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Platelet-derived Growth Factor (PDGF) Autocrine Signaling Regulates Survival and Mitogenic Pathways in Glioblastoma Cells: Evidence That the Novel PDGF-C and PDGF-D Ligands May Play a Role in the Development of Brain Tumors

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ABSTRACT

Glioblastoma multiforme, the most common form of malignant brain tumor, is resistant to all forms of therapy and causes death within 9–12 months of diagnosis. Glioblastomas are known to contain numerous genetic and physiological alterations affecting cell survival and proliferation; one of the most common alterations being platelet-derived growth factor (PDGF) autocrine signaling characterized by coexpression of PDGF and its receptor. The PDGF family consists of four members, PDGF-A, -B, -C, and -D, that signal through the α and β PDGF receptor (PDGFR) tyrosine kinases. Numerous studies have demonstrated expression of PDGF-A, PDGF-B, and the PDGFRs in gliomablastomas, but such studies have not been conducted for the newly identified PDGF-C and -D. Therefore, we examined the expression of all PDGF ligands and receptors in 11 glioma cell lines and 5 primary glioblastoma tumor tissues by quantitative reverse transcription-PCR. Expression of PDGF/PDGFR pairs that are known to functionally interact were identified in all of the samples. Interestingly, PDGF-C expression was ubiquitous in brain tumor cells and tissues but was very low or absent in normal adult and fetal brain. PDGF-D was expressed in 10 of 11 brain tumor cell lines and 3 of 5 primary brain tumor samples. As a strategy for blocking PDGFR signaling, CT52923, a potent selective small molecule piperazinyl quinazoline kinase inhibitor of the PDGFR, was identified. In model systems using NIH/3T3 cells, CT52923 blocked PDGF autocrine-mediated phosphorylation of PDGFR, Akt, and mitogen-activated protein kinase (MAPK), while having no effect on v-*fms* or V12-*ras*-mediated Akt or extracellular signal-regulated protein kinase (Erk) phosphorylation. More importantly, p.o. administration of CT52923 to nude mice caused a significant 61% reduction ($P < 0.006$) in tumor growth of NIH/3T3 cells transformed by PDGF, whereas tumor formation by cells expressing v-*fms* was unaffected. We next characterized PDGF autocrine signaling in five glioblastoma cell lines. In all of the cases, PDGF autocrine signaling was evident because treatment with 1–10 μM CT52923 inhibited PDGFR autophosphorylation when present at a detectable level and blocked downstream Akt and/or Erk phosphorylation. The functional significance of PDGF autocrine signaling in these cells was demonstrated by the fact that the CT52923 inhibited soft agar colony formation, and, when given p.o. to nude mice, it effectively reduced tumor formation by 44% ($P < 0.0019$) after s.c. injection of C6 glioblastoma cells. This study of glioblastoma cells and primary tissues is the first to implicate PDGF-C and -D in brain tumor formation and confirms the existence of autocrine signaling by PDGF-A and -B. More importantly, treatment with the PDGFR antagonist CT52923 inhibited survival and/or mitogenic pathways in all of the glioblastoma cell lines tested and prevented glioma formation in a nude mouse xenograft model. Together these findings demonstrate the potential therapeutic utility of this class of compounds for the treatment of glioblastoma.

INTRODUCTION

Approximately 17,500 primary central nervous system tumors occur annually in the United States. The majority of these are malignant gliomas that are among the most aggressive and highly invasive of human cancers (1). After diagnosis of glioblastoma multiforme, the median survival time of 9–12 months has remained unchanged despite multiple clinical trials designed to optimize radiation and/or chemotherapy (2). These cytotoxic treatment strategies kill cells by damaging DNA or by disrupting pathways required for cell division but do not target the underlying oncogenic defects in signal transduction that are now being more fully defined. These genetic alterations affecting genomic stability, cell cycle progression, and cell survival pathways begin to be observed in low-grade astrocytomas and become more frequent in the more advanced anaplastic astrocytomas and glioblastoma multiforme (3). One of the most common defects observed in brain tumors at all stages is the establishment of a putative PDGF[3]-autocrine loop attributable to the coexpression of PDGF and its receptor (4–6). The importance of PDGF signaling in brain tumors is underscored by the fact that retrovirus-mediated expression of PDGF-B in the brain of neonatal mice results in the formation of astrocytomas (7).

The PDGF family consists of four members, PDGF-A, -B, -C, and -D, which signal through the α and β PDGF-receptor (PDGFR) tyrosine kinases. Biosynthesis and processing of the PDGFs results in the formation of full-length disulfide-linked homodimers PDGF-AA, BB, CC, and DD and the heterodimer PDGF-AB (reviewed in Ref. 8). Although PDGF-AA, BB, and AB undergo additional processing it is not required for their biological activity. In contrast, PDGF-CC and -DD require proteolytic cleavage for activity (9–12). The PDGF-A and -C chains selectively bind α PDGFR, whereas PDGF-D preferentially binds β PDGFR, and PDGF-B displays similar affinity for both receptors (8–12). Numerous studies have demonstrated expression of PDGF-A, PDGF-B, and the PDGFRs in glioblastomas, but such studies have not been conducted for the newly identified PDGF-C and -D. Here, we demonstrate that these novel PDGFs are routinely expressed in glioma cell lines and in primary glioblastoma tissues along with their cognate PDGFR, which indicates a potential role in the development of brain tumors.

