Coadministration of Sorafenib with Rottlerin Potently Inhibits Cell Proliferation and Migration in Human Malignant Glioma Cells

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ABSTRACT

Mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) are activated in the majority of gliomas and contribute to tumor cell growth and survival. Sorafenib (Bay43-9006; Nexavar) is a dual-action Raf and vascular endothelial growth factor receptor inhibitor that blocks receptor phosphorylation and MAPK-mediated signaling and inhibits growth in a number of tumor types. Because our initial studies of this agent in a series of glioma cell lines showed only partial growth inhibition at clinically achievable concentrations, we questioned whether inhibition of PKC signaling using the PKC-δ inhibitor rottlerin might potentiate therapeutic efficacy. Proliferation assays, apoptosis induction studies, and Western immunoblot analysis were conducted in cells treated with sorafenib and rottlerin as single agents or in combination. Sorafenib and rottlerin reduced proliferation in all cell lines when used as single agents, and the combination produced marked potentiation of growth inhibition. Flow-cytometric measurements of cells stained with Annexin V-propidium iodide and immunocytochemical assessment of cytochrome *c* and apoptosis-inducing factor release demonstrated that addition of rottlerin resulted in significantly higher levels of apoptosis than sorafenib alone. In addition, the combination of sorafenib and rottlerin reduced or completely inhibited the phosphorylation of extracellular signal-regulated kinase and Akt and down-regulated cell cycle regulatory proteins such as cyclin-D1, cyclin-D3, cyclin-dependent kinase (cdk)4, and cdk6 in a dose- and time-dependent manner. Our results clearly indicate that inhibition of PKC-δ signaling enhances the antiproliferative effect of sorafenib in malignant human glioma cell lines and support the examination of combinations of signaling inhibitors in these tumors.

A characteristic feature of malignant gliomas, shared by many other types of cancer, is dysregulation of signal transduction pathways that control cell proliferation and promote survival (Hunter, 1997; Hanahan and Weinberg, 2000). Malignant glioma presents a particular therapeutic challenge because these lesions are invasive and not amenable to complete surgical removal, and they are typically refractory to radiotherapy and conventional chemotherapy. In contrast to the improvements in outcome that have been achieved with new treatment approaches in many other cancer types, the

duration of survival for patients with malignant gliomas has not changed in decades (Nagane et al., 1997; Maher et al., 2001), which highlights the need for novel therapies that target signaling pathways that underlie abnormal cellular growth. Protein kinase C (PKC) and Raf-1 represents two such targets in malignant gliomas. PKC comprises a family of phospholipid-dependent serine-threonine kinases that play important roles in signal transduction and in the regulation of cell survival, growth, differentiation, transformation, and apoptosis (Nishizuka, 1984, 1988, 1992). PKC isozymes are grouped on the basis of their structural and biochemical properties as either "classic" or calcium-dependent PKCs (α, βI, βII, and γ), novel or calcium-independent PKCs (δ, ε, η, and θ), or atypical PKCs (ζ and λ/ι). In various cell types, certain PKC family members stimulate mitogen-

ABBREVIATIONS: PKC, protein kinase C; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; HUVEC, human umbilical vein endothelial cell; DMSO, dimethyl sulfoxide; Bay43-9006, sorafenib; MTS, 3-[4,5-dimethylthiazol-2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H, tetrazolium; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; AIF, apoptosis-inducing factor; PAGE, polyacrylamide gel electrophoresis; TNS, Tris-buffered saline; EGFR, epidermal growth factor receptor; JNK, c-Jun NH$_2$-terminal kinase; RTK, receptor tyrosine kinase; cdk, cyclin-dependent kinase; PI3K, phosphatidylinositol 3-kinase; E-64, N-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide; CCI 779, temsirolimus.

esis, whereas others are preferentially growth inhibitory, contributing to delicate regulation of signaling pathways by individual PKC isoforms, which may reflect a distinctive pattern of intracellular compartmentalization and access to targets (da Rocha et al., 2002). For example, PKC-α activity levels seem to be increased in breast cancers (Carey and Noti, 1999) and malignant gliomas (Leirdal and Sioud, 1999) but underexpressed in colon cancers (Assert et al., 1999), suggesting that this PKC isoform acts as an oncogene in the former cases and as an antioncogene in the later cases. Studies by our group (Bredel and Pollack, 1997; Bredel et al., 1999) and others (von Deimling et al., 1995; Baltuch and Yong, 1996) indicate that the aberrant proliferation of malignant gliomas may result in part from excessive activation of PKC, which is driven by dysregulated upstream growth-factor mediated signaling cascades (Moriya et al., 1996; Bornancin and Parker, 1997; Edwards and Newton, 1997; da Rocha et al., 2002), and culminates in activation of downstream signaling modules (Ueda et al., 1996; Ueffing et al., 1997; Schonwasser et al., 1998) and inhibition of apoptotic signaling (Haldar et al., 1995).

The Raf-1 cascade is one of the main systems for the transduction of proliferative signals through the cytoplasm (Moriya et al., 1996; Ueda et al., 1996; Edwards and Newton, 1997; Ueffing et al., 1997; Schonwasser et al., 1998; Schenk and Snaar-Jagalska, 1999). Raf family members are intermediate molecules in the MAPK [Ras/Raf/MAPK kinase/extracellular regulated kinase (ERK)] pathway, which relays extracellular signals from cell membrane-based tyrosine kinase receptors to the nucleus via a series of consecutive phosphorylation events (Mercer and Pritchard, 2003; Satyamoorthy et al., 2003; Smalley, 2003). Sorafenib (Bay43-9006; Nexavar) is one of the most promising agents of the class of Raf kinase inhibitors and was recently approved by the Food and Drug Administration for treatment of renal cell carcinoma (Lyons et al., 2001; Awada et al., 2005; Sridhar et al., 2005; Strumberg et al., 2005). It is undergoing phase II evaluation in glioma as a single agent and in combination with other novel agents, including CCI 779, tipifarnib, and erlotinib. This compound inhibits multiple kinase targets in addition to Raf, thereby not only inhibiting the ERK pathway but also interfering with angiogenesis and growth signaling by platelet-derived growth factor receptor (PDGFR)-β, vascular endothelial growth factor receptor (VEGFR)-2, VEGFR-3, Flt-3, and c-Kit (Ahmad and Eisen, 2004; Wilhelm et al., 2004). Given that pharmacological inhibition of the PKC pathway has demonstrated independent activity in glioma cells (Bredel and Pollack, 1997; Bredel et al., 1999), in addition to potentiating the efficacy of apoptosis-promoting strategies (Drew et al., 2001; Kim et al., 2005), and the fact that Raf is phosphorylated by PKC at multiple sites (Kolch et al., 1993; Cai et al., 1997; Marais et al., 1998), we reasoned that the combination of PKC and Raf-1 inhibition might cooperate synergistically to block glioma cell proliferation and induce apoptosis. To test this hypothesis, we examined the interactions between rottlerin, which has demonstrated selective inhibition of PKC-δ activity (Pongracz et al., 1999; Frasch et al., 2000; Basu et al., 2001; Vancurova et al., 2001; Tillman et al., 2003), and sorafenib in glioma cell lines. Here, we show synergistic potentiation by the PKC inhibitor rottlerin of the growth inhibition and apoptosis in human malignant glioma cells induced by sorafenib.

