Natural product derivative Bis(4-fluorobenzyl)trisulfide inhibits tumor growth by modification of β-tubulin at Cys 12 and suppression of microtubule dynamics

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Abstract
Bis(4-fluorobenzyl)trisulfide (BFBTS) is a synthetic molecule derived from a bioactive natural product, dibenzyltrisulfide, found in a subtropical shrub, Petiveria allieacea. BFBTS has potent anticancer activities to a broad spectrum of tumor cell lines with IC50 values from high nanomolar to low micromolar and showed equal anticancer potency between tumor cell lines overexpressing multidrug-resistant gene, MDR1 (MCF7/adr line and KBv200 line), and their parental MCF7 line and KB lines. BFBTS inhibited microtubule polymerization dynamics in MCF7 cells, at a low nanomolar concentration of 54 nmol/L, while disrupting microtubule filaments in cells at low micromolar concentration of 1 μmol/L. Tumor cells treated with BFBTS were arrested at G2-M phase, conceivably resulting from BFBTS-mediated antimicrotubule activities. Mass spectrometry studies revealed that BFBTS bound and modified β-tubulin at residue Cys12, forming β-tubulin-S- fluorobenzyl. The binding site differs from known antimicrotubule agents, suggesting that BFBTS functions as a novel antimicrotubule agent. BFBTS at a dose of 25 mg/kg inhibited tumor growth with relative tumor growth rates of 19.91%, 18.5%, and 23.42% in A549 lung cancer, Bcap-37 breast cancer, and SKOV3 ovarian cancer xenografts, respectively. Notably, BFBTS was more potent against MDR1-overexpressing MCF7/adr breast cancer xenografts with a relative tumor growth rate of 12.3% than paclitaxel with a rate of 43.0%. BFBTS displays a novel antimicrotubule agent with potentials for cancer therapeutics. [Mol Cancer Ther 2009;8(12):3318–30]

Introduction
Microtubules are dynamic polymers of α-tubulin and β-tubulin heterodimers, arranged in the form of slender filaments whose polymerization dynamics are tightly regulated both spatially and temporally. Microtubules are involved in a variety of cellular processes, making them an important target for anticancer drugs (1). In interphase cells, microtubules exchange their tubulin with soluble tubulin in the cytoplasmic pool with half times of ~3 minutes to several hours (2, 3). With the onset of mitosis, the interphase microtubule network disassembles and is replaced by a population of highly dynamic microtubules, forming mitotic spindle, driving the intricate movement of chromosomes and separation of sister chromatids. Mitotic spindle microtubules with a short tubulin half-life of ~15 seconds are 20 to 50 times more dynamic than interphase microtubule (3–5). Therefore, the dynamics of mitotic spindle microtubules are especially sensitive to modulation by microtubule-binding proteins and to microtubule-active drugs (6). Chemically diversified group of microtubule-targeted drugs can alter microtubule polymerization and dynamics in a wide variety of ways (1) and are classified based on their distinct tubulin-binding domains, including Vinca domain, colchicine domain, taxane domain, and other domains distinct from the above three domains (1, 7).

Bis(4-fluorobenzyl)trisulfide (BFBTS) is a synthetic analogue of an organosulfur compound, dibenzyltrisulfide (DBTS; ref. 8). DBTS is a bioactive natural compound found in a subtropical shrub, Petiveria Allieacea, which had been used as a folk medicine for antitumor and antirheumatic effects in Central and South America since the Aztecs (9). Primary bioactive compounds in the essential oil of the roots of this shrub include benzyldehyde, dibenzyl disulfide, DBTS, cis- and trans-stilbene, and 2[(phenylmethyl)dithio]-ethanol.
DBTS is the principle bioactive compound in the lipophilic extract of plant roots (10, 11) and its reported biological activities include enhancement of phagocytosis in treated mice (12), in vitro antifungal action mainly mediated by its derivative, methyl benzy sulphonic anhydric (13), inhibition of neurite outgrowth and proliferation in neuroblastoma cell line, SH-SY5Y (9, 11, 14), and induction of monocytoid-like differentiation of promyelocytic acute leukemia cell line, HL-60 (15). The molecular mechanisms underlying the biological activities of DBTS remain largely unknown, although it was reported that DBTS induced disassembly of microtubules and decreased the total expression of tubulin in SH-SY5Y cells, indicating that the antiproliferative activity of DBTS may result from its potential antimitotic effect (9).

