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An Orally Bioavailable c-Met Kinase Inhibitor Potently Inhibits Brain Tumor Malignancy and Growth

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Abstract

The receptor tyrosine kinase, c-Met and its ligand hepatocyte growth factor (HGF) are important regulators of malignancy in human cancer including brain tumors. c-Met is frequently activated in brain tumors and has emerged as a promising target for molecular therapies. Recently, an orally bioavailable small molecule kinase inhibitor of c-Met (SGX523) was developed by SGX Pharmaceuticals. We tested the effects of this inhibitor on c-Met brain tumor cell activation, c-Met-dependent malignancy, and *in vivo* glioma xenograft growth. SGX523 potently inhibited c-Met activation and c-Met-dependent signaling at nanomolar concentrations in glioma cells, primary gliomas, glioma stem cells and medulloblastoma cells. SGX523 treatment inhibited c-Met-dependent brain tumor cell proliferation and G1/S cell cycle progression. SGX523 also inhibited brain tumor cell migration and invasion. Furthermore, systemic delivery of SGX523 via oral gavage to mice bearing orthotopic human glioblastoma xenografts led to a significant decrease of *in vivo* tumor growth. These studies show that c-Met activation and c-Met-dependent brain tumor cell and stem cell malignancy can be inhibited by small molecules. The study also shows for the first time that oral delivery of a small molecule kinase inhibitor of c-Met inhibits intracranial tumor growth. These findings suggest that targeting c-Met with small molecule kinase inhibitors is a promising approach for brain tumor therapy.

Keywords

c-Met; glioblastoma; hepatocyte growth factor; kinase inhibitor; medulloblastoma; small molecule; scatter factor

INTRODUCTION

Gliomas are extremely aggressive brain tumors that account for the majority of deaths due to primary CNS neoplasms. Glioblastoma multiforme (Grade IV glioma) is the most commonly diagnosed glioma and has an average life expectancy of only 14 months [1]. Medulloblastoma is the most common malignant brain tumor in children [2]. Although

average 5-year progression-free survival of medulloblastoma patients has surpassed 60%, a number of patients still succumb to the disease and a large number of survivors suffer severe life-long neurocognitive deficiencies due to radiation-induced damage of the developing brain [3–7]. Human and experimental gliomas and medulloblastoma express a number of growth factors and corresponding receptors which contribute to their malignancy by enhancing cell mitogenicity and motogenicity, inhibiting apoptosis and stimulating angiogenesis [8,9]. Among these growth factors, hepatocyte growth factor (HGF), also called scatter factor, and its tyrosine kinase receptor c-Met play important roles.

Inappropriate c-Met activation in cancer occurs through autocrine and paracrine activation, transcriptional overexpression, gene amplification, and activating mutations and has been observed in virtually all types of solid tumors including brain tumors [8–10]. c-Met and HGF are highly expressed in brain tumors and their expression levels frequently correlate with tumor grade and poor prognosis [11–15]. Overexpression of HGF and/or c-Met in brain tumor cells enhances their tumorigenicity, tumor growth, and tumor-associated angiogenesis [11,16,17]. Activation of c-Met in brain tumor cells induces tumor cell proliferation, migration, and invasion and inhibits apoptosis and resistance to death induced by chemotherapy and radiation [8,11,13,17–21]. Conversely, inhibition of HGF and c-Met in experimental tumor xenografts leads to inhibition of tumor growth and tumor angiogenesis, suggesting that c-Met is a promising target for brain tumor therapy [22–24].

Recently, a very potent, remarkably selective and orally bioavailable small molecule inhibitor of c-Met kinase, SGX523, has been developed by SGX Pharmaceuticals (San Diego, CA). In purified enzyme assays and various cell-based assays, SGX523 inhibited c-Met at low nanomolar concentrations. SGX523 was screened against a panel of 213 human kinases of which only c-Met was inhibited to any substantial (>40%) degree at 1 μ M. *In vivo*, evaluation of SGX523 demonstrated that it is orally bioavailable and has good pharmacokinetic properties in mice, rats and dogs. SGX523 demonstrated potent anti-tumor activity when dosed orally in human gastric tumor xenograft model with no overt toxicity. Pharmacodynamic studies showed a close correspondence between *in vivo* anti-tumor activity and inhibition of c-Met phosphorylation. However, SGX523 has not been tested in brain tumor models. Notably also, no small molecule kinase inhibitor of c-Met has to our best knowledge been tested by systemic delivery in orthotopic brain tumor models in which blood brain barrier-associated limitations play a critical role.

