Synergistic combination of YS-1 and adriamycin inhibits human renal cancer through ERK1/2 signaling pathway *in vitro* and *in vivo*

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Abstract. Adriamycin (ADM) is a principal drug for the treatment of renal cell cancer (RCC). Due to its limited response and high renal and cardiac toxicity, synergistic effects of ADM in combination with other drugs have been widely researched. In this study, we found the combination between YS-1 and ADM, performed higher anticancer activity on 786-O human RCC cells *in vitro* and *in vivo*, than that reported on its anti-angiogenesis effect compared with monotherapy of ADM. Our data showed that when combined with ADM, YS-1 promoted the sensitivity of 786-O cells to ADM. The combination of YS-1 and ADM also inhibited cell proliferation, but without affecting cell apoptosis. We found that ADM monotherapy treatment notably upregulated the activity of extracellular signal-regulated kinase ERK1 and ERK2 (ERK1/2), but when combined with YS-1, the p-ERK1/2 level was reduced; then inhibited the Ras/Raf/MEK pathway. Additionally, the synergistic effects on cell cycle arrest inhibition were eliminated when ERK1/2 was silenced using siRNA. Our combination therapy of YS-1 with ADM showed the strongest antitumor effects *in vivo* (inhibition ratio: 5 mg/kg YS-1 combined with 1 mg/kg ADM, 68.19%) in comparison with individual effects (inhibition ratio: 5 mg/kg YS-1, 30.07%; 1 mg/kg ADM, 50.42%). Collectively, these findings indicated that YS-1 did not only enhance the ability of ADM to inhibit tumor proliferation, but also reduce the renal toxicity to protect the normal renal tissues.

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Introduction

It is well-known that renal cell carcinoma (RCC) is a high-risk and high-mortality cancer and is notoriously resistant to traditional chemotherapies and radiotherapies (1), marked by adverse tumor biology and its low CSS ranking among urological malignancies (2). RCC patients either receive surgery or chemoradiotherapy, have poor prognosis and low five-year survival rate (3,4). Because of the outcomes of chemotherapy, radiotherapy and hormone therapy are unsatisfactory, it is necessary to develop an effective adjuvant therapy.

Adriamycin (ADM), a kind of antitumor antibiotic, inhibits the synthesis of DNA and RNA, has strong cytotoxicity (5) and induces toxicity through oxidative stress (6). In clinic, ADM has been used for sarcomatoid-type tumors, including RCC (7,8); however, the chronic toxicity and acute toxicity of ADM limit its clinical application (9,10). YS-1, a recombinant human p43 protein, not only has been confirmed to have anti-angiogenesis and antitumor effects, *in vitro* and *in vivo* (11); but also showed potential antitumor properties for primary and metastatic solid tumors. In our previous study, YS-1 could directly inhibited angiogenesis through Dll4-Notch1 signal transduction pathway (12), and caused renal toxicity mainly by the activation of ERK1/2 in kidney cells (13). Thus, inhibiting the effects of ADM on ERK1/2 pathway may be a key mechanism to improve the anticancer effect of ADM. According to our previous research, YS-1 might inhibit ERK1/2 activation through affecting the Notch1 pathway. Taking advantage of combination therapies (i.e., avoiding the risk of the development of resistance, increasing the effectiveness of the therapy, and the effectiveness of clinical combination therapies with ADM), we designed YS-1 combined with ADM, to reduce the expression of p-ERK1/2 and increase the effectiveness of ADM therapy for RCCs.

The ERK1/2 cascade regulates a variety of cellular processes by phosphorylating multiple target proteins (14). The outcome of its activation ranges from stimulation of cell survival and proliferation to triggering tumor suppressor responses such as cell differentiation, cell senescence, and apoptosis (15). Recent studies have shown that inhibition of ERK1/2 can effectively reverse the multidrug resistance of prostate cancer, gastric cancer, and cancer of the blood system (16-18). The signaling

via the ERK cascade is mediated by sequential phosphorylation and activation of protein kinases in the different tiers of the cascade, and the main core phosphorylation chain of the cascade includes Raf kinases, MEK1/2, ERK1/2 (ERKs) and RSKs. The Ras/Raf/MEK/ERK pathway has been reported to be activated in over 50% of acute myelogenous leukemia and acute lymphocytic leukemia and is also frequently activated in other cancer types (e.g., breast and prostate cancers). Importantly, this increased expression is associated with a poor prognosis (19). The Ras/Raf/MEK/ERK interacts with each other to regulate growth and in some cases tumorigenesis, and it is commonly thought to have anti-apoptotic and drug resistance effects on cells (20,21).

In this study, we assessed the antitumor activity of ADM alone and in combination with YS-1 on RCCs. We also identified the optimal dose of YS-1 which can decrease p-ERK1/2 expression without causing toxicity. We propose that inhibition of the ERK1/2 activation by YS-1 may be a promising therapeutic target for enhancing the sensitivity of cancer cells to ADM. These findings highlighted the possibility of using such natural, safe, relatively inexpensive compounds as potential adjunct treatment in improving the overall treatment response of patients with RCC.

