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Inhibition of cell proliferation by a selective inhibitor of the Ca^{2+}-activated Cl^- channel, Ano1

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ABSTRACT

Background: Ion channels play important roles in regulation of cellular proliferation. Ano1 (TMEM16A) is a Ca^{2+}-activated Cl^- channel expressed in several tumors and cell types. In the muscle layers of the gastrointestinal tract Ano1 is selectively expressed in interstitial cells of Cajal (ICC) and appears to be required for normal gastrointestinal slow wave electrical activity. However, Ano1 is expressed in all classes of ICC, including those that do not generate slow waves suggesting that Ano1 may have other functions. Indeed, a role for Ano1 in regulating proliferation of tumors and ICC has been recently suggested. Recently, a high-throughput screen identified a small molecule, $T16A_{inh}$-A01 as a specific inhibitor of Ano1.

Aim: To investigate the effect of the $T16A_{inh}$-A01 inhibitor on proliferation in ICC and in the Ano1-expressing human pancreatic cancer cell line CFPAC-1.

Methods: Inhibition of Ano1 was demonstrated by whole cell voltage clamp recordings of currents in cells transfected with full-length human Ano1. The effect of $T16A_{inh}$-A01 on ICC proliferation was examined *in situ* in organotypic cultures of intact mouse small intestinal smooth muscle strips and in primary cell cultures prepared from these tissues. ICC were identified by Kit immunoreactivity. Proliferating ICC and CFPAC-1 cells were identified by immunoreactivity for the nuclear antigen Ki67 or EdU incorporation, respectively.

Results: $T16A_{inh}$-A01 inhibited Ca^{2+}-activated Cl^- currents by 60% at 10 μM in a voltage-independent fashion. Proliferation of ICC was significantly reduced in primary cultures from BALB/c mice following treatment with $T16A_{inh}$-A01. Proliferation of the CFPAC-1 human cell-line was also reduced by $T16A_{inh}$-A01. In organotypic cultures of smooth muscle strips from mouse jejunum, the proliferation of ICC was reduced but the total number of proliferating cells/confocal stack was not affected, suggesting that the inhibitory effect was specific for ICC.

Conclusions: The selective Ano1 inhibitor $T16A_{inh}$-A01 inhibited Ca^{2+}-activated Cl^- currents, reduced the number of proliferating ICC in culture and inhibited proliferation in the pancreatic cancer cell line CFPAC-1. These data support the notion that chloride channels in general and Ano1 in particular are involved in the regulation of proliferation.

1. Introduction

Alteration of membrane potential through changes in extracellular ion concentration is a crucial regulator of proliferation, performing important roles in the progression of cell cycle at multiple key checkpoints (reviewed in [1]). Many ions are involved in these processes; the role of chloride ions in regulating cell cycle has been studied mostly as a driving force in cytoplasmic condensation [2,3]. Ano1 is a Ca^{2+} activated Cl^- channel expressed in secretory epithelia of lung, salivary glands and kidney [4–6]. Expression of Ano1 is up-regulated in several cancers including esophageal cancer [7] and gastrointestinal stromal tumors (GISTs), the most common mesenchymal tumors in the gastrointestinal tract [8,9]. In the muscle layers of the gastrointestinal tract Ano1 expression is restricted to interstitial cells of Cajal (ICC [10]), pacemaker and neuromodulator cells of the gut. Ano1 appears to be required for normal gastrointestinal function [11,12] and has been proposed to play a key role in the pacemaker activity of ICC [12,13]. Ano1 is expressed in all classes of ICC, including those that do not generate slow waves [10] suggesting that Ano1 may have other functions. A possible role of Ano1 in regulation of proliferation was suggested by its expression in GISTs. We investigated this

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possibility and showed that Ano1 activity contributes to the proliferation of Ano1 positive cells by acting at the G1/S phase of cell cycle. This was demonstrated by showing reduced proliferation of ICC in Ano1$^{(-/-)}$ mice, in the presence of chloride depleted culture medium and by the use of three structurally different chloride channel blockers [14]. Also, in a human head and neck squamous cell carcinoma cell line (UM-SCC1) knockdown of Ano1 resulted in a decrease in xenograft growth in nude mice [15].

