Small Molecule Therapeutics

The Dual Pathway Inhibitor Rigosertib Is Effective in Direct Patient Tumor Xenografts of Head and Neck Squamous Cell Carcinomas

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Abstract

The dual pathway inhibitor rigosertib inhibits phosphoinositide 3-kinase (PI3K) pathway activation as well as polo-like kinase 1 (PLK1) activity across a broad spectrum of cancer cell lines. The importance of PIK3CA alterations in squamous cell carcinoma of the head and neck (HNSCC) has raised interest in exploring agents targeting PI3K, the product of PIK3CA. The genetic and molecular basis of rigosertib treatment response was investigated in a panel of 16 HNSCC cell lines, and direct patient tumor xenografts from eight patients with HNSCC [four HPV-serotype16 (HPV16)-positive]. HNSCC cell lines and xenografts were characterized by pathway enrichment gene expression analysis, exon sequencing, gene copy number, Western blotting, and immunohistochemistry (IHC). Rigosertib had potent antiproliferative effects on 11 of 16 HPV⁻ HNSCC cell lines. Treatment sensitivity was confirmed in two cell lines using an orthotopic in vivo xenograft model. Growth reduction after rigosertib treatment was observed in three of eight HNSCC direct patient tumor lines. The responsive tumor lines carried a combination of a PIK3CA-activating event (amplification or mutation) and a p53-inactivating event (either HPV16- or mutation-mediated TP53 inactivation). In this study, we evaluated the in vitro and in vivo efficacy of rigosertib in both HPV⁺ and HPV⁻ HNSCCs, focusing on inhibition of the PI3K pathway. Although consistent inhibition of the PI3K pathway was not evident in HNSCC, we identified a combination of PI3K/TP53 events necessary, but not sufficient, for rigosertib sensitivity. Mol Cancer Ther; 12(10); 1994–2005. ©2013 AACR.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth-most frequent cancer worldwide by incidence. Originating in the mucosa of the upper airways, one third of patients present with early-stage disease, whereas the majority present with advanced disease and lymph node metastasis (1). Estimates have suggested that more than 600,000 cases arose globally in 2012 with a 5-year patient survival rate of 40% to 50% (2), which has not improved substantially despite advances in anticancer therapy. Tobacco and alcohol consumption have historically been the most important risk factors, and incidence of HNSCC in the western world had been on a slow decline over the past decade correlated with decline in tobacco use (3). However, a subgroup of HNSCCs associated with high-risk human papillomavirus (HPV) infection, notably in the oropharynx, is becoming more prevalent (4).

Meta-analysis data suggest that HPV is detectable in 26% to 35% of patients with HNSCC (5). Data from the Surveillance, Epidemiology, and End Results (SEER) program documented an increase in HPV-serotype16 (HPV16) oropharyngeal squamous cell carcinomas from 16% to 72%, particularly in young (<60 years of age) Caucasian males between 1984 and 2004 (6).

High-risk HPV infection leads to genetic instability by impairing the tumor suppressors TP53 and retinoblastoma (Rb) via the E6 and E7 viral oncoproteins, respectively (7). Because of their distinct molecular driver, the progression of HPV⁺ HNSCCs is different from cancers associated with alcohol and tobacco use (8). The rate of mutation in HPV⁺ tumors was half the rate observed in HPV⁻ tumors (9). Although HPV⁺ HNSCC has a more favorable prognosis than HPV⁻
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(10), it can be hypothesized that this dramatically different pathogenesis will require alternative therapeutic approaches. Expression of the HPV16 oncoprotein E7 upregulates Akt activity in human keratinocytes, which is likely a contributing factor to transformation (11, 12), and E7 enhances keratinocyte migration in a PI3K/Akt-dependent manner (13). There is evidence that E6, in addition to labeling p53 for degradation, differentially modulates the phosphoinositide 3-kinase (PI3K) signaling pathway (14), and that E6-driven activation of PI3K/Akt confers resistance to cisplatin in HPV+ cancers (15). Furthermore, HPV+ cases have a higher rate of activating canonical PI3K catalytic α (PIK3CA) mutations (9, 16). The link between PI3K/Akt activation and HPV infection suggests that PI3K inhibition may be an appropriate therapeutic approach for HPV+ HNSCC.

