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## Subsets of ATP-Sensitive Potassium Channel ( $K_{ATP}$ ) inhibitors increase gap junctional intercellular communication in metastatic cancer cell lines independent of SUR expression

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### Abstract

Gap junctional intercellular communication (GJIC) regulates cellular homeostasis by propagating signaling molecules, exchanging cellular metabolites, and coupling electrical signals. In cancer, cells exhibit altered rates of GJIC which may play a role in neoplastic progression.  $K_{ATP}$  channels help maintain membrane polarity; and, linkages between  $K_{ATP}$  channel activity and rates of GJIC have been established. The mechanistic relationship has not been fully elucidated. We report the effects of treatment with multiple  $K_{ATP}$  antagonist compounds on GJIC in metastatic cell lines demonstrating an increase in communication rates following treatment with compounds possessing specificities towards the SUR2 subunit of  $K_{ATP}$ . These effects remained consistent using cell lines with different expression levels of SUR1 and SUR2, suggesting possible off target effects on GJIC by these compounds.

### Keywords

Sulfonylurea;  $K_{ATP}$ ; gap junction; glibenclamide

## 1. Introduction

Gap junctional intercellular communication (GJIC) is a process by which cells communicate soluble factors and electrical signals through physical interactions at the plasma membrane via regulated channels known as connexons (1). Connexons are hexameric structures comprised of a family of monomeric proteins, connexins. More than 20 connexin proteins have been reported in mammalian cells, each with varying specificities of regulation. Once assembled in the plasma membrane connexon channels can be opened or closed through a variety of signals including cellular pH, ion concentrations, and ATP levels (2–4). From a

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metabolic perspective, exchange of small molecules (<1.5kDa) between these channels allows cells to share nutrients and secondary signaling molecules (e.g. IP<sub>3</sub>), in addition to regulating multiple cell types (e.g. cardiac, neuronal, epithelial) through electrical communication which contributes to the coordination of tissue function.

While GJIC is a means for normal cellular homeostasis, in cancer cells this communication is often dysregulated. Initially, reports demonstrated that during neoplastic progression, GJIC between cancer cells was often reduced compared to non-transformed cells of the same origin (5–7). More recently however, it is appreciated that GJIC may also be increased in cancer cells, or between cancer cells and stromal cells at secondary metastatic sites, highlighting cell and tissue context specific events (8–10). In any case, alterations of the level of GJIC between cancer cells is commonly observed.

The ATP sensitive potassium channel (K<sub>ATP</sub>) regulates K<sup>+</sup> conductance in cells and is closed by increasing concentrations of ATP. Closure of these channels results in depolarization of the plasma membrane due to reduced potassium conductance. The structure of the K<sub>ATP</sub> channel is composed of an inwardly rectifying potassium channel K<sub>IR</sub> (K<sub>IR</sub>6.1, K<sub>IR</sub>6.2), and a sulfonylurea subunit (SUR1, SUR2A and SUR2B) which regulates the activity of K<sub>IR</sub> through sensitivity to ATP levels, as well as other metabolites (e.g. PIP<sub>2</sub>) (11). Growing evidence has supported a role for K<sub>ATP</sub> in the regulation of GJIC activity. Vera *et al.* initially demonstrated that reduction of endogenous ATP levels (which would relieve K<sub>ATP</sub> inhibition) decreased GJIC in astrocytes and that this effect was reversible (12). Further work showed that closure of K<sub>ATP</sub> channels by the sulfonylurea receptor inhibitors tolbutamide and glibenclamide lead to increases in GJIC, suggesting that K<sub>ATP</sub> may play a regulatory role in opening of connexon channels, possibly through mechanisms related to membrane depolarization (13,14). Collectively these studies proposed evidence that inhibition of K<sub>ATP</sub> channels leads to greater rates of GJIC between cells, while opening of the same channels decreases GJIC. These data provide a link to the metabolic regulation of gap junctions through ATP. Additionally, development of tolbutamide as a therapeutic agent for cancer treatment through its effects on connexin regulation and gap junction modulation remains promising (15–18). Interestingly however, an increased risk of cancer mortality in Type 2 diabetes patients administered sulfonylureas has been reported (19,20), implicating context-dependent mechanisms and a possible role for modulation of K<sub>ATP</sub> conductance in the progression of cancer.

