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 employing cisplatin to treat patients with advanced stage head and neck squamous cell carcinoma (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 while mutation or loss of TP53 is associated with cisplatin resistance. We also demonstrate 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 Chk1/2 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 demonstrating 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 pre-clinical data provide evidence that a personalized approach to the treatment of HNSCC based on Chk inhibition in p53 mutant tumors may be feasible.
Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cause of cancer death worldwide and globally afflicts roughly 500,000 new persons each year. Approximately 40,000 new cases of this devastating and often fatal disease will be diagnosed in the United States alone in 2012 (1). Surgery and radiation therapy have long been standard treatment regimens for HNSCC, but increasingly chemotherapy is being added as an adjuvant to treat patients with advanced disease. Despite the widespread use of cisplatin in HNSCC, only a subset of patients respond favorably, while a significant number have treatment resistance tumors (2-4). A clear absence of reliable markers predicting cisplatin response has been a major hindrance towards making therapeutic advances in HNSCC (5). Recently, whole exome sequencing of HNSCC performed by our group and others has confirmed TP53 to be the most frequently altered gene in primary HNSCC tumors making it a potential biomarker for predicting therapeutic response (6, 7). A number of studies have reported that mutation in p53 is associated with poor response to therapy and decreased survival in HNSCC, while the presence of wtp53 predicts for better therapeutic response and improved survival (8-11). Poor response to therapy and adverse prognosis in p53 mutant HNSCC tumors suggests that p53 dependent response mechanism(s) following cisplatin treatment are altered in p53 mutant HNSCC.

The role of p53 as a mediator of chemosensitivity is an area of active investigation, with several in vitro studies producing contrasting results (12-20). It has been shown in a variety of cell types that the cytotoxic effect of cisplatin is due to activation of p53 and induction of apoptosis (15, 21-23). Previous studies with HNSCC cells have also reported a similar finding (2, 4, 24-26). However, the cisplatin doses used in all of these studies were 10-50 folds higher than the clinically achievable dose of cisplatin. Hence, it is currently unclear what mode of cell 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 non-proliferative state,
or mitotic catastrophe, which is characterized by the presence of giant multi-nucleated 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 like cell cycle arrest and DNA repair hinge on the function of Chk1 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 demonstrated that TP53-deficient cells can be sensitized to genotoxic agents in a synthetic lethal strategy by inhibiting DNA repair enzymes such as the checkpoint kinases (Chk). Indeed, human clinical cancer trials employing 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 regarding 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 front 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 demonstrate 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 if 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 Astra Zeneca (Södertälje, Sweden). The drug powder was dissolved in 100% DMSO to a stock concentration of 10 mM and stored in aliquots. Intermediate drug concentration of 500 μM was prepared from this stock and aliquoted. A final drug concentration of 100 nM (0.1% DMSO) in culture medium was used for all experiments. HNSCC cell lines (HN30, HN31, UMSCC17A, SCC61, Cal27) 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 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 Modified Eagle Medium (DMEM) containing fetal bovine serum, glutamine, sodium pyruvate, penicillin/streptomycin, non-essential 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 performed 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, 500-600 cells per well was 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. 24hrs later cisplatin was removed by sucking off the media and cells were washed with phosphate buffered saline (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 on June 21, 2017. © 2013 American Association for Cancer Research. mct.aacrjournals.org Downloaded from mct.aacrjournals.org on June 21, 2017. © 2013 American Association for Cancer Research.
For clonogenic assays with cisplatin plus AZD7762 combination treatment, cells were treated with cisplatin and AZD7762 concurrently for 24 hrs. After 24 hrs, media was sucked off and cells were washed with PBS for three times. Cells were then supplied with fresh media containing AZD7762 (100nM) for another 24 hrs. 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 above.

**Cell Cycle Analysis**

HNSCC cells (3 - 5 x 10⁴) were plated in 60mm 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 min at room temperature. Cells were then stained with propidium iodide (PI) and processed in BCL-xL analyzer (Beckmann Coulter) for FACSCAN analysis. The data was analyzed on Flo-Jo software (Flo-Jo).

