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The combined activation of $K_{Ca}3.1$ and inhibition of $K_v11.1$/hERG1 currents contribute to overcome Cisplatin resistance in colorectal cancer cells

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Running title: K^+ channels and Cisplatin resistance

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ABSTRACT

Background: Platinum-based drugs such as Cisplatin are commonly employed for cancer treatment. Despite an initial therapeutic response, Cisplatin treatment often results in the development of chemoresistance. To identify novel approaches to overcome Cisplatin resistance, we tested Cisplation in combination with K+ channel modulators on colorectal cancer (CRC) cells.

Methods: The functional expression of Ca^{2+}-activated ($K_{Ca}3.1$, also known as KCNN4) and voltage-dependent ($K_v11.1$, also known as KCNH2 or hERG1) K^+ channels, was determined in two CRC cell lines (HCT-116 and HCT-8), by molecular and electrophysiological techniques. Cisplatin and several K^+ channel modulators were tested *in vitro* for their action on K^+ currents, cell vitality, apoptosis, cell cycle, proliferation, intracellular signaling and Platinum uptake. These effects were also analyzed in a mouse model mimicking Cisplatin resistance.

Results: Cisplatin-resistant CRC cells expressed higher levels of $K_{Ca}3.1$ and $K_v11.1$ channels, compared to Cisplatin-sensitive CRC cells. In resistant cells, $K_{Ca}3.1$ activators (SKA-31) and $K_v11.1$ inhibitors (E4031) had a synergic action with Cisplatin in triggering apoptosis and inhibiting proliferation. The effect was maximal when $K_{Ca}3.1$ activation and $K_v11.1$ inhibition were combined. In fact, similar results were produced by Riluzole, which is able to both activate $K_{Ca}3.1$, and inhibit $K_v11.1$. Cisplatin uptake into resistant cells depended on $K_{Ca}3.1$ channel activity, as it was potentiated by $K_{Ca}3.1$ activators. $K_v11.1$ blockade led to increased $K_{Ca}3.1$ expression, and thereby stimulated Cisplatin uptake. Finally, the combined administration of a $K_{Ca}3.1$ activator and a $K_v11.1$ inhibitor also overcame Cisplatin resistance *in vivo*.

Conclusions: Since Riluzole, an activator of $K_{Ca}3.1$ and inhibitor of $K_v11.1$ channels, is in clinical use, our results suggest that this compound may be useful in the clinic to improve Cisplatin efficacy and overcome Cisplatin-resistance in CRC.

Keywords: Riluzole, SKA-31, E4031, Cisplatin uptake, preclinical mouse models.

INTRODUCTION

Platinum-based drugs and, in particular, *cis*-diamminedichloridoplatinum (II), best known as Cisplatin, are employed for the treatment of a wide range of solid malignancies, including colorectal cancer (CRC). Cisplatin exerts anticancer effects by inducing the formation of platinum-DNA adducts (Huang *et al*, 1995), which in turn trigger the apoptotic process (Wang and Lippard, 2005). Cisplatin also produces "non-genomic effects", affecting plasma membrane proteins, including ion channels and transporters, and cytoskeletal components. Such effects are often related to Cisplatin side effects, such as peripheral neuropathy (Carozzi *et al*, 2015).

Despite a consistent rate of initial responses, Cisplatin treatment often results in the development of resistance, leading to therapeutic failure. Intense research has identified several mechanisms underlying Cisplatin-resistance (Galluzzi *et al*, 2014). Among them, reduced uptake of Cisplatin through the plasma membrane is one of the most critical "pre-target" steps of resistance development. Cisplatin is taken up by both simple and facilitated diffusion (Yoshida *et al*, 1994; Ishida *et al*, 2002). Relevant in the latter process are the copper transporters CTR1 and CTR2, encoded by the *SLC31A* genes *1* and *2*, respectively. Altered levels or mis-functionality of CTR1 and CTR2 are consistently associated with Cisplatin resistance (Katano *et al*, 2002; Huang *et al*, 2014). Moreover, extrusion of the drug by two P-type ATPases, ATP7A and ATP7B is also operant. Altered expression and cellular localization of such ATPases has been linked to the occurrence of Cisplatin resistance in ovarian cancer (Kalayda *et al*, 2008). Plasma membrane transporters are not only involved in the transport of the drug, but can also be affected by Cisplatin itself (Shimizu *et al*, 2008). For example, when apoptosis is triggered, an early persistent shrinkage (named apoptotic volume decrease (AVD)) results as a consequence of efflux of K^+ and the activation of an outwardly rectifying Cl^- current. The latter has the electrophysiological and pharmacological characteristics of Volume Regulated Anion Channels (VRAC) (Lang and Hoffman, 2012). Recently VRAC, and in particular the LRCC8A and LRCC8D molecular

components, have been shown to influence Cisplatin uptake (Jentsch *et al*, 2016). Not surprisingly, a reduction of anion currents through VRAC has been linked to Cisplatin chemoresistance (Lee *et al*, 2008; Poulsen *et al*, 2010).

K^+ channels are frequently dysregulated in cancer (Arcangeli *et al*, 2009; D'Amico *et al*, 2013). In particular, $K_{Ca}3.1$ and $K_v11.1$ are up-regulated during tumor progression (Lastraioli *et al*, 2004; Muratori & Petroni *et al*, 2016) and contribute to increased tumor malignancy, which includes chemoresistance (Pillozzi *et al*, 2011). Interestingly, Cisplatin sensitivity is related to K^+ channel expression and activity in several cancer cell lines (Lee *et al*, 2008; Zhang *et al*, 2012; Leanza *et al*, 2014; Hui *et al*, 2015; Samuel *et al*, 2016). For example, increased activity of intermediate-conductance $K_{Ca}3.1$ (KCNN4) calcium-activated K^+ currents (I_{IK}) contributes to Cisplatin sensitivity in epidermoid cancer cells (Lee *et al*, 2008) and I_{IK} activation consistently potentiates Cisplatin-induced cytotoxicity. In contrast, expression of large-conductance $K_{Ca}1.1$ (KCNMA1, BK) calcium-activated channels is reduced in cisplatin-resistant ovarian cells (Samuel *et al*, 2016). In patients with ovarian cancer treated with cisplatin-based adjuvant chemotherapy, decreased expression of $K_v10.1$ (KCNH1, Eag1) correlates with favorable prognosis and predicts cisplatin-sensitivity in ovarian cancer cells (Hui *et al*, 2015). $K_v11.1$ (hERG1) channels have been found to be upregulated by Cisplatin in gastric cancer cells, and their silencing decreases the cytotoxic effects of the drug (Zhang *et al*, 2012). Moreover, a clear correlation between K^+ channel expression and Cisplatin sensitivity was shown in cancer cell lines of different histogenesis (Leanza *et al*, 2014).

Here, we investigated the role of K^+ channels in Cisplatin resistance in CRC and tested the possibility of overcoming Cisplatin resistance with K^+ channel modulating agents.

MATERIALS AND METHODS

Chemicals and Antibodies

Unless otherwise indicated, all chemicals, drugs and antibodies were from Sigma-Aldrich, Milan, Italy. For *in vitro* experiments Riluzole, SKA-31 and TRAM-34 were dissolved in DMSO, at a concentration of 5 mM, whereas for *in vivo* experiments Riluzole was dissolved in 5% Kolliphor in 0.9% NaCl. E4031 dihydrochloride, Cisplatin and Oxaliplatin were dissolved in bi-distilled water. All stock solutions were stored at -20°C. The list of antibodies and the concentrations used for western blot experiments are reported in Supplementary Methods section.

Cell culture

All the Colorectal cancer (CRC) cell lines were cultured in RPMI-1640 medium (Euroclone; Milan, Italy), supplemented with 2% L-Glut, 10% fetal bovine serum (Euroclone) and 1% penicillin/streptomycin (complete medium). HCT-116 cells were obtained from the American Type Culture Collection (ATCC); HT-29 cells were kindly provided by Dr. R. Falcioni (Regina Elena Cancer Institute, Roma); HCT-8 and H630 were kindly provided by Dr. E. Mini (University of Florence).