Materials and methods

Cell culture and siRNA. Human cancer (95D, SGC-7901, BEL-7402, MDA-MB-435 and 786-O) cell lines were purchased from the Shanghai Institute of Life Science, Chinese Academy of Sciences. BEL-7402 cells were maintained in DMEM with 10% fetal bovine serum (FBS, Gibco) and antibiotics (100 U/ml penicillin, and 100 mg/ml streptomycin). The other cells were grown in RMPI-1640 with 10% FBS and antibiotics (100 U/ml penicillin, and 100 mg/ml streptomycin). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37˚C. ERK1/2 siRNA was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Lipofectamine 2000 reagent was obtained from Invitrogen (Shanghai, China).

Cell growth inhibition assay. Cells were seeded in 96-well plates in 180 μl of medium and incubated for 12 h. These cells were then cultured in the presence of YS-1, ADM individually as well in combination for 72 h. Afterwards, 5 mg/ml MTT solution (20 μl/well) was added and cultured in 5% CO_2 incubator at 37˚C for 4 h, then the supernatant was discarded and DMSO was added (150 μl/well). The suspension was placed on a micro-vibrator for 5 min and the absorbance was measured at 570 nm using a Universal Microplate Reader (EL800; Bio-Tek Instruments Inc.). Experiments were performed in triplicate in a parallel manner for each concentration and the results are presented as the mean ± SD. The inhibitory ratio was calculated by the following formula: inhibitory ratio (%) = (1 - average absorbance of treated group/average absorbance of control group) x100.

Colony formation assays. The 786-O cells were seeded in a dish (100 mm x 20 mm) at a density of 300 cells per dish and cultured in the presence of YS-1, ADM individually as well in combination for two weeks. At the end of the incubation, the cells were fixed with 4% paraformaldehyde and stained with Giemsa. Megascopic cell colonies were counted using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). Each measurement was performed in triplicate and the experiments were conducted at least three times. The clone formation rate was calculated by the following formula: clone formation rate (%) = (number of colonies cells / number of vaccination cells) x100.

Cell cycle analysis. The 786-O cells ($1x10^6$) were seeded into dish (100 mm x 20 mm) and incubated overnight, and then cultured in the presence of YS-1, ADM individually as well in combination for 6 h. Next, the cells were harvested, washed with cold PBS, and then fixed with 70% cold ethanol at 4˚C overnight. After being washed twice with cold PBS, fixed cells were resuspended with 100 μg/ml RNase, incubated with 50 μg/ml PI at 37˚C for 30 min in the dark. Data acquisition and analysis were performed in Becton Dickinson FACS Calibur flow cytometer using Cell Quest software (Franklin Lakes, NJ, USA).

Wound closure assay. The 786-O cells were cultured to confluence or near confluence (>90%) in a 6-well dish. A sterile 10 μl pipette tip was used to scratch a cross-shaped wound through the cells. Cells were rinsed with PBS, and were cultured in the presence of YS-1, ADM individually and in combination. Wounds were imaged at 0, and 6 h under a microscope with an attached camera. The TScratch program (Computational Science and Engineering Laboratory, Zurich, Switzerland) (22) was used to measure the open areas and analyze the data.

Migration assay. Cells ($5x10^4$) were suspended in 200 μl serum-free RMPI-1640 medium and seeded into the upper chamber of each insert of Transwell (Millipore). Then, 700 μl of RMPI-1640 containing 10% FBS was added to a 24-well plate. After incubation at 37˚C for 6 h, the cells that migrated were fixed and stained for 30 min in a 0.1% Crystal Violet solution in PBS. The migrated cells were quantified by manual counting by using Image-Pro Plus 6.0 software (Media Cybernetics), and five randomly chosen fields were analyzed for each group.

Annexin V/PI double staining. Cells were incubated for 6 h with YS-1, ADM separately or in combination. Apoptotic cells were identified by the Annexin V-FITC Apoptosis Detection kit (Vazyme) in accordance with the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining.

Cell transfection. Cells were transfected with ERK1/2 siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfection medium (OptiMEM; Gibco) was replaced with complete medium 12 h after transfection, and the cells were incubated for the indicated times.

