Suppression of SRC Signaling Is Effective in Reducing Synergy between Glioblastoma and Stromal Cells

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Abstract

Glioblastoma cells efficiently interact with and infiltrate the surrounding normal tissue, rendering surgical resection and adjuvant chemo/radiotherapy ineffective. New therapeutic targets, able to interfere with glioblastoma’s capacity to synergize with normal brain tissue, are currently under investigation. The compound Si306, a pyrazolo[3,4-d]pyrimidine derivative, selected for its favorable activity against SRC, was tested in vitro and in vivo on glioblastoma cell lines. In vitro, combination treatment with Si306 and radiotherapy was strongly active in reducing U-87 xenograft growth with respect to control and single treatments. The histology revealed a significant difference in the stromal compartment of tumoral tissue derived from control or radiotherapy-treated samples with respect to Si306-treated samples, showing in the latter a reduced presence of collagen and α-SMA-positive cells. This effect was paralleled in vivo by the capacity of Si306 to interfere with myofibroblastic differentiation of normal fibroblasts induced by U-87 cells. In the presence of Si306, TGF-β released by U-87 cells, mainly in hypoxia, was ineffective in upregulating α-SMA and β-PDGF in fibroblasts. Si306 efficiently reached the brain and significantly prolonged the survival of mice orthotopically injected with U-87 cells. Drugs that target SRC could represent an effective therapeutic strategy in glioblastoma, able to block positive paracrine loop with stromal cells based on the β-PDGF axis and the formation of a tumor-promoting microenvironment. This approach could be important in combination with conventional treatments in the effort to reduce tumor resistance to therapy.

Introduction

Glioblastoma is the most frequent primary brain tumor in adults. At diagnosis, glioblastoma usually demonstrates very aggressive cellular features, with infiltration of normal brain (1). Glioblastoma is characterized by increased proliferation and demand of nutrient and oxygen as revealed by frequent evidence of microvascularity and necrosis (2). The prognosis of glioblastoma is poor: median survival time is less than one year since the diagnosis and patients usually die within two years (3). In fact, also in presence of aggressive standard therapy, that is, surgical resection, followed by concomitant radiotherapy and temozolomide therapy, fatal relapses could not be avoided.

New therapeutic targets for glioblastoma, including receptors for growth factors and other tyrosine kinases involved in intracellular signal transduction processes, are actively studied (4). Among these, SRC-family members have been proposed as key kinases able to drive glioblastoma carcinogenesis and progression (1). Initial studies have found elevated SRC activity in glioblastoma compared with normal brain samples, and have revealed its oncogenic properties for brain tumors (5). SRC is a key downstream intermediate of growth factor receptors frequently over-expressed in brain tumors, including EGFR and platelet-derived growth factor receptor (PDGFR), allowing, in association with FAK kinase, cytoskeletal-linked cell survival and migration (6). In preclinical models of glioblastoma, genetic and pharmacologic blockade of SRC resulted effective in inhibiting proliferation and invasion (7, 8).

In the current race for new anticancer therapies, the tumor microenvironment is increasingly recognized as promising target. Tumor microenvironment consists of a dynamic mixture of stromal cells and extracellular matrix (ECM), and plays a critical role during tumor initiation, angiogenesis, progression, and metastasis (9). The carcinoma-associated fibroblasts (CAF), an important component of the tumor stroma, are able to protect cancer cells against drugs (10). In fact, the release of growth factors by CAFs can activate alternative pathways contributing to drug resistance. They participate in each step of angiogenesis and may be responsible for mediating resistance to chemotherapeutic, antiangiogenesis drugs and other targeted therapies (11). Stromal cells, through cytokines secretion, can modulate the ECM remodeling, suppress immune responses, and affect tumor growth and...
invasiveness (9). A fibroblast-like cell population was recently identified in computer-guided stereotaxic biopsies from glioblastoma surrounding tissue. These cells express CAFs associated markers, including fibroblast surface protein, alpha-smooth muscle actin (α-SMA), and β-PDGFR (12). The role of these cells in glioblastoma progression is under investigation.

