# Imatinib Mesylate Inhibits Platelet-Derived Growth Factor Receptor Phosphorylation of Melanoma Cells But Does Not Affect Tumorigenicity *In Vivo*

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**Platelet-derived growth factor (PDGF) and its cognate receptor are widely expressed on melanomas. Coexpression of the growth factor and receptor suggests their role in autocrine or paracrine growth mechanisms. Imatinib mesylate was previously reported to have specific activity in inhibiting select tyrosine kinase receptors, including PDGF and c-Kit. Melanoma cells express abundant levels of the PDGF receptor (PDGFR). Nevertheless, c-Kit expression is progressively lost as the cells take on a more highly metastatic phenotype. To investigate the potential of imatinib mesylate as a therapy for melanoma, we studied its effect on the growth of melanoma cells using an *in vivo* mouse model. Melanoma cells with high malignant potential (PDGFR-positive, c-Kit-negative) or low malignant potential (PDGFR-positive, c-Kit-positive) were injected subcutaneously into athymic nude mice. Mice were treated with imatinib mesylate (100 mg/kg three times weekly) or with phosphate-buffered saline for 4 to 6 wk. PDGFR-α and -β were expressed on all melanoma cell lines tested. The level of PDGFR expression correlated with the metastatic potential of the melanoma cells: higher levels of PDGFR-α were expressed on cells with higher metastatic potential, and higher levels of PDGFR-β were expressed on cells with lower metastatic potential. There was no significant difference in tumor size between treated and control mice. Immunohistochemical studies demonstrated inhibition of PDGFR phosphorylation on the tumors from mice treated with imatinib mesylate but not from control mice, suggesting that the receptors were functional and that the concentration of drug used was appropriate. Our data demonstrated that imatinib mesylate blocked both PDGFR-α and PDGFR-β *in vivo*. It did not, however, affect the growth of melanoma cells expressing PDGFR, regardless of whether the cells expressed c-Kit.**

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In the United States, melanoma is the eighth most prevalent cancer (Wingo *et al*, 1995). Melanoma is predominantly diagnosed in the third and fourth decades of life; thus, the disease is a significant public health concern. Nevertheless, in women 25 to 29 y old, melanoma is the most common cancer, and in women between 30 and 34 y old, it is the second most common type of malignancy after breast cancer (Brochez and Naeyaert, 2000). In 1998, the age-adjusted incidence of invasive cutaneous melanoma was 18.3 in 100,000 men and 13.0 in 100,000 women in the United States (Jones *et al*, 1999). Melanoma is largely curable if identified at any early stage. Unfortunately, patients with locally advanced or metastatic disease are left with few medical treatment options. Therefore, there is a growing need to investigate novel agents that are both efficacious and minimally toxic.

Abbreviations: PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor.

In recent years, increasing attention has been directed at small molecules that specifically inhibit tyrosine kinase receptors, many of which are dependent on growth factors that act by autocrine or paracrine mechanisms (Baserga, 1994). The development of compounds that selectively inhibit growth-factor-signaling loops may present new treatment strategies for the management of several tumors. One such compound is imatinib mesylate (Gleevec, formerly known as STI571 and CGP57148B, Novartis Pharmaceuticals, Basel, Switzerland). Imatinib mesylate was developed as an ATP-competitive inhibitor of ABL tyrosine kinase (Druker *et al*, 1996). This molecule also inhibits other tyrosine kinases, including platelet-derived growth factor receptor (PDGFR) and c-Kit, at concentrations required to inhibit Bcr-ABL (Carroll *et al*, 1997). Recently, imatinib mesylate has been approved by the United States Food and Drug Administration for the treatment of c-Kit-positive gastrointestinal stromal tumors and Philadelphia-chromosome-positive chronic myelogenous leukemia.

