Forced Mitotic Entry of S-Phase Cells as a Therapeutic Strategy Induced by Inhibition of WEE1

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INTRODUCTION

Cell-cycle progression is regulated by multiple overlapping checkpoints that stall the cell cycle in response to DNA damage or other cellular insults. Functionally distinct checkpoints have been described that block the initiation of S-phase (G₁–S checkpoint), the progression through S-phase (intra–S-phase checkpoint), the initiation of mitosis (G₂–M checkpoint), and the initiation of anaphase (spindle checkpoint; ref. 1). The disruption of these checkpoints provides a potential approach to cancer treatment, synergizing with chemotherapy to drive cells inappropriately onward through the cell cycle without first repairing DNA damage (2).

Entry into mitosis is a tightly regulated process ultimately driven by the cyclin B-CDK1 complex (3). In normal cells, cyclin B expression is low at the start of S-phase and peaks at the end of G₂ phase, leading to the build-up of cyclin B-CDK1 complexes. However, until the end of G₂ phase, cyclin B-CDK1 is kept inactive through inhibitory phosphorylation of cyclin-dependent kinase (CDK)1 at Thr14 and Tyr15 by WEE1 (predominantly Tyr15) and MYT1 (Thr14 and Tyr15). Mitotic entry is ultimately initiated by dephosphorylation of these residues by the CDC25 family of phosphatases, initiating a positive feedback loop, in which cyclin B-CDK1 inhibits its inhibitors (WEE1 and MYT1) and activates its activators (CDC25 phosphatases), ensuring rapid amplification of CDK1 activity (reviewed in ref. 3). In response to DNA damage, checkpoint kinases such as CHK1 (4) mediate the inhibition and/or degradation of the CDC25 phosphatases to inhibit CDK1 activation until the damage is repaired, a safeguard mechanism termed the G₂–M checkpoint (1).

Inhibitors of WEE1 have been developed as potential cancer therapeutics, originally conceived to synergize with chemotherapy by abrogating the G₂–M checkpoint and drive cells into mitosis with unrepaired DNA damage (reviewed in ref. 5). Previous work with chemical inhibitors of WEE1 and siRNA-mediated depletion (6) suggested that WEE1 inhibition abrogated the G₂ checkpoint and selectively sensitized p53-deficient cells to various DNA-damaging agents, such as gemcitabine, carboplatin, and cisplatin (7, 8), and inhibited tumor growth in vivo models (7, 9). In addition, WEE1 inhibition or depletion sensitized tumor cells to ionizing radiation (10–13).

However, the proposed mechanism of sensitization via abrogation of the G₂–M checkpoint, discounts the importance of the intra–S-phase checkpoint that is engaged when replication fork progression is hampered in response to stresses such as depletion of deoxyribonucleotides [deoxyribonucleotide triphosphate (dNTP)] or DNA damage. The checkpoint machinery inhibits the initiation of DNA replication from unfired replication origins and protects the integrity of stalled replication forks (14). Here, we show that WEE1 inhibitors have a distinct and novel mechanism of action, forcing cells arrested in S-phase directly into G₂ phase, cyclin B-CDK1 is kept inactive through inhibitory phosphorylation of cyclin-dependent kinase (CDK)1 at Thr14 and Tyr15 by WEE1 (predominantly Tyr15) and MYT1 (Thr14 and Tyr15). Mitotic entry is ultimately initiated by dephosphorylation of these residues by the CDC25 family of phosphatases, initiating a positive feedback loop, in which cyclin B-CDK1 inhibits its inhibitors (WEE1 and MYT1) and activates its activators (CDC25 phosphatases), ensuring rapid amplification of CDK1 activity (reviewed in ref. 3). In response to DNA damage, checkpoint kinases such as CHK1 (4) mediate the inhibition and/or degradation of the CDC25 phosphatases to inhibit CDK1 activation until the damage is repaired, a safeguard mechanism termed the G₂–M checkpoint (1).

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ABSTRACT

Inhibition of the protein kinase WEE1 synergizes with chemotherapy in preclinical models and WEE1 inhibitors are being explored as potential cancer therapies. Here, we investigate the mechanism that underlies this synergy. We show that WEE1 inhibition forces G₂-phase-arrested cells directly into mitosis without completing DNA synthesis, resulting in highly abnormal mitoses characterized by dispersed chromosomes and disorganized bipolar spindles, ultimately resulting in mitotic exit with gross micronuclei formation and apoptosis. This mechanism of cell death is shared by CHK1 inhibitors, and combined WEE1 and CHK1 inhibition forces mitotic entry from S-phase in the absence of chemotherapy. We show that p53/p21 inactivation combined with high expression of mitotic cyclins and EZH2 predispose to mitotic entry during S-phase with cells reliant on WEE1 to prevent premature cyclin-dependent kinase (CDK1) activation. These features are characteristic of aggressive breast, and other, cancers for which WEE1 inhibitor combinations represent a promising targeted therapy.

SIGNIFICANCE: Here, we describe a novel mechanism of inducing cancer cell death by WEE1 inhibition, forcing mitotic entry directly from S-phase. This mechanism represents a potential therapeutic approach for aggressive breast cancers, and in particular triple-negative and basal-like breast cancers, as WEE1 inhibition specifically targets the features inherent in these cancers: frequent TP53 mutation and high expression of mitotic cyclins and the polycomb protein EZH2. Cancer Discov, 2(6); 524–39. © 2012 AACR.
mitosis. We go on to show that this mechanism is also shared, although to a lesser extent, by inhibitors of CHK1.

We explore the pathophysiologic changes that underlie the reliance on WEE1 and CHK1 to prevent inappropriate engagement of mitosis while arrested in S-phase, and provide evidence that forced mitosis targets the oncogenic phenotype of poor prognosis cancers characterized by TP53 mutation and high expression of mitotic cyclins and polycomb protein EZH2.

