Chk1/2 Inhibition Overcomes the Cisplatin Resistance of Head and Neck Cancer Cells Secondary to the Loss of Functional p53

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Abstract

Despite the use of multimodality therapy using cisplatin to treat patients with advanced stage squamous cell carcinoma of the head and neck (HNSCC), there is an unacceptably high rate of treatment failure. TP53 is the most commonly mutated gene in HNSCC, and the impact of p53 mutation on response to cisplatin treatment is poorly understood. Here, we show unambiguously that wild-type TP53 (wtp53) is associated with sensitivity of HNSCC cells to cisplatin treatment, whereas mutation or loss of TP53 is associated with cisplatin resistance. We also show that senescence is the major cellular response to cisplatin in wtp53 HNSCC cells and that cisplatin resistance in p53-null or -mutant TP53 cells is due to their lack of senescence. Given the dependence on checkpoint kinase (Chk)1/2 kinases to mediate the DNA damage response in p53-deficient cells, there is potential to exploit this to therapeutic advantage through targeted inhibition of the Chk1/2 kinases. Treatment of p53-deficient HNSCC cells with the Chk inhibitor AZD7762 sensitizes them to cisplatin through induction of mitotic cell death. This is the first report showing the ability of a Chk kinase inhibitor to sensitize TP53-deficient HNSCC to cisplatin in a synthetic lethal manner, which has significance given the frequency of TP53 mutations in this disease and because cisplatin has become part of standard therapy for aggressive HNSCC tumors. These preclinical data provide evidence that a personalized approach to the treatment of HNSCC based on Chk inhibition in p53-mutant tumors may be feasible.
death is contributing to treatment responses when clinically relevant doses of cisplatin are used. Interestingly, emerging reports have shown that genotoxic stresses could also culminate in alternative cellular responses—such as senescence, which is characterized by a nonproliferative state, or mitotic catastrophe, which is characterized by the presence of giant multinucleated cells (27–30). Regardless, the role of p53 in mediating cisplatin therapy response in HNSCC cells still remains unresolved.

In response to DNA damage, p53 can cause cell-cycle arrest and execute DNA repair. In the absence of functional p53, however, the critical responses such as cell-cycle arrest and DNA repair hinge on the function of checkpoint kinase (Chk)1 and Chk2. Although it has proven difficult to therapeutically exploit the loss of TP53 tumor suppressor function for cancer treatment, a synthetic lethal strategy involving targeted inhibition of Chk1/2 could be exploited for therapeutic benefit in p53-mutant HNSCC. Several studies have shown that TP53-deficient cells can be sensitized to genotoxic agents in a synthetic lethal strategy by inhibiting DNA repair enzymes such as the Chk. Indeed, human clinical cancer trials using small-molecule inhibitors in combination with various chemotherapeutic agents have been conducted and are still ongoing. As these trials are still in early stages, important questions still exist about whether the TP53 mutational status of tumors should be a factor in selecting patients, which chemotherapies work with Chk inhibitors, and which types of cancers this therapeutic strategy would benefit. There are currently no clinical trials or preclinical studies combining Chk inhibitors with chemotherapeutic agents specifically in HNSCC, and there is a paucity of studies examining the efficacy of Chk inhibitors as sensitizing agents to cisplatin, which is the first-line chemotherapy for treating aggressive HNSCC. A few studies in other cancer models have reported enhancement in cisplatin toxicity upon inhibition of Chk (31–34), other studies in contrast have found no therapeutic benefit with this combination treatment (35, 36). Regardless, whether the addition of Chk inhibitor would potentiate the cisplatin killing of p53-deficient HNSCC cells is currently unclear.

Using an isogenic pair of HNSCC cell lines, we show that HNSCC cells respond to cisplatin in a p53-dependent manner. Furthermore, we show that HNSCC cells bearing wtp53 undergo senescence rather than apoptosis in response to cisplatin treatment, whereas HNSCCs deficient or mutant for TP53 are less sensitive to cisplatin and fail to undergo senescence. Finally, we sought to determine whether p53-mutant or -deficient cells that evade cisplatin-induced senescence can be directed to die through inhibition of Chk.

Materials and Methods

Reagents and cell lines

Cisplatin was purchased from the institutional pharmacy (MD Anderson Cancer Center, Houston, Texas) and staurosporine was purchased from Sigma. The Chk1/2 inhibitor drug (AZD7762) was obtained from AstraZeneca. The drug powder was dissolved in 100% dimethyl sulfoxide (DMSO) to a stock concentration of 10 mmol/L and stored in aliquots. Intermediate drug concentration of 500 μmol/L was prepared from this stock and aliquoted. A final drug concentration of 100 nmol/L (0.1% DMSO) in culture medium was used for all experiments. HNSCC cell lines (HN30, HN31, UMSCC17A, SCC61, and CaI27) used for this study were obtained from an established cell bank in the laboratory of Dr. Jeffrey Myers (University of Texas MD Anderson Cancer Center, Houston, TX) under the approved institutional protocols. These cell lines were tested and authenticated against the parental cell lines using short-tandem repeat analysis within 6 months of use for the current study (37). All cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM) containing FBS, glutamine, sodium pyruvate, penicillin/streptomycin, nonessential amino acids, and vitamins. HN30-shp21 and HN30-shp53 cell lines were generated from a lentiviral stable knockdown of p21 and p53, respectively, in HN30 cells as described previously (5, 38).

