Doxycycline sensitizes renal cell carcinoma to chemotherapy by preferentially inhibiting mitochondrial translation

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Abstract

Objectives: The anti-cancer activity of doxycycline has been reported in many cancers but not renal cell carcinoma (RCC). This study aimed to determine the efficacy of doxycycline alone and in combination with paclitaxel and analyze the underlying mechanism in RCC.

Methods: Proliferation, colony formation and apoptosis assays were performed in RCC cell lines after drug treatments. An RCC xenograft mouse model was generated, and tumor growth was monitored. Mechanistic studies focused on mitochondrial translation and functions.

Results: Doxycycline at clinically achievable concentrations inhibited proliferation and colony formation and induced apoptosis in RCC cell lines. In normal kidney cells, doxycycline at the same concentrations either had no effect or was less effective. The combination index value demonstrated that doxycycline and paclitaxel were synergistic *in vitro*. Consistently, this combination therapy was significantly more effective than the monotherapy in RCC xenograft mice without causing significant toxicity. Mechanistic studies revealed that doxycycline acts on RCC cells via preferentially inhibiting mitochondrial DNA translation, thereby disrupting multiple mitochondrial complexes and impairing mitochondrial respiration.

Conclusions: Doxycycline is a useful addition to the treatment strategy for RCC. Our work also highlights the therapeutic value of mitochondrial translation inhibition in sensitizing RCC to chemotherapy.

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Introduction

Renal cell carcinoma (RCC), an epithelial tumor derived from the proximal tubules of nephrons, is a common and deadly disease worldwide.[1] RCC is characterized by distinct histological subtypes, extensive inter- and intra-heterogeneity and varying clinical responses to therapies.[2] The treatments include cytoreductive nephrectomy in combination with chemotherapy, radiotherapy, immunotherapy and targeted therapy.[3] Although the relative 5-year survival rates at diagnosis have increased, the overall prognosis remains poor.[4] Therefore, the identification of novel treatment options is required to improve the clinical management of RCC. We and others recently demonstrated that targeting mitochondrial respiration is an attractive therapeutic strategy in RCC because these tumor cells largely depend on mitochondrial respiration for survival and maintenance.[5–7] The mitochondrial respiratory chain subunits critically involved in mitochondrial respiration are encoded by mitochondrial DNA; therefore, the inhibition of mitochondrial protein translation results in deficient mitochondrial respiration.[8]

Tigecycline and doxycycline are tetracyclines that block bacterial protein synthesis by binding to small ribosomal subunits, preventing the attachment of aminoacyl-tRNA.[9] Tetracyclines also interfere with mitochondrial protein synthesis in eukaryotic cells.[10] Tigecycline has been shown to inhibit mitochondrial protein translation, leading to cell death in RCC, leukemia and hepatocellular carcinoma.[5,11,12] The anti-cancer activities of doxycycline have been demonstrated in a range of tumor systems but not RCC.[13,14] A recent study revealed the contribution of mitochondria to the mechanism of action of doxycycline in cancer.[15] We hypothesized that doxycycline inhibits mitochondrial inhibition in RCC cells and sensitizes them to chemotherapy. In this work, we systematically investigated the efficacy of doxycycline alone and in combination with paclitaxel in cell cultures and xenograft mouse models and analyzed the underlying mechanism of doxycycline in RCC.

Materials and methods

Drugs, cell lines and treatments

Doxycycline and paclitaxel (Selleckchem, Houston, TX, USA) were reconstituted in water and dimethyl sulfoxide. Three human RCC cell lines were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Beijing, China. The immortalized normal renal cell line HK-2 was obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured using medium based on the manufacturer's instructions and maintained at $37^{\circ}C$ in 5% CO_2. The authentication of cell lines was performed using short tandem repeat profiling (XP Biomed, Shanghai, China). Cells were examined for mycoplasma using a MycoAlert Mycoplasma Detection kit (Lonza, Basel, Switzerland) prior to experiments. Cells were seeded at a density

that allowed logarithmic growth during the treatment period. Specific drug treatment durations for each experiment were indicated in the figure legends.

Cell proliferation and combination index (CI) measurements

Cell proliferation was determined using the bromodeoxyuridine (BrdU) labeling method and quantified using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]. To measure cell proliferation, a Proliferation Assay kit (Abcam, Branford, CT, USA) was used as per the manufacturer's protocol. Absorbance at 490 nm was measured on a microplate reader (Biotek, Winooski, VT, USA). Combination studies were performed using the Chou–Talalay method.[16] Briefly, the half-maximum inhibitory concentration (IC_{50}) of doxycycline and paclitaxel was determined using GraphPad Prism (version 9.0, San Diego, CA, USA). The cells were treated with increasing doses of each drug alone or an equipotent constant-ratio concentration of two drugs based on the IC_{50} of each drug. After treatment, cell proliferation was determined, and the CI for drug-drug interactions was calculated using CompuSyn (Cambridge, UK). $CI < 1, = 1$ and > 1 indicate synergism, additivity and antagonism, respectively.[17]

Measurement of apoptosis

Cell apoptosis was assessed by flow cytometry analysis of Annexin V staining as indicated in our previous study.[5]

Anchorage-independent colony formation

An anchorage-independent colony formation assay was performed using soft agar. Briefly, 0.7% Bacto agar (Sigma Aldrich, Saint Louis, MO, USA) was plated and solidified as a base layer. Then, 0.3%

Bacto agar containing 5000 cells and drugs were plated onto the base layer. The medium was replenished twice a week. After 10 to 14 days, colonies were stained with crystal violet (Sigma Aldrich) and counted under a microscope (Zeiss, Berlin, Germany).

