Imatinib mesylate (STI571; Glivec)—a new approach in the treatment of biliary tract cancer?

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Non-resectable biliary tract cancer is associated with poor prognosis due to widespread resistance to chemotherapeutic agents and radiotherapy. It is therefore essential to explore new therapeutic approaches like the inhibition of tyrosine kinases. The aim of this study was to determine the expression of c-*kit* and platelet-derived growth factor (PDGF) receptors (PDGFRs) and the effects of the tyrosine kinase inhibitor imatinib ± 5-fluorouracil (5-FU) on proliferation and apoptosis in biliary tract cancer cell lines. The expression of c-*kit* and PDGFR mRNA was examined in 12 biliary tract cancer cell lines using RT-PCR. Cells were treated with imatinib (1, 10, 20 and 50 μmol/l) ± 5-FU (0.1 μg/ml) for 6 days and inhibition of cell growth was assessed by manual cell counting. Cell proliferation and apoptosis were analyzed by flow cytometry of BrdU and Annexin-V/propidium iodide-stained cells. c-*kit* and PDGF mRNA expression was detected in 50 and 75%, respectively. Imatinib (10 and 20 μmol/l) alone inhibited cell growth significantly higher in c-*kit*$^+$ cell lines ($p < 0.02$) and inhibition was independent of PDGFR status. The combination with 5-FU increased the effect of imatinib mesylate in all cell lines. Treatment of cells with imatinib ± 5-FU was associated with a significant induction of apoptosis, but no inhibition of proliferation. We conclude that imatinib alone exerts marked effects on c-*kit*$^+$ biliary tract cancer cell lines only at intermediate and high concentrations, but there is a potential role of low-dose imatinib in combination with 5-FU for the treatment of biliary tract cancers. *Anti-Cancer Drugs* 14:751–760 © 2003 Lippincott Williams & Wilkins.

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Introduction

Biliary tract cancer has an incidence of about 3/100 000 [1] and is therefore a rare tumor. About two-thirds of the tumors originate from the gall bladder, while about one-third are bile duct cancers. Of the latter, about two-thirds are perihilar, about a quarter are distally extrahepatic and the rest are primarily intrahepatic [2].

At present, only surgical excision of all detectable tumor is associated with improvement in 5-year survival [3–5]. Unfortunately, almost 40% of patients with gall bladder cancer are diagnosed in an advanced stage and in the case of patients with hilar cholangiocarcinoma, only 20–30% are candidates for potentially curative resection. Palliative chemotherapy is only marginally effective and associated with considerable toxicity [6–9]. The role of radiation therapy is controversial and most of the studies are retrospective or comprise only a small number of patients. At best, there is a small prolongation of survival [10–17]. Therefore, new reagents for palliative therapy should be investigated.

c-*kit* (CD-117) is a class III receptor tyrosine kinase with a physiological role in the development of mast cells, melanocytes and hematopoetic stem cells, gametogenesis, and brain and spinal cord development. Recent publications report an increased c-*kit* receptor expression in benign and malignant human endometrium [18], acute myelogenous leukemia and chronic myelogenous leukemia (CML) [19]. c-*kit* is activated by binding of its ligand stem cell factor (SCF) [20], which leads to dimerization of the receptor and activation of the tyrosine kinase, followed by auto-phosphorylation. Signaling from c-*kit* involves activation of Jak kinases (Jak2), phospholipase Cγ, phosphoinositol-3-kinase and the Ras/Raf/mitogen-activated protein (MAP) kinase kinase/MAP kinase cascade [19]. In 1998, Hirota *et al.* described mutations of the Kit tyrosine kinase in patients with gastrointestinal stromal tumors (GISTs) [21]. These mutations involve the juxtamembrane domain and lead to constitutive activation of the kinase via receptor dimerization in the absence of ligand. Point mutations involving the kinase domain of the receptor were discovered in malignant mastocytosis and some patients with acute myelogenous leukemia [22].

Imatinib mesylate (STI571, Glivec), a selective inhibitor of c-*abl*, *bcr–abl*, c-*kit* and the platelet-derived growth

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factor (PDGF) receptors (PDGFRs) [23], is effective for the treatment of CML [24,25], chronic myelomonocytic leukemia [26], some cases of hypereosinophilic syndrome [27] and GISTs [28,29]. These tumors carry abnormal fusion genes, generated by chromosome translocations, or activating gene mutations, involving Abl, PDGFR β and α, and Kit, respectively. As in the case of the c-*kit*, PDGFR is activated by ligand-induced dimerization. PDGFR consists of two subunits, α and β, which form homo- or heterodimers (αα, ββ and αβ). PDGF consists of 60% homologous α and β chains, which also form homo- or heterodimers (αα, ββ and αβ).

In the gastrointestinal tract c-*kit* is expressed in the interstitial cells of Cajal, from whose stem cell GISTs are thought to originate [30–32]. Expression was also observed in hepatocytes during fulminant liver failure [33], invasive ductal carcinoma of the pancreas [34] and human colorectal tumors [35], but no data in relation to expression in biliary epithelial cells have been presented. In this study we investigated the expression of c-*kit* and PDGFRs in biliary tract cancer cell lines, and evaluated the effect of imatinib mesylate upon the growth of these lines.

