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Wee-1 Kinase Inhibition Overcomes Cisplatin Resistance Associated with High-Risk *TP53* Mutations in Head and Neck Cancer through Mitotic Arrest Followed by Senescence

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Abstract

Although cisplatin has played a role in “standard-of-care” multimodality therapy for patients with advanced squamous cell carcinoma of the head and neck (HNSCC), the rate of treatment failure remains particularly high for patients receiving cisplatin whose tumors have mutations in the *TP53* gene. We found that cisplatin treatment of HNSCC cells with mutant *TP53* leads to arrest of cells in the G₂ phase of the cell cycle, leading us to hypothesize that the wee-1 kinase inhibitor MK-1775 would abrogate the cisplatin-induced G₂ block and thereby sensitize isogenic HNSCC cells with mutant *TP53* or lacking p53 expression to cisplatin. We tested this hypothesis using clonogenic survival assays, flow cytometry, and *in vivo* tumor growth delay experiments with an orthotopic nude mouse model of oral tongue cancer. We also used a novel *TP53* mutation classification scheme to identify which *TP53* mutations are associated with limited tumor responses to cisplatin treatment. Clonogenic survival analyses indicate that nanomolar concentration of MK-1775 sensitizes HNSCC cells with high-risk mutant p53 to cisplatin. Consistent with its ability to chemosensitize, MK-1775 abrogated the cisplatin-induced G₂ block in p53-defective cells leading to mitotic arrest associated with a senescence-like phenotype. Furthermore, MK-1775 enhanced the efficacy of cisplatin *in vivo* in tumors harboring *TP53* mutations. These results indicate that HNSCC cells expressing high-risk p53 mutations are significantly sensitized to cisplatin therapy by the selective wee-1 kinase inhibitor, supporting the clinical evaluation of MK-1775 in combination with cisplatin for the treatment of patients with *TP53* mutant HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) affects over 500,000 patients worldwide annually and half this number of patients will die from the disease each year (1). Multimodality chemotherapy employing cisplatin in the neoadjuvant setting or given concurrently with radiation has become a standard of care for patients with locally advanced HNSCC (2–4). Despite advances in therapy, there is a high rate of treatment failure and the long-term survival in patients with advanced-stage head and neck cancer remains poor (5). Recent genomic data have revealed that *TP53* is the most frequently mutated gene in HNSCC, occurring in up to 85% of non-human papillomavirus-positive primary tumors (6–8). Several reports have shown that *TP53* mutation is associated with poor therapeutic response and decreased survival in HNSCC (9–12).

Recently, we developed an evolution-based scoring algorithm, called evolutionary action (EA), which stratifies *TP53* mutations based upon scores (i.e., high risk vs. low risk) that correlate with HNSCC patient clinical outcomes and response to treatment (unpublished observations). This system (EA_{p53}) has been further validated to predict response to cisplatin-based therapy in patients with HNSCC and in preclinical models of oral tongue cancer using established HNSCC cell lines where we have shown tumors with high-risk *TP53* mutations were resistant to cisplatin relative to those with low-risk mutations or wild-type *TP53* (unpublished observations). Decreased cisplatin sensitivity associated with these high-risk mutations is driven by their inability to undergo cellular senescence, the primary response for cells with wild-type *TP53* (13). Therefore, an important clinical objective is to develop therapeutic strategies for overcoming inherent chemotherapy resistance in tumors

from patients with high-risk *TP53* mutations. Tumors with loss of p53 function are dependent on activation of the S- and G₂-phase checkpoints for mediating the growth arrest needed to repair DNA damage and survive genotoxic stress, making these cells potentially sensitive to G₂ checkpoint abrogation (14–17). Conceptually, abrogation of the G₂ checkpoint could sensitize cisplatin-resistant mutant *TP53* HNSCC cells to DNA-damaging agents and spare normal cells with intact p53 function (18). Thus, developing novel molecularly targeted drugs that abrogate the G₂ checkpoint has become an intense area of research.

Wee-1 is a tyrosine kinase involved in DNA damage–induced G₂–M arrest, owing to its ability to inactivate the CDC2 also known as cyclin-dependent kinase 1 (CDK1) through phosphorylation of the Tyr15 residue (19). Inhibition of Wee-1 kinase activity can override a G₂ cell-cycle arrest, causing an accumulation of cells with extensive DNA damage in the M-phase which can lead to mitotic catastrophe or death (20). Therefore, inhibitors of Wee-1 have been developed as potential anticancer therapeutics (21). Recent work with MK-1775 (currently known as AZD-1775), a specific inhibitor of Wee-1, and siRNA-mediated depletion of this gene (22) suggests that Wee-1 inhibition abrogated the G₂ checkpoint and selectively sensitized p53-deficient cells to various DNA-damaging agents, such as gemcitabine, carboplatin, and cisplatin (23, 24), and inhibited tumor growth in *in vivo* models (24, 25). In light of these preclinical findings, MK-1775 has entered phase I and II clinical trials as a chemosensitizer in combination with gemcitabine, carboplatin, or cisplatin in patients with advanced solid tumors and shows good tolerability and less cytotoxicity (26, 27).

The exact molecular mechanism(s) through which MK-1775 enhances the antitumor efficacy of cisplatin in tumor cells is not completely understood. In addition, the single-agent efficacy of MK-1775 or in combination with cisplatin therapy has not been carefully evaluated in HNSCC. Therefore, we hypothesized that the Wee-1 inhibitor MK-1775 will sensitize HNSCC-bearing high-risk mutant *p53* stratified by EAp53 system to cisplatin treatment both *in vitro* and *in vivo* in preclinical models of oral cancer. Our data demonstrate that MK-1775 sensitizes high-risk p53 mutant HNSCC cell lines to cisplatin *in vitro* through abrogation of G₂ arrest and accumulation of cells harboring unrepaired DNA lesions in mitosis. Interestingly, the combination therapy leads to aberrant mitosis associated with a senescence-like rather than an apoptotic phenotype. MK-1775 significantly potentiates the efficacy of cisplatin in high-risk mutant p53 *in vivo*. Furthermore, tumor cells bearing wild-type p53 displayed minimal response to MK-1775 addition, indicating that cisplatin sensitization was linked to p53 loss of function and that MK-1775 may have clinical utility to overcome drug resistance associated with cisplatin-based therapy in patients with HNSCC whose tumors have absent or mutated *TP53*.

Materials and Methods

Cell culture and reagents

The HNSCC cell line PCI-13 lacking endogenous p53 was obtained from the laboratory of Dr. Jennifer Grandis (University of Pittsburgh, Pittsburgh, PA) in August 2008 and engineered to stably express constructs containing wild-type p53, high-risk EA score mutant

p53 (C238F and G245D), which were generated and inserted into a pBabe retroviral vector (pBaBepuro; Addgene) by using standard cloning techniques. The naturally occurring HNSCC cell lines, HN30 (wtp53) and HN31 (mutp53), were obtained in December 2008 from the laboratory of Dr. John Ensley (Wayne State University, Detroit, MI). The cell lines and their isogenic derivatives were tested and authenticated against the parental cell lines by our group using short-tandem repeat analysis within 6 months of use for the current study. All cell lines were maintained in DMEM supplemented with 10% FBS, L-glutamine, sodium pyruvate, nonessential amino acids, and vitamins. The Wee-1 inhibitor MK-1775 was provided by Merck Corp. (currently licensed by AstraZeneca and known as AZD-1775), and its chemical structure has been described previously (23). Cells were trapped in mitosis using 0.2 $\mu\text{g}/\text{mL}$ of nocodazole (Sigma-Aldrich). For *in vitro* studies, MK-1775 was prepared as 10 mmol/L stock solution in DMSO and stored at -20°C and diluted in culture medium (0.25 $\mu\text{mol}/\text{L}$) immediately before use. Staurosporine was purchased from Sigma and used at 1:1,000 final concentration

Clonogenic survival assay

For synergy analysis between cisplatin and MK-1775, 500 to 800 cells/well were seeded in 6-well plates and exposed concurrently to different fixed-ratio combinations of cisplatin (dose range, 0.01–2 $\mu\text{mol}/\text{L}$) and MK-1775 (dose range, 0.01–1 $\mu\text{mol}/\text{L}$) for 24 hours. Cells were then washed with $1\times$ PBS to remove the cisplatin followed by addition of fresh MK-1775 for 24 hours and a second washout before culturing 10 to 14 days. Colonies were counted and survival fraction (IC_{50}) was determined as previously described (28).

