

# WD-3 inhibits the proliferation of breast cancer cells by regulating the glycolytic pathway

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## ABSTRACT

Number 3 Prescription (WD-3) is an herbal remedy used in traditional Chinese medicine that has been shown to improve the outcomes of patients with advanced colon and gastric cancers. This study aimed to investigate the effect of WD-3 on proliferation, glycolysis, and hexokinase 2 expression in breast cancer cells. Four breast cancer cell lines (MDA-MB-231, BT-549, MCF-7, and MCF-7/ADR-RES) were treated with different concentrations of WD-3 compared with blank control (phosphate-buffered saline). Each of the breast cancer cell lines was also divided into WD-3, paclitaxel, and blank control group. Cell proliferation and morphology were assessed by MTT assay, nuclear Hoechst 33258 staining, or immunofluorescence. Apoptosis was analyzed by flow cytometry. High performance liquid chromatography was used for measurement of ATP, ADP, and AMP. Hexokinase 2 expression was analyzed by Western blot and quantitative reverse transcription PCR. WD-3 inhibited proliferation and increased apoptosis in all four breast cancer cell lines, in a dose-dependent manner. ATP and EC (energy charge) were significantly decreased in WD-3-treated BT-549 and MDA-MB-231 cells. WD-3 significantly downregulated the protein and mRNA expression of hexokinase II in BT-549 cells, however, not in the other three breast cancer cell lines. Our findings indicate that WD-3 targets the glycolytic pathway in breast cancer cells to exert its antitumor activity.

KEYWORDS: Breast cancer; WD-3; glycolysis; hexokinase 2; apoptosis; ATP; antitumor activity

## INTRODUCTION

Breast cancer is a life-threatening disease that predominantly affects women. The Global Cancer Statistics 2018 report estimated 2,089,000 new cases of breast cancer in 2018 globally [1]. In females, breast cancer was the leading cause of mortality (15.0%) among the ten most common cancers [1]. Breast cancer is also on the rise in China. Moreover, the age of onset of breast cancer is dropping in China. In early 2019, the National Cancer Center of China reported that there were 304,000 breast cancer cases in 2015 and that breast cancer was the first cause of morbidity and the fifth cause of mortality among all cancers in females [2].

Traditional Chinese Medicine (TCM) has been widely used as an alternative therapy for breast cancer. Some TCM treatments can significantly improve the quality of life and overall survival of breast cancer patients [3-5]. TCM is

related to the unity and integrity of the human body and its relationship with nature. As the basic substance of which the human body is made, Qi is involved in promoting, warming, defending, and consolidating and governing action in the body. Qi stems from the congenital essence of parents before birth and is converted from daily diet as well as natural air that we breathe in through the lung. Qi deficiency is common in clinical practice and typically presents as pale complexion, shortness of breath, limb weakness, dizziness, sweating, and low voice [6-7]. Zhao Jingfang, a well-known oncologist and practitioner of traditional Chinese medicine, proposed a therapy called "Balance via Minor Manipulation," characterized by accurate pinpointing, simple prescriptions, small doses, and regulation of spleen and stomach. Zhao believes that the treatment of breast cancer should rely on regulating the circulation of Qi, blood, and fluid in the middle energizer (spleen and stomach) of the body. Only through the harmony between the three the immune system of the body can strengthen and pathogenic factors can be eliminated. Number 3 Prescription (WD-3) is a prescription developed according to the Zhao's philosophy of "Balance via Minor Manipulation" in 1997. Several previous studies showed that the treatment with WD-3 could improve the quality of life of patients with advanced colon and gastric cancer. Namely, the achieved disease control rate (88.16%) and the 3-year overall survival rate (61.18%) among patients with advanced gastric cancer receiving treatment with WD-3 were significantly improved in comparison with untreated patients [8,9]. WD-3 has been used in the treatment of breast cancer for a long time in our hospital,

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showing good therapeutic efficacy. However, the specific therapeutic mechanism of WD-3 in breast cancer needs to be further clarified.

The energy metabolism of breast cancer cells largely differs from that of normal cells, which is mainly manifested in the increase of glycolysis. As breast cancer progresses, the cells become increasingly dependent on adenosine triphosphate (ATP) produced by the glycolytic pathway [10-13]. Thus, inhibition of the glycolytic pathway by targeting key enzymes and metabolic factors has become a viable strategy for developing antitumor drugs, which has been achieved in some *in vitro* and *in vivo* experiments [14]. Hexokinase is the first rate-limiting enzyme in the glycolytic pathway and is highly expressed in many types of tumors [15]. It is generally believed that hexokinase 2, the most common subtype of hexokinases in tumor cells, not only regulates glycolysis, but also inhibits apoptosis by binding to voltage-dependent anion channel (VDAC) on the mitochondrial outer membrane [16]. This study aimed to investigate the effect of WD-3 on proliferation, glycolysis, and hexokinase 2 expression in breast cancer cells.

## MATERIALS AND METHODS

### Drug preparation

WD-3 prescription (Table 1), which is mainly composed of *Codonopsis pilosula*, *Poria cocos*, *Atractylodes macrocephala* Koidz, *Ophiopogon japonicus*, *Poria cum Radix Pini*, *Schisandra chinensis*, *Eriobotrya japonica*, *Pinellia ternata*, *Caulis perillae*, *Coix lacryma-jobi*, *Citrus aurantium*, *Semen Raphani*, *Setaria italic*, *Hordeum vulgare*, and *Glycyrrhiza uralensis*, was prepared by the Reagent Preparation Room, Wuxi Hospital Affiliated to Nanjing University of Chinese Medicine (Jiangsu Food and Drug Administration [2005] No. 401, Jiangsu medicine license 204002111). The herbs comprising WD-3 were

identified and decocted together. In the first step, herbs were placed in a gallipot, soaked in water eight times for 1 h, heated, and kept boiling for 1 h, resulting in distilled Liquid 1. In the second step, the herbs were immersed in five times the volume of water in a pan for 30 min and cooked for 1 h, and Liquid 2 was filtered. In the third step, Liquid 1 and Liquid 2 were combined and left for over 12 h. The resulting Liquid 3 was filtered and concentrated to one tenth of the original solution [17]. White granulated sugar (20 g), sodium benzoate (0.4 g), and ethylparaben (0.05 g) were added to the Liquid 3. Finally, the mixture were boiled for 15 min, sterilized at 110°C, and stored in the refrigerator at 4°C. Paclitaxel injection (5 ml: 30 mg × 1 bottle/box, lot number: 17060411), provided by Yangtze River Pharmaceutical Group Co., Ltd was used as positive control. Phosphate buffered saline (PBS) was used in blank control group.