Total RNA extraction, reverse transcription (RT) and RQ-PCR

RNA extraction, RT and RQ-PCR were as described in Pillozzi *et al,* 2007. The primers relative to *ATP7A*, *ATP7B*, *KCNA3, KCNH1*, *KCNH2, KCNMA1*, *KCNN3*, *KCNN4, SLC31A1, SLC31A2, LRCC8A and LRCC8D* are shown in Supplementary Table S1.

Silencing of HCT-116 cells

Silencing of HCT-116 cells was done as in Crociani *et al*, 2013, using the following siRNAs: 1) K_V11.1/KCNH2-siRNAs (44858 anti-Kv11.1 siRNA1 and 44762 anti-Kv11.1 siRNA3, Ambion; Austin TX, USA) (total 100 nM final concentration) 2) K_{Ca}3.1/KCNN4-siRNAs (7801 anti-kcnn4 siRNA1 and 7803 anti-kcnn4 siRNA3, Ambion; Austin TX, USA) (total 5 nM final concentration). As negative controls cells were treated with Lipofectamine only.

Cell viability assay, IC_{50} and Combination Index (CI) calculation

Cell viability was measured through the Trypan Blue exclusion test, following the procedure described in Pillozzi *et al*, 2016. IC_{50} and CI calculations were as in Pillozzi *et al*, 2011. Cells were seeded at $1x10^4$/well in 96-well plates (Costar Corning) in complete medium; Cisplatin and the other drugs were added at their final concentration after 24 hours incubation, and further incubated for different times.

Cell cycle analysis

Cell cycle distribution was assessed by flow cytometry after staining cells with propidium iodide (PI) as in Pillozzi *et al*, 2016.

Annexin/ propidium iodide (PI) assay

Apoptosis was determined using the Annexin V/ propidium iodide (PI) test (Annexin-V FLUOS staining kit; Roche Diagnostics, Mannheim, Germany) as described in Pillozzi *et al*, 2011. According to this procedure, (i) viable cells are negative for both Annexin V and PI (Q3 quadrant gate in the dot plots); (ii) cells that are in the early phases of apoptosis are Annexin V positive and PI negative (Q4 quadrant gate in the dot plots); (iii) cells that are in the late phases of apoptosis are both Annexin V and PI positive (Q2 quadrant gate in the dot plots); (iv) dead (necrotic) cells are Annexin V negative and PI positive (Q1 quadrant gate in the dot plots).

Patch-clamp experiments

Membrane currents were recorded at room temperature (25°C), with the whole-cell configuration of the patch-clamp technique. $K_v11.1$ currents were elicited by a two-step protocol, conditioning the cell at 0 mV and testing the tail current at -120 mV (Gasparoli *et al*, 2015). $K_{Ca}3.1$ currents were elicited by 200-ms voltage ramps from -120 to +40 mV applied every 10 s, and the fold increase of slope conductance by drug was taken as an indication of channel activation (Sankaranarayanan *et al*, 2009). All the solutions are in Supplementary Table S2. The effects of Riluzole, SKA-31 and Cisplatin were determined on maximal $K_v11.1$ tail currents. The effects of Cisplatin and TRAM-34

were determined on the maximal $K_{Ca}3.1$ activation induced by Riluzole and SKA-31. Drugs were applied at the concentrations indicated in the figure legends for 2 minutes before recording their effects. Resting potential (V_{REST}) values were measured in I= 0 mode, in the presence of the extracellular solution N° 1 (see Supplemetary Table S2).

Immunofluorescence

Immunofluorescence was performed applying the procedures detailed in Lastraioli *et al,* 2015, using the antibodies reported in the Supplementary Methods section.

Protein extraction and western blot (WB)

Protein extraction and western blots (WBs) relative to cell lines and tumor masses were performed as described in Crociani *et al,* 2013, using the antibodies reported in the Supplementary Methods section.

Cisplatin uptake measurement

HCT116 cells were incubated in complete medium containing the different compounds as reported in the legend to Figure 4 for 3 or 24 hours. For incubation in high extracellular K^+, NaCl in the RPMI medium was substituted in part (to obtain 40 mM KCl) or totally (to obtain 108 mM KCl) with KCl. At the end of the uptake period, cells were quickly washed 3 times with ice cold PBS, collected, gently spun down (1.000 g for 5 min at 4°C), and the pellet suspended in 1 ml ice cold PBS. Part (50 µl) of this suspension was taken for protein concentration determination and part (50 µl) for cell viability assay. The remaining 900 µl were spun down at 400 g for 5 min, and processed for ICP-AES analysis as described in Marzo *et al,* 2015.

***In vivo* experiments**

Experiments were performed at the Animal House of the University of Florence (CESAL). Mice were housed in filter-top cages with a 12h dark-light cycle, and had unlimited access to food and water. Procedures were conducted according to the laws for experiments on live animals (Directive

2010/63/EU), and approved by the Italian Ministry of Health (1279/2015-PR). All the procedures are detailed in the Supplementary Methods section.

Statistical analysis

Unless otherwise indicated, data are given as mean values ± standard error of the mean (SEM), with n indicating the number of independent experiments. Statistical comparisons were performed with OriginPro 2015 (Origin Lab, Northampton, Massachusetts). The normality of data distribution was checked with Kolmogorov-Smirnov (K-S) test. In case of unequal variances, the Welch correction was applied. For comparisons between two groups, we used Student's *t* test. For multiple comparisons, one-way ANOVA followed by Bonferroni's post-hoc test was performed to derive P values. The individual P values are reported in the Figures.

RESULTS

Effects of Cisplatin on different CRC cell lines: identification of Cisplatin-resistant and Cisplatin-sensitive cell lines

We investigated the response of four CRC cell lines (HCT-116, HCT-8, HT-29 and H-630) to Cisplatin treatment by measuring cell viability by Trypan Blue exclusion test. The Inhibiting Concentration 50 (IC_{50}) values determined after 24h of treatment (Figure 1A) show that HCT-116 cells are the most resistant and HCT-8 the most sensitive (see also Table 1A and Table S3). Cisplatin, added at its IC_{50} value to the two cell lines, triggered apoptosis (with a higher percentage of cells in late apoptosis in HCT-8 cells than HCT-116 cells (Figure 1B, Table 1A), and increased the percentage of G0/G1 cells in both cell types (Table 1A and Supplementary Figure S1), after 24h of treatment. Cisplatin blocked cell proliferation in HCT-8 at 1 μM (Figure 1C, left panel), while in HCT-116 at 20 μM (Figure 1C, right panel). In summary, HCT-116 is a Cisplatin-resistant line, while HCT-8 is Cisplatin-sensitive.

We next determined the expression of different K^+ channel genes (Spitzner *et al*, 2007; D'Amico *et al*, 2013; Huang *et al*, 2014) and Cisplatin transporter systems (Owatari *et al*, 2007; Pedersen *et al*, 2015; Barresi *et al*, 2016; Jentsch *et al*, 2016) in the two CRC cell lines, focusing on those already reported to be expressed in CRC cells and primary samples. RQ-PCR data are shown in Figure 1D. K_v11.1 (KCNH*2*, hERG1) was expressed at higher levels in HCT-116 than HCT-8 cells. The K_{Ca}3.1 (*KCNN4*) transcript was also highly expressed in HCT-116 cells, and much less so in HCT-8 cells. All other tested K^+ channel transcripts were negligible in both cell lines. The copper transporter CTR1 (*SLC31A1*) was highly expressed in both cell lines; the two P-type ATPases, ATP7A (*ATP7A*) and ATP7B (*ATP7B*) displayed a higher amount in HCT-8 cells; the LRRC8A/D (*LRCC8A* and *LRCC8D*) components of VRAC were only found in HCT-116 cells.

The higher expression of both K_v11.1 (*KCNH2,* hERG1) and K_{Ca}3.1 (*KCNN4*) in HCT-116 compared to HCT-8 cells, was confirmed by western blot (Figure 1E), immunofluorescence (Figure 1F) and patch-clamp experiments. Larger K_v11.1 currents were previously reported in HCT-116

than HCT-8 cells (Crociani *et al*, 2013, and Supplementary Figure S2). A calcium-activated K^+ current with characteristics of $K_{Ca}3.1$ was detected only in HCT-116 cells, but only after the channel was activated by Riluzole or SKA-31 (Figure 2A). HCT-116 cells showed a significantly hyperpolarized V_{REST} (-38.5 ± 2.9 mV, n= 11) compared to HCT-8 cells (-13.1 ± 2.5 mV, n= 12, $p < 0.01$), consistent with their higher expression of K^+ channels.