Analysis of combined drug effects

Drug synergy was determined by the combination index (CI) and isobologram analyses, according to the median-effect method of Chou and Talalay (29) using the CalcuSyn software (Bio-soft). The CI is a quantitative representation of the degree of drug interaction. Although a $\text{CI} < 1.0$ can be considered as synergy, we chose a cutoff point of <0.75 to more rigorously define synergy. Details of the analyses are provided in Supplementary Materials and Methods.

Antibodies and immunoblotting

Cells were treated with cisplatin (1.5 $\mu\text{mol}/\text{L}$), MK-1775 (0.25 $\mu\text{mol}/\text{L}$) either alone or in combination as previously indicated. Cell extracts were prepared and Western blot analysis was conducted as described previously (28). Membranes were blocked for 1 hour at room temperature using 1% powdered milk in 0.1% Tween-20 in TBS, and incubated overnight with the following primary antibodies, including phospho- γH2AX (Ser139; #2577), phospho-CDC2-Tyr15 (#9111), CDC2 (#9112), cyclin B1 (#4138), phospho-CDC25C-Ser216 (#4901), phospho-Histone H3 (p-HH3, #9701), PARP-1, CHK1 (#2345), and CHK1-Ser345 (#2341), all from Cell Signaling Technology; β -actin (#A5316; Sigma-Aldrich). Membranes were then washed with 0.1% Tween-20 in TBS and incubated for 1 hour at room temperature with species-specific horseradish peroxidase-conjugated secondary antibodies, and protein signals were developed using the SuperSignal West

chemiluminescent system (Pierce Biotechnology). Membranes were stripped and reprobed with anti- β -actin to verify equal protein loading.

Cell-cycle analysis and apoptosis detection

HNSCC cells (50,000) were seeded in 60-mm dishes, treated the next day with 1.5 μ mol/L cisplatin, 0.25 μ mol/L MK-1775 either alone or in combination for 48 hours, and then harvested 0, 24, or 48 hours later. Cells were fixed in ice-cold 70% ethanol, permeabilized with 0.25% Triton X-100 in PBS, and incubated with phospho-Histone H3 (Ser10) antibody conjugated to Alexa Fluor 488 (#9708; at concentration 1:100, from Cell Signaling Technology) for 2 hours at 4°C. DNA was stained with 20 μ g/mL propidium iodide (PI; Sigma-Aldrich) in the presence of 100 μ g/mL RNase A (Sigma-Aldrich). For apoptosis assessment, cells were treated as indicated above and DNA strand breaks detected with an APO-BrdU TUNEL assay kit (Life Technologies) according to the manufacturer's instructions. Samples were analyzed on a Gallios Flow Cytometer (Beckman Coulter, Inc.) combined with Flo-Jo software (FloJo).

Immunofluorescence

Cells were plated on glass coverslips and treated with drugs the following day as described above. Cells were then fixed in 2% paraformaldehyde for 1 hour, washed, permeabilized in 0.2% Triton X-100 in PBS for 20 minutes, washed, and blocked for 1 hour at room temperature in 1 \times PBS buffer containing 2% normal goat serum, and 0.3% Triton X-100. Next, cells were incubated with primary phospho-H3 (Ser10) antibody overnight at 4°C. After washing with PBS, primary antibody was visualized with secondary Alexa Fluor-conjugated antibody. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were acquired on a Leica confocal microscope. For assessment of mitotic catastrophe, the number of cells with multilobulated nuclei or 2 or more micronuclei per high-power field (hpf) were identified by DAPI and phalloidin (Molecular Probes) staining, counted from four quadrants (in duplicates) and reported as a percentage of total cells per field (800 cells per coverslip).

Senescence-associated- β -gal staining

Briefly, PCI-13 cells plated in 6-well plates were treated with cisplatin and MK-1775 and cultured normally for 72 hours after treatment. Senescence-associated (SA)-beta-gal staining was performed as previously described (28).

Reactive oxygen species measurement

Intracellular reactive oxygen species (ROS) levels were measured according to a published protocol using 5-(and-6)-carboxy-2',7'-dichlorofluorescein (CM-H2DCFDA) dye (28). Briefly, after treatment with drugs, cells were loaded with CM-H2DCFDA for 60 minutes in culture media and excessive dye removed. Cells were trypsinized, and fluorescence analyzed by flow cytometry was normalized to the control condition and cell number was assayed by total DNA.

Orthotopic mouse model of oral cavity cancer and tumor growth delay

All animal experimentation was approved by the Institutional Animal Care and use Committee of the University of Texas MD Anderson Cancer Center. Our orthotopic nude mouse tongue model has been previously described (30). PCI-13 cells expressing either a high-risk mutant p53, pBabe *TP53* null, or wild-type *TP53* were injected into the tongues of male athymic nude mice, and 8 days after injection mice were randomized into different groups. Treatment was initiated when tumors were less than 0.5 cm³ in size. Mice were treated with 4 weekly cycles consisting of cisplatin at a dose of 4 mg/kg administered i.v. on day 1, followed by MK-1775 at a dose of 30 mg/kg (in 0.5% methylcellulose) given by oral gavage (p.o.) on days 2 and 4. Tongue tumor size was measured with microcalipers, and tumor volume calculated as previously described (30). For phospho-H3 immunohistochemical analysis, cisplatin treatment was followed by MK-1775 for 3 days (3 doses), with tumors collected 12 hours after the final dose, fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned. Tissue sections were then stained with phospho-H3 (Ser10) rabbit polyclonal antibody (Cat # 06-570; Millipore) at 1:50 concentration using the DAKO Envision + system (Dako Corp.). The percentage of area positively stained in each tumor was calculated for each field.

Statistical analysis

The Student *t* test was carried out to analyze *in vitro* data. For mouse studies, the two-tailed *t* test was used to compare tumor volumes between control and treatment groups. All data were expressed as mean \pm SE, and *P* values <0.05 were considered significant.

Results

The Wee-1 inhibitor MK-1775 synergizes with cisplatin to inhibit *in vitro* growth of HNSCC cells expressing high-risk *TP53* mutations

The impact of MK-1775 on HNSCC PCI-13 cells expressing wild-type *TP53* or high-risk *TP53* mutants treated with cisplatin was assessed using clonogenic survival assays. As shown in Fig. 1A, irrespective of the *TP53* mutational status, the PCI-13 isogenic cells displayed similar sensitivity to MK-1775 as a single agent with an average IC₅₀ of 250 nmol/L. We next investigated whether MK-1775 treatment was synergistic with cisplatin treatment in the isogenic PCI-13 cell lines, using the CI method of Chou and Talalay (29). Figure 1B shows representative images of clonogenic survival assays demonstrating the relative resistance of PCI-13 cells expressing the high-risk mutant (C238F) or lacking p53 (pBabe), which could be overcome by addition of MK-1775. In PCI-13 cells expressing wild-type *TP53*, MK-1775 did not cause shift of the cisplatin response curve and these agents were found to be antagonistic; the CI value [fraction affected (Fa) 0.5, \pm SD] was 2.22 ± 0.44 (Fig. 1C, top plot). The results are also displayed as a CI plot (Fig. 1C, bottom plot) and show no synergistic interaction at the more relevant FA values. However, MK-1775 significantly enhanced the cytotoxic effect of cisplatin in p53-deficient HNSCC cells carrying only pBabe control vector (Fig. 1D, top plot) and those expressing the high-risk *TP53* mutation (C238F; Fig. 1E, top plot). The combination effect reveals strong synergism manifested by the shift of cisplatin response curves and the IC values (Fa 0.5, \pm SD) of 0.35 ± 0.08 , and 0.07 ± 0.07 , respectively. The CI plots (Fig. 1D and E, bottom plots)

in the pBabe p53 null and high-risk p53 mutant cells show a clear synergistic effect at the more relevant FA values (> 50%). The degree of synergy between cisplatin and MK-1775 was also determined in other HNSCC cell lines derived from human tumors with known *TP53* status, wild-type HN30 (CI = 1.75 ± 0.32 , antagonism), and mutant *TP53* HN31 (CI = 0.12 ± 0.15 , strong synergism; Supplementary Fig. S1). These data clearly demonstrate that MK-1775 sensitizes the HNSCC tumor cells to cisplatin therapy in a p53-dependent manner.