**Immunoblotting**

HNSCC cells were subjected to different treatments, and at indicated time points cells were lysed with RIPA lysis buffer (Tris 50mM (7.4 pH), 150mM NaCl, 1mM EDTA, 1% NP40, 0.1% SDS, 0.5% deoxycholate, 10% glycerol, 10% mercaptoethanol, 10 mM NaF, 1mM orthovanadate, 2.5 mM pyrophosphate, and proteinase inhibitors). Scrapped cells were pulse sonicated and proteins were allowed to solubilize in the buffer for 20 min. The cell lysates were then centrifuged at 14,000 rpm at 4°C for 20 min and supernatant was collected. Protein quantification was carried out using BCA protein assay kit (Thermo Scientific, IL, USA). The western blotting was performed as described previously (39). After performing the transfer, membrane blocking was done in 5% milk in Tris Buffered Saline with 0.1 % Tween 20 (TBST). The membranes were incubated overnight at 4°C with the following primary antibodies - PARP (#9542), Phospho-Chk1 (S345)(#2348), Phospho-Chk1 (S296) (#2349), Total Chk1(#2345), Phospho-Chk2 (Thr68)(#2661), Total Chk2 (#2662), Phospho-H3(S10)(#9701),Total H3 (#9715) γH2AX(S139)(#9718), Phospho-p53(S15)(#9284), p21(#2946)- all
purchased from Cell Signaling. After 1hr incubation at room temperature with 2° antibody in 2.5% milk (TBST 0.05%), the blots were briefly incubated with ECL reagent (GE Healthcare Life Sciences) and developed by exposing to X-ray film.

**Senescence β-Galactosidase Assay**

HNSCC cells (4x10^4) were seeded in a 6 well plate and allowed to attach overnight. Next day, cells were treated with the cisplatin at 1.5μM. After treatment, cisplatin was washed out using PBS. Cells were then supplied with fresh media. At day 4 or 6, cells were fixed in a fixative for 10 mins and incubated with staining solution following manufacturer’s instructions (#9860, Cell Signaling). In each treated or untreated well, four random field selections were made and the number of B-Gal positive (staining blue) were counted under high powered microscope (Olympus, IX71). Percentage B-Gal positive cells were measured as a ratio of blue cells to the total cell counted in each field multiplied by 100.

**Immunofluorescence**

HNSCC cells were seeded on glass coverslips and allowed to attach overnight. Next day, cells were subjected to treatments as indicated. Cells were then fixed in a 1:1 mixture of methanol/acetone for 10 mins. Cells were then washed several times with PBS and permeabilized using 0.1% Triton-X for 5 mins. Cells were again washed with TBST and incubated with Fluorescein Isothiocyanate (FITC) conjugated to Phalloidin (Sigma Aldrich) for 45 min at room temperature. Cells were then washed with TBST three times. The cover slips were then mounted on standard glass slides using 4’, 6-diamidino-2-phenylindole (DAPI)– Vectashield (Vector Laboratories). Using high powered microscope (Hamamatsu orca ER, Leica), multiple representative photographs in the field were taken. For quantification of mitotic catastrophe, a total of about 200 cells were counted across four different fields under each treatment condition in two independent experiments. Multinucleated cells were counted as cells containing greater than two nuclei and graphed as a percent of total number of cells.
TUNNEL assay

DeadEnd™ Fluorometric TUNEL System (Promega Corp., Madison, WI) was utilized for apoptosis detection. Briefly, 40 x 10^3 HN30 cells were seeded on cover glass slips inside 6 well plates. Cells were allowed to attach overnight. Next day, cells were treated with 1.5 μM cisplatin for 24hrs. At indicated time points, cells were washed 3X in PBS, fixed in 4% methanol-free formaldehyde for 25 min at 4°C, washed again, then permeabilized in 0.2% Triton X-100 in PBS for 5 min. After permeabilization, cells were equilibrated in equilibration buffer for 5 min at room temperature and then incubated with a buffer containing nucleotide mix and rTdT enzyme for 1hr. Cell nuclei were stained with DAPI. Fluorescence microscopy was performed 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 #SI00299859; siRNA for Chk2, #SI02224271) and non-silencing siRNA was purchased from Ambion (#4390846). HN31 cells were electroporated with 1.5μg of each siRNA using nucleofector II apparatus (Amaxa), and proteins were harvested 48hrs later, resolved by SDS-PAGE and analyzed by western blotting.