In this study, we aimed to investigate the therapeutic role of Si306 in glioblastoma, a new compound chosen among our family of pyrazolo[3,4-d]pyrimidines (structure of Si306 in Fig. 1A) thanks to its favorable and previously characterized set of ADME and activity properties (13). The compound exhibited the ability to inhibit c-SRC tyrosine kinase in cell-free assays at micromolar concentration. Moreover, Si306 demonstrated a good aqueous solubility profile and a high metabolic stability determined in vivo after incubation with Human Liver Microsomes (HLM; Supplementary Table S1).

Materials and Methods

Cell culture

U-87 MG and U-251 MG (formerly known as U-373 MG), human glioblastoma cell lines, were purchased from European Collection of Authenticated Cell Cultures in 2014 (ECACC, Public Health England). Cell lines were cultured in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% FBS, 2 mmol/L glutamine, 100 IU/mL penicillin, 100 µg/mL
streptomycin, 1% non-essential amino acids, and 1 mmol/L sodium pyruvate. NIH/3T3, mouse fibroblasts, were cultured in DMEM with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in culture for no longer than 20 passages. DNA profiling was carried out to authenticate resuscitated cells. Short tandem repeat profiling was performed by using GenePrint 10 System that permitted co-amplification and detection of eight human loci required by the guidelines ASN-0002 (Promega Corporation). To evaluate cell proliferation, the number of cells was measured by direct cell counting assay. Cells were plated at a density of 10^3 cells/cm², incubated in 5% CO₂ at 37°C and recovered after different times of incubation. At the endpoint, cells were harvested, centrifuged, and aliquots of cell suspensions were counted using a Neubauer hemocytometer chamber. Dead cells were assessed by the trypan blue dye exclusion test. For the hypoxia, cells were cultured in a Galaxy 48 R CO₂ Incubator equipped with O₂ control module (New Brunswick Scientific).

Migration assay
Exponential growing cells were trypsinized, washed twice with PBS, rinsed in complete medium and incubated at 37°C for 30 minutes to reconstitute the membrane structures, washed again for FCS removal, and then added to the upper compartment of collagen-coated (PTFE) Transwell insert (Corning Inc.). Cells were allowed to migrate through coated filters for 6 hours. The cells attached on the lower membrane surfaces were stained with 0.1% crystal violet w/v in 0.1 mol/L borate, pH 9.0 and 2% ethanol v/v for 20 minutes at room temperature. Cells were counted at 400 magnifications in standard optical microscopy and the average number of cells per field in 5 random fields was recorded. Triplicate filters were used and the experiments were repeated three times.

Time-dependent cell response profiling using the real-time RTCA iCELLigence system
The RTCA iCELLigence system (ACEA, Biosciences, Inc.) is a microelectronic biosensor system for cell-based assay. Cell viability, cell number, cell morphology, and degree of adhesion are evaluated by real-time electrode impedance. A dimensionless parameter termed Cell Index (CI) is used to measure the relative change in electrical impedance and to quantify cell status. For time-dependent cell response profiling, 150 μL of media was added to 8-well electric plates (E-Plates) to obtain background readings followed by the addition of 300 μL of cell suspension at the density of 35,000 cells/mL. The E-Plates containing the cells were incubated at room temperature for 30 minutes and placed on the reader in the incubator for continuous recording of impedance as reflected by CI. After 20 to 24 hours, the cells were subjected to specific treatment. Coculture experiments were performed by adding 10,000 cells growing in an insert that is sealed at microporous polyethylene terephthalate (PET) membrane. The cells were monitored every hour starting from 1 hour after compound addition to about 100 hours to capture the long term response. A "Normalized Cell Index" at a given time point, just following the treatment, is calculated by dividing the CI at the time point by the CI at a reference time point. Thus, the Normalized Cell Index is 1 at the reference time point.

Low density growth assay
U-87 cells capacity for growth at clonal density, was evaluated by plating cells at density of 10 cells/cm² in 10% FBS supplemented in DMEM. Treated cells received one irradiation (4 Gy, 3 days after plating) in combination or not with 1 or 10 μmol/L Si306 every other day. After 2 weeks of culture, adherent cells were fixed with cold methanol, washed with PBS/BSA and air-dried. Adherent cells were stained with 0.5% crystal violet for 15 minutes at room temperature. The stained colonies were photomicrographed and analyzed by number and size with the public domain software ImageJ (by Wayne Rasband, http://rsb.info.nih.gov/ij/).