PIK3CA copy number changes have also been documented in HPV− tumors, making PIK3CA relevant in both HPV+ and HPV− HNSCC subtypes (17). PI3K transduces stimuli involved in the regulation of several processes involved in transformation including neovascularization, proliferation, cell motility, adhesion, survival, and apoptosis (18, 19). A direct association between enhanced PI3K/Akt pathway activation and tumor formation within in HNSCC has been identified (17, 20, 21), and dysregulation and/or genetic aberrations of the PIK3CA, Akt, and PTEN have been associated with HNSCC development (22). Targeted therapeutic agents to members of this pathway are currently being evaluated in several cancer types (23).

Direct binding of p53 to the promoter induces transcriptional inhibition of PIK3CA (24). TP53 is the most commonly altered gene in HPV− HNSCCs, with mutations found in 78% of patients not infected by a high-risk HPV subtype (16). It has been well established that mutations within the DNA-binding domain result in a loss-of-function phenotype and correlate with a more advanced tumor stage at diagnosis, a high incidence of lymph node metastasis, and may predict suboptimal patient response to traditional therapeutic treatment regimens (25–27). TP53 status is an important diagnostic consideration, especially in HPV− HNSCCs. Patients infected with HPV have nonfunctioning p53 due to E6-driven destruction (7).

Rigosertib (ON 01910.Na, Estybon) is a non-ATP competitive small-molecule targeted agent that inhibits PI3K/Akt pathway activation and disrupts polo-like kinase 1 (PLK1)–mediated G2–M transition (28, 29). Although it was initially thought that direct inhibition of PLK1 was responsible for the observed antimitotic activity, subsequent studies did not support a direct effect on PLKs (30). Direct inhibition of PI3K has been observed in mantle cell lymphoma (MCL) cell lines treated with rigosertib (31). Inhibition of PI3K signaling was later confirmed in chronic lymphocytic leukemia cells (28). This agent is unique in its ability to impair both cell signaling and mitosis. Rigosertib is currently being evaluated in phase II clinical trials as a single agent for squamous cell carcinomas and hematologic malignancies, and with gemcitabine for pancreatic cancer.

In this study, we aimed to evaluate the efficacy of PI3K inhibition by rigosertib in HNSCC both in vitro and in vivo. Furthermore, we investigated the differential response to treatment in correlation to HPV status, genetic aberrations, and signaling pathway modulation in an effort to identify biologic markers predictive of treatment outcome.

Materials and Methods

Cell lines and in vitro drugs

HNSCC cell lines were commercially acquired and/or obtained from David Sidransky (Johns Hopkins University, Baltimore, MD) and Barbara Frederick (University of Colorado, Aurora, CO); cell lines were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS, 200 μg/mL penicillin, and 200 μg/mL streptomycin. Low serum media (LSM) contained 0.5% FBS. Cell lines were authenticated after receipt by mitochondrial DNA sequencing, and passaged for less than 6 months following authentication. Mycoplasma was tested by using the MycoAlert Mycoplasma Assay (Lonza). All cultures tested negative. ZSTK474 was acquired commercially. The chemical structure of rigosertib has been published previously (32), and the drug was supplied by Onconova Therapeutics, Inc.

In vitro colony formation assay

Cells were seeded at 300 cells per well in 6-well plates and incubated for 24 hours. Media containing either vehicle or 1.0 μmol/L drug was added and plates were incubated for 7 days. Resulting colonies (>50 cells) were fixed with 4% formalin and stained using 0.1% crystal violet.

Sulfurodamine B colorimetric assay

Cells (2,500–5,000) were plated in 96-well plates and incubated overnight. Drug was added and plates were incubated for 96 hours. Cells were fixed with 50 μL of 10% trichloroacetic acid (TCA) at 4°C (30 minutes) and washed five times with distilled water (dH₂O). Next, 70 μL/well sulfurodamine B (SRB) reagent was added, wells were washed five times with 1% acetic acid, 200 μL/well 10 mmol/L Tris base was added, plates were shaken at 40 rpm at room temperature (15 minutes), and absorbance was measured using a Synergy 2 Microplate Reader (BioTek).

