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Differential effects of anti-metastatic mechanism of Tian-Xian liquid (TXL) and its bioactive fractions on human colorectal cancer models☆

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ABSTRACT

Aim of study: This study aimed to elucidate and compare the anti-metastatic mechanism of Tian-Xian liquid (TXL) and its bioactive components namely butanol (BU), ethyl-acetate (EA) and aqueous (WA) fractions on human colorectal cancer *in vitro* (HT-29 cancer cells) and *in vivo* (nude mouse xenografts).
Materials and methods: The anti-proliferative effects of TXL and its bioactive components in HT-29 cells were determined by MTT assay. Their modulations on the potential angiogenic and metastatic marker expressions on HT-29 cells and xenografts were investigated by real-time PCR and Western blot at transcriptional and translational levels, respectively. For the *in vitro* study, migration abilities of HT-29 cells were determined using wound healing assay. For the *in vivo* study, daily measurements of the tumor size and volume of the xenografts were also performed.
Results: TXL, BU, EA and WA effectively inhibited the proliferation of HT-29 cells in a dose- and time-dependent manner. The IC_{50} value of TXL on HT-29 cells was obtained after incubation with 1% (v/v) TXL for 4 h; whereas IC_{50} values were obtained for the following bioactive components: BU at 1.25% (v/v); EA at 5% (v/v); and WA at 0.3125% (v/v). It was found that 1% (v/v) TXL significantly down-regulated MMP2 and MMP7 expression at both transcriptional and translational levels and it reduced MMP9 and VEGF protein expression *in vitro*. TXL decreased the metastatic ability of HT-29 cells as demonstrated by wound healing assay. TXL and its bioactive fractions caused no significant changes in the body weight indicating lack of toxicity to the xenografts.
Conclusions: In summary, TXL multi-targeted to down-regulate the metastatic markers in both *in vitro* and *in vivo* models. However, the effects of its bioactive fractions were not obvious. This study profoundly elucidated the anti-proliferative mechanism of TXL, which is vital for the development of future anti-cancer regime in Chinese medicinal formulations.

1. Introduction

Traditional Chinese medicines (TCMs), also known as folk medicines with their unique systematic approach, have been used in China for several thousand years. These herbal medications exhibit anti-neoplastic properties and have long been applied for cancer treatment (Jonas, 1998; Yuan and Lin, 2000; Gao et al., 2007; Huang et al., 2008). However, the scientific mechanisms of their efficacy in cancer treatment remain to be investigated (Cho and Chen, 2009). In order to advance our knowledge of TCM in cancer studies, the attributes of these herbal agents and conflicting issues related to their efficacy deserve further investigations before clinical consumption to restore human health (Ruan et al., 2006).

Tian-Xian liquid (TXL) has been commercially used as an anti-cancer dietary supplement for more than 10 years without known adverse effects (Sun et al., 2004). TXL is an aqueous extract from a Chinese herbal mixture consisting mainly of 10 Chinese medicinal herbs: *Radix Ginseng*, *Cordyceps*, *Radix Astragali*, *Radix Glycyrrhizae*, *Rhizoma Dioscorea*, *Margarita*, *Fructus Lycii*, *Ganoderma*, *Fructus Ligustri Lucidi*, and *Herba Scutellariae Barbatae*. Previous experiments reported that TXL had inhibitory effects on different carcinomas, including cervical carcinoma, lung carcinoma and breast cancer (Sun et al., 2005a,b; Chia et al., 2010). Our group has demonstrated the quality control of TXL and studied the cytotoxic effect of TXL in colorectal carcinoma *in vitro* and *in vivo* (Sze

Abbreviations: FBS, fetal bovine serum; MMP2, matrix metalloproteinase 2; MMP7, matrix metalloproteinase 7; MMP9, matrix metalloproteinase 9; PBS, Dulbecco's phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBS-T, Tris-buffered saline Tween-20; TXL, Tian-Xian liquid; VEGF, vascular endothelial growth factor.

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et al., 2011). Here we further investigate and compare the anti-metastatic effects of TXL and its fractions in human colorectal carcinoma cells *in vitro* and *in vivo* in order to demonstrate the multi-targeting characteristics of TXL in colorectal cancer.

Colorectal carcinoma remains the second leading cancer in Hong Kong and ranks the third leading cause of death in the West (Hong Kong Cancer Registry, 2007). In the last decade, irinotecan, oxaliplatin and the standard fluorouracil-based chemotherapy regimens have set the new benchmark of survival for patients with metastatic colorectal cancer (Prat et al., 2007). Despite these therapeutic advances, there is a strong medical need for more effective and well-tolerated therapies for colorectal cancer. Therefore, it is imperative to develop new therapeutic regimes for colorectal cancer.

Angiogenesis is required for the initiation of metastasis, whereas migration is required for invasion. Identifying key proteins involved in these processes, such as matrix metalloproteinases (MMPs), is important for new and effective cancer treatment regimes. Tumor metastasis is a complex and multi-stage process present in the tumor microenvironment (Polette et al., 2004). One of the critical factors for tumor metastasis and invasion is the large production of proteolytic enzymes, matrix metalloproteinases (MMPs) that principally participate in the degradation of basement membranes and stromal extracellular matrix (ECM) (Eccles and Welch, 2007). MMPs belong to a large family of zinc- and calcium-dependent enzymes. MMPs are initially expressed in an enzymatically inactive state due to the interaction of a cysteine residue of the pro-domain with the zinc ion of the catalytic site. Only after disruption of this interaction by cysteine switch, which is usually mediated by proteolytic removal of the pro-domain or chemical modification of the cysteine residue, does the enzyme become proteolytically active. The pro-domain requires proteolytic cleavage by convertases, which occurs intracellularly by furin or extracellularly by other MMPs or serine proteinases such as plasmin (Sternlicht and Werb, 2001). The major MMPs involved in tumor angiogenesis are MMP2, 9 and 14, and to a lesser extent MMP1 and 7 (Kessenbrock et al., 2010). MMP2 is a prevalent type IV collagenase named gelatinase A that will congregate at the leading edge of a metastatic cell in order to facilitate confined and coordinated breakdown of the ECM barrier. It has been found that a high level of tissue MMP2 was associated with poor survival in gastric carcinomas (Turpeenniemi-Hujanen, 2005). MMP9 has a distinct role in tumor angiogenesis. It mainly regulates the bioavailability of vascular endothelial growth factor (VEGF), the most potent inducer of tumor angiogenesis and a major therapeutic target (Turpeenniemi-Hujanen, 2005). Some reports demonstrated that herbal extracts have the potential to inhibit the metastatic and angiogenic effects in hepatocellular carcinoma cells by down-regulation of MMP2 and VEGF expression *in vitro* and *in vivo* (Huang et al., 2009; Zhang et al., 2009). These studies suggest that TCM could play a vital role in preventing metastasis and anti-angiogenesis in cancers by elucidating the molecular pathways in different cancers that are modulated by TCM.