Western blot analyses

Treated cells were harvested and lysed using radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktails and phosphatase inhibitor (Invitrogen, Waltham, MA, USA). The cell lysate of each sample was loaded in sodium dodecyl sulfate-polyacrylamide gels and separated via electrophoresis. Western blot was performed using a standard protocol with antibodies against cyclooxygenase 1 (COX-1), COX-2, COX-4, glucose regulatory protein 78 (GRP78) and β-actin (Santa Cruz Biotechnology, CA, USA). The signal was developed using the enhanced chemical luminescence method (Pierce Biotechnology Inc., Rockford, IL, USA), and western blot bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Quantitative reverse transcription polymerase chain reaction (PCR)

RNA extraction, cDNA preparation and quantitative reverse transcription PCR were performed using the protocol reported in our previous study.[5] The cDNAs encoding *COX-1, COX-2, COX-4* and *GRP78* were amplified using the following primer pairs: (*COX-1*) 5′-CTA TAC CTA TTA TTC GGC GCA TGA-3′ and 5′-CAG CTC GGC TCG AAT AAG GA-3′; (*COX-2*) 5′-CTG AAC CTA CGA GTA CAC CG-3′ and 5′-TTA ATT CTA GGA CGA TGG GC-3′; (*COX4*) 5′-GCC ATG TTC TTC ATC GGT TTC-3′ and 5′-GGC CGT ACA CAT AGT GCT TCT G-3′; and

(*GRP78*) 5′-TGG GTC GAC TCG AAT TCC AAA G-3′ and 5′-GTC AGG CGA TTC TGG TCA TTG G-3′.

Mitochondrial complex activities

Complex I, II, IV and V activities were assessed using total cell lysates and Mitochondrial Complex I, II, III, IV and V Activity Assay Kits (Novagen, Madison, WI, USA) as per the manufacturer's protocol. The complex activities were determined colorimetrically using a microplate reader (Biotek).

Mito stress assay

Cells were seeded, cultured, treated with doxycycline and equilibrated in XF96 cell culture plates prior to performing oxygen consumption rate (OCR) assays using the Seahorse XF96 analyzer (Seahorse Bioscience, North Billerica, MA, USA) as per the standard protocol. Oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), rotenone and antimycin A were purchased from Seahorse Bioscience. Both basal and maximal OCRs were measured. The specific experimental conditions were indicated in the figure legends.

In vivo RCC mouse model

All procedures were conducted in accordance with the China Animal Protection Law, and the protocols were approved by the Animal Care Committee of The First People's Hospital of Jiangxia District Wuhan City (Protocol No. 2018061). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Severe combined immunodeficient mice (SJA Laboratory Animal Co., Ltd., Changsha, China) were maintained under specific pathogen-free conditions. The mice were subcutaneously inoculated with 10^7 786-O cells. After the development of a palpable

tumor, mice were randomized into different treatment groups. The administration routes, drug dose and duration were indicated in the figure legends. Tumor width and length were measured every 5 days, and the volume was calculated using the following formula: width$^2 \times$ length $\times 0.52$. Mice were euthanized using CO_2 inhalation when tumors reach $\sim 1500\,mm^3$.

Statistical analyses

All data were obtained from at least three independent experiments with duplicate or triplicate repeats and expressed as the mean and standard deviation. Data were compared by an unpaired Student's t test and analysis of variance with p-values <0.05 considered statistically significant. All statistical analyses were performed with GraphPad, version 9.0.