Materials and Methods

Cell Culture. The established malignant glioma cell lines U87, T98G, A172, human pulmonary fibroblasts, and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (Manassas, VA). Human astrocytes and human cerebellar astrocytes were obtained from ScienCell Research Laboratories (San Diego, CA). LN18, LNZ308, and LNZ428 were generously provided by Dr. Nicolas de Tribolet. U87, T98G, and human pulmonary fibroblasts were cultured in growth medium composed of minimum essential medium supplemented with sodium pyruvate and nonessential amino acids; A172, LN18, LNZ308, and LNZ428 were cultured in α-minimal essential medium supplemented with L-glutamine; human astrocytes were cultured in astrocyte growth medium; and HUVECs were cultured in endothelial cell medium (ScienCell Research Laboratories). All growth media contained 10% fetal calf serum, L-glutamine, ribonucleosides, deoxynucleosides, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin (Life Technologies, Inc., Bethesda, MD). These cell lines were chosen because they are widely available and incorporate cells having, in various combinations, a range of genomic alterations commonly seen in malignant gliomas, such as p53 mutations, phosphatase and tensin homolog deleted on chromosome 10 deletions, and p16 deletions. Cells were grown in 75-cm² flasks at 37°C in a humidified atmosphere with 5% carbon dioxide and were subcultured every 4 to 7 days by treatment with 0.25% trypsin in Hanks' balanced salt solution (Life Technologies, Inc.).

Inhibitors and Reagents. Sorafenib was kindly provided by Bayer Pharmaceutical Corporation (West Haven, CT). Rottlerin was purchased from Calbiochem (San Diego, CA). Materials were dissolved in sterile dimethyl sulfoxide (DMSO) and stored frozen under light-protected conditions at −20°C.

Cell Proliferation and Cytotoxicity Assays. Cells (5×10^3/well) were plated in 96-well microtiter plates (Costar, Cambridge, MA) in 100 μl of growth medium, and after overnight attachment, were exposed for 3 days to a range of concentrations of sorafenib and rottlerin, alone and in combination. Control cells received vehicle alone (DMSO). After the treatment interval, cells were washed in inhibitor-free medium, and the number of viable cells was determined using a colorimetric cell proliferation assay (CellTiter96 Aqueous NonRadioactive Cell Proliferation Assay; Promega, Madison, WI), which measures the bioreduction of the tetrazolium compound 3-[4,5-dimethylthiazol-2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H tetrazolium (MTS) by dehydrogenase enzymes of metabolically active cells into a soluble formazan product, in the presence of the electron coupling reagent phenazine methosulfate (Riss, 1992; Riss and Moravec, 2004). All studies were conducted in triplicate and repeated at least three times independently.

To perform the assay, 20 μl of MTS/phenazine methosulfate solution was added to each well, and after 1 h of incubation at 37°C in a humidified 5% CO$_2$ atmosphere, absorbance was measured at 490 nm in a microplate reader. Triplicate wells with predetermined cell numbers were subjected to the above-mentioned assay in parallel with the test samples to normalize the absorbance readings.

To directly assess cellular toxicity, 2.5×10^5 cells were seeded in 60-mm Petri dishes and treated with selected concentrations of inhibitors or vehicle. Cells were harvested, stained with trypan blue, and counted using a hemacytometer. All samples were tested in triplicate. Viable (trypan blue-excluding) and dead cell numbers were plotted as a function of inhibitor concentration.

Clonogenic Growth Assay. The effect of different inhibitor concentrations on cell viability was also assessed using a clonogenic assay. For this analysis, 250 cells were plated in six-well trays in growth medium, and after overnight attachment, they were exposed to selected inhibitor concentrations or vehicle for 24 h. The cells were then washed with inhibitor-free medium and allowed to grow for 2 weeks under inhibitor-free conditions. Colonies of a diameter of

approximately 2 to 4 mm were counted directly. All studies were performed in quintuplicate.

Annexin V Apoptosis Assay. Apoptosis induction in control (DMSO-treated) or inhibitor-treated cells was assayed by the detection of membrane externalization of phosphatidylserine with Annexin V-FITC conjugate using an Annexin V assay kit according to manufacturer's protocol (Molecular Probes). In brief, 2×10^5 cells were harvested at various intervals after treatment and washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 200 μl of binding buffer. Both adherent and floating cells were harvested for the apoptosis assay. Annexin V-FITC and 1 μg/ml propidium iodide were added to individual samples and incubated for 15 min in a dark environment. The reaction was stopped by adding 300 μl of 1$\times$ binding buffer. Then, the cells were analyzed by flow cytometry with FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Cell Cycle Analysis. The effect of varying concentrations of inhibitors on cell cycle distribution was determined by flow cytometric analysis of the DNA content of cell nuclei following staining with propidium iodide. In brief, cells grown exponentially to 40 to 50% confluence were exposed to the inhibitors or DMSO for a range of intervals, harvested, washed briefly in ice-cold PBS, and fixed in 70% ethanol. DNA was stained by incubating the cells in PBS containing 50 μg/ml propidium iodide and RNase A (1 mg/ml) for 60 min at room temperature, and fluorescence was measured and analyzed using a Becton Dickinson FACScan and the CellQuest software (BD Biosciences).

Hoechst Staining for Nuclear Fragmentation. Cells were seeded and allowed to grow overnight in complete media on glass slides. The following day, cells were treated with inhibitors for a range of intervals. Then, cells were fixed in 3.7% formaldehyde in PBS for 30 min. Finally, cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked in 1% bovine serum albumin in PBS for 1 h. Cells were washed with PBS and incubated with 0.2 μg/ml Hoechst 33342 in PBS for 1 h. Cells were washed with PBS and observed under a 20$\times$ objective (Olympus, Tokyo, Japan). Samples were examined and images were collected. The figures were prepared using Photoshop software (Adobe Systems, Mountain View, CA).

Immunocytochemistry and Fluorescence Microscopy. Cells were grown on chamber slides (Nalge Nunc, Naperville, IL) in growth medium, and, after an overnight attachment period, were exposed to selected concentrations of inhibitor or vehicle (DMSO) for various durations. Cells were washed once with PBS, fixed with 3.7% formaldehyde for 30 min, washed two times in PBS, and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. After being washed three times with PBS, the cells were blocked with 0.5% bovine serum albumin for 1 h and then incubated with anti-cytochrome c monoclonal antibody (BD Biosciences PharMingen, San Diego, CA) at 1:100 and rabbit anti-AIF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (at 1:250) overnight at 4°C. After PBS wash, the slides were incubated with secondary antibody (tetramethylrhodamine B isothiocyanate-goat anti-mouse and FITC-goat anti-rabbit immunoglobulin G; Invitrogen) and Hoechst 33342 (Invitrogen) for 2 h at room temperature. The slides were then washed in PBS, mounted, and examined under a fluorescent microscope.

