

Chemosensitizing Effect and Efficacy of Wilforlide A in Combination With Docetaxel in Drug-resistant Prostate Cancer

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Abstract. *Background/Aim:* Prostate cancer is currently the second most common cancer in men and chemotherapy is the main treatment for metastatic castrate-resistant prostate cancers (mCRPC). However, chemoresistance leading to treatment failure is inevitable. Thus, therapeutic approaches that can overcome chemoresistance are important areas of research for cancer chemotherapy. *Materials and Methods:* In the present study, six components of tripterygium wilfordii including celastrol, triptolide, pristimerin, triptonide, demethylzeylasteral, and wilforlide A were screened for their chemosensitizing effect on drug-resistant prostate cancer cell lines PC3 and DU145. The most active compound was further investigated on its potential mechanism of action and in vivo efficacy using a SCID mouse model. *Results:* Among the six components only wilforlide A significantly enhanced sensitivity to docetaxel (by reducing the IC₅₀ in resistant prostate cancer cell lines). Wilforlide A inhibited P-glycoprotein efflux transporter and downregulated cyclin E2

splice variant 1 mRNA, both have been known as mechanisms of resistance. The chemosensitizing effect was further verified using a xenograft mouse model. In the high-dose treatment group, the combination of wilforlide A and docetaxel significantly retarded tumor growth of resistant prostate cancer, although neither docetaxel nor wilforlide A monotreatment groups showed any effect. *Conclusion:* Wilforlide A was found to enhance the chemosensitizing effect of docetaxel both in vitro and in vivo. Further studies are warranted to verify wilforlide A as a new drug candidate to overcome docetaxel resistance in prostate cancer.

Prostate cancer is currently the second most common cancer in men. About 248,530 new cases of prostate cancer are estimated to occur in the US during 2021 with 34,130 estimated deaths (1). The recommended treatment for newly diagnosed (predominantly localized early stage) prostate cancer is radiation, radical prostatectomy, brachytherapy, or active monitoring (2). Those with metastatic disease are usually recommended for androgen deprivation therapy (ADT) and possibly radiation therapy (2). However, treatment with ADT (to achieve a castration effect) has a high rate of resistance, usually occurring within a year (3). Subsequently, these metastatic castrate-resistant prostate cancers (mCRPC) are usually treated with androgen receptor targeted therapy, chemotherapy, immunotherapy, or bone targeted therapy (2-6).

Docetaxel (Dtx) is a chemotherapeutic agent and is considered to be a first-line agent for mCRPC treatment. However, its efficacy in increasing the overall survival is only about 2.4 months and the effect by other chemotherapeutic agents is also similar, ranging from 2.2 to 4.8 months (5, 7, 8). The short duration of overall survival is invariably due to chemoresistance. Clinically, chemoresistance is known to be

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a major problem responsible for over 90% of drug treatment failures in cancer treatment (9). Thus, therapeutic approaches that can overcome chemoresistance is an important area of research for cancer chemotherapy (10-12).

One strategy to overcome chemoresistance is to utilize drugs that can induce chemosensitizing effect of an existing resistant anticancer drug. This approach has the benefit of significantly reducing the time and cost of developing new anticancer drugs. If the chemosensitizer is also known and relatively safe, such a combination can be considered as an attractive “new product” for development with a significantly reduced development time and cost.

Chemosensitizers can be either synthesized or identified *via* screening the natural products. Many compounds, especially those from natural products, can sensitize anticancer drugs that have become resistant (12, 13). In our previous study, tripterygium wilfordii (TW) extract, a well-known Chinese medicine used extensively for inflammatory diseases such as arthritis and systemic lupus in China since 1960, has been found to possess chemosensitizing effects, *i.e.*, restoring the sensitivity of Dtx in resistant prostate cancer cells (PC3-TxR cells) both in cultured cell lines and animal studies (when using a xenograft model) (14). In addition, TW was found to be associated with at least 4 genes contributing chemosensitizing effect using microarray analysis (14). The extensive usage experience of TW in patients (15), together with the above chemosensitizing effect, provided a strong and attractive lead to further develop TW and its active components as possible chemosensitizing agents to overcome docetaxel resistance in prostate cancer therapy. Since TW has been associated with numerous adverse effects and as an herbal extract, it is also difficult to develop a quality control method that is practical and yet meets drug regulatory agency approval (*e.g.* FDA) (16). Thus, screening its active components will be a more attractive endeavor aiming for drug development.

Although TW extract is known to contain more than 300 active components, 6 components are well documented (17). These include celastrol, triptolide, pristimerin, triptonide, demethylzeylasteral, and wilforlide A (WA). Thus, in the present study, we investigated 1) the chemosensitizing effect of these 6 potential active components, 2) the *in vitro* safety profile of the best chemosensitizing compound, WA, 3) the potential mechanisms of action of WA, and (4) the *in vivo* efficacy of combination of WA+Dtx in suppressing resistant tumors using a xenograft mouse model.

Materials and Methods

Materials. The six active components, celastrol, triptolide, pristimerin, triptonide, and demethylzeylasteral, were purchased from PI & PI Biotech Inc (Guangzhou, PR China) (Purity >95%, HPLC), while wilforlide A was obtained from the National Institutes

for Food and Drug Control (Beijing, PR China) (Purity >95%, HPLC). Docetaxel was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The human androgen-independent prostate cancer cell line PC-3 and normal human prostate epithelial cells (HPrEpC) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The Dtx-resistant cell lines (PC3-TxR and DU145-TxR), that were established using a stepwise increase in exposure to Dtx were kindly provided by the Department of Medicine, University of Michigan. All the other solvents used were of HPLC grade purity or higher (VWR, Brisbane, CA, USA). Cell culture medium RPMI-1640, DMEM, fetal bovine serum (FBS), pyruvic acid, none-essential amino acids, penicillin-streptomycin, and 0.25% trypsin-EDTA solution were purchased from Invitrogen (Carlsbad, CA, USA).

Cytotoxicity of individual compounds. The cytotoxicity (IC₅₀) of each individual component and Dtx was determined in PC3 and DU145 and their corresponding drug resistant cell lines (PC3-TxR and DU145-TxR). The two cell lines were maintained in RPMI1640 medium supplemented with 10% FBS, 100 µM streptomycin and 100 units penicillin at 37°C in a 5% CO₂ humidified atmosphere. Upon reaching 80% confluence, the cells were trypsinized and seeded onto 96-well plates (3,000 cells/well) and incubated for another 24 h. The cells were then treated with 6 compounds at various concentrations for an additional 72 h (Dtx: 0.01, 0.1, 0.33, 1, 3.3, 10, 33, and 100 nM; pristimerin, triptolide, celastrol, and triptonide (0.1 to 100 ng/ml); WA (0.05 to 20 µg/ml); demethylzeylasteral (0.005 to 10 µg/ml).

