Glutamatergic Pathway Targeting in Melanoma: Single-Agent and Combinatorial Therapies

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Abstract

Purpose: Melanoma is a heterogeneous disease where monotherapies are likely to fail due to variations in genomic signatures. B-RAF inhibitors have been clinically inadequate but response might be augmented with combination therapies targeting multiple signaling pathways. We investigate the preclinical efficacy of combining the multikinase inhibitor sorafenib or the mutated B-RAF inhibitor PLX4720 with riluzole, an inhibitor of glutamate release that antagonizes metabotropic glutamate receptor 1 (GRM1) signaling in melanoma cells.

Experimental Design: Melanoma cell lines that express GRM1 and either wild-type B-RAF or mutated B-RAF were treated with riluzole, sorafenib, PLX4720, or the combination of riluzole either with sorafenib or with PLX4720. Extracellular glutamate levels were determined by glutamate release assays. MIT assays and cell-cycle analysis show effects of the compounds on proliferation, viability, and cell-cycle profiles. Western immunoblotting and immunohistochemical staining showed apoptotic markers. Consequences on mitogen-activated protein kinase pathway were assessed by Western immunoblotting. Xenograft tumor models were used to determine the efficacy of the compounds in vivo.

Results: The combination of riluzole with sorafenib exhibited enhanced antitumor activities in GRM1-expressing melanoma cells harboring either wild-type or mutated B-RAF. The combination of riluzole with PLX4720 showed lessened efficacy compared with the combination of riluzole and sorafenib in suppressing the growth of GRM1-expressing cells harboring the B-RAF mutation.

Conclusions: The combination of riluzole with sorafenib seems potent in suppressing tumor proliferation in vitro and in vivo in GRM1-expressing melanoma cells regardless of B-RAF genotype and may be a viable therapeutic clinical combination. Clin Cancer Res; 17(22): 7080–92. ©2011 AACR.

Introduction

The incidence of melanoma has increased rapidly in the past 3 decades and has become a significant health risk in the United States (1). The treatment of early-stage melanoma is surgical resection, with more than 85% of patients in the early stages of disease experiencing long-term survival.

However, when melanoma metastasizes, the prognosis is poor, with few patients with a diagnosis of stage IV disease surviving past 5 years (2). Standard cytotoxic chemotherapeutic regimens have failed to alter the outcome in patients with advanced disease (3), and only the use of biological therapies based on interleukin-2 (IL-2) shows any effect in extending long-term survival (4). Over the past decade, our understanding of the genetic alterations that lead to melanogenesis and melanoma progression has advanced rapidly. Key signaling pathways involved in the pathogenesis and progression of melanoma, including the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/AKT, TGF-β, NF-kB, and others (5–7), suggest a molecularly complex and heterogeneous disease.

Our group has added to the understanding of aberrant signaling in melanoma by discovering that the ectopic expression of a G-protein–coupled receptor (GPCR), metabotropic glutamate receptor 1 (GRM1), in melanocytes was sufficient to induce spontaneous melanoma development in vivo with 100% penetrance (8). We also confirmed ectopic expression of GRM1 in a subset of human melanoma cell lines and biopsy samples (8). To date, we have
Inhibitors of GRM1 and B-RAF\textsuperscript{V600E} Suppress Melanoma Growth

Translational Relevance

Recently, PLX4720/PLX4032, the potent inhibitor of B-RAF\textsuperscript{V600E}, was reported to induce regression of melanocytic lesions in patients with late-stage melanoma. However, a significant number of these patients became refractory to the treatment, experiencing recurrence in less than 1 year. Previously, we identified aberrantly expressed metabotropic glutamate receptor 1 (GRM1) in approximately 65% of human melanoma biopsies and cell lines, suggesting the potential of developing GRM1-targeted therapies. A phase II trial with riluzole, a U.S. Food and Drug Administration–approved inhibitor of glutamate release, showed mixed clinical responses, with 42% of the patients exhibiting stable disease. Here, we show that the combination of riluzole plus sorafenib reduces the growth of wild-type or B-RAF\textsuperscript{V600E} human melanoma cells in vitro and in vivo. The PLX4720 and riluzole combination has additive effects in suppressing growth of B-RAF\textsuperscript{V600E} melanoma cells but less so than the sorafenib combination in the same cells in vitro and in vivo. These results propose a therapeutic regimen using riluzole and sorafenib to circumvent the drug resistance arising with the use of PLX4032 in patients with melanoma.