### Cell lines

Breast cancer cell lines MDA-MB-231, BT-549, MCF-7, and MCF-7/ADR-RES and fluorescently labeled MDA-MB-231/RFP (red fluorescent protein), BT-549/RFP, MCF-7/RFP, and MCF-7/ADR-RES/RFP were provided by Anticancer Biotechnology (Beijing) Co., Ltd, Beijing, China.

### Reagents

Rabbit monoclonal anti-Hexokinase II (Cat. No. 2867) was purchased from Cell Signaling Technology, Co. Ltd, USA; anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cat. No. YM3029) from Immunoway, USA; ATP (Cat. No. A7699), adenosine diphosphate (ADP, Cat. No. A2754), adenosine monophosphate (AMP, Cat. No. A6885), and MTT (Cat. No. M2128) from Sigma-Aldrich, USA; perchloric acid (Cat. No. 10019318), potassium dihydrogen phosphate (Cat. No. 10017618), sodium hydroxide (Cat. No. 10019762), and potassium hydroxide (Cat. No. 10017018) from Sinopharm

**TABLE 1.** Herbs included in WD-3

Chinese name	Latin name	Locality in China	Lot no.	Dosage (g)
Dang-shen	<i>Codonopsis pilosula</i>	Shanxi	160702	30
Fu-ling	<i>Poria cocos</i>	Yunnan	160726	10
Bai-zhu	<i>Atractylodes macrocephala</i> Koidz	Zhejiang	160729	10
Mai-dong	<i>Ophiopogon japonicus</i>	Hebei	160625	10
Fu-shen	<i>Poria cum Radix Pini</i>	Yunnan	16052001	10
Wu-wei-zi	<i>Schisandra chinensis</i>	Heilongjiang	160625	6
Pi-pa-ye	<i>Eriobotrya japonica</i>	Jiangsu	160605	10
Zhi-ban-xia	<i>Pinellia ternata</i>	Sichuan	160710	6
Su-geng	<i>Caulis perillae</i>	Hubei	160608	10
Yi-yi-ren	<i>Coix lacryma-jobi</i>	Hebei	160715	30
Zhi-qiao	<i>Citrus aurantium</i>	Fujian	160703	10
Lai-fu-zi	<i>Semen Raphani</i>	Hebei	160815	10
Gu-ya	<i>Setaria italic</i>	Jiangsu	16061901	10
Mai-ya	<i>Hordeum vulgare</i>	Hebei	160726	10
Gan-cao	<i>Glycyrrhiza uralensis</i>	Inner Mongolia	160625	3

Chemical Reagent Co., Ltd, China; RPMI 1640 culture medium (SH30809.01) from Hyclone, USA; fetal bovine serum (FBS, 040011ACS) from Biological Industry, Israel; Penicillin-Streptomycin (Pen-Strep, Cat.No. 15140122) from Gibco, USA; Hoechst 33258 (Cat. No. H21491) and Dead Cell Apoptosis Kit with Annexin V FITC/PI (Cat. No. V13242) from Invitrogen, USA; 2 × T5 Fast qPCR Mix (Cat. No. TSE202) and Golden star RT6 cDNA Synthesis kit (Cat. No. TSK301S) from TSINGKE, China; Trizol reagent (Cat. No. 15596018) from Life Technologies, USA; M-MLV Reverse Transcriptase (Cat. No. M5313) from Promega, USA; PowerUp™ SYBR® Green Master Mix (Cat. No.A25742) from ABI and 5 × RNA Loading Buffer (Cat. No. CW0611A) from CWBio Co.Ltd, China.

## Instruments

The instruments used were OLYMPUS IMT-2 fluorescence microscope (OLYMPUS, Japan), laser confocal scanning microscopy (LCSM, Thermo scientific, USA), Agilent 1200 high performance liquid chromatography (HPLC, Agilent, USA), ELx800 microplate reader (BioTek, USA), flow cytometry (BD Biosciences, USA), Roche LightCycler 480II real-time PCR (Roche, Switzerland), PowerPac Basic electrophoresis instrument (BIO-RAD, USA).

## MTT assay

The breast cancer cells in the logarithmic phase were seeded in a 96-well plate ( $4 \times 10^3$  cells per well) for cell adhesion. After 48 h treatment with WD-3 (paclitaxel positive control and blank control were also set), 0.5 mL MTT solution was added. Two hours later, the supernatant was discarded and 150  $\mu$ L of dimethyl sulfoxide (DMSO) was added. The samples were then oscillated at low speed on the shaking table for 10 min to fully dissolve the crystals. OD<sub>450</sub> was measured and the half maximal inhibitory concentration (IC<sub>50</sub>) was calculated for each sample as follows:  $IC_{50} = (\text{OD}_{450} \text{ of positive control group} - \text{OD}_{450} \text{ of samples}) / \text{OD}_{450} \text{ of positive control group} \times 100 \%$ . Each experiment was performed in triplicate.