Transwell Migration Assay. Transwell inserts (Corning Life Sciences, Acton, MA) with 8-μm pore size, precoated with collagen, were used to assess glioma cell migration. In brief, cells were seeded at 60% confluence and allowed to attach for 18 h. The cells were then serum-starved for 12 h. After this interval, cells were trypsinized, and 200 μl of cell suspension (5×10^5 cells/ml) containing different concentrations of inhibitors were added in triplicate to the upper wells. VEGF was added to the lower wells as a chemoattractant. After 12 h, cells that migrated through the membrane were fixed and stained in thiazine and eosin using Diff-Quik II solution (Dade Behring, Marburgh, Germany), and these cells were sealed on slides. Migrated cells were counted by microscopy.

Western Blotting Analysis. Treated and untreated cells were washed in cold PBS and lysed in buffer containing 30 mM HEPES, 10% glycerol, 1% Triton X-100, 100 mM NaCl, 10 mM MgCl$_2$, 5 mM EDTA, 2 mM Na$_3$VO$_4$, 2 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, and 10 μM pepstatin A for 15 min on ice. Samples were centrifuged at 12,000g for 15 min, supernatants were isolated, and protein was quantified using Protein Assay Reagent (Pierce Chemical, Rockford, IL). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto a nylon membrane (Invitrogen). Nonspecific antibody binding was blocked by incubation of the blots with 2% bovine serum albumin in Tris-buffered saline (TBS)/Tween 20 (0.1%) for 1 h at room temperature. The blots were then probed with appropriate dilutions of primary antibody overnight at 4°C. The antibody-labeled blots were washed three times in TBS/Tween 20 for 15 min and then incubated with a 1:1500 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) in TBS/Tween 20 at room temperature for 1 h. After additional washing in TBS/Tween 20, the proteins were visualized by Western Blot Chemiluminescence Reagent (Cell Signaling Technology Inc., Beverly, MA). Where indicated, the blots were reprobed with antibodies against β-actin (Sigma-Aldrich, St. Louis, MO) to ensure equal loading and transfer of proteins.

The primary antibodies to epidermal growth factor receptor (EGFR) and phospho-EGFR (Tyr^{845}); PDGFR and phospho-PDGFR; ERK1/2 and phospho-p44/42 ERK (Thr^{202}/Tyr^{204}); p38 and phospho-p38 (Thr^{180}/Tyr^{182}); JNK and phospho-JNK (Thr^{183}/Tyr^{185}); Akt and phospho-Akt (Ser^{473}); and CDK4, CDK6, cyclin-D1, and cyclin D3 were obtained from Cell Signaling Technology, Inc. VEGFR and phospho-VEGFR antibodies were obtained from BioSource International (Camarillo, CA).

Analysis of Combinatorial Effects. Unless otherwise stated, data are expressed as mean $\pm$ S.D. The significance of differences between experimental conditions was determined using the two-tailed Student's t test. To characterize IC$_{50}$ concentrations and synergistic effects between the agents, a commercially available software program was used (Calcusyn; Biosoft, Ferguson, MO) (Chou and Talalay, 1984; Zhao et al., 2004).

Results

Sorafenib Inhibits VEGF and PDGF Receptor Phosphorylation. Sorafenib has been reported to inhibit several receptor tyrosine kinases, including VEGFR-2, mouse VEGFR-3, mouse PDGFR-β, Flt-3, c-KIT and fibroblast growth factor receptor-1, but not EGFR, insulin-like growth factor receptor, c-met, or HER-2 (Wilhelm et al., 2004). To confirm the specificity of inhibition in U87 and T98G cells, we examined the effect of sorafenib on several tyrosine kinase receptors that have been implicated in glioma growth. Treatment with PDGF or VEGF rapidly induced tyrosine phosphorylation of PDGF and VEGF receptors (Fig. 1), and this effect was inhibited in the presence of sorafenib. In contrast, sorafenib did not directly inhibit phosphorylation of EGFR by epidermal growth factor.

