

## Original Article

# Bornyl caffeate induces apoptosis in human breast cancer MCF-7 cells via the ROS- and JNK-mediated pathways

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**Aim:** The purpose of the present study was to investigate the anticancer activity of bornyl caffeate in the human breast cancer cell line MCF-7.

**Methods:** The cell viability was determined using the MTT assay, and apoptosis was initially defined by monitoring the morphology of the cell nuclei and staining an early apoptotic biomarker with Annexin V-FITC. The mitochondrial membrane potential was visualized by JC-1 under fluorescence microscopy, whereas intracellular reactive oxygen species (ROS) were assessed by flow cytometry. The expression of apoptosis-associated proteins was determined by Western blotting analysis.

**Results:** Bornyl caffeate induced apoptosis in MCF-7 cells in a dose- and time-dependent manner. Consistently, bornyl caffeate increased Bax and decreased Bcl-xl, resulting in the disruption of MMP and subsequent activation of caspase-3. Moreover, bornyl caffeate triggered the formation of ROS and the activation of the mitogen-activated protein (MAP) kinases p38 and c-Jun N-terminal kinase (JNK). Antioxidants attenuated the activation of MAP kinase p38 but barely affected the activation of JNK. Importantly, the cytotoxicity of bornyl caffeate was partially attenuated by scavenging ROS and inhibited by MAP kinases and caspases.

**Conclusion:** The present study demonstrated that bornyl caffeate induced apoptosis in the cancer cell line MCF-7 via activating the ROS- and JNK-mediated pathways. Thus, bornyl caffeate may be a potential anticancer lead compound.

**Keywords:** cytotoxicity; apoptosis; human breast cancer MCF-7 cells; bornyl caffeate

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## Introduction

Alteration of the physiological apoptotic pathways and disruption of normal homeostasis are known to cause the initiation, progression and metastasis of different cancers<sup>[1, 2]</sup>. Pharmacological induction of apoptosis in cancer cells has emerged as a key anticancer strategy over the past several decades<sup>[3, 4]</sup>. Drug-induced apoptosis is readily characterized by microvilli, cell shrinkage, chromatin condensation, nuclear collapse and cellular fragmentation into apoptotic-bodies. The anticancer activity of current anticancer drugs is mediated by multiple apoptotic mechanisms, for example, the activation of mitogen-activated protein (MAP) kinases and caspases. The MAP kinases ERK, p38, and JNK are involved in the regulation of cell proliferation, differentiation and cell death<sup>[5, 6]</sup>. ERK

isoenzymes are mainly regulated by the ras/raf/MEK pathway but are also activated by MEK-1-dependent signals<sup>[7-9]</sup>. Activation of ERKs promotes the proliferation and survival of most cell types<sup>[7, 10]</sup> and regulates cell differentiation and apoptosis<sup>[11, 12]</sup>. In contrast, the MAP kinases JNK and p38 are often activated by oxidative stress and xenobiotics, and they subsequently induce apoptosis and promote the production of pro-inflammatory cytokines<sup>[7, 13]</sup>. Interestingly, JNK and p38 exist in multiple isoforms and function in a cell-type-specific manner<sup>[14]</sup>. Moreover, their individual isoenzymes may reside in different intracellular compartments and regulate different biological events<sup>[15, 16]</sup>. Under certain circumstances, p38 and JNK could exert opposing functions and even attenuate cellular apoptotic signals<sup>[17]</sup>. Nevertheless, recent studies suggest that p38 and/or JNK directly activate the caspase cascade, thereby mediating the activation of the apoptotic transcription factor c-jun<sup>[18, 19]</sup>. Activation of the caspase cascade actu-

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ally hallmarks cell apoptosis<sup>[20]</sup>, and notably, many anticancer drugs kill tumor cells by mainly activating caspases, especially caspase-3<sup>[3, 21, 22]</sup>.