We have worked to unravel the causes and consequences of GRM1 signaling in this disease (9), as well as design therapeutic interventions that target GRM1 signaling. Earlier, we reported in vitro and in vivo preclinical findings using human melanoma cell lines that are wild type in B-RAF and N-RAS (C8161) or contain an N-RAS\textsuperscript{Q61R} mutation (WM239A). We showed that MAPK signaling is critical in GRM1-mediated oncogenesis (9) and have also shown that activation of the receptor using known GRM1 agonists results in an upregulation of the activated (phosphorylated) form of extracellular signal–regulated kinase (ERK; ref. 9). In addition, the majority of GRM1-expressing human melanoma cell lines tested exhibited elevated levels of extracellular glutamate, which promotes growth by activation of a glutamate autocrine loop. Suppression of GRM1 signaling by either GRM1 antagonists or a reduction in the levels of GRM1 ligand, glutamate, with a glutamate release inhibitor riluzole, resulted in decreased cell proliferation in vitro and tumorigenesis in vivo (9).

The U.S. Food and Drug Administration (FDA)-approved riluzole is a member of the benzothiazole class of compounds and acts as an inhibitor of glutamate release for the treatment of amyotrophic lateral sclerosis (ALS). The ability of riluzole to block the release of the ligand (glutamate) for GRM1 allows it to act functionally as a putative antagonist and interfere with intracellular events that follow stimulation of this receptor. With a low toxicity profile (11, 12), riluzole was deemed an excellent compound for use in preliminary studies on the effects of glutamate signaling inhibition on melanoma cells (9). To date, the reported modes of actions of riluzole in humans are inhibition of glutamate release, inactivation of voltage-dependent Na\textsuperscript{+} channels, and interference with G-protein–dependent signaling (11). In melanoma cells expressing GRM1, riluzole has been shown to inhibit cell proliferation in vitro and in vivo (9) as well as migration and invasion (13). Recently, a phase 0 clinical trial of riluzole in patients with advanced melanoma was conducted, with 34% of patients given riluzole showing measurable clinical responses. Some tumors decreased in size by more than 90% and exhibited suppression of MAPK and PI3K/AKT signaling pathways in posttreatment tumor samples (14). A recently completed phase II trial showed no Response Evaluation Criteria in Solid Tumors (RECIST); however, 42% of the patients exhibited stable disease, suggesting that riluzole has overall modest antitumor activity whose potential could be realized by combination with other anti-cancer agents (15).

As we continue with studies that target GRM1 signaling in melanoma, it is important to conduct preclinical studies using potential therapeutic agents that reflect the genetic diversity of this disease. Mutations in B-RAF have been identified in 8% of all cancers including more than 50% of melanomas (16). Most of these mutations are due to the substitution of a single amino acid at residue 600 in the B-RAF kinase domain resulting in constitutive activation of the RAF/MAP/ERK kinase (MEK)/ERK signaling pathway (16).

The small-molecule multikinase inhibitor sorafenib (17) has proven to be ineffective against melanoma as a single agent, but its use in combinatorial therapies may prove more effective in the clinic. A recently described specific small-molecule inhibitor specific to B-RAF kinase, PLX4720/PLX4032, was shown to have potent anti-melanoma activity in preclinical and clinical studies (18–20). However, its effectiveness has been hampered by the acquisition of drug resistance mechanisms including involvement of other RAF isoforms (21–24).

Given the high incidences of B-RAF\textsuperscript{V600E} mutations and GRM1 expression in numerous melanomas, we investigate cellular responses for the combination of an RAF inhibitor with riluzole, the putative antagonist of GRM1 signaling. Here, we provide data that show that combining inhibitors of RAF and GRM1 results in the suppression of human melanoma cell growth in vitro as well as tumorigenicity.
in vivo, suggesting that such a combinational therapy may be superior to either modality alone in patients with melanoma. The following report describes in vitro and in vivo preclinical experiments using GRM1-expressing human melanoma cell lines that harbor the most common mutation B-RAFV600E found in human melanomas. We show that the combination of riluzole with sorafenib seems potent in suppressing cell proliferation in vitro and in vivo in GRM1-expressing cells regardless of B-RAF status and may be a viable therapeutic clinical combination.

Materials and Methods

Antibodies and reagents
Antibodies against activated caspase-3, Ki-67, PARP, phospho- and total ERK, cleaved PARP, and Mcl-1 were obtained from Cell Signaling; antibody for α-tubulin, MTT cell viability assay solution 1, iodonitrotetrazolium chloride, and riluzole were obtained from Sigma; sorafenib was obtained from LC Laboratories; and PLX4720 was a gift from Plexxikon Inc.

