

Novel multi-kinase inhibitor, T03 inhibits Taxol-resistant breast cancer

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Abstract. Activation of kinase-associated signaling pathways is one of the leading causes of various malignant phenotypes in breast tumors. Strategies of drug discovery and development have investigated approaches to target the inhibition of protein kinase signaling. In the current study, the anti-tumor activities of a novel multi-kinase inhibitor, T03 were evaluated in breast cancer. T03 inhibited Taxol-resistant breast cancer cell proliferation and induced cell cycle arrest and apoptosis *in vitro* and *in vivo*. The current results demonstrated that T03 downregulated c-Raf, platelet-derived growth factor receptor- β and other kinases, thus inhibited Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase and Akt/mechanistic target of rapamycin survival pathways in MCF-7 and MCF-7/Taxol xenograft tumors. At a dose of 100 mg/kg, T03 inhibited tumor growth by 62.90 and 59.98% in tumor weight in MX-1 and MX-1/T xenograft models, respectively and by 62.60 and 60.22% in MCF-7 and MCF-7/T tumors, respectively. These data indicate that the novel multi-kinase inhibitor, T03, may present as a potential compound to develop novel treatments against breast cancer and Taxol-resistant breast tumors.

Introduction

Breast cancer is the most common type of invasive cancer in women. Approximately 1.7 million women are diagnosed with breast cancer annually, and >500,000 succumb to it worldwide (1). While surgery, traditional chemotherapy and radiotherapy are commonly used to treat breast cancer; targeted therapy has drawn the attention of clinicians and researchers in the past decade for its improved therapeutic effect in metastatic cancer, as compared with traditional chemotherapy. Thus, numerous multi-kinase inhibitors have been adopted in the targeted therapy of metastatic breast cancer (2-4). However, the resistance of breast cancer to these inhibitors and drugs remains challenging in chemotherapy and targeted therapy. Therefore, continuing to develop novel anti-cancer drugs is necessary in cancer therapy.

In tumorigenesis of breast and other types of tissue, protein kinases are important in the regulation of proliferation, apoptosis and migration (5). For example, platelet-derived growth factor receptor- β (PDGFR β), a member of the tyrosine kinase receptors type III family, is associated with the malignancy of breast carcinoma (6-8). In response to survival signals, PDGFR β activates Akt by upregulating phosphoinositide 3-kinase (PI3K) and phosphoinositide-dependent protein kinase-1 (PDK1) (9). Thus, inhibition of PDGFR β activity by TKI inhibitor(s) may suppress breast tumor growth (10).

In addition, the Ras/Raf/mitogen-activated protein kinases (MAPK) signaling pathway is critical in breast tumorigenesis (11). Studies have demonstrated that constitutive activation of the MAPK signaling pathway is associated with the progression of breast cancer via the induction of chemoresistance and distant metastases (12-16). Thus, the MAPK signaling pathway may be a potent target for breast cancer chemotherapy (17).

Currently, certain drugs, such as Trastuzumab, Lapatinib, Bevacizumab and Taxol have been identified for breast cancer targeted therapy. Of them, Taxol is the commonly administered drug for the treatment of breast cancer. However, continuous use of Taxol results in acquired drug-resistance of breast cancer (18). Therefore, development of novel drugs is essential to improve targeted therapy of breast cancer and Taxol-resistant

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breast cancer. In the current study, a novel multi-kinase inhibitor, T03 is reported. T03 is a novel multi-kinase inhibitor against PDGFR β and c-Raf, and inhibition of PDGFR β and c-Raf by T03 may downregulate the Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and PDGFR/Akt/mechanistic target of rapamycin (mTOR) survival pathway. In the present study, the anti-tumor activity and underlying mechanism of T03 in regular and Taxol-resistant breast cancer were investigated *in vitro* and *in vivo*.

Materials and methods

Cell culture. Breast cancer cell line MCF-7 was obtained from the cell center of Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC; Beijing, China). Cell lines MX-1 and MX-1/T (Taxol-resistant) A549, A549/T (Taxol-resistant) were obtained from the Professor Yongkui Jing (Mount Sinai School of Medicine, New York, NY, USA). The MCF-7/T (Taxol-resistant) cell was established in our laboratory (Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing China) (19). MCF-7/ADM (Adriamycin-resistant) was obtained from the Assistant Professor Hongbo Wang (Yantai University, Shandong, China). The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 μ g/ml streptomycin in a humidified incubator containing 5% CO₂ at 37°C. In the present study, four specific cells (MX-1/T, MCF-7/T, MCF-7/ADM, A549/T) were used.

Drugs. T03, a small molecule compound containing 2-picolinylhydrazide moiety (Chinese patent application no. 201110129115.7) was synthesized by the Department of Pharmacology at the Institute of Materia Medica, CAMS and PUMC (Purity >97%; high-performance liquid chromatography). For *in vitro* experiments, T03 and Taxol (Beijing Union Pharmaceutical Factory, Beijing, China) were dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C until use. DMSO served as a vehicle control in all experiments at a final concentration of 0.1%. For the *in vivo* experiments, T03 was dissolved in a solution of Cremophor EL (Aladdin Industrial Corporation, Shanghai, China; cat no. C107105)/ethanol/water (12.5:12.5:75) (20).

Cell viability assay. MX-1, MX-1/T, MCF-7 and MCF-7/T cells (2,500 cells per well) were seeded in a 96-well plate. After 24 h of incubation at 37°C, cells were treated with various different concentrations of Taxol and T03. After 72 h of incubation at 37°C, the Cell Counting kit-8 (CCK-8; cat. no. C0037; Beyotime Institute of Biotechnology, Shanghai, China) assay was performed to evaluate cell viability. Absorbance values which was measured using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm were normalized to the values obtained for vehicle-treated cells to determine the percentage of surviving cells. The median inhibitory concentration (IC₅₀) was defined as the drug concentration at which cell growth was inhibited by 50%. Each assay was performed in triplicate.