A significant problem in investigating the role of Ano1 in proliferation is the lack of selectivity of many Cl^- channel inhibitors for Cl^- channels and transporters and for Ano1. Compounds such as DIDS, niflumic acid and tamoxifen decrease ICC proliferation in culture; however, they are not selective and have multiple effects on other targets that can indirectly alter chloride secretion [16–19].

Recently, a high throughput screen of 110,000 compounds revealed a novel small molecule, an aminophenylthiazole named T16A$_{inh}$-A01 as a specific inhibitor of Ano1 [20]. This compound was reported to inhibit Cl^- efflux due to Ano1 (human) with an IC50 of 1.1 μM but to have little effect on CFTR and no effect on cytoplasmic calcium [20]. However, direct effects on Ano1-mediated Cl^- currents have not been reported.

The aim of this study was to confirm T16A$_{inh}$-A01's inhibitory effect on Ano1-mediated Cl^- currents in an heterologous expression system and to investigate the effect of the inhibitor on the proliferation of ICC and an Ano1-expressing human pancreatic cancer cell line.

2. Material and methods

2.1. Animals

BALB/c mice were obtained from Harlan (Indianapolis, IN). Mice were killed by CO_2 inhalation and cervical dislocation at post-natal day 3 (PND 3). The mice were maintained and the experiments were performed with approval from the Institutional Animal Care and Use Committee of the Mayo Clinic.

2.2. Cell cultures

Primary cultures enriched in ICC were obtained by enzymatic dissociation of the mouse small intestines and co-cultured in the presence of *Sl/Sl*^{4} mSCF248, murine stem cell factor—secreting fibroblasts as previously described [21]. M199 media without phenol red (Invitrogen) supplemented with 1% antibiotic–antimycotic (Invitrogen) was used for the co-cultures. Cells were allowed to incubate for 1 h at 37 °C/5% CO_2 before adding 2 ml of the culture medium to the well.

CFPAC-1 is a human pancreatic duct cell line that endogenously expresses Ano1. This cell line was derived from a cystic fibrosis patient and as a consequence expresses the most common cystic fibrosis mutation in CFTR, a deletion of three nucleotides, resulting in the absence of phenylalanine at position 508. CFPAC-1 cells were grown in Iscove's Modified Dulbecco's Media (IMDM, ATCC) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

HEK293 cells were maintained in Minimum Essential Medium (MEM, Invitrogen) supplemented with 10% heat inactivated horse serum (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% non-essential amino acids (Invitrogen) and1% penicillin/streptomycin. Cells were transfected with a vector bearing the full length Ano1 cDNA using LIPOFECTAMINE™ 2000 Reagent (Invitrogen).

2.3. Electrophysiology

Currents were recorded by standard whole cell voltage clamp recordings at room temperature (22 °C) from HEK293 cells expressing Ano1 and the fluorescent marker GFP. 2–5 MΩ glass patch clamp pipettes in standard whole cell configuration were used. Glucose-free *N*-methyl d-glucamine containing extracellular solutions (in mM: $NMDG^+$ 149.2, K^+ 4.74, Ca^{2+} 2.54, Gd^{3+} 0.01, Cl^- 159, HEPES 5; pH 7.35, osmolality 290 mmol/kg) and CsCl and 500 nM free Ca^{2+} intracellular solutions (in mM: Cs^+ 145, Na^+ 5, Mg^{2+} 5, Ca^{2+} 1.27, Cl^- 162.5, EGTA 2, HEPES 5; pH 7.25, osmolality 300 mmol/kg) were used. Data were collected and analyzed using an Axopatch 200B, Digidata 1322A, and pCLAMP 9 software (Molecular Devices). Free Ca^{2+} was calculated online at <http://www.stanford.edu/~cpatton/CaMgATPEGTA-TS-Plot.htm>. Under these conditions, Cs^+, $NMDG^+$, and Gd^{3+} block K^+, Na^+, and non-selective cation currents, and equimolar Cl^- results in a predicted reversal potential of 0 mV. Cells were held at −100 mV between 1 s long voltage steps from −100 to + 120 mV. Start-to-start time between sweeps was 5 s. Data were analyzed using Clampfit and Excel (Microsoft). In current–voltage (*I–V*) relationships (Fig. 1), Cl^- currents at 1 s are shown as a fraction of total cell capacitance (pA/pF). Previously we found non-transfected cells to have currents <2 pA/pF in 500 nM free Ca^{2+} [22]. Significance was determined by 1-way repeated measures ANOVA with Dunnett post-test. A *P*-value less than 0.05 was considered significant.

