Results

Elevated circulating plasma glutamate levels in a Grm1-driven melanoma model

We derived TGS mice from crosses between melanoma-prone TG-3 (11, 36–38) with hairless SKH-1. Onset and progression of the pigmented lesions are very similar in TG-3 and TGS mice; in the absence of hair the pigmented lesions are readily visible in TGS mice. Homozygous TGS mice that harbor two copies of the disrupted endogenous *Grm1* gene succumb to large tumor burden by 4 to 5 months old; thus, they are not included in our studies. Heterozygous $Grm1^{+/-}$ TGS mice with only one copy of the disrupted endogenous *Grm1* are viable, show highly pigmented tumors, and bear large tumor burden by 11 to 12 months of age, indicating that Grm1 signaling stimulates melanomagenesis. An image addressing the visual (phenotypic) difference between heterozygous a TGS mouse and a wild-type TGS mouse is shown in Fig. 1A. Comparison of glutamate levels in circulating blood plasma between 6-month-old heterozygous TGS and wild-type (no disrupted *Grm1*) mice, heterozygous TGS mice showed elevated glutamate levels (Fig. 1B), suggesting aberrant Grm1 expression may promote an increase in its natural ligand, glutamate, in circulation to ensure constitutive activation of Grm1 receptor; similar observation was made in *in vitro* culture cells (17).

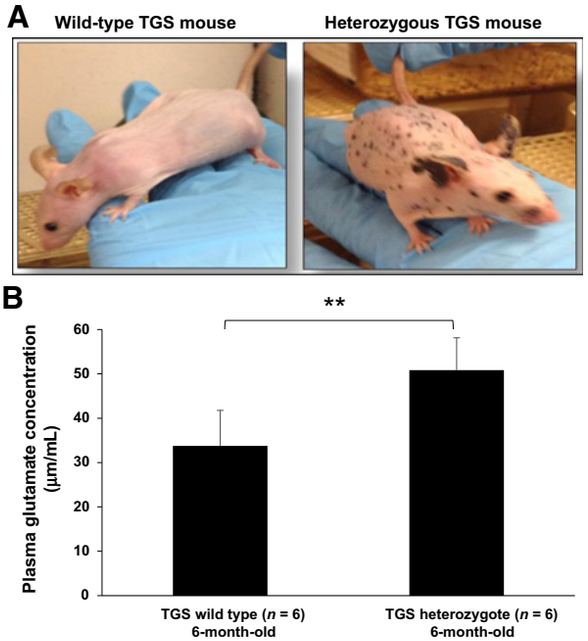

Figure 1.

A, Phenotypes of wild-type and heterozygous TGS genotypes. Pigmented lesions on the skin of heterozygous TGS mice are easily visualized compared with the wild type without any copies of the disrupted endogenous metabotropic glutamate receptor 1 (*Grm1*) gene. Homozygous and heterozygous TGS mice are indistinguishable in the progression of the disease; the major difference is the onset of the disease, 2 to 3 months for homozygous TGS and 5 to 7 months for heterozygous TGS. **B,** Elevated circulating glutamate levels in plasma isolated from heterozygous TGS mice. Glutamate concentration in plasma isolated from wild-type (6-month-old) or heterozygous (6-month-old) TGS mice was measured using the Glutamate Determination Kit (GLN1, Sigma-Aldrich) according to the manufacturer's instructions. Data are given as μmoles of glutamate per mL of plasma and are represented as mean ± SD ($n = 6$). Student *t* test was used to calculate statistical significance. **, $P < 0.01$.

Elevated GLS detected in GRM1$^+$ human melanoma cell lines

Ectopic expression of GRM1 is sufficient to induce cellular transformation *in vitro* and spontaneous melanoma development *in vivo* (11). To investigate a possible relationship between GRM1 expression, altered glutamate bioavailability, and glutaminase (GLS), we first confirmed GRM1 expression in C8161, UACC903, and 1205Lu human melanoma cells, plus immortalized normal human melanocytes, *TERT*/*CDK4*^{R24C}/*TP53*^{DN} (AR7119). C8161 is a malignant human melanoma cell line that expresses wild-type B-Raf proto-oncogene, serine/threonine kinase (BRAF). UACC903 and 1205Lu are other malignant melanoma cell lines that harbor the *BRAF*^{V600E} mutation. C8161, UACC903, and 1205Lu demonstrated significantly elevated levels of GRM1 and GLS compared with AR7119 human melanocyte control cells with almost undetectable GRM1 and much lower GLS expression (Fig. 2).