Materials and methods

Cell lines and treatment

Twelve biliary tract cancer cell lines—five extrahepatic bile duct cancer cell lines (EGI-1, TFK-1, CC-SW-1, CC-LP-1 and SK-ChA-1) [36–40], three intrahepatic bile duct cancer cell lines (NEC, RBE and H-1) [41] and four gall bladder cancer cell lines (Mz-ChA-1, MzChA-2, GB-CL-1 and Wittier) [39,40,42]—were examined. All cell lines were cultured in a 37°C incubator with 5–10% CO_2 in appropriate media. Treatment of the cells was performed by culturing 0.25×10^6 cells in T-25 cell culture flasks in duplicates in the presence of 0, 1, 10, 20 and 50 μmol/l imatinib mesylate (Novartis Pharma, Basel, Switzerland) ± 0.1 μg/ml (2.5 ng/ml for TFK-1) 5-fluorouracil (5-FU; Roche, Mannheim, Germany) for 6 days. The medium was changed once after 3 days.

RT-PCR analysis

Total RNA was extracted from 5×10^5 cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. First-strand cDNA synthesis was performed with 1 μg of total RNA and oligo(dT)$_{12–18}$ using the Superscript Kit (Gibco/BRL, Rockville, MD). Ten percent of cDNA products were used for PCR amplification in 50-μl reactions containing 1 × Taq polymerase buffer (Qiagen), 50 pmol each of the upstream and downstream primers, 0.2 mmol/l of dNTP, and 2.5 U of Taq DNA polymerase. The primers used for PCR were as follows: (i) human c-*kit* receptor [43]: sense 5′-AGGAGATAAATGGAAACAATTATGT-3′, nucleotides 1703–1727/antisense 5′-AAAATCCCATAGGACCAG-3′, nucleotides 2577–2594 or sense 5′-GTTCAGAGTTCTATAGATTCTAGTG-3′, nucleotides 1441–1465/antisense 5′-TTGAGCATCTTTACAGCGACAGTC-3′, nucleotides 1875–1898, (ii) human SCF [43]: sense 5′-ATGAAGAAGACACAACTTG-3′, nucleotides 184–203/antisense 5′-AAGGCATCAATGGATCTATT-3′ nucleotides 616–635, (iii) human PDGFR-α [44]: sense 5′-CTGGAAGAAATCAAAGTCCCATCC-3′, nucleotides 1175–1198/antisense 5′-TGAGCCATGGTGATCATCGACC-3′, nucleotides 1654–1675, (iv) human PDGFR-β [45]: sense 5′-TGACCACCCAGCCATCCTTC-3′, nucleotides 3296–3315/antisense 5′-GAGGAGGTGTTGACTTCATTC-3′, nucleotides 3503–3523, (v) human PDGF-α [46]: sense 5′-CTCCCGCGTCCACCACCGCAGCGTC-3′, nucleotides 1264–1288/antisense 5′-GCTGCGGCTCATCCTCACCTCA-3′, nucleotides 1479–1500, (vi) human PDGF-β [47]: sense 5′-CCCGGAGTCGGCATGAATCG-3′, nucleotides 971–990/antisense 5′-TGGCCGTCCGAATCAGGCAT-3′, nucleotides 1819–1838, and (vii) human β-actin [48]: sense 5′-AACCGCGAGAAGATGACCCAG-3′ nucleotides 384–404/antisense 5′-CTCCTGCTTGCTGATCCACAT-3′, nucleotides 1104–1124. The PCR was performed using 30 s for denaturation at 94°C, 1 min annealing at 55°C and 90 s for extension at 72°C for a total of 40 cycles. The PCR products were fractionated in 1.5% agarose gels.

Sequencing of c-*kit* cDNA

In order to detect c-*kit* mutations, cDNA products were amplified using the two sets of primers for c-*kit* receptor as mentioned above. The amplified PCR products were gel purified (QIAquick Gel Extraction Kit; Qiagen) and directly sequenced from both directions. Results were compared with published wild-type sequence bp 1441–2594 (amino acids 481–864) using the BLAST search program (Gen Bank accession no. X06182) covering exons 9–17 of the gene.

Inhibition of cell growth

After 6 days of treatment (see above) cells were trypsinized, washed and counted in triplicates in a Neubauer chamber after staining with Trypan blue.

Apoptosis and proliferation assays

Apoptosis was assessed using Annexin-V/propidium iodide staining kit (Annexin Apoptosis Detection Kit I; BD Biosciences, Heidelberg, Germany), with the method adapted for adherent cells according to the instructions of the manufacturer. Cell proliferation was measured with the *In situ* Cell Proliferation Kit, FLUOS (Roche, Mannheim, Germany). In brief, 1/10 volume BrdU-labeling solution was added to the cells followed by 60 min incubation at 37°C. Cells were then washed 3 times in phosphate-buffered saline (PBS), trypsinized and pelleted. Pellets were resuspended in 0.5 ml PBS and fixed for 30 min at 4°C with 0.5 ml 70% ethanol in

50 mmol/l glycine buffer, pH 2.0. Cells were then centrifuged and resuspended in 1 ml PBS/EDTA containing 50 µg/ml RNase A (Sigma, St Louis, MO), followed by another wash step with PBS and denaturation in 500 µl HCl (4 mol/l) for 20 min at room temperature. After centrifugation the cells were resuspended in incubation buffer for 2 × 5 min to neutralize the pH and block nonspecific binding. Cells were then centrifuged and resuspended in 50 µl Anti-BrdU-FLUOS working solution. After 45 min incubation at 37°C, cells were washed twice in PBS and counterstained with propidium iodide (1 µg/ml). Flow cytometric analysis was performed after resuspension in 1 ml PBS.

