The c-kit Tyrosine Kinase Inhibitor STI571 for Colorectal Cancer Therapy

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Abstract

The c-kit tyrosine kinase inhibitor STI571 exhibits a substantial therapeutic activity in patients with chronic myeloid leukemia and gastrointestinal stromal tumors respectively associated with constitutive activation of the BCR-ABL and c-kit tyrosine kinases. Human colorectal tumors also express the c-kit proto-oncogene. The present study focuses on the anticancer activity of STI571 in human colorectal tumor cells in vitro and in vivo. The c-kit receptor was identified as a M, 145,000 immunoreactive band in human colon cancer cells HT29, HCT8/S11, and HCT116. Cellular invasion induced by 10 ng/ml stem cell factor (EC_{50} = 3 ng/ml) in HT29 cells was blocked by 1 \mu M STI571 (IC_{50} = 56 nm) and pharmacological inhibitors of several oncogenic signaling pathways, namely, phosphatidylinositol 3-kinase 3-kinase (LY294002), Rho GTPases (Clostridium botulinum exoenzyme C3 transference), and Rho-kinase (Y27632). STI571 inhibited HT29 cell proliferation (IC_{50} = 6 \mu M) and induced apoptosis in vitro. These cellular effects were associated with a decrease in tumor growth. We also demonstrated that stem cell factor is a proangiogenic factor in vitro and in vivo. These encouraging results warrant further preclinical investigations and clinical trials on the use of the c-kit inhibitor STI571 as a chemotherapeutic agent in colon cancer prevention and in treatment of advanced colorectal cancers associated with liver metastases.

Introduction

The c-kit proto-oncogene encodes a transmembrane tyrosine kinase receptor. Activation of c-kit by SCF, its natural ligand, promotes its dimerization and autophosphorylation at specific tyrosine residues Tyr^{567} and Tyr^{719}. Signaling by c-kit plays an important role in cellular transformation and differentiation, including proliferation, survival, adhesion, and chemotaxis (1). These pleiotropic functions of c-kit are mediated through STAT3, PI3K, PLC, and the MAPK cascade (2-5).

The expression of the c-kit proto-oncogene has been reported in hematopoietic cells, small cell lung cancer, and gastrointestinal stromal tumors (6-8). Colorectal cancer is one of the leading causes of cancer death in Western countries, with approximately 50% of colon cancer-related deaths due to liver metastasis. Recent studies have demonstrated that human colorectal tumors express c-kit transcripts and protein by immunohistochemistry (9). Accordingly, c-kit mRNA expression has been detected in the human colonic adenocarcinoma cell lines HT29 and DLD-1 (9, 10). Furthermore, it has been shown that activating mutations of c-kit protect human colon adenocarcinoma cells against apoptosis and enhance their invasive potential (10). The c-kit ligand SCF has been also detected in normal intestinal epithelial cells (11), suggesting autocrine and paracrine control of transforming functions by SCF in human colon cancer, including the regulation of cell proliferation, survival, invasion, and tumor angiogenesis.

The c-kit tyrosine kinase inhibitor STI571 has shown a significant activity in CML patients bearing the BCR-ABL tyrosine kinase produced by the Philadelphia chromosome (12, 13). Furthermore, STI571 also blocks the autophosphorylation of the platelet-derived growth factor receptors (12). This drug (Glivec; Novartis, Basel, Switzerland) is now being tested in clinical trials for treatment of metastatic gastrointestinal stromal tumors and gliomas (14-16). This study focuses on the transforming functions exerted by SCF and the anticancer activity of STI571 in vitro and in vivo, using human colorectal tumor cell lines that express c-kit.

Materials and Methods

Cell Culture and Reagents. Human colorectal cancer cell lines HT29, HCT116, and HCT116p21 were cultured in DMEM (Life Technologies, Inc., Cergy Pontoise, France) supplemented with 10% FBS and antibiotics (Roche Molecular Biochemicals, Meylan, France). HCT8/S11 colorectal cancer cells were cultured in RPMI 1640 (Life Technologies, Inc.) containing 10% FBS containing bovine serum albumin, insulin (5 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and neomycin (100 μg/ml). The human erythroleukemia cell line K562 was established from a patient with CML (18). Philadelphia chromosome-positive K562 cells were grown in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, amphotericin B (2 μg/ml), and glutamine (2 mM). Primary cultures of HUVECs were obtained from Clonetics-BioWhittaker (Emerainville, France) and maintained at 37°C using the EBMM-2 bullet kit from Clonetics-BioWhittaker supplemented with 2% FCS, endothelial cell growth supplement (containing VEGF, human fibroblast growth factor B, insulin-like growth factor I, and human epidermal growth factor), 50 μg/ml heparin, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and the supplements hydrocortisone, ascorbic acid, gentamicin, and insulin (Clonetics-BioWhittaker). Cells used in the experiments were between passages 5 and 15.

