Original Paper

A Novel Cinnamide YLT26 Induces Breast Cancer Cells Apoptosis *via* ROS-Mitochondrial Apoptotic Pathway *in Vitro* and Inhibits Lung Metastasis *in Vivo*

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Key Words

YLT26 • Breast cancer • Apoptosis • ROS • Metastasis • MDSCs

Abstract

Background: Breast cancer is the leading cause of cancer death among women worldwide and metastasis is the major cause of treatment failure. Thus, new treatment options for breast cancer, especially, drugs which could prevent metastasis, are pressing needed. **Methods:** In the present study, we designed and synthesized a novel cinnamide derivative, (E)-N-(4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl)-3-(3,4,5-trimethoxyphenyl)acrylamide (YLT26), which displayed potent inhibitory effects on breast cancer cells. The cell viability, apoptosis-inducing effect and reactive oxygen species (ROS) production were examined in 4T1 cells following treatment with YLT26. Meanwhile, apoptosis-related proteins levels were determined by western blotting. Finally, we evaluated the effects of YLT26 on breast tumor growth, lung metastases *in vivo* and the infiltration of myeloid-derived suppressor cells (MDSCs) in lung tissue. **Results:** Our results showed that the proliferation inhibitory effects of YLT26 were correlated with its apoptosis-inducing effect. Exposure to YLT26 induced mitochondrial transmembrane potential ($\Delta\Psi$ m) change, activated caspase-9, and downregulated the Bcl-2 expression, as well as enhanced ROS accumulation in 4T1 cells. Moreover, YLT26 significantly inhibited tumor growth without obvious side effects in the 4T1 tumor-bearing mice model. Immunohistochemistry analyze revealed YLT26 also induced apoptosis *in vivo*. More importantly, YLT26 also significantly inhibited lung metastases, which may be associated with the reduction of MDSCs. **Conclusion:** The present study suggested that YLT26 could inhibit breast cancer cells proliferation *via* ROS-mitochondrial apoptotic pathway, delay breast tumor progression, and suppress lung metastases by impacting on the immunologic microenvironment *in vivo*.

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Introduction

Breast cancer is a widely recognized type of cancer around the world and remains the leading cause of cancer death among women [1]. Recent statistics shows that approximately 232,000 American women are still being diagnosed with breast cancer and about 64,000 cases of breast cancer are expected to be newly diagnosed, accounting for 29% of all new cancer cases among women [2, 3]. Despite recent progress in early detection and adjuvant chemotherapy, a considerable number of patients receiving chemotherapy will relapse as a result of distant metastasis [4]. Breast tumor metastases remains a mostly incurable form of disease and is the major cause of mortality among breast cancer patients [5, 6]. Therefore, efforts are needed to develop novel therapeutic approaches to inhibit the tumor progression and metastasis.

Apoptosis, a form of programmed cell death, is controlled by a diverse range of cell signals [7]. Molecular biological studies indicate that two major apoptotic pathways are characterized: cell death receptor-mediated extrinsic pathway and mitochondrial-mediated intrinsic pathway [8]. In the extrinsic pathway, activation of death receptors leads to the cleavage of caspase-8, and then the activation of downstream effectors caspase-3 and/or -7 [9]. In the intrinsic pathway, change in mitochondrial membrane results in the release of cytochrome c and the subsequent activation of caspase-9, leading to cleavage of the executioner caspase-3 and/or -7 [10].

Reactive oxygen species (ROS) are the byproducts of cell metabolism, and many studies have showed that disruption of the ROS homeostasis plays a critical role in mitochondrial dysfunction and apoptotic events [11, 12]. ROS can directly result in activation of the mitochondrial membrane permeability and induce loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) [13]. Recently, some small molecular compounds, such as piperlongumine and millepachine, have been demonstrated that they could induce apoptosis through accumulation of ROS and inhibit the growth of spontaneously formed malignant breast cancer and their associated metastases in mice [14, 15]. Thus, development of novel small molecular compounds which can upregulate the intracellular ROS level provide us a new avenue to combat malignancy tumors.

