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

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Received May 29, 2006; accepted September 5, 2006

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-α 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-α 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 represent 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-depend-ent PKCs (α, βI, βII, and γ), novel or calcium-independent PKCs (δ, ε, η, and θ), or atypical PKCs (ζ and λ). In various cell types, certain PKC family members stimulate mitogen-
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 (Leiralid 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; Bor- nancin 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 (Meyer 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 rottrlerin, 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 rottrlerin of the growth inhibition and apoptosis in human malignant glioma cells induced by sorafenib.
approximately 2 to 4 mm were counted directly. All studies were performed in quintuplicate.

Anaxinn 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× 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 propidium iodide. In brief, cells grown exponentially to 40 to 50% confluence were exposed to the inhibitors or DMSO for a range of propidium iodide concentrations. In brief, cells grown exponentially to 40 to 50% confluence were exposed to the inhibitors or DMSO for a range of propidium iodide concentrations. In brief, cells grown exponentially to 40 to 50% confluence were exposed to the inhibitors or DMSO for a range of propidium iodide concentrations.

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 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× objective (Olympus, Tokyo, Japan). Samples were examined and images were collected. The figures were prepared using Photoshop software (Adobe Systems, Mountain View, CA).

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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× objective (Olympus, Tokyo, Japan). Samples were examined and images were collected. The figures were prepared using Photoshop software (Adobe Systems, Mountain View, CA).

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Analysis of Combinatorial Effects. Unless otherwise stated, data are expressed as mean ± 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).
No significant inhibition was seen in control cells treated with equivalent concentrations of vehicle (DMSO) in the absence 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).
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 G1-to-S transition as well as disable pro-apoptotic molecules, thus leading to dysregulated proliferation and enhanced tumor cell survival (Yarden and Sliwkowski, 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 G1 phase in a dose- (Fig. 3A) and time-dependent (Fig. 3B) manner with a concomitant decline in the percentage of cells in S and G2/M relative to controls. Simultaneous with the accumulation of cells in G1 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 G1 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.](https://jpet.aspetjournals.org/doi/10.1124/jpet.112.206822)
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\textsubscript{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

![Graphs showing the relationship between colony counts and concentration of the inhibitors.](image-url)
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 G1 Arrest and Apoptosis in Glioma Cells.** It has been shown that the PKC-δ signaling network activates several signaling pathways that subvert the G1-to-S transition as well as disable prosapoptotic 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 G1 phase in a dose- and time-dependent manner with a concomitant decline in the percentage of cells in S and G2/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 G1 arrest but also to cell death by apoptosis, indicated by an increase in the sub-G0/G1 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 VEGF165 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
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 G1 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-G1 components, suggesting that the cells were undergoing apoptosis.

Western blot analysis showed that sorafenib affected phos-
rottlerin. The normal distribution of mitochondrial cytochrome 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.
Acknowledgments

We thank Beth Arnold and Naomi Agostino for technical assistance.

References


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