Antitumor activity of Src inhibitor Saracatinib (AZD-0530) in preclinical models of biliary tract carcinomas

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The authors declare that they have no competing interests.

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ABSTRACT

Biliary tract carcinoma (BTC) has a poor prognosis due to limited treatment options. There is therefore urgent need to identify new targets and to design innovative therapeutic approaches. Among potential candidate molecules, we evaluated the non-receptor tyrosine kinase Src, observing promising antitumor effects of its small molecule inhibitor Saracatinib in BTC preclinical models.

The presence of an active Src protein was investigated by immunohistochemistry in 19 surgical samples from BTC patients. Upon Saracatinib treatment, the phosphorylation of Src and of its downstream transducers was evaluated in the BTC cell lines TFK-1, EGI-1, HuH28 and TGBC1-TKB. The effect of Saracatinib on proliferation and migration was analyzed in these same cell lines, and its antitumor activity was essayed in EGI-1 mouse xenografts. Saracatinib-modulated transcriptome was profiled in EGI-1 cells and in tumor samples of the xenograft model.

Src was activated in about 80% of the human BTC samples. In cultured BTC cell lines, low-dose Saracatinib counteracted the activation of Src and of its downstream effectors, increased the fraction of cells in G0/G1 phase, and inhibited cell migration. At high concentrations (median dose from 2.26 to 6.99 µM), Saracatinib was also capable of inhibiting BTC cell proliferation. In vivo, Saracatinib treatment resulted in delayed tumor growth, associated with an impaired vascular network.

We here provide a demonstration that the targeted inhibition of Src kinase by Saracatinib is of therapeutic benefit in preclinical models of BTC. We propose our results as a basis for the design of Saracatinib-based clinical applications.
INTRODUCTION

BTC is a highly malignant epithelial cancer with a poor prognosis, due to its aggressiveness and to the availability of limited therapeutic options. At present, only surgical resection is associated with an improvement in patients’ outcome, with 5-year survival rates of 20–40% (1). However, the presence of distant metastases, extensive regional lymph node metastases and vascular encasement or invasion precludes resection. Consequently, patients with unresectable disease have a survival of less than 12 months from the diagnosis.

Chemotherapy has been commonly used to manage BTC patients, with the dual intention of improving their outcome and controlling tumor progression (2). Among the chemotherapeutic agents employed in BTC, gemcitabine has shown efficacy both as a single agent and in combination with other cytotoxic drugs. The response rates of single-agent gemcitabine range from 8 to 60%, depending on the cohort reported (3). The best result of combination chemotherapy in a phase II study was obtained for a regimen based on gemcitabine plus oxaliplatin (GEMOX), for which a 36% of response rate and 15.4 months of median survival have been achieved (4). Recently, the UK ABC-02 randomized phase III trial demonstrated that the association of gemcitabine with cisplatin increased both progression free (PFS) and overall survival (OS), compared to gemcitabine alone (median, 8 vs 5 months and 11.7 vs 8.1 months, respectively) (5). Although significant, the results achieved in these clinical trials witness a modest increase in both progression free and overall survival rates, evidencing that current therapies have a limited potential of improving a patient’s outcome. Thus, there is an urgent need to develop novel therapeutic strategies for the treatment of BTC, based on specific molecules on cancer cells as targets for innovative therapies.

Src is a non-receptor tyrosine kinase associated with growth factor/cytokine receptors, and plays a key role in the regulation of multiple cellular mechanisms in both normal and cancer
cells, including migration, adhesion, invasion, survival, proliferation, angiogenesis and inflammation (6). Src regulates important signaling cascades, including the focal adhesion kinase (FAK), the phosphatidylinositol 3-kinase (PI3K), and the signal transducer and activator of transcription 3 (STAT3) pathways (7-10). An increase in Src expression has been reported in several human cancers including colorectal, pancreatic and breast carcinomas (11-14). Recently, the small molecule Src inhibitor AZM555130 has been demonstrated to significantly reduce the proliferative and invasive potential of the HuCCA-1 human cholangiocarcinoma cell line (15), suggesting that targeting Src tyrosine kinase might provide a therapeutic benefit for the management of cholangiocarcinoma.

