CRISPR Screening Identifies WEE1 as a Combination Target for Standard Chemotherapy in Malignant Pleural Mesothelioma

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Abstract

Malignant pleural mesothelioma (MPM) is an aggressive cancer with dismal prognosis, largely due to poor response rates to and rapid relapse after first-line pemetrexed (MTA)/cisplatin chemotherapy. A better understanding of the molecular mechanisms underlying chemotherapy sensitivity and duration represents a significant but still unmet clinical need. In this study, we reported on a kinome CRISPR/Cas9 knockout screen that identified several G2–M checkpoint kinases, including WEE1, whose loss of function sensitizes MPM cells to standard chemotherapy. We further showed that deregulation of the G2–M checkpoint contributes to chemotherapy resistance, and that WEE1 inhibition synergizes with cisplatin/MTA, leading to enhanced MPM cell death in vitro and potent antitumor effects in vivo. Mechanistically, WEE1 blockage overrides chemotherapy-induced G2–M cell-cycle arrest and promotes premature mitotic entry, which causes DNA damage accumulation and ultimately apoptosis. Our results suggest a new therapeutic combination for MPM, and support the application of CRISPR/Cas9-based functional genomics in identifying novel therapeutic targets to potentiate existing cancer therapies.

Introduction

Malignant pleural mesothelioma (MPM) is a rare but highly aggressive cancer, with a median survival time shorter than 1 year and less than 5% of patients live up to 5 years after diagnoses. MPM etiology is closely associated with asbestos and despite the ban in most developed countries, MPM incidence still increases worldwide because of the long latency (40–60 years) of the disease (1, 2). Patients with early-stage MPMs are treated with surgical resection, radiotherapy and chemotherapy (3). However, a majority (>80%) of MPM is diagnosed with an advanced disease (4), for which a chemotherapy regimen that combines cisplatin with pemetrexed (MTA) is the only clinically approved treatment (3, 5–7). Disappointedly, cisplatin/MTA only marginally improves patients’ outcome by increasing median survival from 9 to 12 months (5). The underlying causes that preclude clinical efficacy of the first-line chemotherapy include poor response rates (40%) and swift but inevitable development of resistance in the minority of patients who initially respond to the treatment (5, 8). Hence, there is a desperate need for better understanding of the molecular mechanisms that limit clinical efficacy of chemotherapy and for development of new strategies to treat MPM more effectively.

The cell cycle is exquisitely controlled by cyclin-dependent kinases (CDK) that promote cell-cycle progression, and by several checkpoints positioned at the G1–S transition, S phase, and G2–M transition that halt cell-cycle progression, serving to censor genomic integrity by allowing for damaged DNA to be repaired (9). Progression through the G2–M transition is driven by CDK1 (also known as CDC2) and cyclin B1, but counteracted by WEE1, a nuclear tyrosine kinase that inactivates CDK1 via an inhibitory phosphorylation at tyrosine 15 (10). The G2–M checkpoint plays a pivotal role in response to genotoxic stress, for example, chemotherapy, as cancer cells recurrently deficient in the G1–S checkpoint rely to a greater degree on the G2–M checkpoint mechanism to arrest cell-cycle progression premitotically, which enables repair of therapy-induced DNA damage and thereby escapes apoptosis (11). Indeed, multiple tumor lineages, including glioblastoma, head and neck squamous carcinoma, breast, lung, and pancreatic cancers, can exploit WEE1-mediated G2–M checkpoint mechanism to mitigate DNA damage load and evade current therapies, and abrogation of WEE1 renders tumor cells particularly susceptible to genotoxic insults (12–18). AZD1775, a pyrazolo-pyrimidine derivative (12), potently inhibits WEE1 kinase activity, exerts promising antitumor efficacy in preclinical tumor models, and has advanced to multiple clinical trials as single agent or in combination with DNA-damaging therapies (www.ClinicalTrials.gov; refs. 19–21).

CRISPR/Cas9 is a powerful gene-editing technology, which enables rapid and accurate assessment of gene functions (22, 23), which is particularly useful for identifying genetic determinants of therapy resistance (24–27). Here, by implementing a kinome-wide CRISPR knockout screen, we identify WEE1 whose deletion or inhibition by AZD1775 sensitizes MPM cells to cisplatin/MTA chemotherapy. Mechanistically, WEE1 inhibition abrogates chemotherapy-induced G2–M arrest and forces a premature mitotic entry, which translates to more pronounced DNA damage and apoptosis in vitro and more...
potent antitumor effect in vivo than chemotherapy alone. We further show that chemotherapy resistance is associated with deregulated G2–M checkpoint and AZD1775 resensitizes resistant MPM cells to standard chemotherapy.