PDGF is a potent mitogen for cells of mesenchymal origin. Acting through its cognate receptor, PDGF has been shown to affect cellular proliferation, chemotaxis, and cell survival (Heldin and Westermark, 1999). Therefore, inhibition of PDGFR activation may inhibit the growth of several PDGFR-expressing tumors. Indeed, the effect of imatinib mesylate on *in vivo* tumor growth and PDGFR signaling has been studied in various types of tumors (Tomasson *et al*, 1999; Sjoblom *et al*, 2001; McGary *et al*, 2002; Uehara *et al*, 2003; Zhang *et al*, 2003). Several studies have confirmed the expression of PDGF and its cognate receptors on melanoma cells, suggesting that inhibition of these receptors may affect the growth of melanoma cells *in vivo* (Westphal *et al*, 2000; Barnhill *et al*, 1996; Kubo *et al*, 1998).

The expression of a second target for imatinib mesylate, c-Kit, has been extensively studied in melanoma. Both c-Kit and its ligand play important roles in the normal growth and differentiation of melanoblasts. As melanoma cells acquire a more malignant phenotype, as determined by the extent of local tumor growth, invasion, and the ability to metastasize, c-Kit expression is downregulated (Lassam and Bickford, 1992; Natali *et al*, 1992; Zakut *et al*, 1993). Indeed, the majority of highly metastatic human melanomas do not express detectable levels of the c-Kit receptor (Lassam and Bickford, 1992; Natali *et al*, 1992; Zakut *et al*, 1993). We have previously shown that enforced c-Kit expression significantly inhibits melanoma growth *in vivo* and that exposure of c-Kit-positive cells to stem cell factor (SCF) led to apoptosis of these cells (Huang *et al*, 1996). These findings raise the question as to the whether malignant transformation of melanocytes may be associated with changes in c-Kit expression. Several studies have shown that imatinib mesylate inhibits tumor growth of several nonmelanoma c-Kit-positive tumor types (Krystal *et al*, 2000; Merchant *et al*, 2002; Vitali *et al*, 2003). Nevertheless, our prior studies suggest that functional blockade of c-Kit by imatinib mesylate may possibly promote the growth of c-Kit-positive melanoma cells with low metastatic potential. Furthermore, treatment with imatinib mesylate could potentially select for the more aggressive c-Kit-negative melanoma cells *in vivo*.

The purpose of this study was to determine whether imatinib mesylate would affect the growth of melanoma xenografts *in vivo* and whether the effect, if any, was dependent on the level of PDGFR and c-Kit expressed on those cells.

## Results

**Expression of PDGFR-α and -β on human melanoma cells correlates with their metastatic potential** Specific tyrosine kinase receptors are targets for imatinib mesylate. For example, imatinib mesylate inhibits PDGFR at concentrations similar to those that inhibit Bcr-Abl (Carroll *et al*, 1997). A prior study demonstrated the presence of PDGFR-α but not PDGFR-β on human melanoma specimens (Barnhill *et al*, 1996). To determine whether human melanoma cells express adequate levels of PDGFR on their cell surface, we determined their expression by western blotting with primary antibodies specific for either PDGFR-α or PDGFR-β. PDGFR-α was expressed on all five human melanoma cell lines tested. Nevertheless, the level of expression was significantly greater on melanoma cell lines with high metastatic potential (A375SM, TXM-13, and WM-266-4) than it was on those with low metastatic potential (SB-2 and MeWo; Fig 1). Similarly, PDGFR-β was expressed on all five cell lines tested; however, unlike PDGFR-α, PDGFR-β expression was significantly greater on melanoma cell lines with low metastatic potential (SB-2 and MeWo) than it was on those with high metastatic potential (A375SM, TXM-13, and WM-266–4; Fig 1). We have previously shown that c-Kit is expressed on the nonmetastatic cell lines SB-2 and MeWo but its expression is lost on the highly metastatic A375SM, WM-266-4, and TXM-13 cells (Huang *et al*, 1996, 1998).

