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Research article

Synergistic effects of MK-1775 and gemcitabine on cytotoxicity in non-small cell lung cancer

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ABSTRACT

Background: Non-small cell lung cancer (NSCLC) remains a leading cause of cancer-related mortality. Chemotherapy is crucial in NSCLC treatment, and targeting Wee1 kinase, a key regulator of the G2/M cell cycle checkpoint, may enhance the efficacy of cytotoxic agents. This study investigates the potential of the Wee1 inhibitor MK-1775 in combination with gemcitabine and pemetrexed to enhance cytotoxicity in NSCLC cell lines.
Methods: Human NSCLC cell lines H1975, HCC827, A549, and H460 were treated with MK-1775 and chemotherapeutic agents, both alone and in combination. Growth inhibitory effects were assessed using the CCK8 assay. Apoptotic markers were evaluated via Western blotting, and cell cycle distribution was analyzed using FACS. *In vivo* efficacy was assessed using xenograft mouse models with H1975 and H460 cells, monitoring tumor growth and treatment toxicity.
Results: MK-1775 combined with gemcitabine or pemetrexed significantly decreased cell survival rates and IC_{50} values in A549 and HCC827 cell lines. Increased levels of phosphorylated cdc2, γ-H2AX, and PARP indicated enhanced apoptosis. Cell cycle analysis revealed G2/M phase arrest in p53-mutant HCC827 and H1975 cells treated with MK-1775 and gemcitabine. In xenograft models, the combination significantly inhibited tumor growth without significant toxicity.
Conclusions: MK-1775 enhances the cytotoxic effects of gemcitabine and pemetrexed in NSCLC cell lines and effectively inhibits tumor growth *in vivo*. These findings suggest that Wee1 inhibition by MK-1775, combined with chemotherapy, represents a promising therapeutic strategy for NSCLC treatment.

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1. Introduction

Chemotherapy has remained the standard treatment for non-small cell lung cancer (NSCLC), even with the advent of targeted therapies and immunotherapies. The epidermal growth factor receptor (EGFR) mutation is the most common driver gene mutation in Asian NSCLC patients [1,2]. Activating mutations in the EGFR gene result in increased growth factor signaling activity, regarding the cancer cells responsive to tyrosine kinase inhibitors (TKIs) [3,4]. Consequently, first-generation EGFR TKIs, such as gefitinib and erlotinib, were developed as the initial treatment for patients with EGFR-mutated NSCLC [5–8]. Afatinib, a second-generation EGFR TKI, acts as a pan-HER family inhibitor that irreversibly binds to EGFR and has been approved for the treatment of EGFR-mutated NSCLC, based on results from key randomized clinical trials [9–11]. Osimertinib, a third-generation EGFR TKI, has shown superior survival outcomes compared to first-generation EGFR TKIs (gefitinib or erlotinib) when used as frontline treatment [12,13]. It also demonstrated significantly greater efficacy than platinum therapy plus pemetrexed in patients with T790M-positive NSCLC whose disease had progressed during initial EGFR TKI therapy [14,15]. Targeted therapies are also indicated for NSCLC patients with other driver mutations, such as ALK, ROS-1, BRAF, RET, MET, and NTRK [16]. For patients without driver gene mutations or those who become refractory to frontline TKIs, chemotherapy, with or without immunotherapy, is recommended [17,18]. Unfortunately, patients often experience disease progression after receiving targeted therapy and chemotherapy. Ultimately, these patients receive supportive care and eventually succumb to the disease. Despite significant advancements in targeted therapies through preclinical and clinical studies, an unmet need remains: patients eventually develop treatment resistance and die. Therefore, novel therapeutic strategies are essential to improve treatment outcomes and patient survival.

Cell cycle dysregulation is a hallmark of tumor cells [19]. The tumor suppressor gene TP53 regulates G1 arrest via p21, and several studies have explored targeting p53 in cancer treatment [20–24]. In contrast, Wee1 kinase is essential for the G2/M cell-cycle checkpoint, which allows for DNA repair before mitotic entry. Normal cells repair damaged DNA during G1 arrest, but p53-mutated cancer cells often have a defective G1/S checkpoint and depend on the G2/M checkpoint for DNA repair under cytotoxic conditions. Wee1 inhibitors, when combined with cytotoxic agents, prevent G2/M arrest for DNA repair, causing cells to re-enter the G1 phase and undergo apoptosis [25,26]. For patients with advanced NSCLC without actionable genomic alterations or those resistant to current targeted therapies, targeting Wee1 to disrupt the G2/M checkpoint presents a promising therapeutic strategy.

Adavosertib (MK-1775), a Wee1 inhibitor, is currently under clinical investigation. Preclinical studies have reported that MK-1775 selectively enhances the radiosensitivity of p53-mutant ($p53^{mut}$) cell lines while having no effect on p53-wild-type ($p53^{wt}$) cell lines [27]. Conversely, another study indicated that MK-1775 can potentiate the effects of antimetabolite chemotherapeutics independently of TP53 status in both hematologic and solid tumor models [28]. Thus, the dependence on p53 may be limited to specific cancers and/or chemotherapeutics. Biomarker analysis in phase I studies of MK-1775 revealed response rates of 21 % in TP53-mutant ($TP53^{mut}$) patients compared to 12 % in wild-type TP53 ($TP53^{wt}$) patients, suggesting a slight enrichment of responders with TP53 mutations [29]. Phase II studies of MK-1775 alone and in combination with other cytotoxic agents are ongoing, and larger cohort studies are needed to confirm whether p53 mutation is a predictive factor for the use of MK-1775.

The study aims to evaluate MK-1775 in combination with cytotoxic agents such as gemcitabine and pemetrexed to determine if Wee1 inhibition can synergistically enhance their efficacy. Our data showed that MK-1775 significantly increased the cytotoxic effects of gemcitabine and pemetrexed in NSCLC cell lines, particularly A549 and HCC827, by decreasing cell survival rates and IC_{50} values, and inducing apoptosis. These findings suggest that MK-1775 may enhance the therapeutic efficacy of these chemotherapeutic agents in treating NSCLC.

2. Material and methods

2.1. Cell lines and compounds

Human NSCLC cell lines H1975, HCC827, A549, and H460 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Grand Island, NY, USA) at 37 °C in a humidified atmosphere with 5 % CO_2. MK-1775 was obtained from AstraZeneca under material transfer agreement (MTA) with the certificate of analysis (COA) confirming a purity greater than 98 %. Gemcitabine was purchased from Selleckchem, and pemetrexed was purchased from MedChemExpress.

