Inhibition of the MET Receptor Tyrosine Kinase as a Novel Therapeutic Strategy in Medulloblastoma¹²³

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Abstract
Medulloblastoma is the most common pediatric posterior fossa malignancy, with a 5-year overall survival of only 60% and many survivors experiencing treatment-related morbidity secondary to current therapeutic regimens. With an improved understanding of the molecular basis for this disease, the opportunity to develop novel treatments with more tolerable toxicity profiles that target key molecular pathways, now exists. Recently, the hepatocyte growth factor (HGF)/MET signaling pathway has been implicated in medulloblastoma pathogenesis. Several therapeutic strategies targeting this pathway exist, including small molecule inhibitor therapy against the MET receptor tyrosine kinase. We examined the in vitro efficacy of targeting the MET receptor using the highly specific small molecule inhibitor PHA665752 as a novel treatment strategy in medulloblastoma. MET inhibition using PHA665752 was effective at reducing the proliferative capacity of the D283, ONS76, and MED8A medulloblastoma cell lines as assessed by MTS assay. Furthermore, PHA665752 treatment reduced D283 and ONS76 cell motility and impaired the growth of D283 cells in soft agar. Pretreatment of D283, ONS76, and MED8A cells with PHA665752 blocked exogenous recombinant human HGF-induced up-regulation of the downstream RAS/mitogen-activated protein kinase signaling pathway in D283, ONS76 and MED8A cell lines. Similarly, PHA665752 prevented HGF-induced phosphatidylinositol 3-kinase/AKT signaling in ONS76 and MED8A cells. These results highlight the efficacy of targeting the MET receptor tyrosine kinase therapeutically in medulloblastoma and provide support for further preclinical testing of small molecule inhibitors targeting the MET receptor in medulloblastoma.

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Introduction
Medulloblastoma (MB) is the most common malignant pediatric posterior fossa brain tumor, affecting young children with a peak incidence at 7 years [1–3]. Current therapy involves a combination of surgery, radiation, and conventional chemotherapeutic strategies—therapies with significant inherent risks of neurocognitive, endocrinologic, hematologic, and oncologic adverse effects [4–7]. Furthermore, current 5-year survival rates are approximately 60% despite aggressive therapy. An increased understanding of the molecular pathogenesis of this tumor has offered the promise of therapeutic strategies with improved toxicity profiles, targeting key signaling pathways implicated in MB formation or progression. The hepatocyte growth factor (HGF)/MET signaling pathway has been implicated in the pathogenesis of a variety of human malignancies including MB, and it represents a novel therapeutic target in this disease [8–13]. Several strategies aimed at limiting HGF/MET signaling are available, including ATP-competitive small molecule inhibitors of the MET receptor tyrosine kinase [14]. The use of small molecule MET inhibition has been evaluated in other human malignancies such as neuroblastoma; however, it has yet to be reported as a therapeutic strategy in MB [13]. Here, we have examined the in vitro efficacy of targeting the MET receptor using the highly specific small molecule inhibitor PHA665752 in medulloblastoma cell lines as assessed by MTS assay. MET inhibition using PHA665752 was effective at reducing the proliferative capacity of the D283, ONS76, and MED8A medulloblastoma cell lines. Pretreatment of D283, ONS76, and MED8A cells with PHA665752 blocked exogenous recombinant human HGF-induced up-regulation of the downstream RAS/mitogen-activated protein kinase signaling pathway in D283, ONS76 and MED8A cell lines. Similarly, PHA665752 prevented HGF-induced phosphatidylinositol 3-kinase/AKT signaling in ONS76 and MED8A cells. These results highlight the efficacy of targeting the MET receptor tyrosine kinase therapeutically in medulloblastoma and provide support for further preclinical testing of small molecule inhibitors targeting the MET receptor in medulloblastoma.

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efficacy of the MET-selective small molecule inhibitor PHA665752 (Pfizer, La Jolla, CA) against human MB.

