Human Schwannomas Express Activated Platelet-Derived Growth Factor Receptors and c-kit and Are Growth Inhibited by Gleevec (Imatinib Mesylate)

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Abstract

Schwannomas, although benign, can be fatal or give rise to significant morbidity due to an unpredictable growth rate. They can reoccur after surgery or radiation, current treatments each with significant inherent risks. These risks are further amplified in neurofibromatosis type 2 (NF2), a germline predisposition syndrome characterized by multiple schwannomas, underlying the need for biological targeted therapies. Gleevec (STI571, imatinib mesylate), in addition to the bcr-abl oncogene in chronic myelogenous leukemia, inhibits c-kit and platelet-derived growth factor receptor (PDGFR) signaling, thereby expanding its use to several malignant and benign human diseases. In the present study, we show that human sporadic and NF2-associated schwannomas have increased expression along with activation of PDGFR-α, PDGFR-β, and c-kit receptors, compared with normal or traumatic nerve. Using the human NF2-null HEI-193 schwanna cell line, Gleevec inhibited schwanna viability, proliferation, and anchorage-independent growth, as well as induced apoptosis in a dose-dependent manner (IC₅₀ 5–10 μmol/L). These antitumorigenic effects were correlated to inhibition of PDGFR-α, PDGFR-β, and c-kit activation/phosphorylation and major downstream signaling pathways. Lack of robust xenograft or transgenic models of schwannomas prevents extension of these studies in vivo. However, the established long track record and tolerable toxicity of Gleevec already in clinical use and our preclinical data lead us to propose that Gleevec should be evaluated in human schwannomas with shown progressive growth.

Introduction

Schwannomas are benign tumors originating from Schwann cells of peripheral or cranial nerves (1). Vestibular schwannomas (VS) or acoustic neuromas have an estimated incidence of 0.7 to 1 per 100,000 population, but in 5% of cases they occur early in life and are often bilateral in the germline cancer predisposition syndrome neurofibromatosis type 2 (NF2), with an incidence of ∼1/40,000 live births (2) Although the growth of majority of sporadic and NF2-associated VS is insidious and slow (1–2 mm/y), in a subset growth can be in unpredictable spurts (>1 cm/y; ref. 3). These growth spurts can be associated with clinical symptoms due to brain stem compression, compromise of adjacent cranial nerve function (Fig. 1), and often precipitate active intervention in the form of surgery or radiosurgery, each with their short- and long-term risks (4–9). The etiology of these growth spurts, which rarely represents malignant transformation, is not well understood. In contrast, our understanding of the molecular alterations involved in initiation of schwannomas has been enhanced by elucidation of the NF2 tumor suppressor gene on chromosome #22q12, which encodes for the protein Merlin or Schwannomin (10, 11). Merlin is a 65- to 70-kDa protein belonging to the band 4.1 protein superfamily, with implicated roles in establishing cytoskeletal dynamics, cell growth, ion transport, proliferation, and cell motility. Cell-specific knockout in Schwann cells supports the critical role of NF2 in the initiation of schwannomas (12). In contrast to initiation, the molecular factors that promote the usual indolent growth of schwannomas and the occasional growth spurts are not well studied. Aberrant expression and activation of growth factors and receptors are prime candidates and are potential biological targets, which may be therapeutically exploited to either negate or delay active intervention in selected cases (13–18).

The clinical use of Gleevec (imatinib mesylate, STI571) was first shown in chronic myelogenous leukemia (CML), where it inactivated the characteristic bcr-abl oncprotein, making it the first and still the best example of the utility of biological targeted therapies in human cancer (19, 20). The demonstration that Gleevec inhibited the activity of other receptor tyrosine kinases such as c-kit, platelet-derived growth factor receptor (PDGFR)-α, and PDGFR-β (21) has led to its investigation in a variety of human cancers (22–25).

In this study, we analyzed and showed increased expression and activation of c-kit, PDGFR-α, and PDGFR-β and their respective ligands in flash-frozen surgical specimens of sporadic and NF2-associated human peripheral schwannoma and VS. We then analyzed the in vitro therapeutic efficacy of Gleevec in the immortalized human NF2-null VS cell line (HEI-193; ref. 26). Gleevec inhibited cell growth and proliferation while increasing apoptosis and cell cycle arrest, leading to decreased anchorage-independent growth. These antitumorigenic effects were associated with decreased activation of c-kit, PDGFR-α, and PDGFR-β and major downstream effector pathways. Although desirable, these in vitro results are not readily tested in vivo due to lack of robust in vivo schwannoma models. However, we suggest that Gleevec, based on its long clinical track record and associated minimal toxicities, warrants evaluation in human schwannomas.
toxicity, warrants clinical evaluation in growing and symptomatic schwannomas.

