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Inhibiting prenylation augments chemotherapy efficacy in renal cell carcinoma through dual inhibition on mitochondrial respiration and glycolysis

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ABSTRACT

Prenylation is a posttranslational lipid modification required for the proper functions of a number of proteins involved in cell regulation. Here, we show that prenylation inhibition is important for renal cell carcinoma (RCC) growth, survival and response to chemotherapy, and its underlying mechanism may be contributed to mitochondrial dysfunction. We first demonstrated that a HMG-CoA reductase inhibitor pitavastatin inhibited mevalonate pathway and thereby prenylation in RCC cells. In addition, pitavastatin is effective in inhibiting growth and inducing apoptosis in a panel of RCC cell lines. Combination of pitavastatin and paclitaxel is significantly more effective than pitavastatin or paclitaxel alone as shown by both *in vitro* cell culture system and in vivo RCC xenograft model. Importantly, pitavastatin treatment inhibits mitochondrial respiration *via* suppressing mitochondrial complex I and II enzyme activities. Interestingly, different from mitochondrial inhibitor phenformin that inhibits mitochondrial respiration but activates glycolytic rate in RCC cells, pitavastatin significantly decreases glycolytic rate. The dual inhibitory action of pitavastatin on mitochondrial respiration and glycolysis results in remarkable energy depletion and oxidative stress in RCC cells. In addition, inhibition of prenylation by depleting Isoprenylcysteine carboxylmethyltransferase (Icmt) also mimics the inhibitory effects of pitavastatin in RCC cells. Our work demonstrates the previously unappreciated association between prenylation inhibition and energy metabolism in RCC, which can be therapeutically exploited, likely in tumors that largely rely on energy metabolism.

1. Introduction

Prenylation is a three-step posttranslational lipid modification process in the maturation of many proteins. It involves the covalent addition of either farnesyl or geranylgeranyl isoprenoids to conserved cysteine residues, proteolytic removal of the −aaX sequence, and the methylation of the carboxyl group of the prenylated cysteine residue by isoprenylcysteine carboxyl methyltransferase (Icmt) [1]. Since the discovery that prenylation is required for proper function of many oncogenic proteins, such as Ras and Raf [2,3], prenylation inhibitors targeting farnesylation, geranylgeranylation or Icmt have been hotly evaluated in various pre-clinical cancer models [4]. Prenylation inhibition prevents tumorigenesis and growth in cancer cells of different tumor origins [5–7]. Many studies demonstrate that the inhibitory roles of prenylation inhibition on tumor growth are via impairment of K-Ras activity [8,9] unraveling other possible impacts of prenylation inhibition is fundamentally important.

Since mevalonate is an essential intermediate in the synthesis of farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), statins that prevent mevalonate production via suppressing 3-hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA) reductase

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activity have been shown to inhibit prenylation [10]. Statins are clinically used to treat hypercholesterolemia and reduce the incidence of cardiovascular events. Clinical data suggest that statins may have a role in the prevention of cancer but the mechanisms are largely unknown [11].

In this work, we investigated the impact of prenylation inhibition in renal cell carcinoma (RCC) growth, survival, mitochondrial function, glycolytic rate and energy status. Our findings demonstrate that prenylation inhibition has a previously unappreciated role in energy metabolism, which account significantly for its impact on RCC growth, survival and response to chemotherapy.

2. Materials and methods

2.1. Cell lines, drugs and cell treatment

All human RCC cell lines used in our study were obtained from the Institute of Cell Research, Chinese Academy of Sciences, Shanghai, China. Cells were cultured in RPMI 1640 medium (Life technologies, US) supplemented with 10% fetal bovine serum (Hyclone, US), 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco, US). Pitavastatin, paclitaxel, farnesol (FOH) and mevalonate (MV) were purchased from Sigma, US. Geranylgeraniol (GGOH) was purchased from ICN Biomedicals, The Netherlands. Cells were treated with pitavastatin at concentrations ranging from 5 to 20 μM. Treatment duration for each experiment is described in the figure legends.

2.2. Measurement of cell proliferation and apoptosis

Cell proliferation was carried out using MTS Cell Proliferation Colorimetric Assay Kit (Abcam, US). Cell apoptosis was determined by flow cytometry of Annexin V-FITC and 7-AAD (BD Pharmingen, US) staining. Flow cytometry was performed on a MACSQuant Analyser (Miltenyi Biotec, US).