Because of the numerous genetic and physiological alterations observed in human glioma, the relative importance of PDGF signaling is not fully understood. To demonstrate a causative role, reversion of the transformed phenotype of glioblastoma cells or tumor growth has been achieved with antibodies that neutralize PDGF, with dominant negative mutants of PDGF or PDGFR or with the small molecule PDGFR kinase inhibitor STI571 (13–17). Recently, we identified CT52923, a potent selective small molecule antagonist of the PDGFR that effectively inhibits PDGF-induced cell proliferation and migration in cultured cells and the *in vivo* PDGF-mediated response to

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[3] The abbreviations used are: PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; PI3k, phosphatidylinositol 3′-kinase; Cyc, cyclophilin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ECL, enhanced chemiluminescence; RT-PCR, reverse transcription-PCR.

vascular injury in rats (18). Using a panel of five glioblastoma cell lines, we, demonstrate in all cases, that treatment with CT52923 inhibits PDGFR autophosphorylation, blocks the ras/MAPK proliferative pathway or the PI3k/Akt survival pathway and causes a reversion of the transformed phenotype. Oral administration of CT52923 to nude mice in a xenograft tumor model produces a significant inhibition of glial cell solid-tumor growth demonstrating the therapeutic potential of this small molecule PDGFR kinase inhibitor.

MATERIALS AND METHODS

Chemicals. CT52923 was synthesized as described previously (18). Its chemical structure is shown in Fig. 1. A stock solution of 3 mM CT52923 was prepared in DMSO and stored at −20°C. Dilutions for all assays were made before use.

Cells and Reagents. Mouse NIH/3T3 fibroblasts, human glioblastoma-derived A172, T98G, U251, human carcinoma-derived A431, MDA468, and the glial rat C6 tumor cell lines were obtained from American Type Culture Collection. Human glioblastoma-derived SF188 cell line was obtained from Mark Israel (University of California-San Francisco, San Francisco, CA). Human PDGF-AA and -BB were purchased from R&D Systems. Expression vectors MMTneo-PDGF-B, (19) PCV2-*Ras*-V and LTR-2-gpt-v-*Fms* were gifts from Sylvio Gutkind (NIH, Bethesda, MD).

Cell Transfections. Fugene 6 (Roche Molecular Biochemicals) was used to transfect the above expression vectors into NIH/3T3 cells according to the manufacturer's protocol, and cells stably expressing each construct were selected by growing them in 750 μg/ml G418 in DMEM, 10% calf serum, 1% penicillin/streptomycin, and 1% L-glutamine.

Western Blotting. Cells were plated in 10-cm Petri dishes in the absence or presence of the indicated amounts of CT52923 (from a 3-mM stock in DMSO) for 5 h in serum-free medium. Cell lysates were prepared as described earlier (18), and proteins were immunoprecipitated when indicated on Figures 2 and 4, using standard techniques, separated by SDS-PAGE (Novex; 4–20% gradient gels), and transferred onto nitrocellulose. Blots were blocked in 5% milk in TTBS (Tris-buffered saline with 0.02% Tween 20) and immunoblotted with antiphosphotyrosine antibodies, phospho-Akt, phospho-Erk, PY99, or phospho-βPDGFR-PY769, as specified in Figure legends 2, 4, and 5. Anti-phospho-Erk antibody was purchased from UBI; anti-Erk antibody, anti-Akt, anti-phospho-Akt antibodies from New England Biolabs; and anti-βPDGFR-PY769 from UBI. Appropriate horseradish peroxidase-labeled secondary antibodies (Roche Molecular Biochemicals) were used followed by chemiluminescent detection (Amersham). All of the blots were stripped and reblotted with anti-Erk, anti-Akt, or anti-PDGFR antibodies, as noted in Figure legends, to determine total protein amounts loaded.

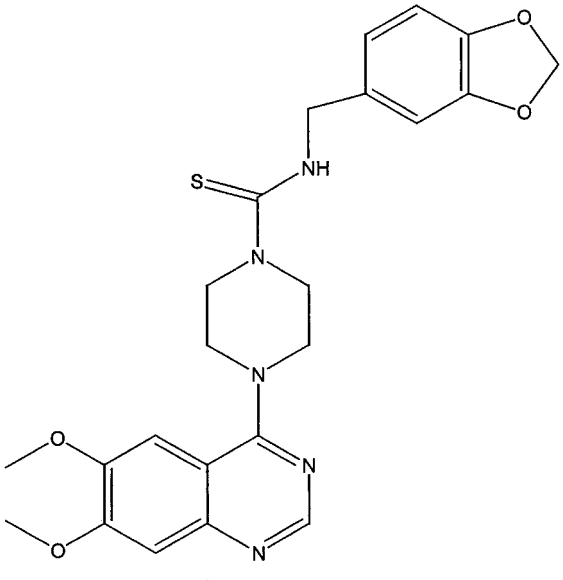

Fig. 1. Chemical structure of CT59223. CT52923 is [(2H-benzo[*d*]1,3-dioxalan-5-ylmethyl)amino][4-(6,7-dimethoxyquinazolin-4-yl)piperazinyl]methane-1-thione. Molecular formula, $C_{23}H_{25}N_5O_4S$; M_r, 467.5.

