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Metabotropic Glutamate Receptor-1: A potential therapeutic target for the treatment of breast cancer

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Introduction

The global health burden of breast cancer as measured by incidence, mortality, and economic costs, is substantial [1]. In the United States alone, it is estimated that breast cancer will account for 26% (182,460) of new cancer cases in women and that 15% (40,480) of these women will ultimately die from their disease [2]. Although there exist therapies directed at molecular targets such as the estrogen receptor (ER) and HER2/neu, a significant percentage of breast cancers (15%), dubbed “triple negative” breast cancer (TNBC), express neither of these proteins nor the progesterone receptor and are aggressive, difficult-to-treat tumors [3]. Currently there exist no validated targeted therapies directed against TNBC, leaving cytotoxic chemotherapy as the only option for adjuvant or primary treatment of women with TNBC. Consequently, the identification and validation of additional molecular targets against which targeted therapies can be directed, most likely in combination with existing chemotherapy or targeted therapies, are critical to improving outcomes in women with TNBC. Given the problem of resistance to antiestrogen therapy [4] and HER2-targeted therapy [5] even in non-TNBC, the identification of such targets may have the potential to improve the care of women with other subtypes of breast cancer as well.

Glutamate, a major neurotransmitter in mammals, functions by binding to either ionotropic or metabotropic receptors on the cell surface of neurons in the central nervous system (CNS). Metabotropic glutamate receptors (genes: *GRM1-GRM8*; receptors: mGluR1-mGluR8) belong to the family of G-protein-coupled seven transmembrane domain receptors (GPCRs) [6], which mediate responses to a diverse array of signaling molecules that include hormones, neurotransmitters, and chemokines and can act in an autocrine or paracrine fashion [7-10]. In the mammalian CNS, mGluRs, which are categorized into group I, II, or III based on sequence homology, agonist selectivity, and effector coupling, are essential for normal neuronal function. These receptors have been implicated in diverse neurological pathology, including amyotrophic lateral sclerosis (ALS) [8,11-13] and Fragile X syndrome [14,15]. Of these, mGluR1 and mGluR5 comprise Group I mGluRs and are mainly involved in excitatory responses induced by strong presynaptic stimulation. Coupled to a G_{aq}-like protein, they activate pro-proliferative signaling cascades, such as phospholipase C (PLC), which converts phosphatidylinositol to IP₃ and DAG, the latter of which activates MAPK and PI3K [16]. PI3K is a key enzyme in the regulation of phosphoinositide metabolism, with its activation resulting in PIP₃-mediated phosphorylation of Akt. The PI3K/Akt pathway has

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been implicated in a variety of cellular processes, such as cell cycle regulation and activation of pro-apoptotic and pro-survival proteins. Indeed, the PI3K/Akt pathway has been shown to play a role in the initiation and progression of several human malignancies, including breast cancer [17,18].

A link between mGluR1 and cancer was serendipitously discovered when the creation of a transgenic mouse for a different purpose unexpectedly resulted in a transgenic strain in which melanoma formation developed at high penetrance at a young age. In this transgenic strain, melanoma formation was also associated with the overexpression of mGluR1 [19]. Construction of a second transgenic strain expressing *Grm1* under the control of a melanocyte-specific promoter recapitulated this melanoma-susceptible phenotype, and ectopic expression of mGluR1 was later detected in 60% of human melanomas but not in normal skin or benign nevi [20,21].

Riluzole, an FDA-approved orally available drug for the treatment of amyotrophic lateral sclerosis (ALS) [22], functions in part by blocking mGluR1 signaling. Consistent with a role in melanoma progression, this drug was shown to block proliferation of melanoma cell lines expressing it *in vitro* and *in vivo* while inhibition with a dominant negative construct resulted in apoptosis [23]. Recently, mGluR1 stimulation in melanoma cells was demonstrated to activate MAPK [24] and Akt [25] signaling and, in conditional *Grm1* knockout mice, melanoma growth *in vivo* was shown to require mGluR1 activity [26]. More promisingly, a recent phase 0 trial of Riluzole in melanoma showed promise [27].