Western blot analysis. Cells were treated with YS-1 alone, ADM alone or combination both for 6 h. As previously described (23), then cellular protein extraction and Western blot analysis were performed. All first antibodies were purchased from Cell Signaling Technology, Inc. Horseradish peroxidase (HRP) linked anti-mouse immunoglobulin G
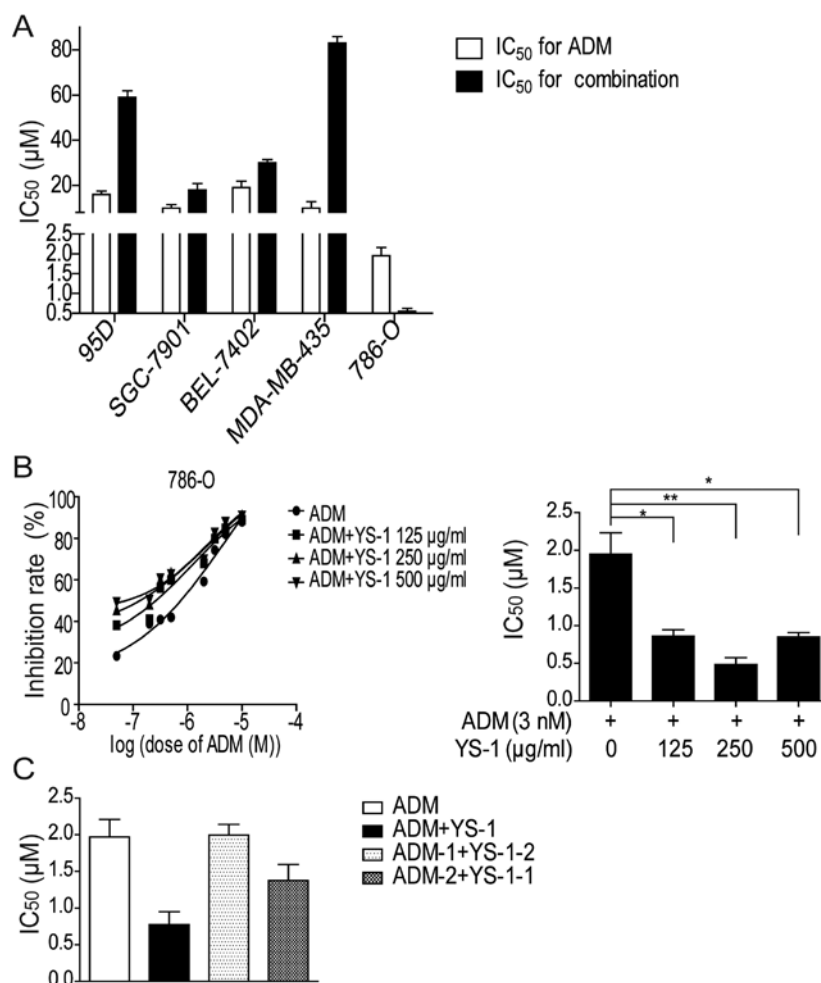
Figure 1. YS-1 combined with ADM had a synergistic anticancer effect on cell death in RCC. (A) MTT assay was used to measure the inhibitory effect of YS-1 combined with ADM on the survival of different cancer cell lines: 95D, SGC-7901, BEL-7402, MDA-MB-435, 786-O. (B) MTT assay was used to determine the appropriate concentration of YS-1 when combined with ADM (3 nM). (C) The inhibitory effect of the different drug delivery order of ADM (3 nM). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test, *P<0.05; **P<0.01, for B statistical analysis was performed using at least three independent replicates.

(Sigma) and anti-rabbit immunoglobulins G (CST) were used as the secondary antibodies. Different protein bands were made visible by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Quantitative real-time PCR. Total RNA was extracted using TRIzol reagent (Invitrogen). Complimentary DNA (cDNA) was synthesized with the Prime-Script RT reagent kit (Takara). The mRNA level was measured with the SYBR Green master mix (Vazyme). The amount of mRNA for each gene was standardized with internal control (GAPDH mRNA). Each treatment group was compared with the control group to show the relative mRNA level. The gene-specific primer pairs were as follows: GAPDH (F) 5'-GGTGGTCTCCTCTGACTTCA ACA-3', GAPDH (R) 5'-GTTGCTGTAGCCAAATTCGT TGT-3'; hERK1 (F) 5'-CATGAGAATGTCATCGGCATCC-3', hERK1 (R) 5'-CCATCAGGTCCTGCACAATGTAG-3'; hERK2 (F) 5'-GACATTATTCGAGCACCAACCATC-3', hERK2 (R) 5'-GAGGTGTTGTGTCTTCAAGAGCTTG-3'.

In vivo tumorigenicity. Female athymic BALB/c nude mice (5-6 weeks old) with body weight from 18 to 22 g were supplied by the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. 786-O cells were collected in serum-free RMPI-1640 medium and cell suspension (10^7 cells/100 μl), then cell suspension was injected subcutaneously into mice in one flank (n=6). Animal care and surgery protocols were approved by Animal Care Committees of China Pharmaceutical University. All animals were treated appropriately and used in a scientifically valid and ethical manner.

Statistical analysis. All of the results are presented as mean ± SD from triplicate experiments which were performed in a parallel manner unless otherwise indicated. Statistically significant differences (one-way ANOVAs followed by Bonferroni's multiple comparison test) were determined using GraphPad Prism 6 software. P<0.05 was considered significant, and P<0.01 and P<0.001 were considered highly significant.