Clonogenic assay

Clonogenic assays were conducted on 6-well plates. Plating efficiency for each HNSCC cell line was determined by seeding different cell numbers in wells and allowing them to form colonies over 14 days incubation. Roughly, 500 to 600 cells per well were found to be good plating number for each cell line. For cisplatin titration experiments, HNSCC cells in logarithmic growth phases were trypsinized, counted, and seeded. Cells were then allowed to attach overnight. Next day, serial dilutions of cisplatin were prepared in DMEM and cells were exposed to different cisplatin concentrations. Twenty-four hours later, cisplatin was removed by sucking off the media and cells were washed with PBS three times. Cells were then supplied with fresh media and allowed to form colonies. At day 14, media was removed and cell colonies were fixed in cold methanol and stained with crystal violet (1.5%). The colony pictures were taken (Alpha Innotech), and analyzed using ImageJ software (NIH, Bethesda, MD). For clonogenic assays with cisplatin plus AZD7762 combination treatment, cells were treated with cisplatin and AZD7762 concurrently for 24 hours. After 24 hours, media was sucked off and cells were washed with PBS for three times. Cells were then supplied with fresh media containing AZD7762 (100 nmol/L) for another 24 hours. At the end of the treatment, cell were washed with PBS and fed with fresh media. Colonies were allowed to form and processed as described earlier.

Cell-cycle analysis

HNSCC cells (3–5 × 10⁴) were plated in 60-mm dish and subjected to various treatments as indicated. At each indicated time point, cell media was collected and saved. Cells were then washed with PBS and trypsinized. The collected media was used to neutralize the trypsin, and cells were spun down into a pellet. Then cell pellet was
washed with cold PBS and the resulting cell suspension was fixed in 70% ethanol for 30 minutes at room temperature. Cells were then stained with propidium iodide (PI) and processed on an XL flow cytometer analyzer (Beckmann Coulter) for FACScan analysis. The data were analyzed on Flowjo software (TreeStar).

**Immunoblotting**

HNSCC cells were subjected to different treatments, and at indicated time points, cells were lysed with radioimmunoprecipitation assay buffer (RIPA) lysis buffer [Tris 50 mM/L (pH 7.4), 150 mM/L NaCl, 1 mM/L EDTA, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, 10% glycerol, 10% mercaptoethanol, 10 mM/L NaF, 1 mM/L orthovanadate, 2.5 mM/L pyrophosphate, and proteinase inhibitors]. Scapped cells were pulse sonicated and proteins were allowed to solubilize in the buffer for 20 minutes. The cell lysates were then centrifuged at 14,000 rpm at 4°C for 20 minutes and supernatant was collected. Protein quantification was carried out using BCA protein assay kit (Thermo Scientific). The Western blotting was conducted as described previously (39). After conducting the transfer, membrane blocking was done in 5% milk in TBS with 0.1% Tween 20 (TBST). The membranes were incubated overnight at 4°C with the following primary antibodies: PARP (sc-511), phospho-Chk1 (S345; sc-2348), phospho-Chk1 (S296; sc-2349), total Chk1 (sc-2345), phospho-Chk2 (Thr68; sc-2661), total Chk2 (sc-2662), phospho-H3 (S10; sc-9701), total H3 (sc-7751), γ-H2AX (S139; sc-9718), phospho-p53 (S15; sc-9264), p21 (sc-2946), all purchased from Cell Signaling Technology. After 1-hour incubation at room temperature with 2% antibody in 2.5% milk (TBST 0.05%), the blots were briefly incubated with enhanced chemiluminescence (ECL) reagent (GE Healthcare Life Sciences) and developed by exposing to X-ray film.

**Senescence β-galactosidase assay**

HNSCC cells (4 × 10^6) were seeded in a 6-well plate and allowed to attach overnight. Next day, cells were treated with the cisplatin at 1.5 μM/L and allowed to attach overnight. The next day, the cells were treated with 1.5 μM/L cisplatin for 24 hours. At indicated time points, cells were washed three times in PBS, fixed in 4% methanol-free formaldehyde for 25 minutes at 4°C, washed again, and permeablized in 0.2% Triton X-100 in PBS for 5 minutes. After permeablization, cells were equilibrated in equilibration buffer for 5 minutes at room temperature and then incubated with a buffer containing nucleotide mix and rTdT enzyme for 1 hour. Cell nuclei were stained with DAPI. Fluorescence microscopy was conducted using Olympus IX81-DSU Spinning disk confocal microscope and images were taken with Hamamatsu ORCA II ER camera.