**Materials and Methods**

**Cell culture and reagents**

MPM cells (H28, MESO-1, MESO-4, MSTO-211H, JL-1) were described (28–30) and cultured in RPMI1640 medium (catalog no. 8758; Sigma-Aldrich) with 10% FBS (catalog no. 10270-106; Life Technologies) and 1% penicillin/streptomycin solution (catalog no. P0781; Sigma-Aldrich) at 37°C with 95% air/5% CO2. H28, H2452, and H2052 were obtained from ATCC in 2018, and MSTO-211H and JL-1 from DSMZ in 2017. Meso-1 and Meso-4 were provided by L. Cortes-Dericks, Bern, Switzerland, in 2012. All the cells were authenticated by DNA fingerprinting using highly-polymorphic short tandem repeat (STR) analysis (Mycosynth) and confirmed free from mycoplasma contamination. Cells with 3 to 15 passages were used in this study.

Primary cell cultures (BE261T) were established from surgically resected MPM tumors of a 67-year-old male patient after neoadjuvant chemotherapy (pemetrexed plus carboplatin) using the described protocol (29, 31). Cells within three to eight passages were used in this study. The human study was performed under the auspices of institutional review board (KEK number: 042/15 and 200/2014), and written informed consent was obtained from patients.

Chemotherapy-resistant (H28-R, MESO-1-R, and MESO-4-R) cells were generated by chronic exposure to cisplatin/MTA following a weekly schedule of 4-day treatment and 3-day recovery as described (29, 31). Brieﬂy, H293T cells (1 × 104 cells/mL in total 10 mL antibiotic-free media) were cultured for 24 hours before transfection. For each 10-cm dish, 9 mL of the transfection mixture (MegaTran 1.0 (catalog no. TT200003; Origene) diluted in OptiMEM) and 1% penicillin/streptomycin solution (catalog no. 8758; Sigma-Aldrich) with 10% FBS (catalog no. 10270-106; Life Technologies) were added and mixed, and 9 g of psPAX2 (Addgene, catalog no. 12260), and 54 g of pCMVdeltaR8.2 (Addgene, catalog no. 8854), and 9 µg of psPAX2 (Addgene, catalog no. 12260), and 54 µL of MegaTran 1.0 (catalog no. TT200003; Origene) diluted in OptiMEM were mixed and added to cells. After 48 hours, the supernatant was collected and filtered through a 0.45-µm strainer.

**Lentivirus production**

The lentivirus was produced as described (22). Brieﬂy, H293T cells seeded at 3.8 × 106 cells/mL in total 10 mL antibiotic-free media were cultured for 24 hours before transfection. For each 10-cm dish, 9 µg of pLentiCRISPR plasmid library, 0.9 µg of pVSyVg (Addgene, catalog no. 52962), 9 µg of psPAX2 (Addgene, catalog no. 12260), and 54 µM of MegaTran 1.0 (catalog no. TT200003; Origene) diluted in OptiMEM were mixed and added to cells. After 48 hours, the supernatant was collected and filtered through a 0.45-µm strainer.

**CRISPR/Cas9 knockout screening with LentiCRISPRv2 library**

Kinome-wide CRISPR screen was essentially performed as described (22). Brieﬂy, 50 × 105 Meso-1 cells plated in eight 15-cm dishes were transduced in triplicates with kinome-wide sgRNA lentiviral pool (MOI ~0.3). Twenty-four hours later, cells were selected with puromycin (1 µg/mL) for 3 days and survival cells were divided into two groups (4 × 15 cm dishes/group) following treatment with vehicle (PBS) or chemotherapy (0.5 µmol/L cisplatin plus 1 µmol/L pemetrexed) for 21 days. Cells were subcultured every 3 days and at least 3 × 106 cells were collected for genomic DNA isolation at the end of treatment.

**Genomic DNA sequencing and data analysis**

Genomic DNA (gDNA) was isolated from vehicle- and chemotherapy-treated Meso-1 cells using the QiAamp DNA Blood Maxi Kit (catalog no. 51192; Qiagen), followed by a two-step PCR procedure to amplify sgRNAs as described previously (22). The first PCR was performed in 100 µL reactions for 20 cycles with 5 µg gDNA and the first PCR primers (forward: 5′_F_ont-7nt; reverse: 5′_R; Supplementary Table S2). The second PCR was done in 100 µL reaction for 25 cycles by mixing 5 µL of the first PCR product and the second PCR primers containing unique barcodes and sequences for deep sequencing (Supplementary Table S2). The resulting PCR amplicons were purified and analyzed by HiSeq 3000 (Illumina). Raw FASTQ files were demultiplexed and trimmed to contain unique sgRNA sequence only. The number of reads for each sgRNA was quantified, and normalized to total reads of all sgRNAs using the equation: normalized counts of each sgRNA = (total reads per sgRNA in each sample/reads mapped to target library of each sample) × 106 + 1. Student t test and Benjamini-Hochberg adjustment for multiple comparisons were used to determine P values.