Our research group has been engaged in the design, synthesis and biological evaluation of novel compounds as potential anticancer agents. In a cell-based screening study, we found a novel cinnamide derivative, (E)-N-(4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl)-3-(3,4,5-trimethoxyphenyl)acrylamide (YLT26), efficiently inhibiting the growth of breast cancer cells *in vitro*. In order to provide the theoretical basis for YLT26 development and further optimization of this kind of anticancer agent, it is necessary to investigate the molecular mechanism of YLT26 and its antitumor activity *in vivo*.

In this study, we investigated the effects of YLT26 on breast cancer cells and mice bearing highly metastatic 4T1 tumor. We found that YLT26 inhibited proliferation of 4T1 cells by causing apoptosis *via* ROS mitochondria apoptotic pathway. In addition, YLT26 inhibited tumor growth and had considerable activity in impairing formation of pulmonary metastases *in vivo*, which might be associated with the reduction of myeloid-derived suppressor cells (MDSCs) in the lung. Its potential to be a candidate against metastatic breast cancer is worth further investigation.

Materials and Methods

Preparation of YLT26

(E)-N-(4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl)-3-(3,4,5-trimethoxyphenyl)acrylamide (YLT26) was initially synthesized by our group (State Key Laboratory of Biotherapy, Sichuan University Sichuan, China) according to Scheme 1. Briefly, commercially available 3,4,5-trimethoxybenzaldehyde (2.94g, 15 mmol) was first reacted with malonic acid (1.87g, 18mmol) in the presence of piperidine (176 μ L, 1.80mmol) as the base in pyridine under reflux to give trans-3,4,5-

Scheme 1. Synthetic route for YLT26. Reagents and conditions: (a) $\text{CH}_2(\text{COOH})_2$, pyridine, cat. piperidine, 100 °C, 1 h; (b) 1. CH_2Cl_2 , cat. DMAP, EDCI, rt, 1 h, 2. 4-(hexafluoro-2-hydroxyisopropyl)aniline, rt, 24 h.

Fig. 1. HPLC data about compound YLT26 purity.

trimethoxycinnamic acid. The compound YLT26 was prepared by reacting trans-3,4,5-trimethoxycinnamic acid (500mg, 2.1mmol) with 4-(hexafluoro-2-hydroxyisopropyl)aniline (544.2, 2.1mmol) in the presence of *n*-(3-dimethylaminopropyl)-*n*'-ethylcarbodiimidehydrochloride (EDCI, 644mg, 3.36 mmol) and 4-Dimethylaminopyridine (DMAP, 12.8mg, 0.105 mmol) in dichloromethane at room temperature. The crude product was purified by column chromatography on silica gel using petroleum ether/ethyl acetate as eluent (543.2mg, 54% yield). The chemical structure of YLT26 was determined by ^1H -NMR, ^{13}C -NMR and ESI-MS. ^1H -NMR (DMSO- d_6) δ : 10.42 (1H, s), 8.64 (1H, s), 7.82 (2H, d, J = 8.9 Hz), 7.64 (2H, d, J = 8.6 Hz), 7.56 (1H, d, J = 15.6 Hz), 6.98 (2H, s), 6.74 (1H, t, J = 27.7 Hz), 3.84 (6H, s), 3.70 (3H, s); ^{13}C -NMR (DMSO- d_6) δ : 163.87, 153.08, 140.82, 138.97, 130.14, 127.44, 124.95, 124.39, 121.52, 121.17, 118.89, 105.16, 60.07, 55.99, 55.84; ESI-MS m/z : 478.2 [$\text{M} - \text{H}$] $^-$. Purity (>98%) was determined by high-performance liquid chromatography (HPLC) (Fig. 1). HPLC analysis of YLT26 was performed on an UltiMate 3000 HPLC system (Dionex, USA) equipped with a variable wavelength UV detector and an autosampler system. The detection wavelength was set at 317 nm, experiments were performed on a 5 μm Thermo C_{18} column (200 mm $\times$ 4.6 mm, Waters Technologies, Ireland) operated at 35 °C, and a mobile phase of methanol/water (70:30, v/v) was used, and the flow rate was 1.0 mL/min.

