Abstract

**Background:** Pancreatic cancer is an aggressive malignancy characterized by low responsiveness to chemotherapy and radiotherapy. This resistance is partly due to the overexpression of several tyrosine kinase receptors and their ligands. STI571 has specific activity in inhibiting c-kit, PDGF and Abl receptor tyrosine kinases and has proven successful in the treatment of CML and GIST patients. Here, we investigated the potential role of STI571 in pancreatic cancer.

**Results:** The GI\textsubscript{50} of STI571 as well as the effects of STI571 on growth factor actions in pancreatic cell lines were analyzed using the MTT assay. FACS analysis using Annexin and PI staining was performed to study cell cycle, apoptosis, and cell death. Western blot analysis was carried out to investigate MAP kinase and receptor tyrosine kinase phosphorylation. STI571 inhibited cell proliferation in pancreatic cancer cell lines with GI\textsubscript{50} concentrations ranging from 17 to 31.5 microM. EGF, IGF-1, and FGF-2 but not PDGF exerted growth stimulatory effects in pancreatic cancer cell lines. STI571 only partly blocked these effects on cell growth, and did not abrogate growth factor-induced receptor and MAPK phosphorylation.

**Conclusion:** Our data demonstrate that STI571 inhibits pancreatic cancer cell growth with high GI\textsubscript{50} concentrations through tyrosine-kinase receptor independent pathways. The clinical application of STI571 in pancreatic cancer is therefore rather doubtful.
In recent years, increasing attention has been directed towards the role of growth factors in the pathogenesis of human tumors. Human pancreatic cancers overexpress a number of important tyrosine kinase growth factor receptors and their ligands, such as those belonging to the epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF-1), and vascular endothelial growth factor (VEGF) families [10,11]. In addition, expression of both PDGF and PDGF receptors (PDGFRs) has been observed in pancreatic cancer [12]. It is thought that these growth factors act in an autocrine and/or paracrine manner to stimulate pancreatic cancer growth. Binding of growth factors to their receptors results in receptor autophosphorylation and subsequent signal transduction via an array of different molecules.

Small molecule drugs that can selectively inhibit tyrosine kinases are likely to be of benefit in a number of neoplastic diseases. Although tyrosine kinase inhibitors have been studied for many years, they often had little specificity and thus were unlikely to be suitable for clinical applications. Recently, more selective tyrosine kinase inhibitors have been developed, one of them being STI571 (Gleevec, imatinib mesylate and also known as CGP57148B; Novartis Pharmaceuticals). STI571 belongs to the 2-phenylaminopyrimidine class, and has selectivity for Abl, PDGF receptor [13–15] and c-kit receptor tyrosine kinases [16]. The effectiveness of this drug in blocking Bcr-Abl and c-kit tyrosine kinases has led to Food and Drug Administration (FDA) approval for the treatment of Philadelphia chromosome-positive chronic myelogenous leukemia and c-kit-positive gastrointestinal stromal tumors.

STI571 could inhibit pancreatic cell growth dose-dependently [17]. Since most human pancreatic cancer tissues express PDGFR, STI571 might exert its function through this pathway in pancreatic cancer cells. The purpose of this study was to gain insight into the mechanisms of STI571 action in pancreatic cancer cells, with special emphasis on receptor tyrosine kinase signaling.