Results

YS-1 combined with ADM has a synergistic anticancer effect on cell death in RCCs. We measured the inhibitory effect of YS-1 combined with ADM on the survival of different cancer
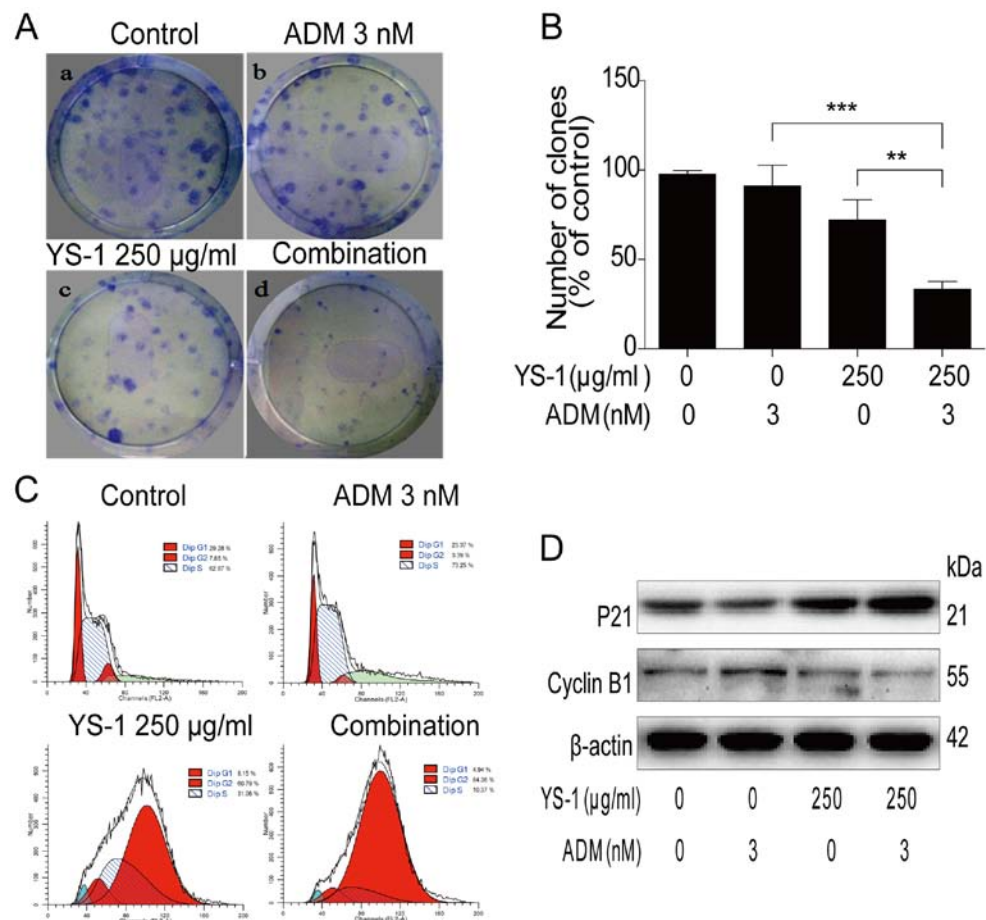
Figure 2. YS-1 combined with ADM promotes the anti-proliferative activity in 786-O cells. (A and B) The combination treatment of YS-1 and ADM could inhibit clone formation activity of 786-O cells. (C) The combination treatment of YS-1 and ADM induced G2/M cell cycle arrest of 786-O cells. (D) The G2/M cell cycle arrest related proteins were detected by western blot analysis. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test, **P<0.01; ***P<0.001, for B, G statistical analysis was performed using at least three independent replicates.
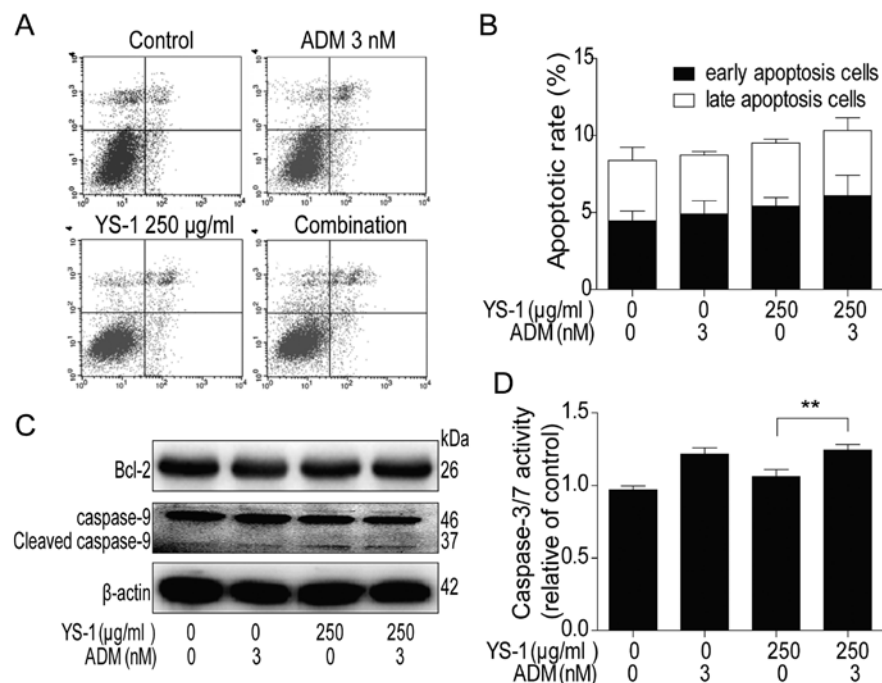

