Cooperation between c-Met and Focal Adhesion Kinase Family Members in Medulloblastoma and Implications for Therapy

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Abstract

We previously showed the involvement of the tyrosine kinase receptor c-Met in medulloblastoma malignancy. The nonreceptor tyrosine kinases focal adhesion kinase (FAK) and Pyk2 are key players in the progression of different cancers. However, their role in medulloblastoma malignancy is not well understood. In this study, using a protein array approach, we found that c-Met induces FAK and Pyk2 phosphorylation in medulloblastoma cells. We therefore studied the interactions between c-Met and FAK/Pyk2 and their implications for medulloblastoma therapy. We found that c-Met activates FAK and Pyk2 in several medulloblastoma cell lines. We also found that FAK and Pyk2 mediate the malignant effects of c-Met on medulloblastoma cell proliferation, migration, and invasion. On the basis of these findings, we hypothesized that combined c-Met and FAK inhibitions would have additive effects on the inhibition of medulloblastoma malignancy. To test this hypothesis, we assessed the effects on medulloblastoma malignancy parameters of single or combined treatments of medulloblastoma cells with c-Met and FAK small-molecule kinase inhibitors. We found a significant increase in the inhibitory effect of both inhibitors on medulloblastoma cell migration and cell invasion as compared with single inhibitions (P < 0.05). In addition, oral gavage treatment with c-Met inhibitor of mice bearing medulloblastoma xenografts significantly reduced in vivo tumor growth. Therefore, combining c-Met inhibitors with FAK inhibitors constitutes a new potential strategy for medulloblastoma therapy. Altogether, our study describes a role for FAK and Pyk2 in medulloblastoma malignancy, uncovers new interactions between c-Met and FAK/Pyk2, and proposes for the first time combining anti-c-Met and anti-FAK inhibitors as a new strategy for medulloblastoma therapy. Mol Cancer Ther; 11(2); 288–97. ©2011 AACR.

Introduction

Medulloblastoma is the most common brain tumor in children with an incidence of 0.6 per 100,000 patient-years according to the Central Brain Tumor Registry of the United States. It is an embryonal brain tumor that arises in the cerebellum, where it is thought to originate from primitive pluripotent precursor cells of the ventricular zone and cerebellar external germinal layer (1). Multiple signaling pathways have been associated with medulloblastoma formation and growth. These include the developmental pathways Hedgehog (Hh), Notch, and Wnt as well as the receptor tyrosine kinases (RTK) erbB2, insulin-like growth factor receptor (IGF-R), and TrkC, and the oncprotein Myc (2).

Our laboratory recently showed the involvement of the receptor tyrosine kinase c-Met and its ligand hepatocyte growth factor (HGF) in medulloblastoma malignancy (3). Inappropriate activation of the HGF/c-Met signaling pathway has been shown to be involved in the etiology of various human cancers including brain tumors, conferring them with invasive and metastatic properties (2, 4, 5). On the basis of the widespread and profound involvement of c-Met in cancer, several c-Met pathway inhibitors have been recently developed. These include ribozymes, HGF kringle variants/NK4, decoy receptors, HGF or c-Met neutralizing antibodies, and small-molecule kinase inhibitors (4, 6, 7). One such small-molecule kinase inhibitor, PF-2341066, was recently identified as a potent, orally available, ATP-competitive, and selective inhibitor of the catalytic activity of the c-Met receptor (8). PF-2341066 strongly inhibits c-Met phosphorylation and signal transduction, as well as c-Met oncogenic phenotypes of tumor cells and endothelial cells, and exerts its cytoreductive effect through antiproliferative and antiangiogenic mechanisms in different cancers (9).
The small-molecule c-Met kinase inhibitor PF-2341066 (siRNA)/control-siRNA, FAK-siRNA, and Pyk2-siRNA from Sigma-Aldrich. The scrambled short interfering RNA R&D systems. Human type IV collagen was from Sigma-Aldrich. The scrambled short interfering RNA R&D systems. Human type IV collagen was from Sigma-Aldrich.