Colony formation assay. MCF-7 and MCF-7/T cells were trypsinized to single-cell suspensions, and resuspended in DMEM culture medium containing 10% FBS. Approximately 500 cells were plated in 6-well tissue culture plates. After a 24-h incubation at 37°C, the cells were treated with either T03, 0.1% DMSO, or nothing. Cells were incubated in 5% CO₂ at 37°C for 14 days, and the colonies were washed, fixed and stained with 0.005% crystal violet in methanol. The number of colonies was manually counted without a microscope, and experiments were performed in triplicate and repeated three times.

Apoptosis analysis. MX-1, MX-1/T, MCF-7 and MCF-7/T cells were treated with either T03 or 0.1% DMSO for 72 h. Apoptotic cells were measured using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (cat. no. 556570; BD Biosciences, Franklin Lakes, NJ, USA). Briefly, cells were trypsinized and washed with PBS following treatment and stained with Annexin V-FITC according to the manufacturer's protocol, then analyzed by ACCURI C6 flow cytometry with BD Accuri C6 software (BD Biosciences).

Cell-cycle analysis. MX-1, MX-1/T, MCF-7 and MCF-7/T cells were incubated at 37°C with either T03 or 0.1% DMSO for 72 h. Cells were washed in PBS and fixed with 4°C ice-cold 70% ethanol overnight. The cells were then suspended in PBS containing RNase A (100 μ g/ml; cat. no. R1030; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), propidium iodide (50 μ g/ml; cat. no. P8080; Beijing Solarbio Science and Technology Co., Ltd.), Triton X-100 (0.1%), and incubated on the ice in the dark for at least 1 h (21). The cell cycle profiles were determined by flow cytometric analysis.

Tumor implantation and growth in MX-1, MX-1/T, MCF-7 and MCF-7/T xenografts. All animal studies were performed in compliance with the policies of the Institute of Materia Medica Animal Care and Use Committee. Six-week-old, female BALB/c/nu nude mice were used in the present study (all had 20 mice/experiment). The body weight was 15-16 g for MX-1 and MX-1/T xenograft model, and 16-22 g for MCF-7 and MCF-7/T xenograft model. They were purchased from Vital River Laboratory Animal Technology Co., Ltd., (Beijing, China) and housed in the controlled environment at 25°C on a 12-h light/dark cycle (5 mice per group). When tumors grew to an average volume of 100-250 mm³, tumor-bearing mice were randomly separated into four groups of five animals. A total of one group received per os Cremophor EL/ethanol/water and served as a vehicle control; the other groups received injections of 5 mg/kg Taxol (twice per week), or received an oral dose of 50 or 100 mg/kg T03 six times per week for 13 days (MX-1 and MX-1/T) and 33 days (MCF-7, MCF-7/T). Mice were euthanized at the end of the treatment period. Tumors were removed and weighed, and samples of all of the sections were stored at -80°C for western blot analysis.

Kinase inhibition assay. Inhibition of kinase activity against target kinases was measured using Caliper and Glo-ATP assays (ADP-Glo assay buffer: 25 mM HEPES, 10 mM MgCl₂, 0.01% Triton X-100, 100 μ g/ml BSA, 2.5 mM DTT, (pH 7.4); Caliper assay buffer: 100 mM HEPES, 10 mM MgCl₂, 100 μ l/l Brij35

Table I. Effect of T03 on proliferation in breast cancer cells.

Treatment	Median inhibitory concentration, mol/l			
	MX-1	MX-1/T	MCF-7	MCF-7/T
T03	$8.46 \pm 0.28 \times 10^{-6}$	$11.65 \pm 2.19 \times 10^{-6}$	$6.39 \pm 1.15 \times 10^{-6}$	$10.95 \pm 0.49 \times 10^{-6}$
Taxol	$4.44 \pm 0.30 \times 10^{-8}$	$1.52 \pm 0.50 \times 10^{-6}$	$1.83 \pm 0.36 \times 10^{-9}$	$7.66 \pm 2.06 \times 10^{-7}$

Table II. Effect of T03 on proliferation in resistant cells.

Treatment	Median inhibitory concentration, mol/l			
	MCF-7	MCF-7/ADM	A549	A549/T
T03	7.91×10^{-6}	1.83×10^{-5}	9.83×10^{-6}	2.76×10^{-5}
Taxol	5.97×10^{-9}	4.37×10^{-7}	5.09×10^{-9}	2.30×10^{-7}