RESULTS

WEE1 Inhibition Triggers Mitotic Entry in Early S-phase–Arrested Cells

We investigated whether synergy occurred between gemcitabine and the WEE1 inhibitor MK-1775 in a panel of 25 breast cancer cell lines (23 breast cancer cell lines and 2 breast epithelial cell lines; Fig. 1A; Supplementary Table S1), using the combination index (CI) method of Chou and Talalay (15). Gemcitabine inhibits DNA synthesis by targeting ribonucleotide reductase, resulting in depletion of the dNTP pool and by competing with endogenous dCTPs for incorporation into DNA. A synergistic interaction between gemcitabine and MK-1775 was found in 44% (11 of 25) of the cell lines. As shown previously, TP53 wild-type cell lines displayed no evidence of substantial synergy (7), nor did TP53 wild-type cell lines (Fig. 1E). MK-1775 treatment alone displayed no evidence of substantial synergy (7), nor did TP53 wild-type cell lines (Fig. 1E). MK-1775 treatment alone displayed no evidence of substantial synergy (7), nor did TP53 wild-type cell lines (Fig. 1E). MK-1775 treatment alone displayed no evidence of substantial synergy (7), nor did TP53 wild-type cell lines (Fig. 1E).

To examine the mechanisms underlying synergy, we initially chose to study 2 model cell lines: MCF7 (nonsynergistic, CI = 1.04; TP53 wild-type) and CAL120 (synergistic, CI = 0.61; TP53-mutant; Fig. 1B). MK-1775 treatment alone increased the proportion of cells in mitosis with a full 4N complement of DNA (CAL120 4N mitotic cells: 3.9%–27.8%, MCF7 4N mitotic cells: 1.9%–4.9%; Fig. 1C), most likely reflecting a subpopulation of G2 phase cells undergoing appropriately scheduled mitosis. In response to gemcitabine, both cell lines arrested in early S-phase (Fig. 1C). Unexpectedly, the addition of MK-1775 to gemcitabine-arrested cells triggered a large proportion of CAL120 cells to engage mitosis without completing DNA synthesis (46.9% mitotic cells with <4N DNA content), an effect that was not seen in MCF7 cells (1.4% mitotic cells with <4N DNA content). Addition of MK-1775 triggered mitosis in CAL120 cells, but not in MCF7 cells, that were arrested in early S-phase by hydroxyurea treatment or by a double thymidine block (Fig. 1D), blocking DNA synthesis by depleting the dNTP pool through inhibition of ribonucleotide reductase, suggesting that mitotic entry was independent of the mechanism of inducing S-phase arrest. Mitosis without completing S-phase was also observed with other chemical inhibitors of WEE1 (PD0166285, WEE1 inhibitor II, and PHCD; Supplementary Fig. S1A) and siRNA-mediated silencing of WEE1 (Supplementary Fig. S1B), confirming the specificity of the results obtained with MK-1775.

We assessed mitotic entry in an expanded set of breast cancer cell lines (Fig. 1E; Supplementary Fig. S2A and S2B), confirming the specificity of the results obtained with MK-1775. MK-1775 and gemcitabine, with the variation in synergy explained by the susceptibility to forced mitotic entry. There was no correlation between cell doubling time and synergy as assessed by CI (r = 0.15, P = 0.63; Pearson correlation coefficient).

Taken together, these results show that after stalling in S-phase, p53-deficient cell lines rely, in part, on WEE1 activity to delay mitosis until DNA replication is complete. Inhibition of WEE1 by MK-1775 in combination with DNA-damaging agents overrides the induced early S-phase arrest and forces cells directly into mitosis without completing DNA synthesis.

WEE1 Inhibition Induces Highly Abnormal Mitoses in S-phase–Arrested Cells

We examined nuclear morphology in response to gemcitabine, MK-1775, and the combination of both drugs. After gemcitabine treatment, only interphase nuclei with uncondensed chromatin were observed, whereas cells with condensed chromosomes typically aligned at the metaphase plate were observed after MK-1775 treatment (Fig. 2A). In contrast, gemcitabine and MK-1775 treatment resulted in highly abnormal mitoses with unaligned and partially condensed chromosomes (Fig. 2A and B), showing characteristics of S-phase premature chromosome condensation (PCC; ref. 16). To assess this further, we examined microtubule organization by labeling cells treated with hydroxyurea and MK-1775 for α-tubulin and phospho-histone H3 (phH3; Fig. 2C). After hydroxyurea and MK-1775 combination treatment, 65% of the phH3-positive cells showed highly disorganized bipolar spindles with dispersed chromosomes and little evidence of alignment to the metaphase plate, a phenotype that was only rarely seen in control cells or with MK-1775 alone (Fig. 2C and D). The occurrence of mono- or multipolar spindles was not affected by WEE1 inhibition. A small number of interphase nuclei in control cells stained positive for focal phH3, indicative of pericentromeric H3 phosphorylation in cells with incipient mitotic entry (17).

Unscheduled Mitosis Results in Mitotic Slippage, Apoptosis, and Gross Micronuclei Formation

To assess the consequences of unscheduled mitosis, CAL120 cells were arrested in early S-phase by hydroxyurea, followed by addition of MK-1775 for 8 hours or continued hydroxyurea treatment alone. Treatments were washed out and cell-cycle progression was monitored by flow cytometry. Hydroxyurea-arrested control cells readily reentered the cell cycle and reached late S-G2 phase after 8 hours, went through mitosis at 16 hours after release, and subsequently progressed...
Forced Mitotic Entry by WEE1 Inhibition

