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PRECLINICAL STUDY

Riluzole mediates anti-tumor properties in breast cancer cells independent of metabotropic glutamate receptor-1

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Abstract Riluzole, the only drug approved by the FDA for treating amyotrophic lateral sclerosis, inhibits melanoma proliferation through its inhibitory effect on glutamatergic signaling. We demonstrated that riluzole also inhibits the growth of triple-negative breast cancer (TNBC) and described a role for metabotropic glutamate receptor-1 (*GRM1*) in regulating TNBC cell growth and progression. However, the role of *GRM1* in mediating riluzole's effects in breast cancer has not been fully elucidated. In this study, we seek to determine how much of riluzole's action in breast cancer is mediated through *GRM1*. We investigated anti-tumor properties of riluzole in TNBC and ER+ cells using cell growth, invasion, and soft-agar assays and compared riluzole activity with *GRM1* levels. Using Lentiviral vectors expressing *GRM1* or sh*GRM1*, these studies were repeated in cells expressing high or low *GRM1* levels where the gene was either silenced or overexpressed. Riluzole inhibited proliferation, invasion, and colony formation in both TNBC and ER+ cells. There was a trend between *GRM1* expression in TNBC cells and their response to riluzole in both cell proliferation and invasion assays. However, silencing and overexpression studies had no effect on cell sensitivity to riluzole. Our results clearly suggest a *GRM1*-independent mechanism through which riluzole mediates its effects on breast cancer cells. Understanding the mechanism by which riluzole mediates breast cancer progression will be useful in identifying new therapeutic targets for treating TNBC and in facilitating stratification of patients in clinical trials using riluzole in conjunction with conventional therapy.

Keywords Metabotropic glutamate receptor-1 · Breast cancer · Riluzole · Voltage-gated sodium channels

Introduction

Breast cancer is a global concern, accounting for nearly a quarter of all cancers in women. [1]. According to the American Cancer Society, approximately one in eight women in the U.S. will develop invasive breast cancer at some point in their life [2]. This toll will continue to increase as the number of women in age groups at risk for breast cancer increases. Of women diagnosed with breast cancer, 12–15 % will be diagnosed with triple-negative breast cancer (TNBC), an aggressive subtype of breast cancer with a higher mortality rate [3, 4]. TNBC refers to breast tumors that lack the receptors for estrogen (ER) and progesterone (PR), and lacking amplification of the gene for epidermal growth factor receptor 2 (HER2). These tumors do not respond to currently existing targeted therapy, including hormonal (e.g., tamoxifen and aromatase inhibitors) or *HER2*-targeting drugs (e.g., trastuzumab and

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pertuzumab). Decreasing this high rate of recurrence and mortality is currently difficult, because there is no effective targeted therapeutic treatment for TNBC, and the only treatment options currently available include chemotherapeutic drugs that target replicating cells and cause severe toxicities [5]. Being able to directly target TNBC tumors has the potential to significantly reduce mortality and toxicity associated with chemotherapeutics, while addressing a critical unmet medical need [6]. Also, given the problem of resistance to anti-estrogen and *HER2*-targeted therapies, identification of new targets will have the potential to improve care even in hormone receptor-positive breast cancer.

Recently, our laboratory and others have identified the metabotropic glutamate receptor-1 (gene: *GRM1*; protein: mGluR1) as a promising molecular target for the treatment of TNBC [7–10]. Metabotropic glutamate receptors (genes: *GRM1-GRM8*; receptors: mGluR1-mGluR8) belong to the family of G-protein-coupled seven transmembrane domain receptors which, upon binding glutamate, cause G proteins bound to the intracellular region to be phosphorylated, affecting multiple downstream signaling pathways and ion channels in the cell [11, 12]. Of these receptors, mGluR1 and mGluR5 comprise Group I mGluRs. They are mainly involved in excitatory responses and activate pro-proliferative signaling cascades, such as phospholipase C (PLC), PI3 K, and Akt [13]. In our studies, we have detected *GRM1* expression in TNBC cell lines and demonstrated their ability to regulate cell growth and survival via signaling through the ERK and Akt pathways [7, 8]. Indeed, when we overexpressed *GRM1* in MCF10AT1 cells, part of the MCF10A progression series representing atypical ductal hyperplasia, and implanted them in the mammary fat pads of nude mice, we observed formation of locally invasive tumors in 91 % of mice [8]. We have also observed that inhibiting mGluR1 activity with the non-competitive antagonist BAY36-7620 or indirectly with the glutamate release inhibitor riluzole significantly inhibits the growth of 4T1 mouse tumors and MDA-MB-231-derived xenografts in mice [7, 9].

In many ways, riluzole is a near-ideal drug to be repurposed for the treatment of breast cancer and a potential candidate to fill the role of a "tamoxifen for TNBC." First, it is an oral drug that is already approved by the U.S. Food and Drug Administration for the treatment of the degenerative neurological disease amyotrophic lateral sclerosis (ALS) [12]. In addition, it has very low toxicity, arguably less than tamoxifen (which, in addition to menopausal symptoms, can cause thrombosis and uterine cancer) or aromatase inhibitors (e.g., debilitating arthralgia and loss of bone density). Its most significant adverse effects include asthenia, somnolence, vertigo, and nausea, all dose-related and generally tolerable [12, 14]. Finally, given that riluzole is already FDA-approved for one indication, it could potentially be much more rapidly translated from the preclinical arena to the clinic than a new experimental drug.

Preclinical studies have demonstrated anti-tumor effects due to riluzole in melanoma. In melanoma, riluzole appears to act primarily by inhibiting mGluR1 signaling [15–20], but it has other pharmacological properties as well, which include inactivation of voltage-dependent sodium channels and an ability to interfere with intracellular signaling events due to its lipophilic nature [12, 21–23]. Unlike the case in melanoma, we found that in breast epithelium, pre-existing expression of an oncogene was required for mGluR1-mediated transformation [8]. Although elevated *GRM1* expression has been reported to correlate with poorer outcome in ER(+) breast cancer patients treated with tamoxifen [24], riluzole activity does not associate as tightly with mGluR1 expression in breast cancer as it does in melanoma [7, 8]. Thus, the purpose of this study is to determine the extent to which riluzole mediates its anti-tumor properties and whether it is through mGluR1-dependent or independent mechanisms. Our results suggest that riluzole's anti-tumor properties in breast cancer are largely mediated through a mechanism independent of mGluR1.

