDC7i) 18–24 h. Following treatment, cells were allowed to recover in fresh media for 6 days. Plates were analyzed for DNA content with Hoechst 33258 dye. The GI50 graph represents the mean ± S.D. of the concentration of gemcitabine that resulted in 50% growth inhibition. #, p value < 0.0001. C, MDA–MB-231 cells were incubated with gemcitabine 0–6 h, MK-8776 (CHK1i) 18–24 h, and NSC663284 (pan-CDC25i) 18–24 h. Lysates were analyzed by Western blotting.
in gemcitabine-treated cells consistent with CDK2 inhibition and was rapidly depleted upon addition of CHK1i (Fig. 8A). However, we noted that low concentrations of CVT-313 did not rescue cyclin E levels, and $10 \mu M$ only partially rescued it. These observations led us to escalate the concentration of CVT-313 further, and it then inhibited all the expected consequences of CDK2 action; i.e. cyclin E degradation, CDC45 chromatin loading, and the formation of ssDNA and γH2AX.

Using an antibody that detects CDK substrates, we clearly demonstrated that various substrates are differentially sensitive to the concentration of CVT-313. This could be due to the confounding issue that CVT-313 is only selective for CDK2 at low concentrations and inhibits CDK1 at higher concentrations. However, many of the events inhibited at the higher concentrations, such as CDC45 loading, have consistently been considered as CDK2-dependent effects. Furthermore, analysis of pY15-CDK1/2 showed that only CDK2 was dephosphorylated upon addition of CHK1i suggesting that only CDK2 was activated. Further support for the premise that these high concentrations of CVT-313 are inhibiting CDK2 comes from ongoing studies in which high concentrations are also required to prevent S phase progression when CHK1i is added to cells arrested in S phase by a topoisomerase I inhibitor. Unfortunately, complementary studies with siRNA to CDK2 are uninformative as this causes $G_1$ arrest thereby preventing cells progressing into S phase and activating the DNA-damage response (46–48).

An explanation for the differential sensitivity of substrates to CVT-313 can be found in experiments using a monomolecular CDK/cyclin module in *Schizosaccharomyces pombe* (49, 50). Those experiments demonstrated that CDK activity increases as cells pass through S and $G_2$ and that CDK activity thresholds exist for specific substrates thereby providing temporal ordering of S phase and mitosis. The sensitivity of the various substrates to CDK activity was also reflected in the concentration of CDKi required to inhibit phosphorylation, with S phase substrates being up to 1000-fold more resistant to CDKi than M phase substrates (49). This is consistent with our observations that S phase effects (e.g. CDC45 loading) are the most resistant to CVT-313 and therefore supports the hypothesis that these are indeed CDK2-dependent. To our knowledge, this is the first report of CDK2 activity thresholds in a mammalian system.

Although our results ruled out premature mitosis as a critical mechanism of cytotoxicity from the drug combination, we noted that some HT29 cells did undergo premature mitosis from S phase. Surprisingly, this premature mitosis was inhibited by very low concentrations of CVT-313 thought to be selective for CDK2 (Fig. 2), and only CDK2 appeared to be activated (Fig. 7C). These observations suggest that under some circumstances, CDK2 can catalyze mitosis in the absence of CDK1 activity.

This investigation provides additional evidence that delayed CHK1i is more effective at sensitizing cancer cells to gemcitabine than concurrent treatment (4–6). Other reports suggest that checkpoint inhibition can cause replication catastrophe in a few hours, but those reports rely on cell lines that are sensitive to single-agent CHK1i (e.g. U2OS) (23, 24, 27, 51). The U2OS cells are similar to AsPC-1 (Fig. 4) in that extensive γH2AX is observed within 6 h of addition of CHK1i as a single agent. Consequently, their observed catastrophe may be primarily due to single-agent activity. The three main cell lines used in this study all have a single-agent GI50 (50% growth inhibition) of
>10 μM MK-8776 following a 24-h incubation (7); here, we used a 6-h incubation with 2 μM MK-8776 to elicit sensitivity to gemcitabine. These results suggest that tumors resistant to CHK1i monotherapy may still benefit from CHK1i in the context of combination therapy.

Our findings also suggest that DNA breaks caused by gemcitabine plus CHK1i differ from those from CHK1i alone. Here, we show an increase in DNA breaks via the alkaline comet assay, which identifies both single- and double-strand breaks, but we did not see significant comets with the neutral comet assay (data not shown). However, CHK1i as a single agent elicited robust induction of neutral comets (7, 8). This difference may explain why only the single-agent activity of CHK1i is inhibited by MRE11i. Overall, our data demonstrate that sensitivity to CHK1i as a single agent or in combination with gemcitabine can be discriminated by the requirement of MRE11, CDK2 activity thresholds, and type of DNA break.

