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Pre-clinical evidence that salinomycin is active against retinoblastoma via inducing mitochondrial dysfunction, oxidative damage and AMPK activation

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Abstract
The poor outcomes in retinoblastoma necessitate new treatments. Salinomycin is an attractive candidate, and has demonstrated selective anti-cancer properties in different cancer types. This work addressed the efficacy of salinomycin in retinoblastoma models and probe the associated mechanisms. Cellular functional assays were conducted to determine the effects salinomycin in vitro. Xenograft retinoblastoma mouse model was established to investigate the efficacy of salinomycin in vivo. Biochemical assays were conducted to analyze the mechanism of salinomycin's action focusing on mitochondrial functions, energy reduction-related signaling pathways. Salinomycin has positive effects towards retinoblastoma cells regardless of heterogeneity through suppressing growth and inducing apoptosis. Salinomycin also specifically inhibits cells displaying stemness and highly invasive phenotypes. Using retinoblastoma xenograft mouse model, we show that salinomycin at non-toxic dose effectively inhibits growth and induces apoptosis. Mechanistic studies show that salinomycin inhibits mitochondrial respiration via specifically suppressing complex I and II activities, reduces mitochondrial membrane potential and decreases energy reduction, followed by induction of oxidative stress and damage, AMPK activation and mTOR inhibition. Our study highlights that adding salinomycin to the existing treatment armamentarium for retinoblastoma is beneficial.

Keywords Salinomycin · Retinoblastoma · Mitochondria respiration · AMPK/mTOR

Introduction

Retinoblastoma is one of many different malignant intraocular diseases affecting children. This is the most common condition in infants that has very poor prognosis (Kivela 2009). Standard of care (SOC) options include enucleation, laser photocoagulation, cryotherapy, transpupillary thermotherapy and chemotherapy (Cassoux et al. 2017). Although the loss of retinoblastoma 1 (RB1) is a key factor, which has a critical role in retinoblastoma initialization and progression, there exists other genetic and epigenetic modifications that affects RB1. This may in turn produce different clinical outcomes (Stenfelt et al. 2017). Furthermore, a significant fraction of aggressive tumor results from MYCN amplification independent of RB1 (Theriault et al. 2014). Transcriptomics analysis together with known genetic mutations show that retinoblastoma is extensively heterogeneous (Winter et al. 2020). Therefore, targeting common rather than differential molecular "driver" mutations may represent a promising therapeutic strategy for the treatment of retinoblastoma.

Disrupting the metabolic pathways to aid in cancer treatment has garnered attention with discovery of aerobic glycolysis (Luengo et al. 2017). Further work reveal that metabolic activities in tumor cells are particularly dependent on oxidative phosphorylation (mitochondrial respiration) to generate ATP for energy production (Zheng 2012). This is because mitochondrial metabolic properties differ significantly among cancerous and normal cells. Many cancers, including leukemia, breast cancer and retinoblastoma, exhibit increased mitochondrial biogenesis as well as oxygen consumption (Lagadinou et al. 2013; Skrtic et al. 2011; Yu et al. 2016). Of note, stem-like tumor initiating cells are rely on mitochondrial metabolism (Lagadinou et al. 2013; Luca et al. 2015). Salinomycin, an antibiotic, is identified

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to be an attractive candidate for cancer chemoprevention and therapy with board mechanisms of action against cancer (Tung et al. 2017; Ko et al. 2016; Markowska et al. 2019). Salinomycin has been shown to have negative impact on mitochondrial bioenergetic function (Manago et al. 2015; Mitani et al. 1976). We hypothesized that salinomycin is a promising drug for retinoblastoma treatment.

In the current study, we examined the effects of salinomycin on retinoblastoma using both in vitro and in vivo retinoblastoma models. We systematically analysed mechanisms of the action of salinomycin. We demonstrate that salinomycin inhibits retinoblastoma growth, survival and colony formation, and furthermore that these inhibitory properties of salinomycin in retinoblastoma are contributed to inhibition of mitochondrial respiration and energy production, induction of oxidative stress and damage, activation of AMPK and inhibition of mTOR.

Materials and methods

Cell culture

RB 383, WERI-Rb-1 and RB116 cell lines (the Cell Bank of Type Culture Collection of Chinese Academy of Sciences) were grown as suspension cultures using DMEM media containing 10% FBS (Hyclone), 50 µM β-mercaptoethanoal (Sigma), 10 µg/ml insulin, 1% penicillin–streptomycin and 4 mM L-glutamine and. All related culturing reagents were procured from Invitrogen unless otherwise indicated.

Mitochondrial DNA-deficient cell line generation (ρ^0 cells)

The RB 383 ρ^0 cell line was derived using processes detailed by Hashiguchi and Zhang (Hashiguchi and Zhang-Akiyama 2009). Culturing of RB 383 cells were performed and further selected using 2 µg/ml ethidium bromide (EtBr). Uridine at 50 µg/ml and sodium pyruvate (Sigma) at 2 mM were added to the selection medium. Cells were cultured in the above media for a duration of 8 weeks. Cells were thereafter cultured in normal culture media in the absence of EtBr.

Bromodeoxyuridine (BrdU) assay

A total of 10^4 cells/well were incubated with dimethyl Sulfoxide (DMSO; Sigma) or salinomycin (MedChemExpress) at different concentrations. After drug treatment for 3 days, proliferative activities were assessed by BrdU Cell Proliferation Colorimetri Assay kit (Abcam, Catalog No. ab126556) and quantified on a microplate reader based on the absorbance at 450 nm.

Flow cytometry

To determine cell apoptosis, cells at 10^5/well were treated with drugs. After 3 days, these cells were counterstained with Annexin V-FITC and 7-AAD (Abcam, Catalog No. ab214663). Each sample was incubated with 10 µl of Annexin V-FITC and 5 µl of 7-AAD for 15 min prior to being analysed. For determining the cell intracellular reactive oxygen species (ROS) and mitochondrial membrane potential, cells at 10^5/well were treated with DMSO or salinomycin for 24 h. These cells were counterstained with 10 µM CM-H2DCFDA (Life Technologies, Catalog No. C6827) at 37 °C for 30 min for ROS and 10 µg/ml 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolylcarbocyanine iodide (JC-1, Invitrogen, Catalog No. T3168) at 37 °C for 20 min for mitochondrial membrane potential, respectively. The percentage of Annexin V, intensity of CM-H2DCFDA and JC-1 dye were determined by flow cytometry analysis on Beckman Coulter FC500.