2.4. Organotypic cultures

The tunica muscularis from the jejunum of PND 3 mice was quickly dissected out, flushed with ice-cold calcium-free Hanks balanced salt solution (Invitrogen, Carlsbad, CA) and pinned on to a sterile Sylgard lined petri dish. Whole-mount preparations were incubated for 24 h in the presence of vehicle (DMSO) or T16A$_{inh}$-A01 inhibitor diluted to a final concentration of 10 μM in medium composed of M199 without phenol red supplemented

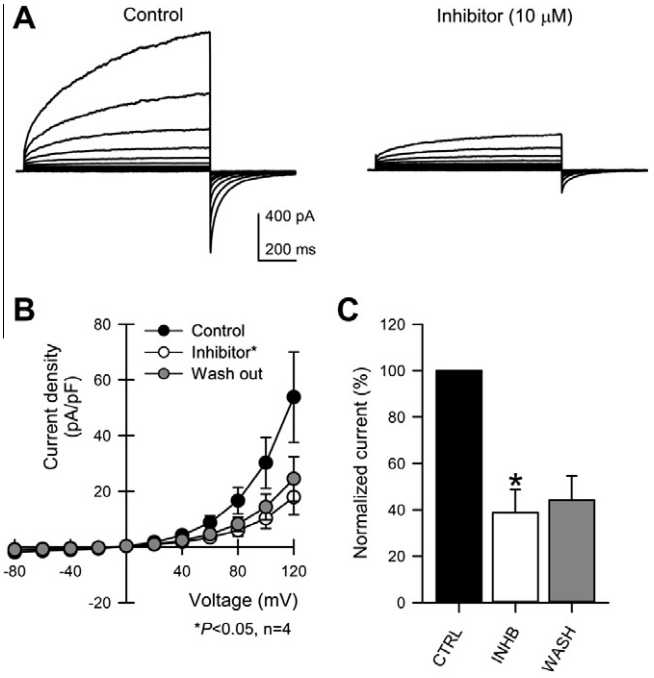

Fig. 1. T16A$_{inh}$-A01 inhibits Ano1 currents in transfected cells. (A) Representative traces of Ano1-mediated currents evoked by depolarization from the holding potential of −100 mV. Outwardly rectifying chloride currents were recorded from HEK293 cells transfected with full-length human Ano1 in control conditions or in the presence of T16A$_{inh}$-A01 inhibitor (10 μM). (B) Current–voltage graph for chloride currents. Note reduced current densities after exposure to T16A$_{inh}$-A01. (C) Graph showing quantification of normalized currents. Data are means ± SEM, $n = 4$, $^*p < 0.05$.

with 4.5 g/l of glucose (Sigma–Aldrich), 10% FBS and 2% antibiotic–antimycotic (Invitrogen).

2.5. Immunohistochemistry

For primary cultures, immunohistochemistry was performed as previously described [23] and proliferating ICC were identified for immunoreactivity for the nuclear antigen, Ki67 (Abcam). Proliferating CFPAC-1 were identified by detection of incorporated 5-ethynyl-2'-deoxyuridine (EdU) using the "Click-iT" technology (Invitrogen) according to the manufacturer's instructions. Immunostained cultures were examined with the use of a fluorescence microscope (BX51WI, Olympus). Proliferating ICC were identified by co-immunoreactivity for Kit and Ki67, while proliferating CFPAC-1 cells were identified by positivity to EdU. Cells were counted using a 20X objective on two separate coverslips per condition.

For whole-mount staining, following incubation with either vehicle or $T16A_{inh}$-A01 inhibitor, tissues were incubated with EdU (10 μM diluted in culture medium) for 2 h and then fixed in 4% paraformaldehyde for 30 min, washed with PBS and permeabilized in 0.3% Triton X-100 for 30 min. After washing, tissues were incubated in "Click-iT" reaction buffer for 2 h at room temperature protected from light, washed in PBS and then incubated with 10% normal donkey serum (NDS, Jackson Immunoresearch Laboratories) and 0.3% Triton-X-100 (Sigma–Aldrich) in PBS (4 °C, overnight) to minimize nonspecific antibody binding. Tissues were then incubated with Kit antibody (0.4 μg/ml in 5% NDS, R&D Systems) for 8 h at 4 °C. Following washings in PBS, the tissues were incubated with donkey anti-goat IgG conjugated with CY3 (Jackson Immunoresearch Laboratories, 1.8 μg/ml in 2.5% NDS, 4 °C, 24 h). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 0.01 μg/ml in dH2O, 30 min). The labeled tissues were mounted using Slowfade mounting medium (Invitrogen). Whole-mounts were imaged using an FV1000 confocal microscope (Olympus). Four image stacks (field size: 211.5 × 211.5 μm) representing the entire thickness of the muscle were collected from each tissue using a 60X (NA 1.2) objective.