Materials and Methods

Quantitative Real-time PCR
Total RNA was extracted (RNAeasy Mini prep kit, Qiagen) from five specimens, normal nerves, traumatic neurona, NF2-associated VS, NF2 peripheral schwannoma, sporadic VS, sporadic peripheral schwannoma, plus one stomach and gastrointestinal stromal tumor (GIST) specimen. The flash-frozen surgical specimens were obtained from the research ethic board–approved tumor bank. RNA (0.2 μg) was subjected to cDNA synthesis using the One-Step RT-PCR kit (Qiagen) in 20 μL reactions, diluted to 100 μL using double-distilled water. Two microliters cDNA were used with the PCR primers for PDGFR-α, PDGF-A, PDGFR-β, c-kit, stem cell factor (SCF), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) listed in Supplementary Table S1. Each reaction was set up in triplicate with 96-well microtitre plates, using the PCR SYBR Green kit and an Applied Biosystems model 7700 sequence detector. The PCR program was 94°C for 5 min, 94°C for 30 s, 58°C for 30 s, and 68°C for 1 s for 45 cycles. Expression of the test gene in each specimen was normalized as the ratio of test gene expression to the level of expression of the GAPDH gene as a control, followed by a final normalization step of expression relative to normal nerve. The statistical significance of differences in expression was discerned by pairwise Student’s t test (Fig. 2).

Receptor Expression and Phospho-immunoblot Activation Assays
Surgical specimens were lysed as described previously (27), and 800 μg of tissue lysate, measured by bicinchoninic acid (BCA) assay (Pierce Chemical), were incubated overnight at 4°C with 5 μg of respective primary antibody (all from Santa Cruz Biotech): c-kit goat polyclonal, PDGFR-α goat polyclonal, and PDGFR-β rabbit polyclonal. Protein-G-agarose (Sigma; 100 μL) was added to the preincubated tissue lysates, incubated at 4°C for 4 h, and processed using standard immunoprecipitation pull-down procedures. The immunoprecipitated tissue precipitation pull-down lysates were subjected to Western blot analyses using 1:1,000, phosphoryrosine primary antibody (Upstate Biotech) to detect activated receptors or the primary antibodies to detect total receptor levels, with 1:4,000 protein-G horseradish peroxidase as the secondary antibody (Bio-Rad). In summary, five such blots were undertaken, each with lysates from separate controls (normal nerves and traumatic neurona), but the same lysate from the single sample of GIST and normal stomach. Densitometric analyses were undertaken within the linear range and analyzed with the Fluor-Chem software for each of the five blots, and the median value was calculated. Fold changes were calculated by using the Preceptor/total receptor ratio in normal nerves as the standard value of 1 and comparing it to Preceptor/total receptor ratio from each specimen tested. The statistical significance of differences in expression was evaluated by pairwise Student’s t test (Fig. 3).

Tumorigenicity Assays on HEI-193 NF2-Null Human Schwannoma Cells
Cell viability assay. HEI-193 cells (1 × 10^5 per well; ref. 26) were plated in 96-well plates for 24 h, followed by varying concentrations of Gleevec [vehicle (0 μmol/L), 1, 5, and 10 μmol/L]. Trypan blue dye was added at 24-h intervals (day 1–5), and direct cell counts of both dead (positive) and viable cells (negative) were undertaken using the Vi-CELL Beckman Coulter analyzer. The percentage of viable cells (viable/viable + dead) was plotted against each dose level for days 1 to 5, and normalized to HEI-193 cells incubated with vehicle alone, representing 100% viability. HEI-193 cell viability subjected to Gleevec was also evaluated by the 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (Promega) according to the manufacturer’s instructions. Briefly, HEI-193 cells were seeded in 96-well plates at a density of 1 × 10^4 per well, with Gleevec added (vehicle, 1, 5, and 10 μmol/L) after 24 h. After every 24 h, the MTS labeling reagent was added, and the absorbance at 490 nm determined 4 h later, for the subsequent 5 d. The corrected absorbance (control blanks) was used to determine the number of live/dead cells. The mean value from each of six wells with a similar dose of Gleevec was obtained, and three such independent experiments were undertaken for both the trypan blue exclusion and MTS assays, with subsequent analysis using the Student t test (Fig. 4A and B).

