

# Metabotropic Glutamate Receptor 1 and Glutamate Signaling in Human Melanoma

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## Abstract

Recently, several laboratories have started to investigate the involvement of glutamate signaling in cancer. In previous studies, we reported on a transgenic mouse model that develops melanoma spontaneously. Subsequent studies in these mice identified that the aberrant expression of metabotropic glutamate receptor 1 (GRM1) in melanocytes played a critical role in the onset of melanoma. Confirmation of the etiologic role of GRM1 in melanoma development was shown in a second transgenic line with GRM1 expression under the regulation of a melanocyte-specific dopachrome tautomerase promoter. Ectopic expression of GRM1 was also detected in a subset of human melanoma cell lines and biopsies, suggesting that aberrant expression of GRM1 in melanocytes may contribute to the development of human melanoma. GRM1, a seven-transmembrane domain G protein-coupled receptor, is normally expressed and functional in neuronal cells, and its ligand, glutamate, is the major excitatory neurotransmitter. Human melanoma cells are shown here to release elevated levels of glutamate, implying a possible autocrine loop. Treatment of GRM1-expressing human melanoma cells with a GRM1 antagonist (LY367385 or BAY36-7620) or a glutamate release inhibitor (riluzole) leads to a suppression of cell proliferation as well as a decrease in levels of extracellular glutamate. Treatment of human melanoma cell xenografts with riluzole for 18 days via p.o. gavage or i.v. injection leads to inhibition of tumor growth by 50% in comparison with controls. These data suggest the importance of glutamate signaling in human melanoma and imply that the suppression of glutamate signaling may be a new target for melanoma therapy. [Cancer Res 2007;67(5):2298–305]

## Introduction

The incidence of melanoma has been increasing for the past several years. In the United States, more than 60,000 patients are estimated to be diagnosed with melanoma with ~8,000 deaths in 2006 (1). The overall lifetime risk of developing melanoma is 1 in 77 for women and 1 in 52 for men (1). Early detection is the key for increased chance of survival; however, for late-stage melanoma, currently there is no assuring therapeutic means. Animal models have been a valuable tool to understand human disorders and to

identify new therapeutic targets for the treatments of a given disease. Our laboratory has developed a mouse model for melanoma and identified that the aberrant expression of metabotropic glutamate receptor 1 (GRM1) in melanocytes induces spontaneous melanoma development *in vivo* (2, 3). The neoplastic role of GRM1 is verified by a second transgenic line with GRM1 expression targeted to melanocytes by the use of a melanocyte-specific dopachrome tautomerase promoter (4). Furthermore, we showed that a subset of human melanoma cell lines and biopsy samples displays aberrant GRM1 expression, suggesting involvement of GRM1 in some cases of human melanoma (4).

GRM1 is normally expressed and functional in the mammalian central nervous system (CNS) and is implicated in learning and memory formation (5). GRM1 belongs to the glutamate receptor family, which is divided into two major groups: ionotropic glutamate receptors (iGluR) and metabotropic glutamate receptors (mGluR). mGluRs are seven-transmembrane domain G protein-coupled receptors and are further subdivided into three groups based on sequence homology and downstream signaling. GRM1 and GRM5 belong to group I mGluRs. Expression of GRM1 has not been detected in melanocytes, whereas expression of GRM5 has been detected in melanocytes (6). Previously, our laboratory has shown that GRM5 does not play any role in melanoma development (7). Several pharmacologic agents have been used extensively as agonists and antagonists of GRM1 to elucidate the functions of GRM1 in the CNS (8). L-quisqualate is an agonist for group I mGluRs (8), whereas either LY367385 or BAY36-7620 is identified as a competitive or noncompetitive antagonist of GRM1, respectively (9, 10). These pharmacologic agents were crucial in previous studies on GRM1 signaling in mouse melanoma cell lines (11).

Our laboratory has shown that stimulation of GRM1 by its agonist, L-quisqualate, leads to the activation of mitogen-activated protein kinase (MAPK) in mouse melanoma cell lines (11). Constitutively activated MAPK pathway, specifically extracellular-signal regulated kinase (ERK), has been reported for human melanoma cell lines and biopsies (12–14). In these earlier studies, the presence of BRAF-activating mutation (V600E) is the driving force for the activation of ERK. In this current report, we show that MAPK pathway can be activated in GRM1-positive human melanoma cell lines by GRM1 agonist in the absence of the V600E BRAF-activating mutation.

In recent years, glutamate signaling in cancer has been a focus of investigation by several laboratories. The antiproliferative potential of iGluR antagonists has been examined in several types of cancers, including breast, colon, astrocytoma, and lung (15). Treatment of tumor cells with iGluR antagonists results in inhibition of tumor cell proliferation and motility (15). Dizocilpine, the iGluR antagonist, can suppress the growth of metastatic lung adenocarcinoma through inhibition of the MAPK signaling

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pathway (16). In addition to iGluR and GRM1, GRM4 has been reported to be overexpressed in colorectal cancer (17). These studies implicate the involvement of glutamate signaling in tumor development through mGluRs and/or iGluRs. In this current report, we show the release of glutamate by several human melanoma cell lines. Treatment of these melanoma cells with GRM1 antagonists or an inhibitor of glutamate release, riluzole, suppresses glutamate release and cell growth. In addition, riluzole diminishes tumor growth in a xenograft model of human melanoma cells. Taken together, these results suggest that GRM1 and glutamate signaling may be used as novel targets for melanoma therapy.

## Materials and Methods

**Antibodies and reagents.** Anti-phosphorylated ERK, anti-ERK, and anti-poly(ADP-ribose) polymerase (PARP) were purchased from Cell Signaling (Danvers, MA); GRM1 antibodies were purchased from BD Biosciences (Franklin Lakes, NJ) and ImmunoStar, Inc. (Hudson, WI); and monoclonal  $\alpha$ -tubulin antibody, myo-inositol, and riluzole were obtained from Sigma (St. Louis, MO). DMSO was purchased from Fisher Scientific (Pittsburgh, PA). L-quisqualate [(L)-(+)-a-amino-3,5-dioxo-1,2,4-oxadiazolidine-2-propanoic acid] and LY367385 [(S)-(+)-a-amino-4-carboxy-2-methylbenzeneacetic acid] were purchased from Tocris (Ellisville, MO). BAY36-7620 [(3aS,6aS)-6a-naphthalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[c]-furan-1-on] was obtained from Bayer (West Haven, CT).