Cell lines
UACC903 and UACC930 cells were provided by Dr. Jeffery Trent (The Translational Genomics Research Center, Phoenix, AZ) and 1205Lu cells were provided by Dr. Mary Hendrix (Children's Memorial Research Center, Chicago, IL). SKMEL2, SKMEL 187, and A2058 cells were purchased from American Type Culture Collection. The cells were maintained in RPMI plus 10% FBS. Human epidermal melanocytes were maintained in medium 254 (Invitrogen) supplemented with human melanocyte growth supplement (Invitrogen). Human epithelial kidney cells were maintained in Dulbecco's Modified Eagle's Medium plus 10% FBS.

MTT assays, cell-cycle analysis, and glutamate release
MTT cell viability assays were conducted as previously described (9). C8161, UACC903, 1205Lu, SKMEL 187, and A2058 melanoma cell lines were used in the MTT assays. Each cell line was cultured in 96-well plates (10^3 cells per well) with the following conditions: no treatment, vehicle alone (dimethyl sulfoxide, DMSO), riluzole (1, 5, 10, 25, or 50 μmol/L), sorafenib (1, 3, 5, or 7 μmol/L), or the combination of riluzole (10 μmol/L) and sorafenib (5 μmol/L), PLX4720 (0.01, 0.05, 0.1, or 0.3), or the combination of riluzole (10 μmol/L) plus PLX4720 (0.05 μmol/L). Viable cells were measured every day for 4 or 7 days. For cell-cycle analysis, UACC903, 1205Lu, and A2058 melanoma cell lines were used. Cell-cycle analysis was conducted at 24 and 48 hours of incubation of the cell lines in monolayer culture with no treatment, vehicle alone (DMSO), or 10 μmol/L riluzole. Cells were harvested at each time point and examined by propidium iodide staining followed by flow cytometry carried out by the Flow Cytometry Facility Core at Rutgers University as previously described (9). The Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (Invitrogen) was used to measure levels of glutamate.

Three-dimensional anchorage-independent assays
We conducted 3-dimensional colony assays using C8161, UACC903, SKMEL2, and 1205Lu human melanoma cell lines in the presence of vehicle (DMSO), riluzole (10 μmol/L), sorafenib (5 μmol/L), or the combination of riluzole (10 μmol/L) and sorafenib (5 μmol/L). The cells were suspended in 0.35% agar in RPMI supplemented with 10% FBS and plated on a layer of 0.75% agar in the same medium in 12-well culture plates (1 × 10^4 to 1.5 × 10^5 cells per well). Vehicle, riluzole alone, sorafenib alone, or riluzole and sorafenib were added in the agar underlay, as well as to the cells suspended in agar, on day 1. Every other day, the vehicle, or drug(s) was again added with 250 μL of complete medium. After 14 days, the colonies were stained with iodonitrotetrazolium chloride (Sigma) and photographed. The numbers of colonies were counted with the ImageJ software. Quantitation was done by comparing the total number of colonies from 3 representative photomicrographs from each experiment. The histograms represent the average of 3 independent experiments.

Western immunoblotting
Protein lysates were prepared as described previously (9). Briefly, media were removed and cells were washed once with ice-cold PBS. After removal of PBS, the extraction buffer was added directly to the plates and cells were collected with a cell scraper. Cells were incubated on ice for 20 minutes. Cell debris was removed by centrifugation at 25,000 × g at 4°C for 20 minutes and supernatant taken for Western immunoblot analysis. Western blotting was carried out with anti-PARP, anti-cleaved PARP, anti-phospho ERK, anti-total ERK, and anti-α-tubulin antibodies.

Xenografts in immunodeficient nude mice
The Institutional Review Board approved all animal studies for the Animal Care and Facilities Committee of Rutgers University. Nude mice were purchased from TacOnc. Cells were injected into 2 dorsal sites of each mouse at 10^6 cells per site. Tumor size was measured twice a week with a Vernier caliper and calculated as described (9). Once tumor volumes reached 6 to 10 mm^3, mice were divided into no treatment and treatment groups. The treatment groups received either vehicle (DMSO), riluzole (10 mg/kg), sorafenib (24 mg/kg), PLX4720 (20 mg/kg), or the combination of riluzole (5 mg/kg) and sorafenib (12 mg/kg) or riluzole (5 mg/kg) and PLX4720 (10 mg/kg) by oral gavage daily. The doses of oral riluzole, sorafenib, and PLX4720 were based on published reports (11, 12, 20). The experiments were terminated when the xenografts on the no-treatment group reached the maximum permitted size.

Immunohistochemistry
Tissue Analytical Services at the Cancer Institute of New Jersey carried out immunohistochemical staining on
excised tumor xenografts to detect changes in the number of apoptotic and proliferating cells (activated caspase-3 and Ki-67, respectively).