Sorafenib Inhibits Glioma Cell Proliferation and Induces Mitochondrial Injury and Apoptosis. We examined the effect of the Raf inhibitor sorafenib on the cellular proliferation of a panel of glioma cell lines. Cells were cultured with increasing concentrations of sorafenib for 3 days, and cell proliferation was assessed by MTS assay. Sorafenib inhibited cell proliferation in a dose-dependent manner (Fig. 2A). The sensitivity, as assessed by the IC$_{50}$, ranged from 5 to 20 μM for sorafenib (U87, 17.2 μM; T98G, 8.1 μM; A172, 5.4
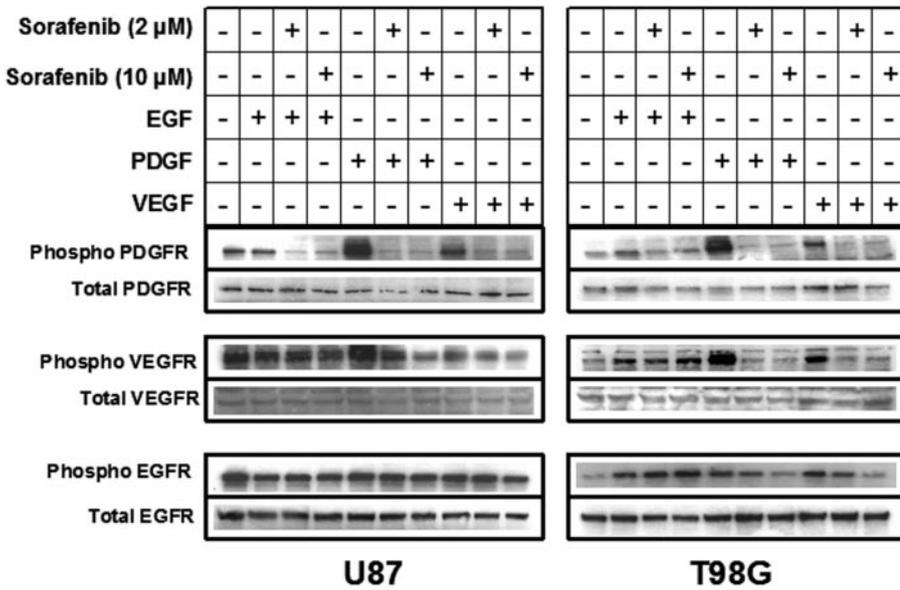
Fig. 1. Effects of sorafenib on receptor phosphorylation. U87 and T98G were seeded at 60% confluence and allowed to attach. Then, the cells were serum-starved 24 h and pretreated with 0 to 10 μM sorafenib for 1 h and then left untreated or treated with 50 ng/ml epidermal growth factor (EGF), 50 ng/ml PDGF, and 100 ng/ml VEGF for 30 min. Cell extracts were prepared, and equal amounts of protein (50 μg/lane) were separated by SDS-PAGE analysis and subjected to Western blotting analysis with the indicated primary antibodies.
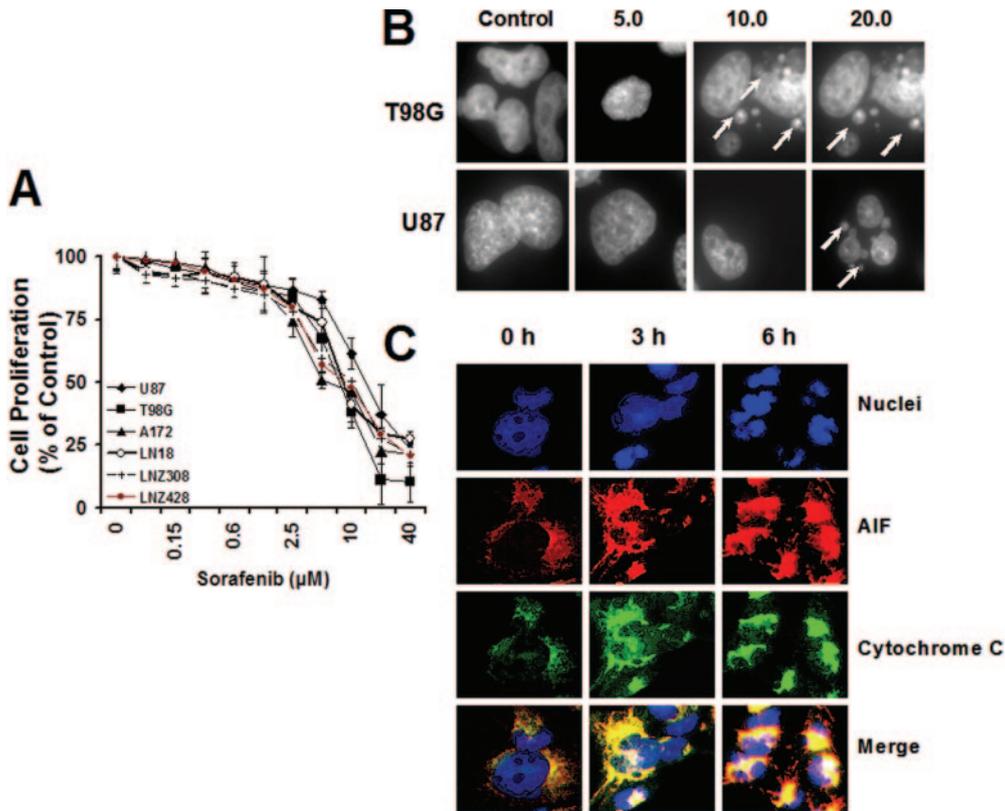
Fig. 2. Effects of sorafenib on cellular proliferation and apoptosis. A, logarithmically growing glioma cell lines were incubated with varying concentrations of sorafenib for 3 days. The relationship between sorafenib and cell numbers was assessed semiquantitatively by spectrophotometric measurement of MTS bioreduction in six established malignant human glioma cell lines. Points represent the mean of four measurements ± S.D. There was a dose-dependent reduction in cell growth. Control cells were treated with equivalent concentrations of vehicle (DMSO). B, logarithmically growing U87 and T98G cells were incubated with varying concentrations of sorafenib for 24 h. Cells were fixed, permeabilized, and stained with Hoechst 33342 to visualize nuclear morphology. The nuclei showed changes consistent with nuclear fragmentation typical of apoptosis. The arrows point to fragmented nuclei. C, T98G cells were incubated for the designated interval in the presence of 5 μM sorafenib. Cells were fixed, permeabilized, and stained with antibodies specific for cytochrome *c* and AIF as described under *Materials and Methods*. The cells were counterstained with Hoechst 33342 to visualize nuclei. Immunofluorescence detection of AIF and cytochrome *c* normally yields a punctate cytoplasmic staining (0-h control). Cells exposed for 6 h resulted in diffuse staining for cytochrome *c* and nuclear translocation of AIF in the majority of the cells. This experiment was repeated three times, yielding comparable results.

μM; LN18, 8.2 μM; LNZ308, 10.2 μM; and LNZ428, 8.8 μM). No significant inhibition was seen in control cells treated with equivalent concentrations of vehicle (DMSO) in the ab-

sence of sorafenib (data not shown). Exposure to 5 μM sorafenib for varying durations revealed a time-dependent induction of apoptosis as assessed by Annexin V analysis (data

not shown). This apoptotic effect was confirmed by morphological analysis of nuclei stained with Hoechst 33342. Microscopy demonstrated that sorafenib treatment induced nuclear fragmentation (Fig. 2B). Because redistribution of cytochrome c and AIF has been reported to be an early event in the apoptotic process associated with mitochondrial damage (Daugas et al., 2000; Arnoult et al., 2002; Joseph et al., 2002; Premkumar et al., 2006), we examined the localization of cytochrome c and AIF after sorafenib treatment by immunofluorescence microscopy. Immunofluorescence detection of AIF and cytochrome c in untreated control cells showed a punctate cytoplasmic staining pattern with some preference for the perinuclear area (Fig. 2C), typical for mitochondrial localization (Daugas et al., 2000). In contrast, cells incubated with 5 μM sorafenib showed an increased diffuse staining of cytochrome c in the cytoplasm and translocation of AIF from the mitochondria into the nucleus as early as 3–6 h (Fig. 2C). Together, these findings indicate that treatment of glioma cells with sorafenib results in an induction of mitochondrially mediated apoptotic cell death.

Sorafenib Modulates Cell Cycle and Survival Regulatory Molecules. The receptor tyrosine kinase (RTK) signaling network activates several key signaling pathways that subvert the G_1-to-S transition as well as disable proapoptotic molecules, thus leading to dysregulated proliferation and enhanced tumor cell survival (Yarden and Sli-

wkowski, 2001). In view of its ability to inhibit a broad spectrum of RTKs, we examined the impact of sorafenib on cell cycle progression, assessed by flow cytometry, in the T98G glioma cell line. Treatment with sorafenib induced accumulation of cells in G_1 phase in a dose- (Fig. 3A) and time-dependent (Fig. 3B) manner with a concomitant decline in the percentage of cells in S and G_2/M phase relative to controls. Simultaneous with the accumulation of cells in G_1 was complete elimination of both active Akt and ERK (Fig. 3C), as measured with phosphospecific antibodies, without changes in the content of total Akt and ERK protein levels. The sorafenib-induced G_1 arrest was further confirmed by examining the effect on the expression of several key cell cycle regulatory proteins. Western immunoblot analysis confirmed that treatment with varying concentrations of sorafenib decreased the expression of cyclin-D1, cyclin-D3, CDK4, and CDK6 (data not shown).