In this study, we assessed the therapeutic efficacy of SGX523 in human brain tumors. We found that SGX523 inhibits c-Met, AKT and MAPK phosphorylation, cell proliferation, cell cycle progression, migration and invasion in different human glioblastoma cell lines, glioblastoma primary cells, glioblastoma stem cells and medulloblastoma cell lines. We also found that oral delivery of SGX523 to mice bearing intracranial human glioma xenografts leads to inhibition of *in vivo* tumor growth. We therefore conclude that c-Met kinase inhibition is a feasible and promising approach for brain tumor therapy.

MATERIALS AND METHODS

Cell Culture and Reagents

Except for stem cells, all cell culture media, sodium bicarbonate, sodium pyruvate, nonessential amino acids and HEPES buffer used in this study were purchased from Cellgro Mediatech (Washington, DC). Neurobasal media, N2, B27, penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). Human recombinant HGF, bFGF and EGF were purchased from R&D systems (Minneapolis, MN). Fetal bovine serum (FBS) was purchased from Gemini BioProducts (West Sacramento, CA). Crystal Violet was purchased from Promega Corp (Madison, WI). Propidium iodide (PI) was purchased from BD

Pharmingen (San Diego, CA). The c-Met kinase inhibitor SGX523 was provided by SGX Pharmaceuticals (San Diego, CA).

The glioblastoma cell lines U87 and A172 and the medulloblastoma cell line DAOY were obtained from American Type Culture Collection (Manassas, VA). Primary glioblastoma cells (GBM10) were isolated from surgical specimens of patients who underwent surgical treatment at the Mayo Clinic and who consented to the use of the tissue for research. The primary cells were propagated in animals via heterotopic implantation in the flanks of immunodeficient mice [25]. Glioma stem cells 1228 were a kind gift from Dr. Howard Fine (NIH) [26]. U373 cells were grown in DMEM (1 g/L glucose with L-glutamine) supplemented with HEPES buffer, and 10% FBS. U87 cells were grown in Eagle's MEM supplemented with 1 mmol/L sodium pyruvate, 0.15% sodium bicarbonate, 0.1 mol/L nonessential amino acids and 10% FBS. A172 cells were grown in DMEM (4.5 g/L glucose with L-glutamine) and 10% FBS. DAOY cells were grown in RPMI 1640 and 10% FBS. Primary glioblastoma cells were grown in α -MEM with 2.5 % FBS. Glioma stem cells 1228 were grown in neurobasal media with N2 and B27 supplements (0.5X each), and supplemented with human recombinant EGF, bFGF (50 ng/ml each). All cells were grown at 37°C in 5% CO₂-95% O₂.

Treatment with the c-Met Inhibitor SGX523

SGX523 was dissolved in dimethyl sulfoxide (DMSO, vehicle) at 1 mg/ml stock, aliquoted and stored at room temperature in a desiccator until used. SGX523 was added to the cells at the indicated concentrations for 1 hour prior to further experimentation. Control cells were treated with the medium containing equal volume of the vehicle.

Immunoblotting

Immunoblotting was used to assess the effects of c-Met kinase inhibition on basal and HGF-induced c-Met, MAPK and AKT activations. Immunoblotting was performed as previously described using antibodies specific for phospho-c-Met, total c-Met, phospho-MAPK, total MAPK, phospho-AKT and total AKT (Cell Signaling, CA) [27]. All cells were exposed to 0.1% FBS medium for 24 h prior to treatment with SGX523 at different concentrations ranging from 30 nM to 1.5 μ M for 1 hour prior to treatment with or without HGF (50 ng/ml) for 10 minutes. The cells were subsequently lysed with 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 and equal amounts protein were electrophoretically separated in a polyacrylamide gel (Lonza, MD) and then trans-blotted to a nitro-cellulose membrane. The membrane was then incubated overnight with primary antibodies at 4°C. The primary antibody-bound membranes were washed three times with tween-PBS before incubation with the corresponding horseradish-conjugated antibodies. After the final wash, the immunoreactive signals were detected by enhanced chemiluminescence.