In the present study we examined the effect of treatment of highly metastatic cancer cell lines (MDA-MB-231, MDA-MB-435, C8161.9), which exhibit low baseline gap junction activity, with K<sub>ATP</sub> inhibitory compounds. Treatment of cells with the sulfonylurea receptor inhibitor glibenclamide produced a robust and consistent increase in calcein dye transfer indicative of GJIC between cancer cells expressing detectable protein levels of SUR2 with little to no detection of SUR1. Upon further examination of additional K<sub>ATP</sub> inhibitors with the MDA-MB-231 breast carcinoma cell line, we found that inhibitors with dual specificities to both the SUR1 and SUR2 subunits increased GJIC while those with primary specificities to SUR1 had little to no effect on GJIC suggesting that inhibition of SUR2 K<sub>ATP</sub> channels was responsible for the increase in GJIC. To evaluate this hypothesis, we screened additional breast cancer cell lines and identified the SUM159 as expressing inverse levels of SUR1 and SUR2 compared to MDA-MB-231, MDA-MB-435 and C8161.9. Treatment of SUM159 with the K<sub>ATP</sub> inhibitors resulted in the same pattern of GJIC. These data suggest the possibility of novel effects on GJIC by K<sub>ATP</sub> inhibitors that is independent of their K<sub>ATP</sub> specificity and will be important for future studies involving these compounds.

## 2. Materials and methods

### 2.1 Cell lines

MDA-MB-231, MDA-MB-435 and C8161.9 were grown in Dulbecco's-modified Eagle's medium mixed 1:1 (v:v) with Ham's F-12 medium (DMEM/F12, Invitrogen #11330) supplemented with 2 mmol/L of L-glutamine, 0.2 mmol/L of nonessential amino acids with 5% fetal bovine serum (FBS). MDA-MB-231 and MDA-MB-435 are human breast carcinoma-derived cell lines. For the origin of MDA-MB-435 the reader is referred to *Chambers, Can Res, 2009* (21). The C8161.9 is a clone derived from the C8161 human melanoma. The SUM159 cell line was a generous gift provided by the laboratory of David Salomon, National Cancer Institute. SUM159 were maintained in Ham's F12 media (Invitrogen #11765) supplemented with 5 µg/ml insulin, 0.1 µg/ml epidermal growth factor, 10 mM HEPES and 10% fetal bovine serum. All cell lines were tested for *Mycoplasma spp.* contamination using PCR (#302108; Aligent Technologies, Santa Clara, CA).

### 2.2 Chemicals

The following chemicals were obtained from Sigma (chlorpropamide #C1290, glibenclamide #G0639, gliclazide #G6127, glimepiride #G2295, repaglinide #R9028, tolbutamide #T0891). Calcein-AM #C1430 and 1,1'-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) #C7001 were purchased from Invitrogen.

All compounds were dissolved in DMSO, aliquoted and frozen at -20°C. At time of experiments aliquots were thawed and working concentrations made fresh. DMSO alone (NT, non-treated) had no effect on dye transfer. No morphological changes or signs of toxicity were observed for each compound/dose reported in this study.

### 2.3 GJIC assay

Gap junction assays were performed as previously described (22). "Donor" cells were loaded with Calcein-AM and DiI, a lipophilic dye that does not transfer between cells used to mark donor cells. After washing 3 times with Dulbecco's phosphate buffered saline (DPBS), donor cells were plated with nonlabeled "acceptor" cells for 6 hours. Calcein spread from donor to acceptor cells was indicative of GJIC. All experiments reported herein were conducted in serum-free media in order to observe the effects of  $K_{ATP}$  inhibitors in the absence of additional growth factors. Flow cytometry with a BD LSR II Cell analytic flow cytometer using BD FACS Diva software was used to calculate the average number of cells that received calcein per donor cell and represented as fold change.