Modulators of $K_{Ca}3.1$ and $K_v11.1$ channels affect viability, apoptosis and cell cycle phases of CRC cells

Next, we tested on our cell lines the effects of activators or inhibitors of $K_{Ca}3.1$ and inhibitors of $K_v11.1$. Riluzole was used as a broad modulator of ion channels, as it activates K_{Ca} currents (both intermediate-conductance $K_{Ca}3.1$ and small- conductance $K_{Ca}2.1$, $K_{Ca}2.1$ and $K_{Ca}2.3$ currents), and inhibits $K_v11.1$ (Sankaranarayanan *et al*, 2009), voltage-gated sodium (Wang *et al*, 2008) and voltage-gated calcium channels (Stefani *et al*, 1997). SKA-31 is a specific $K_{Ca}3.1$ activator (Sankaranarayanan *et al*, 2009), and TRAM-34 a specific $K_{Ca}3.1$ inhibitor (Wulff *et al*, 2000). E4031 inhibits $K_v11.1$ (Sanguinetti *et al*, 1990). We first tested these compounds on HCT-116 cells. Both Riluzole and SKA-31 increased the $K_{Ca}3.1$ current (reversal potential at -80 mV; inhibition by TRAM-34 IN DIDASCALIA?) respectively 2.11 ± 0.46 (n=9) and 4.36 ± 1.67 (n=10) fold (Figure 2A and 2C), and induced cell hyperpolarization (Figure 2A). Riluzole also inhibited $K_v11.1$ currents (by 23% and 44%, with 10 μM and 45 μM, respectively) (Figure 2B and 2C), in keeping with previous reports (Sankaranarayanan *et al*, 2009). SKA-31 had no effect on $K_v11.1$ currents (Figure 2B and 2C).

In current-clamp experiments, both Riluzole and SKA-31 strongly hyperpolarized V_{REST} (Figure 2E, right panel). In contrast, TRAM-34 (Figure 2D) and E4031 (Figure 2E) depolarized V_{REST}. Cisplatin did not significantly affect $K_{Ca}3.1$ currents either in control conditions, or after SKA-31 stimulation (Figure 2B and 2F), and slightly inhibited $K_v11.1$ (Figure 2B and 2G). Cisplatin addition rapidly and reversibly depolarized V_{REST} (Figure 2H).

Next, we tested the effects of Riluzole, SKA-31, E4031 and TRAM-34 on cell viability, apoptosis and cell cycle phases of HCT-116 and HCT-8 cells. All these compounds reduced cell viability but, differently from Cisplatin, their IC_{50} values were generally lower in HCT-116 than in HCT-8 cells, except for TRAM-34 (Table 1A and Supplementary Table S3). All the K^+ channel modulators triggered apoptosis in HCT-116 cells, and the effect was smaller in HCT-8 cells (Table 1A). They also increased the percentage of cells in G0/G1 phase, in both cell lines, with the exception of Riluzole, which caused a strong G2/M block in HCT-8 cells (Table 1A), as reported by Khan *et al,* 2011. All drugs reduced HCT-116 cell proliferation when added at time zero at their specific IC_{50} values (Figure 3A). Less evident effects were observed in HCT-8 cells (Supplementary Figure S3).

K_{Ca}3.1 activation and K_v11.1 block have a synergistic activity with the pro-apoptotic effects of Cisplatin

We tested the different K^+ channels modulators in combination with Cisplatin in HCT-116 and HCT-8 cells, and measured the combination index (CI) to assess synergistic, antagonistic or additive effects of the different combinations (Pillozzi *et al*, 2011). Riluzole, SKA-31 and E4031 synergized with Cisplatin in decreasing viability of HCT-116 cells after a 24 h incubation, whereas TRAM-34 was antagonistic (Table 1B and Figure 3B). A synergistic effect of Riluzole, SKA-31 and E4031 was also observed with Oxaliplatin (Table 1B and Supplementary Table S6), which was weakly efficacious when tested alone on HCT-116 cells (Table 1A). All drugs increased the pro-apoptotic effect of Cisplatin in HCT-116 cells (Table 1B), while the effects of the combination treatments on cell cycle were less homogeneous (Supplementary Table S7). TRAM-34, which was antagonistic in HCT-116 cells, only slightly increased the percentage of cells in early apoptosis and the percentage of cells in G2/M (Supplementary Table S7). In HCT-8 cells, all the K^+ channel modulators were antagonistic to Cisplatin (Supplementary Table S5), and decreased apoptosis (Supplementary Table S8).

Next, we studied the signaling pathways underlying such effects, focusing on Caspase 3 activation, and the inhibition of anti-apoptotic molecules, such as ERK1/2 and Akt (Wong *et al*, 2011). Cisplatin activated Caspase 3, with no or only a small effect on ERK and Akt phosphorylation. The combination of Cisplatin with $K_{Ca}3.1$ activators (Riluzole or SKA-31) further activated Caspase 3 and reduced Akt phosphorylation, without affecting the ERK1/2 pathway. Cisplatin and TRAM-34 decreased ERK1/2 phosphorylation and increased Caspase 3 activation, but did not affect Akt phosphorylation. The combination of Cisplatin with E4031 strongly decreased ERK1/2 and Akt phosphorylation, and activated Caspase 3 (Figure 3C). Overall, the synergistic pro-apoptotic effects of Cisplatin with Riluzole, SKA-31 and E4031 were mediated by both the Akt and Caspase 3 pathways. E4031 also modulated the MAPK pathway. Cisplatin and TRAM-34 were antagonistic because of a lack of convergence on the Akt pathway, which could impair completing the apoptotic process (see the relatively low percentage of cells in late apoptosis observed in cells treated with Cisplatin and TRAM-34 in Table 1B).

Riluzole, SKA-31 and E4031 had a synergic effect with Cisplatin also on the inhibition of HCT-116 cell proliferation. This effect was particularly evident with low Cisplatin concentrations, and the strongest effect was obtained with E4031 (Figure 3D).

We then studied whether the anti-proliferative effects and the synergy with Cisplatin of drugs that activate $K_{Ca}3.1$ (Riluzole, SKA-31) or inhibit $K_v11.1$ (E4031) depended on their effects on either $K_{Ca}3.1$ or $K_v11.1$ currents. Hence the drugs were tested in $K_v11.1$- and $K_{Ca}3.1$-silenced HCT-116 cells. The silencing of $K_v11.1$ and $K_{Ca}3.1$ by specific siRNAs is shown in Supplementary Figure S4. Silencing $K_v11.1$ potentiated the Cisplatin effects in combination with Riluzole or SKA-31, while abrogated the effects of E4031 (Figure 3E, left panel). Conversely, silencing $K_{Ca}3.1$ reversed the effects of both Riluzole and SKA-31 (Figure 3E, right panel). We conclude that specific activation of $K_{Ca}3.1$ and/or inhibition of $K_v11.1$ underlie the synergistic anti-proliferative effects of Riluzole, SKA-31 or E4031 in combination with Cisplatin.

K^+ channel modulators increase Cisplatin uptake in CRC cells.