Anchorage-independent growth in soft agar

Anchorage-independent growth assay was conducted using method previously described (Gao et al. 2005). In brief, 1000 tumor cells-salinomycin mixture was seeded in 12-well plate. The top layer consists of 0.3% Bacto agar and the bottom layer consists of 0.7% Bacto agar. Cell culture media was subsequently added onto the top layer. Culture media in the wells were removed and fresh solutions were added twice each week. Associated cell colonies were counterstained with crystal violet and colony numbers were counted after 2 weeks.

Mito stress and glycolytic stress test assays

10^4 cells/well were seeded in each XF24 cell culture plate (Seahorse Bioscience, Catalog No. 101848-400) and treated with drugs for 24 h. Media were replaced with the XF assay medium. For pH stabilization, the plate was then placed at 37 °C incubation chamber in a CO_2-free environment. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) was then measured using a Seahorse XF24 extracellular flux analyser (Seahorse Bioscience). Respiratory capacity was examined after injection of 1 µg/ml oligomycin, 0.4 µM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 2.5 µM antimycin A and rotenone. Optimal FCCP concentration was determined based on its efficacy in stimulating OCR level. Glucose (10 nM), oligomycin (1 µM) and 2deoxy-D-glucose (2-DG, 50 mM) were injected sequentially for

ECAR measurement. OCR and ECAR values were normalized to protein mass.

Measurement of mitochondrial complex I, II, IV and V activities

Cells at 10^5/well were treated with DMSO or salinomycin for 24 h. In vitro mitochondrial complex I (Abcam, Catalog No. ab109721), II (Abcam, Catolog No. ab109908), III (Sigma, Catalog No. MAK360), IV (Abcam, Catalog No. ab109909) and V (Abcam, Catalog No. ab109907) activities were assessed using total cell lysates and were measured using kits as per the manufacturer's instructions. Protein lysates of each sample were adjusted to the concentration of 5 mg/ml prior to being added to microplates coated with antibodies against each electron transport chain complex. The complex activities were determined calorimetrically on microplate reader.

Western blot (WB) anlaysis

Cells at 10^6/well were incubated with DMSO or salinomycin for 24 h. Samples of cell lysate were prepared, resolved using denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by WB using the standard protocol (Mahmood and Yang 2012). Antibodies against p-AMPK (Catalog No. #2531), AMPK (Catalog No. 2532), p-ACC (Catalog No. 11818), ACC (Catalog No. 3662), p-mTOR (Catalog No. 2971), mTOR (Catalog No. 2983), p-rS6 (Catalog No. 2211), rS6 (Catalog No. 2217), p-4EBP1 (Catalog No. 2885), 4EBP1 (Catalog No. 9644) and β-actin (Catalog No. 4970) were obtained from Cell Signaling.

Measurement of cellular ATP

10^5 cells/well were seeded in a 24-well and exposed with drug for a duration of 24 h. ATP levels were quantified using ATPlite Luminiescent Assay kit (Perkin Elmer, Catalog No. 6016941) as per manufacturer's instructions. ATP values were normalized to protein mass.

Measurement of oxidative DNA damage assays

10^5 cells/well were seeded in a 24-well and exposed with drug for a duration of 24 h. Oxidative DNA damage was assessed by quantifying 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels using the OxiSelect Oxidative DNA Damage ELISA kit (Cell Biolabs, Catalog No. STA-320-T) as per manufacture's instructions.

Retinoblastoma xenograft in SCID mouse

The xenograft experiments were approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology. RB 383 cells suspended in 100 µl 50%/50% PBS/Matrigel were subcutaneously injected into flank of 6-week-old NOD/SCID mice (Hunan SJA Laboratory Animal Co., Ltd). When tumor reached ~200 mm^3, mice were randomized into two groups and administrated for vehicle (20%/80% DMSO/PBS) and salinomycin (2 mg/kg/day) by intraperitoneal injection (n = 10 per group). After 2 weeks of drug treatment, mice were euthanatized using CO_2 inhalation. Tumours were excised and sectioned for immunostaining. Sections were fixed with 4% paraformaldehyde (Sigma). The sections were subsequently exposed to Ki67 and active caspase 3 (Cell Signalling) primary antibodies, and counterstained using secondary antibody conjugated with horseradish peroxidase-DAB (3,3′-diaminobenzidine). Cell nuclei were stained with hematoxylin.

Results

Salinomycin effectively inhibits biological functions of retinoblastoma cells

Using multiple retinoblastoma cell lines (RB383, RB116 and WERI-Rb1) that represent different cell origins and covers a broad range of genetic profiles (Busch et al. 2015), we first studied the effects of salinomycin on retinoblastoma cell growth, associated forming of colonies and survival. We showed that salinomycin at 5, 10 and 15 µM exhibited antiproliferative effects against three retinoblastoma cell lines dose-dependently ($p < 0.05$; Fig. 1A). The IC_{50} for salinomycin as determined by the BrdU assay is comparable among retinoblastoma cell lines (Supplementary Table 1), suggesting that salinomycin is effective against retinoblastoma regardless of cell heterogeneity.

Retinoblastoma cell lines selected in our study present with certain stem cell (eg, Nanog and Oct4) and retinal progenitor markers (eg, PAX6) (Hu et al. 2012; Bejjani et al. 2012). This subpopulation with stemness and highly invasive phenotypes can be examined by anchorage-independent colony formation assay (Gao et al. 2005). We showed salinomycin potently inhibited the anchorage-independent growth of retinoblastoma cells ($p < 0.05$; Fig. 1B). Of note, we observed that salinomycin at the same concentration (eg, 10 µM) decreased growth and colony formation by ~60% and ~90%, respectively (Fig. 1A, B), suggesting that this subpopulation is more sensitive to salinomycin than the whole cell population.