### 2.4 Immunoblot assay and antibodies

Cells were lysed in buffer containing 25 mmol/L Tris (tris(hydroxymethyl)aminomethane), 1% Triton-X100, 500 mmol/L β-glycerolphosphate, 0.5 mM EDTA and 5% glycerol on ice followed by sonication to disrupt cell membranes. Lysates were resolved by 12% SDS-PAGE and transferred to PVDF membranes. Antibodies to SUR1 (Abcam #ab32844) and SUR2 (BD Pharmingen # 550429) were incubated at 1:1000 in 5% bovine serum albumin (BSA) in Tris-buffered saline Tween-20 (TBST) overnight at 4°C followed by incubation with horse radish peroxidase (HRP) conjugated anti-mouse (GE Healthcare #NXA931) or conjugated anti-rabbit (GE Healthcare #NA934) at 1:2500 in 5% non-fat dry milk in TBST for 3 hours at room temperature. Membranes were developed with ECL (Thermo Scientific #32209). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) HRP-conjugated antibody (AbCam #ab9385) and Alpha Actin (Millipore #1501) were used for equal loading control.

## 2.5 Statistical analysis

Data for GJIC are presented as mean  $\pm$  standard deviation and represented as fold change compared to non-treated (NT) group. Student t-test was used for statistical analysis between groups.

## 3. Results and Discussion

Dysregulation of gap junction coupling is a phenotypic alteration commonly observed in neoplastic cells. We and others have previously demonstrated a specific loss of homotypic and heterotypic GJIC in metastatic cells (22–24). While the dysregulation of GJIC in neoplastic cells is apparent, the specific signaling events and the mediators of those signaling events between malignant cells or between malignant cells and the surrounding stromal compartment appear to be largely context dependent (24,25). Further, restoration of GJIC appears to reduce metastatic ability of cancer cells in some cases (26,27). The ability of clinically used pharmacologic agents to alter GJIC in cultured astrocytes was the first indication to topically relate the pharmacology of sulfonylureas with specific alterations in GJIC (13).

Increased risk for cancer in patients with Type-2 diabetes is thought to be mediated through the development of metabolic syndrome which encompasses hyperinsulinemia and insulin resistance (28,29). Sulfonylureas are used as a treatment option for Type-2 diabetes and recent reports show epidemiological evidence for increased cancer-related mortality in patients treated with sulfonylureas rather than biguanides (19). In this report, we demonstrate that treatment of cancer cells in vitro with sulfonamides results in a consistent increase in GJIC, a phenomenon that has been associated with a reduction in metastatic potential (6,9,23,24,26,27). Our results highlight a germane paradox where a supposed reduction in metastatic potential due to increased GJIC conflicts with clinical data showing increased mortality from the use of sulfonamide agents. Importantly, our results appear to indicate that further investigation needs to address specific mediators and context dependency rather than the generic process of GJIC itself between cancer cells.

For our experiments, we utilized first generation sulfonylureas tolbutamide and chlorpropamide and second generation agents glibenclamide and gliclazide. To initially evaluate the effect of sulfonamides on GJIC, metastatic cancer cell lines of breast and melanocytic origin which exhibit basally low gap junction coupling were treated with 10  $\mu$ mol/L of glibenclamide. A consistent increase in GJIC as measured by the passage of fluorescent calcein dye passing from labeled donor cells to co-cultured acceptor cells over 6 h was observed visually (Figure 1A) and quantified by flow cytometry (Figure 1B). Since glibenclamide consistently increased GJIC following  $K_{ATP}$  channel inhibitor treatment, the metastatic breast cancer cell line MDA-MB-231 was utilized for further investigation. Similar to the addition of glibenclamide, treatment of MDA-MB-231 cells with glimepiride (a sulfonylurea  $K_{ATP}$  channel inhibitor) and repaglinide, an unrelated meglitinide class  $K_{ATP}$  channel inhibitor resulted in a dose-dependent increase in the passage of calcein from labeled donor cells to acceptor cells indicative of increased GJIC (Figure 2B). These results taken together initially indicated that an increase in GJIC may be dependent on inhibition of  $K_{ATP}$  channels and independent of structure and therefore class of these inhibitors.