**Cell culture.** Primary human epidermal melanocytes (HEM) were purchased from Cascade Biologics (Portland, OR) and maintained in Medium 254 and human melanocyte growth supplements. UACC930, UACC903, and A2058 were provided by Dr. Jeffrey M. Trent (Translational Genomics Research Center, Phoenix, AZ). WM239A and WM35 were from Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). C8161 and C81-61 were from Dr. Mary J.C. Hendrix (Children's Memorial Research Center, Chicago, IL). Melanoma cells were grown in RPMI 1640 plus 10% fetal bovine serum (FBS). For glutamate measurement or induction experiments with GRM1 agonist, to minimize glutamate in the medium, customized glutamine- and glutamate-free RPMI 1640 (Invitrogen-Life Technologies, Carlsbad, CA) was used with 10% dialyzed FBS (Invitrogen-Life Technologies) and supplemented with 2 mmol/L GlutaMax (Invitrogen-Life Technologies). For the measurement of inositol-1,4,5-triphosphate (IP3), customized glutamine- and glutamate-free RPMI 1640 was additionally deprived of inositol (Invitrogen-Life Technologies).

**Western immunoblots.** Protein lysates were prepared as described previously (18). In short, cells were washed with ice-cold PBS. Extraction buffer was added and cells were collected. After incubation on ice for 20 min, supernatants were collected by centrifugation at 4°C. Protein concentration was determined using DC protein assay kit (Bio-Rad, Hercules, CA). Routinely, 25  $\mu$ g of protein lysates were loaded for Western immunoblots.

**IP3 measurements.** IP3 was measured as described previously with minor modifications (11, 19). After overnight incubation in the presence of 3  $\mu$ Ci of myo-[<sup>3</sup>H]inositol (3.22 TBq/mmol; GE Healthcare, Piscataway, NJ), cells were incubated in fresh glutamate/inositol/serum-free RPMI 1640 with LiCl (10 mmol/L) for 15 min in the presence or absence of LY367385 (10  $\mu$ mol/L) before stimulation with L-quisqualate (10  $\mu$ mol/L) for 15 min. The reactions were terminated and samples were either washed with the addition of 1 mL of a 1:1 mixture of 1,1,2-trichlorotrifluoroethane (Sigma) and tri-n-octylamine (Sigma) or washed twice with water-saturated diethyl ether (Sigma). Levels of incorporated [<sup>3</sup>H]inositol in IP3 were measured by a scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

**DNA transfection.** Transfections of DNA were done with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate liposomal transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Dominant-negative GRM1 (dnGRM1) constructs were provided by Dr. Anna Francesconi (Albert Einstein College of Medicine, Bronx, NY; refs. 11, 20). DNA transfections were done with 0.5  $\mu$ g of DNA per 60-mm plate.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assays.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done according to the manufacturer's protocol (Roche). Briefly, 10<sup>3</sup> cells were plated in 96-well plate and treated with various compounds as indicated. Absorbance was measured by GENios plate reader (Tecan, Durham, NC) for the time points indicated.

**Measurement of extracellular glutamate.** Amplex Red Glutamic Acid/Glutamate Oxidase assay kit (Invitrogen-Molecular Probes) was used to measure the amount of glutamate released in the medium. Cells were grown in medium devoid of glutamate and glutamine but supplemented with GlutaMax (2 mmol/L) for 3 days. Cells were plated at 10<sup>3</sup> cells per well with 200  $\mu$ L of medium containing specific compounds with concentration as indicated in 96-well plate. After specified time, 100  $\mu$ L of medium were collected for measurement of the amount of glutamate released according to the manufacturer's protocol. Cells left with ~100  $\mu$ L of medium in the wells were used to confirm the viability of cells by MTT cell proliferation assays.

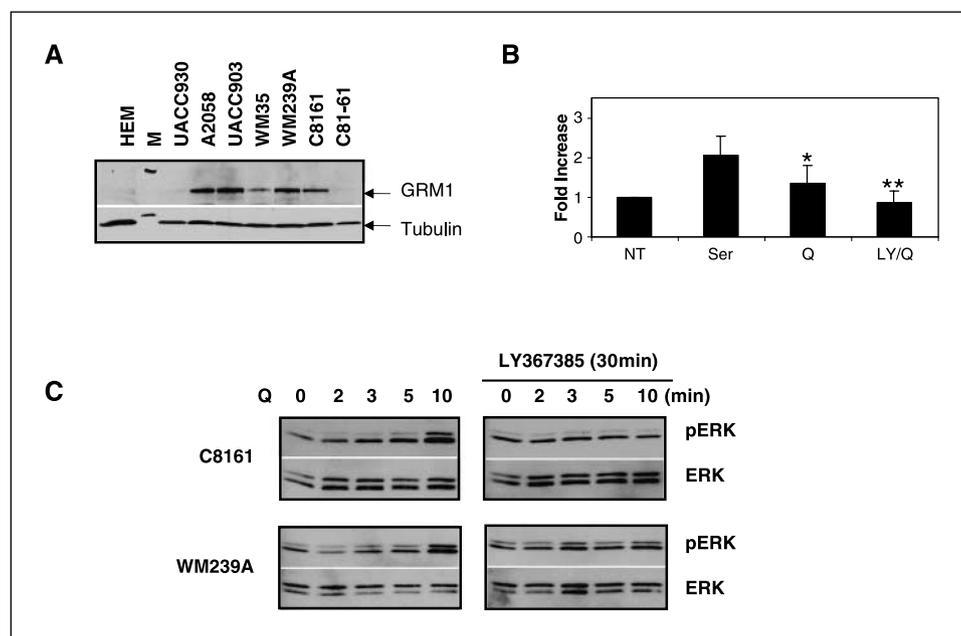
**Cell cycle analysis.** Cells were plated at 2  $\times$  10<sup>6</sup> per 100-mm culture plate and treated as indicated. After 24 and 48 h, cells were collected and washed twice with ice-cold PBS. Cell pellets were fixed by drop-wise addition of ice-cold 70% ethanol and incubated for 20 min at 4°C. Fixed cells were washed twice with ice-cold PBS and resuspended in 500  $\mu$ L PBS. Cells were treated with RNase A solution (20  $\mu$ g/mL; Sigma) and labeled with propidium iodide (50  $\mu$ g/mL; Sigma) for 30 min. Cell cycle analysis was done by the Flow Cytometry Facility Core at Rutgers University (Piscataway, NJ) using a Beckman Coulter system (Epics XL-MCL model).

**Xenografts in immunodeficient nude mice.** All animal studies were approved by the Institutional Review Board for the Animal Care and Facilities Committee of Rutgers University. Nude mice were purchased from Taconic (Hudson, NY). Human melanoma cells, C8161, were injected into the dorsal area at 10<sup>6</sup> cells per site. Tumor size was measured twice weekly with a Vernier caliper and calculated as described (16). Treatment with either vehicle (DMSO) or 7.5 mg/kg riluzole was given daily via p.o. gavage or i.v. when tumor volumes reached 6 mm<sup>3</sup>. After 18 days of treatment, experiments were terminated due to tumor burden, as tumor volume had reached 300 mm<sup>3</sup> in some animals.

