MEK Inhibition Overcomes Cisplatin Resistance Conferred by SOS/MAPK Pathway Activation in Squamous Cell Carcinoma

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Abstract

Genomic analyses of squamous cell carcinoma (SCC) have yet to yield significant strategies against pathway activation to improve treatment. Platinum-based chemotherapy remains the mainstay of treatment for SCC of different histotypes either as a single-agent or alongside other chemotherapeutic drugs or radiotherapy; however, resistance inevitably emerges, which limits the duration of treatment response. To elucidate mechanisms that mediate resistance to cisplatin, we compared drug-induced perturbations to gene and protein expression between cisplatin-sensitive and -resistant SCC cells, and identified MAPK–ERK pathway upregulation and activation in drug-resistant cells.

Introduction

Lung cancer remains a leading cause of cancer-related mortality, accounting for an estimated 1.4 million deaths in 2010 (1). Squamous cell carcinoma (SCC) arises primarily from cigarette smoking and is the second most common lung cancer subtype. Major advances have been made in the identification of oncogenic driver mutations in adenocarcinoma of the lung, but these efforts have been less successful in SCC. Although targeting EGFR mutations in the tyrosine kinase domain, ROS1 and EMLA-ALK rearrangements have yielded impressive gains in therapeutic efficacy in adenocarcinoma of the lung, as have the use of bevacizumab and pemetrexed chemotherapy for non-SCC histologies (2–5); for SCC histologies, however, ERK-induced resistance appeared to be activated by Son of Sevenless (SOS) upstream, and mediated through Bim degradation downstream. Clinically, elevated p-ERK expression was associated with shorter disease-free survival in patients with locally advanced head and neck SCC treated with concurrent chemoradiation. Inhibition of MEK/ERK, but not that of EGFR or RAF, augmented cisplatin sensitivity in vitro and demonstrated efficacy and tolerability in vivo. Collectively, these findings suggest that inhibition of the activated SOS–MAPK–ERK pathway may augment patient responses to cisplatin treatment.

In this study, we performed exome sequencing on lung SCC tumors to define the mutational profile of SCC. Using several genomics and proteomics-based technologies, we conducted parallel investigations to (i) elucidate the pathways associated with cisplatin resistance in lung SCC and (ii) dissect potential chemosensitizing mechanism(s) that enhance cisplatin cytotoxicity. These two approaches led us to the discovery that activation of the MAPK–ERK pathway under cisplatin treatment could confer cisplatin resistance. Importantly, we showed that disruption of the ERK signaling pathway via pharmacologic inhibition improved both in vivo and in vitro efficacy of cisplatin treatment. As SCC of the lung shares common etiology, demographics, and molecular biology with SCC of the head and neck (HNSCC), we extended these
investigations to include HNSCC. Finally, our study also provided conclusive evidence for the potential role of ERK phosphorylation as a predictive biomarker of clinical outcomes for cisplatin-treated SCC patients.

**Materials and Methods**

**Cell lines**

All lung cell lines were purchased from the ATCC. Lung SCC lines H226, H2170, H520, H596, and ChaGo-k-1 were cultured in RPMI-1640, Calu-1 in McCoy’s 5a, SK-MES-1 in DMEM, SW900 in Leibovitz’, H1869 in ACL-4 and H2066 in HITES medium. Lung normal fibroblast cells MRC-5, IMR-90, and WI-38 were cultured in EMEM medium. Head and neck SCC (HNSCC) cell lines were provided by Dr Timothy A. Chan from Memorial Sloan –Kettering Cancer Center. HNSCC cell lines UMSCC1, SCC13, and 93-VU-147 were cultured in DMEM. All media were supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. ACL-4 and HITES were further supplemented with other nutrients as recommended by the ATCC. All cell lines were authenticated by short tandem repeat analysis using GenePrint 10 System (Promega) in 2013.

**Cell viability assay**

Cells were cultured (2,000–4,000 cells/well) on 96-well plates and coincubated with cisplatin for 72 hours. At the end of the treatment, 20 μL/well of CellTiter 96 AQuos One Solution (MTS) solution (Promega) was added and incubated at 37°C for 3 hours. Absorbance was measured at 490 nm. All data points were set up with four replicates for each experiment. The IC50 was calculated by GraphPad Prism software (GraphPad Software).