Cell-cycle analysis by flow cytometry

Cells (1 × 10⁶) were trypsinized, centrifuged, and resuspended in cold PBS, then fixed by adding cold 100% ethanol drop-wise until reaching a final concentration of 70%, and then incubated for 20 minutes at 4°C. The cells were suspended in propidium iodide buffer (50 μg/mL) in PBS and incubated for 1 hour at room temperature. Samples were analyzed on a CyAn flow cytometer (Beckman Coulter).
Microarray analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Samples were analyzed on the Human Gene 1.0 Array (Affymetrix). Arrays were normalized with the robust multichip average (RMA) algorithm, and probe sets were collapsed to genes by finding the maximum signal for each gene (33). Gene set enrichment analysis (GSEA, v. 2.07) was conducted on the collapsed data using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and BioCarta pathway definitions obtained from the Molecular Signatures Database v. 3.1 (34). All analyses except GSEA were conducted in R/Bioconductor v. 2.11. Relevant pathways with a nominal P value 0.1 or less for the KEGG and 0.05 or less for the BioCarta databases were included in the analysis. The microarray datasets were uploaded to the Gene Expression Omnibus (GEO) repository (accession number: GSE47864).

Human xenograft generation and in vivo studies

Fresh tumor tissue was collected from patients with HNSCC consented at the University of Colorado Hospital (Aurora, CO) in accordance with the protocol approved by the Colorado Multiple Institutional Review Board (COMIRB # 08–0552). Tissue was prepared from 3 mm × 3 mm × 3 mm tumor pieces dipped in Matrigel (BD Biosciences) and inserted subcutaneously into both hind flanks of nude mice. Upon reaching 1,500 mm³, tumors were passed to a second colony of animals for therapeutic studies.

Eight patient cases were treated with rigosertib. For each case, we implanted 20 tumors in 10 mice. When tumors reached 200 mm³, mice were randomly distributed into two groups (at least n = 8 tumors/group) and treated: control or rigosertib, 250 mg/kg administered 5×/week intraperitoneally (i.p.) for 4 weeks. Tumor size was evaluated twice weekly using the formula: volume = (length × width²)/2. Six hours after the last drug administration, tumors were extracted and portions were flash-frozen and embedded in paraffin.

Cancer cell lines were injected orthotopically in the floor of the mouth of athymic nude mice at a concentration of 10⁶ cells/50 μL suspended in 50:50 DMEM to Matrigel (BD Biosciences). Once tumor volumes reached an average of 100 mm³, mice were randomly distributed into two groups (at least n = 7 tumors/group) and evaluated as previously described.

FISH

Slides were incubated at 56°C for 4 hours, soaked in CitriSolv three times for 5 minutes each, dehydrated, and allowed to air dry. The tissue area to be hybridized was marked with a diamond pen. The slides were incubated in pretreatment solution at 80°C for approximately 12 minutes, in protease solution IV at 37°C for approximately 20 minutes, washed in Milli-Q water at room temperature, dehydrated, and air-dried. The 3-color probe mixture was applied to the selected hybridization areas, covered with coverslips, and sealed with rubber cement. DNA codenaturation was conducted at 76°C for 5 minutes in a thermocycler, and hybridization was allowed to occur at 37°C for 40 to 48 hours. Posthybridization washes were conducted through incubations in 2× SSC/0.3% NP-40 at 74°C for 2 minutes and 2× SSC/0.3% NP-40 at 37°C for 20 minutes each, followed by dehydration. Finally, 14 μL of DAPI/anti-fade (0.3 μg/mL in Vectashield mounting medium) was applied to the slide and the area was covered with a 22 mm × 50 mm coverslip. Analysis was conducted on epifluorescence microscopes using single interference filters sets for green [fluorescein isothiocyanate (FITC)], red (Texas red), blue [4',6-diamidino-2-phenylindole (DAPI)], gold, dual (red/green), and triple (blue, red, and green) band pass filters. For each interference filter, monochromatic images were acquired and merged using CytoVision (Leica Microsystems, Inc.).

Human papillomavirus detection

In situ hybridization for HPV low- and high-risk types was conducted using the Ventana INFORM HPV III automated assays (Ventana Medical Systems) on 4-μm paraffin-embedded tissue sections. The Benchmark system uses the Ventana ISH-Protease 3 enzyme to remove proteins surrounding the target DNA. A biotinylated antifluorescin antibody is used to detect the hybridized probe, followed by streptavidin to bind biotin and then a chromagen reaction with nitroblue tetrazolium and 5-Bromo-4-chloro-3-indolyl-phosphate for detection.

