

Tetrandrine suppresses metastatic phenotype of prostate cancer cells by regulating Akt/mTOR/MMP-9 signaling pathway

BO KOU^{1*}, WEI LIU^{2*}, WENBO HE^{3*}, YUANYUAN ZHANG⁴, JIANJIE ZHENG¹,
YANG YAN¹, YONGJIAN ZHANG¹, SUOCHUN XU¹ and HAICHEN WANG¹

Departments of ¹Cadiovascular Sugery and ²Urology, First Affiliated Hospital of Medical School, Xi'an Jiaotong University; ³Medical School, Xi'an Jiaotong University; ⁴Department of Burn and Plastic Surgery, Xi'an Central Hospital, Xi'an, Shaanxi 710061, P.R. China

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Abstract. Tetrandrine (TET), a bisbenzylisoquinoline alkaloid found in traditional Chinese medicines, exerts anticancer activity *in vitro* and *in vivo*. However, its potential role in the prostate cancer metastatic process has not yet been elucidated. Thus, we investigated the inhibition effect of tetrandrine on prostate cancer migration and invasion and the corresponding molecular basis underlying its anticancer activity. Cell migration and invasion were determined using the Transwell chamber model. The protein expression of Akt, phosphorylated Akt, the mammalian target of rapamycin (mTOR), phosphorylated mTOR and matrix metalloproteinases 9 (MMP-9) was detected by western blot in the presence or absence of tetrandrine or in the group tetrandrine combination with LY294002 (inhibitor of Akt) and rapamycin (inhibitor of mTOR). Our studies showed that excluding the effect of tetrandrine on cell proliferation, tetrandrine significantly inhibited cell migration and invasion in prostate cancer DU145 and PC3 cells. Furthermore, tetrandrine decreased the protein levels of p-Akt, p-mTOR, and MMP-9. While the inhibition of Akt or mTOR by the respective inhibitors could potentiate this effect of tetrandrine on prostate cancer cells, the studies indicate that tetrandrine inhibits the metastasis process by negatively regulating the Akt/mTOR/MMP-9 signaling pathway. These results suggest that tetrandrine might serve as a potential metastasis suppressor to treat cancer cells that have escaped surgical removal or that have disseminated widely.

Introduction

Prostate cancer (PCa), one of the most prevalent malignancies in male patients worldwide, causes considerable morbidity and mortality, and a great deal of attention is currently given to its tumorigenesis. Androgen signaling based on the androgen receptor is an essential oncogenic pathway for PCa progression (1) and deprivation of androgen is used widely as the basic therapeutic strategy for patients with androgen-dependent PCa (2). Nevertheless, most patients have a recurrence with a more aggressive form, known as castration-resistant prostate cancer (CRPC) (3), with 80% of CRPC patients experiencing the presence of bony metastases. Due to its frequent metastasis and significant heterogeneity, CRPC is difficult to cure, and it has become a vexing problem for the oncologists and clinicians (4). Therefore, it is of vital importance to identify appropriate agents to kill selectively or sensitize prostate cancer.

Tetrandrine (TET) is a bisbenzylisoquinoline alkaloid with the molecular structural formula $C_{38}H_{42}N_2O_6$ and a molecular weight of 622.74988 g/mol. Tetrandrine isolated from the Chinese herbal medicine *Stephaania* has been applied to a very broad spectrum of pharmacological events (5), such as antihypertension, antiarrhythmia, and antirheumatism. In recent decades, tetrandrine has been used as an antifibrotic agent in the treatment of silicosis (6), and accumulating evidence suggests that tetrandrine exerts strong anticancer effects on diverse cancers *in vitro*, including colon (7,8), hepatoma (9), bladder (10), and lung cancer (11). The beneficial impact of tetrandrine on tumor cell multidrug resistance (12), radiosensitization (13), and angiogenesis (14) has also attracted a great deal of attention. Also, tetrandrine has been shown to modulate multiple cellular signaling events, including the Wnt/ β signaling pathway (15), mitogen-activated protein kinase activation (16), and NF- κ B signaling pathway.