## Results

**Functional GRM1 in human melanoma cells.** Earlier, we showed GRM1 expression in several human melanoma cell lines and biopsy samples by reverse transcription-PCR and Western immunoblots (4). Subsequent analysis of additional human melanoma cell lines and biopsies showed that ~40% of them were positive for GRM1 expression. An example of immunoblots of several human melanoma cell lines and normal primary HEMs is shown (Fig. 1A). Expression of GRM1 was detected in some human melanoma cell lines but not in HEM. Previously, our laboratory showed MAPK to be an important signaling pathway in melanoma cell lines derived from our transgenic lines (11). MAPK is one of the key signaling pathways in human melanoma (21). We were interested to know if MAPK pathway was also critical in GRM1-positive human melanoma cells. It is well known that the common BRAF-activating mutation (V600E) constitutively stimulates MAPK signaling (14); therefore, genotypes of BRAF and N-Ras were assessed by DNA sequencing in GRM1-positive human melanoma cell lines. C8161 did not have the most common mutations at either BRAF (codon 600) or N-Ras (codons 12, 13, and 61); however, WM239A displayed a mutation in BRAF (V600D). Most of the other cell lines showed the most common BRAF mutation (V600E; data not shown).<sup>5</sup> In human melanoma cell lines that bore the

<sup>5</sup> M. Herlyn and S. Kazianis, personal communication.



**Figure 1.** A, GRM1 protein expression was detected in several human melanoma cell lines by immunoblots. HEM was used as a normal melanocyte control, and  $\alpha$ -tubulin was used as a loading control. B, the functionality of GRM1 was examined by GRM1 agonist-induced IP3 accumulation. Two human melanoma cell lines (C8161 and WM239A) were stimulated with L-quisqualate alone (10  $\mu$ mol/L; Q) for 15 min or pretreated with LY367385 (10  $\mu$ mol/L) for 15 min followed by stimulation with L-quisqualate (LY/Q) for 15 min. Data were expressed relative to no treatments (NT). FBS (10%) was used as a positive control (Ser). Columns, average of three independent experiments of a representative cell line (WM239A); bars, SD. \*,  $P < 0.05$ , compared with no treatment ( $t$  test); \*\*,  $P < 0.05$ , compared with L-quisqualate ( $t$  test). Statistically significant elevated levels of accumulated IP3 are detected in L-quisqualate-treated cells. Pretreatment of these cells with specific antagonist of GRM1 LY367385 abolished L-quisqualate-induced IP3 accumulation. C, stimulation of GRM1 in human melanoma cell lines led to the activation of ERK. Treatments of human melanoma cells with GRM1 agonist (10  $\mu$ mol/L; Q) up to 10 min resulted in ERK phosphorylation. Pretreatment of these cells with LY367385 (10  $\mu$ mol/L) for 30 min before L-quisqualate induction resulted in no modulation of phosphorylated ERK (pERK; C8161 and WM239A). The membrane was stripped and reprobbed with total ERK.

most common activating mutation in BRAF (V600E), such as UACC903, MAPK pathway was constitutively activated. As a consequence, stimulation with GRM1 agonist did not lead to further activation of ERK (data not shown). Therefore, cell lines bearing the V600E BRAF mutation were excluded from further studies. C8161 and WM239A were selected for subsequent analysis of the involvement of GRM1 signaling in human melanoma.

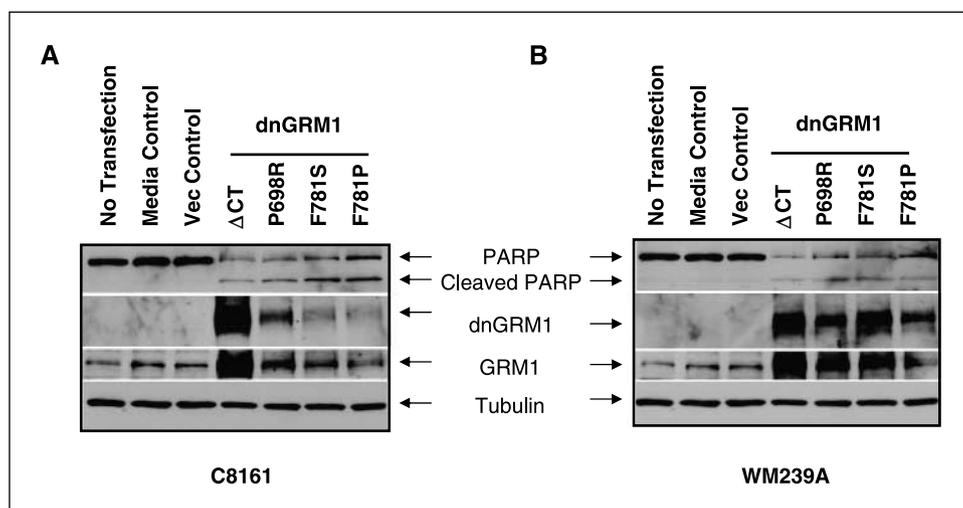
Previously, we showed in mouse melanoma cell lines that GRM1 signals through Gq protein followed by phospholipase C, leading to activation of second messengers, diacylglycerol and IP3 (11). To examine the functionality of GRM1 in C8161 and WM239A, the cells were stimulated with GRM1 agonist, L-quisqualate (Q), and the accumulation of IP3 was measured (Fig. 1B). Cells were divided into four groups: no treatment, serum as a positive control, treatments with group I mGluR agonist (L-quisqualate), or preincubation with GRM1-specific antagonist, LY367385, followed by induction with L-quisqualate. To minimize the amount of glutamate, the natural ligand of GRM1, glutamate- and glutamine-free media were used for the measurement of IP3 supplemented with GlutaMax. We did three independent experiments with C8161 and WM239A human melanoma cell lines and representative data are shown. In the presence of serum, an increase in levels of IP3 accumulation was detected when compared with no treatment. Treatment with GRM1 agonist (L-quisqualate) for 15 min resulted in a statistically significant increase in the accumulation of IP3. The specificity of L-quisqualate-induced increase in IP3 accumulation was shown by the absence of IP3 accumulation when these cells were pretreated with GRM1 antagonist (LY367385) followed by induction with L-quisqualate. The functionality of GRM1 in these

human melanoma cells was further confirmed by GRM1 agonist-induced ERK phosphorylation (Fig. 1C). L-quisqualate-induced ERK activation was inhibited when these cells were pretreated with LY367385 for 30 min before induction with L-quisqualate. Taken together, these results showed that GRM1 receptors in these human melanoma cell lines were functional and responded to GRM1 agonist and antagonist.

