Beni-Suef University Journal of Basic and Applied Sciences

RESEARCH

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The anti-tumor drug 2-hydroxyoleic acid regulates the oncogenic potassium channel Kv10.1

Rita Morán-Zendejas and Aldo A. Rodríguez-Menchaca*

Abstract

Background 2-hydroxyoleic acid (2OHOA) is a synthetic fatty acid with antitumor properties that alters membrane composition and structure, which in turn influences the functioning of membrane proteins and cell signaling. In this study, we propose a novel antitumoral mechanism of 2OHOA accomplished through the regulation of Kv10.1 channels. We evaluated the effects of 2OHOA on Kv10.1 channels expressed in HEK-293 cells by using electrophysiological techniques and a cell proliferation assay.

Results 2OHOA increased Kv10.1 channel currents in a voltage-dependent manner, shifted its conductance-voltage relationship towards negative potentials, and accelerated its activation kinetics. Moreover, 2OHOA reduced proliferation of cells that exogenously (HEK-293) and endogenously (MCF-7) expressed Kv10.1 channels. It is worth noting that the antiproliferative effect of 2OHOA was maintained in HEK-293 cells expressing a non-conducting mutant of Kv10.1 channel (Kv10.1-F456A), while it did not affect HEK-293 cells not expressing Kv10.1 channels, suggesting that 2OHOA interferes with a non-conducting function of Kv10.1 channels involved in cell proliferation. Finally, we found that 2OHOA can act synergistically with astemizole, a Kv10.1 channel blocker, to decrease cell proliferation more efficiently.

Conclusion Our data suggest that 2OHOA decreases cell proliferation, at least in part, by regulating Kv10.1 channels.

Keywords Potassium channels, Synthetic lipids, Electrophysiology, Cell proliferation

1 Background

2-hydroxyoleic acid (2OHOA) is a synthetic structural analogue of oleic acid [1]. Insertion of 2OHOA into the plasma membrane alters its structure and biophysical properties [2–4], which in turn influences the functioning of membrane proteins and cell signaling [5–9]. In vitro and in vivo studies have shown that 2OHOA displays antitumor properties in different cancer cell lines, but neither toxic nor antiproliferative effects on non-tumor cells [10, 11]. Several mechanisms have been proposed to explain the action of 2OHOA; for example, by modifying the membrane microdomain organization, 2OHOA affects the recruitment and activity of G-proteins, protein kinase C (PKC) and adenylyl cyclases [7, 12, 13]. It has been demonstrated that 2OHOA increases the sphingomyelin content via sphingomyelin synthase activation [8, 10, 14, 15]. Additionally, this drug induces endoplasmic reticulum stress, autophagy, cell cycle arrest, and uncouple oxidative phosphorylation [6, 13, 16–18]. Hitherto, it is unknown whether the changes on the plasma membrane properties induced by 2OHOA affect the functioning of transport proteins, like ion channels.

In this scenario, ion channels are transmembrane proteins highly susceptible to alterations of the plasma membrane. Increasing evidence supports the sensitivity of ion channels to the membrane environment [19, 20], whether by specific lipid-protein interactions or by changes in the

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membrane biophysical properties, but most likely as a mixture of both [21].

K_V10.1 is a voltage-gated potassium channel associated to specific membrane microdomains and it is affected by strategies that disturb such domains [22]. This ion channel has been associated with oncogenesis and tumor progression [23, 24]. Remarkably, K_V10.1 is ectopically expressed in over 70% of human non-central nervous systems cancers [25–27], whereas in healthy tissue is mainly found in the central nervous system [28], but also at low levels in myoblasts, placenta, testis, and adrenal glands [25, 29, 30]. K_V10.1 is involved in cell proliferation and survival [31–33], angiogenesis [34], and cell migration and invasion [35, 36]. Blocking the potassium flow through K_V10.1 channels disrupts the cell cycle progression and induces a significant decrease in cell proliferation and migration [27, 31, 36, 37]; however, is noteworthy that the oncogenic potential of K_V10.1 channel is also independent of its ion-conductive activity, as non-conductive mutants still preserve the ability to induce cell proliferation and a transformed phenotype [34, 38].

This study aimed to explore if the plasma membrane targeting drug, 2OHOA, modulates the functioning of K_V10.1 channels as part of its mechanisms to regulate cell proliferation.

