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Disruption of GRM1-mediated signalling using riluzole results in DNA damage in melanoma cells

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Summary

Gain-of-function of the neuronal receptor, metabotropic glutamate receptor 1 (Grm1), was sufficient to induce melanocytic transformation in vitro and spontaneous melanoma development in vivo when ectopically expressed in melanocytes. The human form of this receptor, GRM1, has been shown to be ectopically expressed in a subset of human melanomas but not benign nevi or normal melanocytes, suggesting that misregulation of GRM1 is involved in the pathogenesis of certain human melanomas. Sustained stimulation of Grm1 by the ligand, glutamate, is required for the maintenance of transformed phenotypes in vitro and tumorigenicity in vivo. In this study we investigate the mechanism of an inhibitor of glutamate release, riluzole, on human melanoma cells that express metabotropic glutamate receptor 1 (GRM1). Various in vitro assays conducted show that inhibition of glutamate release in several human melanoma cell lines resulted in an increase of oxidative stress and DNA damage response markers.

Keywords

Melanoma; DNA damage; riluzole; glutamate signalling

Introduction

The incidence of melanoma has increased about 600% from 1950 to 2004 (Tsao et al., 2004). Despite recent treatment advances the mortality and morbidity of melanoma remain high. Systemic therapies induce tumor response in only 5–20% of patients and combinatorial treatments have yet to improve survival significantly (Tsao et al., 2004, Doble, 1996, Khan et al., 2011, Pollock et al., 2003, Weber, 2011, Simeone and Ascierto, 2012). Recent therapies that enhance the immune system and inhibit protein kinase pathway signalling are encouraging, but durable benefits remain elusive due to the relative resistance built by melanoma cells (Flaherty et al., 2012). Precise mechanisms of resistance are currently being debated, but appear to comprise an innate ability of melanoma cells to avoid immune

surveillance and recognize and export drugs (Weber, 2011, Zabierowski and Herlyn, 2008, Sosman et al., 2012).

It has been demonstrated that expression of metabotropic glutamate receptors upregulate proliferation and tumor progression both in vitro and in vivo by the establishment of autocrine/paracrine loops to assure constitutive activation of the receptor, as well as that of downstream effector proteins leading to enhanced proliferation and reduced apoptosis (Burger et al., 1999). In mouse and human melanoma cell lines, stimulation of GRM1 by glutamate results in activation of the MAPK and PI3K/AKT pathways (Ferraguti et al., 1999, Thandi et al., 2002). Preliminary data shows that treatment of melanoma cells with the glutamate release inhibitor riluzole led to a reduction of GRM1 activity resulting in decreased tumor cell proliferation and combining riluzole with irradiation enhances selective cancer cell cytotoxicity (Namkoong et al., 2007, Khan et al., 2011, Le et al., 2010). However, a complete mechanistic understanding of this effect remains unknown. Riluzole has been shown to inhibit the intracellular release of glutamate from the cell acting as a putative antagonist of GRM1 (Doble, 1996). Currently riluzole is approved for use in treating neurodegenerative disease, Amyotrophic Lateral Sclerosis (ALS) (Levine et al., 2010). In this report we show that treatment of human melanoma cells with riluzole promotes selective cancer cell death via increases in oxidative stress by interfering with detoxification pathways resulting in DNA damage and cell death.

Results

Riluzole induces DNA damage in human melanoma cells

We have previously shown that the combination of riluzole and ionizing radiation results in an increase in the number of apoptotic cells in a xenograft model of tumorigenesis using GRM1-expressing human melanoma cells (Khan et al., 2011). Elevated levels of phosphorylation of histone H2AX (γ -H2AX) staining were detected in excised tumor samples, however, the precise mechanism by which riluzole enhances this effect remains unknown. Phosphorylation of histone H2AX (γ -H2AX) is among the earliest responses to DNA double strand breaks (DSBs). Upon insult to the DNA, histone H2AX is phosphorylated on serine 139 and is sequestered into the nucleus to sites of insult. To assess if riluzole elicited DNA damage, we treated GRM1-positive C8161 and GRM1-negative UACC930 melanoma cells with DMSO (Veh), etoposide (Etop), or riluzole (Ril) for 24 hours after which nuclear proteins were extracted (Khan et al., 2011, Namkoong et al., 2007). Etoposide, a widely prescribed chemotherapeutic agent used in the treatment of various human cancers, was administered to cultured cells as a positive control for DNA DSBs. Nuclear proteins from both cell lines were analysed by Western immunoblots using an antibody against γ -H2AX as a measure of DNA damage. Only GRM1-positive C8161 cells and not GRM1-negative UACC930 cells (Figure 1A) demonstrate activation of H2AX in the presence of riluzole in comparison to the vehicle treated cells. To confirm DNA damage, a second assay was performed Single Cell Gel Electrophoresis assays (COMET assay) with both cell lines (Figure 1B). Again the presence of damaged DNA was only detected in riluzole treated, GRM1 positive cells and not in GRM1 negative UACC930 cells, confirming the observations from the Westerns.

