Development, Characterization, and Reversal of Acquired Resistance to the MEK1 Inhibitor Selumetinib (AZD6244) in an In Vivo Model of Childhood Astrocytoma

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Abstract

Purpose: The BT-40 low-grade childhood astrocytoma xenograft model expresses mutated BRAFV600E and is highly sensitive to the MEK inhibitor selumetinib (AZD6244). In this study, we developed and characterized selumetinib resistance and explored approaches to circumventing the mechanisms of acquired resistance.

Experimental Design: BT-40 xenografts were selected in vivo for selumetinib resistance. Resistant tumors were obtained and characterized, as were tumors that reverted to sensitivity. Characterization included expression profiling, assessment of MEK signature and compensatory pathways, MEK inhibition, BRAF expression, and cytokine levels. Combination treatment of BT-40/AZD-resistant tumors with the MEK inhibitor and a STAT3 inhibitor (LLL12) was assessed.

Results: Resistance was unstable, tumors reverting to selumetinib sensitivity when passaged in untreated mice, and MEK was equally inhibited in sensitive and resistant tumors by selumetinib. Drug resistance was associated with an enhanced MEK signature and increased interleukin (IL)-6 and IL-8 expression. Selumetinib treatment induced phosphorylation of STAT3 (Y705) only in resistant xenografts, and similar results were observed in BRAFV600E astrocytic cell lines intrinsically resistant to selumetinib. Treatment of BT-40-resistant tumors with selumetinib or LLL12 had no significant effect, whereas combined treatment induced complete regressions of BT-40/AZD-resistant xenografts.

Conclusions: Resistance to selumetinib selected in vivo in BT-40 tumor xenografts was unstable. In resistant tumors, selumetinib activated STAT3, and combined treatment with selumetinib and LLL12 induced complete responses in resistant BT-40 tumors. These results suggest dual targeting BRAF (V600E) signaling and STAT3 signaling may be effective in selumetinib-resistant tumors or may retard or prevent onset of resistance.

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Introduction

Astrocytomas are the most common tumors of the central nervous system in children and are subdivided according to histologic subtypes, grades 1–4 (1). The majority of astrocytomas are grade 1 and 2 tumors, yet the biologic behavior of low-grade astrocytomas reflect a heterogeneous spectrum. Radical surgical resection is the standard therapy; however, whereas most cerebellar pilocytic astrocytomas (WHO I) do not frequently involve the diencephalon, those that do are not resectable due to the tumor’s involvement in the diencephalon and surrounding eloquent structures. Thus, adjuvant therapy is warranted, which includes chemotherapy and/or radiation therapy. The 5-year progression-free survival rate for chemotherapy plus radiotherapy has been reported as 68%, which is superior to chemotherapy alone 38% (2). However, significant morbidity is associated with the presence of residual tumor and the current therapy that includes neuroendocrine cognitive deficits, visual deficits, vasculopathy, and secondary tumors (3, 4). Moreover, the metastatic potential and transformation to a high-grade astrocytoma further contributes to the poor prognosis (5, 6). In recent years, there have been considerable advances in defining subsets of pediatric tumors by...
Cell lines harboring BRAFV600E may be highly sensitive to the MEK inhibitor selumetinib. We have developed resistance to selumetinib in the xenograft model and have characterized the mechanism for resistance. Specifically, as MEK is inhibited, there is a compensatory activation of STAT3 signaling mediated by increased interleukin (IL)-6 expression and activation of NF-kB. Combined therapy targeting both MEK and STAT3, but not either single pathway, resulted in complete regression of selumetinib-resistant tumors. Our results suggest that STAT3 activation may account for both acquired and intrinsic resistance to selumetinib in BRAF-mutant astrocytoma and suggests a novel therapeutic approach to therapy of these brain tumors.

**Translational Relevance**

Activation of the BRAF oncogene occurs frequently in childhood low-grade astrocytomas. Our studies show that a patient-derived xenograft model that expresses the V600E mutant is highly sensitive to the MEK inhibitor selumetinib. We have developed resistance to selumetinib in the xenograft model and have characterized the mechanism for resistance. Specifically, as MEK is inhibited, there is a compensatory activation of STAT3 signaling mediated by increased interleukin (IL)-6 expression and activation of NF-kB. Combined therapy targeting both MEK and STAT3, but not either single pathway, resulted in complete regression of selumetinib-resistant tumors. Our results suggest that STAT3 activation may account for both acquired and intrinsic resistance to selumetinib in BRAF-mutant astrocytoma and suggests a novel therapeutic approach to therapy of these brain tumors.
from R&D systems (Cat no ARY005). Primary antibodies to β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphorylated STAT3 (Y705), STAT3, phosphorlated ERK, and extracellular signal–regulated kinase (ERK) were purchased from Cell Signaling Technology and Ki67 antibody was purchased from DAKO.

Selumetinib was provided by AstraZeneca. Selumetinib was dissolved in 0.5% hydroxypropyl methyl cellulose, 0.1% Polysorbate 80 and administered per os, using a twice daily schedule [excepting weekends, for which a once daily (SID) schedule was used] for a scheduled 6 or 12 weeks at a dose of 75 mg/kg.

LLL12 was synthesized in the Laboratory of Dr. Pui-Kai Li (College of Pharmacy, The Ohio State University, Columbus, OH). The powder was dissolved in sterile dimethyl sulfoxide (DMSO) to make a 5 mg/mL stock solution. Aliquots of the stock solution were stored at −20°C. LLL12 was administered by intraperitoneal (i.p.) injection at 5 mg/kg daily, for 6 weeks.