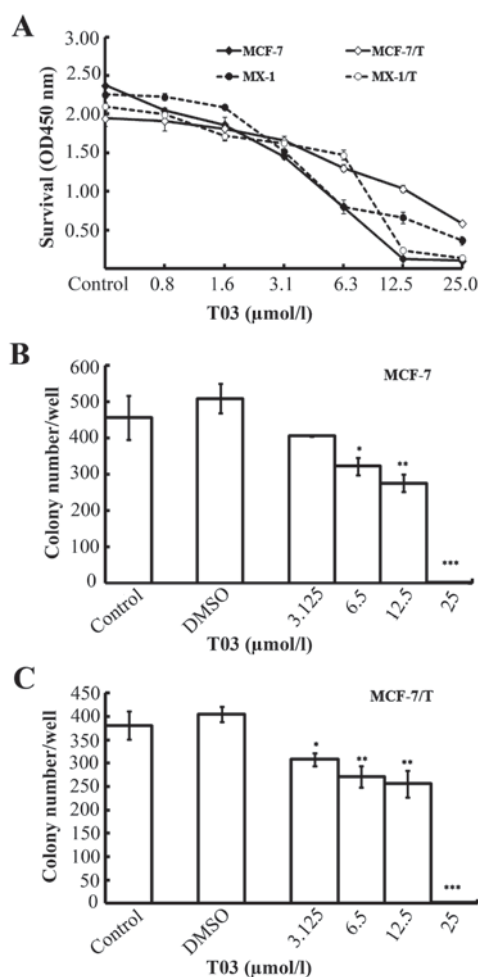


Figure 1. T03 inhibited proliferation of breast cancer cells. (A) T03 decreased the viability of human MX-1, MX-1/T, MCF-7 and MCF-7/T breast cancer cells. Cells were treated with various concentrations (0.8–25.0 $\mu\text{mol/l}$) of T03 for 72 h. Cell viability was determined by Cell Counting kit-8 assay and based on absorbance. Error bars represent standard deviation. T03 suppressed colony formation of (B) MCF-7 and (C) MCF-7/T cells. Cells were treated with T03, at the indicated concentrations, or with DMSO (0.1%) for the controls followed by 14 days of culture in full-serum media. Colonies were counted and the bars represent standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the control. DMSO, dimethyl sulfoxide; OD, optical density.

(30%), 1 mM DTT, (pH 7.4); Other reagents: ATP (cat. no. A7699 Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); ADP Glo reagent (cat. no. V9102; Promega Corporation, Madison, WI, USA). The Biochemical assay was performed according to the manufacturer's protocol. The assay was performed by HD Biosciences (China) Co., Ltd. (Shanghai, China).

Western blot analysis. Lysates (portions of two or three randomly selected tumors from MCF-7 and MCF-7/T xenograft mice) were prepared as previously described. The protein extraction buffer was a radioimmunoprecipitation buffer (1 mM phenylmethylsulfonyl fluoride) (21). Protein concentration was determined using the bicinchoninic acid method. Total proteins (50 μg) were separated by 10.0% SDS-PAGE and transferred to a nitrocellulose membrane by semi-wet electrophoresis were incubated with primary antibodies overnight at 4°C following blocking with TBS containing 1% Tween-20 and 5% skimmed dry milk for 1 h at room temperature. The antibodies were as follows: Rabbit anti-phosphorylated (p)-c-Raf (Ser259; cat. no. 9421), c-Raf (cat. no. 9422), p-MEK (cat. no. 9127), MEK (cat. no. 9903), p-ERK (cat. no. 4370), ERK (cat. no. 9101), p-PDGFR β (cat. no. 3170), PDGFR β (cat. no. 3169), p-PDK (cat. no. 3061), PDK (cat. no. 3062), p-Akt (Thr 308; cat. no. 9275), Akt (cat. no. 4691), p-mTOR (cat. no. 2971), mTOR (cat. no. 2983), p-AuroraA (cat. no. 2914), AuroraA (cat. no. 14475) (all from Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse anti-actin (cat. no. 3700; CST Biological Reagents Co., Ltd., Shanghai China). All the primary antibodies were used at 1:1,000. The samples were detected with peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5,000, cat. no. sc-2004, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature and developed using an enhanced chemiluminescence system western blot detection and analysis system (Applygen Technologies, Inc., Beijing, China). The membranes were assessed for equal loading by probing for β -actin.

Statistical analysis. Data were expressed as means \pm standard deviation. Statistical analysis of the results was performed

