Suppression of proliferation and migration in highly-metastatic lung cancer cells as well as tumor growth by a new synthesized compound TBrC and its molecular mechanisms of action

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Abstract To develop new anticancer agents has been considered as a useful and necessary strategy to suppress highly-metastatic lung cancer, the leading cause of cancer-related deaths in the world. In this study, we synthesized a new compound ethyl 6-bromocoumarin-3-carboxylate L-theanine (TBrC) and studied the anticancer activity of TBrC and its molecular mechanisms of action. Our results show that TBrC remarkably inhibits the proliferation and migration in highly-metastatic lung cancer cells by inducing apoptosis and cell cycle arrest as well as regulating related protein expressions. Further study indicated that TBrC not only enhances the protein levels of Bax, cytosolic cytochrome *c*, caspase-3 and

PARP-1 but also reduces the protein expressions of Bcl-2, cyclin D1, VEGFR1 and NF- κ B as well as inhibits the phosphorylation and expressions of VEGFR2 and Akt in the cancer cells. More importantly, TBrC displays strong suppression of highly-metastatic tumor growth and reduces the tumor weight by 61.6 % in tumor-bearing mice without toxicity to the mice. Our results suggest that TBrC suppresses the proliferation and migration of lung cancer cells via VEGFR-Akt-NF- κ B signaling pathways; TBrC may have a wide therapeutic and/or adjuvant therapeutic application in the treatment of lung cancer.

Keywords TBrC · Lung cancer cells · Proliferation and migration · VEGFR1/VEGFR2-Akt-NF- κ B pathways · Tumor growth

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Abbreviations

TBrC	Ethyl 6-bromocoumarin-3-carboxylate L-theanine
NF- κ B	Nuclear factor κ B
VEGFR	Vascular endothelial growth factor receptor
Ly	Ly294002
Bay	Bay 11-7082
PARP	Poly (ADP-ribose) polymerase
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
Cyto C	Cytochrome <i>c</i>
LLC	Lewis lung cancer
HUVECs	Human umbilical vein endothelial cells

Introduction

It is estimated that there are more than 226,000 new cases and about 160,000 deaths from Lung and bronchus cancer in the U.S. in 2012 (Siegel et al. 2012). Lung adenocarcinoma has become the leading cause of cancer-related deaths in the world (Jemal et al. 2008, 2011; Siegel et al. 2011, 2012). Despite recent advances in diagnosis and treatment, overall 5-years survival of lung cancer patients is only about 15 %. The patients with advanced disease have a median survival of approximately 10 months when treated with standard platinum-based therapy. Hence, there is an imminent need for better therapies and/or anticancer agents for lung cancer. Theanine (γ -glutamylethylamide) is a characteristic amino acid in tea and has been widely used as a safe food additive without limitation to its dose. The anticancer activities of theanine against some cancers have been reported (Sugiyama and Sadzuka 1998; Sugiyama et al. 1999). Our previous study indicated that theanine and the sera from theanine-fed rats inhibited the invasion of rat hepatoma cells in vitro and ex vivo (Zhang et al. 2001) and hepatoma growth in vivo (Zhang et al. 2002). In order to further enhance the anticancer activity of theanine and develop a new anticancer agent targeting lung cancer, we have synthesized a new compound TBrC based on the structure of theanine and investigated the effects of TBrC on induction of apoptosis and cell cycle arrest, migration, and tumor growth as well as the related receptors-mediated signaling pathways in highly-metastatic lung cancer cells.

Materials and methods

Chemicals

Fibronectin and Boyden chambers were purchased from BD Inc. (BD Biosciences, San Jose, CA, USA) and Corning Inc. (Corning, NY, USA), respectively. DMEM, penicillin, streptomycin, fetal bovine serum (FBS), trypsin/EDTA, propidium iodide, Ly294002 (Ly), Bay 11-7082 (Bay), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), collagenase, and all other chemicals including materials for the synthesis of TBrC were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of ethyl 6-bromocoumarin-3-carboxylate L-theanine (TBrC)

All materials we used were purchased from commercial suppliers (Sigma Chemical Co.) and used without further purification. Solvents were distilled prior to use and flash chromatography was performed using silica gel (60 Å, 200–300 mesh). 6-bromo-2-oxo-2H-chromene-3-carboxylic acid (BrC) was obtained according to the literature method (Deshmukh et al. 2003). All reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60GF-254) and visualized with UV light, or Ninhydrin Spray (0.5 % in butanol). Melting points were determined on an electrothermal melting point apparatus. ^1H , ^{13}C NMR spectra were determined on a Bruker Avance 500/125 spectrometer using TMS as an internal standard in $\text{DMSO}-d_6$ (Hexadeuterodimethyl sulfoxide) or CDCl_3 (Chloroform-d) solutions. Chemical shifts are reported in delta (δ) units, parts per million (ppm) downfield from trimethylsilane. ESI-MS were determined on an API 4000 spectrometer (AB SCIEX, Concord, Ontario, Canada).