Treatment of MDA-MB-231 cells with tolbutamide, gliclazide, and chlorpropamide failed to cause a consistent increase in GJIC (Figure 2A). Glibenclamide, glimepiride and repaglinide show comparable efficacy in the inhibition of SUR1 and SUR2, while tolbutamide, gliclazide, and chlorpropamide are selective for SUR1 inhibition at low doses, suggesting a possible differentiation of effects through these different agents (references for specificities of compounds 30–33, summarized in 34). To determine the expression of SUR1 and SUR2,

whole cell lysates from MDA-MB-231, MDA-MB-435, and C8161.9 cells were probed with antibodies directed towards SUR1 and SUR2. SUR2 was evident in each cell line, while expression of SUR1 could not be detected in MDA-MB-231 and MDA-MB-435 with low levels of detection in C8161.9 (Figure 3). These results suggested a mechanistic explanation for the pattern of GJIC changes observed following treatment with  $K_{ATP}$  inhibitors specific for SUR1 and SUR2, and why we did not observe a consistent increase in GJIC with tolbutamide (although used at lower concentrations in our studies (i.e., 50  $\mu$ M vs. 400  $\mu$ M)).  $K_{ATP}$  channels of the SUR2 subtype (SUR2A, SUR2B) are most commonly paired with  $K_{IR6.2}$  channels as in cardiac, skeletal, and smooth muscle. However, although expression analysis of  $K_{IR}$  subunits was not performed in this study, treatment of MDA-MB-231 with cibenzoline succinate (50 – 200  $\mu$ mol/L), a  $K_{ATP}$  inhibitor that binds directly to the  $K_{IR}$  subunit (35), did not induce GJIC, but rather slightly decreased GJIC levels (Figure 2C), suggesting possible secondary effects on GJIC of glibenclamide, glimepiride and repaglinide that are not related to the inhibition of  $K_{ATP}$  channels.

To further explore this possibility, we used the SUM159 breast cancer cell line which expresses inverse levels of SUR1 and SUR2 by comparison to MDA-MB-231, MDA-MB-435 and C8161.9 (Figure 4A). Interestingly, treatment of SUM159 with the SUR1 specific  $K_{ATP}$  channel inhibitors chlorpropamide and gliclazide (10, 25, 50 $\mu$ M) failed to induce GJIC while glibenclamide and glimepiride (10, 25, 50 $\mu$ M) significantly increased GJIC similar to treatment in MDA-MB-231, MDA-MB-435 and C8161.9 (Figure 4B). Although an explanation for the latter result could indicate that inhibition of SUR1  $K_{ATP}$  in SUM159 by glibenclamide and glimepiride was responsible for GJIC, failure of chlorpropamide and gliclazide to increase GJIC contradicts these results. The data propose the possibility of novel  $K_{ATP}$  independent effects on GJIC by glibenclamide, repaglinide and glimepiride. A second explanation would be that inhibition of the levels of SUR2 expressed in SUM159 is sufficient to increase GJIC but that this effect is not shared by inhibition of SUR1, although the SUR1 specific inhibitor tolbutamide has been shown to increase GJIC in other cell types (15–18), suggesting possible cell context specificities.

