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SRC Family Kinase Inhibition Through a New Pyrazolo[3,4-*d*]Pyrimidine Derivative as a Feasible Approach for Glioblastoma Treatment

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ABSTRACT

Glioblastoma (GB) is the most common and aggressive primary tumor of the central nervous system. The current standard of care for GB consists of surgical resection, followed by radiotherapy combined with temozolomide chemotherapy. However, despite this intensive treatment, the prognosis remains extremely poor. Therefore, more effective therapies are urgently required. Recent studies indicate that SRC family kinases (SFKs) could represent promising molecular targets for GB therapy. Here, we challenged four GB cell lines with a new selective pyrazolo[3,4-*d*]pyrimidine derivative SFK inhibitor, called SI221. This compound exerted a significant cytotoxic effect on GB cells, without significantly affecting non-tumor cells (primary human skin fibroblasts), as evaluated by MTS assay. We also observed that SI221 was more effective than the well-known SFK inhibitor PP2 in GB cells. Notably, despite the high intrinsic resistance to apoptosis of GB cells, SI221 was able to induce this cell death process in all the GB cell lines, as observed through cytofluorimetric analysis and caspase-3 assay. SI221 also exerted a long-term inhibition of GB cell growth and was able to reduce GB cell migration, as shown by clonogenic assay and scratch test, respectively. Moreover, through in vitro pharmacokinetic assays, SI221 proved to have a high metabolic stability and a good potential to cross the blood brain barrier, which is an essential requirement for a drug intended to treat brain tumors. Therefore, despite the need of developing strategies to improve SI221 solubility, our results suggest a potential application of this selective SFK inhibitor in GB therapy. J. Cell. Biochem. 116: 856–863, 2015.

KEY WORDS: SRC FAMILY INHIBITION; GLIOBLASTOMA; APOPTOSIS; CDK1; BLOOD BRAIN BARRIER

Elisa Ceccherini and Paola Indovina contributed equally to this work.

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Glioblastoma (GB) is the most common and aggressive primary tumor of the central nervous system (CNS). According to the World Health Organization classification, GB is a grade IV astrocytoma [Louis et al., 2007] and can develop either de novo or through malignant progression of lower-grade astrocytomas. Histologically, GB is characterized by a high degree of proliferation, angiogenesis, necrosis, and invasiveness into the surrounding brain tissue [Omuro and DeAngelis, 2013].

The current standard of care for GB consists of surgical resection, followed by radiotherapy combined with temozolomide (TMZ) chemotherapy and, then, by an adjuvant course of chemotherapy with TMZ [Stupp et al., 2005]. However, despite this intensive therapy, the prognosis remains extremely poor, with a median survival ranging between 12 and 15 months [Wen and Kesari, 2008]. Indeed, following the first-line treatment, nearly all the patients with GB experience recurrence and the available salvage therapies have not been proven to improve survival [Omuro and DeAngelis, 2013]. Therefore, more effective therapies are urgently needed.

A thorough understanding of the molecular mechanisms underlying GB development, progression, and resistance to therapy is fundamental to identify new possible therapeutic targets.

Key molecular targets in cancer therapy are the SRC family kinases (SFKs), including non-receptor tyrosine kinases such as SRC, YES, FYN, LYN, HCK, BLK, FGR, and LCK. These kinases are hyperactivated in many cancer types and are involved in the regulation of cell proliferation, survival, invasion, and angiogenesis, thus playing a crucial role in tumor development and progression [Yeatman, 2004]. Recently, SFKs have been shown to be frequently hyperactivated or overexpressed also in GB cell lines and tumor samples [Stettner et al., 2005; Du et al., 2009; Lu et al., 2009]. Interestingly, SFK inhibition by dasatinib, a multitargeted ATP-competitive tyrosine kinase inhibitor, reduced GB cell growth, viability and migration both in vitro and in mouse models [Du et al., 2009; Lu et al., 2009; Milano et al., 2009]. Furthermore, dasatinib increased the efficacy of TMZ in GB cells [Milano et al., 2009] and trials on dasatinib in combination with radiotherapy and TMZ are ongoing [Wick et al., 2011]. Therefore, all these data indicate that SFKs could represent promising molecular targets for GB therapy.

