

# Chemical-Genetic Screen Identifies Riluzole as an Enhancer of Wnt/ $\beta$ -catenin Signaling in Melanoma

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## SUMMARY

To identify new protein and pharmacological regulators of Wnt/ $\beta$ -catenin signaling, we used a cell-based reporter assay to screen a collection of 1857 human-experienced compounds for their ability to enhance activation of the  $\beta$ -catenin reporter by a low concentration of WNT3A. This identified 44 unique compounds, including the FDA-approved drug riluzole, which is presently in clinical trials for treating melanoma. We found that treating melanoma cells with riluzole *in vitro* enhances the ability of WNT3A to regulate gene expression, to promote pigmentation, and to decrease cell proliferation. Furthermore riluzole, like WNT3A, decreases metastases in a mouse melanoma model. Interestingly, siRNAs targeting the metabotropic glutamate receptor, GRM1, a reported indirect target of riluzole, enhance  $\beta$ -catenin signaling. The unexpected regulation of  $\beta$ -catenin signaling by both riluzole and GRM1 has implications for the future uses of this drug.

## INTRODUCTION

Wnt proteins act as ligands for members of the Frizzled family of serpentine receptors and for the LRP5/6 coreceptors. Activation of the pathway results in  $\beta$ -catenin (CTNNB1) stabilization and nuclear accumulation. Aberrant Wnt/ $\beta$ -catenin signaling arising from either hyper- or hypoactivation has been linked to numerous clinical conditions, most notably cancers (Moon et al., 2004). Several types of cancer have been linked to mutations in core Wnt/ $\beta$ -catenin pathway genes that result in constitutive ligand-independent activation of the pathway (Moon et al., 2004). However, as described below for melanoma, elevation of Wnt/ $\beta$ -catenin signaling correlates with or may promote favorable biological or clinical outcomes.

Wnt/ $\beta$ -catenin signaling is a major regulator of melanocyte differentiation (Thomas and Erickson, 2008), so it is not surprising that this pathway is also involved in the pathogenesis of malignant melanoma. In a mouse model of malignant melanoma, Wnt/ $\beta$ -catenin signaling itself is insufficient for melanocyte transformation, but activation of this pathway can work synergistically with active Ras signaling to promote tumor formation (Delmas et al., 2007). By contrast, several studies using patient-derived tumor samples have reported that melanoma progression is associated with the loss of nuclear  $\beta$ -catenin, a clinical marker of Wnt/ $\beta$ -catenin activation. Furthermore, improved survival is seen in patients with higher levels of cytosolic or nuclear  $\beta$ -catenin (Bachmann et al., 2005; Chien et al., 2009; Kageshita et al., 2001; Maelandsmo et al., 2003), suggesting that active Wnt/ $\beta$ -catenin signaling in patients predicts improved prognosis. Interestingly, activation of Wnt/ $\beta$ -catenin signaling by Wnt3a in a mouse melanoma model results in decreased proliferation *in vitro* and *in vivo*, along with increased expression of melanocyte differentiation markers usually lost with melanoma progression, pointing to potential mechanisms that might explain the improved prognosis of patients with elevated  $\beta$ -catenin (Chien et al., 2009). Collectively, these observations underscore the potential importance of determining whether any existing approved drugs enhance Wnt/ $\beta$ -catenin signaling and whether they may have therapeutic benefit in treating melanoma.

In the present study, we used a cell-based screen to identify several small molecule enhancers of Wnt/ $\beta$ -catenin signaling. We focused on one enhancer compound, riluzole, an FDA approved therapeutic for amyotrophic lateral sclerosis that is under investigation in clinical trials as a melanoma therapy (Yip et al., 2009).

## RESULTS AND DISCUSSION

Using the  $\beta$ -catenin-activated luciferase reporter (BAR) (Biechele and Moon, 2008) (see Figure S1A available online) in a cell-based assay, we screened for small molecules that enhance the activation of the reporter by exogenous WNT3A (Figure 1 inset). We optimized the WNT3A stimulus to obtain an EC<sub>20</sub> (Figure S1B)



in both the number of Venus positive cells and their mean fluorescent intensity (MFI) by FACS analysis (Figure 1B). To validate that Venus expression is tightly linked to  $\beta$ -catenin signaling, the expression of endogenous  $\beta$ -catenin target genes in Venus-positive and -negative cells was compared. Consistent with a positive correlation, the Venus-positive population of untreated cells, or the Venus-positive population of cells that had been treated with WNT3A-conditioned media prior to FACS sorting, showed elevated expression of the  $\beta$ -catenin target genes *AXIN2* and *TNFRSF19* when compared with Venus-negative cells (Figure 1C). In this assay, several primary screen hits enhanced the WNT3A-mediated increase in the percentage of Venus-positive cells and their MFI (Figure 1D).