**siRNA-mediated knockdown**

Commercially available siRNA against Chk1 and Chk2 were purchased from Qiagen (siRNA for Chk1 #S10299859; siRNA for Chk2, #S10222471) and nonsilencing siRNA was purchased from Ambion (#4390846). HN31 cells were electroporated with 1.5 μg of each siRNA using nucleofector II apparatus (Amaxa), and proteins were harvested 48 hours later, resolved by SDS-PAGE and analyzed by Western blotting.

**Statistical analysis**

Data for analysis were pooled from three independent experiments and each experiment was carried out in triplicates. For clonogenic survival assays, data were transformed with the square root function to correct for deviations from normality and analyzed for statistical differences by conducting an ANOVA test, followed by the Bonferroni multiple comparison test using GraphPad Prism version 6 software. Two-tailed Student t tests were conducted for senescence and other unpaired group comparisons, also using GraphPad Prism. For all comparisons, P < 0.05 was considered statistically significant.
Results

HNSCC cells respond to cisplatin in a p53-dependent manner

To examine the cisplatin responses of HNSCC cells differing in the p53 status, an isogenic pair of HNSCC lines originally derived from the same patient that differ in the p53 status, namely HN30 (wtp53) and HN31, which bears mutant p53 (mutp53; C176F and A161S), as well as an additional wtp53 cell line (UMSCC17A) were exposed to various concentrations of cisplatin and their survival was assessed by clonogenic assay. The clonogenic survival of the wtp53 UMSCC17A and HN30 cells was significantly lower than HN31 cells (Fig. 1A). The IC_{50} for cisplatin was determined to be 0.16 μmol/L in UMSCC17A and 0.14 μmol/L in HN30 cells, and 0.60 μmol/L in HN31. To examine the relationship between functional p53 and cisplatin sensitivity, wtp53-expressing HN30L (i.e., HN30 cells infected with a lentiviral devoid of short hairpin RNA (shRNA)) and a derivative of the wild-type cell line referred to as HN30-shp53 in which p53 has been stably knocked down following infection with an shRNA lentivirus, were treated with different concentrations of cisplatin and their survival was assessed by clonogenic assay. The clonogenic survival of HN30L cells (IC_{50} = 0.12 μmol/L) was found to be much lower than HN30-shp53 cells (IC_{50} = 0.32 μmol/L) in response to cisplatin treatment (Fig. 1B and C) supporting that cisplatin sensitivity in HNSCC cells is p53-dependent. Parental HN30 cells were used for the remaining study as the cisplatin sensitivity of HN30L and HN30 cells was found to be similar (Fig. 1A and B).

Senescence, not apoptosis, is the main mechanism for cisplatin sensitivity in wtp53 HNSCC cells

To investigate the mechanism of cell death in response to cisplatin treatment in HNSCC cells, we assayed cells for apoptosis induction using three complementary assays. First, PI-based cell-cycle analysis was conducted and sub-G1 values were determined. The percentage sub-G1 values for HN30 and HN30-shp53 were found to be less than 10% at all the time points (Fig. 2A) and did not differ much from each other despite their difference in cisplatin sensitivity. Also, the percentage sub-G1 values for HN31 were found to be less than 10% at all-time points (data not shown).
shown). In addition, cell lysates from HN30, HN30-shp53, and HN31 cells were collected at 24 and 48 hours post-cisplatin treatment and probed for the presence of cleaved PARP by Western blotting. Cell lysates from staurosporine (1 μmol/L)–treated HN31 and HN30-shp53 cells were used as a positive control for apoptosis. We failed to detect PARP cleavage in all three cell lines after cisplatin treatment at both the time points (Fig. 2B). These results suggested that apoptosis is unlikely to be the prominent mode of cell death in response to cisplatin treatment in these HNSCC cells. Finally, to confirm absence of apoptosis in HNSCC cells upon cisplatin treatment, we investigated whether cisplatin-treated HNSCC cells show the typical morphologic changes that accompany apoptosis. The characteristics of apoptosis such as membrane blebbing and nuclear fragmentation were absent in cisplatin-treated HN30 and HN30-shp53 cells (Fig. 2C). In addition, no evidence of apoptosis was detected when cisplatin-treated HN30 cells were assayed for positive terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (Supplementary Fig. S1). Thus, we conclude that wtp53 HN30, its p53-deficient derivative HN30-shp53, and p53-mutant isogenic variant HN31 undergo minimal apoptosis in response to cisplatin treatment. For all the above experiments, we used a clinically relevant dose of cisplatin (1.5 μmol/L) at which no apoptosis could be detected. However, when HN30 cells were treated with cisplatin at a high dose (20 μmol/L), we were able to detect PARP cleavage and also observed an increase in sub-G1 values, which suggests that HN30 cells...
undergo apoptosis when exposed to high doses of cisplatin (Fig. 2D).