Results

The oncogenic transformation of various cell types by ectopic expression of GPCRs is characterized by the development of autocrine and paracrine loops that enhance cellular proliferation (25). Three melanoma cell lines (UACC903, 1205Lu, and A2058) containing the activating B-RAFV600E mutation exhibited elevated levels of extracellular glutamate similar to that previously described for wild-type B-RAF melanoma cells, C8161 and mutant RAFV600E cells WM239A (9), compared with cells that do not express the receptor or cells that contain a truncated, nonfunctioning GRM1 receptor, UACC930 melanoma cells (Fig. 1A). MTT cell viability assays were conducted to rule out that the increase in glutamate observed was not attributable to cell lysis, establishing that the cells themselves must be excreting glutamate into their surroundings in an attempt to establish autocrine activity (Fig. 1B). We next assessed the effects of the glutamate release inhibitor riluzole on the growth of human melanoma cells in monolayer culture. Standard MTT assays were conducted with 4 GRM1-expressing melanoma cell lines harboring wild-type forms of B-RAF and N-RAS (C8161 and SKMEL 187) or B-RAFV600E mutation (UACC903, 1205Lu, and A2058). We found that riluzole at a concentration of 25 or 50 μmol/L significantly decreased the number of viable cells as compared with no-treatment or vehicle (DMSO)-treated cells (Fig. 2A). Melanoma cells harboring a wild-type B-RAF (C8161 and SKMEL 187) were found to be much more sensitive to riluzole than those that contained a mutant copy of B-RAF (1205Lu and UACC903). This is in support of earlier reports that indicated that because both GRM1 and B-RAFV600E stimulate MAPK signaling, one of the key signaling pathways in human melanoma leading to metastasis, abolishing GRM1 signaling alone in cells that bear B-RAFV600E would not abolish overactivated MAPK (9).

We next obtained the cell-cycle profiles of riluzole (10 μmol/L)-treated UACC903, 1205Lu, and A2058 melanoma cells to assess the effects that it had on cell-cycle progression over time. All 3 cell lines yielded very similar results, with an example of UACC903 shown. At 24 hours posttreatment, about half of the cells were found to accumulate in the G2–M phase. By 48 hours, there was a 10- to 20-fold shift of the cell population to the sub-G1 phase of the cycle, indicative of apoptotic cell response (Fig. 2B). This apoptotic response was confirmed by an increase in the cleaved form of PARP by Western blot analysis. Control samples showed negligible amounts of cleaved PARP at 24 and 48 hours (Fig. 2C). These results were very similar to our previous report showing a similar G2–M cell-cycle arrest followed by apoptotic shift in GRM1-expressing human melanoma cell lines harboring wild-type B-RAF and N-RAS (C8161) or mutated N-RAS (WM239A) in the presence of riluzole (9), suggesting that depletion of the ligand (glutamate) to the receptor GRM1 by riluzole induces cell-cycle arrest and promotes apoptosis in GRM1-positive melanoma cells regardless of B-RAF genotype. To confirm this observation in vivo, we carried out xenograft experiments using single-agent riluzole as described (ref. 9; Fig. 2D). Briefly, UACC903 cells were injected into the dorsal flanks of nude mice. Tumors were allowed to grow to approximately 6 to 10 mm³, and mice were divided into groups to obtain relatively constant tumor volumes between each group (12 mice per group). Animals were treated daily with riluzole or vehicle (DMSO) by oral gavage. At day 18, there was a substantial difference between the tumor sizes of riluzole-treated animals compared with controls (Fig. 2D). Although riluzole on its own seems effective in inhibiting proliferation and inducing apoptosis in melanoma cells harboring activating B-RAF mutations in vivo, it is
less effective at doing so than in melanoma xenografts harboring wild-type B-RAF (9). Clinically, these observations suggest it is likely that administration of a single-agent riluzole will not be as effective in patients whose melanomas contain a mutated form of B-RAF.