Recombinant human SCF, leptin, and VEGF-165 were from R&D Systems Europe Ltd. (Oxon, United Kingdom). HGF was a generous gift from Prof. Paolo Comoglio (University of Turin, Turin, Italy). The 2-phenylnaminoypirimidine derivative STI571 was a generous gift from Dr. E. Buchdunger (Novartis, Basel, Switzerland). The pI2/4 MAPK inhibitor PD98059 and the PLC inhibitor U-73122 were from Calbiochem (Meudon, France). Pharmacological inhibitors of PI3K (LY294002) and mTOR/FRAP kinase (rapamycin) were from Sigma (Saint-Quentin Fallavier, France). C3T, which ADP-ribosylates and inactivates the small Rho GTPases, was a generous gift from Dr. Gilles Flattau (INSERM U452, Nice, France). The ROCK inhibitor Y27632 was kindly provided by Yosshimi Chemicals (Osaka, Japan). Collagen type I was from Upstate Biotechnology (Lake Placid, NY).

Immunoblotting. Confluent cells were lysed at 4°C in radiomunnunoprecipitation assay buffer containing 0.1 mg/ml phenylmethylsulfonyl fluoride, 100 μM benzamidine, and 100 mM NaVO₃, as protease inhibitors. Detergent-insoluble material was removed by centrifugation at 12,000 x g for 15 min at 4°C. About 100 μg of total cellular proteins were resolved by SDS-PAGE.

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3 The abbreviations used are: SCF, stem cell factor; PI3K, phosphatidylinositol 3-kinase; STAT, signal transducers and activators of transcription; PLC, phospholipase C; MAPK, mitogen-activated protein kinase; CML, chronic myelogenous leukemia; FBS, fetal bovine serum; HUVEC, human umbilical vascular endothelial cell; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; C3T, Clostridium botulinum exoenzyme C3 transference; ROCK, Rho-kinase; CAM, chorioallantoic membrane.
calculated using the formula

\[
\text{invasion index} = \frac{\text{number of cells invading}}{\text{total number of cells}}
\]

Five days after cell injection, STI571 treatment was started (day 1) at a dose

ty

Collagen Invasion Assay. For invasion of collagen type I by HT29 cells, Petri dishes were filled with 1.35 ml of neutralized type I collagen (Upstate Biotechnology) and incubated for 2 h at 37°C to allow gelation. Cells were harvested using Moscona buffer and trypsin/EDTA and seeded on top of the collagen gels. Cultures were incubated for 24 h at 37°C in the presence or absence of the indicated agents. The depth of cell migration inside the gels was measured using an inverted microscope, as described previously (19). Invasive and superficial cells were counted in 12 fields of 0.157 mm². The invasion index corresponds to the ratio of the number of cells invading the gel to the total number of cells counted in each field.

Apoptosis. HT29 cells (1 × 10⁶) were plated and cultured for 24–72 h at 37°C on 100-mm Petri dishes in DMEM and 10% FBS with or without STI571 (10 μM). For flow cytometric analysis, adherent and floating cells were combined, washed once with PBS, and fixed overnight at 4°C in 70% ethanol. Fixed cells were washed with PBS, incubated for 30 min at 37°C with 1 μg/ml RNase A, and stained with propidium iodide. The stained cells were analyzed on a FACScan flow cytometer for relative DNA content (FACSCalibur; Becton Dickinson, Le Pont de Claix, France). About 10,000 cells were recorded per assay.

Cell Proliferation. HT29 cells (1 × 10⁵) were plated on 35-mm dishes in DMEM supplemented with 10% FBS. After 24 h, cells were treated for 48 h with increasing concentrations of STI571 (1–50 μM). Total cells were then trypsinized, taken up in 1 ml of DMEM, and counted using a cell counter (Coulter Counter, Northwell, United Kingdom).