The nonreceptor tyrosine kinases, focal adhesion kinase (FAK) and the proline-rich tyrosine kinase-2 (Pyk2), have emerged as key players in the progression of different cancers. FAK and Pyk2 are important signaling effectors linking integrins and growth factor signaling to cell adhesion, invasion, proliferation, migration, survival, and apoptosis in many cancers (10). Similar to FAK, which undergoes autophosphorylation at the tyrosine residue, autophosphorylation of Pyk2 at residue leads to the recruitment of Src family kinases, activation of extracellular signal–regulated kinase (ERK), regulation of ion channels, cell adhesion, and motility (11). FAK expression and/or phosphorylation is elevated in a variety of cancers and frequently correlates with malignant or metastatic disease and poor patient prognosis (12). Many studies have shown the association between FAK expression and malignancy grade, angiogenesis, invasion, and migration in gliomas (13–15). However, their role in invasive medulloblastoma is not well understood. Recently, a novel small-molecule FAK inhibitor, PF-573228 (FAKi), 3,4-dihydro-6-[4-[[3(methylsulfonyl)phenyl methyl]amino]-5-(trifluoromethyl)-2-pyrimidinyl]amino] (1H) quinolinone, was purchased from Tocris Bioscience. The chemical structures of PF-2341066 and PF-573228 are shown in Fig. 1A. Panorama antibody microarray cell signaling kit, Cy3 and Cy5 fluorescence dyes, and all chemicals and solvents were from Sigma-Aldrich.

**Protein microarrays**

Protein arrays were conducted to identify cell signaling molecules that are modulated by HGF in medulloblastoma cells. The Panorama antibody microarray contains 336 antibodies representing cell signaling molecules in their total and active (usually phosphorylated) forms. The antibodies are printed in duplicates on 4 × 8 grids. Each grid contains 7 antibody duplicates plus a Cy3- and Cy5-conjugated bovine serum albumin (BSA) positive control as well as a nonlabeled BSA negative control resulting in a total of 512 spots. ONS-76 medulloblastoma cells were treated with HGF (20 ng/mL) for 30 minutes and untreated cells were used as a control. The cells were processed for the microarray hybridization according to the manufacturer’s instruction. Briefly, proteins were extracted by an extracting/labeling buffer containing Benzonase, protease inhibitors, and phosphatase inhibitors. One milligram of protein extract from HGF-treated and nontreated cells was labeled with Cy3 or Cy5, respectively, according to the manufacturer’s instructions and used at dye/protein ratio >2. Free nonincorporated Cy3 and Cy5 dyes were separated by applying the labeled extracts to SigmaSpin Post- Reaction Clean-Up Columns. An equal amount of labeled protein of both extracts (10 µg/mL) was incubated on the Panorama Ab Microarray slide for 30 minutes; all washes were done in PBS-Tween 0.05%. The slides were air-dried before scanning with the GSI Lumonics scanner, and images were generated with the Panorama software (GSI Lumonics). The slide scanning was conducted using a microarray scanner (ProScanArray Scanner, Perkin Elmer). The results were analyzed using standard median normalization between both channels Cy3 and Cy5 using the “ScanArray Express Software.” All experiments were carried out in triplicates.

**Immunoblotting**

Immunoblotting was conducted using antibodies specific for phosphorylated and nonphosphorylated forms of FAK, Pyk2 (Cell Signaling), and β-actin (Santa Cruz Biotechnologies), the loading control. To assess the effects of HGF on FAK and Pyk2 activations, D425, DAOY, and ONS medulloblastoma cells were treated with 20 ng/mL HGF for various time points (5 minutes to 6 hours) and subsequently immunoblotted for phospho-FAK on