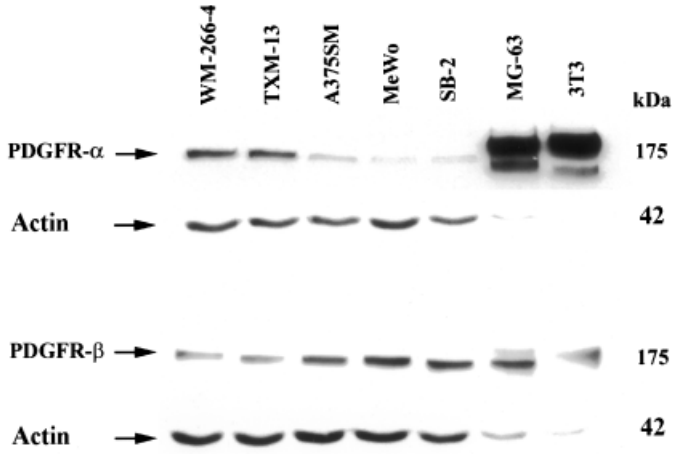


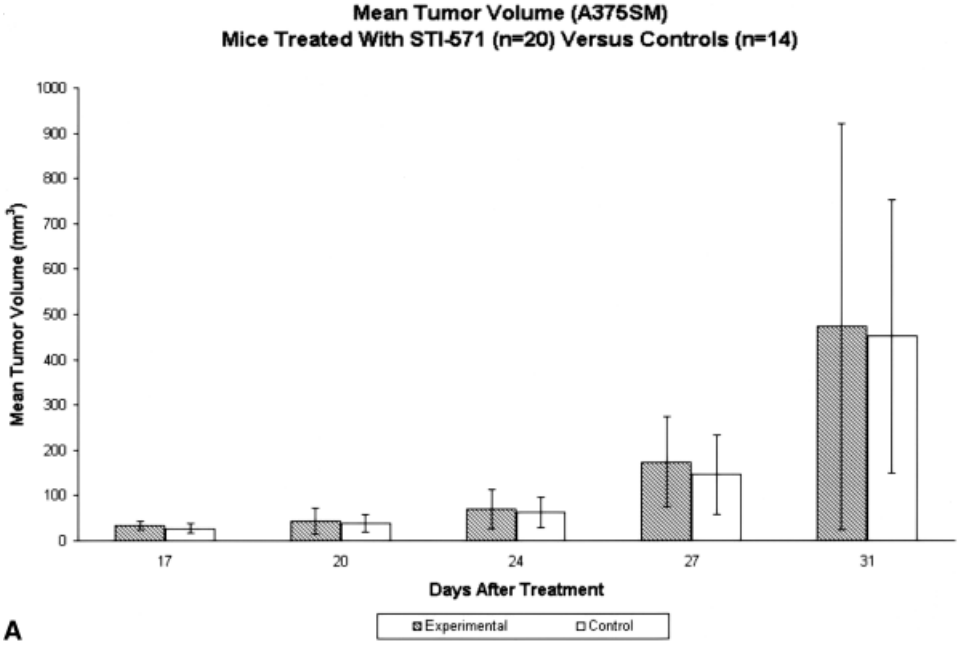

**Figure 1**
**Western blot analysis for the expression of PDGFR-α and PDGFR-β on human melanoma cell lines.** Equal loading of gels was determined by actin expression. Total cell lysates from MG-63 osteosarcoma cells (6 μg) and 3T3 fibroblast cells (20 μg) were used as positive controls for PDGFR expression. The level of PDGFR-α and PDGFR-β expression was determined after loading 30 μg of total protein lysates from various human melanoma cells: WM-266-4, TXM-13, A375SM, MeWo, and SB-2.

**Imatinib mesylate does not affect the growth of A375SM or MeWo human melanomas *in vivo*** To study the effect of imatinib mesylate on melanoma growth *in vivo*, either A375SM or MeWo melanoma cells were orthotopically introduced into nude mice by subcutaneous injection. Three days after injection, mice were randomized to the control arm (untreated) or the imatinib mesylate treatment arm. Tumor growth was assessed in both groups twice weekly. We have previously shown that c-Kit expression is inversely correlated with the metastatic potential of human melanomas (Huang *et al*, 1996, 1998). At concentrations required for inhibition of Bcr-Abl, imatinib mesylate also inhibits c-Kit (Carroll *et al*, 1997). Therefore, it is plausible that in c-Kit-positive melanomas with low metastatic potential (e.g., MeWo), treatment with imatinib mesylate could promote tumorigenicity through functional blockade of c-Kit. This is of particular importance because imatinib mesylate is currently being investigated in several human clinical trials.