Based on these findings, we decided to study the role of mGluR1 in regulating proliferation and progression of breast cancer. Our results implicate mGluR1 as a potential new molecular target for the treatment of breast cancer. Because it is an oral drug with low toxicity, Riluzole may well represent a promising new approach to the treatment of breast cancer by targeting glutamate signaling especially triple negative breast cancer which, in marked contrast to hormone receptor-positive breast cancer, there is currently no orally available targeted therapy.

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). Mammary epithelial and the SUM breast cancer cell lines were a kind gift from Dr. Stephen P. Ethier, also an investigator at Karmanos Cancer Institute in Detroit, MI, and used within six months upon receipt. These cells are authenticated by their lab periodically by morphological assessment and their ability to grow in serum-free medium. MDA-MB-231 and BT549 cells were purchased from ATCC where their authenticity was verified by cytogenetic analysis and the cells were used within six months of purchase. Nonmalignant AB589 cells and triple negative MDA-MB-231 cells were grown in DMEM supplemented with 10% FBS. BT549 cells were grown in RPMI1640 supplemented with 10% FBS and 5 µg/ml insulin. SUM159 cells were grown in Hams F12 supplemented with 5% FBS, 5 µg/ml insulin, and 1 µg/ml hydrocortisone. Media was supplemented with penicillin and streptomycin, and cells were maintained at 37° C in 5% CO₂. For experiments measuring L-quisqualate-induced Akt activity, cells were incubated in customized RPMI containing no glutamate (Invitrogen-Life Technologies) and 10% dialyzed FBS supplemented with 2 mM GlutaMaxTM (Invitrogen-Life Technologies) for at least 5 days prior to experiments.

Cell Proliferation

To determine the effect of inhibiting glutamate signaling on cell growth, cells were plated at 1×10^5 cells per well in 96-well plates in reduced serum (5%) and exposed to either Riluzole (Sigma-Aldrich, 10-50 μM), BAY36-7620 (Tocris Bioscience, Ellisville, MS, 10-50 μM), or vehicle (0.05% DMSO), and cell proliferation was determined on day three by measuring the conversion of tetrazolium salt into a formazan product using the CellTiter Non-Radioactive Cell Proliferation Assay (MTT Assay) according to manufacturer's protocol (Promega). In some of the experiments, cell numbers were also determined in parallel with the MTT assay by counting isolated nuclei with a Coulter counter or by counting manually on a hemacytometer.

Protein expression

At various time points after treatment of cells with Riluzole (0-50 μM), BAY36-7620 (0-50 μM), or the mGluR1 agonist, L-quisqualic acid (10 μM), 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. 50-80 μg of protein was separated by SDS-polyacrylamide gel electrophoresis (4-20%) and transferred to polyvinylidene fluoride membranes. Immunodetection of phosphorylated or total Akt, PARP or cleaved PARP was performed using primary antibodies to these antigens (Cell Signaling, Canton, MA) with appropriate secondary antibodies and detected by chemiluminescence. Primary blots were stripped and reprobed with antibody against α -tubulin (Sigma-Aldrich, St. Louis, MO).

Stable transduction of MDA-MB-231 cells with shRNA for GRM1

Karmanos Cancer Institute (KCI) has a subscription to the Thermo Scientific Open Biosystems Human GIPZ Lentiviral shRNAmir library, which includes ~130,000 shRNA constructs covering every known gene in the human genome. There are at least five shRNA constructs available for human GRM1, which were tested for their ability to knock down GRM1 expression prior to use, with a non-silencing control sequence used as control. Approximately 5×10^6 TU/ml were used to infect MDA-MB-231 cells. A stable culture was generated by growing these cells in the presence of 10 $\mu\text{g}/\text{ml}$ puromycin, the lowest concentration observed to kill 100% of non-transduced MDAMB-231 cells (data not shown). Confirmation of GRM1 silencing was confirmed by RT-PCR analysis.

RT-PCR Analysis of GRM1 Expression

Total RNA was extracted from TNBC cell lines 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, Carlsbad, CA) according to manufacturer's instructions. PCR was performed using ABsolute QPCR Mix (Thermo Scientific) according to manufacturer's protocol using the following sense/anti-sense oligonucleotide primers for GRM1 and housekeeping gene GAPDH:

GRM1	sense	5'-GCA CGG CCT GCA AAG AGA ATG AAT-3'
	antisense	5'-TCC ACT CAA GAT AGC GCA CAG GAA-3'
GAPDH	sense	5'-ACA ACT TTG GTA TCG TGG AAG G-3'
	antisense	5'-CAG TAG AGG CAG GCA TGA TGT TC-3'

Thermal cycling was performed under the following conditions: 15 minute denaturing step at 95° C, followed by 40 cycles of denaturation consisting of 15 seconds at 95° C; annealing/extension at 60° C for 60 seconds. Product sizes were 126 bp for GRM1 and 138 bp for GAPDH. The products were analyzed by agarose gel (1%) electrophoresis, and no-RT controls were used to confirm lack of contaminating genomic DNA.

