Preclinical Development

Enhanced Efficacy of IGF1R Inhibition in Pediatric Glioblastoma by Combinatorial Targeting of PDGFRα/β

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Abstract

Pediatric glioblastoma (pGBM), although rare, is one of the leading causes of cancer-related deaths in children, with tumors essentially refractory to existing treatments. We have identified IGF1R to be a potential therapeutic target in pGBM due to gene amplification and high levels of IGF2 expression in some tumor samples, as well as constitutive receptor activation in pGBM cell lines. To evaluate the therapeutic potential of strategies targeting the receptor, we have carried out in vitro and in vivo preclinical studies using the specific IGF1R inhibitor NVP-AEW541. A modest inhibitory effect was seen in vitro, with GI50 values of 5 to 6 µmol/L, and concurrent inhibition of receptor phosphorylation. Specific targeting of IGF1R with short interfering RNA decreased cell viability, diminished downstream signaling through phosphoinositide 3-kinase (PI3K), and induced G1 arrest, effects mimicked by NVP-AEW541, both in the absence and presence of IGF2. Hallmarks of PI3K inhibition were observed after treatment with NVP-AEW541 by expression profiling and Western blot analysis. Phospho–receptor tyrosine kinase (RTK) arrays showed phosphorylation of platelet-derived growth factor receptor (PDGFR) α/β in pGBM cells, suggesting coactivation of an alternative RTK pathway. Treatment of KNS42 with the PDGFR inhibitor imatinib showed additional effects targeting the mitogen-activated protein kinase pathway, and cotreatment of the PDGFR inhibitor imatinib with NVP-AEW541 resulted in a highly synergistic interaction in vitro and increased efficacy after 14 days therapy in vivo compared with either agent alone. These data provide evidence that inhibition of IGF1R, in combination with other targeted agents, may be a useful and novel therapeutic strategy in pGBM. Mol Cancer Ther; 10(8); 1407–18. ©2011 AACR.

Introduction

High-grade glioma in children continues to have a dismal clinical outcome, with a median overall survival for patients with supratentorial WHO grade IV glioblastoma multiforme of 11 months (1). Current standard of care is still questionable, with the Stupp protocol (2) for adult cases of radiotherapy plus temozolomide (TMZ) commonly used despite an absence of published data showing a clearly beneficial therapeutic effect (3). Novel, molecularly targeted agents have shown little efficacy in early-phase clinical trials (4–6), a problem exacerbated by concerns over drug delivery coupled with an incomplete knowledge of the importance of the specific target in the childhood setting.

Recent molecular profiling data have shown that there is a spectrum of genomic aberrations in high-grade glioma covering patients of all ages, with pediatric cases tending to have an overlapping but distinct biology from their adult counterparts (7–11). Such differences can be shown at the levels of both large-scale and highly focal DNA copy number alterations, as well as gene expression signatures defining molecular subclassifications. In particular, pediatric high-grade glioma appear to be both preferentially and differentially driven by platelet-derived growth factor receptor (PDGFR) signaling (10), with only a minority of cases showing the epidermal growth factor receptor (EGFR)-driven subtype largely present in adults (12–14).

In adults, high-grade glioma with EGFR amplification/overexpression was reported to be mutually exclusive, with a smaller subset of tumors that had extremely high levels of the ligand IGF2 (15). IGF2 was found to convey a strong growth-promoting effect in tumor-derived neurospheres, signaling through PI3K (15). Given the distinctions between EGFR and PDGFR in adult and...
pediatric high-grade glioma, we reasoned that dysregulation of the IGF signaling system was likely to coexist with alterations in PDGFR and thus may be a useful target in a significant proportion of childhood cases.