In addition to the complexity of  $K_{ATP}$  channel composition, the roles of  $K_{ATP}$  channels located other than the cell membrane in the mitochondria, sarcolemma, and nucleus beg further investigation, although our preliminary results involving the treatment of cells with 5-hydroxydecanoate (30 – 300  $\mu$ mol/L), a compound with specificity towards the mitochondrial- $K_{ATP}$  channel produced no effect on GJIC in our experiments (data not shown). Although much work remains to further understand the role of  $K_{ATP}$  channels with GJIC and importantly in identifying the key players that define a paradoxical relationship between pharmacological  $K_{ATP}$  channel inhibition and patient outcome, especially in cancer, we report that  $K_{ATP}$  inhibitors with SUR2 specificity increase GJIC independently of differences in SUR1 and SUR2 expression between cell lines and warrant further molecular investigation. Our results provoke thought in the role of conventional therapy not only for the treatment of Type-2 diabetes, but also cancer and eventual metastasis.

### Highlights

- Inhibition of  $K_{ATP}$  channels in metastatic breast and melanoma cells increases gap junction communication
- Evaluation of seven  $K_{ATP}$  inhibiting compounds on GJIC showed that only compounds inhibiting the SUR2 subunit of  $K_{ATP}$  increased GJIC
- Cibenzoline, which acts by binding  $K_{IR}$ , fails to increase GJIC, suggesting alternative mechanisms of action for the  $K_{ATP}$  channels and their association with GJIC

- Novel characterization of  $K_{ATP}$  subunits in breast and melanoma cell lines

## Abbreviations

<b><math>K_{ATP}</math></b>	ATP-sensitive $K^+$ channel
<b>BSA</b>	bovine serum albumin
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>FBS</b>	fetal bovine serum
<b>GJIC</b>	gap junction intercellular communication
<b>SDS-PAGE</b>	sodium dodecyl sulfate polyacrylamide electrophoresis
<b>NFDM</b>	non-fat dry milk
<b>TBST</b>	TRIS-buffered saline Tween-20
<b>TRIS</b>	tris(hydroxymethyl)aminomethane