Bornyl caffeate was initially isolated as an anti-inflammatory and antibacterial compound from several plants, such as *Piper caninum* (Piperaceae), *Piper philippinum*, *Coreopsis mutica* var *mutica* and *Verbesina turbacena* Kunth<sup>[23–26]</sup>. Recent studies have further demonstrated that bornyl caffeate inhibits human neutrophil elastase, HIV-1 integrase and trypanosome cysteine protease<sup>[26–28]</sup>. The chemical structure of bornyl caffeate represents a combination of two naturally occurring, anti-inflammatory compounds, namely, borneol and caffeic acid. Borneol is widely used to treat against microorganisms, inflammation and pain in Traditional Chinese medicine and other folk medicines<sup>[29, 30]</sup>. However, borneol could be cytotoxic and genotoxic, depending on its concentration<sup>[30, 31]</sup>. At non-toxic concentrations, borneol attenuates the cytotoxicity and genotoxicity of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), whereas borneol at higher concentrations manifests synergy with H<sub>2</sub>O<sub>2</sub>, primarily by potentiating the DNA-damaging effects of H<sub>2</sub>O<sub>2</sub>. Interestingly, borneol and its derivative MT103 inhibited 7, 12-dimethylbenz(a)anthracene-induced carcinogenesis and tumor growth, while both compounds barely showed toxicity in normal cells<sup>[32, 33]</sup>. It is believed that borneol kills cancer cells largely by inducing apoptosis. However, caffeic acid and its derivatives are known for their antioxidant, anti-inflammatory and antiviral activities<sup>[2, 34–38]</sup>. For example, caffeic acid phenethyl ester (CAPE) from insect propolis specifically induces apoptosis in tumor or virally transformed cells but not in parental, normal cells<sup>[39, 40]</sup>. Octylcaffeate, another example, was recently synthesized and evaluated for its antioxidant, anti-inflammatory and anticancer activities<sup>[35, 41, 42]</sup>. Octylcaffeate also induces apoptosis in human cancer cells and prevents experimental lung metastasis of murine colon 26-L5 carcinoma cells<sup>[43, 44]</sup>.

The present study was designed to explore the anticancer potential of bornyl caffeate. We recently synthesized bornyl caffeate via direct esterification of caffeic acid with borneol and found that bornyl caffeate strongly induced cell death in human breast cancer MCF-7 cells. The research focus of the present study was to understand the molecular mechanism underlying the cytotoxicity of bornyl caffeate in breast cancer MCF-7 cells.

## Materials and methods

### Chemicals and antibodies

The antibodies against phospho-Akt, phospho-ERK, phospho-p38, phospho-JNK, Akt, ERK, p38, and JNK were purchased from Cell Signaling Technology (Boston, MA, USA). The antibodies against Bax, Bcl-2, Bcl-xl, caspase-3, PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against GAPDH and the goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate antibody were purchased from Sigma-Aldrich Co (St Louis, MO, USA). Other chemicals were obtained from Sigma-Aldrich Co (St Louis, MO, USA) unless indicated otherwise.

Bornyl caffeate was synthesized from caffeic acid by exhaustive esterification with borneol following a modified procedure that was described previously<sup>[45]</sup>. At the end of reaction, bornyl caffeate was purified by silica gel chromatography and characterized by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). In this study, bornyl caffeate was diluted in dimethylsulfoxide (DMSO) to make stock solutions of 1 mmol/L and was filtrated using a sterile filter (Millipore, USA). For cell culture, bornyl caffeate was diluted to the appropriate final concentrations in cell-culture medium. Control cells were treated with the same amount of vehicle. The DMSO concentration in the cell-culture medium was controlled below 0.1% (*v/v*).

### Cell culture

MCF-7 and several other cell lines (T47D, HepG2, HeLa and PC12) were obtained from the American Type Cell Culture Collection (Manassas, VA, USA). MCF-7, T47D, HepG2, and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) and 1% penicillin/streptomycin (Invitrogen, USA). PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA), 5% horse serum (Invitrogen, USA) and 1% penicillin/streptomycin (Invitrogen, USA). All cells were cultured at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere.