Several strategies to block HGF/MET signaling have been explored, including interfering with ligand-receptor interaction, inhibition of MET tyrosine kinase activity, and disruption of MET interaction with downstream effector molecules [15]. Antagonism of the ligand-receptor interaction may be effective in tumors where MET signaling is driven in either a paracrine or an autocrine manner by HGF ligand. Strategies to block ligand-receptor binding have included the use of truncated or uncleavable HGF (NK2 [N-terminal hairpin, first two Kringle domains], NK4 [N-terminal hairpin, first four Kringle domains], uncleavable pro-HGF), neutralizing antibodies against HGF or the MET receptor, and decoy MET receptors [16–24]. Decay MET receptors also interfere with endogenous MET receptor dimerization and subsequent downstream signaling [14]. With respect to MB specifically, preclinical evidence demonstrating efficacy of the HGF-neutralizing monoclonal antibody L2G7 at improving the survival of mice with MBs induced by a combination of Sonic Hedgehog (SHH) and HGF compared with control antibody therapy exists [8,17]. MET signaling can be upregulated in a ligand-independent fashion through wild-type receptor overexpression or through activating translocation or mutation events. In such cases, strategies targeting the MET receptor may directly prove to be more efficacious.

One method of inhibiting MET receptor tyrosine kinase activity is to use small molecule inhibitor therapy. The effectiveness of small molecule inhibitor therapies has been demonstrated by the clinical success achieved with agents such as Gleevec for chronic myelogenous leukemia and gastrointestinal stromal tumors, Herceptin for human epidermal growth factor receptor 2–positive breast cancer, and Iressa for non–small cell lung cancer [25]. A small molecule inhibitor targeting the SHH pathway (HhAntag) has shown effectiveness in preclinical Patched-deficient murine models of MB [26]. Several small molecule inhibitors targeting the MET kinase have been developed, but their efficacy in MB has yet to be reported [27,28].

We have examined the effectiveness of MET kinase inhibition in vitro in MB using the small molecule inhibitor PHA665752. PHA665752 is an ATP-competitive small molecule inhibitor, which exhibits more than 50-fold selectivity for MET compared with other tyrosine and serine-threonine kinases [29]. It has been shown to inhibit MET-dependent functions including cell proliferation, migration, and branching morphogenesis. In cells that lack MET expression, PHA665752 treatment at concentrations of up to 18 μM has no biologic effect [29]. Its effectiveness has been demonstrated in animal xenograft models of small cell and non–small cell lung cancers, in which PHA665752 treatment reduced xenograft size and impaired tumor angiogenesis [30]. In a model of esophageal adenocarcinoma, PHA665752 treatment reduced cell viability and cell motility [31]. In this model, PHA665752 treatment was associated with reduced downstream MET-mediated mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathway activation [31]. In the current study, we show that anti-MET small molecule inhibitor therapy represents a novel targeted treatment strategy that warrants further investigation in preclinical models.

Materials and Methods

Cell Lines, Cell Culture, and PHA665752 Treatment

D283 MB cells were obtained from the American Type Culture Collection (ATCC, Rockland, MD). The ONS76 MB cell line was obtained from the Institute for Fermentation (Osaka, Japan). The MED8A MB cell line was obtained from Dr. R. Gilbertson (St Jude Children’s Research Hospital, Memphis, TN). The D283 cell line was initially isolated from a patient with metastatic MB [32]. ONS76 cell line was isolated from a MB from a 2-year-old girl [33]. MED8A was isolated from a patient younger than 2 years at the time of diagnosis [34]. Taken together, all three cell lines are from high-risk invasive MB disease.

D283 and ONS76 were maintained in culture in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS and 1× antibiotic/antimycotic solution. MED8A was cultured in DMEM supplemented with 20% FBS and 1× antibiotic/antimycotic solution. All cell lines were kept at 37°C, in 5% CO₂. Media and reagents for cell culture were purchased from Wisent, Inc (St Bruno, Quebec, Canada). The small molecule MET inhibitor PHA665752 was purchased from Tocris Bioscience (Ellisville, MO).