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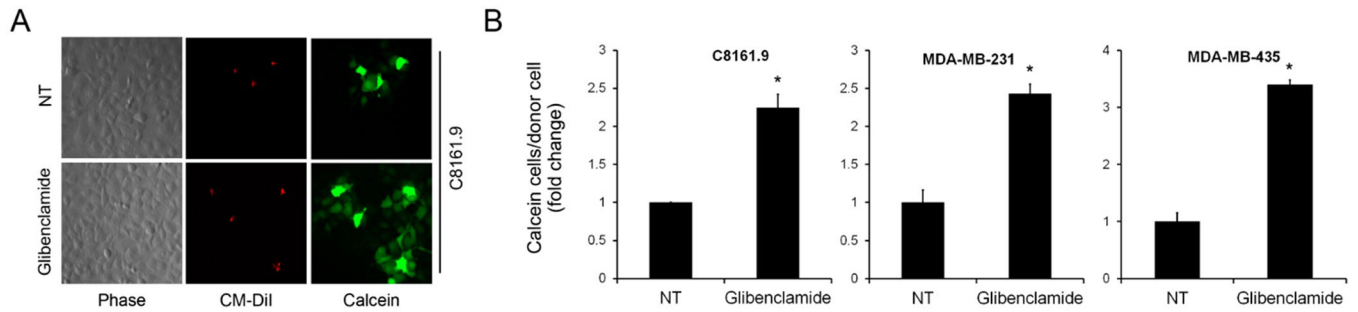
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