This study has outlined several differences in the regulation of replication replication that occur at early and late time points following gemcitabine. We re-confirmed that RAD51 foci indicative of homologous recombination are induced after longer incubation with gemcitabine, but we now show that it is not required for CHK1i-mediated sensitization. One of the most striking observations was the apparent dissociation of DNA-bound PCNA 24 h after gemcitabine, with a dramatic increase following addition of CHKi suggesting new origin firing (Fig. 7A). A similar time-dependent dissociation of PCNA and other components of the replication machinery was previously reported in hydroxyurea-treated cells (40). Hence, the replication machinery appears to dissociate from stalled replication forks with delayed kinetics, and subsequent replication can only be reinitiated by firing dormant origin. This is consistent with a report that cells recovering from 24 h of incubation with hydroxyurea predominantly fire dormant origins to reinitiate replication (25). Therefore, the need for delayed CHK1i could be explained if some replication components are limiting, as suggested for CDC45 (52); dormant origins of replication cannot fire until the replication machinery dissociates from stalled replication forks.

An intriguing aspect of the mechanism presented here, but not previously discussed, is that inhibition of CHK1i appears to bypass ATR-ATR-mediated replication checkpoints. ATR is reported to directly repress DNA replication by phosphorylating several essential replication proteins, including DBF4, FANCI, MCM2, and TopBP1 (36, 53–55). Although we have not confirmed that these targets are phosphorylated on inhibitory sites during gemcitabine plus CHK1i treatment, we have observed CDC7/DBF4-dependent activation phosphorylation of MCM2–pSer-53 (Fig. 5, A and B) under the same conditions as CHK1i–pSer-345 (Fig. 1B), with the latter indicative of ATR activity. It is possible that ATR activity predominates in local areas of replication stress but relies on CHK1 to extend its regulatory effects to distant origins of replication. Not surprisingly, inhibition of ATR also induces replication catastrophe (20).

Toledo et al. (20) proposed that basal expression levels of RPA subunits may predict response to treatments that cause replication catastrophe. This postulate was based on overexpression or siRNA knockdown of RPA subunits in U2OS cells; increased RPA expression conferred resistance to replication catastrophe, whereas knockdown conferred sensitivity. In a panel of 12 cell lines, we did not find a correlation of RPA32 protein levels with induction of RPA32 phosphorylation (Fig. 6). MDA–MB-231 cells exhibited the greatest basal RPA32 but had a high ratio of phosphorylated to unphosphorylated RPA32 following hydroxyurea plus CHK1i (Fig. 6). Conversely, EKVX cells exhibited the lowest RPA32 expression but had a relatively low level of RPA32 phosphorylation. We conclude that basal RPA protein expression is not a robust biomarker to predict patient response to gemcitabine plus CHK1i. One cell line, ATLD1, exhibited a very different phenotype in that it exhibited no RPA32 phosphorylation when incubated with hydroxyurea plus CHK1i, but it also failed to phosphorylate MCM2. This cell line is defective for MRE11i, which could potentially impact the formation of ssDNA at stalled forks, but would not explain the lack of MCM2 phosphorylation.

This study comprehensively evaluated the mechanism of action of combined gemcitabine plus CHK1i in cell lines of various phenotypes. We ruled out homologous recombination and premature mitosis as contributing to the mechanism of sensitivity to this combination. In contrast to CHK1i as a single agent, the MRE11i nuclease played no role in generating ssDNA, which exceeded the protective capacity of RPA and led to replication catastrophe. Additionally, analysis of CDK substrates demonstrated differential sensitivity of phospho-proteins to CDK2i. We conclude that different substrates are involved in these two distinct mechanisms of CHK1i action. The sensitization of gemcitabine occurs in multiple cell lines, demonstrating that the CHK1–CDC25–CDK2 pathway is functional, yet CDK2 is only activated in a few cell lines by single-agent CHK1i. We hope these findings will aid further clinical development of CHK1i as a single agent and in combination.

Experimental procedures

Cell culture

The human cell lines MDA–MB-231, SF295, HT29, EKVX, HOP62, PC3, and DU145 are a part of the NCI60 panel of cell lines and were obtained from the Developmental Therapeutics Program, NCI, National Institutes of Health. ASPC1, MiaPaCa, and U2OS cells were obtained from ATCC. ATLD1 cells were a generous gift from Dr. Matthew Weitzman at the Salk Institute (San Diego). HCC1937 cells were a generous gift from Dr. Todd Miller, Dartmouth College (Lebanon, NH). Cells were grown in RPMI 1640 media (Corning/Mediatech, Corning, NY) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT) and 1% antibiotic/antimycotic (Gibco) at 37 °C with 5% CO₂. All cells lines tested negative for mycoplasma contamination prior to experimentation.