Wee-1 inhibition attenuates cisplatin-induced CDC2 phosphorylation and triggers a general DNA damage response

The Wee-1–induced G₂ phase arrest results from phosphorylation and inactivation of CDC2 (31). To confirm that MK-1775 affects its downstream target, the isogenic PCI-13 cells were treated as previously described and phosphorylation of CDC2 was examined by Western blot. Following cisplatin, increased phosphorylation of CDC2 was apparent irrespective of p53 status. MK-1775 alone caused substantial suppression of CDC2 phosphorylation accompanied by decreased protein levels of Cyclin B1 (Fig. 2), indicating effective engagement of downstream targets. Combination treatment attenuated CDC2 phosphorylation, indicating that MK-1775 inhibited Wee-1 activity. The combination of cisplatin and MK-1775 significantly increased the levels of phosphorylation of the DNA damage markers, γ H2AX and CHK1, at ser139 and ser345, respectively, indicating an increase and persistence of unrepaired DNA damage in all the PCI-13 clones (Fig. 2).

Wee-1 inhibition induces prolonged mitotic arrest in high-risk *TP53* mutant HNSCC cells harboring unrepaired DNA lesions

To examine if MK-1775 abrogates a cisplatin-mediated G₂ checkpoint in HNSCC PCI-13 cells, unsynchronized cells were treated, and the cell-cycle progression and entry into mitosis were measured as described in Materials and Methods. In response to cisplatin alone, all PCI-13 cells arrested at the G₂–M phase (43.6%, 60.9%, and 57.9%, respectively) regardless of their p53 status. However, the total percentage in G₁ phase following cisplatin treatment was higher in wtp53 cells (30.9%) compared with cells lacking p53 (pBabe; 5.8%) or mutant p53 cells (10.33%), suggesting that a portion of the wild-type cells may have arrested in G₁ phase (Fig. 3A and Supplementary Fig. S2). Addition of MK-1775 to cisplatin did not trigger entry into mitosis in the PCI-13 cells carrying wild-type *TP53* (6.91% vs. 6.3% mitotic cells with 4N DNA content; Fig. 3A and Supplementary Fig. S2). However, a substantial proportion of PCI-13 cells carrying (pBabe) and high-risk mutant p53 (C238F) progressed through the G₂ checkpoint (18.7% vs. 28.3%, and 18.1% vs. 30.5% mitotic cells with 4N DNA content respectively; Fig. 3A and B and Supplementary Fig. S2) in response to combination treatment. These p53-altered PCI-13 cells did not completely progress to the next cell-cycle phase at 48 or 72 hours following addition of MK-1775. This suggests that abrogation of the G₂ block is likely followed by prolonged mitotic arrest and perhaps an exit of some cells with 4N DNA content to the G₁ phase through mitotic slippage.

To determine whether addition of MK-1775 to cisplatin results in premature mitotic entry, we used mitotic trapping followed by phospho-H3 immunostaining analysis to quantitate cells in mitosis. Representative fluorescence images of PCI-13 cell expressing high-risk

mutant p53 are shown in Fig. 3C along with quantification in Fig. 3D. The addition of MK-1775 to cisplatin produced significantly higher phospho-H3 (Ser10) staining and expression level (Fig. 3E) in PCI-13 cells null for *TP53* (pBabe) and with high-risk mutant *TP53* (C238F) compared with cisplatin treatment alone, indicating premature entry of a large proportion of cells into mitosis consistent with the G₂ abrogation and prolonged mitotic arrest observed during the cell-cycle analysis (Fig. 3A and B and Supplementary Fig. S2). No significant increase in phospho-H3 staining was seen in PCI-13 cells carrying wild-type *TP53* with the drug combination, suggesting that the G₂ block in these cells is substantially preserved. No sub-G₁ peaks were observed following treatment of any of the cells evaluated, indicating that the cell growth inhibition resulting from the synergistic interaction of cisplatin and MK-1775 is unlikely to be mediated through apoptosis.

Wee-1 inhibition sensitizes high-risk mutant p53 HNSCC cells to cisplatin therapy through induction of mitotic catastrophe associated with a senescence-like phenotype

Our data show that MK-1775 addition to cisplatin in p53-mutant or -deficient HNSCC cells results in premature mitotic entry and prolonged mitosis suggestive of mitotic catastrophe, a type of abnormal mitosis. To test this, the isogenic PCI-13 cell lines were grown on glass coverslips and treated as indicated. Cells were stained with phalloidin and DAPI and scored for the presence of multinucleated cells (Fig 4A and B). Treatment of PCI-13 cells deficient for *TP53* (pBabe) or expressing high-risk mutant *TP53* (C238F or G245D) with the combination of cisplatin and MK-1775 leads to significantly increased number of multinucleated and often giant lobulated multinucleated cells (45.5%, 57%, and 58.3%, respectively) compared with cell treated with cisplatin alone (27.7%, 27.1%, and 29.5%, respectively). These findings are consistent with aberrant mitosis or mitotic catastrophe. However, in PCI-13 cells expressing wild-type *TP53*, the addition of MK-1775 to cisplatin did not result in more multinuclei formation compared with cisplatin alone (Fig. 4A). Taken together, these results indicate an association between multinuclei formation, *TP53* status, and the synergism observed with the combination therapy in the HNSCC PCI-13 cells. Representative immunofluorescence images illustrating the presence of gross multi- and giant lobulated nuclei in isogenic PCI-13 cells following drug treatments are presented in Fig. 4B.

It has been shown that unscheduled mitosis results in micro-nuclei formation and apoptosis in tumor cells treated with gemcitabine and MK-1775 (32, 33). Therefore, we tested whether sensitization of the isogenic PCI-13 cells to cisplatin by addition of MK-1775 led to apoptosis induction. Treated cells were assessed for apoptosis by examining PARP-1 protein cleavage 48 hours after treatment in Western blots. No PARP-1 cleavage was identified in any of the cells, indicating that there was no induction of apoptosis (Supplementary Fig. S3A). The absence of apoptosis was also confirmed with the APO-BrdU tunnel assay, where the percentages of apoptotic cells with positive APO-BrdU staining were very low (Supplementary Fig. S3B). These results further corroborate the absence of the sub-G₁ peaks during the cell-cycle analysis in these cells following all treatments. Our results indicate that the underlying synergy between cisplatin and MK-1775 is an abnormal mitosis in the absence of apoptosis.