It was noted that after cisplatin treatment wtp53 HN30, but not HN30-shp53, have flat and enlarged cell morphology reminiscent of senescence (Figs. 2C and 3A). Thus, we next hypothesized that the reduction in colonies following cisplatin treatment in HN30 could be due to induction of senescence. HN30 and UMSCC17A were treated with cisplatin (1.5 μmol/L) and 4 days posttreatment, cells were stained for SA-β-Gal activity, a hallmark of senescence. High levels of senescent cells (30%–60%) were seen in cisplatin-treated UMSCC17A and HN30 cells (Fig. 3B and C). A profoundly reduced (30%–60%) were seen in cisplatin-treated UMSCC17A and HN30 cells, which most likely accounted for induction of senescence. HN30 and UMSCC17A were treated with cisplatin (1.5 μmol/L) and 4 days posttreatment, cells were stained for SA-β-Gal activity, a hallmark of senescence.

In addition, we conducted Western blot analysis using protein lysates from cisplatin-treated HN30 and HN30-shp53 cells and found that p21, a p53-regulated mediator of senescence, was highly induced in cisplatin-treated HN30 cells, but no induction of p21 was seen in cisplatin-treated HN30-shp53 cells (Fig. 3D). Furthermore, no p21 induction was detected in HN31 cell treated with cisplatin (data not shown). To determine whether p21 was required for induction of senescence in HN30 cells, we created a stable lentiviral knockdown of p21 in HN30-shp53 cells (HN30-shp53 cells were treated with cisplatin (1.5 μmol/L) and 4 days posttreatment, cells were stained for SA-β-Gal activity, a hallmark of senescence. High levels of senescent cells (30%–60%) were seen in cisplatin-treated UMSCC17A and HN30 cells (Fig. 3B and C). A profoundly reduced (30%–60%) were seen in cisplatin-treated UMSCC17A and HN30 cells, which most likely accounted for induction of senescence. HN30 and UMSCC17A were treated with cisplatin (1.5 μmol/L) and 4 days posttreatment, cells were stained for SA-β-Gal activity, a hallmark of senescence.

Inhibition of Chk1/2 sensitizes p53-mutant HNSCC cells to cisplatin

HN31 cells harboring mutp53 were far more resistant to cisplatin-induced senescence, which most likely accounted for their reduced sensitivity to the drug. Given that as many as 70% of patients with HNSCC have tumors with mutp53, we investigated whether cisplatin-resistant HNSCC lacking wtpt53 could be rendered more sensitive through a different mechanism, as a prelude to improving outcomes for those with this disease. To this end, we next evaluated whether cisplatin-resistant HNSCC cells having wtpt53 could be directed toward mitotic catastrophe. As Chk1/2 play an important role in mediating the DNA damage response in cancer cells treated with DNA-damaging agents, we hypothesized that inhibition of Chk1/2 could sensitize p53-mutant HNSCC cells to cisplatin-induced cell death. To test this hypothesis, HN31 (mutp53) and HN30 (wtpt53) cells were treated with cisplatin at two different concentrations and in combination with AZD7762, a pan Chk1/2 inhibitor, and survival was assessed by clonogenic assays. In HN31 cells, at both concentrations of cisplatin tested, the combination treatment induced greater colony killing than cisplatin or AZD7762 treatment alone (P < 0.003). In contrast, the combination treatment induced only marginal killing above single-agent treatment with cisplatin in HN30 cells (P > 0.999; Fig. 4A). When tested in HN30-shp53 (p53-knockdown cells), the combination treatment was significantly more lethal than AZD7762 or cisplatin treatment alone (P < 0.0003; Fig. 4A, second row). Thus, cisplatin resistance in p53-mutant and -null background of HNSCC cells can be overcome by addition of Chk inhibitor to the chemotherapy.

To confirm that sensitization of p53-mutant cells to cisplatin is specifically due to inhibition of Chk, we conducted a siRNA knockdown of Chk1, Chk2, or both in HN31 cells. The Chk knockdown cells were then exposed to cisplatin for 24 hours and cell survival was assessed by clonogenic assay. Cisplatin response of HN31 mock-treated or transfected with control scrambled siRNA transfected HN31 were similar to each other, suggesting no real effects from the control siRNA knockdown. Overall, the knockdown of Chk1 or Chk2 alone lowered the clonogenic survival compared with scramble at both doses of cisplatin. However, the differences reached statistical significance with Chk2 knockdown alone only at lower dose of cisplatin (P < 0.0001). The Chk2 knockdown resulted in greater colony killing than the Chk1 knockdown at lower dose of cisplatin (P < 0.0001). At higher dose of cisplatin, the double knockdown significantly reduced the survival compared with scramble suggesting that Chk1 knockdown improved the results over Chk2 knockdown alone (P < 0.0001; Fig. 4B). Western blot analyses to confirm the knockdown of Chk1 and Chk2 are shown in Fig. 4C along with the chemical structure of AZD7762 (Fig. 4D).