The dependence of tumor growth and metastasis on blood vessels makes angiogenesis a rational target for cancer therapy (Carmeliet and Jain, 2000). Angiogenesis is a critical step for tumor growth and metastatic spread. New blood vessel formation depends on the regulation of angiogenic proteins. Among these, vascular endothelial growth factor (VEGF) and its receptors are present in one of the major pathways involved in angiogenesis (Hicklin and Ellis, 2005). VEGF plays a vital role in tumor angiogenesis and its expression is inversely correlated with patient survival in many human cancers, including colorectal carcinoma (Logan-Collins et al., 2008; Zafirellis et al., 2008). VEGF is also associated, not only with cancer angiogenesis, but also with those involved in wound healing and other important pathologies (Ferrara, 1995). VEGF and its receptors are coordinated with integrins to promote cell adhesion to the extracellular matrix (ECM). Studies demonstrated that expression of both VEGF and integrin has a critical role in promoting ECM degradation and in mediating tumor cell invasiveness via MMP9 in colon and gastric cancers (Wang et al., 2008; Zhao et al., 2010). VEGF expression is regulated by various signalling pathways induced by external stimuli and the cellular context (Xu et al., 2005). Cascio et al. (2009) reported that VEGF expression in the colorectal cell line (HT-29) was induced by the epidermal growth factor signalling pathway (Cascio et al., 2009). Reports also demonstrated that herbal extracts and decoctions targeted tumor angiogenesis by inhibition of VEGF expression in various tumors (Lee et al., 2006; Hsu et al., 2009).

Therefore, this study aimed at determining the efficacy of TXL decoction and its bioactive fractions by modulating MMP2, MMP7, MMP9 and VEGF expression in colorectal cancer cells and xenografts at molecular and protein levels as they are the indicative metastatic markers for indicating the efficacy of cancer treatment.

2. Materials and methods

2.1. Herbal materials and preparation

Tian-Xian liquid (TXL) is a commercially available Chinese medicine decoction. It was kindly provided by China-Japan Feida Union Company Limited. It is an aqueous extract of a Chinese herbal formulation consisting mainly of 10 Chinese medicinal herbs: 12.5% *Radix Ginseng*, 24% *Cordyceps*, 15% *Radix Astragali*, 5% *Radix Glycyrrhizae*, 11% *Rhizoma Dioscorea*, 4% *Margarita*, 9% *Fructus Lycii*, 17% *Ganoderma*, 0.5% *Fructus Ligustri Lucidi*, and 2% *Herba Scutellariae Barbatae*. The sample was kept in the School of Chinese Medicine, LKS Faculty of Medicine, The University of Hong Kong.

The TXL sample (100 mL) was dissolved in water and partitioned with ethyl acetate and butanol, successively. The aqueous (WA), ethyl-acetate (EA) and butanol (BU) fractions were collected separately in different bottles. The aqueous, ethyl acetate and butanol fractions were evaporated to yield 60, 8 and 30 g, respectively for subsequent experiments. The quality control of the fractions was performed as described in our previous publication (Sze et al., 2011).

2.2. Nude mice xenografts, drug administration and excised tumor specimens

The experiment was approved by the Department of Health, Hong Kong SAR and Committee on the Use of Live Animals in Teaching and Research (CULATR) of Li Ka Shing Faculty of Medicine, The University of Hong Kong. Seven to eight-week-old female nude mice were purchased from the Animal Laboratory Unit, The University of Hong Kong. The mice were kept under sterile conditions in isolated pathogen-free ventilation chambers at an ambient temperature of 22–24 °C and 50–65% relative humidity with automatic 12 h light:dark illumination cycles. Cell suspension was obtained by trypsinization of confluent HT-29 cells. The HT-29 carcinoma was established subcutaneously in nude mice by injecting 1×10^5 cells into the right thigh of each animal. When the tumors became palpable after xenografting, mice were randomly divided into two groups of 6 animals each. TXL, BU, EA, and WA were orally administered at a volume of 200 μL everyday for 16 days. The control group received orally an equal volume of water instead of the drugs. Tumor volume was measured using a digital caliper every day and calculated using the formula = (length in mm × width in mm × height in mm). The body weights of all animals were recorded throughout the

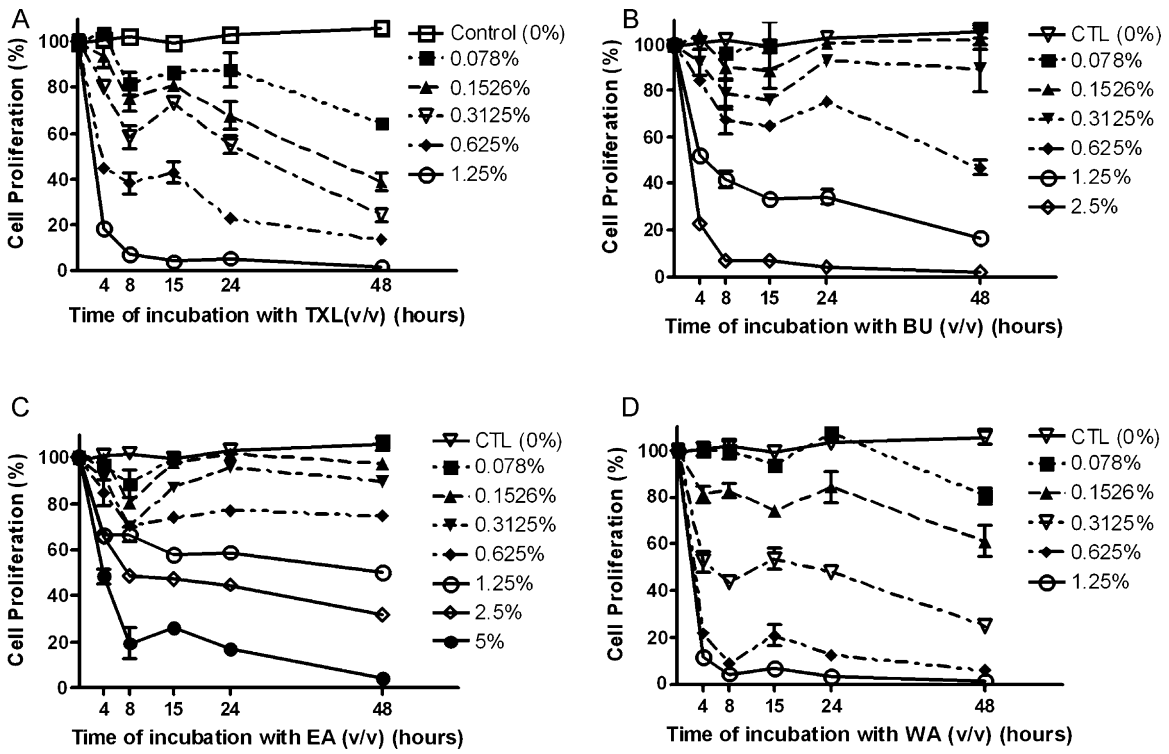

Fig. 1. Anti-proliferative effect of TXL and its bioactive fractions in HT-29 cells, HT-29 cells were treated with a range of concentrations of TXL, BU, EA and WA. MTT assay was performed at 4, 8, 15, 24 and 48 h. Untreated HT-29 cells were included as control. The IC_{50} values of TXL and its bioactive fractions on HT-29 cells were obtained with 1% (v/v) TXL (A), 1.25% (v/v) BU (B), and 0.3125% (v/v) WA (D) after 4-h incubation and 5% (v/v) EA (C) after 8-h of incubation. The results demonstrated that TXL and its bioactive fractions produced anti-proliferative effects on HT-29 cells in a dose- and time-dependent manner. The data were obtained from three independent experiments.

whole experiment as an assessment of drug toxicity. At the end of the experiment, all animals were sacrificed by cervical dislocation. Their tumor specimens were collected and stored at −80 °C for further transcriptional and translational analysis by real-time PCR and Western blot, respectively.

2.3. Cell line and culture

Human colon carcinoma HT-29 cell line (HTB-38, ATCC, Rockville, MD, USA) was cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 1% antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin sulfate) (Hyclone, Logan, UT, USA). Cells were incubated at 37 °C in a humidified 5% CO_2 incubator and sub-cultured when the cells confluent.