Immunohistochemistry

For immunohistochemical staining, slides were deparaffinized and rehydrated in graded concentrations of alcohol by standard techniques before antigen retrieval in citrate buffer pH 6.0 (Dako Cooperation) at 105°C for 20 minutes. Next, slides were cooled for 20 minutes before washing. All staining was done in a Dako Autostainer. Slides were incubated in 3% H₂O₂ for 10 minutes, followed by primary 2211 pS6K, 3787 pAkt, 9559 PTEN, or 4376 pMAPK (Cell Signaling Technology), and incubated for 60 minutes at room temperature. Staining was developed by EnVision+ Dual Link System-HRP (Dako) for 30 minutes and substrate-chromogen [3,3'-diaminobenzidine−positive (DAB⁺)] solution (Dako) for 7 minutes. Slides were then counterstained with automated hematoxylin (Dako) for 5 minutes. The intensity (0, 1+, 2+, and 3+) and the percentage (0%–100%) of cells positive were interpreted blinded and in triplicate to the case and treatment. Reported immunohistochemical scores were calculated by multiplying the population% by the intensity (0, 1, 2, and 3) then averaging the values for the triplicate reads for each sample.

DNA sequencing

PCR amplification reactions were carried out in 96-well ABI Veriti thermocycler (Applied Biosystems) using a touchdown PCR protocol as described previously (35).
DNA sample quality and concentrations were assessed by gel electrophoresis. PCR amplification and Sanger sequencing were conducted using primer sets referenced in Agrawal and colleagues (16). PCR product was directly sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems). The standard sequencing thermo-cycling parameters were as follows: denaturation for 5 minutes at 94°C, followed by 30 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension/termination at 60°C for 4 minutes, followed by incubation at 10°C until the samples were processed. Residual dye-labeled dideoxynucleotides (dye-terminators) were removed from the cycle-sequencing reaction products using the paramagnetic bead technology CleanSEQ from Agencourt Bioscience Corporation or PureSeq from Aline Biosciences, and a modification of the manufacturers’ recommended protocol. The products were sequenced on a fluorescent capillary automated sequencer—an Applied Biosystems/Hitachi 3730 Genetic Analyzer with a 50 cm long, 48-capillary array containing POP7 polymer. Analyses of DNA sequences were conducted with Sequencing Analysis version 5.2 and Sequence Scanner version 1.0 (both from Applied Biosystems). Alignments of DNA sequences were done with Sequencher 4.8 (Gene Codes Corporation) and/or Seqcape (Applied Biosystems). PCR product sequence comparison with GenBank reference sequence and mutation identification was accomplished using Mutation Surveyor version 4.0.4 (SoftGenetics).

**SNaPshot assay**

The Colorado Molecular Correlates (CMOCO) SNaPshot panel tests for 68 discrete mutational loci, 8 of which are validated for treatment guidance in non–small cell lung carcinoma. Briefly, after nucleic acid is extracted, target regions of the 15 tested genes are PCR amplified with multiplex primer sets. After phosphatases and exonuclease treatment to remove unincorporated dNTs and primers, the PCR products are then used as a template for labeled single base extension using matched probe sets. The resulting SNaPshot products are analyzed with capillary electrophoresis and resultant data analyzed for the presence of mutations. Determination of the presence or absence of mutation is carried out by manual evaluation of the data examined through GeneMapper software.

**Western blotting**

Cell pellets were lysed in 30 to 100 μL radioimmunoprecipitation assay (RIPA) lysis buffer containing 5 μL/mL phenylmethylsulfonylfluoride (PMSF). Tissue sample (50 mg) portions were thawed in a 4× volume of RIPA buffer and homogenized using single-use plastic pestles. Protein was measured using the ELx800 absorbance microplate reader (BioTek) according to the manufacturer’s instructions. Thirty nanogram of protein was loaded per well into NuPage Novex 4% to 12% Bis–Tris Midi Gel (Invitrogen), transferred using the iBlot Gel Transfer Stack System (Invitrogen), and then processed. Primary antibodies purchased from Cell Signaling Technology were: 4060 pAkt, 4821 Akt, 4094 pMAPK, 9102 MAPK, 2215 pS6RP, 2217 S6RP, and 4968 pan-actin. Antibody specificity as determined by an isotype control was completed by the manufacturer. Secondary anti-rabbit immunoglobulin G (IgG; Jackson Immunoresearch) was used at a 1:50,000 dilution. The signal was visualized using Immobilon Western chemiluminescent horse-radish peroxidase (HRP) substrate (Millipore).