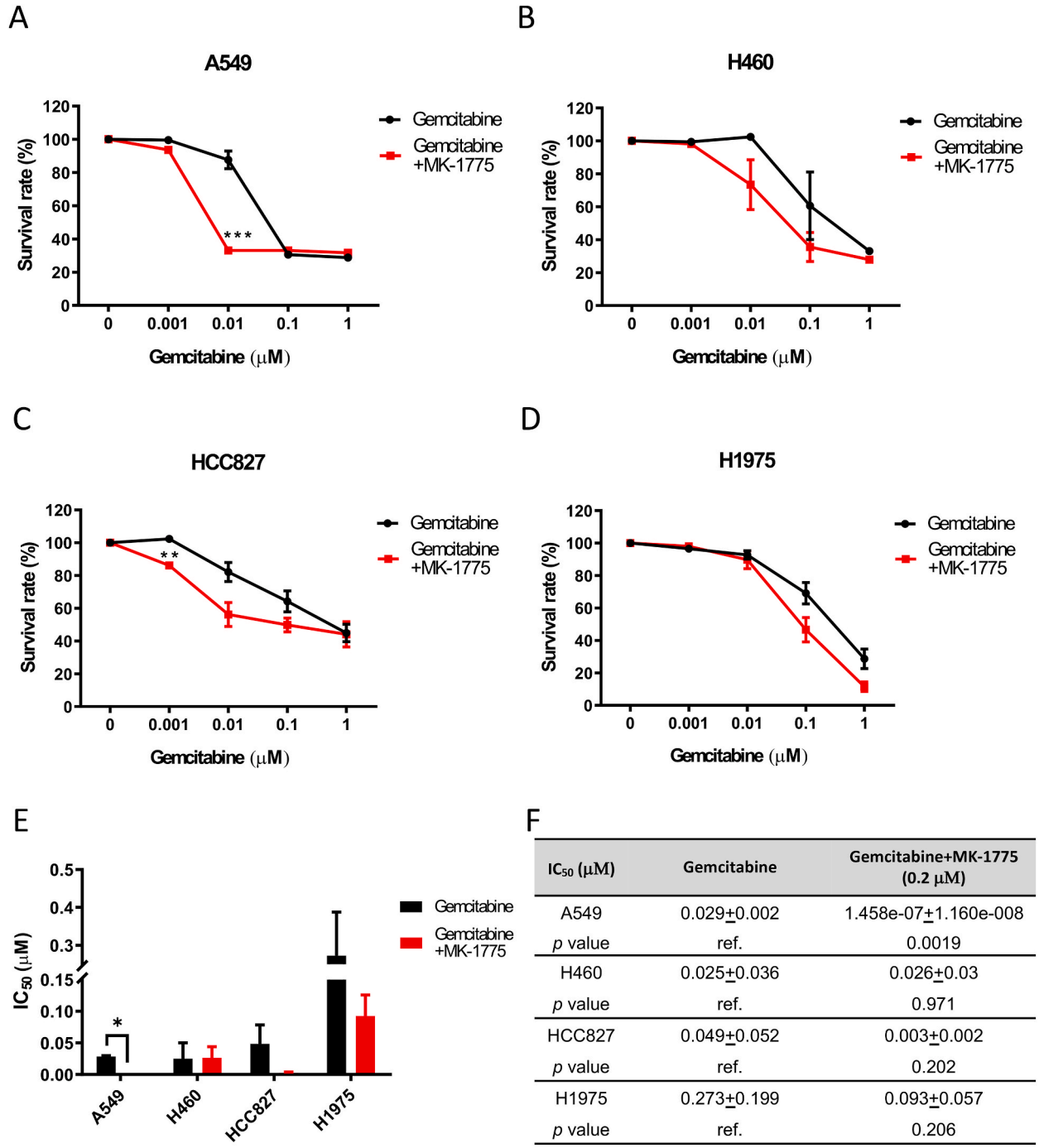
2.2. Growth inhibitory assay

Cells were plated at a density of 3000 cells per well in 96-well plates and incubated overnight before being treated for 72 h with afatinib, osimertinib, gemcitabine, cisplatin, MK1775, and their combinations. The CCK8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was conducted according to the manufacturer's protocol, and absorbance was read at 450 nm using a microplate reader.

2.3. Western blotting

Following treatment with the specified inhibitor for the indicated times (typically 6 and 24 h), cell lysates were prepared using Pierce™ RIPA Lysis and Extraction buffer (Thermo Scientific, NY, USA) with added protease and phosphatase inhibitors (Roche) on

ice. Total protein concentration was measured with a Pierce™ bicinchoninic acid kit (Thermo Scientific, NY, USA). Equal amounts of protein were then loaded onto SDS-polyacrylamide gels for separation. The proteins were subsequently transferred to and immobilized on Amersham™ nitrocellulose membranes (GE Healthcare Life Science, UK). Primary antibodies against Wee1 (GTX111392, 1:1000, GeneTex), phospho-Wee1 (#4910, 1:1000, Cell Signaling Technology), cdc2 (GTX108120, 1:1000, GeneTex), phospho-cdc2 (GTX128155, 1:1000, GeneTex), phospho-histone H2AX Ser139 (γ-H2AX; #05–636, 1:1000, EMD Millipore), poly (ADP-ribose)



IC_{50} (μM)	Gemcitabine	Gemcitabine+MK-1775 (0.2 μM)
A549	0.029±0.002	1.458e-07±1.160e-008
p value	ref.	0.0019
H460	0.025±0.036	0.026±0.03
p value	ref.	0.971
HCC827	0.049±0.052	0.003±0.002
p value	ref.	0.202
H1975	0.273±0.199	0.093±0.057
p value	ref.	0.206

Fig. 1. MK-1775 in combination with gemcitabine increased the cytotoxic activity in NSCLC. The survival rate of gemcitabine alone or plus wee1 inhibitor in $p53^{wt}$ NSCLC A549 (A) and H460 (B). The $p53^{mut}$ NSCLC HCC827 (C) and H1975 (D) cells treated with the same drug therapy for 72 h. The IC_{50} is displayed in bar graph (E) or detailed data in the table (F). The *p* values (mean ± SEM) presented are from three independent experiments. *$p < 0.05$ by Student's t-tests.

polymerase (PARP; #9532, 1:1000, Cell Signaling Technology), α-Tubulin (T9026, 1:1000, Sigma-Aldrich), along with secondary goat anti-mouse/rabbit horseradish peroxidase-conjugated antibodies (#115-035-003/111-035-003, Jackson ImmunoResearch Laboratories), were used. All antibodies were diluted in 5 % (w/v) non-fat milk in TBS-T. Protein detection was carried out using enhanced chemiluminescence (Merck Millipore, Darmstadt, Germany) and visualized with a UVP ChemStudio PLUS detector [30].

2.4. Cell cycle distribution by fluorescence activated cell sorting (FACS)

After treating the cells with the specified agents or combinations for 24 and 48 h, both floating and adherent cells were collected and fixed with 70 % cold ethanol. The samples were then incubated in PI/RNase Staining Solution (Cell Signaling) for 20 min at room temperature in the dark. Following this, they were analyzed using a FACSCalibur™ flow cytometer with CellQuest Pro software (Becton Dickinson, Oxford, UK). Cell cycle distribution was assessed based on DNA content using FlowJo vX software.

2.5. Xenograft mouse model

In vivo studies were approved by the Institutional Animal Care and Use Committee (IACUC, No. 2022032302) of the Chang Gung Memorial Hospital at Linko. H1975 cells (2×10^6) and H460 cells (1×10^6) were inoculated subcutaneously into the flanks of 4-week-old null male mice in 50 μl PBS. Tumor growth was monitored until the tumors reached an average volume of 100 mm^3. At this point, mice were randomly assigned to different treatment groups, receiving either vehicle (0.5 % methylcellulose), gemcitabine (50 mg/kg), pemetrexed (100 mg/kg), MK-1775 (50 mg/kg), or a combination of these drugs. Treatments were administered via intraperitoneal injection according to the schedule depicted in Figs. 5A and 6A. Tumor size was measured using calipers, and tumor volume was calculated using the formula: $V = (1/2) \times L \times W^2$, where L is the length and W is the width of the tumor. Body weight and any observable adverse events were recorded throughout the treatment period to assess toxicity. Mice were observed daily for signs of distress, weight loss, or other adverse effects. At the end of the treatment period, mice were sacrificed, and tumors were carefully excised, weighed, and photographed. Tumor volumes and weights were compared across different treatment groups to evaluate the efficacy of the drug combinations.

2.6. Statistical analysis

The data are shown as the mean ± SEM. Statistical analyses were performed using Student's *t*-test and two-way ANOVA where applicable, with GraphPad Prism 8 software. A *p*-value of less than 0.05 was deemed statistically significant.