It has been proposed that cells undergoing mitotic arrest can eventually become senescent (reviewed in ref. 34). To examine for senescence, isogenic PCI-13 cells, treated as indicated, were stained and scored for the SA- β -gal positivity by microscopy. As shown in Fig. 4C and D, cisplatin alone leads to significantly increased levels of SA- β -gal activity only in cells expressing wild-type *TP53* but not in cells null for *TP53* (pBabe) or with C238F mutation. These results agree with our previously published work (13). Interestingly, a significant increase in the SA- β -gal staining associated with large, flattened cell shape was observed in PCI-13 cells carrying pBabe and in those expressing C238F following combination treatment when compared with cisplatin alone (Fig. 4C and D). A little increase in the SA- β -gal staining was seen in wtp53 cells following combination treatment. These data suggest that inhibition of Wee-1 in the *TP53*-altered PCI-13 cells exposed to cisplatin results in mitotic arrest associated with senescence-like phenotype and not apoptosis.

MK-1775–induced senescence is not associated with p21 expression and dependent on sustained ROS production in high-risk p53 mutant HNSCC cells treated with cisplatin

Senescence has also been linked to induction of p21 expression and ROS production, both of which are believed to be necessary for maintenance of the senescent phenotype in HNSCC cells (13, 28). To determine if cisplatin-induced p21 expression or ROS production correlates with the observed senescence phenotype following MK-1775 addition, we assayed p21 protein and ROS levels in cell lines expressing representative *TP53* mutations. Compared with cisplatin alone, addition of MK-1775 to cisplatin had no effect on p21 protein expression in cells deficient for *TP53* (pBabe) or expressing high-risk mutant *TP53* (Fig. 5A). However, production of ROS was significantly increased following the combination treatment in these cells (Fig. 5B–F). Inhibition of ROS using N-acetyl cysteine (NAC) dramatically decreased senescence in cells deficient for *TP53* (pBabe) or expressing high-risk mutant *TP53* (Fig. 5G and H). These results suggest that sustained ROS production following combination treatment with cisplatin and MK-1775 plays a key role in senescent phenotype.

MK-1775 enhances antitumor efficacy of cisplatin *in vivo*

To determine whether MK-1775 could sensitize HNSCC cells to cisplatin in an orthotopic mouse model of oral cancer, PCI-13 cells expressing the pBabe, wild-type *TP53*, or high-risk mutant *TP53* (C238F) were injected into the tongues of nude mice as previously described (30), and effects of MK-1775 or cisplatin, alone or in combination, on tumor growth over time were examined. MK-1775 did not provide significant improvement over the suppressive effect of cisplatin alone on tumor growth in mice bearing PCI-13 cells with wild-type *TP53* (Fig. 6A). However, the combination of cisplatin and MK-1775 displayed significant improvement over cisplatin alone in tumors of mice bearing PCI-13 cells with high-risk mutant *TP53* (C238F; Fig. 6C) or absent *TP53* (pBabe; Fig. 6B), supporting our previous *in vitro* findings. To confirm that the enhancement of cisplatin antitumor efficacy by MK-1775 was associated with entry into mitosis, phospho-H3 was evaluated in tongue xenografts bearing tumors with high-risk mutant *TP53* (C238F) treated with MK-1775 and/or cisplatin. MK-1775 induced an increase in phospho-H3–positive cells *in vivo*, suggestive of premature mitotic entry (Fig. 6D and E).

Discussion

We investigated the chemo-sensitizing abilities of a highly selective inhibitor of the Wee-1 kinase MK-1775 in HNSCC cells treated with cisplatin because platinum-based chemotherapy typically plays a key role in the management of patients with these tumors. We focused our investigations of MK-1775 on isogenic cell lines expressing different high-risk *TP53* mutations in human HNSCC tumors. We found that the *TP53*-deficient (pBabe null) and high-risk mutant *TP53* cells are more sensitive to clinically relevant doses of cisplatin when cotreated with nanomolar concentrations of MK-1775. On the other hand, tumor cells expressing wild-type *TP53* are already sensitive to cisplatin (13) as a single agent and they were only minimally further sensitized by MK-1775 addition, supporting the concept that the chemo-sensitizing effect of MK-1775 is dependent on *TP53* mutational status. We also obtained similar results with HNSCC cells with naturally occurring wild-type or high-risk *TP53* mutations, confirming that the chemo-sensitizing effect of MK-1775 depends on the *TP53* mutational status. Most tumor cells that harbor p53 alterations are heavily dependent on the G₂ checkpoint for DNA repair. We show that cisplatin arrests HNSCC cells deficient for p53 or expressing high-risk mutant p53 at the G₂ phase and that addition of MK-1775 abrogates the G₂ block and pushes the cells prematurely in mitosis. Not surprisingly, these *TP53*-altered HNSCC cells enter mitosis with extensive DNA lesions and the majority of them suffered prolonged mitotic arrest 48 hours after the addition of MK-1775, accompanied by phosphorylation of the Wee1 downstream target, CDC2. There was no significant increase in the sub-G₁ fractions in these cells following combination treatment, indicating that in HNSCC the synergistic interaction of cisplatin and MK-1775 is not mediated through apoptosis, in contrast with what is seen with gemcitabine in colon cancer and sarcoma cell lines (23, 33).

Our recent publication has shown that senescence, rather than apoptosis, is the major mechanism of cisplatin-induced response in wild-type *TP53* HNSCC cells and that cisplatin resistance in *TP53* null or high-risk mutant *TP53* cells is due to a lack of senescence (13). In this study, we found that addition of MK-1775 to cisplatin caused increased growth inhibition only in *TP53* null (pBabe) or high-risk mutant *TP53* HNSCC cells as a result of premature mitotic entry and prolonged mitosis. No alteration in the mitotic phenotype is seen in wild-type *TP53* cells with the combination treatment, implying that wild-type p53 directs HNSCC cells treated with cisplatin alone to undergo senescence. We detected significant induction in phospho-H3 levels with the combination treatment in *TP53* null (pBabe) and high-risk mutant *TP53* cells, indicating forced entry into mitosis. Furthermore, combination therapy significantly increased the number of giant lobulated multinuclei in *TP53* null (pBabe) and high-risk mutant *TP53*, which is suggestive of mitotic catastrophe.

Aarts and colleagues have shown that unscheduled mitosis results in gross multinuclei formation leading eventually to apoptosis in tumor cells treated with gemcitabine and MK-1775 (32). Recent report has also shown that Wee-1 kinase inhibition with high doses of MK-1775 leads to unscheduled mitotic entry associated with apoptosis in p53 mutant HNSCC cells (35). In our study, neither PARP-1 cleavage nor APO-BrdU tunnel staining was detected in our isogenic cell HNSCC lines with combination therapy, confirming no engagement of apoptosis. Our results strongly suggest that targeted inhibition of Wee-1

kinase with more physiologic dose of MK-1775 preferentially sensitizes *TP53*-deficient or high-risk mutant *TP53* HNSCC cells to cisplatin through induction of mitotic arrest without progression to apoptosis. One possible explanation for such discrepancy is that cisplatin and gemcitabine work through different mechanism(s) when sensitized with MK-1775 addition or perhaps the phenotype is specific for the type of tumor or nature of p53 mutation. In fact, evidence is now accumulating that different p53 mutations possess different functions and responses to therapy in different tissues, potentially reflecting differences in the expression of their cellular targets (36). In addition, the higher doses of MK-1775 used in previous studies may have accounted for extensive DNA damage leading to induction of apoptosis. We have shown that higher concentrations of the drug are extremely toxic to HNSCC cells *in vitro*. It is also possible that the mutant *TP53* HNSCC cells possess major alterations in the BH3 proapoptotic signaling pathways which render them resistance to apoptosis upon chemotherapy (37, 38).