**Anchorage-independent soft agar assay**

Soft agar was mixed with culture media to form agar layers with different concentration: A bottom layer with 0.6% agar; a middle layer comprised of 5,000 to 10,000 cells suspended in 0.36% agar; a top layer with complete media containing compound or compound combinations at various doses. Colonies were allowed to form for 2 to 4 weeks. On the final day of assay, 40 μL of MTT solutions (5 mg/mL in PBS) were added and incubated at 37°C for 4 hours. Images of each well were acquired with Epson V330 Photo scanner. The number and size of the colonies were analyzed and quantified using ImageJ (NIH). The percentage of cell colony formation was calculated relative to vehicle control treated cells.

**Microarray**

Total RNA from cell lines were harvested with the RNeasy Mini Kit (Qiagen) 8 hours post-cisplatin treatment to capture cisplatin-induced transcriptional changes before activation of apoptosis. Gene-expression profiling was conducted in technical duplicates with Affymetrix Human GeneChip 1.0ST array (Affymetrix) as per the manufacturer’s instructions. Data normalization and analysis were performed with GeneSpring Software V12. Genes with an absolute fold change ≥1.5 across the comparison groups were denoted as commonly altered genes (Fig. 1C, Supplementary Table S1). Pathway analysis was conducted with the publicly available Database for Annotatin, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 Software (12). The gene-expression data have been submitted to GEO repository (GEO accession number: GSE66549).

**Proteome profiler antibody array**

Whole-cell lysates from cells treated with cisplatin or vehicle controls were prepared 48 hours after drug incubation and normalized according to protein concentrations. Cell lysates (250 μg) were mixed with biotinylated detection antibodies and subjected to Proteome Profiler Phospho-MAPK arrays (R&D Systems). Signal detection was performed with Chemiluminescence detection system (GE Healthcare) and densitometric data was analyzed with ImageJ. The analyses were performed by subtracting the background density signal, and normalizing with positive controls on each membrane. Comparisons between the treatment groups where then made using normalized values, and expressed in fold change to the vehicle control group.

**Total protein extraction and Western blotting**

Cultured cells were rinsed with PBS and lysed with Cell Lytic buffer (Sigma; 150 mmol/L NaCl, 0.41% bicine, 2% EDTA) supplemented with complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). Protein concentration was quantified and normalized samples were resolved with Bio-Rad SDSPAGE system on 8% to 12% protein gels. Signal detection was performed with chemiluminescence detection system (GE Healthcare). Blotting was performed with the following antibodies: PARP, Caspase-3, p-ERK1/2 (Thr202/Tyr204), total ERK1/2, p-p38 (Thr180/Tyr182), total p38, p-BRAF (Ser445), p-MEK1/2 (Ser217/221), total MEK1/2, SOS1, p-JNK (Thr183/Tyr185), total JNK, p-Src (Tyr416), total Src, β-actin, and HRP-conjugated anti-rabbit from Cell Signaling Technology; SOS2 from Abcam.

**Immunofluorescent staining and image analysis**

Cells were seeded and treated with cisplatin at the indicated doses for 48 hours. At the assay endpoint, cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton-X prior to staining. Samples were then immunostained with p-ERK1/2 antibody (Cell Signaling Technology; 1:500), followed by Alexa-Fluor 488 secondary antibody (Life Technologies; 1:1,000), and Hoechst 33342 nuclear counterstain (Sigma Aldrich; 1:2,000). Protocols for image acquisition and analysis were adapted from image cytometry methods as described previously (13).