We recently synthesized new pyrazolo[3,4-*d*]pyrimidine derivative ATP-competitive SFK inhibitors, which showed excellent antiproliferative and proapoptotic properties in several tumor types [Schenone et al., 2010; Cozzi et al., 2012; Indovina et al., 2012]. Here, we focused on the SI221 molecule, whose selectivity towards the SFK members was previously verified through a kinase activity screening assay (unpublished data from our group). Our results showed that this compound was able to reduce significantly cell viability and migration in four GB cell lines, without affecting non-tumor cells. Moreover, through in vitro pharmacokinetic assays, SI221 proved to have a high metabolic stability and a good potential to cross the blood brain barrier (BBB), which is an essential requirement for a drug intended to treat brain tumors. Therefore, although this study also pointed to the need of developing strategies to improve SI221 solubility, our results suggest a potential application of this selective SFK inhibitor in GB therapy.

MATERIALS AND METHODS

CELL LINES AND CULTURE CONDITIONS

The human GB cell lines U-373MG (European Collection of Cell Cultures) and PRT-HU2 [Bacciocchi et al., 1992] were kindly provided by Prof. Sergio Comincini (University of Pavia, Italy), whereas U-87MG and T98G (American Type Culture Collection) were a kind gift of Prof. Annamaria Cimini (University of L'Aquila, Italy). Primary human skin fibroblasts [Pianigiani et al., 2010] were kindly provided by Michele Fimiani, Giancarlo Mariotti, and Stefania Mei (University of Siena, Italy). Cells were grown in DMEM containing 10% fetal bovine serum, 0.5% penicillin-streptomycin, and 1% glutamine at 37°C in a humidified atmosphere containing 5% CO_2. Cells were maintained at low passage numbers.

CELL TREATMENT AND MTS ASSAY

The pyrazolo[3,4-*d*]pyrimidine derivative SFK inhibitor SI221 was synthesized as reported by Radi et al. (2013). This SFK inhibitor and the SFK inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine, Calbiochem) were dissolved in DMSO (Sigma-Aldrich), to make 20 mM stock solutions, and then diluted in culture medium before use.

GB cell lines and fibroblasts were seeded in 96-well culture plates and, after 24 h, treated with the SFK inhibitors, at concentrations ranging from 1 to 25 μM, or with DMSO (at the maximum amount used to deliver the SFK inhibitors), as a control. DMSO had no toxic effect on the cell lines (data not shown). Cell viability was evaluated 72 h after treatment by MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega), following the manufacturer's instructions. To evaluate the half maximal inhibitory concentration (IC_{50}) values, the GraphPad Prism Software, version 5.01 for Windows was used.

WESTERN BLOTTING ANALYSIS

Cells were collected and suspended in lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 2 mM Na_3VO_4, 0.3 mM PMSF, 1% NP40, 50 mM EDTA pH 8, the phosphatase inhibitor cocktail 3 (Sigma-Aldrich) and a protease inhibitor cocktail (Roche). Fifty μg of proteins per sample were subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes (Bio-Rad). The membranes were then probed overnight at 4°C with primary antibodies against: phospho-SRC family (this antibody detects human SRC when phosphorylated at Tyr419. It cross-reacts with the other SFK members when phosphorylated at equivalent sites), total SRC, phospho-cyclin-dependent kinase 1 (CDK1) Tyr15, total CDK1 (Cell Signaling) and β-actin (Sigma-Aldrich). After 1 h of incubation at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibodies, immunoreactive bands were detected using the Supersignal West Pico Chemiluminescent Substrate (Pierce) and autoradiography films (Pierce).

CLONOGENIC ASSAY

U-373MG, U-87MG, T98G, and PRT-HU2 cells were seeded in 60 mm culture plates at a density of 3000 cells/plate, at least in duplicate, and allowed to attach overnight. The GB cells were then incubated

for 14 days with SI221, at its IC_{50} values, or DMSO, renewing the medium every four days. Cells were then fixed with methanol and stained with crystal violet.

CYTOFLUORIMETRIC ANALYSIS

U-373MG, U-87MG, T98G, and PRT-HU2 cells were plated in 10 mm dishes and treated the next day with SI221 at its IC_{50} values or DMSO. After 72 h of treatment, cells were collected, washed with PBS and then fixed in 70% ice-cold ethanol at −20°C for 20 min. Nuclei were stained with 5 μg/mL propidium iodide plus 20 μg/mL RNase (Sigma-Aldrich) at 4°C overnight in the dark and then analyzed with a BD FACS Calibur Flow Cytometer (Becton Dickinson). Data were analyzed using the BD CellQuest Pro Software (Becton Dickinson).

CASPASE-3 ACTIVITY

Caspase-3 enzymatic activity was evaluated 72 h after treatment of U-373MG, U-87MG, T98G, PRT-HU2 cells with either SI221, at its IC_{50} values, or DMSO. We used the Colorimetric Caspase-3 Assay Kit (Sigma-Aldrich), according to the manufacturer's instructions. During the assay, activated caspase-3 cleaves the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide, releasing the p-nitroaniline (pNA) moiety, the absorbance of which is measured by spectrophotometry at 405 nm through a microplate reader. pNA pmols were determined by a calibration curve prepared with defined standard solutions. Caspase-3 activity is expressed as pmol pNA/μg protein × h.