We next tested whether Riluzole, SKA-31 or E4031 affected Cisplatin uptake, measured as intracellular accumulation of Platinum (Pt), in HCT-116 cells. We first analyzed the role of Cu transporters, determining the dose dependence of Cisplatin accumulation in the absence or presence of 1 mM $CuSO_4$, to inhibit Cu transporters (Matsumoto *et al*, 2007). Unexpectedly, $CuSO_4$ had no effect. Hence, Cu transporters do not play a significant role in the intracellular accumulation of Cisplatin in these cells. The same experiment was performed in the presence of 25 μM TRAM-34, a dose that fully inhibits $K_{Ca}3.1$, without affecting cell viability after a 3 h incubation (Supplementary Figure S5A). Blocking $K_{Ca}3.1$ channels reduced Cisplatin uptake (Figure 4A). This result was confirmed by incubating the cells for 3 h with the IC_{50} dose (22 μM) of Cisplatin (Figure 4B). Hence the activity of $K_{Ca}3.1$ channels determines or at least contributes to the uptake of Cisplatin in HCT-116 cells. Consistently, Riluzole increased Cisplatin accumulation (Figure 4B). A similar mechanism was observed for Oxaliplatin uptake (Figure 4B). Recalling the opposing effects of TRAM-34 (depolarization) and Riluzole (hyperpolarization) on V_{REST} of HCT-116 cells (Figure 2), we also evaluated the effect of membrane depolarization on Cisplatin uptake. Cells were depolarized by exposure to a high K^+ medium (either 40 or 108 mM), which increased Pt uptake. The effect depended on $K_{Ca}3.1$ activity (since it was reduced by TRAM-34), but not on Ca^{2+} influx through voltage-gated Ca^{2+} channels (since 200 μM $CdCl_2$ had no effect (Becchetti *et al*, 1992)).

Cisplatin uptake was then determined after longer (24 h) treatment with Cisplatin (at the IC_{50} dose), alone or in combination with our K^+ current modulators. Riluzole, SKA-31 and E4031 increased, while TRAM-34 decreased, Cisplatin accumulation (Figure 4C). TRAM-34 inhibited the potentiation of Cisplatin-uptake induced by SKA-31 or Riluzole, while it did not affect the potentiation by E4031. Finally, the combination of SKA-31 and E4031 induced the largest increase in Cisplatin uptake (Figure 4C). These data suggest that Cisplatin uptake in HCT-116 cells is facilitated by $K_{Ca}3.1$ channels, being increased by their activation and reduced by their inhibition.

To determine why $K_v11.1$ inhibition alone increased Cisplatin uptake, we examined if $K_v11.1$ activity was related to $K_{Ca}3.1$ expression and/or activity. $K_{Ca}3.1$ channel activity (Figure 4D) and expression (Figure 4E) were analyzed in HCT-116 cells treated for 24 h with E4031. Treatment with TRAM-34 was included as a control. E4031 augmented both $K_{Ca}3.1$ current amplitude (Figure 4D) and membrane expression (Figure 4E), both when it was applied alone and in the presence of Cisplatin (Figure 4E). TRAM-34 blocked $K_{Ca}3.1$ activity, as expected (Figure 4D), but scarcely affected its surface expression (Figure 4E). Neither TRAM-34, nor E4031 altered $K_v11.1$ expression (Supplementary Figure S5B). Hence, in HCT-116 cells, inhibition of $K_v11.1$ currents was compensated by increased expression of $K_{Ca}3.1$. TRAM-34 (25 μM) did not completely block $K_{Ca}3.1$ currents in E4031-treated cells (Figure 4D), as it did not completely block Cisplatin uptake in E4031-treated cells (Figure 4C). Overall, we attribute the increase in Cisplatin uptake in the presence of $K_v11.1$ inhibitors to the upregulation of $K_{Ca}3.1$ currents less sensitive to TRAM-34 inhibition, possibly a splice variant or a post-translationally modified form (see Discussion).

K^+ channel modulators overcome Cisplatin resistance *in vivo*.

We next hypothesized that combining a $K_{Ca}3.1$ activator and a $K_v11.1$ inhibitor could potentiate the effect of low doses of Cisplatin (1 μM). Using Cisplatin in the presence of SKA-31, or E4031, or a compound with both effects (Riluzole) reduced cell viability to a greater extent than observed with each compound alone (Figure 5A). The most effective combination was Cisplatin+Riluzole+E4031 (see also the CI in Table 1B; note that both Riluzole and E4031 are $K_v11.1$ inhibitors, and their effects are likely to be additive, Figure 2C).

Finally, we tested whether the above synergistic effects also occurred in a preclinical *in vivo* model of chemoresistance. HCT-116 cells were xenografted subcutaneously into immunodeficient nude mice. Both Cisplatin (0.35 mg/kg, twice a week) and Riluzole (10 mg/kg, daily) reduced HCT-116 tumor growth, when added as single agents (Figure 5B and inset). The inhibitory effects

of E4031 on tumor growth in the same mouse model have already been reported (Crociani *et al*, 2013). Moreover, we tested Riluzole and E4031 in a mouse model we have developed to mimic chemoresistance (see Supplementary Materials and Methods). To this purpose, xenografted mice were first treated with Cisplatin (0.35 mg/kg, twice a week) for one week (phase 1), and then with a 10 times lower dose (0.035 mg/kg) for a further two weeks (phase 2). During phase 2, Riluzole+E4031 were included in the treatment schedule (see the scheme in Figure 5C). Tumors decreased their growth by Cisplatin treatment during phase 1, but recovered their growth during Cisplatin treatment in phase 2, with approximately the same rate as displayed by the controls. In contrast, when both Riluzole and E4031 were included in the treatment schedule of phase 2, both the growth rate and the final volume of tumors were significantly reduced (Figure 5C). Consistently, such treatment induced a strong decrease of ERK1/2 and Akt phosphorylation and an increased activation of caspase 3 (Figure 5D).

DISCUSSION

In search of novel strategies to overcome Cisplatin resistance in CRC, we tested the effects of K^+ channel modulators in combination with Cisplatin. We show that compounds that activate $K_{Ca}3.1$ (SKA-31) or inhibit $K_v11.1$ (E4031) or have both effects (Riluzole) promote Cisplatin uptake and enhance apoptosis of Cisplatin-resistant cells both *in vitro* and in a preclinical mouse model. Our results highlight the translational potential of using K^+ channel modulators to overcome Cisplatin resistance in CRC.

Among the different K^+ channel-encoding gene tested, Cisplatin-resistant (HCT-116) cells exhibited higher functional expression of $K_{Ca}3.1$ and $K_v11.1$ (hERG1, KCNH2) channels, compared to Cisplatin-sensitive HCT-8 cells. Moreover, the two channels are functionally related in these cells: 1) they set V_{REST} in HCT-116 cells to more hyperpolarized (-38.5 vs -13 mV) vales compared to HCT-8 cells; 2) their expression is coordinated in HCT-116 cells, one compensating for the other. In fact, prolonged (24 h) inhibition of $K_v11.1$ currents leads to up-regulation of functional $K_{Ca}3.1$ channels (Figure 4D and E). Since all described CRC cell lines express $K_v11.1$ (D'Amico *et al*, 2013), and $K_{Ca}3.1$ inhibition has no effect on $K_v11.1$ (Supplementary Figure S5B), we hypothesize that $K_v11.1$ drives the expression of the other K^+ channel. The coordinated and balanced expression of the two K^+ channels has two consequences in HCT-116 cells: (A) blocking $K_v11.1$ increases the uptake of Cisplatin, which relies on the activity of $K_{Ca}3.1$ channels, and (B) the concomitant activation of $K_{Ca}3.1$ and inhibition of $K_v11.1$ potentiates the pro-apoptotic activity of Cisplatin. Cisplatin uptake into HCT-116 cells is reduced by TRAM-34, a specific $K_{Ca}3.1$ blocker, and it is increased by SKA-31 and Riluzole, which activate $K_{Ca}3.1$. These results suggest a requirement for $K_{Ca}3.1$ in Cisplatin uptake. Blocking $K_v11.1$ (hERG1) with E4031 also enhances Cisplatin uptake, an effect that can be explained by E4031-induced up-regulation of $K_{Ca}3.1$. Notably, the $K_{Ca}3.1$ up-regulation triggered by E4031 is not completely blocked by TRAM-34. This decreased TRAM-34 sensitivity may be due to up-regulation of a post-translationally modified $K_{Ca}3.1$ protein (see $K_{Ca}3.1$ band of higher molecular weight, i.e. more glycosylated, in Figure 4D)

or a $K_{Ca}3.1$ splice variant with reduced TRAM-34 sensitivity as reported in the rat colon (Barmeyer *et al*, 2010). This could also explain why increased Cisplatin uptake in cells treated with E4031 was not completely reversed by TRAM-34 addition.