MTS Cell Proliferation Assay

The Promega CellTiter 96 AQueous One Solution Cell Proliferation Assay was used, as per the manufacturer’s instructions (Promega, Madison, WI). Cells were seeded into 96-well plates (1000 cells/well) in triplicate. Absorbance at 490 nm was measured 2 hours after the addition of 20 μl of MTS reagent/well, every 24 hours during a 96-hour period. PHA665752 or DMSO (vehicle control) was added to the media in respective wells at the time of cell plating. The cells were incubated in the presence of the inhibitor for the entire course of the experiment; however, the medium was not changed or supplemented with additional inhibitor.

Detection of Apoptosis through Production of Cleaved Caspase-3

A cleaved caspase-3 assay was performed with untreated cells and with cells treated with 2.5 μM PHA665752 for 12, 24, 36, or 48 hours. After treatment, cell lysates were prepared, and Western blot analysis was performed. Full-length intact caspase-3 or the cleaved caspase-3 product indicative of apoptosis pathway activation was detected with caspase-3 antibody (1:1000; Cell Signaling Technology, Beverly, MA). Jurkat cells untreated or treated with cytotoxome served as negative and positive lystate controls, respectively (Cell Signaling Technology).

Artificial Wound Healing Assay

An artificial wound healing assay was performed to assess cells’ migratory ability. Cell lines were grown to confluence in a medium containing 10% FBS, in triplicate in six-well plates. A uniform scratch defect (“wound”) was created across the monolayer using a p10 pipette tip. Wells were then washed with 1× phosphate-buffered saline, followed by the addition of serum-free medium containing either 2 μM PHA665752 or DMSO control. Plates were imaged immediately (T = 0 hour) and at 18 hours (T = 18 hours), and the degree to which cells at the wound margin had migrated in to close the initial defect was assessed.

Colony Formation in Soft Agar Assay

A base layer of 1.5% agar/2× DMEM/FBS was prepared in 35-mm plates. The upper-layer agar was made using 1.5% agar, 2× DMEM, FBS, and sterile water and was kept in the liquid phase in a 42°C water bath. Cells were resuspended in 0.75% agar/DMEM/FBS and overlaid on the base layer with 1000 cells/35-mm plate. PHA665752 or DMSO was added to both base- and upper-layer agars before plating. Once the agar had solidified, plates were overlaid with phosphate-buffered saline containing either PHA665752 or DMSO of the same concentration as in the agar layers. Plates were kept in a humidified chamber at 37°C for 2 weeks, stained with crystal violet, and digitally imaged. Each
cell line was plated in triplicate, and the data presented are representative of three separate experiments. Of the MB cell lines examined, only the D283 line grew readily under anchorage-independent conditions at baseline.

Western Blot Analysis

MB cell lines were cultured overnight in serum-free DMEM. Cells were then pretreated with either 2 μM PHA665752 or DMSO control for 3 hours, followed by the addition of 20 ng/ml of recombinant human HGF (Sigma-Aldrich, St Louis, MO) for 20 minutes before obtaining protein cell lysates with radioimmunoprecipitation assay buffer. Primary antibodies were used at a 1:1000 dilution, hybridized overnight at 4°C. Anti-AKT and anti-π-AKT antibodies were purchased from Cell Signaling Technology. Anti-ERK1/2 (extracellular regulated kinase 1/2) and anti-π-ERK1/2 antibodies were purchased from Santa Cruz Biotechnology.

Results

PHA665752-Mediated MET Inhibition Reduces MB Cell Proliferation In Vitro

Given that HGF/MET signaling activates downstream mitogenic effectors, we examined the effect of PHA665752 treatment on MB cell proliferation by MTS assay. The D283 MB cell line showed a dose-response effect with greater reduction in proliferative capacity as assessed by MTS assay during a 96-hour period with increasing doses of PHA665752 (range = 1.0–2.0 μM) compared with untreated or vehicle control (DMSO)–treated cells (Figure 1A). PHA665752 doses between 1.5 and 2.0 μM were particularly effective at limiting D283 cell growth. Similarly, both ONS76 and MED8A MB cells exhibited a dose-response effect of reduced cell proliferation in response to increasing dose of PHA665752 (range = 1.5–2.5 μM; Figure 1, B and C). The ONS76 cell line was sensitive to MET inhibition by PHA665752, with doses between 1.75 and 2.5 μM reducing cell proliferation (Figure 1B). In fact, ONS76 cells failed to proliferate when treated with doses greater than 2.0 μM. MED8A cells were sensitive to PHA665752 doses of 1.5 μM and greater and failed to proliferate in doses greater than 2.0 μM (Figure 1C).