**Cell culture**

AM38c1 and DBTRG-05MG glioblastoma cell lines (generously provided by T. Nicolaides, UCSF) were maintained in antibiotic-free RPMI (Invitrogen) in high glucose supplemented medium with 10% FBS and 2 mmol/L glutamine at 37°C with 5% CO₂. All cells were maintained as subconfluent cultures and split 1:3, 24 hours before use. Experiments were carried out within 10 passages of receiving the cells.

**Genomic DNA extraction and sequencing**

Genomic DNA was extracted using a Puregene DNA Isolation Kit (Gentra Systems) and quantified spectrophotometrically. The genomic DNA from BT-40 xenografts and cell lines AM38c1 and DBTRG-05MG were screened for BRAF mutations with primers designed to amplify the exons 1 to 18 using primers described previously (24). Big Dye Terminator Chemistry was used for sequencing.

**RNA isolation and quantitative reverse transcriptase PCR**

Total RNA was extracted from three independent tumors per condition (parental, resistant, or revertant) before treatment, or at 72 hours of AZD6244 treatment, using TRizol (Life Technologies)/chloroform/ethanol precipitation, as per the manufacturer’s instructions. Before cDNA synthesis, 5 µg of total RNA per condition was treated with 2.5U of DNase I (Life Technologies) to remove any contaminating genomic DNA. The reverse transcription reaction contained 2.5 µg of pure total RNA, 500 ng of anchored oligo(dT) primer (Life Technologies), as well as 25 ng of random hexamer primers (Life Technologies) to reverse transcribe the control 18S ribosomal RNA. The resulting cDNA was purified using QIAquick PCR cleanup columns (Qiagen) and quantified by spectrophotometry at 260 nm. Each real-time PCR reaction contained 75 ng of the purified cDNA brought up to a final volume of 9 µL. To this, 10 µL of the TaqMan master mix was added, along with 1 µL of the transcript specific or 18S TaqMan probe. All aliquot pipetting was conducted on a Biomek 3000 (Beckman Coulter) to ensure accuracy. Quantitative reverse transcriptase (RT-PCR) amplification was conducted on an ABI 7300 Real Time PCR System, under the following conditions: 2 minutes at 50°C (uracil DNA glycosylase activation), 10 minutes at 95°C, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative gene expression was calculated using the ΔΔCt method, with each Ct value for the probes set relative to the internal 18S control Ct value. The ΔCt values were then compared between the conditions.

**Gene expression analysis by microarray**

Total RNA was prepared from snap-frozen subcutaneous BT-40 tumor xenografts using the RNeasy Kit (Qiagen). Gene expression analysis was done in the Medical Genomics Core Laboratory, Nationwide Children’s Hospital (NCH), using the Affymetrix HG-U133Plus2 GeneChip (54,613 probe sets). RNA quality was confirmed by UV spectrophotometry and by analysis on the Agilent 2100 Bioanalyzer. Processing of RNA samples was done according to the Affymetrix gene expression protocol. Expression signals were calculated using the MAS5 statistical algorithm within the Affymetrix GCOS software (version 1.4). Signal values were scaled using the global normalization method with the 2% trimmed mean set to 500. Detection calls for each transcript (absent, marginal, or present) were determined using the default variables within the GCOS software.

**Gene set enrichment analysis**

Four BT-40 tumor replicates (4 per condition), each from independent mice, were measured using the Agilent Sureprint G3 version 1 human gene-expression array. The biologic replicates were used to measure differences between conditions given xenograft biologic variability. Gene set enrichment analysis (GSEA; ref. 39) was applied to measure the overall activity of an MEK activity and compensatory signature. To gauge significance of enrichment, we used GSEA v2.08 desktop application (39, 40) and applied default algorithm settings, which included 1,000 gene set permutations, condition difference by signal-to-noise, and a weighted enrichment statistic.

**Western blotting**

Cell lysis, protein extraction, and immunoblotting were as described previously (41). Immunoreactive bands were visualized by using Super Signal Chemiluminiscence substrate (Pierce) and Biomax MR and XAR film (Eastman Kodak Co.). Fifteen microliters of total sample was resolved for 1% to 12% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, and immunodetection was conducted with specific primary antibodies.

**Cell viability/proliferation assay**

AM38c1 and DBTRG-05MG cells were seeded on 6-well plates at a density of approximately 1 × 10⁴ cells per well in
their respective media. Cells were treated with 10 nmol/L to 10 μmol/L AZD2644 or LLI12 (100 nmol/L to 5 μmol/L) to determine IC50 concentrations. For combination studies, cells were exposed to 10 μmol/L selumetinib combined with 2 μmol/L LLI12 one day after seeding. After 2 days, Alamar Blue (AB) was added directly into culture media at a final concentration of 10% and the plates were incubated at 37°C. Optical density was measured spectrophotometrically at 540 and 630 nm within 3 to 4 hours after adding dye. As a negative control, AB was added to medium without cells.

**In vivo tumor growth inhibition studies**

CB17SC-M scid−/− female mice (Taconic Farms) were used to propagate subcutaneously implanted BT-40 tumors. All mice were maintained under barrier conditions and experiments were carried out using protocols and conditions approved by the institutional animal care and use committee of NCH. Mice were randomized into groups of 10 when tumors were 100 to 200 mm3. Tumor volumes (cm³) were determined as previously described (42).