Small molecule inhibitors

Gemcitabine (Lilly) was diluted in media prior to each experiment. Hydroxyurea (Sigma) was dissolved in water. CVT-313 (Sigma), MK-8776 (Merck, Kenilworth, NJ), CHK2 inhibitor II (Cayman Chemical, Ann Arbor, MI), and NSC663284 (Cayman Chemical) were dissolved in DMSO. Cells were treated with 2 μM MK-8776 for up to 6 h to mimic the pharmacokinetic profile in humans (9). XL413 (Tocris, Bristol, UK) was dissolved in...
water. Mirin was synthesized at Dartmouth College as described previously (56).

**Antibodies**

The following antibodies were used at the indicated dilutions: Abcam (Cambridge, MA): RPA32(blotting) (1:5000, RRID: AB_302873); Bio-Rad: anti-mouse HRP (1:3000, RRID: AB_11125547); Cell Signaling (Danvers, MA)–dyLight680 (1:20000, RRID: AB_10696895); anti-mouse DyLight800 (1:20000, RRID: AB_106957505); CDK1/2-pY15 (1:1000, RRID: AB_331460); CDK substrates/pTPXK (1:1000, catalog no. 14371); CHK1–pSer-345 (1:3000, RRID: AB_331212); CHK1–pSer-296 (1:1000, RRID: AB_2080323); CHK2–pSer-516 (1:1000, catalog no. 26695); H2AX–pSer-139 (1:2000, RRID: AB_2118009); H2AX–pSer-139–Alexa488 (1:100, RRID: AB_10694488); HH3–pS10 (1:3000, RRID: AB_1549592); HH3–pS10-Alexa647 (1:200, RRID: AB_1549592); MCM2 (1:5000, RRID: AB_2687884); MEK1/2 (1:1000, RRID: AB_823567); ORC2 (1:1000, RRID: AB_10694717); PCNA-Alexa488 (1:1000, RRID: AB_11178664); RPA32(microscopy) (1:1000, RRID: AB_2238543); GE Healthcare: BrdU (1:2500, RRID: AB_11178664); ORC2 (1:1000, RRID: AB_10694717); pSer-53 (1:3000, RRID: AB_669843); RPA70 (1:1000, RRID: 1131294); Bethyl Laboratories (Montgomery, TX): MCM2–Alexa488 (1:1000, RRID: AB_627357); vinculin (1:3000, RRID: AB_2078507); CHK1 (1:2000, RRID: AB_627257); cyclin E (1:1000, RRID: AB_627257); vinculin (1:3000, RRID: AB_1131294); Bethyl Laboratories (Montgomery, TX): MCM2–pSer-53 (1:3000, RRID: AB_669843); RPA70 (1:1000, RRID: AB_2180681); Sigma: actin-HRP (1:1000, RRID: AB_2620101); ThermoFisher Scientific (Carlsbad, CA): anti-mouse Alexa635 (1:1000, RRID: AB_2536185); anti-rabbit Alexa555 (1:1000, RRID: AB_141784); anti-rat Alexa488 (1:1000, RRID: AB_2535873); and anti-rat-HRP (1:1000, RRID: AB_2535648).

**Western blotting**

Cells were plated at 200,000–300,000 per well in a 6-well plate. Following treatment, cells were rinsed with phosphate buffered saline (PBS) then directly lysed in the well by the addition of Laemmli sample buffer. Samples were boiled for 5 min and stored overnight at −20 °C. The lysates for Figs. 6 and 8C and Fig. S6 were normalized to total protein using the Pierce BCA Protein Assay (components) and stored overnight at 4 °C. Following treatment, cells were rinsed with phosphate buffer without Triton X-100 and re-centrifuged at 13000 g for 5 min to reduce residual pellet. The supernatants (soluble fraction) were transferred to new tubes and centrifuged at 17,000 × g for 5 min to reduce residual pellet. The supernatants (200 µl; soluble fraction) were combined with 40 µl of Laemmli sample buffer. The original pellets were washed with fractionation buffer without Triton X-100 and re-centrifuged at 1300 × g. The pellets (chromatin-bound fraction) were suspended in 120 µl of Laemmli sample buffer. All samples were boiled and stored at −20 °C, then run on a Western blotting as described above; 10 µl of whole-cell extract and soluble fractions and 5 µl of chromatin-bound fractions were loaded into each well. MEK1/2 was used as a control for fractionation, and ORC2 was used as a DNA-bound control.

**Immuno-Fluorescence**

Cells at 200,000 per well were grown on 18-mm square no. 1.5 glass coverslips in 6-well plates and washed with PBS following treatment. Soluble RAD51, RPA, and phospho-MCM2 proteins were extracted with 20 mM HEPES, 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose, 0.5% Triton X-100, pH 7.4 (5 min on ice), as described previously (27). Cells were washed with PBS (two times), fixed with 1.5% formaldehyde in PBS (10 min, room temperature), and rewashed with PBS (two times). Fixation was quenched with 30 mM NH4Cl in PBS (5 min, room temperature) and washed off with PBS (two times). Plates were sealed with paraffin and stored overnight at 4 °C in PBS. The
cells were permeabilized in PBS, 0.5% Triton X-100 (15 min, room temperature), and blocked with 3% FBS, 0.1% Triton X-100 in PBS (30 min, room temperature).