**Induction of apoptosis by dnGRM1.** To further investigate GRM1 functionality and activity in human melanoma cells, two different but complimentary means were used to suppress GRM1 function. First, we took advantage of dnGRM1. These mutants have a small deletion ( $\Delta$ CT 694-695,  $\Delta$ CT) or single base substitutions (P698R, F781S, and F781P) in the intracellular loop 2 or 3, which had been shown to be critical in GRM1 signaling (11, 20). Human melanoma cell lines C8161 (Fig. 2A) and WM239A (Fig. 2B) were transfected with vector control or four different dnGRM1 mutants. At 24 h after transfection, protein lysates were collected for Western immunoblots. PARP cleavage is a well-known apoptotic marker by the appearance of the cleaved form at 89 kDa. PARP cleavage was detected only in dnGRM1-transfected samples but not in vector control (Fig. 2, top). Second panels show the levels of exogenously transfected GRM1 to verify the presence of dnGRM1 in these cells. dnGRM1 clones were made from a wild-type GRM1 cDNA from rat brain (20); therefore, anti-GRM1 antibody that only recognizes the rodent forms of GRM1 was used. Apoptotic marker was only observed in samples that had been transfected with dnGRM1.

**Inhibition of human melanoma cell proliferation by GRM1 antagonists.** As a second approach, we used GRM1 antagonists to

**Figure 2.** dnGRM1 induced apoptosis in C8161 (A) and WM239A (B). Human melanoma cells were transfected with vector control or dnGRM1. Apoptosis was assessed by the presence of the cleaved form of PARP at 89 kDa at 24 h after transfection. dnGRM1 constructs were derived from cloned wild-type rat GRM1 cDNA (20). GRM1 antibody that only recognizes the rodent form of GRM1 was used to show the levels of transfected GRM1 (dnGRM1). The membrane was stripped and reprobed with GRM1 antibody that recognized both endogenous (human) and transfected GRM1 (GRM1). Negative controls included no transfection and medium control, where growth media were changed to Opti-MEM for 6 h during the transfection.  $\alpha$ -Tubulin was used as a loading control.



examine GRM1 functionality and activity in human melanoma cells. Several GRM1 antagonists had been described previously (8). They are divided into two major groups: competitive and noncompetitive. LY367385, a competitive antagonist, binds to the same site as the natural ligand glutamate (9). BAY36-7620, one of the noncompetitive antagonists, binds to the transmembrane domain of the receptor resulting in stabilization of inactive conformation (10). We used MTT cell proliferation assay to assess growth response of human melanoma cells to the presence of competitive or noncompetitive antagonist.

Human melanoma cells were grown in the presence of different concentrations of LY367385 in RPMI 1640 devoid of glutamate and glutamine supplemented with GlutaMax because LY367385 competes with the natural ligand glutamate for the binding to GRM1 receptor (Fig. 3A). The growth of primary HEMs requires special medium supplemented with several growth factors, which contain  $\sim 70$   $\mu\text{mol/L}$  glutamate. In the absence of these factors and glutamate, the growth of HEM was inhibited.<sup>6</sup> Therefore, human embryonic kidney (HEK) cells were used as a normal control instead of HEM. Cell proliferation was measured for 4 days, and the growth of C8161 was inhibited by 70% in the presence of 500  $\mu\text{mol/L}$  LY367385. There was only a negligible effect on the growth of HEK cells at this concentration. In a parallel set of cells under same conditions, protein lysates were prepared and levels of phosphorylated ERK were examined (Fig. 3B). A dose-dependent decrease in levels of phosphorylated ERK was detected in cells treated with 100 or 500  $\mu\text{mol/L}$  of LY367385 in comparison with no treatment or vehicle treated. These results showed that treatment of GRM1-positive human melanoma cells with LY367385, a competitive GRM1 antagonist, resulted in suppression in cell proliferation and that this suppression is likely, in part, due to inhibition of MAPK signaling as indicated by a decrease in levels of activated phosphorylated ERK.

Next, the growth of human melanoma cells was examined in the presence of a GRM1 noncompetitive antagonist, BAY36-7620. As a noncompetitive antagonist, BAY36-7620 does not compete for the binding site with the natural ligand glutamate; therefore, regular growth media were used for both human melanoma cells and

HEM. MTT cell proliferation assays were done with different concentrations of BAY36-7620 (10–50  $\mu\text{mol/L}$ ) for 4 days and only data for day 4 are shown (Fig. 3C). At 30  $\mu\text{mol/L}$  BAY36-7620, only 30% of C8161 cells were viable, whereas  $>60\%$  of HEM cells were viable. Cell cycle analysis indicated that BAY36-7620-treated C8161 cells showed an increase in the sub- $G_1$  phase after 48 h of treatment, suggesting an induction of apoptosis by BAY36-7620 (data not shown). Protein lysates were prepared under the same conditions, and PARP cleavage was used as an apoptotic marker. Apoptosis was induced by BAY36-7620 at 50  $\mu\text{mol/L}$  after 48 h as shown by the cleaved form of PARP in comparison with controls (Fig. 3D). These data indicated that a noncompetitive antagonist of GRM1, BAY36-7620, inhibited human melanoma cell growth and induced apoptosis, suggesting that GRM1 could be a target in human melanoma therapy.

**Inhibition of glutamate release by GRM1 antagonists in human melanoma cells.** It was puzzling to see only 15% inhibition of melanoma cell growth by 100  $\mu\text{mol/L}$  LY367385 in C8161 cells (Fig. 3A), whereas 10  $\mu\text{mol/L}$  LY367385 was sufficient in the suppression of L-quisqualate-induced ERK activation (Fig. 1C). Studies on mouse melanoma cells and mouse melanocytic clones stably expressing GRM1 showed higher levels of released glutamate than normal mouse melanocytes or vector controls.<sup>7</sup> In light of these results, levels of released glutamate by several human melanoma cell lines were examined. Each day, half of the media were collected and the amount of released glutamate was determined. MTT assays were done to ensure that these cells were viable. Again, because HEM, normal human melanocytes, required growth factors as well as glutamate to grow, HEK cells were used as a control. Regardless of whether they express GRM1, all human melanoma cells examined released more glutamate than HEK. In fact, substantial amount of glutamate was released into the medium, especially by C8161 cells. An example of glutamate released by HEK, C8161, and WM239A is shown (Fig. 4A, left). After 4 days, C8161 released  $\sim 200$   $\mu\text{mol/L}$  glutamate into the medium, which was  $\sim 10$  times the amount released by HEK. Although very little glutamate was released by HEK cells, MTT assay showed their vigorous growth (Fig. 4A, right). Next, we were interested to know if

<sup>6</sup> J. Namkoong, unpublished observation.