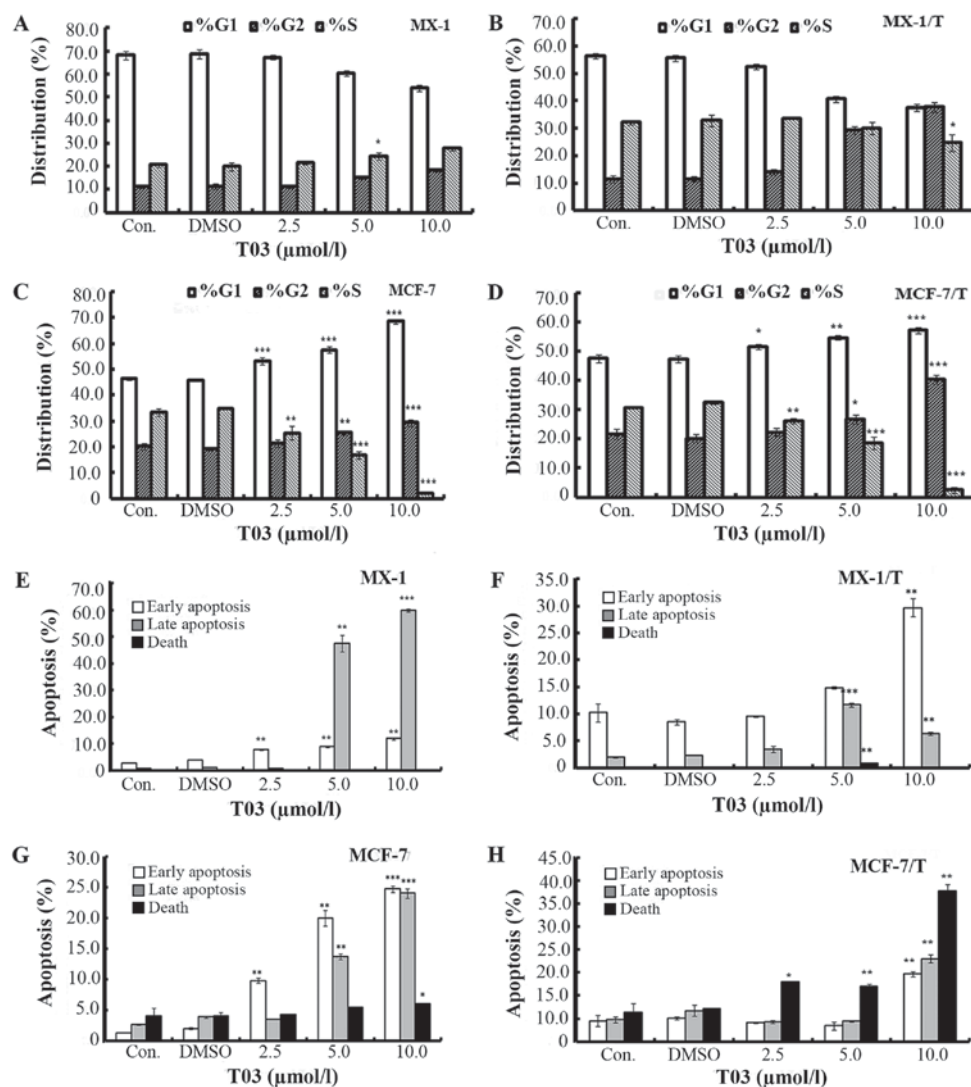


Figure 2. T03 treatment induced cell cycle arrest and apoptosis in breast cancer. T03 induced cell cycle arrest in (A) MX-1, (B) MX-1/T, (C) MCF-7 and (D) MCF-7/T cells. Cells were incubated with the indicated concentration of T03 or DMSO (0.1%) for 72 h, stained with propidium iodide and analyzed by flow cytometry. Error bars represent standard deviation. T03 treatment induced apoptosis in (E) MX-1, (F) MX-1/T, (G) MCF-7 and (H) MCF-7/T cells. Cells were incubated with the indicated concentration of T03 or DMSO for 72 h. Cells of early apoptosis, late apoptosis and death were stained with Annexin V-FITC and determined by flow cytometry. Quantization of the Annexin V-FITC staining data was presented based on the percentages of the cells for early apoptosis, late apoptosis, and death. Each graph represents the mean, and the error bars represent standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the control cells. DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; Con., control.

using one-way analysis of variance followed by a Bonferroni post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical software used was Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and SPSS software (version 19.0; SPSS, Inc., Chicago, IL, USA).

Results

T03 inhibits the proliferation of breast cancer cells. To evaluate the suppressive efficacy of T03 on breast cancer cells *in vitro*, the regular (MX-1, MCF-7) and Taxol-resistant (MX-1/T, MCF-7/T) breast cancer cells were treated with various concentrations of T03 (0.8–25.0 $\mu\text{mol/l}$) for 72 h. The CCK-8 assay results showed that T03 inhibited the growth of the breast cancer cells in dose-dependent manner (Fig. 1A). The T03 IC_{50} values were 8.46 ± 0.28 (MX-1), 11.65 ± 2.19 (MX-1/T), 6.39 ± 1.15 (MCF-7), and 10.95 ± 0.49 $\mu\text{mol/l}$

(MCF-7/T; Table I). These results indicated that T03 effectively inhibited the growth of breast cancer cells, as well as the Taxol-resistant breast cancer cells. To establish whether T03 inhibits other drug-resistant cells, similar experiments on Doxorubicin-resistant breast cancer cells (MCF-7/ADM) were conducted. Similarly, T03 inhibited Adriamycin-resistant cells in a dose-dependent manner (Table II).

T03 suppresses colony formation in breast cancer cells. To confirm the suppressive ability of T03 on tumor cell proliferation, a colony formation assay was conducted in MCF-7 and MCF-7/T cells. The data revealed that T03 effectively inhibited the colony formation in MCF-7 and MCF-7/T colony cells in a dose-dependent manner (Fig. 1B and C). T03 inhibited colony formation by ~50.0% (IC_{50}) at a concentration of 7.61 $\mu\text{mol/l}$ in MCF-7 and 7.45 $\mu\text{mol/l}$ in MCF-7/T cells. The data were consistent with the results of the CCK-8 assay.

T03 treatment led to cell cycle arrest in breast cancer cells. T03 was observed to cause cell cycle arrest in breast cancer cells. T03 treatment led to elevated numbers of G₂-phase cells and decreased G₁-phase cells in a dose-dependent manner in MX-1 and MX-1/T cells (Fig. 2A and B). With a treatment of 5.0 μmol/l T03, the percentage of G₂-phase cells increased to 15.23±0.83% (MX-1) and 29.30±1.37% (MX-1/T), while it was 11.37±0.90 and 11.47±1.26% in the untreated controls. Concomitantly, the percentage of G₁ phase cells reduced to 60.37±1.01% (MX-1) and 40.60±1.06% (MX-1/T) compared with 68.10±1.65 and 56.33±0.86% in the untreated controls. Furthermore, while T03 treatment induced cell accumulation at the G₂ and G₁ phases, it caused the decrease of the S phase cells in MCF-7 and MCF-7/T (Fig. 2C and D). The percentage of G₂ phase cells was 25.50±0.92 and 26.80±1.51% in MCF-7 and MCF-7/T cells, respectively, when they were treated with 5.0 μmol/l T03. By contrast, the percentages were 20.20±1.25% (MCF-7) and 21.73±1.66% (MCF-7/T) in the controls. The percentages of S phase cells decreased from 33.43±1.45 and 30.77±0.46% to 16.90±1.49 and 18.53±2.10% (following treatment with 5.0 μmol/l T03), respectively (Fig. 2C and D).

T03 induced apoptosis in breast cancer cells. T03 treatment was demonstrated to induce apoptosis in MX-1, MX-1/T, MCF-7, and MCF-7/T breast cancer cells. Upon treatment with 10.0 μmol/l T03, the early apoptosis increased by 4.16- and 2.90-fold, and late apoptosis increased by 67.25- and 3.23-fold in MX-1 and MX-1/T cells (Fig. 2E and F). In MCF-7 and MCF-7/T cells, early apoptosis increased from 1.28 and 4.51% (control) to 24.75 and 14.70%, respectively, while late apoptosis increased from 2.67 and 4.73% (control) to 24.05 and 18.00%, respectively (Fig. 2G and H).