IGF2 signals through its receptor IGF1R, which plays important roles in development, with constitutive abnormalities in the IGF system causative in fetal and postnatal growth syndromes (16, 17). In the central nervous system, IGF1R promotes cell growth and survival as well as cooperating with growth and morphogenetic factors that induce cell fate specification and selective expansion of specified neural cell subsets (18). In cancer, there is widespread overexpression and occasional DNA copy number gains of IGF2/IGF1R present in a number of disparate pediatric tumor types (19–25), with amplifications reported in pediatric glioblastoma (pGBM; refs. 8, 11). Considerable promise targeting the receptor in childhood cancers has been shown in the preclinical setting by small molecules and in early-phase clinical studies by humanized monoclonal antibodies, particularly in Ewing’s sarcoma (26–29).

In the present study, we have sought to investigate the potential usefulness of targeting IGF1R in pediatric high-grade glioma. By utilizing a small panel of well-characterized glioma cell lines derived from children (30), we have screened the small molecule inhibitor NVP-AEW541, which as a tool compound has shown efficacy in a range of tumor types (31–33). We have further explored the mechanisms by which its effectiveness may be enhanced, alighting on a clinically relevant combinatorial strategy with anti-PDGFR therapy, which produces a synergistic interaction in vitro and in vivo due to differential inhibition of PI3K and mitogen-activated protein kinase (MAPK) pathways by the 2 compounds in pGBM cells.

Materials and Methods

Cell lines and reagents
Glioblastoma cell lines were obtained and cultured as previously described (30) and identity reauthenticated by DNA copy number and gene expression profiling. R−/C0 (IGF1R-null) and R+ (IGF1R-overexpressing) mouse fibroblasts were provided by Renato Baserga, Thomas Jefferson University, Philadelphia, PA. NVP-AEW541 and imatinib mesylate were synthesized by Novartis. TMZ was obtained from Sigma. Chemical structures are provided in Figure 1.

Growth inhibition
Growth inhibition was determined using the MTS assay as previously described (34). For the assessment of combination effects, cells were treated with increasing concentrations of drugs either alone or concurrently at their equipotent molar ratio and combination indices calculated by the method of Chou and Talalay (35). All values are given as mean ± SD of at least 3 independent experiments.

Short interfering RNA
Predesigned short interfering RNA (siRNA) duplexes directed against IGF1R were purchased from Qiagen. Cells were transfected with 100 nmol/L IGF1R siRNAs as well as with a scrambled sequence control duplex using HiPerFect transfection reagent (Qiagen).

Western blot analysis
Immunodetection was carried out as previously described (30) using antibodies against IGF1R (3027), phospho-Akt (ser473) (9271), Akt (9271), phospho-Erk1/2 (9101), Erk1/2 (9102), PARP (9542), caspase-3 (9662), LC3B (2775), and β-actin (9315), all at 1:1,000 dilution and purchased from Cell Signaling Technology.

Figure 1. Chemical structures of NVP-AEW541, imatinib, TMZ, LY294002, and wortmannin.
Electrochemiluminescent immunoassay

Mesoscale Discovery system (MSD) 96-well multispot assays for total/phospho-IGF1R/IR/IRS-1, total/phospho-Akt, and total/phospho-Erk1/2 were carried out as per the manufacturer’s protocol. Plates were washed 4 times, read buffer was added, and the plates were analyzed on a SECTOR 6000 instrument (MSD).

Detection of phosphorylated receptor tyrosine kinases

A phospho-RTK assay (R&D Systems) was used to screen multiple receptor tyrosine kinases (RTK) according to manufacturer’s instructions. Phosphorylated PDGFRA/β were measured in a sandwich ELISA assay (Cell Signaling) as previously described (36).

Assessment of autophagy

KNS42 cells were grown on a glass coverslip and after 24 hours treated with 5 × GI50 of NVP-AEW541 or equivalent dimethyl sulfoxide concentration for 1 hour. Cells were fixed with 2.5% glutaraldehyde fixative (pH 7.3) at 4°C, postfixed in osmium tetroxide, dehydrated, and embedded in epoxy resin. The resin blocks were cut on a Leica ultratcut machines and picked on to 150-mesh Guilders Grids with a support film of Pioloform. Grids were stained with uranyl acetate and lead citrate and viewed in the Hitachi H7600 transmission electron microscope. Autophagy was also assessed in cells cultured in the presence or absence of NVP-AEW541 by measuring levels of cytosolic LC3-I and autophagosome-associated LC3-II by Western blot analysis.