Moreover, the inhibitory effect of TRAM-34 was not related to its depolarizing action. In fact, exposing the cells to high extracellular K^+ concentrations (40 and 108 mM, which set V_{REST} at -10 mV and 0 mV, respectively) increased Cisplatin uptake. One possibility to explain this effect is that $K_{Ca}3.1$ modulates VRAC, which was found to mediate Cisplatin uptake (Jentsch *et al*, 2016). HCT-116 cells do show a substantial expression of *LRRC8D,* the main molecular component of VRAC implicated in cell volume regulation (Planells-Cases *et al*, 2015; Syeda *et al*, 2016). In this case, Cisplatin uptake through VRAC would be modulated by the activity of $K_{Ca}3.1$. Another possibility is that the depolarization caused by high extracellular K^+ also decreases the driving force for Cl^-, leading to a smaller ratio between the outward and the inward flux. The consequent relative increase of inward Cl^- flux would facilitate Pt entry, compared to the basal conditions.

In Cisplatin-resistant CRC cells, $K_{Ca}3.1$ activators (SKA-31), $K_v11.1$ inhibitors (E4031), and compounds with both activities (Riluzole) displayed a synergistic action with Cisplatin. In fact, they restored the pro-apoptotic and cytotoxic effects of Cisplatin, even when the latter was tested at very low doses. The effect of E4031 in CRC cells is the opposite of that observed after silencing $K_v11.1$/hERG1 in gastric cancer cells (Zhang *et al*, 2012), suggesting that different channel-dependent mechanisms are operant in CRC cells. The effects of our K^+ channel-modulating drugs on apoptosis and cell cycle phases were generally stronger in Cisplatin-resistant cells. Moreover, in these cells, only $K_{Ca}3.1$ activators and $K_v11.1$ inhibitors were synergistic with Cisplatin, thus increasing the percentage of apoptotic cells, and affecting the relative intracellular signaling pathways.

Although we studied mainly Cisplatin, the effects we observed were also evident with Oxaliplatin, stressing the translatability of our data. Overall, we believe that the results discussed herein may be of relevance for overcoming chemoresistance to Pt-based drugs, one of the major

challenges in cancer treatment (Kartalou and Essigmann, 2001; Siddik *et al*, 2003; Wang and Lippard, 2005).

In our CRC model, HCT-116 cells, although expressing $K_{Ca}3.1$, have a low Cisplatin uptake because the activity of $K_{Ca}3.1$ is kept low by the concomitant $K_v11.1$ activity. The combination of $K_{Ca}3.1$ activation with $K_v11.1$ inhibition, improving Cisplatin uptake, allows also low doses of the drug to trigger apoptosis and reduce HCT-116 cell growth. This interpretation explains why a $K_{Ca}3.1$ activator and a $K_v11.1$ inhibitor can be combined to trigger a cooperative effect with Cisplatin. The best combination includes Riluzole, which has a mild $K_v11.1$ inhibitory activity, besides activating $K_{Ca}3.1$. In the present paper we provide evidence that such cooperation occurs both *in vitro* and *in vivo*, in preclinical models (subcutaneous xenografts of HCT-116 cells into immunodeficient mice) and contributes to overcome Cisplatin resistance. In these models we tested Riluzole and E4031, applying dosages and routes of administrations already used and proven to be efficacious (Yip *et al*, 2009; Crociani *et al*, 2013; Speyer *et al*, 2016). We showed the capacity of the combination of the two drugs to improve Cisplatin antineoplastic effects. In particular (Figure 5C), we mimicked in mice the onset of chemoresistance in mice, treating the xenografted animals with full Cisplatin doses, first, and then with very low doses. In the latter case, tumors started to grow again, except when Riluzole and E4031 were included in the chemotherapeutic regimen. It is worth noting that the mouse model of chemoresistance we produced allowed us to unravel the effects of the combination, which was however masked by the intense effect of Cisplatin at full doses.

During CRC adjuvant therapy, the combination of Cisplatin (Oxaliplatin) with drugs that activate $K_{Ca}3.1$ and inhibit $K_v11.1$, such as Riluzole, may improve Cisplatin (Oxaliplatin) efficacy and overcome resistance in the clinical setting. Such combination would represent an example of personalized medicine in those patients, that co-express $K_{Ca}3.1$ and $K_v11.1$ (Muratori & Petroni *et al*., 2016). Importantly, Riluzole is already in clinical use for the treatment of Amyotrophic Lateral Sclerosis (ALS) and is being investigated for the treatment of solid tumors in several clinical trials

(https://clinicaltrials.gov/, NCT00903214 and NCT0086684). Of interest, Riluzole, showed preliminary benefit in a Phase 0 trial in patients with advanced melanoma and is currently in Phase 2 clinical trials (NCT0086684; Yip *et al*, 2009) and in Phase 1 for breast cancer (NCT00903214). Besides its effect on $K_{Ca}3.1$ and $K_v11.1$ in CRC cells, Riluzole may also enhance anti-tumor T cell activity by overcoming the recently described ionic immune checkpoint (Eil *et al*, 2016). A combination of Riluzole with Cisplatin may show clinical benefit.

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Author contributions: A.A. designed and supervised the whole study; S.P., M.D'A, and G.B. performed cell viability, Annexin/PI assay and cell cycle analysis; G.B. and S.P. performed RQ-PCR analyses; L.G. and M.D'A. performed patch clamp experiments; S.P. and G.B. performed Pt uptake experiments; T.M., M.S., R.U. and L.M. performed Pt uptake measurements; S.P., G.B. and G.P. performed WB experiments; A.G. prepared silenced cells; S.P. O.C. and G.P. performed *in vivo* experiments; A.B. revised patch-clamp data; G.C. revised the paper and originally suggested the idea of evaluating riluzole in colorectal tumors; A.B. and H.W. revised the paper ; A.A. wrote the paper.

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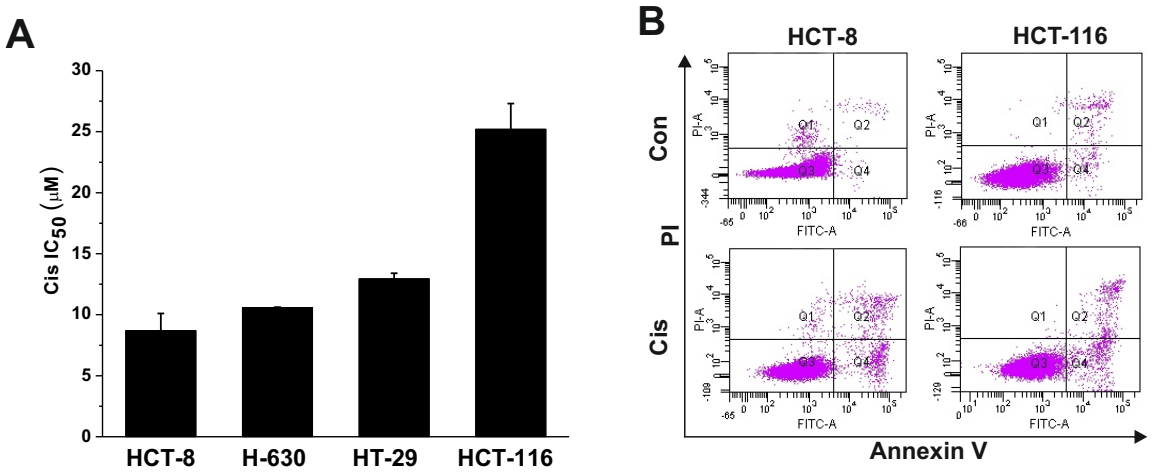
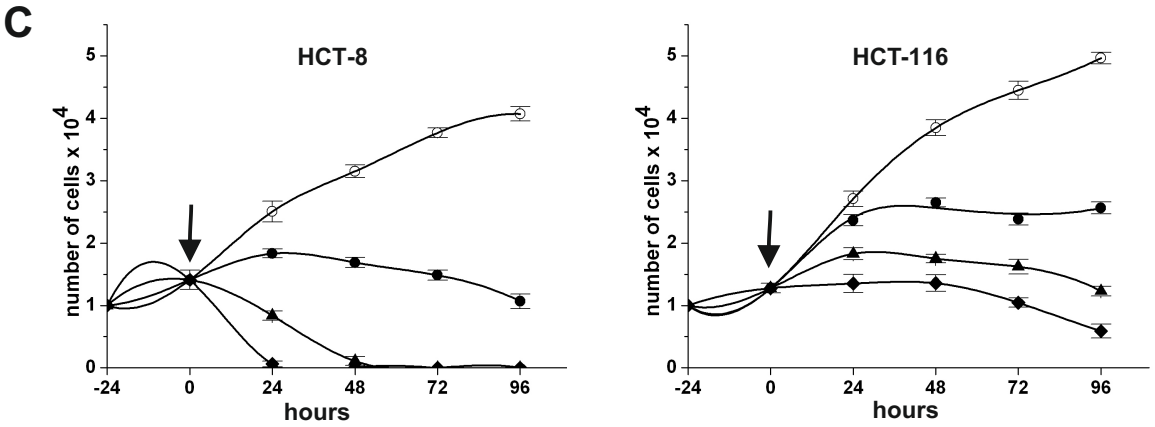
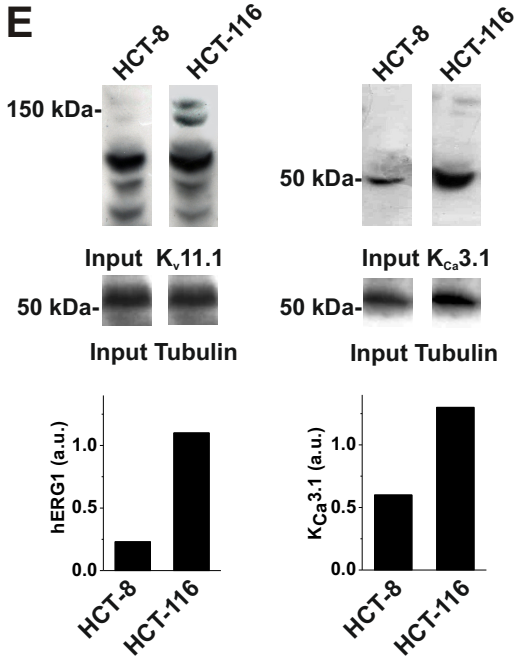
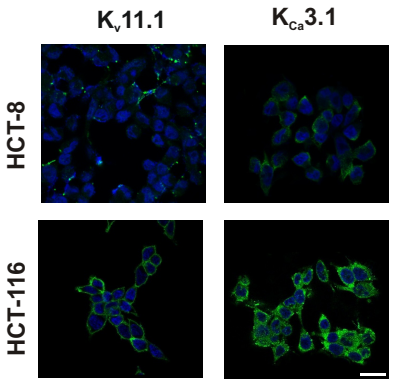