Figure 1. WEE1 inhibition forces S-phase–arrested cancer cells into mitosis. A, assessment of the degree of synergy between gemcitabine and MK-1775 in 25 breast cell lines (23 breast cancer cell lines and 2 breast epithelial cell lines), displayed according to TP53 mutational status. Drug interactions are expressed as a CI. B, SF50 isobolograms of gemcitabine and MK-1775 combination in MCF7 (wild-type TP53) and CAL120 (mutant TP53) cells. C, CAL120 and MCF7 cells were treated with gemcitabine (Gem), MK-1775, or the combination and subjected to dual pH3 (mitotic cells) and PI flow cytometric analysis (FACS). Top, PI alone; bottom, pH3/PI. 2N DNA content indicates cells in G1 phase. 4N DNA content indicates cells in either G2 or M phase. D, CAL120 and MCF7 cells were treated with hydroxyurea (HU) for 24 hours or synchronized (synchr) by a double thymidine block (T) after which MK-1775 was added for an additional 8 hours. Mitotic index was determined by pH3/PI FACS analysis for cells with <4N DNA content (green bars) or cells with 4N DNA content (red bars). E, mitotic index after gemcitabine and MK-1775 combination treatment in various breast cancer cell lines. TP53 status (mutant or wild-type, WT) and CI are indicated for each cell line (*, P < 0.01; Student t test compared with <4N DNA mitotic index of MCF7 cells).
Figure 2. WEE1 inhibition induces highly abnormal mitoses in S-phase–arrested cells. A, nuclear morphology of DAPI-stained CAL120 cells after treatment with gemcitabine (Gem) for 24 hours followed by MK-1775 for an additional 8 hours or after treatment with each drug alone. Ctr, untreated control cells. B, chromosome spreads of CAL120 cells treated as described in (A). C, immunofluorescent images of untreated pH3-positive CAL120 cells in interphase (i) and in metaphase (ii) or after hydroxyurea (HU) and MK-1775 combination treatment (iii). Cells were stained for pH3 (red), α-tubulin (microtubules, green), and DAPI (DNA, blue). Representative images of cells with monopolar (iv) or multipolar spindles (v) and early prophase cells without spindle (vi) are shown. D, quantification of phenotypes in pH3-positive CAL120 cells that were either untreated (white bars), treated with MK-1775 alone for 8 hours (green bars), or HU and MK-1775 combination (red bars). Cells with disorganized spindle are seen in 2% untreated, 9% MK-1775, and 65% HU + MK-1775 (*, P < 0.004 untreated vs. HU + MK-1775 and MK-1775 vs. HU + MK-1775; Student t test).

into the next cell cycle (Fig. 3A; Supplementary Table S2). In contrast, cells treated with hydroxyurea and MK-1775 progressively exited mitosis, 8 hours after release the percentage of pH3-positive cells had dropped from 60.1% to 8.5%, but did not progress into S- or G2 phase for up to 48 hours after release (Fig. 3A and data not shown).

Normally, entry into mitosis is triggered by progressive activation of cyclin B1-CDK1 at the end of G2 phase (3). Cyclin B1-CDK1 activity remains high until anaphase onset, when cyclin B1 is targeted for degradation by anaphase-promoting complex or cyclosome (APC/C) after the spindle assembly checkpoint (SAC) is satisfied by proper attachment of all chromosomes to the mitotic spindle. Cyclin B1 degradation results in inactivation of CDK1, which is required for anaphase progression and exit from mitosis. To gain more insight into the kinetics of unscheduled entry and exit from mitosis, we conducted a detailed time course analysis (Fig. 3B and C). After MK-1775 addition, cells gradually entered mitosis during the first 6 hours and subsequently exited mitosis many hours later, regardless of whether WEE1 inhibition was maintained (Fig. 3B). As the majority of cells exited mitosis, 10 to 14 hours after MK-1775 addition (Fig. 3B), cyclin B1 levels decreased and CDK1-Y15 phosphorylation partially increased (Fig. 3C), suggesting that after a prolonged mitotic arrest, loss of cyclin B1-CDK1 activity allowed cells to exit mitosis without undergoing complete chromosome segregation and cell division, a process termed “mitotic slippage” (18).

The nuclear morphology of cells exiting unscheduled mitosis was highly abnormal with gross micronuclei formation (Fig. 3D and E). Accompanying mitotic exit, PARP-1 was cleaved, indicating engagement of apoptosis (Fig. 3C). Substantial induction of apoptosis was confirmed with both Annexin V staining (Supplementary Fig. S4A and S4B) and
Figure 3. Unscheduled mitosis results in mitotic slippage and apoptosis. A, CAL120 cells were treated with hydroxyurea (HU) alone for 24 hours with or without the addition of MK-1775 for further 8 hours. Drugs were washed out and mitotic index was assessed by pH3/PI FACS at indicated time points after washout. B, time course analysis of mitotic index of cells with <4N DNA content. MK-1775 was added to HU-arrested CAL120 cells for 8 hours, after which drugs were washed out (W) or in the continuous presence of both drugs. Mitotic index was determined by pH3/PI FACS. C, CAL120 cells were treated as described in (B), lysed at indicated time points, and analyzed by Western blotting for PARP-1, cyclin B1, CDK1 (phospho-Y15 and total), and β-tubulin as loading control. Asynchronously (async) growing cells were included as controls. D, CAL120 cells were untreated (white bar) or treated with HU alone (green bar), MK-1775 alone (red bar), or HU and MK-1775 combination (purple bar). Drugs were washed out and at 8 hours after washout (w8h), cells were stained for α-tubulin (green) and DAPI (blue). Quantification of proportion of micronucleated cells by immunofluorescent microscopy (untreated, 6%; HU, 8%; MK-1775, 14%; HU and MK-1775, 44%, * P = 0.008 one-way ANOVA). E, representative immunofluorescent images of normal and micronucleated cells taken at 8 hours after washout of HU and MK-1775 combination treatment as described in (D).
abrogated the camptothecin-induced S-phase delay, causing of the intra–S-phase checkpoint [Fig. 4F (iii)]. AZD7762 induced a substantial S-phase delay due to engagement cycle progression of the BrdU-labeled cells. Camptothecin lication in S-phase cells was determined by monitoring cell- I poison camptothecin (Fig. 4F). The recovery of DNA rep- dination (BrdU) followed by treatment with the topoisomerase point, CAL120 cells were pulse-labeled with bromodeoxyuri- with induction and maintenance of the intra–S-phase check- point, CAL120 cells were pulse-labeled with gemcitabine for 24 hours followed by CHK1 inhibitor AZD7762 (21) alone or in combination with MK-1775. Inhibition of CHK1 in gemcit- abine-arrested cells induced premature mitosis in 33% of the CAL120 cells and only 2% in the MCF7 cells (Fig. 4A). Time course analysis revealed that within 1 hour after addition of both inhibitors, 52% of the cells had entered mitosis, compared with 4% and 14% treated with AZD7762 or MK-1775 alone, respectively (Fig. 4B).