## Nuclear Hoechst 33258 staining

Dual-color fluorescent cells in the logarithmic phase were prepared as a suspension at  $4 \times 10^5$  cells/mL. Cell suspension was seeded in a 96-well plate (100  $\mu$ L per well) for cell adhesion. WD-3 treatment group, paclitaxel treatment group, and blank control group were set. After a treatment for 24 h, the cells were incubated with 0.5  $\mu$ g/mL Hoechst 33258 solution for 20 min at room temperature, followed by PBS wash 3 times with 5 min interval. Finally, cell morphology was observed under LCSM.

## Transfection and screening of dual-color fluorescent cells and immunofluorescence

Dual-color fluorescent cells expressing RFP in the cytoplasm and green fluorescent protein (GFP) in the nucleus were used. GFP was stably transfected into RFP-positive cells. Briefly, PT-67 H2B/GFP cells were packaged and grown to the logarithmic phase. The supernatant was collected and filtered (pore size: 0.22  $\mu$ m). MDA-MB-231/RFP, BT-549/RFP, MCF-7/RFP, MCF-7/ADR-RES/RFP cells were transfected with a filtration repeated 2–4 times. At 48 h, GFP-positive nuclei were partially observed under a fluorescence microscope. RFP-positive cells were screened by treatment with 200, 400, and 800  $\mu$ g/mL G418. After 1–2 weeks, the cells in a good condition and with relatively high ratio of dual-color fluorescent proteins were selected for monoclonal screening. These cells were inoculated into a 96-well plate with one cell per well. One week later, the cells with monoclonal formation were subjected to amplification culture. The clones with the brightest fluorescence were preserved.

Dual-color fluorescent cells in the logarithmic phase were prepared as a suspension at  $4 \times 10^5$  cells/mL. Cell suspension was injected into a 96-well plate (100  $\mu$ L per well) for cell adhesion overnight. After a treatment with WD-3 or paclitaxel for 24 h and 48 h, cell morphology was observed under a fluorescence microscope.

## Apoptosis determination

The cells were seeded in a 6-well plate ( $1 \times 10^5$  cells per well). After culture for 18–24 h, different concentrations of WD-3 were added, and paclitaxel positive control and blank control groups were set. Then the cells were collected and centrifuged at 1500 r/min for 10 min. The precipitant was fixed in 1 ml of 75% ethanol at 4°C overnight. After 24 h treatment, fixed cells were centrifuged at 1000 r/min for 10 min, and the precipitant was incubated with 200  $\mu$ L of binding buffer, 10  $\mu$ L of Annexin V/FITC (1  $\mu$ g/mL), and 5  $\mu$ L of propidium iodide [PI] (2  $\mu$ g/mL) at 4°C, in dark for 15 min. Cell apoptosis was determined using a flow cytometry.

## HPLC

Cells in the logarithmic phase were seeded into 100-mm culture dishes and treated with different doses of WD-3. The same amount of cells was collected from each sample, the cells were quantified for protein concentration and subjected to HPLC. The following equations were used: Total adenine nucleotides (TAN) = ATP + ADP + AMP; Energy charge (EC) = (ATP + 0.5 ADP)/(ATP + ADP + AMP).

## Western blot

A suspension of cells in the logarithmic phase (2 ml,  $5 \times 10^4$  cells/mL) was injected into each well in a 6-well

plate. After specific treatment, the cells were lysed for total protein extraction using radioimmunoprecipitation assay (RIPA) buffer and quantified by bicinchoninic acid (BCA) method. The protein sample was loaded for electrophoresis and transferred on a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in 5% skim milk for 2 h and subjected to incubation with primary (anti-hexokinase 2 [HK2] antibody) and secondary antibodies (mouse monoclonal anti-GAPDH). Bands were detected by enhanced chemiluminescence (ECL) and analyzed by ImageJ Software, NIH, USA.

### Quantitative reverse transcription PCR (qRT-PCR)

Breast cancer cells (2 mL of suspension,  $5 \times 10^4$  cells/mL) in the logarithmic phase were seeded into a 96-well plate. WD-3 group and blank control group were established. After 24 h of culture, TRIzol kit was used to extract total RNA, and M-MLV kit was used for reverse transcription, according to the manufacturer's protocols. The RT-PCR steps were 16°C for 30 min, 42°C for 30 min, and 70°C for 15 min, total of 30 cycles. The samples were stored in a refrigerator at -20°C. For each sample, 2 µl of cDNA was amplified with target gene primers and reference gene primers (Table 2) for 40 cycles (95°C, 1 min; 95°C, 15 sec; and 60°C, 50 sec). The relative expression was quantified with the formula  $2^{-\Delta\Delta CT}$  [18].

### Statistical analysis

Data analysis was performed using IBM SPSS Statistics for Windows, Version 19.0. (IBM Corp. Armonk, NY). Qualitative data were analyzed by chi-square and rank sum test. Quantitative data were analyzed by the Student's *t*-test and analysis of variance (ANOVA). A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### WD-3 treatment inhibited the proliferation of breast cancer cells

Breast cancer cells MDA-MB-231, BT-549, MCF-7, and MCF-7/ADR-RES were treated with different concentrations of WD-3 (0, 0.0128, 0.064, 0.32, 1.6, 8, 40, and 200 mg/mL). Proliferation inhibition rate was determined by MTT assay. WD-3 treatment markedly inhibited the proliferation of the four breast cancer cell lines (Figure 1). The inhibition rate gradually increased in a dose-dependent manner. IC<sub>50</sub> values of the four breast cancer cell lines were calculated and shown in Table 3. The inhibitory effect of WD-3 on the proliferation rate was much more pronounced in MCF-7/ADR-RES cells, the lowest inhibition rate was observed in the hormone-dependent MCF-7 cell line.