Bromodeoxyuridine proliferation assay. HEI-193 cells were seeded at a density of 1 × 10^5 per well in 96-well plates for 24 h, followed by Gleevec and then incubation with bromodeoxyuridine (BrdUrd) for 18 h. BrdUrd-positive cells were evaluated every 24 h (day 1–5) by removing the labeling solution and addition of 200 μL of FixDenat (Roche Diagnostic) solution for 30 min at room temperature. After removing FixDenat, 100 μL of anti-BrdUrd-POD solution were added and incubated for 90 min at room temperature, followed by three washes with 300 μL/well of washing buffer.
Substrate solution (100 μL) was then added and incubated for 10 min at room temperature, with the absorbance of the samples measured by an ELISA reader at 450 nm within 10 min. The average of six wells per Gleevec dose for three independent experiments was undertaken and values obtained were compared with HEI-193 cells that received only vehicle, with statistical analysis by Student’s t test (Fig. 4C).

**Cell cycle and apoptosis analysis.** Cell cycle analysis was assessed with propidium iodide (BD Pharmingen) staining and fluorescence-activated cell sorting analysis (FACScan, Becton Dickinson). Briefly, cells were fixed with 4% paraformaldehyde for 15 min at room temperature, centrifuged at 1,500 rpm, and resuspended in propidium iodide solution containing propidium iodide and RNase A and incubated for 40 min at 37°C. After another centrifugation, the cells were resuspended in 500 μL PBS for fluorescence-activated cell sorting analysis. Samples were analyzed using the CellQuest Pro software. Sub-G0 and S-phase subfractions were calculated as averages of duplicate experiments.

Apoptosis was analyzed by measuring caspase-3/7 activity using the Apo-One Homogeneous Caspase-3/7 assay (Promega) on HEI-193 cells seeded at a density of $1 \times 10^5$ per well in a 96-well plate and subjected to a one-time dose of Gleevec (vehicle, 1, 5, and 10 μmol/L). After every 24 h, 100 μL of Apo-One were added in each well, incubated for 3 h, and then fluorescence levels were measured at 485 Ex/527 Em for 5 d.

**Soft agar clonogenic assay.** To the bottom of each 60-mm well plate were added 1.5 mL of 0.5% agar medium. HEI-193 cells ($5 \times 10^3$) were suspended in 0.7% low melting point agarose (Cambrex) and dissolved in equal amounts of whole-cell lysates were separated on 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membrane (NEN) using a semi-dry transfer apparatus (Bio-Rad). Membranes were probed overnight with activation-specific phosphotyrosine antibodies to PDGFR-α (Upstate Biotech; Tyr754, 1:500); PDGFR-β (Upstate Biotech; Tyr716, 1:250), c-kit (Cell Signaling Tech; Tyr719, 1:250), extracellular signal–regulated kinase (ERK)-1/2 (Santa Cruz; Tyr204, 1:500), AKT (Zymed; Ser473, 1:2,500), p-38 (Cell Signaling Tech; Tyr716, 1:250), and Fak (Abcam; Tyr397, 1:1,000). Total receptor levels were derived using non–activation-specific antibodies: PDGFR-α (Santa Cruz; 1:200); PDGFR-β (Santa Cruz; 1:200), c-kit (Santa Cruz; 1:100), ERK1/2 (Millipore; 1:2,500), Akt (Cell Signaling Tech; 1:1,000), Fak (Abcam; 1:1,000), and β-actin (Sigma; 1:20,000). Horseradish peroxidase–conjugated secondary antibody (Bio-Rad) was used, with visualization using the Chemiluminescence Reagent Plus (Perkin-Elmer). Densitometric analysis was undertaken for the phosphorylated/total receptor levels. These experiments were repeated.