**Ethics statement**

All animal experiments were carried out in accordance with institutional animal care and use committee of The Research Institute at NCH-approved protocols, designed to minimize the numbers of mice used and to minimize any pain or distress. The named institutional review board or ethics committee specifically approved this study.

**Human cytokine array**

Proteome profiler antibody array (R&D Systems; Cat no ARY005) was used according to manufacturer’s instructions to detect the relative levels of expression of 36 cytokines, chemokines and acute phase proteins in a single sample in control and treated tumors. After blocking, the membranes 300 μg of protein from the tumor lysate from control and selumetinib-treated BT-40 (drug-sensitive), BT-40/AZD (drug-resistant), and BT-40/REV (drug-revertant) groups were added and incubated for overnight at 4°C. After 16 to 18 hours, the membranes were washed and streptavidin/ horseradish peroxidase (HRP) was added or 30 minutes. Immunoreactive signals were visualized by using Super Signal Chemiluminescence substrate (Pierce) and Biomax MR and XAR film (Eastman Kodak Co.). Array data on developed X-ray film were quantified by scanning the film using Biorad Molecular Image Gel Doc XR+ and data using were analyzed using Image Lab software.

**Immunohistochemistry**

For immunohistochemistry, formalin-fixed tumors were sectioned at 5 μm and then dewaxed and soaked in alcohol. After microwave treatment in antigen unmasking solution (Vector Laboratory) for 10 minutes, endogenous peroxidase activity was inactivated by incubating in 3% hydrogen peroxide (H2O2) for 15 minutes, and sections were incubated with primary antibody in PBS at 4°C overnight. After washing with PBS, immunostaining was conducted using the Vectastain Universal Quick Kit and DAB Peroxidase Substrate Kit (Vector Laboratories) according to the manufacturer’s instructions. Antiserum was omitted in the negative control. The number of cells staining positive was counted by a blinded observer in 5 random 40× fields and treated versus controls compared (Student t test). Images were obtained with an Olympus AX70 fluorescence microscope and Spot v2.2.2 (Diagnostic Instruments) digital imaging system. Proliferation was measured by Ki67 staining. Apoptosis was detected in deparaffinized tumor sections by TUNEL assay using the In Situ Cell Death Detection Kit (Gene Script USA Inc.) according to manufacturer’s instructions.

**Detection of mutant BRAFV600E in tumor tissues**

The VE1 antibody that can differentiate between wild-type and mutant BRAF (43) was used to determine expression of mutant BRAF. Total BRAF (wild-type plus mutant) was detected using rat monoclonal antibody pBR1 (43). The expression levels of mutant BRAFV600E and total BRAF were assessed in parental BT-40, in BT-40/AZD derivatives (A1 and B4) lines, and BT-40/REV revertant tumor models. FFPE tissues from each individual xenograft tumor were processed as previously described (43). Paraffin blocks or slides were coded and scored “blinded.”

**Statistical analysis**

Data are presented as mean ± SE. Differences were analyzed with the Student t test, and significance was set at P < 0.05. Unpaired t tests were used for the continuous variables comparisons, such as expression level differences shown in Fig. 2D. For in vivo testing against pediatric glioma xenograft models, criteria for defining an event (4 times the tumor volume at the start of treatment) were similar to that used by the PPTP (20). Log-rank test was used to compare the time-to-event curves between groups and Holm’s method was used to adjust for multiplicity within each xenograft model. SAS 9.3 was used for this analysis (SAS, Inc.).

**Results**

**Development of resistance in vivo**

The BT-40 astrocytoma was derived from a tumor diagnosed as a juvenile pilocytic astrocytoma (JPA; ref. 29). As a xenograft, Ki67 staining showed a proliferating fraction that was high for a JPA with Ki67 index of 16.8 ± 7.2, 16.2 ± 7.1, and 4.26 ± 6.1 on passages 5, 10, and 20, respectively. In addition, BT-40 is homozygous for mutant BRAFV600E, suggesting that it is a low-grade astrocytoma rather than a JPA. Our initial screen of selumetinib against the solid tumor and ALL panels within the PPTP showed minimal single-agent activity despite clear evidence of MEK inhibition (42). In contrast, BT-40 astrocytoma xenografts that harbor the BRAFV600E mutation were highly sensitive to treatment with selumetinib, completely regressing during the period of treatment (6 weeks). However, although treatment of BT-40 xenografts induced complete tumor regression, tumors regrew after discontinuing therapy (Supplementary Fig. S1). Two tumors designated A5 and A6...
B4 were selected for further transplant and retreatment. As shown in Fig. 1A, A5 tumors were essentially resistant to selumetinib treatment on passage 2, whereas B4 tumors showed some degree of response but then progressed during selumetinib treatment (Fig. 1B). Tumor line A5/A2 was further transplanted in selumetinib-treated mice. In addition, A5/A2 was transplanted and maintained in untreated mice for 3 passages, then retested for sensitivity to selumetinib. As shown in Fig. 1C, after 3 passages in untreated mice, the A5/A2 tumor was somewhat sensitive to treatment with selumetinib, whereas by the fifth passage, it had regained full sensitivity to selumetinib treatment (Fig. 1D). BT-40 parental, the A5/A2-resistant line (designated BT-40/AZD) and the revertant line from passage 5 in untreated mice (BT-40/REV) were further characterized.