YLT26 was prepared as 40 mM stock solution in dimethyl sulfoxide (DMSO) and diluted with the relevant medium for the *in vitro* experiments. For *in vivo* assays, YLT26 was prepared in 10% (v/v) aqueous cremophor EL (CrEL) containing 5.0% (v/v) alcohol and dosed at 0.1 ml/10g of body weight.

Materials

DMSO, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), Rhodamine-123 (Rh123), 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma (St Louis, MO). The Annexin V-FITC Apoptosis Detection Kit was purchased from KeyGen Biotech (Nan-jing, China). Hoechst 33342 was purchased from Beyotime Institute of Biotechnology (Shanghai, China). TUNEL assay kit was

purchased from Roche (Shanghai, China). FITC-CD11b, PE-Gr1 conjugated antibodies were obtained from BD Biosciences. The antibodies against caspase-9 and Bcl-2 were purchased from Cell Signaling Technology Company (Beverly, MA). Antibody against β -actin was obtained from Santa Cruz Biotechnology Company (Santa Cruz, CA).

Cell lines and animals

4T1 (mouse breast cancer cell line), MCF-7, MDA-MB-231 (human breast tumor cell lines) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI 1640 or DMEM containing 10% fetal bovine serum (FBS; Gibco, Auckland, N.Z.) and 1% antibiotics (penicillin and streptomycin) in 5% CO₂ at 37°C. Female BALB/c mice used in this study were obtained from Beijing HFK bioscience CO. Ltd, Beijing, China.

Cell proliferation assay

The cell viability of YLT26 treated cancer cells were determined using MTT assay as previously described [16]. Briefly, cells (3–5×10³/well) were seeded in 96-well culture plates. After 24 h incubation, the cells were treated with vehicle (0.1% DMSO) and various concentrations of YLT26 for 24, 48 and 72 h, respectively. Then, 20 μ L of 5 mg/mL MTT was added to each well and the plates were incubated for an additional 2 h at 37 °C. The medium was subsequently discarded, and 150 μ L DMSO was added to dissolve the formazan. Absorbance was measured at 570 nm using a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, CA, USA) and the IC₅₀ values were calculated.

Colony formation assay

Briefly, 4T1 cells (400–500 cells/well) were seeded in a 6-well plate [17]. After 24 h incubation, the cells were treated with various concentrations of YLT26 and then cultured for another 10 days. After washing by phosphate buffer saline (PBS), colonies were fixed with 4% paraformaldehyde and stained with a 0.5% crystal violet solution. Finally, the colonies with > 50 cells were counted under an inverted microscope.

Morphological analysis by Hoechst staining

Morphological changes associated with apoptosis in 4T1 cells were detected by Hoechst 33342 staining [18]. Briefly, 4T1 cells (1–2×10⁵ cells/well) were plated in a 6-well plate for 24 h. The cells were treated with YLT26 for another 48 h and then washed with cold PBS. Finally, the cells were stained with the Hoechst 33342 solutions (5 μ g/ml) according to the manufacturer's instructions. Then nuclear morphology of cells was examined under an inverted fluorescence microscopy (Zeiss, Axiovert 200, Germany).

Mitochondrial membrane potential ($\Delta\Psi$ m) assay

The changes of mitochondrial transmembrane potential were evaluated using rhodamine 123 (Rh123) as described previously [19]. Briefly, 4T1 cells (1–2×10⁵ cells/well) were plated in a 6-well plate and allowed to attach overnight. Cells were treated with 0–10 μ M YLT26 for 36 h. 4T1 cells were washed with cold PBS and incubated with Rh123 (5 μ g/ml) for 30 min in the dark and then detected by flow cytometry (FCM, ESP Elite, Beckman-Coulter, Miami, FL).

Western blotting analysis

The western blotting analysis was performed as described previously [18]. 4T1 cells were plated (1–2×10⁵ cells/well) in a six-well plate and incubated overnight. After 48 h exposure to various concentrations of YLT26, cells were washed with PBS twice and lysed in RIPA buffer. The protein concentration was measured by the Lowry method. Equal amounts of total protein from each sample were subjected to SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Bioscience, Piscataway, NJ). After incubation with the primary and secondary antibodies, the bands were visualized using the enhanced chemiluminescence method (Amersham, Piscataway, NJ).