Measurements of PDGF and PDGFR Expression Using TaqMan PCR. Total RNA from glioblastoma cell lines or frozen human primary glioma tissues were prepared using Tri-reagent (Molecular Research Center, Inc.) and mRNA from RNA of tissues using Fastrack kit (Invitrogen). To quantify PDGF-A, -B, -C, -D and α- and β-PDGFR expression in multiple cell lines and tissues, we used the ABI Prism 7700 Sequence Detection System (PE Biosystems) that uses TaqMan (real-time quantitative PCR) chemistry. cDNA was synthesized in a 50-ul reaction using a TaqMan reverse transcription kit (PE Biosystems). RNAs were normalized using primers, and a TaqMan probe corresponding to human *Cyc* for glioblastoma cell lines and *GAPDH* for primary glioma tissues and expression of each *PDGF* or *PDGFR* gene was measured relative to these housekeeping genes. Because each experimental sample has this endogenous reference standard, correction for the amount of cDNA added to any individual reaction is not needed. Primers and TaqMan probes to PDGF ligands and receptors were designed using the Primers Express software (PE Biosystems) and synthesized by ABI Prism Primers and Probes (PE Biosystems). TaqMan probes were labeled with 6-carboxy-fluorescein phosphoamidite (FAM) at the 5′ end and as quencher 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3′ end (Table 1).

Each 50-μl PCR reaction for any *PDGF* or *PDGFR* gene consisted of 400 nM forward primer, 400 nM reverse primer, 200 nM TaqMan probe, cDNA (200 ng of total RNA from glioblastoma cell lines or 10 ng of polyadenylated RNA equivalents from glioma tissues), and 1× (final concentration) TaqMan Universal Master Mix (PE Biosystems). Identical but separate TaqMan PCR reactions were performed for housekeeping gene controls *Cyc* or *GAPDH* expression for each experimental cell line or tissue, respectively. All of the reactions were done in duplicate. PCR parameters were 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 10 s and at 60°C for 1 min. We used the TaqMan software to calculate a threshold cycle (*Ct*) value for each reaction according to the manufacturer's recommendations. We confirmed that no amplification of fragments occurred from genomic DNA contaminants by performing a cDNA control sample without reverse transcriptase for each sample tested ($Ct = 40$). We normalized *Ct* values by using the following ratio: $[40 - Ct_{PDGF}]:[40 - Ct_{CYC\ or\ GADPH}]$, where Ct_{PDGF} represents the *Ct* values of any PDGF or PDGFR amplicon studied and $Ct_{CYC\ or\ GADPH}$ represents the housekeeping gene *Ct* value for *Cyc* or *GADPH*. For data representation, *Ct* numbers were converted to percentage expression, relative to the sample exhibiting the highest level of expression (=100%).

Colony Soft Agar Assay. Glioma cells (10^5) were cultured in 60-mm dishes in 0.5% low gelling agarose (Sea Plaque) on a base layer of 1% noble agar (Difco) in the presence of indicated amounts of CT52923 (added on day 1 only) or vehicle control in complete medium (according to American Type Culture Collection recommendations) and colonies were scored after 21 days. During the experiment, 0.5 ml of fresh complete medium (without CT52923) was added every 5 days.

Xenograft Tumor Growth in Nude Mice. Athymic nude mice ($n = 15$) received injections s.c. in the left flank region with 2×10^6 PDGF/3T3 or Fms/3T3 cells, or 3×10^6 C6 glial tumor cells. Animals were given CT52923 at 60 mg/kg twice a day by p.o. gavage. For the PDGF/3T3 and Fms/3T3 studies, CT52923 treatment began when tumors reached about 50 mg in size, between day 15 and day 20 and continued for up to 17 days when control tumors reached in average 800 mg. For the C6 glial cells, dosing started on the day of cell injections (day 1) and the study ran for 18 days, when control tumor weights reached in average 1250 mg.

RESULTS

Expression of Novel PDGF-C and -D in Glioblastoma-derived Cell Lines and Primary Human Tumor Tissues. A PDGF autocrine loop involving PDGF-A or -B and the PDGFRs has been shown to exist in many malignant gliomas (20–28) but a role for the newly discovered PDGF-C and -D ligands in brain tumor signaling has not been investigated. Therefore, we used real-time quantitative RT-PCR (TaqMan) to measure the level of expression of all of the PDGFs and PDGFRs in 11 different human glioblastoma cell lines. Interestingly, PDGF-C was expressed in all of the cell lines, as was PDGF-A and

Table 1 *Primers and TaqMan probes (5′ to 3′) to PDGF ligands and receptors*

PDGF ligands and receptors	Forward primer	Reverse primer	TaqMan probe
PDGF-A	TCGATGAGATGGAGGGTCG	ACCCGGACAGAAATCCAGTCT	CGTGGGATGGAAGTGCAGAGGTCTCA
PDGF-B	AGGAGGGAGACTGTGGTAGGG	GAGGACTTTGGGAAATGGAGG	CAGGGAGGCAACACTGCTGTCCACAT
PDGF-C	GGAGCACCATGAGGAGTGTGA	GAGCTGCTGGTGGTGATGC	TGTGTGCAGAGGGAGCACAGGAGGATA
PDGF-D	CGAGGCAGGTCATACCATG	CGCTTGGCATCATCATTG	CCGGAAGTCAAAAGTTGAACCTGGATAGG
αPDGFR	TTCCCTTGGTGGCACCC	GGTACCCACTCTTGATCTTATTGTAGAA	TACCCCGGCATGATTGGTGGATTCTAC
βPDGFR	GCCTTACCACATCCGCTC	TCACACTCTTCCGTCACATTGC	TGCACATCCCCAGTGCCGAGTTAGA