A growing number of pharmacological Src inhibitors are currently being tested in clinical trials (16, 17). The dual Src/Abl inhibitor dasatinib (BMS-354825) has shown preclinical efficacy in solid tumors and clinical activity in leukemia (18). In vitro, the Src inhibitor Saracatinib reduces migration and, in combination with tamoxifen, blocks proliferation of breast cancer cells (14). In orthotopic prostate cancer models, Saracatinib inhibits bone metastasis formation (19). This molecule is currently being tested in phase I-II clinical trials for various solid tumors (20, 21).

Here we investigate the effects of targeting Src with Saracatinib in preclinical models of BTC, and we report that this inhibitor is capable of reducing cell migration and proliferation in vitro, and of delaying tumor growth in vivo, in human BTC xenografts.

**MATERIALS AND METHODS**

**Tissue specimens**

The study was conducted on formalin-fixed tissue derived from 19 patients with BTC including 4 intrahepatic cholangiocarcinomas (ICC), 10 extrahepatic cholangiocarcinomas
(ECC) and 5 gallbladder carcinomas (GBC). All patients were of Italian origin and were 14 males and 5 females aged from 46 to 75 years (median 63 years). Surgical samples were obtained from the patients before any systemic treatment was administrated and collected according to conventional histopathological diagnostic protocols. Histological diagnosis and grading were performed according the *World Health Organization Classification of Tumors* (2002), and staging was determined according to the TNM system (22). The clinical profiles of patients as well as their pathological features are reported in supplementary table 1.

**Immunohistochemistry, immunocytochemistry and immunofluorescence**

For tumor patients, histological sections of 4 µm were mounted on glass slides. Sections were decorated with primary mouse anti-p-Src polyclonal (Tyr419, Cell Signaling, Inc.3 Trask LaneDanvers, MA 01923) and then incubated with a Dextran polymer conjugated to horseradish peroxidase. The final reaction was visualized using 3,3’-diaminobenzidine. Finally, sections were counterstained with Harris's hematoxylin, dehydrated, and mounted in DPX. Staining scores were established semi quantitatively from the percentage of p-Src+ cells and the staining intensity. Tumors were graded as negative (less than 1% positive cells), + (low intensity, 1-10% to positive cells), ++ (moderate intensity, >25% to <50% positive cells), and +++ (high intensity, >50% positive cells). For OCT-frozen tissues, sections of 10 µm were mounted on SuperFrost Plus glass slide (Menzel-Gläser, Braunschweig, Germany). Sections were decorated with rabbit polyclonal anti-HIF-1α (Upstate Millipore) and Dako EnVision+ System-HRP Labelled Polymer Anti-Rabbit; the Dako AEC+ High Sensitivity Substrate Chromogen was used to visualize the reaction. For immunofluorescence, sections were decorated with the purified rat anti-mouse CD31 clone MEC 13.3 primary antibody (BD Pharmingen) and with goat anti-rat secondary antibody (Invitrogen). Apoptosis was evaluated by TUNEL assay using In Situ Cell Death Detection Kit, TMR red (Roche), using
manufacturer’s instructions. To evaluate proliferation, slides were decorated with primary antibody anti-Ki-67 clone MIB1 (Dako) and with donkey anti-mouse secondary antibody (Invitrogen). For immunocytochemistry, 100,000 cells were spotted per glass slide (Menzel-Gläser, Braunschweig, Germany) and formalin fixed. Apoptosis was performed by TUNEL assay as previously described. Fluorescent images were acquired with a DMIRE2 confocal microscope from "Leica TCS SP5 II confocal microscope". The images were analyzed with the Image Processing and Analysis software in Java (ImageJ), version 1.44h.

**Cell lines and drug preparation**

The ECC cell lines EGI-1 and TFK-1 were kindly provided by Scherubl from the Institute of Physiology, Charité-Universitätsmedizin Berlin, Germany; the ICC cell line HuH28 and the GBC cell lines TGBC1-TKB were obtained from Cell Bank, RIKEN BioResource Center, Tsukuba, Japan. All the cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Murine endothelial cells were cultured in DMEM 10% FBS addicted of 4000 glucose (23).