GLS inhibition reduces proliferation/viability of GRM1$^+$ human melanoma cells

In *in vitro* tetrazolium-based proliferation/viability assays, all three GRM1-expressing human melanoma cells, C8161, 1205Lu, and UACC903 displayed modest efficacy in suppressing cell growth in the presence of CB-839 as compared with the control vehicle (DMSO) group regardless of their *BRAF* genotypes (Fig. 3; Supplementary Fig. S1A). It is noteworthy that a considerably higher concentration of CB-839 (10–50 μmol/L) is required to observe a reduction in UACC903 cell proliferation, likely due to the presence of other driver mutations including *BRAF*^{V600E} (Supplementary Fig. S1A). Furthermore, to determine if GRM1 expression modulates the responsiveness to GLS inhibition, exogenous human GRM1 cDNA was introduced into an early-stage melanoma cell line, C81-61, which does not express endogenous GRM1 (see profiling data below). Characterization of several GRM1-expressing C81-61 clones confirmed that these clones were transformed and tumorigenic (34). We compared the growth rate of the parental C81-61 cell line to the C81-61OE cell line in the presence of CB-839. A marked reduction in the cell proliferation of C81-61OE was seen with 0.5 μmol/L CB-839 as compared with the vehicle (DMSO) control (Fig. 3). Strikingly, very minute if any changes were detected in growth of the parental C81-61 cells with analogous treatment conditions (Fig. 3). These results suggest that GRM1 expression may influence the responsiveness of melanoma cells to GLS inhibition.

Combinatorial treatment with CB-839 and riluzole leads to enhanced inhibition of GRM1$^+$ melanoma cell proliferation

Suppressive effects of riluzole on GRM1$^+$ melanoma cell proliferation were reported earlier (17, 39, 40). Here, the consequences of including both CB-839 and riluzole on cell growth of two GRM1-expressing human melanoma cell lines were investigated. As shown in Fig. 4, C8161 and 1205Lu cells were treated for 7 days with 0.5 μmol/L CB-839, 10 μmol/L riluzole, or 0.5 μmol/L CB-839 + 10 μmol/L riluzole. Treatment with either CB-839 or riluzole reduced C8161 cell proliferation by ∼40%, while combining both CB-839 and riluzole

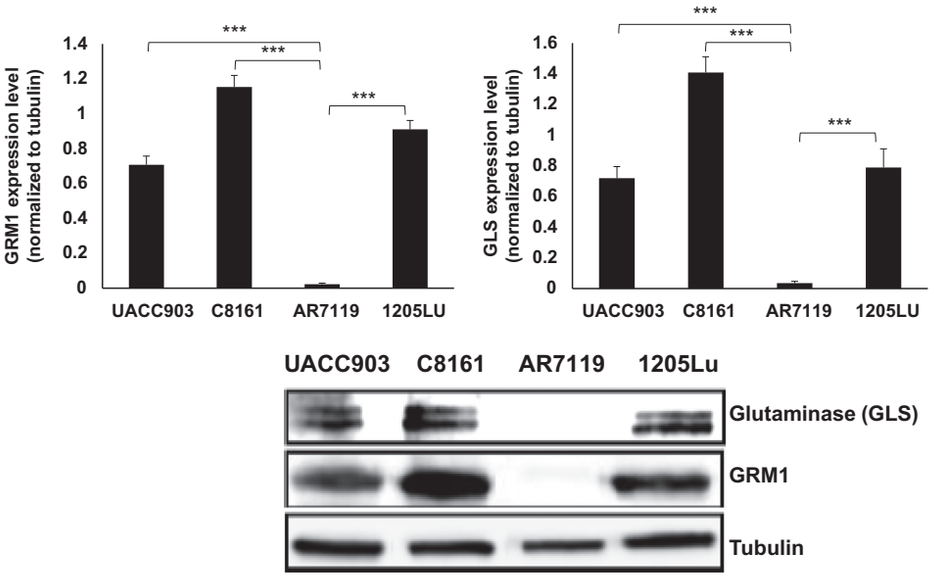

Figure 2.
A link between GLS and GRM1 levels in human melanoma cells. Western immunoblots of metabotropic GRM1 and GLS on protein lysates from three different human melanoma cell lines (C8161, UACC903, and 1205Lu) and the human normal immortalized melanocyte cell line, *TERT/CDK4*^{R24C}*/TP53*^{DN} (AR7119). Lysates were probed with the indicated antibodies. GLS expression correlated with GRM1 levels. Quantification of the intensity of GRM1 and GLS bands normalized to tubulin is displayed above the gel on a bar graph. Data are expressed as a mean ± SD of three independent experiments. Student *t* test was used to calculate statistical significance. ***, $P < 0.001$.