2.4. Anti-proliferative effect of TXL and its fractions on HT-29 cells

HT-29 cells were cultured in 96-well plates at the density of 1×10^4 cells/0.1 mL/well. The cells were serum starved for 24 h, and then incubated with serial concentrations of TXL, BU, EA, and WA (5, 2.5, 1.25, 0.625, 0.3125, 0.1526 and 0.078%) for 4, 8, 15, 24, 33 and 48 h. Inhibition of cell proliferation was assessed by MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay (Chu et al., 2006). After lysing cells with DMSO, the absorbance was recorded using a spectrophotometer at 595 nm and 655 nm as reference. The data obtained were in triplicate.

2.5. Determination of mRNA expressions of TXL- and bioactive fractions-treated HT-29 cells and xenografts using real-time PCR

The HT-29 cells were treated with TXL (1%), BU (1.25%), EA (2.5%) and WA (0.6%) for 4, 8, 24 and 48 h and the xenografts were harvested from TXL- and bioactive fractions-treated nude mice. Extraction of total RNA was performed for each sample using High Pure Isolation kit (Roche Applied Science, USA). Control samples (without drug treatment) were also included. 2 μg total RNA of each sample was reverse-transcribed into cDNA with Oligo-dT primers using Revert First strand cDNA synthesis kit (Fermentas). Primer pairs and probes for real-time PCR were designed from the Assay Design Centre of the Universal ProbeLibrary (Roche Applied Science, USA). The target genes included MMP2 (NM_004530.4; 63 nt; forward: ataacctggatgccgtcgt; reverse: aggcacccttgaagaagtagc; Probe ID: #70, cat. no. 04688937001), MMP7 (NM_002423.3; 128 nt; forward: tggacggatggtagcagtct; reverse: tctccatttccataggttggat; Probe ID: #6, cat. no. 04685032001), MMP9 (NM_004994.2; 67 nt; forward: gaaccaatctcaccgacagg; reverse: gccacccgagtgtaaccata; Probe ID: #6, cat. no. 04685032001) and VEGF (NM_001025370.1; 74 nt; forward: ctacctccaccatgccaagt; reverse: ccacttcgtgatgattctgc; Probe ID: #29, cat. no.: 04687612001). GAPDH was included as the internal control. Real-time PCR was carried out in a 384-multiwell plate of LightCycler® 480 system. The results were analyzed using LightCycler® 480 Software, Version 1.5.

2.6. Western blotting analysis of TXL- and bioactive fractions-treated HT-29 cells and excised tumor specimens

HT-29 cells that had been treated with TXL (1%), BU (1.25%), EA (2.5%) and WA (0.6%) for various time points, ranging from 0, 4, 8, 15, 24, 33, 48 h, and the drug-treated excised tumor specimens were lysed and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). A 15 μg of the denatured protein from the untreated and TXL-treated cells were separated on a 10% SDS-polyacrylamide gel, and the

resolved proteins were transferred to a polyvinylene difluoride (PVDF) membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA). The membrane was blocked in 5% bovine serum albumin (Sigma) for 1 h at room temperature, then incubated overnight at 4 °C with primary antibodies specifically recognizing MMP2, MMP7, VEGF (Santa Cruz Biotechnology) and MMP9 (Millipore). The membrane was then washed with TBS-T three times, and the membranes were incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies (Santa Cruz Biotechnology and Dako Cytomation) in TBS-T buffer for 1 h. Chemiluminescence detection (GE Bio-health) was accomplished with horseradish peroxidase-conjugated secondary antibody (Santa-Cruz Biotechnology). The detection was performed using the Advanced Chemiluminescence Western blotting detection system (GE Bio-health). The band intensities were quantified by a Bio-Rad Chemi Doc™ EQ densitometer and Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, USA). Each band intensity was normalized to its anti-*GADPH* (MAB374, Millipore) band intensity.

2.7. Wound healing assay

The procedures of the wound-healing effect were modified (Chu et al., 2008). In brief, HT-29 cells (3×10^4) were seeded in 24-well plates overnight at 37 °C with 5% CO_2. After the cells had reached confluence, a wound was made with a 200-μL pipette tip. The detached cells and debris were removed and washed with PBS. The cells were then treated with TXL, BU, EA and WA; control cells were included. The closure of the wound by migrating cells, if any, was observed at 24 and 48 h post-drug treatment using an inverted-phase contrast microscope (Carl Zeiss) coupled with a CCD camera. The wounded area of each sample was determined by NIH Image Software. The dose-dependent effect of the drugs on the wounded HT-29 cells at a particular time point was expressed as a migration rate compared with the untreated control cells, and the data were analyzed by two-way ANOVA.

$$\text{Migration rate (\%)} = \left[\frac{\text{mean of treatment area}_{24\,h} - \text{mean of treatment area}_{0\,h}}{\text{mean of control area}_{24\,h} - \text{mean of control area}_{0\,h}}\right] \times 100\%$$

2.8. Statistical analysis

The GraphPad Prism Version 5.02 was used to analyze the results obtained. All data were presented as mean and standard error (S.D.). A P value smaller than 0.05 ($P < 0.05$) was considered statistically significant.

3. Results

3.1. Anti-proliferative effect of TXL and its fractions

The anti-proliferative effects of TXL and its bioactive fractions, BU, EA and WA on HT-29 cells were determined by the MTT assay. Fig. 1 presents the anti-proliferative effects of TXL (Fig. 1A), BU (Fig. 1B), EA (Fig. 1C) and WA (Fig. 1D) on HT-29 cells at various concentrations (5%, 2.5%, 1.25%, 0.625%, 0.3125%, 0.1526% and 0.078%) and different time points (0, 4, 8, 15, 24 and 48 h). Cells treated with 0% (without any drug) were included as control. The results indicated that TXL and its bioactive fractions exerted an anti-proliferative effect on HT-29 cells in a dose- and time-dependent manner. The IC_{50} values in HT-29 cells were 1% (v/v) TXL, 1.25% (v/v) BU and 0.3125% (v/v) WA after 4 h of incubation and 2.5% (v/v) EA after 8 h of incubation. These values were employed for subsequent experimental investigation.