Candidate hits from the primary screen were analyzed with respect to their being mentioned in the literature and in clinical trials databases, enabling us to generate a list of diseases where these bioactive molecules were being studied. We compared this list to the diseases being treated with lithium chloride, a classic inhibitor of GSK3 which is the only available patient-experienced drug that is known to activate Wnt/ $\beta$ -catenin signaling. As a result of this analysis, we focused on the drug riluzole (brand name Rilutek) (Figure S1D), an FDA-approved drug used to slow the progression of amyotrophic lateral sclerosis (ALS) (Aggarwal and Cudkovic, 2008). Riluzole is under intensive evaluation as a treatment for bipolar disorder, a condition where lithium chloride is a first-line treatment (Pittenger et al., 2008). Lithium is also being evaluated as a treatment for ALS (Fornai et al., 2008), which is also consistent with the possibility that these two unrelated compounds may regulate the same signaling pathway(s).

Riluzole enhanced Wnt/ $\beta$ -catenin signaling in both the primary screen in HT22 neuronal cells (Figure S1E) and in adult hippocampal progenitor cells (Figure S1F). Furthermore, riluzole enhanced the stabilization and nuclear localization of  $\beta$ -catenin in U2OS cells (Figures S2A and S2B). As riluzole is in clinical trials for treatment of metastatic melanoma (Yip et al., 2009) and decreased Wnt/ $\beta$ -catenin in clinical samples has been associated with melanoma progression (Chien et al., 2009), we focused on validating this hit and investigating its mechanism of action in the context of melanoma. We first demonstrated that riluzole enhances Wnt/ $\beta$ -catenin signaling in a dose-dependent manner in A375 melanoma cells stably expressing BAR confirming the observations in neuronal cells (Figure S2C). As a control, we showed that riluzole does not activate a control reporter construct (fuBAR) in which the TCF/LEF sites required for responding to  $\beta$ -catenin have been mutated (Figure S2C). We next demonstrated that riluzole enhances the WNT3A-dependent activation of the promoter of a known direct target gene of  $\beta$ -catenin, *Axin2* (Figure 1E). Moreover, riluzole, like WNT3A, upregulates several genes involved in melanocyte development (*Trpm1*, *Met*, *Mitf*, *Sox9*, *Kit*, and *Si*) alone (Figure 2A) and in synergy with exogenous WNT3A (Figure 2B).

Consistent with the reported ability of WNT3A to elevate levels of transcripts involved in melanocyte differentiation (Chien et al., 2009), treating B16 melanoma cells with WNT3A leads to a dose- and  $\beta$ -catenin-dependent increase in cellular pigmentation (Figure 2C; Figure S2D). Treatment of these cells with riluzole also increases pigmentation, and does so in a  $\beta$ -catenin-dependent manner that is synergistically enhanced by cotreatment with