Saracatinib was from Sequoia (Sequoia Research Products, Pangbourne, UK), dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20° C. For *in vivo* experiments Saracatinib was dissolved in Cremophor EL (Sigma-Aldrich)/95% ethanol (50:50).

**Cell growth and colony formation assay**

Cells (3000 cells/well) were seeded onto 96-well tissue culture plates and treated 24 hours later with different drug doses, (0.625-10 μM) for 72 hours. Proliferation was evaluated with Cell Titer-Glo® cell viability assay (Promega, Corporation, Madison, WI, USA), following the manufacturer’s protocol. The luminescent signal was measured by GloMax (GloMax®-Multi Detection System, Promega). All tests were performed in quadruplicate and repeated in three independent experiments. The median dose (Dm) value (concentration inhibiting 50% of
cell growth compared with 0.001% of DMSO control) was calculated for each cell line after 72 h of treatment using CalcuSyn software (Biosoft, Cambridge, UK) based on Chou-Talalay method. Colonies derived from single cells were obtained by plating 300 cells/well on 24 well tissue culture plates in RPMI plus 10% FBS, and treated with different doses of Saracatinib (from 80 nM to 5 μM). After 3 days, colonies were stained with 0.1% crystal violet (Sigma Aldrich).

**Cell cycle measurements and flow cytometry**

Cell cycle distribution was determined by flow cytometry. Briefly: 1×10⁶ harvested cells were fixed with 70% cold ethanol at -20°C for 16 hours. After washing with PBS, cells were resuspended in staining solution (50µg/ml Propidium Iodure PI + 100µg/ml RNaseA in PBS) (all from Sigma–Aldrich) and incubated at 4°C over night. The analysis was performed by FACS using Summit Research Software (Becton Dickinson).

**Wound-healing assay**

Cells were seeded in triplicate in 6-well tissue culture plates and allowed to grow until 100% confluence. The cell layer was gently “wounded” through the central axis of the plate using a pipette tip, then treated with different doses of Saracatinib in RPMI plus 10% FCS. Cell migration toward the scraped area was observed in 9 randomly selected microscopic fields for each condition and time point (up to 24 h). Images were acquired with a Leica DM13000B inverted microscope (Leica).

**Western Blot analysis**

Cells were lysed with lysis buffer (10% SDS, 0.5M Tris-HCl pH 6.8) at 100°C, 20 µg of proteins were electrophoresed on 7.5% SDS-PAGE and transferred to 0.45-µm nitrocellulose
membranes (Hybond™-C Extra, Amersham Biosciences). Luminescence was revealed with a chemiluminescence reagent (Euroclone, Milan, Italy). Anti-mouse and anti-rabbit antibodies linked with horseradish peroxidase, anti p-Src (Tyr 419), Src, p-paxillin (Tyr 118), p-p38 (Tyr180-182), p38, p-MAPK (Tyr 202-204), MAPK, p-Akt (Ser 473), Akt and Cyclin D1 were from CellSignaling Technology (Beverly, USA); antibodies anti p-FAK (Tyr 861), FAK and paxillin were MILLIPORE (Temecula, CA). For blot protein quantification, densitometric analysis of the detected bands was performed with the QuantityOne software (BioRad, Hercules, CA); band intensities of total proteins were normalized to the intensity of corresponding actin bands; band intensities of phosphorylated proteins were normalized to the intensity of corresponding normalized total proteins values.

**VEGF ELISA assay**

Cells were plated at 600,000 cells/well on 24-well tissue culture plates in appropriate medium for 24 h, then cultured in serum-free medium for 24 h; fresh medium supplemented with 5% FBS and containing various doses of Saracatinib (from 10 µM to 0.625 µM), was added for additional 24 h. The supernatant was harvested and secreted VEGF was measured by ELISA using the human VEGF Quantikine ELISA immunoassay kit (R&D Systems, Minneapolis, USA) according to the manufacturer’s protocol.

**Mice xenograft models**

NOD (Non-Obese Diabetic)/Shi-scid (severe combined immunodeficient) IL2rgnull female mice (5–6 weeks old) were used for in vivo experiments. Animals were maintained at the animal facilities of our institution (IRCC, Candiolo, Torino, Italy) and handled according to institutional regulations. EGI-1 cells were used for these experiments because their tumorigenicity.

