Terrestrosin D, a steroidal saponin from *Tribulus terrestris* L., inhibits the growth and angiogenesis of human prostate cancer *in vitro* and *in vivo*

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Introduction

Prostate cancer (PCa) is a frequently diagnosed cancer and leading cause of cancer-related death in males. There is currently no systemic and effective therapy for castration-resistant PCa (CRPC). In this study, we are the first to describe that terrestrosin D (TED), a steroidal saponin from *Tribulus terrestris* L., exhibits antitumor and antiangiogenic potential through induction of apoptosis and cell cycle arrest in PCa and endothelial cells *in vitro* and is effective in PC-3 cells-bearing nude mouse xenograft model.

Materials and Methods

Firstly, to investigate the anti-cancer activity of TED, we assessed its repression on the growth of three androgen-independent PCa cells, PC-3, PC-3M and DU145, and two androgen-dependent PCa cells, LNCaP and 22RV1. Secondly, to access the antiangiogenic property of TED, we examined its inhibitory effect on the viability of two endothelial cells, HUVEC and HMVEC-Bd.

1 Cell Viability Assay

After 24 hours exposure to TED, 3-(4, 5-dimethyl-thiogol-2-yl)-2, 5-diplenyltetrazollium (MTT) was added to each well and incubated for 3 hours to allow the mitochondria dehydrogenase to covert MTT into an insoluble formazan product. The medium was then aspirated, and the sediment was dissolved in DMSO. Absorbance was detected at 570 nm for cell viability.

2 Flow Cytometric Analysis

After treatment, the cells were trypsinized and washed with PBS. The samples were stained with propidium iodide (PI, 50 mg/mL), Annexin-V and PI, and tetramethylrhodamine methyl ester (TMRM, 50 nM) and then analyzed by FACScan flow cytometry (Becton Dickinson) for cell cycle distribution, apoptosis and mitochondrial membrane potential assay, respectively. Data analysis was performed with ModFIT software.

3 Caspase-3 Activity Assay

The cells were harvested and the lysates were prepared after treatment with TED. Then the lysates were mixed with reaction buffer containing 20 mM HEPES buffer (pH 7.5), 0.1 M NaCl and 10 uM of Ac-DEVD-MCA as a substrate. Fluorescence of released 7-amino-4-methylcoumarin (AMC) was measured using a fluorescence spectrophotometer with the excitation and emission wavelength of 355 and 460 nm, respectively.

4 Quantification of VEGF Secretion

After treatment, the cells were washed with PBS and fresh medium were added to each well. Twenty-four hours later, VEGF in culture supernatants were measured as described by the manufacturer’s introduction using a quantitative enzyme-linked immuosorent assay (ELISA).

5 *In Vivo* Tumor Xenograft Study

To investigate whether TED inhibited tumor growth through the inhibition of tumor angiogenesis, we used anti-CD31 antibody to stain solid tumor sections in the xenograft mouse model. PC-3 cells were injected subcutaneously into the flasks of the mice (2×10^6 cells in 100 uL of medium). To study nonestablished tumors, the treatment was initiated after tumor implantation. TED (25 or 50 mg/kg body weight) in 100 uL saline was administrated intraperitoneally 3 times a week, 4 weeks. The control group was treated with an equal volume of saline. Tumor volume was monitored using calipers and established according to the following formula: tumor volume (mm^3) = $\pi ab^2/6$, where is the length and b is the width. Proliferation and apoptosis of cancer cells, and microvessels density were determined using immunohistochemistry with antibody for single stranded DNA (ssDNA) and CD31, respectively.

Results

1 TED Results in Cell Cycle Arrest and Growth Inhibition in PC-3 Cells and HUVECs

After treatment with for 24 hours, TED induced a dose-dependent decrease in cell viability. In PC-3 cells, TED caused significant increases G1 populations and concomitant decreases in S phase populations. TED exhibited potential growth inhibition of endothelial cells in a dose-dependent manner. In HUVECs, TED induced cell cycle arrest in the S phase.

2 TED Induces Apoptosis in PC-3 Cells and HUVECs

Treatment with TED for 24 hours induced apoptosis in a dose-dependent manner in PC-3 cells. However, caspase inhibitor (z-VAD) did not reverse TED-induced decreases in cell viability else apoptosis. Additionally, analysis of the activity of caspase-3, a major effector caspase involved in caspase-dependent apoptosis, did not show any increase after treatment with TED. In HUVECs, TED induced apoptosis in a dose-dependent manner. No effect of z-VAD on TED-induced apoptosis and reduced activity of caspase-3 indicated TED induced caspase-independent apoptotic cell death in HUVECs.

3 TED Inhibits Tumor Growth *in Vivo*

Administration of 50mg/kg TED substantially suppressed tumor growth. The average tumor volume in the control mice was 315.75 ± 108.54 mm^3 after 28 days, whereas the average tumor volume in the TED-treated mice was 127.41 ± 51.65 mm^3. However, the same dose of TED had no significant effect on the body weight of mice. A lower dose of TED (25mg/kg) had little effect on the tumor growth. Immunohistochemical analysis of tumors with ssDNA showed that TED induced apoptotic death in xenograft tumor tissues. Microvessel density (MVD) counted per 400× field in TED-treated group was 17.6 ± 1.6 compared with 31.5 ± 5.8 in the control group, indicating that TED significantly inhibited tumor angiogenesis.

4 TED Increases VEGF Secretion in PC-3 Cells and HUVECs

Treatment with TED for 24 hours increased VEGF secretion in a dose-dependent manner in PC-3 cells and HUVECs. The ELISA analysis showed that treatment with 5 uM TED resulted in a 1.86-fold increase of VEGF levels. And at a dose of 3 uM,

increase of 11.21-fold in VEGF secretion was observed in HUVECs.

Discussion

Attention has been focused on natural products as potential sources of novel anticancer drugs over the last few decades. In this study, we elevated the effect of TED on the growth of human PCa. TED effectively targeted and inhibited the growth of PCa cells and enthothelial cells by inducing cell cycle arrest and apoptosis, which led to the suppression of tumor growth and angiogenesis. Like *T. terrestris* extracts, TED can inhibit the cancer cells growth by induction of cell cycle arrest and apoptosis. Considering that TED is the main constituents of *T. terrestris*, TED may partly represent the pharmacological effects, especially anticancer effect of *T. terrestris*. Our study also reveals that TED has antiangiogenic property, which may suggest the anticancer effect of *T. terrestris* is also due to inhibition of tumor angiogenesis. Currently, maximal androgen blockade (MAB) therapy is an effective treatment for prostate cancer. But it induces sexual dysfunction that troubles most of patients, especially for young patients. TED treatment may be a potential therapy for young patients to escape from sexual dysfunction. We suggest TED treatment could be as first line therapy to young patients who suffer prostate cancer. Additional studies will focus on dissecting the mechanism of its action on the apoptotic pathway and antiangiogenesis in detail.