**RNA interference**

For gene knockdown, MEK1 siRNA (sequence: 5′-GLCAGAIAAAALUCGUAIAIAATT-3′), MEK2 siRNA (sequence: 5′-GCAIUUGUCAGGCAAGAATT-3′), ERK1 siRNA (sequence: 5′-CGUCIAAAAIUAIAIAAAATT-3′), ERK2 siRNA (sequence: 5′-GIUIGGAGUAGGCAAGAATT-3′), SOS1 siRNA (sequence: 5′-GAACGCUCAACGAUAATATT-3′), SOS2 siRNA (sequence: 5′-UCAIUAAAUCUAGCUCUUATT-3′), Bim siRNA (sequence: 5′-AGAGAGGACUJIAACCUAAAT-3′), Src siRNA (sequence: 5′-AAGCAGTCGCTGCCATATGAAA-3′), and AllStar negative control siRNA were obtained from Qiagen. Transfection (50 nmol/L siRNA for each target in each reaction) was conducted with JetPRIME reagent (Polyplus Transfection). For gene overexpression, MAP2K1 (D67N) and empty vector (pCMV-entry) plasmid constructs were obtained from OriGene Technologies. Plasmid transfection was conducted with ViaFect reagent (Promega).

**Clinicopathologic and immunohistochemistry analyses**

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded (FFPE) sections of HNSCC and xenograft tumors.
as described in the Supplementary Methods. Preparation and scoring of clinical samples was performed by two pathologists in a blinded manner based on the staining intensity in the cytoplasm and/or the nucleus (0, negative; 1, weak; 2, moderate; and 3, strong) and the fraction of stained tumor cells (0%–100%). The total immunoreactive score (IRS) was determined by multiplying the intensity score with the fraction of stained cells (0–300). Survival outcomes were defined by disease-free survival (DFS). Photomicrograph images acquired from xenograft tumors for each of the treatment groups were processed with an adapted image processing workflow using ImageJ as described previously (14).

**Xenograft studies**

Patient-derived xenograft (PDX) model SCC10-0508 was established and maintained in 9- to 10-week-old SCID mice as described previously (15). In brief, SCC10-0508 tumors were excised and minced into small 1 to 2 mm³ dices before being implanted s.c. into left and right flanks of SCID mice. Mice were assigned treatment in to four stratified groups based on average tumor volume from the left and right flanks of the mouse: Vehicle (30% w/v Captisol), PD0325901, Cisplatin, PD0325901 + Cisplatin (n = 5 animals/group). Cisplatin was provided in 0.9% NaCl and given at 2 mg/kg by i.p., once weekly for 3 weeks. PD0325901 was formulated in 30% (w/v) Captisol (CyDex) at 10 mg/kg and given orally, once daily until mice were terminated. Mice were treated 9 days after tumor implantation (tumor volume ~200 mm³). Tumor growth (length × width × width × 3.14159/6) was monitored. Bodyweight at sacrifice and tumor samples were collected when mice were sacrificed 26 days after treatment commenced. Animals were housed in accordance to IACUC guidelines.

**Statistical analysis**

All experiments were conducted three times unless stated otherwise. Results are expressed as mean ± SD. Statistical analysis for the comparison between two groups was conducted using the Student t test, whereas comparisons between multiple groups was conducted using ANOVA. The Gehan–Breslow test was performed to test the statistical differences in DFS. All tests were two-sided and the significance level was set at P < 0.05.

**Results**

**Paucity of somatic DNA alterations and presence of cisplatin-resistant subtypes in lung SCC tumors**

To look for oncogenic mutations in SCC tumors among Asian patients that would be responsive to molecular targeting, targeted exome sequencing were performed on 45 lung SCC samples after pathway-specific PCR-based exon enrichment. Consistent with the findings by The Cancer Genome Atlas (TCGA; ref. 16), common oncogenic lesions in lung adenocarcinoma were rarely
found in SCC tumors. Despite observing EGFR mutations in 82% of patient samples (37/45), these alterations were almost exclusively codon-silent events (30/37); whereas nonsynonymous mutations in KRAS, BRAF, AKT1, and PIK3CA were rare (<10%; Supplementary Fig. S1). Moreover, oncogenic mutations were rare among the 10 lung SCC cell lines used in this study as curated from the Sanger Catalogue of Somatic Mutations in Cancer (COSMIC) Database, with only two cell lines (Calu-1 and SW900) harboring mutated KRAS and none with mutated EGFR.