2.6. $T16A_{inh}$-A01 inhibitor

T16Ainh-A01 inhibitor compound was provided by Dr. Verkman (UCSF). Stock solutions (5 mM) were made in DMSO (Sigma–Aldrich) and stored at −20 °C. The compound was diluted fresh on the day of the experiment to a final concentration of 10 μM. DMSO was used at a final concentration of 1:500 for the vehicle control.

3. Results

3.1. $T16A_{inh}$-A01 inhibits Ano1 currents in transfected cells

To test if the $T16A_{inh}$-A01 inhibitor blocked Ano1 currents we recorded whole cell patch clamp currents from HEK293 cells transfected with a plasmid containing the full-length human Ano1 in the presence or absence of the inhibitor. As previously shown in Mazzone et al. [22], transfection with the Ano1 vector resulted in expression of channels that produced Ca^{2+}- and voltage-dependent, outwardly rectifying currents (30.2 ± 9.1 pA/pF at 100 mV, Fig. 1(A)). When the transfected cells were treated with the Ano1 inhibitor at a final concentration of 10 μM, current densities markedly decreased (10.3 ± 3.7 pA/pF at 100 mV, $p < 0.05$, student t-test, $n = 4$, Fig. 1(A)). Treatment with the inhibitor reduced the current density of Ano1 at all the voltages examined (Fig. 1(B), $p < 0.05$, $n = 4$). The effect of $T16A_{inh}$-A01 was relatively voltage-independent with inhibition ranging from 44 ± 7% at −100 mV to 67 ± 5% at −20 mV and 53.7 ± 3.4% at 20 mV to 63 ± 7% at 100 mV (Fig. 1C). Washing out the inhibitor did not restore the original current density (14.3 ± 4.6 pA/pF at 100 mV, $n = 4$, Fig. 1(B) and (C)).

3.2. $T16A_{inh}$-A01 reduces the number of proliferating ICC in primary cultures from mouse small intestine

To study the effect of the $T16A_{inh}$-A01 inhibitor on the proliferation of ICC we tested the effect of this inhibitor in primary cultures. Proliferation of ICC was significantly reduced in primary cultures from BALB/c mice following treatment with $T16A_{inh}$-A01 (10 μM, Fig. 2B), when compared to controls (Fig. 2A). This was observed as a decrease in number of ICC displaying nuclear immunostaining for Ki67 (arrows Fig. 1A) from 16.14 ± 2.3% per field-of-view to 7.5 ± 3.5% ($p < 0.03$, paired t-test, $n = 4$, Fig. 2C).

3.3. $T16A_{inh}$-A01 reduces the number of proliferating cells in the human pancreatic cancer cell line, CFPAC-1

We also tested the effect of the $T16A_{inh}$-A01 inhibitor on CFPAC-1, a human pancreas cell line endogenously expressing Ano1. To assess proliferation we counted the number of nuclei that incorporated EdU in each field. Proliferation of CFPAC-1 cells was significantly reduced when cultured in the presence of 10 μM $T16A_{inh}$-A01 compared to vehicle (vehicle: 27.9 ± 0.4, Fig. 3A; inhibitor: 17.2 ± 1.2, Fig. 3B; $p < 0.002$, paired t-test, $n = 4$, Fig. 3C). The total number of cells per field was not different between the two conditions (vehicle: 73. ± 3, inhibitor: 72.9 ± 6.2, $p = 0.77$, t-test, $n = 4$, Fig. 3D).