In our previous studies, we reported that BFBTS was more cytotoxic than DBTS against nine human tumor cell lines (8). To further explore the anticancer potency and the mechanism of action, we compared BFBTS to paclitaxel and other antimitotic agents, and found that BFBTS is cytotoxic to a broad range of cancer cell lines including drug-resistant cell lines that overexpress the drug-resistant gene, MDR1. The anticancer activities may result from covalent modification of β-tubulin at a unique site of Cys 12, culminating in microtubule inhibition and leading to suppression of microtubule dynamics. BFBTS inhibited tumor growth in four human cancer xenografts including one MDR1-expressing breast cancer xenografts.

**Materials and Methods**

**Chemicals**

BFBTS was synthesized using the method reported previously (8). Other chemicals used in the study were purchased from Sigma. For all in vitro experiments, BFBTS and paclitaxel were dissolved in DMSO, and for all in vivo animal experiments, BFBTS and paclitaxel were dissolved in cremaphor/ethanol (w:v 4:6). BFBTS-injectable solution in cremaphor/ethanol (w:v 4:6) were prepared using the process reported previously (16, 17).

**Cell Lines and Reagents**

MCF7, MCF7/adr, KB, and KBv200 cell lines were cultured in DMEM medium, 5% CO2, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco/ThermoFisher, Waltham, MA), 4% fetal bovine serum (Gibco/ThermoFisher, Waltham, MA), and 2% non-essential amino acid (Gibco/ThermoFisher, Waltham, MA). MCF7 cells stably expressing green fluorescent protein (GFP) α-tubulin were from Dr. Steinfeind of University of California Santa Barbara, Santa Barbara, CA (18). All other cell lines used were purchased from American Type Culture Collection. Cell culture reagents were purchased from Hyclone. All cancer cells were cultivated in 10% fetal bovine serum DMEM media at 37°C, 5% CO2.

**Cell Proliferation, Viability, and Apoptosis Analysis**

Label-free real-time detection technology developed by ACEA Biosciences (19, 20) was used for continuously monitoring cell proliferation and viability after BFBTS addition.

The system xCELLigence RTCA system (Roche Applied Sciences) was operated based on the instruction in the user manual. In brief, cells were seeded into wells of 96-well microtiter E-plate (Roche Applied Sciences) at cell concentration of 5,000 cells per well, cultured in CO2 incubator at 37°C, 5% CO2, and continuously monitored on xCELLigence SP system. At about 20 h after cell seeding, the cells in exponential growth phase were treated with different concentrations of BFBTS. The cell response to BFBTS treatment was continuously monitored by xCELLigence SP system for a period of 48 h.

Cell apoptosis and death detection was measured by Cell Death Detection ELISA kit (Roche Applied Sciences), according to assay protocol for the kit. Briefly, cells were seeded into the 96-well plate. Then cells were treated for 48 h with BFBTS at 1 μmol/L, a concentration close to the IC50 value of cell proliferation. Cells were harvested and lysed. Twenty microliters of lysate were removed and transferred to streptavidin-coated microplate and then incubated with anti–histone-biotin and anti–DNA-POD antibodies for 2 h followed by adding 2,2′-Azinobis-3-ethylbenzthiazoline-sulphonic-acid substrate for color development. The plate was measured at an absorbance of 405 and 490 nm in Beckman Multimode DTX880 plate reader.

**Phosphorylated Histone H3 Staining, Mitotic Index, and Flow Cytometry**

Cultured A549, MCF7, and MCF7/adr cells were treated with 2 μmol/L BFBTS or 30 nmol/L paclitaxel for 24 h (this concentration are close to compound IC50 value in cell proliferation), fixed with ice-cold methanol for 3 min, permeabilized with PBS/0.25% TX-100 for 10 min, followed by blocking in PBS/1% bovine serum albumin/0.1% TX-100 for 30 min at room temperature. The cells were stained with anti–phosphorylated histone H3 (Ser-10) polyclonal antibody (Upstate) followed by Rhodamine-labeled anti-rabbit IgG (Chemicon). Mitotic index was determined by calculating the ratio of the number of phosphorylated histone H3–positive cells versus total 4′,6-diamidino-2-phenylindole–positive cells. For flow cytometry (fluorescence-activated cell sorting) analysis, A549 cell were cultured in Petri dishes until reaching ~40% confluence. Cells were treated with BFBTS at 2 μmol/L for 12 or 24 h, were harvested, and then followed by fixation in 100% ethanol and fluorescence-activated cell sorting analysis.