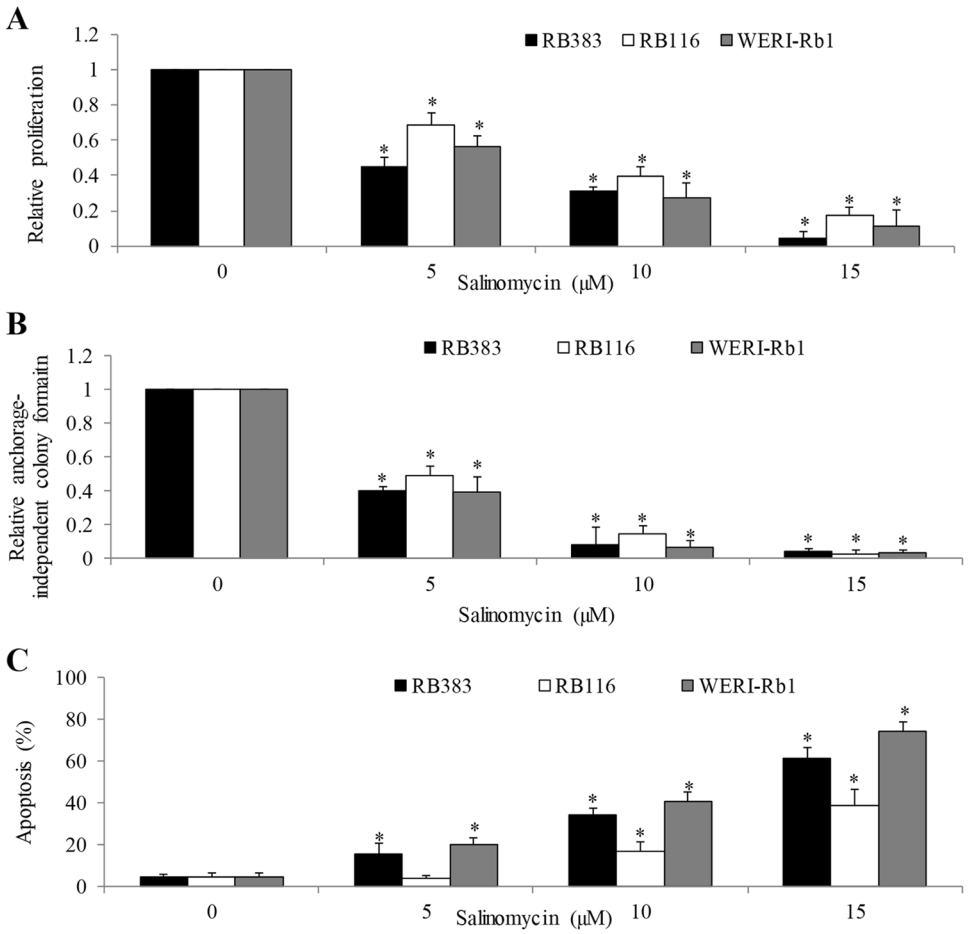

Fig. 1 Salinomycin is active against retinoblastoma cells in vitro. Salinomycin at 5, 10 and 15 μM inhibits proliferation (**A**), suppresses anchorage-independent colony formation (**B**) and induces apoptosis (**C**) in RB 383, RB116 and WERI-Rb1 cell lines. The results were obtained from at least three independent experiments with triplicate. The results were presented as average ± SD and were shown as relative to 0 μM salinomycin. *$p < 0.05$, compared to 0 μM salinomycin

Apoptosis assay was performed by flow cytometry of Annexin V/7-AAD. The results were shown in Fig. 1C and supplementary Fig. 1. Pro-apoptotic effects of salinomycin were demonstrated in all cell lines at high concentration.

Salinomycin impairs mitochondrial function, induces oxidative damage, activates AMPK and inhibits mTOR in retinoblastoma cells

We analysed the effects of salinomycin on mitochondrial bioenergetic performance in RB 383 cells. We measured the rate of oxygen consumption by the cells, an indicator of mitochondrial respiration, under basal and electron transport chain (ETC) accelerator-stimulated conditions. We observed a reduced basal respiration rates and decreased ATP coupler response in salinomycin-treated cells (Fig. 2A–C). Trifluorocarbonylcyanide phenylhydrazone (FCCP), an ETC accelerator, is often used to assess the maximal respiratory capacity of cells. We found that salinomycin-treated cells were not responsive to FCCP stimulation and there was a dose-dependent reduction of ETC accelerator response (Fig. 2A, D), indicative of reduction in the maximal respiratory capacity and potential rate for ATP production. We performed glycolytic stress test assay in cells after salinomycin treatment. We found that there was no significant difference in the basal and maximal glycolytic capacity between control and salinomycin-treated cells (Fig. 2E), indicating that salinomycin does not compromise glycolysis. We next measured mitochondrial complexes activities in cells after salinomycin treatment. We found that salinomycin significantly decreased activities of complex I and II without affecting activities of complex III, IV and V (Fig. 3), suggesting that salinomycin inhibits mitochondrial respiration through suppressing mitochondrial complex I and II in retinoblastoma cells.

Mitochondrial membrane potential was quantified using flow cytometry of JC-1 dye. We showed that salinomycin significantly decreased mitochondrial membrane potential in retinoblastoma cells (Fig. 4A and supplementary Fig. 2). As expected, ATP levels were observed in cells after salinomycin treatment (Fig. 4B). To understand the sequences of mitochondrial membrane potential disruption, ATP reduction and apoptosis, we performed time course analysis. Reduction of membrane potential and ATP levels were initialized after 0.5 h and 6 h whereas apoptosis was initialized after 24 h addition of salinomycin (Fig. 4C–E and supplementary Fig. 3), suggesting that salinomycin treatment