**MEK inhibition in BT-40 parental and BT-40/AZD-resistant tumors**

Several studies have suggested that the BRAF/MEK pathway becomes reactivated in cell lines selected for resistance to BRAF and MEK inhibitors (31, 33–35). We examined inhibition of MEK by selumetinib in BT-40 (Fig. 2A) and BT-40/AZD (Fig. 2B) xenografts. Inhibition of MEK signaling resulted in significant hyperphosphorylation of MEK in both sensitive and resistant tumor models, whereas phospho-ERK1/2 was markedly inhibited in both tumor lines by selumetinib treatment. Quantitation of phospho-MEK and phospho-ERK2 is shown for BT-40 xenografts in Fig. 2C. These data are consistent with reduced ERK-mediated negative feedback on RAS or upstream RTKs (44). Phospho-ERK1/2 was also significantly reduced by selumetinib treatment; however, in resistant tumors, there was a statistically significant increase in phosphorylation of ERK1 compared with that in BT-40 xenografts after treatment (Fig. 2D), although there was no substantial difference in the level of inhibition of the MEK/ERK pathway that could account for resistance to selumetinib.

**Mutant BRAF expression in BT-40 and derived sublines**

BT-40 and derivative tumor lines are heterozygous for mutant BRAFV600E (29). We wondered, initially, whether selumetinib-selected resistant sublines expressed predominantly wild-type BRAF and hence became insensitive to MEK inhibition as we found for other tumor models (29). To assess expression of BRAFV600E protein, tumor sections were processed and stained using VE1 antibody that reacts only with the mutant protein (43). As shown in Supplementary Fig. S2, staining for mutant BRAF was generally homogeneous with moderate intensity in both parental and AZD-resistant xenografts. The intensity of staining was slightly less in the BT-40/REV tumors that had reverted to sensitivity to selumetinib. Genomic profiling showed that mutant and wild-type BRAF alleles were represented in equal proportions in all tumor sublines and also showed no amplification of the BRAF locus in BT-40 or BT-40/AZD xenografts.

**“MEK-Functional” expression profile is differentially affected by selumetinib in sensitive and resistant xenografts**

Gene expression changes between sensitive and resistant tumors were examined in xenografts carefully staged to be between 200 and 300 mm^3 at time of harvest. Four replicates from independent mice for the sensitive (BT-40) and resistant (BT-40/AZD) groups were profiled using the Agilent platform. Variance between replicate tumors in each group was small [coefficient of variation (CV) < 5%]. We determined whether the “MEK-Functional” signature, derived predominantly from cell culture experiments (45), distinguished between parental BT-40 and BT-40/AZD tumors. Enrichment of transcriptional pathway signatures was proposed to predict “addiction” to MEK signaling and response to selumetinib. Rather surprisingly, the “MEK-Functional” expression signature was enriched in BT-40/AZD tumors resistant to selumetinib relative to the parental
tumor. As shown in Fig. 2E, we observed that genes driven by MEK activity (45) were significantly enriched (NES = 1.55, ES = 0.53, nominal P = 0.045, FDR q = 0.13, FWER P = 0.061) in BT-40/AZD tumors relative to parental BT-40 tumor. The core enrichment was largely driven by SLCO4A1 (signal-to-noise = 0.51) and DUSP4 (signal-to-noise = 0.50) as well as supported by 6 additional core enriched genes (LGALS3, PROS1, SERPINB1, DUSP6, MAP2K3, and SPRY2) with moderate signal-to-noise (0.11–0.19).

**Quantitative real-time PCR of the MEK and compensatory signatures**

Microarray data indicated that the MEK functional activation signature was significantly enriched, whereas the compensatory resistance signature was not enriched in the BT-40/AZD-resistant tumors. qRT-PCR was carried out using TaqMan probes specific for the genes in the signatures. (Note that a probe for the compensatory gene, STAC, was unavailable.) The purpose of this analysis was 3-fold: to determine the validity of the microarray results, to examine the effects of selumetinib treatment on gene expression, and to incorporate the revertant tumor line gene expression into the data set. From the microarray results, 3 MEK functional activation genes were found to be upregulated in the resistant BT-40 tumors relative to the parental tumors: DUSPM, LGALS3, and SLCO4A1. The ΔΔCt values calculated from the qRT-PCR results confirmed that in the resistant tumors, the same 3 genes were driving the enrichment of the MEK signature, with values of 138.8-, 70.5-, and 9.2-fold elevation in BT-40/AZD tumors, respectively (Supplementary Table S1). The qRT-PCR results of the compensatory signature differed from the microarray results; in that the microarray showed 2 genes, COL5A1 and LOX, were significantly downregulated, and 2 genes, interleukin-6 (IL-6)