α-PDGFR (Table 2). Because PDGF-C and -A preferentially bind the α-PDGFR, they may cooperate to enhance α-PDGFR autocrine signaling. PDGF-D was widely expressed in all of the cells with the exception of SF763 as was its preferred β-PDGFR (11), which was undetectable in only SF763 and SF767 (Table 2). These findings are of particular interest because the other β PDGFR ligand, PDGF-B, was not expressed in six of the cell lines that expressed PDGF-D and β-PDGFR which suggests a previously unrecognized PDGF autocrine loop. To extend these studies, primary glioblastoma multiforme tissues obtained from five patients were evaluated by a similar TaqMan analysis of PDGF and PDGFR expression (Table 3). As with the glioma cell lines, PDGF-C was expressed with PDGF-A and α-PDGFR in all of the primary glioblastoma tissues but was undetectable in normal fetal and adult brain tissues (Table 3). PDGF-D mRNA was detected in three of five primary tumors, whereas β-PDGFR was expressed in all; a lower level of PDGF-D was observed in normal brain tissue. This study demonstrates that the novel PDGF-C and -D ligands are coexpressed with the PDGFRs in primary glioblastoma tissues and cell lines, which implies a potential role in mediating PDGF autocrine signaling.

CT52923 Selectively Inhibits Proliferation and Survival Pathways and Tumor Growth of NIH/3T3 Cells Expressing a PDGFR Autocrine Loop. On forced expression of PDGF-B, NIH/3T3 cells undergo a loss of contact-inhibited growth and acquire the ability to grow in soft agar and form tumors in nude mice as the result of PDGFR autocrine signaling (29–33). Therefore, we established a model system for PDGFR autocrine signaling by transforming NIH/3T3 cells through stable expression of PDGF-B. For controls, we transformed NIH/3T3 cells with oncogenic V12-*ras* and the viral oncogene v-*fms*, a constitutively active variant of colony-stimulating factor-1 receptor (CSF-1R), which is a closely related member of the PDGFR family (34, 35). These cell lines designated PDGF/3T3, Ras/3T3, and Fms/3T3 were used to evaluate further the selective inhibitory activity of CT52923 toward PDGFR autocrine signaling as compared with signaling mediated by V12-*ras* or v-*fms*. CT52923 is a piperazinyl quinazoline kinase inhibitor (Fig. 1) that is highly selective for PDGFR and c-kit, with little or no activity against the other PDGFR family members nor against a wide range of additional receptor tyrosine kinases or cytoplasmic kinases (18). We previously demonstrated that CT52923 inhibited PDGFR phosphorylation with an IC_{50} of 100–200 nM when Chinese hamster ovary (CHO) cells expressing recombinant β-PDGFR were treated with PDGF-(18). To assess the ability of the compound to block autocrine-mediated PDGFR phosphorylation, serum-starved PDGF/3T3 cells were incu-

Table 2 *Expression of PDGF ligands and receptors mRNAs in various human glioblastoma cell lines*

Quantitative RT-PCR analysis of PDGF ligands and receptors was measured using TaqMan chemistry. The threshold cycle (*Ct*) value of each PDGF product was normalized using the *Ct* value of the *Cyc* housekeeping gene for the same sample, and normalized ratios were converted to percent expression, relative to the sample exhibiting the highest level of expression.

	PDGF-A	PDGF-B	PDGF-C	PDGF-D	αPDGFR	βPDGFR
Glioma cell line						
A172	++[a]	++	++	++	+	++
U87	++	−	++	+	+	++
U251	++	++	++	++	++	+
SF763	++	++	++	−	+	−
SF767	++	++	++	++	+	−
SF188	++	++	++	++	++	++
SW1088	++	−	++	++	++	++
ST11G1	++	−	++	++	++	++
SW783	+	−	+	+	+	+
T98G	+	−	+	++	+	+
U183	+	−	++	+	+	+
Carcinoma cell line						
MDA468	++	++	++	−	−	−

[a] ++, Ct ratio similar to highest expresser (≥80%); +, Ct ratio lower expression than the highest expresser (<80%); −, not expressed.

Table 3 *Expression of PDGF ligands and receptors mRNAs in various human primary glioblastoma multiform tissues*

Quantitative RT-PCR analysis of PDGF ligands and receptors was measured using TaqMan chemistry. The threshold cycle (*Ct*) value of each PDGF product was normalized using the *Ct* value of the *GADPH* housekeeping gene for the same sample, and normalized ratios were converted to percent expression, relative to the sample exhibiting the highest level of expression.

	PDGF-A	PDGF-B	PDGF-C	PDGF-D	αPDGFR	βPDGFR
Glioma tissue						
40L	++	++	++	++	++	++
90A20	++	++	++	++	++	++
64A44	++	++	++	−	++	++
52A28	++	++	+	−	++	+
5A4	+	+	++	++	++	++
Normal tissue						
Normal adult human brain	++	++	−	+	++	++
Normal fetal human brain	++	++	−	+	++	++

[a] ++, similar expression to highest expresser (≥80%); +, lower expression than highest expresser (<80%); −, not expressed.