In our previous study, we found that tetrandrine exhibited anticancer effects against PCa *in vitro* by suppressing cell proliferation, inducing apoptosis and inhibiting cell migration and invasion. Despite its potential as an anticancer constituent, the underlying mechanism of tetrandrine on PCa metastasis has not yet been elucidated. Therefore, we investigated the possible mechanism of tetrandrine on the inhibitory effect of metastasis in PCa DU145 and PC-3 cells.

Correspondence to: Dr Suochun Xu or Dr Haichen Wang, Department of Cadiovascular Sugery, First Affiliated Hospital of Medical School, Xi'an Jiaotong University, #277 Yanta West Road, Xi'an, Shaanxi 710061, P.R China
E-mail: xusuochun@163.com
E-mail: whccvscd@126.com

*Contributed equally

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Materials and methods

Cell culture. Human PCa cell lines DU145 and PC-3 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium/1640 supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), in a humidified atmosphere with 5% CO₂ at 37°C.

Reagents. Tetrandrine (C₃₈H₄₂N₂O₆) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tetrandrine was diluted with 0.1 mol/l HCl at a concentration of 25 mg/ml⁻¹, then added to the cell culture supernatant in appropriate proportions. Antibodies against Akt, phospho-Akt, the mammalian target of rapamycin (mTOR), matrix metalloproteinase-9 (MMP-9) and peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). LY294002 (Akt inhibitor) and rapamycin (mTOR inhibitor) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) detection system was obtained from Amersham Life Science, Inc. (Arlington Heights, IL, USA).

MTT assay. A modified MTT assay was used to assess cell proliferation viability. Briefly, DU145 and PC-3 cells were seeded in 96-well plates (8×10³ cells/well, 90% density) and incubated in the presence or absence of tetrandrine for various periods of time. Then, 0.5 mg/ml MTT dye solution was added to each well and incubated at 37°C for 4 h. After incubation, the culture medium was discarded and the cells were lysed with dimethyl sulfoxide to dissolve the formazan crystals. Absorbance at a wavelength of 490 nm was detected using a 96-well microplate reader (Bio-Rad, Hercules, CA, USA). The experiments were performed in triplicate.

Transwell migration assay. Transwell migration assays were performed using PCa DU145 and PC3 cells after treatment with tetrandrine. Cells (DU145: 6×10⁴ or PC-3: 5×10⁴ in 200 µl medium of serum starvation, respectively) were seeded into the top chamber, and 800 µl of medium supplemented with 10% fetal calf serum were added to the lower chamber. After incubation at 37°C for various times, cells adhering to the top chambers were removed with a cotton swab. The migratory cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, Shanghai, China). Cells that migrated to the lower surface were counted in five randomly chosen visual fields under a microscope at x100 magnification. The data were obtained from three independent experiments.

Matrigel invasion assay. The Transwell chambers (polycarbonic membrane, 6.5-mm diameter, 8 µm pore size) were coated with 50 µl Matrigel (Matrigel:serum-free medium 1:5). After incubation at 37°C for five hours, the cells (DU145: 10×10⁴ or PC-3: 12×10⁴ in 200 µl medium of serum starvation, respectively) were treated according to the protocol procedure, which was similar to the Transwell migration assay.

Western blotting. The PCa cells were harvested 24 h after the tetrandrine treatment, and the total cell lysates were denatured with lysis buffer [10 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/l⁻¹ ethylenediaminetetraacetic acid, 1 mmol/l ethylene glycol tetraacetic acid, 0.3 mmol/l phenylmethylsulfonyl fluoride, 0.2 mmol/l sodium orthovanadate, 1% NP-40, 10 mg/ml leupeptin, and 10 mg/ml aprotinin]. Then clarified protein lysates (~30-60 µg whole-cell lysates, mitochondrial and cytosolic fractions) were resolved electrophoretically on denaturing SDS-polyacrylamide gel (10%) and transferred to nitrocellulose membranes. Immunoblotting was performed with the primary antibody, anti-MMP-9, as well as anti-mTOR, and p-Akt overnight at 4°C. The membranes were washed and incubated with peroxidase-conjugated secondary antibody at room temperature (25°C). Ultimately, the proteins of interest were visualized with ECL Substrate and exposed to X-ray film.