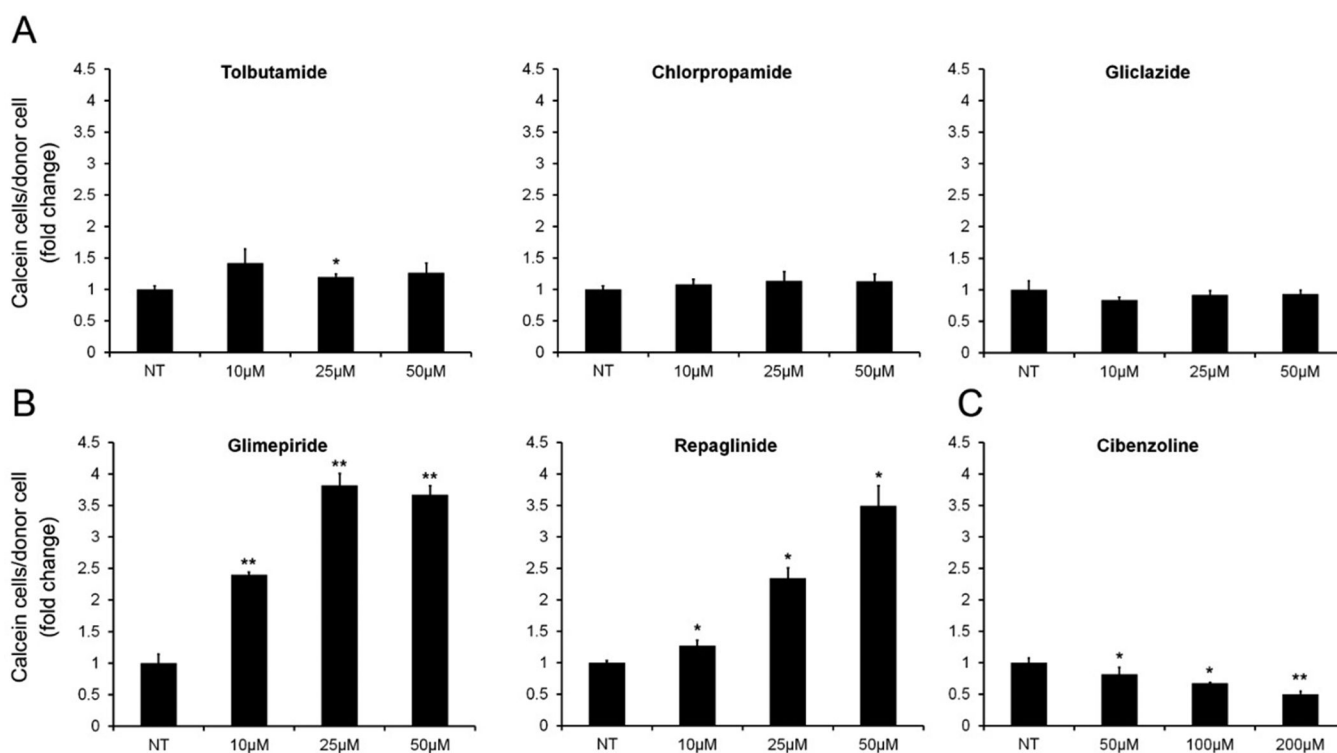
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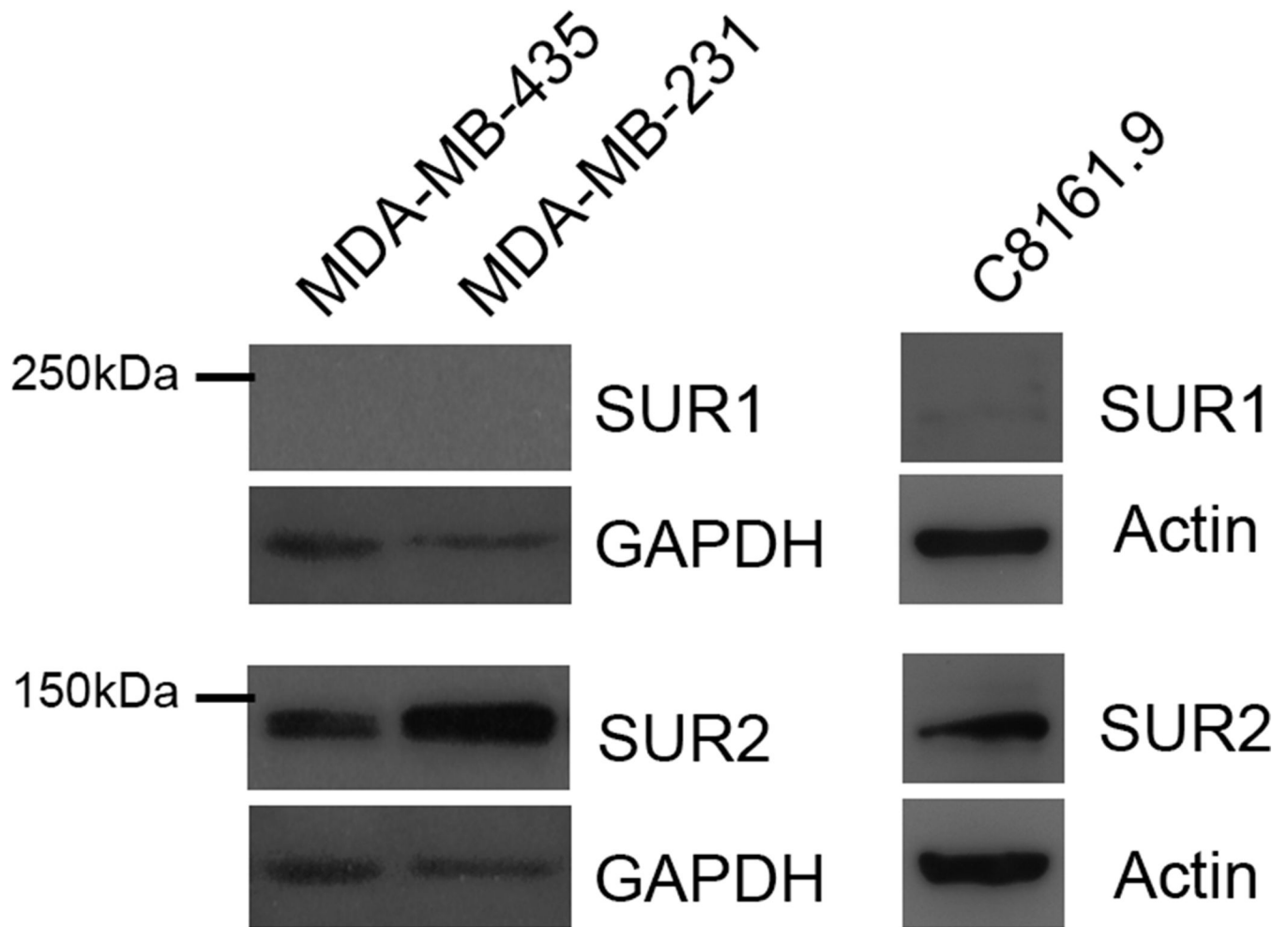
**Figure 1.**