Western blotting
Total cell lysates were obtained by incubating cells in a lysis buffer containing 1% triton, 0.1% SDS, 2 mmol/L CaCl₂, 10 mg/mL orthovanadate, and 1x protease inhibitors cocktail (Sigma). Protein content was determined using the Protein Assay Kit 2 (Bio-Rad Laboratories). Sixty micrograms of proteins were electrophoresed in 10% SDS-polyacrylamide gel. After electrophoresis gels were placed onto Trans-Blot Turbo mini nitrocellulose transfer pack and transferred using Trans-Bolt Turbo Transfer System (Bio-Rad Laboratories). The membrane was incubated with 1 μg/mL primary antibody and then with the appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies. Primary antibodies α-SMA were purchased from Sigma-Aldrich; PDGFR, p-PDGFR, SRC, p-SRC, histone γH2AX were purchased from Cell Signaling Technology; β-actin was purchased from Santa Cruz Biotechnology. Protein bands were visualized using a chemiluminescent detection system (Thermo Scientific) and signals were digitally acquired by Chemidoc XRS system (Bio-Rad Laboratories).

Histology assays
Slide-mounted tissue sections (4-um-thick) were deparaffinized in xylene and hydrated serially in 100%, 95%, and 80% ethanol. For immunohistochemistry Endogenous peroxidases were quenched in 3% H₂O₂ in PBS for 1 hour and then slides were incubated with a primary antibody for 1 hour at room temperature. Sections were washed three times in PBS and antibody binding was revealed using the Sigma fast 3,3-diaminobenzidine tablet set (Sigma). For Masson’s Trichrome Staining sections were stained in Weigert’s iron hematoxylin working solution for 10 minutes, washed and stained in Biebrich scarlet-acid fuchsin solution for 10 to 15 minutes. Then slides were differentiated in phosphomolybdic-phosphotungstic acid solution until collagen was not red and then transferred to aniline blue solution for 5 to 10 minutes. The sections were washed, dehydrated, and mounted with resinous mounting medium.

TGFβ measurement
Conditioned media (CM) were collected, centrifuged for eliminating cells and were analyzed by the Human TGFβ 1 ELISA Kit according to the manufacturer’s instructions (Boster biological technology). Briefly, 100 μL CM were added to each well of a precoated 96-well plate, a biotinylated detection polyclonal antibody specific for TGFβ was added subsequently and then washed with PBS. Avidin–biotin–peroxidase complex was added and unbound conjugates were washed away with PBS. TMB (3,3',5,5'-tetramethylbenzidine), HRP substrate, was used to visualize HRP enzymatic reaction. HRP catalyzed the conversion of TMB producing a blue color (due to the product: 3,3',5,5'-tetramethylbenzidine diimine) that turned into yellow after adding the acidic stop solution. The optical density of yellow was proportional to the amount of human TGFβ 1 in the sample. The
concentration was calculated by applying the sample reading to a standard curve.

**Animal glioblastoma models**

Male CD1 nude mice (Charles River Laboratories) were maintained under the guidelines established by our Institution (University of L’Aquila), complying with the Italian government regulation for the use of laboratory animals. Before any invasive manipulation, mice were anesthetized with a mixture of ketamine (25 mg/mL)/xylazine (5 mg/mL). Tumor grafts were obtained by injecting s.c. a total of 1 × 10^6 U-87 cells in 100 μL of 12 mg/mL Matrigel (Becton Dickinson). Tumor growth was monitored daily by measuring the average tumor diameter. The tumor volume was expressed in mm^3 according to the formula 4/3πr^3. In vivo orthotopic tumor growth experiments were performed in nude mice by injecting intracranially 3 × 10^6 U-87 cells. The surgical zone was swabbed with Betadine solution, the eyes coated with Lacri-lube. The head was fixed in a stereotactic frame (mouse stereotaxis instrument, Stoelting Europe, Dublin, Ireland) and a midline scalp incision was made. A small hole was made at 1.0 mm anterior and 2 mm lateral to the exposed bregma. A sterile 5 μL Hamilton syringe with a 26 gauge needle was inserted at a depth of 3.0 mm from the skull surface and withdrawn by 0.5 mm to inject U-87 cells in a volume of 3 μL. The injection rate was set up to 1 μL/min. The needle was then completely withdrawn from the brain over the course of 4 min (1.0 mm/min), and the skin was sutured. Just before treatment initiation (5 days after injection), animals were randomized to treatment groups of 7 mice each. Mice were euthanized when they displayed neurological signs (e.g., altered gait, tremors/seizures, lethargy) or weight loss of 20% or greater of pre-surgical weight. For each sample 1 mL of acetonitrile (with 10 mmol/L internal standard) was added to denature proteins and to extract the drug. For each sample 1 mL of acetonitrile (with 10 mmol/L internal standard) was added to denature proteins and to extract the drug.