It is possible that the PHA665752 is mediating its effect by activating apoptosis in the treated MB cells. Therefore, we checked for activation of the apoptotic pathway by Western blot analysis for cleaved caspase-3 in lysates from untreated or treated D283 or ONS76 cells. We observed no cleavage of the intact caspase-3 protein and no detection of the cleaved caspase-3 product that is indicative of apoptotic pathway activation (Figure W1).

PHA665752-Mediated MET Inhibition Reduces MB Cell Migration In Vitro

HGF/MET signaling is known to promote cell motility. The effect of MET inhibition on MB cell motility was assessed using an artificial wound healing assay. MET inhibition by PHA665752 reduced D283 MB cell motility in comparison to DMSO-treated controls, as evidenced by the impaired closure of an artificial wound at 18 hours after creation of the defect in a confluent D283 cell monolayer (Figure 2A). Similarly, ONS76 cells migrated more slowly with PHA665752 treatment, demonstrating complete closure of the defect at 18 hours in control-treated cells but persistence of a visible defect in the cell monolayer for PHA665752-treated cells (Figure 2B). The MED8A cell line was excluded from this experiment because there was a significant loss of cell adherence when plated in serum-free conditions when setting up the wound healing assay.

PHA665752-Mediated MET Inhibition Abrogates D283 Cell Capacity for Anchorage-Independent Growth

One key characteristic of transformed cells is the acquisition of the capability to proliferate in an anchorage-independent manner. We tested the effects of MET inhibition on MB cell capacity to evade anoikis using a colony formation assay in soft agar. Of the three MB cell lines examined, D283 MB cells possess the capacity to form colonies readily under conditions of anchorage-independent growth. When grown under anchorage-independent conditions, D283 MB cells treated with DMSO alone readily form macroscopically visible colonies after 2 weeks in culture (Figure 3). In comparison, when grown under similar conditions but in the presence of 1.5 μM PHA665752, macroscopic colonies fail to form (Figure 3). The experiment was repeated three times, and enumerated colonies between dose conditions were evaluated for statistical significance. Treatment with 1.5, 1.75, and 2 μM PHA665752 was determined to be statistically different compared with untreated (P = .0021).

Discussion

We observed a reproducible dose-response effect of PHA665752 treatment on reducing cell proliferation in vitro, across several MB cell lines examined (Figure 1). With increasing dose of PHA665752, D283 cells showed progressive impairment of cell proliferation as measured by the MTS assay (Figure 1A). ONS76 and MED8A cell lines also demonstrated a dose-response reduction in proliferative capacity.
Figure 1. MTS proliferation assay for D283 MB cells (A), ONS76 MB cells (B), and MED8A MB cells (C) treated with an increasing dosage of PHA665752, in comparison to untreated cells and DMSO vehicle control–treated cells. A dose-response effect was observed for each cell line, with an increasing dosage of PHA665752 resulting in a greater reduction in cell proliferation during the 96-hour culture.
in response to PHA665752-mediated MET inhibition (Figure 1, B and C). PHA665752 treatment was also effective at limiting MB cell motility as determined by an artificial wound healing assay (Figure 2). PHA665752 treatment impaired MB cell capacity for anchorage-independent growth, with no colonies visible after 2 weeks of growth in soft agar (Figure 3). At the molecular level, MET signaling results in upregulated downstream MAPK kinase signaling in the D283, ONS76, and MED8A cell lines and in upregulated PI3K signaling in the D283–ONS76, and MED8A cell lines and in upregulated PI3K signaling.