Tumors are composed of heterogeneous cell populations. For this reason, we started to explore potential combinatorial therapies that would include riluzole as one of the components to treat heterogeneous tumor populations in an attempt to slow the progression of this disease. We choose sorafenib, a multikinase inhibitor that has been shown to inhibit RAF signaling and whose toxicity profile is known in vivo (17, 26), and PLX4720, a recently described specific small-molecule inhibitor for B-RAFV600E (18–20). We treated 3 GRM1-expressing human melanoma cell lines (C8161, UACC903, and 1205Lu) with riluzole, sorafenib, or a combination of both riluzole and sorafenib for 7 days and assessed cell proliferation and viability by MTT assays (Fig. 3). In the presence of riluzole alone, C8161 cell line has the highest reduction in the number of viable cells, confirming our earlier report (ref. 9; Fig. 3A). UACC903 and 1205Lu cells are also positive for GRM1 expression and harbor a mutated B-RAF (B-RAFV600E). These cell lines were not as sensitive to riluzole (Fig. 3B and C). In the presence of sorafenib, the opposite responses were observed; UACC903 and 1205Lu cells displayed a substantial decrease in the number of viable cells in comparison with C8161 cells. A combination of 10 µmol/L riluzole with 5 µmol/L sorafenib led to synergistic, inhibitory effect on the proliferation C8161 cells (Fig. 3A), and
an additive, inhibitory effect on UACC903 and 1205Lu cells (Fig. 3B and C) when analyzed as described (27). To assess whether combining PLX4720 with riluzole would also yield the additive effect observed with sorafenib, we treated UACC903 and C8161 cells with riluzole, PLX4720, or the combination of both. The IC50 for PLX4720 in UACC903 cells was determined to be 0.1 μmol/L (Fig. 3B). UACC903 cells treated with a combination of 10 μmol/L riluzole and half the IC50, 0.05 μmol/L PLX4720 exhibited additive inhibitory activity when compared with either single agent alone (Fig. 3B). As expected wild-type B-RAF, GRM1-positive C8161 cells show only slight inhibition in cell proliferation with higher concentrations of PLX4720 (10 μmol/L) and no increase in efficacy when combined with riluzole (data not shown).

To further predict the results obtained in 2-dimensional assays in a model more closely related to in vivo, we carried out 3-dimensional, anchorage independence assays using 4 GRM1-positive melanoma cell lines: C8161 (wild-type B-RAF and N-RAS wild type), UACC903 (B-RAF

\[ RF \text{V600E} \]), and 1205Lu (B-RAF

\[ RF \text{V600E} \]) melanoma cells treated with different concentrations of riluzole, sorafenib, PLX4720, or the combination of the compounds at half of the concentration of each one alone. NT, no treatment; DMSO, vehicle-only treatment: Ril, riluzole; Sor, sorafenib; PLX, PLX4720.
colonies in UACC903 (Fig. 4A and B). In 1205Lu cells, riluzole or sorafenib alone yielded a 30% reduction in colony formation whereas the combination of both resulted in a 55% decrease in the number of colonies (Fig. 4A and B). In SKMEL2, riluzole alone had a modest effect, decreasing colony formation by 18%, whereas sorafenib was more efficacious at decreasing colony formation. The combination treatment yielded a 62% decrease (Fig. 4A and B) compared with the control group. These observations further strengthen our hypothesis that a combination of riluzole and sorafenib would be able to inhibit tumor cell proliferation more effectively than either agent alone, regardless of the presence or absence of activating mutations in B-RAF or N-RAS in the cells. Given these findings, we carried out combinatorial in vivo experiments using C8161, UACC903, and 1205Lu xenografts.