The cell viability was measured using a proliferation assay with sulforhodamine B (SRB). The IC₅₀ was calculated using an Emax sigmoid model with the aid of GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Chemosensitizing effect. The IC₅₀ of Dtx was determined for each combination of the herbal component with Dtx. The concentrations of each herbal component selected for the combination were 12.5, 25 or 50% of individual IC₅₀ values. The cytotoxicity of Dtx in combination with each herbal component was determined using a similar method as mentioned above. The drug combination effect of WA, the most chemosensitizing compound, was also determined in the corresponding sensitive cell line (PC3 and DU145) using the same method as above.

For all studies, the chemosensitizing effect (CE) was expressed as $CE = IC_{50D} / IC_{50HD}$, where IC_{50D} was the Dtx concentration alone that inhibited 50% proliferation, whereas IC_{50HD} was the Dtx concentration that inhibits 50% proliferation when used in combination with the herbal component.

Cytotoxicity of WA and Dtx in normal prostate cells. The cytotoxicity (IC₅₀) of WA, the best chemosensitizing component, was further determined in normal prostate and cancer cells. Normal prostate cells were cultured in prostate epithelial cell basal medium (PCS-440-030 from ATCC) supplemented with prostate epithelial cell growth kit (PCS-440-010 from ATCC) in a 5% CO₂ humidified atmosphere. Upon reaching 80% confluence, the cells were trypsinized and seeded onto 96-well plates (3,000 cells/well) and incubated for another 24 h. The cells were then treated with these compounds at various concentrations for additional 72 h (Dtx: 0.01, 0.1, 0.33, 1, 3.3, 10, 33, and 100 nM; WA: 0.0008, 0.002, 0.008, 0.02, 0.08, 0.2, 0.8, 2, 8, 20 µg/ml). Cell viability was measured and IC₅₀ calculated using the same method as mentioned above.

Identification of the potential mechanisms. PC3-TxR is a cell line known to overexpress P-glycoprotein (P-gp), which is associated with the mechanism of resistance to Dtx. Thus, this effect of WA on P-gp functional activity was investigated. The inhibition on P-gp activity was determined using a flow cytometry-based drug accumulation assay using a P-gp overexpressed cell line, K562/Dox. Daunorubicin (DNR), a well-known P-gp substrate with fluorescence, was used as the marker and PSC833 was used as a positive control (at a concentration of 2.5 mM). The K562/Dox cells were maintained by suspending culture in RPMI 1640 with 10% FBS and harvested by centrifugation (1,000 rpm for 5 min, room temperature). The cells were then re-suspended and adjusted to the cell density of 5×10^3 cells/ml. The DNR accumulation was measured by incubating with DNR (5 μ M) in the presence of various concentrations of WA (0, 0.675, 1.25, 2.5, and 5 μ g/ml) for 2 h. Afterwards, the incubation was halted by washing with ice-cold PBS. The intra-cellular fluorescent intensity was measured using a Becton Dickinson FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an Ultraviolet Argon laser (excitation at 488 nm, emission at 585/542 nm). The intracellular amount of DNR was expressed as the logarithm of fluorescent intensities of each treatment and normalized as the percentage of the positive control (PSC833, 2.5 mM).

Since we had previously identified several genes that contributed to the sensitizing effect of TW extract, including connective tissue growth factor (CTGF), cyclin E2 splice variant 1 mRNA (CCNE2) and Ankyrin repeat domain containing (ANKRD) (14), the effect of WA on these genes was investigated. The effect of WA on the expression level of these genes was determined using real-time PCR with a SYBR green protocol (Applied Biosystems 7300 Real-Time PCR System, Applied Biosystems, Carlsbad, CA, USA). The PC3-TxR cells were cultured and treated with 2.5 μ g/ml WA for 6 and 24 h ($n=3$). The mRNA was then extracted, and the cDNA was obtained by reverse transcription. The gene expression was determined by using specific primers for each gene. The data were expressed as fold changes of these genes over the non-treated cells with GAPDH as the normalizing control.

In vivo efficacy study. Based on the results of the above *in vitro* studies, WA (the most active component) was selected for the *in vivo* study to investigate its antitumor effect in severe combined immunodeficiency (SCID) mice (Taconic Farms, Inc. Oxnard, CA, USA) implanted with Dtx-resistant tumor.

The dose and route of WA administration were based on our previous study (18). WA was found to be well tolerated in mice [oral dose: >30 mg/kg; intraperitoneal dose (*i.p.*): >6 mg/kg; intravenous dose (*i.v.*): >1.2 mg/kg] (18). However, the maximum concentration of WA following the 30 mg/kg oral dose was less than 30 ng/ml (which was far below the estimated effective concentration based on IC_{50} determination). In view of the low oral bioavailability of WA (0.6%), *i.p.* and *i.v.* injections that produced sufficient *in vivo* plasma concentration in mice were used in the present efficacy study.

The study procedure was similar to our previous report (14). Male SCID mice (15 to 20 g, between 4–6 weeks old) were used. Before tumor implantation with PC3-TxR cells, they were housed in cages with HEPA-filtered air (12-h light/dark cycle) for one week after arrival at our animal vivarium. Then these cells were suspended in a 1:1 mixture of Matrigel (BC Biosciences, Franklin Lakes, NJ, USA) and RPMI 1640 (Mediatech, Manassas, VA, or Life Technologies, Grand Island, NY, USA) and subcutaneously

implanted into planks of mice *via* injection. Mice that had consistently shown tumor growth for 14 days following the injection of cells were used in the efficacy studies. More specifically, the study was initiated when the tumors reached a size of about 120 mm³ (based on a formula for calculating semi-epilipsoid: $Volume = Width^2 \times (Length/2)$).

The tumor-containing mice in the study were randomized into 7 groups with combination treatments consisting of high (H), medium (M), and low (L) dose WA as shown below ($n=8$). Since Dtx 20 mg/kg was effective in PC3 tumors (14), this dose was selected as the reference treatment. For WA, the *i.p.* dosing was chosen for convenient daily maintenance injection and the high dose (6 mg/kg) was chosen based on maximum solubility. This dose was expected to yield a WA plasma concentration of about 0.625 μ g/ml (chemosensitizing concentration) based on our *in vitro* study.