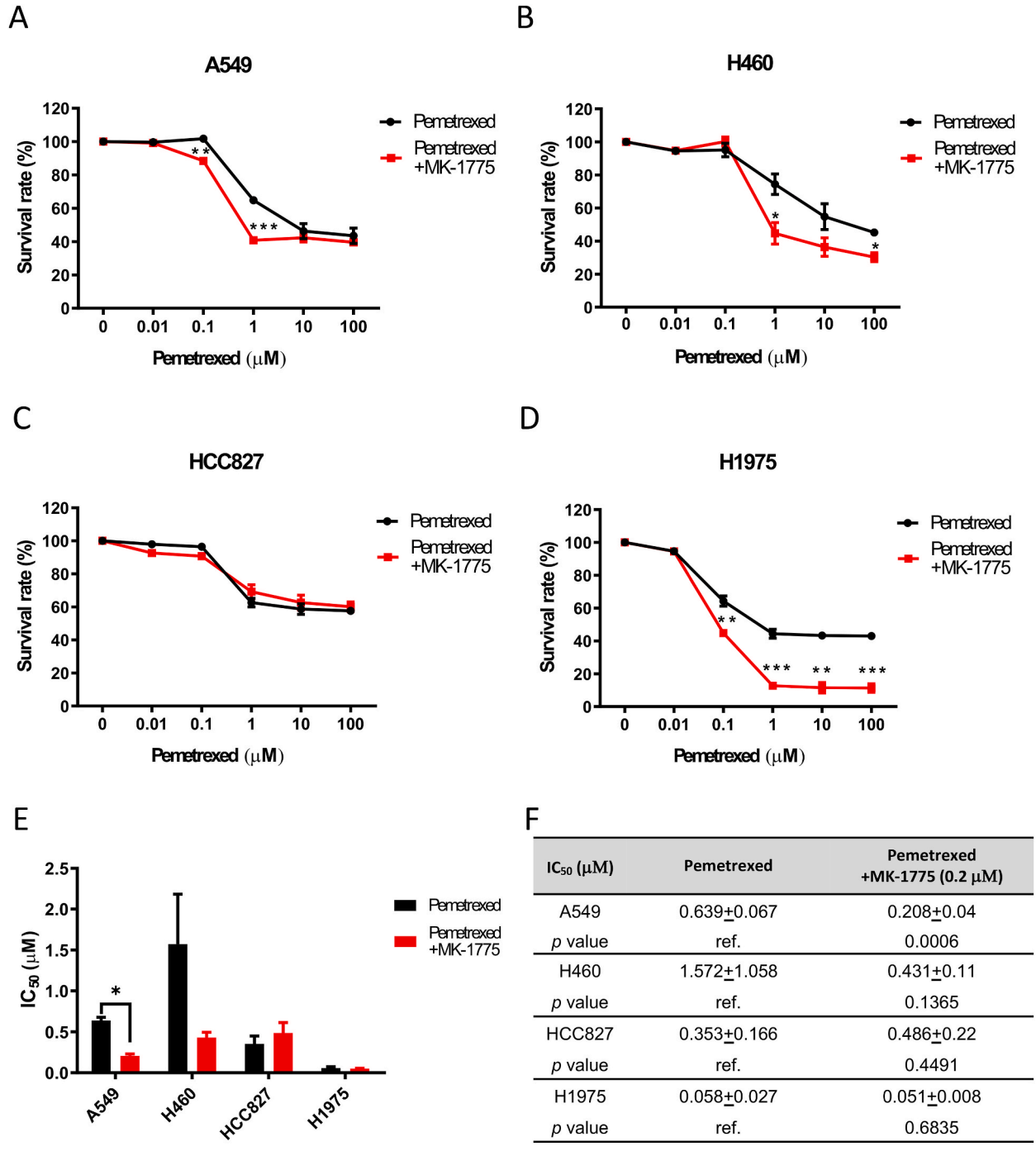
3. Results

3.1. The combination of MK-1775 with gemcitabine increased cytotoxic activity in NSCLC cell lines

Chemotherapy and EGFR TKIs are the most commonly used drugs in NSCLC. We firstly evaluated whether Wee1 inhibition by MK-1775 enhances the cytotoxic activity of afatinib or osimertinib, most commonly used EGFR TKIs in clinical practice, in EGFR mutated NSCLC cell lines HCC827 (harbors the EGFR L858R/T790M double mutations and p53 R273H mutation) and H1975 (harbors the EGFR E746-A750 deletion and p53 V218 deletion). Survival rates were similar whether afatinib or osimertinib were used alone or combined with MK-1775 (Supplementary Figs. 1A and B). The half-maximal inhibitory concentration (IC_{50}) values showed no significant differences between monotherapy and combination treatments in both cell lines (Supplementary Figs. 1C and D). These findings suggest MK-1775 does not augment the effectiveness of afatinib and osimertinib in these cell lines, indicating alternative strategies may be needed. Next, we evaluated the cytotoxic effect of combining chemotherapy with MK-1775 in NSCLC cells. The effects of varying concentrations of gemcitabine, both alone and in combination with MK-1775, on the survival rates of two EGFR and p53 wild type NSCLC cell lines (A549 and H460) and two mutation NSCLC cell lines (HCC827 and H1975) were evaluated (Fig. 1A–D). In the A549 (Fig. 1A) and HCC827 (Fig. 1C) cell lines, the combination treatment significantly decreased cell survival rates compared to gemcitabine alone. In contrast, although the combined treatment group in the H460 (Fig. 1B) and H1975 (Fig. 1D) cell lines showed lower cell survival rates, the differences were not statistically significant. The IC_{50} values across the four cell lines were summarized (Fig. 1E). For the A549 and HCC827 cell lines, the IC_{50} values in the combined treatment group were significantly lower than those for gemcitabine alone, indicating an enhanced anticancer effect. In the H460 and H1975 cell lines, the IC_{50} values for the combined treatment were lower but not significantly different from those for gemcitabine alone. Specifically, in the A549 cell line, the IC_{50} for gemcitabine alone was 0.029 ± 0.002 μM, while it was significantly reduced to 1.458e-07 ± 1.160e-08 μM in the combined treatment group. For the H460 cell line, the IC_{50} for gemcitabine alone was 0.025 ± 0.036 μM, and for the combined treatment, it was 0.026 ± 0.03 μM, with no significant difference. In the HCC827 cell line, the IC_{50} for gemcitabine alone was 0.049 ± 0.052 μM, while it was significantly reduced to 0.003 ± 0.002 μM in the combined treatment group. In the H1975 cell line, the IC_{50} for gemcitabine alone was 0.273 ± 0.199 μM, and for the combined treatment, it was 0.093 ± 0.057 μM, with no significant difference (Fig. 1F). These results demonstrated that combining gemcitabine with MK-1775 significantly decreased cell survival rates and IC_{50} values in the A549 and HCC827 NSCLC cell lines, indicating enhanced anticancer effects, while the H460 and H1975 cell lines showed decreased cell survival and IC_{50} values without statistically significant differences.