led to an ~85% decrease when compared with vehicle-treated control cells. 1205Lu cells also displayed significant reduction in cell proliferation in the presence of both CB-839 and riluzole as compared with either agent alone (Fig. 4). As mentioned above, a higher dose of CB839 and riluzole was needed to diminish cell proliferation in UACC903 cells (Supplementary Fig. S1B), similar to our earlier observations (41). The GRM1-negative C81-61 and human immortalized melanocyte AR7119 cells were used as negative controls—these cells did not respond to riluzole plus CB-839 treatment (Fig. 4), confirming that GRM1 expression is required to be responsive to these compounds at the indicated doses. Furthermore, increasing evidence illustrates that the presence of a mutation in BRAF oncoprotein frequently makes some cancer cells less responsive to various targeted treatments (42). Taken together, our results suggest that CB-839 combined with riluzole can enhance the antiproliferative properties of GRM1$^+$ human melanoma cells and that higher doses are needed for some *BRAF*-mutated cells.

CB-839 treatment leads to inhibition of glutamate release from GRM1$^+$ human melanoma cells

Inclusion of riluzole in cultured media modulated the amount of glutamate released by melanoma cells (17). To determine the consequences on the level of glutamate released by GRM1$^+$ melanoma cells upon treatment with CB-839 only or riluzole + CB-839, C8161 cells were plated in glutamate-free MEM media followed by collection of conditioned media at days 0, 2, and 4. We plated a different number of C8161 cells, so at the time of collecting the conditioned media samples, cell numbers were very similar among the different days (Fig. 5A). In parallel, we also performed cell viability/cell proliferation MTT assays to ensure that the treated cells were viable, as the levels of glutamate release were determined. We showed that extracellular glutamate levels were significantly reduced in the conditioned culture media isolated from CB-839, riluzole, or CB-839 + riluzole-treated C8161 cells compared with the untreated cells (Fig. 5B).

Concurrent administration of CB-839 and riluzole diminishes *in vivo* tumorigenicity

Next, to confirm our *in vitro* antitumorigenic potential in GRM1$^+$ melanoma cells upon combining CB-839 with riluzole, we conducted *in vivo* experiments on established C8161 xenografts. C8161 cells were inoculated into the dorsal flanks of immunodeficient nude mice and when the tumor volumes reached approximately 10 to 20 mm^3, the mice were randomly divided into 4 treatment groups: vehicle (DMSO), riluzole (10 mg/kg), CB-839 (200 mg/kg), or the combination of riluzole (10 mg/kg) and CB-839 (200 mg/kg) by oral gavage, daily. In support of the encouraging *in vitro* findings, we showed considerable suppression of tumor progression in mice treated with the combination of CB-839 plus riluzole as evident by the decrease in tumor volumes (Fig. 6A and B). Interestingly, CB-839 plus riluzole mediated enhanced suppression of tumor growth in female mice ($P < 0.05$) but only moderate in male mice ($P = 0.113$) when compared with either agent alone. It is worth noting that C8161 cells were isolated from a female human patient, and recent reports support the importance of the cell lines' gender when screening for anticancer agents (43). In addition, our coworkers have previously demonstrated the prevalent cross-talks between GRM1 and estrogen signaling (44), thereby providing some clarification on the sex-biased differences observed in this study. Most importantly, all of the treatment groups did not significantly affect the body and liver weights of the mice (Figs. 6C–E), highlighting that these compounds are not toxic and well tolerated even when administered together. The absence of liver toxicity in all treatment groups was confirmed by hematoxylin and eosin (H&E) staining of liver tissue followed by exhaustive histopathologic evaluation of the slides (Supplementary Fig. S2).

Modulation of GRM1 alters the intracellular production of glutaminolytic and glycolysis metabolites in human melanoma cells

We next asked whether modulation of GRM1 expression affects the intracellular levels of key glutaminolytic and glycolytic