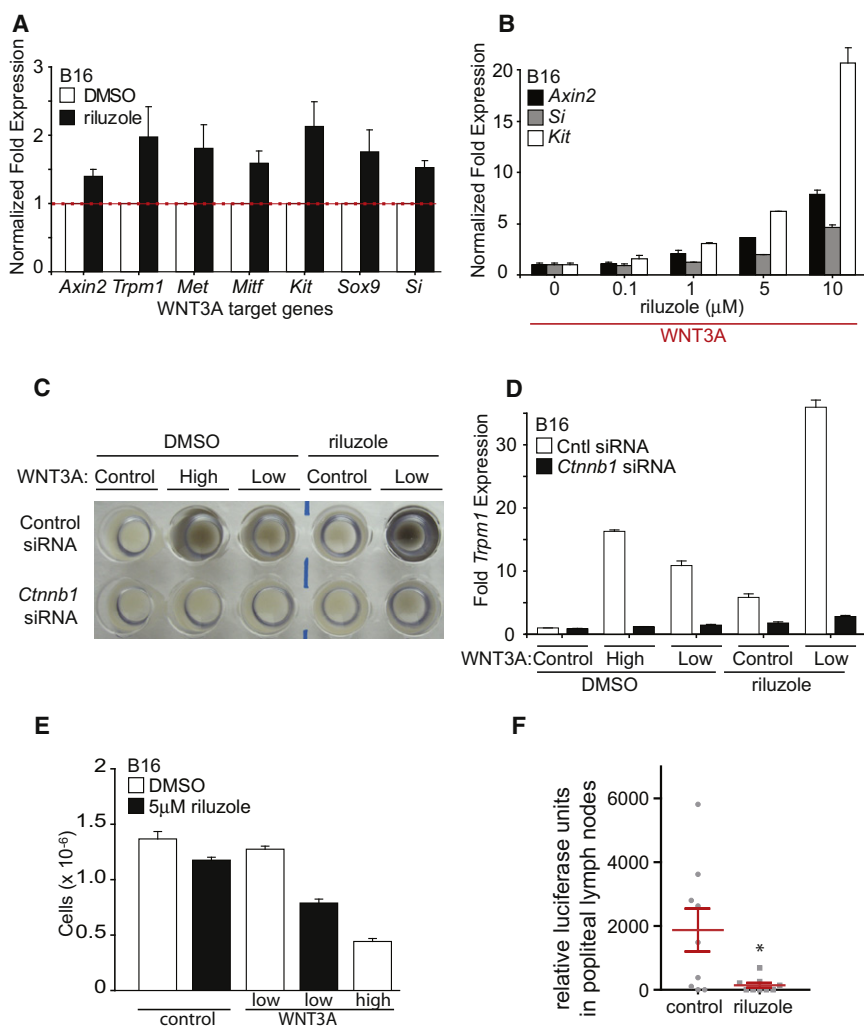
WNT3A (Figure 2C; Figure S2D). Analysis of riluzole-treated B16 cells in which siRNAs have been used to deplete endogenous  $\beta$ -catenin showed that Wnt-3a and riluzole synergize in a  $\beta$ -catenin-dependent manner to elevate the expression of *Trpm1* (Figure 2D).

We have previously demonstrated that elevated Wnt/ $\beta$ -catenin signaling in melanoma cell lines cultured in vitro, and in melanoma patients, correlates with a reduction in cell proliferation (Chien et al., 2009), raising the question as to whether riluzole would also affect proliferation. Synergy between riluzole and WNT3A was observed in the reduction of proliferation in B16 cells (Figure 2E). We also observed a decrease in the proliferation of several human melanoma cell lines in the presence of riluzole (Figure S2E).

We then tested whether riluzole inhibits melanoma progression in vivo using B16 melanoma cells implanted into the footpads of mice, followed by treating the mice with riluzole. There was a striking reduction in metastases compared with vehicle-treated mice, as measured by the detection of cells in the sentinel popliteal lymph node (Figure 2F). Collectively, the data show that riluzole both mimics and enhances the ability of WNT3A to promote differentiation of melanoma cells toward a more melanocyte-like state, that riluzole decreases the proliferation of melanoma cells in vitro, and that riluzole reduces the metastasis of melanoma cells in vivo.

To better understand the mechanism by which riluzole enhances Wnt/ $\beta$ -catenin signaling in melanoma cells, we explored known signaling targets and pathways previously linked to riluzole. In a phase 0 trial of riluzole for treatment of melanoma, it was shown that tumors from patients treated with riluzole exhibited decreased phosphorylation of ERK (Yip et al., 2009). However, treatment of A375 human melanoma cells with riluzole resulted in no change in ERK phosphorylation (Figure S3A). Another possible mechanism for riluzole to enhance Wnt/ $\beta$ -catenin signaling involves the activation of PKA through a riluzole-mediated increase in cAMP levels (Duprat et al., 2000; Taurin et al., 2006). To test this mechanism, A375 cells were treated with riluzole or with forskolin, a small molecule known to activate adenylyl cyclase and thus PKA (Seamon et al., 1981). Cell lysates from forskolin, but not riluzole-treated cells, showed a significant enhancement in PKA-phosphorylated CTNNB1 as well as other protein substrates relative to controls (Figure S3B).