3.2. The combination of MK-1775 with pemetrexed increased cytotoxic activity in NSCLC cell lines

Pemetrexed is a chemotherapy drug used to treat various types of cancer, including non-small cell lung cancer (NSCLC). The effects of varying concentrations of pemetrexed, both alone and in combination with MK-1775, on the survival rates of four NSCLC cell lines (A549, H460, HCC827, and H1975) were evaluated. In the A549 cell line, the combination treatment significantly decreased cell



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IC_{50} (μM)	Pemetrexed	Pemetrexed +MK-1775 (0.2 μM)
A549	0.639±0.067	0.208±0.04
p value	ref.	0.0006
H460	1.572±1.058	0.431±0.11
p value	ref.	0.1365
HCC827	0.353±0.166	0.486±0.22
p value	ref.	0.4491
H1975	0.058±0.027	0.051±0.008
p value	ref.	0.6835

Fig. 2. Wee1 inhibition plus pemetrexed improved cell death in NSCLC except for HCC827 cells. The cell survival of pemetrexed only or in combination with MK-1775 in A549 (A), H460 (B), HCC827 (C), and H1975 (D). The bar graph (E) and the table (F) presented the IC_{50} and *p* values (mean ± SEM) after NSCLC treated with the above monotherapy or combination therapy. The *p* values presented are from three independent experiments. *$p < 0.05$ by Student's t-tests.

survival rates compared to pemetrexed alone (Fig. 2A). In the H460 cell line, the combined treatment group showed significantly lower cell survival rates compared to pemetrexed alone (Fig. 2B). In the H1975 cell line, the combination of pemetrexed and MK-1775 significantly reduced cell survival rates compared to pemetrexed alone (Fig. 2D). However, in the HCC827 cell line, there was no significant difference in cell survival rates between the treatment groups (Fig. 2C). The IC_{50} values were summarized across the four cell lines. For the A549 cell line, the IC_{50} in the combined treatment group was significantly lower, indicating an enhanced anticancer effect (Fig. 2E). Specifically, for the A549 cell line, the IC_{50} for pemetrexed alone was 0.639 ± 0.067 μM, while the IC_{50} for the combined treatment was significantly reduced to 0.208 ± 0.04 μM. In the H460 cell line, there was no significant difference in IC_{50} between the two treatment groups, with the IC_{50} for pemetrexed alone being 1.572 ± 0.158 μM, and for the combined treatment, it was 1.058 ± 0.11 μM. In the HCC827 cell line, the IC_{50} values did not show a significant difference between the treatment groups, with the IC_{50} for pemetrexed alone being 0.353 ± 0.166 μM, and for the combined treatment, it was 0.486 ± 0.22 μM. In the H1975 cell line, the IC_{50} in the combined treatment group was not significantly different from that of the pemetrexed alone group, with the IC_{50} for pemetrexed alone being 0.058 ± 0.027 μM, and for the combined treatment, it was 0.051 ± 0.008 μM (Fig. 2F). These data indicated that the combination of pemetrexed and MK-1775 significantly enhanced the anticancer effect in the A549 and H1975 cell lines, as

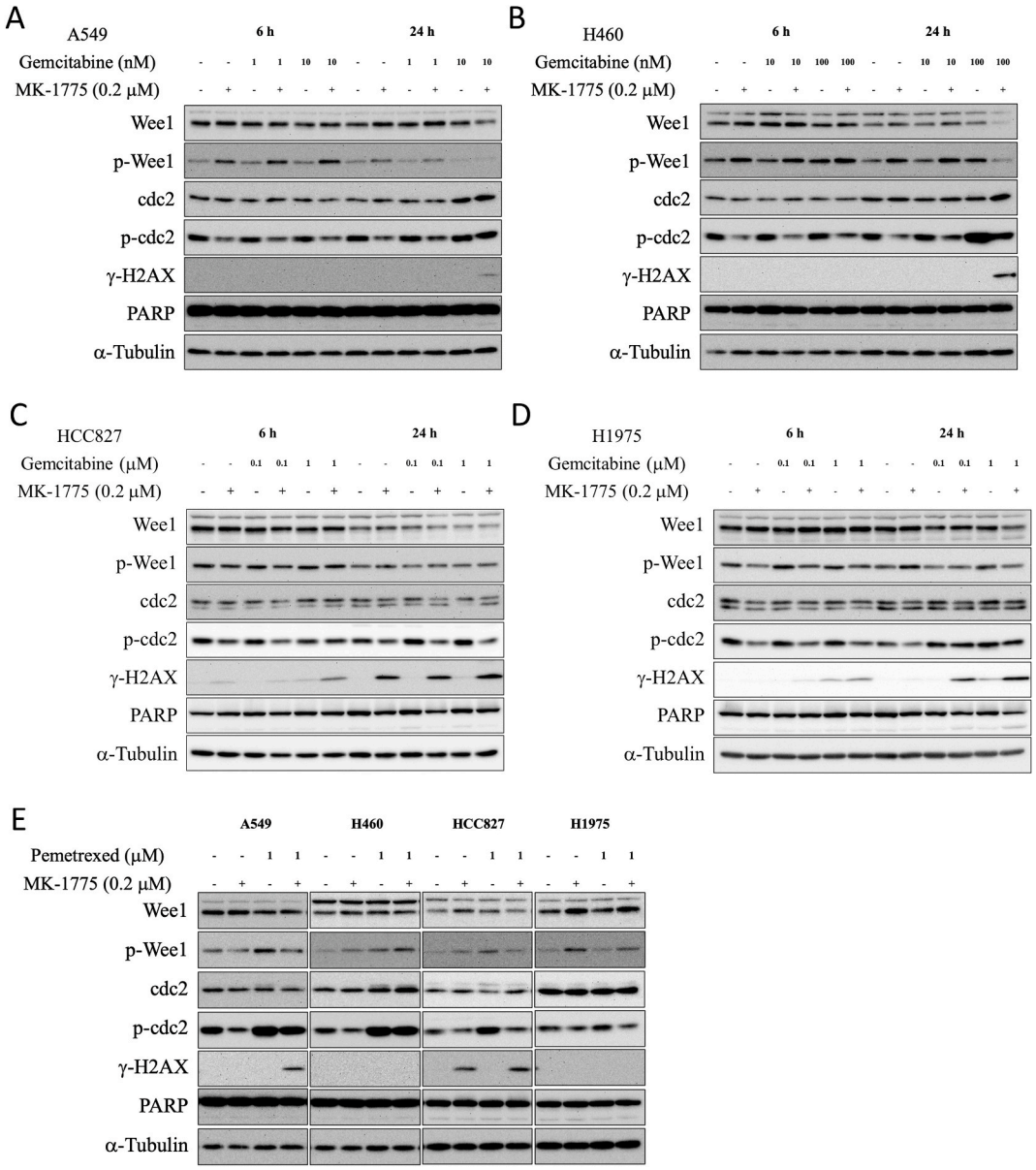

Fig. 3. MK-1775 in combination with chemotherapy induced apoptosis in NSCLC. Western blot analyzed the expression of Wee1, cdc2, γ-H2AX, and PARP after MK-1775 monotherapy or combined with gemcitabine in A549 (A), H460 (B), HCC827 (C), and H1975 (D) or four cells treated MK-1775 plus pemetrexed for 24 h in E (The original uncropped images are provided in the Supplementary file 2).

evidenced by the reduced survival rates and lower IC_{50} values.

3.3. The combination of MK-1775 with cisplatin increased cytotoxic activity in NSCLC cell lines

Wee1 inhibition by MK-1775 shows varying effects on the cytotoxic activity of cisplatin in NSCLC cell lines. The effects of this combinations were evaluated in A549, H460, HCC827, and H1975 cells. The combination with MK-1775 significantly decreased cell survival rates in the A549 and H460 cell lines compared to cisplatin alone, indicating enhanced cytotoxicity (Supplementary Figs. 2A and B). However, in the HCC827 and H1975 cell lines, no significant differences in survival rates were observed between the combination treatment and cisplatin alone (Supplementary Figs. 2C and D). The IC_{50} values also showed a significant reduction in A549 and H460 cell lines with the combination treatment, while no significant differences were observed in HCC827 and H1975 cell lines (Supplementary Figs. 2E and F). These data indicated that the combination of MK-1775 with cisplatin significantly enhanced the cytotoxic effect in A549 and H460 cell lines but not in HCC827 and H1975 cell lines. These findings suggest that the efficacy of MK-1775 in combination with chemotherapy drugs may vary depending on the specific genetic and molecular characteristics of different NSCLC cell lines.

3.4. MK-1775 in combination with chemotherapy induced apoptosis in NSCLC cell lines

Western blot analysis showed that combining MK-1775 with gemcitabine or pemetrexed resulted in reduced levels of phosphorylated cdc2 (p-cdc2), increased level of γ-H2AX, and PARP in A549, H460, HCC827, and H1975 cell lines (Fig. 3A–E). These markers indicate enhanced apoptosis when MK-1775 is combined with these chemotherapy agents. Combining MK-1775 with cisplatin also resulted in similar apoptotic effects, with increased γ-H2AX, and PARP levels across the same cell lines after 24 h of treatment (Supplementary Fig. 3). This further supports the role of MK-1775 in enhancing the apoptotic effects of chemotherapy in NSCLC cell lines.