Results

Doxycycline selectively targets RCC cells

To investigate whether doxycycline has anti-RCC activity, we selected three human cell lines that represent different RCC subtypes, cellular origins and genetic backgrounds.[18] To determine whether doxycycline has selective anti-RCC activity, the immortalized human kidney cell line HK-2 was used as a normal control. To demonstrate the clinical significance, doxycycline was used at a concentration range from 5 to $20\,\mu g/mL$, which corresponds to the serum level in patients receiving doxycycline with the standard recommended dose.[19] We found that doxycycline significantly inhibited the proliferation of RCC cell lines by up to 85%, as assessed by BrdU incorporation assays (Figure 1A, p < 0.05). Anchorage-independent colony formation assays were performed to examine subpopulations with highly proliferative and stem cell-like phenotypes.[20] We found
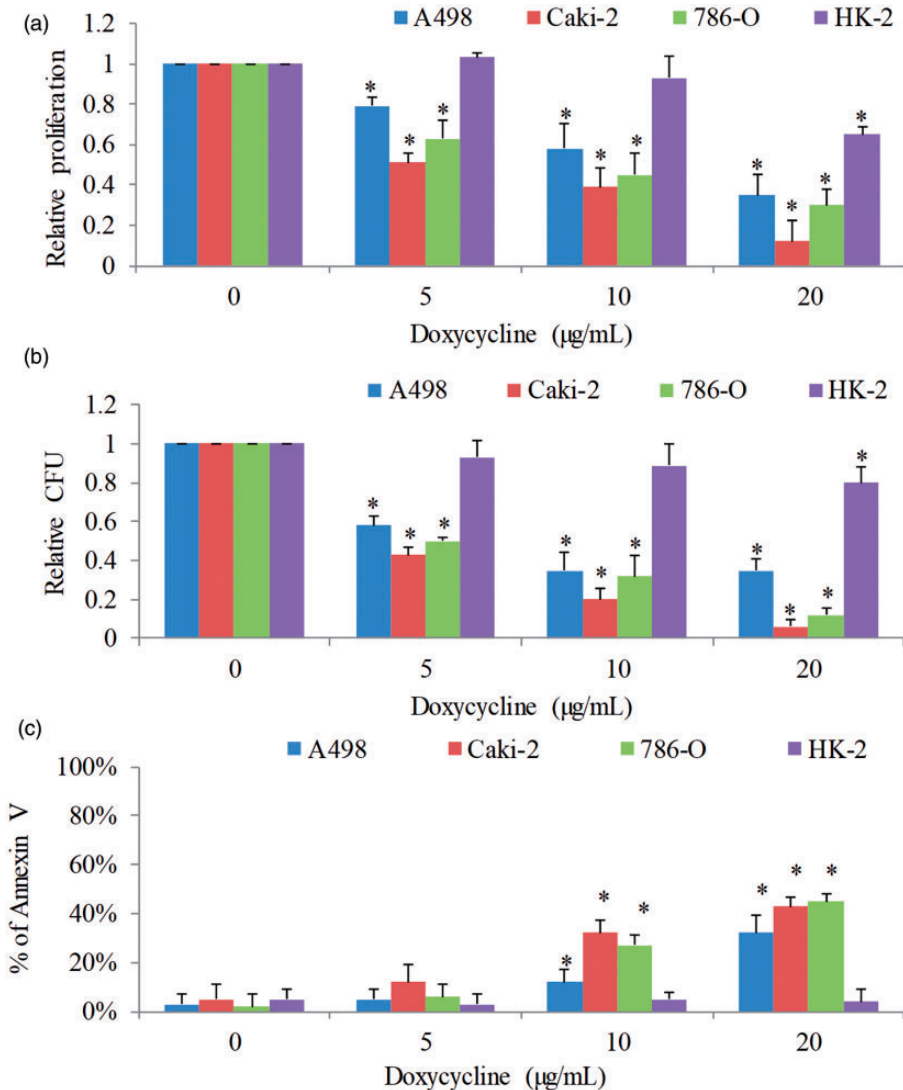
Figure 1. Doxycycline is active against RCC cells but spares normal kidney cells. Doxycycline is significantly more effective at inhibiting proliferation (a) and colony formation (b) and inducing apoptosis (c) in the human RCC cell lines 786-O, A498 and Caki-2 than the normal kidney cell line HK-2. Drugs were added to cell medium for 72 hours prior to MTS and apoptosis assays. Drugs were plated together with cells on soft agar, and colonies were counted after 10 to 14 days. *$p < 0.05$, compared with the control.
RCC, renal cell carcinoma; CFU, colony-forming units.

that doxycycline decreased colony formation up to 90% (Figure 1B, $p < 0.05$), suggesting that doxycycline is effective in inhibiting stem cell-like subpopulations. Doxycycline at 10 and 20 µg/mL significantly increased the level of Annexin V (Figure 1C, $p < 0.05$), demonstrating that doxycycline induces apoptosis in RCC cells. Under the same experimental conditions, doxycycline either had no effect or inhibited

proliferation and colony formation to a lesser extent in HK-2 cells compared with RCC cells (Figure 1A and B). In addition, doxycycline up to 20 µg/mL did not affect HK-2 apoptosis (Figure 1C). These results demonstrate that doxycycline actively targets RCC cells and is less toxic in normal kidney cells.

Doxycycline acts synergistically with paclitaxel in RCC cells

To investigate whether doxycycline enhances the efficacy of paclitaxel in RCC, we performed combination studies and interpreted the proliferation data using the Chou–Talalay method, which uses the median-effect equation to determine drug-drug interactions.[16] The CI-fraction affected (Fa) plot provides algorithms for the automated computer simulation of combinatory effects at any effect and dose level. As shown in the CI-Fa plots (Figure 2), the CI of all Fa values was less than 1 in 786-O, A498 and Caki-2 cells, indicating that doxycycline and paclitaxel synergistically inhibit RCC cell proliferation.

Doxycycline preferentially inhibits mitochondrial translation in RCC cells

We previously demonstrated that tigecycline acted on RCC cells via inhibiting mitochondrial translation.[5] Because both tigecycline and doxycycline are tetracyclines and function by inhibiting bacterial protein synthesis,[21] we investigated whether doxycycline affected mitochondrial translation in RCC cells. COX-1 and COX-2 are mitochondrial proteins synthesized by mitochondrial ribosomes. COX-4 and GRP78 are nuclear and cytosolic proteins synthesized via nuclear and cytosolic ribosomes, respectively.[11,22] Doxycycline treatment did not alter the expression of COX-4 and GRP78 but significantly decreased COX-1 and COX-2 protein levels in 786-O cells (Figure 3A and B, $p < 0.05$). In contrast, doxycycline treatment increased the mRNA level of COX-1 and COX-2 but not COX-4 or GRP78 (Figure 3C, $p < 0.05$). Mitochondrial DNA encodes 13 subunits of the electron transport chain complexes I, III, IV and V.[23] We found that doxycycline significantly decreased the activities of mitochondrial complexes I, III, IV and V in 786-O cells (Figure 4A, C to E, $p < 0.05$). However, doxycycline did not alter the activity of mitochondrial complex II (Figure 4B), which does not contain mitochondrially-encoded subunits. Collectively, our results demonstrate that doxycycline preferentially inhibited the synthesis of proteins encoded by mitochondrial DNA and suppressed the activities of multiple mitochondrial complexes.