Tumor Growth Assay. Six-week-old athymic NMRI nude mice (nu/nu; Elevage Janvier, Le Genest, France) were maintained under specified pathogen-free conditions. Human colorectal HT29 cells were injected s.c. into the lateral flank of the nude mice (5 × 10⁶ cells). Throughout this study, nude mice were housed in filtered-air laminar flow cabinets and manipulated following aseptic procedures. Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies (European Economic Community’s Council Directive 86/609, OJ L 358, 1, December 12, 1987; NIH Guide for Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985). Five days after cell injection, STI571 treatment was started (day 1) at a dose of 50 mg/kg/day delivered by i.p. injections, or vehicle buffer was administered as a control, using six mice for each group. Tumor volumes were calculated using the formula \(V = 0.4 \times a \times b^2\), in which \(V\) represents volume, \(a\) is the largest diameter of the tumor, and \(b\) is the smallest diameter of the tumor. Animals were sacrificed 16 days after treatment initiation, and tumors were excised and weighed.

Angiogenesis Assay in Vivo and in Vitro. The CAM angiogenesis assay was performed as described by Maragoudakis et al. (20), with some modifications (21). The angiogenic response in a given CAM assay was analyzed with the c-kit receptor previously reported in gastrointestinal stromal tumors (16) and the human mast cell leukemia cell line HMC-1 that expresses a constitutively activated c-kit tyrosine kinase (8). As a positive control, cellular extracts prepared from K562 cells showed a signal at \(M_r\) 145,000, in agreement with immunofluorescence studies performed in this human erythroleukemia cell line (25).

The c-kit Ligand SCF Induces Cellular Invasion in HT29 Colon Cancer Cells. To examine whether c-kit may participate in the regulation of cellular invasion in human colon cancer cells, we assayed the proinvasive capacity of the c-kit ligand SCF in HT29 cells. As shown in Fig. 1B, HT29 cells exhibited a remarkable invasiveness in collagen type I in response to SCF (10 ng/ml), as well as to leptin (100 ng/ml) and HGF (10 units/ml). Half-maximal stimulation of invasion was observed at an EC₅₀ of 3 ng/ml SCF (data not shown).

In view of the critical role of PI3Ks in tumor cell invasion (26–28) and because PI3K is also implicated in c-kit signaling (29), we next examined the contribution of this lipid/protein kinase to the regulation of invasiveness by SCF. As shown in Fig. 1C, the PI3K inhibitor LY294002 (10 μM) abolished HT29 invasiveness induced by 10 ng/ml SCF.
SCF. We also assessed whether Rho-like GTPases contribute to the induction of tumor cell invasion by SCF. Rho activation promotes actin stress fiber assembly and focal adhesion formation, common responses to cell adhesion and migration, through the Rho-associated kinase ROCK (30). We found that pharmacological inhibitors of Rho (C3T3, 3 μg/ml) and ROCK (Y27632, 10 μM) both prevented cellular invasion induced by SCF (Fig. 1C). Recent studies have demonstrated that the PLC-β, mTOR/FRAP, and MAPK signaling pathways are new potential therapeutic targets against metastatic disease (31, 32). Accordingly, we have presented evidence that the proinvasive pathways monitored by trefoil factors, thromboxane A2, and thrombin PAR-1 receptors are mediated through PLC-β and/or mTOR/FRAP-dependent pathways in kidney and colonic cancer cells (30, 33, 34). Both PLC and p42/44 are downstream of the c-kit receptor (2, 4). In the present study, SCF-induced invasiveness was not altered by inhibitors of PLC (U-73122, 1 μM), mTOR/FRAP (rapamycin, 20 nM), or the p42/p44 MAPK (PD98059, 50 μM), as shown in Fig. 1C.

We next examined the effects of STI571 on cellular invasion induced by the c-kit activator SCF, leptin, and the Met activator HGF in HT29 cells. As shown in Fig. 1D, 1 μM STI571 selectively abolished invasiveness induced by SCF and leptin, but not by HGF, in a dose-dependent manner. Half-maximal inhibition of invasion was observed at an IC50 of 20–30 nM STI571.

Inhibition of Cellular Proliferation and Induction of Apoptosis by STI571. The antiproliferative effects of STI571 were assayed in a series of human colorectal cancer cell lines, including HT29, HCT8, S11, and HCT116. Cultured cells were incubated in a medium containing 10% FBS and increasing concentrations of STI571 (up to 50 μM). After 48 h, adherent cells were counted. The inhibitory effect of 10 μM STI571 on cellular proliferation varied between 8% and 64%, according to the cell line. At 1 μM, the c-kit inhibitor had no effect on cell growth in vitro. As shown in Fig. 2A, half-maximal inhibition of HT29 cell proliferation by STI571 was observed at IC50 = 6 μM.