A decrease in apoptosis is often compensated in several tumor cell lines by an increase in cellular senescence after treatment with DNA-damaging agents and radiation (13, 28, 34). In addition, it has been proposed that tumor cells undergoing mitotic catastrophe eventually die through terminal growth arrest known as cellular senescence (39). Treatment with cisplatin alone induced senescence in the HNSCC wild-type *TP53* cells, but not in high-risk *TP53* mutant or pBabe null cells. Surprisingly, the addition of MK-1775 partially restored cisplatin ability to induce senescence in cells expressing pBabe or high-risk mutant *TP53*. The induction of ROS appears to be the driving factor for senescence in these cells, whereas the induction of p21 alone does not seem sufficient. ROS induction is most likely caused by excessive DNA damage as it was recently shown that DNA damage induces ROS generation in several tumor cell lines (40).

MK-1775 has shown activity in nude rats bearing WiDr human colon carcinoma xenografts treated with 5-FU, gemcitabine, and cisplatin (23, 24). Moser and colleagues have recently shown that MK-1775 potentiates cisplatin response in mice bearing mutant p53 HNSCC cells established as subcutaneous flanks; however, they have not examined efficacy of MK-1775 in oral tongue xenografts (35). Ectopic subcutaneous xenograft models have proven less useful for studying therapeutic agents than orthotopic models that recapitulate the tumor microenvironment (30). In this study, we examined the antitumor efficacy of MK-1775 in combination with cisplatin in HNSCC xenografts growing in oral tongue of nude mice to mimic the primary tumor site. A significant delay in tumor growth was observed in mice injected with HNSCC cells expressing the pBabe control or high-risk mutant *TP53* following combination treatment compared with either cisplatin or MK-1775 alone. No enhanced treatment effect was seen in mice carrying tumors with wild-type *TP53*. Most likely, tumors harboring wild-type *TP53* undergo a G₁ or G₂ arrest similar to what we demonstrated *in vitro*, which prevents MK-1775-mediated premature mitotic entry. Thus, our data confirm previous findings that MK-1775 is not effective as a single agent and should be used only in combination with cisplatin or other DNA-damaging agents that induce G₂ cell-cycle arrest in HNSCC (23, 24).

Combination of cisplatin and MK-1775 promoted mitotic entry in xenografts obtained from mice bearing HNSCC with high-risk mutant *TP53*, indicating forced mitotic phenotype.

However, the percentage of the phospho-H3 immunostaining *in vivo* is lower than that observed *in vitro*, perhaps due to trapping of the cells with nocodazole under the latter conditions (23, 24, 32). On the basis of current understanding of aberrant mitosis (41–43), it is possible that unscheduled entry into mitosis in the presence of unrepaired DNA damage activates the spindle assembly checkpoint and inhibits activation of the anaphase-promoting complex APC/C-Cdc20. This, in turn, could lead to a delay in anaphase and eventually results in either mitotic death or mitotic slippage into G₁ phase.

In conclusion, we showed that the Wee-1 kinase inhibitor, MK-1775, selectively sensitized HNSCC cells to cisplatin therapy both *in vitro* and *in vivo* based on their *TP53* mutational status. The mechanism to explain this sensitization appears to involve a drug-induced, premature acceleration of G₂-phase cells into abnormal mitosis. Such cells harbor unrepaired DNA lesions that lead to abnormal cell divisions resulting in a senescence-like process. Our study suggests that forced mitosis with MK-1775 represents a novel therapeutic approach that potentially targets the consequences of oncogenic transformation caused by high-risk mutant *TP53* in HNSCC. These pre-clinical data provide compelling evidence that a personalized approach to the treatment of HNSCC based on Wee-1 kinase inhibition in p53-altered cells may be feasible. Further clinical investigations are necessary to determine whether this approach will be useful to improve treatment outcomes for patients with high-risk mutp53 HNSCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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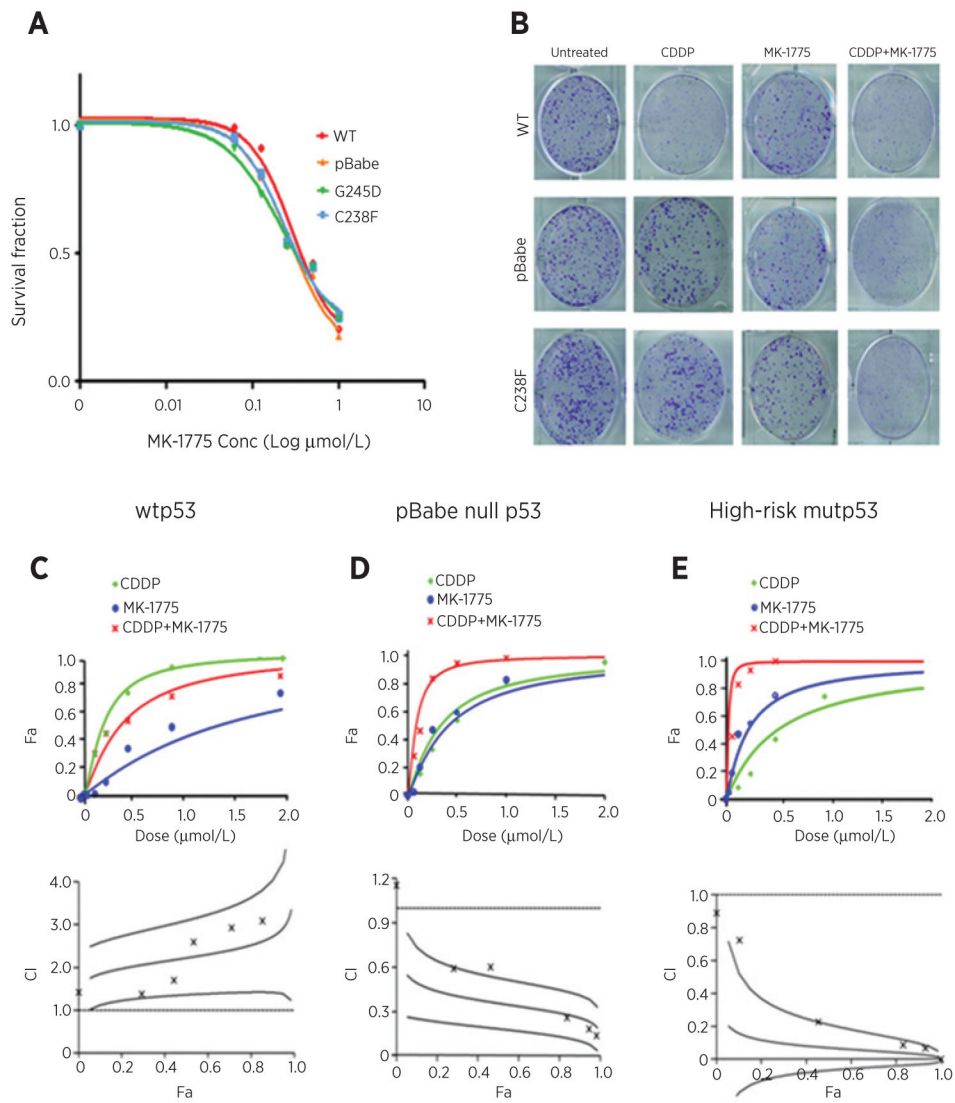
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**Figure 1.**

The Wee-1 inhibitor MK-1775 synergizes with cisplatin *in vitro* in HNSCC cells expressing high-risk *TP53* mutations. A, clonogenic survival curves for HNSCC PCI-13 isogenic cell lines stably expressing wild-type *TP53*, pBabe *TP53* null control, and high-risk mutant *TP53* vectors, treated with a range of MK-1775 concentrations (0–1 $\mu\text{mol/L}$) for 48 hours to determine the drug IC_{50} . All MK-1775 treatments were performed in triplicate, and each experiment was repeated at least three times. B, representative images of clonogenic survival assays for PCI-13 cells treated with cisplatin (CDDP) followed by MK-1775 according to the treatment protocol described in Materials and Methods. C–E, assessment of the degree of synergy between cisplatin and MK-1775 in PCI-13 cells expressing wild-type *TP53*, pBabe null *TP53* control, and high-risk mutant *TP53*, respectively, using the Chou and Talalay method. Drug interactions are expressed as fraction affected (Fa) curves and combination index (CI) plots. The CI values were generated over a range of Fa levels from

growth inhibition percentages. Synergy was clear at relevant Fa values that are greater than 50%.

