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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 BRAF^{V600E} 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 mechanism(s) 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 IL6 and IL8 expression. Selumetinib treatment induced phosphorylation of STAT3(Y705) only in resistant xenografts, and similar results were observed in BRAF^{V600E} astrocytic cell lines intrinsically resistant to selumetinib. Treatment of BT-40 resistant tumors with selumetinib or LLL12 had no

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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.

INTRODUCTION

Astrocytomas are the most common tumors of the central nervous system in children and are subdivided according to histologic subtypes, grades I-IV (1). The majority of astrocytomas are grade I and grade II tumors, yet the biological 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 genotyping and expression profiling (7-9). Whereas histologically, astrocytomas in children and adults are similar, childhood astrocytomas are distinct clinical entities from those in adults and are not associated with many of the critical genetic alterations found in the adult astrocytomas. With the possible exception of *TP53* mutations, frequent genetic alterations detected in adult astrocytomas have been identified at lower frequencies in childhood astrocytomas (10-13). For pediatric astrocytomas, low-grade tumors are associated with activation of BRAF through a tandem duplication that results in the KIAA1549-BRAF fusion (14) or through an activating point mutation of BRAF (predominantly V600E). More recent data suggests that the KIAA1549-BRAF fusion is restricted to grade I pilocytic astrocytoma (100%) whereas BRAF^{V600E} occurs more frequently in grade II-IV gliomas (~23%; although lower frequencies have been reported (15)), and in 60% of xanthoastrocytomas (15, 16). Thus, activating mutation of BRAF appears to be the most common genetic alteration in intermediate grade astrocytoma. Homozygous deletion of the CDKN2A locus is frequent (~70%) in tumors harboring the BRAF^{V600E} mutation (17). Mutations in PIK3CA are reported to be rare in these tumors (18). Findings for BRAF mutation, similar to other tumors with activated BRAF (e.g. melanoma), suggest that activated BRAF may provide a potential drug target (19).

BRAF is a component of the mitogen activated protein kinase (MAPK) signaling pathway that induces multiple proliferative or differentiation signals within tumor cells (20, 21). In many adult carcinomas MAPK activation occurs through activating mutations in RAS or

RAF. The frequency of BRAF^{V600E} mutations range from 90% in Hairy Cell leukemia, and 60-80% in melanoma to around 10% in colon cancer with other tumors in between (22-28). Cell lines harboring BRAF^{V600E} may be highly sensitive to MEK inhibition, and these agents may have significant utility against melanoma and other tumors with similar mutations (19). As part of the Pediatric Preclinical Testing Program (PPTP) we recently reported the activity of the MEK1/2 inhibitor selumetinib (AZD6244) against 43 in vivo pediatric tumor xenografts, and 23 cell lines. At the highest concentration used in vitro (10 μM) selumetinib only inhibited growth by 50% in 5 of the 23 cell lines. Against the in vivo tumor panels, selumetinib induced statistically significant differences in Event-Free Survival (EFS) distribution in 10 of 37 (27%) solid tumor models and 0 of 6 acute lymphoblastic leukemia (ALL) models. However, even at best, growth inhibition was modest with EFS increased <2-fold in all but 3 tumor models (29). There were no objective responses. Pharmacodynamic studies indicated that, at the dose and schedule used, selumetinib completely inhibited ERK1/2 phosphorylation in non-responding tumors. These data imply that MEK signaling is not required for cell proliferation or survival in most of these pediatric tumor models, or that there is rapid compensation and activation of alternative signaling pathways.

In the secondary panel of tumors in the PPTP, we have developed two astrocytomas as direct patient/mouse heterografts. Characterization of these models showed BT-35 tumor cells have 2-5 copies of wild type BRAF, whereas BT-40 tumor cells have 5 copies of activated BRAF^{V600E} (29). Further characterization of BT-40 revealed loss of the CDKN2A locus, and wild type p53, typical of many grade II astrocytomas (17). BT-40 xenografts were highly sensitive to selumetinib, regressing completely during the period of treatment (6 weeks), but recurring in a dose-dependent manner after treatment was finished. In contrast BT-35 xenografts progressed on selumetinib treatment without any significant drug-induced growth delay.