Materials and methods

Reagents and cell culture

All cell culture reagents were purchased from Invitrogen-Life Technologies (Carlsbad, CA) except fetal bovine serum (FBS), which was purchased from Thermo Fisher Scientific (Waltham, MA). The human SUM159 breast cancer cell line was a kind gift from Dr. Stephen P. Ethier [25, 26] and the mouse mammary carcinoma cell line 4T1 was a kind gift from Fred Miller (Karmanos Cancer Institute) [27]. All cell lines were used within 6 months upon receipt or stored in liquid nitrogen for later use. These cell lines were authenticated by their respective labs periodically by morphological assessment and their ability to grow in serum-free medium. The remaining human breast cancer cell lines (MCF7, ZR75-1, MDA-MB-361, T47D, BT474, MDA-MB-231, MDA-MB-468, BT549) were purchased from ATCC where their authenticity was verified by cytogenetic analysis and the cells used within 6 months of purchase or stored in liquid nitrogen for later use. All cell lines were grown in RPMI 1640 supplemented with 10 % FBS, 1 % penicillin, and 0.1 % fungizone and maintained at 37 °C in 5 % CO_2.

Cell proliferation assay

To determine the effect of riluzole on cell growth, cells were plated at 1×10^4 cells/well in 96-well plates in reduced serum (5 %) and exposed to increasing concentrations of riluzole (Sigma-Aldrich, 10–50 μM) or vehicle (DMSO). Cell proliferation was determined on day 3 by measuring the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt into a formazan product according to manufacturer's instructions (Life Technologies). The formazan product was detected by measuring absorbance at 540 nm. Initial absorbance values (on day of treatment) were subtracted from absorbance readings on day 3 and results expressed as percentage of control (vehicle treated). In some experiments, cell numbers were also determined in parallel with the MTT assay by counting manually on a hemacytometer and using trypan blue to confirm cell viability.

Soft-agar assay

Anchorage-independent growth of breast cancer cells was determined using the soft-agar assay. Briefly, cells were cultured in 0.8 % SeaPrep agarose (Lonza, Rockland, ME) at concentrations ranging from 1000 to 5000 cells/well and plated onto an already established 0.8 % agarose layer in 24-well plates. The agarose/cell layer is allowed to gel for 30 min and then supplemented with media containing the appropriate dilutions of riluzole (1–50 μM) or vehicle. Plates are incubated at 37 °C in 5 % CO_2 for 1–2 weeks or until colonies are established and visible to the naked eye. Media was replenished with fresh riluzole every 3–4 days. Colonies were stained using MTT substrate and imaged and counted using the Oxford Optronix GelCount system (Oxfordshire, United Kingdom) and results expressed as % control (vehicle treated).

Cell Invasion assay

Invasion assays were performed using BD cell culture inserts with a pore size of 8 μM and coated with 300 μg/ml Matrigel reconstituted basement membrane matrix (BD Biosciences, Franklin Lakes, NJ). Briefly, cells were plated initially in 60-mm tissue culture dishes at a concentration of $3–6 \times 10^5$ cells/dish. When cells reached 70–80 % confluence (approximately 3 days), they were treated with various concentrations of riluzole (1–50 μM) or vehicle (DMSO) overnight and then trypsinized and plated onto inserts. After overnight incubation on inserts, non-invaded cells were removed from the top of the insert using a moistened cotton swab and remaining invaded cells on the bottom of insert were fixed and stained using the Hema 3 Stain Set from Fisher Scientific (Waltham, MA). Brightfield images (using a 4× objective) were taken to capture the entire well (5 image fields/well) with a Nikon Eclipse TE2000-U inverted microscope and the invaded cells were counted using ImageJ64 software. An MTT assay was also plated in parallel at the same time as insert plating using the same treated cells and invasion results normalized according to % viable cells and expressed as % control (vehicle treated).

GRM1 and mGluR1 expression analysis

In some experiments, mGluR1 protein expression was measured by Western blot analysis. Briefly, cells were collected by scraping in RIPA lysis buffer (Santa Cruz, CA) containing 10 mM Tris–HCl, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 0.004 % sodium azide, and supplemented with a protease inhibitor cocktail solution. 20 μg of protein was separated by SDS–polyacrylamide gel electrophoresis (10 %) and transferred to polyvinylidene fluoride membranes. Immunodetection of human mGluR1 protein was performed using anti-mGluR1 antibody purchased from Alamone Labs (Jerusalem, Israel) with rabbit secondary antibodies and detected by chemiluminescence. Primary blots were stripped and reprobed with antibody against GAPDH (Novus Biologics, Littleton, CO).

For real-time reverse transcriptase quantitative PCR (RT-QPCR) analysis of *GRM1* expression, total RNA was extracted from cells using RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Reverse transcription was performed with 2 μg RNA using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems-Life Technologies) according to the manufacturer's instructions. QPCR was performed using FastStart Universal Probe Master Mix with ROX (Roche Diagnostics, Indianapolis, IN) and using the following sets of primers and probes:

GRM1	Forward	5′-ATT TGC ACG GCC TGC AAA GA-3′
	Reverse	5′-ACT GGA GGA TTT GAC CAC TG-3′
	Probe	5′-/56-FAM/CGC TAT CTT/ZEN/GAG TGG AGC AAC ATC/3IABkFQ/-3′
GAPDH	Forward	5′-CAA CGG ATT TGG TCG TAT TGG-3′
	Reverse	5′-GCA ACA ATA TCC ACT TTA CCA GAG TTA A-3′
	Probe	5′-/56-FAM/CGC CTG GTC/ZEN/ACC AGG GCT GCT/3IABkFQ/-3′

Thermal cycling was performed under the following conditions: 10 min activation of FastStart Taq DNA Polymerase at 95 °C followed by 40 cycles of denaturation for 10 s at 95 °C and annealing/extension at 60 °C for 30 s.

No RT controls were used to confirm lack of contaminating genomic DNA. GRM1 expression ratio was determined using the following equation: $2^{Ct(reference)—Ct(target)}$ with GAPDH as the reference gene.

Stable transduction of cells with GRM1 shRNA or GRM1 plasmids

GIPZ Lentiviral particles containing *GRM1* shRNA vector or non-silencing control vector DNA (Thermo Scientific-Open Biosystems) were generated by reverse transfection of these constructs, together with Trans-Lentiviral packaging mix, into HEK293T cells using Arrest-In/Express-In transfection reagent. A dilution (1:1) of the viral supernatant was used to infect high *GRM1*-expressing breast cancer cells (SUM159, T47D) in the presence of polybrene (10 μg/ml). A stable culture was generated by growing these cells in the presence of puromycin (1 μg/ml), the lowest concentration observed to kill 100 % of both non-transduced cells.