The scheme of chemical synthesis of TBrC is shown in the Fig. 1. The targeted compound TBrC was synthesized as follows. To a solution of *L*-Theanine 87 mg (500 μM) in absolute ethanol (1 mL), SOCl_2 0.055 mL (760 μM) was added slowly, and the mixture was stirred at room temperature for 2 h, then the resulting mixture was concentrated under reduced pressure to give ethyl *L*-theanine (ET). To the obtained oily residue ET, CH_2Cl_2 (20 mL) was added, then 6-bromo-2-oxo-2H-chromene-3-carboxylic acid (BrC) (224 mg, 1,000 μM), 1.07 mL DIPEA (N,N-Diisopropylethylamine) (791.8 mg; 610 μM) and 383 mg EDCI (1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide) (200 μM) were added, respectively. The mixture was stirred at room temperature for 1 h, and then was concentrated under reduced pressure to remove the solvent. The crude product was purified by chromatography (acetone/petroleum ether = 1/5) on silica gel to give a light yellow solid (Yield = 84.6 %; purity of 98 %). m.p.: 109–111 °C. ^1H -NMR δ : 1.12 (t, J = 7.2 Hz, 3H, CH_3), 1.28 (t, J = 7.2 Hz, 3H, CH_3), 2.17–2.38 (m, 4H, CH_2), 3.26 (m, 2H, $\text{NH}-\text{CH}_2$), 4.15 (t, J = 5.4 Hz, 1H, $\text{NH}-\text{CH}$), 4.21 (q, J = 7.1 Hz, 2H, $\text{O}-\text{CH}_2$), 5.50 (br, 1H, NH), 6.87 (d, J = 8.5 Hz, 1H, coumarin-8H), 7.39–7.42 (m, 2H, coumarin-5H, 7H), 8.32 (s, coumarin-4H), 12.95

Fig. 1 Scheme of preparation of ethyl 6-bromocoumarin-3-carboxyl L-theanine (TBrC). Targeted compound TBrC was prepared as outlined in the scheme. L-Theanine treated with SOCl_2 in ethanol to generate its ethyl L-theanine, which was

subsequently reacted with ethyl 6-bromocoumarin-3-carboxylic acid (BrC) to give the corresponding compound TBrC. The final product TBrC was purified by column chromatography and the structure was characterized by ^1H NMR, ^{13}C NMR and MS

(s, 1H, NH). ^{13}C -NMR δ : 14.2 (CH_3), 14.8 (CH_3), 29.1 (CH_2), 32.0 (CH_2), 34.4 (CH_2), 61.6 (CH), 70.0 (CH_2), 110.3 (C), 119.2 (CH), 119.9 (C), 133.9 (CH), 139.7 (CH), 160.1 (C), 166.2 (C), 170.7 (C), 171.2 (C). ESI-MS (m/z): 451.0 [$\text{M}-\text{H}$] $^-$.

Cell culture and in vitro proliferation assay

In order to obtain normal primary human umbilical vein endothelial cells (HUVECs), we recruited mothers who assented and gave written consent to contributing 20 cm of umbilical cord postpartum. HUVECs were isolated according to a previous reported method (Jaffe et al. 1973) with minor modifications. Briefly, untraumatized umbilical cord segments were cannulated and perfused with 200–400 mL phosphate buffered saline (PBS) to remove all traces of blood. Then the vein lumen was filled with PBS containing 1 mg/mL collagenase and incubated at 37 °C for 10 min. The contents of the vein were gently flushed with an equal volume of PBS, centrifuged at 1,000 r/min for 10 min and re-suspended in DMEM medium supplemented with 10 % heat-inactivated FBS. After 12 h, fresh medium was added. The cells were grown in DMEM supplemented with 10 % heat-inactivated FBS, glutamine (2,000 microM), penicillin (100 units/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$). The cultures were maintained at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2 . Culture medium was refreshed every 2 days. After 6–8 days, uniform monolayers developed in the primary cultures and 0.25 % trypsin was used to harvest cells. Immunohistochemical staining for CD34 was employed to identify the

specificity of endothelial cells. The HUVECs were used for in vitro proliferation assay described below.

The highly-metastatic Lewis lung cancer (LLC) cell line was obtained from the American Type Culture Collection. The cell line of LLC was cultured in DMEM containing 10 % heat-inactivated FBS, glutamine (2,000 microM), penicillin (100 U/mL) and streptomycin (100 $\mu\text{g}/\text{mL}$) at 37 °C in a humidified incubator with 95 % air /5 % CO_2 atmosphere. The in vitro proliferation assay was performed according to our published methods (Liu et al. 2009; Luan et al. 2010; Zhang et al. 2000; Jiang et al. 2010). LLC cells and HUVECs were, respectively, plated in 96-well plates (Becton–Dickinson, Franklin Lakes, NJ, USA) at 2×10^3 (in the case of LLC cells) or 5×10^3 (in the case of HUVECs) per well and incubated overnight to allow attachment. The cells in the control group were treated with DMSO [0.1 % (v/v), final concentration]. The cells were, respectively, incubated in DMEM medium supplemented with 10 % FBS containing different concentrations of TBrC (16–250 microM), theanine (16–250 microM), or Ly294002 (Ly, 16 microM), Bay (3.2 microM). Ly and Bay are the inhibitors of PI3K/Akt, and NF- κB , respectively. After 48 and 72 h of treatment, 20 μL of MTT (5 mg/mL) was added to each well of cells, and the plate was incubated for 3 h at 37 °C. One hundred and fifty microliters DMSO were added to each well to lyse the cells. Absorbance was measured at a wavelength of 570 nm and a reference wavelength of 630 nm using a Synergy H4 Hybrid Multi-Mode Microplate Reader (Bio-Tek, Instruments, Inc., Winooski, VT, USA). Absorbance values in each test group were normalized to the values of the control

group treated with 0.1 % (v/v) DMSO to determine the relative cell growth (%) of LLC and HUVEC cells. Here the absorbance values are designated as the relative cell growth (%) of the cells. The relative cell growth in the control group was designated as 100 %. Each experiment was performed in triplicate and repeated at least three times.