**Cell viability and clonogenic survival assay**

MPM cells seeded in 96-well plates (2,500 cells/well) were dosed 24 hours later with drugs for 72 hours unless otherwise indicated. Cell viability was determined by acid phosphatase (APH) assay as described (29, 31). Drug effects on cell growth were normalized to untreated control. Each data point was generated in triplicate and each experiment was done three times (n = 3). Unless otherwise stated, a representative result is presented. Best-fit curve was generated in GraphPad Prism [log (inhibitor) vs. response (−variable slope four parameters)]. Error bars are mean ± SD. Combination index (CI) was calculated by ComboSyn software (http://www.combosyn.com/).

Clonogenic assay was done as described (29, 31). In brief, exponentially grown MPM cells seeded in 6-well plates (1,000–2,000 cells/well) were treated with drugs for 3 days and cultured without drugs for 10 to 14 days depending on growth rate, the resulting colonies were stained with crystal violet (0.5% dissolved in 25% methanol). Quantiﬁcation was done by eluting crystal violet staining with 10% acetic acid and measuring absorbance at 590 nm.

**Western blot and IHC**

Cell lysates were prepared and Western blot analysis was performed as described (29, 31). In brief, equal amounts of protein lysates (10–25 µg/lane) were resolved by SDS-PAGE (catalog no. 456103; Bio-Rad Laboratories) and transferred onto nitrocellulose membranes (catalog no. 170-4158; Bio-Rad). Membranes were then blocked in blocking buffer (catalog no. 927-4000; LI-COR Biosciences) for 1 hour at room temperature and incubated with appropriate primary antibodies overnight at 4°C (Supplementary Table S6). IRDye 680LT-conjugated goat anti-mouse IgG (catalog no. 926-68020) and IRDye 800CW-conjugated goat anti-rabbit IgG (catalog no. 926-32211) from LI-COR Biosciences were used at 1:5,000 dilutions. Signals of IRDye 680LT-conjugated goat anti-mouse IgG and IRDye 800CW-conjugated goat anti-rabbit IgG were quantified using ImageJ (https://imagej.nih.gov/).
hematoxylin and eosin (H&E) using standard protocols. FFPE tissue blocks were sectioned at 4 μm, deparaffinized, rehydrated, and subsequently stained with appropriate antibodies (Supplementary Table S6) using the automated system BOND RX (Leica Biosystems). Visualization was performed using the Bond Polymer Refine Detection Kit (Leica Biosystems) as instructed by the manufacturer. Images were acquired using PANNORAMIC whole slide scanners and processed using Case Viewer (3DHISTECH Ltd.). Immunoreactivity was evaluated by IHC Profiler (Image) plug-in in a blinded manner (32).

**Cell-cycle analysis and apoptosis assays**

MPM cells were fixed with 70% cold EtOH, permeabilized with 0.1% Triton X-100, and stained with 0.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; catalog no. D9542; Sigma-Aldrich). For apoptosis assay, cells after treatment in the supernatant and adherent to plates were collected, washed with PBS, and pooled before suspended in 400 μL binding buffer and stained with the Annexin V Apoptosis Detection Kit-FITC (catalog no. 88-8005; Thermo Fisher Scientific) according to the manufacturer’s instructions. Samples were analyzed on a BD Biosciences LSRII flow cytometer.

**In vivo mouse study**

Mouse studies were conducted in accordance with Institutional Animal Care and Ethical Committee-approved animal guidelines and protocols. All mouse experiments were performed in age- and gender-matched NOD-iscid IL2Rγnull mice. Suspensions of tumor cells (in PBS) mixed 1:1 with BD Matrigel Basement Membrane Matrix (catalog no. 356231; Corning) were subcutaneously inoculated in left and right flanks (MESO-1 cells: 1.8 × 10⁶/injection; BE261T cells: 1 × 10⁶/injection). When tumors were palpable, tumors were excised and bisected in the middle to obtain tumor volumes, respectively.

**Public databases (The Cancer Genome Atlas, COSMIC, and genomics of drug sensitivity in cancer)**

Transcriptome profiling of mesothelioma patients was obtained from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/projects/TCGA). Expression of G₂-M kinase genes was dichotomized based on the optimal cut-off value, and patient survival data were extracted for further analysis using R (version 3.4.3). Genes (TP53 and CDKN2A) mutational status was extracted from the Sanger Cancer Cell Line Project COSMIC (https://cancer.sanger.ac.uk/cosmic). The Genomics of the Drug Sensitivity Project (https://www.cancerrxgene.org/) was employed to extract drug sensitivity data of MPM and pan-cancer cell lines.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 6.03 (GraphPad Software Inc., http://www.graphpad.com) unless otherwise indicated. In all studies, data represent biological replicates (n) and are depicted as mean values ± SD or mean values ± SEM as indicated in the figure legends. Comparison of mean values was conducted with unpaired, two-tailed Student t test, one-way ANOVA, or two-way ANOVA with Tukey multiple comparisons test as indicated in the figure legends. In all analyses, P values less than 0.05 were considered statistically significant.