2 Methods

2.1 Cell culture and compounds

HEK-293 [ATCC® CRL-1573™] and MCF-7 [ATCC® CRL-HTB-22™] cells were cultured in 35 mm culture dishes (Corning, Corning, NY, USA) at 37 °C in a humidified air atmosphere containing 5% CO_2. Cells were maintained in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Corning Life Sciences, Manassas, VA, USA) and 1% (v/v) antibiotic–antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA). Sodium salt of 2-hidroxioleic acid (2OHOA) and astemizole were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and Sigma-Aldrich, respectively. Stocks of 2OHOA and astemizole were prepared in aqueous solution and dimethyl sulfoxide (DMSO) respectively and diluted to the final desired concentrations.

2.2 Cell transfection

HEK-293 cells were transiently co-transfected with the cDNAs encoding the Kv10.1 channel (kindly provided by Dr. Michael C. Sanguinetti, University of Utah, USA) and the enhanced green fluorescence protein (EGFP), the Kv10.1 (F546A) mutant channel and EGFP (for Fig. 3D) or transfected only with EGFP (for Fig. 3E) using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's specifications. In all cases the transfection efficiency was of ~ 50–60%. For electrophysiological recordings, cells were used 24 h after transfection.

2.3 Cell proliferation assay

MCF-7, HEK-293 cells and HEK-293 cells expressing Kv10.1 channels were seeded in 96-well plates at different densities (5000–20,000 cells/per well) and cultured overnight. Cells were then treated with 12.5–1000 µM of 2OHOA and/or 5 µM of astemizole for 24 h, 48 h or 72 h before the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT reagent (5 mg/ml in PBS) was then added to each well and incubated for 4 h. Finally, cells were lysed with 10% SDS. A Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the absorbance at 570 nm. The percentage of viable cells after 2OHOA and/or astemizole treatment was calculated by dividing the absorbance of the treated group by that of the control group.

2.4 Electrophysiological recordings

Kv10.1 currents in HEK-293 cells were recorded at room temperature using the whole-cell patch clamp technique with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and a Digidata 1440A interface (Molecular Devices) controlled by the pCLAMP 10 software (Molecular Devices). Currents were filtered with a four-pole Bessel filter at 1 kHz and digitized at 10 kHz. The specific voltage-clamp protocols used are described in the Results section. Recording pipettes were pulled with a micropipette programable puller (Sutter Instruments, Novato, CA, USA) and had a resistance of 1.5–2.5 MΩ when filled with the internal solution. The external solution contained (in mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 10 HEPES, 1.8 $CaCl_2$, and 10 glucose, pH 7.4 (NaOH). The pipette solution contained (in mM): 130 KCl, 5 $MgCl_2$, 10 EGTA, 5K_2ATP, and 10 HEPES, pH 7.2 (KOH). Solutions were applied using the Fast-Step Perfusion System VC-77SP (Warner Instruments, Hamden, CT, USA).

2.5 Data analysis and statistics

Patch clamp data were processed using Clampfit 10 (Molecular Devices) and analyzed in Origin 8.6 (OriginLab Corp., Northampton, MA, USA) software. Conductance-voltage (G–V) relationships were calculated according to the equation:

$$G = \frac{I_{\max}}{V - V_{\text{Krev}}}$$

where $I_{\max}$ is the maximum current amplitude at the test potential V and V_{Krev} is the potassium reversal potential.

The voltage dependence was determined from the G–V curves fitted to a Boltzmann equation:

$$y = \frac{1}{1 + \exp[-(V - V_{1/2})/K]}$$

where V is the test potential, $V_{1/2}$ is the potential at which the conductance was half-activated, and K is the slope.

Data are presented as the mean ± SEM. Statistical comparisons were determined using ANOVA and paired or one-sample Student's t-test. Statistical significance was set at $p < 0.05$.

3 Results

3.1 2OHOA increased Kv10.1 currents amplitude in a voltage dependent manner

The effect of 2OHOA on Kv10.1 channels was explored using the whole-cell configuration of the patch clamp technique. A 200 µM concentration of 2OHOA is commonly employed for in vitro studies and was selected for the electrophysiological experiments.

