Forced mitotic entry of S-phase cells as a therapeutic strategy induced by inhibition of WEE1

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Running title: Forced mitotic entry by WEE1 inhibition

Keywords: WEE1, MK-1775, breast cancer, CHK1, mitosis

Financial support: M.A., R.S., I.G.M., A.A., N.T. were supported by Cancer Research UK (A10038) and Susan G. Komen for the Cure. M.A. is supported by the Netherlands Organisation for Scientific Research (NWO).

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Conflict of interest: M.S.H and S.D.S are employees of Merck Sharp & Dohme Corp. C.T. was previously an employee of Merck Sharp & Dohme Corp.

Word count: 5635

Total no. of figures: 7 figures, 9 supplementary figures and 2 supplementary tables
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Abstract

Inhibition of the protein kinase WEE1 synergises 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 S-phase arrested cells directly into mitosis without completing DNA synthesis, resulting in highly abnormal mitoses characterised by dispersed chromosomes and disorganised 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 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.
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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 checkpoint), the initiation of mitosis (G2-M checkpoint), and the initiation of anaphase (spindle checkpoint) (1). The disruption of these checkpoints provides a potential approach to cancer treatment, synergizing with chemotherapy to drive cells inappropriately onwards 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 CDK1 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 (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
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cells into mitosis with unrepaired DNA damage (reviewed in (5)). Previous work with chemical inhibitors of WEE1 and siRNA-mediated depletion (6) suggested that WEE1 inhibition abrogated the G2 checkpoint and selectively sensitised p53-deficient cells to various DNA-damaging agents, such as gemcitabine, carboplatin and cisplatin (7, 8) and inhibited tumour growth in \textit{in vivo} models (7, 9). In addition, WEE1 inhibition or depletion sensitized tumour cells to ionizing radiation (10-13).

However, the proposed mechanism of sensitisation 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 (dNTPs) 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 demonstrate that WEE1 inhibitors have a distinct and novel mechanism of action, forcing cells arrested in S-phase directly into mitosis. We go on to show that this mechanism is also shared, although to a lesser extent, by inhibitors of CHK1. We explore the pathophysiological 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 characterised by \textit{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 cell lines (23 breast cancer cell lines and 2 breast epithelial cell lines; Figure 1A and Supplementary Table 1), 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% (11/25) of the cell lines. As demonstrated previously, TP53 wild-type cell lines displayed no evidence of substantial synergy (7), nor did the untransformed breast epithelial cell lines. There was a substantial variation in the degree of synergy in the TP53 mutant cell lines (Figure 1A).

To examine the mechanisms underlying synergy, we initially chose to study two model cell lines: MCF7 (non-synergistic, CI=1.04; TP53 wild-type) and CAL120 (synergistic, CI=0.61; TP53 mutant) (Figure 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 to 27.8%, MCF7 4N mitotic cells: 1.9 to 4.9%; Figure 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 (Figure 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 (HU).
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treatment or by a double thymidine block (Figure 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 Figure 1A) and siRNA-mediated silencing of WEE1 (Supplementary Figure 1B), confirming the specificity of the results obtained with MK-1775.

We assessed mitotic entry in an expanded set of breast cancer cell lines (Figure 1E, Supplementary Figures 2A and 2B). Unscheduled mitosis, prior to completion of DNA synthesis, was seen in multiple TP53 mutant cell lines upon gemcitabine and MK-1775 treatment, but not in TP53 wild-type cell lines (Figure 1E). Time course analysis revealed that unscheduled mitosis occurred earlier (after 6 hours; Supplementary Figure 3A) and at lower doses of MK-1775 (300 nM; Supplementary Figure 3B) in the synergistic cell lines, while non-synergistic cell lines responded only to 1 μM MK-1775 at later time points (after 12 hours). This suggests that unscheduled entry into mitosis is the predominant mechanism underlying synergy between 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’s correlation coefficient).