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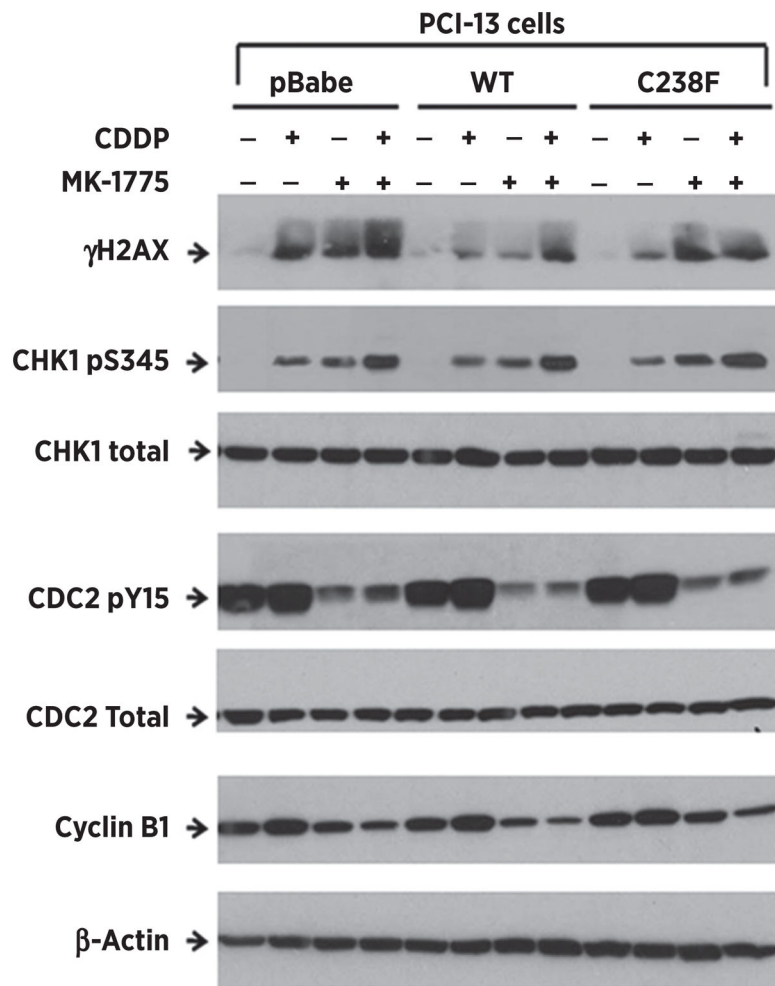
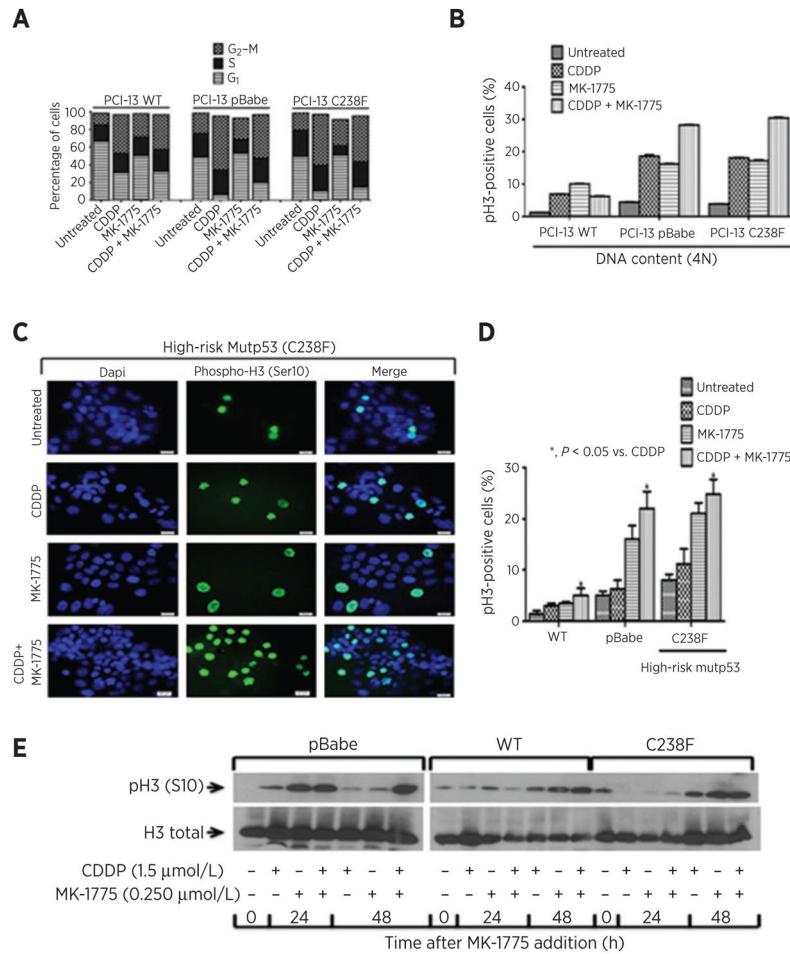


Figure 2.

Wee-1 inhibition attenuates cisplatin-induced CDC2 phosphorylation and triggers a general DNA damage response in high-risk *TP53* mutant HNSCC cells. The isogenic HNSCC PCI-13 cells were treated with 1.5 $\mu\text{mol/L}$ cisplatin alone or in combination with 0.25 $\mu\text{mol/L}$ of MK-1775 as described in Materials and Methods. Cells were harvested and lysates analyzed by Western blot with antibodies to the proteins and phosphoproteins indicated. Increased phosphorylation levels of CHK1 and γ -H2AX indicate persistence of unrepaired DNA damage. The levels of phospho-CDC2 and Cyclin B1 expression were significantly suppressed with the MK-1775 treatment, indicating the successful inhibition of Wee-1 kinase activity by this agent.

**Figure 3.**

Wee-1 inhibition induces prolonged mitotic arrest in high-risk *TP53* mutant HNSCC cells harboring DNA damage. PCI-13 HNSCC cells were treated with cisplatin (CDDP), MK-1775, or the combination as described in Materials and Methods and subjected to dual phospho-H3 staining for mitotic cells and propidium iodide (PI) flow cytometric analysis (FACS). A 2N DNA content indicates cells in G₁ phase and 4N DNA content indicates cells in either G₂ or M phase. A, percentage of cell-cycle distribution of phospho-H3/PI FACS analysis shown in Supplementary Fig. S2. Percentage of G₂-M phase indicates that pBabe *TP53* null and high-risk mutant *TP53* (C238F) progressed into mitosis after the G₂-block abrogation 48 hours following the combination treatment. B, quantification of mitotic phenotype in phospho-H3-positive HNSCC cells presented in Supplementary Fig. S2 and plotted as mitotic index. *TP53* status and treatment type are indicated for each cell line. An increased proportion of cells entering into mitosis with a full 4N complement of DNA was observed. C, representative fluorescence images of PCI-13 HNSCC cells indicating that abrogation of the G₂ block results in prolonged mitotic arrest following addition of cisplatin and MK-1775. Cells were plated on cover-glass slips, treated as previously mentioned and incubated in medium containing nocodazole (mitotic trap) for 16 hours before the end of treatment and then subjected to phospho-H3 immunostaining analysis. Scale bars, 20 μm. D, quantification of fluorescence images (phospho-H3-positive staining) shown in C. Error

bars, SEM. *, $P < 0.05$ vs. cisplatin (CDDP). E, Western blot for PCI-13 HNSCC cells with different *TP53* mutational status treated at indicated time points and analyzed for pH3 (S10) and total pH3 protein expression.