Using 10 tumor-derived and three normal lung fibroblast-derived cell lines as representative study models, we determined the IC50 of cisplatin via cell proliferation and anchorage-independent assays. Whereas lung fibroblast cell lines were sensitive to cisplatin (IC50 < 5 μmol/L), lung SCC cell lines displayed differential cisplatin sensitivity, with a 6-fold difference between the most sensitive and most resistant cells. Four SCC cell lines (H596, H1869, H226, and ChaGo-k-1) had IC50 < 10 μmol/L (cisplatin sensitive) and six cell lines (H2066, SW900, H2170, Calu-1, H520, and SK-MES-1) had IC50 > 10 μmol/L (cisplatin-resistant; Fig. 1A). Consistent with these observations, cisplatin strongly inhibited colony formation of H226 and ChaGo-k-1 cells (IC50 < 100 nmol/L), whereas H520, H2170 and Calu-1 were more resistant to cisplatin (IC50 > 200 nmol/L; Fig. 1B; Supplementary Fig. S2).

**ERK phosphorylation is associated with cisplatin resistance**

Gene-expression profiling was performed to identify putative genes and cellular pathways that may be associated with cellular response to cisplatin. Cell lines were grouped according to their cisplatin IC50 (H596, H1869, H226, and ChaGo-k-1 as sensitive; Calu-1, H2170, SW900, and H2066 as resistant). Upon exposure to cisplatin, a total of 150 and 382 genes were found to be altered in the cisplatin-resistant and -sensitive study sets, respectively (fold change > 1.5; P < 0.05). Of these genes, 123 genes were commonly modulated in both study sets (Fig. 1C). To identify key factors that regulate cellular sensitivities to cisplatin in lung SCC, we compared the mutually exclusive genes that were differentially regulated between the resistant (27) and sensitive (259) phenotypes (Fig. 1C; Supplementary Table S1) by using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (P < 0.05). Cisplatin treatment downregulated genes associated to cellular pathways in cisplatin-sensitive H596 cells (Fig. 2B and C).

To verify the pathways identified from gene-expression profiling in terms of phosphorylated protein regulation, we performed parallel phospho-kinase arrays covering the MAPK and phosphatidylinositol pathways on Calu-1 (resistant) and H596 (sensitive) cells in the presence or absence of cisplatin (Fig. 1D). The signal intensities of each pair of duplicate spots were quantified (Supplementary Fig. S3A and B), and the perturbations to protein expression profiles of the two cell lines were compared. By contrast, p-ERK1/2 showed largest increase in expression (1.87-fold) in cisplatin-resistant cells (Calu-1) after cisplatin treatment, whereas p-p38α showed the most apparent decrease in expression (0.14-fold) in cisplatin-sensitive cells (H596) (Supplementary Fig. S3C). Conversely, cisplatin suppressed Akt phosphorylation in both cell lines (Supplementary Fig. S3A and S3B).

The observed changes in kinase phosphorylation were next validated by Western blot analyses on independent cell lysates of cisplatin-sensitive (H596 and H226) and -resistant (Calu-1, H2170, and H520) cells. Interestingly, ERK1/2 but not p38, were consistently phosphorylated in resistant cells, but dephosphorylated in sensitive cells with increasing cisplatin concentrations (Fig. 1E). Moreover, cisplatin treatment triggered PARP cleavage at lower doses in cisplatin-sensitive cells (H596 and H226) as compared with that in cisplatin-resistant cells (Calu-1 and H520; Fig. 1E). Collectively, these data indicate that p-ERK is positively associated with cisplatin resistance in SCC cells.

**MAPK/ERK upregulation in cisplatin-treated cells is mediated through SOS and leads to Bim degradation**

The RAS–RAF–MEK–ERK signaling axis is a complex network that triggers cell growth and/or cell death through downstream stimulation of transcription factors. Upon activation, RAS binds to RAF and the resulting complex phosphorylates MAPK/ERK kinase (MEK1/2), which, in turn, mediates activation and nuclear localization of ERK1/2 (17, 18). Here, we showed that the phosphorylation patterns of both MEK and BRAF were consistent with that of p-ERK1/2 in both cisplatin-resistant and -sensitive cells (Fig. 2A). Furthermore, cisplatin treatment induced the cytoplasmic accumulation and nuclear translocation of p-ERK1/2 in the cisplatin-resistant Calu-1 cells, but reduced p-ERK1/2 in the cisplatin-sensitive H596 cells (Fig. 2B and C).