Figure 3. Synergistic anticancer effect of YS-1 combined with ADM did not induce pro-apoptotic activity in 786-O cells. (A and B) The early apoptosis was assessed by Annexin-V/PI staining in 786-O cells. (C) The apoptosis related proteins were assessed by western blot analysis in 786-O cells. (D) The activity of caspase-3/7 was determined by Apo-One® homogeneous Caspase-3/7 assay. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test, **P<0.01.
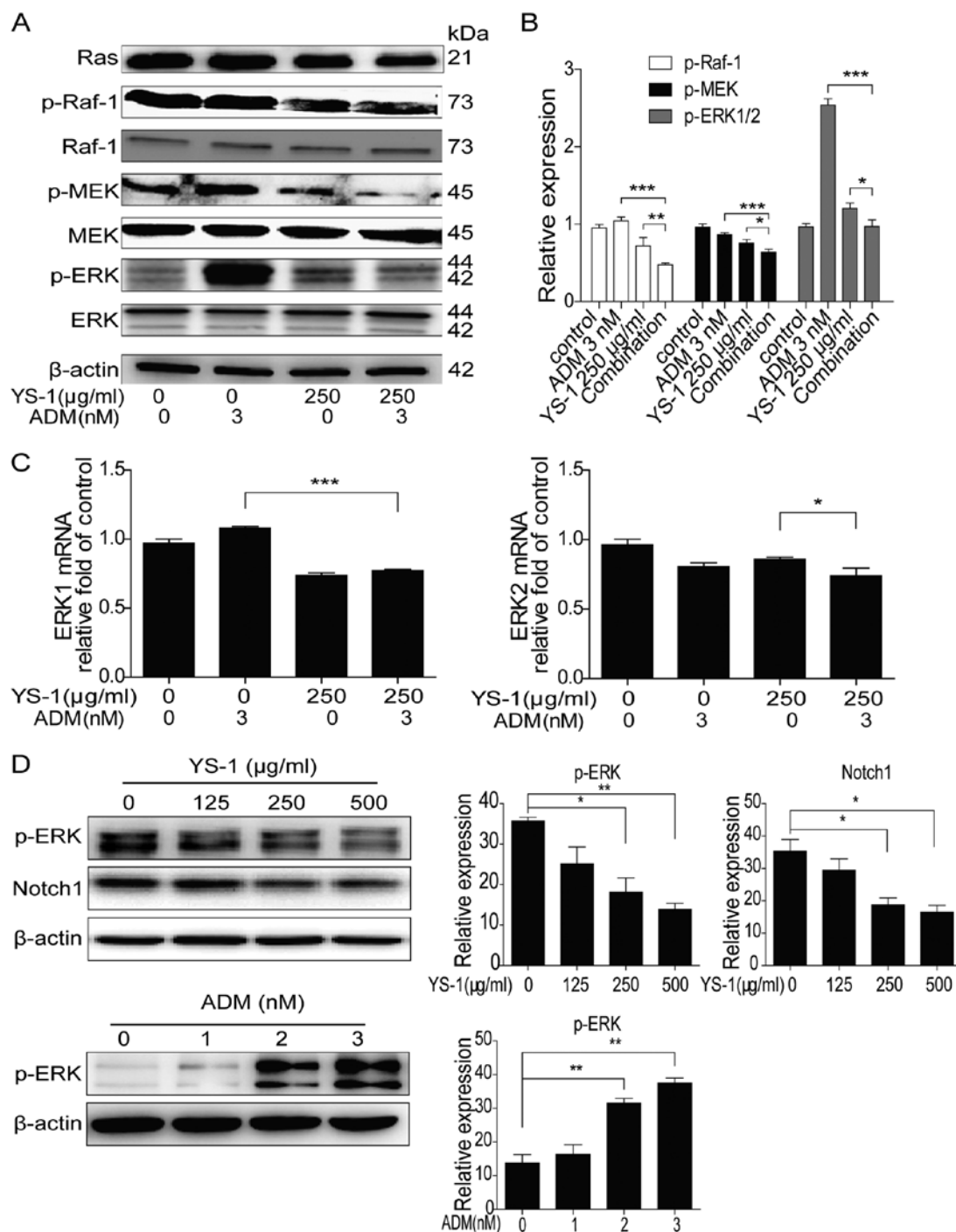
Figure 4. YS-1 combined with ADM down-regulates Ras/Raf/MEK/ERK1/2 pathway. (A and B) The combination treatment significantly reduced the Ras/Raf/MEK/ERK1/2 pathway. (C) The ERK1 and ERK2 mRNA levels were detected by QPCR analysis. (D) The 786-O cells were treated with YS-1 (125, 250, 500 μg/ml), or ADM (1, 2, 3 nM) for 6 h, the levels of p-ERK1/2 and Notch1 were checked by western blot analysis. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test, *P<0.05; **P<0.01; ***P<0.001.

cell lines: 95D, SGC-7901, BEL-7402, MDA-MB-435, 786-O. After 72 h of treatment, we found YS-1 combined with ADM only exhibited a synergistic inhibitory effect on the survival of 786-O cells (Fig. 1A).