### Results

#### Determination of the GI₅₀ concentration of STI571 in pancreatic cancer cells

To determine the GI₅₀ concentration of STI571, pancreatic cancer cells grown in 10% FBS-containing medium were exposed to different doses of STI571. As seen from Table 1 and Figure 1, STI571 inhibited the growth of all tested pancreatic cancer cells in a dose-dependent manner. The concentrations of STI571 required to inhibit cell growth by 50% (GI₅₀) were in the range of 17–31.5 µM, with Colo-357 cells being the most sensitive (17 µM), and Aspc-1 cells the most resistant (31.5 µM) (Table 1 and Figure 1). Thus, the GI₅₀ for pancreatic cells appeared to be higher than the GI₅₀ for other reported cancer cells, such as small cell lung cancer cells, and out of the range of the STI571 plasma concentration reported in patients treated with this drug under clinical conditions [18,19]. Since effects of STI571 were reported to depend on serum concentration, we also tested the GI₅₀ of STI571 under low serum conditions (1% FCS). Here, GI₅₀ concentrations ranged between 9 and 20 µM, with Mia-PaCa-2 cells being the most sensitive (9 µM), and Aspc-1 cells the most resistant (20 µM) (Figure 1). Therefore, increasing concentration of growth factors in serum increased the resistance of pancreatic cancer cells to STI571.

| cancer cell lines | STI571 (µM) |
|-------------------|-------------|
| Aspc-1#            | 31.5        |
| BxPc-3#            | 21          |
| Capan-1#           | 19          |
| Colo-357#          | 17          |
| Mia-PaCa-2#        | 26          |
| T3M4#              | 25          |
| lung cancer (6 cell lines) [16] | ~5         |
| lung cancer (AS49 cell line) [32] | 2–3        |
| colorectal cancer (HT29) [34] | 6          |
| CML (K562 cell lines) [35] | 0.56       |
| STI571 plasma levels[19] | 0.17–5.68  |

#### Mechanism of STI571 action on pancreatic cancer cells

In the next set of experiments, we evaluated the contribution of cytotoxic and cytostatic components to STI571-
Effect of STI571 on pancreatic cancer cell lines. Aspc-1, BxPc-3, Capan-1, Colo-357, Mia-PaCa-2 and T3M4 cells were cultured in medium containing 1% FCS (open triangle) or 10% FCS (solid circle) and incubated in the absence (0) or presence of increasing concentrations of STI571 for 48 hours. Cell growth was determined by the MTT assay. Percent growth inhibition was calculated by comparison with control cell growth. Values shown are the mean ± SEM obtained from three independent experiments.

Figure 1
induced growth inhibition. First, PI staining of cells was performed in order to determine whether cell death occurred in the treated cultures. Results presented in Figure 2 demonstrate that STI571 exerted potent toxic effects towards pancreatic cancer cells: 33.7 ± 16.5% in Mia-PaCa-2 and 26.8% ± 16.5% in T3M4 cultures as compared to 10.7% ± 2.5% and 5.7% ± 2.8% in control cultures, respectively (p < 0.05). Second, simultaneous annexin V/propidium iodide staining was employed to clarify the type of cell death taking place in the cultures. Although we observed progressive accumulation of annexin V-positive cells (Figure 2), the role of apoptosis as a possible mechanism of STI571-induced cell death was not further supported by the results of PI staining of nuclear DNA (Figure 2). Third, we performed cell cycle analysis of STI571-treated pancreatic cancer cell lines, which did not reveal any significant changes in the cell cycle pattern after 48 hours of incubation when compared to untreated cells. Apparently, STI571 treatment caused membrane alterations, leading to the layer flipping and phosphatidyserine translocation, but did not induce caspase-dependent DNA fragmentation. The exact mechanisms of the STI571-induced toxicity towards pancreatic tumor cells remain to be determined.