Combination of Sorafenib and Rottlerin Potentiates Inhibition of Proliferation and Clonogenic Survival in Malignant Glioma Cells. Because Raf is one of the downstream effectors of the PKC signaling pathway (Corbit et al., 1999, 2000, 2003; Monick et al., 2000) and the PKC pathway plays an independent role in glioma cell proliferation, differentiation, and transformation, we speculated that inhibitors of PKC signaling might augment the effect of sorafenib in malignant glioma cell lines. First, we examined the effect of
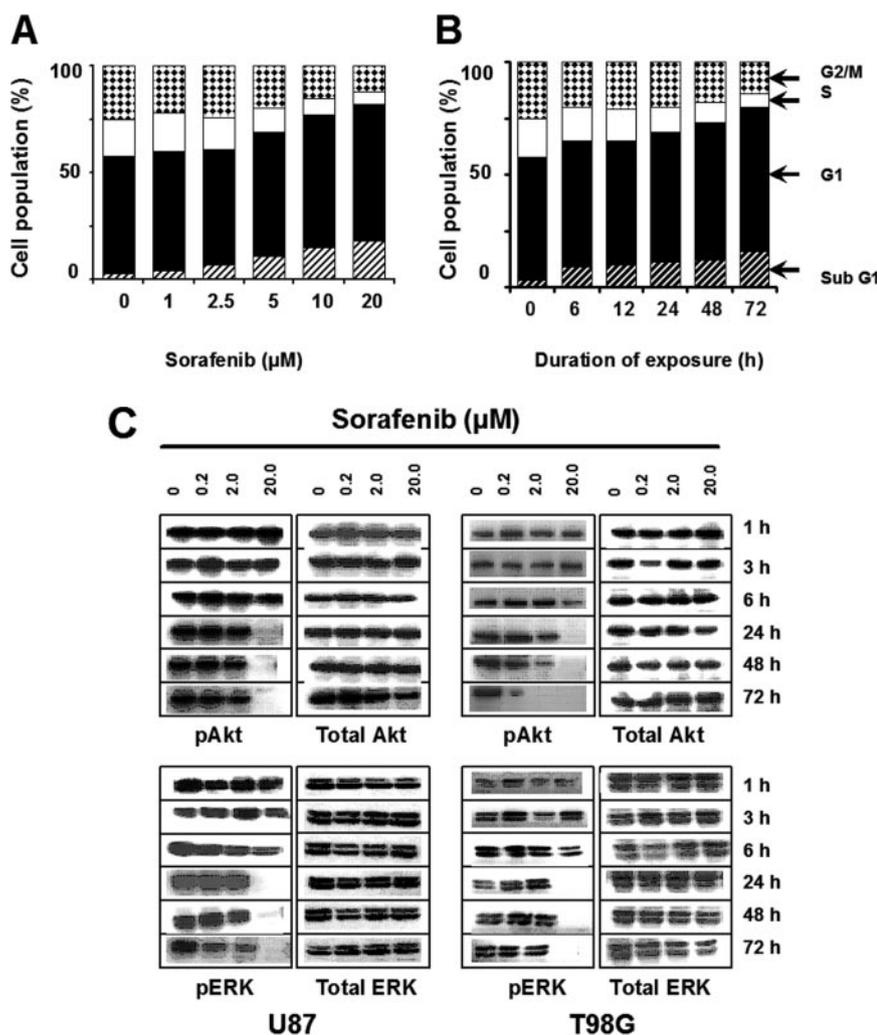

Fig. 3. Effect of sorafenib on T98G cell cycle progression. A, exponentially growing T98G cells were exposed to varying concentrations of sorafenib for 24 h. Cells were harvested, fixed, and DNA-stained with propidium iodide as described under *Materials and Methods*. DNA content was obtained by flow cytometry, and representative histograms for the indicated concentrations are shown with values for each phase of the cell cycle. B, T98G cells, grown to 50% confluence, were exposed to 5 μM sorafenib for different durations (hours). Control cells received DMSO. DNA content (percentage) of the cells was obtained by flow cytometry as described in A. C, logarithmically growing U87 and T98G cells were incubated with varying concentrations of sorafenib for the indicated time. The cells were lysed, and equal amounts of proteins were separated by SDS-PAGE and probed with specific antibodies against phospho-Akt and phospho-ERK (C). Western analysis was performed as described under *Materials and Methods*. The blots were subsequently stripped and reprobed against total Akt or total ERK.

the PKC-δ inhibitor rottlerin on the cellular proliferation of a panel of glioma and normal cell lines. Cells were cultured with increasing concentrations of rottlerin for 3 days, and cell proliferation was assessed by MTS assay. Rottlerin inhibited cell proliferation in a dose-dependent manner, and the IC_{50} values ranged from 2 to 25 μM for glioma cell lines (Fig. 4A) versus 10 to 40 μM for human nonneoplastic cell lines (Fig. 4B). To characterize potential interactions between sorafenib and rottlerin, human glioma cell lines and human astrocytes were exposed to varying concentrations of rottlerin with or without 2 (Fig. 4C) or 5 μM sorafenib (data not shown), and cell viability was assessed after 3 days. These concentrations were selected since they had relatively modest independent effects on cell proliferation and survival in each of the glioma cell lines tested. The combination of both inhibitors was substantially more effective than either single agent and produced a significant decrease in glioma cell survival. In contrast, the combination of these concentrations of rottlerin
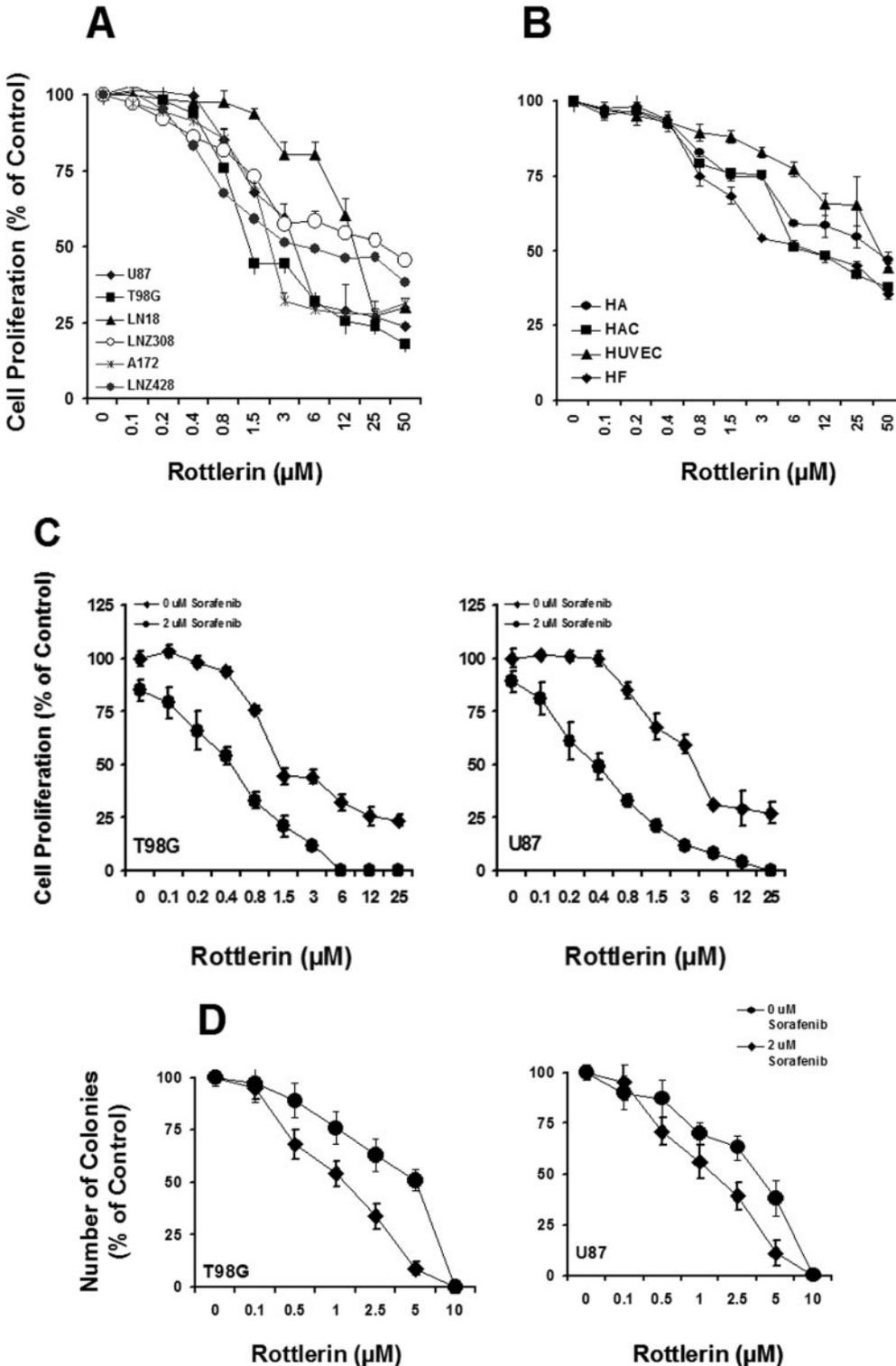