In order to determine the optimum concentration of YS-1 when combined with ADM, 786-O cells were treated with ADM and YS-1 (125, 250, and 500 μg/ml, respectively) for 72 h, we found that when combining ADM with 250 μg/ml YS-1, the IC_{50} value reduced most markedly (Fig. 1B). Next, we investigated the inhibitory effect of the different drug delivery

order of 3 nM ADM with 250 μg/ml YS-1 on 786-O cells, as shown in Fig. 1C, only when YS-1 and ADM were added simultaneously, the IC_{50} value reduced most significantly. These findings clearly showed that 250 μg/ml YS-1 combined with 3 nM ADM exhibited a synergistic inhibitory effect on the survival of 786-O cells.

YS-1 combined with ADM promote anti-proliferative activity in 786-O cells. To investigate the mechanism of the combined treatment on the survival of 786-O cells, we measured the
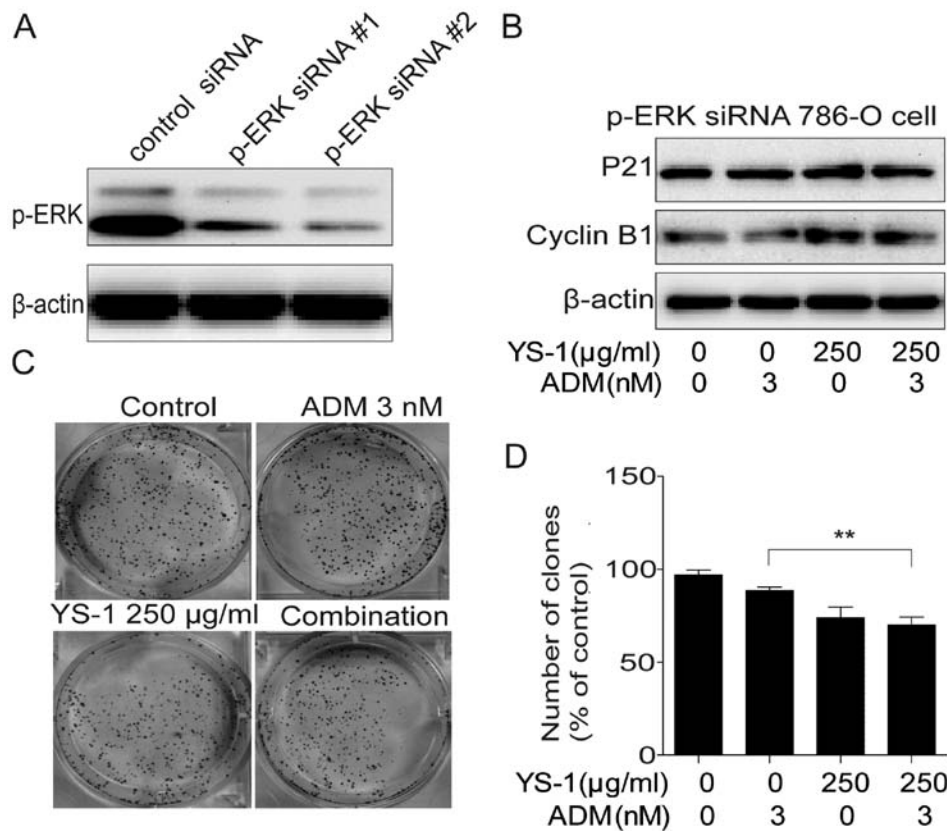
Figure 5. YS-1 combined with ADM play synergistic effects through p-ERK1/2 inhibition. (A) The ERK1/2 and p-ERK1/2 protein levels were examined by western blot analysis in 786-O cells transfected with ERK1/2 siRNA. (B) The ERK1/2 silenced 786-O cells were used to determine the effects of p-ERK1/2 on the combination anti-proliferation activity through cell cycle related protein assay. (C and D) The effects of the combination treatment on the 786-O cell clone formation activity when ERK1/2 was silenced. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test, [**]P<0.01.

effect of combination treatment on anti-proliferative activity. As shown in Fig. 2A and B, the combination treatment group inhibited the clone formation of 786-O cells significantly. Moreover, the combination group induced apparent G2/M cell cycle arrest in 786-O cells as assessed by flow analysis (Fig. 2C). Then we measured the levels of proteins involved in the G2/M cell cycle arrest pathway. The combination treatment increased the expression of P21 and reduced the expression of cyclin B1 (Fig. 2D). These findings clearly showed YS-1 combined with ADM promoted the anti-proliferative activity in 786-O cells.

Synergistic anticancer effect of YS-1 combined with ADM did not increase the pro-apoptotic activity in 786-O cells. Apoptosis is an important mechanism contributing to the cell survival reduction (24), so in our further investigation, we measured the effect of the combined treatment on the pro-apoptotic activity. As shown in Fig. 3A and B, after 6 h treatment, compared with the monotherapy group, the comb-therapy group could not show any synergistic pro-apoptotic effect, by flow analysis in 786-O cells. After that, we analysed the protein levels of Bcl-2 and the cleaved level of caspase-9 by western blot analysis in 786-O cells, and found that the combination group did not downregulate Bcl-2 or upregulate cleaved caspase-9 (Fig. 3C) compared with ADM monotherapy treatment group. Furthermore, the activity of caspase-3/7 was consistent with the results of flow analysis

and western analysis (Fig. 3D). These finding clearly showed YS-1 combined with ADM could not induce pro-apoptotic activity in 786-O cells.