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**Materials and Methods**

**Cell culture and reagents**

Three human medulloblastoma cell lines were used for this study. DAOY and ONS-76 were grown in RPMI-1640 media supplemented with 10% FBS. D425 cells were grown in Improved Modified Eagle Medium Zinc option and 20% FBS. HGF-overexpressing DAOY cells (DAOY-HGF) were generated in our laboratory and were cultured in Improved Modified Eagle Medium Zinc option, supplemented with 10% FBS and the selection antibiotic Zeocin (1 µg/mL; ref. 17). All cells were grown at 37°C in 5% CO2. HGF was purchased from R&D systems. Human type IV collagen was from Sigma-Aldrich. The scrambled short interfering RNA (siRNA)/control-siRNA, FAK-siRNA, and Pyk2-siRNA were purchased from Sigma-Aldrich. Oligofectamine and SDS-PAGE were from Invitrogen. The small-molecule c-Met kinase inhibitor PF-2341066 (METi), also clinically known as crizotinib, (R)-3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-[1-piperidin-4-yl-1H-pyrazol-4-yl]-pyridin-2-ylamin, was from Pfizer. The FAK inhibitor PF-573228 (FAKI), 3,4-dihydro-6-[4-[[3(methylsulfonyl)phenyl methyl]amino]-5-(trifluoromethyl)-2-pyrimidinyl]amino] (1H) quinolinone, was purchased from Tocris Bioscience. The chemical structures of PF-2341066 and PF-573228 are shown in Fig. 1A. Panorama antibody microarray cell signaling kit, Cy3 and Cy5 fluorescence dyes, and all chemicals and solvents were from Sigma-Aldrich.

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different phosphorylation sites Tyr\textsuperscript{397}, Tyr\textsuperscript{576/577}, Tyr\textsuperscript{925}, Ser\textsuperscript{910}, or phospho-Pyk2 on Tyr\textsuperscript{402} site. The cells were subsequently lysed with radioimmunoprecipitation assay (RIPA) buffer (1% Igepal, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) and lysates were collected and cleared by centrifugation. The protein concentration of the supernatant was determined, equal amounts of protein were electrophoretically separated in PAGE, and then transblotted to a nitrocellulose membrane. The membrane was then incubated overnight with primary antibodies at 4°C. The primary antibody-bound membranes were washed 3 times with Tween-PBS before incubation with the corresponding horseradish-conjugated secondary antibodies. After a final wash, the immunoreactive signals were detected by enhanced chemiluminescence and quantified by densitometry on film using computer-assisted image analysis.

siRNA silencing

FAK-siRNA and Pyk2-siRNA were used to knockdown FAK and Pyk2 expressions in DAOY and D425 cells. Cells were transfected with either FAK-siRNA or Pyk2-siRNA using Oligofectamine transfection reagent according to the manufacturer’s instructions. Random/scrambled siRNA (cont-siRNA) was used as a control. The knockdown of FAK and Pyk2 was confirmed by immunoblotting using the FAK and Pyk2 antibodies as described above.

Cell proliferation assays

DAOY and D425 cells were seeded in triplicates, in medium containing 10% FBS and treated with either FAK-siRNA or Pyk2-siRNA (35 nmol/L). Controls were treated with cont-siRNA (35 nmol/L). Twenty-four hours later, the media were changed to low serum media (FBS 0.1%), and cells were treated with HGF (20 ng/mL). The cells were cultured for 48 hours, and cell counts were performed using a haemocytometer.
were trypsinized and harvested every day, for 4 days, counted with a hemocytometer, and growth curves were established.

**Migration assays**

Wound/scratch assay was conducted as previously described (18). DAOY cells were seeded at 80% confluence in low serum media for 24 hours. The cells were treated with 35 nmol/L FAK-siRNA, Pyk2-siRNA, or cont-siRNA and 24 hours later treated with 20 ng/mL HGF. The cells were assessed for migration using the scratch assay. Cells that migrated into the scratch 24 hours posttreatment were photographed at ×40 magnification.