Fig. 4. Sorafenib and rottlerin preferentially inhibit growth and colony formation of glioma cell lines. Logarithmically growing glioma cell lines (A) or human astrocytes (HA), human cerebellar astrocytes (HAC), HUVEC, and human fibroblasts (HF) (B) were incubated with varying concentrations of rottlerin for 3 days. The relationship between rottlerin and cell numbers was assessed semiquantitatively by spectrophotometric measurement of MTS bioreduction in four established malignant human glioma cell lines (A) and human non-neoplastic cell lines (B). Points represent the mean of five measurements ± S.D. Rottlerin inhibited cell proliferation in a dose-dependent manner. C, logarithmically growing glioma (U87 and T98G) were incubated with varying concentrations of rottlerin and sorafenib (2 μM) for 3 days, and MTS assay was performed as described under *Materials and Methods*. Addition of 2 μM sorafenib significantly potentiated the rottlerin-induced glioma cell toxicity, but it had no such potentiation in non-neoplastic cell lines (data not shown). D, Graphs showing the relationship between colony counts (± S.D.) and concentration of the inhibitors. Human glioma cell lines U87 and T98G were exposed to varying concentrations of rottlerin with or without 2 μM sorafenib for 24 h. The following day, the media were changed, and complete media were added. Cells were grown for an additional 14 days in the absence of inhibitors, and colonies were then counted. Points represent the mean of four experiments ± S.D.

and sorafenib had no significant additive effect on human non-neoplastic cell lines (data not shown).

The cytotoxic effect of rottlerin and sorafenib was further confirmed using a clonogenic assay (Fig. 4D). Cells were treated for 1 day with or without compounds, and medium was aspirated and washed with inhibitor-free medium. Cells were allowed to grow for an additional 2-week period. There was a dose-dependent decrease in colony forming ability due to rottlerin (Fig. 4D) and sorafenib (data not shown), when administered independently, with the latter having activity at concentrations above 5 μM. In addition, there was striking potentiation of efficacy when the two agents were administered in combination. Whereas only modest effects were seen at low micromolar concentrations of rottlerin, the addition of 2 μM sorafenib dramatically potentiated the degree of inhibition, despite having no effect on clonogenicity when administered as a single agent at this concentration (Fig. 4D).

Rottlerin and Sorafenib Induces G$_1$ Arrest and Apoptosis in Glioma Cells. It has been shown that the PKC-δ signaling network activates several signaling pathways that subvert the G$_1$-to-S transition as well as disable proapoptotic molecules, thus leading to dysregulated proliferation and enhanced tumor cell survival, depending on the cellular context (Cerda et al., 2006). To elucidate the role of the PKC-δ inhibitor rottlerin and sorafenib in cell cycle control and cell death, asynchronously growing T98G cells were treated with inhibitors, and cell cycle analysis was performed. As noted above, sorafenib alone induced accumulation of cells in G$_1$ phase in a dose- and time-dependent manner with a concomitant decline in the percentage of cells in S and G$_2$/M phase relative to controls (Fig. 3). Comparable effects were observed with low micromolar concentrations of rottlerin. In contrast, exposure of T98G cells to 5 μM sorafenib in conjunction with 2 μM rottlerin led not only to G$_1$ arrest but also to cell death by apoptosis, indicated by an increase in the sub-G$_0$/G$_1$ fraction (Fig. 5A). To further evaluate the enhancement of glioma cell cytotoxicity by the combination of sorafenib and rottlerin, U87 and T98G cells were exposed to 5 μM sorafenib or 2 μM rottlerin or the combination of both, and apoptosis was assessed after 24 h by Annexin V assay. Although only modest effects were seen with both agents alone, at these concentrations, the combination of 2 μM rottlerin and 5 μM sorafenib significantly potentiated glioma cell toxicity (Fig. 5B).

To assess the interaction between sorafenib and rottlerin and proteins implicated in glioma growth signaling, human U87 and T98G glioma cells were exposed to each of these agents alone or in combination for 24 h. The cell lysates were examined for activation of various signaling pathway components. Whereas 5 μM sorafenib resulted in some diminution of phosphorylated ERK1/2 and phosphorylated Akt, combined exposure to these agents resulted in significant to complete decrease of phosphorylated forms of ERK1/2 and Akt (Fig. 5C). In contrast to these findings, neither sorafenib nor rottlerin nor the combination of both modified the phosphorylation status of p38 or JNK/stress activated protein kinase (data not shown). Collectively, these results support the notion that down-regulation or interruption of ERK and Akt pathways by sorafenib and rottlerin play important functional roles in the synergistic induction of glioma cytotoxicity.

Given the striking combinatorial effects of Raf and PKC inhibition on proximal signaling pathway components, we questioned whether this combination would have comparable effects on cell cycle regulatory proteins. Accordingly, we examined the effects of these agents, alone and in combination, on several intermediates that play critical roles in glioma cell cycle progression. U87 and T98G cells were therefore seeded at subconfluence, treated with sorafenib, rottlerin, or the combination of both, and the effects on protein expression levels were assessed. Results from Western blot analysis showed that the combination of sorafenib and rottlerin significantly decreased the levels of cyclin-D1, cyclin-D3, CDK4, and CDK6 (Fig. 5D) compared with controls or each agent individually.

Sorafenib and Rottlerin Reduce Cell Migration. Tumor cell migration and invasion is a characteristic feature of malignant gliomas, typically associated with pathological vascularization (Folkman, 1971; Hamby and Showalter, 1999). Given the contribution of VEGF signaling and PKC to this invasive phenotype (Cho et al., 1999; da Rocha et al., 2000), we questioned whether signaling inhibition with sorafenib and rottlerin might inhibit this process in vitro. Figure 6A demonstrates that stimulation of U87 and T98G cells with recombinant VEGF$_{165}$ protein strongly promotes cell migration. Conversely, the migration of U87 and T98G cells was significantly inhibited by exposure to sorafenib and rottlerin. Both agents not only blocked VEGF-induced migration but also reduced migration to lower than control levels. Moreover, the coadministration of sorafenib and rottlerin showed a supra-additive reduction of U87 and T98G migration, which was virtually eliminated by low concentrations of both agents in combination (Fig. 6B).