Statistics

Data for analysis were pooled from three independent experiments and each experiment was done 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 performing an ANOVA test, followed by the Bonferroni’s multiple comparison test using GraphPad Prism version 6 software. Two tailed student’s t tests were performed 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 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 half maximal inhibitory concentration (IC₅₀) for cisplatin was determined to be 0.16/μM in UMSCC17A and 0.14/μM in HN30 cells, and 0.60/μM 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 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 (IC₅₀ = 0.12 μM) was found to be much lower than HN30-shp53 cells (IC₅₀ = 0.32 μM) in response to cisplatin treatment (Fig. 1B and 1C) 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 1B).

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 performed and subG1 values were determined. The percentage subG1 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 subG1 values for HN31 were found to be less than 10% at all-time points (data not shown). Additionally, cell lysates from HN30, HN30-shp53, and HN31 cells were collected at 24hrs and 48hrs post-cisplatin treatment and probed for the presence of
cleaved PARP by western blotting. Cell lysates from staurosporine (1μM) 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 morphological 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). Additionally, no evidence of apoptosis was detected when cisplatin treated HN30 cells were assayed for positive TUNNEL staining (Supplementary figure 1). 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 above experiments, we used a clinically relevant dose of cisplatin (1.5 μM) at which no apoptosis could be detected. However, when HN30 cells were treated with cisplatin at a high dose (20 μM), we were able to detect PARP cleavage and also observed an increase in subG1 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 (Fig. 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 (1.5μM) and 4 days post treatment, 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, 3C). A profoundly reduced number of SA-β-Gal staining cells (i.e., < 5%) was seen in p53 knockdown HN30-shp53 cells compared to HN30 cells (p<0.01) and fewer than 10% of HN31 cells were senescent after cisplatin treatment (Fig. 3C).

Additionally, we performed western blot 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 if p21 was required for induction of senescence in HN30 cells, we created a stable lentiviral
knockdown of p21 in HN30 cells (HN30-shp21 cells), and subsequently performed senescence assay (after
cisplatin treatment). The basal levels of senescence seen in HN30L cells was completely lost upon p21
knockdown (HN30-shp21 cells), and a significantly lower number of senescent cells (<20%) were detected
in HN30-shp21 cells (p<0.0001) after cisplatin treatment (Fig.3E). Based upon above findings, it appears
that senescence, not apoptosis, is the main mechanism for cisplatin induced response of wtp53 HNSCC
cells and p21 is critical for the cisplatin induced senescence in wtp53 HNSCC cells.

**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 HNSCC patients have
tumors with mutp53, we investigated whether cisplatin resistant HNSCC lacking wtp53 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 mutp53 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. In order to test this hypothesis,
HN31 (mutp53) and HN30 (wtp53) 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 knock down 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.

In order to confirm that sensitization of p53 mutant cells to cisplatin is specifically due to inhibition of Chk, we performed a siRNA knockdown of Chk1, Chk2 or both in HN31 cells. The Chk knockdown cells were then exposed to cisplatin for 24hrs 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 to 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 to scramble suggesting that Chk1 knockdown improved the results over Chk2 knockdown alone (p<0.0001) (Fig. 4B). Western blots to confirm the knockdown of Chk1 and Chk2 are shown in Fig. 4C.

**DNA Damage Response signaling in HNSCC cells**

The ability of AZD7762 to inhibit Chk activity was verified by performing western blot 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 24hr cisplatin treatment and for an additional 24 h 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. Cisplatin treatment lead to an increase in Phospho Chk1 (S345) levels compared to 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 2/3 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 mediated by upstream kinases such as ATR, and facilitates auto phosphorylation on S296 during Chk1 activation. In all three cell lines, levels of phospho H2AX (i.e., γH2AX), a marker of DNA damage, was increased following cisplatin but further elevated by the combination of cisplatin plus AZD7762. Increased γH2AX 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 lead 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 of the treatment conditions (Fig. 5B).