MIGRATION ASSAY (SCRATCH TEST)

U-373MG, U-87MG, T98G, PRT-HU2 cells were grown to confluence on culture dishes and a scratch was made in the cell monolayer using a sterile pipette tip. Cells were washed with PBS and then complete medium containing either SI221 at its IC_{50} values (as previously evaluated 72 h after treatment) or DMSO was added. Cells were photographed at 0 and 24 h after treatment and gap widths were measured through the ImageJ software.

To verify that the number of viable cells was not affected by SI221 treatment at the culture time used for the migration assay, we performed parallel experiments in which cells were identically treated and then assessed for cell viability by trypan blue staining.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION (ADME) ASSAYS

Parallel artificial membrane permeability assay-BBB (PAMPA-BBB). SI221 capability to passively cross the BBB was predicted through a PAMPA-BBB, using a well-standardized protocol [Radi et al., 2011a]. The analysis of the samples collected from both the receiver and donor compartments was performed using liquid chromatography (LC) with UV detection at 280 nm, following a previously optimized method [Radi et al., 2011b]. Three independent experiments were performed. Apparent permeability (Papp), representing the rate of passive penetration, and membrane retention (MR), expressed as the percentage of compound unable to reach the receiver compartment, were calculated as previously described [Vignaroli et al., 2013].

Microsomal stability assay. SI221 was incubated with pooled human liver microsomes (HLM) using a previously validated method [Radi et al., 2011b] and the parent drug and metabolites were subsequently identified and quantified by LC-UV-mass spectrometry (MS) as previously described [Zamperini et al., 2014].

Kinetic solubility assay. SI221 was dissolved in DMSO to make a 2 mM solution, which was then diluted in water to a total volume of 1 mL to reach the DMSO final concentration of 5% (v/v). The sample was shacked in a shaker bath at room temperature for 24 h and then the suspension was filtered through a 0.45 μm nylon filter (Acrodisc). The solubilized compound was determined by LC-tandem mass spectrometry (MS/MS), according to a previously described method [Radi et al., 2012]. The determination was performed in triplicate.

STATISTICAL ANALYSIS

Statistical analysis was carried out using GraphPad Prism Software, version 5.01 for Windows. Statistically significant differences among the means of multiple matched groups were evaluated by one-way repeated measures Anova with Dunnet post-test, to compare all data vs. control (MTS assay). Statistically significant differences between the means of two matched groups (caspase-3 assay) were evaluated using paired Student t test. $P < 0.05$ was considered to be statistically significant.

RESULTS

ANTIPROLIFERATIVE EFFECT OF SFK INHIBITION IN GB CELL LINES

After evaluating the antiproliferative activity of a panel of pyrazolo[3,4-*d*]pyrimidine SFK inhibitors in 4 GB cell lines (U-373MG, U-87MG, T98G, PRT-HU2) and in non-cancer cells (primary human skin fibroblasts) by MTS assay (data not shown), we focused on the SI221 compound (Fig. 1A) because of its effectiveness on GB cells and very low toxicity on normal fibroblasts. Indeed, this molecule significantly reduced cell viability in all the GB cell lines and had almost no toxic effect on fibroblasts, slightly affecting their viability only at the highest concentrations used (Fig. 1B). SI221 IC_{50} values calculated for the 4 GB cell lines 72 h after treatment are reported in Fig. 1C. Since SI221 was nearly ineffective on fibroblasts, the IC_{50} value for these non-tumor cells was not determinable at the range of concentrations used.

We also compared the antiproliferative efficacy of SI221 with that of the well-known SFK inhibitor PP2 [Hanke et al., 1996]. We observed that PP2 was nearly ineffective on U-373MG, U-87MG, and PRT-HU2 cell lines at the concentrations used and had an efficacy lower than SI221 on T98G (Fig. 1B and C).

To verify whether SI221 was indeed able to inhibit SFKs in GB cells, we evaluated the expression of the active phosphorylated form of SFKs and total SRC in the GB cell lines after 72 h of treatment with SI221 at its IC_{50} values. As expected, our molecule caused a sharp decrease in phospho-SFKs in all the GB cell lines (Fig. 1D). Moreover, we analyzed the expression of phospho-SFKs also in fibroblasts. The active form of SFKs was undetectable in these non-tumor cells on which SI221 was nearly ineffective, thus confirming the specificity of SI221 towards active SFKs (Fig. 1E).