**Results**

**Rigosertib induces a potent antiproliferative effect in HNSCC cell lines**

We investigated rigosertib activity in a panel of 16 immortalized HNSCC cell lines. Eleven of 16 cell lines showed potent antiproliferative activity at sub-micromolar concentrations (Fig. 1A). Three cell lines had a moderate response to treatment, and proliferation of 2 cell lines (MSK921 and UMSCC19) was unaffected at concentrations up to 10 μmol/L. All HNSCC cell lines tested HPV−.

We chose two sensitive (584 and HN11) and two resistant (MSK921 and UMSCC19) cell lines for further investigation of changes in gene expression and PI3K pathway activation. Treatment sensitivity for these cell lines was confirmed by clonogenic assay (Fig. 1B). Sensitivity to treatment at 1.0 μmol/L was comparable with the SRB assay results.

**Differential gene set enrichment in the rigosertib-sensitive versus -resistant cell lines**

We then sought to correlate global gene expression to rigosertib sensitivity (<15% or ≥45% proliferation after 1.0 μmol/L rigosertib treatment, respectively) using the Affymetrix microarray platform. GSEA was used to identify pathways enriched in the rigosertib-sensitive and -resistant cohorts. Several cancer-related KEGG gene sets were enriched in the more resistant cohort. KEGG gene sets enhanced in resistant cell lines with a false discovery rate (FDR, q val) of less than 25% included pancreatic cancer and DNA replication (Table 1). BioCarta pathways involved in endocytosis were also enriched. This is particularly interesting because of the overlap between differential phosphorylation patterns of phosphoinositides in both the regulation of endocytic trafficking and their involvement in the PI3K signal transduction pathway (36).

The hedgehog, mitogen-activated protein kinase (MAPK), calcium, and gap junction signaling pathway gene sets, all of which include members of key pathways associated with HNSCC development, progression, and maintenance, were enriched in the sensitive cell lines in the KEGG dataset (Supplementary Table S1; ref. 4). Interestingly, the two pathways enriched in the BioCarta dataset involve antiapoptotic pathway activation through the PI3K/Akt, MAPK, and Src pathways. We then
extracted core genes for PI3K-related pathways within the top 50 pathways identified by GSEA for both datasets. Using heatmaps, we compared sensitive and resistant cell lines revealing enrichment in this gene set in the sensitive cell lines (Supplementary Fig. S1C).

**Antiproliferative effects of rigosertib result from an inhibition of more than one pathway in HNSCC cell lines**

We then investigated the mutation status of our chosen four cell lines by full exon sequencing of PIK3CA and TP53, and by the multiplexed PCR-based SNaPshot assay, a technology capable of simultaneously identifying mutational status at 68 hot spot loci (37). Poly Phen-2 analysis was used to interpret the effect of identified amino acid substitutions (38). The resistant cell line UMSCC19 harbored a H1047R-activating mutation in PIK3CA exon 20 (Table 2). This mutation has been cited as oncogenic in ovarian and breast cancers (39). Two heterozygous mutations were documented in the DNA-binding region of TP53 in the resistant cell line MSK921, both predicted as damaging by Poly Phen-2 analysis.

![Graphs and images corresponding to the text](image-url)
These mutations likely impair DNA binding by p53. Broad genotyping by SNaPshot confirmed the H1047R wild-type. PIK3CA-activating mutations did not correlate with a rigosertib treatment-sensitive phenotype.

We extended our full-exon sequencing and SNaPshot platforms to characterize the mutational status of the full panel of HNSCC cell lines (Supplementary Table S2). Eight of 16 cell lines harbored inactivating mutations in TP53. There was no noticeable trend between TP53 mutation status and treatment sensitivity. Interestingly, Detroit 562 harbored a mutation in both genes and was sensitive to treatment with rigosertib.