Statistical analysis

Data was analyzed with Student's *t*-test and the Friedman test at an exploratory significance level of $p < 0.05$ (two-sided) using SPSS 10.0 software.

Results

RT-PCR analysis

c-*kit* mRNA was detected in 50% of the cell lines (NEC, TFK-1, CC-SW-1, Mz-ChA-1, Mz-ChA-2 and Wittier) and mRNA expression of c-*kit* receptor ligand SCF in all cells lines, respectively (Fig. 1 and Table 1). There was no correlation between c-*kit* expression and the grade of differentiation of the tumors from which the cell lines are derived (data not shown). Since imatinib mesylate has previously been shown not only to inhibit c-*kit*, but also PDGFR, we investigated the expression of PDGFR and its ligand PDGF. We found that nine of 12 cell lines were positive for PDGFR α (NEC, H-1 and Wittier) or β (CC-LP-1 and Sk-ChA-1), or both (RBE, CC-SW-1, Mz-ChA-1 and MzChA-2) (Table 1). All cell lines expressed PDGF α and β mRNA (Table 1).

Sequencing of c-*kit* cDNA

Mutational analysis in the c-*kit* receptor$^+$ cell lines NEC, TFK-1, CC-SW-1, MzChA-1, MzChA-2 and Wittier by direct sequencing of PCR products did not reveal any mutations.

Inhibition of cell growth

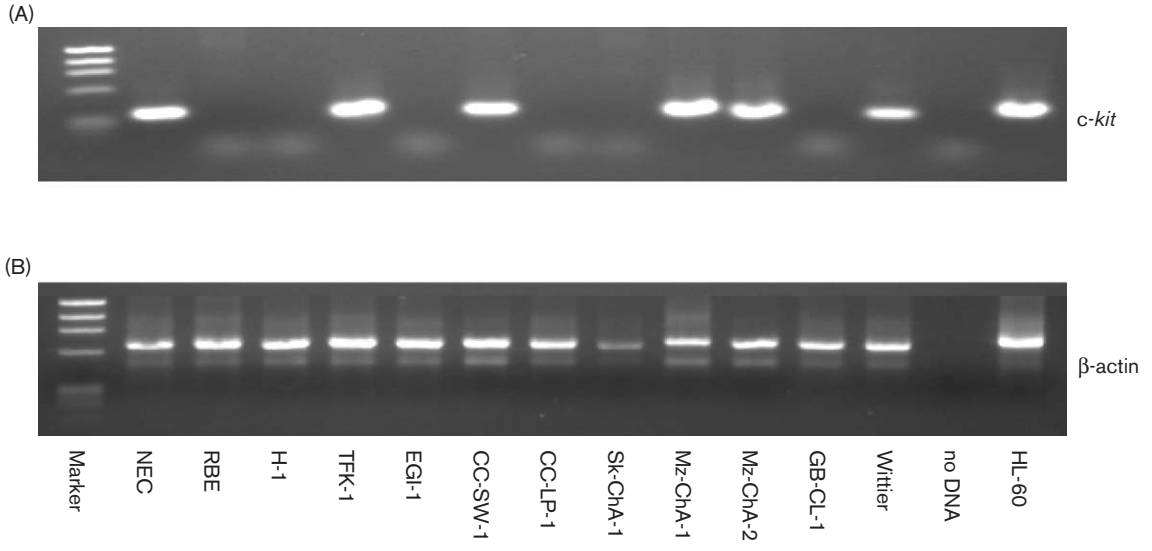
In order to test for physiological function of c-*kit* and PDGFRs in the tumor cell lines, we treated cells with different concentrations of imatinib mesylate

Table 1 mRNA expression of c-*kit* receptor, SCF, PDGFR α/β and PDGF α/β in biliary tract cancer cell lines

Cell line	Source	c-*kit* receptor	SCF	PDGFR α/β	PDGF α/β
NEC	Bd	+	+	+/−	+/+
RBE	Bd	−	+	+/+	+/+
H-1	Bd	−	+	+/−	+/+
TFK-1	Bd	+	+	−/−	+/+
EGI-1	Bd	−	+	−/−	+/+
CC-SW-1	Bd	+	+	+/+	+/+
CC-LP-1	Bd	−	+	−/+	+/+
Sk-ChA-1	Bd	−	+	−/+	+/+
Mz-ChA-1	Gb	+	+	+/+	+/+
MzChA-2	Gb	+	+	+/+	+/+
GB-CL-1	Gb	−	+	−/−	+/+
Wittier	Gb	+	+	+/-	+/+

Gb, cancer of the gall bladder; Bd, bile duct cancer.

