Imatinib Mesylate Inhibits Leydig Cell Tumor Growth: Evidence for In vitro and In vivo Activity

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Abstract

Leydig cell tumors are usually benign tumors of the male gonad. However, if the tumor is malignant, no effective treatments are currently available. Leydig cell tumors express platelet-derived growth factor (PDGF), kit ligand and their respective receptors, PDGFR and c-kit. We therefore evaluated the effects of imatinib mesylate (imatinib), a selective inhibitor of the c-kit and PDGFR tyrosine kinases, on the growth of rodent Leydig tumor cell lines in vivo and in vitro, and examined, in human Leydig cell tumor samples, the expression of activated PDGFR and c-kit and the mutations in exons of the c-kit gene commonly associated with solid tumors. Imatinib caused concentration-dependent decreases in the viability of Leydig tumor cell lines, which coincided with apoptosis and inhibition of proliferation and ligand-stimulated phosphorylation of c-kit and PDGFRs. Mice bearing s.c. allografts of a Leydig tumor cell line treated with imatinib p.o., had an almost complete inhibition of tumor growth, less tumor cell proliferation, increased apoptosis, and a lesser amount of tumor-associated mean vessel density compared with controls. No drug-resistant tumors appeared during imatinib treatment but tumors regrew after drug withdrawal. Human Leydig cell tumors showed an intense expression of the phosphorylated form of c-kit and a less intense expression of phosphorylated PDGFRs. No activating mutations in common regions of mutation of the c-kit gene were found. Our studies suggest that Leydig cell tumors might be a potential target for imatinib therapy. (Cancer Res 2005; 65(5): 1897-903)

Introduction

Leydig cell tumors are tumors of the male gonadal interstitium that comprise ~3% of testicular neoplasms (1). They are usually benign, but ~10% are malignant (2). The etiology of Leydig cell tumors in humans remains unknown. Mutations that render the luteinizing hormone receptor constitutively active or constitutive activation of G protein Leydig cells cause Leydig cell hyperplasia (3, 4). Leydig cell hyperplasia is, however, distinct from Leydig cell tumors and the role of luteinizing hormone receptor and G protein mutations in tumorigenesis has been questioned (5). Therapy consists of inguinal orchectomy and, if the tumor is malignant, a retroperitoneal lymph node dissection is recommended. No known role exists for radiation therapy and chemotherapy has limited efficacy. Prognosis for benign Leydig cell tumors is excellent; however, the mean survival for patients with the malignant variant is 2 years. Alternative treatments are therefore needed to improve the prognosis of Leydig cell tumor patients.

Receptor tyrosine kinases have been proposed as potential targets for antitumor therapy. Imatinib mesylate (also known as STI571 or Gleevec, and hereafter called imatinib) belongs to the group of new drugs classified as signal transduction inhibitors and has been approved as an effective treatment for chronic myeloid leukemia (6). Imatinib inhibits Bcr-Abl kinase activity, inducing cytogenetic remissions in the majority of chronic myeloid leukemia patients (6). Additional tyrosine kinases are inhibited by imatinib: c-kit, the receptor for kit ligand (KL), and the two structurally similar platelet-derived growth factor receptors (PDGFR), PDGFR-α and PDGFR-β (6, 7). Imatinib therapy is well tolerated and leads to remission in patients with c-kit-positive gastrointestinal stromal tumors (GIST) that contain gain-of-function mutations in c-kit (8). Imatinib has also been reported to inhibit the growth of glioblastoma, dermatofibrosarcoma protuberans, neuroblastoma, Ewing’s sarcoma, and small cell lung cancer, all of which may express PDGF/PDGFR or KL/c-kit autocrine growth loops (9–13).

Functional PDGFR-α, PDGFR-β, c-kit and their ligands, PDGFAA (the ligand for PDGFR-α), PDGF-BB (the ligand for both PDGFR-α and PDGFR-β), and KL are expressed in normal Leydig cells and play a crucial role in Leydig cell development (14–17). These data, coupled with the reported expression of PDGFs/PDGFRs and KL/c-kit in Leydig cell tumor samples in animals and in man (18–20), and with the notion that genes identified pertinent to early development may serve as candidate susceptibility genes for various cancers, led us to hypothesize that imatinib might inhibit Leydig cell tumor growth via inhibition of c-kit and PDGFRs. The current study examines the effect of imatinib on the growth of Leydig cell tumors and on c-kit, PDGFR-α, and PDGFR-β phosphorylation in both in vitro and in vivo models. In addition, we evaluated the presence of phosphorylated PDGFR-α, PDGFR-β, and c-kit in human Leydig cell tumors and explored c-kit genomic mutations in exons 9, 11, 13, and 17, which correspond to the c-kit exons containing oncogenic mutations in many GISTs (8).