Group 1 (Control): no treatment;

Group 2 (Dtx only): Dtx 20 mg/kg (*i.v.* once a week);

Group 3 (Dtx+WA(H)): Dtx 20 mg/kg (*i.v.* once a week)+WA 1.2 mg/kg (*i.v.* once a week immediately after Dtx)+WA 6 mg/kg (*i.p.* once daily);

Group 4 (Dtx+WA(M)): Dtx 20 mg/kg (*i.v.* once a week)+WA 0.6 mg/kg (*i.v.* once a week immediately after Dtx)+WA 3 mg/kg (*i.p.* once daily);

Group 5 (Dtx+WA(L)): Dtx 20 mg/kg (*i.v.* once a week)+WA 0.3 mg/kg (*i.v.* once a week immediately after Dtx)+WA 1.5 mg/kg (*i.p.* once daily);

Group 6 (WA(H)): WA 1.2 mg/kg (*i.v.* once a week)+WA 6 mg/kg (*i.p.* once daily);

Group 7 (WA(L)): WA 0.3 mg/kg (*i.v.* once a week)+WA 1.5 mg/kg (*i.p.* once daily).

The changes in the tumor sizes of each mouse were measured over a 14-day time course, beginning on the first day of treatment.

Statistical analysis. All data from the study were expressed as mean \pm standard deviation (SD). The results among different groups were compared using a one-way analysis of variance (ANOVA), followed by the post-hoc Bonferroni test for multiple comparisons. The difference between the two independent groups was compared by the Student *t*-test. $p < 0.05$ was considered statistically significant for all tests. All analyses were performed with the SPSS software (version 12.0; SPSS, Chicago, IL, USA).

Results

Cytotoxicity of the active components of TW. The cytotoxicity of 6 herbal components of TW for Dtx-resistant (PC3-TxR, DU145-TxR) and -sensitive (PC3, DU145) cells are shown in Figure 1. The results showed that WA was least toxic to all 4 prostate tumor cells compared to the other five compounds. Additional studies in normal human prostate epithelial cells found its IC_{50} to be comparable to that for prostate cancer cells (Figure 2) and less toxic than that of Dtx (IC_{50} of WA in μ g/ml range compared to ng/ml range for Dtx) (Table I). These data indicate WA may be non-toxic to normal tissues.

Sensitizing effect of the active components. The combination effect of Dtx with each of 6 herbal components on PC3-TxR

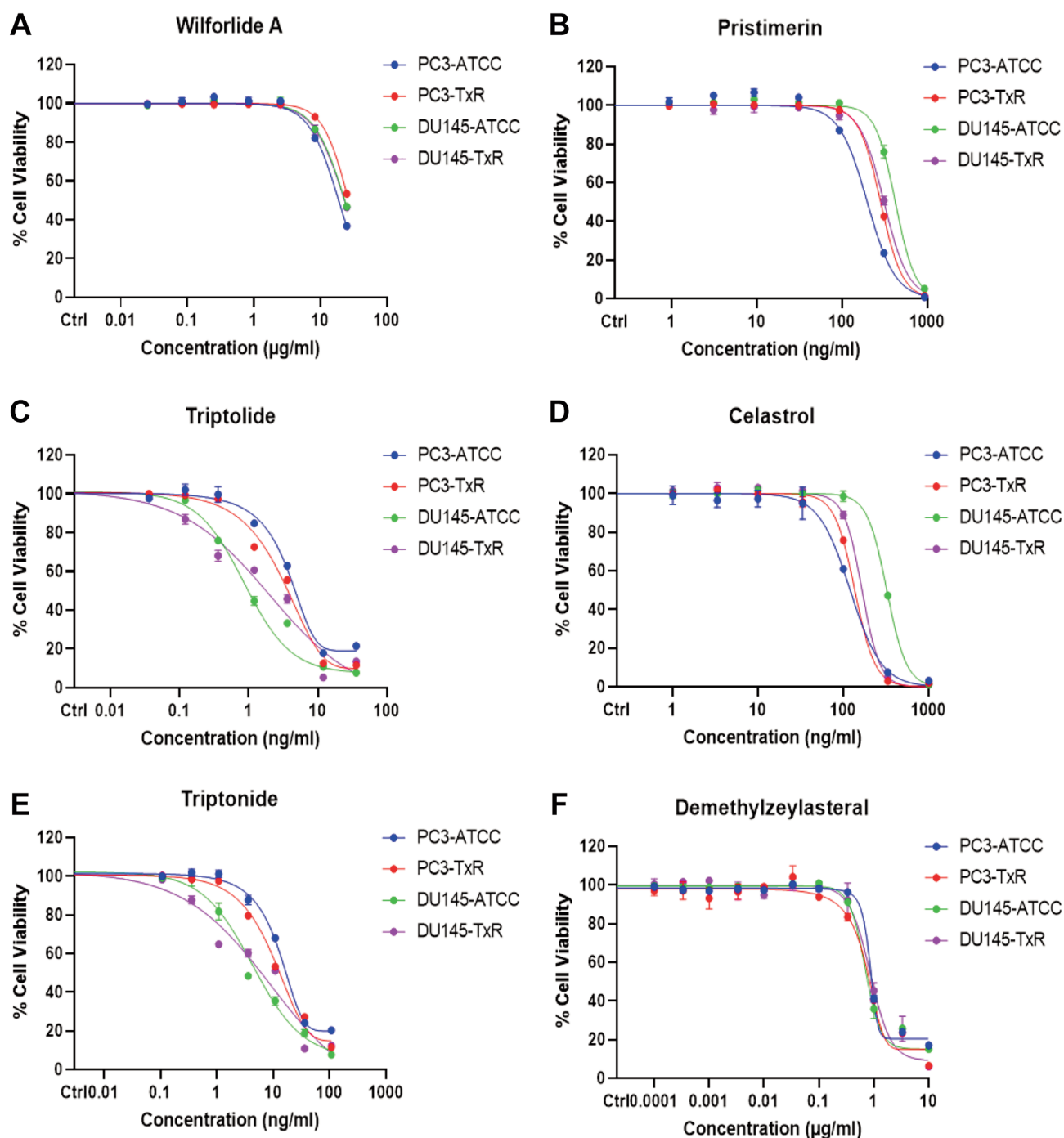


Figure 1. Cytotoxicity of six terpenoids to prostate cancer cell lines (PC3 and DU145) and their corresponding resistant cell lines. (A) IC_{50} of wilforlide A 9.2-13.9 $\mu\text{g/ml}$; (B) IC_{50} of pristimerin 189.7-389.1 ng/ml ; (C) IC_{50} of triptolide 1.0-3.7 ng/ml ; (D) IC_{50} of celastrol 117.5-324.1 ng/ml ; (E) IC_{50} of triptonide 3.4-12.9 ng/ml ; (F) IC_{50} of demethylzeylasteral 0.65-0.85 $\mu\text{g/ml}$.