We then analyzed SI221 ability to exert a long-term inhibition of GB cell growth by clonogenic assay and observed that SI221

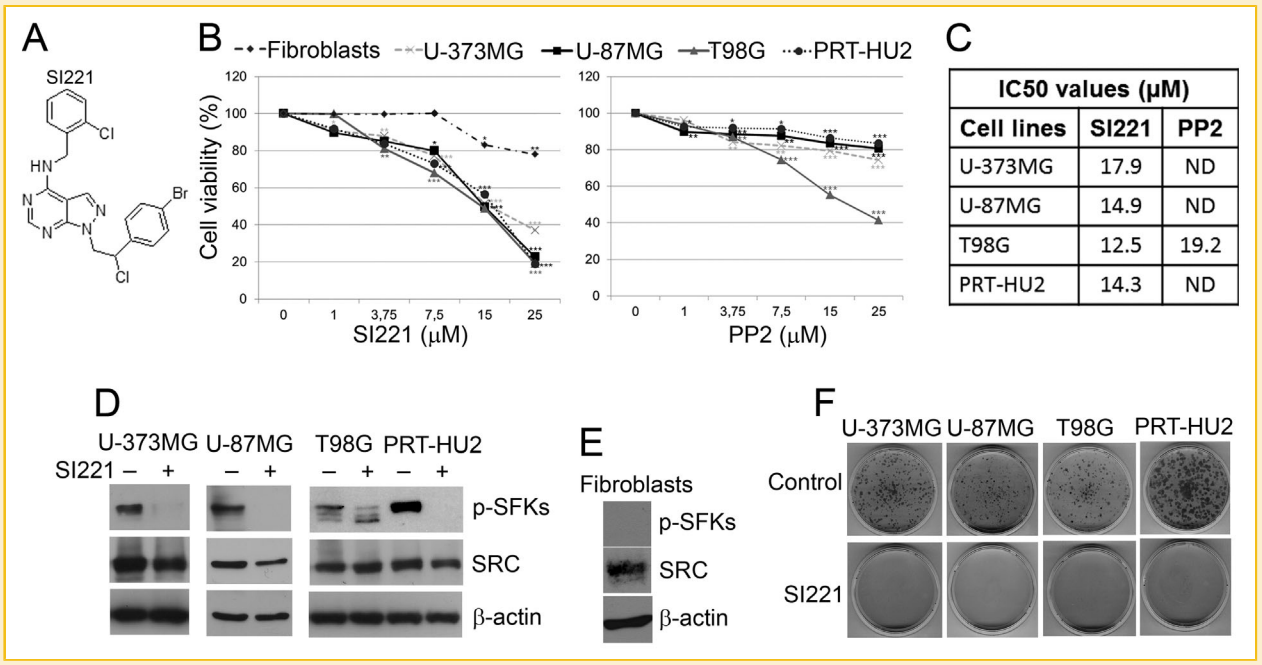


Fig. 1. Effect of SFK inhibition on the viability of GB cell lines. (A) Structure of the pyrazolo[3,4-*d*]pyrimidine derivative SFK inhibitor SI221. (B) Cell viability analysis by MTS assay in primary human skin fibroblasts, U-373MG, U-87MG, T98G, and PRT-HU2 cell lines 72 h after treatment with either SI221 or PP2 at the indicated concentrations. Results are reported as means of three independent experiments, each conducted in triplicate, and expressed as percentages of cell viability calculated with respect to control cells treated with DMSO alone. The absorbance values of treated and control samples were subjected to one-way Anova with Dunnett post-test. Statistically significant differences between treated and control cells are indicated with *: significant ($P < 0.05$), **: very significant ($P < 0.01$), and ***: extremely significant ($P < 0.001$). (C) Table reporting the IC_{50} values of SI221 and PP2 on GB cell lines. PP2 was nearly ineffective on U-373MG, U-87MG, and PRT-HU2 cells at the concentrations used and, thus, the IC_{50} values were not determined (ND). (D) Representative western blotting analyses of phospho-SFKs Tyr419 (p-SFKs) and total SRC in GB cell lines treated with either SI221, at its IC_{50} values, or DMSO, as a control, for 72 h. The anti-β-actin antibody was used for a loading control. (E) Western blotting analysis of phospho-SFKs and total SRC expression in primary human skin fibroblasts. An anti-β-actin antibody was used for a loading control. (F) Representative dishes from clonogenic assays showing the long-term effect of SI221 treatment on GB cell lines. Control cells were treated with DMSO alone.

treatment dramatically inhibited colony formation in all the GB cell lines (Fig. 1F).