Discussion

The majority of human tumors demonstrate activation of the PKC and/or Ras/Raf signal transduction pathways in association with cellular proliferation, survival, and migration (Ahmad et al., 1994). It has been shown that glioma cells express multiple PKC isoforms, and these tumors may be particularly sensitive to PKC inhibition (Ahmad et al., 1994; Yuan et al., 1996). The aim of our investigation was to assess the responsiveness of glioma cells to inhibition of Raf signaling by sorafenib, a novel orally active Raf kinase and VEGFR inhibitor, and to determine whether the combination of this agent with the PKC inhibitor rottlerin could potentiate antiproliferative and cytotoxic efficacy in human malignant glioma cells.

Recent in vitro studies have demonstrated that sorafenib inhibits the proliferative activity of a number of mammalian cells, although there is a broad range of sensitivities between cell types (Ahmad and Eisen, 2004; Rahmani et al., 2005). In this study, we observed that sorafenib was capable of inhibiting the phosphorylation of VEGFR and PDGFR at low micromolar concentrations, and it was capable of inhibiting cellular proliferation in a dose-dependent manner at somewhat higher concentrations in human malignant glioma cells. However, combined treatment with sorafenib and rottlerin significantly enhanced the antiproliferative effect on glioma cells. A potential explanation for this sensitization is that multiple proliferation and survival pathways are typically involved in driving the proliferation of glioma cells, and
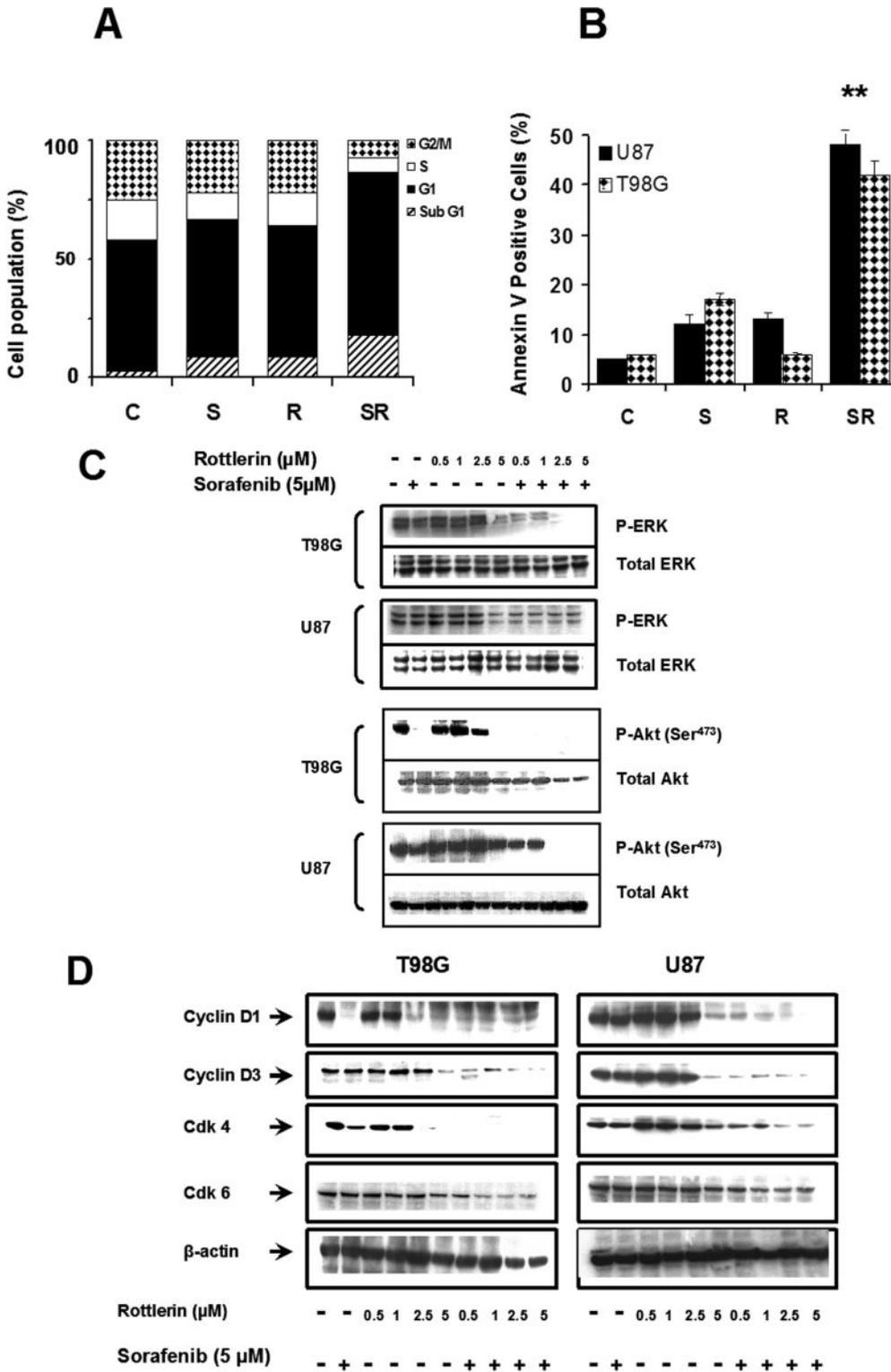
Fig. 5. Combination of sorafenib and rottlerin induces cell cycle arrest and apoptosis and cooperatively modulates survival and cell cycle regulatory molecules. A, exponentially growing human malignant glioma cells were exposed to 5 μM sorafenib or 2 μM rottlerin or the combination of both for 24 h. Cells were harvested, fixed, and DNA-stained with propidium iodide (C, control; S, sorafenib; R, rottlerin; and SR, sorafenib and rottlerin). DNA content of the cells was obtained by flow cytometry, and representative histograms for the inhibitors are shown with values for each phase of the cell cycle. B, logarithmically growing U87 and T98G cells were incubated with 5 μM sorafenib or 2 μM rottlerin or the combination of both for 24 h. Apoptosis was determined by flow cytometry using Annexin V-Alexa Flour 488 as described under *Materials and Methods* following the manufacturer's protocol. Points represent the mean of four experiments ± S.D. **, $P < 0.001$ versus control. C, logarithmically growing U87 and T98G cells were incubated with varying concentrations of rottlerin with or without 5 μM sorafenib for 24 h. The cells were lysed, and equal amounts of proteins were separated by SDS-PAGE and probed with specific antibodies against phospho-ERK, phospho-Akt (C), cyclin-D1, cyclin-D3, cdk4, cdk6, and β-actin (D). Western analysis was performed as described under *Materials and Methods*. The blots were subsequently stripped and reprobed against total Akt or total ERK.

conversely, that inhibition of several of relevant pathways in combination may achieve a synergistic effect on inhibition of cell growth and viability.