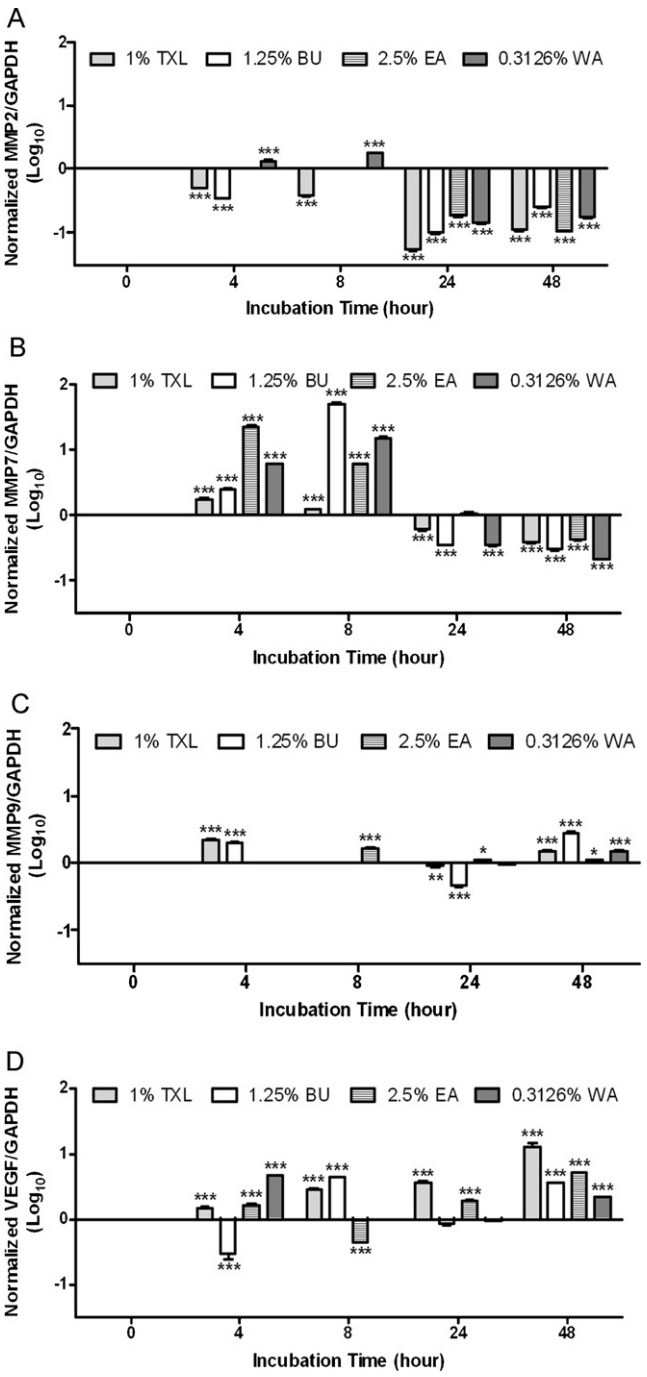

Fig. 2. *In vitro* modulation of MMP2, MMP7, MMP9 and VEGF mRNA expression by TXL and its bioactive fractions. All four drugs statistically demonstrated significant down-regulation in both MMP2 (A) and MMP7 (B) mRNA expression after 24- and 48-h of incubation. (C) There was only a slight down-regulation in MMP9 mRNA expression by BU. (D) All four drugs enhanced VEGF mRNA expression after 48-h of incubation. The data were obtained in three independent experiments by real-time PCR (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; by two-way ANOVA).

3.2. In vitro modulation of MMP2, MMP7, MMP9 and VEGF mRNA expression in HT-29 cells by TXL and its bioactive fractions

TXL and its bioactive fractions-induced modulation of metastatic protein mRNA expression on HT-29 cells were determined by real-time PCR (LC480, Roche) at transcriptional level.

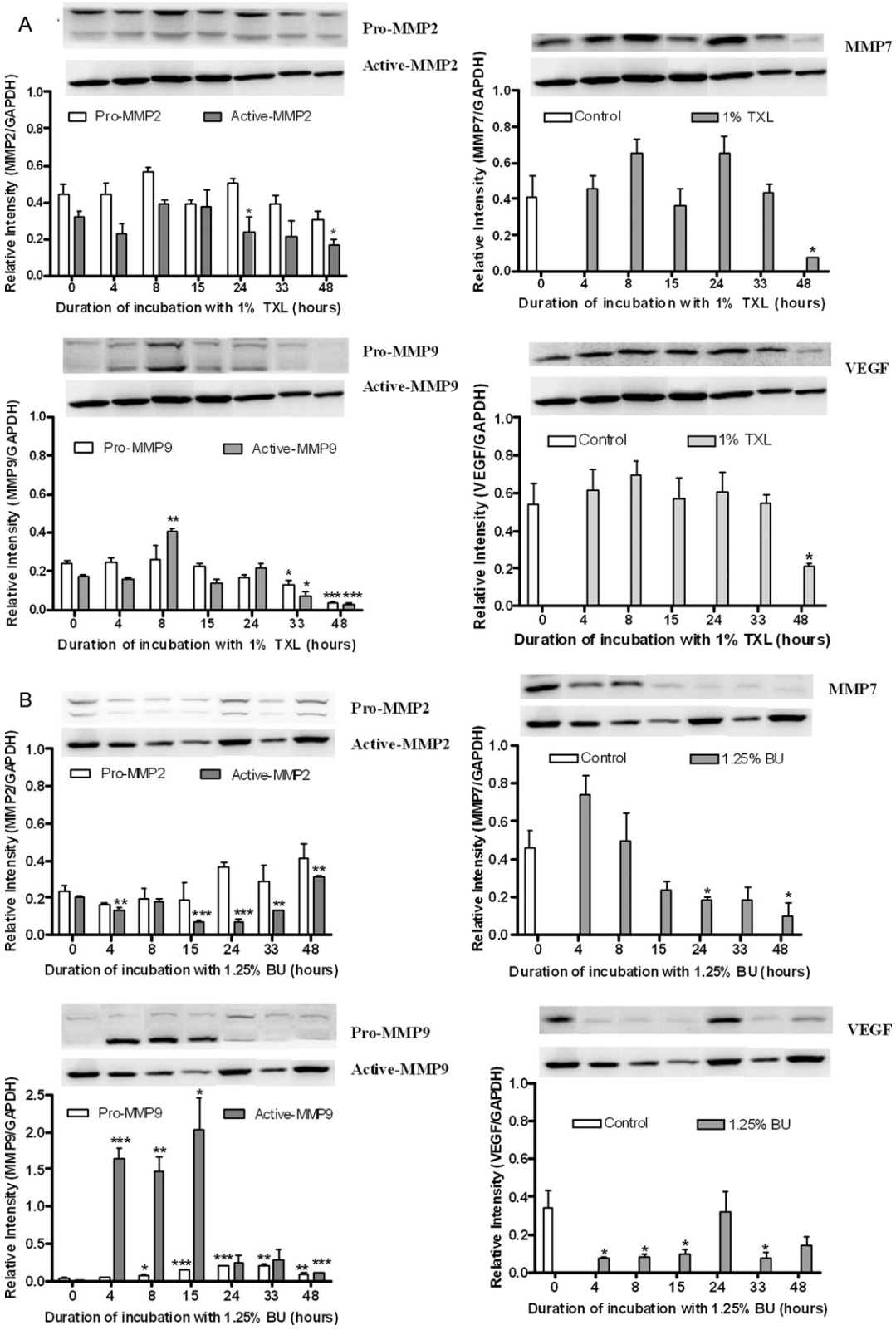

Fig. 3. *In vitro* modulation of MMP2, MMP7, MMP9 and VEGF protein expression by TXL and its bioactive fractions, TXL significantly inhibited MMP2, MMP7 and VEGF protein expression at 48-h incubation. (A) TXL also significantly down-regulated MMP9 protein expression after 33- and 48-h of incubation (*$P<0.05$; ***$P<0.001$; by Student's *t*-test). (B) After 15-, 24- and 33-h incubation, BU significantly lowered MMP2 protein expression. Starting from 15-h of incubation and onward, BU reduced MMP9 protein expression significantly. It also down regulated VEGF protein expression starting from 4-h of incubation (*$P<0.05$; **$P<0.01$; ***$P<0.001$; by Student's *t*-test). (C) EA only significantly down regulated both pro- and active-form of MMP2 protein. For MMP7, MMP9 and VEGF protein expression, EA significantly enhanced expression of all these proteins (*$P<0.05$; **$P<0.01$; ***$P<0.001$; by Student's *t*-test). (D) WA only significantly down regulated MMP2 protein expression after 8-h of incubation, and then gradually increased the expression to its original level. WA intensely induced an up-regulation in MMP9 protein expression and mediated a slight increase in VEGF protein expression (*$P<0.05$; **$P<0.01$; by Student's *t*-test). All the data presented were obtained from three to six individual experiments.