SFK INHIBITION INDUCES APOPTOSIS AND G2/M PHASE ARREST IN GB CELL LINES

To assess whether the antiproliferative effect of SFK inhibition in GB cells was due to cell death or to cell cycle arrest, we analyzed by FACS the cell cycle profile of U-373MG, U-87MG, T98G, PRT-HU2 cell lines 72 h after treatment with SI221 at its IC_{50} values. We observed a marked increase in the sub-G1 peak in all the GB cell lines after treatment with SI221, which could be indicative of apoptosis (Fig. 2A).

To confirm the ability of the SFK inhibitor SI221 to induce apoptosis in GB cell lines, we analyzed the caspase-3 enzymatic activity. We observed, indeed, a significant increase in caspase-3 activity in all the GB cell lines 72 h after treatment with SI221 at its IC_{50} values (Fig. 2B).

Moreover, SI221 treatment induced an accumulation in G2/M phase of U-373MG cells (Fig. 2A). Consistently, in this cell line we also observed a sharp increase in the inactive phosphorylated form of CDK1, which is the main inducer of entry into mitosis (Fig. 2C).

SFK INHIBITION DECREASES GB CELL MIGRATION

To examine the effect of SFK inhibition on GB cell migration, we performed a scratch assay. In particular, after making a scratch in U-373MG, U-87MG, T98G, PRT-HU2 cell monolayers, we treated these cells with SI221 at its IC_{50} values (as previously calculated 72 h after treatment) and evaluated wound closure 24 h after treatment. We observed that SI221 treatment dramatically decreased GB cell ability to migrate into the scratch (Fig. 3).

The number of viable cells was not significantly affected by SI221 treatment at the culture time used for the scratch assay, as verified by trypan blue staining of U-373MG, U-87MG, T98G, and PRT-HU2 cells identically treated in parallel experiments (data not shown).

SI221 ADME PROPERTIES

SI221 was previously subjected to in vitro pharmacokinetic assays in order to preliminarily estimate the overall ADME properties of this candidate drug [Radi et al., 2013]. In the present study, we aimed to predict SI221 ability to passively cross the BBB, since the low permeability of this barrier represents a major hurdle in developing drugs for brain tumor treatment [Siegal, 2013]. To this purpose, we performed a PAMPA-BBB assay and found that SI221 had a good capability to cross the BBB, with an extremely low percentage of membrane retention (Table I).

Our previous ADME studies showed a high SI221 metabolic stability [Radi et al., 2013]. Indeed, through a HLM stability assay, we observed a high percentage of unmodified parent drug (approximately