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**Figure 2.** Inhibition of MEK signaling induces feedback phosphorylation of MEK in BT-40 (A) and BT-40/AZD (B) tumors treated with selumetinib (75 mg/kg twice daily). Tumors were harvested at the times shown after start of treatment (0 hour, no treatment controls). C, quantitation of selumetinib-induced changes in levels of phospho-MEK (pMEK) and phospho-ERK2 (pERK2) in BT-40 xenografts relative to control levels. D, decreases in phospho-ERK2 in BT-40 (A) and BT-40/ AZD (B) xenografts treated with selumetinib (*, P < 0.05). E, left, Blue-Pink O’Gram in the space of the analyzed GeneSet showing increased expression (pink) in the “test set” (BT-40/AZD) compared with control (BT-40). Right, “MEK-functional” signature showing enrichment in the BT-40/AZD-resistant line. Four independent tumors from each group were analyzed. F, IL-6 gene overexpression is maintained in BT-40/AZD tumors resistant to the MEK inhibitor, selumetinib, BT-40, BT-40/AZD, and BT-40/REV tumor xenografts were harvested from mice without selumetinib administration (untreated) or after 72 hours of selumetinib administration (treated) in cohorts of 3 tumors. ΔΔCt and relative quantitation (RQ) analysis was conducted using ABI software, with threshold values of 2.0 (red) and 0.5 (green), relative to the bottom-listed condition. Genes were not considered above threshold unless both ΔΔCt and RQ values were above the range. The average of 3 ΔΔCt values is shown. Level of significance was calculated using Student t-test analysis of both ΔΔCt and RQ analysis. Unpaired t-tests were used for the continuous variable comparisons, such as expression level differences shown in Fig. 2D.
and SERPINE1, were significantly upregulated in the resistant tumors. It should be noted, however, that LOX [fold change (FC) = −2.0] and SERPINE1 [FC = 2.5] were barely above the significance cutoff values of ±2.0. From the qRT-PCR results, only IL-6 was significantly upregulated in the resistant tumors, with a ΔΔCt value of 7.45 (Table 1).

To investigate the effects of MEK1/2 inhibition on the signature gene expression, mice with BT-40- and BT-40/AZD-resistant xenografts were treated with twice daily with selumetinib. Overall, when qRT-PCR was carried out on tumors harvested at 72 hours, gene expressions did not differ greatly when the BT-40- and BT-40/AZD-resistant tumors were compared with their untreated cohorts for either “MEK-Functional” or “compensatory” signatures (Supplementary Tables S1 and S2). It did show, however, that the same 3 genes, DUSP4, LGALS3, and SLCO4A1, that were driving MEK signature enrichment remained upregulated in the resistant tumors under conditions of MEK inhibition. Interestingly, the expression of the compensatory signature gene, IL-6, also remained significantly upregulated in the resistant tumors compared with the BT-40 xenografts (ΔΔCt = 5.1) after treatment. This increased expression was maintained even though IL-6 was shown to be significantly downregulated in both the parental and AZD-resistant tumors after MEK1/2 inhibition, with ΔΔCt values of 0.09 and 0.05, respectively (Fig. 2F).

Cytokine expression in BT-40 xenografts and sublines

Both microarray and qRT-PCR analyses showed that IL-6 expression increased in the selumetinib-resistant xenografts and decreased upon reversion to sensitivity. To examine

| Table 1. Compensatory resistance gene signature qRT-PCR results |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| **Gene probe**    | **Untreated vs. untreated** | **Treated vs. untreated** | **Treated vs. untreated** |
| **BASP**          | 1.58 ± 0.61       | 0.74 ± 0.53       | 8.45 ± 4.34       |
| **CD274**         | 1.22 ± 1.00       | 1.07 ± 0.41       | 1.55 ± 2.99       |
| **CLU**           | 1.05 ± 0.45       | 2.12 ± 1.09       | 1.76 ± 1.30       |
| **COL12A1**      | 0.44 ± 1.32       | 0.68 ± 0.34       | 1.11 ± 6.61       |
| **COL3A1**       | 0.92 ± 0.74       | 0.75 ± 2.31       | 6.21 ± 1.61       |
| **CRMP1**        | 0.49 ± 0.86       | 0.95 ± 0.26       | 1.21 ± 7.08       |
| **FZD2**         | 2.01 ± 3.65       | 3.63 ± 8.16       | 2.72 ± 2.13       |
| **GOS2**         | 0.74 ± 0.91       | 0.51 ± 0.18       | 1.85 ± 6.54       |
| **GPR176**       | 0.98 ± 0.76       | 0.54 ± 0.25       | 4.16 ± 5.85       |
| **IL-6**         | 7.45 ± 64.83      | 0.09 ± 0.12       | 5.10 ± 28.28      |
| **LOX**          | 0.71 ± 0.47       | 5.59 ± 14.85      | 0.35 ± 0.07       |
| **SERPINE1**     | 1.40 ± 1.55       | 0.61 ± 0.05       | 2.26 ± 29.01      |