Cell Proliferation

To assess the effect of c-Met kinase inhibition on cell growth, 30 000 cells/well were incubated in 0.1% FBS media for 24 hours prior to treatment with SGX523 at concentrations of 100 nM for all brain tumor cells except stem cells (1.5 μ M) for 1 hour and then treated with HGF (50 ng/ml). The control group was treated with equivalent volumes of vehicle. The cells were harvested every day, for 5 days and counted with a hemocytometer and growth curves were established.

Propidium Iodide Flow Cytometry

The effects of c-Met kinase inhibition on the cell cycle were analyzed using propidium iodide (PI) flow cytometry as previously described [28]. The cells were exposed to 0.1% FBS medium for 24 h prior to treatment with SGX523 at different concentrations (30, 100 and 300 nM), for 1 hour prior to treatment with or without HGF for 24 hrs. 1228 glioblastoma stem cells were treated with 1.5 μ M SGX523. The treated cells were subsequently washed with PBS, harvested and fixed in 70% (v/v) ethanol. The cells were then treated with 20 μ g DNase-free RNase for 30 min at 37°C, stained with propidium iodide and analyzed on a FACscan (Becton-Dickinson, Fullerton, CA) and G0/G1, S and G2/M fractions were determined.

Migration Assay

Glioblastoma and medulloblastoma cells were seeded in 6-well plates at 80% confluency in low serum media for 24 hours. Scratch wounds were made in the middle of the plates with sterile pipette tips. The cells were then treated with SGX523 (100 nM) for 1 hour followed by treatment with HGF (50 ng/ml) for 24 hours. Control cells were treated with PBS only. Twenty four hours later, pictures of migrating cells were taken for each condition.

Invasion Assay

The effects of c-Met kinase inhibition on cell invasion were tested using Modified Boyden Chambers (Becton Dickinson, MA) that were coated with human type IV collagen (Sigma). Glioma and medulloblastoma cells were treated with SGX523 (100 nM) for 1 hour and then with 50 ng/ml of HGF for 24 hours. Glioma stem cells were seeded in poly-ornithine coated 6-well plates at the density 5×10^5 and allowed to adhere to the plate overnight and treated with 1.5 μ M SGX523 for 1 hour and exposed to HGF 50 ng/ml for 24 hours. The following day, the cells (5×10^5) were resuspended in serum-free media and added to each insert in the presence or absence of HGF. 600 μ l of 10% FBS medium was placed in the lower chamber as a chemoattractant. The control group was treated with PBS only. After 6 hrs of incubation at 37°C in 5% CO₂, invaded cells were stained with 0.1% crystal violet solution and photographed. The cells were then counted under the microscope in five randomly chosen fields.

In Vivo Xenograft Experiments

Glioblastoma U87 cells (3×10^5) were stereotactically implanted in the striatum of immunodeficient mice (n=10). SGX523 was dissolved in 0.5% methylcellulose and 0.05% tween 80. SGX523 treatment started four days post-tumor implantation. The animals were treated by oral gavage of 0.2 ml of 0.5% SGX523 solution (50 mg/kg body weight) every day, twice per day at 12 hour intervals for a period of 3 weeks. Control animals (n=10) were treated with an equal volume of the vehicle solution, 0.5% methylcellulose and 0.05% tween 80. The animals were euthanized 4 weeks after tumor implantation. The brains were removed and frozen. The brains were then cryosectioned and subjected to Hematoxylin-Eosin staining. Tumor sizes were measured on maximal cross-sectional areas using computer-assisted image analysis as previously described [11].

Statistics

All experiments were performed at least in triplicates. Numerical data were expressed as mean $\pm$ standard deviation. Two group comparisons were analyzed by two-sided Student's t test. Multiple group comparisons were analyzed with Bonferroni/Dunn multiple comparisons tests. P values were determined and $p < 0.05$ was considered significant.