Kv10.1 currents were elicited by 500-ms depolarizing pulses from −80 mV to +90 mV (in 10 mV increments) followed by a pulse to −50 mV, from a holding potential of −100 mV. Representative Kv10.1 current traces are shown before (Fig. 1A) and after application of 200 µM 2OHOA (Fig. 1A). 2OHOA increased the Kv10.1 current amplitude in the whole voltage range (Fig. 1C), but with a clear voltage dependence (Fig. 1D), showing the higher effect at voltages close to the activation threshold ($n = 13$). To further characterize the effects of 2OHOA on Kv10.1 channels, we determined the conductance-voltage relationship (G–V curve) from untreated (control) and 2OHOA treated cells (Fig. 2A). The conductance-voltage relationship is displaced towards more negative voltages after application of 200 µM 2OHOA, indicating that Kv10.1 channels are more prone to open. The

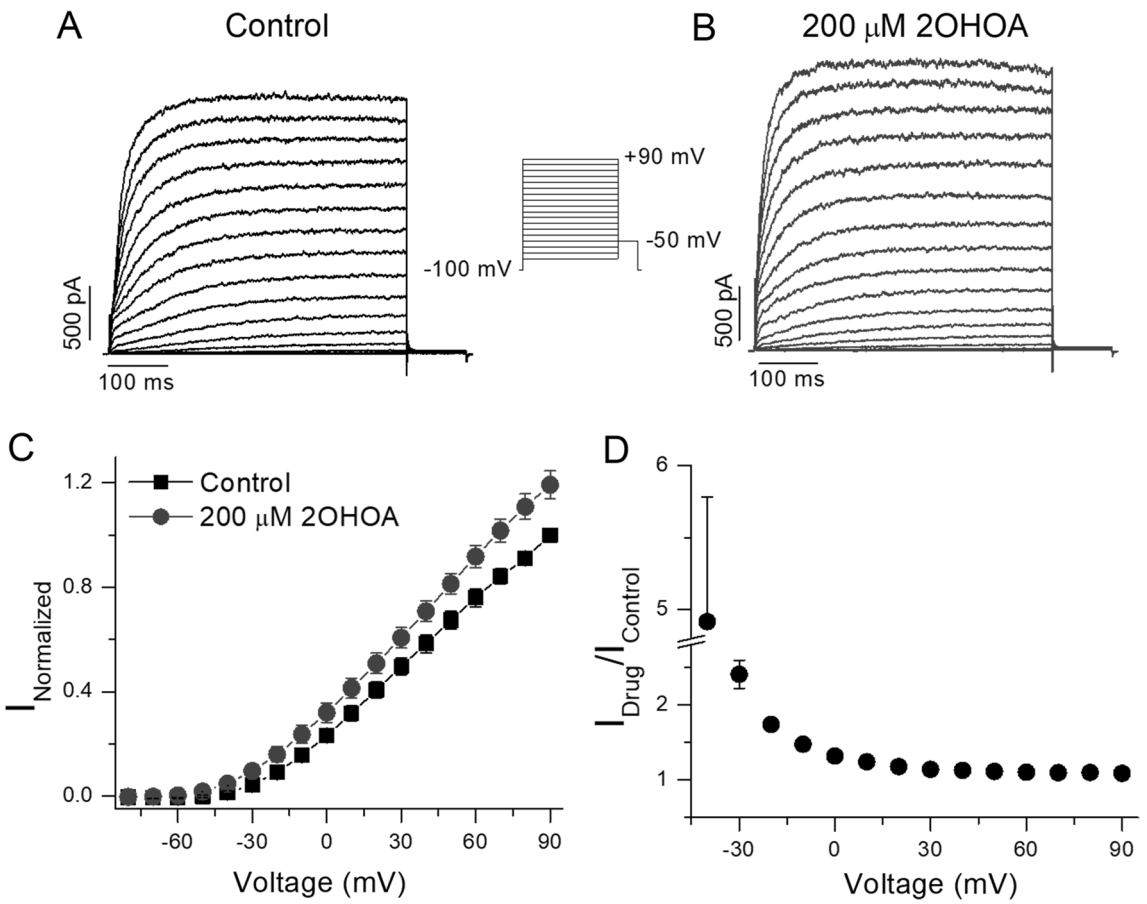

Fig. 1 2OHOA increases Kv10.1 currents in a voltage-dependent manner. **A**, **B** Representative recordings of Kv10.1 currents before (**A**) and after application of 200 µM 2OHOA (**B**), from the same cell. Currents were evoked by 500 ms depolarizing steps to test potentials ranging from -80 to + 90 mV in 10 mV increments from a holding potential of -100 mV. **C** normalized I-V relationships for currents measured at the end of the 500 ms pulses before (squares) and after 2OHOA (circles). **D** Kv10.1 current increase by 2OHOA plotted as a function of the test potential. Data are represented as mean ± SEM ($n = 13$ cells)