Apoptosis

Apoptosis of MDA-MB-231 cells after treatment with Riluzole or BAY36-7620 was measured by dual channel flow cytometry and PE Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA). The fraction of cells positive for PE Annexin V and negative for staining with 7-AAD were considered early apoptotic and cells staining positive for both Annexin V and 7-AAD were either necrotic or late apoptotic. Fas ligand-treated Jurkat cells served as positive controls.

Xenograft model

MDA-MB-231 cells (1×10^6) in matrigel (1:1) were injected into mammary fat pads of female athymic nude (nu/nu) mice, aged between 6 and 8 weeks (Harlan Laboratories, Indianapolis, IN) and allowed to grow until the xenografts reached a mean size of 100 mm³, at which point they were divided into experimental groups, consisting of 10 mice per group, such that the means did not vary by more than 10%. At this point, treatment with either 9 or 18 mg/kg Riluzole or vehicle i.p. once a day was begun. In a second study, BAY36-7620 was given in place of Riluzole i.p. once a day, at a single dose level of 10 mg/kg. Tumor size was measured three times a week using a Vernier caliper and calculated using the following formula: length $\times$ width $\times$ depth/2, and treatment continued until tumors in the control group either reached a volume of 1,000 mm³ or ulcerated, whichever came first. All experimental protocols were approved by the local IACUC (Animal Investigation Committee, Wayne State University, Detroit, MI).

Statistical Analysis

All numerical results are expressed as mean $\pm$ SEM. For these experiments, excluding the xenograft study, statistical analysis was performed by one-way repeated-measures analysis of variance (ANOVA) followed by a multiple comparison procedure with the Student-Newman Keuls method. A value of 0.05 was considered significant. For the xenograft studies, statistical analysis was performed by either one-way (Riluzole treatment) or two-way (BAY36-7620 treatment) ANOVA followed by a multiple comparison procedure with the Student-Newman Keuls method.

Results

GRM1 expression and mGluR1 activity in human breast cancer cells

To determine whether GRM1 is present and its protein product mGluR1 is active in TNBC cells, we first assessed several TNBC cell lines for GRM1 expression. GRM1 message was detected in all five breast cancer cell lines tested at varying levels as well as in normal mammary epithelial cells (Fig. 1A). Previous studies have implicated Akt as a major downstream mediator of mGluR1-mediated signaling [28,29,25]. In one of these studies, a role for Akt2, the dominant isoform of Akt, in regulating mGluR1-mediated melanocyte transformation was demonstrated [25]. Because Akt is a major signaling pathway regulating breast cancer growth and metastasis [17,18], we wished to assess whether it is also a downstream mediator of mGluR1 signaling in breast cancer cells. To determine this, we chose to use MDA-MB-231 cells because of their high tumorigenicity *in vivo* [30]. MDA-MB-231 cells were stimulated with the mGluR1 agonist, L-quisqualic acid, either in the

presence or absence of BAY36-6720 (10 μ M), after which levels of phosphorylated Akt were measured. After stimulation, Akt phosphorylation levels increased dramatically, reaching a maximum by 30 minutes (Figure 1B-C). However, pre-incubation with BAY36-7620 prior to L-quisqualic acid stimulation blocked this increase in phosphorylated Akt levels, demonstrating a role for Akt in mediating mGluR1 signaling. To determine whether mGluR1 signaling is specific to the breast cancer cells, we tested the ability of L-quisqualic acid, in the presence or absence of BAY36-7620, to phosphorylate Akt in the normal AB589 cells. Interestingly, even though mRNA expression levels for GRM1 appear similar in MDA-MB-231 and AB589 cells, L-quisqualic acid had very little effect on Akt phosphorylation in the AB589 cells and was not inhibited by BAY36-7620 at any of the time points tested (Figure 1B-C) suggesting that mGluR1 might be functional in breast cancer cells but not in normal mammary epithelium.