RESULTS

SGX523 Inhibits the Phosphorylation of c-Met, MAPK and AKT in Brain Tumor Cells, and Stem Cells in a Dose-Dependent Manner

The effect of SGX523 on c-Met, MAPK and AKT activation was assessed by immunoblotting for phospho-proteins. SGX523 inhibited HGF-induced c-Met phosphorylation in glioma cells U87 and U373, medulloblastoma cells DAOY, and glioma stem cells 1228 in a dose-dependent manner (Fig. (1)). SGX523 also inhibited basal c-Met phosphorylation in U87 cells, which possess an HGF/c-Met autocrine loop and therefore have significant basal levels of c-Met, MAPK and AKT phosphorylation (Fig. (1)). Interestingly, while c-Met phosphorylation in the established lines U87, U373 and DAOY were completely inhibited at drug concentrations of ~100 nM, glioma stem cells 1228 required more than 10-fold this concentration for complete inhibition. This suggests that cancer stem cells are more resistant to c-Met kinase inhibition. SGX523 also inhibited HGF-induced MAPK activation in all cells and HGF-induced AKT activation in DAOY and U87 cells. Altogether, these data show that SGX523 potently inhibits c-Met activation and c-Met-dependent signaling in brain tumor cells and stem cells.

Inhibition of c-Met Kinase Leads to Inhibition of Cell Proliferation and G1/S Cell Cycle Progression in Brain Tumor Cell Lines, Primary Cells and Stem Cells

c-Met activation has been shown to promote tumor malignancy by inducing tumor cell proliferation and cell cycle progression. Therefore, we tested the effects of SGX523-mediated inhibition of c-Met activation on brain tumor cell and stem cell proliferation and G1/S cell cycle progression using cell counting and propidium iodide flow cytometry, respectively. Based on the phosphorylation inhibition data described above, cell lines and primary cells were pre-treated with 100 nM SGX523 and stem cells were treated with 1.5 μ M SGX523 for 1 hr prior to treatment with or without 50 ng/ml HGF and subsequent assessment of cell proliferation and cell cycle.

c-Met activation by HGF treatment significantly induced cell proliferation in all tested cells. SGX523 significantly inhibited HGF-induced proliferation of U87 and A172 glioma cells, DAOY medulloblastoma cells, and GBM10 primary cells (Fig. (2)). SGX523 also inhibited basal cell proliferation of U87 cells, 1228 stem cells and GBM10 primary glioma cells (Fig. (2)). These results show that SGX523-mediated inhibition of c-Met activation leads to inhibition of brain tumor cell and stem cell proliferation.

c-Met activation by HGF treatment also significantly induced cell cycle progression in all tested cells. SGX523 significantly inhibited HGF-induced cell cycle progression beyond G1/S in a dose-dependent manner in glioma cells U87, U373 and A172 and glioma stem cells 1228 (Fig. (3)). SGX523 also inhibited basal cell cycle progression of U87 cells and 1228 stem cells (Fig. (3)). These results show that SGX523-mediated inhibition of c-Met activation leads to inhibition of glioma cell and stem cell cycle progression.

Inhibition of c-Met Kinase Leads to Inhibition of Brain Tumor Cell Migration and Invasion

c-Met activation has been shown to promote tumor malignancy by inducing tumor cell migration and proliferation. Consequently, we tested the effects of SGX523-mediated c-Met inhibition on brain tumor cell migration and invasion using a scratch assay and a transwell invasion assay, respectively. SGX523 treatment inhibited basal and HGF-induced the migration of A172 glioblastoma and DAOY medulloblastoma cells (Fig. (4A)). Similarly, SGX523 significantly decreased basal and HGF-induced glioma U87 cell invasion from 377 ± 3 invading cells treated with HGF to 39 ± 2 ($p < 0.05$) for cells exposed to SGX523 followed by HGF. SGX523 also significantly decreased DAOY cells invasion from $382 \pm$

16 cells treated with HGF to 42 ± 3 ($p < 0.05$) invading cells exposed to SGX523 followed by HGF (Fig. (4B)). These results show that SGX523-induced inhibition of c-Met activation leads to inhibition of brain tumor cell migration and invasion.