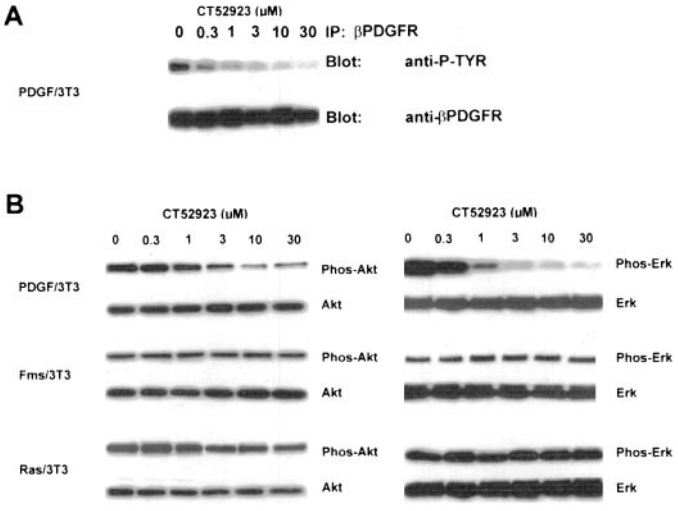

Fig. 2. CT52923 inhibits PDGF-mediated signaling in NIH/3T3 cells. *A*, CT52923 inhibits autocrine PDGFR phosphorylation in PDGF-B-transformed NIH/3T3 cells. PDGF-B-transformed NIH/3T3 cells (*PDGF/3T3*) were incubated with the indicated amount of CT52923 for 5 h in serum-free DMEM. Cell lysates were prepared; and β-PDGFR protein was immunoprecipitated with anti-βPDGFR antibody (sc432) and separated by SDS-PAGE (4–20% gradient gels), transferred onto nitrocellulose, and immunoblotted with antiphosphotyrosine antibody PY99, which was detected with iodinated protein A. The blot was stripped and reblotted with anti-βPDGFR antibody sc432 to control for the amount of β-PDGFR protein loaded. *B*, CT52923 selectively inhibits PDGF-mediated survival and mitogenic pathways. PDGF-B (*PDGF/3T3*)-, v-fms (*Fms/3T3*)-, or V12-ras (*Ras/3T3*)-transformed NIH/3T3 cells were incubated with the indicated amount of CT52923 for 5 h in serum-free DMEM. Lysates were prepared, separated on 4–20% SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with anti-phospho-Akt or anti-phospho-Erk antibodies as well as anti-Akt or anti-Erk antibodies, as indicated; and detection was done using ECL.

bated for 1 h with increasing concentrations of CT52923, and then immunoprecipitated β-PDGFR from cell lysates was evaluated by antiphosphotyrosine Western blot analysis. As shown in Fig. 2*A*, the high level of β-PDGFR phosphorylation detected in PDGF/3T3 cells was readily blocked by CT52923 treatment with an IC_{50} of 300 nM, and nearly complete inhibition was observed at 3 μM. To control for the amount of protein applied to the gels, the blot was also probed with anti-β-PDGFR antibody.

NIH/3T3 cell transformation by PDGF-B, V12-*ras*, or v-*fms* causes constitutive activation of the MAPK and PI3k pathways leading to the phosphorylation of Erk and Akt, respectively (Fig. 2*B*). Therefore, the effects of CT52923 treatment on Erk and Akt phosphorylation was evaluated in PDGF/3T3, Ras/3T3, and Fms/3T3 cells by Western blot analysis of lysates using phospho-specific antibodies. Consistent with its effect on PDGFR autophosphorylation in PDGF/3T3 cells, CT52923 inhibited Erk and Akt phosphorylation at concentrations above 300 nM (Fig. 2*B*). In contrast, treatment with CT52923 at concentrations up to 30 μM had no effect on Erk or Akt phosphorylation in Ras/3T3 or Fms/3T3 cells. These studies demonstrate that CT52923 is a potent inhibitor of PDGFR autocrine activation. Furthermore, the specificity of inhibition was demonstrated by the fact that common signaling pathways, activated at a similar proximal location by v-*fms* or more distally by V12-*ras*, are insensitive to CT52923 treatment.

Once transformed by PDGF, (13, 36) V12-*ras* (13) or v-*fms*, (37, 38) NIH/3T3 cells readily form solid tumors when injected s.c. into nude mice. Therefore, we used this nude mouse tumor model to evaluate the *in vivo* effects of CT52923 at inhibiting a tumor mass. Approximately 15 days after an inoculation with 2×10^6 PDGF/3T3, or Fms/3T3 cells, when tumors had reached ~50 mg in size, CT52923 or vehicle alone was administered 60 mg/kg/twice a day by oral gavage for an additional 17 days at which time the mice were killed and tumor weights were determined. As shown in Fig. 3, average tumor weights for PDGF/3T3 and Fms/3T3 vehicle control groups were in the 700–800-mg range, whereas PDGF/3T3 tumors in the CT52923 treatment group showed a 61% reduction in size ($P < 0.006$). In contrast, CT52923 treatment had no effect on Fms/3T3 tumor growth. These results demonstrate selective *in vitro* and *in vivo* inhibition of PDGFR autocrine signaling by CT52923 and provide the basis for its use to study the role of PDGF autocrine signaling in glioblastoma cells.