Previously, a mouse with a high incidence of spontaneous melanoma-like lesions was found to carry a mutation that led to elevated expression of metabotropic glutamate receptor 1 (*GRM1*) (Pollock et al., 2003). Riluzole has been reported to inhibit glutamate release and reuptake and thereby inhibit activation of metabotropic glutamate receptors (Doble, 1996). If riluzole enhances Wnt/ $\beta$ -catenin signaling through a mechanism involving GRM1 then reducing GRM1 expression might enhance Wnt/ $\beta$ -catenin signaling. To test this hypothesis, we transfected A375:BAR melanoma cells with two independent siRNAs targeting *GRM1*, or with siRNAs targeting *CTNNB* and *AXIN1/2* as controls. Both *GRM1* siRNAs depleted *GRM1* transcript levels to approximately 10% of control siRNA transfected cells (Figure S3C). Strikingly, reduction of *GRM1* transcripts by siRNAs synergized with WNT3A in the BAR luciferase reporter assay, demonstrating that endogenous GRM1 functionally represses Wnt/ $\beta$ -catenin signaling (Figure 3A). *GRM1*-directed siRNAs



**Figure 2. Riluzole Enhances Wnt/ $\beta$ -Catenin Signaling to Promote Markers of Differentiation and Decrease the Proliferation of Melanoma Cells**

(A) B16 melanoma cells were treated with 10  $\mu$ M riluzole or DMSO for 24 hr and qRT-PCR was performed. All data were normalized to *Gapdh* expression and represent the mean value of triplicate experiments  $\pm$  SD.

(B) B16 melanoma cells were treated with a low dose of WNT3A conditioned media and DMSO or the indicated doses of riluzole for 6 hr and profiled for the expression of *Axin2*, *Si*, and *Kit* by qRT-PCR. All data were normalized to *gapdh* expression, expressed as fold over the corresponding DMSO control, and represent the mean value of triplicate experiments  $\pm$  SD.

(C and D) B16 melanoma cells transfected with control or *Ctnnb1* siRNA were treated for 3 days with the indicated conditions and analyzed for pigmentation (C) and *Trpm1* expression (D). The pigmentation data are representative of three independent experiments and *Trpm1* expression represents the mean value of triplicate experiments  $\pm$  SD.

(E) Riluzole synergizes with WNT3A to decrease the proliferation of melanoma cells. B16 cells were treated for 4 days in the indicated conditions and then harvested and counted. Data represent the mean value of six experiments  $\pm$  SD.

(F) B16 cells were injected into footpads of C57BL/6 mice, and treatment with riluzole was initiated 1 week postinjection. Sentinel lymph nodes in the popliteal fossa adjacent to the injected foot were assayed for the presence of metastases as measured by Firefly luciferase. Bars represent the mean and standard deviation of ten mice for each group and indicate that tumors from mice treated with riluzole exhibited significantly decreased metastasis compared to control mice with no treatment (unpaired two-tailed t test) \* $p < 0.02$ . See also Figure S2.

also enhanced WNT3A-mediated upregulation of endogenous *AXIN2* transcripts (Figure S3D). Consistent with these findings, three structurally unrelated GRM1-selective small molecule antagonists enhanced WNT3A-stimulated BAR activity in A375:BAR cells in a dose-dependent manner (Figure 3B). Furthermore, like riluzole, all three GRM1-selective antagonists enhance the pigmentation of B16 melanoma cells alone and synergize with WNT3A (Figures 3C and 3D; Table S1), while antagonists of the related glutamate receptor, GRM5, have no effect on BAR activity or B16 melanoma cell pigmentation (Figure S3E and Table S1). Collectively, these data identify GRM1 and not GRM5 as a novel regulator of Wnt/ $\beta$ -catenin signaling and the likely target of riluzole-mediated enhancement of Wnt/ $\beta$ -catenin signaling.