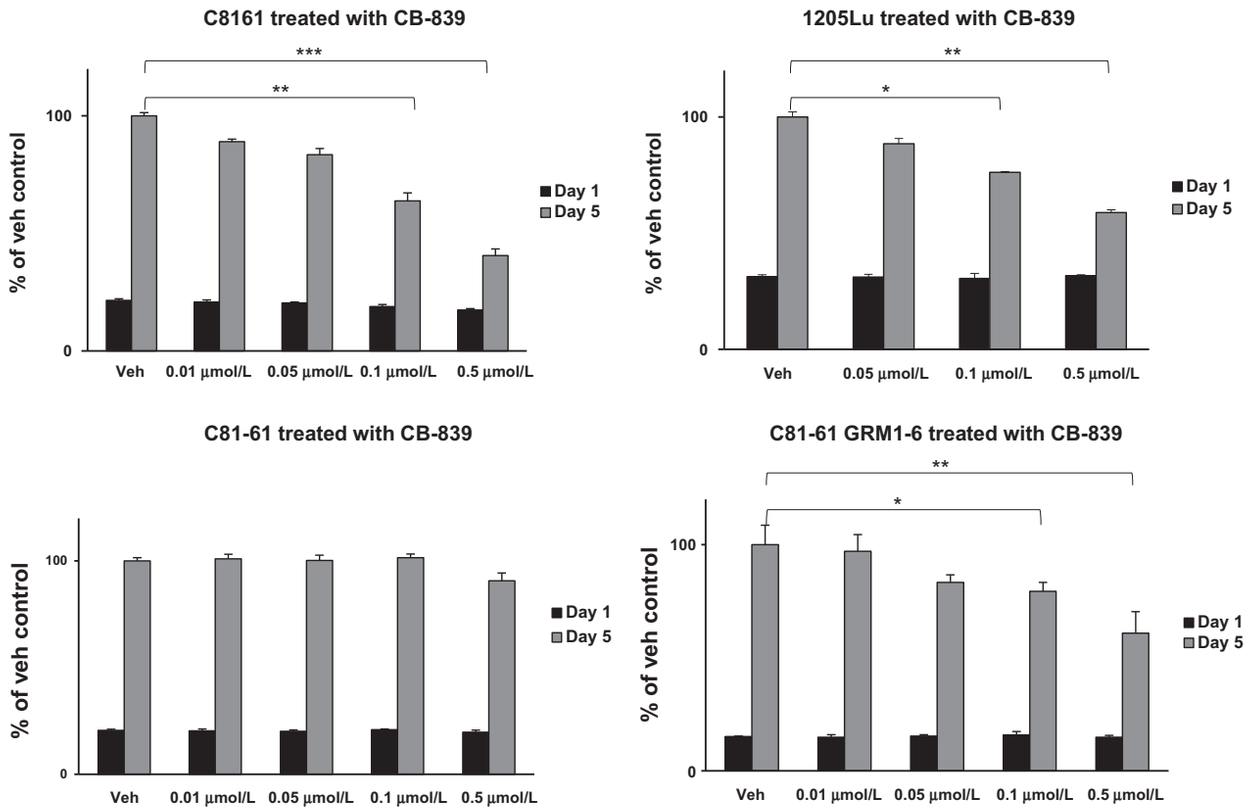

Figure 3.

Inhibition of GLS reduces proliferation of GRM1-expressing melanoma cells. MTT cell viability/proliferation assays were performed on GRM1-expressing C8161, 1205Lu, and C81-61OE (C81-61 GRM1-6) cell lines, and GRM1-negative C81-61 cells. Treatment conditions for all cells were vehicle (DMSO) or CB-839 at 0.01, 0.05, 0.1, and 0.5 μmol/L. Each time point and concentration represents a mean ± SD of four independent measurements of the absorbance. A one-way ANOVA test with Bonferroni *post hoc* analysis was used to calculate statistical significance between experimental and control groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

metabolites. We analyzed both overexpression of GRM1 in a GRM1-low background (parental C81-61 and C81-61OE) and suppression of GRM1 in a GRM1-high background (parental C8161 and C8161si). Although manipulation of GRM1 expression levels failed to alter intracellular lactate concentration (Fig. 7A), higher levels of GRM1 correlated with significantly increased levels of intracellular citrate, α-ketoglutarate, and glutamate ($P < 0.01$; Fig. 7B–D). This indicates that GRM1 expression does not increase lactate fermentation but does increase levels of TCA cycle intermediates. The increased intracellular pool size of glutamate could be a direct result of increased conversion of glutamine into glutamate via the activity of GLS. To determine whether modulating GRM1 expression affects the level of GLS, we assayed GLS protein levels by Western blot. Consistent with our observed elevated glutamate concentrations, cells with higher levels of GRM1 also had higher levels of GLS protein (Fig. 7E). These results suggest that GRM1 expression increases glutamate production by increasing GLS expression.