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

HN30, HN30-shp53, or HN31 cells were treated with cisplatin, Chk inhibitor, or the combination and cell cycle analysis was performed to measure %8N or polyploidy values at various times afterwards. At earlier time points (i.e., 24 and 48hrs), the combination treatment induced negligible polyploidy over cisplatin treatment alone in HN30, HN31 and HN30shp53 cells (p>0.700) (Fig. 5C). However, a significant increase in the 8N values was seen with the combination treatment at 72hrs in HN30, HN31 and HN30shp53 cells compared to cisplatin treatment alone (p<0.0001). Importantly, combination treatment induced significantly higher levels of polyploidy in HN31 and HN30shp53 cells compared to HN30 cells (p<0.0001), with absolute 8N values that were at least double to that observed in HN30 cells at the 72hr 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 post-treatment, cells were
fixed, stained with DAPI DNA and counterstained with FITC-phalloidin. Multi-nucleated 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 multi-nucleated cells observed under each treatment is shown in Fig. 5D. The appearance of multi-nucleated cells and presence of polyploidy in p53-deficient cells after combination treatment suggested the cells were undergoing mitotic catastrophe. Western blotting 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 phopho-H3 compared to 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 96hrs, significantly higher subG1 values were observed with the combination treatment in HN31 and HN30shp53 cells over cisplatin treatment alone (p< 0.0015). On the other hand, the subG1 values observed with the combination or cisplatin treatment alone in HN30 cells at 96hrs 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 if 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; while 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 60-80% of HPV negative HNSCC specimens (6, 42). In studies of p53 in HNSCC patients, the presence of wtp53 has been associated with favorable therapeutic response and improved survival while 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 while loss of wtp53 expression through p53 stable knockdown leads to cisplatin resistance. Further, 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, while 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. In order to eliminate the possibility that the observed sensitization to cisplatin by wtp53 is limited to only one genetic background, a similar experiment was performed 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
24h, 48h and 72 hr. Similarly, there was no significant increase sub G1 fraction of HNSCC cells at these time points. Additionally, cisplatin treated HNSCC cells failed to show morphological characteristics of apoptosis like membrane blebbing or nuclear fragmentation. In contrast, several groups have shown that 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·hr/l (43). This value translates to an equivalent in vitro cisplatin exposure of about 1μM over 24hrs or 24 μM·hr for cultured cells. Other research groups have used cisplatin exposures that were 10-50 folds 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 biological 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 μM over 24 hours). Thus, we believe, our results are reflective of the actual biological outcomes in HNSCC patients.

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 non-proliferative 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 confluency 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 non-senescent cells (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 lab 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 (A.A. Osman; unpublished observations). 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 if 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, 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, checkpoint kinases 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 inhibitor combination treatment have presented contrasting results. In some instances, this was probably due to different genetic backgrounds or tumor types while 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 18-20% p53 knockdown or mutp53 HNSCC cells following the combination treatment. Mitotic catastrophe is a type
of post-mitotic death sometimes manifested by formation of large nonviable cells with multiple nuclei (28).

Although the percentage of polyploidy cells were 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 subG1 values in wtp53 HNSCC cells after the combination treatment compared to cisplatin alone. The increase in subG1 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 which report preferential requirement of either Chk1 or Chk2 for mediating resistance to radiation or other cytotoxic therapy, we report that both Chk1 and Chk2 contribute to cisplatin resistance in p53 knockdown or mutp53 HNSCC cells (31-33, 52, 53). Nonetheless, our data suggests that at lower dose of cisplatin the sensitization of p53 knockdown or mutp53 HNSCC cells seem to be driven by Chk2 and at higher dose of cisplatin the double knockdown of Chk1 and Chk2 results in the greatest sensitization. A spectrum of p53 mutations are seen in HNSCC patients across the 11 exons encoding the p53 protein and it is currently unknown how HNSCC tumors harboring these different p53 mutations would respond to the combination treatment. We demonstrated 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 performed 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 lab 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 mutant setting is due to their inability to activate a senescence program. Finally, we present one promising strategy of sensitizing p53 null or mutp53 cells to cisplatin. These pre-clinical 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.

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References


**Figure Legends:**

**Figure 1.** HNSCC cells respond to cisplatin in a p53 dependent manner. A) HN30, UMSCC17A (wtp53) and HN31 (mutp53) cells seeded for clonogenic assay were treated with cisplatin for 24hrs and surviving colonies were counted after imaging. B) HN30L (wtp53) and HN30-shp53 (p53 knockdown) cells seeded for clonogenic assay were treated with cisplatin for 24hrs at various concentrations. After drug wash out, colonies were allowed to form and counted. Surviving colonies at each cisplatin concentration were normalized to the control and plotted in the graphs. All cisplatin treatments were performed in triplicates and each experiment was repeated at least three times. Note in some cases error bars are not visible because they are smaller than marker symbols C) The images shown are representative of the differential response to cisplatin observed with HN30, HN30-shp53, and HN31 cells.