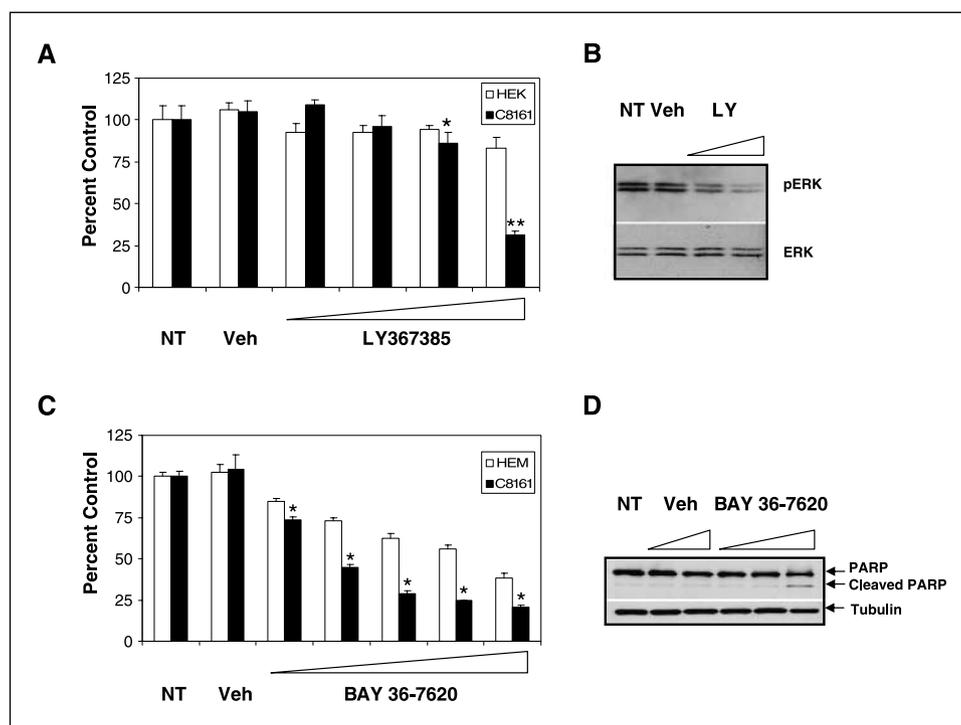
<sup>7</sup> S. Shin, in preparation.

GRM1 antagonists could inhibit the release of glutamate by these cells. Treatment of C8161 cells with LY367385 resulted in a dose- and time-dependent suppression in the levels of released glutamate (Fig. 4B, left). When C8161 cells were treated with 500  $\mu\text{mol/L}$  LY367385, <30% of glutamate was released compared with controls, which correlated with MTT assays. Under the same conditions, very little influence on the glutamate released was detected in LY367385-treated HEK cells (Fig. 4B, right). These results suggested that a higher concentration of LY367385 was required to inhibit GRM1-positive melanoma cell growth due to the constant release of glutamate by these cells. Suppression of glutamate release was also detected in cells treated with BAY36-7620, the noncompetitive GRM1 antagonist (Fig. 4C). BAY36-7620 seemed to be more potent in the suppression of glutamate release than LY367385.

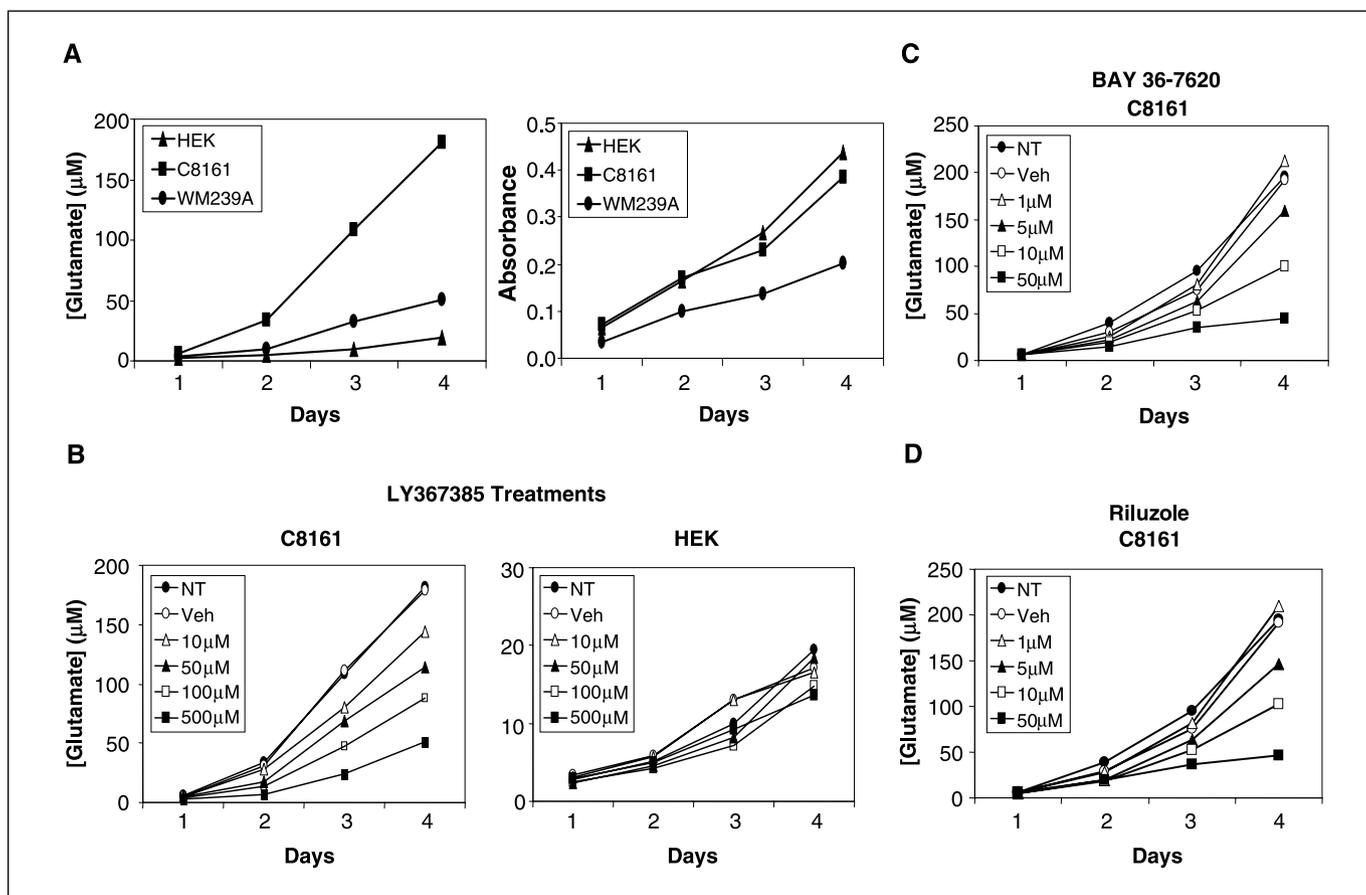
**Inhibition of cell proliferation by glutamate release inhibitor riluzole.** Riluzole is a Food and Drug Administration (FDA)-approved drug for amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease). ALS is a degenerative motor neuron disease that gets progressively worse with time. The actual cause of ALS is not known; however, excessive glutamate, a major neurotransmitter, has been proposed to be one of the factors that promotes neuronal excitotoxicity leading to ALS (22). In clinical trials, the inhibitor of glutamate release, riluzole, is shown to slow down the progression of ALS (23, 24).