mGluR1 regulates growth of triple negative breast cancer cells

We assessed breast cancer cell growth in the presence of the mGluR1 non-competitive antagonists, Riluzole and BAY36-7620, at various concentrations for up to three days. BAY36-7620 is a specific antagonist of mGluR1 that exhibits its effect through association with the transmembrane region of the receptor [31] whereas Riluzole, in addition to inhibiting mGluR1, has been shown to affect other signaling pathways as well, including Ca^{2+} release and PKC [32]. Both Riluzole and BAY36-7620 inhibited cell growth in all three breast cancer cell lines tested (Figure 2). By day three, MDA-MB-231 proliferation was significantly inhibited in a dose-dependent manner by both Riluzole (50%) and BAY36-7620 (70%) at the highest concentrations tested (Figure 2). Interestingly, Riluzole significantly inhibited cell growth of both breast cancer and normal epithelial cells whereas BAY36-7620, the more specific inhibitor of mGluR1, was ineffective at inhibiting the growth of the normal breast epithelial cells. Since Riluzole has other inhibitory effects unrelated to the mGluR1 receptor, these results suggest that breast cancer cell growth might be more sensitive to more tightly targeted mGluR1 inhibition, such as that due to BAY36-7620, than that of normal epithelial cells and further confirm that mGluR1 may not be functional in normal breast epithelial cells.

To confirm the role of mGluR1 in regulating cell growth in breast cancer cells, MDA-MB-231 cells were transduced with Lentiviral vectors expressing one of the five silencing shRNAs previously identified or a non-silencing control after which cell proliferation was measured. GRM1 expression was inhibited by three of these shRNAs (#1, 2, & 5) compared to the non-silencing control shRNA (Figure 3A). By day three, cell growth was significantly inhibited in the MDA-MB-231 cells expressing shRNA targeted to GRM1 compared to non-silencing plasmid vector, with a strong association between the potency of each specific shRNA at knocking down GRM1 with its ability to inhibit MDA-MB-231 growth (Figure 3B).

Inhibition of mGluR1 induces apoptosis in breast cancer cells

The ability of GRM1 to regulate cell growth of TNBC cells suggests that inhibition of its activity may also lead to cell death. To test this hypothesis, we incubated triple negative MDA-MB-231 or BT549 cells with varying doses of Riluzole and assessed the cells for proteolytic cleavage of PARP over time. After an eight hour incubation with Riluzole, total PARP levels began to decrease but no cleavage products were detected in either MDA-MB-231 or BT549 cells (data not shown). However, within 24-48 hours, Riluzole induced PARP cleavage in a dose-dependent manner in both cell lines (Figure 4A). Apoptosis was further assessed by dual channel flow cytometry using the Annexin V/7-AAD staining procedure, where cells staining positive for Annexin V but negative for 7-AAD were identified as being apoptotic. Using this method, we observed that Riluzole increased

apoptosis in the MDA-MB-231 cells by almost ten-fold after 48 hr incubation, (Figure 4B). BAY36-7620 also increased apoptosis of these cells by three-fold compared to vehicle treated, but this effect was not significant before the 48 hr time point.

Riluzole and BAY36-7620 inhibit *in vivo* MDA231 tumor growth

To determine whether mGluR1 can mediate breast cancer cell growth *in vivo*, we used an MDA-MB-231 xenograft model in which tumor-bearing mice were treated with either Riluzole (9 or 18 mg/kg), BAY36-7620 (10 mg/kg), or vehicle (DMSO) by intraperitoneal injection daily for up to 50 days or until tumors ulcerated. In the first experiment, MDA-MB-231 xenografts grew to an average volume of 825 mm³ by day 50 in the vehicle-treated group (Figure 5A). Treatment with the higher dose of Riluzole (18 mg kg⁻¹ d⁻¹), significantly reduced tumor volume by 40% as early as 40 days after treatment with maximal tumor reduction of 80% at day 50, after which the mice were euthanized because the largest tumors in the control group had begun to ulcerate. We conclude that in mice, effective inhibition of tumor growth using Riluzole requires a dose of at least 18 mg kg⁻¹ d⁻¹ which is equivalent to the maximal FDA-approved dose. Consequently, to treat cancer in humans it might be possible to increase the dose beyond the current approved maximum, given the excellent safety profile of orally administered Riluzole at doses currently used in the treatment of ALS. The only adverse effect observed in mice was brief somnolence.