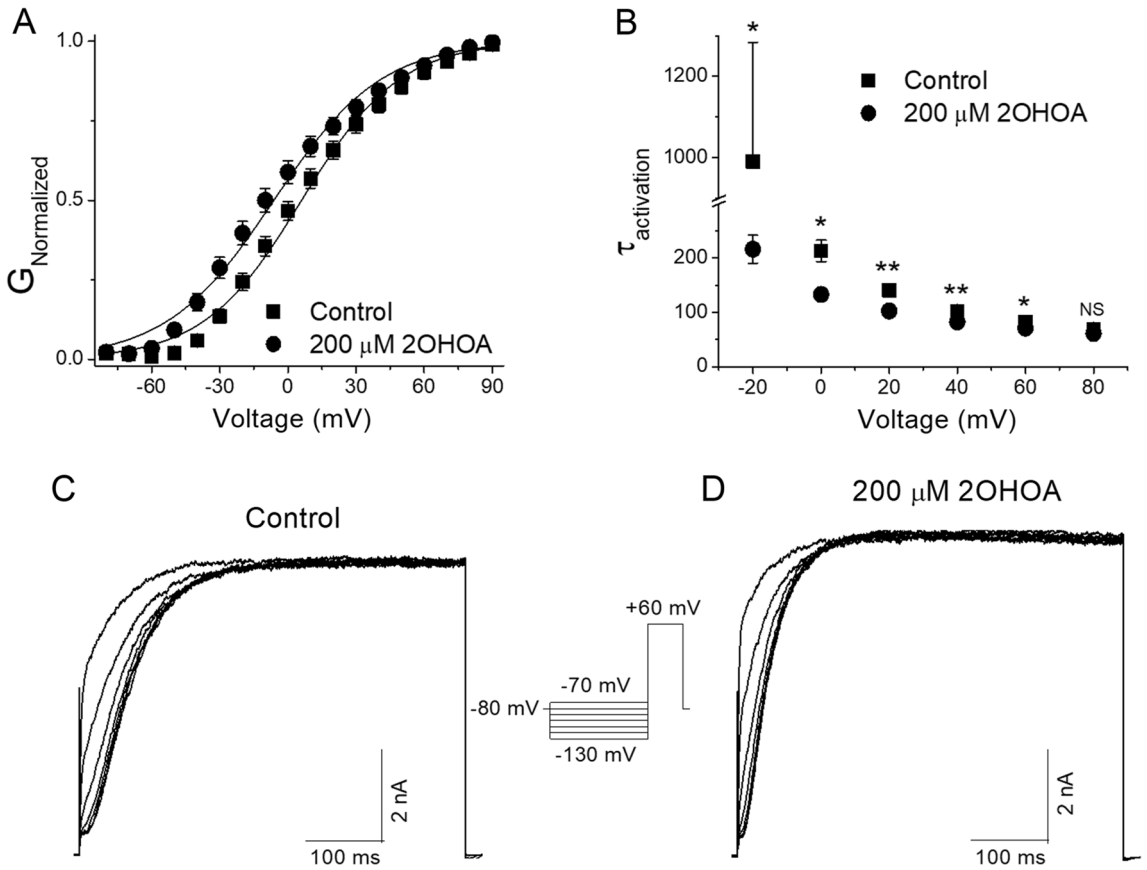

Fig. 2 Effect of 2OHOA on the voltage-dependence of activation, activation kinetics and Cole-More shift of Kv10.1 channel. **A** conductance-voltage (*G–V*) curves before (squares) and after application of 200 μM 2OHOA (circles). The $V_{1/2}$ values were 5.9 ± 0.9 mV and − 6.2 ± 1.1 mV for currents recorded before and after 2OHOA, respectively (n = 13 cells). **B** time constant of activation kinetics as a function of test potential before (squares) and after 2OHOA application (circles; *n* = 9 cells). **C**, **D** Representative Kv10.1 currents at + 60 mV evoked with the protocol shown inset in control (**C**) and 200 μM 2OHOA treated cells (**D**). similar results were obtained in five more cells. Data are represented as mean ± SEM. **p* < 0.05, ***p* < 0.01, NS: Not significant