**Figure 2.** Apoptosis is not likely the main mode of cell death in HNSCC cells. A) HN30 and HN30-shp53 cells were treated with cisplatin for 24hrs. At 24, 48 and 72hrs, cells were fixed and stained with propidium iodide and sub G1 values were quantitated by flow cytometry. B) HN30, HN30-shp53 and HN31 cells were treated with cisplatin (1.5μM) and cell lysates were collected at 24 and 48hrs post treatment. *Lysates from staurosporine treated (1μM) HN30, HN31 and HN30-shp53 cells were collected at 8hrs and used as positive controls for apoptosis. C) HN30 and HN30-shp53 cells were treated with cisplatin (1.5μM) for 24hrs. Later, cells were fixed, stained and counter stained with DAPI and FITC phalloidin, respectively. Staurosporine (1μM) treated HN30-shp53 cells were used a positive control for apoptosis. D) HN30 cells were treated with cisplatin (20μM) for 24hrs and at given time points cells were processed for flow cytometry and subG1 values were quantitated as above. HN30 cells were treated with cisplatin (20μM) for 24hrs. After drug wash out, cell lysates were collected at 30 hrs and WB was performed to probe for PARP cleavage.

**Figure 3.** HNSCC cells undergo senescence in response to cisplatin treatment in a p53 dependent manner. A) HN30 and HN30-shp53 cells were treated with cisplatin for 24hrs and morphological changes were
monitored 4 days later (20X magnification). B) HN30, UMSCC17A and HN30-shp53 cells were treated with cisplatin (1.5μM) for 24hrs, 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 (10X 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 HN30shp53 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. E) Representative photos of cisplatin treated HN30L and HN30-shp21 cells were taken after β-Gal senescent staining (20X magnification) and percentage of β-Gal positive cells were graphed. Western blot was performed to confirm knockdown of p21. §, significantly different from cisplatin treated HN30 cells by two tailed student’s t test, ‡, significantly different from cisplatin treated HN30L cells by two tailed student’s t test.

Figure 4. Inhibition of Chk1/2 sensitizes p53 mutant or p53 knockdown HNSCC cells to cisplatin. A) HN30 and HN31 cells were seeded for clonogenic assay and subjected to following treatments: - DMSO (CNT), AZD7762 alone (100nM for 48hrs), Cisplatin (0.4μM and 0.8μM) (24hrs), and cisplatin plus AZD7762 (combination for 24hrs, then PBS wash, AZD7762 treatment for another 24hrs). Treatments were performed in triplicate wells. Later, surviving colonies were stained and counted as described previously. Surviving colonies in each treatment were normalized to the control 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’s multiple comparison test. B) HN31 cells were electroporated with buffer alone (mock), control scrambled siRNA, Chk1 siRNA, Chk2 siRNA, or Chk1/Chk2 siRNA, and then seeded for clonogenic assay. 24hrs post electroporation, the cells were treated with cisplatin (0.4μM and 0.8μM) (24hrs). After drug wash out, colonies were allowed to form, stained and counted as described earlier. 24hrs after electroporation with
different siRNAs, HN31 cells were treated with cisplatin (1.5 μM) and cell lysates were collected 24hrs later. The knockdown of target genes was confirmed by western blot (C). D) The structure formula of AZD7762 is shown. †, significantly lower than scramble transfected cells treated with cisplatin (0.4μM), ‡, significantly lower than Chk1 knockdown cells treated with cisplatin (0.4μM), §, significantly lower than scramble transfected cells treated with cisplatin (0.8μM), using one way ANOVA and bonferroni’s multiple comparison test.