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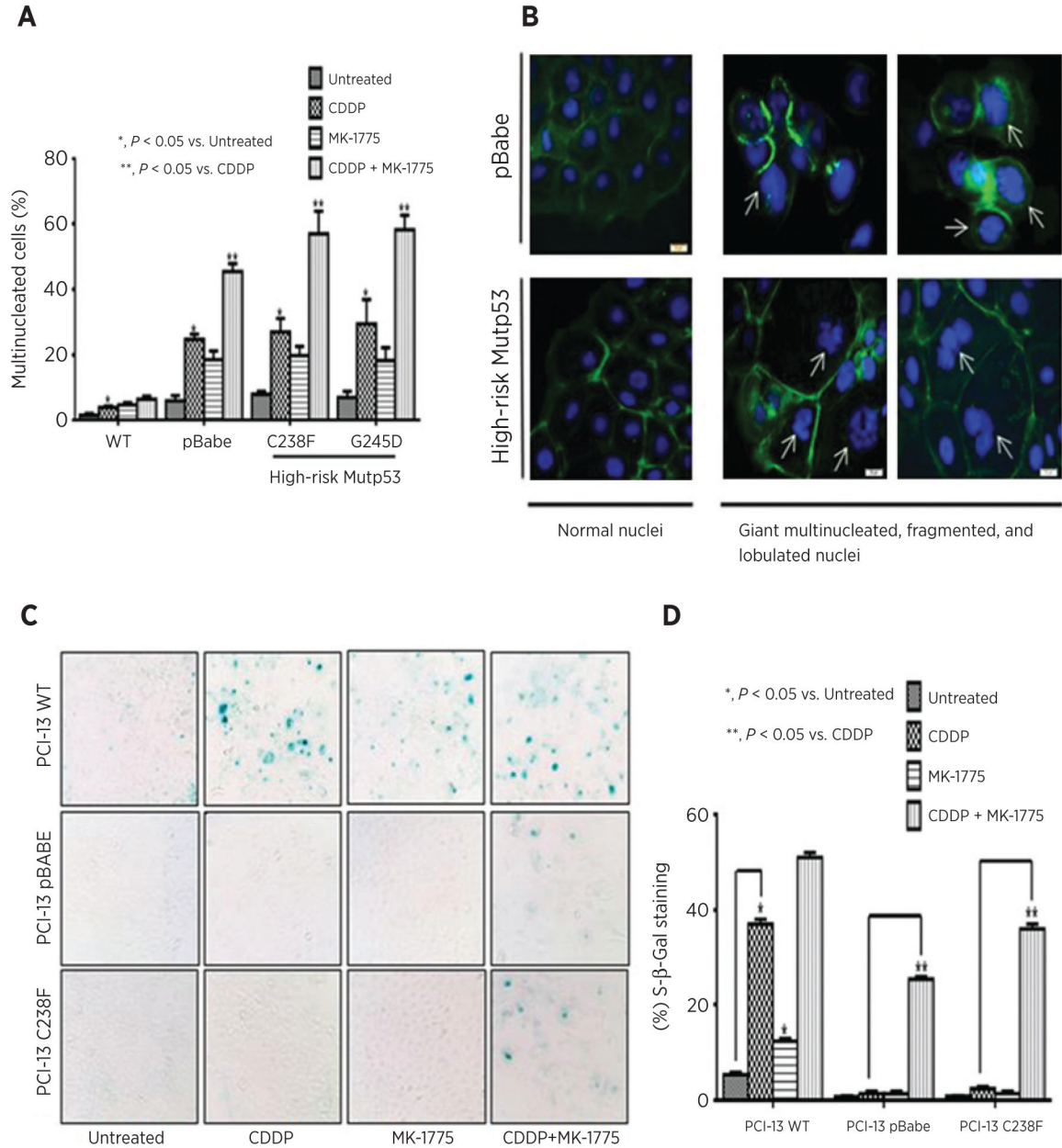


Figure 4.

Cisplatin and MK-1775 synergize in high-risk mutant p53 HNSCC cells and induce mitotic arrest associated with a senescence-like phenotype. **A**, PCI-13 HNSCC cells expressing different *TP53* vectors were treated with cisplatin, MK-1775, or in combination as described in Materials and Methods. The cells were then washed and stained with Phalloidin (green) and DAPI (blue). The proportion of multinucleated and giant lobulated or even fragmented multinucleated cells was determined by immunofluorescent microscopy (pBabe *TP53* null control, C238F, and G245D mutant *TP53*s; CDDP + MK-1775, 45.5%, 57%, and 58.3%, respectively, compared with CDDP alone, 27.7%, 27.1%, and 29.5%, respectively). **B**, representative immunofluorescent images of normal and giant multinucleated cells

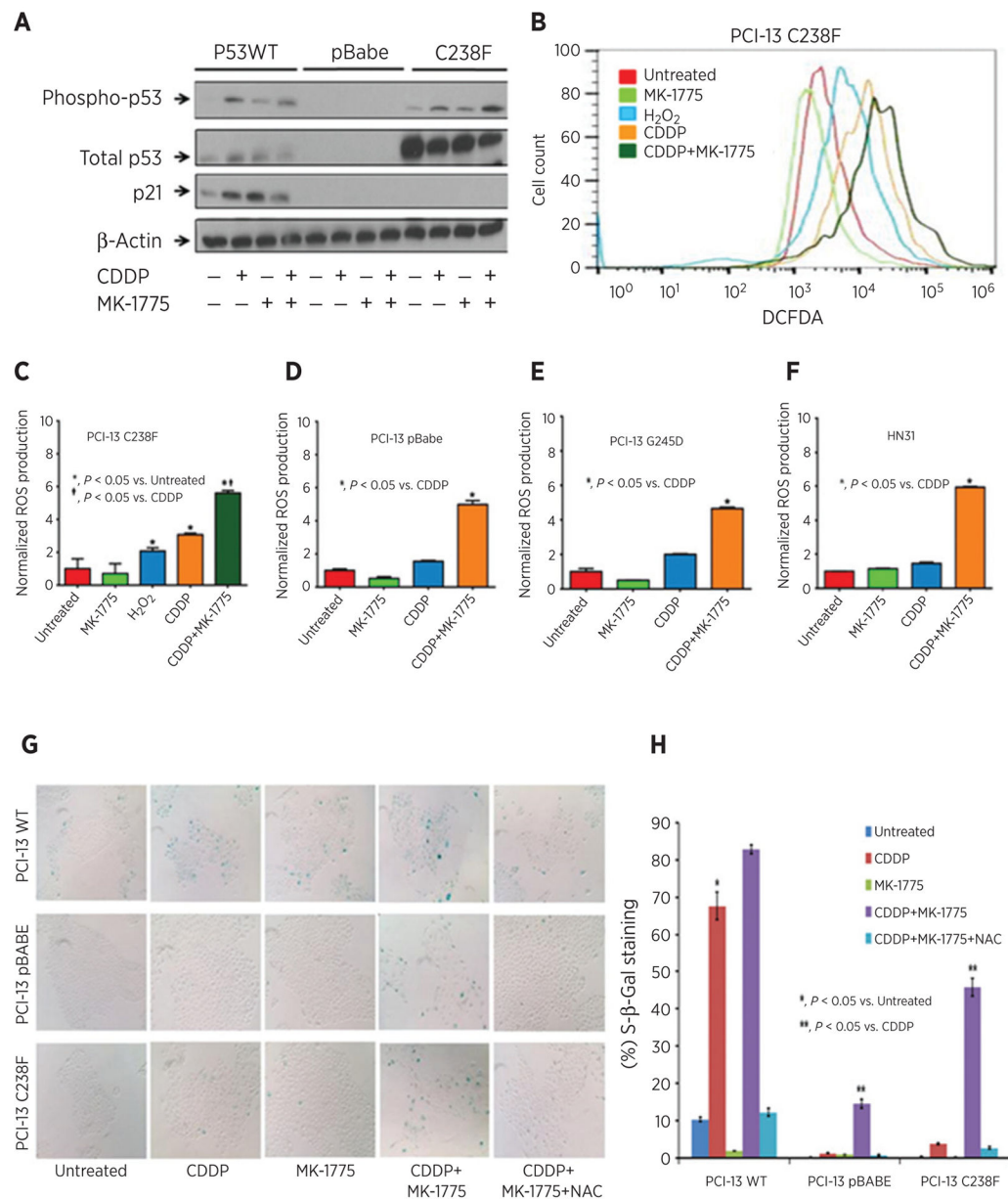
illustrating mitotic catastrophe (as described in A) taken after washout of CDDP and MK-1775 combination treatment. Scale bars, 20 μ m. C, representative light microscopy showing SA- β -gal staining. Cells were treated with cisplatin and MK-1775 as described in Materials and Methods, and morphologic changes were monitored 4 days later (20 \times magnification). D, percentage of SA- β -gal-positive cells per total number of cells in an hpf after treatment is graphed. A substantial proportion of cells stained positive for β -Gal was seen in *TP53* null (pBabe) and high-risk mutant *TP53* cells following combination treatment.