In the xenograft studies, all cell lines used express GRM1 but differ in B-RAF genotype, with C8161 being wild type and UACC903 and 1205Lu containing the activating mutation. In C8161 xenografts, there was a significant decrease in the tumor volumes in animals treated with riluzole alone, confirming our previous report (9). Administration of sorafenib on its own did not yield a significant decrease in tumor size, and the combination of riluzole with sorafenib at half the dose used in either one alone yielded a considerable reduction in tumor volume (Fig. 5A). In the human melanoma cell lines with mutated
Figure 5. Suppression of human melanoma xenograft growth. Xenografts of (A) C8161 (B-RAF and N-RAS wild type), (B) UACC903 (B-RAFV600E), and (C) 1205Lu (B-RAFV600E). The groups were no treatment (NT), vehicle (Veh; DMSO), riluzole (10 mg/kg), sorafenib (24 mg/kg), or the combination of riluzole (5 mg/kg) and sorafenib (12 mg/kg). Tumor volume (mm³) was a mean ± SD of 12 mice per group. *, P < 0.01 when riluzole- or sorafenib-treated UACC903 samples were compared with no-treatment or vehicle controls at day 18. **, P < 0.001 when riluzole-treated C8161 or the combination of riluzole plus sorafenib-treated C8161, UACC903, or 1205Lu samples were compared with no-treatment or vehicle controls. D, xenograft experiments of UACC903 treated with either riluzole (10 mg/kg), PLX4720 (20 mg/kg), or a combination of riluzole (5 mg/kg) and PLX4720 (10 mg/kg). *, P < 0.05 in comparison with no-treatment or vehicle controls. E and F, excised UACC903 xenograft tumors from (B) and stained with activated cleaved caspase-3 (E) and Ki-67 (F). Arrows indicate clusters of caspase-3-positive cells. Below each representative image is the average of positively stained cells from 10 randomly selected fields. *, P < 0.01 comparison between riluzole-treated with no-treatment or vehicle (DMSO) controls.
B-RAF, UACC903 and 1205Lu, differential responses were detected. UACC903 xenografts showed very similar, statistically relevant responses with riluzole or sorafenib alone (Fig. 5B). The combination of riluzole and sorafenib yielded a higher reduction in tumor volume than either compound alone (Fig. 5B). 1205Lu xenografts were found to be more sensitive to riluzole, sorafenib, or the combination of both reagents than UACC903 xenografts (Fig. 5C). It was noted that 1205Lu xenografts were more responsive to the combination therapy than UACC903 xenografts in spite of their common B-RAFV600E genotype, indicating that other mutations persistent in these cells must influence their response. In addition, immunohistochemical analyses were conducted on excised xenografts with antibodies against the cleaved form of caspase-3, to detect apoptotic cell death and Ki-67 to detect changes in cell proliferation. An example of excised UACC903 xenograft tumors is shown. Single-agent riluzole, sorafenib, or the combination of both compounds treated samples showed a substantial increase in the number of positive caspase-3 cells in comparison with the controls (Fig. 5E). Conversely, the number of Ki-67–positive cells was reduced in either single-agent or combined treatments (Fig. 5F). It is equally important to point out that riluzole had a more potent effect on C8161 and 1205Lu cell lines despite the disparity in B-RAF status than UACC903. A combination of riluzole and sorafenib, though at half the concentration when used alone, was effective against all 3 xenografts (Fig. 5A–C). In vivo xenograft studies were also conducted to evaluate the efficacy of riluzole and PLX4720 combination in UACC903 cells. Interestingly, PLX4720 alone was not as potent as riluzole (Fig. 5D); furthermore, when we combined half the doses of riluzole and PLX4720, we did not detect further suppression of tumor progression as we observed with similar dosing with riluzole and sorafenib combination (cf. Fig. 5B with Fig. 5D). Efficacy of combination of riluzole and PLX4720 against the wild-type B-RAF melanoma cell line C8161 was not evaluated with PLX4720 in vivo, as it has been shown by others to be ineffective in inducing apoptosis in vitro and in vivo (20) and has also been shown to promote cell growth through activation of the MAPK pathway in a C-RAF–dependent manner (28).

Preclinical and clinical trials conducted with sorafenib, PLX4720, and riluzole showed a reduction in levels of activated ERK, supporting the notion that MAPK is a target for all 3 compounds (14, 17, 20). We carried out Western immunoblotting with protein lysates prepared from in vitro cultured cells or excised in vivo xenografts treated with sorafenib, PLX4720, and riluzole either alone or in combination as described earlier. Riluzole inhibits the MAPK pathway as measured by a decrease in levels of ERK phosphorylation in a cell line–dependent manner (Fig. 6A and B). Sorafenib was found to highly suppress ERK phosphorylation in UACC903 and 1205Lu cells than in C8161 cells. The combination was, however, capable of suppressing ERK phosphorylation in all 3 cell lines. PLX4720 was found only to suppress ERK activity in the B-RAFV600E cell line UACC903 as a single agent or in combination but not in the C8161 cell line (Fig. 6B). Protein lysates obtained with harvested xenografts showed similar results (data not shown). The effect of the combinational drugs on the proapoptotic protein Mcl-1, which has been shown to be downregulated by sorafenib (29, 30), was investigated as a possible target for additive and synergistic inhibition in tumor growth. A reduction in Mcl-1 levels was detected in sorafenib-treated UACC903 and 1205Lu cells, whereas the combination of riluzole and sorafenib led to a reduction in Mcl-1 in all 3 cell lines (Fig. 6C). PLX4720, however, does not downregulate the levels of Mcl-1 either by itself or in combination with riluzole (Fig. 6C).

Discussion

Several groups have proposed the concept that the glutamatergic system may play a role in tumor biology, and intriguing links between neurodegenerative diseases and cancer have been put forth by several investigators (31, 32). For instance, the incidence of melanoma among patients with ALS or Parkinson disease is 2 to 3 times higher than that of the general population in multicenter studies in Australia and North America (33). These observations are in line with earlier reports that elevated levels of extracellular glutamate have been detected in several human disorders including gliomas, multiple sclerosis, Alzheimer disease, Parkinson disease, and ALS (34, 35), suggesting that the common root of many of these diseases may be glutamate.