T03 inhibited kinases in breast cancer cells. To investigate which kinases T03 inhibited, Caliper and ADP-Glo assays were performed on a panel of kinases in T03-treated breast cancer cells. The results indicated that various oncogenic kinases were susceptible to T03 inhibition. Of these kinases, c-Raf, PDGFRβ and RET proto-oncogene may be suppressed by T03, and the IC₅₀s are 0.78, 0.23 and 0.71 μmol/l, respectively. In addition, T03 may inhibit the activity of PDGFRα, fms related tyrosine kinase 1 (FLT1), kinase insert domain receptor, FLT3, and c-kit with IC₅₀ between 0.05 and 1.0 μmol/l. Furthermore, it was found that T03 downregulates FGFR1, FGFR2 and b-Raf at the micromole level. By contrast, T03 exerted little effect on other tested kinases, such as Aurora A, insulin like growth factor 1 receptor, ERK1, ERK2, Src, PI3K, erb-b2 receptor tyrosine kinase 2 and AMPK (Table III).

T03 inhibited xenograft tumor growth of breast cancer cells. Based on the above results obtained *in vitro*, further experiments were performed to determine whether T03 inhibits xenograft tumor growth from the breast cancer cells. To obtain xenograft tumors, the MX-1, MX-1/T, MCF-7, and MCF-7/T human breast cells were inoculated into BALB/c/nu nude mice. While T03 was used to treat the xenograft tumors, Taxol was adopted as a reference compound. When 5 mg/kg Taxol was applied to MX-1 and MX-1/T xenografts, the treated/control (T/C) ratio were 55.56 and 95.13%, respectively, according to the relative tumor volume (RTV).

Table III. Inhibitory activity of T03 against different kinases (biochemical assay).

A, Median inhibitory concentration of T03 against different kinases

Kinase target	<i>In vitro</i> IC ₅₀ value, μmol/l	Source
c-Raf	0.78	ADP-Glo
PDGFRβ	0.230	Caliper
Ret proto-oncogene	0.71	Caliper
PDGFRα	0.37	Caliper
FLT1	0.93	Caliper
Kinase insert domain receptor	0.504	Caliper
FLT3	0.046	Caliper
c-kit	0.16	Caliper
FGFR1	3.929	Caliper
FGFR2	2.962	Caliper
b-Raf	4.063	ADP-Glo

B, The inhibition rate of T03 against different kinases

Kinase target	Inhibition, 1.0 μmol/l ^a (%)	Source
Aurora A	9.92	Caliper assay
IGFR1	2.57	ADP-Glo
ERK1	9.73	Caliper
ERK2	0.79	Caliper
SRC	-	ADP-Glo
PI3Kα	2.01	ADP-Glo
PI3Kγ	19.33	Caliper
ERBB2	8.91	Caliper
AMPK (A1/B1/G1)	14.65	Caliper
AMPK (A2/B1/G1)	28.76	ADP-Glo

^aInhibition at 1.0 μmol/l. Kinase selectivity profiling was performed at HD Biosciences (China) Co., Ltd. (Shanghai, China) using ADP-Glo and Caliper assays. PDGFR, platelet derived growth factor receptor; FLT, Fms related tyrosine kinase; FGFR, fibroblast growth factor receptor; ERK, extracellular signal regulated kinase; PI3K, phosphoinositide 3-kinase; AMPK, AMP-activated protein kinase; IGFR1, insulin like growth factor 1 receptor; ERBB2, Erb-b2 receptor tyrosine kinase.

Furthermore, the inhibition ratios were 41.56% in MX-1 and 0% in MX-1/T based on the relative tumor weight, indicating that MX-1/T was less sensitive to Taxol at a dose of 5 mg/kg. For T03, the T/C ratio was 46.99% (50 mg/kg) and 34.68% (100 mg/kg) according to RTV, and the inhibition ratio of tumor weight reached 50.00 and 62.90% in MX-1 xenografts (Table IV and Fig. 3A). Furthermore, in MX-1/T xenografts, the T/C ratio of RTV was 45.06% (50 mg/kg) and 31.47% (100 mg/kg), and the inhibition ratio of the tumor weight was 51.02 and 59.98%, respectively (Table V and Fig. 3B),

Table IV. Antitumor activity of T03 on the breast cancer MX-1 xenograft model.

Compound	Dose, mg/kg	Animals, n	Body weight, g		Tumor volume, mm ³		Relative tumor volume		Tumor weight	
			Initial	Final	Initial	Final	$\bar{x}\pm SD$	Treated/control ratio, %	$\bar{x}\pm SD$, g	Inhibition, %
Control		5/5	16.0±1.0	21.8±1.9	122.6±4.2	2,709.5±633.4	22.09±5.12		1.61±0.45	
Taxol	5	5/5	16.0±0.7	20.8±2.2	140.2±13.3	1,714.4±308.2 ^a	12.27±2.33 ^b	55.56	0.94±0.20 ^a	41.56
T03	50	5/5	15.0±1.0	19.2±1.3	121.2±24.9	1,318.2±849.5 ^a	10.38±5.48 ^b	46.99	0.81±0.49 ^a	50.00
	100	5/5	15.2±0.5	18.9±1.4	123.7±6.7	945.7±35.8 ^c	7.66±0.48 ^c	34.68	0.60±0.03 ^b	62.90

^aP<0.05, ^bP<0.001 and ^cP<0.001 vs. control. Means ± standard deviation.

Table V. Antitumor activity of T03 on the breast cancer MX-1/T xenograft model.