Taken together, these results demonstrate 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.
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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, while cells with condensed chromosomes typically aligned at the metaphase plate were observed after MK-1775 treatment (Figure 2A). In contrast, gemcitabine and MK-1775 treatment resulted in highly abnormal mitoses with unaligned and partially condensed chromosomes (Figures 2A and 2B), showing characteristics of S-phase premature chromosome condensation (PCC) (16). To assess this further, we examined microtubule organisation by labelling cells treated with HU and MK-1775 for α-tubulin and phospho-Histone H3 (pH3) (Figure 2C). After HU and MK-1775 combination treatment, 65% of the pH3-positive cells showed highly disorganised 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-1175 alone (Figures 2C and 2D). 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 pH3, 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 HU, followed by addition of MK-1775 for 8 hours or continued HU treatment alone. Treatments were washed out and cell cycle progression was monitored by flow cytometry. HU-arrested control cells readily re-entered the cell cycle and reached late S/G2 phase after 8 hours, went through mitosis at 16 hours after release, and
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subsequently progressed into the next cell cycle (Figure 3A, Supplementary Table 2). In contrast, cells treated with HU and MK-1775 progressively exited mitosis, eight 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 (Figure 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 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 performed a detailed time course analysis (Figures 3B and 3C). 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 (Figure 3B). As the majority of cells exited mitosis, 10-14 hours post MK-1775 addition (Figure 3B), cyclin B1 levels decreased and CDK1-Y15 phosphorylation partially increased (Figure 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 (Figures 3D and 3E). Accompanying mitotic exit, PARP-1 was cleaved indicating engagement of apoptosis (Figure 3C). Substantial induction of apoptosis was confirmed with both Annexin V staining (Supplementary Figures 4A and
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4B) and caspase 3/7 activation (Supplementary Figure 4C). This suggested that on mitotic exit the dispersed chromosomes were unable to assemble into a single nucleus, resulting in apoptosis or long-term cell cycle arrest.

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 dephosphorylation 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 (Figure 3C).

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 (Figure 4A). Time course analysis revealed that within 1 hour after addition of both inhibitors, 52% of the cells had entered mitosis, compared to 4% and 14% treated with AZD7762 or and MK1775 alone, respectively (Figure 4B).

We extended these observations by demonstrating that the combination 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; Figures 4C and 4D). No induction of mitosis was seen with the combination in MCF7 cells (Figure
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AZD7762 increased the sensitivity of CAL120 cells to MK-1775 alone, shifting the MK-1775 IC50 from 329 nM to 75 nM (Supplementary Figure 5A). We explored the molecular basis for the synergistic interaction between CHK1 and WEE1 inhibition in CAL120 cells (Figure 4E, Supplementary Figure 5B). Combined inhibition of CHK1 and WEE1 led to a greater decrease in CDK1-Y15 phosphorylation than WEE1 inhibitor alone (Figure 4E), consistent with the rapid engagement of mitosis with dual inhibition (Figure 4B).

To examine the possibility that WEE1 inhibition interfered with induction and maintenance of the intra-S-phase checkpoint, CAL120 cells were pulse-labelled with BrdU followed by treatment with the topoisomerase I poison camptothecin (CPT) (Figure 4F). The recovery of DNA replication in S-phase cells was determined by monitoring cell cycle progression of the BrdU-labelled cells. CPT induced a substantial S-phase delay due to engagement of the intra-S checkpoint (panel c). AZD7762 abrogated the CPT-induced S-phase delay, causing BrdU-labelled cells to progress into G2 phase at 8 hours post-CPT (panel e). In contrast, MK-1775 treated BrdU-labelled cells showed a similar delay in S-phase progression as controls (panel d versus panel c, respectively), suggesting that WEE1 is not required for maintenance of the intra-S-phase checkpoint.

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 co-treatment with the selective CDK1 inhibitor RO-3306 (Figure 5A). Silencing of CDK1 with siRNA, but not CDK2, blocked unscheduled mitosis (Figure 5B, Supplementary Figure 6A), confirming that CDK1, not traditional S-phase CDKs, triggered entry into mitosis.
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In TP53 wild-type MCF7 cells, silencing of both p53 as well as p21\textsuperscript{Waf1/Cip1}, predisposed to forced mitotic entry by WEE1 inhibition (Figure 5C, Supplementary Figure 6B). This suggested that in TP53 wild-type cells, both p53 and p21\textsuperscript{Waf1/Cip1} 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 (Figure 5D). The expression of mitotic cyclins in normal cells starts at the beginning of S-phase and peaks during G2 phase in preparation for mitotic entry. In response to DNA damage, expression of the mitotic genes CCNB1 and CDC2 is suppressed in a process mediated by p53 and p21\textsuperscript{Waf1/Cip1} (22). After gemcitabine induced early S-phase arrest (Figure 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 (Figure 5D). In CAL120 cells, the expression of CDK1 varied with different cell cycle phases, in a similar manner to cyclin B1 expression (Supplementary Figure 7A).