### Cell morphology changes in breast cancer cells after WD-3 treatment

Cell morphology changes following WD-3 treatment were observed by laser confocal imaging. Breast cancer cells were divided into WD-3 group (80 mg/mL), paclitaxel group (3 µg/mL), and blank control group. Cells were treated with 80 mg/mL WD-3 or 3 µg/mL paclitaxel for 24 h. As shown in Figure 2, chromatin condensation, aggregation, marginalization, and fragmentation were observed in both WD-3 group and paclitaxel group.

Four dual-color fluorescent breast cancer cell lines MDA-MB-231 DUAL, BT-549 DUAL, MCF-7 DUAL, and MCF-7/ADR-RES DUAL were successfully established (Figure 3). These dual-color fluorescent cells were treated with different concentrations of WD-3 (20, 40, and 80 mg/mL) for

**TABLE 2.** Primer sequences used in quantitative reverse transcription polymerase chain reaction (qRT-PCR)

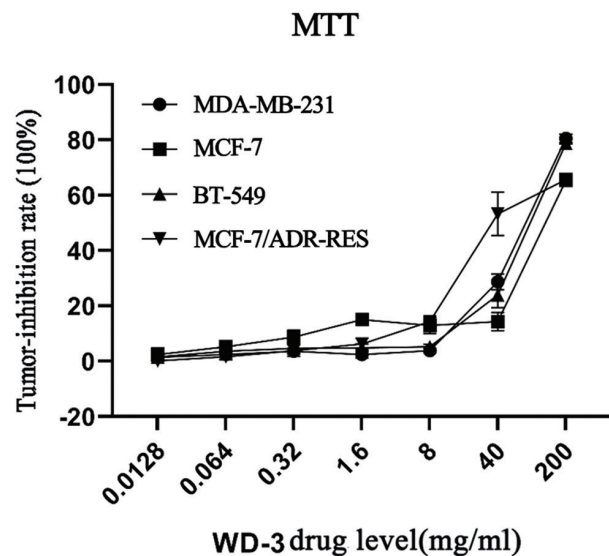
Gene name	Sequence (5' to 3')	Product size (bp)
HK2	F: TGCCACCAGACTAACTAGACG	227
	R: CCCGTGCCCAATGAGAC	
GAPDH	F: AGGTCGGTGTGAACGGATTTG	95
	R: GGGGTCGTTGATGGCAACA	

HK2: Hexokinase 2; GAPDH: Glyceroldehyde 3-phosphate dehydrogenase

**TABLE 3.** IC<sub>50</sub> values of WD-3 (mg/mL) for four breast cancer cell lines

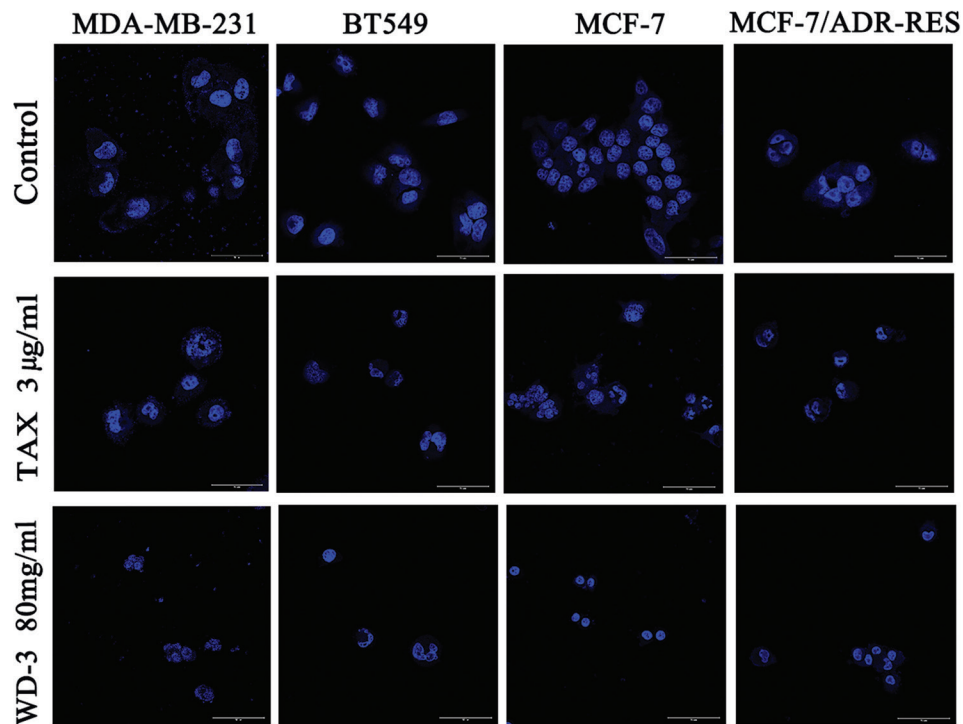
Cell line	MDA-MB-231	BT-549	MCF-7	MCF-7/ADR-RES
IC <sub>50</sub>	78.79±1.34*	90.00±9.16*	146.89±6.69	62.00±14.23*

Data are presented as  $x \pm SD$  (n=3). IC<sub>50</sub>: Half maximal inhibitory concentration. \* $p < 0.05$  vs. MCF-7