### Measurement of cell viability

The cell viability was evaluated by a standard colorimetric assay as described previously<sup>[46]</sup>. In brief, the cells were seeded at 1x10<sup>4</sup>/100 µL per well in a 96-well plate and treated with 0, 10, 25, and 50 µmol/L of bornyl caffeate for 24 h or 50 µmol/L of bornyl caffeate for 0, 3, 6, 12, and 24 h. At the end of the drug treatment, the cell monolayers were incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/mL) in phosphate-buffered saline (PBS) for 4 h. The formation of purple formazan was quantified by measuring the absorbance at 570 nm on a microplate reader (Bio-Rad, USA). To characterize the cytotoxicity of bornyl caffeate, MCF-7 cells were pretreated with 20 µmol/L of different protein kinase inhibitors (LY294002, PD98059, SP600125, and SB203580) or 50 µmol/L of z-VAD-fmk for 1 h and then treated with bornyl caffeate at the indicated concentrations for 24 h. The cell viability was determined using the MTT assay as described above.

### Measurement of lactate dehydrogenase (LDH) release

Extracellular LDH activity was measured using a colorimetric assay and the Cytotoxicity Cell Death Kit (Sigma, USA) according to the manufacturer's instructions. Briefly, MCF-7 cells were seeded in 96-well culture plates at a density of 10000 cells per well. Bornyl caffeate at various final concentrations (0, 10, 25, and 50 µmol/L) or 50 µmol/L of bornyl caffeate was added for the indicated times, and the supernatants were collected to assay LDH activity. The absorbances of

all samples were measured at 490 nm on a microplate reader (Bio-Rad, USA).

#### Morphological changes in MCF-7 cells

Cells were washed twice with PBS and then stained with 5  $\mu\text{mol/L}$  Hoechst 33342 in 1 $\times$ PBS solution for 10 min at room temperature. The cell morphology was examined using a Zeiss fluorescence microscope (Carl Zeiss, Germany), and the apoptotic cells were defined by chromatin condensation and fragmentation of nuclei.

#### Flow cytometric analysis of apoptotic cells

After the treatment with bornyl caffeate, the cells ( $1\times 10^6$ ) were freshly harvested and suspended in a 1:1 (*v/v*) mixture of PBS and 0.2 mol/L  $\text{Na}_2\text{HPO}_4$ -0.1 mol/L citric acid (pH 7.5). Following fixation with ice-cold ethanol at 4°C for 1 h, the cells were resuspended in Annexin V binding buffer and then incubated in a buffer containing 200 ng/mL Annexin V-FITC conjugates at room temperature for 15 min. Subsequently, the cells were stained with PI (300 ng/mL) for 10 min, and the stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

#### ROS Determination

The intracellular ROS level was measured based on the ROS-induced formation of the highly fluorescent product 2',7'-dichlorofluorescein (DCF) from the non-fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA), as previously described<sup>[47, 48]</sup>. Briefly, MCF-7 cells were seeded in a 6-well plate at a density of  $2\times 10^5$  cells per well and incubated for 24 h at 37°C. Following treatment with bornyl caffeate (0, 10, 25, or 50  $\mu\text{mol/L}$ ), the cells were loaded with 20  $\mu\text{mol/L}$  2',7'-dichlorofluorescein diacetate (DCFH-DA) in serum-free DMEM medium for 30 min at 37°C. After the removal of excessive DCFH-DA, the cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, USA).

#### Visualization of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was assessed using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) (Invitrogen, USA) as previously described<sup>[49]</sup>. The cells were cultured in 24-well plates. After a 24-h treatment with bornyl caffeate, the cells were incubated for 30 min at 37°C with 1  $\mu\text{g/mL}$  of JC-1 in growth medium. MMP depolarization was visualized by measuring fluorescence at the emission wavelengths of 530 nm and 590 nm after excitement at a wavelength of 485 nm with a Zeiss fluorescence microscope (Carl Zeiss, Germany).