In Glioma Cells, CT52923 Blocks PDGFR Autophosphorylation and Akt and/or Erk Phosphorylation and Causes a Reversion of the Transformed Phenotype. PDGF autocrine signaling was studied in four human (A172, U251, SF188, and T98G) glioblastoma cell lines and the rat C6 glial tumor cell line. A172, U251, and SF188 were selected because they expressed high levels of all of the PDGFRs and ligands but differ with respect to *PTEN* function (see "Discussion" below), whereas T98G lacks PDGF-B chain expression and has a low level of PDGFR expression (Table 2). Rat C6 cells were selected because they have a well-characterized PDGF autocrine loop and readily form tumors in nude mice (16). The initial event in PDGF autocrine signaling, autophosphorylation of the PDGFR as detected by antiphosphotyrosine Western blot analysis of A172, U251, and C6 cells, was effectively inhibited by pretreatment with 1–3 μM CT52923 (Fig. 4). This antiphosphotyrosine signal was greatly increased by the

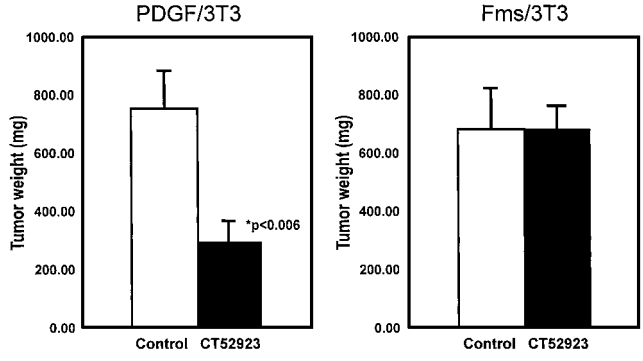

Fig. 3. Selective inhibition of PDGF/3T3 tumor growth by CT52923. Athymic nude mice ($n = 15$) received s.c. injections of 2×10^6 PDGF/3T3 or Fms/3T3 cells. Animals were given 60 mg/kg of CT52923 twice a day via p.o. gavage, beginning when tumors reached about 50 mg in size, between day 15 and day 20, and continuing for up to 17 days, when control tumors reached an average of 800 mg. Shown are mean tumor weights ± SD in the control and the CT52923-treated groups.

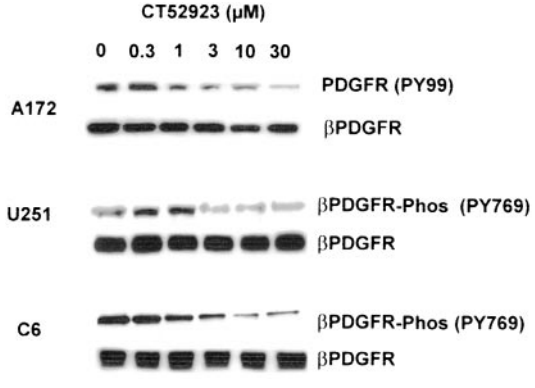

Fig. 4. Effect of CT52923 on autocrine PDGFR autophosphorylation in glioma cells. Human glioblastoma-derived cell lines A172 and U251 and rat C6 glial cells were incubated with the indicated amount of CT52923 for 5 h in DMEM. Cell lysates were prepared, proteins were separated by SDS-PAGE (4–20% gradient gels), transferred onto nitrocellulose, and immunoblotted with an antiphosphotyrosine antibody, PY99, for A172 cells or an anti-phospho-βPDGFR antibody, PY769, for U251 and C6 cells, and followed by ECL detection. Blots were stripped and blotted with anti-βPDGFR sc432 to control for the amount of PDGFR protein loaded.

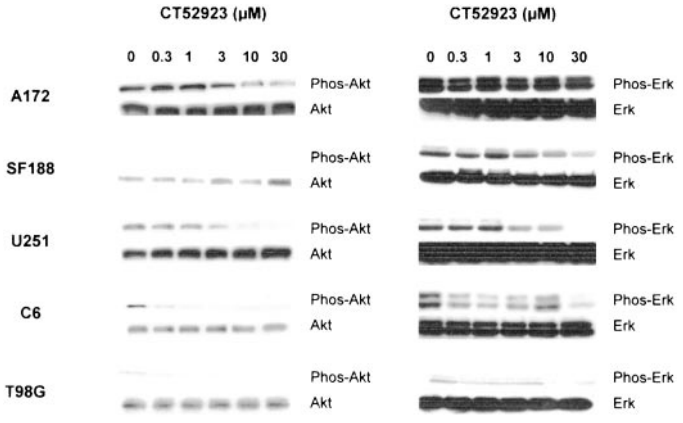

Fig. 5. Effect of CT52923 on constitutive Akt and Erk kinase activation in A172, SF188, U251 and C6 glioma cells. Cells were incubated with the indicated amount of CT52923 for 5 h in serum-free DMEM. Lysates were prepared and proteins separated on 4–20% SDS-PAGE, transferred onto nitrocellulose and immunoblotted with anti-phospho-Akt or anti-phospho-Erk antibodies as well as anti-Akt or anti-Erk antibodies as indicated and detection was done using ECL.

stimulation of these cells with exogenous PDGF, which indicated that only a fraction of the available PDGFR is activated (data not shown).

The best-characterized mechanisms by which PDGF autocrine signaling mediates cellular oncogenic transformation involves the activation of the ras/MAPK pathway, which can increase cellular proliferation, and the PI3k/Akt pathway, which promotes cell survival (39–41). Therefore, we determined the effect of PDGFR inhibition by CT52923 on the phosphorylation of Akt and Erk in each of the glioblastoma cell lines. As shown in Fig. 5, A172, U251, T98G, and C6 cells displayed constitutive Akt phosphorylation that was inhibited by treatment of cells with 0.3–3.0 μM CT52923, as determined by Western blot analysis using anti-phospho-Akt antibodies. PDGF autocrine induction of Akt phosphorylation in these cells is likely to have been exaggerated because of the loss of *PTEN* function in human A172 and U251 cell lines, whereas Akt phosphorylation was below the level of detection in untreated SF188, which has wild-type PTEN (Fig. 5). All of the cell lines demonstrated constitutive Erk phosphorylation that was inhibited in each case by CT52923 (0.3–3.0 μM), with the exception of A172 (Fig. 5). It is possible that A172 cells have additional signaling defects leading to Erk activation that are independent of PDGFR. Remarkably, PDGFR inhibition by CT52923 effectively blocked constitutive Erk and/or Akt signaling in all of the other glioblastoma cell lines studied.