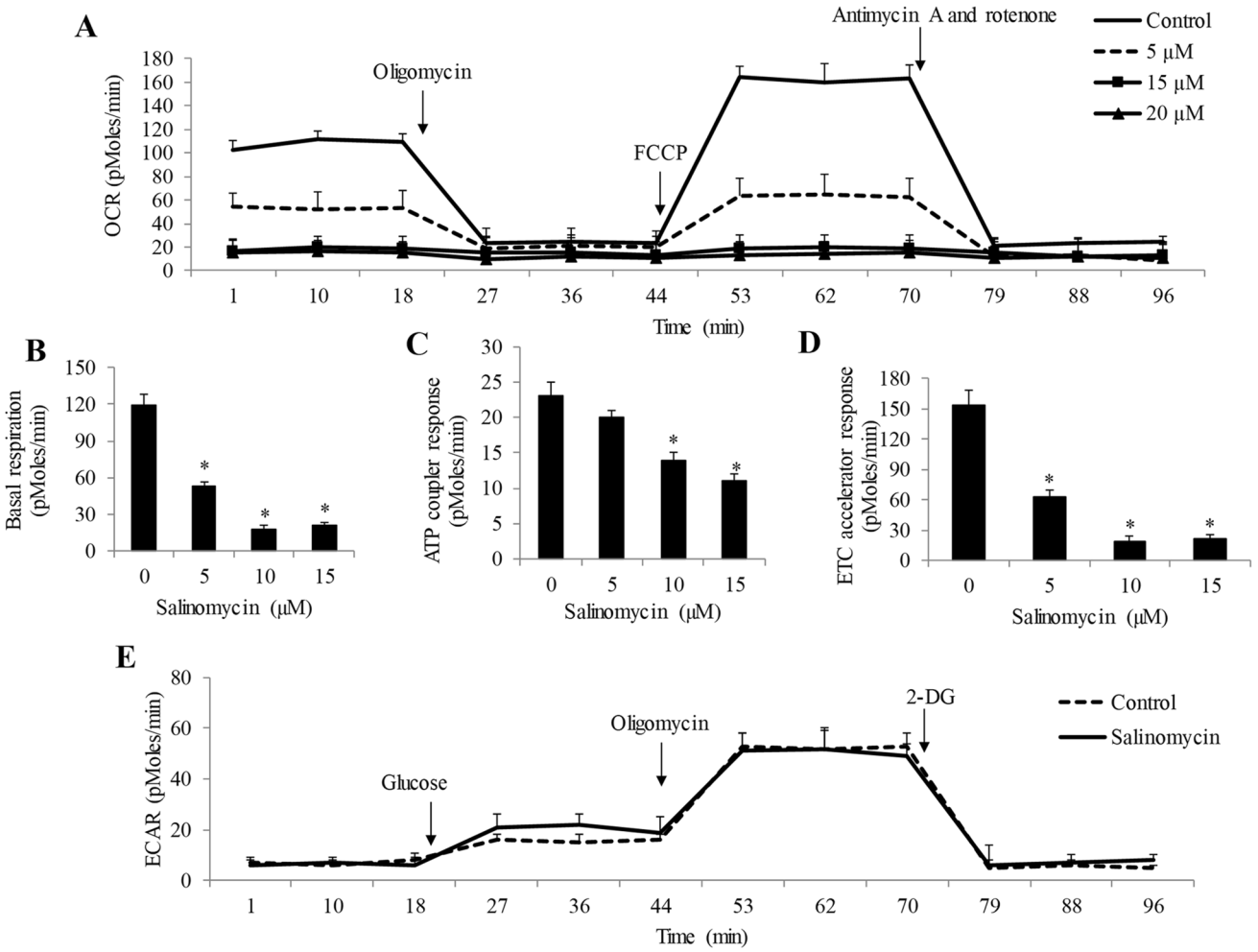

Fig. 2 Salinomycin inhibits mitochondrial respiration in retinoblastoma cells. **A** OCR profile of RB 383 cells after salinomycin treatment. OCRs were measured under basal condition first, followed by that after sequential addition of oligomycin, FCCP, and antimycin A and rotenone. Salinomycin significantly decreases basal respiration (**B**), ATP coupler response (**C**) and ETC accelerator response (**D**) in RB 383 cells. The basal respiration value is the average of measurements 1–3 before oligomycin injection. ATP coupler response value is the average of measurements 4–6 after oligomycin injection. ETC accelerator response value is the average of measurements 7–9 after FCCP injection. **D** ECAR profile of RB 383 cells after salinomycin treatment. ECAR tracings were obtained at baseline, and then after glucose injection, addition of oligomycin and injection of 2-DG. All the OCR and ECAR values presented were normalized to protein mass. The results were presented as average ± SD. $*p < 0.05$, compared to 0 µM salinomycin

leads to membrane potential disruption, followed by ATP reduction and apoptosis.

As a consequence of mitochondrial dysfunction and energy crisis, we observed increased intracellular DCF and 8-OHdG (an oxidized DNA byproduct) levels in salinomycin-treated retinoblastoma cells (Fig. 5A, B and supplementary Fig. 4), indicating that salinomycin induces oxidative stress and damage. We noted that the inhibitory effects of salinomycin on mitochondria respiration reached saturation at 10 µM. Although there is difference on the average levels of ROS and 8-DG between 10 and 15 µM, the difference is not statistically significant ($p > 0.05$). This suggests that mitochondrial respiration inhibition is likely the main cause of oxidative stress and damage in salinomycin-treated cells.

AMP-dependent kinase (AMPK) activity correlates to ATP levels and negatively regulates the mammalian target of rapamycin (mTOR) pathway (Cork et al. 2018). To investigate the consequence of mitochondrial dysfunction and energy reduction by salinomycin, we performed immunoblotting analysis of essential molecules associated in AMPK activity and mTOR signaling pathway. Indeed, treatment of RB383 cells with salinomycin presented elevations in phosphorylated AMPK at T127 (Fig. 5C). Consistent with increased AMPK activity, we observed increased inhibitory phosphorylation of acetycoA carboxylase (ACC) at Ser79, an AMPK phosphorylation site. In addition, decreased phosphorylation of mTOR was observed in RB383 cells after 24 h treatment