Measurement of intracellular ROS

The DCFH-DA method was used to measure the level of intracellular ROS in 4T1 cells [20]. Image analysis of the generation of intracellular ROS was achieved by seeding the cells into a six-well plate at a density of 5–6×10⁵ cells per well and allowed to attach overnight. The next day, cells were pretreated with

10 mM N-acetyl-L-cysteine (NAC, a ROS inhibitor) for 1 h and then treated with 5 μ M YLT26 for 12h. Cells were then washed with PBS and incubated with 10 μ M DCFH-DA for 30 min at 37°C. After washing with PBS, microscopic images of fluorescence were collected using inverted fluorescence microscopy (Zeiss, Axiovert 200, Germany).

Apoptosis analysis by FCM

After 5 μ M YLT26 treatment for 48 h with or without pretreatment of 10 mM NAC, the cells were harvested and washed with cold PBS twice. Then the level of apoptosis was determined using the apoptosis detection kit according to manufacturer's instructions.

Experimental breast tumor metastasis model

All animal assays were approved and conducted by the Institutional Animal Care and Treatment Committee of Sichuan University in China (Permit Number: 20130901). Female BALB/c mice (six- to eight-week-old) were used in present study. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In brief, 1.0×10^6 4T1 tumor cells in 100 μ L PBS were implanted subcutaneously into the right flank of female BALB/c mice. After 7 days inoculation, the tumor-bearing mice were randomized into three groups ($n = 6$ in each group), and received intraperitoneally injection (i.p.) of YLT26 75mg/kg, 150mg/kg or vehicle, respectively every 3 days. Body weight and tumor volumes were measured every 3 days. The tumor volumes was calculated according to the formula: Tumor volume (mm^3) = $0.52 \times L \times W^2$ where L is the length and W is the width. After 21 days treatment, all the mice were euthanized. The lungs were extracted and lung metastases were manually counted by three individuals with the current experiment.

After treatment for 21 days, tumors and lungs of 4T1 models were removed, fixed, routinely processed and embedded in paraffin. Immunohistochemistry staining of tumor sections were described previously [21]. Tumor sections were stained with TUNEL agent. Moreover, to observe metastasis in deep lung, the sections containing lungs were stained with hematoxylin and eosin (H&E).

Flow cytometry

Briefly, we prepared single-cell suspensions of lung according to standard protocols [22]. Then 1×10^6 freshly prepared cells were suspended in 100 μ L PBS and incubated with anti-CD11b and -Gr1 antibodies according to the manufacturer's instructions. Samples were run on a FCM and analyzed using FlowJo software.

Acute toxicity

BALB/c mice were randomly divided into two groups ($n=6$). Animals were given YLT26 (2g/kg) or vehicle by oral administration. Following treatment, the clinical symptoms of the animals, including autonomic effects, changes in the level of activity, mortality and body weight, were observed closely for 14 days. We determined white and red blood cell counts, hemoglobin and platelets levels by Hitachi 7200 Blood Chemistry Analyzer. Serum biochemistry parameters were analyzed by a Nihon Kohden MEK-5216K Automatic Hematology Analyzer.

Statistical analysis

The data were expressed as the means $\pm$ SD. The statistical comparisons were made by Student's T test and a statistically significant difference was considered to be present at $p < 0.05$.

Results

The anti-proliferation effects of YLT26 against breast cancer cells

We investigated the proliferation inhibition caused by YLT26 treatment on several breast cancer cells. After exposure to YLT26 for 72 h, the IC_{50} of MCF-7, MDA-MB-231, 4T1 were 8.3 μ M, 34.1 μ M and 5.2 μ M, respectively (Fig. 2A). Exposure of 4T1 cells to YLT26 for 24h, 48h and 72h, respectively, caused a marked decrease in the viability (Fig. 2B). These results demonstrated that YLT26 inhibited the proliferation of 4T1 cells in a time- and concentration-dependent manner.