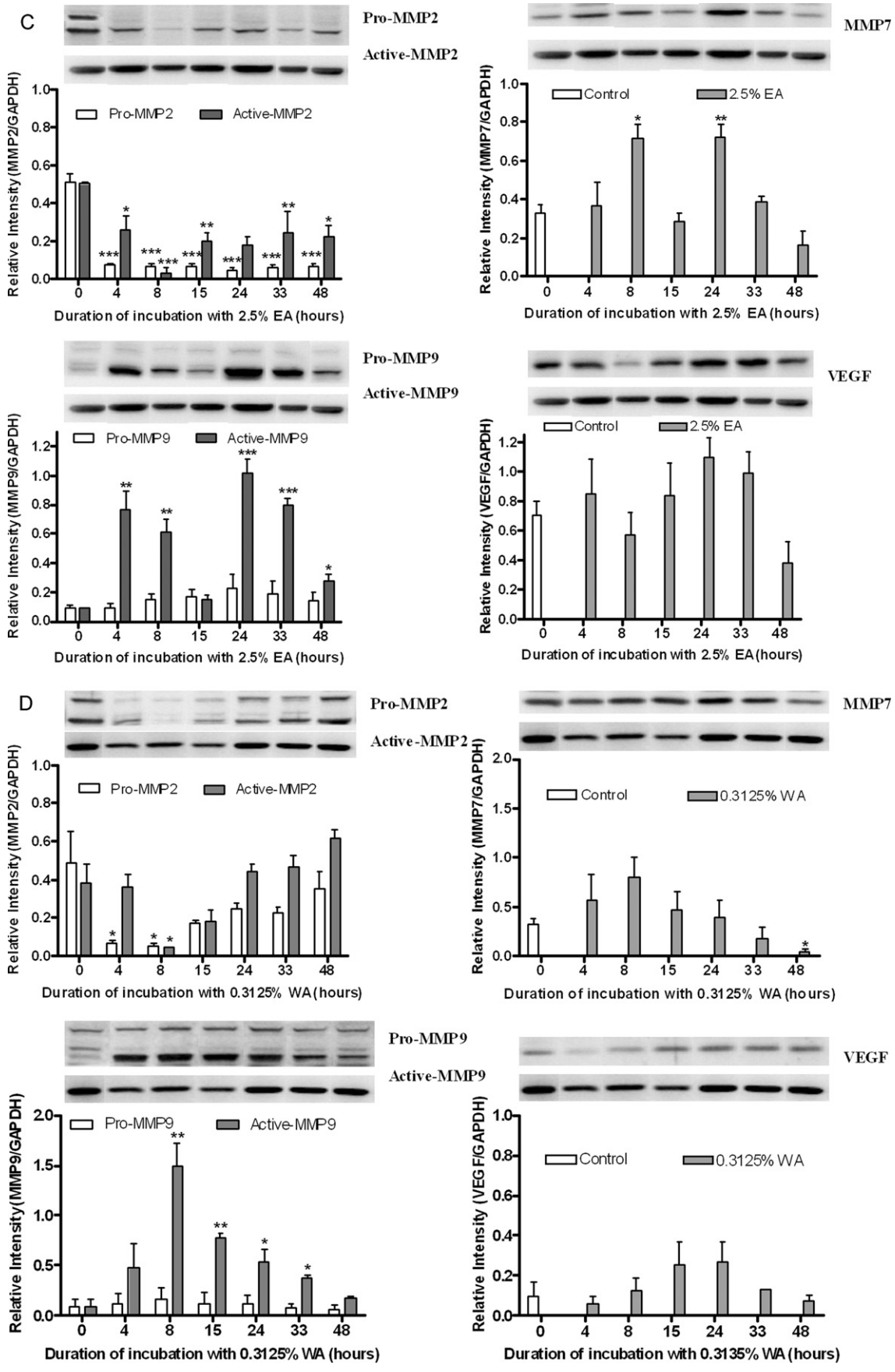

Fig. 3. (*Continued.*)