**Tubulin Polymerization Assay**

Tubulin and the fluorescence-based tubulin polymerization assay kit, and the tubulin polymerization assay was done based on the product instruction (Cytoskeleton). Briefly, 2 mg/mL of tubulin were mixed with BFBTS (at different final concentrations: 30, 300, or 3 μmol/L), paclitaxel (3 μmol/L), vincristine (1.5 μmol/L), or DMSO at final concentration of 0.1%, which served as the solvent control. The polymerization reaction was done at 37°C in 20% glycerol, 80 mmol/L PIPES, 2.0 mmol/L MgCl2, 0.5 mmol/L EGTA (pH 6.9), and 1 mmol/L GTP, and was monitored every minute for 30 min in a temperature-controlled plate reader (BMS) at excitation and emission of 360 and 420 nm, respectively.
Inhibition of tumor cell growth and induction of apoptosis by BFBTS. A, chemical structures of DBTS (a) and BFBTS (b). B, tumor cell growth inhibition dynamics of 10 tumor cell lines by BFBTS. Live cell response to the exposure of BFBTS was monitored in real-time on xCELLigence system. The concentrations of BFBTS used for the treatment of PC3, A549, SH-SY5Y, Bcap-37, Panc-1, MV522, NCI-69AR, SKOV3, HT29, and MKN45 lines were indicated. 0.1% DMSO serves as solvent control. Each curve represented an average of normalized CI from continuous measurement of duplicate wells over 48 h after compound addition (arrow). The representative curves shown were from three repeated experiments.
Mass Spectrometry

Purified tubulin was incubated in the presence or absence of BFBTS at 37°C for 30 min. BFBTS-treated tubulin was digested with trypsin and the peptides were analyzed by liquid chromatography tandem mass spectrometry using reverse phase chromatography generated in Biomolecular Mass Spectrometry Facility, Department of Chemistry and Biochemistry, University of California, San Diego.
California, using a pressure bomb. The microcapillary was packed with Zorbax SB-C18 reversed phase material (Agilent). The peptides were eluted over a 60-min period with a linear gradient 5% to 80% of solvent B going from solvent A (5% acetonitrile, 0.2% formic acid) to solvent B (95% acetonitrile, 0.2% formic acid), with a flow rate of 0.15/min. The tryptic peptide samples were separated and analyzed with a LTQ ion trap mass spectrometer (ThermoFinnigan). Tandem mass spectrometry data obtained were analyzed by using SEQUEST program (Thermo Scientific).

Microtubule Staining and Microtubule Dynamics Analysis

For microtubule staining assay, MCF7 cells were first seeded in the 1 cm² cover glass slide and then placed in tissue culture dishes for cultivation. Cells were then treated with BFBTS, paclitaxel, and vinblastine at the concentration of additional estrogen. The tumor cell implantation protocol was same as above and for the MCF7/adr experiment. Four mice (n = 4) were used for each test group and control group. BFBTS was administrated i.v. with dose regimen of 6.25, 12.5, and 25 mg/kg, respectively. The dose regimens were determined based on the maximum tolerable dose of BFBTS, which was 100 mg/kg (data not shown). Tumors and mouse body weights were measured every 5 d for efficacy and toxicity. Tumor growth was monitored using external measurements with a caliper and tumor volumes were calculated using the formula (width² × length)/2, where the width represents the smaller tumor diameter. For the MCF7/adr xenografts, the tumors were dissected and weighted after termination of the experiment. The relative tumor volume (RTV) was used for evaluating in vivo efficacy of BFBTS, calculated according to relative tumor volume = Vt/Vo, where Vo is the tumor volume before BFBTS treatment. Vt is the tumor volume at a given experiment time point. The relative tumor growth rate (T/C %) is TRTV (treated group)/CRTV (control group) × 100%. Statistical ANOVA was used for statistic analysis.

Results

Inhibition of Cancer Cell Proliferation and Induction of Apoptosis by BFBTS

BFBTS (Fig. 1A, compound b) was derivatized by attaching fluoro-atoms at the para-position of the two benzene rings of DBTS (Fig. 1A, compound a). This modification resulted in improved in vitro cytotoxicity activity compared with DBTS in at least nine tumor cell lines of different origins (8). In these cytotoxicity assays, we also found BFBTS to be the most potent compound among 16 different DBTS derivatives tested (8). In this study, we further explore the antitumor activity of BFBTS by monitoring cell response to BFBTS in additional 10 tumor cell lines, using a real-time cell analysis system (xCELLigence). Unlike the conventional

Table 1. IC50 values of BFBTS in 10 deferent types of tumor cell lines after 48 h of treatment