Gelatin zymography. Gelatin zymography was performed following the standardized protocol (17). Briefly, 5×10⁵ cells were treated with tetrandrine for 24 h, after which the supernatants were collected to load onto 10% polyacrylamide gels and co-polymerized with 0.1% gelatin (Sigma). After undergoing electrophoresis, the gels were washed twice for 40 min in 2.5% Triton X-100 and 50 mmol/l Tris-HCl. Then, the gels were incubated for the next 24 h at 37°C in buffer containing 50 mmol/l Tris-HCl, 5 mmol/l CaCl₂ and 0.02% NaN₃, followed by staining with Coomassie brilliant G-250 and destaining with 20% methanol and 10% acetic acid. Finally, the gels were visualized with a molecular imager (Chemidoc XRS+, Bio-Rad).

Statistical analysis. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Statistical differences between the vehicle and drug treatment groups were compared by one-way analysis of variance, and Dunnett's t-test was used for multiple comparisons. Student's t-test (two-sided) was used for comparisons involving only two groups. A value of P<0.05 was considered statistically significant.

Results

Effects of tetrandrine on cell migration and invasion in prostate cancer DU145 and PC-3 cells. The MTT assays showed that cell proliferation was significantly inhibited by tetrandrine at a concentration of ≥2.5 µM in a cell density >90% (Fig. 1). In view of these results, tetrandrine at 2.5 µM (a <10% inhibitory rate) was chosen as the representative dose in the subsequent *in vitro* studies, to exclude the suppressing interference from PCa proliferation by tetrandrine.

To determine whether tetrandrine regulates cellular metastatic processes, we detected the effects of tetrandrine on PCa cell migration. Using a Transwell migration assay, we found that tetrandrine functioning in the DU145 cell line decreased cell migration approximately 2.5-fold after 24 h (Fig. 2A and C). Similarly, treatment of the PC-3 cells with tetrandrine resulted in a greater than 2.8-fold decrease in migration compared to the control group (Fig. 2B and D).

Next, using a Matrigel invasion assay, we explored whether tetrandrine could affect the invasiveness of PCa

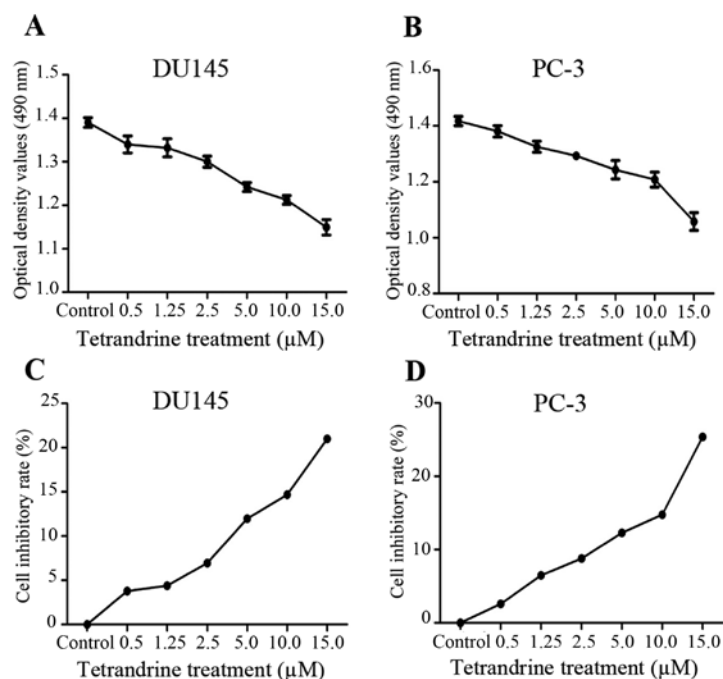


Figure 1. Tetrandrine decreases prostate cancer cell proliferation. The proliferation of cells <90% cell density with various doses of tetrandrine was compared to that of negative-control cells. The mean cell number (\pm SEM) of three independent experiments performed in triplicate is shown for DU145 (A and C) and PC-3 (B and D) cells.