We extended these observations by showing that the combina- tion of AZD7762 and MK-1775, in the absence of DNA-damaging agents, was sufficient to drive late S-phase cells into mitosis (12% mitotic CAL120 cells with <4N DNA content; Fig. 4C and D). No induction of mitosis was seen with the combina- nation in MCF7 cells (Fig. 4C). AZD7762 increased the sensi- tivity of CAL120 cells to MK-1775 alone, shifting the MK-1775 SF<sub>50</sub> value from 329 to 75 nmol/L (Supplementary Fig. S5A). We explored the molecular basis for the synergistic interaction between CHK1 and WEE1 inhibition in CAL120 cells (Fig. 4E; Supplementary Fig. S5B). Combined inhibition of CHK1 and WEE1 led to a greater decrease in CDK1-Y15 phosphorylation than WEE1 inhibitor alone (Fig. 4E), consistent with the rapid engagement of mitosis with dual inhibition (Fig. 4B).

To examine the possibility that WEE1 inhibition interfered with induction and maintenance of the intra–S-phase check- point, CAL120 cells were pulse-labeled with bromodeoxyuri- dine (BrdU) followed by treatment with the topoisomerase I poison camptothecin (Fig. 4E). The recovery of DNA replication in S-phase cells was determined by monitoring cell-cycle progression of the BrdU-labeled cells. Camptothecin induced a substantial S-phase delay due to engagement of the intra–S-phase checkpoint [Fig. 4F (iii)]. AZD7762 abrogated the camptothecin-induced S-phase delay, causing BrdU-labeled cells to progress into G<sub>2</sub> phase at 8 hours after camptothecin [Fig. 4F (v)]. In contrast, MK-1775–treated BrdU-labeled cells showed a similar delay in S-phase progression as controls [Fig. 4F, (iv) vs. (iii), respectively], suggesting that WEE1 is not required for maintenance of the intra–S-phase checkpoint.

### Combined Inhibition of CHK1 and WEE1 Forces Cells into Mitosis

The intra–S-phase checkpoint is predominantly regulated by the checkpoint kinases ATR and CHK1 (4). In response to replicative stress, CHK1 is activated by ATR through phosphorylation at Ser317 and Ser345 (19). CHK1 inhibits the CDC25 phosphatases, keeping CDK1 in its inactive Y15-phosphorylated state (20). Inhibition of WEE1 per se is insufficient to result in CDK1-Y15 dephosphoryla- tion, if CDC25 phosphatases are also inactivated by CHK1. Consistent with this, we observed only partial loss of CDK1-Y15 phosphorylation with WEE1 kinase inhibition alone (Fig. 4D).

To further investigate the role of CHK1 in preventing unscheduled mitotic entry induced by WEE1 inhibition, we treated CAL120 and MCF7 cells with gemcitabine for 24 hours followed by CHK1 inhibitor AZD7762 (21) alone or in combination with MK-1775. Inhibition of CHK1 in gemcitabine-arrested cells induced premature mitosis in 33% of the CAL120 cells and only 2% in the MCF7 cells (Fig. 4A). Time course analysis revealed that within 1 hour after addition of both inhibitors, 52% of the cells had entered mitosis, compared with 4% and 14% treated with AZD7762 or MK-1775 alone, respectively (Fig. 4B).

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### Molecular Determinants of Unscheduled Mitotic Entry

We investigated the molecular changes that predispose to unscheduled mitotic entry. Unscheduled mitotic entry after gemcitabine and MK-1775 combination treatment was blocked by cotreatment with the selective CDK1 inhibitor RO-3306 (Fig. 5A). Silencing of CDK1 with siRNA, but not CDK2, blocked unscheduled mitosis (Fig. 5B; Supplementary Fig. S6A), confirming that CDK1, not traditional S-phase CDKs, triggered entry into mitosis.

In TP53 wild-type MCF7 cells, silencing of both p53 and p21<sup>Waf1/Cip1</sup> predisposed to forced mitotic entry by WEE1 inhibition (Fig. 5C; Supplementary Fig. S6B). This suggested that in TP53 wild-type cells, both p53 and p21<sup>Waf1/Cip1</sup> prevent activation of CDK1 in S-phase. We examined changes in expression of mitotic proteins in MCF7 and CAL120 cells in response to gemcitabine, MK-1775, or the combination (Fig. 5D). The expression of mitotic genes in normal cells starts at the beginning of S-phase and peaks during G<sub>2</sub> phase in preparation for mitotic entry. In response to DNA damage, expression of the mitotic genes CCNB1 and CD22 is suppressed in a process mediated by p53 and p21<sup>Waf1/Cip1</sup> (22). After gemcitabine induced early S-phase arrest (Fig. 1C), TP53 wild-type MCF7 cells expressed relatively low levels of mitotic proteins, whereas TP53-mutant CAL120 cells expressed high levels of mitotic proteins and CDK1-Y15 phosphorylation (Fig. 5D). In CAL120 cells, the expression of CDK1 varied with different cell-cycle phases, in a similar manner to cyclin B1 expression (Supplementary Fig. S7A).

We investigated the factors that promote substantial unscheduled mitosis in some TP53-mutant cell lines, but not in others (Fig. 1E). We have previously published whole-genome gene expression profiles for 20 TP53-mutant breast cancer cell lines used in this study (23). The gene expression levels for each individual probe were correlated with gemcitabine/ MK-1775 CI using Pearson correlation coefficient (Fig. 5E). We hypothesized that genes correlating positively with the CI (correlating with lack of synergy) may be involved in resistance to unscheduled mitotic entry, and conversely genes with a negative correlation (correlating with synergy) may promote unscheduled mitotic entry. Because of the small number of cell lines examined relative to the large number of gene expression probes, this analysis would have a high false discovery rate but could also highlight genes and processes that would be validated by subsequent functional experiments. High expression of cyclin D1 was correlated with lack of synergy (r = 0.46, P = 0.041; Pearson correlation coefficient), whereas high expression of cyclin E1 (r = −0.46, P = 0.040) and B-type cyclins (r = −0.38, P = 0.09) was correlated with synergy.