Resistance to MEK and BRAF inhibitors has been extensively studied in the context of adult cancers (30-35), as has the paradoxical activation of the BRAF pathway by the BRAF inhibitor PLX4032 in the cellular context of wild type BRAF (36). However, as stated above, the genetic features of childhood astrocytomas may be very different from most adult epithelial-derived cancers. Consequently, development of resistance, or development of therapies that retard or prevent emergence of resistance, may be context specific. Our studies, presented here, would support a mechanism of resistance that is unstable (hence unlikely through genetic mutation) not previously identified in BRAF-mutants (31, 35, 37, 38), and mediated by activation of STAT3 signaling in BT-40 xenografts resistant to selumetinib treatment.

MATERIALS AND METHODS

Reagents, Drugs and Formulation

RPMI media, fetal bovine serum (FBS) and Alamar Blue (AB) were purchased from Invitrogen (Carlsbad, CA). Anti-CD34 antibody (ab27448) was from Abcam (Cambridge, MA, USA). TUNEL detection kit was purchased from GenScript USA Inc. Human Cytokine Array from R & D systems (Cat no ARY005) Minneapolis, MN. Primary antibodies to β-

actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphorylated STAT3(Y705), STAT3, phosphorylated ERK, ERK were purchased from Cell Signaling Technology (Beverly, MA) and Ki67 antibody was purchased from DAKO.

Selumetinib was provided by AstraZeneca (Wilmington, DE). Selumetinib was dissolved in 0.5% hydroxypropyl methyl cellulose, 0.1% Polysorbate 80 and administered p.o., 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). The powder was dissolved in sterile dimethyl sulfoxide (DMSO) to make a 5mg/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 mM glutamine at 37°C with 5% CO_2 . All cells were maintained as sub confluent cultures and split 1:3, 24 hours before use. Experiments were conducted within 10 passages of receiving the cells.

Genomic DNA Extraction and Sequencing

Genomic DNA was extracted using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) 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 1-18 using primers described previously (24). Big Dye Terminator Chemistry was used for sequencing.

RNA Isolation and Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from three independent tumors per condition (parental, resistant, or revertant) before treatment, or at 72 hr of AZD6244 treatment, using Trizol™ (Life Technologies)/ chloroform/ ethanol preprecipitation, as per the manufacturer's instructions. Prior to 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 pipeting was performed on a Biomek 3000® (Beckman Coulter, Brea, CA, USA) in order to ensure accuracy. qRT-PCR amplification was performed on an ABI 7300 Real Time PCR System, under the following conditions: 2 mins at 50°C (uracil DNA glycosylase activation), 10 mins at 95°C , and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative gene expression was calculated using

the C_T method, with each C_T value for the probes set relative to the internal 18S control C_T value. The C_T 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 (four per condition), each from independent mice were measured using the Agilent Sureprint G3 version 1 human gene-expression array. The biological replicates were utilized to measure differences between conditions given xenograft biological variability. Gene set enrichment analysis (GSEA) (39) was applied to measure the overall activity of a 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 1000 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 Chemiluminescence substrate (Pierce) and Biomax MR and XAR film (Eastman Kodak Co.). Fifteen μ L of total sample was resolved on a 4-12% SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane and immune-detection was performed with specific primary antibodies.