**Results**

**A CRISPR/Cas9 knockout screen identifies kinase genes whose loss of function sensitizes MPM cells to traditional chemotherapy**

To identify genetic determinants that limit efficacy of cisplatin/MTA chemotherapy in MPM, we took a systematic approach by implementing a CRISPR/Cas9 knockout screen in MPM cells. We transduced MESO-1 cells with the Broad Institute Brunello CRISPR library containing 3052 small-guide RNAs (sgRNA) that target 763 kinase genes in human genome, with four sequence-specific sgRNAs against each single kinase gene (Supplementary Table S1; refs. 26, 27). MESO-1 cells incorporated lentiviral sgRNA vectors were selected by puromycin treatment for 3 days, followed by serial passages and continuous exposure to vehicle control or cisplatin/MTA chemotherapy for 21 days (Fig. 1A). We experimentally determined the concentration of chemotherapeutic agents by choosing the lowest dose of drug combination (0.5 μmol/L cisplatin plus 1 μmol/L MTA), which robustly suppressed MEO-1 cell proliferation or induced cell death. Genomic DNA was isolated from vehicle- and cisplatin/MTA-treated MESO-1 cells, referred to as MESO-1_V and MESO-1_C cells thereafter, and sgRNAs were amplified by two-step PCR (Supplementary Table S2) and subsequently quantified by next-generation sequencing.

We then compared sgRNA abundance and scored those depleted and enriched in MESO-1_C versus MESO-1_V (Fig. 1B; Supplementary Table S3). By focusing on depleted sgRNAs (negative selection) and prioritizing targets based on inter-sgRNA commonality such that all four sgRNAs targeting the same gene significantly decreased (adjusted P-value < 0.05) to minimize off-target effects, we finally identified 33 kinase hits (Supplementary Table S4).

Among the candidate targets, a list of 6 kinase genes, namely WEE1, AURKA, MPP3, MAP3K12, DGGK, and SPEG, met the screening criteria [average cutoff of log₂ fold-change (FC) ≤ −1.5] for follow-up studies (Fig. 1C; Supplementary Table S4). Annotations of these genes indicate that they belong to several functional categories, that is, the G₂-M checkpoint and mitotic regulation (WEE1, AURKA), mitogen-activated protein kinase (MAPK) signaling (MAP3K12), cytoskeleton-associated signaling pathway (MPP3), lipid signaling pathway (GKG), and differentiation signaling (SPEG). Interestingly, CDK11b that is expressed only in the G₂-M phase (9) and CHK1/CHK2 that are involved in DNA damage-induced G₂-M checkpoint and act upstream of WEE1 (33) were also identified as putative targets against MPM, with a median Log₂FC of −1.06, −0.76, and −0.51, respectively (Supplementary Table S4). In line with our functional kinomics, WEE1 and AURKA also scored in RNAi-based kinomic screens aimed at identifying survival kinases in glioblastoma as well as head and neck squamous cell carcinoma cells (13, 34), implying technical feasibility of our screening approach and biological plausibility of the results from this study.

Deregulation of the G₂-M checkpoint is associated with multiple cancers (33). We performed an in silico analysis based on published data (35, 36) and identified several genes regulating the G₂-M transition, that is, CHEK1, AURKA, PLK1, WEE1, CDK1, and CCNB1, were significantly deregulated in MPM tumors compared with normal pleura or autologous nontransformed lung tissues (Supplementary Figs. S1A and S1B). The Kaplan–Meier survival analysis of a cohort of patients with MPM archived in TCGA stratified a subset of patients characterized by significantly poorer overall survival, higher
recurrence rates and aggressive MPM tumors that were defined by high mRNA levels of WEE1, AURKA, CHK1, PLK1, CDK1, and CCNB1 (Supplementary Fig. S2). These data indicate the clinical relevance of our findings, and are consistent with the role of CDK1/cyclin B1 in promoting G2–M progression, and of WEE1 that, despite a negative regulator of CDK1/cyclin B1, has also been proposed to function as an oncogene (14). Because the most significant effect on MPM cell viability was brought about by CRISPR/Cas9-mediated WEE1 knockout in the setting of chemotherapy (Fig. 1C), we focused on WEE1 in the follow-up study.

Figure 1.
A kinome-scale CRISPR/Cas9 screen identifies genes whose loss of function sensitizes MESO-1 cells to cisplatin/MTA chemotherapy. A, Schematic overview of the timeline and experimental procedures of CRISPR/Cas9-based kinomic screening. The scatterplot (B) and ranking list (C) of sgRNAs deleted in chemotherapy-treated versus vehicle-treated MESO-1 cells. The sgRNAs that target WEE1 (red), AURKA (blue), and MPP3 (green) are highlighted (B). The six top-ranking kinase genes and the respective sgRNAs scored in negative selection CRISPR/Cas9 screening are shown in C.
Genetic and pharmacologic inhibition of WEE1 promotes chemotherapy efficacy in MPM cells

To validate WEE1 as a therapeutic target whose inhibition promotes chemotherapy efficacy, we knocked out the WEE1 gene in MESO-1 cells by exploiting two independent sgRNAs (Supplementary Table S5; Fig. 2A). Dose-dependent cell viability assay revealed that MESO-1 cells with WEE1-knockout (KO; designated as WEE1 sgRNA-1, WEE1 sgRNA-2) displayed markedly increased sensitivity to cisplatin/MTA compared with MESO-1 cells transduced with scrambled control sgRNA (Fig. 2B). Flow cytometry-based apoptotic analysis confirmed that cisplatin/MTA treatment induced a significantly greater cell death in WEE1-KO than WEE1-wild-type (WT) MESO-1 cells (Fig. 2C).