Flow cytometry for cell cycle analysis and apoptosis

These were done according to our published methods (Wang et al. 2009; Yu et al. 2009; Zhang et al. 2000, 2012a, b). In brief, LLC cells were treated for 48 h with TBrC (16–250 microM), theanine (16–250 microM), Ly294002 (16 microM), or Bay (3.2 microM). The cells in the control group were treated with DMSO [0.1 % (v/v), final concentration]. The treated cells were detached in PBS/2000 microM EDTA, centrifuged at 300 $\times$ g for 5 min, and then gently resuspended in 250 μ L of hypotonic fluorochrome solution (PBS, 50 μ g propidium iodide, 0.1 % sodium citrate, and 0.1 % Triton X-100) with RNase A (100 units/ml). The function of the fluorochrome solution is to stain cell nuclei. The DNA content was analyzed by flow cytometry (Becton Dickinson FACS Vantage SE, San Jose, CA, USA). Twenty-thousand events were analyzed per sample and the cell cycle distribution and apoptosis were determined based on DNA content and the Sub-G₁ cell population, respectively.

In vitro migration assay

Tumor cell migration was measured by examining cell migration through fibronectin-coated polycarbonate filters, using modified transwell chambers. In brief, LLC cells (5×10^4) were seeded onto the upper chamber in 200 μ L of serum-free medium containing different concentrations of TBrC (4–16 microM) or theanine (4–16 microM); the lower compartment was filled with 0.66 mL of DMEM medium supplemented with 10 % of FBS (as a chemoattractant). The cells in the control group were treated with DMSO (0.1 %, final concentration). After incubation for 6 h at 37 °C, the cells that migrated to the lower surface of the filter were fixed and stained using propidium iodide. The cells on the upper side of the filter were removed using a rubber scraper. The migrated cells on the underside of the filter were counted and recorded for images under a

fluorescent microscope (Nikon, TE2000-U, Tokyo, Japan). Experiments were performed in triplicate.

Western blot analysis

This was performed according to our published methods with some modifications (Wang et al. 2009; Yu et al. 2009; Zhang et al. 2009, Jiang et al. 2010; Zhang et al. 2012a). In brief, LLC cells were treated with different concentrations of TBrC (16–250 microM), theanine (16–250 microM), Ly294002 (Ly, 16 microM), or Bay (3.2 microM). The cells in the control group were treated with DMSO [0.1 % (v/v), final concentration]. The treated cells were collected either at 30 min for detection of phosphorylation ratios of pVEGFR2/VEGFR2 and pAkt/Akt, or at 48 h for detection of protein expressions of Bcl-2, Bax, procaspase/caspase-3, PARP-1, cyclin D1, VEGFR1, NF- κ B (p65), and cytosolic cytochrome *c*. The cells were harvested and washed with PBS. Whole cellular proteins were extracted and cytosolic fractions were prepared following the procedure described by the manufacturer (Beyotime, Haimen City, China). The protein concentration of the extracts was determined using the Bradford method. Equal amounts of cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the primary antibodies to the detected proteins mentioned above, and then horseradish peroxidase-conjugated secondary antibodies, respectively. Anti-b-actin antibody was used as a loading control. The primary and secondary antibodies were used as follows: The primary anti-p-Akt (Ser473) rabbit mAb, anti-Akt rabbit mAb, anti-NF- κ B (p-65) rabbit mAb, anti-p-VEGFR2 (Tyr1175) rabbit mAb, anti-VEGFR2 rabbit mAb, anti-VEGFR1 rabbit polyclonal antibody and the anti-rabbit IgG, HRP-conjugated antibody were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). The primary anti-Bcl-2 rabbit polyclonal antibody, anti-caspase-3 rabbit polyclonal antibody, anti-cyclin D1 rabbit polyclonal antibody, anti-PARP-1 mouse mAb, anti-cytochrome *c* mouse mAb, anti- β -actin mouse mAb and the anti-rabbit IgG, HRP-conjugated antibodies and anti-mouse IgG, HRP-conjugated antibody were purchased from Santa Cruz Technology Inc. (Shanghai, China). Detection was done using an enhanced chemiluminescence system (GE Healthcare Life Sciences, Piscataway, NJ USA).

Subcutaneous tumor model

C57/BL6 mice (age range, 6 weeks) were purchased from the Tumor Research Institute (Chinese Academy of Sciences, Beijing, China). All animal procedures were carried out in accordance with the guidelines established by the Animal Care and Use Committee (ACUC) at the Yantai University and were approved by the ACUC at the Yantai University, China. Single-cell suspensions containing 0.6×10^6 LLC cells in 0.1 mL Hank's balanced salt solution were injected s.c. into the rear left flank of each mouse. About 2 weeks after administration, 120–150 mm³ tumors appeared in all the mice. At this time, mice were randomized into four groups (six mice per group): (1) Control group (vehicle); (2) Cisplatin group (1.5 mg/kg/day); (3) Theanine group (theanine 50 mg/kg/day); (4) TBrC group (TBrC 50 mg/kg/day). The mice were treated with vehicle, cisplatin, theanine, and TBrC by intraperitoneal injection once every day. The therapy was performed for 15 days. Tumor dimensions and mouse body weights were measured every 2 or 3 days by Vernier caliper. Tumor volume was calculated using the formula: tumor size (cm³) = [width (cm)]² × [length (cm)] × 0.52 (Naumov et al. 2009). On the 15th day, mice in all groups were sacrificed. Tumor volumes and weights were compared among the groups using the ANOVA and Bonferroni test. All statistical tests were two-sided.