<table>
<thead>
<tr>
<th>Cell line (tumor type)</th>
<th>IC50 value (μmol/L) ± SD</th>
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<tr>
<td>PC3 (prostate cancer)</td>
<td>0.360 ± 0.117</td>
</tr>
<tr>
<td>Bcap-37 (breast cancer)</td>
<td>0.394 ± 0.321</td>
</tr>
<tr>
<td>NCI-69AR (SCLC)</td>
<td>0.415 ± 0.067</td>
</tr>
<tr>
<td>SKOV3 (ovarian cancer)</td>
<td>0.441 ± 0.012</td>
</tr>
<tr>
<td>SH-SYSY (neuroblastoma)</td>
<td>0.652 ± 0.077</td>
</tr>
<tr>
<td>A549 (NSCLC)</td>
<td>0.768 ± 0.259</td>
</tr>
<tr>
<td>MV522 (NSCLC)</td>
<td>1.295 ± 0.279</td>
</tr>
<tr>
<td>HT29 (colon cancer)</td>
<td>1.678 ± 0.070</td>
</tr>
<tr>
<td>Panc-1 (pancreatic cancer)</td>
<td>2.350 ± 1.202</td>
</tr>
<tr>
<td>MNK45 (gastric cancer)</td>
<td>113.630 ± 16.9</td>
</tr>
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</table>

Abbreviations: SCLC, small cell lung carcinoma; NSCLC, non–small cell lung carcinoma.
end point cell viability assays, the real-time cell detection system continuously monitors live cells in response to the exposure of BFBTS and provides kinetic cell response information from the same cell population. Dose- and time-dependent cell responses to BFBTS were clearly shown in 10 tumor cell lines (Fig. 1B). The IC_{50} values derived from normalized cell index (CI) at 48 hours after BFBTS treatment in 10 tumor cell lines are listed in Table 1, and ranged from high nanomolar concentration (~400 nmol/L for Bcap-37 line) to high micromolar concentration (~113 μmol/L for MNK line). The normalized CI is an arbitrary unit derived from electrical impedance measurement (19–22). The correlation between CI and cell viability is well documented in the literature (23–26). Furthermore, we also tested cell death and apoptosis in three selected tumor lines, A549, HT29, and SH-SY5Y, treated with 1 μmol/L BFBTS, the concentration approximating the IC_{50} value for cell proliferation and cytotoxicity (Table 1). BFBTS induced significant apoptosis in all three tumor lines compared with untreated cells (Fig. 1C).

One major mechanism of acquired drug resistance is the overexpression of efflux pumps, i.e., the p-gp170/MDR (21, 27–31), and many clinically used drugs are good substrate of the drug efflux pump (21, 27). To test the effect of...
MDR expression on the sensitivity of tumor cells to BFBTS, we used MCF7/adr and human epidermoid carcinoma cell line KBv200, where the overexpression of MDR1 is induced by exposure to low dose of vinblastine or daunorubicin (28–31). The MCF7/adr and KBv200 as well as their parental lines (MCF7 and KB) were treated with different concentrations of BFBTS and paclitaxel, and the cell responses were continuously monitored in real-time for 48 hours after treatment. As shown in Fig. 1D, the MDR1-overexpressing cell lines and their parental cell lines were equally sensitive to BFBTS, with IC_{50} values (48 hours after treatment) of 1.31 μmol/L in KB, 1.85 μmol/L in KBV200, 1.09 μmol/L in MCF7, and 3.19 μmol/L in MCF7/adr. In contrast, the cytotoxic effect of paclitaxel in the resistant cell lines was much lower than those in their parent lines, with IC_{50} values (48 hours after treatment) of >50 nmol/L in KBV200 compared with 1.63 nmol/L in KB, and >400 nmol/L in MCF7/adr compared with 6.54 nmol/L in MCF7. The equal inhibitory effect of BFBTS in the MDR1–highly expressing cell lines and their parent cell lines strongly suggests that BFBTS may not be a substrate for the MDR1 transporters.