Discussion

Metabotropic glutamate receptor 1 (GRM1) is an oncogenic driver in the neuroectodermal-derived lineage of melanocytes (45). Aberrant glutamatergic signaling activates mitogenesis and melanomagenesis independent from canonical mitogen-activated protein kinase signaling (4). The high frequency of ectopic GRM1 expression in melanoma and its signaling cascades implicated in cellular transformation has made GRM1 a principal research target to improve treatment of melanoma. In this study, the role of GRM1 in modulating glutamate bioavailability in melanoma cells was explored. Our results suggest that GRM1 expression promotes a metabolic phenotype that supports increased glutamate production and autocrine glutamatergic signaling. Glutamatergic signaling through GRM1 promotes expression of GLS, increasing the conversion of glutamine into glutamate. Melanoma cells heavily depend on anaplerosis via glutamine (46, 47). $GRM1^+$ melanoma cells upregulate GLS and support increased levels of glutamate. Excess amounts of intracellular glutamate are transported to the extracellular environment, where it serves as a trigger for the GRM1 receptor. In neuronal cell lineages, cytoplasmic glutamate is exported via vesicular glutamate transporters or cystine–glutamate exchangers (48).

We demonstrate elevated glutamate levels in systemic circulation of heterozygous TGS mice (which harbor only one copy of the disrupted *Grm1*) compared with that of wild-type TGS

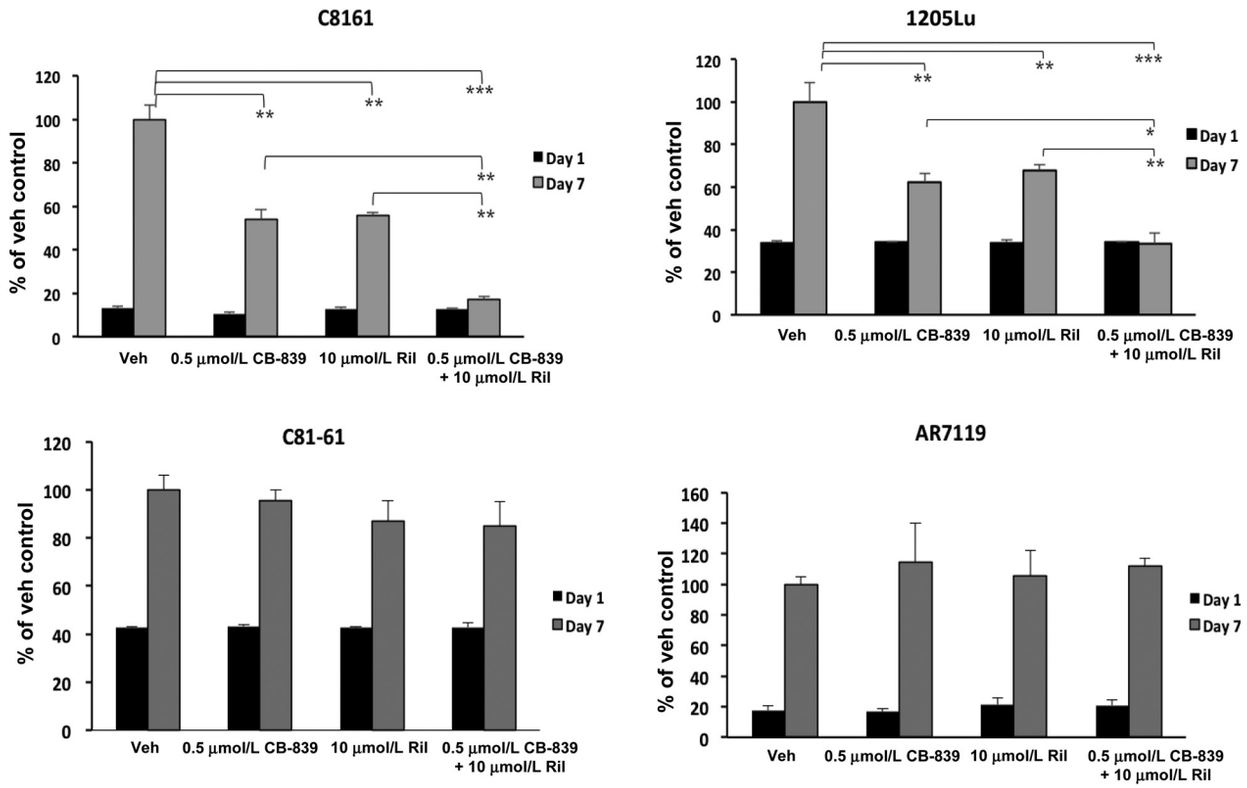

Figure 4.