Phosphorylation patterns of Akt and S6 following rigosertib treatment were not uniformly observed across our panel of HNSCC cell lines (Fig. 1C). The phosphorylation of Akt at Ser473 in HN11 and MSK921 was reduced with treatment, and a corresponding reduction of S6 phosphorylation was observed. However, there was no decrease in phosphorylation in 584 or UMSCC19. Conversely, there was complete inhibition of Akt and S6 phosphorylation following treatment with ZSTK474, which binds directly to the PI3K ATP-binding site, in all four cell lines. Furthermore, the antiproliferative profile of ZSTK474 in these cell lines was very different than that of rigosertib. Our results confirm rigosertib activity in the sensitive HNSCC cell lines using an orthotopic xenograft platform. Cell lines were injected into the floor of the mouth of the mice to better replicate the tumors origin microenvironment (Fig. 2A). After 28 days of treatment, tumor regression and growth reduction were observed in rigosertib-treated 584 and HN11 implanted mice, respectively, compared with the untreated control group. We used FISH to identify if there was gene copy number gain for PIK3CA in these tumors (Fig. 2B and Supplementary Table S3). 584 showed no copy number gain with a representative FISH score of 2. HN11 showed a low copy number gain with a representative FISH score of 3. Western blot analysis of treated tissue did not confer PI3K/Akt pathway inhibition in the xenografted cell lines (Fig. 2C). These observations were confirmed by immunohistochemistry (IHC; Fig. 2D; Supplementary Fig. S4A). This contrasts with the observed Akt and S6 phosphorylation patterns in HN11, although modest, in vitro. Our results confirm rigosertib sensitivity profiles in these cell lines in a xenograft model, and further suggest that rigosertib’s antiproliferative properties do not result solely from an inhibition of the PI3K pathway. Previous reports have documented G2–M arrest and alteration in the mitotic pathway and

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Abbreviations: PI, phosphoinositide; ETC, electron transport chain.

Rigosertib sensitivity confirmed in HNSCC cell lines implanted orthotopically in athymic nude mice

We confirmed rigosertib activity in the sensitive HNSCC cell lines using an orthotopic xenograft platform. Cell lines were injected into the floor of the mouth of the mice to better replicate the tumors origin microenvironment (Fig. 2A). After 28 days of treatment, tumor regression and growth reduction were observed in rigosertib-treated 584 and HN11 implanted mice, respectively, compared with the untreated control group. We used FISH to identify if there was gene copy number gain for PIK3CA in these tumors (Fig. 2B and Supplementary Table S3). 584 showed no copy number gain with a representative FISH score of 2. HN11 showed a low copy number gain with a representative FISH score of 3. Western blot analysis of treated tissue did not confer PI3K/Akt pathway inhibition in the xenografted cell lines (Fig. 2C). These observations were confirmed by immunohistochemistry (IHC; Fig. 2D; Supplementary Fig. S4A). This contrasts with the observed Akt and S6 phosphorylation patterns in HN11, although modest, in vitro. Our results confirm rigosertib sensitivity profiles in these cell lines in a xenograft model, and further suggest that rigosertib’s antiproliferative properties do not result solely from an inhibition of the PI3K pathway. Previous reports have documented G2–M arrest and alteration in the mitotic pathway and

Table 1. GSEA pathways enriched in resistant cell lines

These mutations likely impair DNA binding by p53. Broad genotyping by SNaPshot confirmed the H1047R substitution in UMSCC19, but identified no other mutations. Both the sensitive cell lines were
centrosomal changes as key parts of anticancer effect of rigosertib (40). Thus multiple pathway inhibition is involved in the antiproliferative and biochemical effects of rigosertib.

**Rigosertib is active in direct patient xenografted tumor lines**

We treated eight cohorts of HNSCC direct patient tumor xenografts implanted in immunocompromised mice with rigosertib. Half of the chosen tumor lines were positive for HPV16 infection: CUHN014, CUHN022, CUHN043, and CUHN047. Tumor measurements normalized to the average volume of the control group taken on day 28 from each study are shown in Fig. 3A and Supplementary Fig. S5. Growth reduction more than 50% with rigosertib therapy was observed in CUHN014, CUHN047, and CUHN026. No significant response was observed in the remaining five tumor lines. The two lines most sensitive to treatment, CUHN014 and CUHN047, were HPV⁺. However, two resistant lines, CUHN043 and CUHN022, were also HPV⁺.

We explored whether amplification of *PIK3CA* may yield a more sensitive phenotype to rigosertib treatment. A high prevalence of gene copy number gain abnormalities exists in HNSCC (17). Three of eight tumor lines scored positive for *PIK3CA* gene amplification; a representative FISH score of 5 (Fig. 3B and Supplementary Table S4). Two of them, CUHN014 and CUHN047, were sensitive to treatment with rigosertib. The resistant tumor lines CUHN025, CUHN043, and CUHN022 had either no or low copy gain. High copy number gain was identified in CUHN026 and CUHN040.