Materials and Methods

Cell Culture. Mouse Leydig tumor cells MA10 (supplied by Prof. Mario Ascoli, Department of Pharmacology, the University of Iowa) were grown in Waymouth medium, rat Leydig tumor cells LC540 (American Type Culture Collection) were grown in EMEM in Earle’s balanced salt solution; all media were supplemented with 10% fetal bovine serum (FBS) except where indicated. Purified Leydig cell primary cultures from normal adult C57BL/6J mice and Sprague-Dawley rats (Charles River Laboratories, Calco, Italy)
were obtained from enzymatic disaggregation of the testes followed by Percoll gradient purification (21).

**In vitro Cell Growth Characterization.** MA10 and LC540 cells were plated in 60 mm dishes (5 × 10^5 cells per dish) and incubated for 48 hours in 10% or 0.1% FBS media in the absence or in the presence of various concentrations of imatinib (provided by Dr. Elisabeth Buchdunger, Novartis Pharmaceuticals, Basel, Switzerland), trypsinized and counted. We studied apoptosis by terminal nucleotidyl transferase–mediated nick end labeling (fluorescein in situ detection kit, Roche, Mannheim, Germany) and cell proliferation by 5-bromo-2′-deoxyuridine (BrdUrd) incorporation (BrdUrd immunofluorescence assay, Roche) on 1 × 10^5 MA10 and LC540 cells grown for 48 hours on glass cover slips in the absence or presence of various concentrations of imatinib. Terminal nucleotidyl transferase–mediated nick end labeling and BrdUrd staining was quantitated by assessing a total of 1,000 cells per culture and calculating the percentage of positive nuclei under a fluorescence microscope (Olympus, Tokyo, Japan).

To study the effect of imatinib on the phosphorylation of PDGFRs and c-kit, subconfluent cells shifted to 0.1% FBS overnight, were cultured with or without 10 μmol/L imatinib for 4 hours followed by an additional 10 minutes of incubation in the absence or in the presence of 10 ng/mL human recombinant PDGF-BB (Roche) or for an additional 30 minutes in the absence or in the presence of 100 ng/mL human recombinant KL (PeproTech, London, United Kingdom). In the dose-response studies, the cells were incubated with increasing concentrations of imatinib. Cells were harvested and frozen until protein or mRNA extraction.

**In vivo Tumor Growth.** We injected 3 × 10^5 MA10 cells s.c. in the right flank of 5-week-old C57BL/6j male mice (CharlesRiver Laboratories). When tumors reached a volume of ∼100 mm^3, the animals were randomly assigned to receive either 200 mg/kg imatinib in 200 μL of PBS every 12 hours (n = 6) or 160 mg/kg imatinib in 200 μL of PBS every 8 hours (n = 8) for 15 days by oral gavage. A corresponding number of control animals were treated with vehicle alone. One hour before sacrifice, 100 mg/kg of BrdUrd (Sigma-Aldrich, Milan, Italy) in 0.9% NaCl were given by i.p. injection. To determine the reversibility of the effect of imatinib, 12 animals bearing Leydig cell tumor allografts (volume of ∼100 mm^3) were treated with 160 mg/kg imatinib every 8 hours for 15 days, after which six discontinued the treatment and six executed the treatment for a further period of 7 days. We measured the tumor volume (V) by using the formula \[ V = \pi/6 \times a^2 	imes b; \] where a is the shorter diameter and b is the longer diameter of the tumor. The effect of imatinib on PDGFRs and c-kit phosphorylation in vivo was studied in mice bearing MA10 tumors of 1.5 cm^3. Two hours before sacrifice, two animals received 160 mg/kg imatinib in PBS by oral gavage, and two animals received PBS alone. Treated and control tumors were excised and frozen until protein extraction. All animals were sacrificed by CO2 inhalation. Tumor fragments were either fixed in Bouin's solution and embedded in paraffin or immediately frozen at −80°C for subsequent analysis. The animal studies were approved by the Ethic Committee of the University of Rome "La Sapienza."