Systemic Oral Delivery of SGX523 Significantly Inhibits the *In Vivo* Growth of Intracranial Human Glioblastoma Xenografts

To investigate the *in vivo* anti-tumor properties of SGX523, we generated orthotopic glioma xenografts by implantation of U87 glioblastoma cells in the brains of immunodeficient mice. The animals were treated by systemic administration of SGX523 via oral gavage. The mice were treated for three weeks starting on the fourth day post tumor implantation with 50 mg/kg of body weight SGX523. The control group was treated with equal volume of vehicle only. The animals were sacrificed 4 weeks post-surgery, the brains were removed, cryo-sectioned, H&E stained, and tumor sizes were quantified by computer-assisted image analysis. No obvious drug toxicity-related death or morbidity was observed during the experiment. While control animals developed large tumors averaging $23383 \pm 4167 \mu\text{m}^2$, the SGX523-treated group showed a significant decrease of the cross-sectional area quantified at $2959 \pm 538 \mu\text{m}^2$, ($n=10$, $p<0.05$) (Fig. (5)). These data show for the first time that inhibition of c-Met kinase by a small molecule leads to inhibition of intracranial tumor growth. The data also demonstrate the feasibility of systemic oral delivery of small molecule kinase inhibitors of c-Met and suggest that these molecules can cross the blood brain barrier.

DISCUSSION

In the present study, we established for the first time the anti-tumor properties of a novel, orally bioavailable c-Met kinase inhibitor (SGX523) in medulloblastoma cells and glioma cells, primary cells and stem cells as well as in an orthotopic human glioblastoma xenograft model after systemic drug delivery. We demonstrated that SGX523 strongly inhibits c-Met, AKT and MAPK activations and c-Met-dependent tumor cell proliferation, migration and invasion. We showed that the small molecule inhibitor can be effectively orally delivered to inhibit intracranial tumor growth. We therefore demonstrated the pre-clinical feasibility and efficacy of small molecule kinase inhibitors of c-Met for the treatment of brain tumors.

Given the critical role of the receptor tyrosine kinase, c-Met and its ligand hepatocyte growth factor in the progression of different human cancers including brain tumors, different approaches to inhibiting HGF and c-Met have been developed. These targeted therapies include receptor antagonism with HGF fragments such as NK4, HGF/c-Met expression inhibition with U1snRNA/ribozymes, competitive ligand binding with soluble c-Met receptors, neutralizing antibodies to HGF and c-Met and small molecule kinase inhibitors [8,24,29–35]. Monoclonal antibodies and small molecules are currently perhaps the only clinically applicable anti-HGF/c-Met drugs. Antibodies that target either HGF or c-Met have been developed and one has been tested in a glioblastoma clinical trial. The results from this trial have not been published to date. In comparison with antibodies, small kinase inhibitors can theoretically be expected to achieve greater anti-brain tumor effects. Small molecules are more likely to cross the blood brain barrier than the significantly heavier antibodies. This is supported by our data that show significant inhibition of intracranial glioma growth after systemic oral delivery of SGX523. Additionally, unlike anti-HGF neutralizing monoclonal antibodies, c-Met kinase inhibitors could inhibit basal HGF-independent c-Met activation that has been shown to also play a role in c-Met-dependent malignancy [24]. SGX523 has been recently tested in a phase I trial. The trial was interrupted due to an unexpected kidney toxicity that appears to have resulted from a metabolite of SGX523 which accumulated to higher levels in humans than had been observed in animal toxicology studies. Despite this temporary setback, the data presented in the present manuscript provide proof-of-principle

of the usefulness and efficacy of small molecule kinase inhibitors of c-Met for brain tumor therapy

SGX523 potently inhibited c-Met activation, downstream signaling, and *in vitro* correlates of malignancy in brain tumor cells. To our knowledge, these data are the first to show the effects of a c-Met kinase inhibitor in medulloblastoma and glioma stem cells. Interestingly, more than ten-fold higher concentrations were required to achieve the same inhibitory effects in glioma stem cells than in glioma cells. This suggests that cancer stem cells are more resistant to such therapies and that higher drug concentrations might be required to target this critical cell population. SGX523 also potently inhibited *in vivo* intracranial glioma growth after systemic delivery via oral gavage. This is to our knowledge the first demonstration of intracranial tumor inhibition in response to oral delivery of a c-Met inhibitor. It demonstrates the feasibility of such an approach and suggests that small molecule kinase inhibitors of c-Met are capable of crossing the blood brain barrier.