Fig. 1



RT-PCR-analysis of c-*kit* receptor (A) and β-actin (B) in biliary tract cancer cell lines. (A) c-*kit* receptor mRNA expression was detected in six cell lines (NEC, TFK-1, CC-SW-1, Mz-ChA-1, MzChA-2 and Wittier) and SCF mRNA expression in all cells lines, respectively. HL-60 acute myelogenous leukemic cell line was used as a positive control.

(1–50 μmol/l) for 6 days and examined inhibition of cell growth by cell counting after staining with Trypan blue.

Imatinib mesylate at a low concentration of 1 μmol/l showed a moderate inhibition in all cell lines examined (9 ± 16%). There was no significant difference in inhibition in intergroup comparisons for receptor status, including c-*kit* and PDGFR negative cell lines (Table 2).

Imatinib mesylate at intermediate concentrations of 10 and 20 μmol/l had a more distinct effect (28 ± 23 and 80 ± 20%, respectively) in all cell lines than 1 μmol/l (Table 2). The inhibition of cell growth at these concentrations was significantly higher in c-*kit*$^+$ cell lines (44 ± 23 versus 12 ± 9% and 95 ± 6 versus 68 ± 20%, respectively) ($p < 0.02$) (Fig. 2), but was independent of PDGFR α (37 ± 27 versus 16 ± 7% and 83 ± 23 versus 77 ± 19%, respectively) ($p > 0.05$) or β expression status (39 ± 29 versus 17 ± 11% and 88 ± 17 versus 71 ± 23%, respectively) ($p > 0.05$) (Table 2 and data not shown). Incubation of c-*kit* and PDGFR negative cell lines with imatinib mesylate resulted in a moderate inhibition at 10 μmol/l (14 ± 3%), but was clearly increased at 20 μmol/l (63 ± 24%) (Table 2).

Table 2 Percent inhibition of cell growth by imatinib mesylate depending on c-*kit* and PDGFR status

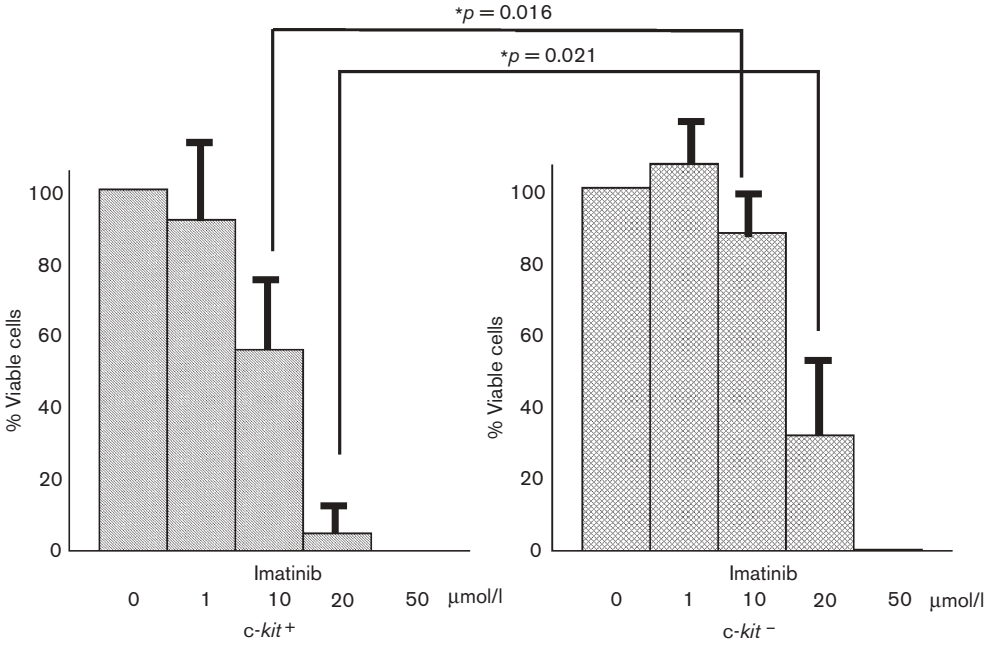
Receptor status	Imatinib mesylate (μmol/l)		
	1	10	20
All	9 ± 16	28 ± 23	80 ± 20
c-*kit*	16 ± 20	44 ± 23	95 ± 6
PDGFRα	14 ± 19	37 ± 27	83 ± 23
PDGFRβ	15 ± 20	39 ± 29	88 ± 17
c-*kit* and PDGFR	19 ± 21	50 ± 19	98 ± 2
Negative	7 ± 11	14 ± 3	63 ± 24

All, all cell lines; c-*kit*, c-*kit* receptor$^+$ cell lines; PDGFRα, PDGFR α$^+$ cell lines; PDGFRβ, PDGFR β$^+$ cell lines; c-*kit* and PDGFR, c-*kit* and PDGFR$^+$ cell lines; negative, c-*kit* and PDGFR$^-$ cell lines.