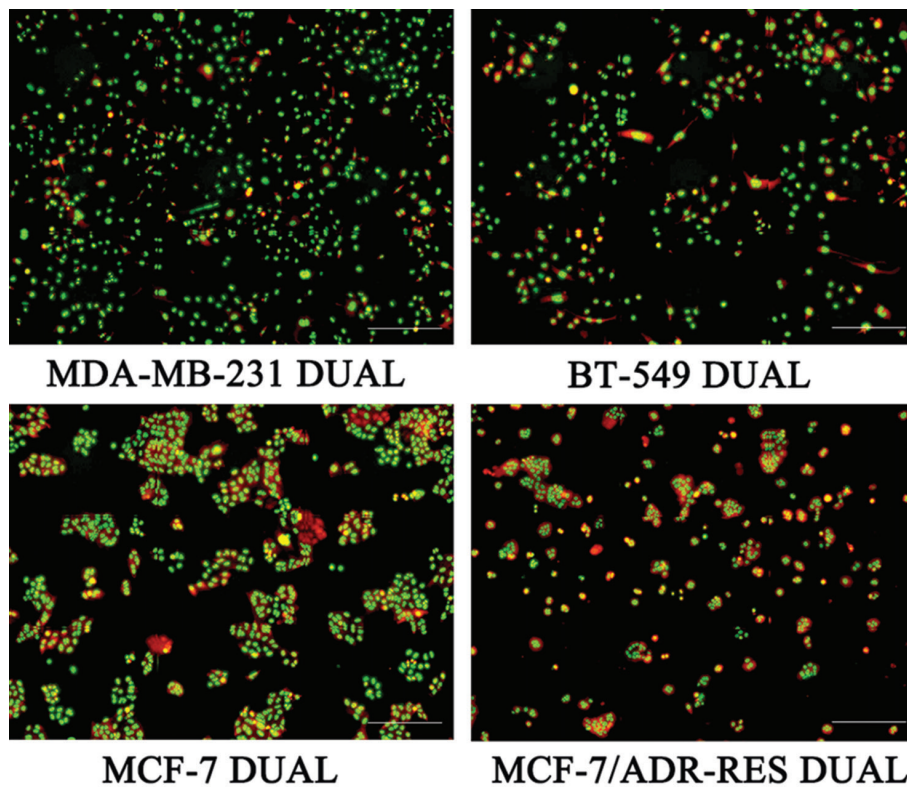


**FIGURE 1.** Proliferation inhibition rate of WD-3 in breast cancer cells by MTT assay. Breast cancer cell lines MDA-MB-231, BT-549, MCF-7, and MCF-7/ADR-RES were treated with different concentrations of WD-3 (0, 0.0128, 0.064, 0.32, 1.6, 8, 40, and 200 mg/mL). WD-3 treatment markedly inhibited the proliferation of the four breast cancer cell lines. The inhibition rate gradually increased in a dose-dependent manner.





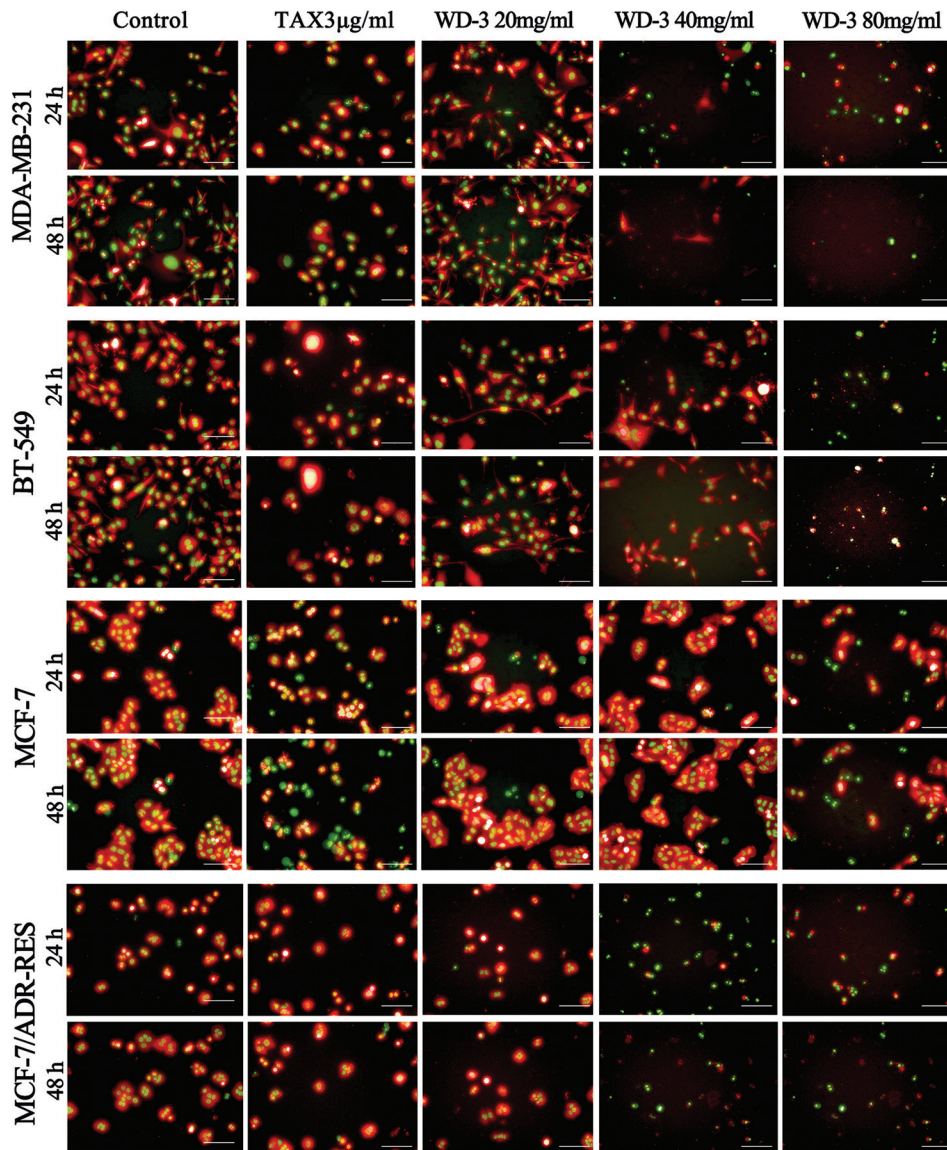
**FIGURE 2.** Laser confocal imaging of four breast cancer cell lines treated with WD-3. Breast cancer cell lines MDA-MB-231, BT-549, MCF-7, and MCF-7/ADR-RES were divided into WD-3 (80 mg/mL), paclitaxel (TAX, 3 µg/mL), and blank control (phosphate-buffered saline) group. Cells were treated for 24 h. Chromatin condensation, aggregation, marginalization, and fragmentation were observed in both WD-3 group and paclitaxel group. Scale bar, 50 µm.