mRNA expression profiling analysis

Expression profiling after treatment of SF188 and KNS42 cells with NVP-AEW541 at 5 × GI50 by Illumina HT-12 BeadChips was carried out according to the manufacturer’s instructions and normalized using the Lumi Package in R2.11 (www.r-project.org). All data have been deposited in the MIAME-compliant ArrayExpress database (accession number E-TABM-889). Time course analysis was carried out using a Pearson correlation coefficient metric. Coordinate gene regulation was identified using Gene Set Enrichment Analysis (GSEA; www.broad.mit.edu/gsea/), with a nominal P value cut-off of 0.001. Gene expression signatures were compared with other drug induced profiles using the Connectivity Network Analysis (DAVID) online software (http://david.abcc.ncifcrf.gov).

In vivo antitumor activity of NVP-AEW541 and imatinib

Six- to 8-week-old female athymic nude mice [CrTac::Ncr-Foxn1nu (www.taconic.com)] were used to establish subcutaneous pGBM xenograft models. Experiments were conducted in accordance with U.K. Home Office regulations under the Animals (Scientific Procedures) Act 1986 and UKCCCR guidelines for animal experimentation (37). A subline of KNS42 was generated using serial xenografting and the addition of Matrigel and growth factors, as the parental cells developed palpable tumors with a low take rate over 235 days. KNS42 A4 cells (growth rate = 36 days, take rate = 100%) were implanted subcutaneously, bilaterally, at 5 × 10⁶ per site. Mice were randomly assigned to 3 groups (n = 6) and treated with NVP-AEW541 (50 mg/kg orally, twice daily, with dose reduced from 75 mg/kg on day 11 due to weight loss) and imatinib mesylate (150 mg/kg orally, twice daily) either alone or in combination as well as vehicle comprising 25 mmol/L L(+)-tartaric acid. Sample extracts were analyzed for drug concentration by liquid chromatography/tandem mass spectrometry (LC/MS-MS).

Results

Pediatric high-grade glioma cell lines show a moderate sensitivity to small molecule inhibition of IGF1R

We reanalyzed our recently compiled molecular profiling studies of pediatric high-grade glioma patient samples (GSE19578) and cell lines (E-TABM-579) to explore the mRNA expression of various components of the IGF signaling network. Specific upregulation of IGF1R was observed in pediatric high-grade versus pediatric low-grade and adult high-grade cell lines (Fig. 2A), and high levels of IGF2, IGF1R, and IRS1 were variously noted in subsets of pediatric high-grade glioma patient samples, including cases with PDGFRα amplification (Fig. 2B). In our cell line panel, mRNA expression corresponded with wild-type receptor expression by Western blot analysis (Fig. 2C) and high levels of phosphorylation were observed in pGBM KNS42 and SF188 cells by an electrochemiluminescent assay (MSD; Fig. 2D). Examining the in vitro growth inhibitory effects of the small molecule inhibitor NVP-AEW541 revealed a relatively modest sensitivity of the pediatric high-grade glioma cell lines (Fig. 2E), with GI50 values of 5.85 to 6.36 μmol/L compared with those of 1.10 and 13.86 μmol/L for the IGF1R-overexpressing R⁺ and null R⁻ cells, respectively (Table 1). Cell survival was diminished in a concentration-dependent manner by NVP-AEW541 both in the absence and presence of the biologically relevant ligand IGF2 (Supplementary Fig. S1).