Thus far, our data have suggested a correlation between ERK and cellular response to cisplatin, but the factor(s) leading to the differential regulation of p-ERK remains unclear. Importantly, the phosphorylation of EGFR was not affected by cisplatin treatment in SCC cells (data not shown). To identify the alternative factor(s)
that modulated MAPK/ERK signaling, we focused on the suppressed genes in cisplatin-treated H596 cells that were associated with MAPK signaling (Table 1). Interestingly, the genes that were profoundly suppressed included Son of Sevenless homolog 2 (SOS2), a key regulator of the MAPK signaling cascade. SOS is a guanine nucleotide exchange factor that facilitates the GDP to GTP exchange of RAS, thereby allowing it to interact with RAF and stimulate the MAPK–ERK signaling cascade (19, 20). Accordingly, we hypothesized that SOS positively modulates p-ERK activity in SCC cells. Cisplatin treatment upregulated both SOS1 and SOS2 in the resistant cell lines that displayed strong ERK activation (H520 and Calu-1) but their expression was either reduced (H596) or unchanged (H226) in sensitive cell lines (Fig. 2D).

Mechanistically, we showed that siRNA silencing of SOS1 and SOS2 was associated with decreased ERK1/2 phosphorylation, with the combined knockdown of both SOS molecules demonstrating the most significant effects (Fig. 2E). Moreover, depletion of SOS abrogated cisplatin-associated ERK phosphorylation in Calu-1 cells (Fig. 2F).

Activation of ERK1/2 is known to promote phosphorylation and degradation of proapoptotic Bim, and eventually confer cisplatin resistance (21, 22). Consistent with these findings, basal p-ERK1/2 led to Bim phosphorylation in Calu-1 and H520 cells transfected with control siRNA, while inhibition of ERK by SOS silencing reduced Bim phosphorylation and increased Bim accumulation (Fig. 2E). Notably, silencing of either Erk1/2 or MEK1/2 reduced mobility shift and increased stabilization of Bim in Calu-1 cells (Supplementary Fig. S4D). Taken together, we postulate a cisplatin-induced signal transduction pathway involving SOS upregulation, activation of the MAPK–ERK axis, and Bim degradation that renders cisplatin resistance in SCC cells through prevention of Bim-induced apoptosis.

Activation of the MAPK–ERK pathway confers survival advantage to SCC cells treated with cisplatin

To address the functional role of the MAPK–MEK–ERK pathway in cisplatin resistance, we found that silencing ERK1/2 increased cisplatin sensitivity in a time- and concentration-dependent manner (Fig. 3A; Supplementary Fig. S4A), with a significant reduction in the cisplatin IC₅₀ by more than 2-fold in all three cisplatin-resistant lung SCC cell lines (Calu-1, H2170, and H520; Fig. 3B; Supplementary Fig. S4B and S4C). Consistent with our findings of upstream activation, siRNA silencing of MEK1/2 abrogated ERK1/2 phosphorylation and
reduced cisplatin IC50 in Calu-1 cells (Fig. 3C). Conversely, as compared with empty vector (EV) control, overexpression of D67N MEK1 mutant constitutively induced p-MEK1 and p-ERK1/2 in H596 cells (Fig. 3D), and this abrogated Bim-induced apoptosis, as indicated by the reduced cleavage of PARP and caspase-3 (Fig. 3D). By generating a H596 cells that rendered cisplatin resistance (CR-H596; 3-fold higher cisplatin IC50 as compared with parental H596) through chronic low-dose exposure to cisplatin (Supplementary Fig. S5A), we demonstrated that acquired resistance was mediated through MAPK/ERK signaling. In comparison with parental H596 cells, p-ERK1/2 and SOS2 were highly expressed in CR-H596 cells, whereas Bim-induced apoptosis was attenuated upon cisplatin treatment (Supplementary Fig. S5B). Together, these observations indicate the critical role of the MAPK pathway in rendering cisplatin resistance.