Statistical analysis

The data were expressed as mean ± standard deviation (SD) and analyzed by the SPSS 13.0 software to evaluate the statistical difference. Statistical analysis was done using the ANOVA and Bonferroni test. Values between different treatment groups at different times were compared. The rate (%) of relative cell growth and migration as well as mean protein levels are shown for each group. For all tests, *P* values <0.05 were considered statistically significant. All statistical tests were two-sided.

Results and discussion

TBrC enhances proliferation inhibition, induction of apoptosis and cell cycle arrest in LLC cells

We first compared the in vitro anticancer activity of TBrC with its parent compound theanine and the

inhibitors of PI3K/Akt (Ly) and NF-κB (Bay), which have a similar function or signaling pathway in anticancer activity against the proliferation inhibition of highly-metastatic LLC cells. The MTT assay confirmed that TBrC increased the inhibition of LLC cell growth by 2–3 fold compared with theanine after the cells were treated with TBrC and theanine (16–250 μM) for 48 and 72 h, respectively (Fig. 2a). In the same conditions, TBrC did not show significant inhibition of the growth of normal human umbilical vein endothelial cells (HUVECs) at concentrations of 16–125 μM although TBrC led to a weak growth inhibition of HUVECs at 250 μM (Fig. 2b). The result indicated that there is no strong toxicity of TBrC on the growth of normal HUVECs. However, theanine led to growth inhibition of HUVECs at 64–250 μM (Fig. 2b). Moreover, the inhibitor of PI3K/Akt (Ly, 16 μM) and NF-κB (Bay, 3.2 μM) remarkably inhibited the growth of both LLC (Fig. 2a) and normal HUVEC cells (Fig. 2b). Furthermore, FACS analysis indicated that TBrC (16–250 μM) exhibited much stronger activity than theanine (16–250 μM) on induction of apoptosis and cell cycle arrest in LLC cells after the cells were treated for 48 h. The apoptotic ratio of LLC cells was 45.95 % in the treatment by TBrC (250 μM) while the apoptotic ratio of LLC cells was 4.62 % in the treatment with the same concentration of theanine (Fig. 2c). The induction of the apoptosis in LLC cells by TBrC could greatly contribute to the proliferation inhibition of LLC cells (Fig. 2a). In addition, the inhibitors of PI3K/Akt (Ly, 16 μM) and NF-κB (Bay, 3.2 μM) also induced the apoptosis in LLC cells. The apoptotic ratios of LLC cells were 17.92 and 39.27 %, respectively. TBrC has shown an activity comparable with Ly and Bay on induction of apoptosis in LLC cells.

TBrC enhances the migration inhibition in LLC cells

The presence of metastasis is the main cause of morbidity and mortality in millions of patients with cancer. During the complicated process of metastasis, the migration of cancer cells is the most important step. Clearly, an agent which could efficiently inhibit the growth and migration of cancer cells would be a hopeful candidate to suppress cancer progression and metastasis and thus could reduce mortality. Therefore, we examined the effects of TBrC and theanine on the

◀ **Fig. 2** Effects of TBrC and theanine (T) on the proliferation and induction of apoptosis and cell cycle arrest in LLC cells as well as the growth of normal human umbilical vein endothelial cells (HUVECs). **a** Effects of TBrC and T on the cell growth of LLC. **b** Effects of TBrC and T on the cell growth of HUVECs. The cells were treated for 48 and 72 h with the indicated concentrations of TBrC and theanine/T (T16–T250/16–250 μ M, TBrC16–TBrC250/16–250 μ M), Ly (16 μ M), and Bay (3.2 μ M). The cells in control group were treated with DMSO. The rate (%) of relative cell growth was determined by the MTT assay. Ly294002 (Ly) and Bay are inhibitors of PI3K/Akt and NF- κ B, respectively. **c** Induction of apoptosis (cells in Sub-G₁ phase) and cell cycle arrest in LLC cells by TBrC and theanine (T) was analyzed by flow cytometry. The cells were treated for 48 h with TBrC16–TBrC250 (16–250 μ M), T16–T250 (16–250 μ M), Ly294002 (16 μ M) and Bay (3.2 μ M) as the positive control. The data are presented as the mean $\pm$ SD (*Bar*) ($n = 6$). The figures are the representative of 3 similar experiments performed. Values with *different letters* (a–e) differ significantly ($P < 0.05$); $*P < 0.05$

migration in LLC cells. The migration assay indicated that TBrC (4–16 μ M) and theanine (8–16 μ M) significantly and dose-dependently suppressed the migration of LLC cells after the cells were treated for 6 h. TBrC displayed a much stronger inhibition than theanine against the migration of LLC cells (Fig. 3a, b). The relative migration rate was reduced by 25–37 % and 10–20 %, respectively by 6 h treatment with TBrC and theanine at the concentrations of 4–16 μ M, which did not significantly inhibit the LLC cell growth (data not shown).