To confirm the observation that high level of mitotic genes may predispose to mitotic entry, we assessed cyclin B1 and CDK1 expression levels in a larger panel of cell lines (Fig. 5F; Supplementary Fig. S7B). Cyclin B1 protein level correlated with CCNB1 mRNA gene expression data (r = 0.7), supporting the gene expression analysis and suggesting that cyclin B1 protein levels were regulated by transcription. Although gemcitabine induced an early S-phase arrest (Supplementary Fig. S2B), a robust induction of cyclin B1 and CDK1 expression was observed, particularly in the cell lines that showed
Figure 4. Combined inhibition of WEE1 and CHK1 forces cells into mitosis. A, mitotic index of CAL120 and MCF7 cells treated with gemcitabine (Gem) for 24 hours followed by AZD7762, MK-1775, or the combination for another 8 hours. B, time course analysis of the mitotic index after CHK1 or WEE1 inhibition in gemcitabine-arrested CAL120 cells. Cells were pretreated with gemcitabine for 24 hours, followed by treatment with AZD7762 (white bars), MK-1775 (green bars), or the combination (red bars). C, mitotic index of CAL120 and MCF7 cells treated with AZD7762, MK-1775, or the combination for 8 hours. Treatment with combination AZD7762 and MK-1775 induces unscheduled mitotic entry in cells with <4N DNA content (AZD7762 0.2% vs. combination 12%, MK-1775 1.1% vs. combination 12%, * P < 0.002; Student t test). D, representative cell-cycle profiles of CAL120 cells treated with AZD7762, MK-1775, or the combination for 8 hours assessed by pH3/PI FACS. E, CAL120 cells were either untreated or pretreated with gemcitabine for 24 hours followed by treatment with AZD7762 and/or MK-1775 for an additional 2 hours (top) or 8 hours (bottom), before lysis. Western blot analysis of Cyclin B1, CDK1 (phospho-Y15 and total) expression, and β-tubulin as loading control. F, induction of the intra–S-phase checkpoint was not affected by WEE1 inhibition. CAL120 cells were pulse-labeled with 10 μmol/L BrdU for 30 minutes, washed (W), and then treated with 1 μmol/L camptothecin (CPT) for 30 minutes. After CPT removal (0 hours), BrdU-labeled S-phase cells (BrdU+, indicated by boxed area) were monitored at the indicated time points in the absence (iii) or presence of MK-1775 (iv) or AZD7762 as a positive control (v). Control cells (ctr) were not exposed to CPT and cultured in the absence (i) or presence of MK-1775 (ii). Arrowheads indicate delayed S-phase progression.
Figure 5. Molecular determinants of unscheduled mitosis after WEE1 inhibition. 

A, premature mitotic entry of gemcitabine-arrested CAL120 cells is blocked by CDK1 inhibitor. CAL120 cells were treated with gemcitabine for 24 hours before addition of MK-1775 alone or in combination with 10 μmol/L RO-3306 (CDK1 inhibitor). Representative histograms show pH3 fluorescent intensity at the indicated time points after MK-1775/RO-3306 addition. 

B, silencing of CDK1, but not CDK2, blocks premature mitotic entry. CAL120 cells were transfected with siCON or siRNA SMARTpools targeting CDK1 or CDK2 for 24 hours before gemcitabine and MK-1775 combination treatment (+Gem/MK). Mitotic cells were quantified by pH3/PI FACS analysis. 

C, MCF7 cells were transfected with siCON or 2 different siRNA duplexes targeting p21Waf1/Cip1 (sip21) or TP53 (siTP53) for 48 hours before gemcitabine/MK-1775 treatment (+Gem/MK). Mitotic index was determined by pH3/PI FACS analysis. p21Waf1/Cip1 silencing was confirmed by Western blotting (Supplementary Fig. S6B). *, P < 0.003 compared with siCON; Student t-test. 

D, Western blot analyses of lysates from CAL120 and MCF7 cells treated with gemcitabine and/or MK-1775, probed for WEE1, cyclin B1, CDK1 (phospho-Y15 and total), p21Waf1/Cip1, and actin as loading control. MCF7 and ZR75.1 are TP53 wild-type and the other cell lines TP53-mutant.

E, correlation of whole-genome gene expression with gemcitabine/MK-1775 CI in TP53-mutant breast cancer cell lines. Displayed are Pearson correlation coefficients for the 8,806 expression probes. Correlation coefficients of CDKN1A (r = 0.81, P < 0.0001), EZH2 (r = -0.58, P = 0.009), and probes for indicated cyclin genes are illustrated. 

F, indicated cells were cultured in the presence or absence of gemcitabine for 24 hours, harvested, and subjected to Western blotting with antibodies against WEE1, cyclin B1, CDK1 (phospho-Y15 and total), p21Waf1/Cip1, CHK1 (phospho-S317), and β-tubulin as loading control. MCF7 and ZR75.1 are TP53 wild-type and the other cell lines TP53-mutant.
Forced Mitotic Entry by WEE1 Inhibition

We treated WiDr xenografts with gemcitabine followed 24 hours later by MK-1775. Within 8 hours of MK-1775 treatment, a substantial increase in pH3-positive cells was seen, with H3 phosphorylation in interphase nuclei indicative of incipient mitotic entry (Fig. 7B and C). Treatment of xenografts with gemcitabine induced an early S-phase arrest (Supplementary Fig. S9B), suggesting that the pH3 induction occurred in early S-phase cells. For efficacy experiments, we used the WiDr derivative HT-29 that grows robustly in nude mice. In this model, prolonged MK-1775 exposure induced mitoses characteristic of forced mitosis following combination treatment (Fig. 7D and E), and the combination was substantially more effective at reducing tumor growth than either treatment given alone (Fig. 7F).

DISCUSSION

In this study, we show that forced mitotic entry of S-phase arrested cancer cells provides a potential novel therapeutic approach for aggressive breast cancers. In cell lines that showed synergy between WEE1 kinase inhibitor MK-1775 and chemotherapy, MK-1775 triggered mitotic entry in S-phase–arrested cells. Forced mitotic entry before completion of DNA synthesis triggered a highly abnormal mitosis characterized by dispersed chromosomes and highly disorganized bipolar spindles. Subsequently, cells “slipped” through the forced mitosis, with mitotic exit resulting in gross micromenuli formation and apoptosis.