Figure 2. Artificial wound healing assay. (A) D283 cells treated with DMSO-vehicle control (D283–control) alone demonstrate near-complete closure of the initial defect in the cell monolayer ($T = 0$ hour) after 18 hours in culture ($T = 18$ hours). In contrast, D283 cells treated with the MET inhibitor PHA665752 (D283–PHA665752) demonstrate reduced migratory ability, with the initial defect ($T = 0$ hour) closed by only approximately 50% after 18 hours in culture ($T = 18$ hours). (B) ONS76 cells treated with DMSO-vehicle control alone (ONS76–control) demonstrate complete closure of the monolayer defect after 18 hours in culture, whereas this closure remains incomplete with PHA665752 treatment (ONS76–PHA665752).

Figure 3. Colony formation in soft agar assay. D283 cells grown under anchorage-independent conditions in soft agar readily form macroscopically visible colonies when treated with DMSO-vehicle control alone (D283–DMSO). Visible colonies fail to form after PHA665752 treatment (D283–PHA665752 1.5 μM). Images are of 35-mm culture plates after 2 weeks’ duration in culture.
in the ONS76 and MED8A cell lines (Figure 4). Pretreatment of these cell lines with PHA665752 before stimulation using exogenous HGF reduces phospho-ERK1/2 levels back to baseline levels in all three cell lines and reduces phospho-AKT levels in the ONS76 and MED8A cell lines (Figure 4).

The HGF/MET pathway is known to play a crucial role in diverse processes including normal development and tissue homeostasis. Dysregulation of HGF/MET signaling has been implicated in several human malignancies, including gastrointestinal, bladder, breast, ovarian, renal, hepatocellular, lung, prostate, and central nervous system tumors [15].

Activation of the MET receptor tyrosine kinase normally results from binding by its ligand, HGF [35,36]. On binding of HGF, MET undergoes autophosphorylation and recruits intracellular adapters that mediate diverse downstream biologic effects through multiple signaling pathways including the RAS/MAPK and PI3K/AKT signaling pathways [15]. In cancer, excess HGF/MET signaling may be secondary to loss of normal pathway inhibition, HGF ligand overexpression (with resultant auto or paracrine pathway activation), MET gene amplification or overexpression, MET gene rearrangements, or activating mutations [14,15]. Pathway activation promotes mitogenesis, morphogenesis, cell

Figure 4. (A) Treatment of serum-starved D283 cells with exogenous recombinant human HGF (rhHGF) stimulates downstream MAPK signaling, as shown by an increase in phospho-ERK1/2. Pretreatment with PHA665752 abrogates this HGF-mediated increase in phospho-ERK1/2. (B) Treatment of serum-starved D283 cells with rhHGF does not alter PI3K activity, as evidenced by high levels of phospho-AKT with or without rhHGF treatment. PHA665752 treatment also does not alter phospho-AKT levels in the D283 cell line. (C) Treatment of serum-starved ONS76 cells with rhHGF results in increased phospho-ERK1/2. Pretreatment with PHA665752 reduces phospho-ERK1/2 levels to baseline. (D) Similarly, rhHGF increased phospho-AKT levels in ONS76 cells, with PHA665752 pretreatment reducing phospho-AKT levels to baseline. (E) Treatment of serum-starved MED8A cells with rhHGF results in increased phospho-ERK1/2. Pretreatment with PHA665752 reduces phospho-ERK1/2 levels to baseline. (F) Similarly, rhHGF treatment increases phospho-AKT levels in MED8A cells, with PHA665752 pretreatment reducing phospho-AKT levels to baseline.
motility, angiogenesis, and resistance to anoikis-induced apoptosis—a coordinated biologic program called invasive growth [14,15,37]. While in the normal context, this program of MET signaling–mediated invasive growth is crucial for tissue and organ morphogenesis; in the context of cancer, it may contribute to tumorigenesis, disease progression, and metastasis [38].