cells are shown in Figure 3. WA is the only compound that showed a significant effect on overcoming chemoresistance of prostate cancer cells to Dtx. WA decreased the Dtx IC_{50} in a concentration-dependent manner from an average of 21.5 nM

to 13.8, 8.8, 5.8, and 2.9 nM at WA concentrations of 0.63, 1.25, 2.5, and 5.0 $\mu\text{g/ml}$, respectively (Figure 4). The corresponding CE was calculated to be 1.56, 2.09, 3.56, and 7.53 folds, respectively (Table I). Although other terpenoids

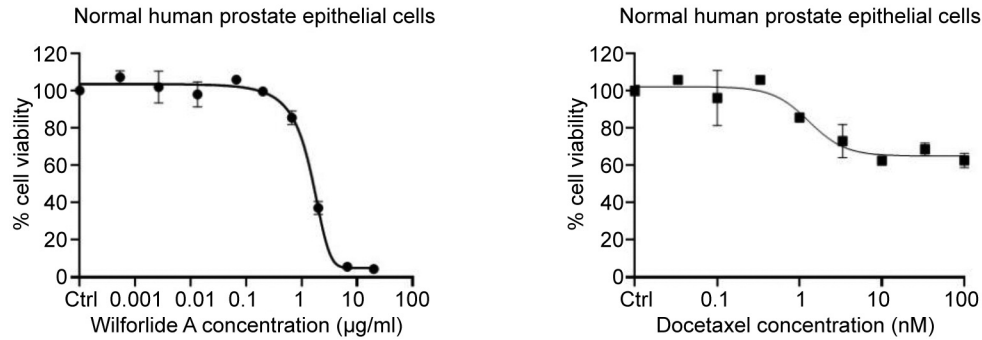


Figure 2. Cytotoxicity of wilforlide A (WA), docetaxel (Dtx) on the normal human prostate epithelial cells (HPrEpC). (A) WA: IC_{50} : 1.4 μ g/ml; (B) Dtx: IC_{50} > 1,000 nM.

Table I. Cytotoxicity of docetaxel (Dtx) on PC3-TxR cells and DU145-TxR alone or in combination with wilforlide A (WA).

Cell	WA concentration (μ g/ml)	IC_{50HD} (nM) (when combined with WA)	IC_{50D} (nM) (Dtx alone)	Chemosensitizing effect
PC3-TxR	0.625	13.75 \pm 1.75	21.19 \pm 1.6	1.56
	1.25	8.77 \pm 0.92		2.09
	2.5	5.75 \pm 0.69		3.56
	5	2.86 \pm 0.36		7.53
Du145-TxR	0.625	990.9 \pm 215.3	>1,000 nM	1.01
	1.25	242.6 \pm 7.3		4.12
	2.5	124.2 \pm 5.2		8.05
	5	48.5 \pm 4.5		20.62

IC_{50HD} is IC_{50D} of docetaxel from herb-docetaxel combination, IC_{50D} is IC_{50} of docetaxel alone in drug resistant PC-3 TxR or Du145-TxR cells; Chemosensitizing effects are calculated as the ratio of IC_{50D} and IC_{50HD} .

such as celastrol, prestimerin, demethylzeylasteral, triptolide, and triptonide showed significant cytotoxicity to prostate cancer cells, none of them showed a significant change in CE. The chemosensitizing effect of WA was further verified using another resistant cell line (DU-TxR): The IC_{50} in DU145-TxR cells decreased from >1,000 nM to 990.9, 242.6, 124.2, and 48.5 nM when combined with Dtx at WA concentrations of 0.625, 1.25, 2.5 and 5 μ g/ml, respectively (Figure 4). The corresponding CE were greater than 1.01, 4.12, 8.05, and 20.62, respectively. These results suggested that among the 6 components of TW, only WA was able to enhance the cytotoxicity of Dtx in the resistant cancer cells in a concentration dependent manner. The concentrations selected were based on the IC_{50} of cytotoxicity of WA (~10 μ g/ml). Therefore, relatively safe concentrations of 5.0, 2.5, 1.25, and 0.63 μ g/ml were selected, which are corresponded to 12.5, 25, or 50% of its IC_{50} . Furthermore, WA did not enhance the cytotoxicity of Dtx in the sensitive cell lines (PC3 and DU145).

Thus, WA is the only one among 6 well-known components of TW to significantly sensitize cytotoxicity of the resistant cell lines but not the sensitive cells when used

in combination with Dtx. In addition, combining WA (0.75 μ g/ml) with Dtx did not appear to enhance the cytotoxicity of Dtx in normal prostate cells.

Mechanistic findings. WA was able to inhibit P-gp mediated DNR efflux in a dose-dependent manner at the concentration range from 0.675 to 5 μ g/ml. The maximum inhibition was 20.5% relative to PSC833, a well-known P-gp inhibitor (Figure 5).

Consistent with the effect of TW, WA suppressed the expression of the CCNE2. However, it had no effect on the other two genes (*ANKRD* and *CTGF*). The changes in CCNE2 were 0.31 and 0.29 folds (or down-regulated by 3.2 and 3.5 folds) in 6 and 24 h, respectively (Figure 6). The inhibition on CCNE2 at mRNA levels was very significant after 6 h incubation, and the effect was not further enhanced when incubated over 6 h.

In vivo efficacy. The changes in tumor volumes are shown in Figure 7. Dtx alone did not show any significant suppression on tumor size, indicating its resistance in the PC3-TxR