We investigated the factors that promote substantial unscheduled mitosis in some TP53 mutant cell lines, but not in others (Figure 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 combination index (CI) using Pearson Correlation Coefficient (Figure 5E). We hypothesised 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. Due to the small number of cell lines examined relative to the large number of gene expression probes this analysis would have a high false discovery...
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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’s Correlation Coefficient), whilst 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 (Figure 5F, Supplementary Figure 7B). 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 Figure 2B), a robust induction of cyclin B1 and CDK1 expression was observed, particularly in the cell lines that showed 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, this data suggest that the switch from expression of G1-S cyclins to late S-G2-M cyclins, that 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
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We noted that in TP53 mutant cell lines high levels of EZH2 expression correlated with synergy (r=-0.58, P=0.009; Figure 6A), and conversely high levels of CDKN1A (p21Waf1/Cip1) expression correlated with lack of synergy (r=0.81, P<0.0001; Figure 6B). We silenced these genes to examine whether they contributed to the forced mitotic phenotype in TP53 mutant cells. Silencing of EZH2 with two independent siRNAs reduced unscheduled mitosis in CAL120 cells (Figure 6C, Supplementary Figure 8A). In contrast, silencing of p21Waf1/Cip1 did not induce unscheduled mitotic entry in TP53 mutant SUM44 and JIMT-1 cells (Figure 6D, Supplementary Figure 8B), 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 (Figure 6E). This suggested that the observed correlation between CDKN1A and CI (Figure 6B) may be reflective of the negative regulation of CDKN1A expression by EZH2 in TP53 mutant cell lines. High expression of the polycomb protein EZH2 is associated with poor prognosis in breast cancer (26), and has been shown to promote mitotic entry (27). Expression of CCNB1 and EZH2 are positively correlated in breast cancers (r=0.51, P<0.0001; Figure 6F). Combined assessment of CCNB1 and EZH2 expression was particularly robust in identifying a poor prognostic group (Figure 6G), suggesting that high expression of both EZH2 and mitotic cyclins may promote an aggressive breast cancer phenotype whilst 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 (Figure 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. Since we were unable to robustly xenograft the synergistic breast cancer cell line CAL120, we utilised the WiDr colon cancer model (7, 8), which demonstrated a substantial degree of forced mitosis in vitro (Figure 7A, Supplementary Figure 9A).

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 (Figures 7B and 7C). Treatment of xenografts with gemcitabine induced an early S-phase arrest (Supplementary Figure 9B), suggesting that the pH3 induction occurred in early S-phase cells. For efficacy experiments, we utilised 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 (Figures 7D and 7E), and the combination was substantially more effective at reducing tumour growth than either treatment given alone (Figure 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 demonstrated synergy between WEE1 kinase inhibitor MK-1775 and chemotherapy, MK-1775 triggered mitotic entry in S-phase arrested cells. Forced mitotic entry prior to completion of DNA synthesis triggered a highly abnormal mitosis characterised by dispersed chromosomes and highly disorganised bipolar spindles. Subsequently, cells
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“slipped” through the forced mitosis, with mitotic exit resulting in gross micronuclei 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 (Figure 1C, Supplementary Figure 2B). 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 p21<sup>Waf1/Cip1</sup> functions protect against unscheduled mitosis (Figure 7G). p53 and p21<sup>Waf1/Cip1</sup> inhibit mitotic gene transcription in response to DNA damage switching the LINC complex from active to repressive states (22, 29) and p21<sup>Waf1/Cip1</sup> directly inhibits CDK1 activity (30-32). In contrast, in TP53 mutant cancer cells, loss of p53 and p21<sup>Waf1/Cip1</sup> function makes cells more reliant on the CHK1-dependent intra-S-phase checkpoint in response to DNA damage (CHK1 pS317, Figure 5F, Supplementary Figure 7B), whilst 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 (Figures 4C and 4D), potentially as CDK1 activity reaches the critical level required to trigger mitosis.
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However, the combination of both inhibitors may have additional effects other than inducing premature mitosis (33), and potential toxicity of the combination would need careful assessment prior to clinical development as the combination modestly sensitises the breast epithelial cell line MCF10A (Supplementary Figure 5A). Our data does emphasize the potential benefit of strategies to enhance forced mitotic entry. Only a restricted set of TP53 mutant cell lines showed evidence of synergy between gemcitabine and WEE1 inhibition in vitro (Figure 1A). Yet, forced mitotic entry could be triggered in the non-synergistic TP53 mutant cell lines SUM44 and MFM223 (Supplementary Figure 3), but only at levels insufficient to result in synergy, which suggests that there may be potential to exploit this mechanism of cell death in all TP53 mutant cancer cell lines.