Fig. 2. The effects of YLT26 on breast cancer cell viability. (A) 4T1, MCF-7 and MDA-MB-231 cells were treated with various concentrations (0–40 μM) of YLT26 for 72 hours, respectively, then, the cell viability was detected by MTT assay. The data are expressed as the means ± SD from three independent experiments. (B) MTT assays showed YLT26 inhibited 4T1 cells proliferation in a concentration- and time-dependent manner. Data are expressed as means ± SD, from three independent experiments. (C) The colony clusters were detected after a 10-day YLT26 treatment. (D) Statistic results of colony-forming assays presented as surviving colonies. Data are expressed as means ± SD, from three independent experiments (* $p < 0.05$, ** $p < 0.01$).

Moreover, to further determine whether YLT26 could inhibit the proliferation of 4T1 cells, we conducted clonogenic assay after YLT26 treatment. Clonogenic assay clearly showed that YLT26 treatment significantly decreased the colony size and number of colonies in a concentration-dependent manner (Fig. 2C, D). The results from clonogenic assay were accorded with the MTT assay. Taken together, these results indicated that YLT26 could inhibit the proliferation of 4T1 cells.

Inducing apoptosis by YLT26

To determine whether reduction of proliferation capacity of the breast cancer cells induced by YLT26 was associated with cells apoptosis, 4T1 cells were treated with YLT26 as described previously. Morphological changes of 4T1 cells treated by YLT26 were performed through Hoechst 33342 staining. Apoptosis cells, with bright-blue fluorescent and condensed nuclei, were observed in 4T1 cells, and the changes were concentration-dependent (Fig. 3A). However, there was no significant apoptosis in untreated 4T1 cells which showed blue, diffusely stained intact nuclei. The results directly suggested that YLT26 induced the apoptosis of 4T1 cells in a concentration-dependent manner.

YLT26 induces apoptosis in 4T1 cells through mitochondrial apoptotic pathway

To investigate the underlying mechanism of 4T1 cells apoptosis induced by YLT26, western blotting analysis was employed to detect the expression of apoptosis-related proteins in the treated cells. As shown in Fig. 3B, following treatment with YLT26 for 48 h, the level of activated caspase-9 was increased. The activation of caspase-9 hinted that the molecular mechanism of apoptosis might be *via* the mitochondrial pathway. A key step in the intrinsic apoptotic pathway is the damage of mitochondria and loss of $\Delta\Psi_m$. To further verify that

Fig. 3. YLT26 induced apoptosis *via* mitochondrial apoptotic pathway in 4T1 cells. (A) The fluorescence microscopic appearance of Hoechst 33342-stained nuclei after incubation with varying concentrations of YLT26 (0 μ M (a), 2.5 μ M (b), 5 μ M (c), 10 μ M (d)) for 48 h ($\times 40$). (B) The levels of caspase-9 and Bcl-2 were measured after an incubation with 0–10 μ M YLT26 for 48 h. (C) 4T1 cells were treated with YLT26 for 36 h, and $\Delta\Psi_m$ was analyzed by flow cytometry. Data are representative of three independent experiments.

YLT26 induces the disruption of the mitochondrial membrane potential, we used a mitochondria-specific and voltage-dependent dye Rh123 to detect alterations in $\Delta\Psi_m$ in 4T1 cells. As shown in Fig. 3C, 19.3% and 43.6% more of cells lost mitochondrial membrane potential, respectively, when cells were treated with 5 μ M and 10 μ M YLT26 for 36 h, compared with control group. Moreover, Bcl-2 family proteins are essential for apoptosis controlled by mitochondria. We examined the expression of Bcl-2 by western blotting analysis after YLT26 treatment for 48 h. As shown in Fig. 3B, the expression of anti-apoptotic protein Bcl-2 in 4T1 cells significantly decreased in a dose-dependent manner. These results suggested that the mitochondria-mediated pathway is involved in YLT26-induced apoptosis.

YLT26 induces ROS generation in 4T1 cancer cells

Mitochondria are considered as the main source of ROS, which was demonstrated to be able to trigger apoptosis [23]. Meanwhile, studies revealed that breast cancer cells were particularly sensitive to oxidative stress because they have persistently high levels of ROS [24]. To investigate the underlying mechanism of anti-proliferative activity, DCFH-DA was applied to detect the change of intracellular ROS level after YLT26 treatment. As compared with control cells, YLT26 treatments caused a stronger fluorescence intensity in 4T1 cells, which indicated an enhanced ROS levels. However, the increased ROS levels were attenuated by treatment with NAC, a powerful antioxidant (Fig. 4A).