Enhanced suppression of proliferation of GRM1-expressing human melanoma cells with CB-839 and riluzole. MTT cell viability/proliferation assays were performed on GRM1-expressing C8161 and 1205Lu cells. GRM1-negative C81-61 and AR7119 cells were used as controls. Because AR7119 cells do not readily take up the tetrazolium MTT reagent, the trypan blue exclusion assay was performed on these cells as an alternative. The treatment conditions were vehicle (DMSO), CB-839, and/or riluzole at 0.5 μmol/L and 10 μmol/L, respectively. Each time point and concentration represents a mean ± SD of four independent measurements of the absorbance. A two-way ANOVA test with Bonferroni *post hoc* analysis was used to calculate statistical significance between experimental and control groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

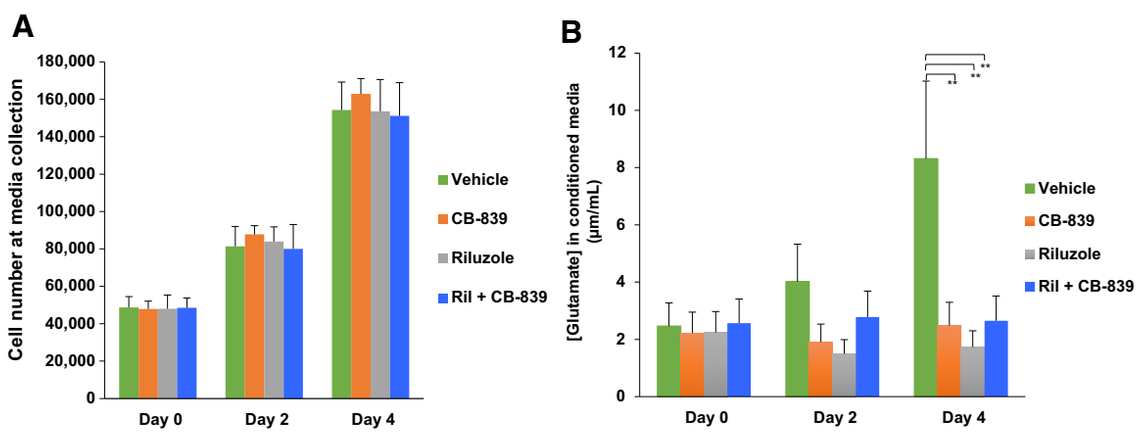

Figure 5.

CB-839 treatment leads to inhibition of glutamate release in GRM1+ human melanoma cells. Human melanoma C8161 cells were assessed for the amount of glutamate they release into the extracellular medium after treatment with CB-839, riluzole, or CB-839 + riluzole. **A,** Different number of cells was plated such that comparable numbers of cells were present at time of sample (conditioned medium) collection. The bar graph represents the number of viable cells during sample collection at day 0, day 2, or day 4. **B,** Concentrations of extracellular glutamate within each treatment group are shown. Statistical analysis was performed between control (vehicle) and treated pairs to show significance. Each bar represents mean ± SD, $n = 3$. **, $P < 0.01$; ns, no significance.