Western blot analysis of PI3K pathway components yielded inconsistent results (Fig. 3C and Supplementary Fig. S6). There was no emerging trend observed after staining for phosphorylated Akt or its negative regulator PTEN (Fig. 3D). Reduction in phosphorylation levels of S6 ribosomal protein were observed in the Western blot analysis staining for CUHN047 and CUHN026, whereas CUHN014 did not have a significant change when compared with total S6 staining. Reduction of phosphorylation of S6 ribosomal protein was observed in all three sensitive lines by IHC, although not to a significant level in CUHN014. Reduced phosphorylation was also observed in the resistant line CUHN013. The Western blot analysis trends observed for the other members of these pathways were confirmed by IHC (Fig. 3D and Supplementary Fig. S7).

**A combination of *PIK3CA* and *TP53* genetic events correlate with a rigosertib-sensitive phenotype**

Exon sequencing of *PIK3CA* and *TP53* was completed for the treated with rigosertib (Table 3). A heterozygous E545K substitution in *PIK3CA* was identified in CUHN026 xenograft. This variant has been cataloged as an activating mutation located within the helical domain (41). Growth of CUHN026 was reduced with rigosertib treatment, consistent with our hypothesis that
Rigosertib treatment will have a more potent effect in the presence of PI3K pathway activation. TP53 somatic mutations were identified in three tumor lines. These mutations are located within the DNA-binding domain, which suggests they are inactivating. Analysis using the PolyPhen-2 program scored all mutations as probably damaging.

Interestingly, all the responsive tumors lines have a PIK3CA genetic event, be it amplification or activating mutation, and are either HPV16-positive or harbor an inactivating TP53 mutation. CUHN013 was not susceptible to rigosertib despite harboring PIK3CA amplification and TP53 mutation. However, CUHN013 is highly EGF receptor (EGFR)-dependent (42), which may explain resistance to a targeting agent that does not directly inhibit EGFR pathway activation. In addition, factors other than these two may influence susceptibility to rigosertib. Although the PI3K pathway may not be the primary target of rigosertib in HNSCC, our data suggest that the combination of a PIK3CA-activating event with a TP53-inactivating event is necessary, but not sufficient, for sensitivity to rigosertib treatment in HNSCC xenografts.

Discussion

Dysregulation of the PI3K pathway in HNSCCs is frequent, and contributes to amplified growth advantage, increased metastatic potential, and resistance to therapeutic regimen. In accordance with published data, our laboratory has identified genomic alterations of PI3K occurring in 20% of our tumor lines generally, and in 40% of HPV+ tumors (16, 43). Furthermore, several studies have evaluated the predictive correlation of common PI3K aberrations and drug-specific treatment outcome (44–46). The established importance of PI3K, as not only a therapeutic target but also a predictive biomarker, confirms the relevance of a multifaceted focus on this pathway in the field of head and neck oncology.

Inhibition of the PI3K/Akt signaling pathway in MCL cell lines and chronic leukemia cell lines, preliminary treatment response data in solid tumors, and clinical relevance supports evaluating rigosertib as a treatment of HNSCC (31, 47, 48). We observed potent antiproliferative activity by rigosertib in our panel of HNSCC cell lines. Only 2 cell lines were resistant to treatment with an IC50 > 10.0 µmol/L. Rigosertib completely inhibited colony formation at 10.0 µmol/L in both sensitive and resistant cell lines. There was no correlation with treatment response in our panel of 17 HNSCC cell lines and mutation profile of either TP53 or PIK3CA alone. However, consistent with the predictive biomarker combination, we identified in our tumor lines, rigosertib-sensitive Detroit 562 harbored an inactivating TP53 mutation in combination with an activating PIK3CA mutation.

Treatment sensitivity of HN11 and 584 was confirmed by in vivo treatment studies when orthotopically xenografted. Regression and significant growth reduction were observed in 584 and HN11 engrafted tumors, respectively. Rigosertib treatment also induced growth
reduction in 3 of 8 HNSCC patient tumor lines. Efficacy both in vitro and in vivo substantiates rigosertib as a promising targeted agent for clinical evaluation in HNSCC.