Riluzole induced DNA damage is dependent on GRM1 expression

To assess if induction of DSBs in human melanoma cells by riluzole is dependent on GRM1 expression, we performed immunofluorescence staining on two GRM1-positive (C8161 and UACC903), and one GRM1-negative (UACC930) human melanoma cell lines. In addition to γ -H2AX, a second protein, 53BP1, was selected. 53BP1 is a checkpoint regulator that binds to the central DNA-binding domain of p53. It relocates to the sites of DNA strand

breaks in response to DNA damage and is a putative substrate for ataxia telangiectasia mutated (ATM), a serine/threonine protein kinase that is recruited and activated by DNA DSBs (Lord and Ashworth, 2012). Both γ -H2AX and 53BP1 are known to co-localize at sites of DNA damage, forming punctated foci visible by immunofluorescence microscopy at sites of DSBs in as little as one hour after exposure to DNA damaging agents (Fernandez-Capetillo et al., 2004, Schultz et al., 2000, Ward et al., 2003). All staining experiments, the positive controls [cells treated with etoposide (ET)] were included, examples of the cells treated are shown in Supplementary Figure 1, quantifications are included in each figure. GRM1-positive (C8161 and UACC903) and GRM1-negative (UACC930) human melanoma cell lines were treated with riluzole (10 μ M) for various time periods (30 min to 48 hours). Staining showed that riluzole treated, C8161 and UACC903 cells had a marked increase in the amounts of both γ -H2AX and 53BP1, an example with C8161 is shown (Figure 2A). In contrast, GRM1-negative UACC930 melanoma cells showed no significant change in levels of γ -H2AX and 53BP1 between riluzole treated and untreated cells. Fluorescence intensities were quantitated for statistical analyses and are shown in Figure 2B.

As a complementary approach, we produced a set of isogenic, GRM1 expressing clones that were derived from immortalized, non-tumorigenic human melanocytes (hTERT/CDKR24C/p53DD) containing either vector (hTERT/CDKR24C/p53DD-Vec) alone or exogenous human GRM1 cDNA (hTERT/CDKR24C/p53DD-GRM1). Functionality of expression was then assessed by the responsiveness of the cells to agonist/antagonist of GRM1 using ERK activities as read-outs by western immunoblot (Figure 3A). An example of clone 1 from Figure 3A was used, downstream activation of the MAPK pathway was assessed by induction using the GRM1 agonist, L-quisqualic acid; this modulation was abolished when cells were pre-treated with the non-competitive inhibitor of GRM1, BAY36-7620 followed by stimulation with L-quisqualic acid (Figure 3B).

For immunofluorescence studies, these cells were either left untreated or treated with riluzole for various time points (30 min to 48 hours) then processed. We found that only hTERT/CDKR24C/p53DD-GRM1 cells but not hTERT/CDKR24C/p53DD-Vec cells displayed an increase in co-localization of discrete foci of 53BP1 and γ -H2AX after exposure to riluzole when compared to untreated control (Figure 3C). Fluorescence intensities were quantitated and are shown graphically (Figure 3D). Only GRM1-expressing hTERT/CDKR24C/p53DD-GRM1 cells not the vector-control cells (hTERT/CDKR24C/p53DD-Vec) show a substantial increase in the amount of co-localization of both γ -H2AX ($p = 0.05$) and 53BP1 ($p = 0.005$) in the presence of riluzole in comparison to no treatment.

To assess the consequence of inhibition of endogenous GRM1 expression in riluzole-mediated DNA damage, we took advantage of several stable C8161 clones that express an inducible silencing RNA to GRM1 (si-GRM1) regulated by the modified mammalian ecdysone-inducible expression vector (Rangasamy et al., 2008) in another study (Wangari-Talbot et al., 2012). In the presence of the inducer, Ponasterone A (PonA), an analogue of ecdysone, the expression of si-GRM1 is turned on and GRM1 protein levels are reduced (Wangari-Talbot et al., 2012). Cells were either induced with PonA or left uninduced and then treated with riluzole (10 μ M) or left untreated and immunofluorescence staining against γ -H2AX and 53BP1 performed. In the absence of the inducer, riluzole treatment resulted in an increase in co-localization of γ -H2AX and 53BP1 when compared to untreated samples (Figure 4A). In contrast, in the presence of the inducer, si-GRM1 is turned on led to decreased GRM1 expression resulted in the reduction in the amounts of co-localized γ -H2AX and 53BP1 foci. These results suggest a decrease in riluzole sensitivity correlated with a decline in functional GRM1 expression. Quantification of fluorescence intensity shows an increase in levels of both DNA damage markers in riluzole treated samples compared to untreated GRM1-expressing cells (Figure 4B, top panel) whereas very similar

levels of DNA damage markers were detected in both untreated and riluzole treated cells in which GRM1 expression was reduced in the presence of the inducer, PonA (Figure 4B, bottom panel).