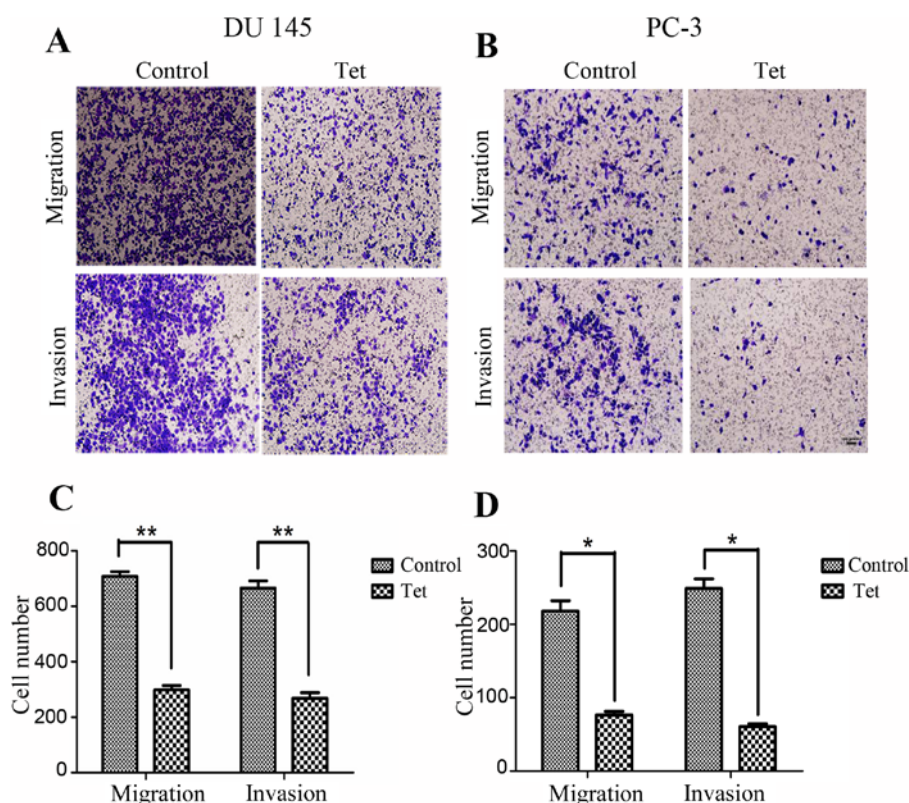


Figure 2. Tetrandrine decreases cell migration and invasiveness in prostate cancer. Transwell invasion assay and Matrigel invasion assay were performed on DU145 (A and C) and PC-3 (B and D) cells treated with tetrandrine. Upper panels show representative regions of the chamber filters with crystal violet-stained cells. The fold change represents the number of migrated or invaded cells per chamber divided by controls from three independent experiments performed in triplicate ($P < 0.05$).

cells. Serum-starved cells were added to the upper chambers of the Transwell and the total number of cells invading through the Matrigel barrier in response to a chemoattractant (serum)

at various times were counted. Tetrandrine significantly decreased the number of invading cells by >2.4-fold and 4-fold in the DU145 and PC-3 cells, respectively (Fig. 2).