Apoptosis and proliferation was studied on deparaffinized sections of allografts excised from animals subjected to 15 days of 3 × 160 mg/kg/day imatinib treatment, by BrdUrd incorporation and terminal nucleotidyl transferase–mediated nick end labeling (Roche, fluorescein detection kits). Nuclear stainings were quantitated as the percentage of positive nuclei of 1,000 counted at ×200 magnification under a fluorescence microscope (Olympus).

For detection of capillary blood vessels, 5-μm-thick deparaffinized sections of allografts of similar size, excised from animals treated for 5 days with 3 × 160 mg/kg/day imatinib or vehicle, were pretreated by boiling in 10 mmol/L citrate buffer (pH 6.0) for 15 minutes and incubated overnight in a moist chamber at 4°C with 1:100 dilution of a goat anti-mouse CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Positive reactions were detected by the streptavidin-biotin immunoperoxidase method using a commercial kit (Zymed Laboratories, San Francisco, CA) and 3,3'-diaminobenzidine as chromogenic substrate. Negative controls were done pre-absorbing the primary antibody with the corresponding immunogen peptide. The preparations were counterstained with hematoxylin and mounted. To calculate microvessel density the images at ×100 magnification of 10 randomly chosen microscopic fields were used to count CD31-positive endothelial cells (22).

**Western Blots.** Tissues or cell proteins were extracted and separated as described (23). Nitrocellulose membranes were incubated with 1:300 dilution of the following primary antibodies reactive in mouse and rat: anti-PDGFR-α, anti-PDGFR-β, anti-phospho-PDGFR-α (anti-p-PDGFR-α), anti-p-PDGFR-β, anti-p-c-kit, and anti-c-kit (Santa Cruz Biotechnology). Thereafter, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (1:10,000 dilution; Santa Cruz Biotechnology) for 1 hour at room temperature. Bound antibodies were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Secondary antibodies alone served as negative controls. Protein bands were quantified by densitometric analysis using a densitometry computer software (Kodak Digital Science, Rochester, NY).

**Reverse Transcription and PCR.** To study mRNA expression for PDGF-A, PDGF-B, PDGF-α, PDGF-β, KL, and c-kit, polyadenylated mRNA extraction and semiquantitative reverse transcription-PCR were done on frozen tissues and cells as described (23). PCR reactions were optimized for the number of cycles to ensure product intensity within the linear phase of amplification. PCR reaction conditions, cycle numbers, and sequences of the primers are shown in the Supplementary Table 1. Quantitation of the signals was done by densitometric analysis (Kodak Digital Science) after normalizing individual bands to the respective β-actin bands.

**PDGFR-α, PDGFR-β, and c-kit Phosphorylation and c-kit Mutational Analysis in Human Leydig Cell Tumors.** Specimens of five paraformaldehyde-fixed and paraffin-embedded Leydig cell tumors from 57- to 65-year-old patients (tissue archive of the Department of Medical Pathophysiology of the University of Rome "La Sapienza"), were examined. All samples had morphologic features predictive of malignant behavior (24), although clinical follow-up was not available. Five micrometer deparaffinized sections were subjected to immunohistochemical staining for p-PDGFR-α, p-PDGFR-β, and p-c-kit by using a 1:100 dilution of polyclonal rabbit antibodies specific for human epitopes corresponding to phosphorylated Tyr574, Tyr575, and Tyr654/670, respectively (Santa Cruz Biotechnology), following the procedure described for the immunohistochemical detection of capillary blood vessels. The specificity of the immunoreactivity was verified by preabsorbing the primary antibodies with the corresponding immunogenic peptides.