**Western blot analysis.** HEI-193 cells were grown in 10% FBS and treated with varying doses of Gleevec for 2 h, lysed in modified PLC lysis buffer supplemented with protease and phosphatase inhibitors (Calbiochem), and concentrations were determined using the BCA assay. Equal amounts of whole-cell lysates were separated on 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membrane (NEN) using a semi-dry transfer apparatus (Bio-Rad). Membranes were probed overnight with activation-specific phosphotyrosine antibodies to PDGFR-α (Upstate Biotech; Tyr754, 1:500); PDGFR-β (Upstate Biotech; Tyr716, 1:250), c-kit (Cell Signaling Tech; Tyr719, 1:250), extracellular signal–regulated kinase (ERK)-1/2 (Santa Cruz; Tyr204, 1:500), AKT (Zymed; Ser473, 1:2,500), p-38 (Cell Signaling Tech; 1:1,000), Fak (Abcam; 1:1,000), and β-actin (Sigma; 1:20,000). Horseradish peroxidase–conjugated secondary antibody (Bio-Rad) was used, with visualization using the Chemiluminescence Reagent Plus (Perkin-Elmer). Densitometric analysis was undertaken for the phosphorylated/total receptor levels. These experiments were repeated.

Figure 2. Increased expression of c-kit, PDGFRs, and PDGF ligands in sporadic and NF2-associated VS and peripheral schwannomas: quantitative real-time RT-PCR expression of c-kit, PDGFR-α, and PDGF-β receptors and their respective ligands, SCF, PDGF-A, and PDGF-B. The data are represented as fold change in expression relative to human normal nerve (NN, n = 5). The median expression of each specimen (n = 5) was normalized to GAPDH (not shown), before normalization to the median value of expression in normal nerves, which was arbitrarily set at 1. Compared with expression in normal nerves or benign human traumatic neuroma (TN; n = 5), there was a significant increase ($P < 0.01$) in expression of all three receptors and ligands in sporadic and NF2-associated VS and peripheral schwannomas.

A single human GIST (GS) specimen, known to express the receptors and ligands, served as a positive control, with corresponding human normal stomach (NS) as negative control. 1, normal nerve (NN); 2, traumatic nerve (TN); 3, normal stomach (NS); 4, GIST (GS); 5, sporadic VS (SV); 6, sporadic peripheral schwannoma (SP); 7, NF2 VS (NV); 8, NF2 peripheral schwannoma (NP).
thrice, and the mean levels of phosphorylated/total receptor as a function of Gleevec dose analyzed by Student’s t test (Fig. 6d).

**Activation of downstream effector pathways.** Ras-GTP was pulled down by the use of lysates containing 2 mg protein and glutathione S-transferase-Ras-binding domain of Raf1 (GIST-RBD; Ras Activation assay Kit, Upstate Biotech; ref. 28). In short, cells were lysed in Mg2+ buffer containing 10% glycerol and protease and phosphatase inhibitors and then incubated for 1 h at 4°C with glutathione-Sepharose beads bound to 7.5 μg of GST-RBD. The beads were then washed thrice in lysis buffer, resuspended, and boiled in 2× Laemmli buffer, and the supernatants subjected to SDS-PAGE followed by immunoblotting with the anti-Ras antibody (1:500; Upstate Biotech; Fig. 6b).

Western immunoblot analysis of whole-cell lysates was undertaken by activation-specific phosphotyrosine antibodies to P-ERK1/2, P-AKT, and P-Fak (Fig. 6b). The blots were also probed by non-activation-specific antibodies to derive total receptor levels. All densitometric analyses were undertaken within the linear range with the AlphaEaseFC software. Fold changes are calculated by using the vehicle-treated ratio as the standard value of 1.

**Results**

**Increased Expression and Activation of Receptors in Human Schwannoma Specimens**

Quantitative real-time PCR showed increased expression ($P < 0.05$) of Gleevec targeted receptors and their ligands in all human schwannoma specimens, compared with normal human nerves (Fig. 2). Levels of receptor/ligand mRNA expression were slightly elevated in a human traumatic neuroma specimens, composed of proliferating nontransformed Schwann cells, but not statistically different ($P > 0.05$) from normal nerves. A human GIST tumor and normal human stomach specimen also served as an internal positive and negative control. Of all three receptor/ligand combinations tested, PDGFR-α and PDGF-A mRNA was increased the most in all four groups of human schwannomas ($P < 0.05$) compared with normal nerves. However, there was no statistical difference ($P > 0.05$) in levels of PDGFR-α, PDGF-A, or in fact c-kit, or SCF expression between schwannomas, irrespective of whether they were sporadic or NF2 associated.