Doxycycline is ineffective in mitochondrial-DNA depleted RCC ρ0 cells

We next investigated the effect of doxycycline on mitochondrial respiration using Mito stress assays. The OCR was measured under both basal and stimulated conditions in doxycycline-treated RCC cells. RCC cells treated with doxycycline had a significantly reduced baseline OCR and were non-responsive to the uncoupling of mitochondrial oxidative phosphorylation via FCCP, leading to a significant reduction in maximal respiration (Figure 5A to C, $p < 0.05$). We next investigated the effect of doxycycline in mitochondrial respiration-deficient RCC ρ0 cells generated and validated in our previous study.[5] As expected, doxycycline was ineffective in inducing apoptosis in RCC ρ0 cells (Figure 5D), confirming that mitochondrial respiration is required for doxycycline activity in RCC cells.
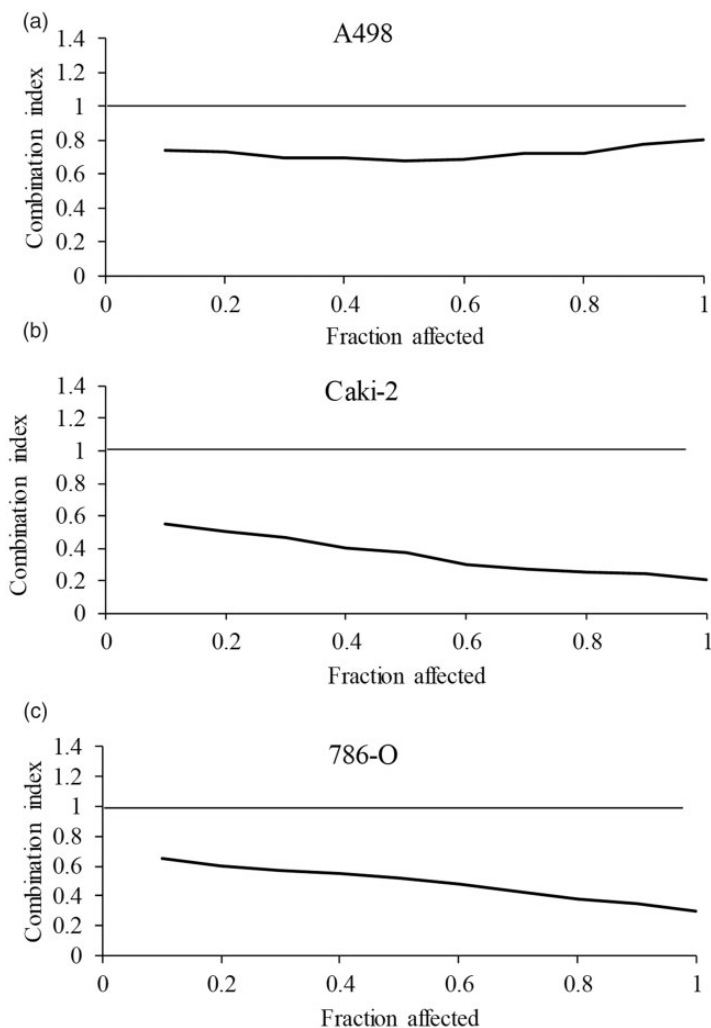
Figure 2. Doxycycline and paclitaxel are synergistic in RCC cells. Cells were treated with increasing doses (2-fold change) of a single drug alone or an equipotent constant-ratio concentration of two drugs based on the IC_{50} of each drug. Doxycycline at 0.5, 1, 2, 4, 8, 16 and 32 µg/mL and paclitaxel at 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 µM were used in combination studies. The CI at 0% to 100% growth inhibition was automatically calculated using CalcuSyn to determine if the combination was synergistic (CI < 1), additive (CI = 1) or antagonistic (CI > 1). Combination index values were all less than 1 in A498 (a), Caki-2 (b) and 786-O (c) cells. The fraction affected represents the fraction affected by a given drug concentration. Proliferation assays were performed for combination studies.