Prior investigations of the combination of MK-1775 and chemotherapy have focused on disruption of the classical G2–M checkpoint as a mechanism by which WEE1 inhibition sensitizes to DNA-damaging agents (6–13, 28). However, we show here that p53-deficient breast cancer cells engage the intra-S-phase checkpoint and arrest in early S-phase in response to gemcitabine (Fig. 1C; Supplementary Fig. S2B). Therefore, understanding the role of WEE1 during S-phase rather than the classical G2–M checkpoint is key to clarifying the mechanism of sensitivity to WEE1 inhibitors. Multiple p53 and p21Waf1/Cip1 functions protect against unscheduled mitosis (Fig. 7G). p53 and p21Waf1/Cip1 inhibit mitotic gene transcription in response to DNA damage switching the LINC complex from active to repressive states (22, 29) and p21Waf1/Cip1 directly inhibits CDK1 activity (30–32). In contrast, in TP53-mutant cancer cells, loss of p53 and p21Waf1/Cip1 function makes cells more reliant on the CHK1-dependent intra–S-phase checkpoint in response to DNA damage (CHK1 pS317; Fig. 5F; Supplementary Fig. S7B), while at the same time, mitotic gene expression is deregulated and WEE1 becomes critical to restrain CDK1 activity.

This model suggests that the primary role of chemotherapy in combination with WEE1 inhibition is to arrest cells in S-phase to allow accumulation of mitotic gene expression to levels that are sufficient to trigger mitosis. WEE1 inhibition alone appears insufficient to trigger mitosis in the absence of S-phase perturbation, potentially as a critical level of CDK1 activity is not achieved. The combination of WEE1 and CHK1 inhibition was sufficient to force mitosis during unperturbed late S-phase (Fig. 4C and D), potentially as CDK1 activity reaches the critical level required to trigger mitosis. However, the combination of both inhibitors may have additional

high levels of premature mitosis. In contrast, TP53 wild-type cell lines MCF7 and ZR75.1 did not show a strong induction of cyclin B1 and CDK1, confirming that p53 is involved in suppressing mitotic gene expression upon DNA damage (22). CHK1 was activated (phospho-Ser317) in response to gemcitabine in all TP53-mutant cell lines but neither of the TP53 wild-type lines.

Taken together, these data suggest that the switch from expression of G1-S cyclins to late S-G2-M cyclins, which is known to occur in poor prognosis breast cancers (24), is associated with synergy, and following S-phase arrest, TP53-mutant cells become reliant on CHK1–WEE1 function to prevent mitotic entry.

WEE1 Inhibition as a Potential Therapeutic Strategy for Poor Prognosis Breast Cancer

We noted that in TP53-mutant cell lines, high levels of EZH2 expression correlated with synergy (r = −0.58, P = 0.0099; Fig. 6A), and conversely high levels of CDKN1A (p21Waf1/Cip1) expression correlated with lack of synergy (r = 0.81, P < 0.0001; Fig. 6B). We silenced these genes to examine whether they contributed to the forced mitotic phenotype in TP53-mutant cells. Silencing of EZH2 with 2 independent siRNAs reduced unscheduled mitosis in CAL120 cells (Fig. 6C; Supplementary Fig. S8A). In contrast, silencing of p21Waf1/Cip1 did not induce unscheduled mitotic entry in TP53-mutant SUM44 and JIMT-1 cells (Fig. 6D; Supplementary Fig. S8B), suggesting that residual p21Waf1/Cip1 in TP53-mutant cell lines was not responsible for preventing premature mitotic entry.

It has previously been shown that EZH2 negatively regulates p21Waf1/Cip1 expression (25), and EZH2 gene expression was negatively correlated with CDKN1A expression in a publicly available data set of TP53-mutant breast cancers (Fig. 6E). This suggested that the observed correlation between CDKN1A and EZH2 expression was particularly robust in identifying a poor prognostic group (Fig. 6G), suggesting that high expression of both EZH2 and mitotic cyclins may promote an aggressive breast cancer phenotype while providing a permissive environment for unscheduled mitosis that could potentially be targeted therapeutically by WEE1 inhibitor combinations. Interestingly, basal breast cancer cell lines showed a greater degree of synergy between MK-1775 and gemcitabine than luminal cell lines (Fig. 6H).

In Vivo Efficacy of Gemcitabine and MK-1775

We examined for potential efficacy of MK-1775 and gemcitabine combinations in vivo and examined for evidence of forced mitosis. Because we were unable to robustly xenograft the syngeneic breast cancer cell line CAL120, we used the WiDr colon cancer model (7, 8), which showed a greater degree of synergy in vitro (Fig. 7A; Supplementary Fig. S9A).
Figure 6. High expression of mitotic cyclins and EZH2 in poor prognosis breast cancer. A, correlation of gemcitabine/MK-1775 CI and EZH2 expression in TP53-mutant breast cancer cell lines with Pearson correlation coefficients, $r = -0.58$, $P = 0.009$. B, correlation of gemcitabine/MK-1775 CI and CDKN1A expression in TP53-mutant breast cancer cell lines with Pearson correlation coefficients. C, CAL120 cells were transfected with siCON and 2 different EZH2 siRNA duplexes or SMARTpool, treated with gemcitabine/MK-1775 48 hours after transfection. Mitotic index was determined by pH3/PI FACS analysis. EZH2 silencing was confirmed by Western blotting (Supplementary Fig. S8A). D, SUM44 and JIMT-1 TP53-mutant cells were transfected with siCON or p21/Waf1/Cip1 siRNA SMARTpool and treated with gemcitabine/MK-1775 48 hours after transfection. Mitotic index was determined by pH3/PI FACS analysis. E, correlation between gene expression of EZH2 and CDKN1A in publicly available series of 251 breast cancers, with TP53 wild-type cancers (left, $n = 193$, $r = -0.09$, $P = 0.2$) and TP53-mutant cancers (right, $n = 58$, $r = -0.47$, $P = 0.0002$), with Pearson correlation coefficients. F, correlation between gene expression of EZH2 and CCNB1 in publicly available series of 251 breast cancers, $r = 0.51$, $P < 0.0001$. G, Kaplan–Meier survival curves of overall survival in publicly available series of 251 breast cancers, according to expression of CCNB1 (left: HR, 2.175; 95% confidence interval, 1.272–3.721; $P = 0.0046$), EZH2 (middle: HR, 2.120; 95% confidence interval, 1.240–3.624; $P = 0.006$), and mean CCNB1–EZH2 expression (right: HR, 2.841; 95% confidence interval, 1.645–4.306; $P = 0.0002$). Comparison is between expression $<$ median and $\geq$ median with log-rank test. H, CI of degree of synergy between gemcitabine and MK-1775, displayed according to cell line subtype, $P = 0.028$; Mann–Whitney U test.
Forced Mitotic Entry by WEE1 Inhibition