To corroborate the genetic results, we tested the WEE1-specific inhibitor AZD1775 (formerly known as MK-1775). Dose–response curves performed on a panel of MPM cell lines (MSTO-211H, H28, MESO-1, MESO-4, JL-1) and a primary MPM cell culture (BE261T) indicated that AZD1775 inhibited MPM cell viability in a dose-dependent manner, with the IC50 ranging from 250 nmol/L to 4.07 μmol/L (Supplementary Figs. S3A–S3C).

Previous studies have proposed that cancer cells deficient in p53, which increases DNA damage at the G2–M transition, are more sensitive to deregulation of the G2–M transition than p53-proficient cells (12, 15, 18). However, this has been poorly explored in MPM and conflicting observations were reported (37, 38). The Genomics of Drug Sensitivity project contains dose–response data on genomically characterized cancer cell lines treated with different compounds (39), including a WEE1 inhibitor (681640). MPM cell lines of different histologic subtypes (WEE1–KO than WEE1-wild-type (WT) MESO-1 cells (Supplementary Fig. S3E)).

Fig. 3A

Next, we assessed whether combined AZD1775 treatment increased cisplatin/MTA toxicity in MPM cells as genetic results indicated (Fig. 2A–C). Although cisplatin/MTA affected MESO-1 cell viability in a dose-dependent manner, combined AZD1775 and cisplatin/MTA produced a strong synergistic effect, resulting in a more pronounced inhibition of cell viability than single regimens alone (Fig. 2D). Synergism between AZD1775 and cisplatin/MTA also held true for MSTO-211H, H28, MESO-4, and JL-1 cells (Supplementary Figs. S4A–S4D). Cytogenetic assay and flow cytometric analyses confirmed that the combination treatment was superior to single agents, manifested by a more pronounced activation of apoptotic cell death induced by combined AZD1775 and cisplatin/MTA than either chemotherapy or AZD1775 alone (Fig. 2E and F; Supplementary Fig. S4E). Together, these results verify that WEE1 inactivation ameliorates chemotherapy toxicity, rendering MPM cells more vulnerable to cisplatin/MTA chemotherapy.

WEE1 inhibition abrogates chemotherapy-induced G2–M checkpoint activation and induces mitotic catastrophe

We then elucidated the molecular mechanisms underlying the synergistic effect between WEE1 inhibition and cisplatin/MTA chemotherapy (Fig. 2). We treated MESO-1 cells with cisplatin/MTA (2.5 μmol/L/7.5 μmol/L) and AZD1775 (0.2 μmol/L), alone or in combination, and probed for perturbations of activities in such cellular processes as G2–M checkpoint control, mitotic progression, and DNA damage. Cisplatin/MTA acutely and substantially induced the phosphorylation of CDC2, p–CDC2 Tyr15 (Fig. 2G), which acts downstream of WEE1 and is a surrogate mark of the G2–M checkpoint activation and cell-cycle arrest, but AZD1775 antagonized this chemotherapy-induced effect, resulting in a time-dependent decrease of p–CDC2 Tyr15 (Fig. 2G). Notably, the expression of cyclin B1 followed a similar pattern of change as p–CDC2 Tyr15 (Fig. 2G). Cyclin B1 expression is known to oscillate during cell-cycle progression, accumulating upon G2–M arrest and diminishing after the G2–M transition (15), thus indicates that these cells had passed through the G2–M checkpoint and entered mitosis. In line with a critical role for WEE1 in the G2–M checkpoint control, AZD1775 substantially increased the phosphorylation of histone H3Ser10 (p–HH3Ser10), a marker indicative of G2–M progression to mitosis (Fig. 2G), suggesting that AZD1775 unleashes the G2–M arrest and induces an unscheduled mitotic entry. Strikingly, AZD1775 in combination with cisplatin/MTA robustly increased the phosphorylation of histone H2AXSer139 (p–H2AX), a DNA damage indicator, compared with chemotherapy or AZD1775 alone (Fig. 2G). Next, we analyzed cell-cycle distribution upon the combination of chemotherapy and WEE1 inhibition. Although chemotherapy alone induced a significant G2–M arrest (Fig. 2H), addition of AZD1775 caused time-dependent accumulation of aneuploid cells (>4N; Fig. 2H), a hallmark of premature G2–M transition and mitotic catastrophe (40, 41). Indeed, the combination treatment augmented apoptosis (sub-G1 population) in a time-dependent manner, and the combinatorial effect was maintained regardless of whether chemotherapy and AZD1775 were concurrently or sequentially applied (Supplementary Fig. S4F and S4G). These results reveal a strong correlation between efficacy of the combined treatment, premature mitotic entry, increased DNA damage load, and induction of apoptosis.