RCC, renal cell carcinoma; IC_{50}, half-maximum inhibitory concentration; CI, combination index.
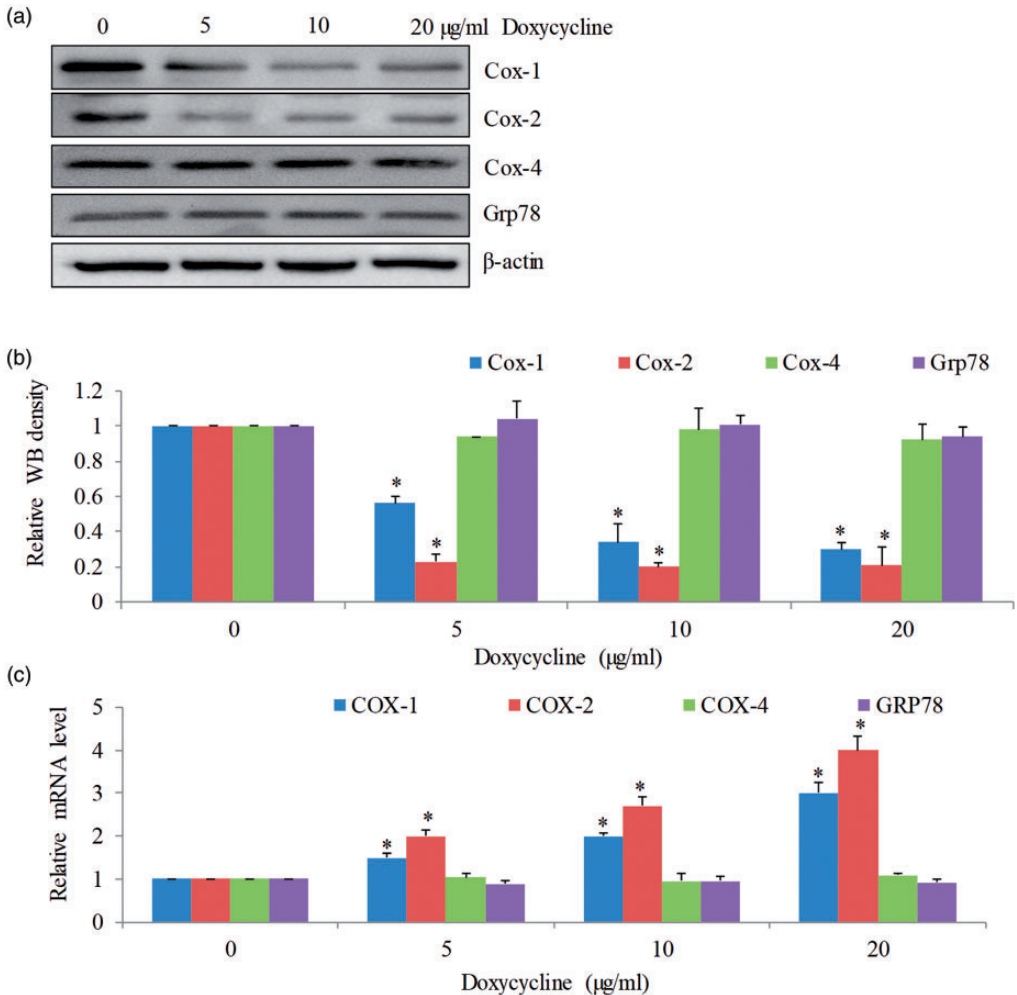
Figure 3. Doxycycline inhibits mitochondrial translation in RCC cells. (a and b) Doxycycline significantly decreases the protein level of COX-1 and COX-2 but not COX-4 or GRP78 in 786-O cells. β-actin was used as the control. (c) Doxycycline increases the mRNA level of *COX-1* and *COX-2* but not *COX-4* or *GRP78* in 786-O cells. The mRNA levels of all genes were quantified using the comparative CT method with 18s rRNA as the control for normalization. Cells were exposed to doxycycline for 24 hours prior to quantitative real-time PCR and western blot analyses. *$p < 0.05$, compared with the control.
RCC, renal cell carcinoma; COX, cyclooxygenase; GRP78, glucose regulatory protein 78; WB, western blot.

Combination therapy with doxycycline and paclitaxel is significantly more effective than monotherapy in an RCC xenograft mouse model

We finally investigated the *in vivo* efficacy of doxycycline alone and in combination with paclitaxel in an RCC xenograft mouse model using 786-O cells. Our data showed that intraperitoneal injection of doxycycline at doses that did not affect body weight slightly inhibited RCC growth in mice (Figure 6A and B). Mice treated with more than 20 mg/kg
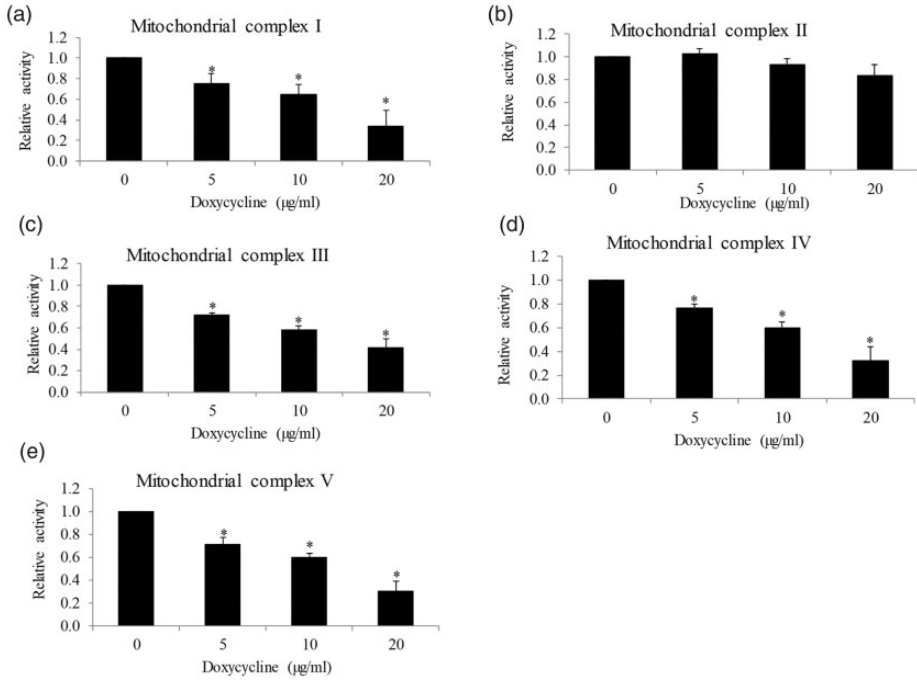
Figure 4. Doxycycline specifically suppresses the activity of mitochondrial complex I, III, IV and V in RCC cells. Doxycycline significantly suppresses mitochondrial respiratory complex I activity (a) in 786-O cells. Doxycycline did not affect respiratory complex II activity (b) in 786-O cells. Doxycycline significantly suppresses mitochondrial respiratory complex III (c), IV (d) and V (e) activities in 786-O cells. Cells were treated with doxycycline for 24 hours prior to measuring mitochondrial complex activities. $*p < 0.05$, compared with the control.
RCC, renal cell carcinoma.

doxycycline displayed some degree of toxicity, such as reduced body weight and gross abnormalities (data not shown). Furthermore, we found that the combination of doxycycline and paclitaxel at nontoxic doses was considerably more effective in inhibiting RCC growth in mice (Figure 6C, $p < 0.05$). Although tumors in single drug-treated groups were smaller than those in the control group, they continuously progressed and reached the same size as control tumors by a later time point. In contrast, the doxycycline and paclitaxel combination completely inhibited tumor growth during the entire treatment period.