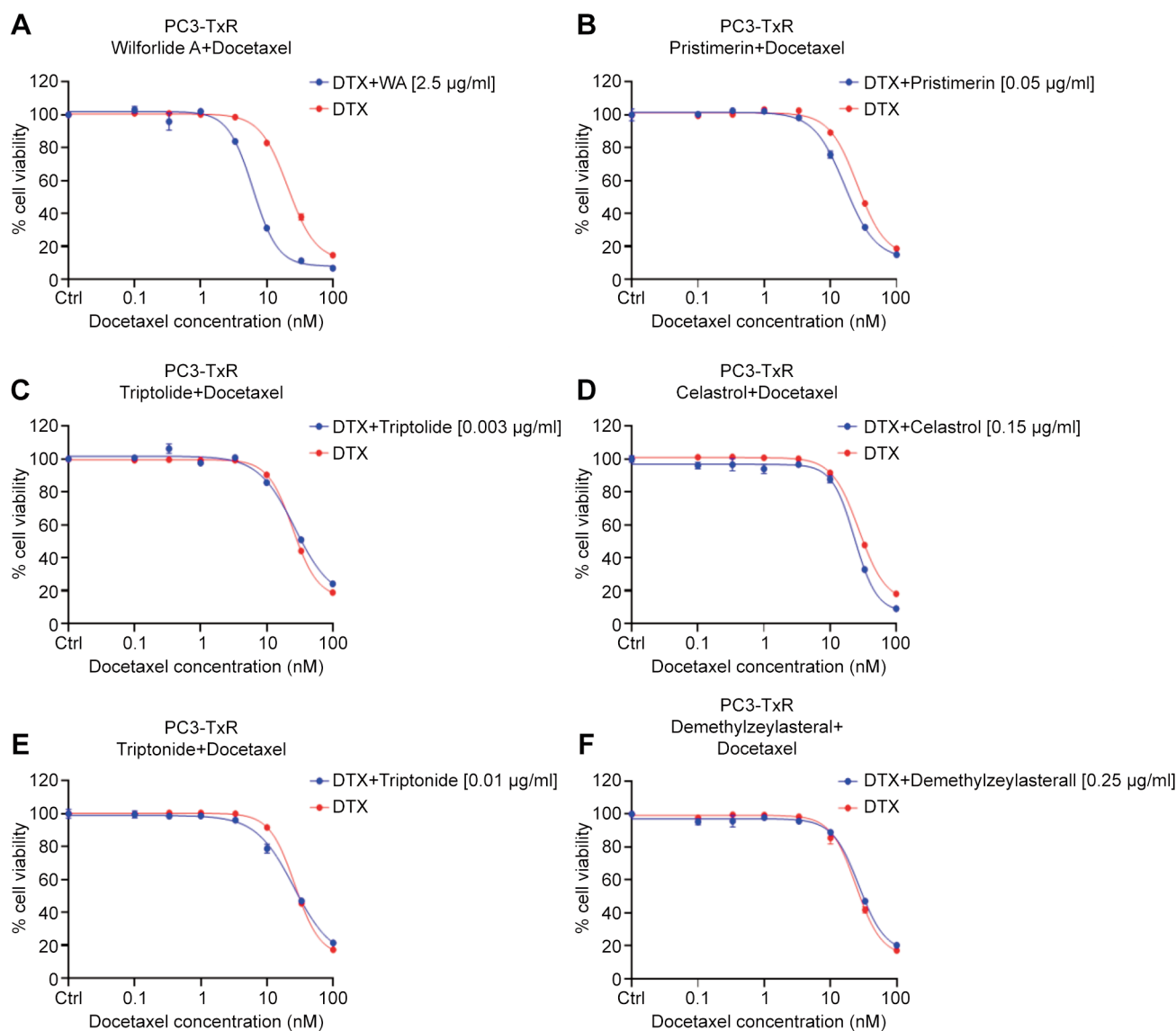


Figure 3. Chemosensitizing effect of six terpenoids to PC3-TxR cells, a PC3 cell line resistant to docetaxel.

xenograft model of the present study (421% versus 330% with and without Dtx, $p=0.51$, 1-way ANOVA). Consistent with the *in vitro* data that WA alone is less toxic, no significant antitumor effect was observed with high or low WA dose. In contrast, the combination of WA (high dose) and Dtx showed significant retardation of tumor growth compared to other treatment groups (101% vs. 421% respectively, $p<0.05$, 1-way ANOVA). However, the combination of WA at low and medium doses did not show any significant effect. Although WA at a medium dose decreased the tumor growth in the initial 10-day period, this effect was not significant after 10 days. These results indicated that dose selection is critical for the combination effect.

During treatment, substantial body weight loss was observed in all groups (Figure 8). Even in the non-treatment group, 3 out of 8 mice experienced more than 25% weight loss in 14 days. Thus, the bodyweight loss observed in the present study was not likely related to the specific drug treatment.

Discussion

In our previous study, TW, a well-known Chinese herbal extract for inflammatory diseases, was found to sensitize Dtx in resistant prostate cancer both *in vitro* and *in vivo* (14). While such activity can be therapeutically important for sensitizing Dtx for resistant prostate cancer, TW as an herbal