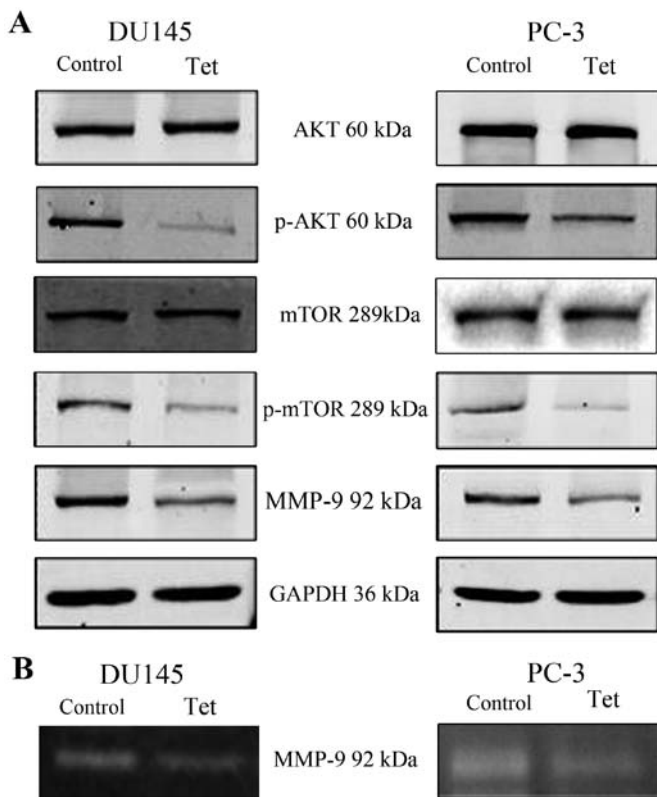


Figure 3. Tetrandrine significantly reduces phospho-AKT, phospho-mTOR and MMP-9 expression and decreases MMP-9 activity in prostate cancer cell lines. (A) DU145 or PC3 cells with tetrandrine treatment were subjected to western blotting for AKT, phospho-AKT, mTOR, phospho-mTOR and MMP-9. GAPDH was used as a loading control. Representative blots from three experiments are shown. (B) Representative gelatin zymography of the enzyme activity of MMP-9 in the supernatants of DU145 and PC-3 exposed to tetrandrine. The experiments were performed in triplicate.

These results suggest that tetrandrine might play a vital role in inhibiting the migration and invasion potential of human PCa cells, as indicated by the observation in the Transwell migration and Matrigel invasion assays.

Identification of the signaling pathway regulated by tetrandrine in prostate cancer. To define the underlying mechanism of tetrandrine-mediated inhibition of metastatic processes in PCa, we investigated the expression of several key factors in cancer cell migration and invasion, including E-cadherin, N-cadherin, vimentin, and MMP-9. To determine the impact of tetrandrine on the production of proteinases by DU145 and PC-3, protein lysates were collected and subjected to SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3, the presence of proteinases led to a clear band at 92 kDa in the nitrocellulose membranes, which was assigned to MMP-9, and tetrandrine reduced MMP-9 protein levels (Fig. 3A) and decreased MMP-9 activity (Fig. 3B), which corresponded to the inhibition of invasiveness in the DU145 and PC-3 cells. However, the protein levels of E-cadherin, N-cadherin and vimentin in the tetrandrine treatment remained the same (data not shown).

To detect the upstream of MMP-9, we found that the pretreatment with tetrandrine resulted in a marked decrease in the protein levels of phosphorylated Akt (p-Akt) and phosphorylated mTOR (p-mTOR) in both the DU145 and

PC-3 cells (Fig. 3). To gain further insights into the molecular mechanisms of tetrandrine in the PCa metastasis process, LY294002 (Akt inhibitor) or rapamycin (mTOR inhibitor) was selected to combine with tetrandrine for functional and mechanism research. The data showed that pretreatment with LY294002 decreased the tetrandrine-inhibited migration and invasion in the DU145 and PC-3 cells (Fig. 4), and reduced the p-mTOR and MMP-9 protein levels (Fig. 5) much more significantly compared with the tetrandrine alone group. This result suggests that Akt protein was passably related to the antimetastasis effect of tetrandrine.

Given that Akt protein might contribute to the anticancer activity of tetrandrine, the downstream of Akt was evaluated to define further the molecular basis of tetrandrine-inhibited metastasis. Similar results were observed in a combination of tetrandrine and rapamycin. The combined effect of the two agents was found to suppress prostate cancer cell migration and invasion (Fig. 6), and diminish of MMP-9 protein levels more effectively (Fig. 7); there was no significant change in NF- κ B protein level (data not shown).

Taken together, our findings show that the Akt/mTOR/MMP-9 signaling pathway was partially associated with tetrandrine-inhibited metastasis in CRPC cells, suggesting that tetrandrine might be a potential promising agents in the suppression of metastatic phenotypes in PCa treatment.

Discussion

In recent decades, the incidence of PCa has increased sharply in Asian countries (18). Until now, the majority of clinical trials have achieved limited benefits in the treatment of advanced PCa, mainly because of the high incidence of invasion and metastasis. Metastasis, a symbol of malignancy, is the migration of cancer cells from the original tumor site to distant organs through the bloodstream or lymph system. Metastasis development is a multi-step process that is implicated in such activities as local invasion, transfer, extravasation, and tumor deposit (19). Shedding light on the mechanisms that facilitate tumor cell migration and invasion is of major concern in cancer research, as approximately 90% of deaths from solid tumors arise from metastasis. The median survival rate for patients with metastatic PCa is far less than five years while the opposite is true for men with localized disease (19). Based on these factors, it is known that the tendency to invade and transfer is one of the obstacles facing PCa treatments. Hence, there is an urgent need to develop new agents for PCa therapy.