To evaluate the clinical relevance of the above results (Fig. 2), we further examined a primary MPM cell culture derived from clinically resected species of a MPM patient (BE261T). AZD1775 and cisplatin/MTA as monotherapy showed dosage-dependent effect, but their combination produced a strong synergism (Fig. 3A), resulting in more pronounced inhibition of cell viability (Fig. 3A and B) and significantly greater apoptotic cell death (Fig. 3C) compared with single agents alone. Importantly, and in line with the results of MPM cell lines (Fig. 2), the escalated sensitivity of BE261T cells to the combination treatment was associated with a reduced activity of the G2–M checkpoint (p–CDC2 Tyr15), catastrophically premature progression into mitosis (p–HH3Ser10), accumulation of damaged DNA (p–H2AX) and increased apoptosis index (cleaved PARP; Fig. 3D).

Together, these results demonstrate that WEE1 inhibition potentiates the efficacy of standard chemotherapy in MPM by overriding cisplatin/MTA-induced G2–M cell-cycle arrest, which causes premature mitotic entry, unrepaired DNA damage and, eventually, apoptotic cell death.

Deregulation of G2–M checkpoint contributes to chemotherapy resistance in MPM

Given our findings that cisplatin/MTA adaptively activates G2–M checkpoint and that WEE1 inhibition potentiates chemotherapy (Figs. 2 and 3; Supplementary Fig. S4), we inferred that G2–M checkpoint enforcement might act as a mechanism by which MPM cells escape chemotherapy. To test this hypothesis, we analyzed G2–M checkpoint signaling in chemotherapy-refractory MPM cells (MESO-1-R, H28-R, and MESO-4-R), generated by chronic exposure of parental cells to stepwise-increasing doses of cisplatin/MTA (Supplementary Fig. S5). Further, chemotherapy-resistant cells unanimously displayed a more pronounced activation of the G2–M checkpoint signaling, characterized by increased levels of p–WEE1 Tyr642.
were treated with increasing doses of chemotherapy (cisplatin/MTA). Cell viability was measured 72 hours after the treatment. Data are presented as mean ± SD (n = 3). Notably, addition of AZD1775 sensitized MESO-1, H28-R, and MESA-4-R cells to cisplatin/MTA, resulting in a strong synergism between AZD1775 and cisplatin/MTA in all three resistant lines as assessed by cell viability (Fig. 4A–F) and clonogenic assay (Fig. 4G).

In line with the results from chemo-naïve MPM cells (Fig. 2G), chemotherapy-resistant cells (MESO-1-R, H28-R) treated with combined AZD1775 and cisplatin/MTA showed decreased WEE1 activity (p-CDC2 Tyr15), enhanced G2-M transition (p-HH3ser10), and augmented DNA damage (γH2AX) compared with those treated with chemotherapy alone (Fig. 4H), although the apoptotic index (cleaved PARP, Bcl-xl) was not significantly changed at this time point. These results indicate that WEE1-mediated G2-M checkpoint activation contributes to chemotherapy resistance, and that abrogation of the refractory PM cell phenotype by AZD1775 sensitizes the refractory MPM cells susceptible to cisplatin/MTA chemotherapy.

Combined WEE1 Inhibition with Standard Chemotherapy Suppresses MPM Tumor Growth in Vivo

Finally, we assessed in vivo efficacy of the combination therapy (WEE1 inhibition plus cisplatin/MTA chemotherapy). In MESO-1 xenograft model, cisplatin/MTA (3.75 and 83 mg/kg), mildly reduced tumor growth, but concomitant treatment with AZD1775 (30 mg/kg), which itself at this dose showed no overt effect, profoundly potentiated chemotherapy efficacy, significantly delaying tumor progression (Fig. 5A). Notably, the dose of chemotherapy and AZD1775 used in our study were below clinically achievable concentrations (5, 42). The tumor size prior to and post (3 weeks) combination therapy was 71 ± 19 and 56 ± 18 mm3, respectively, indicating therapy-induced growth inhibition of MESO-1 xenografts (Fig. 5A). Consistently, tumors after 21 days of treatment weighed 162.4 ± 51 mg (vehicle), 102.9 ± 21 mg (cisplatin/MTA), 143.8 ± 26 mg (AZD1775), and 50.7 ± 8 mg (combination therapy), indicating that tumors had shrunk by about 36.6%, 11.4%, and 68.7% (relative to the vehicle control) after cisplatin/MTA, AZD1775, and the combination therapy, respectively (Fig. 5B and C).