#### Western blotting analysis

The cellular proteins were extracted and analyzed for protein expression as previously described<sup>[47, 50]</sup>. Briefly, 30  $\mu\text{g}$  of cellular proteins were resolved by electrophoresis in a 10% SDS-polyacrylamide gel and subsequently transferred to polyvinylidene difluoride (PVDF) membrane. Following a 1-h incubation in fresh TBS buffer containing 0.1% Tween-20 and

5% skimmed milk powder, the blots were probed with specific antibodies including rabbit anti-Akt, anti-ERK, anti-p38, anti-JNK, anti-caspase-3, anti-PARP, anti-Bax, anti-Bcl-2, anti-Bcl-xl, anti-GAPDH, anti-phospho-Akt, anti-phospho-ERK, anti-phospho-p38, anti-phospho-JNK, or anti-cleaved PARP. The bound primary antibodies were detected using a goat anti-rabbit IgG-HRP conjugate. The activity of peroxidase on the blot was visualized by enhanced chemiluminescence (ECL) detection reagents (GE Healthcare, Sweden). To characterize the effects of the antioxidants on the bornyl caffeate-induced activation of MAP kinases, MCF-7 cells were pretreated with 1 mmol/L of different antioxidants, except for vitamin C (100  $\mu\text{mol/L}$ ), for 1 h, and this was followed by treatment with bornyl caffeate (50  $\mu\text{mol/L}$ ) for 24 h. The intracellular proteins were extracted and analyzed by Western blot analysis using specific antibodies.

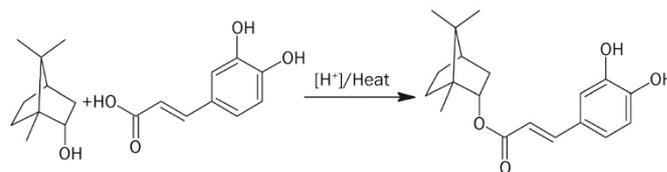
#### Statistical analysis

The data are presented as the means $\pm$ SD for three independent experiments. Statistical analysis was performed using a two-tailed, paired Student's *t*-test, and a *P*-value <0.05 was considered to be statistically significant.