To explore further the functional significance of PDGF autocrine signaling in glioblastoma cells, the ability of CT52923 to inhibit colony growth in soft agar was measured because this property is highly correlated with tumorgenicity (17, 42, 43). As shown in Fig. 6, CT52923 blocked soft agar colony formation of all of the glioblastoma cells with an IC_{50} in the 1–10-μM range, with the most sensitive line, T98G, showing >90% inhibition at 1 μM. As a control, CT52923 treatment at concentrations of up to 30 μM had only minimal effects on colony formation by A431 and MDA468 carcinoma cells, which did not express PDGFR as confirmed by TaqMan analysis (Table 2 and data not shown).

CT52923 Inhibition of C6 Glioma Tumor Growth in Nude Mice. C6 glioma cells are known to reproducibly form s.c. tumors in nude mice, and, therefore, they have been widely used for studies of the *in vivo* formation or inhibition of glioblastomas (16). On the basis of the results of the *in vitro* studies using CT52923 (Figs. 4–6), the C6 xenograft model was chosen to evaluate the ability of CT52923 to block glioblastoma formation *in vivo*. Nude mice received injections s.c. in the left flank with 3×10^6 C6 cells, and CT52923 treatment was initiated by p.o. gavages at 60 mg/kg twice daily for the entire duration of the 18-day study. As shown in Fig. 7, tumors in vehicle control animals reached an average weight of 1.25 grams, and treatment with CT52923 caused a significant 44% reduction in tumor size ($P < 0.0019$). However, optimal treatment may not have been achieved because the monitoring of CT52923 levels during the study indicated that >4-μM (IC_{50}, 2 μM in plasma) concentrations were maintained for 4 h after dosing, but nadir concentrations were undetectable. Thus, adequate levels for inhibiting PDGFR were maintained for up to 12 h/day.

DISCUSSION

One of the most consistent cellular signaling defects observed in malignant gliomas is the establishment of a PDGF autocrine loop

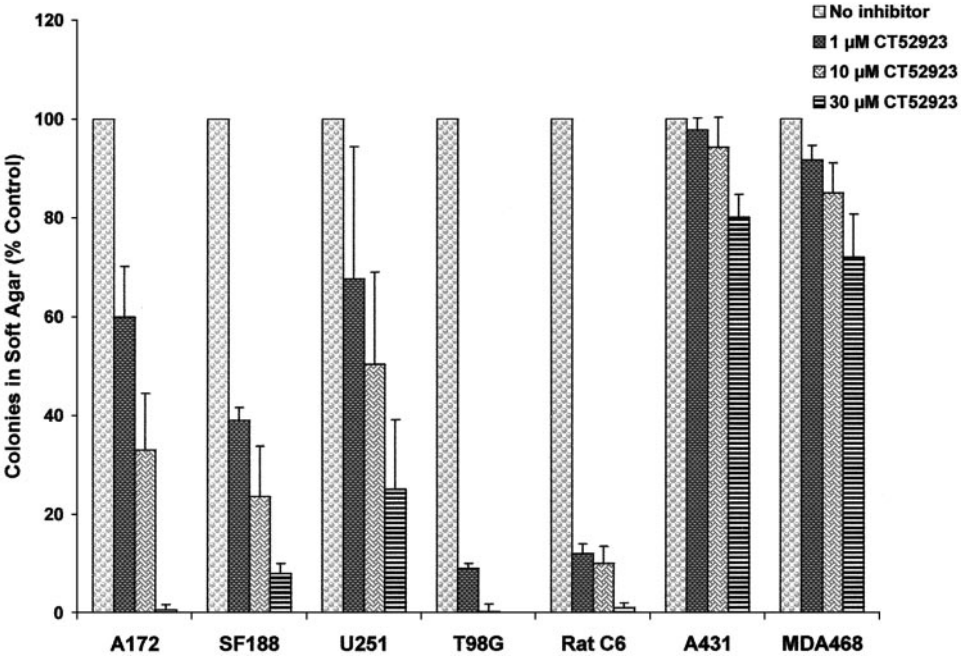

Fig. 6. CT52923 inhibition of glioma colony formation cells in soft agar. Cells (10^5) were cultured in 60-mm dishes in 0.5% low-gelling agarose in the presence of DMEM, 10% fetal bovine serum on a base layer of 1% agar in the presence or absence of the indicated amounts of CT52923; and colonies were scored after 21 days. Shown are the percentage colonies of control (*No inhibitor*, 100%) ± SD ($n = 3$) with each amount of compound tested.