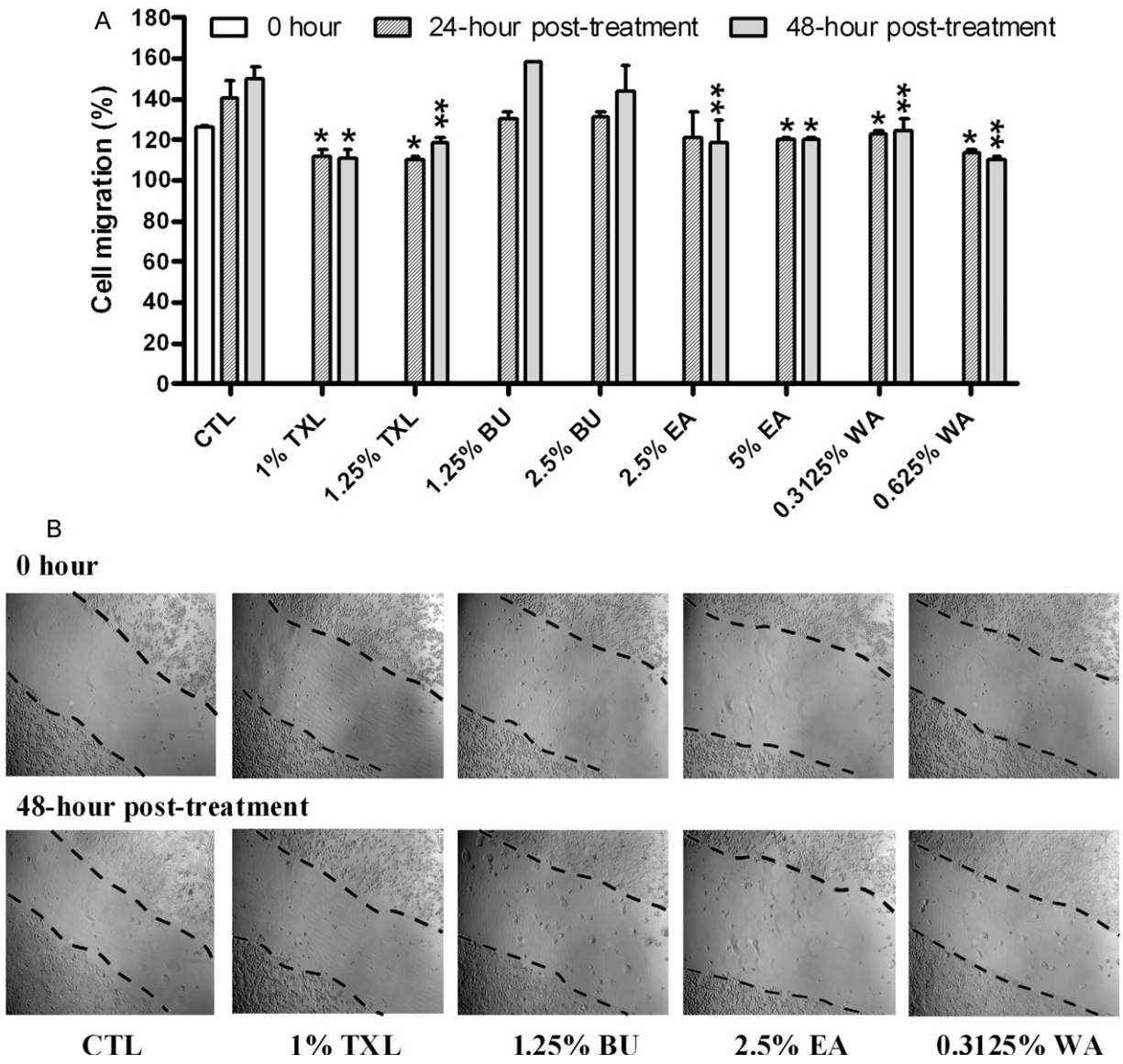

Fig. 4. Reduction of metastatic ability of HT-29 cells by TXL and its bioactive fractions. The metastatic ability of HT-29 cells after treatment with TXL and its bioactive fractions was determined by a functional assay: the wound-healing assay. (A) TXL (1% and 1.25%), EA (5%) and WA (0.3125% and 0.625%) significantly inhibited the wound-healing effect in HT-29 cells when compared with the control cells after incubation for 24- and 48-h (*$P<0.05$ and **$P<0.01$; by Student's *t*-test). Furthermore, BU (1.25% and 2.5%) had no effect on the metastatic ability of HT-29 cells when compared with the control cells at both time points. (B) Representative images of control cells (CTL), TXL and its fractions at 0- and 48-h of incubation are shown for comparison. The data collected were from three independent experiments.

In Fig. 2A, TXL and it bioactive fractions significantly reduced the MMP2 mRNA expression level in HT-29 cells after 24- and 48-h incubation (***$P<0.001$; by two-way ANOVA). In Fig. 2B, TXL and EA down-regulated MMP7 mRNA expression on HT-29 cells in a time-dependent manner (***$P<0.001$; by two-way ANOVA). On the other hand, BU and WA initially increased the MMP7 expression after 4- and 8-h of drug treatment. However, when the drug treatment time was prolonged to 24 and 48 h, MMP7 mRNA expression also decreased by BU and WA (***$P<0.001$; by two-way ANOVA). In Fig. 2C, only BU demonstrated a slight but significant down-regulation of MMP9 mRNA expression in HT-29 cells (***$P<0.001$; by two-way ANOVA). However, TXL and other two fractions induced a slight increase in MMP9 mRNA expression. In Fig. 2D, TXL and its fractions all elicited an increase in VEGF mRNA expression after 48-h of treatment.

3.3. In vitro modulation of MMP2, MMP7, MMP9 and VEGF protein expressions in HT-29 cells by TXL and its bioactive fractions

The modulation of MMP2 (pro- and active-forms), MMP7, MMP9 (pro- and active-forms), and VEGF protein expression by 1% (v/v) TXL, 1.25% (v/v) BU, 2.5% (v/v) EA and 0.3125% (v/v) WA in HT-29 cells was evaluated by Western blotting analysis at various time points (0, 4, 8, 15, 24, 33 and 48 h).

TXL significantly down-regulated MMP2, MMP7, MMP9 and VEGF protein expression in HT-29 cells after 48-h of incubation (*$P<0.05$ and ***$P<0.001$; by Student's *t*-test) (Fig. 3A). Expression of both pro- and active-forms of MMP2 and MMP9 was inhibited as the incubation time increased. Inhibition of MMP7, MMP9 and VEGF protein expression was found in BU-treated

HT-29 cells (*$P<0.05$; **$P<0.01$; ***$P<0.001$; by Student's *t*-test) (Fig. 3B). EA significantly down-regulate both pro- and active-forms of MMP2 protein expression in HT-29 cells (*$P<0.05$; **$P<0.01$; ***$P<0.001$; by Student's *t*-test). In contrast, EA enhanced MMP7, MMP9 and VEGF protein expression after treatment (Fig. 3C). On the other hand, WA only significantly down regulated MMP2 protein expression after 8-h of incubation, and then gradually increased the expression to its original level. WA intensely induced an up-regulation in both MMP7 and MMP9 protein expression and mediated a slight increase in VEGF protein (*$P<0.05$; **$P<0.01$; by Student's *t*-test) (Fig. 3D). This indicated that WA only has a temporary inhibitory effect in MMP2 protein on HT-29 cells.

From the above results, 1% (v/v) TXL demonstrated a significant down-regulation in these four proteins in HT-29 cells *in vitro*, when compared with its bioactive fractions, suggesting that TXL has greater anti-metastatic and anti-angiogenic potential in HT-29 cells.

3.4. Reduction of metastatic ability of HT-29 cells by TXL and its bioactive fractions

The anti-metastatic potentials of TXL, BU, EA and WA on HT-29 cells were determined by the wound-healing assay. TXL (1% and 1.25%), EA (5%) and WA (0.3125% and 0.625%) significantly inhibited the wound-healing effect in HT-29 cells when compared with the control cells after both 24- and 48-h of incubation (*$P<0.05$ and **$P<0.01$; by Student's *t*-test) (Fig. 4A). However, BU (1.25% and 2.5%) had no effect on the metastatic ability of HT-29 cells when compared with the control cells at both time points. The representative images of the control cells (CTL), TXL and its fractions at 0- and 48-h of incubation are shown in Fig. 4B. The results indicated that TXL, EA and WA functionally reduced the metastatic ability of HT-29 cells.

3.5. In vivo modulation of tumor size and body weight of colorectal xenografts by TXL and its bioactive fractions

The colorectal xenografts were treated with TXL, BU, EA and WA consecutively for 16 days. There was no significant change in the body weight of all the treated colorectal xenografts when compared with the control, suggesting all of the drugs had no *in vivo* toxicity (Fig. 5A). TXL and the fractions all produced a significant inhibition of tumor size after 16 days of treatment when compared with the control (CTL vs TXL: *$P<0.05$; **$P<0.01$; CTL vs WA: ●$P<0.05$; ●●$P<0.01$; CTL vs BU: ■■■$P<0.001$; by paired *t*-test). EA showed a gradual increase in tumor size after 14 days of treatment, although it was still lower than that of the control (CTL vs EA: #$P<0.05$; ##$P<0.01$; ###$P<0.001$; by paired *t*-test), indicating that the anti-tumor effect of EA was only a temporary effect (Fig. 5B).

3.6. In vivo modulation of MMP2, MMP7, MMP9 and VEGF expression at transcriptional and translational levels by TXL and its bioactive fractions

The modulation of MMP2, MMP7, MMP9 and VEGF expression in colorectal xenografts at transcriptional and translational levels by TXL and its fractions were determined by real-time PCR (LC480, Roche) and Western blot analysis.

At the transcriptional level, in comparison with the control, TXL, BU and EA that all of them reduced MMP2 and MMP7 mRNA expression, but increased MMP9 and VEGF expression (*$P<0.05$; **$P<0.01$; ***$P<0.001$; by Student's *t*-test). However, WA increased all mRNA expression in the xenografts (Fig. 6A).

On the other hand, at the translational level, TXL only significantly down-regulated VEGF protein expression (*$P<0.05$; by Student's *t*-test) (Fig. 6D) and WA reduced MMP9 and VEGF protein expression (**$P<0.01$; by Student's *t*-test) (Fig. 6D and E). However, there was no significant change in MMP2 and MMP7 protein expression in the xenografts by TXL, BU, EA and WA (Fig. 6B and C).