To assess the effects on downstream signaling of IGF1R abrogation in pGBM, we compared genetic knockdown by specific siRNA with pharmacologic inhibition by NVP-AEW541. siRNA directed against IGF1R in the pGBM cell lines (exemplified by KNS42; Fig. 3A) resulted in efficient reduction of IGF1R, along with inhibition of phospho-Akt (Ser⁴⁷³) with a small increase in phospho-Erk1/2 detected by Western blot analysis (Fig. 3B); induction of apoptosis as measured by PARP cleavage (Fig. 3B); and a profound cell-cycle arrest at G1 determined by fluorescence-activated cell sorting (Fig. 3C). These
observations were replicated in both the absence and presence of IGF2 (Fig. 3D). These effects on protein expression (Fig. 3E) and cell cycle (Fig. 3F) were mimicked by NVP-AEW541 in SF188 and KNS42 cells in both a time- and concentration-dependent manner. NVP-AEW541 blocked IGF2-induced PI3K activation via inhibition of phospho-Akt after 1 hour of treatment and induced apoptosis as seen by PARP and caspase-3 cleavage after 1 and 3 hours in KNS42 and SF188 cells, respectively. As described for the genetic inhibition of IGF1R above, a dose-dependent increase in MAPK activation via phospho-Erk1/2 in a dose-dependent manner was observed. A time- and dose-dependent reduction in phospho-IGF1R and phospho-IRS were also observed by MSD (Supplementary Fig. S2).

NVP-AEW541 acts via downregulation of the PI3K pathway and induces autophagy in pediatric glioblastoma cells

To further explore the mechanism of action of NVP-AEW541 in our pGBM models, we carried out Illumina HT-12 expression profiling for both SF188 and KNS42 cells treated with 5× Gl50 NVP-AEW541 for 1, 6, and 24 hours. In KNS42, genes commonly dysregulated by IGF1R inhibition in a time-dependent manner relative to vehicle-treated control included numerous cyclins (e.g., CCND2/CCNE1), cyclin-dependent kinases

| Table 1. Gl50 values for cell lines treated with NVP-AEW541 as assessed by the MTS assay |
|-----------------|-----------------|-----------------|
| Cell line       | NVP-AEW541      |
|                 | Mean Gl50 (µM)  | Range (µM)      |
| R−              | 13.86           | 13.72–14.50     |
| R+              | 1.10            | 0.50–1.41       |
| U87MG           | 5.51            | 4.96–6.00       |
| KNS42           | 5.85            | 3.89–7.65       |
| SF188           | 5.98            | 3.62–7.35       |
| UW479           | 6.36            | 4.38–7.32       |