Treatment of cancer cells with glibenclamide increases gap junction communication. A) Representative images of metastatic C8161.9 donor cells were labeled with Calcein (green) and CM-DiI (red) and co-cultured with non-labeled acceptor cells in the absence or presence of 10  $\mu$ mol/L glibenclamide. Calcein can be visualized spreading from donor cells to acceptor cells. B) Quantification of calcein/DiI assays measuring dye transfer from donor to acceptor cells using flow cytometry in MDA-MB-231, MDA-MB-435 and C8161.9 cancer cell lines. Results are represented as fold change between non-treated (NT) and glibenclamide treated groups measuring the number of acceptor cells receiving calcein per donor cell. (\*  $P < 0.05$ , error bars represent mean  $\pm$  standard deviation)



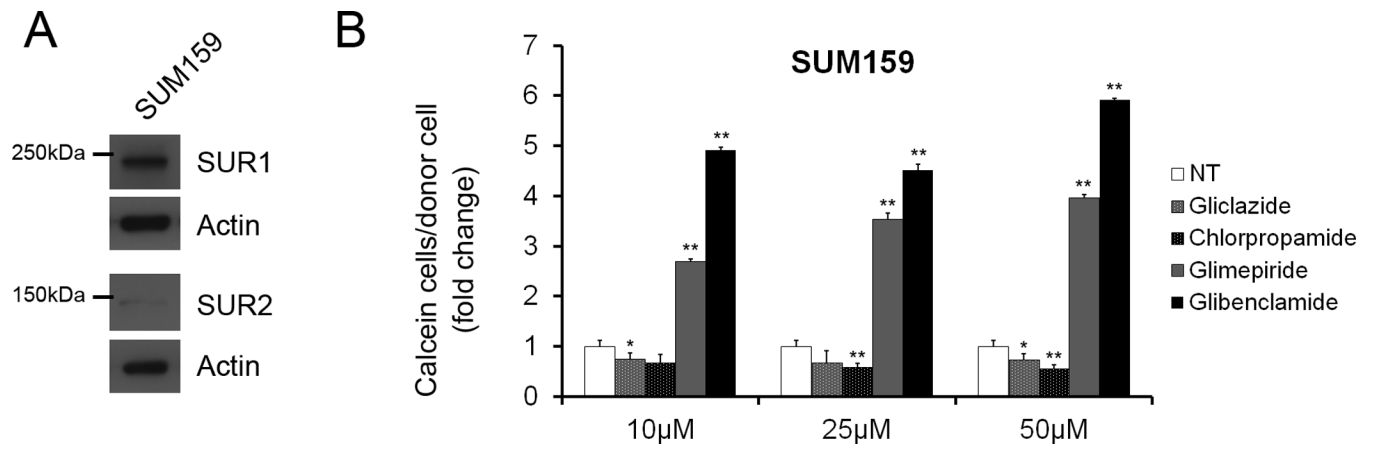
**Figure 2.**

$K_{ATP}$  inhibitors with SUR2 specificity increase GJIC in MDA-MB-231. Flow cytometry quantification of Calcein/CM-DiI assays measuring dye transfer from donor to acceptor cells after 6 hr co-culture. Results are represented as fold change between non-treated (NT) or increasing concentrations of  $K_{ATP}$  inhibitors. (A)  $K_{ATP}$  inhibitors with SUR1 specificity did not increase GJIC in MDA-MB-231, experiments were performed as described in Figure 1 with increasing concentrations of tolbutamide, glizlazide and chlorpropamide (10, 25, 50  $\mu$ mol/L). (B) treatment with glimperide and repaglinide (10, 25, 50  $\mu$ mol/L) significantly increased GJIC. (C)  $K_{ATP}$  inhibitor cibenzoline succinate does not increase GJIC in MDA-MB-231. Cells were treated with increasing concentrations of cibenzoline succinate (50, 100, 200  $\mu$ mol/L) and GJIC assays were performed as described. Minor decreases in GJIC were observed in a dose dependent manner. NT, non-treated. (\*  $P < 0.05$ , \*\*  $P < 0.01$ , error bars represent mean  $\pm$  standard deviation)



**Figure 3.**

Immunoblot analysis of SUR1 and SUR2 in lysates from MDA-MB-231, MDA-MB-435 and C8161.9. Whole cell lysates were collected from indicated cancer cell lines and probed for the expression of SUR1 and SUR2. SUR2 expression was readily detected in each cell line, however SUR1 protein could not be detected in MDA-MB-231 and MDA-MB-435 with minimal detection in C8161.9.



**Figure 4.**

SUR1 and SUR2 protein expression in SUM159. (A) Whole cell lysates from the SUM159 cell line were analyzed via immunoblot analysis for SUR1 and SUR2. SUR1 protein levels were readily detected while SUR2 expression was low by comparison to MDA-MB-231, MDA-MB-435 and C8161.9 probed under the same conditions. (B) Quantified data from GJIC assays demonstrating changes in GJIC with increasing concentrations (10, 25, 50μM) of gliclazide, chlorpropamide, glimepiride and glibenclamide. Significant increases in GJIC were observed with glimepiride and glibenclamide treatments with no increases during treatment with gliclazide and chlorpropamide, similar to results obtained from MDA-MB-231, MDA-MB-435 and C8161.9.