Compound	Dose, mg/kg	Animals, n	Body weight, g		Tumor volume, mm ³		Relative tumor volume		Tumor weight	
			Initial	Final	Initial	Final	$\bar{x}\pm SD$	Treated/control ratio, %	$\bar{x}\pm SD$, g	Inhibition, %
Control		5/5	16.0±1.0	20.8±2.9	91.7±19.7	2,131.3±920.4	23.87±10.22		1.57±0.47	
Taxol	5	5/5	15.6±0.6	20.4±0.9	95.7±17.1	2,183.1±1,091.0	22.71±11.09	95.13	1.58±0.79	-
T03	50	5/5	15.2±0.5	19.0±2.5	98.5±9.8	1,079.1±887.6	10.76±8.66	45.06	0.77±0.62	51.02
	100	5/5	15.8±0.8	18.8±2.2	96.7±6.4	728.6±78.2 ^a	7.51±0.86 ^a	31.47	0.63±0.06 ^b	59.98

^aP<0.05 and ^bP<0.01 vs. control. $\bar{x}\pm SD$, Means ± standard deviation.

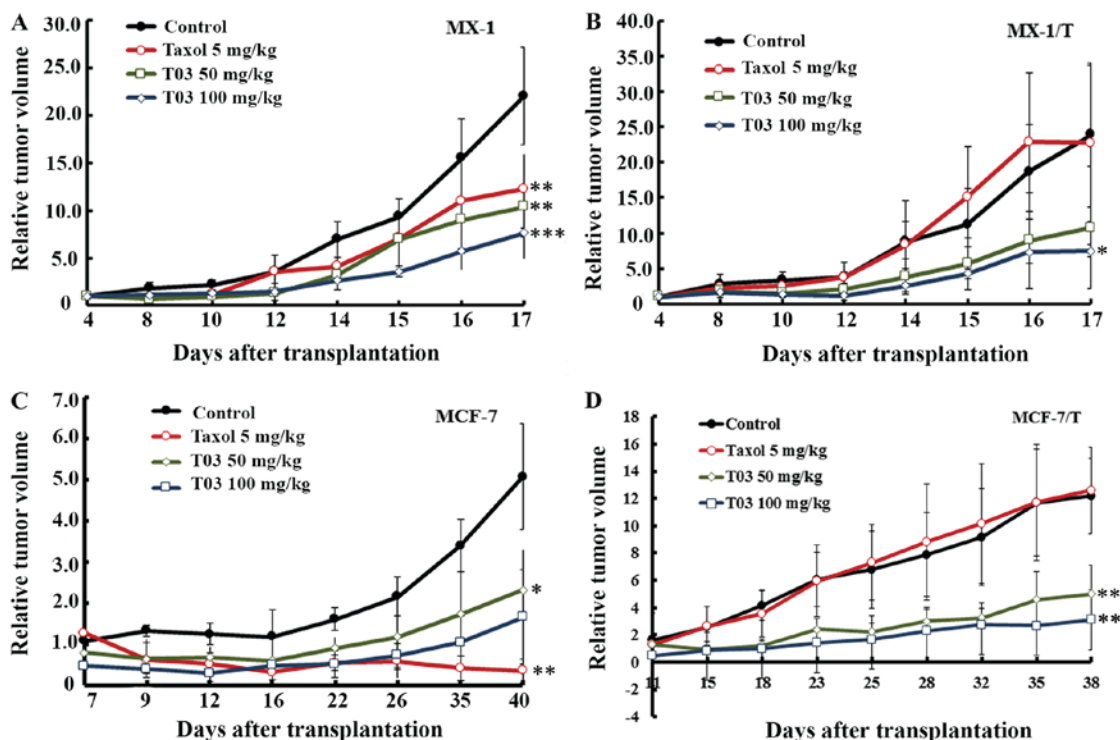


Figure 3. T03 suppressed tumor growth of (A) MX-1, (B) MX-1/T, (C) MCF-7 and (D) MCF-7/T xenografts in nude mice. Animals were randomly divided into four groups when tumor volume reached 100-250 mm³. The dosage of T03 was 50 and 100 mg/kg. Each treatment arm involved 5 independent tumor-bearing mice representing the same xenograft line. Results are presented as mean relative tumor volumes (V_t/V_0) \pm standard deviation at given time-points for MX-1, MX-1/T, MCF-7 and MCF-7/T xenografts. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control. V_t , tumor volume at any given time point; V_0 , tumor volume at the beginning of treatment.

indicating that T03 exerted more effective inhibition on the MX-1 and MX-1/T xenografts.

In addition, it was observed that T03 presented even higher inhibitory ability to MCF-7 and MCF-7/T xenografts compared with MX-1 or MX-1/T models. While Taxol was applied to the MCF-7 xenograft, the T/C ratio of the RTV was 6.75% (5 mg/kg) and the inhibition rate of the tumor weight was 96.36%. By contrast, Taxol exerted almost no inhibitory effect on the MCF-7/T xenograft model. In contrast to this, when T03 was applied to MCF-7 xenografts, the RTV T/C ratio was 45.63% (50 mg/kg) and 32.55% (100 mg/kg), and the inhibition rate of the tumor weight reached 57.09 and 62.60% (Table VI and Fig. 3C). Furthermore, in MCF-7/T xenografts, the RTV T/C ratios were 41.21% (50 mg/kg) and 25.52% (100 mg/kg) while the inhibition rate of the tumor weight attained 44.06 and 60.22%, respectively (Table VII and Fig. 3D). These data indicated that T03 inhibits Taxol-sensitive and -resistant breast cancer tumors.

T03 downregulated the Raf/MEK/ERK and PDGFR β /Akt signaling pathway in MCF-7 and MCF-7/T xenograft nude model mice. Subsequently, whether T03 inactivated the above-mentioned kinase-associated signaling pathways was evaluated. As expected, the phosphorylated levels of PDGFR β decreased in T03-treated tumors of MCF-7 and MCF-7/T xenografts (Fig. 4A and B). As PDK is an important downstream target of PDGFR β /PI3K signaling and a key upstream kinase of the AKT/mTOR signaling pathway, its activation promotes proliferation and inhibits apoptosis in numerous human cancer types (9,22,23). The current study observed that

the activity of PDK, AKT, and mTOR decreased in MCF-7 and MCF-7/T treated with T03 compared with the controls (Fig. 4A and B), indicating that T03 may downregulate PDGFR β and PDK/AKT/mTOR and in MCF-7 and MCF-7/T xenograft models.