Imatinib mesylate at a high concentration of 50 μmol/l caused a general non-specific toxic effect in all cell lines examined (100 ± 0.5%) (data not shown).

Although only marginally effective, in the clinic 5-FU is frequently used for palliation in patients with biliary tract cancer. We therefore tested the combination of imatinib mesylate at a concentration of 1 and 10 μmol/l and 0.1 μg/ml 5-FU (2.5 ng/ml 5-FU were chosen for TFK-1 since this cell line was 40 times more sensitive to 5-FU than the other cell lines). At the selected concentration, 5-FU

Fig. 2



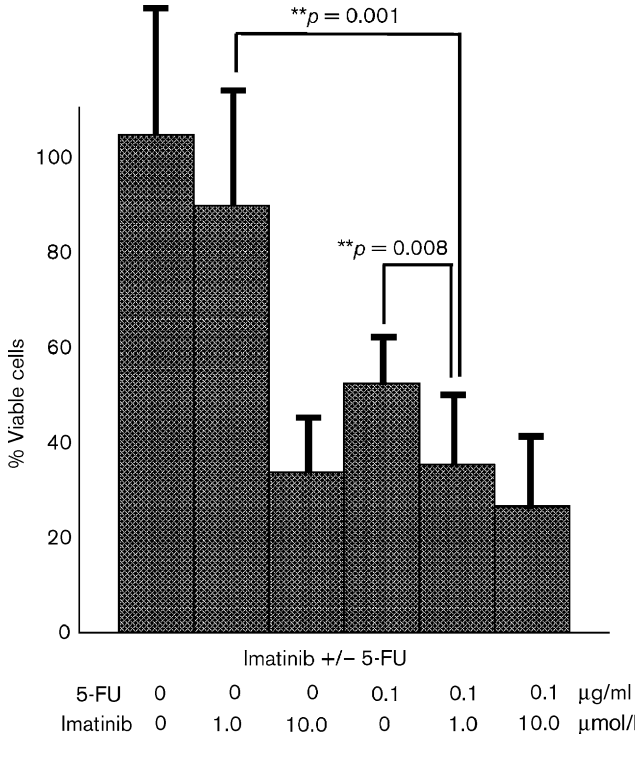
Inhibition of cell growth. Treatment of cells was performed by culturing 0.25 × 10^6 cells in T-25 cell culture flasks in duplicates containing 0, 1, 10, 20 and 50 μmol/l imatinib mesylate for 6 days (medium was changed once after 3 days). Cells were trypsinized, washed and counted in triplicates in a Neubauer chamber after staining with Trypan blue. The number of viable cells (% control) was significantly lower in c-*kit*$^+$ cell lines at concentrations of 10 and 20 μmol/l, respectively (*$p < 0.02$). Graphs depict the mean ± SD of results obtained for each group. Statistical comparisons were made using Student's *t*-tests.

reduced cell growth by 40–50%, while the combination with imatinib mesylate reduced cell growth by another 20% (1 μmol/l) ($p = 0.008$) and 30% (10 μmol/l) ($p = 0.0001$), respectively (Fig. 3).

Apoptosis and proliferation assays

Since imatinib mesylate and 5-FU exhibited inhibition of cell growth in all lines examined, we investigated whether this was caused by induction of apoptosis, inhibition of cell proliferation, or a combination of both. Thus, the proportion of apoptotic cells was assessed by Annexin-V/propidium iodide flow cytometric assay. As shown for the GB-CL-1 cell line (Fig. 4), the rate of apoptotic cells was $10 \pm 2\%$ in untreated cells. Treatment with imatinib mesylate (20 μmol/l) or 5-FU (0.5 μg/ml) alone resulted in 32 ± 4 and $30 \pm 3\%$ apoptotic cells. The combination of imatinib mesylate (20 μmol/l) and 5-FU (0.5 μg/ml) increased the rate of apoptotic cells to $48 \pm 6\%$. The proportion of proliferating cells was estimated by BrdU-FLUOS/propidium iodide flow cytometric assay, a well-established method which detects S phase cells. As shown for the CC-LP-1 cell line (Fig. 5), the proportion of proliferating cells in the untreated control was set to 100%. Treatment with imatinib mesylate (20 μmol/l) or 5-FU (0.5 μg/ml) alone did not result in a significant reduction of proliferation. The combination of imatinib mesylate (20 μmol/l) and 5-FU (0.5 μg/ml) did not cause any significant effect either. Therefore, treatment of cells with imatinib mesylate and/or 5-FU was associated with a strong induction of apoptosis, but proliferation seemed to be unaffected.