ERK1/2 phosphorylation correlates with shorter DFS in HNSCC patients treated with adjuvant chemotherapy

To support the hypothesis that strong ERK activity is responsible for cisplatin resistance in a patient population, we analyzed ERK phosphorylation levels in patients with HNSCC. However, the lung SCC patients at NUH are not treated with single-agent cisplatin, and the correlation of p-ERK expression and patient survival may be confounded by the addition of a second chemotherapeutic agent. Therefore, clinicopathologic analysis was conducted on HNSCC instead where concurrent single-agent cisplatin treatment with radiotherapy is the standard...
first-line regime. It is generally accepted that HNSCC share similar etiology and histopathologic characteristics of SCC of the lung. Among three HNSCC cell lines, we identified UMSCC1 and 93-VU-147 cells to be more tolerable to cisplatin treatment (Supplementary Fig. S6A). Consistent with our hypothesis on lung SCC, cisplatin-sensitive SCC13 cells demonstrated downregulation of SOS2, p-MEK1/2 and p-ERK1/2 upon increasing doses of cisplatin, together with the cleavage of PARP; whereas cisplatin-resistant UMSCC1 cells had sustained activation of p-MEK1/2 and p-ERK1/2 with minimal PARP cleavage (Supplementary Fig. S6B).

The characteristics of the selected HNSCC patient population were presented in Supplementary Table S2. ERK activation was analyzed by immunohistochemistry (Fig. 3E). The DFS—defined as the interval between the chemoradiation completion to the first histologically or radiologically confirmed tumor recurrence locally in the head and neck region or distant metastases—was defined in 45 HNSCC patients. Significantly, shorter DFS was determined for patients with higher expression of p-ERK (log-rank test, \( P = 0.02 \); Fig. 3F), suggesting that activation of the MAPK–ERK pathway is a variable in the association of p-ERK and DFS among the selected HNSCC cases.

MEK inhibition enhances cisplatin cytotoxicity through upregulation of Bim

Our data so far suggest that ERK activation and its downstream signaling events are crucial in mediating resistance to cisplatin. Consequently, the inhibition of ERK signaling may therefore be a potential therapeutic approach in patients with resistant SCC. To investigate this possibility further, Calu-1 cells were treated with several pharmacologic inhibitors of the EGFR–MAPK pathway [i.e., EGFR antibody (cetuximab), RAF inhibitor (GDC-0879), and MEK inhibitors (PD0325901, RDEA119, and GSK1120212)] at the determined sub-lethal doses in combination with cisplatin. Despite inhibiting EGFR and BRAF, cetuximab and GDC-0879 had limited effects on p-ERK in Calu-1 cells (Supplementary Fig. S7A). In contrast, MEK inhibitors abrogated p-ERK expression while simultaneously inducing the accumulation of p-MEK1/2 (Supplementary Fig. S7A). Coincubation of cisplatin and MEK inhibitors, but not cetuximab and GDC-0879, significantly lowered cisplatin IC \(_{50}\) in Calu-1 cells (Fig. 4A; Supplementary Fig. S7B). The failure of cetuximab and GDC-0879 to suppress p-ERK possibly accounts for the lack of chemosensitizing effects in cisplatin-resistant SCC cells that are highly-dependent on MAPK activity. Consistently, effective MEK–ERK inhibition increased cisplatin sensitivity in H520 cells (Fig. 4B; Supplementary Fig. S7C).

Mechanistically, abrogation of p-ERK by MEK inhibitors abolished cisplatin-induced Bim phosphorylation, which prevented mobility shift and increased accumulation of Bim in Calu-1 cells, therefore resulting in the activation of caspase-3 and cleavage of PARP (Fig. 4C). In concordance with these observations, siRNA-silencing of Bim effectively rescued cell death induced by MEK inhibition in cisplatin-treated Calu-1 cells (Fig. 4D). These findings confirm that pharmacologic inhibition of ERK could successfully enhance cisplatin-induced apoptosis in cisplatin-resistant SCC cells.

MEK inhibition augments the antiproliferative activity of cisplatin in xenograft models

Under in vitro anchor- and independent condition, combined treatment of GSK1120212 with cisplatin further suppressed colony formation in H520 cell line (IC \(_{50}\) = 132 nmol/L) as compared with cisplatin treatment alone (IC \(_{50}\) = 193 nmol/L; Supplementary Fig. S8B); whereas GSK1120212 alone effectively inhibited colony formation (at a dose as low as 0.01 pmol/L) in Calu-1 cells (Supplementary Fig. S8A).