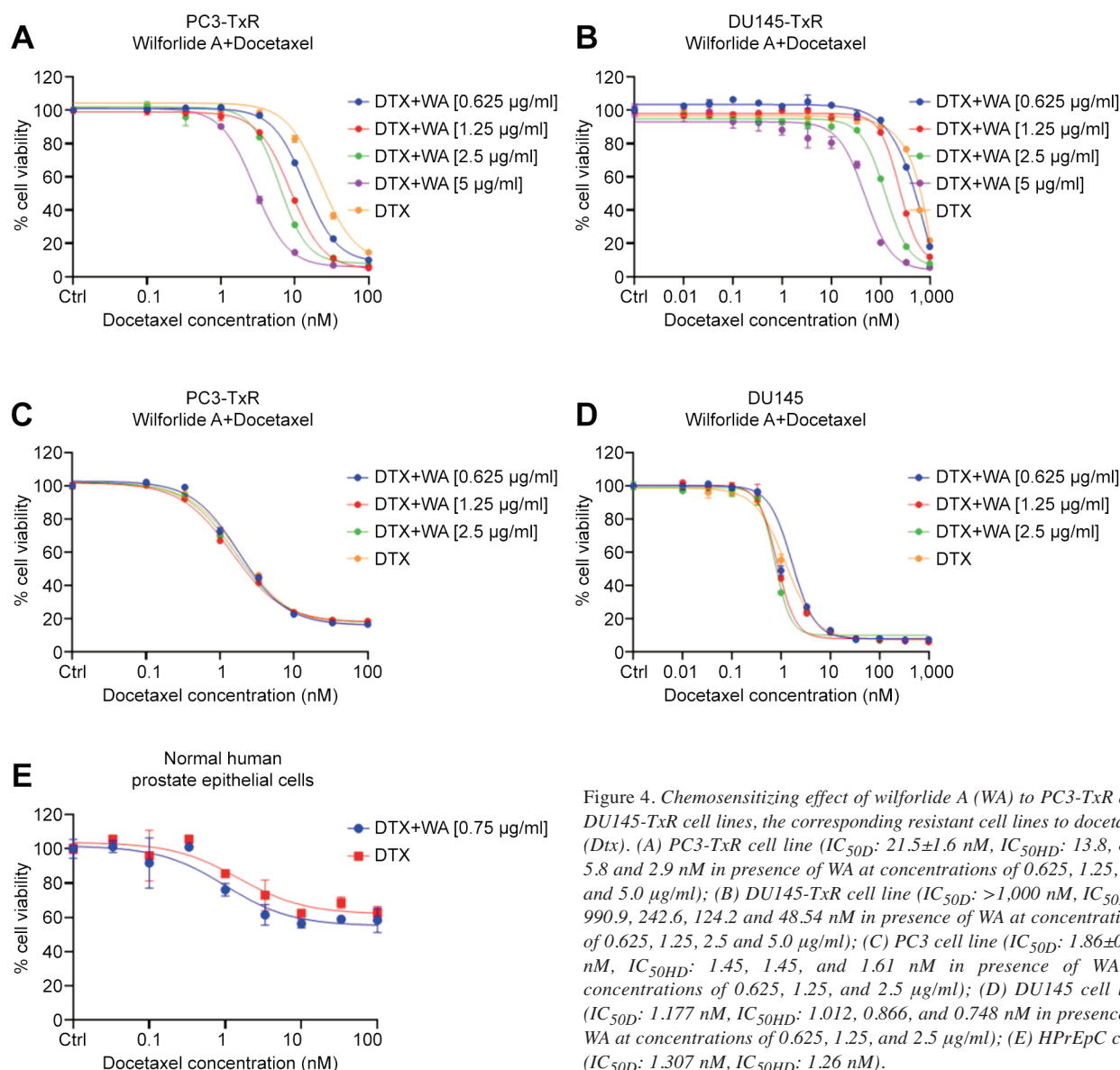


Figure 4. Chemosensitizing effect of wilforlide A (WA) to PC3-TxR and DU145-TxR cell lines, the corresponding resistant cell lines to docetaxel (Dtx). (A) PC3-TxR cell line (IC_{50D} : 21.5 ± 1.6 nM, IC_{50HD} : 13.8, 8.8, 5.8 and 2.9 nM in presence of WA at concentrations of 0.625, 1.25, 2.5 and 5.0 μ g/ml); (B) DU145-TxR cell line (IC_{50D} : $>1,000$ nM, IC_{50HD} : 990.9, 242.6, 124.2 and 48.54 nM in presence of WA at concentrations of 0.625, 1.25, 2.5 and 5.0 μ g/ml); (C) PC3 cell line (IC_{50D} : 1.86 ± 0.12 nM, IC_{50HD} : 1.45, 1.45, and 1.61 nM in presence of WA at concentrations of 0.625, 1.25, and 2.5 μ g/ml); (D) DU145 cell line (IC_{50D} : 1.177 nM, IC_{50HD} : 1.012, 0.866, and 0.748 nM in presence of WA at concentrations of 0.625, 1.25, and 2.5 μ g/ml); (E) HPrEpC cells (IC_{50D} : 1.307 nM, IC_{50HD} : 1.26 nM).

extract is difficult to get regulatory approval by the FDA. One major concern can be related to its quality control and safety that can arise from variations of multiple components from different batches of the final product. Thus, the identification of active ingredients which can significantly sensitize Dtx in PC3-TxR tumor is of considerable interest. In the present study, the discovery of an active compound, WA, among 6 other components, with a significant chemosensitizing effect can be an attractive finding toward developing a chemosensitizing product. Although some of the other components have been widely studied for their cytotoxic effects, such as triptolide, triptonide, and celastrol (12, 19-22), none of them showed any chemo-sensitizing effect.

The *in vitro* cell lines studies showed that constant exposure of PC3-TxR cells for 72 h to Dtx in combination with WA concentration of 0.625 μ g/ml or higher produced a 1.5-fold CE or higher (Figure 4A). Such WA plasma concentration can be achieved with *i.v.* (1.2 mg/kg) or *i.p.* dosing (6 mg/kg) (18). Thus, in the *in vivo* xenograft model, the high dose WA (1.2 mg/kg, *i.v.* once a week+6 mg/kg, *i.p.* once daily) in combination with Dtx appeared to achieve the expected chemosensitizing effect consistent with the *in vitro* CE and concentration of the chemosensitizer.

Cancer chemotherapy resistance usually involves multiple mechanisms, with P-gp considered a key mechanism that can cause resistance. The observed chemosensitizing effect with

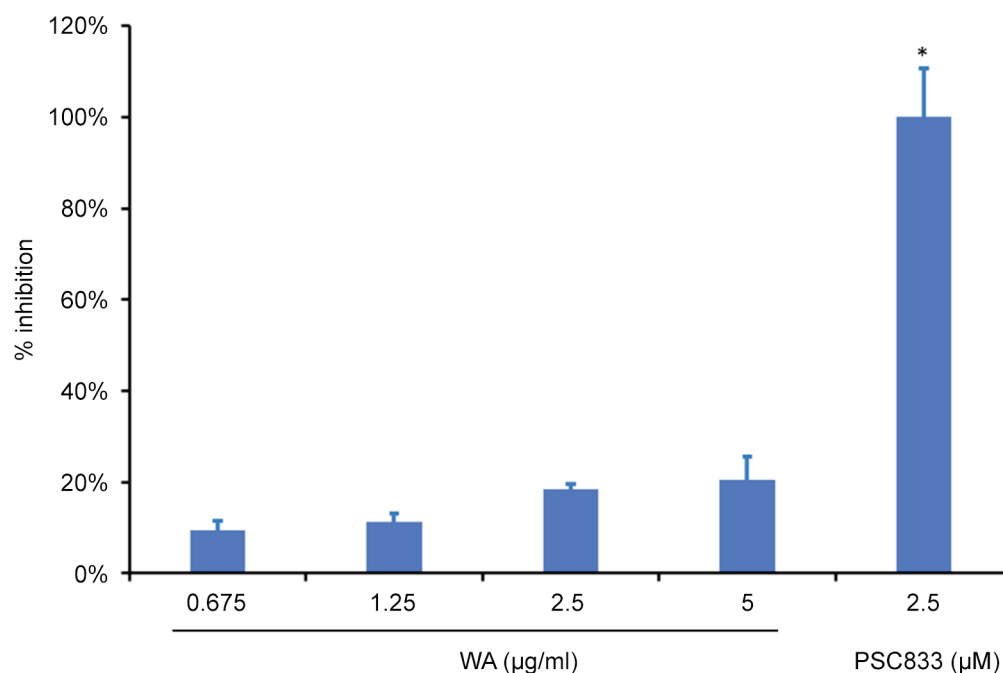


Figure 5. The concentration effect on the inhibition of P-gp mediated daunorubicin efflux in K562/Dox cells. The percent inhibition was normalized to PSC833 (2.5 mM), which is set as 100% inhibition. Data were expressed as mean \pm SD * p <0.05, 1-way ANOVA. WA: Wilforlide A; SD: standard deviation.