Fig. 3



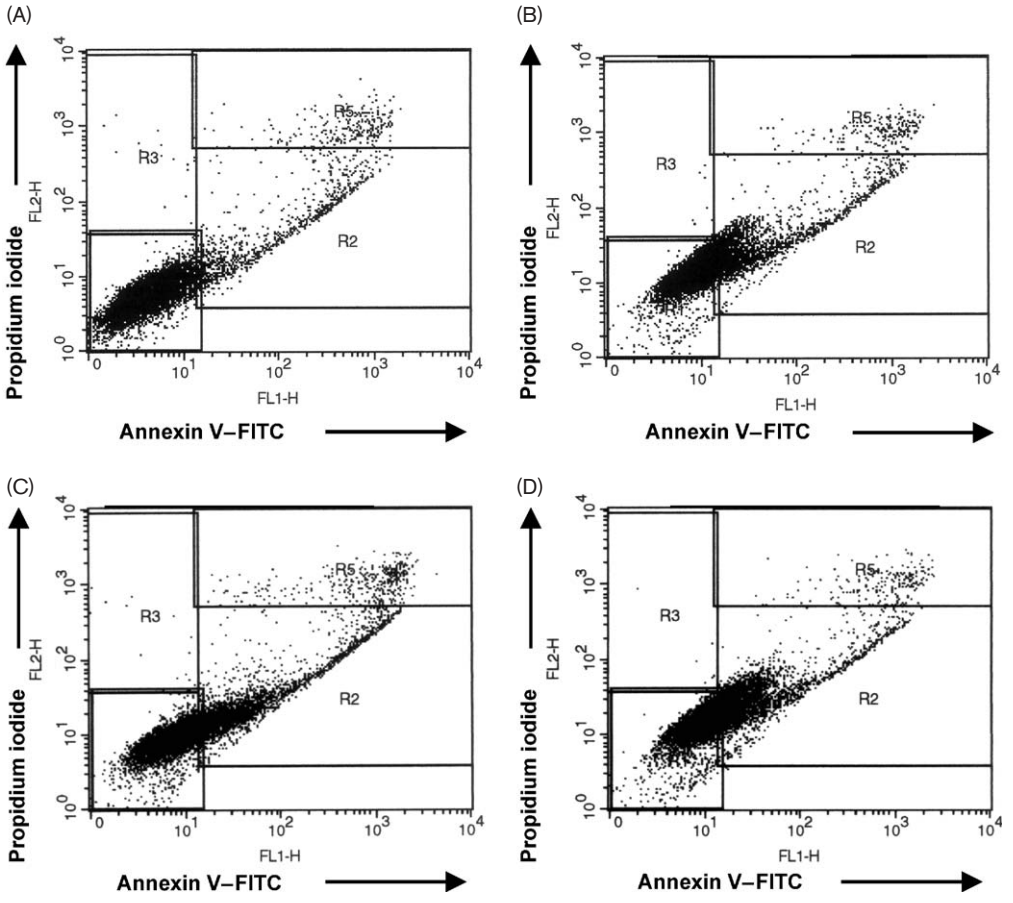
Inhibition of cell growth. Treatment of cells was performed by culturing 0.25×10^6 cells in T-25 cell culture flasks in duplicates containing 0, 1 and 10 μmol/l imatinib mesylate ± 0.1 μg/ml 5-FU for 6 days (medium was changed once after 3 days). Cells were trypsinized, washed and counted in triplicates in a Neubauer chamber after staining with Trypan blue. The number of viable cells (% control) was significantly lower in combination therapy than monotherapy (**$p < 0.01$). Graph depicts the mean ± SD of results obtained for MzChA-2 as an example ($n = 3$). Statistical comparisons were made using Student's *t*-tests.

Discussion

The transformation of normal bile duct tissue into malignancy requires a series of gene mutations similar to the adenoma-carcinoma sequence in colon cancer, although present knowledge is much more limited for this rare type of tumor. Mutations can be subdivided into several gene classes: activating mutations of proto-oncogenes, which promote malignancy, inactivating mutations of tumor suppressor genes, which lose their ability to regulate cell growth, and inactivating mutations of DNA repair genes. Mutations of the k-*ras*, c-*myc*, c-*neu*, c-*erb-B2* and c-*met* oncogenes have been found in bile duct cancer [49–53]. Mutations of tumor suppressor genes involve *p53* and *p16* [40,54–59]. These mutations cause phenotypic changes, but the precise mechanism of cancer development is not yet known.

The goal of this study was to investigate the expression of another proto-oncogene, c-*kit*, in biliary tract cancer and to test if c-*kit* inhibition with a specific inhibitor may have therapeutic potential. Over-expression of SCF and activation of c-*kit* has been found in some patients with primary sclerosing cholangitis and gallstones [60]. Furthermore, CD34 and c-*kit* + cells have been isolated from human liver that are capable of differentiating into bile duct epithelium [61]. It is possible that these pluripotent cells play a role for the development of bile duct cancer. Therefore, the expression of c-*kit* receptor mRNA was examined in 12 biliary tract cancer cell lines (eight bile duct cancer and four gall bladder cancer cell lines) using RT-PCR. c-*kit* receptor mRNA expression was detected in 50% of the cell lines and mRNA expression of c-*kit* ligand SCF in all cells lines, respectively. We reasoned that the proliferation of these cells might be stimulated by an autocrine mechanism. In order to test this hypothesis, we treated cell lines with imatinib mesylate, a selective inhibitor of c-*kit* receptor [23]. We show that imatinib mesylate marginally inhibits cell growth of c-*kit*$^+$ cell lines at a low concentration (1 μmol/l). Cell growth was clearly reduced at higher concentrations (10 and 20 μmol/l). The IC_{50} for growth inhibition of c-*kit*$^+$ cell lines can be estimated as 10 μmol/l and thus is 40–100 times higher than for the inhibition of *bcr-abl*-expressing CML cells, 10 times higher than for the inhibition of c-*kit*$^+$ small cell lung