Formation of γ -H2AX foci after riluzole treatment is independent of apoptosis

The formation of γ -H2AX foci not only reflects the presence of local DNA DSBs following exposure to DNA damaging agents, but can also be observed in cells as a result of apoptosis (de Feraudy et al., 2010). In order to differentiate if the enhanced levels of γ -H2AX foci followed by riluzole treatment was dependent or independent of apoptosis we utilized immortalized baby mouse kidney epithelial cells (iBMK) deficient in apoptosis through the deletion of BAX and BAK (D3 cells) that were established previously by Dr. Eileen White and colleagues (Degenhardt and White, 2006) used by us in another study where introduction of cDNA encoding the murine form of GRM1 (Grm1) into D3 cells resulted in activation of MAPK and PI3K/AKT pathways leading to transformation in vitro as well as tumorigenicity in vivo in contrast to empty vector D3-Vec clones (Martino et al., 2013). Riluzole (10 μ M) treated D3-Vec cells showed scant 53BP1 staining and an overall diffuse γ -H2AX staining patterns (Figure 4C). On the contrary, riluzole-treated D3-Grm1 cells displayed clear co-localization of nuclear 53BP1 and γ -H2AX foci. Quantification of these images show significant differences in the level of fluorescence detected in D3-Grm1 cells treated with riluzole compared to no treatment control or to D3-Vec cells (Figure 4D) supporting the notion that riluzole mediated DNA DSBs is independent of apoptosis.

Riluzole treatment in GRM1 expressing human melanoma cells increases oxidative stress

Results from the above experiments suggest that riluzole induced DNA DSBs are dependent on GRM1-mediated glutamatergic signalling and independent of apoptosis, however, how riluzole elicits DNA damage remains unknown. It has been shown that the export of glutamate is coupled to the import of cystine by plasma membrane transporters, which regulates the bidirectional transfer of specific amino acids across the plasma membrane (Lo et al., 2008). Once inside the cell, cystine is reduced to cysteine, which is the rate limiting amino acid in the synthesis of the antioxidant glutathione (GSH). GSH has a predominant role in cellular defences against oxidative and nitrosative stress, as well as reactive electrophiles such as ROS. We postulated that inhibition of glutamate release from within the cell to the extracellular environment by riluzole could result in a build-up of intracellular glutamate concentrations and alterations in intracellular glutamate/cystine homeostasis. To test this hypothesis, we measured the levels of intracellular ROS in two GRM1-expressing human melanoma cell lines, C8161 and UACC903. Cells were treated with riluzole (10 μ M) for 12, 24, and 48 h followed by incubation with a cell-permeable ROS-specific fluorogenic marker, dihydrorhodamine 123 (DHR123) and analysed by flow cytometry. Examples of one-parameter fluorescence histograms of C8161 at 24 h post-treatment are shown (Figure 5A). We found that 24 h in the presence of riluzole there was a 50% increase in the mean DHR123 fluorescence in C8161 cells when compared to vehicle treated cells (Figure 5A). The antioxidant function of glutathione is well established in physiological and pathological states (Leeuwenburgh and Ji, 1998). GSH can either scavenge radicals by hydrogen donation directly or act as a co-factor for other antioxidant enzymes. Because GSH itself is not effectively transported into cells, compounds such as N-acetyl cysteine (NAC) or GSH ethyl ester (GSH-Et) have been utilized to increase cysteine levels to use for GSH synthesis or GSH-Et hydrolysed to GSH intracellularly (Martensson and Meister, 1989). If the elevated intracellular glutamate levels caused by riluzole treatment perturb the bidirectional transport of glutamate and cystine results in a decrease of GSH synthesis and rise in ROS, we proposed that supplementation of NAC or GSH-Et should at least partially reverse the observed increase in ROS. To test this, C8161 human melanoma cells were either pre-incubated with a 10 mM NAC solution for 30 min prior to riluzole exposure or

inclusion with 1 mM GSH-Et at the time of riluzole treatment (10 μ M). Cells were harvested at 12, 24, and 48 h at which time they were loaded with dihydrorhodamine 123, washed and analysed by flow cytometry. Pre-treatment of C8161 with NAC followed by riluzole exposure led to a 30% reduction in ROS levels respectively (Figures 5A and 5B). Co-incubation of riluzole and GSH-Et resulted in a 49% decrease in ROS levels in C8161 cells (Figures 5A and 5B). These results were confirmed in UACC903, another GRM1-positive cell line (Figure 5B). Immunofluorescent microscopy to demonstrate the reduction in ROS by molecular scavengers correlated with a decrease in co-localization of γ -H2AX and 53BP1 in C8161 cells (Data not shown).

Similar experiments were repeated using the isogenic clones derived from the immortalized non-tumorigenic normal human melanocytes, hTERT/CDKR24C. Cells were loaded with DHR123, incubated with riluzole for 12, 24, and 48 h and fluorescence measurements taken. No significant difference between untreated cells, cells treated with vehicle (DMSO) or riluzole treatment was detected in the parental cells (Figures 5C and D) while the GRM1 expressing isogenic cell line, hTERT/CDKR24C/p53DD-GRM1, showed a 15–34% increase in ROS levels with 24 h post-riluzole treatment having the highest levels in comparison to not-treated. These results demonstrate that (i) an increase in ROS is detected as soon as 12 hours post-treatment and that (ii) riluzole-mediated ROS formation are correlated with GRM1 expression.