**Invasion assays**

Transwell invasion assays were conducted to determine whether FAK and/or Pyk2 mediate the effects of c-Met on medulloblastoma cell invasion. Modified Boyden Chambers (Becton Dickinson) were coated with human type IV collagen (250 mg/mL). DAOY cells (2 × 10⁴) were treated with 35 nmol/L FAK-siRNA, Pyk2-siRNA, or cont-siRNA for 8 hours and then exposed to 20 ng/mL HGF for 24 hours. The following day, the cells were resuspended in serum-free media and added to each insert in the presence or absence of HGF 20 ng/mL. Six hundred microliters of 10% FBS medium was placed in the lower chamber as a chemoattractant. After 8 hours of incubation at 37°C in 5% CO₂, invading cells were stained with 0.1% crystal violet solution and photographed at 40×. The cells were then counted under a microscope in 5 randomly chosen fields.

**Pharmacologic inhibition of FAK and c-Met**

METi was dissolved in sterile water, aliquoted, filtered and stored in the dark at room temperature until use. FAKi was prepared in sterile dimethyl sulfoxide, filtered, aliquoted, and stored at −80°C until use. DAOY cells or DAOY-HGF clones were treated with either FAK inhibitor FAKi (1 μmol/L), c-Met inhibitor METi (100 nmol/L), a combination of both inhibitors, or control vehicle for 24 hours and subsequently treated with 20 ng/mL HGF for 24 hours for cell migration and for 6 hours for cell invasion. The cells were assessed for migration or invasion, as described above. FAK and c-Met inhibitions, as well as p-FAK and p-c-Met inhibitions, were verified by immunoblotting. Signals were quantified by densitometry and the quantification results were reported for each Western blot analysis. For proliferation, DAOY cells and DAOY-HGF clones were seeded in 6-well plates, treated with FAKi or METi or combination of both inhibitors for 24 hours at the same concentrations listed above, and subsequently treated with HGF (20 ng/mL) for 24 hours. Cell growth was monitored by counting the cells for 4 days, and proliferation curves were established. The effects of single treatments on cell migration, invasion, and proliferation were compared with the effects of combined inhibitor treatment.

**In vivo xenograft experiments**

METi was dissolved in sterile water (stock 0.5 mol/L), filtered and stored in the dark at room temperature. Medulloblastoma DAOY-HGF cells (2 × 10⁶) were implanted in the flanks of immunodeficient mice (n = 10). METi treatment started 5 days posttumor implantation. The animals were treated by oral gavage of 0.1 mL of 25 nmol/L METi solution (30 mg/kg body weight) every day for 3 weeks. Control animals were treated with an equal volume of sterile distilled water. The flank tumors were measured with a caliper every day for 2 weeks. At the end of the treatment, the animals were euthanized and the tumors were removed, weighed, and a tumor volume progression curve was established.

**Statistics**

All experiments were carried out at least in triplicates. Numerical data were expressed as mean ± SD. Two group comparisons were analyzed by 2-sided Student t test. Multiple group comparisons were analyzed with Bonferroni/Dunn multiple comparisons tests. P Values were determined for all analyses and P < 0.05 was considered significant.

**Results**

**c-Met activates FAK and Pyk2 as revealed by protein arrays**

We assessed the effects of c-Met activation on global cell signaling in medulloblastoma cells using antibody microarrays containing 336 cell signaling molecule probes in active and total states. We found numerous changes in the activation of signal transduction proteins in response to HGF. Many of these changes were known and expected such as the induction of cyclin D1, cyclin A, and phospho-Akt by HGF. Few other changes showed effects of HGF that had not been described before in medulloblastoma (Supplementary Table S1). One such novel and interesting effect of HGF was the phosphorylation of FAK at Tyr²⁷⁷ and the phosphorylation of the FAK-related protein tyrosine kinase (Pyk2) at Tyr²⁷⁷ (Supplementary Table S1). Interestingly, both proteins are related to each other and play critical roles in cell migration and invasion in other cancers. We therefore hypothesized that c-Met-induced tumor cell invasion and migration could be mediated by FAK and/or Pyk2.