**Effects of STI571 on growth factor-induced proliferation of pancreatic cancer cells**

Pancreatic cancer is characterized by profound disturbances of growth factor signaling pathways. Therefore, we next investigated which particular pathway can be inhibited by STI571 in pancreatic cancer cell lines. Mia-PaCa-2 and T3M4 cell lines were exposed to different growth factors (PDGF, EGF, FGF-2, IGF-1) in the absence or presence of STI571 (at GI50 concentration) for 72 hours and cell growth was assessed by MTT assays (Figure 3). EGF-induced proliferation of both Mia-PaCa-2 and T3M4 cell lines in a dose-dependent manner was seen with maximal effects of +72% ± 7.5% (Mia-PaCa-2) and +52% ± 23% (T3M4). Interestingly, STI571 partially blocked EGF-induced cell growth, with maximal effects reduced to +29% ± 15% (Mia-PaCa-2) and +16% ± 24% (T3M4) following STI571 addition (Figure 3). FGF-2 markedly stimulated cell growth in Mia-PaCa-2 cell lines with maximal effects of +79% ± 6%, and only slightly stimulated T3M4 cell lines, with maximal effects of +7% ± 4%. STI571 also partly blocked FGF-2-induced cell growth, with maximal effects reduced to +28% ± 7% (Mia-PaCa-2) and 2% ± 3% (T3M4) (Figure 3). Both cell lines were less sensitive to IGF-1 stimulation compared with EGF and FGF-2, with maximal effects of +24% ± 2% in Mia-PaCa-2 and +17% ± 4% in T3M4. In addition, STI571 had no significant inhibitory effects on IGF-1-induced proliferation (Figure 3). PDGF did not induce cell proliferation in Mia-PaCa-2 or T3M4 cell lines. Furthermore, in three additional cell lines (RbPc-3, Colo-357 and Capan-1), PDGF also had no effect on cell growth. Thus, although PDGF-mediated growth pathways do not seem to play a role in pancreatic cancer, the above data demonstrate the ability of STI571 to interfere with other growth-stimulatory signaling pathways, such as EGF and FGF-2.

**Effects of STI571 on growth factor-induced receptor and MAP kinase activation**

A common cellular response to a variety of extracellular signals involves the phosphorylation of corresponding receptors and activation of the MAPK pathway. Therefore, we evaluated whether the partial obstruction of EGF, FGF-2, and IGF-1 signaling by STI571 was due to inhibition of receptor- and/or MAPK phosphorylation. As shown in Figure 4, treatment of growth factor-conditioned Mia-PaCa-2 and T3M4 cells with STI571 did not inhibit activation of either MAPK or the EGFR. PDGF did not induce PDG and MAPK kinase phosphorylation in those cell lines (data not shown).

To summarize, pancreatic cancer cells, resistant to growth induction through STI571-sensitive c-kit and PDGF pathways, were still responsive to the inhibitory effects of STI571, although at high GI50 concentrations. Since cell cycle distribution as well as growth factor receptor and MAPK-activation were not disturbed, we assumed that antiproliferative effects of STI571 were apparently of a toxic nature, the exact mechanism of which should be further investigated.

**Discussion**

Protein kinases play a crucial role in signal transduction as well as in cellular proliferation, differentiation, and various regulatory mechanisms. Deregulation of those signaling pathways is frequent during malignant transformation. The inhibition of growth-related kinases, especially tyrosine kinases, might provide new tools in the therapy of cancer. The protein tyrosine kinase inhibitor STI571, which has been effective in CML as well as GIST, is a selective inhibitor of Abl, PDGF and c-kit receptor tyrosine kinases [13,14,20]. This drug has a GI50 for inhibition of Bcr-Abl kinase activity of 0.25 µM and inhibits the growth of cell lines which are dependent on Bcr-Abl kinase activity at 1 µM [13,14]. In addition, STI571 also selectively inhibits the growth of cell lines derived from CML patients, as well as primary CML progenitors in clonogenic assays at low micromolar concentrations [13,21,22]. It also efficiently inhibits SCF-mediated c-kit activation at concentrations similar to those that inhibit Bcr-Abl in cellular assays, with a GI50 in the range of 0.1–0.5 µM [16].