Both MeWo and A375SM melanoma cell lines express PDGFR. The former express higher levels of PDGFR-α, and the latter, higher levels of PDGFR-β (Fig 1). In addition to PDGFR, MeWo melanoma cells express c-Kit, whereas

A375SM melanoma cells do not (Huang *et al*, 1996, 1998). To determine whether imatinib mesylate affects the growth of PDGFR-positive and c-Kit-negative or -positive melanomas, xenografts were established in nude mice by subcutaneous injection with A375SM or MeWo cells (day 0). Mice bearing melanoma xenografts were randomly allocated to one of two treatments: imatinib mesylate or PBS (control group). Tumor growth was periodically determined. After 31 d of treatment, there was no significant difference in the size of tumors (A375SM) in mice treated with imatinib mesylate and in those treated with PBS (Fig 2*A*). Similarly, after 50 d of treatment, there was no significant difference in the size of tumors (MeWo) in mice treated with imatinib mesylate and in mice treated with PBS (Fig 2*B*). Taken together, these data demonstrate that imatinib mesylate had no effect on the growth of PDGFR-positive human melanoma xenografts, regardless of c-Kit expression.

**Imatinib mesylate inhibits PDGFR-α and -β phosphorylation in melanoma xenografts** To determine whether imatinib mesylate was biologically active in mice bearing human melanoma xenografts, PDGFR phosphorylation was determined by immunohistochemistry on frozen sections derived from melanoma xenografts. Receptor phosphorylation was determined by immunohistochemistry with primary antibodies specific for epitopes corresponding to the short amino acid sequences containing phosphorylated Tyr-720