**c-Met activates FAK and Pyk2 in medulloblastoma cells in a time-dependent manner**

To confirm that c-Met activates FAK and/or Pyk2 as observed on the protein arrays, the effect of HGF on FAK and Pyk2 phosphorylations in medulloblastoma cells was assessed by immunoblotting. D425, DAOY, and ONS-76 medulloblastoma cells were treated with 20 ng/mL HGF for various time points (5 minutes to 6 hours) and subsequently immunoblotted for phospho-FAK Tyr²⁷⁷, phospho-FAK Tyr²⁷⁷/³⁰⁷, phospho-Pyk2 Tyr²⁷⁷/³⁰⁷, and phospho-Pyk2 Ser²⁰². HGF treatment led to FAK...
and Pyk2 phosphorylations at multiple sites in all medulloblastoma cells that were examined. c-Met activation with HGF induced a strong phosphorylation of FAK at Tyr397 and Tyr925, which are important for the maximal adhesion-induced activation of FAK and signaling to downstream effectors. c-Met activation with HGF also led to phosphorylation of FAK at Ser910, which is involved in modulating binding/stability of downstream signaling proteins, and phosphorylation of FAK at Tyr576/577, which is strongly associated with migration. Similarly, cell treatment with HGF resulted in a strong and fast phosphorylation of Pyk2 at Tyr402 reported to promote invasion and migration. Phosphorylations started between 5 and 15 minutes after HGF treatment and lasted for several hours (Fig. 1B). Phosphorylations of all amino acids were not detected in all cell lines (not shown). Overall, the above data show that c-Met activates FAK and Pyk2 in a time-dependent manner.

FAK and Pyk2 mediate c-Met–induced cell proliferation in medulloblastoma cells

To determine whether FAK and/or Pyk2 mediate c-Met–dependent cell growth, we assessed the effects of c-Met activation on DAOY and D425 medulloblastoma cell proliferation in the setting of silenced FAK or Pyk2. FAK-siRNA, Pyk2-siRNA, or scrambled control siRNA (cont-siRNA) were transfected into the cells to inhibit FAK and Pyk2 expressions. After 24 hours, the

Figure 2. FAK and Pyk2 mediate the effects of c-Met on medulloblastoma cell proliferation. FAK and Pyk2 expressions were silenced in DAOY and D425 cells by transfection with FAK-siRNA (35 nmol/L) or Pyk2-siRNA (35 nmol/L), respectively. Control cells were transfected with 35 nmol/L scrambled siRNA (Cont-siRNA). After 24 hours, the cells were treated with 20 ng/mL HGF and counted every day for 4 days. The results show that silencing of FAK or Pyk2 significantly decreases basal and c-Met–induced cell growth. D425-FAK silencing (A), D425-Pyk2 silencing (B), DAOY-FAK silencing (C), and DAOY-Pyk2 silencing (D). E, immunoblots show FAK and Pyk2 knockdown in cells treated with respective siRNA as described above. Results are representative of 3 different experiments.
cells were treated with 20 ng/mL HGF for 24 hours and cell proliferation was analyzed by cell counting over 4 days. FAK and Pyk2 silencing was verified by immunoblotting.