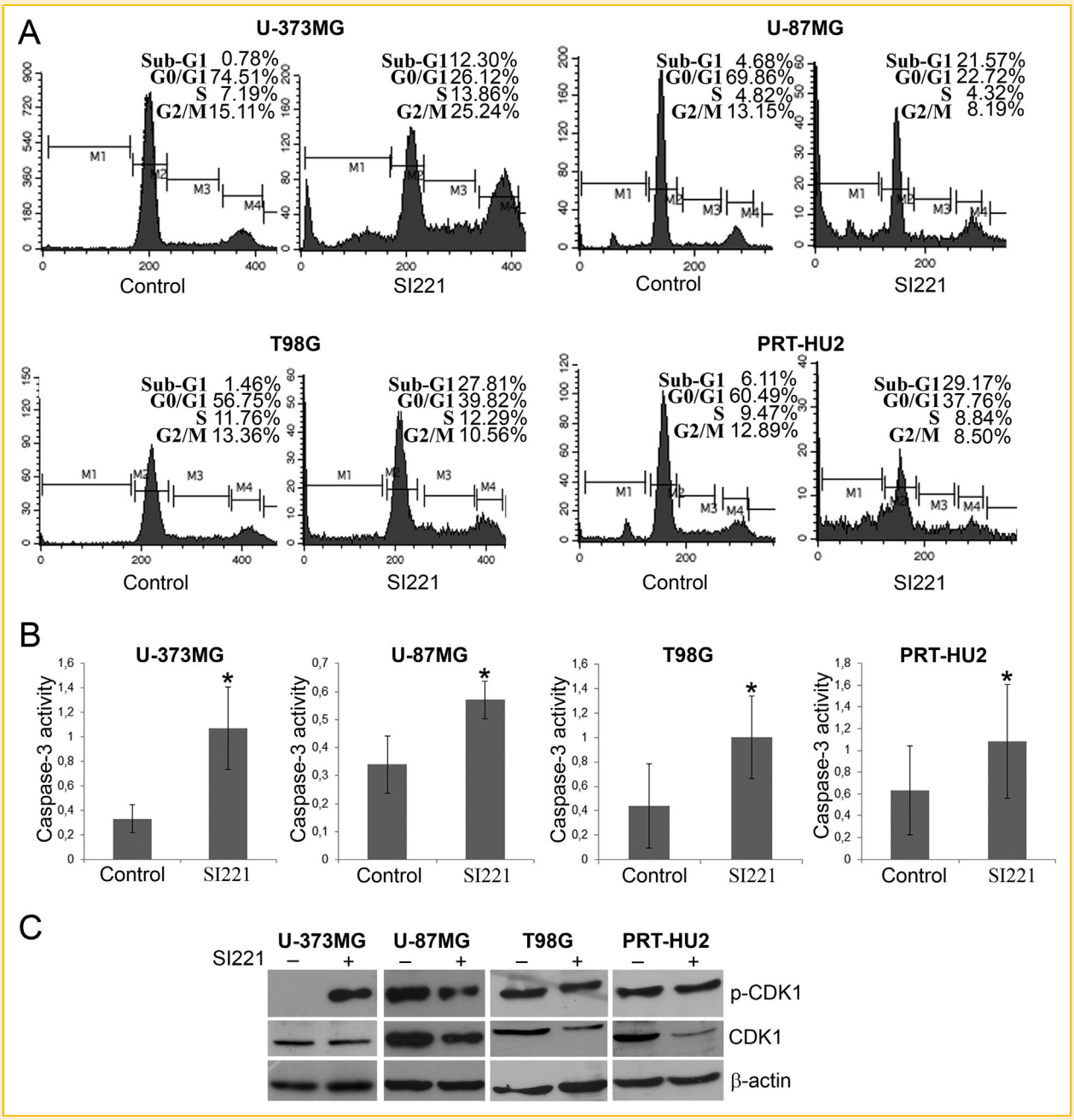


Fig. 2. Apoptosis induction and G2/M phase arrest in GB cell lines treated with SI221. (A) Representative cell cycle analyses by FACS in U-373MG, U-87MG, T98G, and PRT-HU2 cells 72 h after treatment with either SI221, at its IC_{50} values, or DMSO, as a control. (B) Histograms reporting caspase-3 activity in GB cell lines after 72 h of treatment with either SI221, at its IC_{50} values, or DMSO, as a control. Caspase-3 activity is expressed as pmol pNA/μg protein × h. The reported values represent the means and standard deviations of three experiments. Statistically significant differences between treated and control cells were evaluated by Student *t*-test and indicated with *: significant ($P < 0.05$). (C) Representative western blotting analyses of phospho-CDK1 Tyr15 (p-CDK1) and total CDK1 in GB cells treated with either SI221, at its IC_{50} values, or DMSO, as a control. The anti-β-actin antibody was used for a loading control.

95%). Here, we also quantified and characterized by LC-UV-MS the SI221 metabolites resulting from HLM metabolism. We identified two metabolites (one derived from an oxidative dechlorination reaction and the other one from an N-dealkylation reaction), which, as expected, were present in low percentages (Table I), further confirming the high stability of the compound.

Our previous data also showed a poor thermodynamic solubility of SI221 [Radi et al., 2013]. In the present work, we evaluated SI221 solubility by kinetic assessment, which better mirrors the conditions of the in vitro cell assays because the compound was pre-dissolved in DMSO. Although the presence of the organic solvent increased SI221 solubility by one order of magnitude