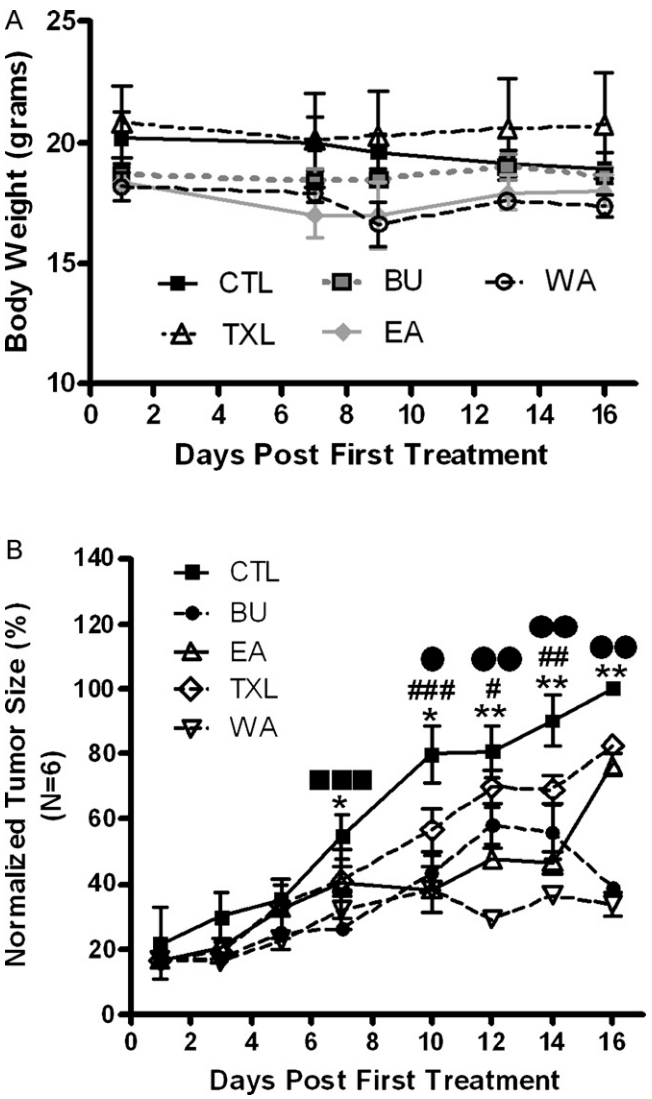

Fig. 5. *In vivo* modulation of tumor size and body weight of colorectal xenografts by TXL and its bioactive fractions. Nude mice were subcutaneously injected with the human colorectal cancer cells. When the tumor xenografts became palpable, TXL, BU, EA and WA were orally fed to the mice. Control mice treated with water instead. (A) The colorectal xenografts were treated with the drugs consecutively for 16 days. There was no significant change in the body weight of all the treated colorectal xenografts when compared with the control, suggesting all of the drugs had no *in vivo* toxicity. (B) For the tumor size, all TXL and the fractions demonstrated a significant inhibition of tumor size after 16 days of treatment when compared with the control. EA showed a gradual increase in tumor size after 14 days of treatment, although it was still lower than that of the control. This indicated that the anti-tumor effect of EA only elicited a temporary response (CTL vs TXL: *$P<0.05$; **$P<0.01$; CTL vs EA: #$P<0.05$; ##$P<0.01$; ###$P<0.001$; CTL vs WA: ●$P<0.05$; ●●$P<0.01$; CTL vs BU: ■■■$P<0.001$; by paired *t*-test). The data obtained from each group were from six mice ($N=6$).

4. Discussion

There has been increasingly important in understanding the molecular mechanisms of Tian-Xian liquid (TXL) as an alternative for cancer therapeutic decoction. Our previous study demonstrated that TXL was effective in inhibiting human colorectal cancer cells (HT-29) by regulating cell cycle and cell cycle related proteins (Sze et al., 2010). In order to further elucidate the anti-cancer mecha-

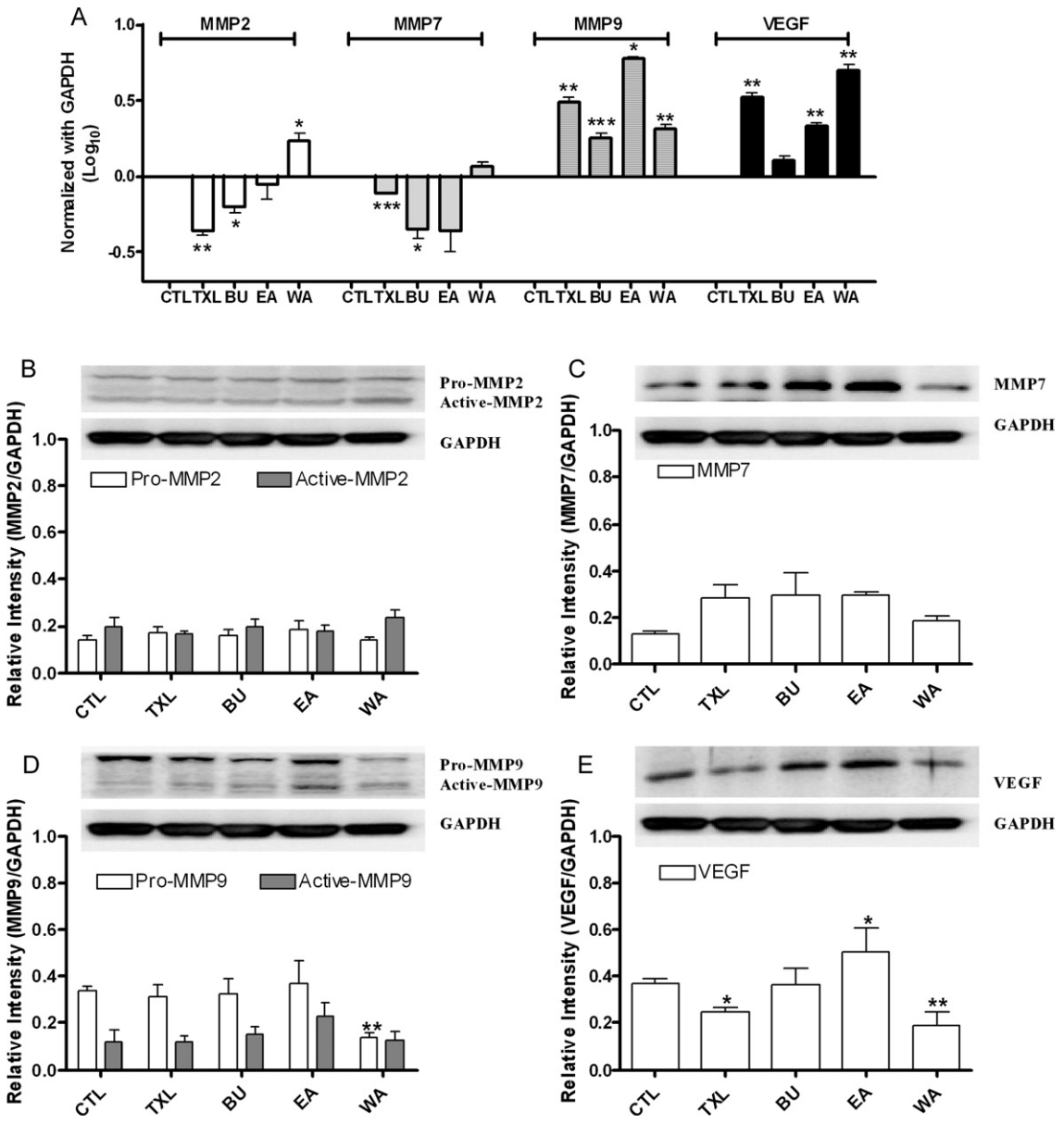

Fig. 6. *In vivo* modulation of MMP2, MMP7, MMP9 and VEGF expression at transcriptional and translational levels by TXL and its bioactive fractions. The metastatic protein markers from TXL- and the bioactive fractions-treated xenografts were determined by real-time PCR and Western blot analysis at transcriptional and translational levels respectively. (A) At transcriptional level by comparing with the control, TXL, BU and EA had similar and significant effects that all of them reduced both MMP2 and MMP7 mRNA expression, but increased MMP9 and VEGF expression (*$P<0.05$; **$P<0.01$; ***$P<0.001$; by Student's *t*-test). However, WA increased all mRNA expression in the xenografts. On the other hand for the translational level, there was no significant change in MMP2 (B) and MMP7 (C) protein expression by TXL, BU, EA and WA on the xenografts. However, TXL significantly down regulated VEGF protein expression (*$P<0.05$; by Student's *t*-test) (D) and WA reduced both MMP9 (D) and VEGF (E) protein expression (**$P<0.01$; by Student's *t*-test). The data obtained were from six individual experiments ($N=6$).