DNA damage response signaling in HNSCC cells

The ability of AZD7762 to inhibit Chk activity was verified by conducting Western blot analysis on HN31 cells treated with cisplatin or cisplatin plus AZD7762 (Fig. 5A). Phosphorylation on S296 of Chk1, a surrogate for Chk1 activity, was detectable after 24 hours of cisplatin treatment and for an additional 24 hours following washout of the drug. As expected, treatment with AZD7762 completely ablated the cisplatin-induced phosphorylation of Chk1 on S296 at both time points, indicating that the inhibitor was working. The molecular signaling changes in the DNA Damage Response pathway for HN30, HN30-shp53, and HN31 cells following treatment with cisplatin, AZD7762, or their combination were examined by Western blot analysis. Cisplatin treatment led to an increase in phosphorylated Chk1 (S345) levels compared with no treatment for all three cell lines, which was enhanced following the combination therapy in most cases (Fig. 5B). Addition of AZD7762 alone also led to increased phosphorylation of Chk1 on S345 in 2 of 3 cell lines, suggestive of a regulatory feedback response in cells where the Chk activity was diminished by drug. Phosphorylation on the S345 residue is thought to be...
Figure 3. HNSCC cells undergo senescence in response to cisplatin (Cisp) treatment in a p53-dependent manner. A, HN30 and HN30-shp53 cells were treated with cisplatin for 24 hours and morphologic changes were monitored 4 days later (×20 magnification). B, HN30, UMSCC17A, and HN30-shp53 cells were treated with cisplatin (1.5 \( \mu \)mol/L) for 24 hours, and 4 days later, cells were assayed for β-Gal enzymatic activity. The β-Gal senescent staining observed in HN30 and UMSCC17A cells is illustrated in the photos (×10 magnification). Comparatively, only a small proportion of HN30-shp53 cells stained positive for β-Gal (data not shown). C, the proportion of β-Gal-positive cells in HN30, UMSCC17A, and HN30-shp53 cells were graphed. Similarly, HN31 cells treated with cisplatin were assessed for β-Gal staining. D, cell lysates from cisplatin-treated HN30 and HN30-shp53 cells were probed for the presence of p21 using Western blot analysis. E, representative photos of cisplatin-treated HN30L and HN30-shp21 cells were taken after β-Gal senescent staining (×20 magnification) and percentage of β-Gal-positive cells were graphed. Western blot analysis was conducted to confirm knockdown of p21. †, Significantly different from cisplatin-treated HN30 cells by two-tailed Student t test; ‡, significantly different from cisplatin-treated HN30L cells by two-tailed Student t test. Con, control.
mediated by upstream kinases such as the ataxia telangiectasia-related (ATR) protein, and facilitates auto phosphorylation on S296 during Chk1 activation. In all three cell lines, levels of phospho-H2AX (i.e., γ-H2AX), a marker of DNA damage, was increased following cisplatin but further elevated by the combination of cisplatin plus AZD7762. Increased γ-H2AX was also accompanied by increased Chk2 phosphorylation on Thr68 following combination therapy, which was more enhanced in mutp53 HN31 cells and in the p53-knockdown HN30-shp53 cells. Cisplatin also induced p53 phosphorylation in HN30 and HN31 cells and there was a moderate enhancement following the combination treatment (Fig. 5B). However, no accumulation of total p53 was observed with either cisplatin or the combination treatment. As expected, cisplatin treatment led to an increase in p21 levels in HN30 cells. A similar level of increase in p21 was also observed in HN30 cells following the combination treatment. In HN31 and HN30-shp53, p21 levels were barely detectable under all the treatment conditions (Fig. 5B).

Cisplatin plus Chk inhibitor induces polyploidy and subsequent mitotic catastrophe in p53-mutant or -knockdown HNSCC cells

HN30, HN30-shp53, or HN31 cells were treated with cisplatin, Chk inhibitor, or the combination and cell-cycle analysis was conducted to measure %8N or polyploidy values at various times afterward. At earlier time points (i.e., 24 and 48 hours), the combination treatment induced negligible polyploidy over cisplatin treatment alone in HN30, HN31, and HN30-shp53 cells (P > 0.700; Fig. 5C). However, a significant increase in the 8N values was seen with the combination treatment at 72 hours in HN30, HN31, and HN30-shp53 cells compared with cisplatin treatment alone (P < 0.0001). Importantly, combination treatment induced significantly higher levels of polyploidy in HN31 and HN30-shp53 cells compared with HN30 cells (P < 0.0001), with absolute 8N values that were at least double to that observed in HN30 cells at the 72-hour time point. Thus, significant polyploidy was induced in HN31 and HN30-shp53 cells treated with cisplatin plus Chk inhibitor.