YS-1 combined with ADM regulate anti-proliferative activity via down-regulating Ras/Raf/MEK/ERK1/2 pathway. Ras/Raf/MEK/ERK1/2 pathway could influence cell growth and drug resistance (19). Recently, research showed that the renal toxicity of ADM was mainly induced by the activation of ERK1/2 signaling pathway in kidney cells (13). Therefore, we investigated the effect of combination therapy on ERK1/2 pathway. As shown in Fig. 4A and B, the combination treatment group significantly reduced Ras/Raf/MEK/ERK1/2 pathway activity in 786-O cells compared with YS-1 or ADM monotherapy treatment group. Furthermore, the mRNA levels of ERK1 and ERK2 were slightly decreased when YS-1 was combined with ADM (Fig. 4C). The combination treatment reduced phosphorylated ERK1/2. However, it is not clear to which drug (or both) the function is due. Thus, we measured the effect of YS-1 and ADM separately on p-ERK1/2 in 786-O cells. As shown in Fig. 4D, a decrease in the expression of p-ERK1/2 as well as Notch1 was observed after 786-O cells were treated with YS-1 for 6 h; similarly, the expression of p-ERK1/2 was significantly increased after treatment of 786-O cells with ADM alone for 6 h. These results indicated that YS-1 promoted ERK1/2 inactivation through regulating Notch1 pathway, and YS-1 combined with ADM exerts its
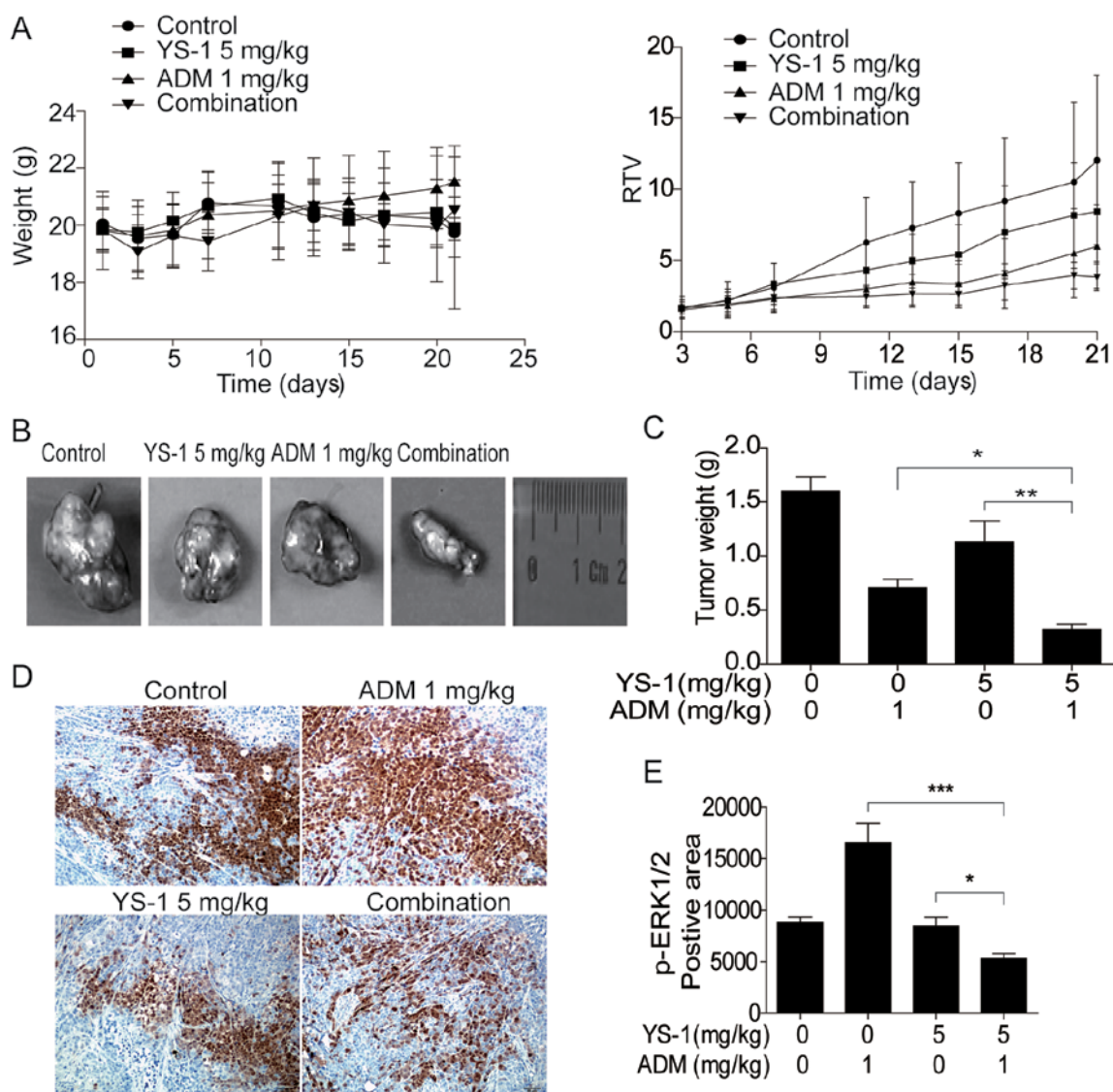
Figure 6. Combination treatment and inhibition of tumor growth in 786-O xenograft nude mice. (A) The animal weight and RTV were examined *in vivo*. (B) Representative photographs of tumors, respectively, in each group at the end of the experiment. (C) The statistical graph of tumor weight when YS-1 combined with ADM. (D and E) Tumor tissues from 786-O-xenografted nude mice after YS-1/ADM combination treatment were analyzed by immuno-histochemistry staining of p-ERK1/2. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test, *P<0.05; **P<0.01; ***P<0.001.