During unperturbed cell cycle progression, CHK1 is localized at the centrosomes where it inhibits cyclin B-CDK1 activity during S-phase via phosphorylation of the CDC25 family phosphatases (34, 35). Chemical inhibition of CHK1 induces premature centrosome separation and activation of centrosome-associated CDK1 (34). Since centrosome separation and positioning are crucial for correct formation of a bipolar spindle, reviewed in (36), premature activation of centrosomal CDK1 could be the underlying mechanism for the disorganized mitotic spindle phenotype observed after WEE1 inhibition (Figure 2C).

Recent evidence has indicated that WEE1 silencing with siRNA results in stalled and collapsed replication forks in S-phase cells (37, 38), potentially resulting from disruption of the interaction between WEE1 and the Mus81-Eme1 endonuclease (39). However, S-phase delay was not observed with WEE1 inhibition by MK-1775 (Figures 1C and 4F, Supplementary Figure 2B). Potentially these differences simply reflect analysis at
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different time points and conditions, or the genetic background of the cell lines examined, although it is possible that inhibition of the kinase with a small molecule and silencing with siRNA may have different phenotypes.

The oncogenic transformation of aggressive breast cancers results in a permissive environment for unscheduled mitotic entry, characterised by TP53 mutation and high levels of cyclin B1 and EZH2 (Figure 7G) (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. This subtype has the highest frequency of TP53 mutation, as well as generally high expression of mitotic B-type cyclins and EZH2 (24, 40-43). In our study, basal breast cancer cell lines show a greater degree of synergy between MK-1775 and gemcitabine than luminal cell lines (Figure 6H). Although it has been reported that WEE1 inhibitors may target triple negative breast cancer cell lines as single agents (44), basal cancer cell lines were not more sensitive to single agent MK-1775 than luminal cell lines (median MK-1775 IC50 basal 310nM versus luminal 320nM, P=0.5 Mann-Whitney U test, data not shown). Therefore, we believe that WEE1 inhibition is a potential therapeutic approach for basal-like breast cancers, but likely only in combination with chemotherapy or CHK1 inhibition.

Our data emphasises the importance of biomarkers in the clinical development of WEE1 inhibitors, as only a restricted proportion of cancer cell lines demonstrate synergy (Figure 1A). WEE1 inhibitors are in early clinical development and we look forward to assessing the expression of CCNB1 and EZH2 in these studies, although further research will be required to ascertain whether similar predictors apply to cancer types other than breast cancer. High expression of the mitotic cyclins, and potentially EZH2, may be a downstream consequence of multiple different oncogenic drivers in aggressive...
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breast cancer. 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.

Materials and Methods

Cell lines and siRNA transfection

Cell lines were obtained from ATCC or Asterand (Supplementary Table 1), and maintained in phenol red free DMEM or RPMI with 10% fetal bovine serum (FBS Gold, PAA Laboratories), and 2 mM L-glutamine (Sigma-Aldrich). All cell lines were banked in multiple aliquots on receipt to reduce risk of phenotypic drift, and identity confirmed by STR profiling with the StemElite ID System (Promega) and arrayCGH profiling. Cell lines were assigned to luminal and basal subtypes as defined by Chin et al. (45),

For synergy analysis between gemcitabine and MK-1775, cells were plated in 96-well plates at 2000-5000 cells per well. The following day, cells were exposed to different fixed-ratio combinations of gemcitabine (dose range: 1.56-100 nM) and MK-1775 (dose range: 31.3 nM-2 μM), and survival was assessed after 72 hours exposure with the Cell Titer-Glo Luminescent Cell Viability assay (Promega). The combination index was determined according to the median-effect model of Chou-Talalay (15). Although a CI<1.0 can be inferred as synergy, we chose a cut-off of <0.75 to more stringently define synergy.