Figure 2. IGF1R as a therapeutic target in pediatric high-grade glioma. A, Affymetrix gene expression data for numerous probesets corresponding to IGF1R in a panel of adult high-grade (dark orange), pediatric high-grade (dark green), and pediatric low-grade (light green) cell lines. Data taken from ArrayExpress E-TABM-579. B, Affymetrix gene expression data for IGF1R (blue), IRS1 (green), and IGF2 (red) in pediatric high-grade glioma patient samples. Asterisks represent cases with known PDGFRA amplification. Data taken from Gene Expression Omnibus GSE19578. C, Western blot analysis for IGF1R protein expression in high-grade glioma cells and the IGF1R-null (R−) and overexpressing (R+) fibroblast lines. D, quantitative measure of phosphorylated IGF1R in high-grade glioma cell lines assessed by the MSD assay. E, effects on cell survival of treatment with high-grade glioma cells with the IGF1R inhibitor NVP-AEW541.
Figure 3. Effects of downstream signaling of genetic and pharmacologic targeting of IGF1R in pGBM cells. A, relative IGF1R mRNA expression in KNS42 cells transfected with siRNA targeting the gene in KNS42 cells as determined by quantitative reverse transcriptase PCR. **, P < 0.01, Student’s t test. B, Western blot analysis showing efficient knockdown of IGF1R protein in association with diminished phospho-Akt and PARP cleavage in KNS42 cells transfected with IGF1R siRNA. A slight increase in phosphorylated versus total Erk1/2 is also seen. C, fluorescence-activated cell sorting analysis of siRNA-transfected KNS2 cells versus scrambled control oligos showing an accumulation of cells in G1 and sub-G1 phases in contrast to a reduction of S-phase and G2 phase. D, Western blot analysis confirming the knockdown of IGF1R, reduction in phospho-Akt, induction of PARP cleavage, and increase in phospho-Erk1/2 after treatment of KNS42 cells with IGF1R siRNA in the presence of the ligand IGF2. E, effects on downstream signaling in KNS42 and SF188 cells after treatment with NVP-AEW541 in the presence of IGF2. Cells were treated for 1, 3, 6, 24, and 48 hours with 1/3/5 GI50 compound (triangle). Treatment with IGF1R inhibitor decreased phospho-Akt, induced PARP and caspase-3 cleavage, and increased phospho-Erk1/2 levels in a time- and concentration-dependent manner. At 48 hours, the highest concentration of compound resulted in a significant cell death with little protein recoverable. F, effects on cell cycle in KNS42 and SF188 cells treated with NVP-AEW541. An accumulation of cells in G1 and sub-G1 phases was induced by NVP-AEW541 in a time- and concentration-dependent manner. At 48 hours, the highest concentration of compound resulted in a significant cell death. DAPI, 4',6-diamidino-2-phenylindole.
(e.g., CDK2/CDK4), cyclin-dependent kinase inhibitors (e.g., CDKN1A/CDKN2A/B), and other cell-cycle regulators (e.g., E2F2/CDC25A) associated with transition through the G1 checkpoint (Fig. 4A). Looking for coordinately regulated transcripts using GSEA highlighted significant effects on numerous groups of genes associated with cell cycle, and in particular the G1–S-phase transition [SERUM_FIBROBLAST_CELLCYCLE, enrichment score (ES) = 0.696, P < 0.00001, false-discovery rate (FDR) q < 0.00001, rank = 2; CELL_CYCLE, ES = 0.619, P < 0.00001, FDR q < 0.00001, rank = 18; CELL_CYCLE - KEGG, ES = 0.610, P < 0.00001, FDR q < 0.00001, rank = 24; G1_TO_S_CELL_CYCLE Reactome, ES = 0.520, P < 0.00001, FDR q = 0.00685, rank = 51; Fig. 4B). Ontology analysis using DAVID further revealed a clustering of genes associated with cell-cycle progression (e.g., GO:0007409:Cell_cycle, enrichment = 5.71, P = 0.00094) and DNA replication (e.g., GO:0006260:DNA_replication, enrichment = 4.06, P = 0.0000051; Fig. 4C) in pGBM cells treated with NVP-AEW541. Probing our specific gene signatures using the Connectivity Map resulted in significant hits for the classical inhibitors of PI3K, whereas the effects with SF188 (PDGFRβ) activated were additive (CI = 1.0; Fig. 6C). Similar additivity was observed with U87MG (PDGFRβ activated) and Res259 (c-Kit activated; data not shown). Analysis of downstream signaling components showed a time- and concentration-dependent inhibition of both phospho-Akt and phospho-Erk1/2, revealing coinhibition of the MAPK pathway in contrast to the PI3K selectivity of NVP-AEW541 (Fig. 6D). Apoptosis via PARP and caspase-3 cleavage (Fig. 6D) and a G1 cell-cycle arrest were also observed (Fig. 6E) in parallel with inhibition of PDGFα/β by ELISA (Supplementary Fig. S4), albeit at relatively high doses of the compound.

Finally, we determined whether these combination effects observed in vitro would translate to the in vivo setting. We thus dosed female athymic nude mice bearing KNS42_A4 subcutaneous xenografts with either 50 mg/kg NVP-AEW541 alone (reduced from 75 mg/kg after day 11), 150 mg/kg imatinib alone, or both compounds in combination, and compared tumor growth with vehicle-treated controls. At these doses, final tumor concentrations of drug exceeded in vitro GI50 values in KN52_A4 cells (PDGFRα coactivated) by 10- and 100-fold, respectively (Fig. 6A). As a result, we hypothesized that at least in part a PI3K-dependent process leading to an upregulation of a HOX/stem cell signature (38), PI3K inhibition is known to induce autophagy in glioblastoma cells (39), and we showed similar effects in KNS42 cells with inhibition of IGFIR by both Western blot analysis for increased LC3B-II (Fig. 5B) and electron microscopy, showing the formation of autophagic structures including autolysosomes and amphiphases upon treatment with NVP-AEW541 (Fig. 5C and D).