**In vivo pharmacokinetics model**

The animal protocols used were reviewed and approved by the Animal Care and Ethics Committee of the Università degli Studi di Siena, Italy. Male BALB/C mice (weight 20–30 g) were obtained from Charles River Laboratories (Milan, Italy). The experiment was performed in triplicate and mice were treated with Si306 (100 μL, DMSO solution), given intraperitoneally. The drug was administered at the dose of 50 mg/kg. Animals were treated with heparin solution and sacrificed under CO₂ at several time points (0.25–4 hours); blood (drawn by cardiac puncture) and brain were collected for the following quantitative analysis. The blood was centrifuged at 4,000 rpm for 20 minutes to separate the plasma fraction, which was subsequently collected in a test tube. For each sample 1 mL of acetonitrile (with 10 mmol/L internal standard) was added to denature proteins and to extract the drug. Samples were centrifuged at 4,000 rpm for 20 minutes, the supernatant was recovered, dried under vacuum, and analyzed. The drug was homogenized using a glass/glass Potter-Elvehjem homogenizer and compounds were recovered using 7 mL of ACN then treated as previously described for blood samples. Each sample was solubilized in 100 μL of methanol and analyzed by HPLC-UV-MS. LC analysis were performed by Agilent 1100 LC/MSD VL system (G1946C, Agilent Technologies) constituted by a vacuum solvent degassing unit, a binary high-pressure gradient pump, a 1100 series UV detector and a 1100 MSD model VL benchtop mass spectrometer was used. The Agilent 1100 series mass spectra detection (MSD) single-quadrupole instrument was equipped with the orthogonal spray API-ES (Agilent Technologies). Nitrogen was used as nebulizing and drying gas. Instrumenting settings are reported in Supplementary Material. The quantification of each compound was performed referring to the appropriate calibration curves. MK MODEL program was used to determine pharmacokinetic parameters.

**Statistical analysis**

Descriptive data are presented as mean ± SD for continuous data and percentages for categorical data. Comparisons between groups have been performed by using the parametric T Student test for continuous measures. For xenograft experiments, the statistical significance was evaluated by the log-rank test. For orthotopic model, data were analyzed through Kaplan–Meier curves considering mean and median survival values (MedCalc software, MedCalc Software bvba). P values <0.05 were considered statistically significant.

**Results**

**In vitro antiproliferative effect of Si306 on glioblastoma cells**

We initially tested Si306 in two different human glioblastoma astrocytoma cell lines, U-87 MG and U-251. Western blot analysis of total lysates demonstrated that both glioblastoma cell lines basally expressed SRC and its active form pY416-SRC. The treatment with 1 μmol/L Si306 for 48 hours in standard culture conditions was effective in downmodulating the phosphorylated forms of SRC (Fig. 1B). In parallel, we observed that SRC inhibition induced the down-modulation of the active form of β-PDGFR. This effect was evident only in U-87 cells that expressed basally significant levels of β-PDGFR (Fig. 1B). Pretreatment of glioblastoma cells with Si306 determined a significant reduction in their migratory ability. Si306 was able to block the migratory stimulus by EGF in a dose-dependent manner and similarly to the reference SRC inhibitor PP2 (Fig. 1C). A prolonged treatment with Si306 determined a significant reduction in glioblastoma cell proliferation and an increased number of dead cells. Dose–response experiment demonstrated that U-87 cells were more responsive to the growth inhibition by Si306 and, in these cells, Si306 resulted more potent than PP2 (Fig. 1D).