Construction and use of our Lentiviral *GRM1* vector have been described before [7–9]. For *GRM1* overexpression, *GRM1* was subcloned from a PCI-Neo vector, a kind gift from Suzie Chen (Rutgers University, New Brunswick, New Jersey), into the pLenti6.3/V5-TOPO cloning vector (Life Technologies). Lentiviral particles containing the *GRM1* vector or *LACZ* control vector DNA (Thermo Scientific) were generated by reverse transfection of these constructs, together with Virapower packaging mix (Thermo Fisher Scientific), into HEK293T cells using Lipofectamine 2000 Reagent (Invitrogen). A dilution (1:1) of the viral supernatant was used to infect low *GRM1*-expressing breast cancer cells (MDA-MB-468, BT474) in the presence of polybrene (10 μg/ml). A stable culture was generated by growing these cells in the presence of blasticidin (0.5 or 5 μg/ml), the lowest concentration observed to kill 100 % of the non-transduced cells.

Statistical analysis

Data were analyzed using GraphPad Prism (v.6.0) for Macintosh (GraphPad Software, San Diego, CA). All numerical results are expressed as mean ± SEM and statistical analysis was performed by either one-way or two-way repeated-measures analysis of variance (ANOVA) using a two-tail p value followed by a multiple comparison procedure with the Student–Newman–Keuls method. A value of $p \leq 0.05$ was considered significant.

Results

GRM1 and mGluR1 are differentially expressed in both TNBC and ER+ cells

GRM1 and its corresponding mGluR1 protein were measured and compared in several human primary ER+ and TNBC cell lines including the mouse 4T1 cell line which is used in generating the well-known orthotopic syngeneic mouse breast cancer model. By RT-QPCR, all human cell lines expressed *GRM1* (average C_t value of 29) with differential expression observed within both subgroups (Fig. 1a). However, on average, the TNBC cells that we examined expressed higher *GRM1* levels with SUM159 and BT549 cells expressing the highest. These results corresponded with mGluR1 protein products (Fig. 1b, c).

Riluzole inhibits cell proliferation in ER+ and TNBC cells independent of mGluR1

To determine if riluzole is capable of inhibiting cell proliferation in both ER+ and TNBC cells independent of mGluR1, first we measured the effect of riluzole on cell proliferation in all the breast cancer cell lines tested above. After treatment of cells with various concentrations of riluzole for 3 days, a significant dose–response decrease in cell proliferation was observed in all the cell lines tested (Fig. 2a, b). The ER+ cells tested appeared to be equally sensitive to riluzole independent of their *GRM1* and mGluR1 protein levels. However, in the TNBC subtype, the SUM159 cells demonstrated maximum sensitivity to riluzole at 50 μM (98 % inhibition), which does correspond with their high *GRM1* levels. However, even in MDA-MB-468 cells that expressed very little *GRM1*, riluzole was still able to significantly inhibit cell proliferation by 99 % at the highest dose tested (50 μM). To better assess this association, the concentration of riluzole shown to inhibit cell proliferation by 50 % (IC_{50}) was calculated for all cells using Prism software. The results of this analysis do suggest a trend between *GRM1* levels and the sensitivity of cells to riluzole in the TNBC cells. However, the magnitude of the difference in IC_{50} values is very small compared to that of the *GRM1* expression levels.

To confirm riluzole is capable of inhibiting cell proliferation in breast cancer cells independent of *GRM1* expression, we repeated the cell proliferation studies using the highest and lowest *GRM1*-expressing cell lines in both the ER+ and TNBC cell subtypes after silencing or over-

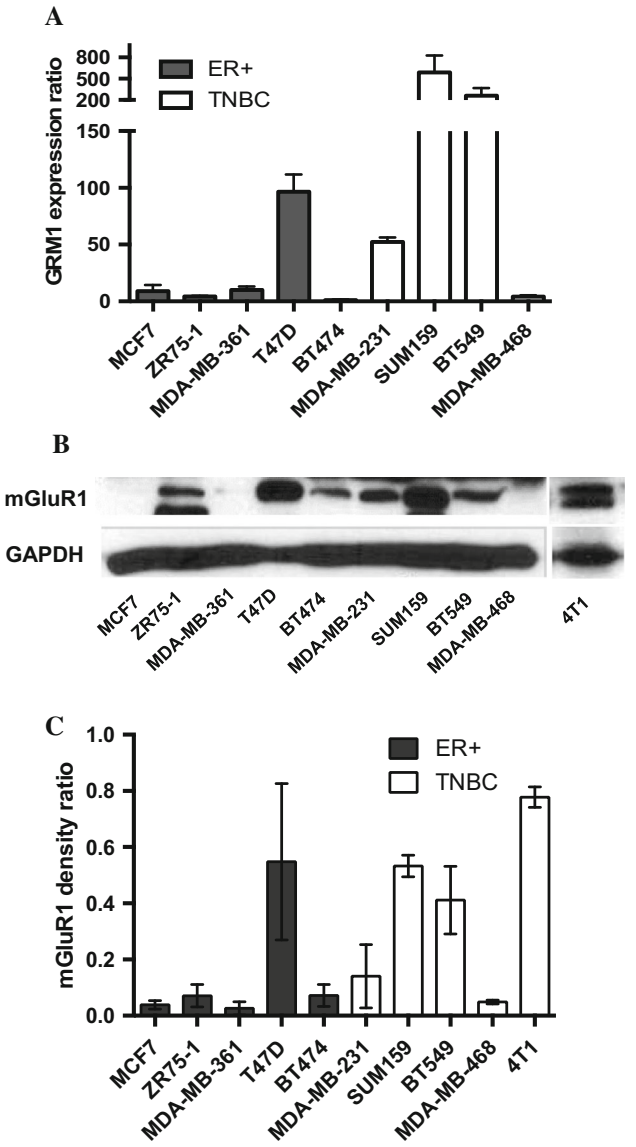

Fig. 1 *GRM1* and mGluR1 expression in various ER+ and TNBC cell lines. **a** *GRM1* message was detected by RT-QPCR and normalized using *GAPDH* as the reference gene. Results are representative of two experiments, performed in triplicate. **b** mGluR1 protein was detected by Western blot analysis. **c** Density graph of the mGluR1 expression, where values represent the mean of two mGluR1 Western analyses where mGluR1 values are normalized to their respective GAPDH values

expressing *GRM1* by transducing with Lentiviral vectors expressing either *GRM*1, sh*GRM1*, or control vectors. After growing in selection media for one week, *GRM1* message was decreased and mGluR1 protein was significantly inhibited by 73 and 90 % in the T47D and SUM159 cells, respectively (Fig. 3). Conversely, *GRM1* message was increased and mGluR1 protein was significantly increased by almost 10-fold and 7-fold in the BT474 and MDA-MB-468 cells, respectively. The cells were then treated with various concentrations of riluzole, and cell proliferation was determined on day three. For both the T47D and SUM159 cells, a small but significant decrease in cell proliferation was detected in the sh*GRM1*-infected vehicle cells when compared to their non-silenced (NS) vehicle cells (33 and 25 %, respectively) demonstrating a *GRM1*-dependent growth mechanism in these cells (Fig. 4a). However, upon treatment with riluzole, cell growth in the *GRM1* silenced cells was further inhibited but to the same extent as the NS cells without any significant differences between them suggesting that this effect of riluzole is independent of *GRM1* (Fig. 4b).