Several PI3K-related gene sets were enriched in the pathways identified by GSEA in the KEGG and BioCarta databases derived from in vitro microarray results. However, PI3K pathway modulation was inconsistent for HNSCCs both in vitro and in vivo. No general inhibitory trend was observed for the phosphorylation of Akt at serine residue 473, or of S6 ribosomal protein at serine residues 240/244, events indicative of PI3K pathway activation. Cell-cycle analysis of HNSCC cell lines treated with rigosertib yielded results more representative of those expected from a cytotoxic drug, a complete G2 block in sensitive lines, than to those obtained with the PI3K-targeting agent ZSTK474 (49). These studies confirm earlier observations indicating that multiple mechanisms are involved in the activity of rigosertib, including effects on signal transduction and mitotic pathways. Such complexity of action likely explains the breadth of activity observed in vitro. Depending on the cell-type and genetic background, inhibition of one pathway may dominate over another.

Merit to the relationship between rigosertib treatment and the PI3K pathway is conferred by the prognostic value of PI3K aberrations. Interactions between TP53 and the PI3K/Akt pathway have been shown to play a significant role in apoptosis and cell survival (50). It has further been documented that amplified PI3K pathway activation correlates with HPV-positivity in HNSCCs due to the loss of attenuation at the PIK3CA promoter (51). Here, we have observed a correlation between rigosertib sensitivity and the coexistence of both a PIK3CA activating event, be it mutation or amplification, and either a deleterious TP53 mutation or

Figure 3. A, mice bearing patient-derived tumors received daily rigosertib infusions or vehicle for 28 days. An asterisk denotes HPV16 positivity. Volumes on the final day of treatment are represented as percentage normalized to the vehicle control group. Growth reduction was identified in CUHN014, CUHN047, and CUHN026. B, gene amplification was characterized by a FISH score above 4 as exemplified by CUHN013. C, Western blot analysis of tissue taken at 28 days treatment shows inconsistent inhibition of the PI3K pathway in the vehicle control- (C) and rigosertib (R)-treated tissue. Inhibition of S6 phosphorylation is observed in CUHN047, CUHN026, and CUHN013. D, immunohistochemical scores plotted for each tumor line from the control and rigosertib-treated tissue. The graphical representations show a correlation; all sensitive lines have a decrease in S6 phosphorylation in the treated tissue. This is also true for CUHN013.
p53 inactivation by HPV16 infection. For example, UMSCC19 harbors a PIK3CA mutation but lacks TP53 inactivation, and is resistant to rigosertib. However, CUHN013 has PIK3CA amplification and is a TP53 mutant but is rigosertib-resistant. Decreased phosphorylation of S6 after treatment with rigosertib was also observed, suggesting that the PI3K pathway is inhibited in this tumor line. However, xenograft treatment studies using the EGFR-targeting agent cetuximab induced remission in a 28-day animal treatment study in CUHN013 (42). This is evidence of EGFR-dependence, and provides a possible mechanism of resistance to rigosertib treatment.

Our data suggest that the combination of a PIK3CA-activating event and p53-inactivating event is necessary, but not sufficient, for a rigosertib-sensitive phenotype. Similarly, cooperation between PIK3CAH1047R and p53 loss-of-function mutations was identified in mouse mammary tumor formation (52). The double-mutant mice formed a spectrum of tumors unique from those of the PIK3CAH1047R single-mutant strain. This indicates that the combination of these events results in a distinct mechanism of PI3K pathway activation, which may explain differential rigosertib treatment response.

A phase I study of oral rigosertib treatment in patients with advanced solid tumors recently concluded at the University of Colorado School of Medicine, and rigosertib showed efficacy in 2 of 8 HNSCC patients treated (48). One of the responsive patients harbored an inactivating TP53 mutation in combination with PIK3CA gene amplification. The second responder was HPV+ and harbored a PTEN loss, highlighting the presence of abnormalities in TP53 function and PI3K/Akt pathway in susceptible cases. The rate of response in patients with HNSCC was very similar in this subset with 8 HNSCC subjects and in the HNSCC xenograft set of 8 cases. However, given the relatively small number of HNSCC cases presented here and the small number of patients in the phase I study, these data are hypothesis-generating. A multicenter phase II study of oral rigosertib treatment in relapsed or metastatic squamous cell carcinoma (SCC) is underway, includes comprehensive genomic testing (SNaPShot and DNAseq in all patients), and will have the statistical power to fully examine this hypothesis.

### Disclosure of Potential Conflicts of Interest

D.L. Aisner has commercial research grant from Onconova Therapeutics, Inc. F. Wilhem has ownership interest (including patents) in Onconova Therapeutics, Inc. A. Jimeno has a commercial research grant, from, has and ownership interest (including patents) in, and is a consultant/advisory board member for Onconova Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

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