Figure 3. Inhibition of microtubule polymerization dynamics and specific modification of β-tubulin at Cys 12 by BFBTS. A, inhibition of microtubule organization in MCF7 cells by BFBTS, paclitaxel, and vinblastine. The drug concentrations were indicated. DMSO served as a solvent control. B, inhibition of tubulin polymerization in cell-free system by BFBTS, paclitaxel, and vincristine at different concentrations as indicated. Tubulin polymerization kinetics of different compounds and DMSO control were recorded and represented by relative fluorescent unit (RFU) in the wavelength range between 360 and 430 nm over time (in minutes). C, life history plots of the microtubule dynamics from DMSO-treated or 54 nmol/L BFBTS-treated MCF7 cells. The curves represent the changes in length (distance) of individual microtubules over time. The bar graph shows a representative data of two separate experiments. The quantitative measurement (columns, mean; bars, SD) of the dynamicity of BFBTS-treated MCF7 cells at 54, 72, and 108 nmol/L are 4.62 ± 2.15, 3.65 ± 0.9, and 2.34 ± 0.64 in comparison with 10.13 ± 2.56 in DMSO-treated cells. *, P < 0.05; **, P < 0.01 in comparison with DMSO treatment.
BFBTS-Mediated Mitotic Arrest and Microtubule Depolymerization

In our previous study, we identified and reported a unique cellular response pattern for cells treated with antimitic agents (32, 33). The kinetic curves of cells treated with antimitic agents declined between 5 to 14 hours after compound addition, and then steadily increased afterwards (32, 33). Interestingly, 7 of 10 tumor cell lines (PC3, A549, SH-SY5Y, Bcap-37, Panc-1, MV522, and HT29) treated with BFBTS also displayed such typical kinetic pattern (Fig. 1C). This finding allowed us to hypothesize that BFBTS may also function as an antimitic agent. To further test whether BFBTS induces antimitic pattern, we treated A549 cells with BFBTS in conjunction with two well-known antimitic agents, paclitaxel and vinorelbine. As a control, we treated A549 cells with compounds that have different mechanisms of action and reveal distinguishable kinetic patterns in live cells, including DNA-damaging agent, etopside, CDK inhibitor, indirubin-3’-oxime, and actin disruption agent, latruculin. Cells were continuously monitored for 48 hours in realtime after the treatment. A549 cells treated with BFBTS generated a mitotic arrest pattern identical to that obtained for paclitaxel and vinorelbine (Fig. 2A), but distinct from those of etopside, indirubin-3’-oxime, and latruculin-treated cells (Fig. 2A), indicating that BFBTS may function as an antimotic agent inducing mitotic arrest.

To further test whether BFBTS induces mitotic arrest, we treated A549, MCF7, and MCF7/adr cells with 2 μmol/L BFBTS, 30 nmol/L paclitaxel, or 0.1% DMSO that served as negative control. The treated cells were then stained with an antibody against phosphohistone histone H3, a marker for cells undergoing mitosis and mitotic arrest (34, 35). As shown in Fig. 2B, increased number of phosphorylated histone H3-positive cells were visualized in BFBTS or paclitaxel-treated A549 cells compared with the DMSO-treated cells. In BFBTS-treated MCF7 and MCF7/adr cells, phosphorylated histone H3-positive cells were increased, and in contrast, such increase can only be seen in paclitaxel-treated MCF7 cells but not in paclitaxel-treated MCF7/adr cells where MDR1 gene was highly expressed. The extent of mitotic arrest was quantified by calculating the mitotic index, which was 48.4 ± 4.49%, 44.92 ± 8.42%, and 53.63 ± 5.98% for BFBTS-treated A549, MCF7, and MCF7/adr, respectively, and 42.35 ± 9.53%, 49.35 ± 12.50%, and 6.44 ± 1.96% for paclitaxel-treated A549, MCF7, and MCF7/adr cells, respectively (Fig. 2C). Consistent with the cytotoxicity assay shown in Fig. 1D, the BFBTS showed equal mitotic arrest potency in the MCF7 cells and MDR1-expressing MCF7/adr cells. Furthermore, using fluorescence-activated cell sorting analysis, we determined that the accumulation of cells with 4N DNA content increased after 12 and 24 hours of BFBTS treatment (Fig. 2D), strongly indicating that BFBTS induced cell cycle arrest at G2-M phase.

Next, we questioned whether BFBTS-induced mitotic arrest resulted from its effect on microtubule polymerization. To address this question, BFBTS-treated MCF7 cells were stained with FITC-conjugated anti-tubulin antibody. As shown in Fig. 3A, MCF7 cells treated with 1 μmol/L BFBTS for 4 hours display a significant reduction in polymerized...
microtubules and microtubule networks, which were similar to MCF7 cells treated with the microtubule inhibitor, vinblastine, but different from MCF7 cells treated with microtubule stabilizer, paclitaxel. This observation suggests that BFBTS may function as a microtubule inhibitor, possibly by preventing polymerization of tubulin into microtubules or by facilitating the disruption of microtubular networks. In cell-free tubulin assembly assay system using purified bovine tubulin, we found that BFBTS at concentration of 3 μmol/L indeed completely inhibited the tubulin assembly into microtubule processes, whereas paclitaxel promoted the assembly (Fig. 3B). Collectively, the results strongly support the notion that BFBTS induces mitotic arrest in the tumor cells by directly inhibiting microtubule polymerization.