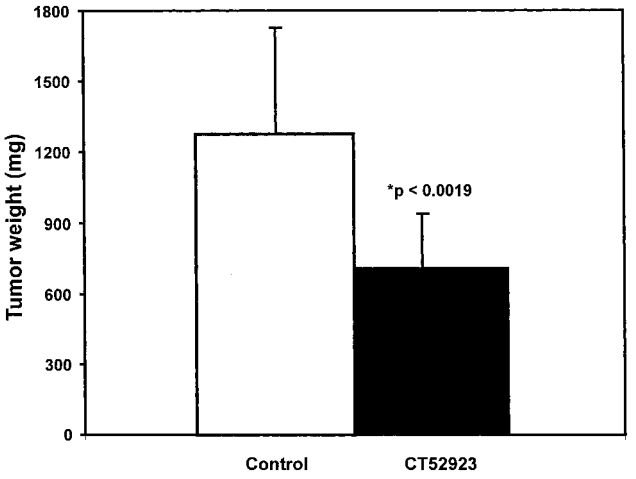

Fig. 7. Inhibition of glioma tumor growth by CT52923. Athymic nude mice ($n = 15$) received s.c. injections of 3×10^6 rat C6 glial tumor cells. Animals were given CT52923 at 60 mg/kg twice a day by p.o. gavage. Dosing started on day 1 and ran for 18 days, when control tumor weights reached in average of 1000–1400 mg. Shown are mean tumor weights ± SD in the control and CT52923-treated groups.

attributable to the coexpression of PDGF-A and -B and their cognate receptors (14, 20, 21, 23, 25, 27, 28). In this study, we extended these observations by demonstrating for the first time that the novel PDGF-C and/or -D ligands were expressed in all of the glioblastoma cell lines and primary glioblastoma multiforme tumors examined, whereas little or no expression was found in normal brain tissue. Because nearly all of the glioblastoma cell lines and primary tumor tissues examined expressed α- and β-PDGFR, PDGF-C or -D expression could induce autocrine signaling through both receptors. This may have important implications, because it was previously thought that PDGF-B expression, observed in only a subset of glioblastoma cells, was required for autocrine signaling through the β-PDGFR (Table 2; Ref. 3). PDGF-C and -D also differ from PDGF-A and -B in that posttranslational cleavage by an unknown protease is required for biological activity, and whether this processing occurs in the tissues of malignant glioma has not been examined (11, 12).

Coexpression of PDGF and PDGFR has been shown at all brain tumor stages including low-grade astrocytomas, anaplastic astrocytomas, and glioblastoma multiforme (3, 25). These observations are consistent with PDGF autocrine signaling being an initiating event, and additional defects in cell signaling are then probably required for progression to glioblastoma multiforme (3, 44, 45). This is underscored by the fact that, as brain tumors become more advanced, they accumulate alterations in additional genes that regulate cell survival and proliferation, including *p53*, *PTEN*, *CDK4*, *Rb*, and *Ink4a-Arf* (3, 44). The importance of additional genetic alterations in combination with PDGF autocrine signaling was recently investigated by Dai *et al.* (46) using retroviral transduction of PDGF-B into glial cells of newborn mice that are wild-type or null for *Ink4a-Arf*. On the wild-type background, PDGF-B expression resulted in low-grade gliomas as compared with high-grade tumors of shorter latency in the *Ink4a-Arf* null mice, which showed the importance of additional genetic defects. Because of the multiple alterations in cell signaling pathways in malignant gliomas, it remains an open question as to the therapeutic effectiveness of blocking a potential initiating event such as PDGFR autocrine signaling. Recent success in the treatment of chronic myelogenous leukemia, either in the acute or in the chronic phase, by targeting the *BCR/ABL* kinase demonstrates that such a strategy can be very effective, even after additional mutations have occurred (47, 48). Because these studies demonstrate that inhibiting an early initiating event can still revert the transformed phenotype even in the late stages of cancer, we chose to investigate the role of PDGF autocrine signaling in glioblastoma multiforme cells using our selective PDGFR antagonist CT52923.

PDGF autocrine expression initially induces PDGFR autophosphorylation on tyrosines, which creates the sites for physical interactions with a number of proteins that contain *Src* homology region 2 domains (reviewed in Ref. 49). These interactions affect the activation of intracellular signaling pathways that are critical for oncogenic transformation, including cell proliferation and survival. Common genetic alterations in glioblastoma multiforme include PTEN mutations that would enhance PDGFR signaling through the PI3k pathway and cell cycle defects that could complement MAPK signaling. In glioblastoma cells A172, U251, and C6, which have a high level of PDGF ligand and receptor expression along with PTEN mutations, it was demonstrated that CT52923 can block autocrine-mediated receptor autophosphorylation and the exaggerated Akt phosphorylation, thereby decreasing signaling through this cell survival pathway. Interestingly, mitogenic signaling through the MAPK pathway was also consistently inhibited by CT52923 in all of the cells tested except A172, in which the activation of alternative convergent pathways may play a larger role. These studies provide evidence that PDGF autocrine signaling continues to play an important role in mediating intracellular events in late-stage glioblastoma multiforme cells, even though multiple genetic alterations have accumulated; and the blockade of this pathway could decrease cell proliferation and survival.

To demonstrate that Akt and Erk signaling had biological significance, we showed that CT52923 significantly inhibited soft agar colony formation by glioblastoma cells but had little effect on A431 and MDA468 carcinoma cells. Similarly, p.o. administration of CT52923 to nude mice significantly inhibited xenograft C6 cell tumor formation. Other studies using neutralizing antibodies, dominant-negative mutants of PDGF or PDGFR, and PDGFR kinase inhibitors have shown the importance of PDGFR signaling for the maintenance of the transformed phenotype of glioblastoma cells and their ability to form tumors in mice (13–17). In conclusion, these studies provide strong evidence that, even in advanced stages of brain tumors, PDGF autocrine signaling still plays a critical role in maintaining cell transformation and that selective blockade of PDGFR with a kinase inhibitor may provide an effective therapeutic intervention. This may be especially true for treatment of low-grade astrocytomas, a situation that may be similar to STI571 treatment of chronic myelogenous leukemia in the early chronic phase that has resulted in long-term remissions.

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