Next, immunofluorescence microscopy was used to confirm polyploidy induction in HN31 and HN30-shp53 following combination treatment with cisplatin and the Chk inhibitor. Four days posttreatment, cells were fixed, stained with DAPI DNA, and counterstained with FITC–phalloidin. Multinucleated polyploidy cells were detected in HN31 and HN30-shp53 cells treated with the combination treatment (Fig. 5D), which were not apparent after exposure to cisplatin or AZD7762 alone. A percentage quantification of multinucleated cells observed under each treatment is shown in Fig. 5D. The appearance of multinucleated cells and presence of polyploidy in p53-deficient cells after combination treatment suggested that the cells were undergoing mitotic catastrophe. Western blot analysis of mitotic marker proteins was used to confirm that AZD7762 indeed abrogated the cell-cycle arrest typically associated with cisplatin treatment in the p53-deficient cells. In wtP53 HN30 cells, cisplatin caused substantial reduction in levels of the mitotic marker protein phoH3 compared with control-untreated cells, and these levels were not restored by the combination with AZD7762 (Fig. 5B). Thus, wtP53 cells remained growth arrested following exposure to cisplatin or the combination therapy. Similarly, cisplatin exposure led to decreased phospho-H3 in HN31 and HN30-shp53 cells as well. However, addition of the Chk inhibitor along with cisplatin completely prevented the drop in phospho-H3, consistent with abrogation of the normal cell-cycle arrest. The abrogation of cell-cycle checkpoint due to inhibition of Chk resulted in forced mitosis, as evident from the increase in phospho-H3 (S10) levels (Fig. 5B). Cells that underwent such abrupt mitosis died via mitotic catastrophe as further evidenced by the accumulation of sub-G1 fraction at later time points (Fig. 5E). At 96 hours, significantly higher sub-G1 values were observed with the combination treatment in HN31 and HN30-shp53 cells over cisplatin treatment alone (P < 0.0015). On the other hand, the sub-G1 values observed with the combination or cisplatin treatment alone in HN30 cells at 96 hours were not much different (P > 0.05). Collectively, the data indicate that inhibition of Chk leads to an override of the cell-cycle checkpoint in p53-deficient HN31 and HN30-shp53 cells in response to cisplatin treatment.

The robust response to cisplatin plus Chk inhibitor treatment is seen in other HNSCC cells expressing mutant p53

To examine whether other HNSCC lines harboring different p53 mutations would respond similarly to cisplatin plus Chk inhibitor combination treatment, we took SCC-61 (R110L) and Cal27 (H193L) and assessed their clonogenic survival in response to the combination treatment. The cisplatin plus Chk inhibitor treatment strongly potentiated the colony killing in both SCC-61 and Cal-27 cells over cisplatin or AZD7762 treatment alone (P < 0.0001) indicating that sensitization to cisplatin induced by inhibiting Chk1 occurs multiple p53-mutant cells (Fig. 6).

Discussion

Despite advances in surgical techniques and multimodality treatments, the survival outcomes have not improved significantly in several decades (12, 40). Cisplatin, which forms the basis of chemotherapy regimens used for HNSCC treatment, is not effective as a single agent in many patients. Multiple studies have attributed resistance of tumors to cisplatin to reduced drug uptake, increased drug detoxification process, enhanced DNA repair, and suppressed apoptotic response of resistant cells, whereas others have sought to link the cisplatin responses in HNSCC to the expression levels of certain biomolecules (4, 19, 21, 22, 41). TP53, the tumor suppressor gene, is one such biomolecule found to be mutated in about 60% to 80% of human papillomavirus (HPV)–negative HNSCC specimens (6, 42). In studies of p53 in patients with HNSCC, the
Figure 4. Inhibition of Chk1/2 sensitizes p53-mutant or -knockdown HNSCC cells to cisplatin (Cisp). A, HN30 and HN31 cells were seeded for clonogenic assay and subjected to following treatments: DMSO (CNT), AZD7762 alone (100 nmol/L for 48 hours), cisplatin (0.4 and 0.8 μmol/L; 24 hours), and cisplatin plus AZD7762 (combination for 24 hours, then PBS wash, AZD7762 treatment for another 24 hours). Treatments were carried out in triplicate wells. Later, surviving colonies were stained and counted as described previously. Surviving colonies in each treatment were normalized to the control (Con) and the data plotted. The clonogenic survival of HN30L (empty lentiviral control) and HN30-shp53 cells in response to the individual treatments was determined and surviving colonies were counted and plotted. * Significantly different from single-agent cisplatin or AZD7762 treatment in the same group using one-way ANOVA and Bonferroni multiple comparison test. (Continued on the following page.)
presence of wtp53 has been associated with favorable therapeutic response and improved survival, whereas the presence of mutp53 has been associated with poor therapeutic response and reduced survival (8–11). Here, using an in vitro model system, we sought to determine the impact of p53 function on the cisplatin sensitivity of HNSCC cells and found that wtp53-bearing HNSCC cells, HN30, are highly sensitive to cisplatin, whereas loss of wtp53 expression through p53 stable knockdown leads to cisplatin resistance. Furthermore, we questioned whether the presence of mutp53 would alter the cisplatin response. HN31, a cell line harboring p53 mutation but isogenic to HN30 was used. HN31 was established from a lymph node metastatic site, whereas HN30 cells were derived from a primary tumor site of the same patient (37). We found that mutp53 HNSCC cells were significantly more resistant to cisplatin. To eliminate the possibility that the observed sensitization to cisplatin by wtp53 is limited to only one genetic background, a similar experiment was carried out with UMSCC17A cells (wtp53).