mean activation parameters in untreated and 2OHOA treated cells were $V_{1/2} = 5.9 \pm 0.9$ mV, $k = 21.6 \pm 0.8$ and $V_{1/2} = -6.2 \pm 1.1$ mV, $k = 23.6 \pm 1.0$, respectively ($n = 13$). Furthermore, we analyzed the effect of 2OHOA on the activation kinetics of Kv10.1 currents like those shown in Fig. 1A, B. A monoexponential function was fitted to the activation recording data. 2OHOA significantly accelerated the activation rate in the range of − 20 to 60 mV (Fig. 2B, $n = 9$). Finally, we explored if the Cole-More shift, a characteristic of Kv10.1 channels which reflect a slowing of current activation kinetics by hyperpolarizing pulses, is affected by 2OHOA. We evaluated this phenomenon by applying prepulses to hyperpolarized potentials from − 130 to − 70 mV (in 10 mV increments), followed by a depolarizing pulse to + 60 mV to open the channels. In drug-free conditions, Kv10.1 currents displayed their characteristic behavior, activating more slowly as the prepulse potential become more negative (Fig. 2C). Notably, although 2OHOA accelerated the current activation, kinetics remained dependent on the prepulse potential, i.e., the Cole-More shift was not affected (Fig. 2D).

3.2 2OHOA inhibited cell proliferation in Kv10.1 expressing cells

We used the MTT assay to determine whether proliferation of Kv10.1 expressing cells was altered by 2OHOA. In addition, we used astemizole, a well-known pore blocker of Kv10.1 channels that decreases cell proliferation by its action on this channel [39]. Initially, HEK-293 cells expressing Kv10.1 channels were treated with 2OHOA, astemizole and a combination of both (2OHOA + astemizole) for 24, 48 or 72 h (Fig. 3A–C). 2OHOA at 200 μM and astemizole at 5 μM significantly reduced cell proliferation after 72 h treatment by 26.7% and 43.2%, respectively (Fig. 3C). Interestingly, the combination of both drugs (200 μM 2OHOA + 5 μM astemizole) significantly