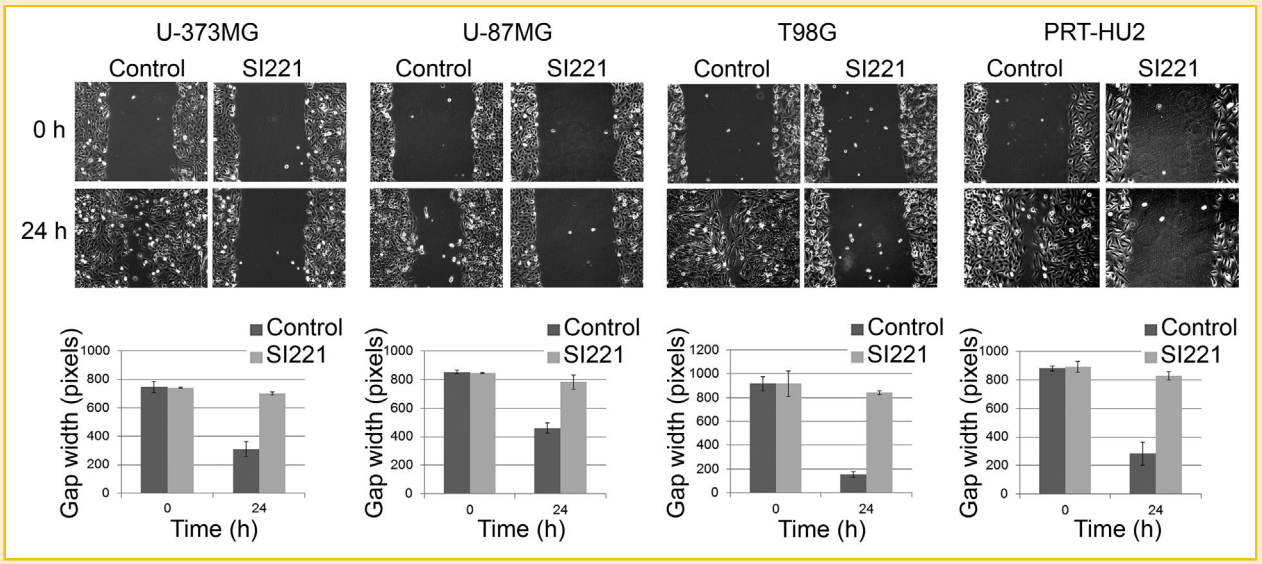


Fig. 3. Effect of SI221 on GB cell migration. Representative micrographs of scratch assays performed on U-373MG, U-87MG, T98G, and PRT-HU2 cells are shown. The micrographs were taken at 0 and 24 h after treatment with either SI221, at its 72-h IC_{50} values, or DMSO, as a control. The histograms report the means and standard deviations of the gap widths measured through the ImageJ software.

compared to the thermodynamic solubility, the compound showed, however, a poor solubility (Table I).

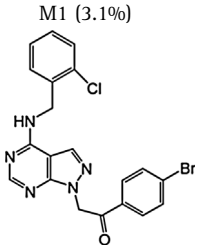
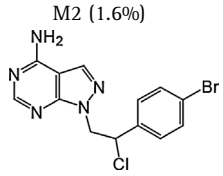
DISCUSSION

Although in recent years significant advances in the treatment and molecular understanding of GB have been achieved, this cancer remains universally fatal. Therefore, more rational therapies targeting the molecular alterations underlying GB are urgently required.

Different studies indicate that SFKs could represent promising molecular targets for GB therapy. Indeed, SFKs are frequently hyperactivated or overexpressed in GB cell lines and tumor samples, suggesting that they act as drivers of tumorigenesis [Stettner et al., 2005; Du et al., 2009; Lu et al., 2009]. Consistently, SFK inhibition through the multitargeted tyrosine kinase inhibitor dasatinib proved to have antitumor activity in GB cells both in vitro and in mouse models [Du et al., 2009; Lu et al., 2009; Milano et al., 2009].

In the present study, we challenged four GB cell lines with new pyrazolo[3,4-*d*]pyrimidine derivative SFK inhibitors, whose efficacy was previously demonstrated in several tumor types [Schenone et al., 2010; Cozzi et al., 2012; Indovina et al., 2012]. We focused in particular on the SI221 molecule, whose selectivity towards the SFK members was previously observed through a kinase activity screening assay (unpublished data from our group). Our results showed that SI221 exerted a significant antiproliferative activity on all the GB cell lines, without significantly affecting non-tumor cells (primary human

TABLE I. SI221 ADME Properties

PAMPA-BBB				
Papp ($\times 10^{-6}$ cm/s)[a]	MR (%)[b]	Metabolites[c]		Kinetic solubility (μg/mL)
13.23 ± 0.3	7.2	M1 (3.1%) 	M2 (1.6%) 	0.11 ± 0.02

[a]Apparent permeability (Papp), representing the rate of passive penetration, was calculated as previously described [Vignaroli et al., 2013].
[b]Membrane retention (MR), expressed as the percentage of compound unable to reach the acceptor compartment, was calculated as previously reported [Vignaroli et al., 2013].
[c]SI221 metabolites derived from human liver microsomal metabolism. M1 is a ketone derivative resulting from an oxidative dechlorination reaction; M2 resulted from an N-dealkylation reaction.

skin fibroblasts). Moreover, SI221 also exerted a long-term inhibition of GB cell growth, as shown by clonogenic assay.

To verify that SI221 effectiveness was indeed dependent on SFK activation status, we analyzed the expression of the active phosphorylated form of SFKs in GB cell lines and fibroblasts. We observed that all the GB cell lines, in which SI221 was effective, expressed active phospho-SFKs. Moreover, as expected, SI221 treatment strongly decreased phospho-SFK levels. Conversely, the active form of SFKs was undetectable in fibroblasts, in which SI221 was nearly ineffective, thus confirming the specificity of SI221 towards active SFKs.