FAK knockdown led to significant inhibition of c-Met–dependent cell proliferation in D425 medulloblastoma cells. FAK knockdown decreased cell numbers at day 4 from 151 ± 9.29 to 36.33 ± 4.48 in HGF-untreated cells (n = 3; P < 0.05). FAK knockdown decreased HGF-induced cell numbers at day 4 from 309 ± 14.57 to 81.33 ± 9.56 (n = 3; P < 0.05; Fig. 2A). Pyk2 knockdown led to significant inhibition of c-Met–dependent cell proliferation in D425 medulloblastoma cells. At day 4 of counting, Pyk2 knockdown reduced cell numbers from 151 ± 9.29 to 18.33 ± 3.18 in HGF-untreated cells (n = 3; P < 0.05). Pyk2 knockdown decreased HGF-induced cell numbers at day 4 from 309 ± 14.57 to 103.00 ± 8.39 (n = 3; P < 0.05; Fig. 2B). FAK silencing also led to significant inhibition of c-Met–dependent cell proliferation in DAOY cells. FAK knockdown decreased cell numbers at day 4 from 105.33 ± 5.70 to 18.08 ± 3.02 in HGF-untreated cells (n = 3; P < 0.05). FAK knockdown decreased HGF-induced cell growth at day 4 from 132.00 ± 7.23 to 61.50 ± 2.41 (n = 3; P < 0.05; Fig. 2C). Pyk2 silencing led to significant inhibition of

Figure 3. FAK and Pyk2 mediate the effects of c-Met on medulloblastoma cell migration and invasion. FAK and Pyk2 expressions were silenced in DAOY and D425 cells by transfection with FAK-siRNA (35 nmol/L) or Pyk2-siRNA (35 nmol/L), respectively. Control cells were transfected with 35 nmol/L scrambled siRNA (Cont-siRNA). After 24 hours, the cells were treated with 20 ng/mL HGF and subsequently assessed for migration (A) and invasion (B) using the scratch assay and the Boyden chamber assay, respectively. The results show that silencing of FAK or Pyk2 significantly decreases basal and c-Met–induced migration and invasion in medulloblastoma cells. The immunoblots at the bottom show the knockdown of FAK and Pyk2 in DAOY cells treated as described above.
c-Met–dependent cell proliferation in DAOY cells. At day 4 of counting, Pyk2 knockdown reduced basal cell numbers from 105.33 ± 5.70 to 15.42 ± 0.87 in HGF-untreated cells (n = 3; P < 0.05). Pyk2 knockdown decreased HGF-induced cell numbers at day 4 from 132.00 ± 7.23 to 52.50 ± 1.84 (n = 3; P < 0.05; Fig. 2D). These data show that FAK and Pyk2 induce cell proliferation and mediate c-Met–dependent cell proliferation in medulloblastoma cells.

**FAK and Pyk2 mediate c-Met–induced migration and invasion of medulloblastoma cells**

To determine whether FAK and/or Pyk2 mediate c-Met–dependent cell migration and invasion, we assessed the effects of c-Met activation on DAOY medulloblastoma cell proliferation in the setting of inhibited FAK or Pyk2 expressions. We tested the effect of FAK and Pyk2 knockdown on basal and HGF-induced cell migration and invasion using the scratch assay and Transwell invasion assay, respectively. DAOY cells were treated with FAK-siRNA and FAK knockdown was confirmed by immunoblotting. The cells were then treated with 20 ng/mL HGF and invasion was quantified. HGF induced DAOY cell invasion in control (Fig. 3A). FAK and Pyk2 silencing significantly decreased HGF-induced cell invasion (Fig. 3A). Similarly, HGF induced DAOY cell migration in control but significantly less in FAK-siRNA or Pyk2-siRNA pretreated cells (Fig. 3B). These data show that FAK and Pyk2 mediate c-Met–dependent migration and invasion in medulloblastoma cells.

**Combined inhibition of c-Met and FAK with small-molecule kinase inhibitors inhibits medulloblastoma cell migration and invasion more than single inhibitions**

On the basis of the above results showing that c-Met activates FAK and Pyk2 and that FAK and Pyk2 mediate the effects of c-Met in medulloblastoma cells, we hypothesized that combined inhibition of c-Met and FAK could have greater inhibitory effects on medulloblastoma malignancy than single inhibitions. To test this hypothesis, we assessed the effects of small-molecule kinase inhibitors of c-Met (METi) and FAK (FAKi) on medulloblastoma cell migration, invasion, and proliferation. We first verified the inhibitory effects of the small molecules on c-Met and FAK activation. DAOY and DAOY-HGF cells were treated with 100 nmol/L METi and/or 1 μmol/L FAKi for 24 hours. METi strongly inhibited HGF-induced c-Met phosphorylation in DAOY and DAOY-HGF cells (Supplementary Fig. S1). FAKi inhibited HGF-induced FAK phosphorylation in DAOY and DAOY-HGF cells (Supplementary Fig. S1). METi did not significantly affect FAK phosphorylation suggesting that FAK is additionally...
activated by factors other than c-Met (Supplementary Fig. S1).