For siRNA transfections, cells were reverse transfected in 6-well plates with 50 nM siRNA using Lipofectamine RNAiMAX (Invitrogen) or DharmaFECT3
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(Dharmacon/Thermo Scientific) according to the manufacturers’ instructions. Drug treatments were started 48 hours post transfection, and protein extracts prepared 72 hours after transfection. siRNAs were obtained from Dharmacon: WEE1 siGENOME SMARTpool (siWEE1, M-005050-02), CDC2 siGENOME SMARTpool (siCDK1, M-003224-03) and individual siRNA duplexes (D-003224-09/10/11/12), CDK2 siGENOME SMARTpool (siCDK2, M-003236-04) and individual siRNA duplexes (D-003236-06/08/09/10), CDKN1A siGENOME SMARTpool (sip21, M-003471-00) and individual siRNA duplexes (D-003471-01/03), TP53 siGENOME SMARTpool (siTP53, M-003329-03) and individual siRNA duplexes (D-003329-05/26), EZH2 siGENOME SMARTpool (siEZH2, M-004218-03) and individual siRNA duplexes (D-004218-01/02), and siGENOME Non-Targeting siRNA #1 (siCON, D-001210-01).

Chemical inhibitors and antibodies

The following chemical inhibitors were used at the indicated concentrations, unless stated otherwise, WEE1 inhibitor MK-1775 (1μM; Merck), CHK1 inhibitor AZD7762 (100nM; Selleck Chem), CDK1 inhibitor RO-3306 (10μM; Tocris), gemcitabine (100nM), camptothecin (CPT; 1μM), hydroxyurea (HU; 3mM), thymidine (2.5mM), nocodazole (200 ng/ml; all from Sigma-Aldrich).

Antibodies used were WEE1 (sc-5285), PARP-1 (sc-8007), CDK2 (sc-163), Actin (sc-1616; all from Santa Cruz Biotechnology), CHK1-Ser317 (A300-163A; Bethyl Laboratories), phospho-Histone H3-Ser10 (pH3, 06-570), phospho-Histone-H2AX-Ser139 (05-636), EZH2 (05-1319; all from Upstate), α-Tubulin (T4026; Sigma-Aldrich), β-Tubulin (T4026; Sigma-Aldrich), cyclin B1 (4135), phospho-CDK1-Tyr15 (9111), CDK1
Forced mitotic entry by WEE1 inhibition

(9112), phospho-CDC25C-Ser216 (4901), CDC25C (4688), p21Waf1/Cip1 (2947; all from Cell Signalling Technology).

Flow cytometry

Cell were seeded in 60 mm dishes (2 × 10⁵ cells per dish), were treated 24 hours after plating with 100nM gemcitabine for 24 hours, after which 1μM MK-1775 or 100nM AZD7762 was added to gemcitabine for another 8 hours. Cells were fixed in ice-cold 70% ethanol, permeabilised with 0.25% Triton X-100 in phosphate-buffered saline (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 20μg/ml propidium iodide (PI) in the presence of 100μg/ml RNase A.

For intra-S-phase checkpoint assessment, CAL120 cells were incubated with 10μM bromodeoxyuridine (BrdU; Sigma-Aldrich) for 30 min, washed and then treated with 1μM CPT 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 (0.4mg/ml in 0.1N HCl) for 30 minutes at room temperature (RT). DNA was denatured using 2N HCl and neutralized with 0.1 M sodium borate. Cells were labelled with FITC-conjugated anti-BrdU antibody (347583, Becton Dickinson) and DNA was stained with PI. Samples were analysed on a BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences).

Western blot analysis

Cells were plated on 100 cm dishes, treated as indicated, and whole-cell extracts were prepared using NP-40 lysis buffer (10 mM Tris-HCl pH8.0, 150 mM NaCl, 1 mM EDTA
Forced mitotic entry by WEE1 inhibition

pH 8.0, 1% NP-40, 5 mM sodium pyrophosphate, 50 mM NaF, 1 mM Na₃VO₄, 5 mM DTT, Complete Protease Inhibitor Cocktail (Roche). Lysates were run on precast 4-12% Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose membranes (Bio-Rad). Signals were visualised using enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagents, GE Healthcare).