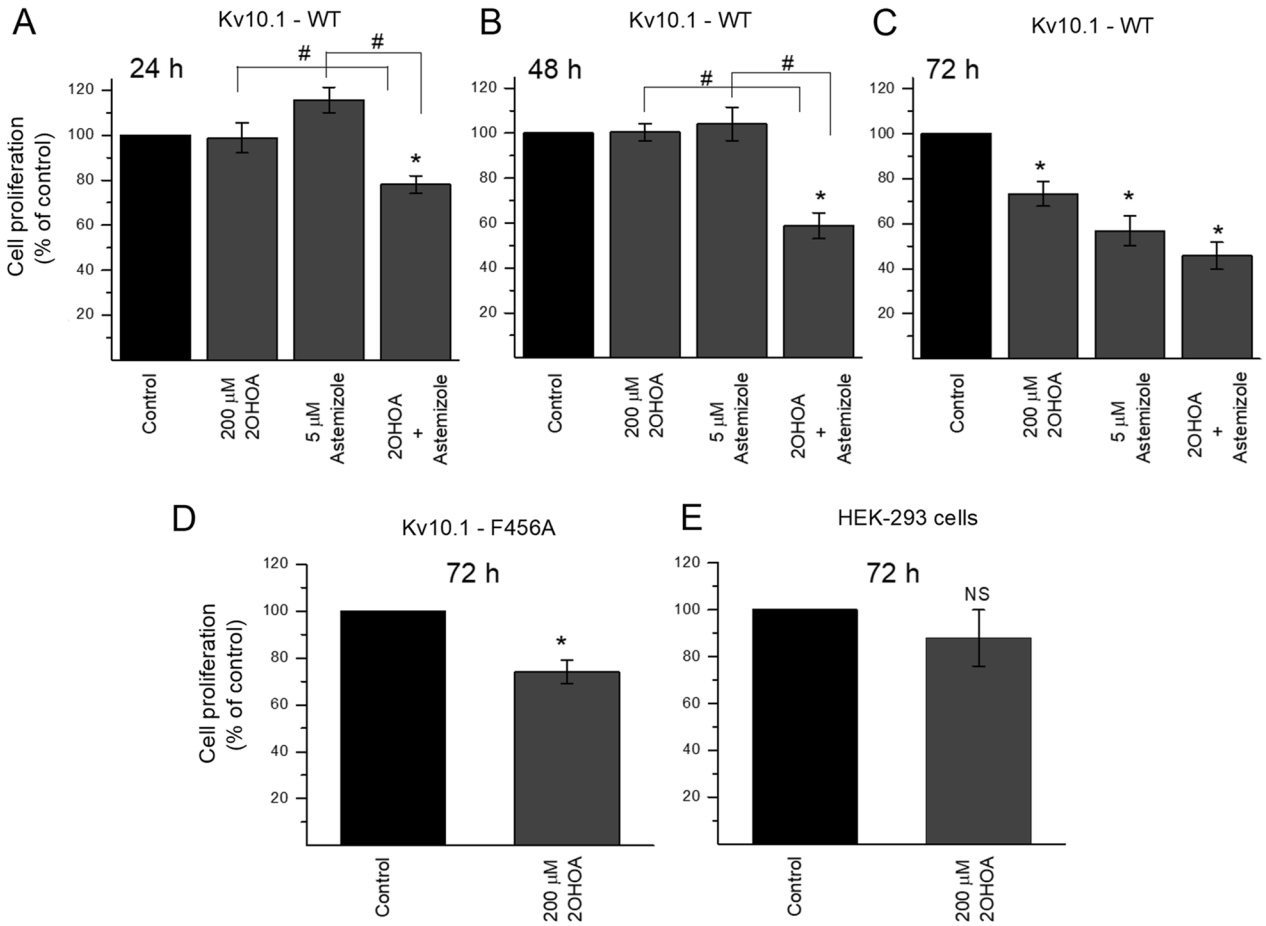

Fig. 3 2OHOA and astemizole synergistically decreases cell proliferation of HEK-293 cells expressing the Kv10.1 channel. **A**–**C** the effect of 200 µM 2OHOA on cell proliferation was determined using the MTT assay at 24 (**A**), 48 (**B**) and 72 h (**C**) on HEK-293 cells expressing the Kv10.1 channel ($n = 6$). **D** Effect of 200 µM 2OHOA on proliferation of HEK-293 cells expressing the non-conducting mutant Kv10.1-F456A ($n = 6$). **E** Effect of 200 µM 2OHOA on proliferation of HEK-293 cells not expressing Kv10.1 channels ($n = 5$). Asterisks indicate significant effects compared with controls ($^{*}p < 0.05$), # indicate a significant effect of the combination 2OHOA + Astemizole compared with both drugs applied alone ($^{\#}p < 0.05$), NS: Not significant. Data are represented as mean ± SEM

reduced cell proliferation by 22%, 41.2% and 54.3% at 24 (Fig. 3A), 48 (Fig. 3B) and 72 h (Fig. 3C), respectively. At 24 and 48 h, the combination of 2OHOA and astemizole induced a higher decrease on cell proliferation than when each drug was applied alone, indicating a synergistic effect (Fig. 3A, B). At 72 h, a small further decrease on cell proliferation was also observed, although was not statistically significant (Fig. 3C).

Additionally, we evaluated the effect of 2OHOA on the proliferation of HEK-293 cells expressing the non-conducting Kv10.1-F456A mutant channel (Fig. 3D). The Kv10.1-F456A channel has been reported to induce cell proliferation even though it is unable to conduct ions [38]. After 72 h treatment, 2OHOA reduced cell proliferation by 26% (Fig. 3D), similar to the effect observed on HEK-293 cells expressing the wild type (WT) Kv10.1 channel. Notably, in HEK-293 cells not expressing Kv10.1 channels, 2OHOA does not significantly inhibited cell proliferation (Fig. 3E).