Overall, the results demonstrate that small molecule inhibitors of c-Met kinase efficiently inhibit c-Met activation and c-Met-dependent malignancy in brain tumor cells and stem cells and that they can be systemically delivered to inhibit brain tumor growth. These molecules could therefore be developed into promising drugs for brain tumor therapy.

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Fig. 1. SGX523 inhibits the phosphorylation of c-Met, MAPK and AKT in brain tumor cells, and stem cells in a dose-dependent manner

The glioblastoma cell lines U87 and U373, the medulloblastoma cell line DAOY and the glioblastoma stem cells 1228 were treated with different concentrations of SGX523 for 1 hour prior to treatment with or without 50 ng/ml HGF for 10 min. Cell lysates were immunoblotted for total and phosphorylated c-Met, MAPK (ERK) and AKT. The results show that SGX523 inhibits HGF-induced c-Met and MAPK in all cells and AKT phosphorylation in DAOY and U87 cells. SGX523 also inhibited basal c-Met, MAPK and AKT phosphorylation in U87 cells that possess a HGF/c-Met autocrine loop.

Fig. 2. Inhibition of c-Met kinase leads to inhibition of cell proliferation in brain tumor cell lines, primary cells and stem cells

The glioblastoma cell lines U87 and A172, the medulloblastoma cell line DAOY, the glioblastoma stem cells 1228, and the glioblastoma primary cells GBM10 were treated with SGX23 (100 nM for all cells except stem cells at 1.5 μ M) for 1 hour prior to treatment with or without 50 ng/ml HGF. The cells were subsequently assessed for cell proliferation by cell counting over 5 days. The results show that SGX23 inhibits cell proliferation in all tested cell lines.

Fig. 3. Inhibition of c-Met kinase leads to inhibition of G1/S cell cycle progression in brain tumor cell lines, primary cells and stem cells

The glioblastoma cell lines U87, U373 and A172 and the glioblastoma stem cells 1228 were treated with different concentrations of SGX523 for 1 hour prior to treatment with or without 50 ng/ml HGF. Twenty four hours later, the cells were assessed for cell cycle status by propidium iodide flow cytometry and the G0/G1, S and G2/M fractions were determined. The results show that SGX523 induces cell cycle arrest in all tested cell lines.

Fig. 4. Inhibition of c-Met kinase leads to inhibition of brain tumor cell migration and invasion
A) A172 and DAOY glioma cells were treated with 100 nM SGX523 for 1 hour and then with 50 ng/ml HGF. The cells were assessed for migration using the scratch/wound assay. Cells that migrated into the scratch were photographed at 40X magnification. The results show that SGX523 inhibits cell migration in both cell lines **B)** U87 glioma and DAOY medulloblastoma cells were treated with 100 nM SGX523 for 1 hour and then with 50 ng/ml HGF. Cell invasion was assessed using a transwell assay. Cells that invaded through the membrane after 6 hrs were fixed and stained with crystal violet and the wells were photographed at 40X magnification. The results show that SGX523 inhibits the invasion of glioblastoma and medulloblastoma cells.

Fig. 5. Systemic oral delivery of SGX523 significantly inhibits the *in vivo* growth of intracranial human glioblastoma xenografts

Human glioblastoma xenografts were generated by intracranial implantation of 3×10^5 U87 cells in immunodeficient mice brains. Four days post-tumor implantation, the animals were treated with SGX523 by oral gavage (50 mg/kg body weight) twice per day for 3 weeks. The control group was treated with equal volume of vehicle. The animals were sacrificed four weeks post-tumor implantation and tumor sizes were assessed on H&E-stained cross sections. Tumor maximal cross-sectional areas were measured with computer-assisted image analysis. The results show that SGX523 treatment inhibits *in vivo* glioblastoma growth (left panel: representative tumors; right panel: quantification of tumor sizes). * = $p < 0.05$.