In this study, we sought to extend the profile of STI571 applications, and tested its effects in pancreatic cancer cell lines. The STI571 target c-kit and its ligand stem cell factor
FACS analysis, Annexin-V and PI staining. Mia-PaCa-2 and T3M4 cell lines were cultured in complete medium overnight, and treated with GI50 concentrations of STI571 for 12 and 48 hours. Annexin and PI staining revealed increasing percentages of Annexin-positive and PI-positive cells with increasing time of STI571 incubation. Cell cycle analysis showed no changes in cell cycle distribution and no apoptosis (lower panel). The figure is representative of three independent experiments.
Figure 3
Effect of STI571 on growth factor-induced cell proliferation. Mia-PaCa-2 and T3M4 cells were cultured in 1% FCS medium and incubated in the absence (solid circle) or presence (open triangle) of STI571 (at GI_{50} concentration for each cell line) and increasing concentrations of the indicated growth factors for 72 hours. Cell growth was determined by the MTT assay. Percent growth inhibition was calculated by comparison with control cell growth. Values shown are the mean ± SEM obtained from three independent experiments.
Effect of STI571 on growth factor-induced MAPK phosphorylation. Mia-PaCa-2 and T3M4 cells were cultured in 1% FCS medium overnight, then treated with 10 ng/ml of the indicated growth factors and GI50 concentrations of STI571 for 5 minutes. Phosphorylation of MAPK was determined by Western blot analysis with antibodies specific for phospho-p44/42 MAPK. Equal loading was determined by stripping the membranes and blotting with an ERK2 (p42) antibody. The figure is representative of three independent experiments.

|       | MiaPaCa2 |       | T3M4 |
|-------|----------|-------|------|
|       | control  | EGF   | STI571 | STI571+EGF |
|       | control  | FGF2  | STI571 | STI571+FGF2 |
|       | control  | IGF   | ST1571+IGF |

Figure 4
(SCF) have been shown to be expressed in pancreatic cancer cells. It has been shown that SCF has no significant effects on pancreatic cancer cell growth [17], whereas STI571 inhibits pancreatic cancer cell growth [17]. Thus, STI571 may exert its effect on pancreatic cancer cell growth through c-kit-independent pathways. For example, STI571 can inhibit PDGF-mediated growth and leads to the apoptosis of osteosarcoma cells in vitro by selective inhibition of the PDGFR tyrosine kinase [15].

PDGF was one of the first polypeptide growth factors identified that signals through a cell surface tyrosine kinase receptor (PDGFR) to stimulate various cellular functions, including growth, proliferation, and differentiation. When PDGF binds to the extracellular portion of the receptor, the receptor undergoes dimerization and autophosphorylation with tyrosine kinase activation. To date, PDGF expression has been shown in a number of different solid tumors, from glioblastomas to prostate carcinomas [23]. The biological role of PDGF signaling can vary from autocrine stimulation of cancer cell growth to more subtle paracrine interactions involving adjacent stroma and angiogenesis. In pancreatic cancers, there is a marked increase in the mRNA levels of PDGFR-alpha and PDGFR-beta in comparison with the normal pancreas, and PDGF and both PDGFR receptors are present in the cancer cells [12]. Thus, STI571 may also inhibit pancreatic cancer cell growth via the PDGFR receptor pathway.

In pancreatic cancer, a variety of other growth factors that signal through tyrosine kinase receptors are also expressed at increased levels. For example, the presence of EGFs, FGFs, PDGFs, and IGFs and their respective receptors has been observed in pancreatic cancer, and these growth factors are thought to contribute to its malignant phenotype [10,11,24]. For example, it has been reported that the mRNA levels of EGF and EGFR are markedly increased in pancreatic cancer tissues in comparison with the normal pancreas [25], suggesting that the coexpression of EGFR and its ligands may contribute to the aggressiveness of human pancreatic cancer. FGF and its receptors are also overexpressed in pancreatic cancer tissues and cell lines [26,27], and cell growth, cell adhesion and invasion are modulated by fibroblast growth factors in pancreatic cancer cell lines [27–29]. IGF-1 and its receptor IGF1R are also overexpressed in pancreatic cancer [30], and they also have the potential to stimulate pancreatic cancer cell growth.