Phospho-immunoblot for activated receptor/total receptor levels normalized to the value obtained from the normal nerve specimen showed severalfold increased activation of c-kit, PDGFR-α, and PDGFR-β in all human schwannoma specimens (Fig. 3). Levels of activated receptors in the nontransformed human traumatic neuroma were the same as normal nerves. Densitometric analysis showed highest levels of all three activated receptors in the GIST specimen (positive control), in keeping with high levels of expression detected at the mRNA level (Fig. 2). Levels of PDGFR-α activation were highest among the three receptors evaluated in all schwannomas specimens (36- to 40-fold increase). Levels of c-kit mRNA expression, modestly elevated in NF2-associated peripheral schwannomas, also corresponded to a comparatively low 8-fold increase in activation of c-kit. The modest but significant increase in levels of PDGFR-β mRNA in sporadic vestibular and NF2-associated peripheral schwannomas was also reflected by a modest increase in activated PDGFR-β of 13- and 15-fold, respectively.
Antitumorigenic Effects of Gleevec on Transformed NF2-Null Human Schwannoma Cells

Reduction in cell viability. HEI-193 human NF2-null schwannoma cells showed decreased cell viability in vitro when subjected to one-time Gleevec administration in a dose-dependent manner. As measured by both trypan blue exclusion and MTS assays (Fig. 4A and B), the percentage of viable HEI-193 cells was statistically lower after receiving 5 μmol/L Gleevec for all 5 days analyzed ($P < 0.05$), with 10 μmol/L Gleevec significantly further decreasing viability ($P < 0.05$). With trypan blue exclusion assay, we extrapolated the IC$_{50}$ to be 6.25 μmol/L for day 1, 5 μmol/L for day 3, and 3.25 μmol/L for day 5, compared with values obtained from vehicle-treated cells only. Similar IC$_{50}$ values were extrapolated with the MTS assay: day 1, 6.25 μmol/L; day 3, 4.50 μmol/L; day 5, 3.8 μmol/L.

Reduction in proliferation. BrdUrd incorporation assay showed significant reduction ($P < 0.05$) in the number of proliferating HEI-193 cells after 5 or 10 μmol/L Gleevec (Fig. 4C), with the greatest reduction with 10 μmol/L Gleevec ($P < 0.05$). At day 1 after 5 and 10 μmol/L Gleevec administration, there was a reduction of ~20% and 35% in proliferating rate, respectively, compared with vehicle. There was an ~50% reduction in proliferating cells after 5 μmol/L by day 5, whereas it was achieved by day 3 after 10 μmol/L Gleevec. The decrease in cellular proliferation was also supported by decreased number of cells entering S phase, as evidenced from lower amounts of cells in M3 quadrant (Fig. 4D). This effect on S phase was also dose and time dependent. For example, there was an ~20% reduction in HEI-193 cells at day 1 after 5 μmol/L Gleevec, with maximal reduction in 5-phase cells at day 5 with ~40% and 42% reductions in 5 μmol/L and 10 μmol/L–treated cells, respectively.

Figure 4. Gleevec decreases growth and increases apoptosis of NF2-null HEI-193 human VS cells. A, viability of HEI-193 cells after single administration of varying doses of Gleevec was evaluated by trypan blue exclusion from day 1 to day 5 and plotted as a percentage of HEI-193 cells receiving vehicle control (0 μmol/L Gleevec). Points, mean value of three wells. There was a significant ($P < 0.01$) dose-dependent decrease even after day 1 of either 5 μmol/L (*) or 10 μmol/L (**) Gleevec administration. B, viability of HEI-193 cells was also evaluated by MTS assay after single administration of Gleevec at varying doses from day 1 to day 5 and plotted as a percentage of cells treated with vehicle only. Columns, mean of six replicate wells from three independent experiments. The number of viable HEI-193 cells decreased at 5 (*) or 10 μmol/L (**) , commencing from day 1 to day 5 ($P < 0.01$). C, BrdUrd proliferation assay on HEI-193 cells also showed decreased proliferation from day 1 to day 5 after single Gleevec administration. Significant ($P < 0.01$) decrease in proliferation was found at 5 μmol/L (*) or 10 μmol/L (**) Gleevec. Columns, average of six replicates from three replicate administration experiments. D, cell cycle analysis by propidium iodide flow cytometry was undertaken day 1 to day 5 after single administration of Gleevec in HEI-193 cells. There was an increase in the sub-G$_0$ phase, representing apoptotic cells, in a dose- and time-dependent manner. In addition, there was a decrease in the number of cells entering S phase. Sub-G$_0$ and S-phase subfractions were calculated as averages of duplicate experiments. E, caspase-3/7 activity showed significant ($P < 0.01$) increase in both intrinsic and extrinsic apoptotic pathways in HEI-193 cells at day 1 after 5 (*) or 10 μmol/L (**) single Gleevec administration.
Induction of apoptosis. Administration of Gleevec to HEI-193 cells increased apoptosis in a dose- and time-dependent manner, as determined by both caspase-3/7 activity and cell cycle flow cytometry. Gleevec administration led to increase in caspase-3/7 activity with significant induction ($P < 0.01$) in 5 or 10 μmol/L Gleevec–treated cells, compared with vehicle- or 1 μmol/L–treated cells (Fig. 4E). The induction of apoptosis was seen as early as day 1 and increased with time after Gleevec. There was no significant difference in caspase-3/7 activity between 5 and 10 μmol/L Gleevec–treated HEI-193 cells, for any of the time points, although it was consistently higher in the 10 μmol/L–treated cells. The induction of apoptosis as measured by caspase-3/7 activity was also supported by an increase in the number of cells in the sub-G₀ phase after Gleevec exposure (Fig. 4D). This increase was observed as early as day 1, and by day 5 up to ~25% of the cells were in the sub-G₀ phase with either 5 or 10 μmol/L Gleevec. Although the increase in sub-G₀ phase was slightly higher in HEI-193 cells treated with 10 μmol/L Gleevec, it was not significantly different from those subjected to 5 μmol/L Gleevec.