During the treatment period, apoptosis marker γ-H2AX represented more sensitive responses in p53-mutant HCC827 and H1975 cell lines. Further analysis using FACS to evaluate cell cycle distribution showed that MK-1775 combined with gemcitabine stimulated the G2/M phase in these two cell lines. Specifically, the combination treatment led to an increased percentage of cells in the G2/M phase at both 24 and 48 h compared to gemcitabine alone (Fig. 4A–B).

In summary, MK-1775 combined with gemcitabine, pemetrexed, or cisplatin enhances apoptosis in NSCLC cell lines, as evidenced by the modulation of key apoptotic markers. Additionally, MK-1775 in combination with gemcitabine stimulates cell cycle arrest in the G2/M phase in p53-mutant HCC827 and H1975 cell lines. These findings suggest that MK-1775 may be a valuable addition to

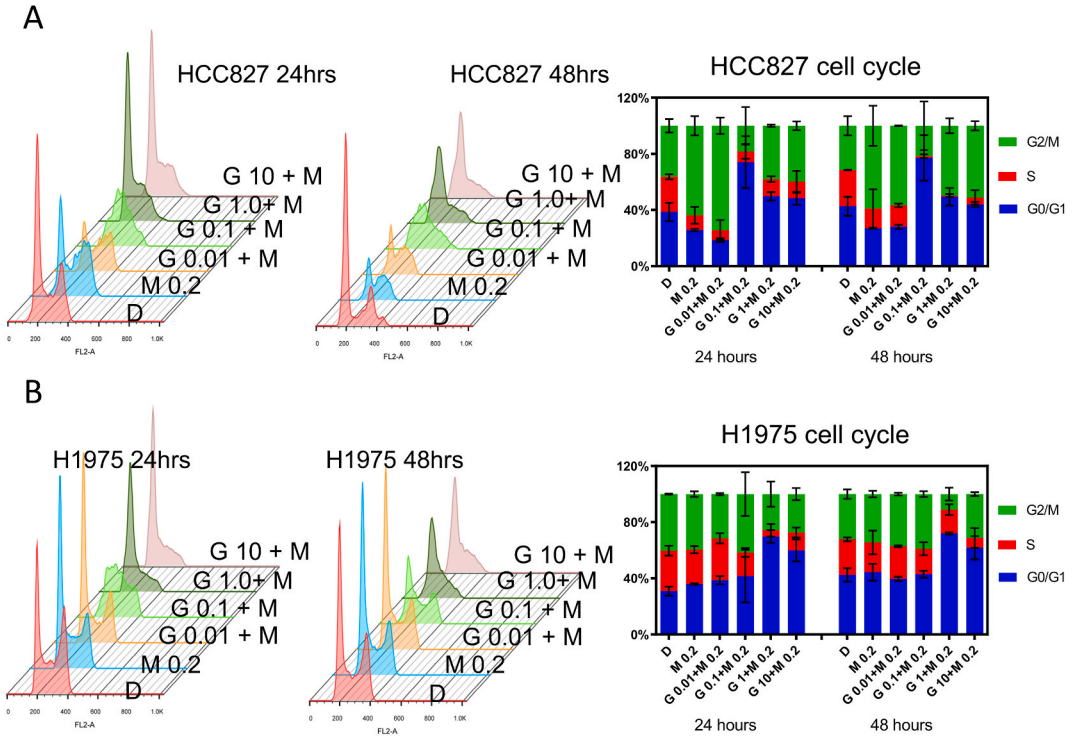

Fig. 4. Wee1 inhibition by MK-1775 plus gemcitabine stimulated the G2/M phase in $p53^{mut}$ HCC827. FACS evaluated the cell cycle after MK-1775 alone or plus gemcitabine in HCC827 (A), and H1975 (B) for 24 and 48 h. The mean ± SEM presented are from three independent experiments.

chemotherapy regimens for improving therapeutic outcomes in NSCLC.

3.5. Effects of MK-1775 with chemotherapy on NSCLC tumor growth

The experimental timeline showed the treatment schedule for H1975 xenograft models with vehicle, gemcitabine, MK-1775, and their combination (Fig. 5A). There were no significant differences in body weights among the different treatment groups, indicating no overt toxicity (Fig. 5B). Tumor volume analysis revealed that the combination of MK-1775 and gemcitabine significantly inhibited tumor growth compared to either treatment alone (Fig. 5C). This is visually confirmed by the harvested tumors (Fig. 5D), where the combination treatment resulted in smaller tumors. The tumor weights were also significantly reduced in the combination treatment group (Fig. 5E).

For the H460 xenograft models, the treatment schedule included vehicle, pemetrexed, MK-1775, and their combination (Fig. 6A). Similarly, there were no significant differences in body weights among the different treatment groups, indicating no overt toxicity (Fig. 6B). Tumor volume analysis showed that the combination of MK-1775 and pemetrexed significantly inhibited tumor growth compared to either treatment alone (Fig. 6C). The harvested tumors confirmed these findings, with the combination treatment group showing the smallest tumors (Fig. 6D). The tumor weights were also significantly reduced in the combination treatment group