Cell viability/proliferation assay

AM38c1 and DBTRG-05MG cells were seeded on 6-well plates at a density of approximately 1×10^5 cells/well in their respective media. Cells were treated with 10 nM – 10 μ M AZD2644 or LLL12 (100 nM to 5 μ M) to determine IC_{50} concentrations. For combination studies, cells were exposed to 10 μ M selumetinib combined with 2 μ M LLL12 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 nm and 630 nm within 3-4 hr 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, Germantown, NY) were used to propagate subcutaneously implanted BT40 tumors. All mice were maintained under barrier conditions and experiments were conducted 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 conducted in accordance with institutional animal care and use committee of the 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, Minneapolis, MN) 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 BT40 (drug sensitive), BT40/AZD (drug resistant), and BT40/REV (drug revertant) groups were added and incubated for overnight at 4°C. After 16-18 hr the membranes were washed and streptavidin-HRP was added for 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 was quantified by scanning the film using Biorad Molecular Image Gel DocTM XR+ and data using were analyzed using Image LabTM software.

Immunohistochemistry

For IHC, formalin fixed tumors were sectioned at 5-µm then dewaxed and soaked in alcohol. After microwave treatment in antigen unmasking solution (Vector Laboratory, Burlingame, CA, USA) for 10 min, endogenous peroxidase activity was inactivated by incubating in 3% hydrogen peroxide (H₂O₂) for 15 min and sections were incubated with primary antibody in phosphate-buffered saline (PBS) at 4°C overnight. After washing with PBS, immunostaining was performed using the Vectastain Universal Quick Kit and DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA, USA) 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 40X 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, Sterling Heights, MI) digital imaging system. Proliferation was measured by Ki67 staining. Apoptosis was detected in deparaffinized tumor sections by TdT-mediated dUTP nick-end labeling (TUNEL) assay using the in situ cell death detection kit (Gene Script USA Inc) according to manufactures instructions.

Detection of mutant BRAF^{V600E} 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 BRAF^{V600E} and total BRAF was 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 $\pm$ SE. Differences were analyzed with the Student *t* test, and significance was set at *P* less than 0.05. Unpaired *t* tests were used for the continuous variables comparisons, such as expression level differences shown in Figure 2.D. For in vivo testing against pediatric sarcoma xenograft models, criteria for defining an event (4 times the tumor volume at the start of treatment) was 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; Cary, NC).

RESULTS

Development of resistance in vivo

The BT-40 astrocytoma was derived from a tumor diagnosed as a Juvenile Pilocytic Astrocytoma (JPA) (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 heterozygous for mutant BRAF^{V600E}, 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 BRAF^{V600E} 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, **Supplemental Figure 1**. Two tumors designated A5 and B4 were selected for further transplant and retreatment. As shown in **Figure 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 (**Figure 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 **Figure 1C**, after three 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 (**Figure 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 (**Figure 2A**) and BT-40/AZD (**Figure 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 **Figure 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 to that in BT-40 xenografts after treatment (**Figure 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 BRAF^{V600E} (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 BRAF^{V600E} protein, tumor sections were processed and stained using VE1 antibody that reacts only with the mutant protein (43). As shown in **Supplemental Figure 2**, 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 200-300mm³ 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 (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 **Figure 2E**, we observed that genes driven by MEK activity (45) were significantly enriched (NES=1.55, ES=0.53, nominal p-value=0.045, FDR q-value=0.13, FWER p-value=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, while the compensatory resistance signature was not enriched in the BT40/AZD resistant tumors. qRT-PCR was performed using Taq-Man® probes specific for the genes in the signatures. (Note: a probe for the compensatory gene, STAC, was unavailable.) The purpose of this analysis was three-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, three MEK functional activation genes were found to be up-regulated in the resistant BT40 tumors relative to the parental tumors: DUSP4, LGALS3 and SLCO4A1. The Δ CT values calculated from the qRT-PCR results confirmed that in the resistant tumors, the same three 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 (**Supplemental Table 1**). The qRT-PCR results of the compensatory signature differed from the microarray results; in that the microarray showed two genes, *COL5A1* and *LOX*, were significantly down-regulated, and two genes, interleukin 6 (*IL6*) and *SERPINE1*, were significantly up-regulated in the resistant tumors. It should be noted, however, that *LOX* (FC = -2.0) and *SERPINE1* (FC= 2.5) were barely above the significance cut-off values of ± 2.0 . From the qRT-PCR results, only *IL6* was significantly up-regulated in the resistant tumors, with a Δ CT value of 7.45 (**Table 1**).