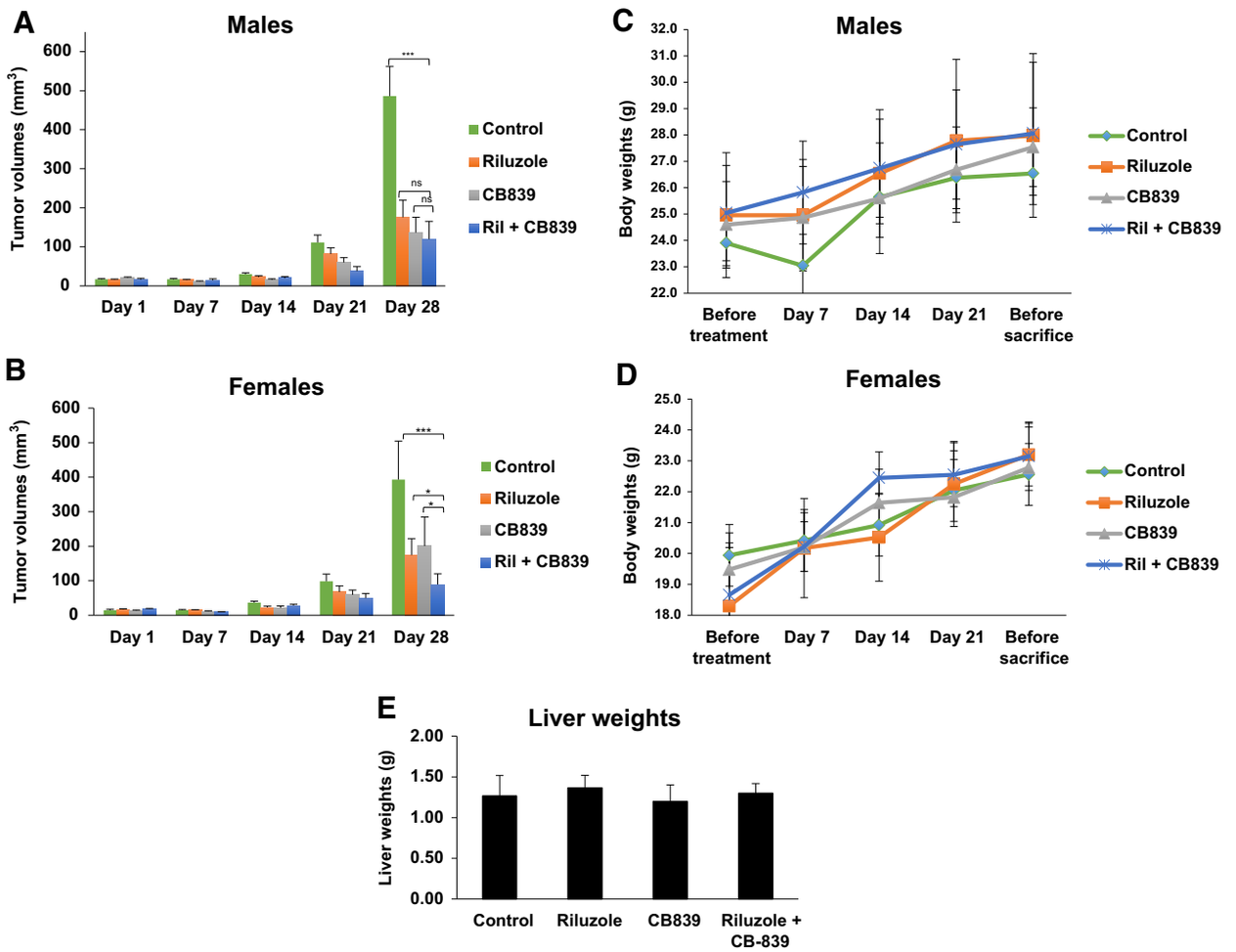

Figure 6.

In vivo xenograft tumorigenicity assay. Xenografts were established in male ($n = 20$) and female ($n = 20$) mice using C8161 cells. The groups were control (vehicle, DMSO + PBS), riluzole (10 mg/kg), CB-839 (200 mg/kg), or the combination of riluzole (10 mg/kg) and CB-839 (200 mg/kg). All agents were given daily by oral gavage. All tumor-bearing mice were euthanized after 28 days due to tumor burden in the control (vehicle) group. A two-way ANOVA test with Bonferroni *post hoc* analysis was used to calculate statistical significance between all treated pairs. Each bar represents volumes of tumors (mean ± SE), $n = 5$ for each of the four treatment groups for males (**A**) or females (**B**). Body weight of male (**C**) and female (**D**) mice was monitored throughout the course of treatment administration. **E,** Liver weights ($n = 3$ for each group) were measured upon termination of the experiment. *, $P < 0.05$; ***, $P < 0.001$; ns, no significance.

mice (no disrupted *Grm1*). The abundant availability of the endogenous ligand for GRM1 leads to constitutive activation of the GRM1 receptor, further promoting cell proliferation and metabolism pathways. To break such positive feedback circuits, we sought after different pharmacologic vulnerabilities of glutamate signaling and metabolism. Although treatment with the GRM1-specific inhibitor riluzole resulted in a significant proliferative disadvantage of tumor cell growth, it is insufficient as single treatment. In contrast, combined treatment of riluzole and CB-839 resulted in substantial tumor cell death. The combined inhibition of glutamatergic signaling via riluzole and of GLS activity via CB-839 efficiently reduced the conversion of glutamine to glutamate and interrupted GRM1 activation. As a consequence, extracellular glutamate was reduced, thus lowering the availability of the natural ligand of GRM1. Such rational combination of two complementary drug-targeting approaches disrupted the ability to circumvent individual blockages yielding a robust response, as evidenced by the enhanced reduction of tumor progression. Although inhibition of glutamatergic signaling by decreasing glutamate release via riluzole or inhibition of GLS activity via CB-839 resulted in reduced cell proliferation as well as diminished tumor growth, the combination of both compounds was most effective. We also expected to see increased reduction of glutamate in the conditioned media after cotreatment with riluzole and CB-839 but this was not the case. Tumor cells have the ability to compensate for GLS inhibition and can overcome glutamate deprivation under such conditions, including through increased anaplerosis, for example, by asparagine synthase (49). This compensatory mechanism may have contributed to