In three different experiments, mice were subcutaneously injected (s.c.) into the right flank with 5x10⁶ EGI-1 cells in 50% growth factor-reduced BD Matrigel basement membrane
matrix. When tumors reached a volume of about 200 mm$^3$ (about two weeks after injection), animals were treated daily with either Saracatinib (25 mg/kg/die) or vehicle by oral gavage for 21 days. S.c. xenograft diameters were measured every 7 days. Ten mice were used for each treatment group. At the end of the treatment, mice were euthanized, tumor diameters measured and volumes calculated using the following formula: $V = A \times B^2/2$ ($V =$ tumor volume, $A =$ largest diameter; $B =$ smallest diameter). Mean volumes of treated and untreated xenografts were compared using an unpaired T test (Student’s T test) considering as statistically significant a p-value $< 0.05$ (C.I. 95%).

**Microarray analysis**

Total RNA was isolated from EGI-1 cells, either untreated or treated with 10 $\mu$M Saracatinib for 24 hours, using TRIZOL (Life Technologies, Gathersburg, MD); for frozen EGI-1 xenograft tissues, RNA was extracted with TriReagent (Sigma, St. Louis, MO, USA). RNA quantification was performed with Bioanalyzer 2100 (Agilent Technologies) and 1 $\mu$g of mRNA was amplified using the MessageAmp II aRNA Amplification kit (Ambion Inc. Austin TX, USA). Ammino-allyl modified nucleotides were incorporated according to the manufacturer’s protocol. Labeling was performed using NHS (N-hydroxysuccinimidy) ester Cy3 or Cy5 dies (GE Healthcare Europe GMBH, Upsala-Sweden). The Dye-Swap replication procedure was applied. Samples were hybridized on 8x60 K glass arrays (Agilent Technologies). Arrays were scanned and images analyzed by the Feature Extraction software Agilent (version 9.5) and the text files were then processed using the Bioconductor package Limma (Linear models for microarray analysis). Microarray data were deposited in GEO (GSE36622).
**Real time quantitative PCR (qRT-PCR)**

RNA was extracted and retro-transcribed in cDNA with the High capacity cDNA reverse transcription kit (Applied Biosystem). The cDNA was used for amplification of CXCL10, CAV1, SAA4, RRM2, CFB genes and PGK housekeeping gene with specific primers (supplementary table 2). Real-time PCR was carried out in triplicate. Quantitative analysis was performed by the measurement of Ct values (24).

**RESULTS**

**Src protein is phosphorylated in human BTC tissues and cell lines**

The presence of an activated Src protein was determined by immunohistochemistry in a panel of 19 BTC specimens revealing a moderate to strong positivity for p-Src expression in 15 samples (five were classified 1+, five 2+ and five 3+); only 4 samples were negative. Supplementary figure 1 shows representative immunostaining of p-Src in BTC samples. We did not find any statistically significant association between p-Src amounts and any clinical-pathological parameter or histological origin (data not shown).

We also evaluated the presence of a p-Src in 4 human BTC lines of different origin (i.e., TFK-1, EGI-1, HuH28 and TGBCl-TKB), observing variable basal levels of Src activation in these cultured cells (supplementary figure 2).

**Saracatinib inhibits the proliferation of BTC cell lines**

To assess the capability of Saracatinib (figure 1A) to interfere with cell proliferation, BTC lines were treated with different concentrations of this Src inhibitor. The ICC-derived HuH28 cell line was the most responsive (Dm=2.26 µM); the ECC cell lines TFK-1 and EGI-1 showed a similar behavior, with Dm of 4.02 µM and 3.82 µM, respectively. The GBC cell line TGBC1-TKB was the less sensitive (Dm=6.99 µM) (figure 1B).
Figure 1C showed representative dose/effect curves of each cell line. A statistical significant decrease in cell proliferation was seen in all the cell lines up to 1.25 μM, except for TGBC1, in which no significant effect was revealed (supplementary figure 3).