Reduction in soft agar colonies. Soft agar assay using HEI-193 cells showed significant reduction in the number of anchorage-independent colonies formed in a dose-dependent manner (Fig. 5). As evaluated and quantified on days 18 and 21, significant ($P < 0.001$) reduction in soft agar colonies was detected after 5 or 10 μmol/L Gleevec, compared with the vehicle control or cells treated with 1 μmol/L Gleevec. Although the increase in sub-G₀ phase was slightly higher in HEI-193 cells treated with 10 μmol/L Gleevec, it was not significantly different from those subjected to 5 μmol/L Gleevec.

Reduction in activation of targeted receptors. Quantification of phosphorylation specific immunoblot assays, normalized to total amount of receptor expressed, showed reduction of c-kit, PDGFR-α, and PDGFR-β activation to varying degrees by Gleevec (Fig. 6A). These assays were undertaken 2 hours after administration of Gleevec to HEI-193 cells growing in 10% FBS. Similar to the quantitative real-time PCR data (Fig. 2) and the receptor activation data (Fig. 3) on human schwannoma specimens, the HEI-193 cells expressed abundant amounts of all three receptors, which were also highly activated. Expression and activation of PDGFR-α was the highest among the receptors in the HEI-193 cells, similar to our findings in the surgical schwannoma specimens (Fig. 3). Activation of all three receptors was significantly ($P < 0.01$) reduced after 5 and 10 μmol/L Gleevec, with highest reduction at 10 μmol/L dose ($P < 0.05$). For example, activation of PDGFR-α was decreased by ~80% at 5 μmol/L, and >90% at 10 μmol/L Gleevec. At 1 μmol/L Gleevec, PDGFR-β activation was inhibited by ~70%, but this dose did not significantly ($P > 0.05$) inhibit PDGFR-α or c-kit activation.

Inhibition of effector pathways after Gleevec administration. Activity assays 2 hours after Gleevec administration to HEI-193 cells inhibited several key protumorigenic effector pathways involved in proliferation (Ras-GTP and P-ERK1/2), cell survival (P-Akt), and migration (P-Fak; Fig. 6B). Fold changes were calculated by normalizing the mean of the two vehicle-treated samples to 1, with experiments repeated thrice. There was a dose-dependent reduction in all four effector pathways examined, although to variable extents. After 10 μmol/L Gleevec, Ras-GTP level was 0.33, P-ERK1/2 level was 0.53, P-Akt level was 0.41, and P-Fak was 0.27, compared with vehicle controls. This inhibition of effector pathways was statistically significant at $P < 0.01$ by Student’s t test.

Discussion

Human schwannomas usually grow in an indolent manner, causing symptoms by compression of adjacent vital structures usually without malignant progression (Fig. 1). They usually arise sporadically in adults, but can arise as multiple tumors early in life in cancer predisposition syndromes such as NF2 and NF3 (also known as schwannomatosis; ref. 29). On occasion, the growth of schwannomas can accelerate and lead to clinical symptoms (3), a scenario that is more critical in NF2 patients harboring multiple
intragranual schwannomas (Fig. 1). Currently, actively growing and clinically symptomatic schwannomas are managed with surgery or radiosurgery, each with some short- and long-term risks (4–9). A management alternative that could blunt or regress the growth of the schwannoma, and be taken on a prolonged basis, would potentially stabilize the clinical symptoms and defray active intervention, especially in high-risk patients.