**Inhibition of Microtubule Dynamics by BFBTS**

Both in cell-free assay system and in cultured cells, microtubules are dynamic polymers that grow and shorten by the addition and loss of tubulin subunits at the microtubule ends (18, 36). In contrast to treatment with relatively high concentrations where microtubule-targeted compounds can be distinguished based on their ability to either promote polymerization of tubulin or induce microtubule disassembly, at low concentrations, they share a common underlying mechanism, which is suppression of microtubule dynamics (6, 36). The suppression of microtubule dynamics, which prevents chromosome alignment at the metaphase plate, results in a sustained block at or before the metaphase-anaphase transition leading to mitotic arrest followed by cell death or apoptosis (18). Previous work has also supported the notion that the concentration of compounds that affects microtubule dynamics, but not microtubule polymerization in live cells, is similar to the concentration that inhibits proliferation (6, 7).

To test the effect of BFBTS on microtubule dynamics, we used MCF7 cells that had been stably transfected with GFP-α-tubulin. At the flat lamellar edge of the interphase cells, GFP-labeled microtubules are readily visible and can be followed for several minutes by time-lapse fluorescent microscopy (data not shown). The position of each microtubule end with time was then determined and graphed to generate a “life history plot” (Fig. 3C), from which parameters of microtubule dynamics were determined. The parameters used here for testing the effect of BFBTS on microtubule dynamics include growth rate, shortening rate, attenuation, time- and length-based catastrophe frequencies, time- and length-based rescue frequencies, and dynamicity (Table 2). The dynamic curves in Fig. 3C, each representing a single microtubule, showed the frequent minute changes in length of the extreme ends of microtubules. In the BFBTS-treated cells, low nanomolar BFBTS (54 nmol/L) significantly stabilized the microtubules compared with the DMSO-treated cells, showing very small changes in microtubule length over time (Fig. 3C). The dynamicity, which represents the changes in length over time (μm/minute), was also significantly inhibited in the cells treated with BFBTS in a dose-dependent manner (Fig. 3), and dynamicity was inhibited by approximately 54%, 64%, and 76% at the concentrations of 54, 78, and 108 nmol/L of BFBTS, respectively.

To further confirm inhibitory effect of BFBTS on microtubule dynamics, we analyzed two microtubule dynamic parameters, the frequencies of catastrophe and rescue, which are important parameters in determining microtubule function (18, 37). The catastrophe frequency is the frequency with which the microtubules switch from either pause or growth to shortening. The rescue frequency is the frequency with which the microtubules switch from shortening to either growth or pause. In BFBTS-treated cells, both the time- and length-based rescue frequencies significantly decreased whereas both the time- and length-based catastrophe frequencies significantly increased compared with DMSO-treated cells that served as the control (Table 2), indicating that BFBTS suppresses the microtubule dynamics by destabilizing the microtubule polymerization.

**Specific Modification of β-tubulin at Cys 12 by BFBTS**

A previous study reported a sulfhydryl group of cysteines in β-tubulin that could be oxidatively modified by the garlic-derived organosulfur compound diallyl trisulfide whose trisulfide group plays a key role in this modification (38). BFBTS contains the same trisulfide group, although the side group of BFBTS is a fluorobenzene ring. Therefore,