YLT26-induced apoptosis is attenuable by antioxidants

To determine whether ROS played an important role in the induction of apoptosis following YLT26 treatment in 4T1 cells, Annexin V assay was conducted. After a 48 h YLT26

Fig. 4. YLT26 induced apoptosis associated with ROS production. (A) Representative fluorescence microscopy images of 4T1 cells treated with YLT26 and YLT26+NAC ($\times 20$). Untreated cells served as the control. (B) Flow cytometric analysis of cells stained with Annexin V-FITC/PI after treatment with 5 μ M YLT26 with or without prior 6 h incubation with NAC (10 mM). (C) Statistic results of apoptosis assays. Data are expressed as means $\pm$ SD from three independent experiments (** $p < 0.01$).

Fig. 5. Effects of YLT26 treatment on primary tumor growth and TUNEL assay *in vivo*. (A) The mean tumor volumes $\pm$ SD of 6 mice every group. (B) The mean mice weights $\pm$ SD of every group. (C) Three independent tumors were taken from the vehicle group or YLT26-treated group for apoptosis detection using TUNEL assay and representative fields are shown ($\times 20$).

(5 μ M) treatment with or without pretreatment of NAC (10 mM), the level of apoptosis was determined by FCM. The apoptosis rates in 4T1 cells of control and YLT26 treatment alone were 1.3% and 57.4%, respectively (Fig. 4B, C). The data indicated that treatment with YLT26 significantly increased the rate of apoptosis of 4T1 cells compared with the controls, which were consistent with the results of Hoechst 33342 staining. However, pretreatment with ROS

Fig. 6. Effects of YLT26 treatment on pulmonary metastasis. (A) Lung metastatic nodules were visualized after 21 days treatment. (B) The mean lung weight of every group. The "Normal" column indicates statistical analysis of lung weight of six normal mice. Bars showed means $\pm$ SD ($n=6$, *** $P<0.001$) (C) The mean lung metastasis nodules of each group. Bars showed means $\pm$ SD ($n=6$; * $p<0.05$, ** $p<0.01$) (D) H&E staining of lung tissues harvested from 4T1 tumor-bearing mice and treated with YLT26 or vehicle for 21 days ($\times 10$).

inhibitor NAC (10 mM) could significantly reduce the amount of apoptosis rate caused by the treatment of YLT26 (Fig. 4B, C). Taken together, these data indicated that the ROS production is critical for the observed YLT26-induced apoptosis. Since NAC did not completely inhibited apoptosis in these cells, it indicated the participation of other pathways in the execution of this cell death program.

Antitumor activity of YLT26 in vivo

To test the antitumor effect of YLT26 *in vivo*, female BALB/c mice were inoculated subcutaneously with 4T1 cells. Mice then received intraperitoneally injection (i.p.) of YLT26 (75 mg/kg, 150 mg/kg, respectively) or vehicle every three days for 21 days. The results showed that the tumor growth of the YLT26 treatment group become significant slowed after 9 days treatment. YLT26 substantially inhibited tumor growth in a dose-dependent manner compared with the control after 21 days treatment (Fig. 5A). Moreover, YLT26 treatment was well tolerated and did not cause significant loss in body weight compared with the vehicle group (Fig. 5B).

To further demonstrate that tumor growth inhibition *in vivo* is a result of apoptosis, immunohistochemical analyses were performed on tumor tissues isolated from 4T1 tumor model. As shown in Fig. 5C, mice treated with YLT26 produced higher apoptosis in a dose-dependent manner than vehicle group. Taken together, these results clearly showed that YLT26 inhibits tumor growth *in vivo* and increased apoptosis in human tumor xenograft model.