TBrC enhances the down-regulation of the protein levels of Bcl-2 and cyclin D1 and increases the up-regulation of the protein levels of Bax, cytosolic cytochrome *c*, caspase-3, and PARP-1 in LLC cells

To understand the molecular mechanisms of action of TBrC on the proliferation and induction of apoptosis and cell cycle arrest in LLC cells, we compared the effects of TBrC with theanine as well as Ly and Bay on the expressions of related protein. Our results showed that TBrC (16–250 μ M) and theanine (16–250 μ M) significantly reduced the Bcl-2 protein levels in LLC cells. TBrC displayed much stronger activity than theanine on the down-regulation of Bcl-2 protein expressions in LLC cells (Fig. 4a). Furthermore, TBrC (16–250 μ M) and theanine (16–250 μ M) remarkably up-regulated the protein expressions of Bax (Fig. 4a), cytosolic cytochrome *c* (Fig. 4b), caspase-3 (Fig. 4c), and the cleavage of poly(ADP-ribose) polymerase-1

(PARP-1) (Fig. 4d) in LLC cells. TBrC showed much stronger activity than theanine on the up-regulation of these protein expressions in LLC cells (Fig. 4a–d). Moreover, TBrC (16–250 μ M) and theanine (16–250 μ M) significantly reduced the cyclin D1 protein expression in LLC cells. TBrC also exhibited much stronger activity than theanine on the down-regulation of cyclin D1 protein expressions in LLC cells (Fig. 4e). In addition, the inhibitors of NF- κ B (Bay) and PI3K/Akt (Ly) showed a significant suppression of the protein expressions of Bcl-2, and cyclin D1 and increase in the protein levels of Bax, cytosolic cytochrome *c*, caspase-3, and cleavage of PARP-1 in LLC cells (Fig. 4). TBrC has shown a comparable activity with Ly and Bay on the regulation of protein expressions of cyclin D1, Bcl-2/Bax, cytosolic cytochrome *c*, caspase-3, and PARP-1 in LLC cells.

One of the main regulatory steps of apoptotic cell death is controlled by the ratio of antiapoptotic and proapoptotic members of the Bcl-2 family of proteins, which determines the susceptibility to apoptosis. Bax, a proapoptotic factor of the Bcl-2 family, is found in monomeric form in the cytosol or is loosely attached to the membranes under normal conditions. Following a death stimulus, cytosolic and monomeric Bax translocates to the mitochondria where it becomes an integral membrane protein and cross-linkable as a homodimer allowing for the release of factors from the mitochondria, such as cytochrome *c*, to propagate the apoptotic pathway (Oltvai et al. 1993). Bcl-2 and its related proteins control the release of cytochrome *c* from the mitochondria (Reed 1997). Hallmarks of the apoptotic process include the activation of cysteine proteases, which represent both initiators and executors of cell death. In the cytosol, cytochrome *c* activates caspase-9, which in turn activates the effector caspase such as caspase-3 and caspase-7 (Stennicke and Salvesen 2000). In the present study, we observed that TBrC significantly enhanced the down-regulation of the antiapoptotic Bcl-2 levels and up-regulation of proapoptotic Bax levels, leading to a reduction of Bcl-2/Bax ratio (Fig. 4a). Furthermore, TBrC significantly enhanced the release of cytochrome *c* from mitochondria (Fig. 4b) and subsequently increased caspase-3 activity (Fig. 4c). Caspase-3 is synthesized as a 35-kDa inactive precursor (procaspase-3), which is proteolytically cleaved to produce a mature enzyme composed of 17-kDa fragments. As shown in Fig. 4c, caspase-3 in cleaved form was enhanced with the

Fig. 3 Inhibition of LLC cell migration by TBrC and theanine (T). The effects of TBrC on the migration of LLC cells were examined by the migration assay as described in the section of “Materials and methods”. The inhibition of LLC cell migration by TBrC and T is shown in (a) and (b). **a** The photomicrographs (200 $\times$) show the propidium iodide-stained LLC cells that migrated through fibronectin coated transwell chambers. **b** The

cells were treated for 6 h with the indicated concentrations of TBrC and theanine T (T4–T16/4–16 μM , TBrC4–TBrC16/4–16 μM). The control received DMSO vehicle. The data are presented as the mean $\pm$ SD (Bar) ($n = 6$). The figure is the representative of 3 similar experiments performed. Values with different letters (a–d) differ significantly ($P < 0.05$)

increase in the concentrations of TBrC. In addition, we observed the up-regulation of cleavage of PARP-1, the substrate of caspase-3, in LLC cells treated with TBrC (Fig. 4d). Moreover, our results also indicated that TBrC significantly enhanced the down-regulation of cyclin D1 protein expression in LLC cells (Fig. 4e).

Taken together, all these results indicated that TBrC significantly suppressed the proliferation of LLC cells and remarkably induced apoptosis and cell cycle arrest by reducing Bcl-2/Bax ratio and activating the mitochondrial and caspase-3 pathway as well as reducing the protein levels of cyclin D1, the cell cycle regulator in LLC cells.

TBrC enhances the down-regulation of the receptor phosphorylation and/or protein expressions of VEGFR1, VEGFR2, Akt and NF- κB in LLC cells

The activated receptors such as VEGFR1 and VEGFR2 play very important roles in the proliferation and migration of cancer cells including lung cancer cells

(Morelli et al. 2006; Naumov et al. 2009; Lee et al. 2010; Zhang et al. 2012a, b). In order to clarify the TBrC-targeted receptors in LLC cells, we studied the effects of TBrC on the related receptor phosphorylation and expression in LLC cells. We have confirmed that TBrC significantly reduced the expression of VEGFR1 (Fig. 5a) and the phosphorylation and expression of VEGFR2 (Fig. 5b) in LLC cells. TBrC showed a much stronger activity than theanine on reduction of the phosphorylation and/or expressions of the receptors in LLC cells. In addition, the inhibitors of PI3K/Akt (Ly) and NF- κB (Bay) also displayed inhibitory effects on the phosphorylation and/or expression of the receptors (Fig. 5a, b) in LLC cells. TBrC has exhibited an activity comparable with Ly and Bay on the down-regulation of the phosphorylation and/or expression of VEGFR1 and VEGFR2 in LLC cells.