<table>
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<tr>
<th>% Time spent</th>
<th>DMSO</th>
<th>BFBTS (54 nmol/L)</th>
<th>BFBTS (72 nmol/L)</th>
<th>BFBTS (108 nmol/L)</th>
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<tr>
<td>Growing</td>
<td>58.92%</td>
<td>32.97%</td>
<td>23.80%</td>
<td>25.14%</td>
</tr>
<tr>
<td>Shortening</td>
<td>29.51%</td>
<td>32.85%</td>
<td>27.56%</td>
<td>17.59%</td>
</tr>
<tr>
<td>Attenuated</td>
<td>11.57%</td>
<td>34.18%</td>
<td>48.64%</td>
<td>57.27%</td>
</tr>
<tr>
<td>Frequency of</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Catastrophe (events/minute)</td>
<td>2.967</td>
<td>2.580</td>
<td>1.572</td>
<td>1.389</td>
</tr>
<tr>
<td>Catastrophe (events/μm)</td>
<td>0.480</td>
<td>0.850</td>
<td>1.010</td>
<td>0.950</td>
</tr>
<tr>
<td>Rescue (events/minute)</td>
<td>6.976</td>
<td>5.134</td>
<td>4.131</td>
<td>5.858</td>
</tr>
<tr>
<td>Rescue (events/μm)</td>
<td>0.403</td>
<td>0.940</td>
<td>0.881</td>
<td>1.138</td>
</tr>
<tr>
<td>Dynamicity</td>
<td>10.130</td>
<td>4.620</td>
<td>3.650</td>
<td>2.430</td>
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BFBTS may also bind sulfhydryl group of cysteines in β-tubulin. To test this hypothesis, we used mass spectrometry to analyze the possible interaction between BFBTS and tubulin. To identify how BFBTS directly interacts with tubulin, we digested the BFBTS-treated tubulin with trypsin and analyzed the digests with liquid chromatography tandem mass spectrometry. Peptide mass mapping of BFBTS-treated tubulin identified >50% of β-tubulin. By comparing with the trypsinized tubulin fragmental mass spectrum of DMSO control samples, the BFBTS-treated β-tubulin peptides showed an increase of 172 kDa in the mass, which corresponded to the expected fragment of BFBTS, containing two sulfur atoms forming β-tubulin-ss-fluorobenzyl. The cysteine residue modification by BFBTS was confirmed by identifying the presence of the peptide of 3EIVHIQAGQCGNQIGAKFW21 ([M+2H]2+ of m/z 1137.18; Fig. 4). Thus, in the β-tubulin of BFBTS-treated samples, the residue Cys 12 was identified to be modified by BFBTS with increased mass of 172 kDa. No modification could be detected in the other cysteine residues either in β-tubulin or in α-tubulin (data not shown), suggesting that the cysteine modification is limited to the indicated peptide region.

**Inhibition of Tumor Growth in Human Tumor Xenografts by BFBTS**

Three human cancer lines, A549, Bcap-37, and SKOV3, were selected for testing BFBTS antitumor activity in xenografts models, using three dose treatment cohorts (6.25, 12.5, and 25 mg/kg) once per day (q.d.), i.v. × 10 days. Mice treated with paclitaxel at 10 mg/kg, i.v., q.d. for 7 consecutive days served as a positive control. Compared with solvent control group, BFBTS in three dose treatment cohorts inhibited tumor growth in A549, Bcap-37, and SKOV3 xenografts, showing significantly low relative tumor growth rates (Figs. 1B, 5A, B, and C). The relative tumor growth rates (T/C %) in BFBTS-treated A549 xenografts were 19.91%, 25.36%, and 31.24% for 25, 12.5, and 6.25 mg/kg, respectively, and the relative tumor growth rate was 26.92% when administrating paclitaxel. In Bcap-37 xenografts, the relative tumor growth rates (T/C %) were 18.57%, 29.23%, and 40.40% when given at doses of 25, 12.5, and 6.25 mg/kg, respectively, and 23.14% of relative...
BFBTS Inhibits Microtubule Dynamics

Discussion

BFBTS is shown to be one of the growing classes of naturally derived microtubule inhibitors with potent antitumor activities. Microtubules are among the most successful targets for anticancer therapy, and antimicrotubule agents are known to directly bind to microtubules and/or soluble tubulin altering highly dynamic microtubule polymerization process (39). The microtubule binding sites of known antimicrotubule agents are diverse and can be classified into Vinca domain, colchicine domain, Taxane domain, and other microtubule binding sites (1, 39). Using mass spectrum assay, we found that the sulphydryl group of Cys12 of β-tubulin was covalently modified and formed a disulfide bridge with BFBTS, resulting in β-tubulin-SS-fluorobenzyl. Notably, among 12 cysteine residues in α-tubulin and 8 cysteine residues in β-tubulin, BFBTS only targeted β-tubulin at Cys12. Interestingly, Cys12 is located in Vinca domain near the exchangeable GTP-binding site (40), and it was reported that C12S mutation of β-tubulin significantly altered tubulin function in Saccharomyces cerevisiae and was lethal, indicating the important role of Cys12 in β-tubulin (41). The covalent modification of Cys 12 in β-tubulin by BFBTS leads to blockage of sulfhydryl group, which is situated in a polar environment to maintain normal tubulin function. Modification by BFBTS is apparently very selective, because no other BFBTS-modified cysteine residues in either α-tubulin or β-tubulin were detected (data not shown). Recently, an antitumor agent, imidazolyl disulfide IV-2, and garlic-derived organosulfur compound, diallyl trisulfide, have been reported to have antimicrotubule activity by modifying cysteine residues in β-tubulin (37, 42). Interestingly, the trisulfide analogue, diallyl trisulfide, modified the tubulin at Cys 12 and at Cys 354 (37). Together with our findings on BFBTS, we suggest that organosulfur compounds may share a common mechanism for microtubule inhibition through modification of sulphydryl groups of cysteine residues in tubulin, and such modification mechanism differs from other microtubule inhibitors. Therefore, this group of compounds may form a new class of antimicrotubule agents.