Our previous experiment suggested a correlation between levels of released glutamate and cell proliferation (see above). Based on these results, human melanoma cells were treated with riluzole. Riluzole-treated C8161 cells released reduced levels of glutamate (Fig. 4D). Suppression of glutamate release by riluzole also reduced the growth of C8161 human melanoma cells (Fig. 5A). At 40  $\mu\text{mol/L}$  riluzole, <25% of C8161 cells were viable, whereas >70% of HEM cells were viable, suggesting that HEM cells were less sensitive under similar conditions. Cell cycle analysis on riluzole-treated C8161 cells showed the accumulation of cells in the G<sub>2</sub>-M phase at 24 h (Fig. 5B, top). By 48 h, there was a substantial increase in cells accumulated in the sub-G<sub>1</sub> phase of the cell cycle, suggesting cellular apoptosis (Fig. 5B, bottom). To confirm these observations, PARP cleavage was examined by Western immunoblots with C8161 cell lysates prepared at 24 and 48 h after riluzole treatment (Fig. 5C). After 24 and 48 h of treatment with 10, 25, and 50  $\mu\text{mol/L}$  of riluzole, cleaved forms of PARP were detected in C8161. Results indicated that treatments with riluzole inhibited growth of human melanoma cells and induced cell cycle arrest leading to apoptosis. These results prompted us to validate the antiproliferative, proapoptotic action of riluzole in human melanoma cells *in vivo*.

**Inhibition of human melanoma cell xenograft growth by riluzole.** C8161 cells were inoculated s.c. into nude mice at  $10^6$  per site. Based on the experiments done previously by others, the



**Figure 3.** A, biological responses of human melanoma cells to LY367385 were measured. Cells were treated with LY367385 at concentrations of 10, 50, 100, and 500  $\mu\text{mol/L}$ , respectively. No treatment (NT) and 500  $\mu\text{mol/L}$  NaOH (Veh) were used as controls. Measurement of cell viability/growth was carried out by MTT assays for 4 d. Only measurements on day 4 are shown here. Growth of C8161 cells was suppressed in a dose-dependent manner, whereas NaOH virtually had no effect on cell growth (black columns). LY367385 had minimal growth-inhibitory effect on HEK cells (white columns). Bars, SD. \*,  $P < 0.05$ , compared with HEK (*t* test); \*\*,  $P < 0.001$ , compared with HEK (*t* test). B, the status of ERK phosphorylation after treatments with LY367385 was examined by immunoblots at day 4. C8161 cells were not treated (NT) or treated with 100 or 500  $\mu\text{mol/L}$  of LY367385 or with 500  $\mu\text{mol/L}$  NaOH (Veh). Protein lysates were prepared for Western immunoblots and probed with phosphorylated ERK. The same membranes were stripped and reprobed with total ERK. C, biological consequences of BAY36-7620 treatments in HEM (white columns) or C8161 (black columns) cells were measured by MTT cell viability assays. Cells were either not treated (NT), vehicle treated (DMSO), or treated with 10, 20, 30, 40, or 50  $\mu\text{mol/L}$  of BAY36-7620, respectively, for 4 d. In the presence of BAY36-7620, the growth of C8161 cells was suppressed in a dose-dependent manner, whereas vehicle treatment had very little or no effect on cell growth. Only measurements on day 4 are shown here. HEM cells were less sensitive to BAY36-7620 in comparison with C8161 cells. Bars, SD. \*,  $P < 0.001$ , compared with HEM (*t* test). D, levels of cleaved PARP were examined by Western immunoblots in C8161 treated with 10, 25, or 50  $\mu\text{mol/L}$  of BAY36-7620 for 48 h. Elevated levels of cleaved PARP were only observed in C8161 cells treated with 50  $\mu\text{mol/L}$  BAY36-7620 compared with DMSO treated (Veh) or no treatment (NT). The same blot was probed with  $\alpha$ -tubulin to show equal loading.



**Figure 4.** A, human melanoma cell lines and HEK cells were examined for levels of released glutamate. *Left*, cells were plated in 96-well plates at  $10^3$  cells per well and half of the media ( $100 \mu\text{L}$ ) were collected to measure the amount of glutamate at indicated times; *right*, cells in the remaining half of the media ( $100 \mu\text{L}$ ) were proceeded to MTT cell proliferation assays. Media without cells were used as controls in each experiment (data not shown). At least three independent experiments were done. All human melanoma cells, especially C8161, displayed elevated levels of extracellular glutamate in comparison with the control HEK. B, the ability of the competitive GRM1 antagonist LY367385 to inhibit the release of glutamate was examined in C8161 and HEK cells at different concentrations for up to 4 d. The vehicle control was  $500 \mu\text{mol/L}$  NaOH. A dose-dependent inhibition of release of extracellular glutamate was observed only in LY367385-treated C8161 cells, whereas very little, if any, inhibition was detected in HEK cells under the same conditions. C, treatment of C8161 cells with BAY36-7620, the noncompetitive GRM1 antagonist, also suppressed glutamate release. DMSO was used as a vehicle control. D, C8161 cells were treated with the glutamate release inhibitor riluzole. Riluzole treatment of C8161 cells suppressed glutamate release. DMSO was used as a vehicle control.

maximum tolerated dose of riluzole<sup>8</sup> was 20 mg/kg. Mice treated with this dose for 2 years had no carcinogenic effects. Based on this information and our pilot experiments, mice were treated with 7.5 mg/kg riluzole either by p.o. gavage or i.v. when tumor volume had reached  $6 \text{ mm}^3$ . Mice were treated every day for 18 days, and tumor sizes were measured twice weekly with a Vernier caliper. A significant reduction in tumor volume was observed only in mice treated with 7.5 mg/kg riluzole by either i.v. (Fig. 6A) or p.o. gavage (Fig. 6B) compared with untreated or vehicle-treated controls. Taken together, these results suggest the possibility of using riluzole to treat human melanoma.