GRM1 expression in human melanoma cells correlates with reduced intracellular GSH levels

Cellular GSH levels reflect a steady state balance between synthesis and loss. A sufficient accumulation in intracellular ROS, in part, is due to an increase in the rate of GSH consumption within the cell. A consequence of this modulation is damage to DNA, protein and lipid membranes (Leeuwenburgh and Ji, 1998). To assess if riluzole altered intracellular levels of GSH in our system, we treated the immortalized non-tumorigenic normal human melanocytes, hTERT/CDKR24C/p53DD and GRM1 expressing hTERT/CDKR24C/p53DD-GRM1, as well the human melanoma cell lines C8161 and UACC930 with 10 μ M of riluzole for the indicated time points. Cells were harvested and assessed for intracellular concentrations of GSH. We found that while riluzole treatment does not reduce the intracellular GSH concentrations in hTERT/CDKR24C/p53DD cells at 24 and 48 hours, hTERT/CDKR24C/p53DD-GRM1 cells displayed statistical significant decreases in intracellular GSH levels (Figure 6A). GRM1-expressing human melanoma cell line C8161 showed a time dependent decrease in levels of GSH while the lack of functional GRM1 in UACC930 cells resulted in very little decrease in GSH concentrations in comparison to not treated cells (Figure 6A).

Riluzole promotes accumulation of intracellular glutamate in GRM1+ human melanoma cells

We have shown that human melanoma cells express functional GRM1 have elevated levels of extracellular glutamate in their surrounding environment (Namkoong et al., 2007). Treatment with riluzole inhibits the release of glutamate from the intracellular to extracellular space thus likely enhancing intracellular accumulation of glutamate. To assess this, C8161 and UACC930 human melanoma cell lines as well as hTERT/CDKR24C/p53DD and hTERT/CDKR24C/p53DD-GRM1 isogenic cell lines were treated with 10 μ M of riluzole for 24 and 48 h after which time, cells were collected and assessed for intracellular concentrations of glutamate. We found that GRM1 expressing C8161 cells treated with riluzole had approximately a two-fold increase in intracellular glutamate concentrations at 48 h after treatment compared to untreated samples (Figure 6B). In contrast, GRM1-negative UACC930 cells showed no alteration in the amount of glutamate

within the cells after treatment with riluzole. The normal human melanocytic cell line, hTERT/CDKR24C/p53DD, showed a decrease of the amount of intracellular glutamate, however, there was a dramatic increase in the amount of glutamate concentrations within riluzole-treated hTERT/CDKR24C/p53DD-GRM1 cells (Figure 6B). A diagram summarizing the proposed metabolic effects of riluzole is shown in Figure 6C.

DNA damage increase in biopsies from patients with biologic responses in riluzole monotherapy trial

Previously, our group had reported on completed Phase 0 and Phase II trials using single agent riluzole in stage III or IV melanoma patients (Yip et al., 2009, Mehnert, 2013). Based on our in vitro results described in this report, we were interested to see if detectable changes in DNA damage markers would present themselves in vivo. We performed IHC staining using γ -H2AX antibody on pre- and post-riluzole treated biopsy samples from the Phase II trial, all samples were positive for GRM1 expression by IHC (Yip et al., 2009, Mehnert, 2013). We detected a significant number of γ -H2AX-positive cells in the post-treatment samples of two stable disease patients with biologic responses compared to pre-treatment samples from the same patients (Figures 7). In contrast, samples from a non-responding, disease progressing patient from the same trial showed similar and low number of γ -H2AX-positive cells in pre- and post-treatment samples. Despite the small number of samples examined, these results strengthen our working hypothesis that responsiveness to riluzole therapy through inhibition of glutamate release in melanoma patients may be modulated by an increase in the DNA damage response and activation of γ -H2AX. However, considering that all patients enrolled in the trial were GRM1 positive as determined by IHC, additional unknown factor(s) must be involved to determine one's sensitivity to riluzole including the ability to elicit DNA damage responses.

Discussion

Our laboratory has demonstrated that aberrant expression of GRM1 in melanocytes is able to induce cellular transformation in vitro and tumorigenesis in vivo (Pollock et al., 2003). We also verified that activation of ectopically expressed GRM1 in melanocytes leads to stimulation of at least two signalling pathways, MAPK and PI3K/AKT, known to be important in melanoma pathogenesis (Namkoong et al., 2007, Shin et al., 2010). Recent reports by others also support the important roles of glutamatergic signalling in the maintenance of transformed phenotypes in cultured melanocytes and progression of melanocytic tumors in vivo (Ohtani et al., 2008, Wei et al., 2011, Choi et al., 2011, Prickett et al., 2011). Therefore it is not surprising that the perturbations in glutamate signalling may result in a decrease in cell proliferation and survival.

Despite the lack of specific GRM1 antagonists with clinical applications, our group has successfully used Rilutek® (riluzole), an FDA approved drug for the treatment of Amyotrophic Lateral Sclerosis (ALS), in clinical trials for stage III/IV melanoma patients (Yip et al., 2009). Riluzole functions as an antagonist to GRM1 by decreasing the levels of free ligand available to bind to the receptor resulting in reduced receptor activity and accumulation of glutamate within the cells (Doble, 1996). Although extensive research has been performed on riluzole, the precise mechanism on inhibition of glutamate release remains largely unknown. In the CNS, the action profile of riluzole is dominated by blockade of glutamatergic transmission mediated by N-methyl-D-aspartate (NMDA) (Song et al., 1997). We demonstrated that, as in the neuronal system, riluzole also suppresses the release of glutamate to the extracellular environment of GRM1-positive melanoma cells (Namkoong et al., 2007). In addition, cell cycle profiling noticeably revealed that riluzole treatment induced cell accumulation in G₂/M phase of the cell cycle followed by a shift to sub G₁ and apoptotic cell death (Namkoong et al., 2007). Subsequent observation of the co-

localization of two well-known DNA damage markers detected only in GRM1-expressing cells following exposure to riluzole suggests that the observed reduction in glutamate export correlated with an increase in intracellular glutamate concentrations may promote DNA damage by disturbing detoxification mechanisms critical for maintaining cancer redox potential, mitigating oxidative damage.