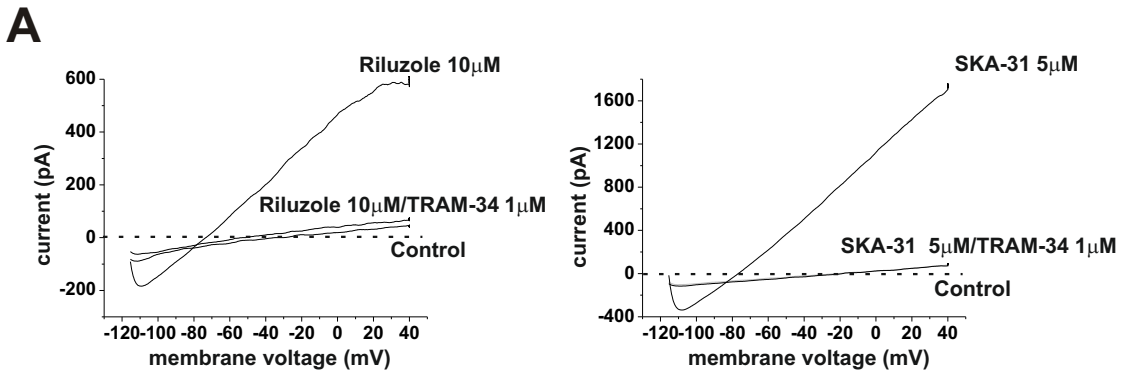


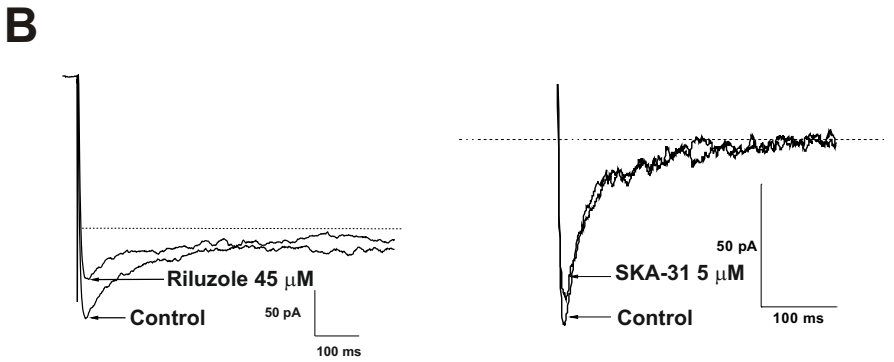
D

	HCT-116		HCT-8	
	Ct value	Score of expression (a.u.)	Ct value	Score of expression (a.u.)
KCNH1	36.33±1.29	0	36.47±1.24	0
KCNH2	25.31±0.66	2	27.57±0.93	1
KCNA3	31.79±0.36	0	29.76±0.28	0
KCNMA1	31.38±1.15	0	32.04±0.53	0
KCNN3	28.93±0.57	0	35.03±2.04	0
KCNN4	22.46±0.44	2	30.04±1.53	0
SLC31A1	22.61±0.26	2	22.31±0.32	2
SLC31A2	32.26±0.71	1	30.54±0.39	0
ATP7A	26.09±0.98	1	23.45±0.29	2
ATP7B	26.71±0.48	1	24.69±0.28	2
LRRC8A	27.15±0.34	1	28.39±0.45	0
LRRC8D	26.38±0.58	1	28.04±0.09	0