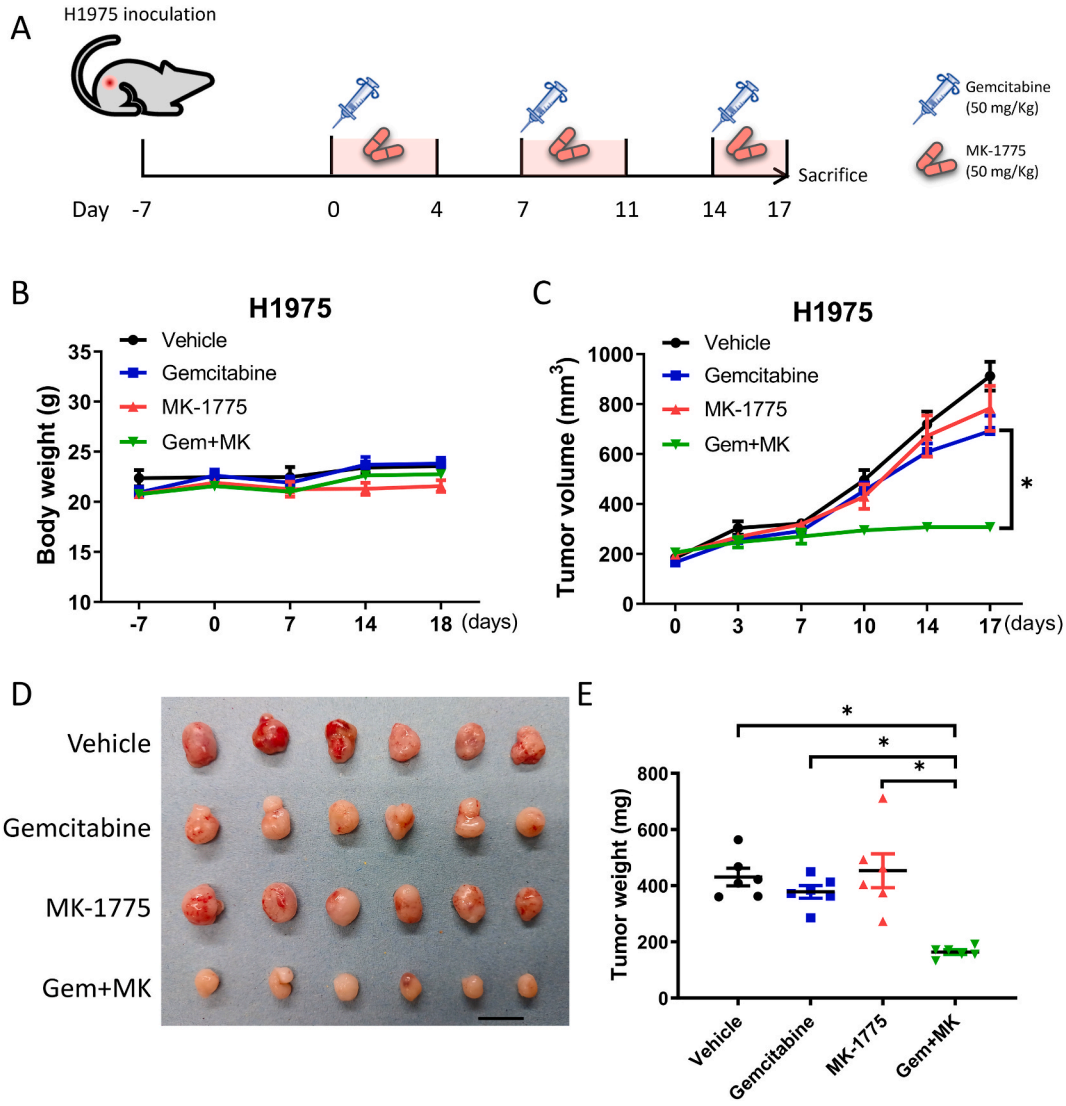

Fig. 5. Wee1 inhibition plus gemcitabine inhibits H1975 tumor formation. (A) Schema showing the experimental timeline. (B) There were no significant differences in the body weights of xenografted mice treated with vehicle, gemcitabine, MK-1775, or combined with these two drugs. (C) Tumor volume raised in the vehicle, gemcitabine, and MK-1775 groups but not in the gemcitabine plus MK1775 group. (D) The harvested tumors are shown. (E) The tumor weights show the mean values of D, along with the standard errors and p values. Data are expressed as mean ± SEM. Scale bar: 1 cm *, $p < 0.05$. Gem: gemcitabine.

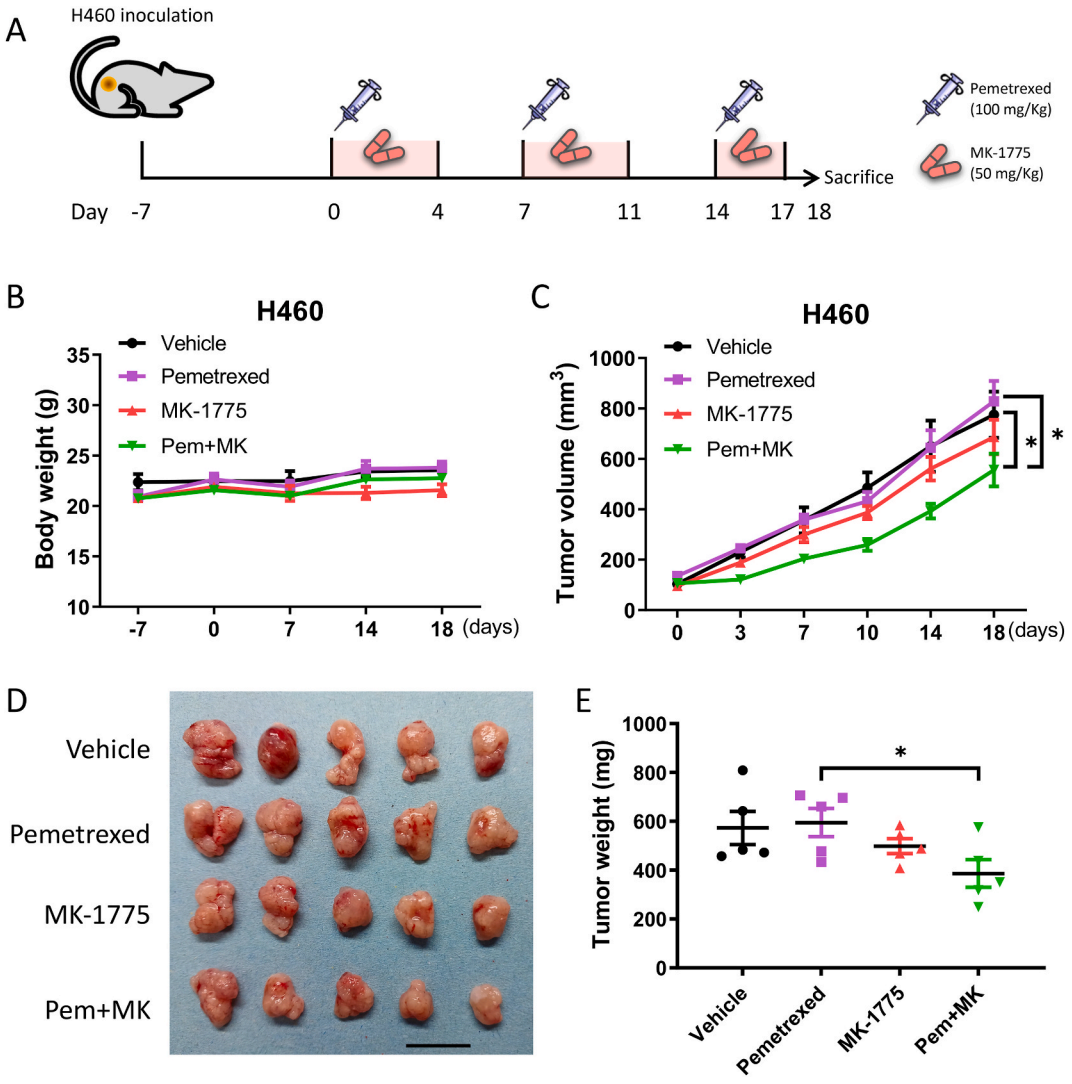

Fig. 6. Wee1 inhibition plus pemetrexed inhibits H460 tumor formation. (A) Schema showing the experimental timeline. (B) There were no significant differences in the body weights of xenografted mice treated with vehicle, pemetrexed, MK-1775, or pemetrexed plus MK-1775. (C) Tumor volume raised in the vehicle and pemetrexed groups but not in the combination group. (D) The harvested tumors are shown. (E) The tumor weights show the mean values of D, along with the standard errors and p values. Data are expressed as mean ± SEM. Scale bar: 1 cm *, $p < 0.05$.

(Fig. 6E). These data indicated that MK-1775 combined with gemcitabine or pemetrexed effectively inhibited tumor growth in NSCLC xenograft models without causing significant toxicity. These findings suggest that MK-1775 may enhance the therapeutic efficacy of gemcitabine and pemetrexed in treating NSCLC.

4. Discussion

This study investigates the potential of the Wee1 inhibitor MK-1775 to enhance the efficacy of chemotherapeutic agents gemcitabine and pemetrexed in NSCLC cell lines. The data demonstrate that MK-1775, in combination with these chemotherapeutic agents, significantly enhances cytotoxic effects, as evidenced by decreased cell survival rates, lower IC_{50} values, and increased apoptosis. The results showed that, compared to using gemcitabine or MK-1775 alone in H1975 cells, the combination therapy significantly reduced tumor growth. Similarly, in the H460 cell line, the combination of MK-1775 with pemetrexed demonstrated a significantly greater inhibitory effect compared to pemetrexed alone. The *in vivo* studies confirmed that the combination therapy effectively inhibits tumor growth without causing significant toxicity. These findings underscore the importance of exploring combination therapies in lung cancer to improve treatment outcomes and overcome resistance to standard therapies. Although the statistical analysis in our study revealed significant differences at only a few concentrations between the two groups, the combination of chemotherapy drugs with MK-1775 exhibited a trend of more effectively inhibiting cancer cell growth compared to chemotherapy alone, as shown in Fig. 1B–D

and 2A-B. This trend may be attributed to the different genetic backgrounds and varying synergistic effects of the drugs across cell lines. Additionally, the decreased p-cdc2 levels confirm that MK-1775 successfully inhibited its downstream targets, as seen in nearly all treatment groups (Supplementary Fig. 4). Therefore, even though certain drug combinations did not reach statistical significance, the combination of MK-1775 with chemotherapy drugs still demonstrates therapeutic potential.