Considering the crucial role of SFKs in cancer development and progression [Yeatman, 2004], several small molecules targeting SFKs have been designed [Schenone et al., 2010]. Here, we compared the efficacy of SI221 with that of the well-known SFK inhibitor PP2. We observed that PP2 was nearly ineffective on U-373MG, U-87MG, and PRT-HU2 cell lines at the concentrations used and had an efficacy lower than SI221 on T98G. These results are consistent with our previous data showing that PP2 was less effective than our compounds in different cancer types [Schenone et al., 2004; Carraro et al., 2006; Spreafico et al., 2008; Cozzi et al., 2012].

We then assessed whether the SI221-induced cytotoxic effect on GB cells was due to cell cycle arrest or to cell death. We observed that SI221 induced apoptosis in all the GB cell lines. The observed induction of apoptosis following SFK inhibition through SI221 is consistent with the SFK role in preventing this cell death process [Karni et al., 1999; Cursi et al., 2006; Laplante et al., 2006; Lue et al., 2007; Yamaguchi et al., 2008]. SI221 ability to induce apoptosis in GB cell lines is particularly important, considering the GB cell intrinsic resistance to apoptosis, which represents a key mechanism whereby GB cells evade cell death induced by anticancer treatments [Ziegler et al., 2008].

Consistent with the established role of SFKs in G2/M transition [Roche et al., 1995; Moasser et al., 1999], we also observed an accumulation in G2/M phase of U-373MG cells following SI221 treatment. We previously showed that our SFK inhibitors induced an arrest in G2/M also in Burkitt lymphoma cell lines through inactivation of CDK1, the main inducer of entry into mitosis [Cozzi et al., 2012]. Consistently, we observed an SFK inhibitor-induced inactivation of CDK1 also in U-373MG cell line, owing to CDK1 phosphorylation on Tyr15.

We also analyzed the effect of SI221 on GB cell migration and found a decrease in this process in all the GB cell lines treated with this compound. This result is consistent with the established role of SFKs in promoting cell motility [Yeatman, 2004; Yadav and Denning, 2011].

The observed antitumor activity of SI221 in GB cells prompted us to assess SI221 drug-like properties through ADME assays. In particular, since the inability to cross the BBB underlies the low efficacy of different therapeutic agents against brain tumors [Siegal, 2013], we analyzed SI221 ability to passively cross the BBB through a PAMPA-BBB assay, which is one of the most powerful and versatile screening tools for CNS-targeted drug discovery [Di et al., 2003]. SI221 showed a good potential to cross the BBB, probably owing to its high lipophilicity.

Moreover, we confirmed the previously observed high metabolic stability of SI221 [Radi et al., 2013] by also quantifying and characterizing the SI221 metabolites derived from HLM metabolism. Indeed, we identified only low percentages of two metabolites, which resulted from two cytochrome P450 (CYP)-dependent metabolic reactions, namely an oxidative dechlorination and an N-dealkylation, as we also previously observed with similar pyrazolo[3,4-*d*] pyrimidine derivatives [Zamperini et al., 2014].

Athough our previous PAMPA data predicted a moderate intestinal absorption (Papp: 8.78×10^{-6} cm/s) of SI221 [Radi et al., 2013], this molecule could still have high in vivo efficacy after per os administration, as occurred previously with similar compounds having comparable predicted intestinal membrane permeability [Manetti et al., 2007].

We also evaluated SI221 kinetic solubility. Although the presence of DMSO, which better mimics the cell assay conditions, increased SI221 solubility with respect to that previously evaluated by thermodynamic assessment [Radi et al., 2013], the compound showed, however, a poor solubility. Since the low solubility of SI221 limits its potential therapeutic utility, studies on SI221 prodrugs are ongoing in order to improve the solubility of the compound, without negatively affecting its ability to cross the BBB. Indeed, we previously developed a prodrug strategy that showed to improve the aqueous solubility of similar pyrazolo[3,4-*d*]pyrimidine compounds [Vignaroli et al., 2013].

In conclusion, our results showed that the selective SFK inhibitor SI221 exerted significant antiproliferative and proapoptotic effects on GB cell lines, without significantly affecting non-tumor cells. Importantly, SI221 also caused a decrease in GB cell migration. Moreover, this compound proved to have a high metabolic stability and a good potential to cross the BBB, which is a fundamental requisite for a drug designed for brain cancer treatment. Therefore, despite the need to develop strategies to improve SI221 solubility, our results suggest a potential application of this SFK inhibitor in GB treatment.

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