Given the improved in vitro efficacy of MEK inhibitor–cisplatin combination, we next used two xenograft models to test the combinatorial effect of the MEK–ERK co-inhibition with cisplatin, using doses previously documented to result in therapeutically relevant concentrations (24, 25). Tumor-bearing mice were randomized to treatment with vehicle, the MEK inhibitor PD0325901, cisplatin, or the combination of PD0325901 and cisplatin. In H520-derived xenografts, PD0325901 and cisplatin attenuated tumor growth as a single agent monotherapy, with the combined treatment demonstrating the most pronounced inhibitory effect (Supplementary Fig. S9A–S9C). The efficacy of this treatment regime was next investigated in a patient-derived HNSCC xenograft model (PDX, SCC10-0508) that harbors wild-type EGFR/RAS/BRAF and expresses high basal p-ERK. Relative to vehicle-treated controls, cisplatin treatment alone had little effect on suppressing tumor growth (Fig. 5A–C). In contrast, treatment with PD0325901 alone dramatically inhibited tumor growth; whereas the combination treatment showed similar tumor inhibition, with one tumor showing regression (Fig. 5A–C). Minimal weight loss was observed in mice in all treatment groups (data not shown). Sustained ERK activation were observed in both xenograft models after cisplatin treatment, whereas PD0325901 treatment suppressed p-ERK and upregulated Bim in tumor tissues (Fig. 5D and E; Supplementary Fig. S9D). Together, these data strongly support the therapeutic potential of combining cisplatin with MEK inhibitors in cisplatin-resistant SCC tumors, including those with wild-type BRAF and RAS.

Discussion

SCC of the lung and head and neck, when recurrent or metastatic, carries a poor prognosis and has limited therapeutic options. Cisplatin chemotherapy is the backbone of treatment against these cancers (8), but patients will eventually develop platinum resistance and very limited or no effective second line treatment exists. To date, only the use of cetuximab, a monoclonal antibody against EGFR, in combination with platinum-based chemotherapy, has been reported to improve their treatment outlook (26, 27). The use of small-molecule inhibitors and monoclonal antibodies have not achieved desirable clinical outcome in patients with lung or HNSCCs. Some recent data have highlighted DDR1 and FGFR1 mutations in SCC lung, but these mutations are still considered rare (28, 29). Our data also confirm the findings of the TCGA group that there is a low incidence of oncogenic mutations in lung SCC despite the detection of a high mutation frequency (16). As such, compared with adenocarcinoma of the lungs, it remains difficult for individualized targeted therapies to be applied for the treatment of SCC.
One likely mechanism of resistance to chemotherapy is the capacity of cancer cells to upregulate signaling pathways such as the MAPK pathway to provide additional survival and proliferative signals. Cisplatin resistance has previously not been known to be associated with any specific signaling pathways directly, and our data provide fresh evidence that whereas both MEK and ERK are rarely activated by mutation in cancers, activation of SOS/MAPK appears to be a drug-associated characteristic of SCC subtypes that are more resistant to cisplatin. In addition, upregulation of MAPK/ERK is seemingly a possible mechanism for acquired resistance to cisplatin. Despite so, contradicting reports have demonstrated both the prosurvival and proapoptotic roles of ERK signaling in cisplatin-treated cells, which are dependent on the downstream components activated by p-ERK (30–32). Here, we found that MEK/ERK silencing and pharmacologic inhibition of MEK in clinically relevant concentrations could act synergistically with cisplatin in cisplatin-resistant cell lines. In contrast, inhibitors targeting components upstream of MEK, such as RAF kinase, were not able to achieve similar synergy possibly due to the activation of alternative pathways that conversely sustained ERK phosphorylation (33, 34). It has been reported that Src kinase could directly regulate MAPK cassettes through RAS (35, 36), but in our study, Src phosphorylation did not seem to correlate with ERK in several lung SCC cell lines (Supplementary Fig. S10A). In addition, inhibition of Src signaling with either Src-specific inhibitor, AZD0530, or Src siRNA did not affect ERK activation and PARP cleavage in Calu-1 cells (Supplementary Fig. S10A and S10B).