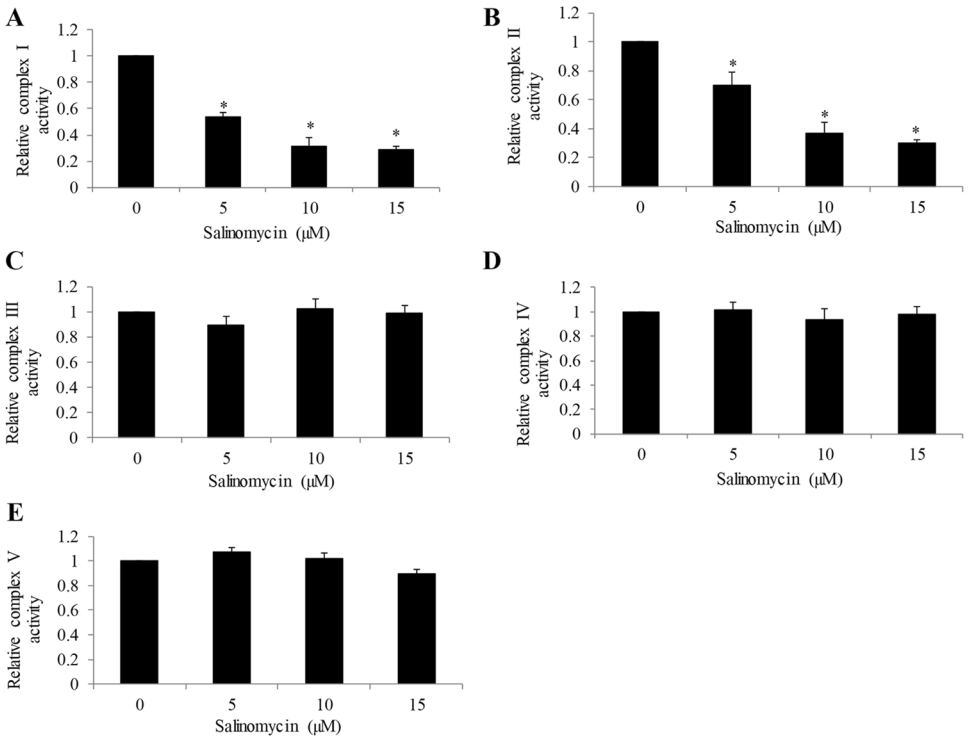

Fig. 3 Salinomycin inhibits mitochondrial complex I and II activities in retinoblastoma cells. Salinomycin significant decreases mitochondrial complex I (**A**), II (**B**) but not III (**C**), IV (**D**) or V (**E**) activities in RB 383 cells. The results were presented as average ± SD and were shown as relative to 0 μM salinomycin. *$p < 0.05$, compared to 0 μM salinomycin

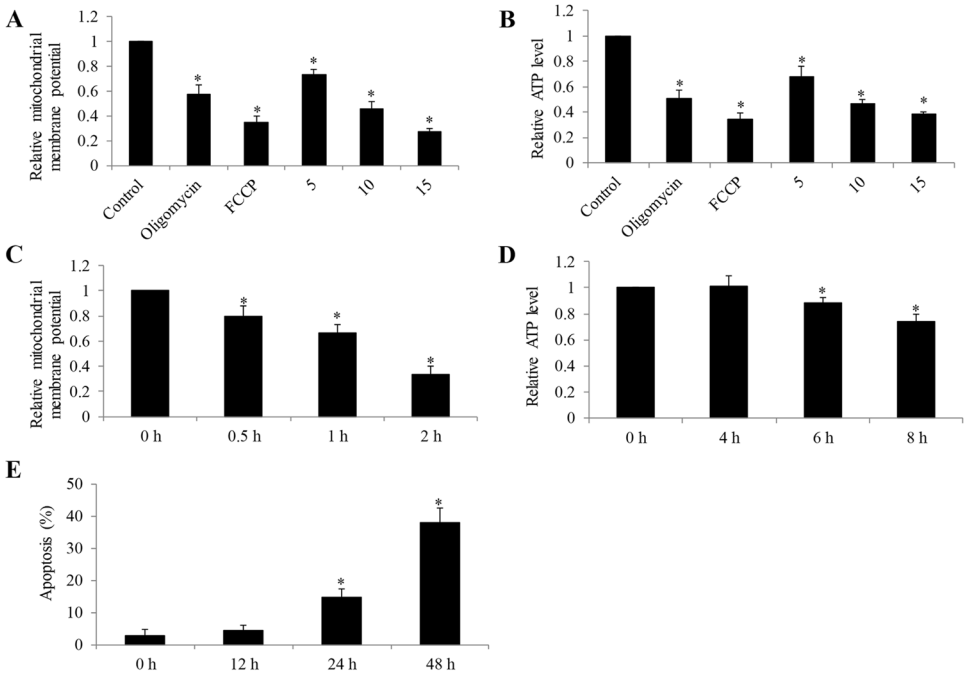

Fig. 4 Salinomycin decreases mitochondrial membrane potential and ATP levels in retinoblastoma cells. **A** Salinomycin at 5, 10 and 15 μM reduces mitochondrial membrane potential in RB 383 cells. **B** Salinomycin decreases ATP levels in RB 383 cells. Membrane potential and ATP level were measured after 24-h salinomycin treatment. 1 ug/ml oligomycin and 1 uM FCCP were used. Time course analysis of mitochondrial membrane potential (**C**), ATP level (**D**) and apoptosis (**E**) in RB 383 cells. The results were presented as average ± SD and were shown as relative to 0 μM salinomycin. *$p < 0.05$, compared to 0 μM salinomycin

of salinomycin, and so do its downstream effectors rS6 and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1). Taken together, our results demonstrate that salinomycin treatment impairs mitochondrial functions, induces oxidative damage, activates AMPK and inhibits mTOR in retinoblastoma cells.

Salinomycin is ineffective in mitochondrial respiration-deficient retinoblastoma ρ0 cells

To confirm that mitochondrial respiration plays an important role in the action of salinomycin in retinoblastoma, we generated RB383 ρ0 cells lacking mitochondrial DNA and thus