anti-proliferative activity via inhibiting the activation of ERK1/2 pathway.

YS-1 combined with ADM exerted synergistic effects through p-ERK1/2 inhibition. To confirm the involvement of p-ERK1/2 in the inhibition of proliferation by YS-1 combined with ADM, we knocked down ERK1/2 by using ERK1/2 siRNA. The ERK1/2 and p-ERK1/2 levels of 786-O cells were decreased remarkably (Fig. 5A). Then we examined the anti-proliferative effect of YS-1 combined with ADM on ERK1/2 knockdown 786-O cells, data showed that the combination treatment could not induce cell cycle arrest (Fig. 5B), and could not inhibit the clone ability of 786-O cells (Fig. 5C and D). These data confirmed that YS-1 combined with ADM exerted synergistic effects through p-ERK1/2 inhibition.

Combination treatment inhibits tumor growth in 786-O-xeno-grafted nude mice. To assess the efficiency of combination

therapy YS-1 and ADM *in vivo*, we established the 786-O cell xenograft model and evaluated the RTV and ERK1/2 activation. As shown in Fig. 6A, the value of RTV was decreased in the combination group, without showing any obvious toxic effect. Results showed that 5 mg/kg YS-1 combined with 1 mg/kg ADM exerted a significant synergistic inhibitory effect on 786-O xenografts (Fig. 6B and C). In tumor tissues from the 786-O-xenografted nude mice treated with YS-1/ADM combination, the positive areas for p-ERK1/2 were reduced significantly (Fig. 6D and E). These results suggested that YS-1 combined with ADM boosted the anti-proliferative effect *in vivo* via p-ERK1/2 inhibition, which was consistent with the *in vitro* data.

Discussion

In the present investigation, we demonstrated that p-ERK1/2 was involved in the chemoresistance to ADM in RCC cells;

ADM monotherapy caused renal toxicity by promoting the activation of ERK1/2 in kidney cells. We found that YS-1, a recombinant human p43 protein, could increase the sensitivity of 786-O cells to ADM by inhibiting the p-ERK1/2 level in 786-O cells. YS-1 promoted ERK1/2 inactivation through regulating the Notch1 pathway. By using ERK1/2 siRNA, we found the synergistic anti-proliferative effect of YS-1 combined with ADM was reversed. In 786-O cell xenograft model, we also found a significantly synergistic inhibitory effect on 786-O xenografts when 5 mg/kg YS-1 was combined with 1 mg/kg ADM. These findings suggested that YS-1 might synergistically combine with ADM to inhibit the RCCs through decreasing p-ERK1/2 modulation of EGFR downstream signaling.

As an important adjuvant treatment, chemotherapy serves as a necessary component of postoperative therapy for renal cancer (25). However, most traditional chemotherapeutic drugs lead to drug resistance and this has become a major obstacle in chemotherapy (26). Therefore, it is imperative to identify novel therapeutic targets which are involved in the acquisition of drug resistance.

Because of the advantages of combination with low toxicity, high efficiency, there have been many studies on the combined application of vascular inhibitors and cytotoxic drugs, such as: Combination of TNP-470 and mitomycin C (MMC), ADM, cisplatin, gemcitabine and 5-fluorouracil (5-FU) in nude mouse xenograft model, which significantly increased its anti-tumor activity (27-29); Doxorubicin with sorafenib augments cytotoxicity to RCC through p-ERK1/2 inhibition, this opposite response of p-eIF2α to sorafenib treatment may determine cell fate after sorafenib administration because increases of p-ERK1/2 and p-eIF2α can rescue cell death (30). In addition, some anticancer agents combined with anti-angiogenic drugs could improve therapeutic index through their anti-angiogenic effects (31). As shown in the mouse model, doxorubicin combined with anti-VEGFR2 antibody exerted an excellent therapeutic effect (32), YS-1 plus ADM might be another novel method in terms of antiangiogenic therapeutics.

However, further investigation of the effect of the YS-1 and ADM combination in clinical trials is necessary to confirm the promising *in vitro* and *in vivo* results reported here.

Acknowledgements

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