We next examined the cellular mechanism underlying the inhibition of cell proliferation by STI571, using the HT29 cell line, which showed the highest sensitivity to the c-kit inhibitor. In the presence of 10 μM STI571, HT29 cells underwent morphological changes and detached from the surface of the dish. Between 48 and 72 h, an increasing fraction of hypoploid cells, from 5.5% to 21%, was detected, suggesting programmed cell death (Fig. 2B). The nonadherent HT29 cells were not viable, probably through induction of anotik, i.e., loss of adherence to the matrix substratum. Accordingly, fluorescence-activated cell-sorting analysis revealed that STI571 induced cellular apoptosis in a time-dependent manner, reaching a level of 21% nonviable cells after 72 h in HT29 cells (Fig. 2B). This could be related to the role of SCF/c-kit signaling on adhesion of intestinal epithelial cells to basement membrane components (11) and mesenchymal-epithelial interactions linked to epithelial cell migration, differentiation, and apoptosis during exfoliation at the tip of the villus. In contrast, there was no significant effect on the cell cycle distribution profile in the presence of 10 μM STI571 (data not shown). For an anticancer drug, cytotoxic and cell death responses would bring additional beneficial effects when combined with cell cycle inhibitors and antiangiogenic agents.

STI571 Slows Down HT29 Cell Tumor Growth in Vivo. The biological relevance of our data was next examined on the in vivo growth of HT29 tumor cells injected s.c. in the nude mice. The growth of the HT29 tumor xenografts was monitored after i.p. injection of 50 mg/kg STI571, once a day, for 16 consecutive days. This treatment reduced by 30% the volume of the HT29 tumor cell xenografts (P < 0.05; Fig. 2C). As shown in Fig. 2D, a similar difference was also found in tumor weight (0.92 ± 0.04 g versus 1.32 ± 0.12 g; P < 0.01), suggesting that the c-kit receptor plays a significant role in the generation and maintenance of human colonic tumor xenografts. There was no undesirable effect of STI571 treatment on animal behavior and body weight.

Induction of Angiogenesis in Vivo and in Vitro by the SCF. Using the CAM assay involving the coordination and integration of multicellular responses during the development of the chick embryo, we demonstrated in Fig. 3A that 100 ng/ml SCF promoted a remarkable stimulation of new vessel formation that was comparable with that induced by the proangiogenic factor VEGF-165 (1 μg/ml).

To assess whether the proangiogenic effect of SCF involves a direct interaction of the c-kit ligand with endothelial cells, we conducted comparative studies on the formation of capillary-like structures in vitro, using HUVECs plated on Matrigel-coated plates. As shown in Fig. 3B, normal HUVECs have the ability to form capillary structures when seeded and cultured for 8 h on top of Matrigel substratum. Control cells move from their initial uniform pattern of dispersed cell layers and associate to form a network of cell clusters connected by long, multicellular processes, leading to the formation of tube-like structures. This spontaneous angiogenic phenotype is enhanced by the addition of 100 ng/ml SCF (P < 0.006 versus control) and 40 ng/ml VEGF-165 (P < 0.006 versus control) in the Matrigel matrix and involves endothelial cell migration and capillary tube formation. Thus, we have demonstrated here the ability of human SCF to function as a proangiogenic factor through a direct interaction on endothelial cells.

Discussion

In this report we presented evidence that human colorectal cancer cell lines express a functional c-kit receptor for the SCF. In our studies, the c-kit proto-oncogene was clearly identified as the mature functional M, 145,000 form of the tyrosine kinase. It has been shown that this fully glycosylated form of the c-kit protein is highly
phosphorylated in gastrointestinal stromal tumors, irrespective of the presence or absence of oncogenic mutations (35). Constitutive activation of the c-kit tyrosine kinase can also be mediated through direct interaction with tumor cell-derived SCF or indirectly via transactivation of the c-kit intracellular domain by transphosphorylation and binding to other signaling adapters, such as STAT3 and STAT1 (5).

The coexpression of SCF and c-kit in human colorectal cancers supports such a hypothesis (9, 10). Therefore, the presence of activating mutations within the c-kit tyrosine kinase gene remains to be explored in human colonic adenocarcinomas.