We have now demonstrated that STI571 has dose-dependent inhibitory effects in all six tested pancreatic cancer cell lines, with GI50 in the range of 17–31.5 µM (10% FCS) and 9–20 µM (1% FCS). Notably, these concentrations are relatively high compared with the concentrations sufficient for the inhibition of Abl and c-kit in other tumors [14,18,31]. Thus, when the drug was used in CML patients, the plasma levels of STI571 inducing hematologic and cytogenetic response in patients were reported to be in the range of 0.17–5.68 µM after treatment with 25–600 mg STI571/day [19]. Concentrations of more than 6 µM under cell culture conditions are unlikely to translate into clinical practice, since the adverse effects of the drug will become intolerable for the patients [32].

In cell cycle analysis we did not see cell cycle change and apoptosis caused by STI571. It has been reported that STI571 did not induce apoptosis in serum-containing medium, and it has been suggested that this is because the cells may be more resistant under these conditions, and serum can persistently activate MAP kinase [16,33]. Based on these data, we treated cells with STI571 in both complete medium (10% FCS) and 1% FCS medium. No apoptotic cell death was observed under either of these serum concentrations, yet prolongation of STI571 treatment led to more cell death under these conditions. In addition, more Annexin-V-positive cells were also observed, suggesting that STI571 can damage the integrity of the cell membrane, causing phosphatidylserine translocation to the outer surface of the cells, and at a late stage, causing cell death without significant apoptosis. Thus, STI571 may exert its effects on cell growth in an unspecific manner.

Conclusion

STI571 inhibits pancreatic cells growth, but these effects are not mediated through blockage of the PDGFR receptor tyrosine kinase, since PDGF did not stimulate pancreatic cancer cell growth and did not lead to MAP kinase or PDGFR receptor phosphorylation. In our previous study, we excluded the SCF/c-kit tyrosine kinase receptor pathway as a possible target of STI571 as well [17]. In the present study, we demonstrate that STI571 partially but not specifically blocks EGF, IGF-1 and FGF-2 mitogenic pathways, which have the potential to stimulate pancreatic cancer cell growth. Since STI571 has relatively high GI50 concentrations in pancreatic cancer cells, its action appears to be of toxic origin and its clinical relevance in pancreatic cancer is rather doubtful. Nevertheless, in view of the extremely poor prognosis of patients with pancreatic cancer, and in view of the fact that pancreatic cancer tissues in vivo indeed express both PDGFR and c-kit receptor tyrosine kinases, it seems worthwhile to further investigate the therapeutic effects of STI571 in small clinical trials.

Material and Methods

RPMI-1640 DMEM, trypsin-EDTA, and penicillin-streptomycin were purchased from Invitrogen (Karlsruhe, Germany); FBS from PAN Biotech (Aidenbach, Germany); human recombinant PDGF, IGF-1 and FGF-2 from R&D...
Systems (Abingdon, United Kingdom), and EGF from Upstate Biotechnology (Hamburg, Germany). Phospho-PDGFR-beta (tyr857) and p-EGFR (tyr1173) polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Phospho-p44/42 MAPK (Thr202/Tyr204) antibodies were purchased from Cell Signaling Technology (Frankfurt, Germany), anti-rabbit IgG HRPO-linked antibodies and ECL immunoblotting detection reagents from Amersham Biosciences (Amersham Life Science, Amersham, UK), and anti-goat IgG-HPRO peroxidase linked antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Complete mini-EDTA-free protease inhibitor cocktails and Annexin-V-Fluos were purchased from Roche GmbH (Mannheim, Germany). All other reagents were from Sigma Chemical Company (Taufkirchen, Germany). STI571 was kindly provided by Novartis Pharma AG (Basel, Switzerland).