In this context, we found that sorafenib and rottlerin, and particularly the combination of both, caused glioma cells to undergo growth arrest in the G_1 phase of the cell cycle. This inhibition was accompanied by a decrease in D-type cyclins shown by Western blot analysis, which may represent an

important downstream target. Loss of cyclin D expression was paralleled by a decrease in other determinants of cyclin-D-associated kinase activity, such as the expression of cdk4 and cdk6. In addition to inhibiting cell cycle progression, the combination of sorafenib and rottlerin showed an increase in the percentage of sub-G_1 components, suggesting that the cells were undergoing apoptosis.

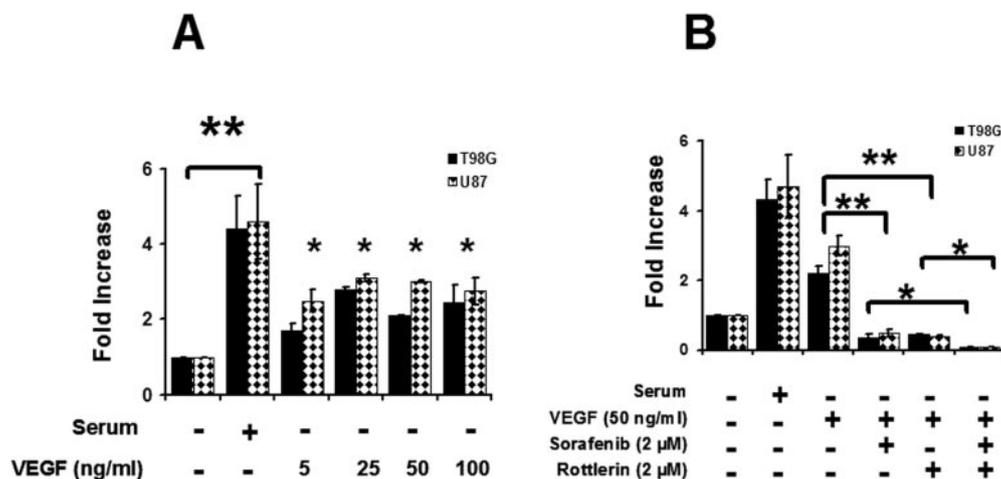Western blot analysis showed that sorafenib affected phos-

Fig. 6. Sorafenib and rottlerin abolish VEGF-induced glioma cell migration. A, logarithmically growing U87 and T98G were seeded at 60% confluence and allowed to attach for 12 h. Then, the cells were serum-starved overnight. Cells were trypsinized, resuspended and treated with medium alone or medium supplemented with varying concentrations of VEGF or 10% fetal bovine serum (serum). Cells were then plated on a polycarbonate membrane precoated with collagen (8-μm pore size) for 12 h. At the end of the experiment, cells on the lower part of the membrane were fixed, stained, and counted as described under *Materials and Methods*. Points represent the mean of four experiments $\pm$ S.D. *, $P < 0.01$ versus control. **, $P < 0.001$ versus control. B, growth factor-deprived U87 and T98G cells were pretreated with 2 μM sorafenib or 2 μM rottlerin or the combination of both. VEGF-induced cell migration was determined. *, $P < 0.01$; **, $P < 0.001$. Values represent the mean $\pm$ S.D. of four separate experiments.

phorylation of ERK and Akt kinases but that combination treatment with the PKC-δ inhibitor rottlerin caused a further reduction in levels of ERK and Akt phosphorylation, with a shift in the response profile to lower concentrations of sorafenib. Recently, Ringshausen et al. (2002) have shown that PKC-δ is a downstream target of PI3K and that blockade of PKC-δ induces apoptosis. Several lines of evidence implicate an important role for PI3K/Akt pathways in tumorigenesis in general and glial tumorigenesis in particular, because of the frequent loss of phosphatase and tensin homolog deleted on chromosome 10, an endogenous inhibitor of Akt (Nagane et al., 1997). Akt represents a major downstream target of PI3K and is linked to a variety of antiapoptotic functions (Datta et al., 1999; Katso et al., 2001). In addition, growth factor-stimulated activation of D-type cyclins has been shown to occur via a phosphatidylinositol 3-kinase/Akt pathway (Muise-Helmericks et al., 1998). Active Akt prevents apoptosis by phosphorylating Bad, caspase-9, Forkhead transcription factors, and IκB kinase (Datta et al., 1997; Cardone et al., 1998). Conversely, inhibition of Akt signaling pathway may potentiate apoptosis induction. Sorafenib- and rottlerin-mediated inhibition of this pathway probably reflects the effects of inhibiting upstream, transmembrane tyrosine kinases as well as downstream components that ultimately regulate cell cycle progression and survival.

The cytotoxicity of this combination for glioma cells was associated with the induction of apoptosis as shown by chromatin condensation and DNA fragmentation. We (Premkumar et al., 2006) and others (Daugas et al., 2000; Arnoult et al., 2002; Joseph et al., 2002) have shown that translocation of AIF and cytochrome *c* from the mitochondria to the nucleus is a critical step for the induction of apoptosis in human malignant glioma cells and that this process initiates nuclear condensation (Susin et al., 1999), chromatin fragmentation, and cell death (Joza et al., 2001). Consistent with these findings, we found translocation of cytochrome *c* and AIF from the mitochondria to the nucleus after treating the cells with sorafenib. Following coadministration of sorafenib and rottlerin, the normal distribution of mitochondrial cyto-

chrome *c* and AIF was lost within a few hours of drug treatment and redistributed to the nucleus.

In addition to the direct effects of oncogenic changes on cell proliferation and survival, the genomic alterations that occur during cancer progression are known to promote invasion and angiogenesis (Bos, 1989). An important factor underlying this phenotype is enhanced VEGF expression, which is commonly observed in malignant gliomas (Pore et al., 2003). The use of anti-VEGF antibodies has been extensively studied in preclinical in vivo models and has demonstrated an inhibition of tumor growth, including growth of glioma (Yuan et al., 1996). Induction of cell migration in response to mitogenic factors such as VEGF is a tightly regulated process requiring the coordination of a complex set of signals involving the extracellular matrix, the integrins and the actin cytoskeletal-associated motile apparatus (Nobes and Hall, 1995). Because of the role of ERK and PKC signaling in modulating cell migration, we examined the effect of sorafenib and rottlerin or the combination of both on glioma cell motility in response to VEGF stimulation. Our data demonstrated that sorafenib and rottlerin each significantly inhibited the VEGF-induced cell migration and that the combination of both agents largely abrogated cell motility, suggesting that these signaling agents may have applicability for blocking not only the proliferative but also the invasive features of malignant glioma cells.

In summary, the results of the present study indicate that treatment with sorafenib, an agent that was recently approved by the Food and Drug Administration for the treatment of patients with renal cell cancer and is undergoing additional phase II/III clinical evaluation in other tumor types (Wilhelm et al., 2004), has independent efficacy in glioma cells and, when administered in combination with the PKC inhibitor rottlerin, results in a striking increase in antiproliferative and cytotoxic activity, associated with mitochondrial injury and apoptosis. Strategies to antagonize PKC and ERK pathways in combination seem to be an attractive approach to enhance therapeutic efficacy in human glioma cells.

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