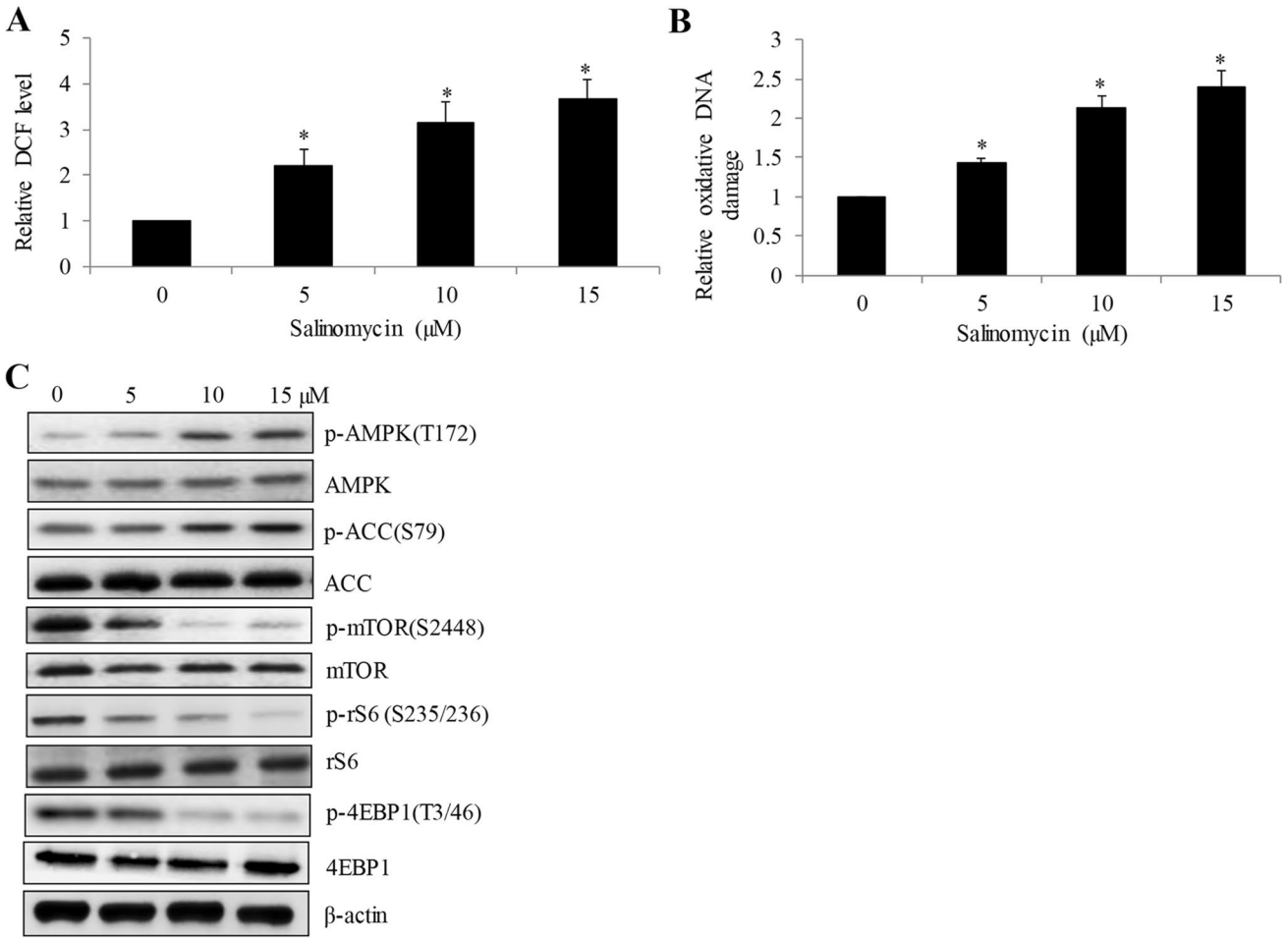

Fig. 5 Salinomycin induces oxidative damage, activates AMPK and inhibits mTOR in retinoblastoma cells. Salinomycin significantly increases intracellular DCF levels (**A**) and increases 8-OHdG (**B**) in RB 383 cells. **C** Representative western blot image of RB 383 cells treated with salinomycin for 24 h. The results were obtained from at least three independent experiments with triplicate. The results were presented as average ± SD and were shown as relative to 0 μM salinomycin. *$p < 0.05$, compared to 0 μM salinomycin

incapable of mitochondrial respiration processes (Chandel and Schumacker 1999). We have attempted to generate ρ0 cells from RB383, RB116 and WERI-Rb1 cell lines but it was only successful on RB383 cells. RB116 and WERI-Rb1 did not survive after mitochondrial respiration depletion. This is consistent with Yasuo et al.'s findings that generation of ρ0 cells were failed in most of tested cell lines (Harada et al. 2012). We validated RB383 ρ0 cells by showing that ρ0 cells display a minimal level of baseline OCR and are non-responsive to FCCP stimulation (Fig. 6A). There was no significant difference on mitochondrial membrane potential, intracellular DCF and 8-OHdG levels between ρ0 and parental cells (Fig. 6B–D). As expected, we observed a remarkable ATP reduction in ρ0 cells compared to parental cells (Fig. 6E). Of note, salinomycin was ineffective in reducing mitochondrial membrane potential, increasing DCF and 8-OHdG levels, and decreasing ATP production in ρ0 cells (Fig. 6B–E). These results suggest that mitochondrial membrane potential disruption, ATP reduction and oxidative stress/damage are the consequence of mitochondrial respiration inhibition by salinomycin. In addition, these ρ0 cells were resistant to salinomycin-induced apoptosis (Fig. 6F). The inhibitory effect of salinomycin on the proliferation of RB 383 ρ0 cells was not determined due to inadequate proliferation of ρ0 cells (Fig. 6G).

Salinomycin inhibits growth and induces apoptosis of retinoblastoma in vivo

Tumor xenograft model in mice is widely applied to understand the anticancer activities of novel compounds. Further examination of the in vivo efficacy of salinomycin uses SCID mice to establish the correlation with in vitro experiments. We established retinoblastoma xenograft mouse model by implanting RB383 cells into the flank of SCID mice. Salinomycin treatment was initialized