3.4. $T16A_{inh}$-A01 reduces the number of proliferating mouse ICC in intact muscle strips

Next, we tested the effect of the $T16A_{inh}$-A01 inhibitor on the proliferation of ICC *in situ* in mouse jejunal tissues maintained in organotypic culture. For this purpose muscle strips were freshly dissociated from the jejunum of PND 3 mice and cultured for 24 h in the presence of either vehicle or inhibitor (10 μM). After fixation and labeling to identify proliferating cells and ICC, high-resolution confocal stacks were acquired to visualize the EdU positive nuclei both in ICC (arrow) and other cell types (asterisks in Fig. 4(A) and (B)). Treatment with the inhibitor significantly reduced the number of proliferating ICC as assessed by EdU incorporation (vehicle: 3.3 ± 0.3 per image stack, inhibitor: 1.4 ± 0.5, $p < 0.03$, paired t-test, $n = 4$, Fig. 4C). This effect appeared to be specific only for ICC, the cells that expressed Ano1, because the total number of proliferating cells in each stack was not statistically different between the two conditions (vehicle: 56.9 ± 3.5, inhibitor: 52.2 ± 4.3, p = 0.12, paired t-test, $n = 4$, Fig. 4D).

4. Discussion

In the present study we show that the inhibitor $T16A_{inh}$-A01 blocked human Ano1 mediated Cl^- currents and that this more selective inhibitor decreased proliferation of Ano1-expressing mouse ICC in dissociated culture and intact tissue. Furthermore, the compound also inhibited proliferation in the human pancreatic cancer cell line CFPAC-1. This supports the work published previously using non-selective Cl^- channel inhibitors, modulation of Cl^- concentration and Ano1 knockout mice to interrogate the role of Ano1 in cellular proliferation [14]. It also addresses the issue regarding the use of broad-spectrum blockers. In the previous study, DIDS, niflumic acid and tamoxifen caused a decrease in ICC proliferation in culture. However, they are not selective and have multiple effects on other targets, including but not limited to effects on Cl^- transport and secretion. For example niflumic acid