Gleevec was the first targeted tyrosine kinase inhibitor to show efficacy in the clinic, revolutionizing the current management of CML, harboring the bcr-abl oncogene (19–21). Gleevec blocks the kinase activity of abl and inhibits the growth of bcr-abl–driven cell lines in vitro with an IC₅₀ of 1 μmol/L (21). This concentration is readily achieved in vivo, with minimal host toxicity. In addition to its minimal toxicity, Gleevec inhibits other receptor tyrosine kinases such as c-kit and PDGFRs, thereby expanding its potential use in other human cancers. For example, Gleevec has shown clinical efficacy in the management of GIST, characterized by c-kit mutational activation or, rarely, PDGFR-α mutational activation (23). Gleevec has also shown efficacy in c-kit–positive small-cell lung cancer cell lines (25), PDGFR-expressing glioblastoma cells (24), and other myeloproliferative disorders characterized by activation of PDGFR-β (22).

Schwannomas do not harbor the bcr-abl oncogenic translocation, but expression of PDGFRs and growth factors has been previously noted (13–18). To determine if c-kit, PDGFR-α, and PDGFR-β are expressed, activated, and play a functional role in promoting growth, we used human surgical schwannoma specimens as well as the HEI-193 immortalized human schwannoma cell line. Quantitative real-time PCR analysis on peripheral schwannoma and VS removed from sporadic and NF2 patients showed increased mRNA expression of all the three Gleevec targeted receptors and their respective ligands (Fig. 2). Phosphorylosomes immunoblots on tumor lysates showed increased expression and activation of all three receptors compared with normal nerve (Fig. 3). Expression and activation of c-kit was moderately increased, compared with the high expression and activation of PDGFR-α in all the schwannoma specimens. There was variable increase in PDGFR-β expression and activation, with relatively lower levels in sporadic VS and NF2 peripheral schwannomas, the reason for which is not currently clear. Of interest, expression of Merlin in the NF2-null HEI-193 cells results in decreased activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase and tumorigenic growth, which partly may be mediated by increasing degradation of cell-surface receptors such as PDGFR (30). Although not statistically significant, there was a trend toward increased expression and activation of all three receptors in the NF2-associated VS, including PDGFR-β, as previously noted in primary schwannoma cultures (13). The expression and activation profile of c-kit, PDGFR-α, and PDGFR-β, in addition to being increased compared with normal human nerve specimens, was also elevated when compared with human traumatic neuroma specimens, the latter implying that the increase in

Figure 6. Growth inhibitory effect of Gleevec in HEI-193 cells is accompanied with decreased activation and downstream signaling of c-kit and PDGFRs. A, Gleevec was administered at varying doses to HEI-193 cells for 2 h and then Western immunoblot analysis of whole-cell lysates was undertaken with activation-specific phosphotyrosine antibodies. The blots were also probed by non–activation-specific antibodies to derive total receptor levels and β-actin as loading control. Densitometric evaluation showed significant inhibition (60–90%; P < 0.01) of c-kit, PDGFR-α, and PDGFR-β at 5 (*) or 10 μmol/L (**) Gleevec. The 1 μmol/L dose inhibited PDGFR-β activation by ~70%, but did not significantly (P > 0.05) inhibit PDGFR-α or c-kit activation. B, Gleevec at varying doses was administered to HEI-193 cells for 2 h, and then p21-Ras activity (Ras-GTP pull-down assay, Upstate Biotech) and P-ERK1/2, P-Akt, and P-Fak levels were measured and normalized to total levels of the respective proteins to obtain activity levels by densitometry. Fold changes were calculated by using the mean of the two vehicle-treated samples (arbitrarily 1) and analyzed using Student’s t test. There was a significant dose-dependent inhibition of all these four major effector pathways analyzed.


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Gleevec, a Candidate Drug for Human Schwannomas

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c-kit, PDGFR-α, and PDGFR-β expression is not associated with proliferating Schwann cells, but rather linked to transformation to schwannomas.