We then assessed the effects of single and combined c-Met and FAK inhibitions on cell migration, invasion, and proliferation using a scratch assay, a Transwell invasion assay, and cell counting, respectively. Single METi and FAKi treatments inhibited basal and HGF-induced migration of DAOY and DAOY-HGF cells. Combined METi and FAKi treatments had greater inhibitory effect on medulloblastoma cell migration and invasion than single inhibitions (Fig. 4). FAKi reduced the number of DAOY-HGF invading cells through the collagen IV matrix from 80.6 ± 10.5 to 40.33 ± 5.77. METi reduced the number of DAOY-HGF invading cells through the collagen IV matrix, from 80.6 ± 10.5 to 29 ± 2.64. The combined effect of both inhibitors significantly decreased the number of invading cells to 10.33 ± 2.08 (P < 0.01; Fig. 5). These data suggest that combining anti-c-Met and anti-FAK approaches might have experimental therapeutic advantage in medulloblastoma. However, such additive effect could not be detected on cell growth in either DAOY or DAOY-HGF cells (Supplementary Fig. S2).

Oral delivery of METi inhibits the in vivo growth of human medulloblastoma xenografts

Our original goal was to test the combined effects of METi and a clinically applicable FAK inhibitor on in vivo medulloblastoma growth. However, the clinically applicable FAK inhibitor is not commercially available and we were not able to obtain it from the manufacturing pharmaceutical company. We therefore assessed the effects of in vivo c-Met inhibition on medulloblastoma xenograft growth.

To test the in vivo antitumor properties of METi, we generated medulloblastoma xenografts by implantation of DAOY-HGF medulloblastoma cells in the flanks of immunodeficient mice. Tumor-bearing animals were treated by oral delivery of METi via gavage for 3 weeks. While control animals developed very large tumors averaging 68.08 ± 9.794 mm³ in volume, the METi-treated group showed a significant inhibition of tumor growth averaging 22.806 ± 2.483 mm³ (n = 10, P < 0.01; Fig. 6). No obvious drug toxicity was observed during the treatment. For the first time, these data show that inhibition of c-Met kinase by a small-molecule inhibitor leads to inhibition of in vivo medulloblastoma tumor growth. The data also show the feasibility of oral delivery of small-molecule kinase inhibitors of c-Met and suggest that these molecules can be combined with FAK inhibitors for improved future medulloblastoma therapies.

Discussion

Our study shows for the first time that c-Met activates FAK and Pyk2 and that FAK and Pyk2 mediate the effects of c-Met in medulloblastoma. The study also establishes a role for FAK and Pyk2 in medulloblastoma malignancy. Furthermore, our study shows for the first time the anti-oncogenic effects of small-molecule kinase inhibitors of c-Met and FAK in medulloblastoma and suggests that combined targeting of c-Met and FAK can be advantageous for medulloblastoma therapy.