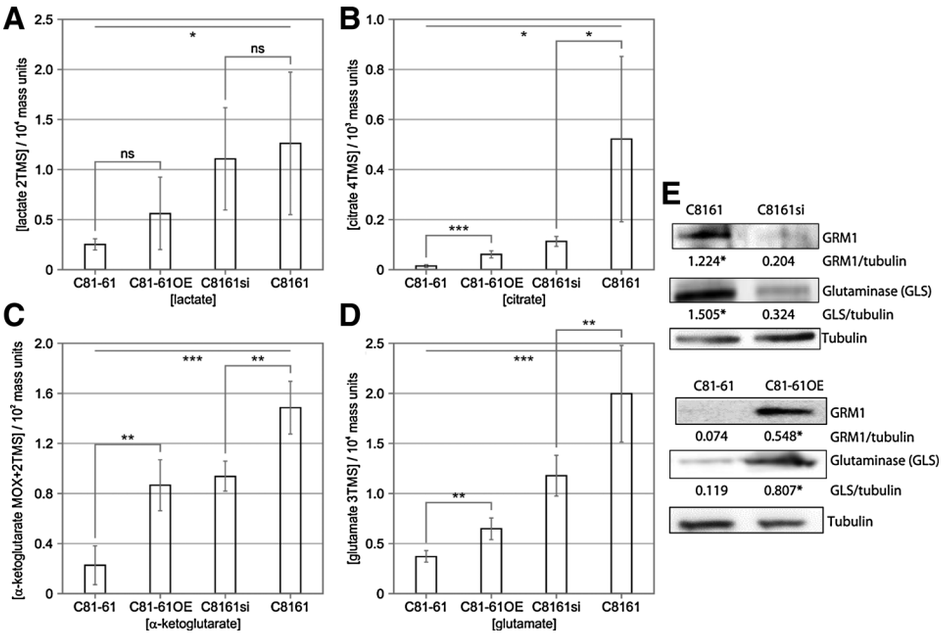

Figure 7.

Modulation of GRM1 alters the intracellular production of glutaminolytic and glycolytic metabolites in human melanoma cells. The intracellular concentrations of lactate (**A**), citrate (**B**), α-ketoglutarate (**C**), and glutamate (**D**) were detected in C81-61, C81-61OE (C81-61 GRM1-6), C8161, and C8161si (C8161 TetR siGRM1 B22-20) cells by GCMS analysis. Data represent the mean of six independent reads ± SD. **E,** Modulations in GRM1 and subsequent changes in glutaminase protein levels in C81-61, C81-61OE, C8161, and C8161si cells were determined by Western blot. Tubulin was used as the loading control. Numerical values indicating quantified intensity of GRM1 and GLS bands normalized to tubulin are displayed below the gel. Data are expressed as a mean ± SD of three independent experiments. Paired, homoscedastic Student *t* test was used to calculate statistical significance with *P* value threshold levels. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, no significance.

intracellular glutamate production and release into the extracellular milieu. Alternatively, other glutamate transport pathways may have been altered to counterbalance the reduced glutamate levels due to burden exhibited by the combinatorial treatment (50). Additional metabolic flux analysis would assist in revealing the source of glutamate before and after treatment with riluzole plus CB-839. Taken together, combined targeting of glutamate production and its eventual release showed highest efficacy *in vitro* and *in vivo*.

The role of glutamine metabolism in cancer cells is well established. However, it is less clear how its role is influenced by the tumor microenvironment, which is often subject to nutrient and oxygen shortage (51). Recently, we reported that one of the consequences of aberrant GRM1 signal transduction is downstream activation of the hypoxia-induced transcription factor 1, HIF1α, which promotes angiogenesis even in normoxic conditions (34). Glutamine metabolism is strongly coupled to HIF1α activity and enhanced in tumors (52). Metabolomics data show that pool sizes of TCA cycle intermediates are increased by GRM1 expression, while glycolytic intermediates are unaffected. This further suggests that GRM1 signaling is tightly connected to glutamine metabolism. Recent reports in ovarian cancer cells demonstrate that GLS inhibition enhances the effectiveness of chemotherapy (53) and also improves the efficacy of other targeted therapies (30, 31), suggesting the critical role of targeting GLS in an attempt to improve overall patient response. These insights, combined with our data, support the rationale to target glutaminolytic glutamate bioavailability by combining riluzole with CB-839 in order to combat GRM1$^+$ human neoplasia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R. Shah, F.V. Filipp, S. Chen
Development of methodology: R. Shah, S.J. Singh, F.V. Filipp, S. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Shah, S.J. Singh, K. Eddy, F.V. Filipp, S. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Shah, S.J. Singh, F.V. Filipp, S. Chen
Writing, review, and/or revision of the manuscript: R. Shah, S.J. Singh, F.V. Filipp, S. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Shah, S. Chen
Study supervision: R. Shah, F.V. Filipp, S. Chen

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