The role of HGF/MET signaling in cerebellar development has been well established [39]. The MET-mediated pattern of invasive growth may be exemplified by the normal proliferation, migration, and invasion of normal cerebellar external granule cell precursors (GCPs; suspected MB cell of origin), as they proliferate and then undergo migration and invade into the developing cerebellum to form the mature internal granule cell layer. Murine cerebellar GCPs express MET and proliferate in response to HGF [39], HGF and MET are expressed in the human fetal and adult cerebella [40,41]. In vitro HGF treatment of GCPs prevents apoptotic cell death [42]. Lastly, mice harboring a hypomorph variant form of Met, in which Met signaling capacity is reduced, display a reduction in overall cerebellar size with foliation defects in the central and posterior vermis [39].

Disregulation of normal developmental signaling pathways, such as Hedgehog and WNT signaling, has been implicated in the pathogenesis of MB. Aberrant MET signaling beyond the developmental period, during which it is required for proper external granule layer cell migration and establishment of the internal granule layer, may contribute to MB pathogenesis by promoting cell proliferation and inhibiting apoptosis and to MB metastasis through its ability to promote cell migration, invasion, and resistance to anoikis-induced apoptosis. MET amplifications have been identified in 38.5% of primary human MB samples by comparative genomic hybridization [43]. In addition, several established MB cell lines and primary patient samples express high levels of HGF and MET proteins [11]. Loss of normal pathway inhibition has also been implicated in aberrant HGF/MET signaling in MB [9]. Furthermore, high MET receptor messenger RNA expression in primary patient samples correlates with reduced overall survival [11]. Experimentally, exogenous HGF treatment of MB cell lines results in downstream phosphorylation of both MAPK and AKT [11]. Exogenous HGF increases cell proliferation, anchorage-independent growth, and cell cycle progression and reduces apoptosis in response to chemotherapy [11]. In vitro, HGF overexpression enhances MB xenograft tumor formation in an immunodeficient mouse model [11]. HGF/MET pathway inhibition limits the growth of intracerebellar MB xenografts [9]. Overexpression of HGF in murine GCPs in conjunction with SHH increases de novo MB formation in comparison with SHH alone [8]. As such, the HGF/MET pathway may represent an effective target for novel therapeutic strategies in MB.

We have demonstrated for the first time that targeting the MET receptor tyrosine kinase using small molecule inhibitor therapy is effective against MB in vitro at reducing cell proliferation, migration, and growth under anchorage-independent conditions. The exact mechanism of how the PHA665752 exerts its dramatic effect on MB cells remains to be determined. Our studies indicate that it is not likely due to the activation of the apoptotic pathway; therefore, it is likely an effect mediated on the cell cycle. The biologic consequences of MET inhibition by PHA665752 may be in part due to inhibition of key downstream onco- genic signaling pathways, including the RAS/MAPK and PI3K/AKT pathways. Of interest was the lack of inhibition of the PI3K/AKT pathway in the D283 cell line, suggesting this pathway is activated by a c-Met–independent signaling event. It is possible that this represents a MB subtype variant and may be of importance when profiling potential candidates for therapeutic responsiveness to this inhibitor. As a proof-of-principle study to determine the utility of targeting the MET kinase in MB, these data demonstrating the effectiveness of PHA665752 in vitro are informative, owing to the high selectivity of this agent for the MET receptor in comparison to other commercially available agents [14,29]. To date, however, the clinical application of PHA665752 has been hindered because of poor pharmacokinetics and low oral bioavailability [14]. With the knowledge that specific targeting of the MET receptor tyrosine kinase in MB limits the malignant phenotype in vitro, future studies using preclinical models of MB to investigate new small molecule MET inhibitors with improved kinetics are warranted.

References


Figure W1. D283 cells (left panel) and ONS76 cells (right panel) were either untreated (−) or treated with 2.5 μM PHA665752 (+) for 12, 24, 36, or 48 hours. Cell lysates were harvested, quantified, and analyzed by Western blot analysis with anti-caspase-3 antibody that will detect the higher–molecular weight intact protein (single arrowhead) and the apoptotic cleaved product (double arrowhead). Jurkat cells either untreated or treated with cytochrome c serve as positive and negative control lysates for caspase-3 cleavage.