GRMs are members of the 7-transmembrane domain GPCR family (36). They are divided into 3 groups on the basis of sequence homology, agonist selectivity, and effector coupling, with all GRMs having glutamate as their natural ligand. GRM1 and GRM5 are composed of group I GRMs and are mainly involved in excitatory responses induced by strong presynaptic stimulation. Group I GRMs are coupled to a Gq-like protein and stimulate phospholipase C beta (PLCβ; ref. 37). It has been reported that in melanoma cells, GRM1 stimulation results in the activation of PLCβ, which, in turn, converts phosphatidylcholinol to 2 second messengers, inositol triphosphate and diacylglycerol. Diacylglycerol activates protein kinase C, which could stimulate both MAPK and PI3K/AKT pathways (38, 39). Activation of these 2 major signaling cascades is central for transformed cell survival, migration, invasion, epithelial–mesenchymal transition, and angiogenesis (40).

Our group described a heretofore unknown component of melanoma pathogenesis. A transgenic murine model of melanoma was constructed by the ectopic expression of GRM1 in melanocytes (8). These mice spontaneously develop melanocytic lesions very similar to human melanoma. We have expanded these original studies and have now shown that more than 60% of human melanomas express the human form of this receptor and that activation of this receptor results in activation of the MAPK and PI3K/AKT pathways in a B-RAF- and N-RAS–independent fashion (9). The functionality of GRM1 in GRM1-expressing human melanoma cells was shown by the
the proliferation of GRM1-positive cells, presumably as a result of glutamate signaling inhibition on melanoma cells expressing GRM1 (9). Depletion of glutamate in human melanoma cells led to reduced extracellular glutamate levels and inhibited responsiveness of these cells to stimuli and inhibitors of GRM1 (9). Studies by others showed that wild-type GPCRs can become tumorogenic when exposed to an excess of locally produced or circulating ligands and agonists (41, 42), whereas other GPCRs harboring mutations in key conserved residues can have transforming activity even in the absence of their ligands (43). It has also been found that the level of expression of GPCRs is not as important to oncogenesis as the fact that the receptor is expressed (44). On the basis of these earlier results, we assessed levels of GPCRs, including GRM1, on Mcl-1 levels. Western immunoblots examining the effect of (A) riluzole (Ril, 10 μmol/L), sorafenib (Sor, 5 μmol/L), or combination of riluzole (10 μmol/L) and sorafenib (5 μmol/L) on MAPK pathway activation as indicated by levels of ERK phosphorylation in C8161 (B-RAF and N-RAS wild type), UACC903, and 1205Lu (B-RAFV600E) cell lines in vitro. B, Western immunoblots examining the effects of riluzole (10 μmol/L), PLX4720 (PLX, 0.05 μmol/L), or the combination 10 and 0.05 μmol/L, respectively, on UACC903 and C8161 cell lines. Samples were normalized to total ERK. NT, no treatment; Veh, vehicle. C, Western immunoblots examining the effects of riluzole (10 μmol/L), sorafenib (5 μmol/L), and PLX4720 (0.05 μmol/L) on UACC903 and 1205Lu or 10 μmol/L C8161 or the combination of 10 μmol/L riluzole and 5 μmol/L sorafenib or 10 μmol/L riluzole and 0.05 μmol/L PLX4720 on Mcl-1 levels.

glomerate exerts its growth-promoting abilities. Riluzole, being FDA approved for the treatment of ALS, was deemed an excellent compound for use in preliminary studies that could be translated into clinically relevant trials assessing the effects of glutamate signaling inhibition on melanoma cells (9).

Phase 0 and phase II clinical trials with riluzole, which functions as a putative antagonist of GRM1 signaling, showed modest antitumor activity as a single agent (14, 15). It is possible that activating mutations in B-RAF, or other unidentified genetic factors, affect how GRM1-expressing tumor cells respond to riluzole therapy because GRM1 signals through other pathways, such as Wnt/β-catenin (45), in addition to the MAPK and PI3K/AKT pathways (9, 39, 46, 47). We therefore extended our preclinical studies to include melanoma cells carrying the most commonly known mutations in B-RAF (B-RAFV600E). We found...
that melanoma cells, which harbor the B-RAFV600E mutation, were less sensitive to the single-agent riluzole in both in vitro MTT cell viability proliferation and anchorage-independent colony assays. We began to examine different combinations of riluzole and other inhibitors of downstream targets. We used sorafenib, a small-molecule inhibitor originally identified as an RAF kinase inhibitor that also inhibits several receptor tyrosine kinases involved in tumor progression and tumor angiogenesis (17). We also investigated PLX4720, a specific B-RAF V600E inhibitor (20). Sorafenib is FDA approved for the treatment of hepatocellular carcinoma and is also a second-line agent in renal cell carcinoma. Recent reports stressing the importance of C-RAF in B-RAF wild-type melanomas has revived interest in the use of sorafenib, in combination with other agents, for the treatment of melanoma. We now report that the combination of riluzole and sorafenib has an additive or synergistic effect in both B-RAF mutant and B-RAF wild-type melanoma cells in vitro and in vivo. In addition to B-RAF inhibition, sorafenib is a well-documented multikinase inhibitor of VEGF and other receptor tyrosine kinases (17). PLX4720/PLX4032 showed remarkable preclinical results in in vitro and in vivo studies in suppressing melanoma cell growth. However, patients from these clinical trials were shown to become resistant to treatment, with recurrence of melanoma occurring 5 to 9 months after start of their treatment. This stresses the need to reexamine the options in targeting melanoma effectively (48).