nism of TXL, the HT-29 cell line was employed as an *in vitro* model for studying its anti-metastatic and angiogenic potential.

This is the pioneer study reporting the anti-metastatic and angiogenic potential of TXL on HT-29 cells. Firstly, our results demonstrated that TXL, BU, EA as well as WA significantly inhibited the proliferation of HT-29 cells in dose- and time-dependent manners (Fig. 1). The inhibitory effect of TXL on HT-29 cells was consistent with our previous publications (Liu et al., 2010; Sze et al., 2011). This provides evidence that the Chinese medicinal formulation, TXL and its bioactive fractions could specifically kill human cancer cells. A recent report studied the growth inhibitory effect of TXL on several human cancer cell lines, such as hepatocellular, cervical and lung carcinoma, indicating TXL effectively killed these cells in a time-dependent manner, and kept the human normal peripheral blood mononuclear cells alive (Sun et al., 2005a). This implied that human normal cells are tolerable to the cytotoxic effect of TXL *in vitro*.

MMP inhibition in tumor therapy takes place at three levels: transcription, pro-MMP activation and MMP inhibition (Leber and Efferth, 2009). Several MMPs trigger cancer cell migration; however, recent evidence suggests that they mediate chemotaxis even without using their proteolytic domain. MMP2 and MMP9 are the prevalent metastatic proteins secreted by cancer cells during tumor cell metastasis and invasion (Leber and Efferth, 2009). It has been documented that VEGF plays a vital role in tumor angiogenesis in various human cancers (Birk et al., 2008). VEGF is a potent angiogenic factor that is up-regulated by a variety of factors, such as hypoxia, growth factors and hormones. The regulation of VEGF may involve other proteins in tissues and treatment-dependent manner, for examples, calcium

signalling, mRNA stability and post-transcriptional regulation; thus could result in reciprocal expression of mRNA and protein levels (Misquitta et al., 2006). MMP2, MMP9 and VEGF are the hallmarks for tumor cell metastasis and angiogenesis, respectively. Dufour et al. (2008) reported that precursor forms of MMP2 and 9 enhance cell migration in a transwell chamber assay. From our *in vitro* study, TXL and EA down-regulated MMP2 expression at both transcriptional and translational levels after 24- and 48-h incubation (Figs. 2A and 3A and C), and in turn, significantly lowering the metastatic ability of HT-29 cells (Fig. 4A). MMP2 is a soluble protein that requires extracellular post-translational modification to gain biological activity (Roberts et al., 2002). Our results suggested both TXL and EA might play a role in controlling the post-translational modification of MMP2 *in vitro*. Also, TXL and the other three fractions inhibited MMP7 expression at both transcriptional and translational levels (Figs. 2B and 3A–D), suggesting that TXL and EA have a multi-targeted role in inhibiting metastasis of HT-29 cells.

It has been reported that inhibition of VEGF expression led to reduction of MMP9 expression in gastric cancer cells, thus inhibited cell migration and vice versa (Zhao et al., 2010). Although there was only a slight alteration of MMP9 mRNA expression by TXL and its fractions (Fig. 2C), an increase in MMP9 protein expression was quantified after treatment with TXL and other three fractions in HT-29 cells (Fig. 3A–D). When compared with other drugs, BU induced a higher protein expression of active-MMP9 in HT-29 cells; therefore it had a lower anti-cell migrating effect (Figs. 3B and 4A). There was a significant reduction in VEGF protein expression in TXL-treated cells (Fig. 3A), which demonstrated that TXL targeted to multiple metastatic proteins for the *in vitro* anti-metastatic effect. Although there were weak correlation between transcriptional and translational levels in MMP2, MMP7, MMP9 and VEGF by TXL and its bioactive fractions, we further demonstrated and confirmed that TXL and its bioactive fractions significantly inhibited HT-29 cells migrated to the wounded area by wound-healing assay (Fig. 4A and B).

A recent study demonstrated that an herbal extract inhibited the tumor growth of human hepatocellular carcinoma xenograft through the down-regulation of MMP2 and VEGF expression (Huang et al., 2009). For the *in vivo* study, our results demonstrated that TXL and its fractions had no toxicity to the animals as determined by the body weight (Fig. 5A). Besides, it was also evident that significant reductions in the tumor size of the drug-treated xenografts were obtained when compared with the control (Fig. 5B). Down regulation of MMP2 and MMP7 mRNA expression was obtained in TXL-, BU- and EA-treated xenografts (Fig. 6A), which were consistent with the findings in our *in vitro* study (Figs. 2A, B and 3A–C). However, there was no obvious reduction in MMP2 and MMP7 protein expression by TXL, BU and EA (Fig. 6B and C). Similarly, TXL mediated an increase in VEGF mRNA expression *in vivo* but not in protein level (Fig. 6E). For MMP9, TXL and its fraction mediated a higher level of mRNA expression in the xenografts; however, a decrease in MMP9 protein (Fig. 6D) indicating TXL and its fractions might have a role in modulating the post-translational control in MMPs. The inconsistent mRNA and protein levels of MMPs and VEGF might be caused by post-transcriptional regulation, mRNA stability, translational efficiency or the presence of microRNAs (Yan and Boyd, 2007; Fanjul-Fernandez et al., 2010). A recent review reported that the microRNAs may also participate in MMP regulation. These microRNAs are capable of negatively regulating gene expression at the post-transcriptional level through either translation repression or degradation of their mRNA targets (Fanjul-Fernandez et al., 2010). Although this study could not confirm what causes these discrepancies in expression levels, we might confirm the xenografts were significantly suppressed as showed by the tumor size (Fig. 5B). Therefore, mechanism of TXL and its fractions on the post-translational control of MMP proteins is still unclear and deserves investigation. However, the results obtained above suggested that the Chinese herbal formulation TXL demonstrated a higher efficacy in lowering the metastatic potential of the human colorectal cancer cells *in vitro* and *in vivo* when compared with its bioactive fractions. This also implied that further investigation of the mechanisms mediated by Chinese herbal formulation in human cancers is worth to be explored.

5. Conclusions

In conclusion, this study reported that TXL exerted a multi-targeting effect on human colorectal cancer cells by lowering the metastatic and angiogenic markers at both transcriptional and translational levels. However, its bioactive fractions have not demonstrated such effects on both study models. This study generated valuable insights of the multi-targeting characteristics of Chinese medicinal formulations, including TXL, and provided significant scientific evidence of Chinese medicinal formulations as a complementary therapeutic regime for human cancers in future clinical settings.

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