**Pediatric glioblastoma cells have constitutive coactivation of IGFIR and PDGFR and cotargeting the receptors shows enhanced efficacy in vitro and in vivo**

Despite the clear targeting of PI3K signaling after inhibition of IGFIR by NVP-AEW541 in pGBM cells, the growth inhibitory effects in vitro were relatively modest. To explore the mechanisms by which the cells may be surviving such targeted drug treatment, we carried out phospho-RTK arrays. Both KNS42 and SF188 had constitutively active IGFIR, confirming the MSD data; however in addition were found to have high levels of PDGFRα and PDGFRβ, respectively (Fig. 6A). Overexpression of wild-type PDGFRα/β was confirmed in these lines by Western blot analysis, as was activation of both PI3K and MAPK pathways upon stimulation with the ligand PDGF-BB (Fig. 6B). Treatment of the cells with the PDGFR (and other RTK) inhibitor imatinib as a single agent resulted in broad insensitivity, with GI50 values in the range 30.6 to 35.0 μmol/L (not shown).

To determine the efficacy of dual targeting of both IGFIR and PDGFRα/β, we carried out a median-effects analysis in vitro. KNS42 cells (PDGFRα coactivated) showed a highly synergistic interaction (CI = 0.44), whereas the effects with SF188 (PDGFRβ activated) were additive (CI = 1.0; Fig. 6C). Similar additivity was observed with U87MG (PDGFRβ activated) and Res259 (c-Kit activated; data not shown). Analysis of downstream signaling components showed a time- and concentration-dependent inhibition of both phospho-Akt and phospho-Erk1/2, revealing coinhibition of the MAPK pathway in contrast to the PI3K selectivity of NVP-AEW541 (Fig. 6D). Apoptosis via PARP and caspase-3 cleavage (Fig. 6D) and a G1 cell-cycle arrest were also observed (Fig. 6E) in parallel with inhibition of PDGFRα/β by ELISA (Supplementary Fig. S4), albeit at relatively high doses of the compound.

Finally, we determined whether these combination effects observed in vitro would translate to the in vivo setting. We thus dosed female athymic nude mice bearing KNS42_A4 subline subcutaneous xenografts with either 50 mg/kg NVP-AEW541 alone (reduced from 75 mg/kg after day 11), 150 mg/kg imatinib alone, or both compounds in combination, and compared tumor growth with vehicle-treated controls. At these doses, final tumor concentrations of drug exceeded in vivo GI50 values in KNS42_A4 cells for both NVP-AEW541 (11.0 μmol/L, 1.5× GI50) and imatinib (38.6 μmol/L, 1.5× GI50). KNS42_A4 xenografts treated with both compounds showed significantly reduced tumor volumes starting day 10 posttreatment and maintained until sacrifice compared with either agent alone (P = 0.0265–0.0218, ANOVA; Fig. 7A). Final tumor weights showed a similar trend toward enhanced efficacy of the combination treatment (P = 0.0863, Student’s t test; Fig. 7B). Pharmacodynamic endpoints assayed by MSD showed specific downregulation of phospho-IGFIR by NVP-AEW541 (P < 0.0001, Student’s t test) in contrast to imatinib (Fig. 7C), although imatinib did cause a significantly greater reduction in phospho-IRS1 levels (P = 0.007, Student’s t test) than the IGFIR inhibitor (P = 0.0267, Student’s t test; Fig. 7D). The most profound parallel inhibition of the PI3K and MAPK pathways was achieved in the combination (P = 0.0002 and P < 0.0001, respectively, Student’s t test), with only modest effects observed.
Figure 4. Expression profiling of KNS42 cells treated with NVP-AEW541 highlights gene signatures associated with a PI3K-mediated cell-cycle arrest. A, heat map showing expression of genes associated with the G1–S-phase transition of the cell cycle in KNS42 cells treated with 5× GI50 NVP-AEW541 for 1, 6, or 24 hours. Genes are colored according to global, not relative, expression values. B, GSEA of NVP-AEW541–treated KNS42 cells identified numerous groups of genes associated with the cell cycle. Enrichment score plots show strong negative correlation (downregulation) with treatment with inhibitors given. C, gene ontology analysis using the DAVID bioinformatics tool. A decrease in expression of numerous genes associated with processes such as cell-cycle control and DNA replication are seen in KNS42 cells treated with NVP-AEW541. Bars represent fold enrichment of the most significant gene ontology groups, colored by Bonferroni-corrected P values. D, Connectivity Map analysis of NVP-AEW541–induced gene signatures in KNS42 cells. The bar view is constructed from 6,100 horizontal lines representing individual treatment instances ordered by their corresponding enrichment with the NVP-AEW541 signature (positive, green; negative, red; no enrichment, gray). All instances involving the PI3K inhibitors LY294002 and wortmannin are colored black, with corresponding highest scoring treatment instances given in the table.
on phospho-Akt by either compound alone \( (P = 0.162, \text{NVP-AEW541} \) and \( P = 0.0496, \text{imatinib} \), Student’s \( t \) test; Fig. 7E), and only imatinib showing inhibition of phospho-Erk1/2 \( (P < 0.0001, \text{Student’s} \ t \) test; Fig. 7F).