In cultured cell studies, sorafenib was not very effective in suppressing C8161 cell growth whereas it was effective in reducing the number of viable cells in both UACC903 and 1205Lu melanoma cell lines with mutated B-RAF. Surprisingly, the combinatorial in vitro studies in C8161 cells using riluzole and sorafenib showed a synergistic reduction in the number of viable cells while exerting an additive effect detected in UACC903 and 1205Lu cell lines under similar conditions. These results were again observed in in vivo xenograft studies where the combination of riluzole and sorafenib again led to a considerable reduction in tumor progression as evident by the decrease in tumor volumes over time in all 3 cell lines compared with controls. It is thus possible that sorafenib enhances the cytotoxic effects of riluzole through suppression of downstream targets of GRM1 signaling including the MAPK pathway.

Stimulation of GRM1 was shown to modulate MAPK via the ERK-mediated signaling pathway in GRM1-expressing human melanoma cells. We postulate that riluzole decreases the levels of glutamate released from the cells disrupting the autocrine loops, whereas sorafenib mediates its activities through inhibition of MAPK signaling, resulting in a more profound inhibition in tumor cell growth and progression than with either agent alone in GRM1-expressing melanoma cells. It is however important to point out that riluzole seems to suppress the MAPK pathway in a cell line–dependent manner, suggesting that it is not the main pathway suppressing proliferation with riluzole treatment. Recently, an alternative mode of action of riluzole has been described, with riluzole serving as an enhancer of the Wnt/β-catenin signaling pathway, which induces melanoma cells to revert to a more normal melanocytic phenotype promoting hyperpigmentation and reducing their proliferation and metastasis (45).

PLX4720 displayed remarkable clinical responses as a single agent. Surprisingly, when combined with riluzole, we did not detect further reduction in tumor cell growth in MTT or xenograft studies. This is in variance with the remarkable results observed with the combination of riluzole and sorafenib in vivo. We hypothesize that the encouraging results observed with the combination of sorafenib and riluzole is likely due to the role of sorafenib as a chemosensitizer by elimination of the proapoptotic protein Mcl-1, resulting in enhanced cytotoxic response to riluzole, which has modest efficacy as a single agent. Elimination of Mcl-1 by sorafenib has been shown to be through translational inhibition in a variety of cancer cell lines (29, 49). In melanoma, depletion of Mcl-1 enhances melanoma cell death by therapeutic compounds such as temozolomide and melphalan (30), sensitizes apoptosis resistance melanoma cells to Fas-mediated apoptosis (50), and renders melanoma cells susceptible to anoikis (51). Similar to other reports, we detected reduced levels of Mcl-1 only in sorafenib-treated B-RAFV600E human melanoma cells. Surprisingly, in C8161 melanoma cells with wild-type BRAF, a decrease in Mcl-1 was also detected in the presence of riluzole and sorafenib, suggesting that the reduced tumorigenicity observed in vivo may be mediated via a decline in Mcl-1. In light of these results, it is not surprising that sorafenib but not PLX4720 sensitizes the cells to riluzole.

Considering that the majority of human melanomas harbor B-RAF mutations, agents used to treat melanoma in the clinical setting need to function in the presence of these mutations. Our findings suggest that the combination of riluzole and sorafenib would be a reasonable, combinatorial therapy for the treatment of patients with advanced melanoma and is currently undergoing clinical testing in a phase I clinical trial in patients with advanced melanomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Correction: Glutamatergic Pathway Targeting in Melanoma: Single-Agent and Combinatorial Therapies

In this article (Clin Cancer Res 2011;17:7080–92), which was published in the November 15, 2011, issue of Clinical Cancer Research (1), Fig. 5D was mislabeled because of a production error. The corrected figure appears below.

Reference

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