YLT26 suppresses lung metastasis in vivo

4T1 breast tumor cells had been confirmed to can metastasize to distant organs such as lung [18, 25]. Therefore, the therapeutic efficacy of YLT26 against lung metastases was evaluated when mice were sacrificed. Lung weight was measured and metastatic lung nodules were counted. As shown in Fig. 6A, B, there was a remarkable decrease in lung weight after YLT26 treatment compared with the untreated control. Importantly, the number of lung metastatic nodules in control group was 65 ± 7 , whereas only 29 ± 5 and 10 ± 2 nodules were observed in 75 mg/kg and 150 mg/kg groups, respectively, further supporting the notion

Fig. 7. Effects of YLT26 treatment on metastasis-associated Gr1⁺/CD11b⁺ MDSCs in lung. Lung CD11b⁺/Gr1⁺ myeloid cells were analyzed by FCM after 21 days of treatment with vehicle (A); or YLT26 at 75mg/kg (B); or YLT26 at 150mg/kg (C). (D) Statistic results of each group. Bars showed means $\pm$ SD (n=6; *p < 0.05, **p < 0.01).

Fig. 8. Evaluation of side effects of YLT26 in mice. (A) The hematological and serum biochemical values were performed with the blood samples collected at the time of sacrifice. (n = 6 for both control and treated group). (B) Data shown are the average body weight changes of each group (n = 6). Values indicated the mean mice weights $\pm$ SD.

that YLT26 treatment inhibited lung metastases (Fig. 6C). In addition, histological analyses demonstrated that the number of micrometastatic nodules per field in the YLT26-treated group at 150 mg/kg was also significant fewer than the other groups (Figure 6D). These results further indicated that high dose of YLT26 could inhibit lung metastasis in breast cancer.

YLT26 reduces the number of lung-infiltrating Gr1⁺/CD11b⁺ MDSCs
MDSCs, as mainly characterized by CD11b⁺ and Gr1⁺ double positive myeloid cells in mice, have been observed to accumulate in highly metastatic breast carcinoma 4T1, and

the excess of MDSCs are closely related to a lung metastatic in patients with breast cancer [17, 26]. Therefore, we further examined the number of MDSCs in lung by flow cytometric analysis. As shown in Fig. 7A, B, the data showed that the percentage of MDSCs decreased in the 75mg/kg -treated group compared with the control group. Moreover, we found that about 2-fold reduction of MDSCs in the lung after 150 mg/kg YLT26 treatment (Fig. 7C). The statistical analysis demonstrated that YLT26 treatment reduced the number of MDSCs in lung in a dose-dependent manner (Fig. 7D). These results suggested that YLT26 inhibited lung metastasis in 4T1 tumors, which might be associated with suppression of lung myeloid cells infiltration.

Acute toxicity

During the 14-day experiment, no death, signs and symptoms of toxicity were observed in any animal. There was no significant difference in hematological and serum biochemical values (Fig. 8A), and body weight (Fig. 8B) after YLT26 treatment compared with control group.

Discussion

Breast cancer is the most common cancer among women and therapeutic options of the modalities available now are very limited, which urges us to develop novel potential drug candidates for breast cancer treatment [1]. In the present study, we firstly described the *in vitro* and *in vivo* effects of a novel small molecular compound YLT26 on breast cancer growth and tumor metastasis.

In a cell-based screening of anticancer drugs, we found YLT26 displayed potent inhibitory effects on breast cancer cells and highly aggressive 4T1 cells were the most sensitive to its treatment, with an IC_{50} value of 5.2 μ M. Our present study showed that YLT26 treatment decreased the viability of 4T1 cells in a time- and concentration-dependent manner. Moreover, the proliferation inhibitory activity of YLT26 against 4T1 cells was further confirmed using clonogenic assays.

Hoechst 33342 staining and FCM assays demonstrated that YLT26 inhibited the viability of 4T1 by inducing apoptosis in a concentration-dependent manner. Generally, two distinct apoptotic pathways are characterized: intrinsic pathway (mitochondrial-mediated) and extrinsic pathway (cell death receptor-mediated); the apoptotic cascade is triggered by activation of caspase-9 and caspase-8, respectively [7]. To investigate the underlying mechanism of YLT26-induced apoptosis, we analyzed the level of proteins involved in apoptosis. Our results showed that caspase-9 was activated, hinting that YLT26 induced apoptosis *via* the mitochondrial pathway.