**FIGURE 3.** Four dual-color fluorescent breast cancer cell lines MDA-MB-231 DUAL, BT-549 DUAL, MCF-7 DUAL, and MCF-7/ADR-RES DUAL were successfully established. Red fluorescent protein (RFP)-positive cytoplasm and green fluorescent protein (GFP)-positive nucleus (yellow-green). Scale bar, 500 µm.

24 h and 48 h. Cell morphology changes were observed under the OLYMPUS IMT-2 fluorescence microscope (Figure 4). The cells in blank control group were normal in morphology.

RFP-positive cytoplasm and GFP-positive nucleus were clear (nuclei were yellow-green due to RFP overlap). Membrane folds were clearly distinguishable.



**FIGURE 4.** Fluorescence imaging of four breast cancer cell lines treated with WD-3 at different time points and concentrations. MDA-MB-231 DUAL, BT-549 DUAL, MCF-7 DUAL, and MCF-7/ADR-RES DUAL were treated with different concentrations of WD-3 (20, 40, and 80 mg/mL) or paclitaxel (TAX, 3  $\mu$ g/mL) for 24 h and 48 h. Blank control was treated with phosphate-buffered saline. WD-3 treatment caused cell surface protrusion and fold disappearance, rounded and smaller cells, excessive intracellular vacuoles, chromatin condensation (fluorescence enhancement), and nuclear decomposition (rippled or creased). Apoptotic bodies were observed at high-dose WD-3. Apoptosis increased in a dose- and time-dependent manner. In particular, WD-3 treatment showed a stronger antitumor effect than paclitaxel in the drug-resistant cell line MCF-7/ADR-RES. Scale bar, 500  $\mu$ m.

The following apoptotic morphology changes were observed in the four breast cancer cell lines after WD-3 treatment: cell surface protrusion and fold disappearance, rounded and smaller cells, excessive intracellular vacuoles, chromatin condensation (fluorescence enhancement), and nuclear decay (rippled or creased). Apoptotic bodies were observed at high-dose WD-3. Apoptosis increased in a dose- and time-dependent manner in all four breast cancer cell lines. Obvious apoptosis was observed in MCF-7 DUAL after 80 mg/mL WD-3 treatment. In addition to MCF-7 DUAL, a decreased number of living cells and an increased number of apoptotic cells were observed in the remaining three breast cancer cell lines following 40 mg/mL and 80 mg/mL WD-3 treatment. Under

the same-dose of WD-3 (80 mg/mL), the lowest number of living cells was observed in MCF-7/ADR-RES DUAL and the highest number in MCF-7 DUAL cell line, which was consistent with the MTT results.

#### WD-3-induced apoptosis in breast cancer cells as determined by flow cytometry

Cells were treated with 40, 80, and 160 mg/mL of WD-3. Paclitaxel (5  $\mu$ g/mL) and blank control group were also set. After treatment for 24 h, the cells were stained with Annexin V/FITC and PI and subjected to flow cytometry (Figure 5A). Apoptotic rate increased in a dose-dependent manner and

living cell ratio decreased (Figure 5B) in all four breast cancer cell lines, suggesting that WD-3 induced apoptosis in breast cancer cells.

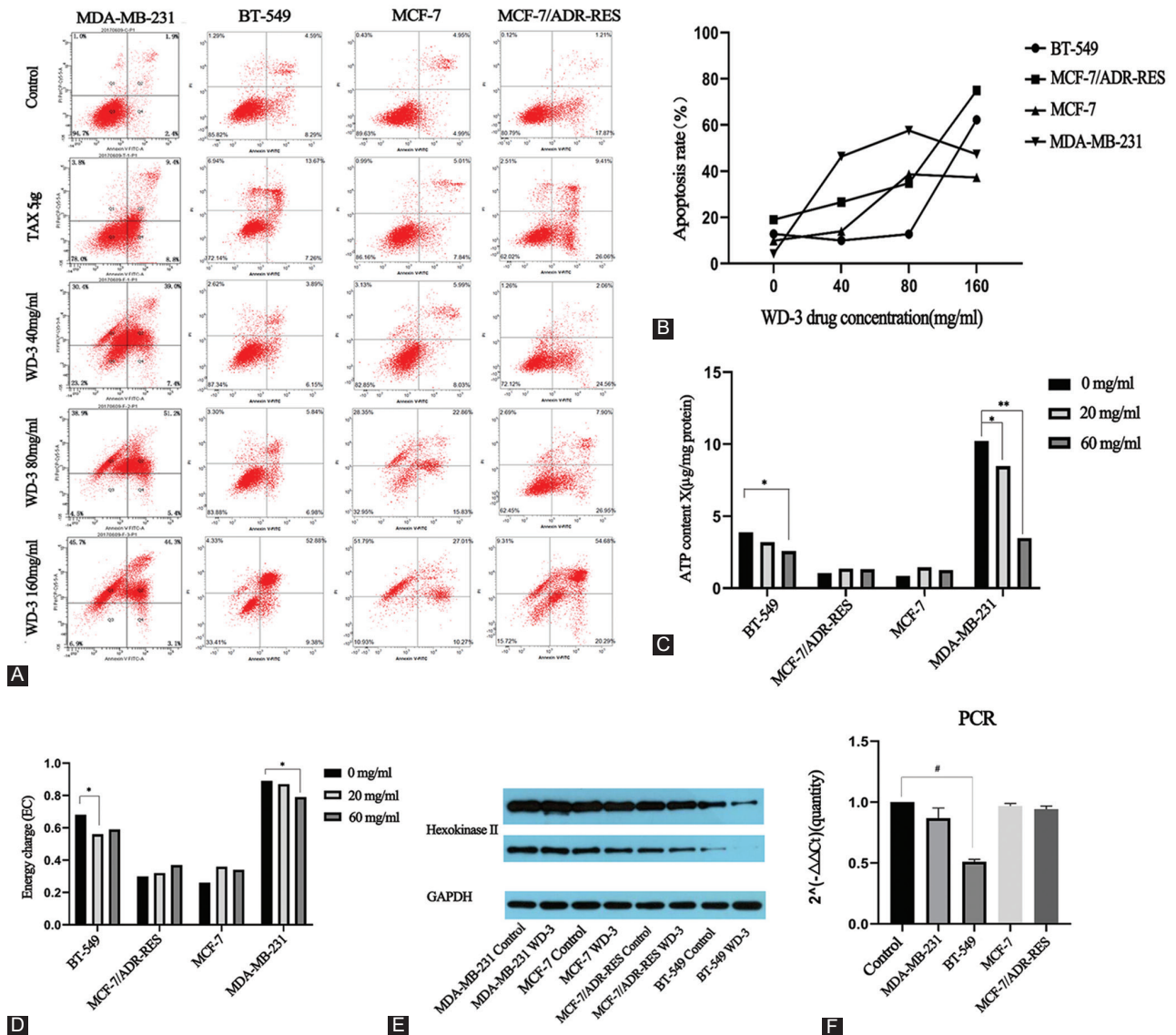
WD-3 affected ATP, ADP, and AMP levels in breast cancer cells