Discussion

Pediatric high-grade glioma, like the disease in adults, is in clear need of novel therapeutic strategies. Early clinical trials with RTK inhibitors directed against EGFR and PDGFR have thus far shown little efficacy \( (4–6) \), although it is not known how much of this is due to poor tumor penetration or inappropriate patient selection. What is clear however, from the experience in numerous other tumor types, is that monotherapy using these selective kinase inhibitors is ultimately unlikely to prove effective due to inherent and/or acquired resistance mechanisms \( (40–42) \). In glioblastoma, one form of resistance which may be either inherent or acquired is the presence of coactivated RTKs, such as EGFR and MET \( (43) \), and it has been suggested that combination treatments targeting these, amongst other kinases may bring more patient benefit \( (44–46) \).

In the childhood setting, in both the primary tumors and cell lines, we have noted the IGF signaling pathway to be a potential therapeutic target, with the presence of gene amplifications, and specific overexpression of the receptor (IGF1R), ligand (IGF2), or adaptor molecule (IR51). In our in vitro model systems, specifically targeting the receptor conveyed only a modest growth inhibitory effect despite constitutive activation of IGF1R. Identification of those additional RTKs that may also be constitutively activated in these pGBM cell lines has provided clues to design appropriate combination strategies to enhance IGF1R inhibitor treatment.

It is significant that we observed coactivation of PDGFR\( \alpha/\beta \) in the cells. PDGFR\( \alpha \) amplification is the...
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Figure 6. Coactivation of IGF1R and PDGFRα/β in pGBM cells and inhibition of both PI3K and MAPK pathways by imatinib. A, phosphorylated RTK assay shows coactivation of IGF1R with PDGFRα in KNS42 cells and PDGFRβ in SF188. B, Western blot confirmation of wild-type protein expression of PDGFRα/β in KNS42 and SF188 cells, respectively, as well as activation of both PI3K and MAPK pathways upon stimulation with ligand PDGF-BB. C, median-effects analysis of combining NVP-AEW541 and imatinib on high-grade glioma cells in vitro. KNS42 cells showed a highly synergistic interaction (CI = 0.44), whereas an additive effect was observed for SF188 (CI = 1.0). D, effects on downstream signaling in KNS42 and SF188 cells after treatment with imatinib in the presence of PDGF-BB. Treatment with imatinib decreased phospho-Akt and phospho-Erk1/2 and induced PARP and caspase-3 cleavage in a time- and concentration (1 x, 3 x, 5 x GI50)-dependent manner. At 48 hours, the highest concentrations of compound resulted in a significant cell death with little protein recoverable. E, effects on cell cycle in KNS42 and SF188 cells treated with imatinib. An accumulation of cells in G1 and sub-G1 phases was induced by imatinib in a time- and concentration-dependent manner. At 48 hours, the highest concentrations of compound resulted in a significant cell death.