Similar to the *GRM1* silencing results, when *GRM1* was overexpressed in the BT474 cells, a small but significant increase in cell proliferation was observed compared to the *LACZ* vehicle-transduced cells, again demonstrating a *GRM1*-dependent growth mechanism in these cells (Fig. 4c). This effect was not observed in the *GRM1*-transduced MDA-MB-468 cells. However, upon treatment of both cell lines with riluzole, cell growth was significantly inhibited to the same extent in the *GRM1*-overexpressing and *LACZ*-transduced cells (Fig. 4d), strongly suggesting lack of direct *GRM1* involvement as the mediator of the anti-proliferative effect of riluzole.

Effects of riluzole on breast cancer cell invasive properties

Since *GRM1* has also been shown to regulate invasion in melanoma, we wanted to determine if riluzole could mediate this effect in breast cancer as well, and, if so, whether this effect is independent of mGluR1 activity. Using the same cell lines as in the cell proliferation assay, we treated cells with riluzole overnight before counting and plating to ensure only viable cells were plated. After plating onto Matrigel-coated invasion inserts, cells were allowed to invade for 24 h. As expected, TNBC cells, on average, were more invasive than ER+ cells. MDA-MB-361 (ER+) and MDA-MB-231 (TNBC) cells are shown in Fig. 5a as a representative sample. Interestingly, riluzole had minimal effect on invasion in the ER+ cell lines, inhibiting invasion only in the MDA-MB-361 and T47D cell lines, maximally inhibiting by 69.8 ± 7.5 and 53.7 ± 2.2 %, respectively (Fig. 5b). In contrast, after treatment of TNBC cells with various concentrations of riluzole for 3 days, a significant dose–response decrease in the number of cells invading was observed in all the TNBC cell lines tested (Fig. 5c).

A role for *GRM1* in mediating riluzole's effect on invasion was also tested using the sh*GRM1*- and *GRM1*-infected cells. Unlike cell growth, silencing or overexpressing *GRM1* had little or no effect on invasion in any of the infected vehicle cells when compared to their respective controls suggesting *GRM1* does not regulate breast

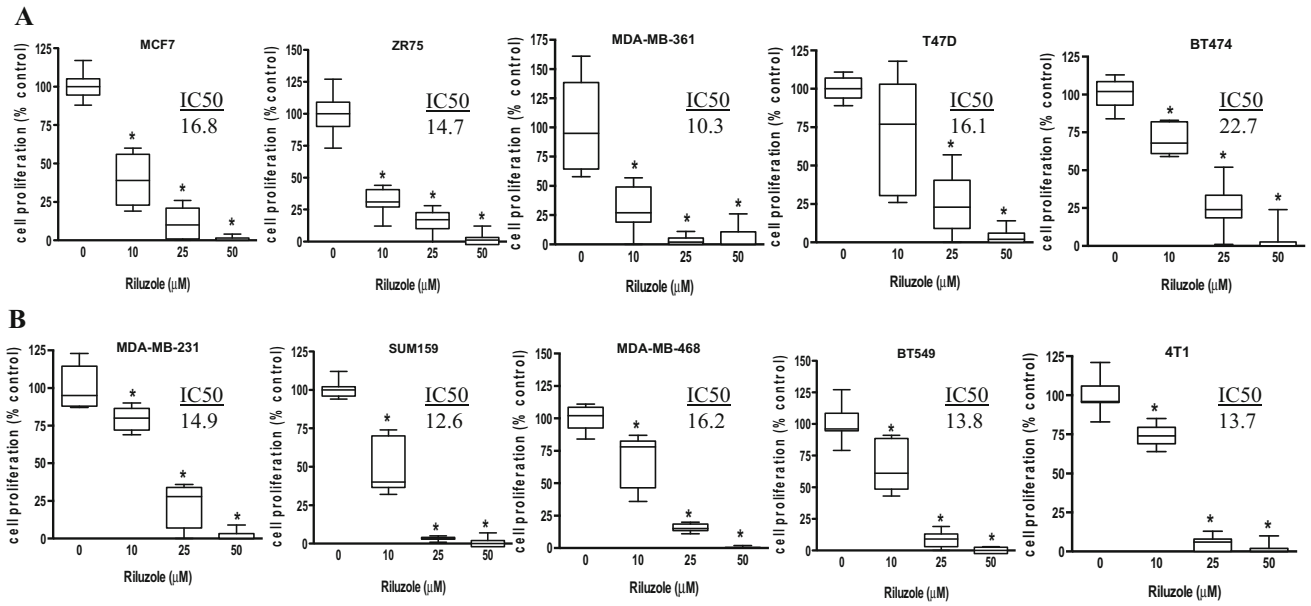

Fig. 2 Riluzole inhibits cell proliferation of ER+ and TNBC cells in a dose-dependent manner. Cell were plated at 1×10^4 cells/well in 96-well plates and treated with varying concentrations of riluzole. Cell proliferation was then determined on day three using MTT assay and results expressed as the percent of vehicle (DMSO)-treated control. **a** ER+ breast cancer cell lines. **b** Triple-negative breast cancer cell lines. Results are expressed as the mean ± SEM of three experiments performed in triplicate where $*p < 0.05$ compared to vehicle control cells. IC_{50} values for riluzole were calculated from the data in **a**, **b** using GraphPad Prism (v.6.0) software

cancer cell invasiveness. However, after treatment with riluzole, there was a significant decrease in invasion in the non-infected cells (T47D, SUM159, MDA-MB-468) with a clear dose–response effect, but to the same extent as the sh*GRM1*- or *GRM1*-infected cells, supporting a lack of *GRM1* involvement in this process (Fig. 5d).