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**Figure 5.**

MK-1775-induced senescence is not associated with p21 expression and dependent on sustained ROS production in high-risk p53 mutant HNSCC cells treated with cisplatin. A, p21 protein expression in PCI-13 cells expressing the indicated *TP53* constructs treated with the indicated doses of cisplatin and MK-1775 unless otherwise stated. B, PCI-13 (C238F) *TP53* mutant cells were exposed to cisplatin in the presence or absence of MK-1775, and ROS levels were analyzed using flow cytometry. Hydrogen peroxide (H₂O₂), a known ROS inducer, was used as a positive control. C, fluorescence from B normalized to control condition and DNA content and presented as ROS levels. D–F, ROS production measured in other cell lines expressing representative *TP53* mutations and cell line with known *TP53* mutational status. G, representative light microscopy showing SA-β-gal staining in HNSCC

cells treated with cisplatin and MK-1775 as previously mentioned in the presence or absence of 10 mmol/L NAC added 2 hours prior to the treatment. H, percentage of cells SA- β -gal staining positive. *, significantly increased over untreated control ($P < 0.05$); **, significantly different from HNSCC cells in the cisplatin group ($P < 0.05$).

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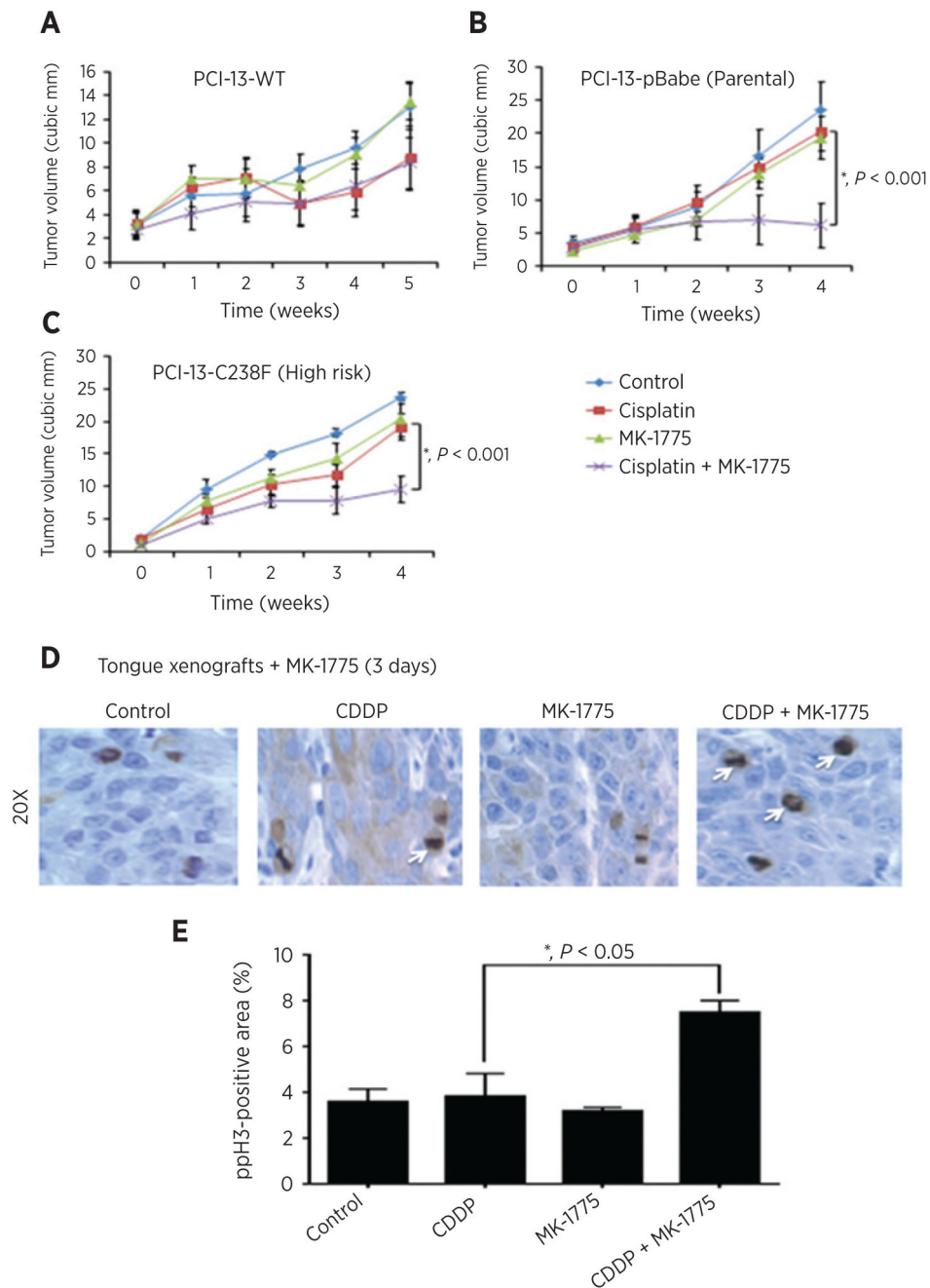


Figure 6. MK-1775 enhances antitumor efficacy of cisplatin *in vivo* in an orthotopic mouse model of oral cancer. A–C, xenograft tumors made by orthotopically injecting wild-type *TP53*, pBabe *TP53* null control, or high-risk mutant *TP53* PCI-13 cells into the tongues of nude mice. The animals were treated with control, cisplatin, MK1775, or the combination of the two drugs once the tumors reached 5 mm in diameter according to protocol outlined in Materials and Methods. Tumor growth was followed for four weeks and tongue tumor size was measured with microcalipers and illustrated as tumor volume curves. Combination treatment reduced tumor growth to a greater extent than individual treatments (repeated measures Student *t*

test: 30 mg/kg, *, $P < 0.001$). D, representative images of phospho-H3 immunofluorescence in PCI-13 mutant *TP53* (C238F) tumors dosed with MK-1775 (30 mg/kg, p.o.) for 3 days (3 doses) after cisplatin treatment (4 mg/kg, i.v.). Arrows, mitotic phenotype phospho-H3-positive cells and histopathologic drug response. E, proportion of mitotic cells with forced mitotic phenotype in tumors treated as described in D, scored blind in 3 tumors per condition. Control vehicles received only 0.5% methylcellulose solution given by oral gavage. *, $P < 0.05$. Error bars, SEM tumor diameter for the group on that day and there were 8 to 9 mice in each group.

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