Emerging evidence suggests that fundamental differences in mitochondrial oxidative metabolism of cancer cells relative to normal cells leads to increased steady-state levels of reactive oxygen species (ROS) (Dayal et al., 2009, Fath et al., 2009). Furthermore, significant evidence suggests that increased glucose metabolism in cancer cells supports not only an increased need for energy, but also the detoxification of ROS via deacetylation reactions involving pyruvate and regeneration of NADPH from NADP⁺. NADPH is the co-factor necessary for maintaining glutathione (GSH) and thioredoxin (Trx) in their reduced state, supporting ROS metabolism by maintaining cancer cell redox potential and mitigate oxidative damage. (Barral et al., 2000, Cheng et al., 2004, Schafer and Buettner, 2001). Many cancer cells including melanoma have been shown to upregulate GSH metabolism and this upregulation has been associated with resistance to radiation and platinum-based therapies (Schafer and Buettner, 2001, Vene et al., 2011). As the importance of GSH for mitigating oxidative stress in cancer cells has emerged, numerous clinical strategies for inhibiting GSH-dependent metabolism have been attempted, with limited success for treating cancer. Based on this it is possible that riluzole has an indirect effect on GSH synthesis and increased ROS by reducing the amount of available cystine within the cell.

Amino acid transporters regulate the bidirectional transfer of specific amino acids across the plasma membrane and are essential for cells that cannot sufficiently synthesize certain amino acids. Normal and cancer cells have a critical requirement for the amino acid cystine or its reduced form, cysteine (Eagle et al., 1966, Iglehart et al., 1977). The glutamate/cystine antiporter (xCT) functions as the exchange system for cystine/glutamate with cystine entry into the cells in exchange for the release of L-glutamate at a 1:1 ratio. Intracellularly, cystine is rapidly reduced to cysteine, the rate-limiting substrate for the biosynthesis of GSH (Figure 6C). Earlier studies by others have shown that elevated levels of intracellular glutamate leads to interference of the system antiporter, inhibiting entry of cystine resulting in reduced GSH synthesis and disparity in cellular redox homeostasis (Okuno et al., 2003, Sato et al., 1999). The xCT antiporter is expressed in various malignant tumors and our laboratory is currently investigating its role and implication in our system. We have detected various levels of xCT in several human melanoma cell lines with no apparent correlations amongst mutations in *BRAF/N-RAS* or stage of the disease (Shin, S. Unpublished observation). To further assess the role of xCT in our system, we have attained xCT-null mouse melanocytes derived from a mouse model of Hermansky-Pudlak syndrome, a rare autosomal recessive disorder which results in oculocutaneous albinism, platelet abnormality, and lysosomal accumulation of ceroid lipofuscin (Oh et al., 1998). By introducing either exogenous GRM1 alone or functional xCT, we can further assess the involvement of xCT in glutamatergic signalling by examining requirement for the maintenance of cellular homeostasis, whether it is a potential target for riluzole in riluzole-mediated inhibition of glutamate release, or if there are other glutamate exchange transporters at play in our system.

DNA-damaging compounds have been the mainstay of cancer treatment for the past century. Many cancer drugs employed in the clinic are highly efficient in producing excessive DNA damage that causes cell death directly or following DNA replication (Powell and Bindra, 2009). Riluzole's ability to induce DSBs depends on a functional receptor that has acquired an oncogenic potential. Cell transformation by GRM1 is mediated in part by the establishment of autocrine/paracrine loops that ensure the receptor is constitutively activated in an aberrant cellular environment where the "normal" cells do not express the receptor.

Riluzole exploits cancer specific differences in oxidative metabolism and could provide long-lasting benefits for the increasing numbers of melanoma patients. The tumor suppressive activity of riluzole can be explained not only by its ability to lower extracellular glutamate level and reduce receptor activity but also by increasing the level of oxidative stress in melanoma cells that express GRM1. Our findings suggest that combining riluzole with other available therapies could deliver enhanced efficacy for a subset of human melanoma.

Materials and methods

Antibodies and reagents

Antibodies against 53BP1 (Bethyl Laboratories Inc. Montgomery, TX); phospho H2AX and H4 antibodies (EMD Millipore Corporation, Temecula, CA); monoclonal α -tubulin antibody, etoposide, glutathione reduced ethyl ester, N-acetyl cysteine, riluzole and dihydrorhodamine 123 (Sigma, St. Louis, MO). Anti-phosphorylated ERK and anti-ERK (Cell Signalling, Danvers, MA (Fisher Scientific, Pittsburgh, PA). L-quisqualic acid [(L)-(+)-a-amino-3,5-dioxo-1,2,4-oxadiazolidine-2-propanoic acid] and BAY36-7620 [(3aS, 6aS)-6a-naphthalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[c]furan-1-on] (Tocris, Ellisville, MO); Alexa fluor 488 goat anti-mouse IgG, alexa fluor 546 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA).