Discussion

RCC constitutes a heterogeneous group of cancers with no effective therapeutic options. In this work, we provide preclinical evidence supporting the inhibition of mitochondrial translation as an approach to preferentially target RCC cells using a combination of paclitaxel and doxycycline. This strategy involves the simultaneous inhibition of microtubule function and mitochondrial translation. As an effective sensitizing therapeutic strategy, the inhibition of mitochondrial translation has been well documented in some cancers but not in RCC.[11] Doxycycline is an
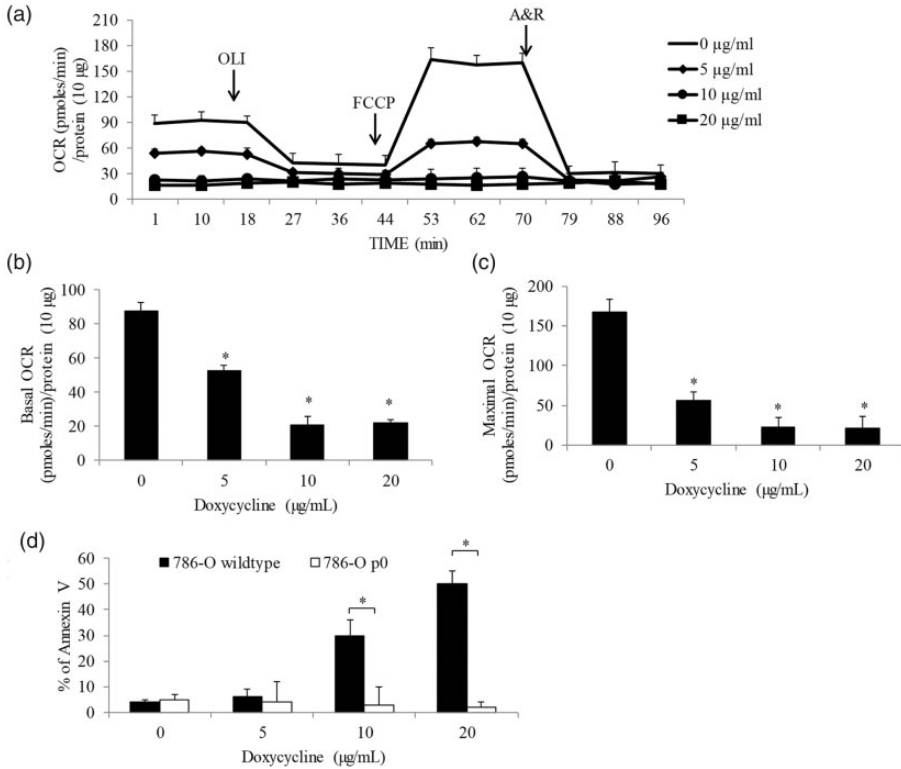
Figure 5. Doxycycline acts on RCC cells in a mitochondrial respiration-dependent manner. (a to c) Doxycycline inhibits basal and maximal mitochondrial respiration in 786-O cells. Cells were exposed to doxycycline for 24 hours prior to OCR measurements. The OCR was measured in the absence (first three measurements) and presence (measurements 4–12) of OLI (1 μg/mL), FCCP (0.4 μM) and A&R (both 2.5 μM). OLI, FCCP and A&R were injected into the wells at the indicated times (arrows). The basal OCR was calculated as the first three measurements. The maximal OCR was calculated as the three measurements after FCCP injection. After measuring OCR levels, cells were lysed, followed by protein concentration measurements for each sample. All OCRs presented were normalized to protein mass. (d) Doxycycline is ineffective in inducing apoptosis in 786-O ρ0 cells. *p < 0.05, compared with the control. RCC, renal cell carcinoma; OCR, oxygen consumption rate; OLI, oligomycin; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; A&R, antimycin A and rotenone combination.

attractive candidate for RCC treatment because it is a well-tolerated drug, almost completely absorbed after oral administration and has good tissue penetration.[11]