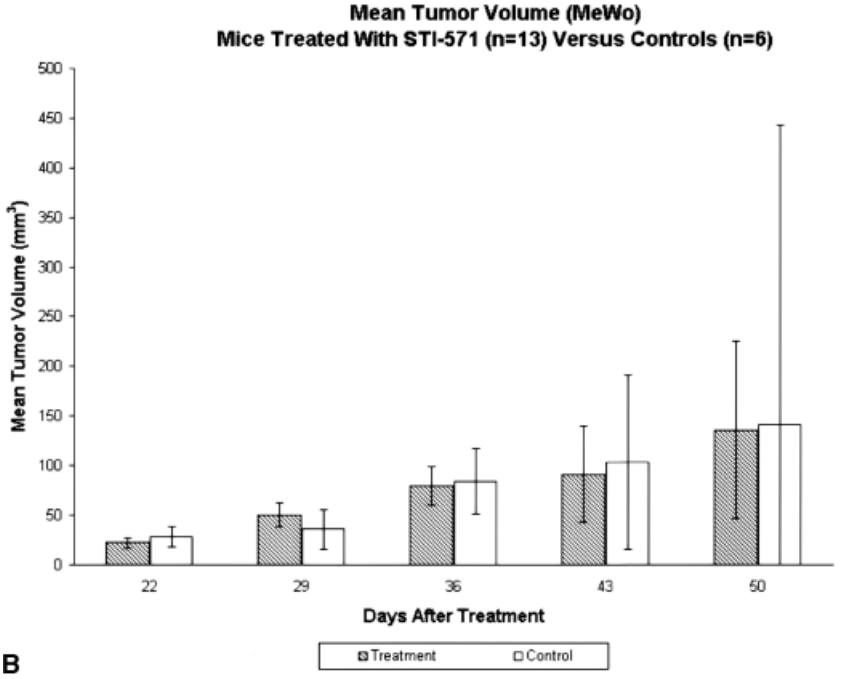




**Figure 2**
**Effect of imatinib mesylate on the growth of melanoma xenografts *in vivo*.** Nine-wk-old nude mice were injected subcutaneously with $1 \times 10^6$ MeWo or A375SM cells and treated with imatinib mesylate (100 mg/kg) or PBS three times weekly for 4 to 6 weeks. Tumor growth was periodically determined. (*A*) There was no significant difference in the growth of A375SM (PDGFR-positive, c-Kit-negative) xenografts among mice treated with imatinib mesylate or control at any point measured. (*B*) There was no significant difference in the growth of MeWo (PDGFR-positive, c-Kit-positive) xenografts among mice treated with imatinib mesylate or control at any point measured. At each interval, mean tumor volume was compared between the treated and control mice with a two-tailed Student's t test. The level of significance was set at $p < 0.05$.