To investigate the effects of MEK 1/2 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 performed on tumors harvested at 72h, gene expressions did not differ greatly when the BT-40 and BT-40/AZD resistant tumors were compared to their untreated cohorts for either ‘MEK-Functional’ or ‘compensatory’ signatures (**Supplemental Table 1** and **Table 2**). It did show, however, that the same three genes, *DUSP4*, *LGALS3*, and *SLCO4A1*, that were driving MEK signature enrichment, remained up-regulated in the resistant tumors under conditions of MEK inhibition. Interestingly, the expression of the compensatory signature gene, *IL6*, also remained significantly up-regulated in the resistant tumors compared to the BT-40 xenografts (Δ CT=5.1) after treatment. This increased expression was maintained even though *IL6* was shown to be significantly down-regulated in both the parental and AZD-resistant tumors after MEK 1/2 inhibition, with Δ CT values of 0.09 and 0.05, respectively (**Figure 2F**). qRT-PCR analysis of untreated BT-40 and BT-40/REV (revertant) tumors showed that the MEK functional and compensatory resistance gene signatures differed very little; the exception being that *IL6* gene expression was reduced approximately 10-fold in the revertant tumors compared to the parental BT-40 xenografts (**Table 1**). Also, as seen in the BT-40 tumors, expression of *DUSP4*, *LGALS3*, *SLCO4A1*, and *IL6*, was significantly upregulated in the untreated resistant tumors *versus* the revertant tumors.

Upon administration of selumetinib, expression of the MEK functional signature genes in the untreated *versus* treated revertant tumors followed the same pattern as seen in the parental and resistant tumors (Supplemental Table 1). However, unlike the BT-40 drug sensitive and BT-40/AZD resistant tumors, the compensatory signature genes in the revertant tumors were greatly affected (Table 1). In the untreated BT-40 or BT-40/AZD tumors *versus* treated BT-40 and BT-40/AZD tumors, only *FZD2* and *IL6* were shown to be differentially expressed due to MEK 1/2 inhibition. In contrast, in the revertant tumors, 9 of 12 genes in the signature were significantly up- or down-regulated in response to drug administration. When the selumetinib-treated BT-40 tumors and treated revertant tumors were compared for changes in MEK signature genes, 7 of 16 genes were down-regulated, and 7 of 12 compensatory genes were differentially expressed. Comparison of treated resistant *versus* treated revertant tumor gene signatures displayed the same pattern. Ten of the 16 MEK functional genes were significantly up regulated in the resistant tumors compared to the revertant, and 5 of the 12 compensatory resistance genes were differentially expressed.

Cytokine expression in BT-40 xenografts and sublines

Both microarray and qRT-PCR analysis demonstrated that *IL6* expression increased in the selumetinib resistant xenografts, and decreased upon reversion to sensitivity. To examine 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 *IL6* were elevated in BT-40/AZD xenografts compared to either parental BT-40 or BT-40/REV tumors, **Figure 3A**. In untreated BT-40/REV xenografts, *IL6* was not detected. Relative to control BT40 xenografts, selumetinib treated tumors showed a dramatic decrease of *IL6* and *IL8*. Similarly, treatment decreased *IL6* levels in BT-40/AZD xenografts. However, although *IL6* 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 **Figure 3B**.

Consistent with increased *IL6* transcripts and protein, there was clear activation of the NFκB 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, **Figure 3C**. Examination of *IL6Rα* 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 (**Supplemental Figure 3**).

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

The above results suggested that *IL6* potentially could maintain STAT3 signaling leading to resistance. We examined the effect of selumetinib in two human astrocytoma lines with BRAF^{V600E} mutations. By sequencing, AM38c1 cells are heterozygous for the BRAF^{V600E} mutant whereas DBTRG-05MG has only the mutant gene (data not shown).