Riluzole inhibits anchorage-independent growth in ER+ and TNBC cells independent of GRM1

Since riluzole has been shown to inhibit colony formation in melanoma cells [17], we wanted to test whether riluzole could mediate this effect in breast cancer cells as well and, if so, to determine whether this effect is independent of *GRM1*. To this end, we plated the ER+ and TNBC cells in an agarose matrix in the presence of increasing concentrations of riluzole and allowed them to grow until colonies reached a detectable size. Once detectable, colonies were stained, imaged, and counted. As expected, the ER+ cells, which are typically of the luminal subtype that derive from the inner epithelial cells [28], were capable of forming much larger colonies than the TNBC cells, which are typically of the basal subtype that derive from the outer mesenchymal cells. MCF7 (ER+) and MDA-MB-468 (TNBC) are shown as representative examples (Fig. 6a). After treatment with riluzole, there was a significant dose–response decrease in the formation of colonies in both the ER+ and TNBC cells (Fig. 6b, c). Based on IC50 values calculated, riluzole was equally effective in inhibiting anchorage-independent colony formation of both ER+ and TNBC cells.

To confirm a lack of *GRM1* involvement, *GRM1*- and sh*GRM1*-infected cells were plated in this assay and treated with riluzole. Similar to the invasion assay, in this assay silencing or overexpressing *GRM1* had no detectable effect on colony formation in any of the infected vehicle cells when compared to their respective controls suggesting *GRM1* does not regulate colony formation in breast cancer. However, after treatment with riluzole, there was a significant dose response decrease in colony formation in the non-infected cells but to the same extent as the sh*GRM1*- or *GRM1*-infected cells, consistent with a lack of *GRM1* involvement (Fig. 6d, e).

Discussion

Riluzole, currently the only FDA-approved drug to treat the fatal neurodegenerative disease ALS, has shown anti-tumor activity suggesting that it can be repurposed to treat breast cancer, particularly TNBC [7–9]. In melanoma, the major mechanism through which riluzole acts is by indirectly blocking glutamatergic signaling, particularly through mGluR1, by blocking glutamate release and inhibiting autocrine signaling [16–18, 29]. Evidence implicating glutamatergic signaling as the primary mechanism through

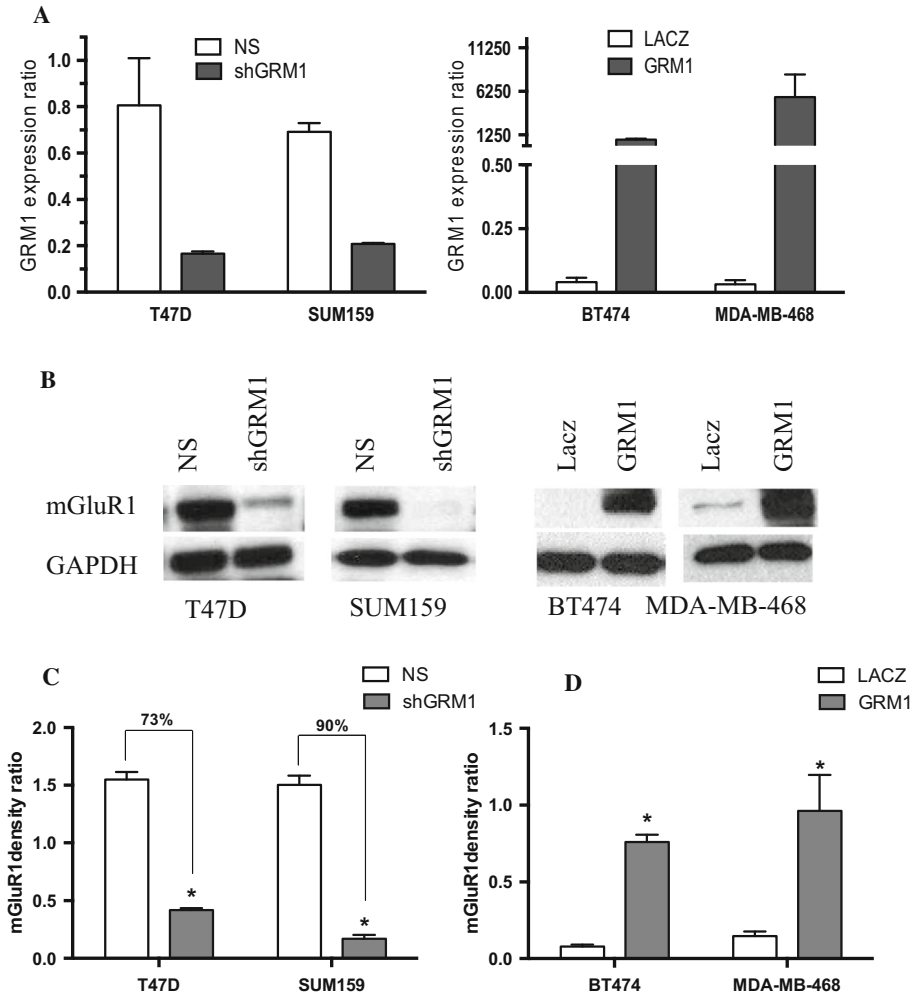

Fig. 3 *GRM1* silencing and overexpression in ER+ and TNBC cells. **a** *GRM1* message is silenced and overexpressed in breast cancer cell lines. Through stable transduction, our Lentiviral *GRM1*-shRNA construct was used to silence *GRM1* in high *GRM1*-expressing T47D (ER+) and SUM159 (TNBC) cells, and our Lentiviral *GRM1* expression construct used to overexpress *GRM1* in low *GRM1*-expressing cell lines BT474 (ER+) and MDA-MB-468 (TNBC) as described in "Materials and methods" section. **b** Confirmation of mGluR1 silencing or overexpression. Cells were treated as in (**a**), after which protein was isolated from them and subjected to Western blot analysis. Cells were analyzed for both *GRM1* and mGluR1 expression 1 week after stable selection with either puromycin (sh*GRM1* infected) or blasticidin (*GRM1* infected) just prior to use. **c** Density graph of the mGluR1 expression, where values represent the mean of three mGluR1 Western analyses where mGluR1 values are normalized to their respective GAPDH values

which riluzole acts in melanoma includes (1) a transgenic mouse strain in which overexpression of *GRM1* unexpectedly resulted in melanocytic tumors [15, 30–32]; (2) detection of mGluR1 expression in 60 % of human melanomas but not benign nevi [15, 16], and (3) transformation of melanocytes by *GRM1* [33]. We also note that the non-competitive mGluR1 inhibitor BAY36-7620 also inhibits TNBC growth in vitro and in vivo [7], suggesting that glutamatergic signaling is a potential therapeutic target in breast cancer as well. However, because in breast cancer, riluzole activity does not correlate as closely with mGluR1 expression as it does in melanoma, where its activity strongly depends on and correlates with mGluR1 expression, we came to question whether mGluR1 is the main target of riluzole in breast cancer cells. We thus hypothesized that a significant fraction of riluzole's anti-tumor activity in breast cancer likely derives from mechanisms other than the inhibition of glutamatergic signaling. Consistent with this hypothesis, the results of this study represent strong preclinical evidence that suggests riluzole is capable of inhibiting the growth and progression of breast cancer, at least in vitro, by a mechanism independent of *GRM1*.