The down-regulation of the receptor phosphorylation and protein expression in LLC cells by TBrC could greatly contribute to the suppression of their downstream targets and the receptors-mediated PI3K/Akt as well as the related signaling pathways, and thus

Fig. 4 Effects of TBrC on protein expression of Bcl-2/Bax (a), cytosolic cytochrome *c* (cyto *c*) (b), caspase-3/pro-caspase-3 (c), PARP-1 (d) and cyclin D1 (e) in LLC cells. The cells were treated for 48 h with the indicated concentrations of theanine (T) and TBrC (T16–T250/16–250 μ M, TBrC16–TBrC250/16–250 μ M), Bay (3.2 μ M) and Ly (16 μ M). The expression of protein was analyzed by Western Blotting. The optical density (OD) of the band was normalized with respect to β -actin and is

expressed as relative optical density (OD). The OD value of the band shown as mean $\pm$ SD is relative to that of the control (DMSO vehicle) designated as 100 %. Bay and Ly are the inhibitors of NF- κ B and PI3K/Akt, respectively. For one experiment, three assays were carried out and only one set of gels is shown. Values with different letters (a–g) differ significantly ($P < 0.05$)

Fig. 5 Effects of TBrC on protein expression and/or phosphorylation of VEGFR1 (a), pVEGFR2/VEGFR2 (b), pAkt/Akt (c), and NF-κB (d) in LLC cells. The cells were treated with the indicated concentrations of theanine (T) and TBrC (T16–T250/16–250 μM, TBrC16–TBrC250/16–250 μM), Bay (3.2 μM) and Ly (16 μM). The expression and phosphorylation of protein were analyzed by Western Blotting. The optical density (OD) of

the band was normalized with respect to β-actin and is expressed as relative optical density (OD). The OD value of the band shown as mean ± SD is relative to that of the control (DMSO vehicle) designated as 100 %. Bay and Ly are the inhibitors of NF-κB and PI3K/Akt, respectively. For one experiment, 3 assays were carried out and only one set of gels is shown. Values with different letters (a–f) differ significantly ($P < 0.05$)

leads to the inhibition of the proliferation and migration in LLC cells.

In order to further confirm the inhibitory effects of TBrC on the receptors-mediated signaling pathways related to the proliferation and migration of LLC cells, we investigated the effects of TBrC on the suppression of the phosphorylation and/or expression of Akt and NF-κB in LLC cells. Our results indicated that TBrC significantly reduced the phosphorylation and expression of Akt (Fig. 5c) and the NF-κB (p65) expression (Fig. 5d) in LLC cells. TBrC also showed a much stronger activity than theanine on reduction of the

phosphorylation and/or expression of the Akt and NF-κB proteins in LLC cells. In addition, the inhibitors of PI3K/Akt (Ly) and NF-κB (Bay) displayed a significant suppression of the phosphorylation and protein expression of Akt and the NF-κB (p65) expression in LLC cells, respectively (Fig. 5c, d). TBrC has shown an activity comparable with Ly and Bay on the down-regulation of the Akt phosphorylation and expression and NF-κB expression in LLC cells.

Akt is a cytosolic signal transduction protein kinase that plays an important role in cell survival pathways (West et al. 2002). Induction of Akt activity is

Fig. 6 In vivo inhibition of LLC xenograft tumor growth in tumor-bearing mice by TBrC. LLC cells (0.6×10^6) were xenotransplanted into the rear *left* flank of each C57/BL6 mouse. After visible tumor formation ($120\text{--}150\text{ mm}^3$), mice were randomly divided into four groups: (1) Control group (vehicle); (2) Cisplatin group (1.5 mg/kg/day); (3) Theanine group (50 mg/kg/day); (4) TBrC group (50 mg/kg/day). The mice were treated with vehicle, cisplatin, theanine, and TBrC by intraperitoneal injection once every day. The therapy was performed for 15 days. Tumor dimensions and mouse weights were measured every 2 or 3 days by Vernier caliper. Tumor

volume was calculated using the formula: tumor size (cm^3) = [width (cm)]² × [length (cm)] × 0.52. **(a)** Average tumor volumes in each treatment group. **(b)** Average tumor weights in each treatment group. **(c)** Excised tumors derived from the mice in each treatment group. On the 15th day, mice in all groups were sacrificed. Tumor volumes and weights were compared among groups using the ANOVA and Bonferroni test. Values are shown as mean $\pm$ SD for each group ($n = 6$). Values with *different letters* (a–c) differ significantly ($P < 0.05$). All statistical tests were two-sided. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$

primarily dependent on the PI3K pathway. Akt can be activated by many forms of cellular stress, such as those observed under treatment with anticancer agents (West et al. 2002). Once activated, Akt controls cellular functions such as apoptosis, cell cycle, gene transcription, and protein synthesis through the phosphorylation of downstream substrates such as NF- κ B (West et al. 2002). NF- κ B is a nuclear transcription regulator with a specific motif for Bcl-2 transcription (Wang et al. 1996; Marsden et al. 2002). Activation of p-Akt and the NF- κ B/Bcl-2 pathway leads to inhibition of chemotherapy-induced apoptosis, which results in treatment resistance (Wang et al. 1996). Our previous results confirmed that suppression of Akt and the NF- κ B/Bcl-2 pathway by anticancer agents inhibited the growth and migration as well as induced apoptosis and cell cycle arrest in human lung cancer A549 cells and breast cancer MDA-MB-231 cells (Liu et al. 2009; Wang et al. 2009; Yu et al. 2009; Jiang

et al. 2010; Zhang et al. 2012a). In the present study, we indicated that TBrC significantly enhanced the inhibition of the proliferation and migration of LLC cells and the PI3K/Akt and NF- κ B pathway by suppressing the phosphorylation and expression of Akt and the NF- κ B expression and activating the apoptotic pathway related to changes of Bcl-2/Bax ratio, mitochondrial cytochrome C and caspase-3 in LLC cells.