Neither cell line was sensitive to selumetinib, ($IC_{50} > 10 \mu M$), **Figure 4A**. Increasing concentrations of selumetinib progressively inhibited phosphorylation of ERK1/2 with 50% inhibition at 5 μM for AM38c1 cells and ~500 nM for DBTRG-05MG cells, **Figure 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, **Figure 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 a potent inhibitor of STAT3 phosphorylation (46). As shown in **Figure 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, IL6 activates the JAK-STAT3 signaling pathway (47), resulting in cell survival and increased angiogenesis (48-50). Our data showed that IL6 levels are elevated in BT-40/AZD xenografts, and that levels are maintained in the selumetinib-resistant tumors after 72h of drug treatment. We hypothesized that as in the cell lines, the transcription factor, STAT3, may be activated in BT-40/AZD xenografts post-treatment with the MEK 1/2 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 prior to treatment (0 hr) and 24, 72, and 144hr 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 144h time-point (**Figure 5A**). The BT40/AZD drug resistant tumors, however, showed a marked increase in phosphorylation of STAT3 after 24hr of the MEK 1/2 inhibitor that was maintained above the levels in untreated tumors at the 144hr time-point. Densitometric analysis of the immunoblots (n=3 per condition; **Figure 5B**) confirmed that after selumetinib treatment at 24h, 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 144h 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 (51). As a proof of principle experiment, using subcutaneous tumors, we examined LLL12, selumetinib and the combination against BT-40/AZD xenografts. As shown in **Figure 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, **Figure 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 BRAF^{V600E} 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 utility (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 < 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. Further, responsiveness to inhibitors may be context specific, as colon carcinoma with BRAF^{V600E} mutations is unresponsive to BRAF inhibitors (44), possibly a consequence of 'ERK rebound' associated with activation of 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 BRAF^{V600E} mutations is approximately 23% in low-grade astrocytomas of childhood, and ~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 I 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 a MEK inhibitor. Previously, we characterized two 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 BRAF^{V600E} allele, were highly responsive to treatment. Neither model has the KIAA1549-BRAF fusion, suggesting that these tumors are low-grade astrocytomas rather than JPA's. Consistent with this, we found that the Ki67 proliferation index was relatively high in early passage xenografts, compared to published data on JPA clinical

samples where labeling index was calculated using bromodeoxyuridine incorporation (0.22 to 4.3%) (55) or Ki67 staining (56).

In this study we sought to identify mechanism(s) 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 BRAF^{V600E} 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 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) (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 to BT-40/REV, where 10/16 genes were expressed at significantly higher levels in resistant tumors. In contrast, expression profiling did not demonstrate significant changes in genes associated with the 'compensatory' profile. However, by qRT-PCR transcripts for *IL6* were clearly enhanced in BT-40/AZD tumors compared to parental (7.5-fold) or revertant tumors (65-fold), and thus correlated with drug sensitivity. Other studies have implicated elevated *IL6* expression in resistance to MEK inhibitors (45, 58)

Examination of *IL6* in parental, resistant and revertant tumors showed that *IL6* protein tracked with the qRT-PCR data. *IL6* was elevated ~5-fold in resistant tumors compared to parental tumors, whereas it was not detected in revertant xenografts. Of importance, selumetinib abrogated *IL6* detection in BT-40 tumors, whereas *IL6* was decreased but detectable in BT-40/AZD treated tumors, being ~75% the level determined in untreated BT-40 xenografts. Although several signaling pathways are implicated in *IL6* transcription, we found that NFκB 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 *IL6* receptor α were decreased 4-fold in BT-40/REV

xenografts, suggesting that in these selumetinib-sensitive revertants, the IL6/STAT3 pathway was abrogated.