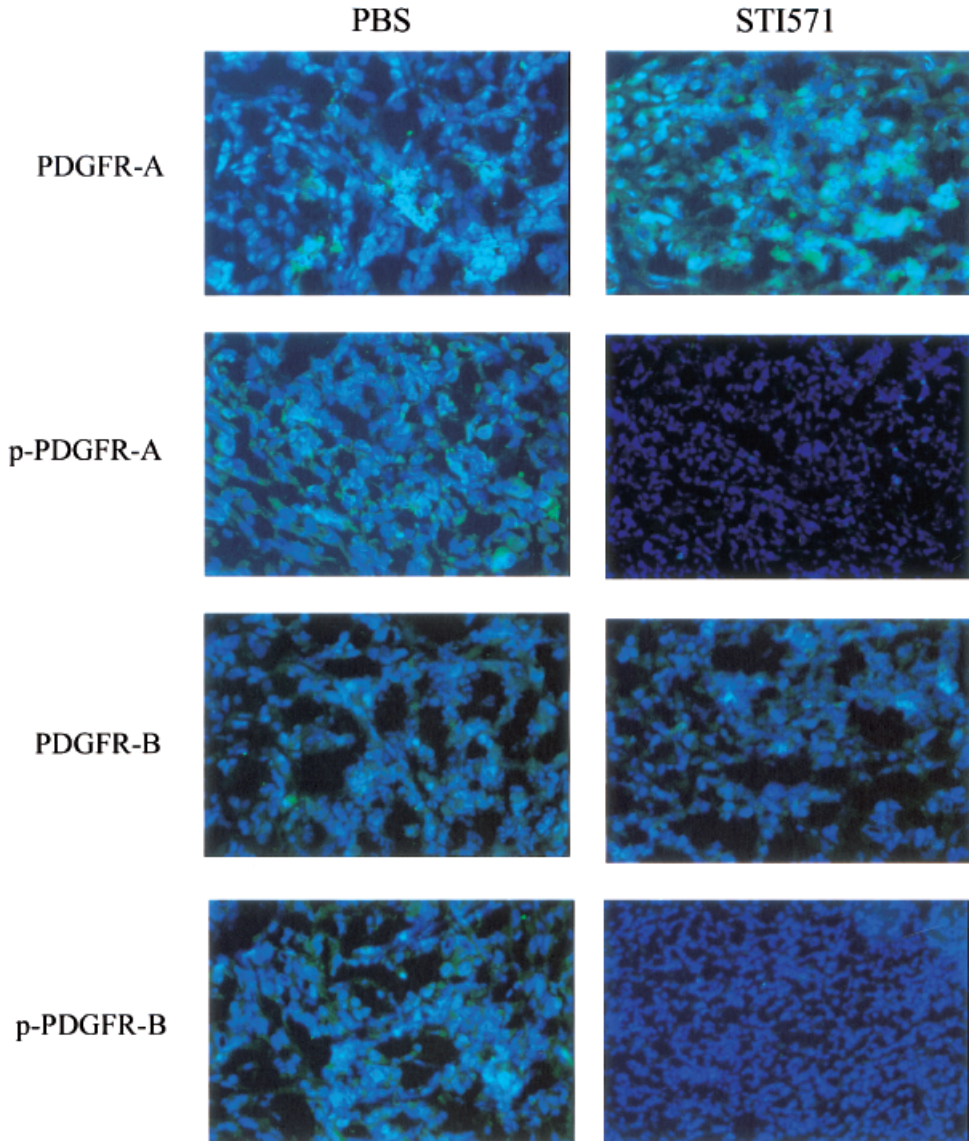



**Figure 3**
**Imatinib mesylate inhibits PDGFR-α and PDGFR-β phosphorylation in melanoma xenografts.** Immunohistochemistry was performed on frozen sections obtained from A375SM xenografts harvested from nude mice. The mice were treated with either imatinib mesylate (treatment arm) or PBS (control arm). PDGFR-α and PDGFR-β phosphorylation was determined using primary antibodies specific for epitopes corresponding to phosphorylated Tyr-720 and Tyr-1021, respectively. Cells taken from treated and untreated mice are presented with the same magnification.

and Tyr-1021 of PDGFR-α and PDGFR-β, respectively. Both PDGFR-α and PDGFR-β were expressed on A375SM melanoma xenografts in both imatinib mesylate- and control-treated mice (Fig 3), suggesting that imatinib mesylate had no effect on the level of PDGFR expression. Nevertheless, in mice treated with imatinib mesylate, there was a significant reduction in PDGFR-α and PDGFR-β phosphorylation. There was no effect on PDGFR phosphorylation in control mice treated with PBS. These data indicate that in the treatment group, 100 mg per kg imatinib mesylate provided adequate *in vivo* plasma levels to inhibit both PDGFR-α and PDGFR-β phosphorylation. These findings demonstrate that the PDGFR on the melanoma cells tested were functional and that the concentration and dosing schedule of imatinib mesylate were appropriate for our *in vivo* studies.

## Discussion

Our *in vitro* studies demonstrated that both PDGFR-α and PDGFR-β were widely expressed on all melanoma cell lines tested. A previous study had shown that only PDGFR-α was expressed *in vivo* on human primary and metastatic melanoma specimens (Barnhill *et al*, 1996). Possibly, our ability to detect PDGFR-β resulted from our using a different primary antibody with a different binding affinity. Furthermore, it is possible that *in vivo*, PDGFR-β is downregulated through epigenetic, transcriptional, or posttranscriptional mechanisms. Nevertheless, we also studied the expression of PDGFR-α and PDGFR-β on human primary and metastatic melanoma specimens. Using the same primary antibodies that had been used previously to detect PDGFR-α and PDGFR-β expression on melanoma cell lines *in vitro*, we were also able to detect expression of both PDGFR-α and PDGFR-β on the majority of human specimens tested (M. Bar-Eli, unpublished data).

We also showed that the level of PDGFR correlates with the metastatic potential of the melanoma cell lines tested. The level of PDGFR-α expression was greater on melanoma cells with high metastatic potential, whereas the level of PDGFR-β expression was greater on melanoma cells with low metastatic potential. No correlation has been established between PDGFR-α expression and the V599E BRAF

mutant. For example, we detected V599E mutation in the WM2664 cells but not in TXM-13 (data not shown). Nevertheless, both cell lines express high levels of PDGFR-$\alpha$. As the malignant phenotype of melanoma cells changes from the noninvasive radial growth phase to the vertical growth phase, which has high metastatic potential, so does the repertoire of the molecules expressed on the cells' surface. We have previously shown that expression of the cellular adhesion molecule MCAM/MUC18 is increased in melanoma cells with high metastatic potential (Xie *et al*, 1997). Another study demonstrated that the expression of the tyrosine kinase receptor c-Kit is downregulated in melanoma cells with high metastatic potential (Natali *et al*, 1992). Nevertheless, to the best of our knowledge, we are unaware of any studies that have shown a correlation between PDGFR expression and the metastatic potential of melanoma cells *in vitro*. We are currently investigating whether changes in PDGFR expression may play a role in the malignant transformation of melanocytes.