Performing the colony formation assay, we demonstrated that Saracatinib had the ability to reduce the formation of cellular foci in all the cell lines starting from 1 μM (supplementary figure 4). We further evaluated the effect of a proliferation-inhibitory dose (5 μM) of Saracatinib on cell cycle progression. After 24 hours of drug treatment, an increase in the fraction of cells in G0/G1 was revealed in TFK-1, EGI-1 and HuH28 (figure 1D); this effect was not improved by longer treatments (data not shown). In HuH28 cells, which proved to be the most sensitive to Saracatinib in terms of growth inhibition, this drug also increased the fraction of apoptotic cells, while in the less responsive TGB1-TKB cells the effect on G1 checkpoint arrest was weak (figure 1D). Moreover, we investigated the effect of Saracatinib on a key cell-cycle regulator, Cyclin D1. In agreement with the weakly effect on BTC growth, a slight inhibition of Cyclin D1 expression was detected only in EGI-1 cells, while in the other models Saracatinib was not able to inhibit this protein (data not shown). We have also evaluated the effect of Saracatinib on apoptosis by TUNEL assay. After 24 hours of treatment, a significant increase in the number of apoptotic cells was revealed in all cell lines, but the effect was prominent only in TGB1 cells (p-value < 0.05) (supplementary Figure 5).

Saracatinib inhibits BTC cell migration

We investigated the impact of Saracatinib on the migration potential of the cited BTC cell lines using the wound healing assay. A wound was produced on a layer of confluent cells, followed by treatment with different doses of Saracatinib (5-1-0.5-0.1 μM) for 24 hours. This assay revealed a significant dose-dependent inhibition of migration by Saracatinib, which was
induced in all the cell lines (data not shown). Figure 2 shows a representative assay performed with HuH28.

Saracatinib inhibits the activation of Src and of its downstream effectors

To evaluate the target effect of Saracatinib on Src and on its main transducers, all the cell lines were treated with 5 μM of inhibitor for 2 hours, followed by western blot analysis for the presence of the cognate phosphoproteins. This assay revealed that a switch-off of the pathway involving Src, FAK and Paxillin was induced in all the cell lines as a consequence of Saracatinib treatment (figure 3A-3B).

We further evaluated whether Saracatinib was capable of interfering with the MAPK and PI3K/Akt signaling cascades. In this case, we observed that only the downstream effector of MAPK, p-p38 was switched-off in all the cell lines examined (figure 3C). A weak down-regulation of p-MAPK was revealed in EGI-1 and HuH28 only; conversely, no down-regulation of p-Akt was seen in any cell line (data not shown).

Saracatinib delays tumor growth of EGI-1 xenografts

Having observed a targeted antitumor effect of Saracatinib in BTC cell models, we evaluated an in vivo antitumor activity on the xenograft implantation of the highly tumorigenic EGI-1 cell line. In three independent experiments, NOD/SCID IL2rgnull mice were s.c. injected with EGI-1 cells, and 10 mice/group were orally treated daily for 21 days, with Saracatinib or with drug vehicle. Saracatinib was able to significantly delay the growth of EGI-1 xenografts in comparison with control mice; the median fold-increase in tumor volume were 4.9 and 13.6 respectively (figure 4A) (p-value=0.06). Values were obtained from the ratio of volume at d21 and volume at d0 from each cohort of mice. In figure 4B, representative tumors harvested from control and Saracatinib-treated EGI-1 xenografts are
shown. On the same tumors, to evaluate the effect of Saracatinib on cell proliferation, the Ki-67 staining was performed. A statistically significant decrease of cell proliferation was revealed in treated mice (p-value=0.0003) (supplementary figure 6 panel A-B-C).