These data suggested that enhanced IL6 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 cell lines (59), and in melanoma cell lines selected for vemurafenib resistance in vitro (60). In NSCLC cell lines STAT3-regulated microRNA miR-17 mediated resistance to selumetinib by preventing drug-induced BIM and PARP cleavage. We examined two additional BRAF^{V600E} mutant astrocytoma cell lines. Both were intrinsically resistant to selumetinib (IC₅₀ > 10 μ M). 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 BT-40/AZD tumors compared to parental or revertant xenografts (data not shown). However, combination of selumetinib with a STAT3 inhibitor (LLL12) gave only additive effects against the two 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, phospho-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 phospho-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 et al (59) where the JAK/STAT3 inhibitor (JS-124, cucurbitacin I) (62) exerted growth delay as a single agent and was essentially additive when combined with selumetinib. In BT-40/AZD xenografts harvested at the end of treatment there was a modest decrease in Ki67 staining and increase in TUNEL-positive cells in tumors treated with selumetinib or LLL12 as single agents. However, for tumors treated with the combination approximately 90% of cells were apoptotic, and the proliferation index was dramatically decreased. Thus, residual tumor had few viable cells, and inflammation/edema may have contributed to the measured tumor volume.

In summary, in one of the only xenograft models of pediatric low-grade astrocytoma we selected resistance to the MEK inhibitor selumetinib. Resistance was unstable, which may suggest epigenetic rather than a genetic basis for resistance. By comparing parental, resistant

and revertant sublines, our studies pointed to enhanced IL6-STAT3 signaling as a potential mechanism for resistance. 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 resistance to MEK inhibitors (58, 59) that is amenable to pharmacological modulation for treatment of childhood low-grade astrocytoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of 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 IL6 expression and activation of NF κ B. 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.

Figure 1.

Development of resistance to selumetinib (AZD6244). A5 and B4 tumors from the first treatment (see Supplemental Figure 1) were transplanted into naïve mice and treatment with selumetinib (75 mg/kg BID) repeated. Arrows show the duration of treatment. **A.** Graphs show growth curves for individual tumors in untreated (control) or selumetinib-treated mice for tumor A5. **B.** Responses of tumor B4; **C.** Response of tumor A5 after passage for 3 generations in untreated mice, then rechallenged with selumetinib (75 mg/kg BID); **D.** Response of tumor A5 after passage for 5 generations in untreated mice, then rechallenged with selumetinib (75 mg/kg BID).

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 BID). Tumors were harvested at the times shown after start of treatment (0 hr, no treatment controls). C. Quantitation of selumetinib induced changes in levels of phospho-MEK (■) and phospho-ERK2 (□) in BT-40 xenografts relative to control levels. D. Decreases in phospho-ERK2 in BT-40 (□) and BT-40/AZD (■) xenografts treated with selumetinib (* p<0.05); E. Left panel: Blue-Pink O'Gram in the space of the Analyzed GeneSet showing increased expression (pink) in the 'test set' (BT-40/AZD) compared to control (BT-40); Right panel: 'MEK-Functional' signature showing enrichment in the BT-40/AZD resistant line. Four independent tumors from each group were analyzed. F. Interleukin-6 (IL6) gene overexpression is maintained in BT40/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 72h of selumetinib administration (Treated) in cohorts of three tumors.

CT and relative quantitation (RQ) analysis was performed 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 CT and RQ values were above the range. The average of three CT values is shown. Level of significance was calculated using student t-test analysis of both CT and RQ analysis. Unpaired t tests were used for the continuous variables comparisons, such as expression level differences shown in Figure 2.D.

Figure 3.

A. Increased IL6 is associated with resistance to selumetinib. Levels of cytokines in tumor lysates were assayed in untreated or from selumetinib treated xenografts (72 hr treatment). Upper panel: cytokine array cropped to show IL6 levels in BT-40, BT-40/AZD and BT-40/REV (arrows mark IL6). **B.** Quantitation. Note IL6 (black bars) could not be detected in BT-40/REV xenografts. **C.** NFκB signaling is enhanced in BT-40/AZD xenografts. Tumor lysates from tumors treated as described in Figure 2 were probed for Rel-A and phospho-Rel-A as markers of NFκB activation. β-actin was used as a loading control.

Figure 4.