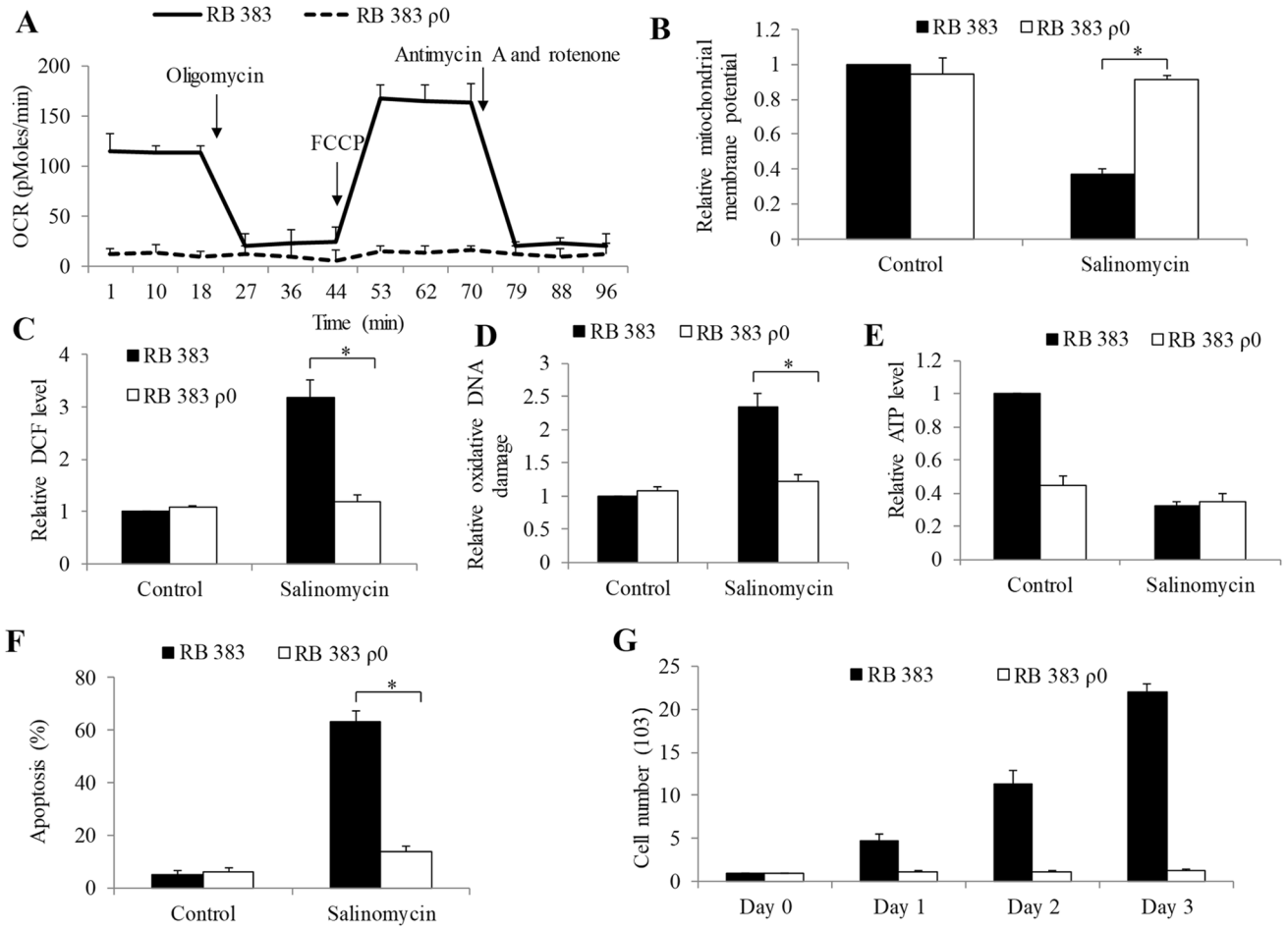

Fig. 6 ρ0 retinoblastoma cells are resistant to salinomycin treatment. a RB 383 ρ0 cells displays minimal basal and maximal OCR. Oligomycin, FCCP and antimycin A and rotenone combination were added as indicated time points. Salinomycin is ineffective in decreasing membrane potential (**B**), increasing DCF (**C**) and 8-OHdG (**D**) levels, decreasing ATP levels (**E**). **F** Salinomycin is ineffective in inducing apoptosis in RB 383 ρ0 cells. Salinomycin at 15 μM was used. **G** Minimal growth rate in RB 383 ρ0 cells. The results were obtained from at least three independent experiments with triplicate. The results were presented as average ± SD. $*p < 0.05$, compared to control

after the development of palpable tumors. After 2 weeks treatment, we did not observe a significant reduction on body weight and abnormal appearance in mice treated with salinomycin compared to control (data not shown), suggesting that salinomycin at 2 mg/kg per day via intraperitoneal administration is not toxic to mice. In contrast, we observed the significant reduction of tumor size and weight in mice treated with salinomycin (Fig. 7A, B). In addition, salinomycin delayed tumor growth starting at 2 days post treatment.

We next performed immunohistochemistry staining of Ki67 and active caspase 3 on tumor sections to determine if the reduced tumor growth was the result of declines in cell proliferation, increased apoptosis or both. The results showed ~ 60% decrease in tumor cell proliferation and ~ 10 times increase in tumor cell apoptosis in mice receiving salinomycin, as compared to the control (Fig. 7C–E).

Discussion

Higher levels of mitochondrial biogenesis is associated with retinoblastoma, and this includes elevated levels of mitochondrial respiration, membrane potential and mass when compared with normal retina cells (Ke et al. 2018). The unique dependency for retinoblastoma on mitochondrial metabolism to maintain cell survival and growth can be exploited therapeutically. In the current work, we demonstrate that mitochondrial functions can be impaired by salinomycin, leading to retinoblastoma models with decreased growth and increased apoptosis. Although salinomycin is a widely used antibiotic in veterinary medicine, substantial pre-clinical evidence has highlighted salinomycin as a potential anti-cancer agent (Markowska et al. 2019).

In agreement with numerous reports on inhibitory effects of salinomycin on colorectal and lung cancer, head and neck