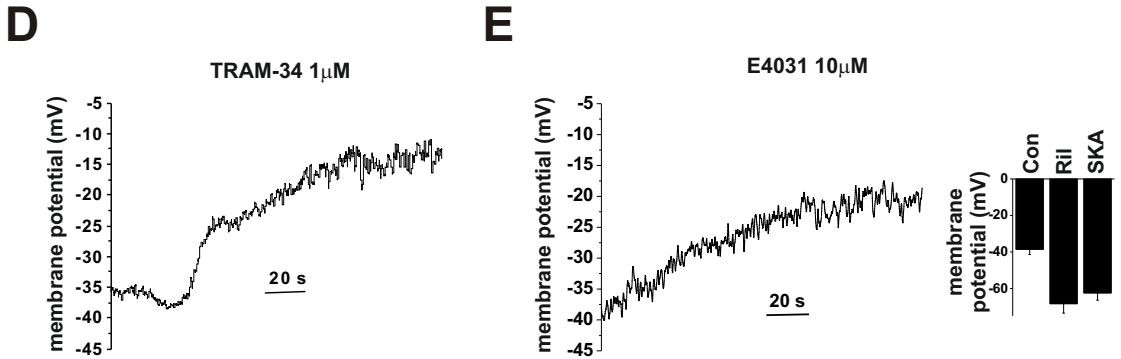


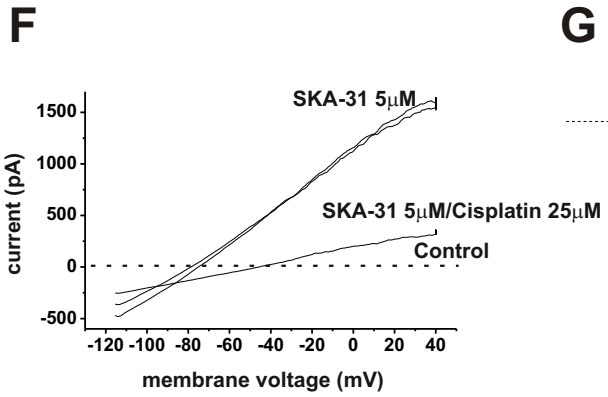


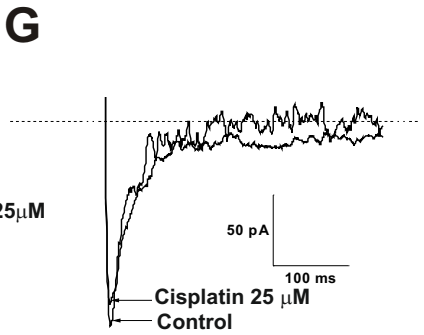


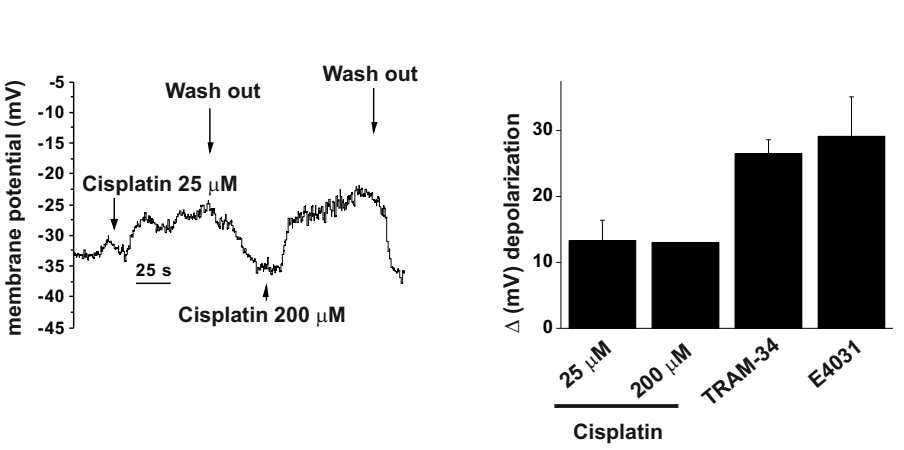
C

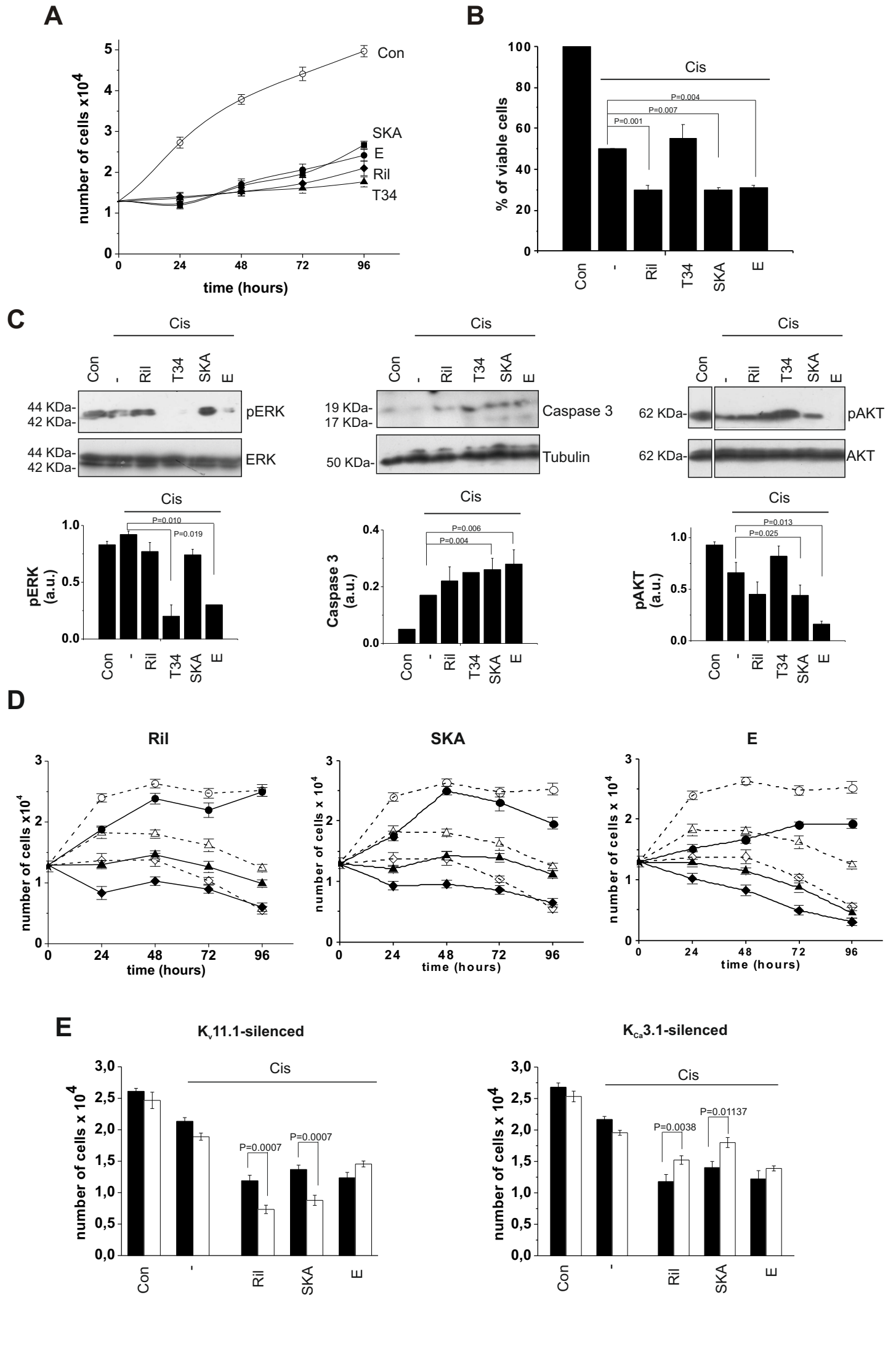
Drug	K_{Ca}3.1 slope fold variation	hERG1 current inhibition (%)
Riluzole	2.11 ± 0.46 (n=9)	23.26 ± 4.49 (n=6)
SKA-31	4.36± 1.67 (n=10)	7.53 ± 1.81 (n=11)
TRAM-34	0	NA
E4031	NA	93.31±1.29 (n=8)
Riluzole (45 μM)	6.74 ± 3.10 (n=6)	43.83 ± 7.02 (n=6)
Cisplatin	1.04 ± 0.25 (n=9)	5.75 ± 2.16 (n=10)
Cisplatin after SKA-31	1.10 ± 0.12 (n=10)	NA