We further evaluated the combinatorial effect of AZD1775 and cisplatin/MTA in a patient (BE261T)-derived xenograft (PDX) model. The combined therapy remarkably attenuated PDX tumor growth (Fig. 5D). Consistently, tumors harvested after a 4-week treatment weighed 202.8 ± 118 mg (vehicle), 165.7 ± 23 mg (cisplatin/MTA), 268.8 ± 68 mg (AZD1775), and 69.9 ± 30 mg (combination therapy), indicating that the combination therapy exerts significantly greater antitumor efficacy than single drugs (Fig. 5E and F). These results indicate that WEE1 inhibition strongly synergizes with standard chemotherapy in preclinical MPM tumor models, which is in line with our in vitro results (Figs. 2 and 3, Supplementary Fig. S4). Notably, PDX tumors administered by the combination therapy showed markedly higher levels of DNA damage, gauged by IHC staining of γH2AX, than those treated with monotherapies (Fig. 5G and H). Concomitant with the increase of damaged DNA, PDX tumors after the combination therapy showed decreased proliferation (Ki-67) but an augmented apoptotic index as indicated by caspase-3 (Fig. 5G and H). Taken together, these in vivo results validate a rational strategy by combining WEE1 inhibition with standard chemotherapy to treat MPM.

Discussion

Here, by implementing a systematic approach for negative selection CRISPR/Cas9 screening, we identified several kinases, prominently the G2-M checkpoint kinase WEE1, whose deficiency improves chemotherapy efficacy in MPM. We show that WEE1 inactivation sensitizes MPM cells to cisplatin/MTA, and that the WEE1 inhibitor AZD1775 strongly synergizes with chemotherapy, resulting in enhanced apoptotic cell death in vitro and potent antitumor efficacy in preclinical MPM models, including PDX tumors. We further demonstrate that the antitumor effect of the combination treatment is associated with AZD1775-mediated abrogation of cisplatin/MTA-induced G2-M checkpoint functionality. This aberrant G2-M checkpoint control promotes catastrophically premature mitotic entry, leading to augmented DNA damage and increased sensitivity to apoptosis. Finally, we provide evidence that associates acquired chemoresistance with aberrant activation of the G2-M checkpoint, which renders chemotherapy-refractory MPM cells particularly susceptible to WEE1 inhibition, alone or in combination with chemotherapy. Overall, these results accommodate strong mechanistic evidence for combining WEE1 inhibitors with standard chemotherapy to treat MPM, and support the application of CRISPR/Cas9 functional genomics towards identifying novel therapeutic targets to potentiate existing therapies.

Our findings are in accord with previous studies in cancers other than MPM, which reported that the G2-M checkpoint plays a pivotal role in response to genotoxic stresses and that unshuffling the G2-M checkpoint control brought about by genetic or chemical inactivation...
Figure 3.
WEE1 inhibition synergizes with standard chemotherapy in a primary MPM cell culture. A, Viability of BE261T cells after treated for 72 hours with the indicated doses of cisplatin, MTA, and AZD1775, alone or in combination. The plot of fraction affected versus combination index is shown underneath. CI < 1.0, synergism. Data are presented as mean ± SD (n = 3). **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001 by two-way ANOVA with Tukey multiple comparisons test. B, Clonogenic assay of BE261T cells treated with vehicle or the indicated drugs. Viable cells after 14 days were stained by crystal violet. Representative images (top) and quantification (below) of three independent experiments (n = 3) are shown. ***, P < 0.001 and ****, P < 0.0001 by one-way ANOVA with Tukey multiple comparisons test. C, Flow cytometry-based apoptotic assay of BE261T cells after treated the indicated drugs or drug combination for 72 hours. Data are presented as mean ± SD (n = 3). ***, P < 0.005 and ****, P < 0.0001 by two-way ANOVA with Tukey multiple comparisons test. D, Immunoblots of BE261T cells after treated for 24 hours with chemotherapy (0.625 μmol/L cisplatin/1.25 μmol/L MTA, AZD1775 (1 μmol/L), or the drug combination.
Figure 4.
WEE1-mediated G2–M checkpoint activation mediates chemotherapy resistance in MPM cells. A–C, Immunoblots of parental cells (P) and chemotherapy-resistant populations (R) derived from MESO-1, H28, and MESO-4 cells. D–F, Dose–response curves determined on chemotherapy-resistant MESO-1-R, H28-R, and MESO-4-R cells treated with the indicated drugs or drug combination for 72 hours. Plots of fraction affected versus combination index are shown underneath. CI < 1.0, synergism. Data are presented as mean ± SD (n = 3). ** P < 0.01; *** P < 0.001; and **** P < 0.0001 by two-way ANOVA with Tukey multiple comparisons test. G, Clonogenic assay of MESO-1-R, H28-R, and MESO-4-R cells after treated with indicated drugs or drug combination. Viable cells after 14 days were stained by crystal violet. Representative images (top) and quantification (below) of three independent experiments (n = 3) are shown: ** P < 0.01; *** P < 0.001; and **** P < 0.0001 by one-way ANOVA with Tukey multiple comparisons test. H, Immunoblots of MESO-1-R and H28-R cells treated with chemotherapy (2.5 μmol/L cisplatin/5 μmol/L MTA) and AZD1775 (0.2 μmol/L) alone or the combination for 72 hours. Signal intensity was quantified by ImageJ and normalized to the loading control β-actin, with values of the vehicle group set as 1.
Figure 5.
Combined WEE1 inhibition with standard chemotherapy suppresses MPM tumor growth in vivo. A, Growth curves of MESO-1 xenograft tumors treated with vehicle, cisplatin/MTA (3.75/83 mg/kg), AZD1775 (30 mg/kg), alone and in combination for the indicated time. Data are presented as mean ± SEM. **, * P < 0.01 and ***, * P < 0.001 by unpaired two-sided t test. Images (B) and weights (C) of MESO-1 xenograft tumors after treated for 21 days. **, * P < 0.05; ***, * P < 0.01; ****, * P < 0.001; and ***, ***, ***, * P < 0.0001 by one-way ANOVA with Tukey multiple comparisons test. D, Development of a patient (BE261T)-derived xenograft (PDX) tumor model after treated with vehicle, cisplatin/MTA (3.75/83 mg/kg), AZD1775 (30 mg/kg), alone and in combination for the indicated time. Data are presented as mean ± SEM. ***, ***, ***, * P < 0.001 by unpaired two-sided t test. E and F, Images and weights of PDX tumors after treated for 28 days. *, * P < 0.05; ***, * P < 0.01; ****, * P < 0.0001 by one-way ANOVA with Tukey multiple comparisons test. G and H, Representative images (G) and quantification (H) of H&E staining and IHC analysis for γH2AX, Ki-67, and caspase-3 of PDX tumors after treated with vehicle, cisplatin/MTA (3.75/83 mg/kg), AZD1775 (30 mg/kg), alone and in combination. Original overall magnification, ×400. **, * P < 0.05; ***, ***, ***, * P < 0.001; and ***, ***, ***, ***, * P < 0.0001 by two-way ANOVA with Tukey multiple comparisons test.
of G2–M kinases, such as CHK1 and WEE1, enhances toxicity of DNA-damaging therapies (14, 16). In MPM, a potential link between deregulation of the G2–M cell-cycle transition, tumor pathogenesis, and progression, as well as sensitivity to single chemotherapeutic agent cisplatin has been proposed (37). However, the molecular underpinnings that render MPM generally insensitive to genotoxic agents have been poorly investigated (29, 31). In addition, it remains unclear whether and how deregulation of the G2–M checkpoint function translates into an animal model of MPM (38).