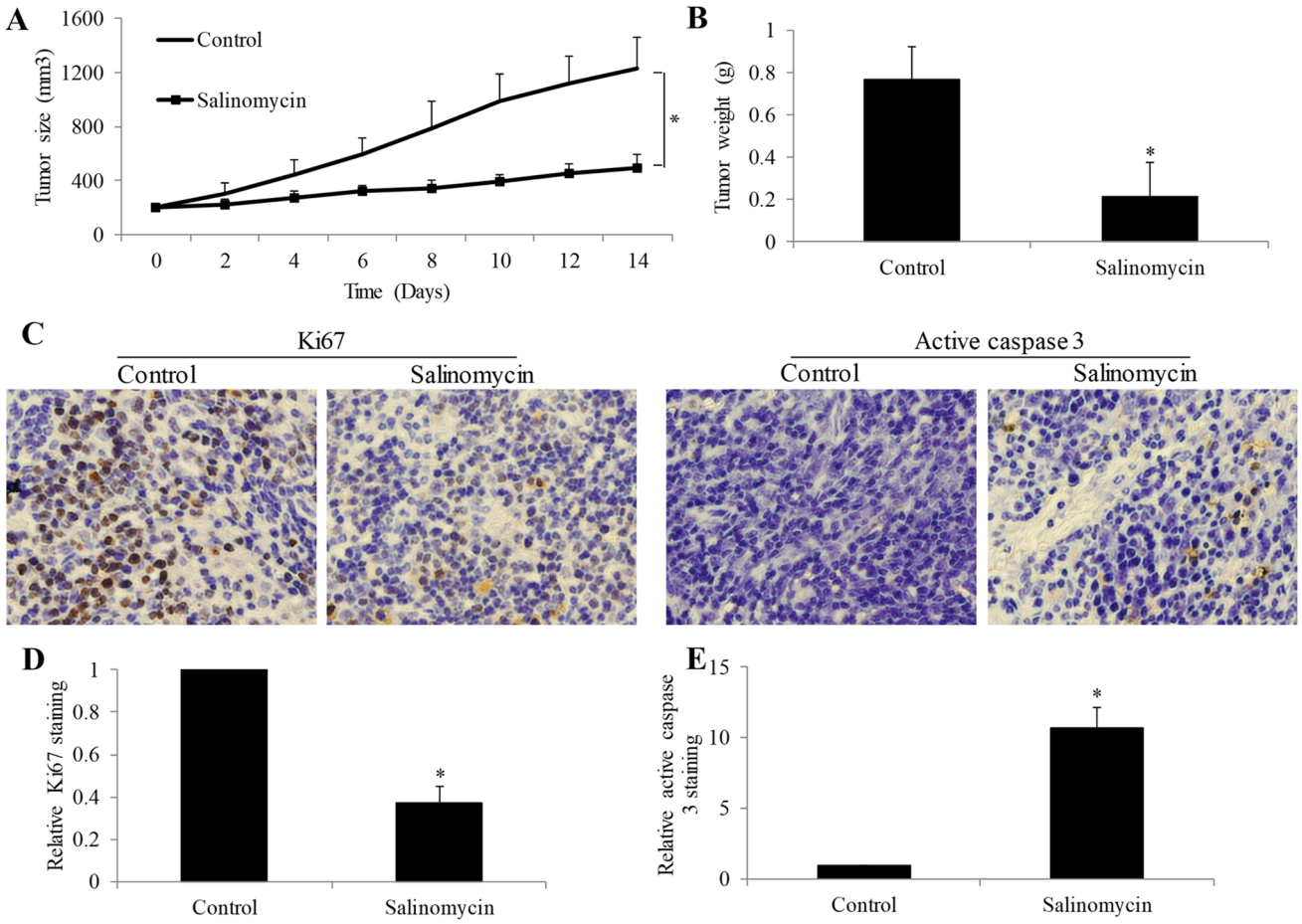

Fig. 7 Salinomycin inhibits retinoblastoma growth in mice. **A** Salinomycin significantly inhibits retinoblastoma growth in xenograft mouse model (n = 10 mice in each group). **B** Salinomycin significantly decreases retinoblastoma weight. **C** Representative photos of immunostaining of Ki67 and active caspase 3. Salinomycin significantly decreases Ki67 staining levels (**D**) and increase active caspase 3 staining levels (**E**) in retinoblastoma. Intraperitoneal salinomycin at 2 mg/kg once per day were given to the mice. Cell proliferation and apoptosis were shown by the average number of proliferative and apoptotic cells per microscopic filed. *$p < 0.05$, compared to control

squamous cell carcinoma (Hochmair et al. 2020; Klose et al. 2019; Gehrke et al. 2018), our findings show that salinomycin displays pro-apoptotic and anti-proliferative activities in a panel of retinoblastoma cell lines that represent a board range of cellular origin and genetic profiles. In addition, our anchorage-independent colony formation analysis indicates that salinomycin preferentially inhibits retinoblastoma subpopulations with stemness and highly invasive phenotypes. This is consistent with the previous work that salinomycin targets human cancer stem cells via reducing stemness and motility, and inducing death (Mai et al. 2017; Sun et al. 2017; Lee et al. 2017). We further validated our findings using retinoblastoma xenograft mouse model and found that the in vitro findings were reproducible in in vivo. We show that salinomycin at non-toxic dose effectively reduces retinoblastoma size and weight via suppressing tumor cell growth and survival. Our findings add retinoblastoma to the long list of salinomycin-targeted cancers and demonstrate the therapeutic window of salinomycin in retinoblastoma. Increasing evidence has shown the synergistic effect of salinomycin with anti-cancer agents and the ability of salinomycin in overcoming therapy-resistant cancer cells (Zhou et al. 2019; Skeberdyte et al. 2018; Manmuan et al. 2017; Dewangan et al. 2017; Gruber et al. 2020). To further characterize the potential application of salinomycin in retinoblastoma, it is worthy of investigating the combinatory effect of salinomycin with chemotherapy on retinoblastoma models.

The underlying mechanisms of salinomycin's anti-cancer action are complex, including inhibition of amplified breast 1 (AIB1) and focal adhesion kinase (FAK), induction of NF-kB degradation, suppression of extracellular signal-regulated kinase (ERK1/2) (Sun et al. 2017; Manmuan et al. 2017; Tyagi and Patro 2019). Although Manago et al. have demonstrated that salinomycin exerts its effect at the mitochondrial level that subsequently changes the bioenergetic performance in B cell chronic lymphocytic leukemia

(Manago et al. 2015), our study further extends the previous findings by showing that besides mitochondrial dysfunction, ATP reduction, oxidative stress and damage, AMPK activation and mTOR inhibition contribute to salinomycin's action in retinoblastoma cells. Salinomycin is ineffective in decreasing ATP levels, increasing oxidative damage and inducing apoptosis in mitochondrial respiration-deficient cells. As a consequence of mitochondrial dysfunction and energy crisis by salinomycin, AMPK is activated and mTOR signaling pathway is inhibited, leading to inhibition of cellular functional activities. Together with the mechanism of action of salinomycin, our observation that the effective dose of salinomycin in retinoblastoma xenograft mouse model is not toxic to mice correlates well with the previous finding that retinoblastoma cells are more dependent on mitochondrial respiration than normal cells (Ke et al. 2018).

In summary, we report that salinomycin is active against retinoblastoma. We show that the activity of salinomycin in retinoblastoma is dependent on its ability to inhibit mitochondrial respiration. Our findings highlight that targeting mitochondrial respiration is an effective therapeutic strategy for the treatment of retinoblastoma.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10863-021-09915-2.

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Authors' contribution JL and YM designed the experiments; JL performed the experiments and wrote the manuscript; All authors interpreted the data, supervised the project and revised the manuscript.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Research involving human participants and/or animals This work was approved by the institutional review board of Tongji Medical College, Huazhong University of Science and Technology on 26 August 2018 (Approval No. 2018-C05). This study was conducted in accordance with the Declaration of Helsinki.

Conflict of interest All authors declare no conflict of interest.

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