To investigate the effect of WD-3 on intracellular levels of ATP, ADP, and AMP breast cancer cells were treated with 0 (control), 20 (low-dose), and 60 mg/mL (high-dose) WD-3

for 24 h. Compared with control, ATP levels and EC dose-dependently decreased in BT-549 and MDA-MB-231 cells. In MCF-7 and MCF-7/ADR-RES cells, the changes in ATP, ADP, and AMP levels were not significant (Figure 5 C and D).

WD-3 downregulated hexokinase 2 protein level in BT-549 cells

Hexokinase 2 protein levels were quantified in the four breast cancer cell lines. WD-3 treatment (60 mg/mL,



**FIGURE 5.** WD-3 inhibited the growth of human breast cancer cells. (A and B) Flow cytometry was used to analyze cell apoptosis. Breast cancer cell lines MDA-MB-231, BT-549, MCF-7, and MCF-7/ADR-RES were treated with 40, 80, and 160 mg/mL of WD-3 or 5 µg/mL of paclitaxel for 24 h. Blank control was treated with phosphate-buffered saline. Apoptotic rate increased in a dose-dependent manner and living cell ratio decreased in all four breast cancer cell lines. (C and D) Changes in ATP and EC after WD-3 treatment in the four breast cancer cell lines. Breast cancer cells were treated with 0 (control), 20 (low-dose), and 60 mg/mL (high-dose) WD-3 for 24 h. Compared with control, ATP levels and EC dose-dependently decreased in BT-549 and MDA-MB-231 cells. (E) The protein levels of hexokinase 2 were determined by Western blot. GAPDH was used an internal control. WD-3 treatment (60 mg/mL, high-dose) significantly downregulated hexokinase 2 protein level in BT-549 cells. (F) The mRNA levels of hexokinase 2 were analyzed by qRT-PCR. WD-3 treatment (60 mg/mL, high-dose) significantly decreased the mRNA level of hexokinase 2 in BT-549 cells. \**p* < 0.05 and \*\**p* < 0.01 vs. 0 mg/mL; #*p* < 0.05 vs. control group. ATP: Adenosine triphosphate; EC: Energy charge; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR: Quantitative reverse transcription polymerase chain reaction.



high-dose) significantly downregulated hexokinase 2 protein level in BT-549 cells. Downregulation of hexokinase 2 was not significant in the other three breast cancer cell lines (Figure 5E).

### WD-3 downregulated hexokinase 2 mRNA level in BT-549 cells

Hexokinase 2 mRNA levels were quantified in the four breast cancer cell lines. WD-3 treatment (60 mg/mL, high-dose) significantly decreased the mRNA level of hexokinase 2 in BT-549 cell line (Figure 5F), but not in the other three cell lines.

## DISCUSSION

There are various TCM theories on the pathogenesis of breast cancer. Accordingly, an increasing number of *in vitro* studies have been conducted to verify the anti-breast-cancer effects of Compound Chinese Traditional Medicines (CCTMs) that function through the dissipation of blood stasis, detoxification, warming Yang, relief of liver symptoms, and enhancement of body resistance [19]. It was reported that Xiaojindan, a CCTM for blood stasis dissipation and detoxification, remarkably inhibited cell invasion and epithelial–mesenchymal transition (EMT) in breast cancer by regulating p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) MAPK pathways [20]. The traditional formula Tongluosanjie Pill could suppress cell growth and estrogen synthesis in breast cancer cells MCF-7 [21]. Wang *et al.* [22] demonstrated that a yang-warming CCTM could significantly arrest MCF-7 cells in S phase and inhibit metastasis by mediating matrix metalloproteinase-2 (MMP-2)/tissue inhibitor of MMP-2 (TIMP-2) balance. Another liver-soothing and spleen-enhancing detoxification prescription could also suppress the proliferation of MCF-7 cells by inducing cell apoptosis [23]. In this study we investigated the effect of WD-3 on breast cancer, a disease that we considered to be a result of spleen deficiency. As suggested by Zhao, breast cancer treatment should act on the spleen and stomach to regulate Qi, eliminate dampness, and resolve phlegm. WD-3 is mainly composed of *Codonopsis pilosula*, *Poria cocos*, *Atractylodes macrocephala* Koidz, *Ophiopogon japonicus*, *Poria cum Radix Pini*, *Schisandra chinensis*, *Eriobotrya japonica* Thunb, *Pinellia ternata*, *Caulis perillae*, *Coix lacryma-jobi*, *Citrus aurantium*, *Semen Raphani*, *Setaria italica*, *Hordeum vulgare*, and *Glycyrrhiza uralensis*. All the ingredients are mild and non-toxic and suitable for long-term regulation of the spleen and stomach [24]. We found that WD-3 treatment inhibited the proliferation, improved the morphology, and increased the apoptosis of breast cancer cells.