As WEE1 controls mitotic entry, abrogation of WEE1-mediated G2–M checkpoint has been proposed to be particularly effective for tumors with p53 deficiencies, which inactivates the G1–S checkpoint, resulting in a greater extent of DNA damage at and a more exquisitely reliable on the G2–M checkpoint (12, 15, 18). In MPM, p53 signaling is frequently inactivated, although TP53 mutations are relatively rare (43–46). One mechanism that confers p53 malfunction is inactivation of p14ARF, a tumor suppressor that protects p53 from proteasome-mediated degradation. Genetic alterations in CDKN2A, encoding p14ARF and p16INK4a, are found in about 50% to 100% of MPM (47, 48). Therefore, our results link sensitivity of G2–M cell-cycle transition, tumor pathogenesis, and deregulation of the G2–M checkpoint has been proposed to be particularly effective for tumors with p53 deficiencies, which inactivates the G1–S checkpoint, resulting in a greater extent of DNA damage at and a more exquisitely reliable on the G2–M checkpoint (12, 15, 18). In MPM, p53 signaling is frequently inactivated, although TP53 mutations are relatively rare (43–46). One mechanism that confers p53 malfunction is inactivation of p14ARF, a tumor suppressor that protects p53 from proteasome-mediated degradation. Genetic alterations in CDKN2A, encoding p14ARF and p16INK4a, are found in about 50% to 100% of MPM (47, 48). Therefore, our results link sensitivity of G2–M checkpoint to combined chemotherapy and WEE1 inhibition with abrogation arrest mechanism (47, 48). Therefore, our results link sensitivity of G2–M checkpoint to combined chemotherapy and WEE1 inhibition with abrogation of the critical safeguard mechanism at G2–M transition imposed by WEE1, whose inactivation predisposes to catastrophically premature mitotic entry and exacerbates chemotherapy toxicity in MPM. Given the prevalence of genetic alterations causing deficient p53 signaling (41–46), which brings about a profound G2–M checkpoint dependency of MPM, targeting WEE1 seems to be of particular interest in MPM therapy.

Expanding therapeutic arsenal to combat MPM has been significantly hampered by paucity of functional studies in clinically relevant setting (49). CRISPR screens for chemotherapy modifiers have been reported in several cancers (50), and our study represents one of the first attempts in MPM. Given that WEE1 inhibitors are under clinical development, our results provide a readily translatable therapeutic combination for patients with newly diagnosed MPM or refractory to first-line chemotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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