Tetrandrine, a traditional Chinese medicine, has been shown to exhibit a wide range of uses. Accumulating evidence indicates that tetrandrine exerts antitumor effects against various cancer cells *in vitro* by inducing cell cycle arrest and inhibiting angiogenesis. In our previous study, we found that the induction of PCa cell apoptosis by tetrandrine might be mediated partially by the activation of caspase cascade. Nevertheless, it is conceivable that the anticancer activity of tetrandrine might be involved with many other signaling pathways. It has been reported that tetrandrine induces apoptosis in hepatocellular carcinoma cells by generating reactive oxygen species, followed by the repression of Akt activity (20). Tetrandrine also exhibits anti-proliferation effects by targeting β -catenin activity to a certain extent. In addition, it has been

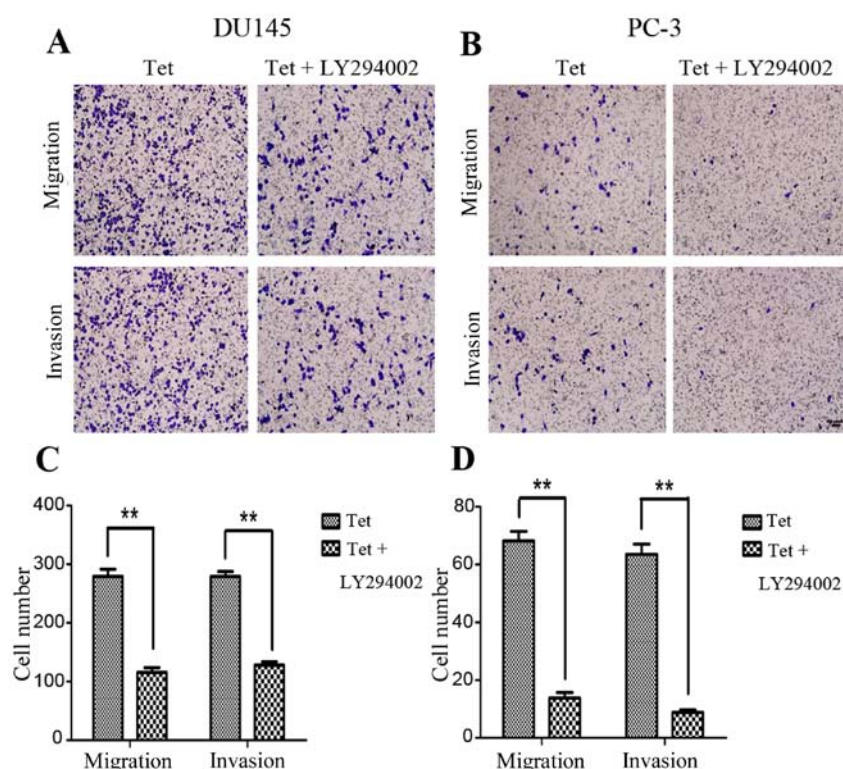


Figure 4. Tetrandrine combined with Akt inhibitor LY294002 significantly inhibits cell migration and invasiveness in castration-resistant prostate cancer cell lines. DU145 (A and C) and PC-3 (B and D) cells that penetrated the membrane were counted after 24 h as described in Materials and methods. Quantification of three independent experiments is shown with error bars representing SEM.

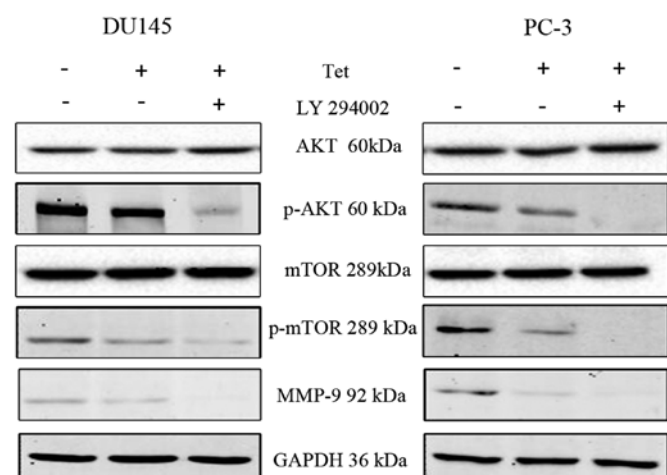


Figure 5. Tetrandrine and Akt inhibitor LY294002 synergistically decreased phospho-mTOR and MMP-9 protein levels more effectively than tetrandrine alone. DU145 and PC-3 cells treated with tetrandrine or LY294002 were subjected to western blotting for phospho-Akt, mTOR, phospho-mTOR, MMP-9, and GAPDH. Representative blots from three experiments are shown.