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most common genomic alteration seen in pediatric high-grade glioma (7–11), and we have shown a specific PDGFRA-driven transcriptional program to be active in these tumors even in the presence of normal gene copy number (10). We have also noted less frequent alterations targeting the ligands PDGFA, PDGFB, and the receptor PDGFRB (10, 11). In pGBM, PDGF/IGF dysregulation may cosegregate, showing that the co-RTK activation observed in our models has clinical relevance. In fact, this mechanism of PDGFR coactivation with RTKs in pediatric high-grade glioma may be a more general mechanism, as we have previously noted in the small subgroup of childhood cases to harbor the EGFRvIII deletion mutation, which notably included anaplastic oligodendroglioma and gliosarcoma samples (36). Treatment with a PDGFR inhibitor alone was ineffective in our models, and only in combination with IGF1R inhibition did we observe efficacy in vitro and in vivo. Of note, greater combinatorial efficacy was observed with activation on PDGFRα than PDGFRβ or c-Kit. By exploring the mechanisms of action of the small molecule inhibitors targeting the specific receptors, we have been able to identify the key roles played by the different components of downstream signaling pathways in this synergistic interaction.

Numerous lines of evidence point to NVP-AEW541 acting highly specifically as an inhibitor of PI3K in the pGBM cells. As well as observing downregulation of phospho-Akt and G1 arrest, gene expression signatures showed a high degree of overlap with published data on PI3K inhibitors and the Connectivity Map database; we further showed the induction of autophagy, a known consequence of PI3K inhibition in U87MG glioma cells (39). Our previous report of a differential combinatorial response to PI3K/TMZ in pGBM cells (38) was replicated with NVP-AEW541, presumably at
least in part through inhibition of high HOX gene expression in KNS42, but not SF188 cells, a process known to be controlled by a PI3K-mediated epigenetic mechanism (47).

NVP-AEW541 elicited an adaptive upregulation of MAPK signaling at the highest doses and longest drug exposure times in the presence of the biologically relevant ligand IGF2. This is a potential source of the limited in vitro efficacy of the IGF1R inhibitor, as MAPK has been reported to contribute to a negative regulatory feedback loop promoting IGF-driven Akt activation via Erk/IRS1 (48), which may counterbalance the inhibition through PI3K. Imatinib, on the other hand, inhibited both PI3K and MAPK pathways equally in the presence of PDGF-BB, and it appears that it is this dual inhibition which is of key importance, resulting in a synergistic interaction in vitro and in vivo when the 2 small molecules were combined in cells with constitutive coactivation of IGF1R and PDGFRa.

It is notable that in our subcutaneous xenografts experiments, as well as the specific inhibition of phospho-IGF1R and phospho-Erk with NVP-AEW541 and imatinib alone, there was enhanced downregulation of both phospho-Akt and phospho-IRS1 in the combination treatments.

As we move toward combinatorial targeted therapies in high-grade glioma of all ages, such data provide a preclinical rationale not only for the use of agents targeting coactivated RTKs themselves but also for the addition of a PI3K or MEK inhibitor to the anti-PDGFR/IGF1R compounds. In the case of adding a PI3K inhibitor to anti-PDGFR therapy, this may obviate any concerns harbored about side effects from coinhibiting the structurally homologous insulin receptor, whereas the addition of an anti-MEK compound to agents targeting IGF1R may be more clinically effective than the disappointing results of inhibitors of PDGFR itself. It is worth noting that neither of the small molecule signaling inhibitors used in the present study crosses the blood–brain barrier, a further key consideration when developing novel strategies to treat these highly malignant central nervous system tumors.

Disclosure of Potential Conflicts of Interest

S. Jeay and F. Hofmann are employees of Novartis.

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subtypes of glioblastoma characterized by abnormalities in PDGFRα, ID1H, EGFR, and NF1. Cancer 2010;17:98–110.


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Enhanced Efficacy of IGF1R Inhibition in Pediatric Glioblastoma by Combinatorial Targeting of PDGFR α/β

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