**Figure 7.** In vivo efficacy of gemcitabine and MK-1775. 
A, WiDr cells were treated with gemcitabine (Gem), hydroxyurea (HU) for 24 hours, or synchronized (Synch) by a double thymidine block (T) after which MK-1775 was added for an additional 8 hours. Mitotic index was determined by pH3/PI FACS analysis. 
B, quantification of pH3 immunofluorescence in WiDr xenografts. Mice bearing WiDr xenografts (6 for each group) were dosed with gemcitabine (Gem; 100 mg/kg, i.p.) and MK-1775 (MK; 30 mg/kg, p.o.) 24 hours later. Tumors were taken 8 hours after MK-1775 administration and analyzed by pH3 immunofluorescence. Assessment was blinded to treatment allocation. Combination (Gem/MK) treatment increased pH3-positive cells compared with all other treatments ($P < 0.05$; Student t test). Ctr, control. 
C, representative immunofluorescent images of tumors treated as described in (B) red, pH3; blue, DAPI (DNA). 
D, representative images of pH3 immunofluorescence in WiDr (HT-29) tumors dosed with MK-1775 (MK; 15 or 30 mg/kg, p.o.) for 2.5 days (5 doses) after gemcitabine (Gem; 100 mg/kg, i.v.). Arrows indicate forced mitotic phenotype. 
E, proportion of mitotic cells with the forced mitotic phenotype in tumors treated as described in (D), scored blind in 3 tumors per condition. Veh, vehicle. 
F, mice bearing WiDr (HT-29) tumors were dosed with vehicle, gemcitabine once a week at 100 mg/kg (i.v.), MK-1775 (15 or 30 mg/kg, p.o.), or the combination (10 mice for each group). Combination treatment reduced tumor growth to a greater extent than individual treatments (repeated measures ANOVA: 15 mg/kg, $P = 0.011$; 30 mg/kg, $P = 0.008$). 
G, regulation of CDK1 activity in normal and TP53-mutant cancer cells in S-phase. In normal cells, CDK1 activity is negatively regulated by multiple mechanisms including Y15 phosphorylation by WEE1 and restriction of high cyclin B1 levels to G2 phases of the cell cycle. After DNA damage in S-phase, the CDC25 phosphatases are negatively regulated by CHK1, and p53 and p21 suppress mitotic gene expression. In TP53-mutant cancer cells, the functions of p53 and p21 are lost. In cancers with an aggressive oncogenic phenotype, this is combined with high expression of mitotic genes and EZH2. After S-phase delay, cyclin B1 levels reach a level sufficient to trigger mitotic entry during S-phase and cancer cells become reliant on WEE1 to restrain CDK1 activity to prevent unscheduled mitotic entry direct from S-phase.
Inhibitors are in early clinical development and we look for proportion of cancer cell lines show synergy (Fig. 1A). WEE1 clinical development of WEE1 inhibitors, as only a restricted CHK1 inhibition. cancers but likely only in combination with chemotherapy or U test, data not shown). Therefore, we believe that WEE1 inhibition with siRNA may have different phenotypes.

The oncogenic transformation of aggressive breast cancers results in a permissive environment for unscheduled mitotic entry, characterized by TP53 mutation and high levels of cyclin B1 and EZH2 (Fig. 7G; refs. 24, 40–43). A current major focus of breast cancer research is triple-negative and basal-like breast cancers because of the lack of effective therapies. Therefore, forced mitotic entry with WEE1 inhibitors represents a novel therapeutic approach that potentially targets the consequences of oncogenic transformation without requiring a direct understanding of the individual oncogenic mutations present.

METHODS

Cell Lines and siRNA Transfection

Cell lines were obtained from American Type Culture Collection or Asterand (Supplementary Table S1) and maintained in phenol red-free Dulbecco’s Modified Eagles’ Media (DMEM) or RPMI with 10% FBS (FBS Gold, PAA Laboratories) and 2 mM L-glutamine (Sigma-Aldrich). All cell lines were banked in multiple aliquots on red–free Dulbecco’s Modified Eagles’ Media (DMEM) or RPMI with 10% FBS (FBS Gold, PAA Laboratories) and 2 mM L-glutamine (Sigma-Aldrich). The following chemical inhibitors were used at the indicated concentrations, unless stated otherwise, WEE1 inhibitor MK-1775 (1 μmol/L; Merck), CHK1 inhibitor AZD7762 (100 μmol/L, Selleck Chem), CDK1 inhibitor RO-3306 (10 μmol/L, Torcis), gemcitabine (100 μmol/L), camptothecin (1 μmol/L), hydroxyurea (3 μmol/L), thymidine (2.5 μmol/L), and nocodazole (200 ng/mL; all from Sigma-Aldrich).