In our study, regardless of the p53 status, we failed to detect apoptosis in HNSCC cells after cisplatin treatment. When assayed for PARP cleavage after cisplatin treatment, we could not detect cleaved PARP at 24, 48, and 72 hours. Similarly, there was no significant increase in sub-G1 fraction of HNSCC cells at these time points. In contrast, when p53 was either lost or mutated, the cisplatin response in cancer cells is due to the induction of apoptosis. One explanation for the discrepancy between our results and those from other groups may be the concentration of cisplatin used. Cisplatin, which is usually given as a bolus infusion to patients, has an area under the curve (AUC) value of 3.98 mg·h/L (43). This value translates to an equivalent in vitro cisplatin exposure of about 1 ± 0.01 mol/L over 24 hours or 24 ± 0.01 mol/L·h for cultured cells. Other research groups have used cisplatin exposures that were 10- to 50-fold higher than the clinically relevant exposures of cisplatin. It is likely that at such a high dose of cisplatin, apoptosis could be triggered, but this may not reflect the actual biologic outcome of cisplatin treatment in patients. In our study, for all experiments, we have used a physiologically relevant dose of cisplatin (i.e., 1.5 μmol/L over 24 hours). Thus, we believe, our results are reflective of the actual biologic outcomes in patients with HNSCC.

Two alternative cellular responses to cisplatin have been previously described in the literature—namely senescence and mitotic catastrophe (28, 44). Senescence, a metabolically active but nonproliferative cellular state, is characterized by enlarged flat, “pancake-like” cell morphology and characteristically show enhanced SA-β-Gal activity at pH 6. Accordingly, upon treatment with cisplatin, we observed that wtp53 HNSCC cells became large and had a “pancake-like” appearance characteristic of senescence and stained for the senescent marker β-galactosidase. Despite its widespread use, the SA-β-Gal activity as a marker of senescence has some limitations. Culture conditions such as serum starvation and increased cell confluence are known to enhance SA-β-Gal activity (45). Furthermore, it has been proposed that SA-β-Gal activity is actually a surrogate marker for increased lysosome number or activity. Consequently, enhanced SA-β-Gal activity has been detected in nongenetic cancer cell lines (46). Thus, the presence of SA-β-Gal activity alone is insufficient criteria for cells to be called senescent. In our study, in addition to SA-β-Gal activity, cells were also examined for the presence of characteristic morphology features that accompany senescence. Further support for the senescent phenotype in wtp53 HNSCC cells was induction of p53-regulated p21, a known upstream mediator of senescence. In contrast, when p53 was either lost or mutated, the β-Gal staining was negligible, and no induction of p21 was found. Parallel studies in our laboratory showed that the exogenous expression of wtp53 in p53-null HNSCC cell lines (UMSCC1 and PCI13) made them sensitive to cisplatin, and that the cisplatin sensitivity in these cells was not due to apoptosis but by senescence induction via a p53-p21 axis (Osman; unpublished data). Collectively, these data indicate that wtp53 mediates sensitivity to cisplatin and suggests that HNSCC tumors with wild-type p53 respond to cisplatin by senescence. Therefore, the relative resistance to cisplatin in the mutp53 or p53-knockdown setting is probably due to an inability to activate the senescence program.