U-87 cells were more susceptible to radiotherapy when compared with U-251. In fact, when glioblastoma cells were grown at low density, the number of cell colonies was statistically lesser in the experiment with irradiated U-87 cells than in the one with irradiated U-251 cells, both compared with the non-irradiated control. In the same experiment, we tested the activity of Si306 and radiotherapy in combination, determining a significant decrease in the number of colonies in U-87 cells only (Fig. 2A). To verify whether co-treatment with Si306 contributed to intensify genotoxic effect of radiotherapy, we evaluated the expression of DNA double-strand break marker, histone γH2AX. An increment of γH2AX expression was observed in U-87 cells treated with the combination in respect of the cells treated with only radiotherapy. On the contrary, in U-251 cells, the histone γH2AX increase was not observed (Fig. 2B).

**In vivo antiproliferative effect of Si306 on U-87 glioblastoma cells**

The combination effect of Si306 and radiotherapy was further investigated in vivo using U-87 cells. Glioblastoma cells were...
injected subcutaneously in immunodeflcient mice and tumor growth was monitored for 30 days. Mice were divided in 4 groups receiving vehicle (CTR), 50 mg/kg Si306, radiotherapy and combination of Si306 and radiotherapy. At the endpoint, cancer masses from combination group were considerably smaller respect to those from all other experimental groups (Fig. 3). Both single treatments, with radiotherapy or with Si306, were effective in reducing tumor mass of about 40% with respect to the vehicle treated control. However, the combination treatment determined a reduction of the tumor growth close to the 80% respect to control group. In addition, as shown by images of excised tumors, the antitumor effect of combination treatment was consistently effective in all the treated mice (Fig. 3A).

Pharmacokinetics and orthotopic animal model

To assess whether compound Si306 was able to overcome the blood–brain barrier, we decided to evaluate its pharmacokinetic profile in vivo. Plasma and brain of mice were collected at different time points in a 24 hours experiment, after intraperitoneally injection of 50 mg/Kg Si306. The quantity of Si306 was determined, using the HPLC-MS-UV standardized method, to define the concentration profile of the compound in plasma and brain in 24 hours (Fig. 3B). Plasma concentration of Si306 increased rapidly and reached the maximum level 30 minutes after the injection. The compound was able to reach the brain compartment, its concentration progressively increased during the 24 hours and it appeared to accumulate in the brain. Concentration data were evaluated using a one-compartment model and the resulting pharmacokinetic parameters are displayed in Table 1.

Then we performed a disease-free survival experiment in an orthotopic in vivo model of glioblastoma. U-87 cells were injected in the brain (see Materials and Methods) of nude mice and general health conditions of animal were monitored daily to detect any signs of distress. Mice were treated every other day with 50 mg/Kg Si306 or with vehicle (7 mice each group) starting from 5 days after brain injection of tumor cells. Si306-treated mice showed a significant increment in median survival time respect to mice treated with vehicle (30.6 days vs. 41.0 days, \( P = 0.0397 \); Fig. 3C). The survival time in Si306-treated mice was increased of about 30% respect to control mice. Hystologic evaluation of tumor tissues by trichrome staining revealed important differences in the architecture of the tissues, with collagen deposition visible only in control tumors and in irradiated tumors (Fig. 4A, left). To evaluate the contribution of tumor-associated stromal cells to collagen deposition, we examined by immunohistochemistry the expression of myofibroblast marker \( \alpha\)-SMA. Results were in agreement with trichrome staining, indicating a possible association between collagen deposition and myofibroblast differentiation (Fig. 4A, right). A similar staining pattern was also observed in tissues from orthotopic glioblastoma model (Fig. 4B). In particular, \( \alpha\)-SMA–positive cells resulted more abundant in glioblastoma tissue from untreated mice respect with tissue from Si306-treated mice, and these cells frequently surrounded extracellular matrix–rich zones (Fig. 4B, right).