NOTE: BT-40, BT-40/AZD, and BT-40/AZD tumors were harvested from mice without selumetinib administration (untreated) or after 72 hours of selumetinib administration (treated) in cohorts of 3 tumors. The snap-frozen tissue was ground, mRNA extracted, and reverse transcribed to cDNA. qRT-PCR was carried out on the treated and untreated naïve, resistant, and revertant cDNA using TaqMan probes (Life Technologies) specific for the compensatory resistance genes. ΔΔCt and relative quantitation (RQ) analysis was conducted using ABI software, with threshold values of 2.0 (red) and 0.5 (green), relative to the bottom-listed condition. Genes were not considered above threshold unless both ΔΔCt and RQ values were above the range. The average of 3 ΔΔCt values is shown. Level of significance was calculated using Student t-test analysis of both ΔΔCt and RQ analysis. Expression was considered significant if P < 0.05 for both analyses.
whether targeting MEK/ERK by selumetinib inhibited cytokine production, levels of cytokines in xenografts were evaluated using a Human Cytokine Protein Array II, an assay that can detect 36 different cytokines. Tumor lysates for the antibody array were derived from control and treated BT-40, BT-40/AZD and BT-40/REV tumors. Basal levels of IL-6 were elevated in BT-40/AZD xenografts compared with either parental BT-40 or BT-40/REV tumors (Fig. 3A). In untreated BT-40/REV xenografts, IL-6 was not detected. Relative to control BT-40 xenografts, selumetinib-treated tumors showed a dramatic decrease of IL-6 and IL-8. Similarly, treatment decreased IL-6 levels in BT-40/AZD xenografts. However, although IL-6 decreased during selumetinib treatment in BT-40/AZD tumors, the level in treated tumors was still similar to that in untreated parental BT-40 xenografts. Levels of individual cytokines, and their response to selumetinib, are presented in Fig. 3B. Consistent with increased IL-6 transcripts and protein, there was clear activation of the NF-κB pathway in BT-40/AZD tumors where phospho-Rel-A was detected. In contrast, phosphorylated Rel-A was not detected in BT-40 xenografts and was significantly reduced by selumetinib treatment in BT-40/REV tumors relative to the levels detected in BT-40/AZD tumors (Fig. 3C). Examination of IL-6Rα transcript levels by RT-PCR, using human-specific primers, showed a slight increase in BT-40/AZD over parental BT-40 xenografts, but a 4-fold decrease in receptor transcript levels in BT-40/REV tumors (Supplementary Fig. S3).

STAT3 signaling increases in MEK-inhibited astrocytoma cells intrinsically resistant to selumetinib in vitro

The above results suggested that IL-6 potentially could maintain STAT3 signaling leading to resistance. We examined the effect of selumetinib in 2 human astrocytoma lines with BRAFV600E mutations. By sequencing, AM38c1 cells are heterozygous for the BRAFV600E mutant whereas DBTRG-05MG has only the mutant gene (data not shown). Neither cell line was sensitive to selumetinib (IC_{50} > 10 μmol/L; Fig. 4A). Increasing concentrations of selumetinib progressively inhibited phosphorylation of ERK1/2 with 50% inhibition at 5 μmol/L for AM38c1 cells and about 500 nmol/L for DBTRG-05MG cells (Fig. 4B). Of note, however, was a reciprocal increase in STAT3 phosphorylation (Y705) in both cell lines, suggesting that activation of STAT3 signaling could compensate for MEK inhibition (Fig. 4B). Thus, activation of STAT3 signaling could represent a mechanism for both intrinsic and acquired resistance to selumetinib. To test the effect of inhibiting STAT3 activation in the presence of MEK inhibition, we used LLL12 as a potent inhibitor of STAT3 phosphorylation (46). As shown in Fig. 3C, the effects of selumetinib and LLL12 were essentially additive.

STAT3 signaling increases in MEK-inhibited astrocytoma xenografts with acquired resistance to selumetinib in vivo

Upon binding its receptor, IL-6 activates the JAK/STAT3 signaling pathway (47), resulting in cell survival and increased angiogenesis (48–50). Our data showed that IL-6 levels are elevated in BT-40/AZD xenografts and that levels are maintained in the selumetinib-resistant tumors after 72 hours of drug treatment. We hypothesized that as in the cell lines, the transcription factor, STAT3, may be activated in BT-40/AZD xenografts posttreatment with the MEK1/2 inhibitor.

To test this, protein lysates were extracted from BT-40, BT-40/AZD, and BT-40/REV xenograft tumors, in triplicate,
from mice that had been administered selumetinib (75 mg/kg BID). Tumors were harvested before treatment (0 hours) and 24, 72, and 144 hours after the start of treatment. STAT3 and phospho-STAT3 (Y705) were determined by analysis of the respective lysates. Both the BT-40 and BT-40/REV tumors displayed a progressive reduction of phospho-STAT3 over the course of drug treatment, with the highest levels of phosphorylation in the untreated tumors, and the lowest at the 144-hour time point (Fig. 5A). The BT-40/AZD drug–resistant tumors, however, showed a marked increase in phosphorylation of STAT3 after 24 hours of the MEK1/2 inhibitor that was maintained above the levels in untreated tumors at the 144-hour time point. Densitometric analysis of the immunoblots (n = 3 per condition; Fig. 5B) confirmed that after selumetinib treatment at 24 hours, phospho-STAT3 levels were reduced by approximately 37% in BT-40 and BT-40/REV tumors. In contrast, phospho-STAT3 increased by 100% in BT-40/AZD xenografts. After 144 hours of drug administration, the parental and revertant tumors both showed a greater than 90% reduction in the phosphorylation of the STAT3 protein, whereas phospho-STAT3 levels in BT-40/AZD tumors were elevated 50% above control (untreated) levels.