In this study, both ER+ and TNBC cells differentially expressed *GRM1*, with TNBC cells expressing the highest level, and, on average, *GRM1* expression did appear to correlate with their growth sensitivity to riluzole. Interestingly, expression of mGluR1 was the highest in mouse 4T1 cells, leading us to believe that the 4T1 breast tumor model could serve as a useful model for examining mGluR1 function in vivo. Upon *GRM1* silencing in SUM159 and T47D cells, a significant decrease in cell growth was observed in the vehicle group compared to their NS control cells, suggesting a *GRM1*-dependent mechanism by which these cells proliferate. A role for *GRM1* in mediating cell growth in TNBC was also observed in BT474 cells, where overexpression of *GRM1* resulted in increased growth of these cells. However, upon *GRM1* silencing or overexpression, cells were still able to respond to riluzole and to the same degree as their non-silenced counterparts, suggesting riluzole also mediates cell growth independent of *GRM1*. One caveat is that

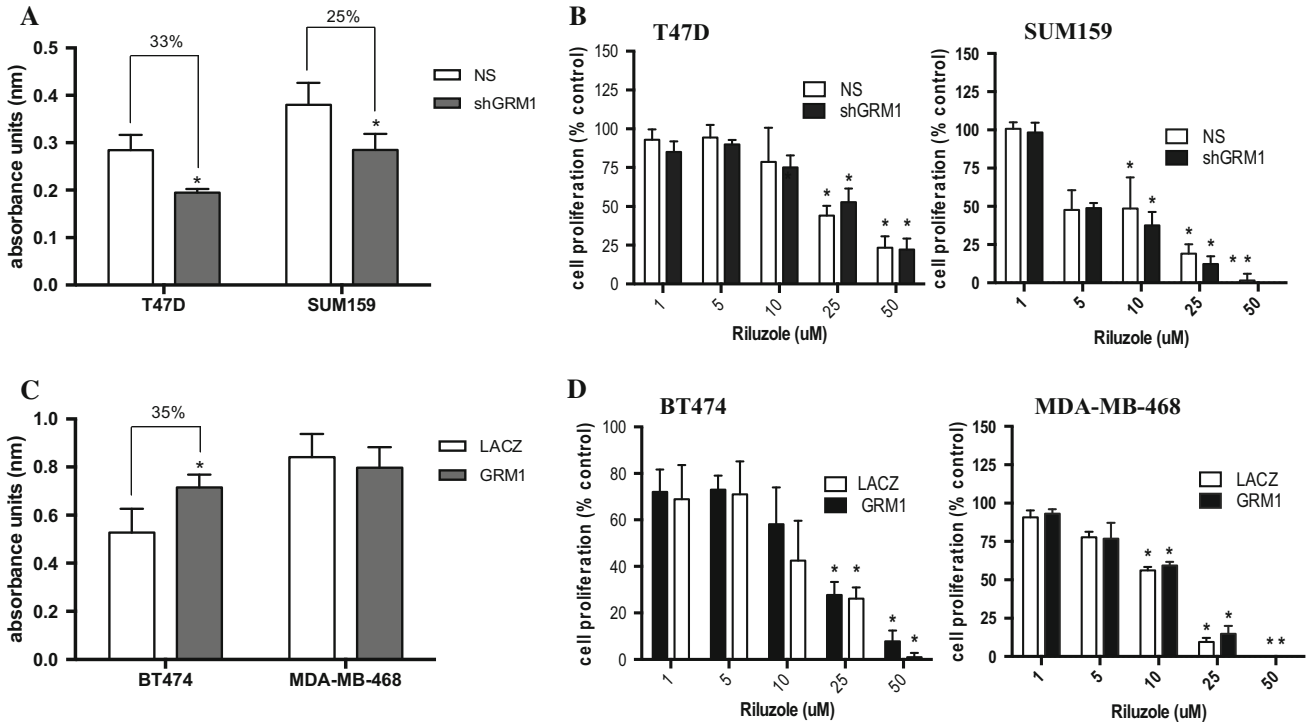

Fig. 4 *Riluzole inhibits cell proliferation independent of GRM1 expression levels.* **a** *GRM1* silencing in T47D and SUM159 cells significantly decreases cell proliferation in vehicle cells when compared to their non-silenced (NS) vehicle cells. **b** Effect of *GRM1* silencing on riluzole activity. Riluzole significantly inhibits cell proliferation in both the T47D and SUM159 *GRM1*-silenced cells to the same extent as their non-silenced (NS) counterparts. **c** *GRM1* overexpression significantly increases cell proliferation in BT474 cells (but not MDA-MB-468 cells) compared to their non-infected (*LACZ*) vehicle cells. **d** Effect of *GRM1* overexpression on riluzole activity. Riluzole inhibits cell proliferation in *GRM1*-overexpressing BT474 and MDA-MB-468 cells to the same extent as their control (*LACZ*) counterpart. For **a**, **c** cell proliferation was determined on day three using MTT assay and raw absorbance units plotted. For **b**, **d**, cell proliferation was determined on day three using MTT assay and results expressed as percentage of vehicle (DMSO)-treated controls. All numerical results represent the average of two experiments performed in triplicate where $*p < 0.05$ compared to their respective NS or *LACZ* controls. For **b**, **d** a two-way ANOVA multiple comparison test was done ($\alpha = 0.05$) between control and GRM1 knockdown or overexpressed cells treated with the same concentrations of riluzole and no significant differences were found at any concentration

GRM1 levels were not completely inhibited in the T47D or SUM159 cells (73 and 90 %, respectively), with some mGluR1 protein remaining; so we cannot completely rule out a role for *GRM1*. On the other hand, when overexpressing *GRM1* in MDA-MB-468 cells, these cells grew at the same rate as their *LACZ* control cells but yet were still capable of responding to riluzole and to the same extent as their *GRM1* normal expressing counterparts confirming lack of *GRM1* involvement.

Further evidence that riluzole can mediate cancer progression independent of *GRM1* was demonstrated in both the invasion and anchorage-independent growth assays, where *GRM1* silencing did not affect invasion or colony formation in SUM159 and T47D cells. However, riluzole was still able to inhibit invasion and colony formation of these cells, suggesting a mechanism independent of *GRM1* by which riluzole regulates these two functions.