Wee1 kinase plays a critical role in regulating the G2/M cell cycle checkpoint, allowing for DNA repair before mitotic entry [31]. Inhibiting Wee1 kinase with MK-1775 prevents G2/M arrest, forcing cancer cells with DNA damage to undergo apoptosis. This study highlights the enhanced cytotoxic effects of MK-1775 when combined with chemotherapeutic agents, compared to the limited efficacy observed with EGFR TKIs (afatinib and osimertinib) [9,10,32,33]. This suggests that the therapeutic potential of MK-1775 may be more pronounced when paired with DNA-damaging agents rather than with TKIs, making it a valuable addition to chemotherapy regimens. TKIs may predominantly induced cell cycle arrest rather than DNA damage associated apoptosis in EGFR mutated NSCLC [34,35], which explained why MK-1775 did not show additional efficacy when combining EGFR TKIs.

The tumor suppressor gene TP53 is crucial in regulating the cell cycle and apoptosis [36]. Many cancer cells, including those in NSCLC, exhibit p53 mutations, leading to defective G1/S checkpoints and increased reliance on the G2/M checkpoint for DNA repair [37]. Although gemcitabine and pemetrexed are both chemotherapeutic agents, their mechanisms of action differ slightly. Previous studies have indicated that both gemcitabine and pemetrexed exert cytotoxic effects on cancer cells via both p53-dependent and p53-independent pathways [38,39]. These studies support with the findings in our study, where both chemotherapeutic agents inhibit the growth of cells, regardless of whether the cells have wild-type or mutated p53. While the results in Fig. 1E show a greater decrease in the average IC_{50} of mutant cells (HCC827 and H1975), the significant effect was observed in the wild-type A549 cells. In Fig. 2E, although the wild-type cells (A549 and H460) displayed a larger decrease in IC_{50}, the mutant H1975 cells exhibited a notable increase in synergistic efficacy, making the combination treatment with pemetrexed more effective across a dosage range of 0.1–100 μM compared to pemetrexed alone. These results indicate that p53 is not the only factor affecting the efficacy of combination therapies; the cancer type and the genetic background of the cancer cells also play critical roles in determining therapeutic success.

Preclinical studies provide robust evidence supporting the combination of MK-1775 with gemcitabine or pemetrexed in enhancing cytotoxicity in pancreatic cancer and sarcoma [27,40]. Early clinical trials of MK-1775, either alone or in combination with other cytotoxic agents, have shown promising results, particularly in patients with advanced solid tumors including lung cancer harboring *TP53* mutations [29]. These above findings and the results of this study highlight the potential clinical benefits of incorporating Wee1 inhibitors into standard chemotherapy regimens to improve therapeutic outcomes in NSCLC patients.

While this study presents compelling evidence for the efficacy of MK-1775 combined with chemotherapy, several limitations exist. The study primarily focuses on preclinical models, and the translatability of these results to clinical settings remains uncertain. Additionally, the heterogeneity of NSCLC and the variability in p53 status across patients may affect the generalizability of the findings. Further research is needed to understand the mechanisms underlying the differential responses to Wee1 inhibition and to identify biomarkers for predicting treatment outcomes. Larger, well-designed clinical trials are essential to validate the preclinical findings and determine the safety and efficacy of MK-1775 in combination with chemotherapy in diverse patient populations.

CRediT authorship contribution statement

Chiao-Ping Chen: Writing – original draft, Visualization, Investigation. **Tsai-Hsien Hung:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Ping-Chih Hsu:** Visualization, Methodology, Investigation. **Chun-Nan Yeh:** Writing – review & editing, Investigation, Conceptualization. **Wen-Kuan Huang:** Supervision, Methodology, Formal analysis. **Yi-Ru Pan:** Methodology, Formal analysis. **Yu-Tien Hsiao:** Methodology, Formal analysis. **Chih-Hong Lo:** Methodology, Formal analysis, Data curation. **Chiao-En Wu:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Ethics statement

N/A.

Data availability statement

The data underpinning the findings of this study can be obtained from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e40299.