Figure 5. Cisplatin plus Chk inhibitor induces polyploidy and subsequent mitotic catastrophe in p53 mutant or p53 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, HN30-shp53 cells under individual treatments were collected at 24hrs and the levels of DNA damage response markers and also the levels of phospho-H3 (S10), a mitotic marker were examined by western blot. p21 levels were examined from cell lysates collected at 72 hrs. C) HN30, HN31 and HN30-shp53 cells were treated with DMSO (CNT), AZD7762 alone (Chk 100nM), cisplatin (Cisp 1.5μM), or cisplatin plus AZD7762 (Cisp plus Chk). The duration of individual treatments was similar to that in clonogenic assays. Cells were collected at 24, 48 and 72 hrs, fixed, stained with propidium iodide and FACSCAN analysis was performed to determine %8N or polyploidy values. Each cell cycle experiment was performed at least two times. In some cases, error bars may not be visible because they are smaller than marker symbols. †, significantly greater than cisplatin treatment alone in each group, *, significantly greater than the combination treatment in HN30 cells at 72hrs time point, using one way ANOVA and bonferroni’s multiple comparison test. D) HN31 and HN30-shp53 cells were subjected to individual treatments as described above. At four days post-treatment, cells were fixed, stained with DAPI and counterstained with FITC-Phalloidin. Light fluorescence microscopy was performed and representative images under each treatment were taken. Red arrow under combination treatment show multi-nucleated cells. Percentage of multinucleated cells under each treatment condition are plotted on graph. *, significantly greater than cisplatin treatment alone using two
tailed student’s t test. E) HN31 and HN30-shp53 cells were exposed to individual treatments as above and harvested at 24, 48, 72 and 96hrs, and sub G1 values were quantitated using flow cytometry. ‡, significantly greater than cisplatin treatment alone in each group using two tailed student’s t test.

Figure 6. HNSCC cells harboring different p53 mutations are also sensitive to cisplatin plus Chk inhibitor treatment. SCC-61 (R110L) and Cal-27 (H193L) cells were seeded for clonogenic assay. Cells were then exposed to the following treatments: DMSO (Con), AZD7762 alone (100nM for 48hrs), cisplatin (0.5μM and 1μM) (24hrs), and cisplatin plus AZD7762 (combination for 24hrs, then PBS wash, AZD7762 treatment for another 24hrs). Each treatment was done in triplicates. Surviving colonies in each treatment were counted, normalized to the control 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’s multiple comparison test.
Figure 1

A

% Colony count (normalized)

\[ \begin{align*}
\text{HN31} & \quad \text{HN30} \\
\text{UMSCC17A} & \quad \text{HN30shp53} \\
\end{align*} \]

Cisplatin concentration (µM)

B

% Colony count (normalized)

\[ \begin{align*}
\text{HN30shp53} & \quad \text{HN30 L} \\
\end{align*} \]

Cisplatin concentration (µM)

C

HN30L

HN30-shp53

HN31

Control

Cisplatin 0.5 µM

Control

Cisplatin 0.5 µM

Control

Cisplatin 0.5 µM

Control
Figure 2

A

![Graph showing % Sub G1 values for HN30 and HN30 shp53](image)

- HN30 Control
- HN30 Cisp 1.5µM
- HN30 shp53 Control
- HN30 shp53 Cisp 1.5µM

B

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![Cleaved PARP (24hrs) and Actin](image)

![Cleaved PARP (48hrs) and Actin](image)

C

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D

![Graph showing % Sub G1 values for HN30 and HN30 Cisp 20µM](image)

- HN30 Con
- HN30 Cisp 20µM

![Cleaved PARP (30hrs) and Actin](image)

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Figure 6

**SCC61**

% Colony count (Normalized)

- Con
- AZD7762 alone (100nM)
- Cisp 0.5 μM
- Cisp 1 μM
- Cis (0.5 μM) + AZD7762 (100nM)
- Cis (1 μM) + AZD7762 (100nM)

**Cal27**

% Colony count (Normalized)

- Con
- AZD7762 alone (100nM)
- Cisp 0.4 μM
- Cisp 0.8 μM
- Cisp (0.4 μM) + AZD7762 (100nM)
- Cisp (0.8 μM) + AZD7762 (100nM)
Molecular Cancer Therapeutics

Chk1/2 inhibition overcomes the cisplatin resistance of head and neck cancer cells secondary to the loss of functional p53

Mayur A Gadhikar, Maria Rita Sciuto, Marcus V Ortega Alves, et al.

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