One mechanism by which riluzole might be mediating its effect on breast cancer progression is by blocking ion channels, specifically TTX-sensitive voltage-gated sodium channels (VGSCs) [12, 21]. It is well established that riluzole blocks the functions of these channels in neurons [12], and over the last decade VGSCs have been shown to be expressed in a range of cell types that are considered "non-excitable," including breast cancer cells [34, 35]. Riluzole has also been reported to directly inhibit the kainate and NMDA ionotropic glutamate receptors [14, 36]. However, the action of riluzole on these receptors, as well as the metabotropic receptors, has been controversial, as no binding of the drug to any known sites has been shown for them [14, 37]. In addition, as its antiglutamatergic action is still detectable in the presence of sodium channel blockers, it is also uncertain whether or not it acts through sodium channels. Rather, its ability to inhibit glutamate release is thought to mediate many of its effects [38, 39]. These effects combined could significantly reduce glutamate signaling and cause indirect antagonism without acting directly on the glutamate receptors themselves.

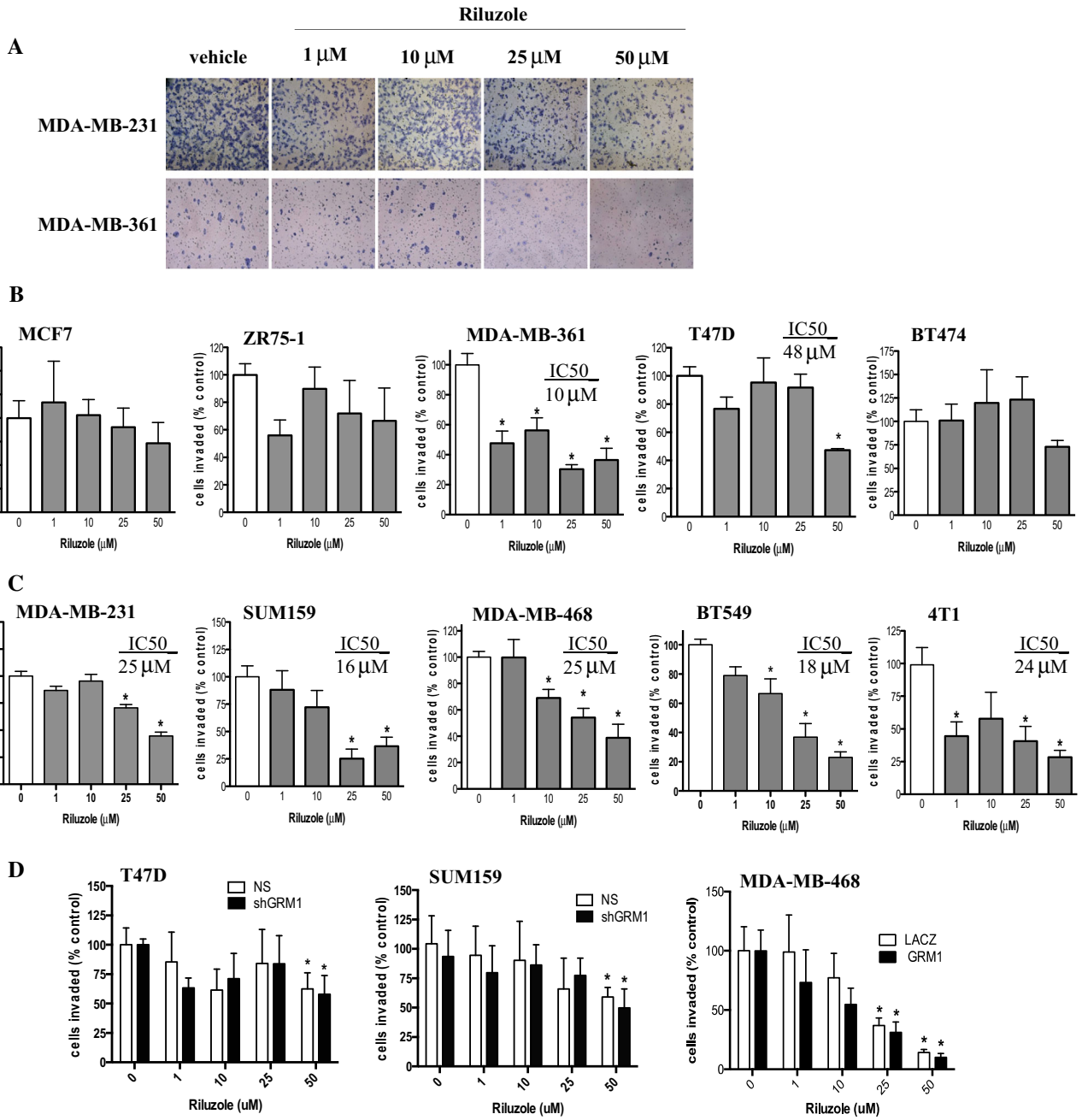

Fig. 5 Riluzole inhibits invasion of breast cancer cells independent of *GRM1* expression. **a** Photomicrograph of invading ER+ (MDA-MB-361) and TNBC (MDA-MB-231) cells. Among the cell lines used in these experiments, TNBC cells were more invasive, with an average of about 1000 cells invading per insert compared to only about 200 cells invading for ER+ cells. Cells were pretreated overnight with riluzole before counting and plating onto Matrigel-coated inserts. After invasion, non-invading cells were removed and the remaining cells fixed, stained, and imaged for cell count analysis using NIH ImageJ64 software. **b**, **c** Effect of riluzole on ER+ and TNBC cells. Riluzole had minimal effect on invasion for ER+ cells (**b**) but significantly inhibited invasion of TNBC in a dose-dependent manner **(c)**. IC_{50} values were calculated using GraphPad Prism (v.6.0) software. **d** Riluzole significantly inhibits cell invasion in both the T47D and SUM159 *GRM1* silenced cells as well as MDA-MB-468 *GRM1* overexpressing cells but to the same extent as their NS or *LACZ* control cells demonstrating lack of *GRM1* involvement. Results for all the invasion assays are expressed as percent of vehicle (DMSO)-treated controls and represent the average of two experiments performed in triplicate where $*p < 0.05$ compared to their respective vehicle, NS, or *LACZ* controls. For **d**, a two-way ANOVA multiple comparison test was done ($\alpha = 0.05$) between control and *GRM1* knockdown or overexpressed cells treated with the same concentrations of riluzole and no significant differences were found at any concentration