Although many PDGFR antagonists exist, we chose to study the effect of imatinib mesylate on the growth of melanoma xenografts. In addition to studying its effect on PDGFR-positive melanoma xenografts, we also studied its effect on melanoma xenografts that were established from c-Kit-positive and -negative melanoma cell lines. Our prior studies suggested that c-Kit expression may inhibit melanoma tumorigenicity (Huang *et al*, 1996, 1998). Therefore, functional blockade of c-Kit-positive melanoma cells could possibly promote tumor growth *in vivo*. This is of particular importance given the fact that imatinib mesylate is currently being investigated in human clinical trials for the treatment of melanoma.

Our data demonstrate that in mice bearing PDGFR-positive, c-Kit-negative xenografts established from the A375SM melanoma cell line, imatinib mesylate had no effect on tumor growth. We further found through immunohistochemical studies that both PDGFR-$\alpha$ and PDGFR-$\beta$ phosphorylation was inhibited in those mice treated with imatinib mesylate. These findings suggest that the concentration of imatinib mesylate used was appropriate and that the PDGFR were functional. Redundancies in growth factor loops have been described (Benini *et al*, 1999) and may partly explain why inhibition of PDGFR activation had no effect on tumor growth. Similarly, in mice bearing PDGFR-positive, c-Kit-positive xenografts established from the MeWo melanoma cell line, imatinib mesylate had no effect on tumor growth. This demonstrates that functional blockade of c-Kit by imatinib mesylate is not sufficient to promote tumor growth in mice bearing xenografts established from c-Kit-positive melanoma cells that exhibit low metastatic potential. This finding suggests that the transformation from melanoma cells with low metastatic potential to cells with high metastatic potential is not solely attributable to the inhibition of a single molecule but rather is a multistep process involving many signal-transduction pathways.

In conclusion, our data demonstrate that melanoma cell lines widely express both PDGFR-$\alpha$ and PDGFR-$\beta$ tyrosine kinases. Furthermore, the expression of these receptors appears to correlate with the metastatic potential of the melanoma cell lines tested. In mice bearing PDGFR-positive xenografts, imatinib mesylate blocked phosphorylation of these targets but had no effect on tumor growth, regardless of whether these xenografts expressed c-Kit.

## Materials and Methods

**Cell culture** The A375-p human melanoma cell line was established in culture from a human lymph node metastasis (Kozlowski *et al*, 1984). The highly metastatic A375SM cell line was established from pooled lung metastases produced by A375-p cells intravenously injected into nude mice (Li *et al*, 1989). The TXM-13 melanoma cell line was established from a patient with a melanoma brain metastasis at The University of Texas M.D. Anderson Cancer Center. The MeWo melanoma, WM-266-4 melanoma, 3T3 fibroblast, and MG-63 osteosarcoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The SB-2 cell line was isolated from a primary cutaneous lesion and was a gift of B. Giovanella (St. Joseph's Hospital Cancer Center Research Laboratory, Houston, TX). WM-266-4 cells are highly malignant (Luca *et al*, 1995). In nude mice, both MeWo and SB-2 cell lines are poorly tumorigenic and nonmetastatic (Verschraegen *et al*, 1991; Luca *et al*, 1993; Singh *et al*, 1995).

Cells were grown in 10% fetal calf serum and minimal essential medium made complete by the addition of glutamine, HEPES, streptomycin, nonessential amino acids, and multivitamins. Cells were grown at 37°C with 5% $CO_2$. All cultures were free of mycoplasma and pathogenic murine viruses.