Finally, we determined the effects of 2OHOA and astemizole on the proliferation of MCF-7 cells, a breast cancer cell line that endogenously expresses Kv10.1 channels.

MCF-7 cells were treated with different concentrations of 2OHOA (12.5, 25, 50, 100, 200, 400 and 1000 µM), 5 µM astemizole, and a combination of 200 µM 2OHOA and 5 µM astemizole for 24, 48 and 72 h (Fig. 4). Proliferation of MCF-7 cells was decreased only at high concentrations of 2OHOA (> 400 µM, Fig. 4). Astemizole (5 µM) decreased MCF-7 cells proliferation at 48 and 72 h by 19.4% and 60.9% respectively (Fig. 4B, C), similar to previous reports [39]. Remarkably, as observed in Kv10.1 expressing HEK-293 cells, the combination of 200 µM 2OHOA and 5 µM astemizole has a synergistic

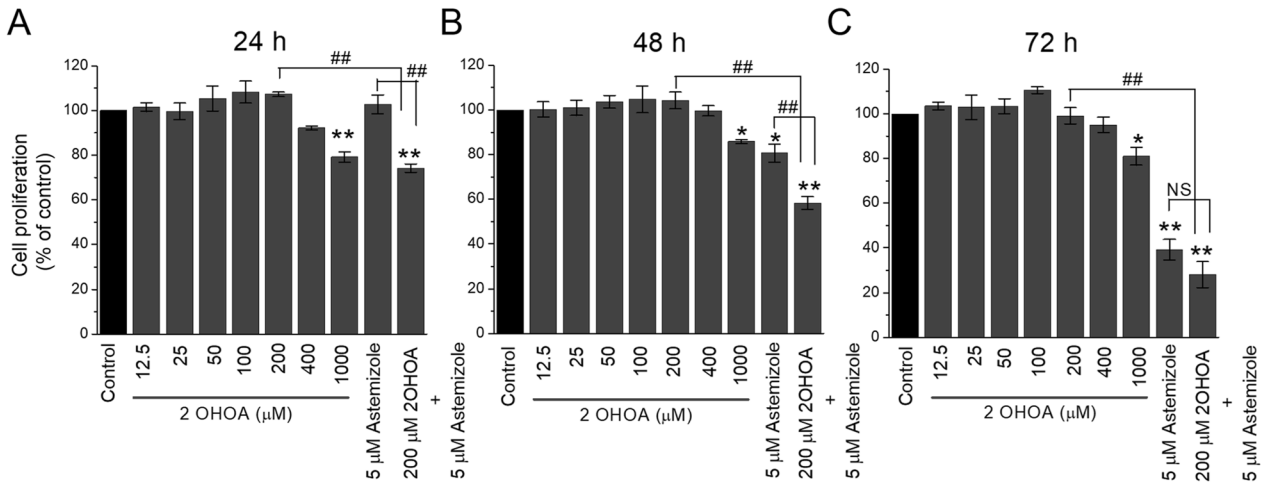

Fig. 4 2OHOA and astemizole synergistically decreases MCF-7 cells proliferation. **A**–**C** proliferation of MCF-7 cells treated with different concentrations of 2OHOA (12.5, 25, 50, 100, 200, 400 and 1000 μM), 5 μM astemizole and the combination 200 μM 2OHOA and 5 μM astemizole determined at 24 (**A**), 48 (**B**) and 72 h (**C**). Asterisks indicate significant effects compared with controls (*$p < 0.05$; **$p < 0.01$), # indicates a significant effect of the combination of 200 μM 2OHOA + 5 μM Astemizole compared to 200 μM 2OHOA or 5 μM Astemizole applied alone ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$), NS: Not significant. Data are represented as mean ± SEM ($n = 5$)

effect in reducing proliferation of MCF-7 cells. Under this condition (2OHOA + astemizole), cell proliferation was reduced by 25.8%, 41.7%, and 71.9% at 24, 48 and 72 h, respectively (Fig. 4A–C).

4 Discussion

2-hydroxyoleic acid (2OHOA) is a synthetic fatty acid with antitumor properties. In contrast with commonly used anticancer drugs, 2OHOA targets the plasma membrane altering its composition and structure, thereby affecting the functioning of membrane proteins and cell signaling [2–9]. In the present study we found another mechanism of action for the antitumoral effects of 2OHOA, resulting from the modulation of Kv10.1 channels.