## Discussion

Glutamate signaling involves a large group of proteins, such as mGluRs, iGluRs, and glutamate transporters with their effectors. Involvement of glutamate signaling in the brain tumor, glioma, has

been under investigation for many years (25, 26). Recently, the association of glutamate receptors with cancer of nonneuronal origin has been the topic of research by several groups. Several antagonists of iGluR were shown to inhibit tumor growth and decrease in tumor cell motility in cancers, including breast, colon, astrocytoma, and lung (15). Dizocilpine, iGluR antagonist, has also been shown to suppress the growth of metastatic lung adenocarcinoma through the inhibition of the MAPK signaling pathway (16). In this report, we showed the importance of GRM1 signaling by GRM1 antagonists or dnGRM1. Treatment of human melanoma cells with LY367385, a GRM1-specific competitive antagonist, exerted dose-dependent inhibition of cell proliferation by suppressing the activation of ERK, suggesting that activated GRM1 in human melanoma cells mediates its signals in part through the MAPK cascade, one of the major cell proliferation pathways. In addition, a noncompetitive GRM1 antagonist, BAY36-7620, inhibited cell growth and promoted apoptosis. Furthermore, transient transfection of dnGRM1 into human melanoma cells induced apoptosis as shown by PARP cleavage.

We showed that human melanoma cells released elevated levels of glutamate, the natural ligand of GRM1, into the medium.

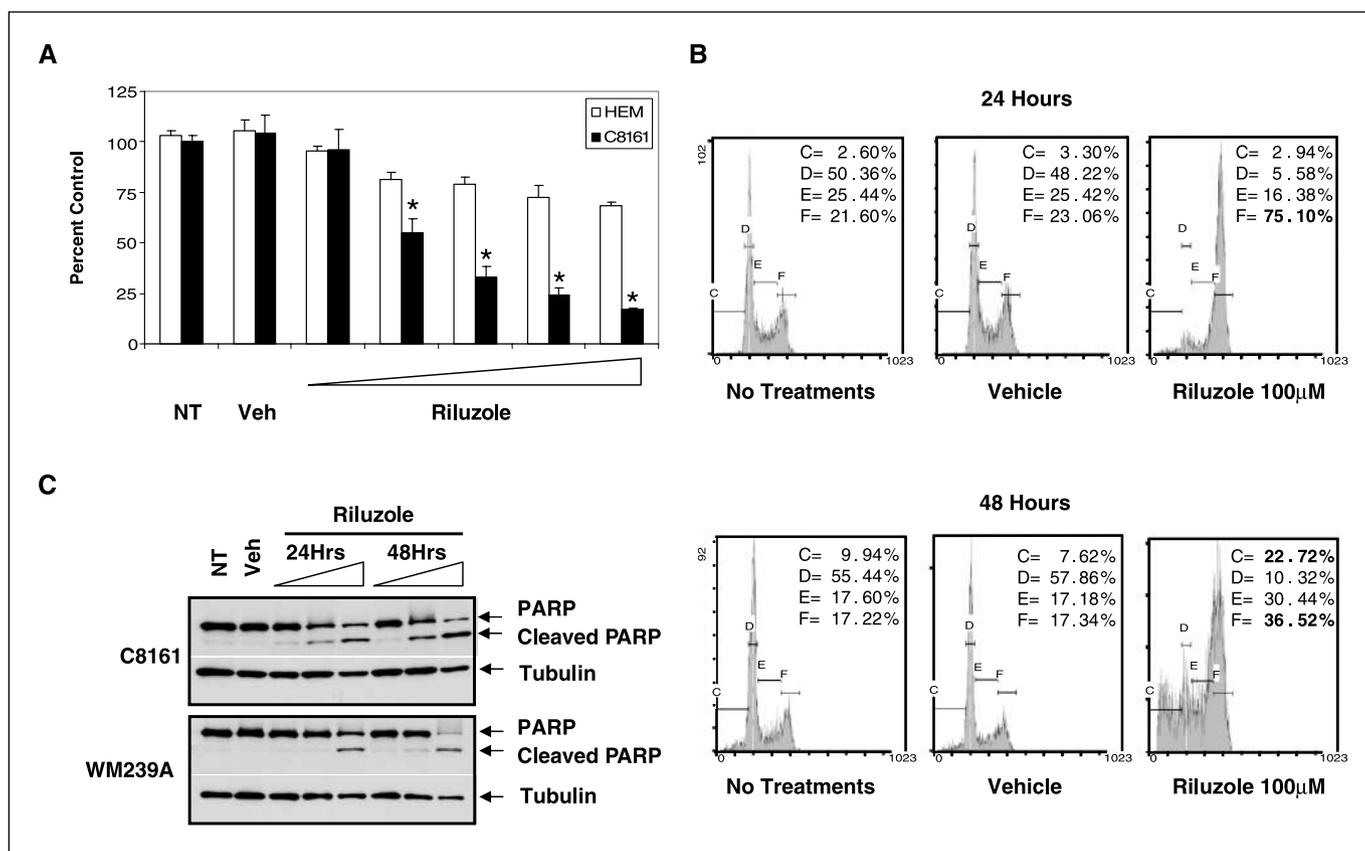
<sup>8</sup> Sanofi-Aventis US. Rilutek (riluzole) prescribing information. c2005-2006 [updated 2006 May 1; cited 2006 Nov 7]. Available from: <http://products.sanofi-aventis.us>.

Suppression of release of glutamate by pharmacologic agents (LY367385, BAY36-7620, and riluzole) correlated with a reduction in human melanoma cell growth. The direct involvement of GRM1 in glutamate release has not yet been investigated extensively. However, it has been reported that the two systems work together to regulate extracellular glutamate levels: glutamate uptake transporters and cystine-glutamate exchangers (27). In studies of the rat prefrontal cortex, suppression of glutamate uptake as well as stimulation of group I mGluRs (GRM1 and GRM5) by their agonist, (*R, S*)-3,5-dihydroxy-phenylglycine, leads to an increase in the accumulation of extracellular levels of glutamate (27). This accumulation is inhibited by LY367385, a GRM1 antagonist. The investigators speculated that LY367385 is also an effective antagonist of cystine-glutamate exchangers; however, the possibility of GRM1-mediated signaling to cystine-glutamate exchangers could not be ruled out. Together, these results suggest that GRM1 or its signaling may be associated with glutamate release.