Using a panel of RCC cell lines and a normal kidney cell line, we demonstrated that doxycycline has preferential toxicity against RCC cells but spares normal kidney cells (Figure 1). Our findings also suggest that doxycycline is likely to be more effective in targeting stem cell-like populations than bulky cells in RCC (Figure 1a and b). This is consistent with the previous reports that doxycycline exerts inhibitory effects on breast cancer stem cells[13] and decreases the tumor-sphere formation efficiency of cancer stem cells.[15] Notably, the ability of doxycycline to eradicate cancer stem cells was validated in a clinical pilot study in which stemness marker levels were significantly reduced in tumor samples obtained from breast cancer
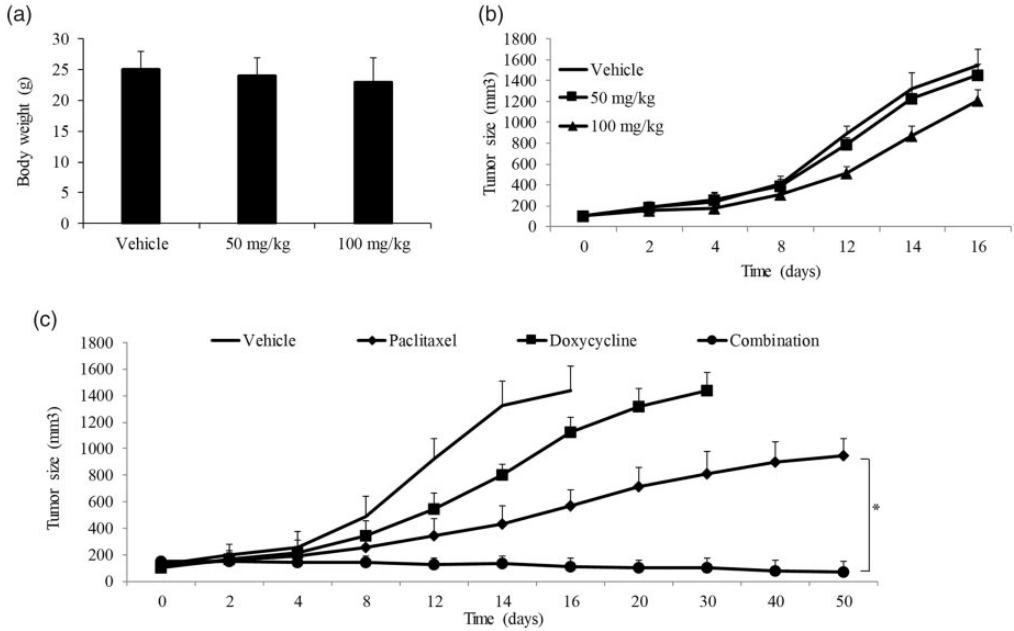
Figure 6. Doxycycline significantly enhances the efficacy of paclitaxel in inhibiting RCC growth *in vivo*. (a and b) The effects of doxycycline on body weight and RCC growth in mice. Doxycycline at 50 mg/kg and 100 mg/kg were given by intraperitoneal injection once per day. (c) The combination of doxycycline and paclitaxel is considerably more effective than paclitaxel alone in inhibiting RCC growth in mice. Mice were treated with vehicle alone, 100 mg/kg doxycycline (once per day), 1 mg/kg paclitaxel (once per week) or a combination of both drugs. Doxycycline and paclitaxel were given by intraperitoneal injection. $*p < 0.05$ compared with paclitaxel.
RCC, renal cell carcinoma.

patients treated with oral doxycycline.[24] As RCC stem cells are essentially involved in the resistance mechanisms to radiation therapies and chemotherapies,[25] the possible inhibitory effects of doxycycline in RCC stem cells suggest that doxycycline may overcome chemoresistance in RCC. This is supported by our results obtained from combination studies in which doxycycline and paclitaxel synergistically inhibited RCC proliferation (Figure 2).

Several studies have revealed the anti-cancer activities of doxycycline. However, most have focused on the efficacy of doxycycline alone in cancer. A significant finding of our work is that the combination of doxycycline with paclitaxel is significantly more effective than the monotherapy.

Using an RCC xenograft mouse model, we observed that the combination of doxycycline and paclitaxel at sublethal doses completely prevented RCC growth during the entire treatment period without causing significant toxicity (Figure 6). This demonstrates the therapeutic window of the doxycycline and paclitaxel combination for the treatment of RCC. An increasing number of clinical trials are investigating the efficacy of doxycycline alone or in combination with anti-cancer drugs for the treatment of various cancers, such as pancreatic cancer (NCT02775695) and breast cancer (NCT02874430). A phase II study showed that a regimen consisting of doxycycline and interferon-alpha was not effective for advanced RCC.[26] Our findings support

the initiation of a clinical trial using a combination of doxycycline and paclitaxel for RCC.

The reported action mechanisms of doxycycline in cancer include the inhibition of matrix metalloproteinase synthesis, suppression of focal adhesion kinase pathways, inhibition of nuclear factor-kappa B and signal transducer and activator of transcription 3 signaling and inhibition of mitochondrial functions.[14,15] We show that doxycycline preferentially inhibits the translation of mitochondrial but not cytosolic or nuclear proteins in RCC, leading to reduced activities of multiple mitochondrial complexes and disrupted mitochondrial respiration (Figure 3 to 5). Using mitochondrial respiration-deficient RCC $\rho 0$ cells, we confirmed the inhibition of mitochondrial translation as the action mechanism of doxycycline in RCC (Figure 5D). We previously showed that tigecycline, a tetracycline antibiotic, acted on RCC via inhibiting mitochondrial protein translation.[5] Given that mitochondria share certain conserved protein translation-related features with bacteria and that tetracycline blocks bacterial protein synthesis, we speculate that other tetracycline antibiotics might also be active against RCC. Similar to several other cancers, RCC cells display increased mitochondrial biogenesis compared with normal renal cells, indicating they are more dependent on mitochondrial respiration for growth and survival.[27] This is also supported by our findings that doxycycline is more effective in targeting RCC compared with normal renal cells.

In conclusion, our findings demonstrate that doxycycline sensitizes RC to chemotherapy in pre-clinical models. Phase II clinical trials are necessary to validate the potential therapeutic efficacy of the combination of doxycycline and paclitaxel in RCC patients.