**Saracatinib inhibits* in vivo* and *in vitro* tumor angiogenesis**

We noticed that Saracatinib-treated tumors appeared macroscopically less vascularized compared to the controls (see figure 4B for examples). This prompted us to verify the status of tumor vasculature at the microscopic level. Sections of tumor tissues from control and Saracatinib-treated EGI-1 xenografts were stained for the endothelial marker CD31. A significant reduction (p<0.0001) in the number of tumor blood vessels was observed in Saracatinib-treated mice. (Figure 5, Panel A-B-C). Confirming the reduced blood support in these tumors, a significant increase of the hypoxia marker HIF-1a was revealed in treated mice (p-value=0.008) (Figure 5, Panel D-E-F). As a consequence, the number of apoptotic cells was significantly enhanced upon Saracatinib treatment (p-value=0.04) (Figure 5 panel G-H-I). Having observed a potent antiangiogenic effect of Saracatinib on tumor xenografts, we further evaluated the *in vitro* effect of Src inhibitor on proliferation and migration of the murine endothelial cell line b-END, and on VEGF secretion by BTC cell lines treated with different doses of the drug. The Dm for b-END cells was 0.97 μM, lower than those found in BTC cells, while the effect on migration is less marked compared with BTC cells (Figure 6). We did not find any statistically significant reduction of VEGF secretion in BTC cells upon Saracatinib treatment (p-value>0.05) (data not shown).

**Saracatinib induces a modulation of gene expression in BTC preclinical models**

The physio-pathological responses observed both *in vitro* and *in vivo* in the described preclinical models of human BTC were suggestive of a transcriptional modulation driven by
Saracatinib treatment. We therefore evaluated Saracatinib-induced gene expression profiles by microarray analysis of samples deriving from cultured EGI-1 cells and corresponding xenografts.

For the *in vitro* experiments, in order to maximize the effect of drug, EGI-cells were treated for 24 hours either with vehicle or with high-dose (10 μM) of Saracatinib, followed by quantification of transcript genes. Raw data were filtered by using a |LogFC| >1 and an adjusted p-value<0.01. The comparison between Saracatinib- and vehicle-treated EGI-1 cells revealed 647 modulated probes, of which 303 up-regulated and 344 down-regulated (supplementary table 2). We found that the expression of 20 genes was commonly regulated both *in vitro* and *in vivo* by Saracatinib treatment (supplementary table 2).

A Gene Ontology analysis with the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (david.abtcc.ncifcrf.gov) revealed that the biological processes significantly perturbed by Saracatinib in cultured cells are related to proliferation, cell cycle, neovascularization, and inflammation/chemotaxis. We further contextualized the deregulated genes in cellular signaling by the use of the PathwayMiner tool (www.biorag.org), finding a significant involvement in the G1/S check point, cyclin/cell cycle, p38 MAPK, and p53 pathways.

For the evaluation of Saracatinib-induced transcriptome *in vivo*, we harvested tumor xenografts after 21 days of treatment. A comparison between tumors from Saracatinib-treated and control mice revealed a modulation of 83 probes only, 46 up and 37 down regulated (supplementary table 2). The Gene Ontology analysis showed that up-regulated genes are involved in inflammation and immune response, while down-regulated genes are generically related to tissue development. Interestingly, the genes modulated by Saracatinib *in vivo* are involved in the metalloproteinase- and the chemokine-related pathways, as revealed by the PathwayMiner analysis.
In order to confirm results obtained by microarray experiments, we selected 5 de-regulated genes for further validation by qRT-PCR, i.e., the up-regulated chemokine (C-X-C motif) ligand 10 (CXCL10), complement factor B (CFB), constitutive serum amyloid A4 (SAA4) transcripts, and the down-regulated ribonucleotide reductase M2 (RRM2) and the caveolin 1 (CAV1). From the two techniques, we obtained the same trend of selected genes (data not shown).

**DISCUSSION**

Growing evidence indicates that a deregulation of Src, a protein implicated in a variety of mechanisms including cell proliferation, survivals and trafficking, is involved in the development and progression of solid tumors. Accordingly, Src inhibition is being actively pursued as a potential therapeutic strategy for many common cancers (10). We here provide a first report of the efficacy of a Src-targeted therapeutic approach in preclinical models of human BTC.

We analyzed a panel of human BTC specimens, showing that in about 80% of the samples an activated Src protein is expressed. Similar results have been previously obtained in other cancer types, particularly in breast, colon and hepatocellular carcinomas (25-27).