Sensitivity of AM38c1 and DBTRG-05MG astrocytoma cell lines to selumetinib. **A.** Cells were cultured in the presence of increasing concentrations of selumetinib for 2 days, and cell number determined by Alamar Blue staining. **B.** Cells were either not treated (control) or treated with increasing concentrations of selumetinib for 24 hr. STAT3, pSTAT3(Y705) pERK1/2(Y204/T202) and ERK1/2 were determined. GAPDH was used as a loading control. Triplicate independent samples were assessed at each concentration. **C.** Cells were cultured in the presence of selumetinib (10 μM) and LLL12 (2 μM) for 3 days, and cell number determined by Alamar Blue staining.

Figure 5.

A. Mice bearing BT-40, BT-40/AZD or BT-40/REV tumors were either not treated (0 hr) or treated with selumetinib (75 mg/kg BID). Tumors were harvested (n=3) at the time points shown. STAT3 and pSTAT3(Y705) were determined. Actin was used as the loading control

B. Quantitation of STAT3 and pSTAT3(Y705). **C.** LLL12 reverses selumetinib resistance in BT-40/AZD xenografts. Mice (n=10 per group) were treated with vehicle (DMSO, control); LLL12 (5 mg/kg/day); selumetinib (75 mg/kg BID); selumetinib and LLL12 combined for up to 6 weeks. **D.** At termination tumors from each control or treatment group were harvested. FFPE sections were stained to assess proliferation (Ki67), apoptosis (TUNEL) or histology (hematoxylin and eosin). Right panels show quantitation of Ki67 and TUNEL staining.

Table 1

Compensatory resistance gene signature qRT-PCR results.

Gene Probe	Untreated vs. Untreated			Treated vs. Untreated			Treated vs. Untreated		
	BT40/AZD vs BT40	BT40/RE vs BT40	BT40/AZD vs BT40/REV	BT40 vs BT40	BT40/AZD vs BT40/REV	BT40/REV vs BT40/REV	BT40/AZD vs BT40	BT40/REV vs BT40	BT40/AZD vs BT40/REV
BASP	1.58	3.28	0.61	0.74	3.71	0.53	8.45	2.10	4.34
CD274	1.22	1.41	1.00	1.07	1.47	0.41	1.55	0.59	2.99
CLU	1.05	3.59	0.45	2.12	3.57	1.09	1.76	1.44	1.30
COL12A1	0.44	0.33	1.32	0.68	3.32	0.34	1.11	0.19	6.61
COL5A1	0.92	2.03	0.74	0.75	12.04	2.31	6.21	4.16	1.61
CRIM1	0.49	0.59	0.86	0.95	2.07	0.26	1.21	0.20	7.08
FZD2	2.01	0.60	3.65	3.63	5.86	8.16	2.72	1.32	2.13
G0S2	0.74	0.89	0.91	0.51	1.47	0.18	1.85	0.32	6.54
GPR176	0.98	1.32	0.76	0.54	1.99	0.25	4.16	0.71	5.85
IL6	7.45	0.12	64.83	0.09	0.05	0.12	5.10	0.19	28.28
LOX	0.71	1.54	0.47	5.59	2.38	14.85	0.35	4.75	0.07
SERPINE1	1.40	1.02	1.55	0.61	0.97	0.05	2.26	0.08	29.01

BT-40, BT-40/AZD and BT-40/REV tumors were harvested from mice without selumetinib administration (Untreated), or after 72h of selumetinib administration (Treated) in cohorts of three tumors. The snap-frozen tissue was ground, mRNA extracted, and reverse transcribed to cDNA. qRT-PCR was performed on the treated and untreated naïve, resistant, and revertant cDNA using Taq-Man® probes (Life Technologies) specific for the compensatory resistance genes. CT and relative quantitation (RQ) analysis was performed 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 CT and RQ values were above the range. The average of three CT values is shown. Level of significance was calculated using student t-test analysis of both CT and RQ analysis. Expression was considered significant if $p < 0.05$ for both analyses.