In a second experiment using the more specific mGluR1 inhibitor, BAY36-7620, the xenografts grew to an average volume of 959 mm³ by day 35 in the vehicle-treated group (Figure 5B). Treatment with BAY36-7620 (10 mg kg⁻¹ d⁻¹) significantly reduced tumor volume by as much as 60% at day 35, after which the mice were euthanized because some of the tumors in the control group had reached a size of 1000 mm³. We therefore conclude that specific targeting of mGluR1 is a potentially effective therapy in breast cancer.

Conclusions

Signaling through metabotropic glutamate receptors plays a critical role in the CNS, but until recently it had not been suspected that dysregulation of the activity of these receptors can result in the growth and progression of cancer. It was first proposed that mGluR1 might be targeted in the treatment of melanoma [19] but we rapidly determined that mGluR1 is also expressed and active in breast cancer. Although mGluR1 is expressed in cell lines representing both basal-like and luminal breast cancer, as yet there are no known molecular targets for therapy in TNBC, the most common form of basal-like cancer. That is why we decided to concentrate on this subtype first. Here we have presented preclinical evidence that strongly suggests that mGluR1 plays an important role in mediating the growth and progression of TNBC, beginning with the detection of expression and functional activity of mGluR1 in various TNBC cells as demonstrated by their ability to regulate cell growth and survival via signaling through the Akt pathway. The lack of a strong L-quisqualic acid-induced Akt phosphorylation and the inability of BAY36-7620 to inhibit cell growth in normal breast epithelial cells, suggests a previously unsuspected pathway that drives the growth of TNBC. Thus, the identification of functional GRM1 receptors on TNBC cells suggests GRM1 as a promising potential target in the treatment of this breast cancer subtype of which there is currently no specific targeted therapy. Current research is currently ongoing in our lab to see if mGluR1 could play a role in mediating growth of hormone receptor-positive and HER2-positive subtypes of breast cancer as well.

In addition to these *in vitro* findings, we have also observed that inhibiting mGluR1 activity with BAY36-7620 or Riluzole, at doses equivalent to doses already being used clinically in human beings to treat ALS, significantly inhibits the growth of MDA-MB-231-derived xenografts in mice, suggesting a potential strategy to target mGluR1 that can be rapidly

translated from the preclinical arena to the clinic. Taken together, our results suggest mGluR1 as a promising molecular target for the therapy of breast cancer, in particular TNBC, that can be inhibited with low toxicity using an existing drug (Riluzole) off-label and potentially through the development of more specific mGluR1 inhibitors. Our results also suggest a role for Akt as a potential mediator of the mGluR1 signaling pathway promoting cell survival in TNBC. Because Riluzole is an FDA-approved drug with low toxicity, we suggest that repurposing Riluzole for the treatment of TNBC may represent a promising treatment. Indeed, it is not difficult to envision Riluzole being used in combination with conventional therapy or in a role similar to the role of aromatase inhibitors and tamoxifen in hormone-responsive breast cancers. In fact, recent observations have suggested that multimodality therapy combining Riluzole with existing therapies, specifically ionizing radiation, shows promise in treating melanoma, both *in vivo* and *in vitro* [33]. Since Riluzole does penetrate the blood-brain barrier and a significant number of TNBC patients do go on to develop brain metastasis, Riluzole, in combination with conventional therapy, may be therapeutically advantageous in the treatment of these patients.

Further studies of the intermediates in the mGluR1/Akt cell survival pathway are currently ongoing and will elucidate in more detail how mGluR1 signaling promotes breast cancer proliferation and how its inhibition might be used for therapy. Most importantly, however, our results suggest that it is worthwhile to consider a clinical trial to test whether adding the inhibition of mGluR1 activity in TNBC by Riluzole to conventional therapy is a viable strategy to improve outcomes in patients with TNBC.