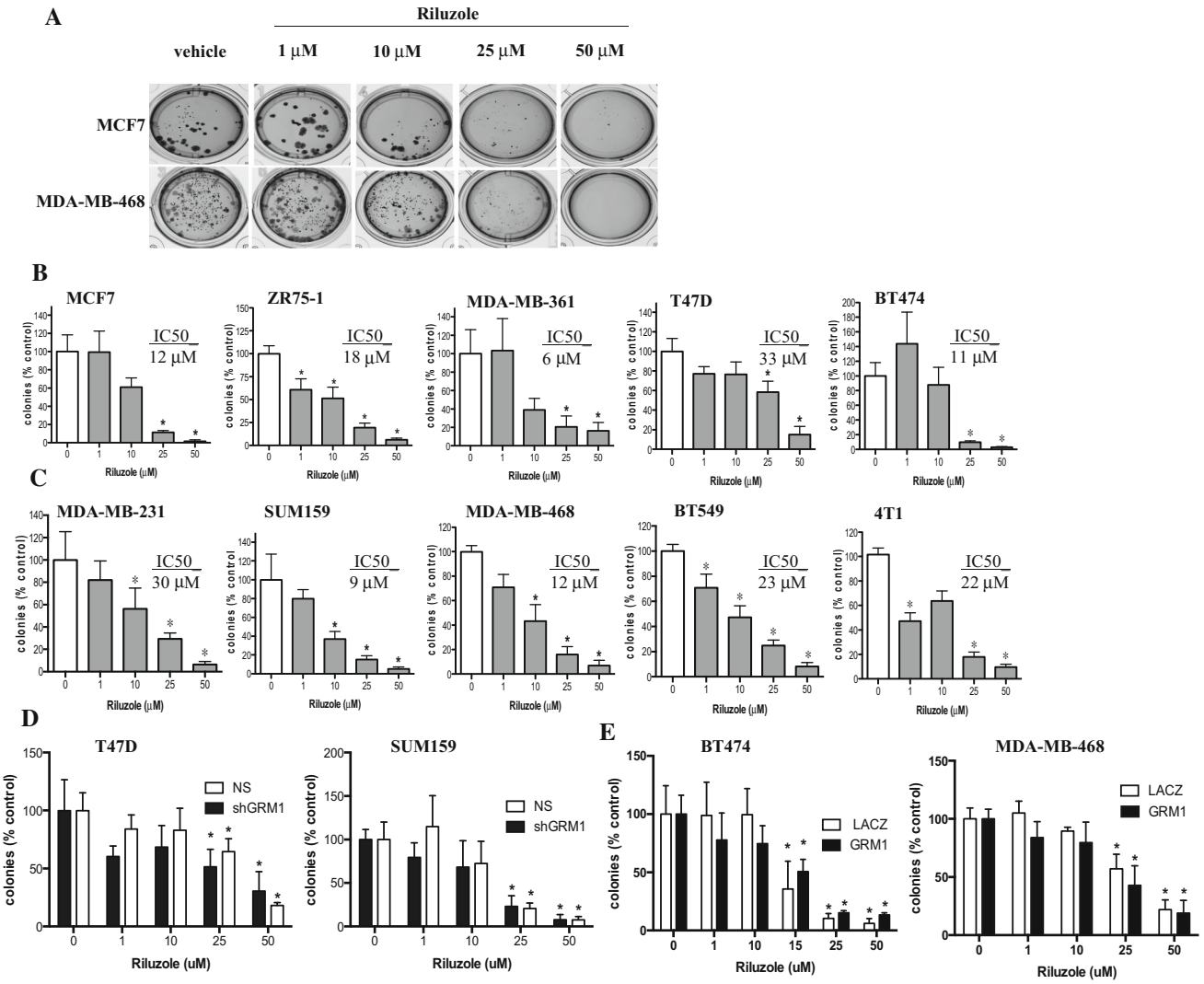

Fig. 6 Riluzole inhibits anchorage-independent growth of breast cancer cells. **a** Representative sampling of colony formation by ER+ (MCF7) and TNBC (MDA-MB-468) cells. On average, ER+ cells formed fewer but larger colonies compared to TNBC cells. **b**, **c** Effect of riluzole on anchorage-independent growth of breast cancer cells. Cells were plated into a soft agarose matrix and treated with various concentrations of riluzole and allowed to form colonies for up to 2 weeks, after which colonies were stained and counted using the Optronix GelCount system. Treatment of ER+ (**b**) or TNBC (**c**) cells with riluzole inhibited colony formation in a dose-dependent manner. IC_{50} values were calculated using GraphPad Prism (v.6.0) software. **d**, **e** Riluzole significantly inhibits colony formation in both ER+ and TNBC cells independent of *GRM1*. Riluzole inhibits colony formation in the T47D and SUM159 *GRM1*-silenced cells (**d**) as well as the BT474 and MDA-MB-468 *GRM1* overexpressing cells (**e**) but to the same extent as their NS or *LACZ* control cells. Results for all colony formation assays are expressed as percent of vehicle (DMSO)-treated controls and represent the average of two experiments performed in triplicate where $*p < 0.05$ compared to their respective NS or *LACZ* controls. For **d**, **e** a two-way ANOVA multiple comparison test was done ($\alpha = 0.05$) between control and *GRM1* knockdown or overexpressed cells treated with the same concentrations of riluzole and no significant differences were found at any concentration

However, the experiments performed in this study were all done in glutamate containing RPMI media (0.02 g/L) with serum that also contains glutamate (0.1 g/L), and thus would likely prevent any effect of riluzole on the binding and activation of metabotropic receptors (including mGluR1) by glutamate. This provides further evidence that mGluR1 was likely not riluzole's target in these experiments.

Another mechanism by which riluzole could be regulating tumor progression is through protein kinase C (PKC) inhibition. PKC is a family of signal transduction molecules that are known regulators of cell cycle and are current targets for development of cancer therapeutics. Riluzole has been shown to directly inhibit PKC activity in cortical cells [22]. Interestingly, PKC has also been shown to be required for regulation of the glutamate transporter EAAT3

in neurons [23]. Further understanding of the mechanism by which riluzole mediates this effect, or its effect on VGSCs, could be beneficial in the development of a target for the therapeutic treatment of cancer.

If riluzole is to be successfully moved from the laboratory to the clinic to treat breast cancer patients, it will be essential to understand its mechanism better. Understanding riluzole's targets and their relative importance would allow for the effective stratification of patients being considered for a clinical trial of riluzole and the intelligent addition of the drug to the multimodality systemic therapy of breast cancer. Until now, it has been generally believed that the anticancer effects of riluzole are almost exclusively through its effect on glutamatergic signaling. Our findings are important because they suggest that in breast cancer cells, riluzole works largely through mechanisms independent of *GRM1*.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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