**Western blot analysis** Melanoma cells were seeded at $1 \times 10^6$ in 100-mm tissue culture plates in 10 mL of complete minimal essential medium. After overnight incubation, the cells were washed with ice-cold phosphate-buffered saline (PBS) solution and lyzed in 0.2 mL of lysis buffer (Cell Signaling Technologies, Beverly, MA) at 4°C for 30 min. Lysates were cleared by a 10-min centrifugation at $10{,}000 \times g$, and protein determination was carried out according to standard methodology. Samples were subjected to 7.5% PAGE analysis after they were boiled for 5 min in sample buffer containing SDS. The separated proteins were transferred to Immobilon-p membranes (Millipore, Bedford, MA) and then blocked for 1 h in $1 \times$ Tris-buffered saline $+$ 0.1% Tween 20 containing 5% nonfat milk. The membrane was then incubated with primary antibody in $1 \times$ Tris-buffered saline $+$ 0.1% Tween 20 overnight at 4°C. Primary antibodies for PDGFR-$\alpha$ and PDGFR-$\beta$ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Equal loading of protein samples was confirmed by incubating membranes with a primary antibody specific for actin (Santa Cruz Biotechnology). Membranes were washed and then incubated for 1 h at room temperature with secondary antibody. Bound antibody was detected using ECL reagent (Amersham Pharmacia, Piscataway, NJ).

**Subcutaneous implantation and treatment of melanoma cells in a nude mouse model** Male athymic nude mice were purchased from the animal production area of the NCI-Frederick Cancer Research Facility (Frederick, MD) and maintained in specific-pathogen-free-barrier animal facilities approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC). They were used for experiments at 9 wk of age.

Cultured A375SM or MeWo cells were harvested at 80% confluence for injection by a brief exposure to 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid. Trypsinization was stopped after 2 min with addition of medium containing 10% fetal calf serum, and the cells were washed once in serum-free medium and resuspended in PBS. Only suspensions consisting of single cells with $>$90% viability were used for the injections. The cells were injected ($1 \times 10^6$ cells/mouse) subcutaneously into the left flank.

Three days after injection, the mice were randomly allocated to one of two treatment groups: one group received PBS administered by oral gavage three times weekly (control group) and the

other, imatinib mesylate (100 mg/kg) by oral gavage three times weekly (treatment group). Imatinib mesylate was provided by Novartis Pharmaceuticals, Basel, Switzerland.

Tumor size was assessed once or twice weekly for 4 to 6 weeks. At each time point, mean tumor volume was compared between the treatment group and the control group with a two-tailed Student's t test. The level of statistical significance was set at $p < 0.05$.

**Immunohistochemistry** Frozen sections of the A375SM cell lines grown orhotopically in nude mice treated with either imatinib mesylate or PBS (control) were sectioned. The sections were mounted on positively charged Plus slides (Fisher Scientific, Pittsburgh, PA) and air-dried for 30 min. The sections were fixed in cold acetone for 5 min followed by 1:1 acetone:chloroform (vol/vol) for 5 min and then acetone for 5 min. The specimens were then analyzed for the expression of PDGFR-$\alpha$, PDGFR-$\beta$, and the corresponding phosphorylated receptors. Samples were incubated with protein-blocking solution containing 4% fish gel and 5% donkey serum in PBS for 20 min at room temperature. They were then incubated at 4°C for 18 h with a 1:100 dilution of the primary antibody (rabbit polyclonal IgG to PDGFR-$\alpha$ (C-20), rabbit polyclonal IgG to PDGFR-$\beta$ (p-20), goat polyclonal IgG to phosphorylated PDGFR-$\alpha$ (Tyr-720), and goat polyclonal IgG to phosphorylated PDGFR-$\beta$ (Tyr-1021), Santa Cruz Biotechnology, Santa Cruz, CA). A positive reaction was visualized by incubating the slides for 1 h with a 1:400 dilution of Alexa Fluor 488-conjugated goat anti-rabbit or Alexa Fluor 488-conjugated rabbit anti-goat (Molecular Probes, Eugene, OR) at room temperature, avoiding exposure to light. Fluorescent bleaching was minimized by covering the slides with 90% glycerol and 10% PBS. Immunofluorescence microscopy was performed using a 20 $\times$ objective and images were captured as previously described (Bruns *et al*, 2000; Solorzano *et al*, 2001).

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