There have been reports showing that suppression of VEGFR1 and VEGFR2 led to the inhibition of cancer cell proliferation, migration and metastasis (Morelli et al. 2006; Naumov et al. 2009; Wang et al. 2009; Jiang et al. 2010; Charpidou et al. 2011; Liu et al. 2011; Pajares et al. 2012; Zhang et al. 2012a). Here we have confirmed that TBrC significantly enhanced the inhibition of the VEGFR1 and VEGFR2 receptors and their downstream targets such as Akt and NF- κ B in LLC cells, which could contribute to the

inhibition of LLC cell proliferation and migration as well as the tumor growth in vivo.

TBrC enhances suppression of tumor growth in vivo

The animal experimental results indicated that TBrC time-dependently inhibited highly-metastatic LLC tumor growth and remarkably reduced the tumor volumes and weights in tumor-bearing mice. TBrC showed much stronger activity than theanine against LLC tumor growth in tumor-bearing mice. The relative inhibitory rates of the LLC tumor volumes (Fig. 6a, c) and weights (Fig. 6b) increased by 63.6 and 61.6 % (in the case of TBrC treatment) and by 34.5 and 29.7 % (in the case of theanine treatment), respectively, after treatment with TBrC and theanine for 15 days. As a positive control, the anticancer drug cisplatin also showed significant inhibition of the LLC tumor growth in the tumor-bearing mice. TBrC has shown an activity comparable with cisplatin on the suppression of LLC tumor growth in the mice (Fig. 6a–c). In addition, TBrC and theanine did not show obvious toxicity to the mice by the analyses of the mouse body weights during the experiment and the pathological dissection of the mice (data not shown).

It should be noted that the higher water solubility of theanine and the structure of coumarin-3-carboxylic acid could hamper the antitumor activity in vitro and in vivo. In order to enhance the anticancer activity of theanine and coumarin-3-carboxylic acid, we synthesized TBrC by acylation of the amino group with 6-substituted coumarin-3-carboxylic acid and esterification of the carboxyl group with ethanol. This structure of TBrC could greatly enhance the concentration and prolong the time of effective compounds in the cancer cells. This increases the anticancer activity in vitro and in vivo as we have observed in the present study. Theanine showed its significant and peak inhibition of cancer cell growth at 1 h after its oral intubation in rats as reported in our previous studies (Zhang et al. 2001; Liu et al. 2009). However, TBrC exhibits its significant inhibition of cancer cell growth for 8 h and the peak inhibition of cancer cell growth at 5 h after its oral intubation in rats in our experiments (data not shown).

In summary, our present results have demonstrated that TBrC significantly suppresses LLC cell proliferation and migration, and induces apoptosis and cell

cycle arrest by targeting the VEGFR1 and VEGFR2 receptors-mediated signaling pathways of Akt and NF- κ B in LLC cells. TBrC has not displayed any toxicity to the mice except for the strong suppression of the tumor growth in the tumor-bearing mice. All these findings suggest that TBrC as a new anticancer compound may have a wide therapeutic and/or adjuvant therapeutic application in the treatment of lung cancer.

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Conflict of interest The authors declare no conflict of interest.

References

- Charpidou A, Gkiozos I, Konstantinou M, Eleftheraki A, Demertzis P, Harrington K, Polyzos A, Syrigos KN (2011) Bronchial washing levels of vascular endothelial growth factor receptor-2 (VEGFR2) correlate with overall survival in NSCLC patients. *Cancer Lett* 304:144–153
- Deshmukh MN, Burud R, Baldino C, Chan PM, Liu JA (2003) Practical and environmentally friendly preparation of 3-carboxycoumarins. *Synth commun* 33:3299–3303
- Jaffe EA, Nachman RL, Becker CG, Minick CR (1973) Culture of human endothelial cells derived from umbilical veins—identification by morphologic and immunologic criteria. *J Clin Invest* 52:2745–2756
- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ (2008) Cancer statistics, 2008. *CA Cancer J Clin* 58:71–96
- Jemal A, Bray F, Center MICROM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. *CA Cancer J Clin* 61:69–90
- Jiang Y, Zhang Y, Luan J, Duan H, Yagasaki K, Zhang G (2010) Effects of bufalin on the proliferation of human lung cancer cells and its molecular mechanisms of action. *Cytotechnology* 62:578–583
- Lee JH, Choi S, Lee Y, Lee HJ, Kim KH, Ahn KS, Bae H, Lee HJ, Lee EO, Ahn KS, Ryu SY, Lü J, Kim SH (2010) Herbal compound farnesiferol C exerts antiangiogenic and anti-tumor activity and targets multiple aspects of VEGFR1 (Flt1) or VEGFR2 (Flk1) signaling cascades. *Mol Cancer Ther* 9:389–399
- Liu Q, Duan H, Luan J, Yagasaki K, Zhang G (2009) Effects of theanine on growth of human lung cancer and leukemia cells as well as migration and invasion of human lung cancer cells. *Cytotechnology* 59:211–217
- Liu W, Xu J, Wang M, Wang Q, Bi Y, Han M (2011) Tumor-derived vascular endothelial growth factor (VEGF)-a