To extract soluble PCNA, an additional permeabilization step was required. Following the Triton-based extraction buffer, cells were incubated with 100% cold methanol (10 min, −20 °C) prior to formaldehyde fixation. For BrdU incorporation, cells were incubated with 10 μM BrdU (Sigma) for 48 h prior to treatment. Immediately before treatment, the cells were washed with PBS, and fresh media were added.

Primary antibodies were suspended in blocking buffer and added to each 6-well dish, with coverslips inverted on top of the antibody solution (4 °C overnight, or 1 h two times at room temperature for BrdU only). Coverslips were washed with PBS (three times) before subsequent staining. The secondary antibodies or conjugated primary antibodies were used to fluorescently label antigens in 6-well plates with inverted coverslips (2−3 h, room temperature). After two PBS washes, DNA was stained with 1 μg/ml DAPI in PBS (Sigma) (5 min, room temperature).

Confocal images were acquired using a Zeiss LSM 510 or LSM 800 microscope. The integrated intensity per nucleus for each antigen was quantified with Cell Profiler software (59), and intensity values were normalized to nucleus/DAPI area for analysis. Cell Profiler was used to calculate the Pearson’s co-localization coefficient for PCNA or RPA with BrdU in cells treated with gemicitabine plus delayed CHK1i. A minimum of 100 cells per sample was included in the analysis for each biological replicate.

Flow cytometry

Cells were plated at 200,000−300,000 per well in a 6-well plate. Following treatment, cells were washed with PBS and then trypsinized (5 min, 37 °C). Cells were pelleted, washed with PBS, resuspended at −20 °C in 70% ethanol, and stored at −20 °C overnight. Cells were rehydrated with PBS and then permeabilized with 0.2% Triton X-100 and 1% FBS in PBS (5 min on ice). The cells were washed with blocking buffer (1% FBS in PBS) and resuspended in blocking buffer with fluorescence-conjugated antibodies (1–2 h, room temperature, in the dark). Cells were then washed with 1% FBS in PBS. DNA was stained with 100 μg/ml propidium iodide (Sigma), 100 μg/ml RNase A (Sigma) in PBS (30 min, 37 °C). Cells were analyzed on either a MACSQuant or Gallios flow cytometer, and the data were analyzed using FlowLogic software.

Single cell gel electrophoresis

Treated cells were washed with PBS and incubated on ice for 10 min, although the positive control sample was treated with 200 μM H2O2. Cells were rewashed with PBS and suspended using a cell scraper. We used an alkaline Comet Assay kit (Trevigen, Gaithersburg, MD) to electrophorese and stain cells according to the manufacturer’s protocol. Cells were imaged on an Olympus IX-73 inverted fluorescence microscope. Comet tail moments were automatically identified and measured using the Open Comet plugin for ImageJ (60). Comets that were mis-aligned, mis-identified, had abnormal morphology, or in clumps of multiple comets were manually excluded from analysis. A minimum of 100 cells were included in the final analysis for each replicate. A threshold of the mean tail moment from untreated cells, plus 1 S.D., was used to score cells positive for DNA breaks.

Growth inhibition assay

Cells were plated at 2,000−5,000 cells per well in 96-well plates and treated with drugs the following day (8 wells/technical replicates per treatment condition). Following treatment, cells were washed with PBS and released into fresh media to recover for 6 additional days. Cells were then lysed, and DNA was stained with 1 μg/ml Hoechst 33258 as described previously (4, 6, 61). DNA content was quantified using a SpectraMax i3x plate reader (Molecular Devices, San Jose, CA).

Quantification and statistical analysis

Quantitative data are shown as mean ± S.D. or range in the case of n = 2 experiments. IC50 and GI50 values were calculated in Microsoft Excel by linear regression of the two closest data points to 50% DNA content of control cells. Values of n reported in the figure legends or identified by scatter plots indicate independent biological repeats. Statistical analysis was performed using one-way analysis of variance with Sidak’s multiple comparisons test in Graphpad Prism 7; significant differences were determined with a threshold of p ≤ 0.05.

Acknowledgments—The Norris Cotton Cancer Center was the recipient of National Institutes of Health Cancer Center Support Grant S10 SA02196-37 from NCI and National Institutes of Health SIG Award S10OD21616 for the confocal microscope. We also acknowledge Katelyn Donahue for technical support with CDK1/2-pY15 experiments.

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