Cell culture and MTT assay

Human pancreatic cancer cell lines were routinely grown in DMEM (Colo-357, and Mia-PaCa-2) or RPMI (Aspc-1, BxPc-3, Capan-1, and T3M4) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete medium). To assess cell proliferation, the MTT test was employed. Briefly, cells were seeded at a density of 5000 cells/well in 96-well plates, grown overnight and exposed to STI571 alone or in combination with growth factors. After 48 or 72 hours of incubation, 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was added (50 µg/well) for 4 hours. Formazan products were solubilized with acidic isopropanol, and the optical density was measured at 570 nm. To determine the GI50 of STI571 (the concentration that causes 50% growth inhibition), graded concentrations of STI571 were added to triplicate wells and GI50 was calculated using 100 × (T-T0)/(C-T0) = 50. T is the optical density of the test well after a 48-hour period of exposure to STI571, T0 is the optical density at time zero, and C is the control optical density after 48 hours. All experiments were performed in triplicate.

FACS analysis of cell death and cell cycle

105 pancreatic tumor cells were seeded into 6-well plates in 1% FBS-containing medium, allowed to adhere overnight and then treated with corresponding GI50 concentrations of STI571. To analyze cell cycle distribution, cells were collected after 48 hours of incubation, washed with PBS and resuspended in 0.5 ml of hypotonic PI buffer (5 µg/ml propidium iodide, 0.1% Triton X100 and 0.1% sodium citrate), stored overnight at 4°C and then analyzed by flow cytometry using BD-LSR (Becton Dickinson and Company, New York, USA). The resulting DNA histograms were interpreted using the Cell Quest Pro software (Becton Dickinson and Company, New York, USA). To determine the degree of cell death, cells were collected after 12, 24 and 48 hours of exposure to GI50 of STI571 and were washed and stained with Annexin-V-FITC (apoptotic death) or PI (necrotic death) according to the manufacturer's instructions (Roche, Mannheim, Germany).

Western blot analysis

Cell culture monolayers were washed twice with ice-cold PBS and lysed with buffer containing Tris-HCl (50 mM, pH 7.4), NP-40 (1%), Na-deoxycholate (0.25%), NaCl (150 mM), EDTA (1 mM), PMSF (1 mM), Na3VO4 (1 mM), NaF (1 mM) and one tablet of complete mini-EDTA-free protease inhibitor cocktail (in 10 ml buffer). Protein concentration was determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA). 30 µg of cell lysates were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Membranes were then incubated in blocking medium, allowed to adhere overnight and then treated with corresponding GI50 concentrations of STI571. To analyze cell cycle distribution, cells were collected after 48 hours of incubation, washed with PBS and resuspended in 0.5 ml of hypotonic PI buffer (5 µg/ml propidium iodide, 0.1% Triton X100 and 0.1% sodium citrate), stored overnight at 4°C and then analyzed by flow cytometry using BD-LSR (Becton Dickinson and Company, New York, USA). The resulting DNA histograms were interpreted using the Cell Quest Pro software (Becton Dickinson and Company, New York, USA). To determine the degree of cell death, cells were collected after 12, 24 and 48 hours of exposure to GI50 of STI571 and were washed and stained with Annexin-V-FITC (apoptotic death) or PI (necrotic death) according to the manufacturer's instructions (Roche, Mannheim, Germany).

Effect of STI571 on growth factor-induced EGFR phosphorylation

Figure 5

Effect of STI571 on growth factor-induced EGFR phosphorylation. Mia-PaCa-2 and T3M4 cell lines were cultured in 1% FCS medium overnight, then treated with 10 ng/ml of EGF and GI50 concentrations of STI571 for 5 minutes. Phosphorylation of EGFR was determined by Western blot analysis with a phospho-EGFR-specific antibody. Equal loading was determined by stripping the membranes and blotting with an antibody to ERK2 (p42). The figure is representative of three independent experiments.
Statistical analysis
Results were expressed as mean ± SEM. For statistical analysis, the Student’s t test was used. Significance was defined as p < 0.05.

Authors Contribution
JL, JC, and LF carried out the cell growth and Western blot experiments. JL and NG carried out the FACS analysis. JK, MWB, and HF conceived of the study, and participated in its design and coordination. JK and NG drafted the manuscript. All authors read and approved the final version.

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