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References

1. Thoenes W, Storkel S and Rumpelt HJ. Histopathology and classification of renal cell tumors (adenomas, oncocytomas and carcinomas). The basic cytological and histopathological elements and their use for diagnostics. *Pathol Res Pract*. 1986; 181: 125–143.
2. Linehan WM and Ricketts CJ. The Cancer Genome Atlas of renal cell carcinoma: findings and clinical implications. *Nat Rev Urol*. 2019; 16: 539–552.
3. Barata PC and Rini BI. Treatment of renal cell carcinoma: Current status and future directions. *CA Cancer J Clin*. 2017; 67: 507–524.
4. Gray RE and Harris GT. Renal Cell Carcinoma: Diagnosis and Management. *Am Fam Physician*. 2019; 99: 179–184.
5. Wang B, Ao J, Yu D, et al. Inhibition of mitochondrial translation effectively sensitizes renal cell carcinoma to chemotherapy. *Biochem Biophys Res Commun*. 2017; 490: 767–773.
6. Jang Y, Han J, Kim SJ, et al. Suppression of mitochondrial respiration with auraptene inhibits the progression of renal cell carcinoma: involvement of HIF-1alpha degradation. *Oncotarget*. 2015; 6: 38127–38138.
7. Chen D, Sun X, Zhang X, et al. Targeting mitochondria by anthelmintic drug

atovaquone sensitizes renal cell carcinoma to chemotherapy and immunotherapy. *J Biochem Mol Toxicol*. 2018; 32: e22195.

8. Chandel NS and Schumacker PT. Cells depleted of mitochondrial DNA (rho0) yield insight into physiological mechanisms. *FEBS Lett*. 1999; 454: 173–176.

9. Chopra I and Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev*. 2001; 65: 232–260; second page, table of contents.

10. Moullan N, Mouchiroud L, Wang X, et al. Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research. *Cell Rep*. 2015; 10: 1681–1691.

11. Skrtic M, Sriskanthadevan S, Jhas B, et al. Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. *Cancer Cell*. 2011; 20: 674–688.

12. Tan J, Song M, Zhou M, et al. Antibiotic tigecycline enhances cisplatin activity against human hepatocellular carcinoma through inducing mitochondrial dysfunction and oxidative damage. *Biochem Biophys Res Commun*. 2017; 483: 17–23.

13. Zhang L, Xu L, Zhang F, et al. Doxycycline inhibits the cancer stem cell phenotype and epithelial-to-mesenchymal transition in breast cancer. *Cell Cycle*. 2017; 16: 737–745.

14. Ali I, Alfarouk KO, Reshkin SJ, et al. Doxycycline as Potential Anti-cancer Agent. *Anticancer Agents Med Chem*. 2017; 17: 1617–1623.

15. Dijk SN, Protasoni M, Elpidorou M, et al. Mitochondria as target to inhibit proliferation and induce apoptosis of cancer cells: the effects of doxycycline and gemcitabine. *Sci Rep*. 2020; 10: 4363.

16. Ashton JC. Drug combination studies and their synergy quantification using the Chou-Talalay method–letter. *Cancer Res*. 2015; 75: 2400.

17. Yadav B, Wennerberg K, Aittokallio T, et al. Searching for Drug Synergy in Complex Dose-Response Landscapes Using an Interaction Potency Model. *Comput Struct Biotechnol J*. 2015; 13: 504–513.

18. Brodaczewska KK, Szczylik C, Fiedorowicz M, et al. Choosing the right cell line for renal cell cancer research. *Mol Cancer*. 2016; 15: 83.

19. van Roeden SE, Bleeker-Rovers CP, Kampschreur LM, et al. The effect of measuring serum doxycycline concentrations on clinical outcomes during treatment of chronic Q fever. *J Antimicrob Chemother*. 2018; 73: 1068–1076.

20. Gao CF, Xie Q, Su YL, et al. Proliferation and invasion: plasticity in tumor cells. *Proc Natl Acad Sci U S A*. 2005; 102: 10528–10533.

21. Zakeri B and Wright GD. Chemical biology of tetracycline antibiotics. *Biochem Cell Biol*. 2008; 86: 124–136.

22. Tam EW, Feigenbaum A, Addis JB, et al. A novel mitochondrial DNA mutation in COX1 leads to strokes, seizures, and lactic acidosis. *Neuropediatrics*. 2008; 39: 328–334.

23. Chen XJ and Butow RA. The organization and inheritance of the mitochondrial genome. *Nat Rev Genet*. 2005; 6: 815–825.

24. Scatena C, Roncella M, Di Paolo A, et al. Doxycycline, an Inhibitor of Mitochondrial Biogenesis, Effectively Reduces Cancer Stem Cells (CSCs) in Early Breast Cancer Patients: A Clinical Pilot Study. *Front Oncol*. 2018; 8: 452.

25. Peired AJ, Sisti A, Romagnani P. Renal Cancer Stem Cells: Characterization and Targeted Therapies. *Stem Cells Int*. 2016; 2016: 8342625.

26. Huie M, Oettel K, Van Ummersen L, et al. Phase II study of interferon-alpha and doxycycline for advanced renal cell carcinoma. *Invest New Drugs*. 2006; 24: 255–260.

27. Zhu M, Li Y and Zhou Z. Antibiotic ivermectin preferentially targets renal cancer through inducing mitochondrial dysfunction and oxidative damage. *Biochem Biophys Res Commun*. 2017; 492: 373–378.