reported that tetrandrine inhibited *in vivo* tumor metastasis in a mouse model of stage IV breast cancer. The inhibitory effect of tetrandrine on breast cancer metastasis might be mediated partly by regulating endothelial cell-specific molecule-1 (ESM-1), integrin $\beta 5$ protein and intercellular cell adhesion molecule-1 (ICAM-1) levels (21).

Metastasis in patients with PCa is one of the main lethal factors blocking direct treatment. In our studies, the impact of tetrandrine resulted in decreased cell migration and invasion in

the metastatic CRPC cell lines DU145 and PC-3. Identifying the molecular mechanisms of tetrandrine on the metastatic process underlying PCa is crucial in order to clarify our understanding of tumorigenesis and metastasis. As such, we have detected the expression of several key factors in the cell metastatic process and found that tetrandrine might decrease MMP-9 protein levels and activity in two independent CRPC cell lines; however, we found no significant changes in MMP-2 protein levels. These findings indicate that MMP-9 deregulation could be a vital event in PCa progression and are consistent with our data that tetrandrine expression resulted in decreased MMP-9 levels and a decline in metastatic traits.

In addition to speculating that MMP-9 might be associated with anti-metastasis activity displayed by tetrandrine, we also measured the Akt signaling pathway, which is upstream of MMP-9. The results showed that p-Akt and p-mTOR protein levels decreased significantly with tetrandrine treatment. Next, to determine whether the ability of tetrandrine to inhibit PCa metastasis was mediated through a decrease in p-Akt and p-mTOR kinase activity, we treated DU145 and PC-3 cells with tetrandrine in combination with LY294002 and rapamycin, respectively, and then documented cell migration and invasion using Transwell assay and western blotting. Our data in the present study demonstrated that the inhibition of metastasis in DU145 and PC3 cells by tetrandrine was markedly enhanced in combination with LY294002 or rapamycin pre-treatment.

Furthermore, tetrandrine and LY294002 synergistically decreased p-mTOR and MMP-9 protein levels, and rapamycin worked in the same manner. However, whether the decrease in p-mTOR protein level is directly related to downregulation of MMP-9 protein needs to be investigated further. These data