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Figure 1. GRM1 expression and activity in triple negative breast cancer cells

A.) GRM1 mRNA expression in triple negative breast cancer cells and normal breast epithelial cells (AB589). GRM1 message was measured using RT PCR analysis. The melanoma cell line SK-MEL was used as a positive control for GRM1 and GAPDH as a loading control. **B.) mGluR1 activity in MDA-MB-231 cells.** mGluR1 activity was assessed by measuring Akt phosphorylation after stimulation with the mGluR1 agonist, L-quisqualic acid (10 μ M) in the presence or absence of BAY 36-7620 (BAY) as described in Materials and Methods. L-quisqualate induced Akt phosphorylation in MDA-MB-231 cells which was inhibited by BAY. L-quisqualate induced only slight Akt phosphorylation in AB589 cells which was not inhibited by BAY demonstrating lack of mGluR1 involvement. Akt activity was normalized to total Akt immunoreactivity (C) and expressed as relative fold change compared to control (no L-quisqualate or BAY). Immunoblots and their corresponding graphs are representative of three separate experiments.

Figure 2. Inhibition of cell growth by mGluR1 antagonists

Triple negative breast cancer cell lines (MDA-MB-231, SUM159, and BT549) or nonmalignant breast epithelial cells (AB589) were plated at 1×10^4 cells per well in 96-well plates and exposed to either vehicle (0.05% DMSO), Riluzole (1-50 μ M), or BAY 36-7620 (1-50 μ M) and cell concentration determined on day 3 by MTT assay. Riluzole (**A**) significantly inhibited cell growth of both breast cancer and normal epithelial cells whereas BAY 36-7620 (**B**), a specific inhibitor of mGluR1, had a significant effect on cell growth only in breast cancer cells. Results are the mean $\pm$ SEM of three experiments performed in triplicate where * is $P < 0.05$ compared to vehicle treated control cells.

Figure 3. shRNA-mediated knockdown of GRM1 inhibits cell proliferation of MDA-MB-231 cells

A. Knockdown of GRM1 expression in MDA-MB-231 cells. MDA-MB-231 cells were infected with GIPZ shRNA Lentiviral clones containing a puromycin resistance gene and either shRNA constructs #1-5 against GRM1 or a non-silencing (NS) shRNA construct. Cells were selected with puromycin (10 μ g/ml) for 10 days and mRNA levels for GRM1 were determined by qPCR. B. Effect of GRM1 knockdown on the growth of MDA-MB-231 cells. MDA-MB-231 cells were infected with GIPZ Lentiviral clones containing either shRNA constructs #1, 2, and 5, or a non-silencing (NS) shRNA construct. Cells were plated in 96-well plates at 1×10^4 cells/well and growth was determined on day three by MTT assay. Results are representative of three experiments performed in triplicate where * is $P < 0.05$ compared to N.S. MDA-MB-231 cells.

Figure 4. Inhibition of mGluR1 induces apoptosis in MDA-MB-231 and BT549 cells

A. Riluzole-induced apoptosis in MDA-MB-231 and BT549 cells. Cells were treated with either 25 or 50 μ M Riluzole for up to 48 hours and apoptosis detected by analysis of cleaved PARP determined by Western analysis. Blots are representative of three separate experiments. B. Riluzole and BAY36-7620 induced apoptosis in MDA-MB-231 cells. Cells were treated with either 50 μ M Ril (Riluzole) or BAY36-7620 and apoptosis was detected at 24 and 48 hours by positive staining for PE Annexin V and negative staining for 7-AAD using dual channel flow cytometry. FasL-treated (100 ng/ml) Jurkat cells were used as a positive control. Results are representative of two separate experiments performed in triplicate where * is $P < 0.05$ compared to vehicle-treated MDA-MB-231 cells and # is $P < 0.05$ compared to untreated Jurkat cells.

Figure 5. Riluzole and BAY36-7620 (BAY) inhibit growth of triple negative breast cancer xenografts

MDA-MB-231 cells (1×10^6) were injected into the mammary fat pads of athymic nude (nu/nu) mice and allowed to grow until the xenografts reached a mean size of 100 mm³ so that the means did not vary by more than 10%, at which point they were divided into experimental groups and treatment with either (A) Riluzole (9 and 18 mg kg⁻¹ d⁻¹) or (B) BAY36-7620 (10 mg kg⁻¹ d⁻¹) began and continued until tumors reached a mean size of 1000 mm³ or began to ulcerate. * is $P < 0.05$ compared to the vehicle-treated group.