Genomic DNA was extracted from 10 μm sections of the Leydig cell tumor samples, using a commercial kit (TaKaRa DEXPAT, Takara Bio, Inc., Japan). Exons 9, 11, 13, and 17 of the c-kit gene were amplified by PCR using TaKaRa Ex Taq DNA polymerase (Takara Bio). Primer sequences were designed based on the human c-kit gene (Supplementary Table 2). The PCR products were purified (NucleoSpin Extract II, Macherey-Nagel, GmbH and Co, Düren, Germany) and screened for mutations by direct sequencing done as described (23). Nitrocellulose membranes were incubated with 1:300 dilution of the following primary antibodies reactive in mouse and rat: anti-CD31, anti-CD117, and anti-c-kit (Santa Cruz Biotechnology) for 1 hour at room temperature. Bound antibodies were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Secondary antibodies alone served as negative controls. Protein bands were quantified by densitometric analysis using a densitometry computer software (Kodak Digital Science, Rochester, NY).

**Results**

Expression of PDGF-A, PDGF-B, PDGF-α, PDGF-β, KL, and c-kit mRNAs in Mouse and Rat Normal Leydig Cells and Tumor Cell Lines. Transcripts encoding PDGF-A, PDGF-B, PDGF-α, PDGF-β, KL, and c-kit were expressed in both rat and mouse Leydig cell primary cultures and tumor cell lines (LC540 and MA10; Fig. 1A and C) with the only exception of the KL mRNA whose signal was not detectable in rat tumor cells LC540 (Fig. 1C). Relative levels of mRNAs are shown in Fig. 1B and D. mRNAs expression in tumor cells were constantly higher than in normal primary cultures in both species except for KL, which was undetectable in LC540 cells and expressed at very low levels by the normal rat Leydig cells and for c-kit whose expression in tumoral and nontumoral rat Leydig cells was comparable.
Imatinib Inhibits Leydig Cell Tumor Growth In vitro by Induction of Apoptosis and Inhibition of Proliferation.

Imatinib treatment (1.25 to 20 × 10⁻⁶ M) for 48 hours reduced the growth rate of both rat and mouse tumor cells grown in the presence of 10% FBS, leading to an 80% to 90% reduction in cell number as compared with untreated cells (Fig. 2A). The concentration of imatinib that was associated with 50% inhibition (IC₅₀) of growth was 5 μmol/L. Imatinib treatment (48 hours) of Leydig tumor cells cultured in 0.1% FBS media reduced the growth rate of MA10 and LC540 cells by 50% to 60% with an IC₅₀ of 10⁻⁵ mol/L (Fig. 2B). These results indicate that tumor Leydig cells are sensitive to imatinib and that PDGFRs and/or c-kit activation likely contributes to the in vitro growth of MA10 and LC540 cells.

Imatinib treatment for 48 hours displayed a dose-dependent antiproliferative (Fig. 2C) and apoptotic (Fig. 2D) effect with an IC₅₀ of ~5 μmol/L. We conclude that proliferation inhibition and apoptosis are the main mechanisms of imatinib-mediated growth inhibition.

Effect of Imatinib on PDGFRs and c-kit Phosphorylation In vitro. We examined PDGFRs and c-kit protein expression and the effect of imatinib on ligand-induced PDGFRs and c-kit phosphorylation in LC540 and MA10 cells by Western blot (Fig. 3A and B). PDGFR-α, PDGFR-β, and c-kit were expressed by both cell lines. Low baseline PDGFR-α, PDGFR-β, and c-kit phosphorylation was detected in tumor lines cultured in 0.1% FBS. Treatment with PDGF-BB (the PDGF isoform that activates all PDGFRs) or KL substantially increased PDGFR-α, PDGFR-β, and c-kit phosphorylation. Pretreatment with imatinib blocked KL-mediated, PDGF-mediated, and basal receptor phosphorylation (Fig. 3A). The imatinib inhibition of PDGFRs and c-kit phosphorylation was concentration-dependent, with an IC₅₀ value of 0.1 to 0.5 μmol/L (Fig. 3B). None of the treatments affected the expression level of the receptors. The IC₅₀ of imatinib for ligand-treated cells cultured in media containing 10% serum was < 1 μmol/L (data not shown).