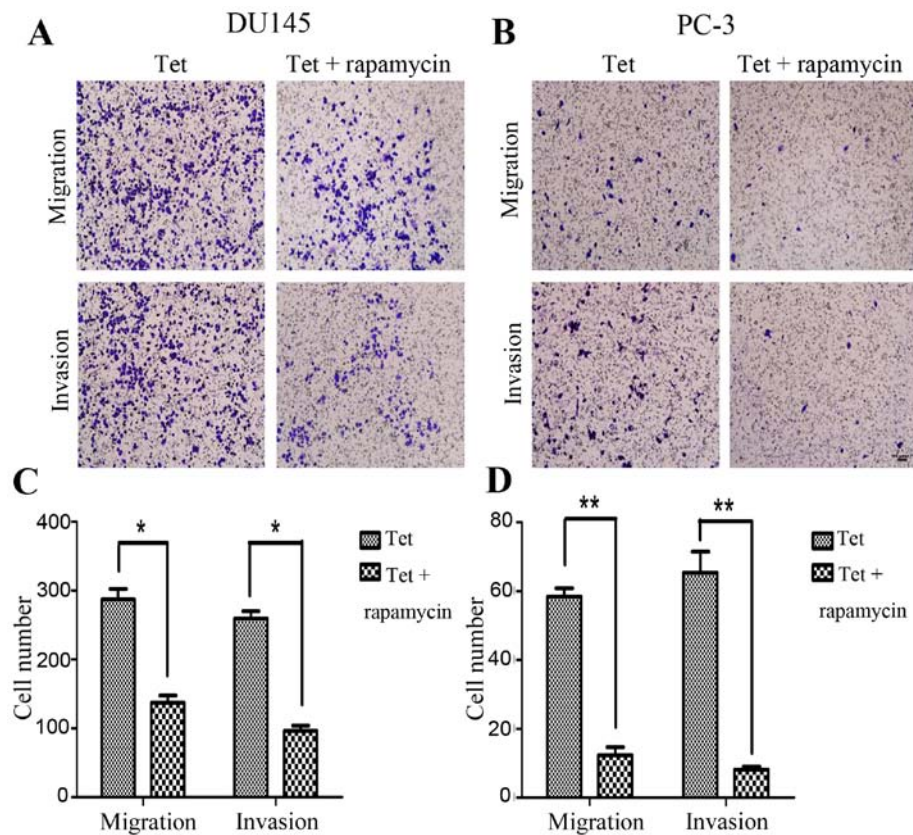


Figure 6. Pretreatment with mTOR inhibitor rapamycin more significantly decreased tetrandrine-inhibited migration and invasion in castration-resistant prostate cancer cell lines. Transwell migration assay and Matrigel invasion assay were performed on DU145 (A and C) and PC-3 (B and D) cells treated with tetrandrine or rapamycin. Five fields were randomly chosen and visualized by microscopy to evaluate the ability of cell migration and invasiveness. Quantification of three independent experiments is shown, with error bars representing SEM.

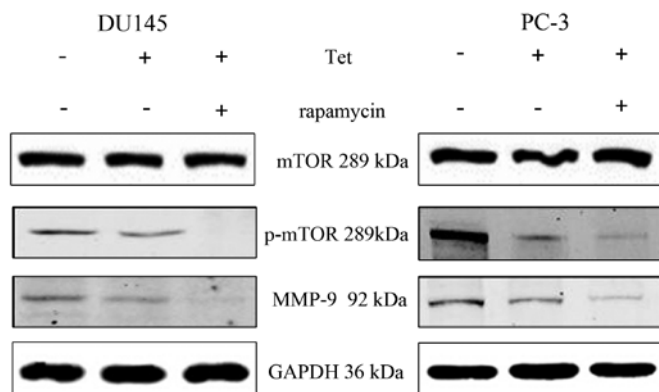


Figure 7. The combined effect of the two agents, tetrandrine and mTOR inhibitor rapamycin, were found to diminish levels of protein MMP-9 more effectively compared with tetrandrine alone. Western blotting was used to detect the levels of mTOR, phospho-mTOR and MMP-9 protein with various treatments in DU145 and PC-3 cells. Representative blots of three experiments are shown.

suggest that, at least partially, the Akt/mTOR/MMP-9 signaling pathway is involved in the tetrandrine-mediated metastatic inhibition of PCa. However, several limitations exist in our study. On the one hand, the lack of animal models in our studies is a short coming, and further considerations should be taken about tetrandrine's function on animal models in our future study. On the other hand, we only observe the anti-metastatic effect

of tetrandrine on prostate cancer cells by negatively regulating Akt/mTOR/MMP-9 signaling pathway. Whether alternative signaling pathways participated in this effect of tetrandrine has not yet been elucidated. Hence, it is of necessity for us to resolve these questions in further research.

While our studies have shown that tetrandrine is valid as a single agent for tumor therapy, it might be rational to speculate that it will be used widely in combination with other agents in the clinical setting. Our findings demonstrated that tetrandrine decreased not only p-Akt and p-mTOR activity, but also showed good synergy with LY294002 or rapamycin in the inhibition of PCa cells. Thus, our studies suggest that tetrandrine might be a stronger chemotherapeutic agent when combined with LY294002 or rapamycin.

To the best of our knowledge, our studies provide the first evidence that tetrandrine inhibits PCa cells metastasis by repressing Akt/mTOR and inactivating MMP-9 and that Akt/mTOR appear to be the upstream regulators of MMP-9 inactivation. In conclusion, it is suggested that tetrandrine suppresses metastasis by negatively regulating the Akt/mTOR/MMP-9 signaling pathway, and that it could serve as a potential inhibitor of tumor metastasis in future therapeutic interventions in PCa patients.

Acknowledgements

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