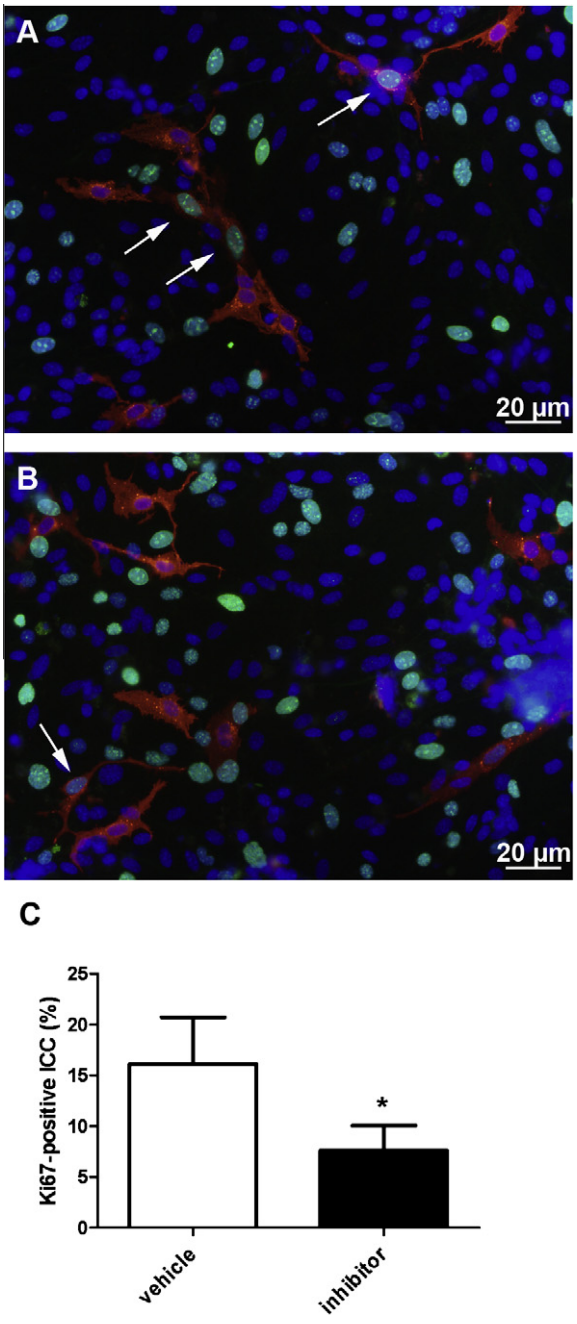

Fig. 2. T16A$_{inh}$-A01 reduces the number of proliferating cells in primary cultures. Representative pictures of freshly dissociated ICC from PND2–3 BALB/c mice after 24 h in culture in the presence of vehicle (A) or 10 μM concentration of T16A$_{inh}$-A01 inhibitor (B). Kit (red) was use as a marker of ICC and Ki67 (green) as a marker of proliferation. The cells nuclei were identified by DAPI staining (blue). Arrows indicate proliferating ICC. Treatment with T16A$_{inh}$-A01 inhibitor (10 μM) significantly reduced the percentage of proliferating ICC (C, $^*p < 0.03$, Paired *t*-test, $n = 4$). Data are means ± SEM.

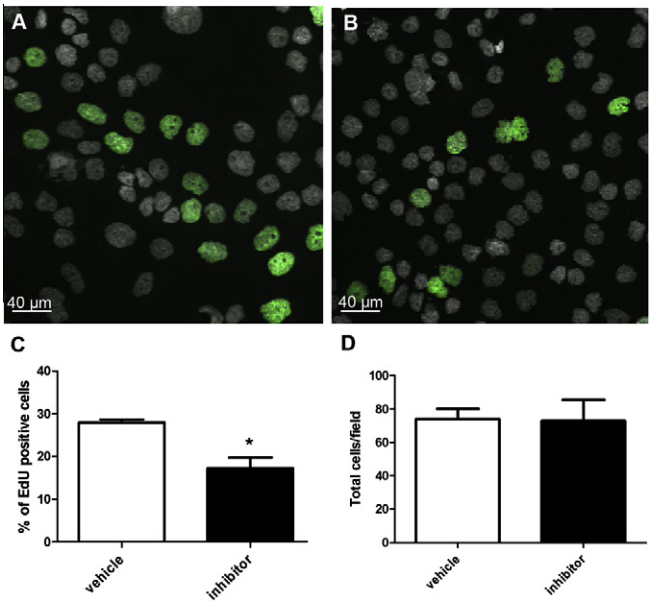

Fig. 3. T16A$_{inh}$-A01 reduces the number of proliferating cells in human cell line CFPAC-1. Representative pictures of CFPAC-1 cells after 24 h in culture in the presence of vehicle (A) or 10 μM T16A$_{inh}$-A01 Ano1 inhibitor (B). EdU incorporation was used as a marker of proliferation (green). Nuclei were identified by DAPI staining (gray). Treatment with T16A$_{inh}$-A01 reduced proliferation of CFPAC-1 cells (C), $^*p < 0.002$, paired *t*-test, $n = 4$), but had no effect on the total number of cells per field (D). Data are means ± SEM.

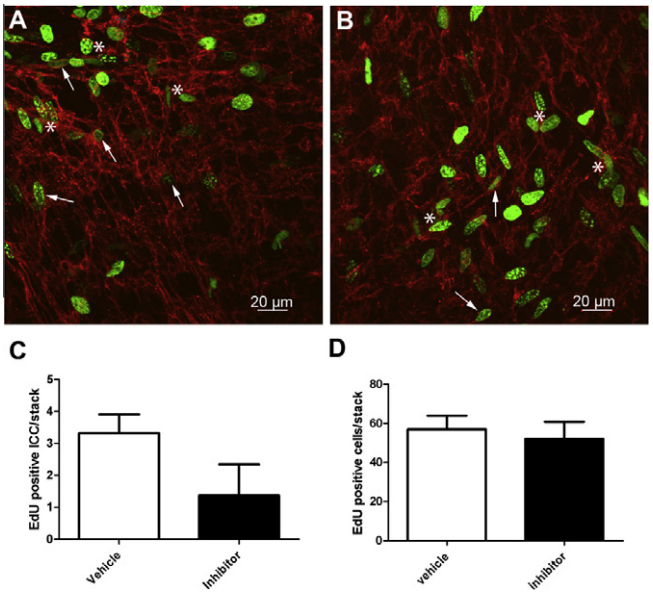

Fig. 4. T16A$_{inh}$-A01 reduces the number of proliferating ICC in intact smooth muscle strips. Representative images from immunolabeled muscle strips dissected from PND3 BALB/c mice after 24 h in culture in the presence of vehicle (A) or 10 μM T16A$_{inh}$-A01 Ano1 inhibitor (B). Kit (red) was used as a marker of ICC and EdU incorporation (green) to identify proliferating nuclei. Arrows indicate proliferating ICC and asterisks indicate the proliferating nuclei of other, unidentified cell types. Treatment with T16A$_{inh}$-A01 inhibitor significantly reduced the percentage of proliferating ICC (C), $^*p < 0.03$, paired *t*-test, $n = 4$ mice). There was no difference in the total number of ICC per field between the two conditions (D). Data are means ± SEM.