Imatinib Inhibits Leydig Cell Tumor Growth and PDGFRs and c-kit Phosphorylation In vivo. We assessed the in vivo effects of imatinib treatment in mice bearing s.c. Leydig cell tumor allografts. All untreated control mice had developed tumors with a mean volume of 2,400 ± 420 mm³ (Fig. 4A and B) and expressed the complete set of genes of the PDGF/PDGFR and KL/ c-kit systems (Fig. 4D). Oral treatment with imatinib every 12 hours reduced the tumor volume to 42% the size of control (Fig. 4A). Tumors of mice receiving imatinib every 8 hours were stable throughout the course of treatment (Fig. 4B). The cessation of drug administration was followed by a resumption of tumor growth (Fig. 4C). These data indicate that imatinib inhibits Leydig cell tumor growth in vivo and suggest that continuous inhibition of the target tyrosine kinases is needed to produce major biological effects. Imatinib significantly reduced tyrosine phosphorylation of tumor-derived PDGFRs and c-kit without affecting the expression levels of the receptors (Fig. 4E). These findings confirm that PDGFRs and c-kit are expressed and functional in Leydig cell tumor allografts and show that the concentration and dosing schedule of imatinib were appropriate for in vivo inhibition of receptor phosphorylation.

**Figure 1.** PDGFs, PDGFRs, KL, and c-kit mRNAs are expressed in rat and mouse Leydig tumor cell lines and normal Leydig cells. Reverse transcription-PCR of PDGF-A, PDGF-B, PDGFR-α, PDGFR-β, KL, and c-kit mRNA expression in mouse and rat adult Leydig cell primary cultures, mouse (MA10), and rat (LC540) tumor Leydig cell lines and respective β-actin bands (A and C). Densitometric analysis of reverse transcription-PCRs expressed as a ratio between the gene being analyzed and the constitutive gene β-actin in mouse (B) and rat (D). Columns, mean; bars, ± SE (n = 4) of two experiments done in duplicate. N.D., not detectable; *, P < 0.05; **, P < 0.001, tumor cells versus normal controls.

**Figure 2.** Imatinib induces concentration-dependent growth inhibition of Leydig tumor cells through inhibition of proliferation and apoptosis. Mouse (MA10, ) and rat (LC540, ) Leydig tumor cells were incubated for 2 days with imatinib at the indicated concentrations in media containing 10% FBS (A) or 0.1% FBS (B). The results are plotted as the percentage of control untreated cells. Proliferation (C) and apoptosis (D) were evaluated in LC540 (solid columns) or MA10 (open columns) cells grown for 48 hours in 10% FBS in the absence or presence of various concentrations of imatinib. Indices are expressed as a percentage of BrdUrd or terminal nucleotidyl transferase–mediated nick end labeling positive nuclei. Columns, mean; bars, ± SE of triplicate samples; similar results were obtained in at least two different experiments.

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inhibition of proliferation and apoptosis, an antiangiogenic effect of mean microvessel density (Fig. 5).

Human Leydig Cell Tumors. and PDGFRs and Mutational Analysis of the c-kit Gene in analyzed for immunohistochemical expression of p-PDGFR-

potentially active in man, human Leydig cell tumor samples were the majority of the cells (Fig. 6).

p-PDGFR- in tumor cell apoptosis (Fig. 5).

Treatment with imatinib resulted in a 5-fold decrease of proliferation (Fig. 5).

Reduction of Microvessel Density.

Imatinib inhibits PDGFR-α, PDGFR-β, and c-kit phosphorylation. A, mouse (MA10) and rat (LC540) Leydig tumor cell lines were incubated for 4 hours in medium 0.1% FBS either in the presence or in the absence of PDGF-BB at 10 ng/mL or KL at 100 ng/mL. Immunoblots were probed with anti-p-PDGFR-α, anti-p-PDGFR-β, and anti-p-c-kit antibodies; B, MA10 and LC540 cells were incubated for 4 hours with 0 to 5 μmol/L imatinib and for additional times with PDGF-BB (10 ng/mL) or KL (100 ng/mL). The protein extracts were subjected to immunoblot analysis as in A. The immunoblots were also reprobed with anti-PDGFR-α, anti-PDGFR-β, and anti-c-kit antibodies.

Imatinib Reduces the In vivo Growth of Leydig Cell Tumor by Induction of Apoptosis, Inhibition of Proliferation, and Reduction of Microvessel Density. Treatment with imatinib resulted in a 5-fold decrease of proliferation (Fig. 5A), 5-fold increase in tumor cell apoptosis (Fig. 5B) and induced a significant reduction of mean microvessel density (Fig. 5C) in the tumor allografts as compared with untreated controls. We conclude that, in addition to inhibition of proliferation and apoptosis, an antiangiogenic effect contributes to the in vivo antitumor action of imatinib.