As T03 inhibited c-Raf in the current study, whether T03 downregulates the Ras/Raf/ERK signaling pathway in MCF-7 and MCF-7/T xenografts was examined. Fig. 4C and D demonstrate that T03 treatment significantly and dose-dependently decreased p-c-Raf, p-MEK, and p-ERK in MCF-7 and MCF-7/T tumors, while the basal levels of c-Raf, MEK and ERK were only reduced in the group treated with 100 mg/kg T03.

Furthermore, T03 downregulated Aurora A, a downstream effector of the Ras/Raf/MEK/ERK signaling pathway (24), in MCF-7 and MCF-7/T xenografts. Aurora A and p-Aurora A were markedly downregulated upon T03 treatment, particularly in MCF-7 tumors, which were consistent with the previous study (24) that Aurora A and p-Aurora A were frequently activated by the Ras/Raf signaling pathway (Fig. 4E and F).

Discussion

Despite improvements in prevention, early detection, and treatment, breast cancer remains one of the most common malignant tumors affecting women in western countries (25). Drug-resistance of breast cancer to Taxol has limited its effect and application in clinical treatment. In the present study, the anti-tumor activity of the novel multi-kinases inhibitor, T03 was investigated, as well as its potential in breast cancer treatment.

Table VI. Antitumor activity of compounds on the breast cancer MCF-7 xenograft model.

Compound	Dose, mg/kg	Animals, n	Body weight, g		Tumor volume, mm ³		Relative tumor volume		Tumor weight	
			Initial	Final	Initial	Final	$\bar{x} \pm SD$	control ratio, %	$\bar{x} \pm SD$, g	Inhibition, %
Control		5/5	21.8±0.8	26.0±1.2	237.5±32.6	1,163.8±503.2	5.06±2.46		1.02±0.46	
Taxol	5	5/5	21.0±1.6	22.8±2.3	227.4±74.6	68.3±32.5 ^a	0.34±0.22 ^a	6.75	0.04±0.02 ^a	96.36
T03	50	5/5	20.6±1.1	26.0±4.6	230.6±65.3	521.2±658.1	2.31±2.50	45.63	0.44±0.27 ^b	57.09
	100	5/5	21.0±2.2	23.2±3.1	240.9±38.6	419.3±413.6 ^b	1.65±1.55 ^b	32.55	0.38±0.49	62.60

^aP<0.01 and ^bP<0.05 vs. control. $\bar{x} \pm SD$, Means \pm standard deviation.

Table VII. Antitumor activity of compounds on the breast cancer MCF-7/T xenograft model.

Compound	Dose, mg/kg	Animals, n	Body weight, g		Tumor volume, mm ³		Relative tumor volume		Tumor weight	
			Initial	Final	Initial	Final	$\bar{x} \pm SD$	control ratio, %	$\bar{x} \pm SD$, g	Inhibition, %
Control		5/5	18.0±1.4	18.6±2.4	112.0±5.8	1,373.5±363.1	12.19±2.78		1.26±0.24	
Taxol	5	5/4	16.8±1.9	17.5±2.1	132.5±45.3	1,871.0±981.5	12.59±3.17	-	1.36±0.48	-
T03	50	5/5	17.4±1.2	17.4±2.5	142.4±38.7	621.2±243.3 ^a	5.02±2.11 ^a	41.21	0.71±0.18 ^a	44.06
	100	5/5	17.3±1.1	15.6±2.7	148.1±37.1	442.6±266.0 ^a	3.11±2.19 ^a	25.52	0.50±0.21 ^b	60.22

^aP<0.01 and ^bP<0.001 vs. control. $\bar{x} \pm SD$, Means \pm standard deviation.