Cell culture

Immortalized non-tumorigenic human melanocytes, hTERT/CDKR24C/p53DD were provided by Dr. David Fisher (Harvard Medical School, Boston, MA) and maintained in Medium 254 with human melanocyte growth supplements (Invitrogen, Carlsbad, CA). The human melanoma cell lines UACC903 and UACC930 were provided by Dr. Jeffery M. Trent (Translational Genomics Research Center, Phoenix, AZ) (Namkoong et al., 2007). C8161 human melanoma cells were from Dr. Mary J.C. Hendrix (Children's Memorial Research Center, Chicago, IL). Apoptosis deficient D3 iBMK cells were provided by Dr. Eileen White (Cancer Institute of New Jersey, New Brunswick, NJ) and derived as described previously (Degenhardt et al., 2002). Melanoma cell lines were grown in RPMI 1640 plus 10% fetal bovine serum (FBS).

Single Cell Gel Electrophoresis (COMET)

Cells were either treated with either DMSO, etoposide (10 μ M) for three hours, riluzole (10 μ M) for 24 hours or left untreated. Cell monolayers are detached using 0.005% trypsin (to prevent trypsin-induced DNA damage) and resuspended in PBS (Ca^{2+} , Mg^{2+} free) at a density of 10^4 cells per 100 μ l then mixed with an equal volume of 1% low melting point agarose (LMPA) made in PBS. 80 μ l of the cell suspension is added to a glass slide pre coated with 1% normal melting agarose (NMA) and rectangular cover slip placed on top to evenly spread the gel. Slides are cooled at 4°C until the agarose hardens (5–10 min). Coverslips are gently removed and 80 μ l of 1% LMPA is added to the layer, coverslip added and slides cooled. Coverslips are then removed and slides placed in chilled, freshly prepared lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% TritonX-100, pH 10.0) for a minimum of 1 hr at 4°C protected from light. Slides are then placed inside a gel box and electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) added to cover the slides and incubated for 20 min. Slides are then electrophoresed at 24 volts (~0.74 V/cm) and current adjusted to 300 milliamperes by raising or lowering buffer level for 30 min. The slides are removed and coated by drop wise application of neutralization buffer (0.4 M Tris, pH 7.5), drained and neutralization repeated two more times. Slides are stained with ethidium bromide solution (2 μ g/ml in dH₂O) and scored (50–100 randomly selected cells).

Images were quantified using NIH ImageJ by calculating the tail moments of comets in 10 random fields containing ~ 50 cells per treatment.

Western immunoblots

Whole cell protein lysates are prepared as described previously (Cohen-Solal et al., 2002). Briefly, cells are washed with ice-cold PBS, extraction buffer is added and cells are collected. After incubation on ice for 20 min, supernatants are collected by centrifugation at $25,000 \times g$ at 4°C for 20 min. Nuclear extracts for histone protein determination are prepared by collection of cells in PBS by centrifugation at $200 \times g$ at 4°C for 10 min. Pellets are washed twice in 10 volumes of ice-cold PBS followed by centrifugation and resuspended in extraction buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM DTT, 1.5 mM PMSF) and HCl is added to a final concentration of 0.2 N. After incubation on ice for 30 min, supernatants are collected by centrifugation at $11,000 \times g$ for 10 min. Protein concentration is determined using DC protein assay kit (Bio-Rad, Hercules, CA). Routinely, 25 μg of protein lysates are loaded in each lane for Western immunoblots. Blots are quantified using ImageJ software (NIH, Bethesda, Maryland) and are shown as a fold difference against relative density of loading control.

Ponasterone A inducible system

C8161 cells were transfected with pVgRXR plasmid DNA or siGRM1 RNA cloned in the pIND vector as described (Rangasamy et al., 2008). Induction of siGRM1 was achieved by treating the cells with 10 μM of Ponasterone A (PonA) for 7 days as described (Wangari-Talbot et al., 2012).

DNA transfection

DNA transfections were done either with N-[1-(2, 3-dioleoyloxy) propyl]-N, N, N-trimethylammoniummethyl sulphate liposomal transfection reagent (Roche, Basel, Switzerland) according to the manufacturers' instructions. Coding sequence for the full length (alpha) form of the murine form of the receptor (Grm1) was identified and cloned from mouse brain cDNA library (Zhu et al., 1999) into a mammalian expression vector, pCI-neo (Promega, Madison, WI, USA). HTERT/CDKR24C/p53DD cells are transfected with pCI neo plasmid with and without cDNA encoding human GRM1 α (NCBI accession NM_001278064.1). Receptor expression is confirmed by Western blotting.

Immunohistochemistry (IHC)

IHC for $\gamma\text{-H2AX}$ is performed by the Tissue Analytic Services at the Rutgers Cancer Institute of New Jersey, and unbiased quantitative assessment of IHC staining is completed using a digital Aperio ScanScope GL system and Aperio ImageScope software (v 10.1.3.2028) (Aperio Technologies Inc., Vista, CA) according to the manufacturer's protocol.