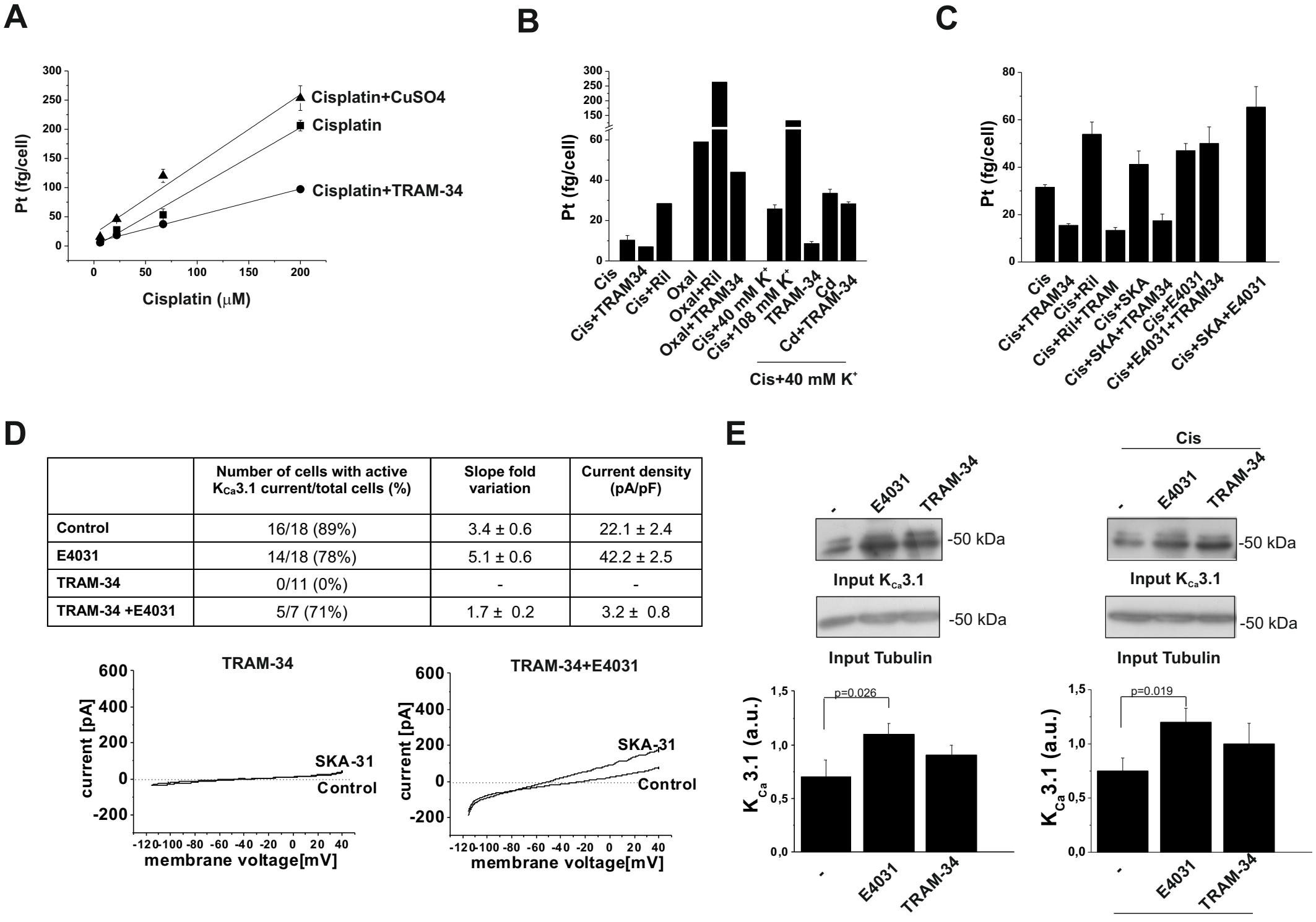




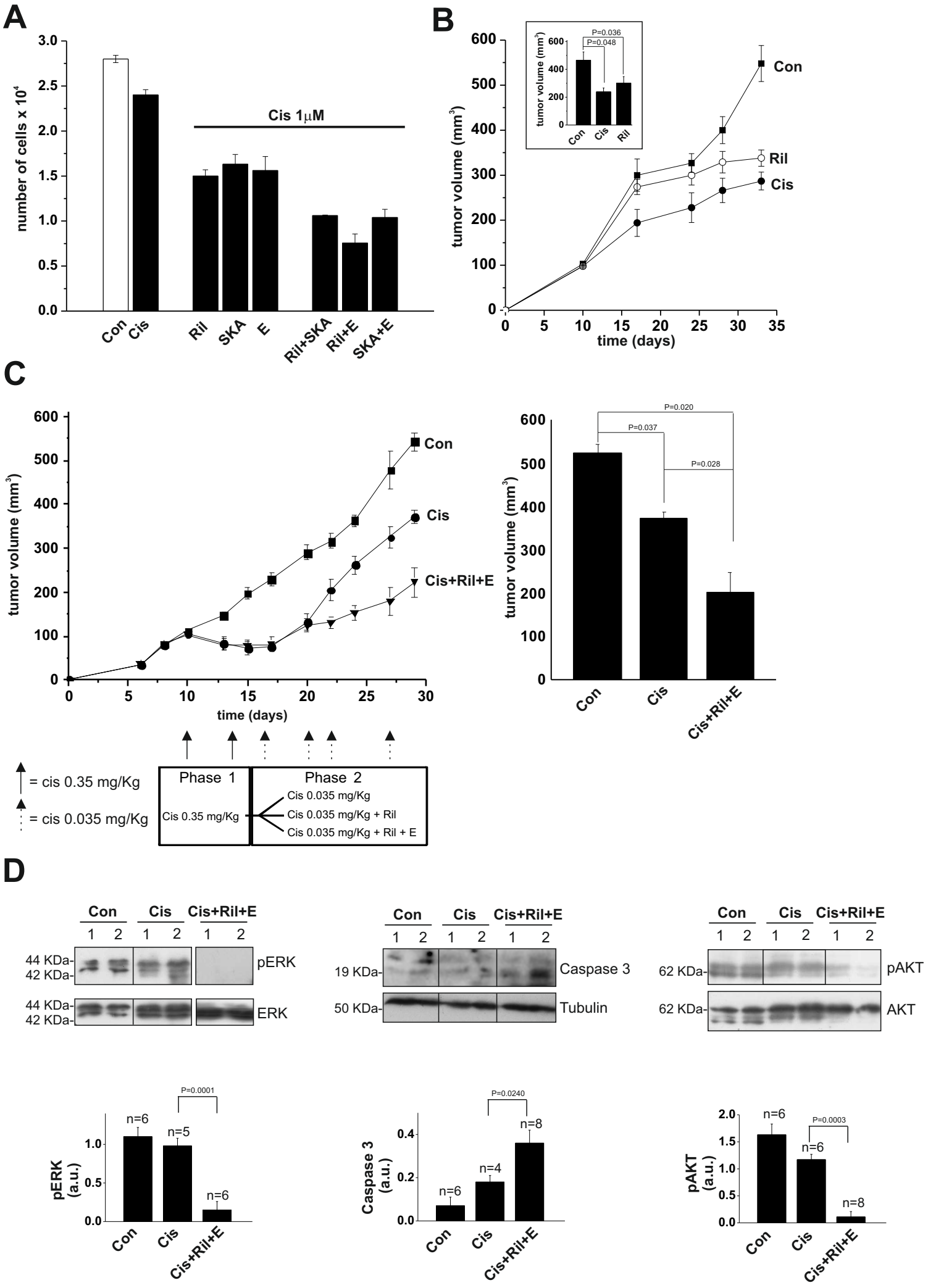





A
number of cells x10^4
time (hours)
Con
SKA
E
Ril
T34
B
% of viable cells
Cis
P=0.004
P=0.007
P=0.001
Con
-
Ril
T34
SKA
E
C
Cis
Con
-
Ril
T34
SKA
E
44 KDa-
42 KDa-
pERK
ERK
19 KDa-
17 KDa-
Caspase 3
50 KDa-
Tubulin
62 KDa-
pAKT
AKT
pERK (a.u.)
P=0.010
P=0.019
Caspase 3 (a.u.)
P=0.006
P=0.004
pAKT (a.u.)
P=0.013
P=0.025
D
Ril
SKA
E
number of cells x10^4
number of cells x 10^4
time (hours)
E
K_v11.1-silenced
K_ca3.1-silenced
number of cells x 10^4
P=0.0007
P=0.0007
P=0.0038
P=0.01137



	Number of cells with active $K_{Ca}3.1$ current/total cells (%)	Slope fold variation	Current density (pA/pF)
Control	16/18 (89%)	3.4 ± 0.6	22.1 ± 2.4
E4031	14/18 (78%)	5.1 ± 0.6	42.2 ± 2.5
TRAM-34	0/11 (0%)	-	-
TRAM-34 +E4031	5/7 (71%)	1.7 ± 0.2	3.2 ± 0.8


A
number of cells x 10^4
Cis 1μM
Con
Cis
Ril
SKA
E
Ril+SKA
Ril+E
SKA+E
B
tumor volume (mm^3)
time (days)
Con
Ril
Cis
P=0.036
P=0.048
C
tumor volume (mm^3)
time (days)
Con
Cis
Cis+Ril+E
P=0.020
P=0.037
P=0.028
= cis 0.35 mg/Kg
= cis 0.035 mg/Kg
Phase 1
Cis 0.35 mg/Kg
Phase 2
Cis 0.035 mg/Kg
Cis 0.035 mg/Kg + Ril
Cis 0.035 mg/Kg + Ril + E
D
Con
Cis
Cis+Ril+E
44 KDa-
42 KDa-
pERK
ERK
19 KDa-
Caspase 3
50 KDa-
Tubulin
62 KDa-
pAKT
AKT
pERK (a.u.)
P=0.0001
n=6
n=5
n=6
Caspase 3 (a.u.)
P=0.0240
n=6
n=4
n=8
pAKT (a.u.)
P=0.0003
n=6
n=6
n=8