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, permeabilised in 0.2% Triton X-100 in PBS for 20 minutes, washed and blocked in IFF (1% BSA, 2% FBS in PBS) followed by incubation with primary and secondary AlexaFluor-conjugated antibodies for 1-2 hours at RT each. DNA was stained with DAPI. Coverslips were mounted on glass slides using Vectashield. Images were acquired on a Leica confocal microscope (63x oil immersion objective). Mitotic and nuclear phenotypes of at least 200 cells per condition were scored for two 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 mM NaCitrate for 15 min 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, NOD SCID female mice aged 5-6 weeks were obtained from Charles River Laboratories (CRL). Mice were inoculated with 5 x 10⁶ WiDr
Forced mitotic entry by WEE1 inhibition

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.). Tumours were taken 8 hours post MK-1775 administration for FACS analysis and pH3 immunofluorescence. For efficacy experiments, we utilised the WiDr derivative HT-29 that grows robustly in nude mice. CD-1 Nu/Nu female mice aged 4-6 weeks were obtained from CRL. Mice were inoculated with 2.5-5 x 10^6 WiDr (HT-29) cells in 100 μL (1:1 Matrigel:PBS) S.C. into the right flank. When tumour 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). Tumour measurements were recorded bi-weekly and tumour volume was calculated as (width*length*length)/2. For pH3 immunofluorescence analysis, gemcitabine treatment was followed by MK-1775 for 2.5 days (5 doses), with tumours 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 performed with GraphPad Prism version 5.0. Unless stated otherwise, P values were two tailed and considered significant if P<0.05. Error bars represent SEM of three experiments unless stated otherwise.

Cell line whole genome gene expression data was assessed on Illumina human-ref6 v2 BeadChips as previously described (23). Gene expression, from probes detectable in >90% of cell lines, was correlated with gemcitabine - MK-1775 combination index using Pearson Correlation Coefficient. Gene expression data on a publically available data set
Forced mitotic entry by WEE1 inhibition

of TP53 wild type and mutant cell lines was analysed from Miller et al. (46). Kaplan Meier survival curves were plotted for EZH2, CCNB1, and mean of EZH2 and CCNB1, in two groups gene expression <median versus >= median.

Grant Support

This work was supported by Cancer Research UK grant A10038, Susan G. Komen for the Cure, and the Netherlands Organisation for Scientific Research (NWO; M. Aarts). We acknowledge NHS funding to the NIHR Biomedical Research Centre.

Acknowledgements

We thank Asha Kigozi, Antoinette van Weverwijk for their assistance with the mouse xenograft experiments and the Breakthrough Centre Histopathology Core Facility for processing the xenograft tumours.

References

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Figure Legends

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 were expressed as a combination index (CI).

B. IC50 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 phospho-histone H3 (pH3; mitotic cells) and propidium iodide (PI) flow cytometry analysis (FACS). Top panels PI alone, and 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 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 (grey bars) or cells with 4N DNA content (black 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 combination index (CI) are indicated for each cell line (* denotes P<0.01, Student’s t-test compared to <4N DNA mitotic index of MCF7 cells).

Figure 2. WEE1 inhibition induces highly abnormal mitoses in S-phase arrested cells
Forced mitotic entry by WEE1 inhibition

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 indicates untreated control cells.

B. Chromosome spreads of CAL120 cells treated as described in part A.

C. Immunofluorescent images of untreated pH3-positive CAL120 cells in interphase (panel a) and in metaphase (panel b) or after HU and MK-1775 combination treatment (panel c). Cells were stained for pH3 (red), α-tubulin (microtubules, green) and DAPI (DNA, blue). Representative images of cells with monopolar (panel d) or multipolar spindles (panel e) and early prophase cells without spindle (panel e) 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 (grey bars) or HU and MK-1775 combination (black bars). Cells with disorganized spindle are seen in 2% untreated, 9% MK-1775, and 65% HU + MK-1775 (* denotes P<0.004 untreated versus HU + MK-1775, and MK-1775 versus HU + MK-1775, Student’s t-test).

Figure 3. Unscheduled mitosis results in mitotic slippage and apoptosis

A. CAL120 cells were treated with HU alone for 24 hours with or without the addition of MK-1775 for a further 8 hours. Drugs were washed out and mitotic index was assessed by pH3/PI FACS at indicated time points after wash-out.