Detection of reactive oxygen species (ROS)

Cells are seeded at 3×10^5 per well in a 6 well plate and treated as indicated. After treatment cells are loaded with dihydrorhodamine 123 (DHR123) for at least 20 min at 37°C in a humidified 5% CO_2 /95% air incubator. The plates are washed by several changes of growth medium, trypsinized and cells collected by centrifugation at $300 \times g$, washed, and resuspended in PBS. ROS levels are immediately measured by the Flow Cytometry Facility Core at Rutgers University using a Beckman Coulter FC500 Analyser (Epics XL-MCL model). Rhodamine 123 derived from DHR123 by oxidation is excited with an air-cooled argon ion laser at 488 nm. Fluorescent emission of the marker is detected between 525 and 535 nm. Cell debris and multi-cell aggregates are electronically gated out. For ROS rescue

studies, cells are either pre-treated with N-acetyl cysteine pH 7.4 at a final concentration of 10 mM for 30 min prior to the addition of riluzole or co-incubated riluzole with reduced glutathione ethyl ester at a final concentration of 1 mM.

Measurement of intracellular glutathione (GSH)

Intracellular levels of total glutathione are measured using a GSH/GSSG quantification kit (Dojindo Laboratories, Kumamoto, Japan). Cells are seeded at 5×10^5 cells in 100 mm culture dishes. Cells are treated, collected and pelleted by centrifugation at $200 \times g$ for 5 min at the indicated time points. Pellets are washed with ice cold PBS and resuspended in 80 μ l of 10 mM HCl solution. Cells are lysed by freezing and thawing twice and 5% sulfasalicylic acid solution is added to a final concentration of 1%. Cells are pelleted by centrifugation at $8000 \times g$ for 10 min and supernatant is collected. Each sample (20 μ l) is placed in a 96 well plate in triplicate in combination with 120 μ l of buffer solution from the kit and incubated for 1 hour at 37° C after which time 20 μ l of substrate, coenzyme, and enzyme working solutions from the kit are added. Samples are incubated for 10 min at 37° C and the absorbance read at 405 nm in a Tecan infinite M200 plate reader (Tecan, Durham NC). Concentration of glutathione is calculated using calibration curve and presented as fold difference compared to untreated cells.

Measurement of intracellular glutamate levels

Amplex Red Glutamic Acid/Glutamate Oxidase assay kit (Invitrogen-Molecular Probes) is used to measure intracellular levels of glutamate. 10^5 cells are seeded per 60 mm plate in media alone or media containing 10 μ M of riluzole, the effective concentration previously established by our laboratory (Namkoong et al., 2007). At specified time points, cells are trypsinized. Cells are counted and pelleted at $300 \times g$ for 10 min. Pellets are rinsed twice using PBS then resuspended in 200 μ l of PBS. Cell membranes are weakened by three freeze and thaw cycles. Cells are transferred to a pre-cooled dounce homogenizer and mitochondrial membrane ruptured by 40 strokes. The suspension is then measured for the amount of glutamate according to the manufacturer's protocol.

Immunofluorescent staining

Cells are grown on glass coverslips in RPMI 1640 and 10% FBS, synchronized by serum starvation for 48 h and treated as indicated. At each time point, cells are fixed with 4% paraformaldehyde in PBS and permeabilized for 10 minutes in 0.5% Triton X-100 in PBS at room temperature. Cover slips with cells are then washed with PBS and blocked in 5% goat serum/PBS. Primary antibodies (53BP1 and γ -H2AX) are incubated at room temperature in blocking solution. Cover slips containing cells are washed with PBS for 5 min and incubated with Fluorophore-conjugated secondary antibody for 1 h in blocking solution. Cover slips with cells are washed with PBS for 5 min and mounted using Vectashield. DAPI is used to show nuclear stains. Etoposide treatment (10 μ M) is used as a positive control for DSBs. Images are captured using a Nikon Eclipse microscope coupled with a Coolsnap EZ camera powered by NIS-Elements BR 3.1 software. All quantifications are performed using ImageJ software on 10 random fields (n=10) each field containing ~30–50 cells.

Statistics

Statistical analyses were calculated using two-sample student t-tests, comparisons are made between treated and either not treated or vehicle treated samples. $P < 0.05$ is considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

We uncovered a previous unknown consequence of inhibition of glutamate release in a subset of human melanoma cells. Our results reinforce the notion that glutamate signalling plays a role in the maintenance of transformed phenotypes of human melanoma cells *in vitro* and *in vivo* and could be a potential therapeutic target for the treatment of melanoma.

Figure 1.

Riluzole induces DNA damage in GRM1 expressing human melanoma cells. **(A)** Western immunoblots of GRM1-positive C8161 and GRM1-negative UACC930 melanoma cells treated with vehicle (DMSO), riluzole (10 μ M), or the positive control for DSBs, etoposide (10 μ M). H4 is used as a loading control. Blots were quantitated and are shown as a fold difference over vehicle control normalized to loading control. **(B)** DNA damage was confirmed using COMET assays.

Figure 2. Riluzole induces double strand DNA breaks in GRM1 expressing human melanoma cells. (A) Immunofluorescence staining was performed with antibodies against 53BP1 (red) and γ -H2AX (green) to show co-localization to sites of DNA DSBs in GRM1-positive C8161 and GRM1-negative UACC930. Quantifications are shown in (B) with each bar representing mean $\pm$ SEM (* $p < 0.05$; ** $p < 0.005$) with etoposide (ET) used as a positive control.

Figure 3. Expression of GRM1 is required for riluzole induced DNA damage. (A) Western immunoblots of stable GRM1-human melanocytic (hTERT/CDKR24C/p53DD) clones.