Immunohistochemical Expression of Phosphorylated c-kit and PDGFRs and Mutational Analysis of the c-kit Gene in Human Leydig Cell Tumors. To investigate if a mechanism of tumor growth similar to that observed in the animal models is potentially active in man, human Leydig cell tumor samples were analyzed for immunohistochemical expression of p-PDGFR-α, p-PDGFR-β, and p-c-kit. All five of the samples tested showed comparable intense positive p-c-kit cytoplasmatic staining in the majority of the cells (Fig. 6A) and a less intense staining for p-PDGFR-α (Fig. 6C) and p-PDGFR-β (Fig. 6E) in some scattered cells. Negative controls were constantly negative (Fig. 6B, D, and F).

Adjacent nonmalignant testicular tissue showed a barely detectable positive staining for the phosphorylated forms of the receptors in the interstitial Leydig cells (data not shown). No mutation was identified in the sequence of the c-kit domains (exons 9, 11, 13, and 17) previously recognized as regions of activating mutations in GISTs.

Discussion

In this study, we show that imatinib inhibits the growth of mouse and rat Leydig tumor cells in vitro and in vivo. This inhibition is associated with the suppression of PDGFRs and c-kit phosphorylation. The suppressive effect of imatinib on Leydig cell tumor development occurs through inhibition of cell proliferation, induction of apoptosis and, in vivo, also through reduction of microvessel density. Furthermore, we show that human Leydig cell tumor samples express activated PDGFRs and c-kit and no mutation in exons 9, 11, 13, and 17 of the c-kit gene. Both mouse and rat tumor Leydig cell lines expressed PDGFRs, c-kit, and their ligands even though with differences in the levels of expression of the mRNAs between species. Mouse malignant cells showed higher levels of PDGFs, PDGFRs, KL, and c-kit genes compared with normal Leydig cells. This pattern of PDGF and PDGFR mRNA expression is novel. The concomitant expression of KL/c-kit and their increased levels in Leydig tumor cells confirm a previous report of spontaneous or hormonally induced mouse Leydig cell tumors (19). In the rat, PDGF and PDGFR expression resembled.

![Figure 3](image-url) Imatinib inhibits PDGFR-α, PDGFR-β, and c-kit phosphorylation. A, mouse (MA10) and rat (LC540) Leydig tumor cell lines were incubated for 4 hours in medium 0.1% FBS either in the presence or in the absence of PDGF-BB at 10 ng/mL or KL at 100 ng/mL. Immunoblots were probed with anti-p-PDGFR-α, anti-p-PDGFR-β, and anti-p-c-kit antibodies; B, MA10 and LC540 cells were incubated for 4 hours with 0 to 5 μmol/L imatinib and for additional times with PDGF-BB (10 ng/mL) or KL (100 ng/mL). The protein extracts were subjected to immunoblot analysis as in A. The immunoblots were also reprobed with anti-PDGFR-α, anti-PDGFR-β, and anti-c-kit antibodies.