Differentiative potential of U-87 cells on fibroblasts

Si306 was able to partially interfere with TGF\( \beta \) release in U-87 cells, in basal conditions. However, in hypoxia conditions,
the level of TGFβ released from U-87 cells was significantly upregulated, also in presence of Si306 (Table 2). To recapitulate in vitro what we observed in vivo, we considered the potential interaction between U-87 and NIH/3T3 fibroblasts. First, we evaluated real-time cell viability of NIH/3T3 cells in the presence of conditioned medium from U-87 cells (Fig. 5A). In this condition, we observed a slight increase in the proliferative rate of NIH/3T3 fibroblasts, with respect to the untreated cells, this increase was intensely counteracted by the addition of 0.1 µmol/L Si306. The treatment with 1 ng/mL TGFβ determined a more evident effect on the proliferative rate of fibroblasts and, also in this case 0.1 µmol/L Si306 was very effective in reducing the growth effect (Fig. 5A). Significantly, conditioned medium from U-87 cells cultured in hypoxia (1% O₂) represented a better proliferative stimulus compared with the conditioned medium from U-87 cells cultured in normoxia as demonstrated by the significant decrease in doubling time of fibroblasts (Fig. 5B). Also, in the presence of conditioned medium from hypoxic U-87, 0.1 µmol/L Si306 was sufficient to significantly decrease the doubling time of fibroblasts. The addition of TGFβ or conditioned medium from U-87 to serum-free culture medium determined the upregulation of β-PDGFR and α-SMA in NIH/3T3 fibroblasts. The specific inhibitor of TGFβ receptor II, LY2109761, effectively counteracted this phenotypic switch. In the same experimental conditions, also Si306 resulted effective in reducing the expression of β-PDGFR and α-SMA (Fig. 6A). The phenotypic switch of fibroblasts was more evident when we cultured cells with conditioned medium from hypoxic U-87 cells (U-87 CM-h), and it was contrasted by the addition of 0.1 µmol/L Si306 (Fig. 6A). To verify the possible effect of differentiated fibroblasts on U-87 cells we performed a real-time proliferation assay measuring the variation in doubling time of U-87 cells when cultured with NIH/3T3 previously conditioned for 72 hours with U-87 CM-h (cancer-associated NIH/3T3, CA-3T3). U-87 cells received a proliferative stimulus by the presence of CA-3T3 that was evident by a reduction of the doubling time starting from 60 hours of co-culture (doubling time = 69 vs. 60 hours; Fig. 6B).

### Discussion

The current vision of antitumoral-targeted therapy is evolving in the concept of tumor-type tailored therapy that deals with the genetic and epigenetic characteristics of the whole tumor.
tissue, including the associated non-tumoral cells. Tyrosine kinases are a large family of antitumoral targets with a specific tissue distribution and involved in key pathogenic processes. Their constitutive activation not only determines the self-sufficiency in growth, but also the prevalent tumor phenotype and the tumor ability to modify the associated microenvironment. Members of SRC family are often overactivated in glioblastoma. This is not surprising because in glioblastoma the high frequency of EGFR amplification and mutation leads to an overactivation of downstream signaling intermediates, including SRC (14). SRC activation determines increased migration ability and desensitization to radiation therapy. In our study, we confirmed that SRC inhibition increased radiosensitivity in glioblastoma cells, and this effect was evident in vitro in U-87 cells that basally expressed high levels of PDGFR. Only in U-87 cells, we observed an upregulation of the double-strand break marker γH2AX and a significant reduction in clonal growth in association with radiotherapy. Interestingly, it was demonstrated that PDGF overexpression in brain tissue determines the activation of an aggressive phenotype mainly in association with SRC (15). In addition, imatinib, an inhibitor of PDGF has demonstrated radiosensitizing activity (16). Our data suggested that concomitant overactivation of SRC and PDGFR in the same tumor phenotype could contribute to glioblastoma progression after radiotherapy treatment. Molecular interaction between SRC and PDGFR might occur at different levels. PDGFR binds SRC homology 2 (SH2) domain-containing proteins, including SRC, FYN, and YES, allowing the activation of signal-transduction mitogenic pathway (17). However, SRC phenotype was associated with a continuous release of growth factors by autocrine mechanism (18). The cooperation between SRC and PDGF in determining an aggressive phenotype involves also the regulation of cell migration. In fact, PDGF stimulation of integrin-mediated motility is dependent on activation of SRC (19). However, our data indicated that Si306 was able to inhibit glioblastoma cell migration in a manner that was independent from the expression of PDGFR. In fact, also U-251 cells that expressed low levels of PDGFR, were effectively inhibited in their motility by Si306. In agreement, SRC-family inhibitor PP2 resulted similarly effective in modulating EGF-induced glioblastoma migration.