Fig. 4



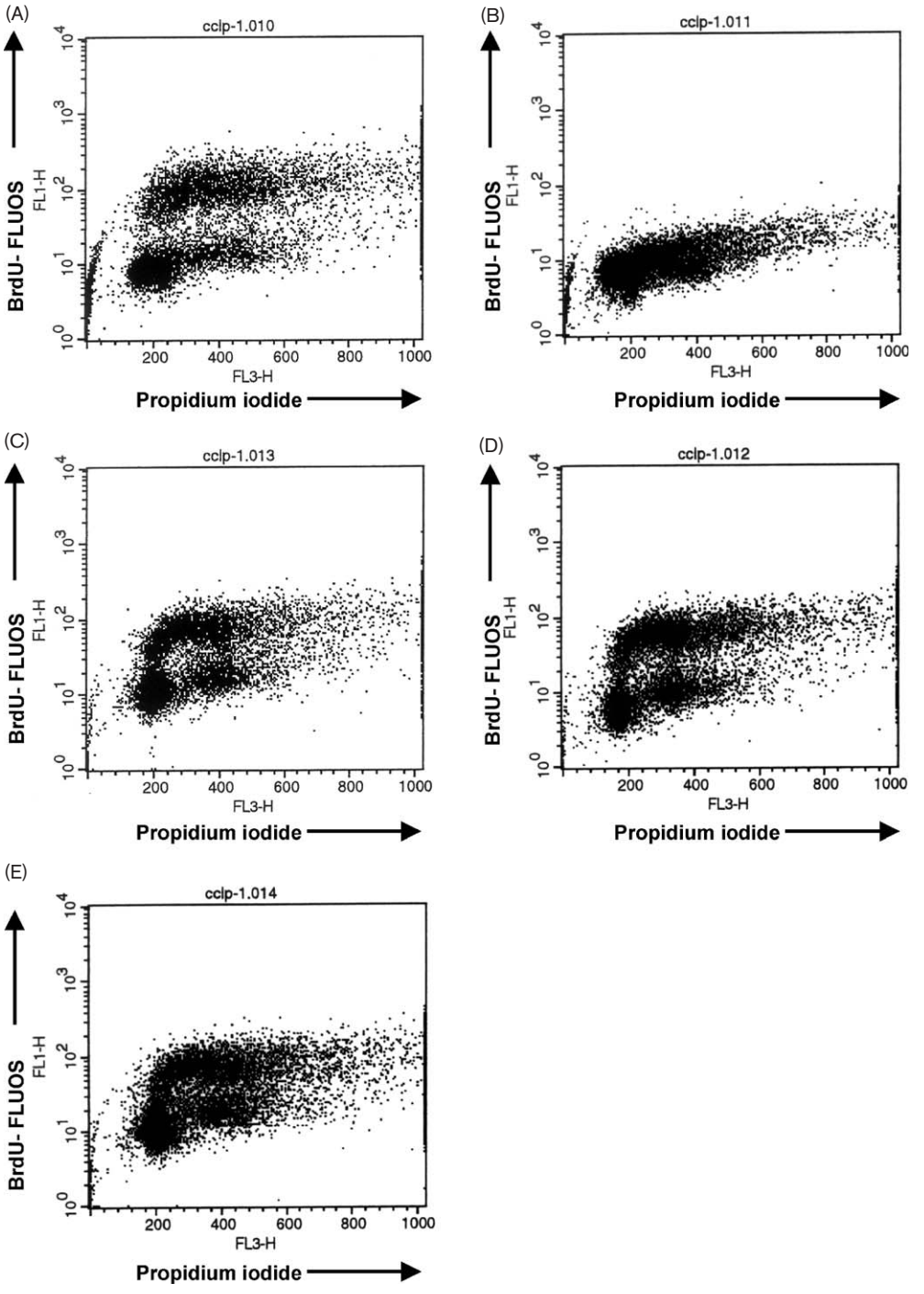
Apoptosis assay of cell lines. Rate of apoptotic cells was assessed by Annexin-V/propidium iodide flow cytometric assay (Annexin-V–FITC Apoptosis Detection Kit I; BD Bioscience). Cells were stained with Annexin-V and propidium iodide, and the rate of apoptotic cells (Annexin-V^+, PI^-) was quantified with a FACScan flow cytometer (Becton Dickinson). In untreated cells (A), the rate of apoptotic cells was 10 ± 2%. Treatment with imatinib mesylate (20 μmol/l) (B) or 5-FU (0.5 μg/ml) (C) alone resulted in 32 ± 5% apoptotic cells. The combination of imatinib mesylate (20 μmol/l) and 5-FU (0.5 μg/ml) (D) increased the apoptosis rate to 38 ± 6% (GB-CL-1 cell line is shown as an example).

cancer cell lines NCI-H69, NCI-H146 and NCI-H209 [62,63], and 1.6 times higher than for the inhibition of c-*kit*$^+$ colorectal cancer cell line HT29 [35]. The reason for such disparity might be explained by such conditions as high-level expression of drug efflux pumps in tumor cells, but needs further investigation. Thus, if imatinib monotherapy is considered, it may not be possible to achieve therapeutically relevant concentrations without causing severe side-effects [64]. However, dose reduction might be possible by combination therapy as discussed below.