2OHOA was rationally designed to treat a condition by modulating membrane lipid composition and structure, the so-called membrane lipid therapy (MLT) [40]. Two main mechanisms have been described for 2OHOA action: (1) either free or in phospholipids, 2OHOA regulates the order of membrane lipids, the membrane structure, and the balance between raft and non-raft domains (type-1 MLT); and (2) by its activation of the sphingomyelin synthase, 2OHOA increases the levels of sphingomyelin, changing the membrane lipid composition (type-2 MLT) [7, 10, 12]. These effects induced by 2OHOA on the plasma membrane could affect the functioning of lipid-sensitive proteins, as is the case of Kv10.1, an ion channel that has been shown to be regulated by different membrane lipids [22, 41]. Notably, the perturbation of membrane structure by methyl-beta-cyclodextrin (MBCD) treatment increases the current density of Kv10.1 channels [22], evidencing the channel sensitivity to alterations on the plasma membrane. Similarly, here we found that 2OHOA application increased the current amplitude of Kv10.1 channels (Fig. 1) and modulate its kinetics and voltage dependence (Fig. 2). The known sensitivity of Kv10.1 to plasma membrane alterations lead us to propose that the effects induced by 2OHOA on the functioning of this channel are likely generated by the type-1 MLT mechanism. Although, our experiments cannot completely discard the tipe-2 MLT mechanism.

Studies have shown that Kv10.1 channel blockage decreases cell proliferation [35], reflecting the importance of ion permeation through this channel on this process. However, non-conducting Kv10.1 mutants also support cell proliferation by regulating intracellular signaling pathways, a process attributed to voltage-dependent changes on the protein conformation rather than ion permeation [34, 38]. In this work, astemizole, a Kv10.1 channel blocker, decreased proliferation of HEK-293 (transfected with Kv10.1) and MCF-7 cells (Figs. 3 and 4), which agree with previous reports [39]. On the other hand, 2OHOA did not block ion conduction through Kv10.1 channels but regulated its voltage-dependent gating; the voltage-dependence of activation was shifted toward negative voltages (Fig. 2A) and the kinetics of channel activation was accelerated (Fig. 2B). 2OHOA induced a reduction of cell proliferation on Kv10.1 expressing cells (Figs. 3 and 4), whereas Kv10.1 non-expressing cells (HEK-293 cells transfected only with EGFP) were not affected by 2OHOA (Fig. 3E).

Interestingly, 2OHOA also reduced proliferation of HEK-293 cells expressing the non-conducting Kv10.1-F456A mutant (Fig. 3D). Altogether, these results suggest that 2OHOA could be interfering with a non-conducting function of Kv10.1 channels involved in cell proliferation, possibly, with an intracellular signaling pathway regulated by voltage-dependent changes in the channel conformation.

Remarkably, 2OHOA and astemizole synergistically decreased cell proliferation of Kv10.1 expressing cells (Figs. 3, 4), suggesting that simultaneously targeting the conducting and non-conducting functions of Kv10.1 channels could be a more effective antiproliferative strategy.

In conclusion, 2OHOA decreases cell proliferation by several mechanisms, including the regulation of Kv10.1 channels. Moreover, 2OHOA can act synergistically with drugs that block ion permeation through Kv10.1 channels to reduce more effectively cell proliferation.

Abbreviations

2OHOA	2-Hydroxyoleic acid
PKC	Protein kinase C
DMSO	Dimethyl sulfoxide
EGFP	Enhanced green fluorescence protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide
MBCD	Methyl-beta-cyclodextrin

Acknowledgements
We thank Xóchitl Ordaz Ruiz for technical assistance.

Author contributions
RM-Z and AAR-M designed the study. RM-Z conducted the experiments and collected the data. RM-Z and AAR-M analyzed the data and drafted the manuscript. Both authors read and approved the final manuscript.

Funding
This work was supported by the SEP-CONACYT grant CB-284443 (to AAR-M), and Universidad Autónoma de San Luis Potosí C18-FRC-08-03.03 (to AAR-M). RM-Z were supported by a Graduate Student Fellowship (#461406) from CONACYT, México.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 21 July 2022 Accepted: 29 January 2023
Published online: 03 February 2023

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