C8161 human melanoma cells and IMR32 human neuroblastoma cells (+) are GRM1 positive controls; vector indicates vector control transfectants while 1, 2, 3, 4 and 5 are independent GRM1 human melanocytic (hTERT/CDKR24C/p53DD-GRM1) clones. α -tubulin was used as a loading control. **(B)** An example of clone 1 of hTERT/CDKR24C/p53DD-GRM1 is shown. Modulation of downstream MAPK was carried out using GRM1 agonist L-quisqualic acid and inhibited by pre-treatment with the agonist BAY 36-7620 for 30 min then induced with the agonist L-quisqualic acid for various time points. **(C)** Immunofluorescence staining was performed to show DNA damage in immortalized non-tumorigenic human melanocytes, hTERT/CDKR24C/p53DD-Vec and isogenic hTERT/CDKR24C/p53DD-GRM1-clone 1 either not treated (NT) or treated with riluzole. Quantifications are shown in **(D)** with each bar representing mean $\pm$ SEM (* p 0.05; ** p 0.005) with etoposide (ET) used as a positive control.

Figure 4.

Knock down of GRM1 expression reduces amount of DSBs in riluzole treated human melanoma cells independent of apoptosis. (A) Co-localization of γ -H2AX and 53BP1 following riluzole treatment using stable C8161 clones harboring either empty vector or PonA-inducible siGRM1 clones. (C) Stable clones isolated from apoptosis deficient (D3) containing either empty vector (D3-Vec) or exogenous murine Grm1 cDNA (D3-Grm1). Quantifications are shown in (B and D). Each bar represents mean $\pm$ SEM (* $p < 0.05$; ** $p < 0.005$) with etoposide (ET) used as a positive control.

A.

B.

Cell line	No treatment (NT)	Vehicle (DMSO)	Riluzole (24 h)	Riluzole + NAC (24 h)	Riluzole + GSH ester (24 h)
C8161	1	1	1.51 *	1.21 *	1.02
UACC903	1	1.02	1.14	1.08	1.04

* = $p \leq 0.05$

C.

D.

Cell lines	No treatment (NT)			Vehicle (DMSO)			Riluzole		
	12h	24h	48h	12h	24h	48h	12h	24h	48h
hTERT/CDKR ^{24C} /p53 ^{DD}	1.00	1.00	1.00	1.00	1.03	0.97	0.94	1.08	0.94
hTERT/CDKR ^{24C} /p53 ^{DD} GRM1	1.00	1.00	1.00	1.10	0.95	0.98	1.21	1.34**	1.15*

* = $p \leq 0.05$ ** = $p \leq 0.005$ **Figure 5.**

Riluzole treatment results in an increase in ROS in human melanoma cells and is rescued by inclusion of NAC or GSH-ester. (A) GRM1-positive C8161 and UACC903 were either not treated, treated with vehicle or riluzole for 12, 24 or 48 h. Levels of ROS is measured as described in materials and methods. Examples at 24 h are shown. ROS scavenger was included either pre-incubated with 10 mM NAC for 30 min before adding riluzole or co-incubation in riluzole and 1 mM reduced GSH-ester. The results of both melanoma cell lines are summarized in (B) as fold differences over the not treated samples, which is set as 1. Open histograms show total number of cells plotted against log rhodamine fluorescence. (C) FACS plots showing levels of ROS formation following treatment with riluzole in immortalized, non-tumorigenic human melanocytes hTERT/CDKR24C/p53DD and hTERT/CDKR24C/p53DD-GRM1 cells treated with 10 μ M riluzole for various time-points. (D) Results are summarized as the fold differences over not treated cells, which is set at 1. Student T-test was performed to show statistical significance of three independent experiments. Open histograms show total number of cells plotted against log rhodamine fluorescence.

C.

Figure 6.

Riluzole treatment of GRM1-positive human melanoma cells causes a reduction in glutathione levels and an increase in intracellular glutamate levels. GRM1-positive C8161, GRM1-negative UACC930, hTERT/CDKR24C/p53DD and isogenic hTERT/CDKR24C/p53DD-GRM1 were not treated or treated with 10 μ M riluzole for 24 and 48 h. Total intracellular glutathione (**A**) and intracellular glutamate (**B**) concentrations were assessed and shown as a percent of no treatment cells (NT). Quantifications were performed with all samples and each bar representing mean $\pm$ SEM (* $p < 0.05$; ** $p < 0.005$). (**C**) A schematic showing known roles of glutamate in cellular physiology. Inhibition of glutamate release

could affect cystine transport in the cells causing a reduction in GSH synthesis. This results in an increase of intracellular ROS resulting in DNA damage and eventually cell death.

Figure 7.

DNA damage marker is only detected in post-treatment biologic response specimens from Phase II trial of riluzole. IHC of γ -H2AX in pre- and post-riluzole treated specimens from biologic responder, stable disease patients (**patients 6 and 13**) and non-responder, disease progressing (**patient 9**) of a recently completed Phase II riluzole trial. Top panels are 10X magnifications and bottom panels are 40X magnifications. All slides were scanned and unbiased quantitative assessment of IHC staining was completed using a digital Aperio ScanScopeGL system and ImageScope software (Aperio Technologies Inc.) as described (Wangari-Talbot et al., 2012). Percent of IHC positive nuclei is indicated below each image.