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 part B, lysed at indicated time points, and analysed by Western blot for PARP-1, Cyclin B1, CDK1 (phospho-Y15 and total), and β-
Forced mitotic entry by WEE1 inhibition

tubulin as loading control. Asynchronously (async) growing cells were included as controls.

D. CAL120 cells were untreated (white bar), or treated with HU alone (light grey bar), MK-1775 alone (dark grey bar) or HU and MK-1775 combination (black bar). Drugs were washed out and at 8 hours after wash-out (w8h) cells were stained for α-tubulin (green) and DAPI (blue). Quantification of proportion of micronucleated cells by immunofluorescence microscopy (untreated 6%, HU 8%, MK-1775 14%, HU and MK-1775 44%, * denotes P=0.008 one way ANOVA).

E. Representative immunofluorescent images of normal and micronucleated cells taken at 8 hours after wash-out of HU and MK-1775 combination treatment as described in part D.

Figure 4. Combined inhibition of WEE1 and CHK1 forces cells into mitosis

A. Mitotic index of CAL120 and MCF7 cells treated with gemcitabine 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 pre-treated with gemcitabine for 24 hours, followed by treatment with AZD7762 (white bars), MK-1775 (grey bars) or the combination (black 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% versus combination 12%, MK-1775 1.1% versus combination 12%; * denotes P<0.002, Student’s 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.
Forced mitotic entry by WEE1 inhibition

E. CAL120 cells were either untreated or pre-treated with gemcitabine for 24 hours followed by treatment with AZD7762 and/or MK-1775 for an additional 2 hours (top panels) or 8 hours (bottom panels), prior to 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-labelled with 10 μM BrdU for 30 min, washed (W), and then treated with 1 μM camptothecin (CPT) for 30 min. After CPT removal (0 h), BrdU-labelled S-phase cells (BrdU+, indicated by boxed area) were monitored at the indicated time points in the absence (panel c) or presence of MK-1775 (panel d) or AZD7762 as a positive control (panel e). Control cells (ctr) were not exposed to CPT and cultured in the absence (panel a) or presence of MK-1775 (panel b). 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 prior to addition of MK-1775 alone or in combination with 10 μM RO-3306 (CDK1 inhibitor). Representative histograms show pH3 fluorescence 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 prior to 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 two different siRNA duplexes targeting p21Waf1/Cip1 (sip21) or TP53 (siTP53) for 48 hours prior to gemcitabine/MK-1775
Forced mitotic entry by WEE1 inhibition

treatment (+Gem/MK). Mitotic index was determined by pH3/PI FACS analysis. p21\textsuperscript{Waf1/Cip1} silencing was confirmed by Western blotting (Supplementary Figure 6B). * denotes P<0.003 compared to siCON, Student’s t-test.

D. Western blots of lysates from CAL120 and MCF7 cells treated with gemcitabine and/or MK-1775, probed for WEE1, Cyclin B1, CDK1 (phospho-Y15 and total), p21\textsuperscript{Waf1/Cip1}, and Actin as loading control.

E. Correlation of whole genome gene expression with gemcitabine/MK-1775 combination index in \textit{TP53} mutant breast cancer cell lines. Displayed are Pearson correlation coefficients for the 8806 expression probes. Correlation coefficients of \textit{CDKN1A} (r=0.81, P<0.0001), \textit{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), p21\textsuperscript{Waf1/Cip1}, CHK1 (phospho-S317), and β-tubulin as loading control. MCF7 and ZR75.1 are \textit{TP53} wild type and the other cell lines \textit{TP53} mutant.

Figure 6. High expression of mitotic cyclins and EZH2 in poor prognosis breast cancer

A. Correlation of gemcitabine/MK-1775 combination index and \textit{EZH2} expression in \textit{TP53} mutant breast cancer cell lines with Pearson correlation coefficients, r=-0.58, P=0.009.

B. Correlation of gemcitabine/MK-1775 combination index and \textit{CDKN1A} expression in \textit{TP53} mutant breast cancer cell lines with Pearson correlation coefficients.

C. CAL120 cells were transfected with siCON and two different EZH2 siRNA duplexes or SMARTpool, treated with gemcitabine/MK-1775 48 hours post transfection. Mitotic index
Forced mitotic entry by WEE1 inhibition

was determined by pH3/PI FACS analysis. EZH2 silencing was confirmed by Western
blotting (Supplementary Figure 8A).