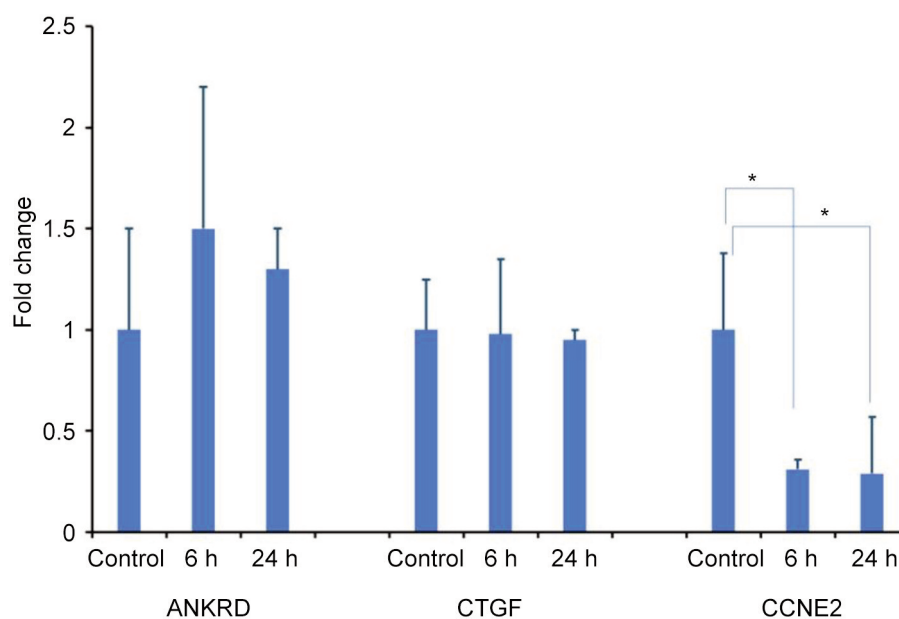


Figure 6. Fold change of ankyrin repeat domain containing (ANKRD), connective tissue growth factor (CTGF) and cyclin E2 splice variant 1 mRNA (CCNE2) expression after treatment of 2.5 µg/ml WA for 6 and 24 h, respectively. * p <0.05 (Student's *t*-test).

improved *in vivo* efficacy appears to be consistent with our mechanistical studies. WA at a concentration of 0.625 µg/ml can inhibit P-gp, although the effect is weak compared to the

potent Pgp inhibitor, PSC833 (Figure 5). However, WA can also down-regulate CCNE2. CCNE2 is G₁ cyclin that can bind Cdk2 and the overexpression of CCNE2 could lead to

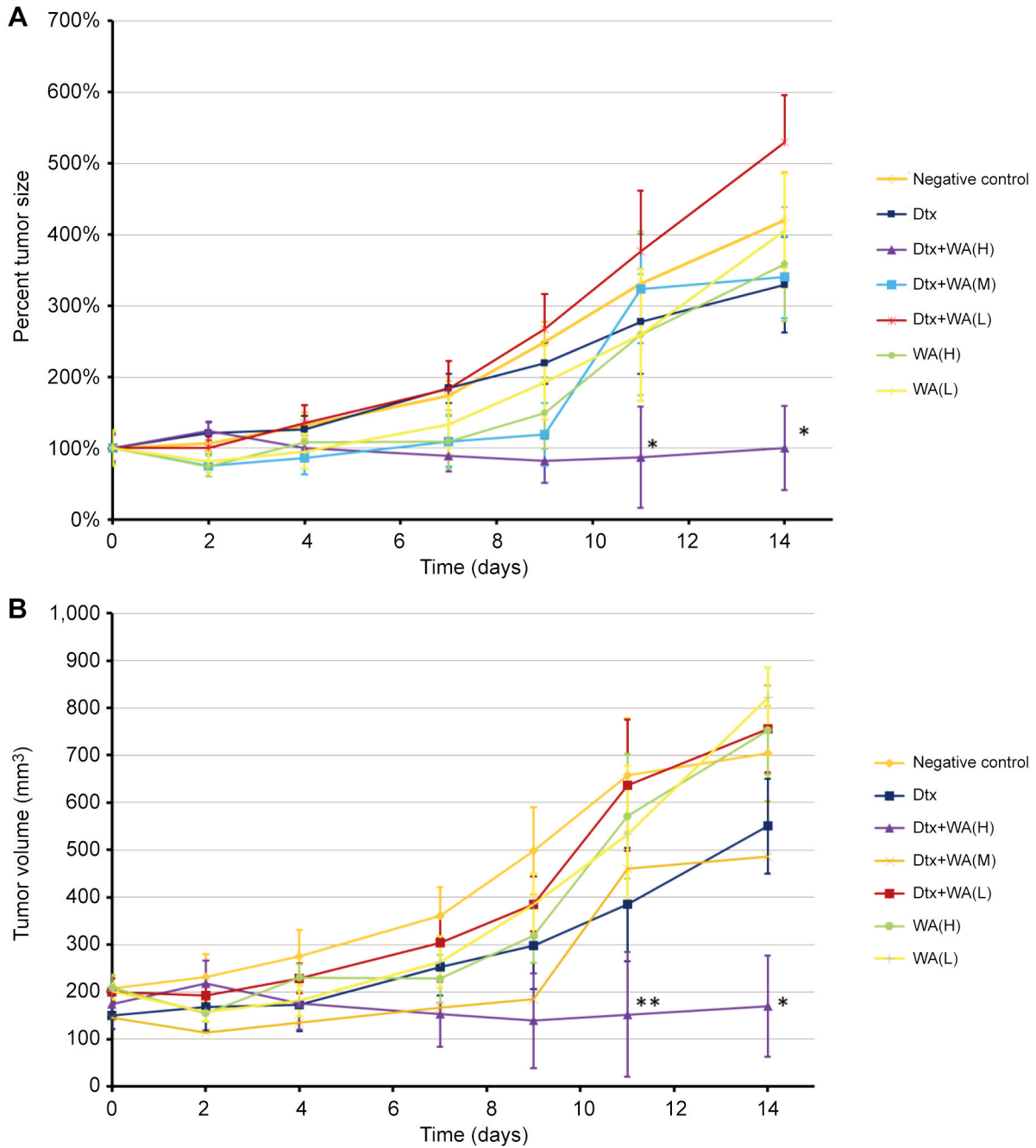


Figure 7. (A) Percent tumor growth of xenografted PC3-TxR cells following various treatments and (B) growth in the size of xenografted PC3-TxR cells following various treatments for groups (1-7) labeled as the negative control, Dtx, Dtx+WA(H), Dtx+WA(M), Dtx+WA(L), WA(H), and WA(L). * $p<0.05$; ** $p<0.01$ (1-way ANOVA). WA: Wilforlide A; Dtx: docetaxel; H: high dose; M: medium dose; L: low dose.

resistance in prostate and breast cancer cells (23). The inhibition of CCNE2 by WA might be a potential mechanism for the sensitizing effect, although other mechanisms need to be further investigated.