We therefore evaluated the *in vitro* activity of the small molecule Src inhibitor, Saracatinib on a panel of BTC cell lines. Although all these cell lines expressed activated Src, the anti-proliferative effect of Saracatinib in culture was moderate and resulted in a broad range of responses, from very low to moderate. However, these results are consistent with data present in the literature: Chang and collaborators (28) reported median doses ranging from 1 to 16
µM for Saracatinib-mediated growth inhibition in a panel of prostate cancer cell lines; in another study (29), the anti-proliferative effect of Saracatinib on cell lines from different tumor types was achieved with very variable median doses (from 0.2 to 14 µM). Our analysis on BTC cell cycle progression demonstrated that Saracatinib is very specific in blocking cells in the G0/G1 phase, but not in apoptosis. Similar results were observed by Ammer and collaborators in head and neck squamous cell carcinoma and in colorectal cancer (30, 31). The Saracatinib-modulated gene expression model supported these observations by demonstrating a down-regulation of genes involved in mitosis and in cell cycle check point.

In the evaluation of Saracatinib-modulated pathways in BTC cell lines, we observed only a partial inhibition of the MAPK pathway in HuH28 and EGI-1 cells (essentially, an inactivation of p38 MAPK), and no effect on Akt (data not shown). These findings indicate that the inhibition of Src by Saracatinib is probably counteracted by compensatory mechanisms involved in cell proliferation and survival. We have previously demonstrated, in BTC preclinical models, that the EGFR/HER2 pathways are inhibited by small molecules targeted to these tyrosine kinase receptors (erlotinib, gefitinib, lapatinib) or to their downstream effectors (everolimus, sorafenib), leading to an inhibition of the proliferation mediated by MAPK and/or PI3K/AKT pathways (32). Together, these data suggest that an association of Saracatinib with one or more of these inhibitors might be a future strategy for combined inhibition of cell migration and proliferation in BTC treatment.

Despite having a poor anti-proliferative effect, the antitumor activity of Saracatinib might be explained by other mechanisms. In fact, we demonstrated that Saracatinib is an efficient inhibitor of BTC cell migration. Accordingly, we evidenced a Saracatinib-mediated switch-off of the Src/FAK/Paxillin signaling pathway, which is a main player in the regulation of cell adhesion and migration. The capability of a cell to detach from the primary tumor mass and to move into the surrounding stroma and vasculature is a necessary step towards cancer invasion
and progression to distant metastasis. Indeed, very recent works proposed that Saracatinib could reduce the metastatic potential of cancer cells, at least in preclinical models of fibrosarcoma (33) and of non-small cell lung carcinoma (34). Our data support the hypothesis that such a role might be exerted by Src in BTC models as well.

A further step in the evaluation of Src inhibition in BTC preclinical models was the assessment of Saracatinib effects in an in vivo BTC model obtained by subcutaneous implant of EGI-1 cells. In mice treated with Saracatinib, a significant delay in xenograft tumor growth was measurable after 21 days of drug administration. These data confirm the efficacy of a Src-targeted approach as an efficient strategy to counteract tumor progression, and are consistent with previous works performed on gastric (35) and pancreatic (8) carcinoma xenograft models.