![Figure 4](image-url) Figure 4. Effect of oral treatment with imatinib on growth of Leydig cell tumor allografts, PDGFRs, and c-kit phosphorylation and effects of withdrawal of therapy. A, allografts were established with MA10 Leydig tumor cells in male mice. One group was treated with 200 mg/kg imatinib (●; n = 6 mice) or vehicle (□; n = 6 mice) every 12 hours for 15 days. B, a second group was treated with 160 mg/kg imatinib (●; n = 8 mice) or vehicle (□; n = 8 mice) every 8 hours for 15 days. C, a third group (●; n = 12 mice) was treated with 160 mg/kg imatinib every 8 hours for 15 days, after which imatinib was withdrawn (arrow) in half of the animals and vehicle alone was given on days 16 to 22 (Δ; n = 6 mice), whereas the remaining (●; n = 6 mice) continued the treatment. Points, mean; bars, ± SE; *, P < 0.05; **, P < 0.01; ***, P < 0.001, treated versus control group. D, representative reverse transcription-PCR showing PDGF-A, PDGF-B, PDGFR-α, PDGFR-β, KL, and c-kit mRNA expression in MA10 tumor allografts excised from untreated animals bearing 2.0 cm² tumors. mRNAs obtained from three randomly selected tumors gave identical results; E, Western blots on protein extracts from 1.5 cm³ allografts excised from one control mice (●) and one mice treated with 160 mg/kg p.o. imatinib (+ imatinib) 2 hours before sacrifice. The filters were probed with anti-p-PDGFR-α, anti-p-PDGFR-β, or anti-p-c-kit antibodies to reveal the status of PDGFRs and c-kit signaling after treatment. The immunoblots were also probed with anti-PDGFR-α, anti-PDGFR-β, or anti-c-kit antibodies. Similar results were obtained in two different experiments.
that described in the mouse; on the contrary, according to previous studies (20), we found a lack of KL expression in tumor cells and comparable levels of c-kit expression between normal and tumor cells. We conclude that, although with differences in the expression intensity, mouse and rat Leydig cell tumor lines express the imatinib-sensitive receptor kinases PDGFR and c-kit. Western blot experiments showed that PDGFRs and c-kit found in tumor Leydig cells are functional. In fact, a basal phosphorylation of PDGFRs and c-kit was seen in the lines supplemented with FBS as well as in serum-free conditions. Additional evidence that Leydig tumor lines are dependent on PDGFRs and c-kit activation is given by the marked decrease in c-kit and PDGFRs phosphorylation induced by imatinib that, in turn, provokes growth inhibition through concentration-dependent proliferation arrest and apoptosis. Although the suppression of PDGFRs and c-kit phosphorylation in our studies (IC$_{50}$ = 0.1-0.5 μmol/L) occurred at concentrations similar to the IC$_{50}$ values reported for tyrosine kinase inhibition (6), the inhibition of Leydig cell tumor growth in vivo required an higher concentration of imatinib (IC$_{50}$ = 5 μmol/L) than that required in studies on chronic myeloid leukemia or GIST cells (IC$_{50}$ = 1 μmol/L; refs. 6, 8), lower than that required in studies on neuroblastoma (11), or Ewing’s sarcoma (IC$_{50}$ = 10 μmol/L; ref. 12) and in the same order than the concentrations achieved in studies on small cell lung cancer (IC$_{50}$ = 5 μmol/L; ref. 13). We also found higher IC$_{50}$ values for imatinib-mediated apoptosis induction (IC$_{50}$ = 5 μmol/L) and proliferation inhibition (IC$_{50}$ = 5 μmol/L) compared with the IC$_{50}$ for PDGFRs and c-kit phosphorylation suppression. The reason for the discrepancy between the pharmacologic IC$_{50}$ and the biological IC$_{50}$ of imatinib is unclear. One possible explanation is that, as reported in different cell models (13, 25), an additional target or targets, which require higher concentrations of imatinib to be suppressed, could be expressed in Leydig tumor cells.

Imatinib treatment led to marked abrogation of Leydig tumor growth and PDGFRs and c-kit phosphorylation of already established and actively growing tumor allografts. A treatment schedule administering imatinib twice a day produced 58% inhibition of tumor growth. A treatment regimen assuring the continuous block of the PDGFRs and c-kit phosphorylating activity (p.o. administration every 8 hours) produced an almost complete inhibition of tumor growth, indicating that the continuous block of tyrosine kinases is needed to produce important biological effects in vivo. The requirement of three daily administrations to obtain major antitumor effects in animals, was already observed in nude mice bearing Bcr/ abl-positive human leukemia cell lines and has been explained with the pharmacokinetic profile of imatinib in mouse (26). Analogously to what is described in chronic myeloid leukemia patients (27), discontinuation of therapy was followed by a rapid recurrence of tumor growth, confirming the need for continued treatment. Similar to observations in vitro, the growth-inhibitory effects of imatinib in vivo are achieved through reduction of proliferation and induction of apoptosis. Moreover, our characterization of the microvessel density provide evidence for an antiangiogenic effect of imatinib. This action of imatinib has been observed in various cancers (22, 28) and it has been explained with the inhibition of PDGFRs and c-kit, which are important survival factors for endothelial cells (29, 30). A further

Figure 5. Imatinib induces inhibition of proliferation, apoptosis, and decrease of vascularization of Leydig cell tumors in vivo. Proliferating (A) and apoptotic cells (B) were scored and indices are expressed as a percentage of positive nuclei of 1,000 counted cells. C, microvessel density was quantitated by counting the number of CD31-positive cells for every 10 randomly chosen 0.159-mm$^2$ microscope fields. Columns, mean; bars, ± SE of triplicate samples; *, P < 0.05; **, P < 0.01, imatinib versus control. D, representative vision fields from imatinib-treated and control tumors stained for proliferation (BrdUrd; left), apoptosis (terminal nucleotidyl transferase–mediated nick end labeling; middle), and capillary endothelial cells (CD31; right). Arrows, positive cells; bars, 50 μm.