2.3. Western blot analyses

Total proteins were isolated by resuspending cells (one million) in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton-X-100, and a cocktail of protease inhibitors). Protein concentrations were determined by the BCA assay (Pierce, US). Equal amounts of protein (20 μg) were loaded on sodium dodecyl sulphate-polyacrylamide gels, then electrically transferred to nitrocellulose membranes and analyzed by western blot using anti-Ras (Becton Dickinson), anti-Rap1A and anti-β-actin (Santa Cruz Biotechnology, US) antibodies.

2.4. Measure of mitochondrial complex I, II, IV and V activities

Complex I, II, IV and V activities were determined using total cell lysates and were measured using corresponding kits (Novagen, US) as previously described [12]. The optimal concentrations of samples are determined using solution buffer provided in the kit. The activity levels were assessed using Infinite200 Microplate Reader (Mannedorf, Switzerland) in kinetic mode at absorbance in mOD/min at 340 nm (I), 600 nm (II), 550 nm (IV) and 340 nm (V).

2.5. Measurement of mitochondrial respiration

Oxygen consumption rate (OCR) was performed using Mito stress assay kit on Seahorse XF24 analyser (Seahorse Bioscience, US). Briefly, after drug treatment, cell media were replaced by XF assay medium and incubated for 30 min at 37 °C in a CO_2-free environment for equilibration. The following Mito stress assay for OCR was conducted according to XF24 analyser standard protocol. OCR was measured under basal conditions, in the presence of oligomycin or Antimycin A, or in the presence of Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) to assess maximal mitochondrial respiration.

2.6. Measurement of ATP, mitochondrial super oxidase level and glycolysis rate

ATP levels were measured by ATPlite Luminiescent Assay kit (Perkin Elmer, US) according to the manufacturer's protocol. Mitochondrial super oxidase levels were determined by staining cells with MitoSox Red at 37 °C for 20 min and read the absorbance at ex/em of 510/580 nm using Spectramax M5 microplate reader (Molecular Devices). The rate of glycolysis was determined using Glycolysis Assay Kit (Extracellular Acidification, Abcam, Catalog No. ab197244) according to manufacturer's instructions. This assay measures cellular glycolytic flux and the rates of extracellular acidification are calculated from the changes in lactate production over time.

2.7. RCC cancer xenograft mouse model

All procedures with mice were approved by the Institutional Animal Care and Use Committee of Yangtze University. 786-O cells in log phase suspended in PBS were subcutaneously injected into the right flank of SCID mice. Tumor diameter was measured every 3 days and volume was determined by using the formula $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$. When tumor reached 200 mm^3, mice were randomly divided into four treatment groups: Vehicle control (20%/80% DMSO/saline, i.p.), paclitaxel (2 mg/kg, i.p.), pitavastatin (40 mg/kg, i.p.), or combination (paclitaxel + pitavastatin).

2.8. siRNA transfection

ICMT-specific knockdown was achieved by performing transfection using specific siRNA and Lipofectamine TM 2000 (Invitrogen, US) as per the manufacturer's protocol. Optimal cell density and siRNA concentration were firstly determined according to manufacturer's instructions. siRNA targeting Icmt and control siRNA were from Invitrogen.

3. Results

3.1. Pitavastatin inhibits proliferation and induces apoptosis in RCC cells, and augments paclitaxel's efficacy in vitro and in vivo

To investigate whether pitavastatin has tumor-suppressive effects on RCC, proliferation and apoptosis assays were firstly performed on a panel of RCC cell lines which represent different subtypes of *in vitro* RCC models. Although RCC cell lines exhibit different sensitivity, we found that pitavastatin is effective in inhibiting proliferation as well as inducing apoptosis at concentrations ranging from 5 to 20 μM in a dose-dependent manner in all tested RCC cell lines (Fig. 1A and B). In addition, pitavastatin demonstrates enhanced anti-RCC activity when used in combination with paclitaxel as measured by MTS proliferation and Annexin V staining (Fig. 1C and D). The enhanced anti-tumor activity induced by the combination of pitavastatin and paclitaxel are observed in all tested RCC cell lines (Fig. 1C and D). Importantly, pitavastatin significantly potentiated paclitaxel's effect in RCC xenograft mouse model using 786-O cells (Fig. 1E).

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