A series of scientific evidences demonstrated that, besides having a role in tumor progression and metastasis (11), Src is also implicated in the regulation of tumor angiogenesis and inflammatory stromal reaction (17). Consistently, we observed that Saracatinib-treated EGI-1 xenograft tumors were macroscopically less angiogenic compared to vehicle-treated tumor tissues. These considerations are further supported by our analysis of Saracatinib-modulated transcriptome in the BTC xenograft model, which revealed a significant deregulation of genes involved in vasculature/blood vessel development and morphogenesis. We therefore evaluated the presence and number of blood vessels in these tissues by specific staining followed by confocal microscope analysis, confirming a significant reduction of vessel areas in tumor specimens from Saracatinib-treated mice compared to untreated mice (p<0.0001). Such a reduced neovascularization was associated to increased hypoxia of the tumor tissues, with consequent up-regulation of the HIF-1α transcription factor (p=0.0008). To support these in vivo data, we investigated the effect of Saracatinib on murine endothelial cell growth.
and migration in vitro; we found that the Src inhibition affected the endothelial cell proliferation at low doses of the drug, while the effect on migration is less marked. Since the strictly relation of Src with VEGF, we have further evaluated the effect of Saracatinib on its production in BTC in vitro models; no statistical significant inhibition of secreted VEGF was found, as demonstrated by Bai and collaborators (36). This could, at least in part, explain why Saracatinib treatment, albeit inducing a significant reduction of the tumor mass and an increase in the number of tumor apoptotic cells, did not prove to be a long-term curative approach by itself. Similar results are being observed also in studies involving human subjects. Several phase II clinical trials on head and neck, gastric and pancreatic cancer patients revealed the poor efficacy of Saracatinib used as single agent (37-39). Further studies are currently reassessing the role of this drug in combination with chemotherapeutic agents. In particular, it has been shown that the inhibition of Src obtained by a Src family kinase inhibitor, alone or in combination with gemcitabine, results in delayed growth and metastasis of orthotopically-implanted human pancreatic carcinoma cells, as a consequence of a reduction in tumor cell proliferation and microvessel density, and by an increase in the apoptotic index (40). In our in vitro preclinical models, we demonstrated that there is a synergistic effect of saracatinib and gemcitabine in two of the four cell lines (41). In this connection and on the basis of our preclinical results, we can hypothesize to design a clinical study of Saracatinib given as a maintenance therapy to verify the capability of the drug to improve the progression-free survival status in those patients in which a disease control was reached by chemotherapy.

In conclusion, we demonstrated that activated Src is widely present in biopsies from BTC patients and that its small molecule inhibitor, Saracatinib has a potent inhibitory effect on cell BTC migration in vitro, and on tumor growth and neovascularization in vivo. Taken together, our findings encourage further studies in which the combination of Saracatinib with
chemotherapeutic agent or its administration upon the obtaining of a stable disease will be investigated to improve the management of BTC patients.

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Figure Legends:

**Figure 1: Impact of Saracatinib on BTC cell growth.**

**A.** Molecular structure of Saracatinib

**B.** Median proliferation-inhibitory doses (Dm) of Saracatinib in BTC cell lines after 72 hours of incubation with different doses of drug.

**C.** Representative dose/effect curves in BTC cell lines.

**D.** Effect of Saracatinib on BTC cell cycle progression. Each bar represents the average percent cells in each phase. NT: not treated; Sar: Saracatinib treated.
**Figure 2:** representative wound healing assays on HuH28 cells treated with the indicated doses of Saracatinib for 24 hours. Time 0: time of the wound; NT not treated cells.

**Figure 3:** Effect of 5 µM of Saracatinib after 2 hours of treatment on BTC cell lines. A) An evident switching off of p-Src, and p-FAK was observed in all the cell lines. B) A switching off of p-Paxillin was revealed in all the cell lines, more moderate in TFK-1. C) A slight inhibition effect was seen on p-p38 in BTC cell lines.

**Figure 4:** *In vivo* antitumor activity of Saracatinib in EGI-1 xenografts. A: mean tumor volumes of the two classes of mice. After 21d of treatment a slow of tumor growth was shown in treated mice. The graph indicates the mean tumor volume (mm$^3$) measured at 0, 7, 14 and 21 days of treatment with Saracatinib (Error bars: standard deviation). B: representative tumors harvested from EGI-1 xenografts treated with either vehicle or Saracatinib (25 mg/Kg/die).

**Figure 5.** CD31 staining of sections derived from EGI-1 xenograft tumors grown in vehicle-(A) and Saracatinib-treated (B) mice. A statistical significant reduction of vessel formation (CD31 staining) was present in the Saracatinib-treated group (C). HIF-1α staining of tumor sections derived from vehicle- (D) and Saracatinib (E) treated EGI-1 xenografts. A statistical significant increase in the expression of HIF-1α was seen in tumor tissues from Saracatinib-treated mice (F). TUNEL staining of tumor sections derived from vehicle (G) and Saracatinib treated group (H). A statistical significant increase of apoptosis was seen in Saracatinib-treated mice (I). NT: vehicle-treated mice

**Figure 6.** Representative wound healing assays on b-END cells treated with the indicated doses of Saracatinib for 24 hours. Time 0: time of the wound; NT: not treated.
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