References

[1] Y. Lei, K. Wang, Y. Liu, X. Wang, X. Xiang, X. Ning, W. Ding, et al., Various subtypes of EGFR mutations in patients with NSCLC define genetic, immunologic diversity and possess different prognostic biomarkers, Front. Immunol. 13 (2022) 811601.
[2] Y.L. Zhang, J.Q. Yuan, K.F. Wang, X.H. Fu, X.R. Han, D. Threapleton, Z.Y. Yang, et al., The prevalence of EGFR mutation in patients with non-small cell lung cancer: a systematic review and meta-analysis, Oncotarget 7 (2016) 78985–78993.
[3] T.J. Lynch, D.W. Bell, R. Sordella, S. Gurubhagavatula, R.A. Okimoto, B.W. Brannigan, P.L. Harris, et al., Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib, N. Engl. J. Med. 350 (2004) 2129–2139.
[4] T.S. Mok, Y.L. Wu, S. Thongprasert, C.H. Yang, D.T. Chu, N. Saijo, P. Sunpaweravong, et al., Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma, N. Engl. J. Med. 361 (2009) 947–957.
[5] M. Maemondo, A. Inoue, K. Kobayashi, S. Sugawara, S. Oizumi, H. Isobe, A. Gemma, et al., Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR, N. Engl. J. Med. 362 (2010) 2380–2388.
[6] C. Zhou, Y.L. Wu, G. Chen, J. Feng, X.Q. Liu, C. Wang, S. Zhang, et al., Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study, Lancet Oncol. 12 (2011) 735–742.
[7] R. Rosell, E. Carcereny, R. Gervais, A. Vergnenegre, B. Massuti, E. Felip, R. Palmero, et al., Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial, Lancet Oncol. 13 (2012) 239–246.
[8] T. Mitsudomi, S. Morita, Y. Yatabe, S. Negoro, I. Okamoto, J. Tsurutani, T. Seto, et al., Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial, Lancet Oncol. 11 (2010) 121–128.
[9] L.V. Sequist, J.C. Yang, N. Yamamoto, K. O'Byrne, V. Hirsh, T. Mok, S.L. Geater, et al., Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations, J. Clin. Oncol. 31 (2013) 3327–3334.
[10] Y.L. Wu, C. Zhou, C.P. Hu, J. Feng, S. Lu, Y. Huang, W. Li, et al., Afatinib versus cisplatin plus gemcitabine for first-line treatment of Asian patients with advanced non-small-cell lung cancer harbouring EGFR mutations (LUX-Lung 6): an open-label, randomised phase 3 trial, Lancet Oncol. 15 (2014) 213–222.
[11] K. Park, E.H. Tan, K. O'Byrne, L. Zhang, M. Boyer, T. Mok, V. Hirsh, et al., Afatinib versus gefitinib as first-line treatment of patients with EGFR mutation-positive non-small-cell lung cancer (LUX-Lung 7): a phase 2B, open-label, randomised controlled trial, Lancet Oncol. 17 (2016) 577–589.
[12] J.C. Soria, Y. Ohe, J. Vansteenkiste, T. Reungwetwattana, B. Chewaskulyong, K.H. Lee, A. Dechaphunkul, et al., Osimertinib in untreated EGFR-mutated advanced non-small-cell lung cancer, N. Engl. J. Med. 378 (2018) 113–125.
[13] S.S. Ramalingam, J. Vansteenkiste, D. Planchard, B.C. Cho, J.E. Gray, Y. Ohe, C. Zhou, et al., Overall survival with osimertinib in untreated, EGFR-mutated advanced NSCLC, N. Engl. J. Med. 382 (2020) 41–50.
[14] T. John, H. Akamatsu, A. Delmonte, W.C. Su, J.S. Lee, G.C. Chang, X. Huang, et al., EGFR mutation analysis for prospective patient selection in AURA3 phase III trial of osimertinib versus platinum-pemetrexed in patients with EGFR T790M-positive advanced non-small-cell lung cancer, Lung Cancer 126 (2018) 133–138.
[15] T.S. Mok, Y.L. Wu, M.J. Ahn, M.C. Garassino, H.R. Kim, S.S. Ramalingam, F.A. Shepherd, et al., Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer, N. Engl. J. Med. 376 (2017) 629–640.
[16] G. Lamberti, E. Andrini, M. Sisi, A. Rizzo, C. Parisi, A. Di Federico, F. Gelsomino, et al., Beyond EGFR, ALK and ROS1: current evidence and future perspectives on newly targetable oncogenic drivers in lung adenocarcinoma, Crit. Rev. Oncol. Hematol. 156 (2020) 103119.
[17] M.C. Garassino, S. Gadgeel, G. Speranza, E. Felip, E. Esteban, M. Domine, M.J. Hochmair, et al., Pembrolizumab plus pemetrexed and platinum in nonsquamous non-small-cell lung cancer: 5-year outcomes from the phase 3 KEYNOTE-189 study, J. Clin. Oncol. 41 (2023) 1992–1998.
[18] S. Novello, D.M. Kowalski, A. Luft, M. Gumus, D. Vicente, J. Mazieres, J. Rodriguez-Cid, et al., Pembrolizumab plus chemotherapy in squamous non-small-cell lung cancer: 5-year update of the phase III KEYNOTE-407 study, J. Clin. Oncol. 41 (2023) 1999–2006.
[19] Z.A. Stewart, M.D. Westfall, J.A. Pietenpol, Cell-cycle dysregulation and anticancer therapy, Trends Pharmacol. Sci. 24 (2003) 139–145.
[20] C.E. Wu, C.Y. Huang, C.P. Chen, Y.R. Pan, J.W. Chang, J.S. Chen, C.N. Yeh, et al., WIP1 inhibition by GSK2830371 potentiates HDM201 through enhanced p53 phosphorylation and activation in liver adenocarcinoma cells, Cancers (Basel) 13 (2021).
[21] C.E. Wu, Y.R. Pan, C.N. Yeh, J. Lunec, Targeting P53 as a future strategy to overcome gemcitabine resistance in biliary tract cancers, Biomolecules 10 (2020).
[22] C.E. Wu, T.S. Koay, A. Esfandiari, Y.H. Ho, P. Lovat, J. Lunec, ATM Dependent DUSP6 modulation of p53 Involved in synergistic targeting of MAPK and p53 pathways with trametinib and MDM2 inhibitors in cutaneous melanoma, Cancers (Basel) 11 (2018).
[23] C.E. Wu, A. Esfandiari, Y.H. Ho, N. Wang, A.K. Mahdi, E. Aptullahoglu, P. Lovat, et al., Targeting negative regulation of p53 by MDM2 and WIP1 as a therapeutic strategy in cutaneous melanoma, Br. J. Cancer 118 (2018) 495–508.
[24] C.E. Wu, T.S. Koay, Y.H. Ho, P. Lovat, J. Lunec, TP53 mutant cell lines selected for resistance to MDM2 inhibitors retain growth inhibition by MAPK pathway inhibitors but a reduced apoptotic response, Cancer Cell Int. 19 (2019) 53.
[25] J.J.J. Geenen, J.H.M. Schellens, Molecular pathways: targeting the protein kinase Wee1 in cancer, Clin. Cancer Res. 23 (2017) 4540–4544.
[26] C.J. Matheson, D.S. Backos, P. Reigan, Targeting Wee1 kinase in cancer, Trends Pharmacol. Sci. 37 (2016) 872–881.
[27] N.V. Rajeshkumar, E. De Oliveira, N. Ottenhof, J. Watters, D. Brooks, T. Demuth, S.D. Shumway, et al., MK-1775, a potent Wee1 inhibitor, synergizes with gemcitabine to achieve tumor regressions, selectively in p53-deficient pancreatic cancer xenografts, Clin. Cancer Res. 17 (2011) 2799–2806.

[28] A.A. Van Linden, D. Baturin, J.B. Ford, S.P. Fosmire, L. Gardner, C. Korch, P. Reigan, et al., Inhibition of Wee1 sensitizes cancer cells to antimetabolite chemotherapeutics *in vitro* and *in vivo*, independent of p53 functionality, Mol. Cancer Therapeut. 12 (2013) 2675–2684.
[29] S. Leijen, R.M. van Geel, A.C. Pavlick, R. Tibes, L. Rosen, A.R. Razak, R. Lam, et al., Phase I study evaluating WEE1 inhibitor AZD1775 as monotherapy and in combination with gemcitabine, cisplatin, or carboplatin in patients with advanced solid tumors, J. Clin. Oncol. 34 (2016) 4371–4380.
[30] C.E. Wu, C.P. Chen, Y.R. Pan, S.M. Jung, J.W. Chang, J.S. Chen, C.N. Yeh, et al., In vitro and in vivo study of GSK2830371 and RG7388 combination in liver adenocarcinoma, Am. J. Cancer Res. 12 (2022) 4399–4410.
[31] A. Ghelli Luserna di Rora, C. Cerchione, G. Martinelli, G. Simonetti, A WEE1 family business: regulation of mitosis, cancer progression, and therapeutic target, J. Hematol. Oncol. 13 (2020) 126.
[32] K. Park, E.H. Tan, K. O'Byrne, L. Zhang, M. Boyer, T. Mok, V. Hirsh, et al., Afatinib versus gefitinib as first-line treatment of patients with EGFR mutation-positive non-small-cell lung cancer (LUX-Lung 7): a phase 2B, open-label, randomised controlled trial, Lancet Oncol. 17 (2016) 577–589.
[33] E. Gourd, Overall survival with osimertinib in untreated NSCLC, Lancet Oncol. 21 (2020) e15.
[34] P.A. Janne, J.C. Yang, D.W. Kim, D. Planchard, Y. Ohe, S.S. Ramalingam, M.J. Ahn, et al., AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer, N. Engl. J. Med. 372 (2015) 1689–1699.
[35] G.J. Riely, K.A. Politi, V.A. Miller, W. Pao, Update on epidermal growth factor receptor mutations in non-small cell lung cancer, Clin. Cancer Res. 12 (2006) 7232–7241.
[36] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.
[37] S. Barajas, W. Cai, Y. Liu, Role of p53 in regulation of hematopoiesis in health and disease, Curr. Opin. Hematol. 29 (2022) 194–200.
[38] R. Hill, M. Rabb, P.A. Madureira, D. Clements, S.A. Gujar, D.M. Waisman, C.A. Giacomantonio, et al., Gemcitabine-mediated tumour regression and p53-dependent gene expression: implications for colon and pancreatic cancer therapy, Cell Death Dis. 4 (2013) e791.
[39] T.Y. Yang, G.C. Chang, K.C. Chen, H.W. Hung, K.H. Hsu, C.H. Wu, G.T. Sheu, et al., Pemetrexed induces both intrinsic and extrinsic apoptosis through ataxia telangiectasia mutated/p53-dependent and -independent signaling pathways, Mol. Carcinog. 52 (2013) 183–194.
[40] J.M. Kreahling, J.Y. Gemmer, D. Reed, D. Letson, M. Bui, S. Altiok, MK1775, a selective Wee1 inhibitor, shows single-agent antitumor activity against sarcoma cells, Mol. Cancer Therapeut. 11 (2012) 174–182.