In terms of downstream mechanisms, we found that inhibition of MEK–ERK signaling induced Bim expression and
enhanced cisplatin cytotoxicity; whereas constitutive stimulation of ERK activity through ectopic expression of MEK1 abrogated Bim-induced apoptosis. This suggests a mechanism for dysregulation of cisplatin cytotoxicity via ERK activation in SCC cells, which is consistent with previous reports in ovarian cancer cell lines where Bim degradation was associated to cisplatin resistance (21), and this process could be reversed by MEK inhibition (30, 31). It is widely accepted that MEK inhibitors exert a cytostatic, rather than cytotoxic, effect when used in cancer cells (37). Concordantly, we showed that GSK1120212 alone predominantly induced G1 arrest in SCC cells, but this effect was attenuated in the presence of cisplatin (Supplementary Fig. S11A–S11D). Furthermore, apoptosis was triggered in GSK1120212/cisplatin-treated cells, suggesting that MEK inhibition leads to cytotoxic instead of cytostatic effect in the presence of cisplatin.

SCC head and neck are similar to lung SCC in etiology, histology, and mutational landscape (38–40). Thus, to determine whether MEK/ERK activation occurs in patient tumor samples and could potentially reduce the antitumor effect of cisplatin, we conducted a clinicopathologic analysis on samples from patients with newly diagnosed chemo-naive HNSCC, where the primary treatment included concurrent standard radiotherapy with single-agent cisplatin chemotherapy. There was wide inter-individual variability of p-ERK expression in tumors, and DFS was shown to be shorter for the high ERK-expressing patients, suggesting p-ERK may be an important predictor of response to cisplatin treatment in HNSCC. Given there is lacking of driver mutations to guide therapy in lung SCC, we propose ERK as a potential therapeutic candidate for treatment of relapsed and cisplatin-refractory tumors.

Though the RAF–MEK–ERK pathway may be inhibited at several levels by pharmacologic perturbations, the specific level...
of inhibition may be critical. For instance, tumors with wild-type BRAF, including those with mutant RAS, circumvent RAF inhibition through paradoxical stimulation of ERK activity (33). In contrast, obfuscation in several findings has indicated that MEK inhibitors are clinically active in tumors with BRAF mutation as single agent (41), whereas the data from our xenograft models suggest that this class of inhibitors could also inhibit growth of wild-type BRAF tumor that has high ERK activity, and shows promising combinatorial effect with cisplatin. Importantly, this drug combination appeared to be tolerated by the treated animals, without exacerbating the toxicities of cisplatin as no apparent weight loss was observed. Deployed as a single agent, it is likely that MEK inhibitors have limited efficacy in SCC as noted in clinical observations from a phase I study that reported the lack of efficacy of PD0325901 in six patients with lung SCC (42). Our data suggest that, through selection of patients with SCC whose tumors express high p-ERK, treatment with cisplatin and MEK inhibitor may be a promising combination strategy.

In summary, we have found that upregulation of MEK/ERK is associated to cisplatin resistance. Although there are other known mechanisms of cisplatin resistance, including reduced influx transport via the copper transporter, conjugation by glutathione and methionine, and enhanced DNA repair (43), the relative importance of each of these pathways is still not defined in SCC. More crucially, our results suggest a role for the combined use of cisplatin with the MEK inhibitor for patients with upregulated MAPK/ERK signaling in SCC of the head and neck. Several MEK inhibitors currently undergoing clinical development have shown promising endpoint results in various trials on NSCLC (44–46). Furthermore, MEK inhibitors have demonstrated favorable tolerability profiles and side effects consisting mainly of diarrhea, rash, and central serous chorioretinopathy (47). Cisplatin is well established and has a nonoverlapping toxicity profile, which lends its potential to be combined with an MEK inhibitor. Indeed, there is evidence for potential renal tubular protection from cisplatin-induced renal tubulopathy by MEK inhibition (48). For a proof-of-concept, it will be critical to study the combination of cisplatin and an MEK inhibitor in patients whose tumors demonstrate evidence of ERK phosphorylation. Such studies are currently being planned.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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