In contrast to the lines that express c-*kit*, c-*kit*$^-$ cell lines did not exhibit a reduction in cell number at the lowest concentration of imatinib mesylate, and showed a significantly lower growth inhibition at 10 and 20 μmol/l. At 50 μmol/l, a general non-specific toxic effect occurred in all cell lines. Thus, growth inhibition by imatinib mesylate was correlated with the expression of c-*kit* mRNA, suggesting that inhibition of c-*kit* signaling may be responsible for the observed effect. Sequence analysis of c-*kit* exons 9–17 showed only wild-type which excludes mutational activation of the kinase by juxtamembrane 'gain of function mutations' as previously described for GISTs [21,65]. However, the fact that c-*kit* ligand mRNA is expressed in the cell lines suggests that an autocrine loop may contribute to the proliferation and viability of the c-*kit*$^+$ lines. This is similar to imatinib mesylate-sensitive small cell lung cancer cell lines which co-express c-*kit* and SCF [66]. However, the partial response of c-*kit*$^-$ cell lines indicates that c-*kit* independent mechanisms might play a role.

In contrast to c-*kit*, we did not find a correlation between the expression of PDGFR α and β and sensitivity to imatinib mesylate, although nine of 12 cell lines

Fig. 5



Proliferation assay of cell lines. The rate of proliferating cells was assessed by BrdU-FLUOS/propidium iodide flow cytometric assay (*In situ* Cell Proliferation Kit; FLUOS; Roche). Cells were stained with BrdU and propidium iodide, and the rate of proliferating cells ($BrdU^+$) was quantified with a FACScan flow cytometer (Becton Dickinson). In untreated cells (A), the rate of proliferating cells was set as 100%; the omission of BrdU treatment served as negative control (B). Treatment with imatinib mesylate (20 μmol/l) (C) or 5-FU (0.5 μg/ml) (D) alone resulted in a 3% reduction of S phase cells. The combination of imatinib mesylate (20 μmol/l) and 5-FU (0.5 μg/ml) (E) did not further reduce proliferation (CC-LP-1 cell line is shown as an example).

exhibited a positive PDGFR status, and PDGF α and β mRNA was present in all cell lines. Thus, the PDGFR/PDGF system does not appear to be involved in the proliferation of biliary tract cancer cell lines. The fact that imatinib mesylate at higher doses causes growth inhibition even in cell lines that express neither c-*kit* nor PDGFR α and β is an indication that other, yet unidentified, imatinib mesylate targets may exist. One candidate may be the flt-3 ligand/flt-3 system [67].

Data from mostly uncontrolled studies have failed to demonstrate a clear benefit of systemic chemotherapy in patients with unresectable gall bladder carcinoma. 5-FU has been the most common agent in most series, with response rates ranging from 5 to 30% [68]. For the palliation of cholangiocarcinoma, an overall response rate of 21% was reported in one study of 14 patients treated with a regimen of 5-FU, leucovorin and carboplatin [69]. This encouraged us to test a combination of low-dose 5-FU and imatinib mesylate *in vitro*. At the selected concentration of 0.1 μg/ml, 5-FU alone reduced cell growth by 40–50%. Combination with imatinib mesylate at 1 and 10 μmol/l reduced cell growth significantly by an additional 20 and 30%, respectively. Thus, 5-FU and imatinib mesylate seem to have additive effects at concentrations of imatinib that are achievable in patients within the therapeutic range [25]. These data suggest that this combination should be tested in future *in vivo* studies.

Imatinib mesylate effects on cell growth are dependent on the cell type. In GIST cell lines and *bcr–abl*$^+$ leukemia cell lines imatinib inhibited proliferation and induced apoptosis [70,71]. In contrast, in small cell lung cancer cells, proliferation was inhibited, but no apoptosis induced [66], and in dermatofibrosarcoma protuberans the major mechanism of *in vitro* growth inhibition is by induction of tumor cell apoptosis, whereas proliferation is unaffected [72]. The latter observation was similar to our own results where 10 and 20 μmol/l imatinib mesylate clearly induced apoptosis, examined by Annexin-V/propidium iodide flow cytometric assay, but did not seem to affect cell proliferation, determined by BrdU staining. The combination of imatinib mesylate and 5-FU increased the rate of apoptotic cells further, but did not reduce proliferation, supporting a previous *in vivo* study of colon cancer [73].

In summary, imatinib mesylate alone induces apoptosis in biliary tract cancer cell lines at intermediate concentrations, which are hard to achieve in humans. This effect is correlated with c-*kit* expression and increases in combination with 5-FU, suggesting a potential role of low-dose imatinib in combination with 5-FU for the treatment of biliary tract cancers.

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