In the intrinsic apoptotic pathway, mitochondria play a central role. Disruption of the mitochondrial membrane potential is known to induce release of cytochrome c from mitochondria into the cytosol, where cytochrome c leads to the generation of caspase-9 and activates downstream caspases, which ultimately induces the cell to undergo apoptosis [27]. In our experiment, a loss of $\Delta\Psi_m$ in 4T1 cells was also observed after YLT26 treatment. Moreover, Bcl-2 family proteins are known to regulate mitochondrial membrane permeability and polarization. Anti-apoptotic protein Bcl-2 is a major member of the Bcl-2 family [28]. The results showed that YLT26 decreased the expression of Bcl-2 in 4T1 cells in a concentration-dependent manner. Therefore, the data indicated that YLT26 treatment induced apoptotic death in 4T1 cells through the mitochondrial pathway, which was mediated by Bcl-2.

Mitochondria are the main source of ROS [29]. Excess levels of ROS can result in apoptosis and many anti-cancer drugs have been reported to induce apoptosis through the generation of ROS in mitochondria [30, 31]. In this study, we found that 5 μ M YLT26 treatment markedly induced the production of ROS in 4T1 cells. However, the enhanced ROS levels were attenuated by pretreatment with antioxidant NAC, which indicated that the effect of YLT26 on 4T1 cells may be associated with cell redox systems imbalance. In addition, we

found that the ability of YLT26 to induce apoptosis could be attenuated by pretreatment with NAC. From these results, we demonstrated that YLT26 induced 4T1 cells apoptosis through the ROS-mediated-mitochondrial apoptotic pathway.

For further study, we confirmed the effect of YLT26 *in vivo* using the spontaneous lung metastasis 4T1 model. For the first time, our study demonstrated that YLT26 resulted in significant inhibition of tumor growth. Immunohistochemical study revealed that the regression of tumor size in mice models by YLT26 was also associated with the activation of apoptosis, as demonstrated by the presence of TUNEL-positive cells in tumor xenograft samples. Furthermore, YLT26 dramatically suppressed the metastasis of 4T1 mice breast carcinoma to lung tissue in a dose-dependent manner. According to the statistical analysis, 150mg/kg YLT26 treatment resulted in a significant reduction in the lung metastatic nodules, an approximately 84% decrease compared with the control group. In addition, we found that YLT26 did not exhibit toxicity *in vivo*. Therefore, the property of high safety made YLT26 suitable for anticancer therapy.

Mounting studies reported that a major barrier to effective breast cancer therapy was immune suppression, and the accumulation of MDSCs has been deemed to be a major mechanism in promoting immune suppression [32, 33]. Moreover, MDSCs, a cellular component of the tumor microenvironment, has been reported to strongly enhance metastasis in a murine breast cancer model [34]. In our study, we also found that YLT26 significantly decreased the percentage of MDSCs in the lung microenvironment compared with the control group in 4T1 breast tumor model. Thus, these results indicated that YLT26 exerted its anti-metastasis effects by stimulating immune responses.

In conclusion, this study indicated for the first time that a novel compound YLT26 inhibited the proliferation of breast cancer cells and induced 4T1 cells apoptosis *via* the ROS-mediated-mitochondrial pathway. Moreover, YLT26 could significantly inhibit breast cancer growth *in vivo* and dramatically block lung metastasis of 4T1 cells by reducing the number of Gr1⁺/CD11b⁺ (MDSCs) without obvious toxicity. Therefore, our studies provided strong evidence that YLT26 may be a new lead compound as breast cancer drug candidate.

Abbreviations

DMSO (Dimethyl Sulfoxide); MTT (3-(4, 5)-dimethylthiazol-2, 5 -diphenyltetrazolium bromide); PBS (phosphate buffer saline); PI (propidium iodide); Rh123 (Rhodamine-123); DCFH-DA (2',7'-dichlorofluorescein diacetate); DMEM (Dulbecco's Modified Eagle's Medium); FBS (fetal bovine serum); $\Delta\Psi_m$ (mitochondrial transmembrane potential); PVDF (polyvinylidene difluoride); ROS (reactive oxygen species); NAC (N-acetyl-L-cysteine); H&E (hematoxylin and eosin); MDSCs (myeloid-derived suppressor cells); FCM (Flow Cytometry); HPLC (high-performance liquid chromatography).

Disclosure Statement

None declare.

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