D. SUM44 and JIMT-1 TP53 mutant cells were transfected with siCON or p21\textsuperscript{Waf1/Cip1} siRNA SMARTpool and treated with gemcitabine/MK-1775 48 hours post 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% CI: 1.272 to 3.721, P=0.0046), EZH2 (middle; HR=2.120, 95% CI: 1.240 to 3.624, P=0.006) and mean CCNB1-EZH2 expression (right; HR=2.841, 95% CI: 1.645 to 4.906, P=0.0002). Comparison is between expression < and > median with log rank test.

H. Combination index of degree of synergy between gemcitabine and MK-1775, displayed according to cell line subtype, P=0.028, Mann-Whitney U test.

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 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 (six 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. Tumours were taken 8 hours post MK-
Forced mitotic entry by WEE1 inhibition

1775 administration and analysed by pH3 immunofluorescence. Assessment was blinded to treatment allocation. Combination (Gem MK) treatment increased pH3-positive cells compared to all other treatments (P<0.05, Student’s t-test).

C. Representative immunofluorescent images of tumours treated as described in part B (red, pH3; blue, DAPI (DNA)).

D. Representative images of pH3 immunofluorescence in WiDr (HT-29) tumours 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 tumours treated as described in part D, scored blind in three tumours per condition.

F. Mice bearing WiDr (HT-29) tumours 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 tumour growth to a greater extent than individual treatments (repeated measures ANOVA 15mg/kg P=0.011, 30mg/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/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.
Figure 1 - Aarts et al., 2012

A

B

C

D

E

Figure 1 - Aarts et al., 2012
Figure 3 - Aarts et al., 2012

A

HU release

HU + MK1775 release

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>PI</th>
<th>pH3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2N</td>
<td>2N</td>
</tr>
<tr>
<td>8</td>
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</tr>
<tr>
<td>16</td>
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</tr>
<tr>
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<td>2N</td>
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DNA content (PI)

B

Mitotic cells with <4N DNA content (%)

HU + MK1775:

- continuous
- wash-out (W)

Time after MK1775 addition (h)

C

Time after MK1775 addition (h)

- PARP-1 cleaved
- Cyclin B1
- CDK1 pY15
- CDK1 total
- β-Tubulin

D

pH3 negative cell (%)

- untreated
- HU w8h
- MK w8h
- HU + MK w8h

E

Normal

Micronucleated

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Figure 4 - Aarts et al., 2012

A

CAL120

MCF7

ph3 positive cells (%)

<4N DNA

4N DNA

Gem: + + + + +

AZD7762: - - - - -

MK1775: - - - - -

B

ph3-positive cells (%)

Time after drug addition (h)

0 1 2 4 6 8

Gemcitabine +

AZD7762

MK1775

AZD7762 + MK1775

C

CAL120

MCF7

ph3-positive cells (%)

<4N DNA

4N DNA

AZD7762: - - - - -

MK1775: - - - - -

D

untreated

AZD7762

MK1775

AZD7762 + MK1775

DNA content (PI)

E

W W + AZD7762 + MK1775

BrdU CPT no drugs

2 h

0.7 0.5 0.3 0.2 1.0 1.0 1.0 0.5

ratio pY15/total CDK1

0.7 0.5 0.3 0.2 0.1 0.6 0.5 0.1 0.0

ratio pY15/total CDK1

F

a ctr

b ctr + MK1775

c CPT

d CPT + MK1775

e CPT + AZD7762

DNA content (PI)
Figure 5 - Aarts et al., 2012

A

B

C

D

E

F

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Figure 6 - Aarts et al., 2012

A

B

C

D

E

TP53 wild-type

TP53 mutant

F

G

CCNB1

EZH2

EZH2 and CCNB1

H

Combination Index

Luminal

Basal

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Figure 7 - Aarts et al., 2012

A

B

C Xenografts + MK1775 (8 h)

D Xenografts + MK1775 (2.5 days)

E

F

G

Regulation of CDK1 activity in normal cells

Regulation of CDK1 activity in cancer cells
Forced mitotic entry of S-phase cells as a therapeutic strategy induced by inhibition of WEE1

Marieke Aarts, Rachel Sharpe, Isaac Garcia-Murillas, et al.

Cancer Discovery  Published OnlineFirst April 23, 2012.

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