Compared with clinically used microtubule inhibitors, taxanes and Vinca alkaloids, BFBTS is a moderately potent microtubule inhibitor with a IC50 value at high nanomolar range in the most sensitive cell lines tested in vitro. Interestingly, in tumor xenografts, BFBTS displayed potent tumor growth inhibition activity in A549, Bcap-37, and SKOV3 xenografts with the dose range between 6.25 and 25 mg/kg, similar to paclitaxel, of which the IC50 values were 11.95, 3.48, and 4.55 nmol/L in A549, Bcap-37, and SKOV3 lines, respectively (data not shown). Although in vitro antitumor activities of a given compound is not necessary directly correlated to in vivo potency of tumor growth inhibition in xenografts, the strong potent antitumor growth activity of BFBTS in A549, Bcap-37, and SKOV3 xenografts may indicate its unique in vivo characteristics, such as tumor tissue penetration and/or unique compound metabolism profiles, which deserve further study. The potent inhibitory activity of BFBTS in xenografts may conceivably result from its potent suppression of microtubule dynamics. Indeed, BFBTS suppresses microtubule dynamics in live cells at the concentration of 54 nmol/L (Fig. 3C), which was almost 19-fold lower than the dose needed for disrupting microtubule organization (Fig. 3A). This clearly indicates that a much lower BFBTS concentration is needed to alter cancer cell microtubule dynamics, which are extremely important in the processes of cancer cell division and mitosis (29, 32). Interestingly, by real-time monitoring of live MCF7 cells, cell proliferation inhibition can be clearly detected in response to the exposure of 190 nmol/L BFBTS (Fig. 1D). This result is consistent with previous findings that potent suppression of microtubule dynamic by microtubule inhibitors was more directly correlated to cell proliferation inhibition (6, 7).

In addition, antitumor activities of microtubule inhibitors may also result from their effect on angiogenesis. Inhibition of angiogenesis by disrupting microtubules has also been reported for microtubule inhibitors such as taxanes, vincristine, noscapine, and a new antimicrotubule agent, 2ME2 (34, 36). Microtubule inhibition by these agents downregulates hypoxia-inducible factor-1α the posttranscriptional level and inhibits hypoxia-inducible factor-1α-induced transcriptional activation of vascular endothelial growth factor expression, which plays a key role in cancer angiogenesis (34, 36). It has been reported that inhibition of angiogenesis by other structurally similar natural product diallyl trisulfide resulted from inactivation of Akt and downregulation of vascular endothelial growth factor and vascular endothelial growth factor R2 (37, 38). In this study, we found that BFBTS inhibited the endothelial...
tube formation (data not shown). Therefore, it is plausible that some aspects of BFBTS anticancer activity may be due to disruption of angiogenesis, which warrants further investigation.

In summary, the natural active product derivative BFBTS was shown to have broad anticancer therapeutic potentials, which most likely resulted from its antimitotic effect. BFBTS directly inhibited microtubule polymerization by covalently binding β-tubulin Cys 12, leading to the disruption of the microtubule stability and inhibition of microtubule dynamics. BFBTS inhibited tumor cell growth in A549, Bcap-37, and SKOV3 human tumor xenografts; furthermore, both in vitro cell culture and in MCF7/adr xenografts, the tumor inhibitory effect was independent of the MDR1 expression, suggesting that BFBTS would have clinical benefit of overcoming MDR1-mediated drug resistance. Dissection of underlying mechanism of BFBTS-tubulin interaction and modification and tumor cell apoptosis is warranted and will help to further explore BFBTS anticancer therapeutic potentials.

**Disclosure of Potential Conflicts of Interest**

X. Xu and X. Wang: employees, shareholders, and patent holders, ACEA Biosciences, Inc. No other potential conflicts of interest were disclosed.

**References**


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Natural product derivative Bis(4-fluorobenzyl)trisulfide inhibits tumor growth by modification of β-tubulin at Cys 12 and suppression of microtubule dynamics

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