Figure 6. Immunohistochemical analysis of phosphorylated PDGFR-α, PDGFR-β, and c-kit in human Leydig cell tumor. Representative immunohistochemical staining of the tyrosine phosphorylated (i.e., activated) form of c-kit (A), PDGFR-α (C), and PDGFR-β (E) in a human Leydig cell tumor sample and corresponding negative controls (B, D, and F). p-c-kit is abundantly and widely expressed in the tumoral tissue (A). A staining intensity weaker than that observed for p-c-kit and localized in some cells is seen for p-PDGFR-α (C) and p-PDGFR-β (E). Negative controls are constantly negative. Comparable results were obtained in the five tumors analyzed. Bars, 100 μm.
mechanism may include the inhibition of vascular endothelial growth factor, the prototype angiogenic factor. Accordingly, it has been reported that imatinib inhibits vascular endothelial growth factor expression either directly (11) and/or indirectly through a PDGF inhibition-mediated mechanism (31).

The etiology and pathogenesis of testicular tumors is poorly defined. It is known that estrogen and surgically induced cryptorchidism are associated with Leydig cell tumorigenesis in mice (32, 33). Interestingly, elevated estrogens or hypoxia, which is a consequence of cryptorchidism, are associated with induction of PDGF, robust phosphorylation of PDGFR, and with increased expression of KL and c-kit (34–37). Accordingly, Leydig cell tumors develop with a very high incidence in a transgenic mice line in which c-kit and KL are coexpressed with 100% incidence (19). These data indicate that there is a tight link between the growth-regulatory pathways of KL/c-kit and PDGFRs/ PDGFRs and recognized inducers of Leydig cell tumorigenesis. The oncogenic potential of PDGFRs and c-kit pathways in solid malignancies can be realized through two general mechanisms: autocrine and/or paracrine stimulation of the receptors, and acquisition of activating mutations (7). Mutation analysis of exons 9, 11, 13, and 17 of the c-kit gene was negative in our panel of Leydig cell tumors in spite of a strong p-c-kit immunohistochemical expression. However, the occurrence of PDGFR-α mutations in a subset of c-kit wild-type GISTs, which may represent an alternative event in GIST pathogenesis, has been described (7), and mutant PDGFR-α chains can heterodimerize with c-kit and phosphorylate c-kit in a constitutive manner so as to be oncogenic (7, 38). Therefore, it will be important to examine PDGFR-α mutations in human Leydig cell tumors. Clinical translation of the results presented in this article is difficult. Leydig tumor cells of human origin could not be employed in our experiments because human Leydig tumor cell lines are not available and primary Leydig cell cancer cultures are arduous to obtain due to the rarity of surgical specimens. Furthermore, the use of orthotopic transplantation or spontaneous Leydig cell tumor modeling in rodents would be valuable in a further preclinical development of imatinib therapy for Leydig cell tumors. Nonetheless, the results reported in the present article, and some lines of evidence suggest that human Leydig cell tumors might be targeted by imatinib. First, previous studies have shown that human Leydig cell tumors express high levels of PDGFRs and PDGFRs ligands (18). Second, long-term imatinib treatment in man alters testosterone secretion, indicating that human Leydig cells are sensitive to imatinib administration (39). Finally, we have shown here that human Leydig cell tumor specimens stain positive for the activated, phosphorylated form of PDGFR-α, PDGFR-β, and particularly for c-kit. Given that 10% of Leydig cell tumors in man are associated with a malignant course, the present study provides a rationale for a potential treatment with imatinib of Leydig cell tumors in which, irrespective of mutational status, a c-kit and/or PDGFR activation can be shown.

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References

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