The immortalized HEI-193 cells were derived from a VS in a NF2 patient and immortalized with human papillomavirus E6-E7 viral genes (26). Recently, a point mutation has been identified in the NF2 gene of the HEI-193 cells, resulting in a nonfunctional Merlin with attenuated tumor-suppressive function (31). Although the HEI-193 cells have been noted to have increased proliferative capacity in vitro, they do not grow as in vivo xenografts. Our group has also attempted to grow explant xenografts from NF2-associated or sporadic schwannomas used in this study, without success. The increased receptor expression and activation profile for c-kit, PDGFR-α, and PDGFR-β in HEI-193 cells was similar to our characterization of the human NF2 VS operable specimens (Figs. 3 and 6), validating their use for the functional experiments undertaken (Figs. 4–6). Although schwannoma cell lines and HEI-193 cells specifically have been linked with increased PDGFR expression (13, 18, 32), our study is the first to decipher the profile of c-kit as a well-recognized target of Gleevec.

Cumulatively, our results show the in vitro antitumorogenic effects of one-time administration of Gleevec on HEI-193 schwannoma cells. This results from a decrease in cell viability (Fig. 4A and B), cell proliferation with a block at S phase (Fig. 4C and D), and an increase in apoptosis as measured by an increase in the sub-G0 phase and caspase-3/7 activity (Fig. 4E and F). These multiple antitumorogenic mechanisms of Gleevec has been noted in other human cancer cell lines, including bcr-abl–positive hematopoietic cells, GIST, dermatofibrosarcoma, and PDGFR-expressing glioblastoma cells (23, 24, 33, 34). These experiments were done in the presence of 10% serum with an extrapolated IC50 of ∼5 μmol/L. Gleevec (Fig. 4A and B), which is higher than the 1 μmol/L found in CML and GIST cell lines (21), but lower than those reported in other human cancer cells such as neuroblastoma (9–13 μmol/L) and Ewing’s sarcoma (10–12 μmol/L; refs. 35, 36). The 5 μmol/L Gleevec used in our study is in fact similar to the 4.6 μmol/L obtained in serum from the early reported successful clinical trials in CML (19, 20). However, the maximal tolerated or effective dose of Gleevec is likely higher than these early trials in CML, with the desired effective dose likely varying with the targeted cancer and patient profile, an area of ongoing clinical investigations (37, 38). The 5 and 10 μmol/L doses were equally effective in decreasing anchorage-independent growth in soft agar (Fig. 5). The 5 and 10 μmol/L doses both effectively decreased phosphorylation activation of all three receptors, with the higher dose being slightly more effective (Fig. 6A). These results are similar to the doses reported to inhibit PDGFR phosphorylation in Swiss 3T3 cells transfected to express PDGFRs (39). Decreased activation of these receptors correlated with inhibition of four key signaling pathways (Ras-GTP, P-ERK1/2, P-AKT, and P-Fak), which are well known to be aberrant in tumorigenic cells (Fig. 6B). In addition to these three receptors and major downstream effector pathways, other molecular mechanisms of the antitumorogenic effects of Gleevec likely exist, some of which may play a dominant role and act by in vivo methods such as on angiogenesis (40–42).

In summary, our results support Gleevec as having antitumorogenic effect on human schwannomas in vitro. Majority of schwannomas will not require any intervention other than clinical and radiologic follow-up due to their indolent but progressive growth. However, a few, such as thoseimpinging on vital structures such as the spinal cord or brainstem and especially schwannomas associated with the NF2 or NF3 predisposition syndromes, will require active intervention. Surgery or radiosurgical intervention in this latter group of patients and those who are elderly or have significant concomitant medical issues may be significant. It is these patients who may benefit from chronic administration of a biological therapy with established low toxicity. For example, in NF2 patients, prolonged hearing preservation is a prime management objective due to the bilateral nature of the VS in addition to often multiple other schwannomas. Although in specialized centers technical improvements have occurred in surgery and radiosurgery to minimize risks, there still exists significant limitation in our ability to preserve long-term hearing, in addition to small but potential critical risks such as radiation-induced carcinogenesis (4–9). This study is limited by its in vitro nature due to lack of readily available xenograft or transgenic models of schwannomas. However, based on almost a decade of experience using Gleevec often with chronic administration, we feel that the presented preclinical data warrant further investigation of Gleevec in phase1/2 clinical trials of human schwannomas with shown radiologic and/or clinical progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Human Schwannomas Express Activated Platelet-Derived Growth Factor Receptors and c-kit and Are Growth Inhibited by Gleevec (Imatinib Mesylate)

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