We found that treatment of cultured HT29 cells with SCF induced cell invasion through signaling pathways implicated in tumor cell invasion, namely, PI3K and the Rho/ROCK cascade (26–28, 30). Several other signal transduction pathways have been implicated in SCF/c-kit-mediated signal transduction, including the src/STAT cascade (1). In agreement with a role of the c-kit tyrosine kinase in invasion, STI571 abolished the proinvasive activity of SCF at a concentration (1 μM) that was ineffective on HT29 cell proliferation. Accordingly, Wang et al. (7) showed that similar low concentrations of STI571 decreased the migration of hematopoietic Ba/F3 and small cell lung cancer cell lines. Thus, we found that the c-kit tyrosine kinase is crucial for the determination of the invasive phenotype induced by SCF and leptin, a regulatory hormone implicated in obesity, tumor cell invasion, and angiogenesis (27, 36). In contrast, we have shown here that the c-kit inhibitor STI571 plays a minor role in the proliferative potential in human colon cancer cells cultured in the presence of serum-associated growth factors. Accordingly, several experimental studies indicate that HT29 cancer cells and human digestive tumors in general are controlled in vitro and in vivo by a complex network of autocrine and paracrine mitogenic loops involving transforming growth factor α and other endogenous growth factors (37). Most interestingly, our data indicate that the growth-suppressive effect of STI571 is directly connected with its ability to induce programmed cell death in cultured HT29 cells. This cytoxic effect is probably caused by the implication of the c-kit receptor in cell survival and adhesion (Fig. 2B) through PI3K/Akt signaling (38), as shown previously (5). A recent report by Bellone et al. (10) also presented evidence that aberrant activation of c-kit signaling contributes to cellular invasion and survival in colon carcinoma cells.

In turn, it will be intriguing to determine whether the proapoptotic response induced by the c-kit inhibitor STI571 in colon cancer cells can synergize with other apoptosis-inducing agents or with anticancer drugs acting through other mechanisms, including cell cycle arrest or antiangiogenic responses. Most interestingly, we observed that SCF is a potent proangiogenic factor in vivo and in vitro. Thus, one can postulate that the c-kit pathway and STI571 may exert a significant impact on the growth and dissemination of digestive tumors expressing c-kit, after detection by systematic screening. In agreement with this proposition, we observed in the present model of HT29 xenografts in the nude mice that the pharmacological control of the c-kit tyrosine kinase by STI571 resulted in a significant inhibition of tumor growth. The highly tumorigenic HT29 human colon cancer cell line was previously selected as a pertinent predictive model for the identification of efficient combinations between chemotherapeutic agents for colorectal cancer therapy (39). Previous therapeutic strategies for advanced colorectal cancer were essentially limited to regimens using 5-fluorouracil combined with folinic acid (40, 41). More recently, combinations of 5-fluorouracil with either CPT-11 or oxaliplatin, according to different schedules, clearly improved the results in metastatic patients. Some of these combinations are now used in clinical trials for adjuvant colorectal cancer patients (42, 43). The third step will undoubtedly be based on novel protocols using anticancer drugs targeting signaling molecules implicated in critical oncogenic pathways, alone or combined with conventional chemotherapy. For example, the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 is now in clinical trials against colon cancer and other solid tumors (44, 45).

In summary, our results suggest a promising therapeutic impact for STI571 in the prevention and progression of adenomas in patients at risk for colorectal cancer and in colorectal cancer patients with advanced primary tumors associated or not associated with liver metastases. Such a prediction merits exploration, especially because this compound has been successfully tested as a therapeutic agent for CML and gastrointestinal stromal tumors and is well tolerated. In our studies, we have based the dosage of the anticancer activity of STI571 on data published in earlier preclinical studies showing a therapeutic impact of the c-kit inhibitor STI571 in glioblastoma and dermatofibrosarcoma protuberssions: 50 mg/kg, twice a day (15), and 100 mg/kg, twice a day (46). In that respect, the amount of STI571 used in our studies compares well with that used successfully in other preclinical models. Immunohistochemical analysis of c-kit expression in normal human colonic tissue showed no detectable signal in the surface epithelium of the normal colonic mucosa (9). In contrast, significant staining was observed in mucosa adjacent to tumor tissue, and strong staining was evident in 40% of primary colorectal carcinomas. Thus, clinical trials in c-kit-positive colorectal cancer patients with STI571 combined with conventional chemotherapy or alone as an oral maintenance treatment deserve further evaluation.
References


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