According to TCM, different physical constitutions lead to differences in breast cancer pathogenesis. Multiple pathological subtypes of breast cancer show obvious heterogeneity [25]. In this study, we selected four human breast cancer cell lines with different biological properties. MDA-MB-231 cell line is derived from human breast invasive ductal carcinoma, a highly metastatic cell line characterized as estrogen receptor negative [ER(-)], progesterone receptor negative [PR(-)], and human epidermal growth factor receptor 2 [HER2(-)]. BT-549 cell line is derived from human papillary invasive ductal carcinoma that is ER(-), PR(-), and HER2(-). MCF-7 is derived from human breast invasive ductal carcinoma with ER(+), PR(+), and HER2(-). MCF-7/ADR-RES is a doxorubicin-induced multidrug-resistant breast cancer cell line [26,27]. Although WD-3 treatment inhibited the proliferation of all four breast cancer cell lines in our study, the IC<sub>50</sub> values were significantly different, with the highest in MCF-7/ADR-RES and the lowest in MCF-7 cell line (the IC<sub>50</sub> values were in a descending order for MCF-7/ADR-RES, MDA-MB-231, BT-549, and MCF-7). Obvious apoptosis was observed in MCF-7 DUAL after 80 mg/mL WD-3 treatment. In addition to MCF-7 DUAL, a decreased number of living cells and an increased number of apoptotic cells were observed in the remaining three breast cancer cells following 40 mg/mL and 80 mg/mL WD-3 treatment. Under the same-dose of WD-3, the lowest number of living cells was observed in MCF-7/ADR-RES DUAL and the highest number in MCF-7 DUAL. WD-3 treatment dose-dependently decreased ATP levels and EC in BT-549 and MDA-MB-231 cells, but did not cause significant changes in MCF-7 and MCF-7/ADR-RES cells. Moreover, WD-3 treatment significantly downregulated hexokinase 2 level in BT-549 cells, but not in the other three breast cancer cell lines. Consistent with previous studies [28], breast cancer cell lines with different biological characteristics may use different pathways for proliferation and metabolism.

In TCM philosophy, the spleen absorbs cereal essence to generate body fluid that nourishes the whole body. Mitochondria as the 'engine room' of eukaryotic cells use the major products of glucose, pyruvate and nicotinamide adenine dinucleotide (NADH), to produce ATP by oxidative phosphorylation, thus providing energy for cell differentiation, information transmission, and apoptosis. The TCM function in the spleen is similar to that of mitochondrial oxidative phosphorylation, which led to the proposal of different hypotheses, such as "similar functions of spleen and mitochondria" and "spleen deficiency causing the decline of mitochondrial activity" [29]. Mitochondrial oxidative phosphorylation is the main source of cellular energy in normal cells. In normoxic cells, aerobic respiration produces 36 moles of ATP from 1 mole of glucose. On the other



hand, in tumor cells, only 2 moles of ATP and lactic acid are produced through glycolysis. Compared with normal cells, the glycolysis in tumor cells consumes more nutrients and produces less energy. The spleen functions to nourish the whole body with cereal essence; once diseased, the spleen fails to transform nutrients and produce energy, which in terms of energy production efficiency can be compared to glycolysis. Xu *et al.* [30] indicated that spleen deficiency and respiratory dysfunction lead to hypoxia and upregulation of hypoxia-inducible factor 1- $\alpha$  (HIF-1 $\alpha$ ). They suggested that, in obese people, chronic inflammation and cytokine release caused by spleen deficiency is a major factor triggering glycolysis in colorectal cancer. The proposed hypothesis “spleen deficiency-mitochondria-aerobic glycolysis-tumor” suggests that replenishing Qi to invigorate the spleen could improve mitochondrial metabolism and inhibit aerobic glycolysis in tumor cells, thus suppressing tumor growth [31]. Our study found that WD-3 treatment significantly decreased ATP levels and EC in BT-549 and MDA-MB-231 breast cancer cells, indicating that WD-3 treatment can reduce the energy production for tumor cell proliferation. Hexokinase 2, the first rate-limiting enzyme in the glycolytic pathway of breast cancer, can initiate and maintain the glycolytic rate during proliferation of cancer cells. Therefore, hexokinase 2 is a viable target to inhibit ATP production in breast cancer cells and induce apoptosis. We showed that WD-3 treatment markedly downregulated hexokinase 2 level in BT-549 breast cancer cells. WD-3 may inhibit tumor cell proliferation by intervening with the glycolytic pathway. To some extent, our findings support the hypothesis of “spleen deficiency-mitochondria-aerobic glycolysis-tumor”. However, the regulatory effects of WD-3 on glycolysis-related genes and its pharmacological mechanism need to be further investigated.

## CONCLUSION

Our preliminary results showed that WD-3 affects apoptosis, glycolysis, and hexokinase 2 expression in breast cancer cells. WD-3 inhibited the proliferation and increased apoptosis of breast cancer cells. In BT-549 cell line, WD-3 both decreased the energy production and downregulated hexokinase 2. Our findings indicate that WD-3 targets the glycolytic pathway in breast cancer cells to exert its antitumor activity.

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