The PDGF secreted by cancer cells has a dual role: directly sustains cancer cell survival and migration, but also determines

Table 2. TGFβ concentration (pg/mL) in conditioned medium from U-87 cells

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<tr>
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<th>CTR</th>
<th>Si306</th>
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<tr>
<td>21% O2</td>
<td>251.7 ± 12</td>
<td>186.7 ± 5.0</td>
<td>&lt;0.001</td>
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<tr>
<td>1% O2</td>
<td>522.0 ± 8.0</td>
<td>470.7 ± 7.2</td>
<td>&lt;0.001</td>
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NOTE: Conditioned medium was recovered from U-87 cells treated with vehicle (CTR) or with 1 μmol/L Si306 after 48 hours of culture, and evaluated for the presence of TGFβ. The experiment was performed also in hypoxic condition (1% O2). Mean value and SD of TGFβ was calculated from two different experiments and from triplicates for each experiment.

Figure 4. Histologic pattern of U-87 glioblastoma tumors grown in vivo. On the left, images of subcutaneous (A) or orthotopic (B) xenograft sections stained with Masson’s trichrome solution. The staining shows blue collagen, light red or pink cytoplasm, and dark brown cell nuclei. On the right, images of subcutaneous (A) or orthotopic (B) xenograft sections immunostained for α-SMA and visualized by brown DAB staining. CTR, vehicle-treated control group; RT, 4 Gy radiotherapy-treated group; Si306, samples from 50 mg/kg Si306-treated mice.
the phenotype of associated non-transformed cells. Tumor-induced angiogenesis is the best known way in which the tumor modifies normal surrounding tissues gaining an advantage for its progression. Glioblastoma tumors are characterized by an abundant new vasculature induced by cancer cells. The expression of β-PDGFR in the endothelial cells of hyperplastic capillaries of malignant glioma indicates the presence of PDGF paracrine loop in the angiogenesis process (20). In the last decade, several evidence have strengthened the hypothesis that angiogenesis represents only one aspect of a wider ability of tumor cells to modify in an active manner the microenvironment, inducing differentiation and/or adaptation of associated cells. Recently, diploid cells with phenotypic and functional properties in common with CAFs and that express α-SMA and β-PDGFR have been isolated in the periphery of glioblastoma. Significantly, the subcutaneous injection of stromal cells, together with U-87 cells, into immunodeficient mice resulted in the induction of tumors larger than those produced by U-87 cells alone (12). We have extended these observations demonstrating new properties of glioblastoma cells. First, the capacity to recall and transform normal cells is a phenotypic property of U-87 cells, which is conserved both in vitro and in vivo. The presence of associated fibroblasts is a characteristic of fast growing tumors in vivo and it might be a component of radiotherapy resistance. The use of Si306, counteracting the differentiation of associated fibroblasts, resulted in a significant reduction of tumor growth. Si306 was able to reduce the autocrine activation of glioblastoma cells, as demonstrated by the direct antiproliferative effect observed in vitro. However, the increased therapeutic potential expressed in vivo and the role on fibroblasts differentiation in vitro, makes plausible that in vivo efficacy of Si306 was the result of a synergic action on cancer and cancer-associated cells.

The origin of tumor-associated fibroblasts in brain is not clear. We demonstrated that conditioned medium from U-87 was able to induce myofibroblastic differentiation in mouse embryonic fibroblasts. Also, in tumor xenograft it seems reasonable to assume that the infiltrating cells were fibroblasts recalled from neighbor tissues, although we cannot exclude other hypotheses, including the recall of mesenchymal stem cells (MSC). Recent evidence highlights the importance of tropism of MSCs for

![Figure 5](image-url)