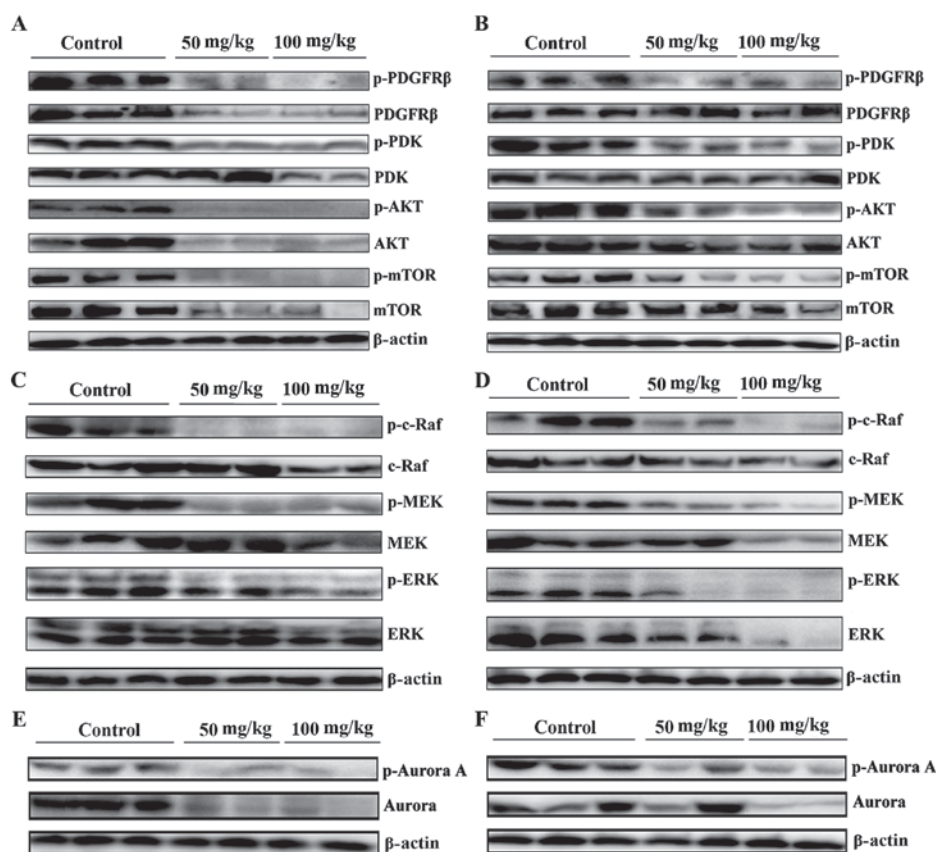


Figure 4. T03 downregulated PDGFR β and c-Raf in MCF-7 and MCF-7/T breast cancer xenograft tumors. Mice bearing MCF-7 and MCF-7/T tumors were randomized and treated with 50 or 100 mg/kg T03, oral gavage once daily (6 days/week). Lysates of two or three tumor tissue samples from each group were pooled and each lane represents one protein pool. Two or three pools per group were subjected to western blot analysis and β -actin served as a loading control. Blots were incubated with the indicated antibodies and the representative blots are shown. Experiments were repeated twice. Western blot analysis of PDGFR β and downstream signaling pathways in (A) MCF-7 and (B) MCF-7/T tumors treated with T03. Western blot analysis of c-Raf, and of downstream signaling pathways in (C) MCF-7 and (D) MCF-7/T tumors treated with T03. Western blot analysis of Aurora A in (E) MCF-7 and (F) MCF-7/T tumors treated with T03. PDGFR β , platelet-derived growth factor receptor- β ; p, phosphorylated; PDK, phosphoinositide-dependent protein kinase-1; mTOR, mechanistic target of rapamycin; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase.

As with Taxol, T03 displayed similar antitumor effects on MX-1 and MCF-7 breast cancer cells. Furthermore, T03 inhibited the growth of Taxol-resistant MX-1/T and MCF-7/T breast tumors *in vitro* and *in vivo*. It caused G₂/M-phase cell accumulation and induced apoptosis, thus resulting in cell growth inhibition. In addition, T03 resulted in tumor regression in MX-1- and MCF-7-derived xenografts, as well as in Taxol-resistant MX-1/T and MCF-7/T tumors, indicating that T03 may exert effects on Taxol-sensitive and -resistant breast cancer cells. In addition, T03 was demonstrated to exert efficient effects on other types of drug-resistant breast cancer cells, such as Doxorubicin (Adriamycin)-resistant cells, which indicated that T03 may be used for treatment of other types of drug-resistant breast cancer.

Previous studies revealed that PDGFR β was overexpressed in breast cancer (26,27). Highly activated PDGFR β promoted tumor cell proliferation via PI3K/Akt and Ras/MEK/ERK signaling pathways (28,29), and resulted in distant metastasis and insensitivity to chemotherapy (30). The current study demonstrated that T03 may downregulate the Akt/mTOR and Ras/MEK/ERK signaling pathways, as well as PDGFR β in MCF-7 and MCF-7/T *in vivo*. In MCF-7 and MCF-7/T xenograft tumors, T03 significantly reduced p-PDGFR β , which was further confirmed by performance of the biochemical assay. Furthermore, T03 downregulated PDK, AKT and

mTOR. As PDK-1, Akt and mTOR are the downstream components of PDGFR β , and are involved in cell growth and apoptosis, T03 may cause cell growth inhibition and apoptosis via downregulation of the PDGFR β /Akt signaling pathway. PDGFR β activation is known to induce Taxol-resistance in breast cancer (31,32). Therefore, T03 may be used for inhibiting Taxol-resistant breast cancer.

Raf kinase is an upstream member of the Raf/MEK/ERK signaling cascade (33). Activation of the Raf/MEK/ERK signaling pathway has been associated with chemoresistance of breast cancer (34-36). The present data indicated that T03 inhibited p-c-Raf, and consequently resulted in downregulation of p-MEK and p-ERK. These results indicate that T03 may inhibit the cell cycle and induce apoptosis via downregulation of the Raf/MEK/ERK signaling pathway in MCF-7 and MCF-7/T breast cancer.

Previous studies observed that activation of c-Raf signaling led to stabilization and accumulation of Aurora A mitotic kinase in breast cancer cells, and deduced that c-Raf may regulate the expression levels of Aurora A (24,37). The current study found that T03 treatment led to inhibition of c-Raf and decreased Aurora A in MCF-7 and MCF-7/T xenografts.

Previous studies have demonstrated that over-expressed Aurora A inhibits apoptosis, promotes cell cycle progression

and metastasis, and mediates Taxol-resistance in breast cancer and other types of cancer (38,39). Inhibition of Aurora A by T03 may cause G₂/M cell accumulation, apoptosis and sensitivity to Taxol in breast cancer. Based on the current results, the therapeutic efficacy of T03 on breast cancer may be partially attributed to the inhibition of c-Raf, PDGFR β and the associated signaling pathways (40,41).

Although T03 presented similar anti-tumor activity in Taxol-sensitive and -resistant breast cancer, and Doxorubicin-resistant breast cancer as well, there are certain efficacy differences, which merit further investigation. In addition, based on our existing data, whether the anti-tumor effects are transient or permanent could not be determined.

In conclusion, the current study demonstrated that T03 induces cell cycle arrest and apoptosis, and inhibits cell proliferation in MX-1, MCF-7, MX-1/T, and MCF-7/T breast cancer *in vitro* and *in vivo*. These results demonstrate that T03 inhibits breast cancer growth by downregulating PDGFR β /Akt and Ras/Raf/ERK signaling pathways, which are regulators of apoptosis, proliferation and chemoresistance. These findings indicate that T03 may be a potential candidate for effective chemotherapy of breast cancer, particularly for Taxol-resistant breast cancer.

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