Medulloblastoma is the most common malignant brain tumor of childhood arising in the cerebellum (19, 20). It has the highest rates of metastasis outside the nervous system (21–23) and tends to spread hematogenously into bones, bone marrow, lymphatic nodes, liver, and lungs (24, 25). To date, surgery and radiation therapy remain the most effective treatment for medulloblastoma. However, craniospinal radiotherapy...
shown the involvement of integrin β1/FAK signaling in migration and invasion in medulloblastoma (26). Few small-molecule inhibitors of FAK have been developed. PF-562271 has shown a highly selective and potent pharmacologic inhibitory effect on FAK catalytic activity, allowing it to be a first-in-class inhibitor reaching clinical trial testing for the cancer therapy (27). Unfortunately, this inhibitor is not commercially available and we were unable to obtain it from the manufacturer. We therefore used the commercially available PF-573228, which cannot be used for in vivo animal experimentation (16). Our study shows for the first time that FAK kinase activity and FAK-associated migration and invasion are inhibited by PF-573228. Therefore, FAK and Pyk2 inhibitions represent a potential novel strategy for medulloblastoma therapy.

Given the critical role of the receptor tyrosine kinase c-Met, and its ligand HGF in the progression of different human cancers including brain tumors, different approaches to inhibiting HGF and c-Met have been developed (4). Among these, the small-molecule kinase inhibitor of c-Met, PF-2341066, has reached phase I and II of clinical trials and therefore represents a promising therapeutic approach to inhibiting c-Met in cancer and medulloblastoma. Our study shows for the first time the usefulness of PF-2341066 in inhibiting c-Met activation and c-Met–dependent oncogenic effects in medulloblastoma cells and tumors.

Our study shows for the first time that HGF/c-Met activates FAK and Pyk2 in medulloblastoma. A connection between c-Met and FAK has been previously described in some cancers such as ovarian cancer (28), breast cancer (29, 30) but not in medulloblastoma. Furthermore, to date, no connection between c-Met and Pyk2 has been described in any cancer. Our findings suggest either a direct or an indirect FAK/Pyk2 activation by c-Met kinase. We used immunoprecipitation of FAK and c-Met upon HGF stimulation of medulloblastoma cells to explore the possibility of direct binding of FAK and c-Met. We could not detect any direct interaction between the 2 proteins (data not shown). The indirect interaction could occur via activation of scaffolding or key adaptor proteins known to mediate FAK effects such as paxillin and p130CAS (31, 32). Considering that the FAK-src complex mediates the phosphorylation of paxillin and p130CAS, and as c-Met is known to signal both upstream and downstream from c-Src, this latter is a possible candidate for mediating FAK activation by c-Met.

Considering that multiple molecular pathways are involved in growth, survival, migration, and invasion of tumor cells, antitumor activity could be improved by simultaneous use of individual agents targeting different pathways such as Raf/MEK/ERK or c-Met to allow vertical or horizontal inhibition of relevant pathways (33, 34). Such simultaneous targeting would have to be based on molecular findings indicating cooperation, redundancy, or compensatory mechanisms involving the targeted molecules. Our study suggests for the first time that combining c-Met and FAK/Pyk2 inhibitors as another approach for achieving improved antitumor effects in medulloblastoma. Our intent was to test this combination approach in vivo in a medulloblastoma animal model, but we were unable to obtain the only FAK inhibitor with proven bioavailability from the manufacturer. However, we did show for the first time the in vivo usefulness of a small-molecule inhibitor of c-Met on medulloblastoma tumor growth in paving the road for a potential testing of combined anti-Met/anti-FAK therapy in medulloblastoma.

Figure 6. Oral delivery of METi significantly inhibits the in vivo growth of human medulloblastoma xenografts. Human medulloblastoma xenografts were generated by flank implantation of 2 × 10⁶ HGF-DAOY cells in immunodeficient mice. Five days posttumor implantation, the animals were treated with METi by oral gavage (30 mg/kg body weight) once per day, for 3 weeks. The control group was treated with equal volume of sterile distilled water. At the end of the treatment, animals were sacrificed and tumor size was assessed by measuring tumor volume. The results show that METi treatment significantly inhibits in vivo medulloblastoma growth (P < 0.01).
Overall, our study uncovers previously unknown interactions between c-Met and FAK/Pyk2 in medulloblastoma and suggests that simultaneous targeting of these molecules might be advantageous for medulloblastoma therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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