alters intracellular Ca^{2+} release and inhibits voltage-gated K^+ channels and tamoxifen is a high-affinity ligand for the estrogen receptor [16–19]. Structurally, the T16A$_{inh}$-A01 compound is unrelated chemically to previously reported chloride channel or CFTR inhibitors. Moreover, it has been shown that T16A$_{inh}$-A01 at a concentration of 10 μM nearly completely inhibited Cl^- flux (using the fluorescence of YFP as an indicator for intracellular Cl concentration) and Cl^- currents, but had little effect on the CFTR transporter and did not alter cytoplasmic calcium, thus differentiating this inhibitor from DIDS and niflumic acid [20].

We also determined that the T16A$_{inh}$-A01 significantly reduced Ca^{2+}-activated Cl^- currents generated by transfection of cells with a vector bearing the full length cDNA encoding human Ano1. The

effect on these characteristically outward rectifying currents [6] was robust in the presence of 500 nM intracellular Ca^{2+}. The inhibition was voltage-dependent of and the inhibitor did not affect the kinetics of the current during the voltage steps. The inhibition was not reversible within the time frame studied (10 min washout). These data indicate that $T16A_{inh}$-A01 is a direct and potent inhibitor of Ano1 but do not clarify the mechanism of action of the molecule.

The use of different but complementary experiments to determine proliferation (Ki67 staining and EdU incorporation) in multiple experimental paradigms including mouse primary cultures enriched in ICC, the human pancreatic cancer cell line, CFPAC-1 and intact mouse muscle strips in organotypic cultures also strengthen the interpretation of our data. Our results show that in all these approaches the use of $T16A_{inh}$-A01 reduced the proliferation of ICC and Ano1 expressing cells when compared to control conditions treated with vehicle only.

In primary cells and tissue strips the $T16A_{inh}$-A01 inhibitor had no effect on the total number of proliferating cells. The lack of effect of $T16A_{inh}$-A01 on the proliferation of Kit-negative cells in the cultured mouse muscle strips is further confirmation that the effects of the compound on proliferation are a consequence of inhibiting Ano1. These findings suggest that the effect of $T16A_{inh}$-A01 is specific for the cells expressing Ano1 in all the substrates studied.

We previously showed that the role of Ano1 in cell proliferation is important for the progression of the cell cycle at the G1/S checkpoint [14]. Consistent with our results, a recent work in a model of head and neck squamous cell carcinoma showed that expression of Ano1 was directly correlated to proliferation of the tumor cells [15]. In this model the mechanism of action of Ano1 appeared to take place through induction of cyclin D1 (which is a key gene in the G1/S checkpoint) and activation of the MAP/ERK pathway and in particular ERK1/2. These results are particularly interesting given the high expression of Ano1 in GIST [8,9]. It has already been suggested that Ano1 could be a new potential target to pursue to arrest proliferation in GIST [24]. Based on these novel results, $T16A_{inh}$-A01 and similar inhibitors represent a further step in this direction. However, a recent paper on vascular tissue described an opposite effect of Ano1 as a negative regulator of cell proliferation in a model of hypertensive rat smooth muscle cells [25]. In this model Ano1 expression affects the proliferation of smooth muscle cells by arresting them at the G0/G1 checkpoint through reduction of cyclin D1 and cyclin E expression [25]. It is thus possible that Ano1 might have different roles in different cell types or in different physiological contexts.

In conclusion, these data add to the emerging evidence that chloride channels in general and Ano1 in particular are involved in the regulation of proliferation. The role of Ano1 in the progression of the cell cycle is confirmed by the effects of the more selective inhibitor $T16A_{inh}$-A01. $T16A_{inh}$-A01 is also shown to directly inhibit Ano1-mediated Cl^- currents. Small molecules of this class with less off-target effects may be novel therapeutic tools for arresting proliferation of Ano1-positive tumors such as GISTs.

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