The STAT3 inhibitor LLL12 reverses selumetinib resistance in vivo

We have shown previously that in an osteosarcoma xenograft model, daily dosing with LLL12 (5 mg/kg) completely abrogates the phospho-STAT3 (Y705) signal (31). As a proof-of-principle experiment, using subcutaneous tumors, we examined LLL12, selumetinib, and the combination against BT-40/AZD xenografts. As shown in Fig. 5C, selumetinib failed to inhibit the growth of BT-40/AZD tumors (P = 0.077), confirming resistance. Similarly, LLL12 had very modest activity as a single agent (P = 0.063). In contrast, combined treatment with selumetinib and LLL12 significantly inhibited growth relative to controls (P < 0.0001). In the combination treatment group, tumors dramatically regressed over the first week of treatment. Interestingly, tumors appeared to regrow but then remained...
essentially static over the 6-week course of therapy. The result suggests that either MEK or STAT3 signaling may maintain proliferation, but inhibition of STAT3 signaling has profound effects only when MEK signaling is simultaneously inhibited, indicating a synthetic lethal interaction. Examination of tumor sections at termination of the respective control or treatment groups showed that LLL12 and selumetinib as single agents had relatively modest effects on reducing proliferation (Fig. 5D). LLL12 reduced Ki67 staining by 28%, whereas selumetinib reduced this index by 10% in BT-40/AZD xenografts. Apoptosis was increased significantly by both LLL12 and selumetinib but was dramatically elevated (~90%) in tumors harvested from mice receiving the combination of these agents.

Discussion

Inhibitors of BRAFV600E such as vemurafenib and to a lesser extent MEK inhibitors such as selumetinib have impacted treatment of BRAF mutant melanoma. However, intrinsic or acquired resistance to each agent, or the combination of BRAF/MEK inhibitors, limits their use (52). Combination treatment with dabrafenib, a selective BRAF inhibitor, and trametinib, a selective MEK inhibitor, was superior to monotherapy; however, median progression-free survival was less than 1 year (52) with objective response rates of 76% versus 54%, and median progression-free survival increased from 5.8 to 9.4 months. Thus, development of acquired resistance remains a significant problem to targeted therapy in melanoma. Furthermore, responsiveness to inhibitors may be context-specific, as colon carcinoma with BRAFV600E mutations is unresponsive to BRAF inhibitors (44), possibly a consequence of “ERK rebound” associated with activation of EGFR receptor (EGFR) signaling (44, 53). Thus, effects of inhibiting the BRAF/MEK pathway may be context-dependent.
The MEK inhibitor selumetinib has recently entered clinical evaluation by the Pediatric Brain Tumor Consortium for treatment of pediatric low-grade astrocytomas (protocol PBTC-029). The incidence of BRAFV600E mutations is approximately 23% in low-grade astrocytomas of childhood and about 60% in xanthoastrocytomas. The tandem duplication of BRAF that results in the KIAA1549–BRAF fusion, considered to activate BRAF, appears to be restricted to grade 1 pilocytic astrocytoma. Thus, there is interest in evaluating both BRAF and MEK inhibitors in these tumors, although there is a lack of preclinical data to support whether these cancers will be responsive, as with melanoma, or unresponsive, as with colon cancer, to these agents. A recent report (54) indicates that in cells expressing KIAA1549–BRAF, the fusion kinase functions as a homodimer that is resistant to vemurafenib and is associated with CRAF-independent paradoxical activation of MAPK signaling. However, there is no reason a priori to anticipate that cells expressing the KIAA1549–BRAF fusion would be resistant to an MEK inhibitor. Previously, we characterized 2 patient-derived JPA xenografts (29) and showed that BT-35 tumors with wild-type BRAF were unresponsive to selumetinib, whereas BT-40 tumors that are heterozygous for the BRAFV600E allele were highly responsive to treatment. Neither model has the KIAA1549–BRAF fusion, suggesting that these tumors are low-grade astrocytomas rather than JPAs. Consistent with this, we found that the Ki67 labeling index was relatively high in early-passage xenografts, compared with published data on JPA clinical samples where the index was relatively high in early-passage xenografts, compared with published data on JPA clinical samples where the labeling index was calculated using bromodeoxyuridine incorporation (0.22%–4.3%; ref. 55) or Ki67 staining (56).

In this study, we sought to identify mechanisms of resistance to a MEK inhibitor selumetinib, with the long-term objective to develop therapeutic strategies to circumvent resistance. By direct transplantation of tumor tissue into SCID mice, we developed a xenograft model of low-grade BRAFV600E astrocytoma and selected for resistance to selumetinib in vivo. The parental BT-40 xenograft was highly sensitive to selumetinib (29), but resistant sublines were readily derived within 2 passages in treated mice. Of interest, further passage in untreated mice led to revertants that were sensitive to selumetinib treatment. These results do not distinguish between an unstable resistance mechanism or overgrowth of a drug-sensitive population of cells when tumors are passaged in untreated mice. Previously, we reported reversion to rapamycin sensitivity in cloned resistant lines, suggesting that epigenetic resistance may occur to signaling inhibitors (57). The levels of mutant BRAF were similar in all tumor lines, indicating that resistance was not associated downregulation of the mutant protein and expression of wild-type BRAF. In contrast to reported mechanisms for acquired resistance to MEK inhibitors selected in vitro, we did not find amplification of the BRAF locus (35) or a failure of drug to inhibit MEK previously reported as a consequence of activation of MAP3K8 (COT; ref. 31) or mutation in MEK (34). Rather, MEK appeared to be equally inhibited in parental and BT-40/AZD selumetinib-resistant xenografts. Thus, we examined changes in the "MEK-functional" and "compensatory" expression signatures (45) that correlated with sensitivity to selumetinib in parental, resistant, and revertant xenograft lines. Rather surprisingly, in BT-40/AZD–resistant xenografts, we found enrichment in gene expression associated with the MEK-functional pathway, considered to reflect "addiction" to MEK signaling and to predict sensitivity to MEK inhibition. Increased expression of these genes was confirmed by qRT-PCR. Three genes (DUSP, LGALS3, and SLCO4A1) were significantly enriched in BT-40/AZD tumors relative to parental BT-40. The differential was larger when BT-40/AZD was compared with BT-40/REV, where 10 of 16 genes were expressed at significantly higher levels in resistant tumors. In contrast, expression profiling did not show significant changes in genes associated with the "compensatory" profile. However, by qRT-PCR, transcripts for IL-6 were clearly enhanced in BT-40/AZD tumors compared with parental (7.5-fold) or revertant tumors (65-fold) and thus correlated with drug sensitivity. Other studies have implicated elevated IL-6 expression in resistance to MEK inhibitors (45, 58).