**Figure 5.** *In vitro* effects of U-87 glioblastoma cells on NIH/3T3 fibroblasts. A, real-time proliferation pattern of NIH/3T3 cells treated with vehicle (CTR), 10 ng/mL TGFβ, 30% conditioned medium from U-87 cells (U-87CM), 10 ng/mL TGFβ plus 0.1 μmol/L Si306, or 30% conditioned medium from U-87 cells plus 0.1 μmol/L Si306. The vertical line indicates the start of the treatments and the point of normalization of Cell Index values. The average ± SD Cell Index recorded each hour from triplicate is shown. B, real-time proliferation pattern of NIH/3T3 cells treated with vehicle (CTR), 30% conditioned medium from U-87 cells (U-87CM-n), 30% conditioned medium from U-87 cells cultured in hypoxia (U-87CM-h), U-87CM-n plus 0.1 μmol/L Si306, or U-87CM-h plus 0.1 μmol/L Si306. The average ± SD Cell Index from triplicate normalized 30 minutes after treatment is shown. The right histogram shows the doubling time in hours calculated by mean Cell Index in the interval 20 to 40 hours. *P < 0.01 according Student t test between experimental points indicated by top line.
A greater effect on NIH/3T3 cells than on cancer cells. In fact, NIH/3T3 cells were greatly affected by TGFβ and reactive to the CAF phenotype (21). In our study, we used mouse embryonic fibroblasts NIH/3T3 that represent a normal cell model highly responsive to U-87 cells. In fact, NIH/3T3 cells were greatly affected by TGFβ regarding their proliferative rate and phenotype. The greatest effect on fibroblasts was obtained when these cells were treated with conditioned medium from U-87 stimulated by hypoxia to secrete more TGFβ. Hypoxia is a common feature of glioblastoma as suggested by frequent presence of central necrotic areas in patients and in vivo models. Thus, hypoxia through the release of TGFβ by cancer cells contributes to determine a response that mimics wound healing, driving the differentiation of myofibroblasts.

The ability of TGFβ to induce myofibroblasts differentiation, together with its profibrotic role is well documented. Our data, obtained by screening for kinase activity, do not support a direct role of Si306 in blocking the signaling downstream of TGFβ (data not shown). Thus, we propose a mechanism in which the activation of SRC and/or PDGFR is required to accomplish the myofibroblastic differentiation. In rodent model of fibrosis, it is well known that pharmacologic inhibition of SRC was associated with suppression of myofibroblast phenotype. A possible mechanism involves SRC-induced activation of FAK, which is required for TGFβ induced α-SMA expression and myofibroblast differentiation (22). In myofibroblasts, the reorganization of filament network with the incorporation of α-SMA is regulated by the formation of large focal adhesions that requires the activation of FAK. In addition, SRC plays a key role also in the epithelial-to-mesenchymal transition. In our previous work, we demonstrated that SRC inhibition is important in the regulation of cell migration and of FAK expression in cancer cells. Importantly, we proved that FAK disturbance by SRC inhibitor was important in reducing tumor-associated angiogenesis by targeting endothelial cells in the tumor (23).

Although the very promising preclinical data, the clinical trials with the SRC inhibitor dasatinib in glioblastoma were frequently disappointing. The reasons that may account for this failure are unknown, but probably reside in two major categories: inadequate pharmacologic characteristics of the drug and non-appropriate patient selection. In the first case, we can cite the low delivery to the brain and the drug resistance, in the second, signaling pathway redundancy and cancer heterogeneity (24). The pharmacokinetic data demonstrated that Si306 was able to reach the brain, overcoming the hurdle represented by the blood–brain barrier. The significant concentration identified 24 hours after administration proved the favorable pharmacokinetic properties of our drug candidate, a crucial feature to further consider this drug as a possible candidate to treat glioblastoma (25). Orthotopic growth of U-87 cells was effectively inhibited by Si306, doubling, in some cases, the survival time of treated mice with respect to control mice. In addition, our data suggest that antitumoral activity of Si306 is particularly effective in interfering with the recruitment of associated non-tumoral cells, a tumor characteristic that was previously neglected in study about SRC inhibition. This is a very promising aspect, although the therapeutic value of this potential drug should be further explored. In fact, since the timing and the biologic features of stroma contribution to cancer progression are largely unexplored, the antitumoral action of Si306 could be limited to particular glioblastoma subtypes or stages. In the next future, a deeper knowledge of glioblastoma biology with particular attention to the phenotype and the origin of glioblastoma-associated cells might confirm the real potential of the proposed therapeutic strategy.

Disclosure of Potential Conflicts of Interest
M. Botti and G. Vignaroli have ownership interest in a patent application for Si306 and related compounds. No potential conflicts were disclosed by the other authors.

Authors’ Contributions
Conception and design: G. Vignaroli, S. Schenone, A. Angelucci
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Mattei

Study supervision: A. Angelucci

References


# Molecular Cancer Therapeutics

## Suppression of SRC Signaling Is Effective in Reducing Synergy between Glioblastoma and Stromal Cells

Alessia Calgani, Giulia Vignaroli, Claudio Zamperini, et al.


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