- facilitates tumor metastasis through the VEGF-VEGFR1 signaling pathway. *Int J Oncol* 39:1213–1220
- Luan J, Duan H, Liu Q, Yagasaki K, Zhang G (2010) Inhibitory effects of norcantharidin against human lung cancer cell growth and migration. *Cytotechnology* 62:349–355
- Marsden VS, O'Connor L, O'Reilly LA, Silke J, Metcalf D, Ekert PG, Huang DC, Cecconi F, Kuida K, Tomaselli KJ, Roy S, Nicholson DW, Vaux DL, Bouillet P, Adams JM, Strasser A (2002) Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome *c*/Apaf-1/caspase-9 apoptosome. *Nature* 419:634–637
- Morelli MP, Cascone T, Troiani T, Tuccillo C, Bianco R, Normanno N, Romano M, Veneziani BM, Fontanini G, Eckhardt SG, De Pacido S, Tortora G, Ciardiello F (2006) Anti-tumor activity of the combination of cetuximab, an anti-EGFR blocking monoclonal antibody and ZD6474, an inhibitor of VEGFR and EGFR tyrosine kinases. *Cell Physiol* 208:344–353
- Naumov GN, Nilsson MB, Cascone T, Briggs A, Straume O, Akslen LA, Lifshits E, Byers LA, Xu L, Wu HK, Jänne P, Kobayashi S, Halmos B, Tenen D, Tang XM, Engelman J, Yeap B, Folkman J, Johnson BE, Heymach JV (2009) Combined vascular endothelial growth factor receptor and epidermal growth factor receptor (EGFR) blockade inhibits tumor growth in xenograft models of EGFR inhibitor resistance. *Clin Cancer Res* 15:3484–3494
- Oltvai ZN, Millman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74:609–619
- Pajares MJ, Agorreta J, Larrayoz M, Vesin A, Ezponda T, Zudaire I, Torre W, Lozano MD, Brambilla E, Brambilla C, Wistuba II, Behrens C, Timsit JF, Pio R, Field JK, Montuenga LM (2012) Expression of tumor-derived vascular endothelial growth factor and its receptors is associated with outcome in early squamous cell carcinoma of the lung. *J Clin Oncol* 30:1129–1136
- Reed JC (1997) Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. *Semin Hematol* 34:S9–S19
- Siegel R, Ward E, Brawley O, Jemal A (2011) Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin* 61:212–236
- Siegel R, Naishadham D, Jemal A (2012) Cancer statistics, 2012. *CA Cancer J Clin* 62:10–29
- Stennicke HR, Salvesen GS (2000) Caspases—controlling intracellular signals by protease zymogen activation. *Biochim Biophys Acta* 1477:299–306
- Sugiyama T, Sadzuka Y (1998) Enhancing effects of green tea components on the antitumor activity of adriamycin against M5076 ovarian sarcoma. *Cancer Lett* 133:19–26
- Sugiyama T, Sadzuka Y, Nagasawa K, Ohnishi N, Yokoyama T, Sonobe T (1999) Membrane transport and antitumor activity of pirarubicin, and comparison with those of doxorubicin. *Jpn J Cancer Res* 90:775–780
- Wang CY, Mayo MW, Baldwin AS Jr (1996) TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* 274:784–787
- Wang S, Liu Q, Zhang Y, Liu K, Yu P, Liu K, Luan J, Duan H, Lu Z, Wang F, Wu E, Yagasaki K, Zhang G (2009) Suppression of growth, migration and invasion of highly-metastatic human breast cancer cells by berbamine and its molecular mechanisms of action. *Mol Cancer* 8:81
- West KA, Castillo SS, Dennis PA (2002) Activation of the PI3 K/Akt pathway and chemotherapeutic resistance. *Drug Resist Updat* 5:234–248
- Yu P, Liu Q, Liu K, Yagasaki K, Wu E, Zhang G (2009) Matrine suppresses breast cancer cell proliferation and invasion via VEGF-Akt-NF-kappaB signaling. *Cytotechnology* 59:219–229
- Zhang G, Miura Y, Yagasaki K (2000) Induction of apoptosis and cell cycle arrest in cancer cells by in vivo metabolites of teas. *Nutr Cancer* 38:265–273
- Zhang G, Miura Y, Yagasaki K (2001) Inhibitory effects of theanine and sera from theanine-fed rats on receptor-mediated cancer cell beneath mesothelial-cell monolayers. *Cytotechnology* 36:195–200
- Zhang G, Miura Y, Yagasaki K (2002) Effects of dietary powdered green tea and theanine on tumor growth and hyperlipidemia in hepatoma-bearing rats. *Biosci Biotechnol Biochem* 66:711–716
- Zhang Y, Zhang H, Yu P, Liu Q, Liu K, Duan H, Luan G, Yagasaki K, Zhang G (2009) Effects of matrine against the growth of human lung cancer and hepatoma cells as well as lung cancer cell migration. *Cytotechnology* 59:191–200
- Zhang G, Wang Y, Zhang Y, Wan X, Li J, Liu K, Wang F, Liu Q, Yang C, Yu P, Huang Y, Wang S, Jiang P, Qu Z, Luan J, Duan H, Zhang L, Hou A, Jin S, Hsieh TC, Wu E (2012a) Anti-cancer activities of tea epigallocatechin-3-gallate in breast cancer patients under radiotherapy. *Curr Mol Med* 12:163–176
- Zhang YM, Dai BL, Zheng L, Zhan YZ, Zhang J, Smith WW, Wang XL, Chen YN, He LC (2012b) A novel angiogenesis inhibitor impairs lovo cell survival via targeting against human VEGFR and its signaling pathway of phosphorylation. *Cell Death Dis* 3:e406