We wondered whether this resistance to cisplatin could be overcome by forcing these cells to die through alternative cell death pathways. It is well known that Chk1/2 are critical enforcers of S and G2-M cell-cycle checkpoints and are rapidly activated to initiate cell-cycle arrest, DNA repair, and inhibit cell death in response to DNA damage (47). Therefore, Chk complement the functions of wtp53 in response to DNA damage. In absence of p53, due to loss or mutation, Chk1/2 assumes a central role in orchestrating these critical functions. Consequently, upon genotoxic treatment, the relaxation of checkpoint functions has been shown to result in increased cytotoxicity in a variety of cell lines with compromised p53 function (48–52). Yet, the studies investigating the effect of cisplatin plus Chk...
Targeting Chk Overcomes Cisplatin Resistance in mutp53 HNSCC

Figure 5. Cisplatin (Cisp) plus Chk inhibitor induces polyploidy and subsequent mitotic catastrophe in p53-mutant or -knockdown HNSCC cells. A, inhibition of Chk1 activity was detected in HN31 cells by measuring the levels of Chk1 autophosphorylation on S296. B, cell lysates from HN31, HN30, and HN30-shp53 cells following treatments: DMSO (Con), AZD7762 alone (100 nmol/L), cisplatin (0.5 μmol/L), cisplatin plus AZD7762 (combination for 24 hours), and cisplatin plus AZD7762 (combination for 24 hours, then PBS wash, AZD7762 treatment for another 24 hours). Each treatment was carried out in triplicate. Surviving colonies in each treatment were counted, normalized to the control (Con), and plotted on the graph as shown. †, Significantly different from single-agent cisplatin or AZD7762 treatment in the same group using one-way ANOVA and Bonferroni multiple comparison test.

inhibitor combination treatment have presented contrasting results. In some instances, this was probably due to different genetic backgrounds or tumor types, whereas in others the p53 status was not considered (34–36).

Here, we show that targeted inhibition of Chk1/2 preferentially sensitizes p53-knockdown or mutp53 HNSCC cells to cisplatin. The abrogation of checkpoints by targeted inhibition of Chk1/2 results in overriding of cisplatin-induced cell-cycle arrest and culminates in forced mitosis. Consistent with these results, we detected no drop in phospho-H3 levels with the combination treatment in p53-null or mutp53 settings. Higher levels of polyploidy or 8N values, suggesting mitotic catastrophe, were detected in 18% to 20% p53-knockdown or mutp53 HNSCC cells following the combination treatment. Mitotic catastrophe is a type of postmitotic death sometimes manifested by formation of large nonviable cells with multiple nuclei (28). Although the percentage of polyploidy cells was also elevated in wtp53 HNSCC cells following combination treatment, the value was considerably lower than in the p53-null or mutp53 setting. This indicates that only a small fraction of wtp53 HNSCC cells undergo polyploidy with the combination treatment and probably explains why there was no significant increase in the sub-G1 values in wtp53 HNSCC cells after the combination treatment compared with cisplatin alone. The increase in sub-G1 values with cisplatin treatment alone suggests that a very small percentage of wtp53 HNSCC cells may be dying through apoptosis. Thus, combination treatment had no apparent sensitization effect in wtp53 HNSCC cells.

Unlike other studies that report preferential requirement of either Chk1 or Chk2 for mediating resistance to radio- or other cytotoxic therapy, we report that both Chk1 and Chk2 contribute to cisplatin resistance in p53-knockdown cells as well.
could be translated in well (54), providing us some confidence that these results of combination treatment using should be conducted in the future to confirm the efficacy of combination of Chk inhibitor and cisplatin, follow-up studies p53-deficient tumor cells could be sensitized by the combination of p53 mutations would respond to the combination treatment. We showed that targeted inhibition of Chk1 and Chk2 sensitized HNSCC cell lines harboring different p53 mutations to cisplatin.

Although we present compelling in vitro evidence that p53-deficient tumor cells could be sensitized by the combination of Chk inhibitor and cisplatin, follow-up studies should be conducted in the future to confirm the efficacy of combination treatment using in vivo mouse models. In other cancer types, Chk inhibitors have been shown to sensitize tumor cells to DNA-damaging agents in in vivo as well (54), providing us some confidence that these results could be translated in in vivo setting. Our data also suggest that HNSCC cells carrying different p53 mutations could be sensitized to the combination treatment. However, in this study we have tested only a subset of p53 mutations in HNSCC, which then leaves a possibility that cells harboring other p53 mutations may behave differently to the combination treatment. Nevertheless, emerging data from our laboratory support our current findings.

Despite these limitations, we have unambiguously shown that HNSCC cells respond to cisplatin in a p53-dependent manner. Next, we present evidence that senescence is a preferred mode of cell death in HNSCC cells, and that cisplatin resistance in p53-null or mutp53 setting is due to its inability to activate a senescence program. Finally, we present one promising strategy of sensitizing p53-null or mutp53 cells to cisplatin. These preclinical data provide evidence that a personalized approach to the treatment of HNSCC based on Chk inhibition in p53-mutant cells may be feasible. Further preclinical and clinical investigation is needed to determine whether this approach will be useful to improve treatment outcomes for patients with p53-mutant HNSCC.

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

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