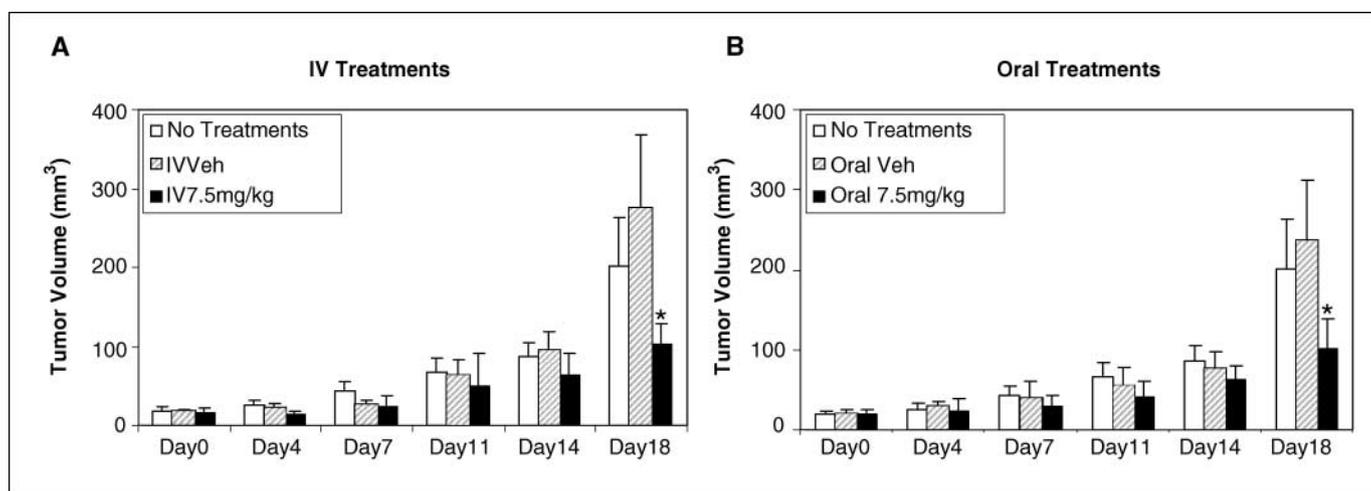
Glutamate is one of the major excitatory neurotransmitter. There are several regulatory mechanisms involved in regulation of release of glutamate, considering excessive glutamate leads to neuronal cell death, excitotoxicity. Even as low as 2 to 5  $\mu\text{mol/L}$  of extracellular glutamate are sufficient to cause excitotoxicity in the

mammalian CNS (22). Regulation of glutamate release in neurons involves iGluRs, glutamate transporters, as well as G protein signaling. In glioma, the skull restricts tumor growth due to spatial restriction (26). It has been hypothesized that, in order for glioma cells to overcome the spatial restriction, excessive glutamate is released to kill nearby neurons, therefore creating enough space for tumor cells to grow. Excitotoxicity of neurons by glioma cells has been shown by coculture of glioma cells with neurons (26). In addition, when glioma cell clones were implanted to rat brain to examine tumor growth, the subclone that release more glutamate developed more aggressive forms of tumors than the original parental glioma cells (25). Interestingly, brain is one of the common metastatic sites for melanoma (28). It is possible that, when melanoma metastasizes to brain, excess glutamate promotes tumor growth and induces excitotoxicity in a very similar mode of action as described for glioma.

Riluzole is the first FDA-approved drug for ALS; however, there are only slight benefits to overall survival (22). The mechanisms of action of riluzole include inhibition of glutamate release, inactivation of voltage-dependent  $\text{Na}^+$  channels, and interference with G protein-dependent signaling (29). Riluzole protects neurons by suppression of glutamate release as well as suppression of ion channels. These neuroprotective properties by



**Figure 5.** A, MTT cell proliferation assays were used to assess the biological consequences of C8161 (black columns) and HEM (white columns) treated with riluzole. Cells were plated on 96-well plate at  $10^3$  cells per well and treated with 10, 20, 30, 40, or 50  $\mu\text{mol/L}$  of riluzole. A dose-dependent suppression of C8161 cell growth by riluzole was detected in comparison with no treatment (NT) and DMSO treated (Veh). Only day 4 measurements are shown here. HEM cells were less sensitive to riluzole under similar conditions. Bars, SD. \*,  $P < 0.001$ , compared with HEM (*t* test). B, cell cycle analysis was done with C8161 cells treated with riluzole at 24 h (top) and 48 h (bottom). At 24 h after treatment, cells were accumulated in the G<sub>2</sub>-M phase of the cell cycle. At 48 h after treatment, a 3-fold increase in the sub-G<sub>1</sub> phase of the cell cycle was detected. DMSO treatment (Vehicle) had little or no effects. Each phase of cell cycle was indicated as C (sub-G<sub>1</sub>), D (G<sub>1</sub>), E (S), and F (G<sub>2</sub>-M), and the percentage of cells in each phase was given. C, to investigate the apoptotic response of human melanoma cells to riluzole, cells were plated and treated with 10, 25, or 50  $\mu\text{mol/L}$  of riluzole for 24 or 48 h. Protein lysates were extracted for Western immunoblots. After 24 and 48 h of riluzole treatments, apoptosis was induced in C8161 cells as evident by PARP cleavage compared with no treatment (NT) or DMSO treated (Veh). The same blot was probed with  $\alpha$ -tubulin to show equal loading.



**Figure 6.** Therapeutic potential of riluzole was investigated by C8161 xenograft model. C8161 human melanoma cells were inoculated s.c. in 6-week-old nude mice at  $10^6$  cells per site. When the tumor volumes had reached  $6 \text{ mm}^3$ , mice were treated with 7.5 mg/kg riluzole daily by i.v. (A) or p.o. gavage (B). Tumor sizes were measured twice weekly with a Vernier caliper. Treatments were terminated after 18 d. Whether given by i.v. or p.o. gavage, a reduction in the tumor volumes was detected in riluzole-treated mice in comparison with untreated or DMSO-treated (Veh) controls. Bars, SD. \*,  $P < 0.01$ , compared with untreated and DMSO treated (*t* test).

riluzole have been used to target several neurologic conditions, including basal ganglion disease (29). We showed that treatment of GRM1-positive human melanoma cells with riluzole results in the reduced levels of released glutamate, the suppression of human melanoma cell growth, as well as induction of apoptosis. Furthermore, riluzole is shown to be effective in suppression of tumor growth in a xenograft model. Taken together, these results suggest the involvement of glutamate signaling in human melanoma and that riluzole maybe a good candidate for human melanoma therapy.

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