## SIGNIFICANCE

**This article describes the identification and characterization of the FDA-approved drug riluzole as an enhancer of Wnt/ $\beta$ -catenin signaling in melanoma cells. In melanoma cells, riluzole enhances the ability of WNT3A to regulate gene expression, to promote pigmentation, and to decrease**

**proliferation. Furthermore, riluzole, like WNT3A, decreased metastases in vivo in a mouse melanoma model. Investigating the mechanisms of action of riluzole revealed that endogenous metabotropic glutamate receptor GRM1 but not GMR5 represses Wnt-mediated activation of  $\beta$ -catenin signaling in melanoma cells. Given that riluzole is in clinical trials for treating melanoma, our data that riluzole modulates  $\beta$ -catenin signaling, combined with prior data that  $\beta$ -catenin signaling has complex effects in different cancers, should stimulate further investigation into the use of riluzole in cancer therapies.**

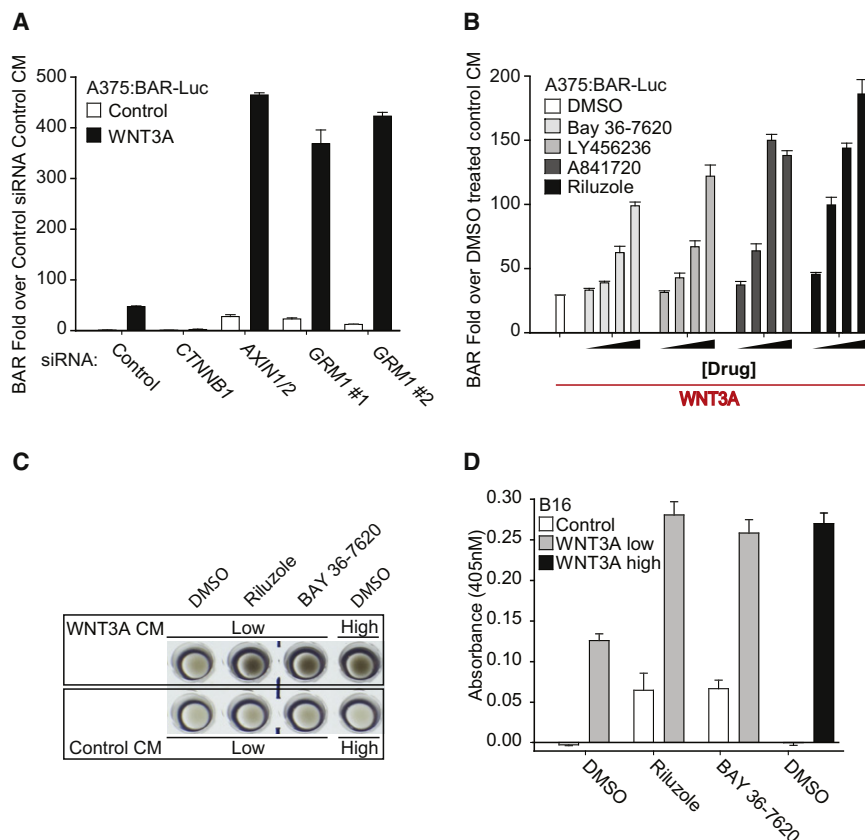
## EXPERIMENTAL PROCEDURES

Full details of materials and experimental procedures used in this study can be found in the Supplemental Experimental Procedures that accompany this Brief Communication (available online).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Database, three figures, and two tables and can be found with this article online at doi:10.1016/j.chembiol.2010.08.012.





**Figure 3. GRM1 Negatively Regulates Wnt/ $\beta$ -Catenin Signaling**

(A) siRNA mediated knockdown of GRM1 enhances Wnt/ $\beta$ -catenin signaling in melanoma cells.

(B) A375:BAR cells were treated with WNT3A CM and several doses of riluzole (1, 5, 10, 20  $\mu$ M), A841720 (0.1, 1, 10, 20  $\mu$ M), LY456236 (0.1, 1, 10, 20  $\mu$ M), or Bay 36-7620 (0.5, 1, 5, 10  $\mu$ M).

(C and D) B16 melanoma cells were treated for 3 days with the indicated conditions. Riluzole and Bay 36-7620 were used at 10  $\mu$ M final concentration. Cells were trypsinized and pelleted for imaging (C) followed by resuspension and 405 nm absorbance measurement (D).

In (A), (B), and (D), data represent the mean value of triplicate experiments  $\pm$ SD. In (C), data are representative of three independent experiments. See also Figure S3.

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