Antibodies were used as WEE1 (sc-5285), PARP-1 (sc-8007), CDK2 (sc-163), and actin (sc-1616; all from Santa Cruz Biotechnology); CHK1-Ser317 (A300-163A; Bethyl Laboratories); phospho-Histone H3-Ser10 (06-570), phospho-Histone-H2AX-Ser139 (05-636), and
Flow Cytometry
Cells were seeded in 60-mm dishes (2 × 10^5 cells per dish), treated for 24 hours after plating with 100 nmol/L gemcitabine for 24 hours, after which 1 μmol/L MK-1775 or 100 nmol/L AZD7762 was added to gemcitabine for another 8 hours. Cells were fixed in ice-cold 70% ethanol, permeabilized with 0.25% Triton X-100 in PBS, incubated with 1 μg/mL anti-pH3 antibody for 2 hours at 4°C, followed by a secondary antibody conjugated to AlexaFluor 488 for 1 hour at 4°C. DNA was stained with 20 μg/mL propidium iodide (PI) in the presence of 100 μg/mL RNase A.

For intra-S-phase checkpoint assessment, CAL120 cells were incubated with 10 μmol/L BrdU (Sigma-Aldrich) for 30 minutes, washed, and then treated with 1 μmol/L camptothecin for 30 minutes, washed, and grown in drug-free medium for 2 hours, after which MK-1775 or AZD7762 were added for another 22 hours. Ethanol-fixed cells were incubated in pepsin (0.4 mg/mL in 0.1N HCl) for 30 minutes at room temperature. DNA was denatured using 2N HCl and neutralized with 0.1 mol/L sodium borate. Cells were labeled with fluorescein isothiocyanate–conjugated anti-BrdU antibody (347583; Becton Dickinson) and DNA was stained with PI. Signals were analyzed on a BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences).

Western Blot Analysis
Cells were plated on 100-mm dishes, treated as indicated, and whole-cell extracts were prepared using NP-40 lysis buffer [10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 8.0, 1% NP-40, 0.5 mmol/L sodium pyrophosphate, 50 mmol/L Na3VO4, 5 mmol/L dithiothreitol (DTT), complete protease inhibitor cocktail (Roche)]. Lysates were run on precast 4% to 12% Bis–Tris gels (Invitrogen) and transferred onto nitrocellulose membranes (Bio-Rad). Signals were visualized using enhanced chemiluminescence (Amersham ECL gen) and transferred onto nitrocellulose membranes (Bio-Rad). Lysates were run on precast 4% to 12% Bis–Tris gels (Invitrogen) and transferred onto nitrocellulose membranes (Bio-Rad). Signals were visualized using enhanced chemiluminescence (Amersham ECL). The authors acknowledge NHS funding to the NIHR Biomedical Research Centre.

Immunofluorescence
CAL120 cells were plated on coverslips and treated with chemical inhibitors the following day as described above. After drug treatment, cells were fixed in 4% paraformaldehyde for 1 hour, washed, permeabilized in 0.2% Triton X-100 in PBS for 20 minutes, washed, and blocked in IFP [1% bovine serum albumin, 2% PBS in PBS] followed by incubation with primary and secondary AlexaFluor-conjugated antibodies for 1 to 2 hours at room temperature each. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Coverslips were mounted on glass slides using Vectashield. Images were acquired on a Leica confocal microscope (×63 oil immersion objective). Mitotic and nuclear phenotypes of at least 200 cells per condition were scored for 2 independent replicate experiments.

For preparation of chromosome spreads, CAL120 cells were treated with KaryoMAX Colcemid solution (20 ng/mL; Gibco-Invitrogen) for 2 hours before harvesting. Cells were treated with 30 mmol/L NaCitrate for 15 minutes at 37°C, fixed in methanol:acetic acid (3:1), and dropped onto glass slides. Chromosomes were stained with DAPI.

Xenograft Experiments
All animal experiments were conducted in accordance with local good animal practice. For the WiDr xenograft experiments, nonobese diabetic/severe combined immunodeficient female mice ages 5 to 6 weeks were obtained from Charles River Laboratories. Mice were inoculated with 5 × 10^6 WiDr cells in 100 μL (1:1 PBS: growth factor–reduced Matrigel; BD Biosciences) subcutaneously (s.c.) into both flanks. Gemcitabine was administered intraperitoneally (i.p.) at 100 mg/kg (in PBS), and 24 hours later, MK-1775 was administered at a dose of 30 mg/kg (in 0.5% methylcellulose) by oral gavage (p.o.). Tumors were taken 8 hours after MK-1775 administration for fluorescence-activated cell-sorting (FACS) analysis and pH3 immunofluorescence. For efficacy experiments, we used the WiDr derivative HT-29 that grows robustly in nude mice. CD-1 nude female mice ages 4 to 6 weeks were obtained from Charles River Laboratories. Mice were inoculated with 2.5 × 10^6 to 5 × 10^6 WiDr (HT-29) cells in 100 μL (1:1 Matrigel–PBS) s.c. into the right flank. When tumor volume reached 325 mm^3 (±70), mice were randomized to treatment groups. For efficacy analysis, gemcitabine was administered i.v. at 100 mg/kg once per week, and 24 hours later, MK-1775 was administered at 15 or 30 mg/kg, p.o., twice daily (3 hours). Tumor measurements were recorded biweekly and tumor volume was calculated as (width × length × length)/2. For pH3 immunofluorescent analysis, gemcitabine treatment was followed by MK-1775 for 2.5 days (5 doses), with tumors collected 2 hours after the final dose, fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned.

Statistical Analysis and Analysis of Gene Expression Data
All statistical tests were conducted with GraphPad Prism version 5.0. Unless stated otherwise, P values were 2-tailed and considered significant if P < 0.05. Error bars represent SEM of 3 experiments unless stated otherwise.

Cell line whole-genome gene expression data were assessed on Illumina human-refs v2 BeadChips as previously described (23). Gene expression, from probes detectable in more than 90% of cell lines, was correlated with gemcitabine/MK-1775 CI using Pearson correlation coefficient. Gene expression data on a publicly available data set of TP53 wild-type and mutant cell lines was analyzed by Miller and colleagues (46). Kaplan–Meier survival curves were plotted for EZH2, CCNB1, and mean of EZH2 and CCNB1, in 2 groups, gene expression < median versus ≥ median.

Disclosure of Potential Conflicts of Interest
M.S. Hurd and S.D. Shumway are employees of Merck Sharp & Dohme Corp. C. Toniatti was previously an employee of Merck Sharp & Dohme Corp. No potential conflicts of interests were disclosed by other authors.

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