The safety of WA is an important consideration for its future development, although herbal medicines are usually

considered to be safer than conventional drugs and be used without prescription. TW products (such as tablets) have been widely used to treat rheumatoid arthritis in China, but some of its components, such as triptolide and celastrol, may be toxic (24). Our *in vitro* cytotoxicity study also found low IC_{50} values for these two compounds. In contrast, WA is

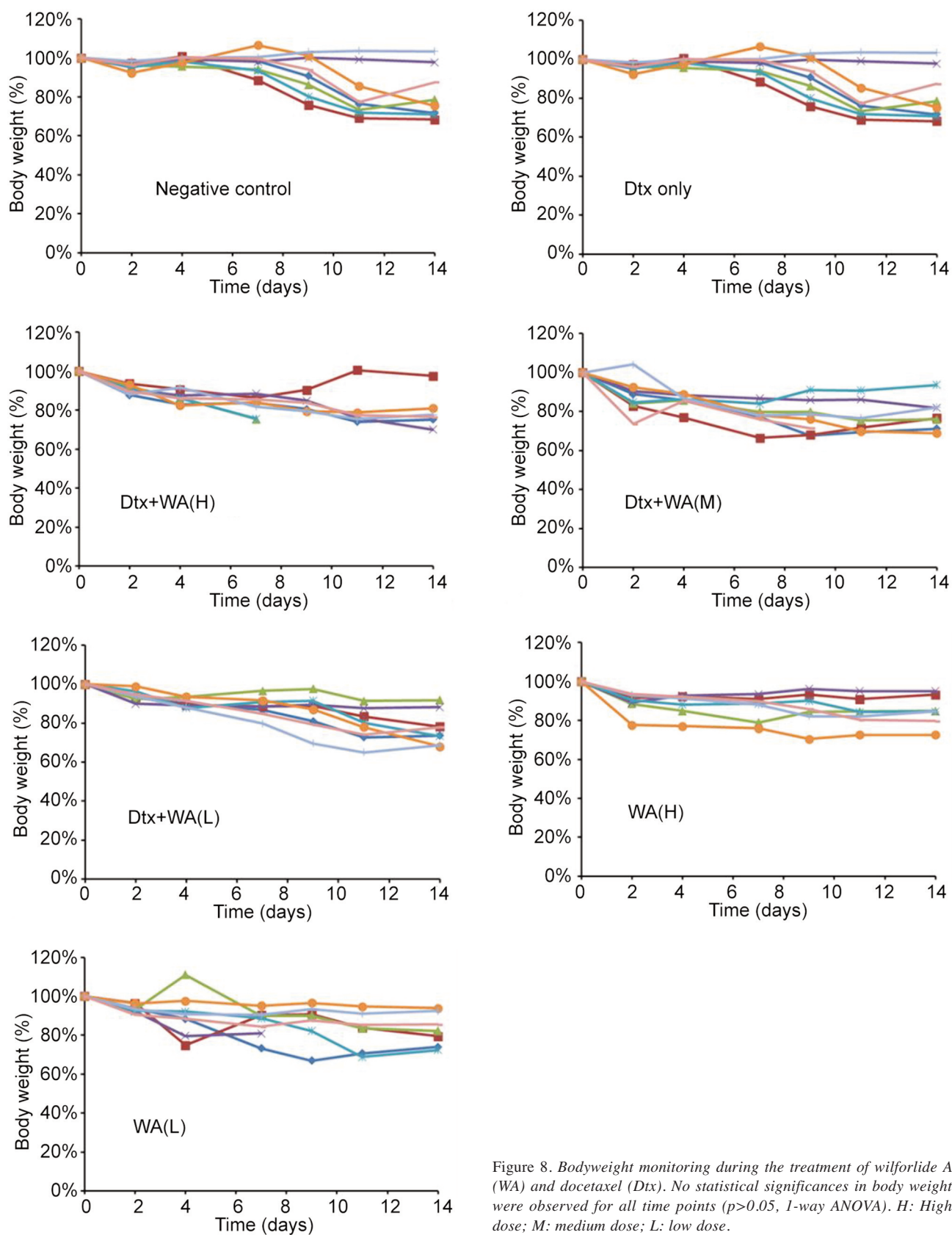


Figure 8. Bodyweight monitoring during the treatment of wilforlide A (WA) and docetaxel (Dtx). No statistical significances in body weight were observed for all time points ($p>0.05$, 1-way ANOVA). H: High dose; M: medium dose; L: low dose.

relatively safer based on its IC₅₀ values in comparison to the other 6 components of TW. Furthermore, our cell line study also found its IC₅₀ in normal prostate cells to be much less potent in comparison to Dtx. When WA at a concentration of 0.75 µg/ml (which is expected from high dose *i.p.* injection) was combined with Dtx, the Dtx IC₅₀ in the normal cell was not changed compared to Dtx alone (Figure 2).

In our previous pharmacokinetic study, WA was identified to be a BSC IV compound (low solubility and permeability) with a very low oral bioavailability (18). We expect that oral administration of WA is not likely to achieve an effective concentration. However, based on our previous pharmacokinetic study, *i.p.* injection with the high dose can achieve effective WA chemosensitizing concentrations. Thus, the combination of *i.v.* and *i.p.* doses were utilized in our efficacy study. In view of its limited solubility, a better drug delivery system to achieve a higher concentration *in vivo* can be explored to produce a better chemosensitizing effect in the future.

Conclusion

Our study identified that the active component (WA) in TW could sensitize the cytotoxic effect of Dtx in resistant prostate cancer cells. Dtx was found to suppress Dtx-resistant (PC3-TxR) tumors in a xenograft model when combined with WA, while Dtx alone did not show any retardation of tumor growth. In addition, WA also appeared to be relatively safe at the doses used for chemosensitization of Dtx. Further studies are warranted to confirm this exciting potential for treating Dtx-resistant patients in the future.

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Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

ZW: Conducted the experiments, data collection and analysis, and manuscript draft; SY: Conducted the experiments and data collection; HY: Assisted in the *in vitro* studies; YH: Supervised the study design, data analysis, and critical revision of the manuscript; MC: Conceived the study, performed data analysis and interpretation, and critical revision of the manuscript.

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