Examination of IL-6 in parental, resistant, and revertant tumors showed that IL-6 protein tracked with the qRT-PCR data. IL-6 was elevated about 5-fold in resistant tumors compared with parental tumors, whereas it was not detected in revertant xenografts. Of importance, selumetinib abrogated IL-6 detection in BT-40 tumors, whereas IL-6 was decreased but detectable in BT-40/AZD–treated tumors, being about 75% of the level determined in untreated BT-40 xenografts. Although several signaling pathways are implicated in IL-6 transcription, we found that NF-kB signaling was activated in BT-40/AZD tumors and was relatively resistant to selumetinib-induced downregulation that occurred in both parental and revertant xenografts. In addition, levels of IL-6 receptor α were decreased 4-fold in BT-40/REV xenografts, suggesting that in these selumetinib-sensitive revertants, the IL-6/STAT3 pathway was abrogated.

These data suggested that enhanced IL-6 secretion may activate STAT3 signaling and circumvent the effect of MEK inhibition. Previously, it was reported that activation of STAT3 correlated with resistance to selumetinib in non–small cell lung cancer (NSCLC) cell lines (59) and in melanoma cell lines selected for vemurafenib resistance in vivo (60). In NSCLC cell lines, STAT3-regulated microRNA miR-17 mediated resistance to selumetinib by preventing drug-induced BIM and PARP cleavage. We examined 2 additional BRAFV600E mutant astrocytoma cell lines. Both were intrinsically resistant to selumetinib (IC50 > 10 μmol/L). In both lines, there was a reciprocal activation of STAT3 as MEK was progressively inhibited, suggesting that STAT3 activation could be mediating intrinsic resistance to the MEK inhibitor. Inhibition of ERK activation by AZD6244 leads to derepression of STAT3, as ERK-mediated STAT3 (S727) phosphorylation negatively modulates STAT3 tyrosine phosphorylation at Y705, which is required for dimer formation, nuclear translocation, and DNA-binding activity of this transcriptional regulator (61). Consistent with this, phospho-STAT3 (S727) was lower in BI-40/AZD
tumors compared with parental or revertant xenografts (data not shown). However, combination of selumetinib with a STAT3 inhibitor (LLL12) gave only additive effects against the 2 BRAF mutant cell lines in vitro, with no obvious increase in apoptosis.

Similar to the in vitro data, where STAT3 was progressively phosphorylated as MEK was inhibited, we found that only in the BT-40/AZD–resistant model was STAT3 phosphorylation increased during treatment with selumetinib. In contrast, phosho-STAT3 decreased more than 90% in both BT-40 and BT-40/REV tumors treated with drug. These data suggested that STAT3 activation could be compensating in the presence of MEK inhibition to maintain proliferation and survival. We have shown previously that the small-molecule STAT3 inhibitor LLL12 (46) completely abrogated phosho-STAT3 (Y705) when administered at 5 mg/kg to mice bearing OS-1 osteosarcoma xenografts (51). We therefore examined the antitumor activity of selumetinib, LLL12, or the combination against BT-40/AZD xenografts. As anticipated, selumetinib had no significant effect in these resistant tumors. Similarly, LLL12 was ineffective single-agent therapy. In contrast, when selumetinib and LLL12 were combined, there was complete regression of BT-40/AZD tumors, with subsequent slight growth, and tumor stasis over the 6 weeks’ treatment. These data are in contrast to that of Dai and colleagues (59) where the JAK/STAT3 inhibitor (JS-124, cucurbitacin I; ref. 62) exerted growth inhibition of Dai and colleagues (59) where the JAK/STAT3 inhibitor (JS-124, cucurbitacin I; ref. 62) exerted growth inhibition.

Both in vivo and in vitro inhibition of MEK signaling was associated with increased STAT3 (Y705) phosphorylation and resistance to selumetinib. In vivo, combination of selumetinib with a STAT3 inhibitor reversed acquired resistance to selumetinib. Although based only on a single low-grade astrocytoma model, the data support the contention that compensatory activation of STAT3 signaling may be a mechanism for intrinsic and acquired resistances to MEK inhibitors (58, 59) that is amenable to pharmacologic modulation for treatment of childhood low-grade astrocytoma.

Disclosure of Potential Conflicts of Interest
D. Capper has ownership interest (including patents) in Ventana Medical Systems Inc. P.D. Smith is an employee of AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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References


Development, Characterization, and Reversal of Acquired Resistance to the MEK1 Inhibitor Selumetinib (AZD6244) in an In Vivo Model of Childhood Astrocytoma

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