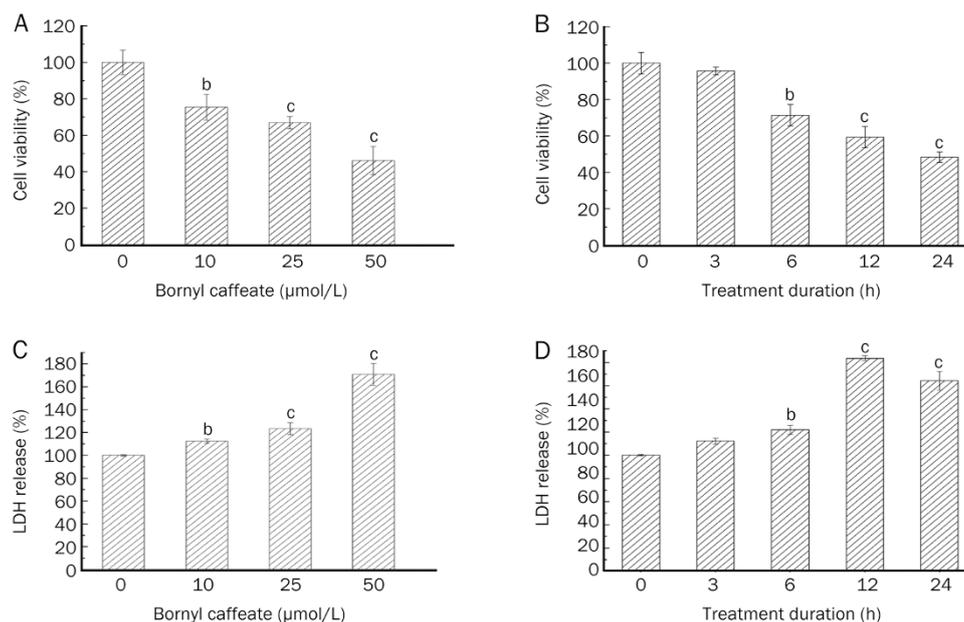
## Results

#### Synthesis and cytotoxicity of bornyl caffeate

Bornyl caffeate was recently synthesized by exhaustive esterification of caffeic acid with borneol as described (Figure 1)<sup>[45]</sup>. The cytotoxicity of bornyl caffeate was evaluated by the standard colorimetric MTT assay in MCF-7 cells, which served as a cell model of human breast cancer<sup>[46]</sup>. Following the exposure to bornyl caffeate at concentrations of 0, 10, 25, and 50  $\mu\text{mol/L}$  for 24 h or at a concentration of 50  $\mu\text{mol/L}$  for 0, 3, 6, 12, and 24 h, cell viability was assayed based on the reduction of MTT by mitochondrial reductase in viable cells. As shown in Figure 2A and 2B, bornyl caffeate reduced the cell viability in a concentration- and time-dependent manner. In similar experiments, caffeic acid and borneol showed no cytotoxicity at a concentration of 50  $\mu\text{mol/L}$  after 24 h (data not shown). Bornyl caffeate was also found to be cytotoxic in other cancer cells such as HepG2, Hela, T47D, and PC12 (data not shown). The cytotoxicity of bornyl caffeate was also verified by measuring LDH release. As shown in Figure 2C and 2D, the LDH activity in the medium was increased in a concentration- and time-dependent manner. Typically, bornyl caffeate at a con-



**Figure 1.** Scheme illustrating the chemical synthesis of bornyl caffeate. Bornyl caffeate was synthesized via acid-catalyzed esterification of caffeic acid with borneol. The structures were generated using the chemistry software ACD/ChemSketch (<http://www.acdlabs.com>).



**Figure 2.** Effect of bornyl caffeate on the cell viability of human breast cancer MCF-7 cells. (A) Dose-dependence of cytotoxicity. The cells were treated for 24 h with bornyl caffeate at the indicated doses. (B) Time-dependence of cytotoxicity. The cells were treated with 50 μmol/L of bornyl caffeate at each time point, whereas the control cells were treated with DMSO. At the end of drug treatment, the cell viability was determined using the MTT assay. The results are expressed as a percentage of the corresponding control. (C) Dose-dependence of LDH release. The cells were treated for 24 h with bornyl caffeate at the indicated doses. (D) Time-dependence of LDH release. The cells were treated with 50 μmol/L of bornyl caffeate at each time point, whereas the control cells were treated with DMSO. At the end of drug treatment, the cell viability was determined using the MTT assay, and the results are expressed as a percentage of the corresponding control. The mean, SEM, was usually within 10% of the mean value ( $n=3$ ). ( $^bP<0.05$ ,  $^cP<0.01$  vs control).

centration of 50 μmol/L significantly induced the release of the LDH enzyme after a 6-h incubation. These results suggest that bornyl caffeate may be a potential cytotoxic reagent for cancer cells.

#### Bornyl caffeate induced apoptosis in human breast cancer MCF-7 cells

To characterize the cytotoxicity of bornyl caffeate, we treated MCF-7 cells with the compound at concentrations of 0, 10, 25, and 50 μmol/L for 24 h. We first examined the nuclear morphology of MCF-7 cells by staining with Hoechst 33342. Upon exposure to bornyl caffeate, the incorporation of fluorescent dye was increased in a concentration-dependent manner (Figure 3A), and it was clear that bornyl caffeate induced the formation of condensed and fragmented cell nuclei (see arrows). Secondly, we detected the appearance of the early apoptosis biomarker phospho-serine at the cell surface by Annexin V-FITC staining. As shown in Figure 3B, bornyl caffeate increased the number of Annexin V-FITC-positive cells in a concentration-dependent manner. Due to the loss of cell-membrane integrity, the incorporation of the fluorescent dye PI was similarly increased. Thirdly, we investigated the effect of bornyl caffeate on the mitochondrial membrane potential (MMP) using a cationic fluorescent probe JC-1. JC-1 is preferably incorporated into the mitochondrion of viable cells, giving rise to a maximal fluorescence emission at ~590 nm (red), whereas the maximal fluorescence emission of JC-1 in the

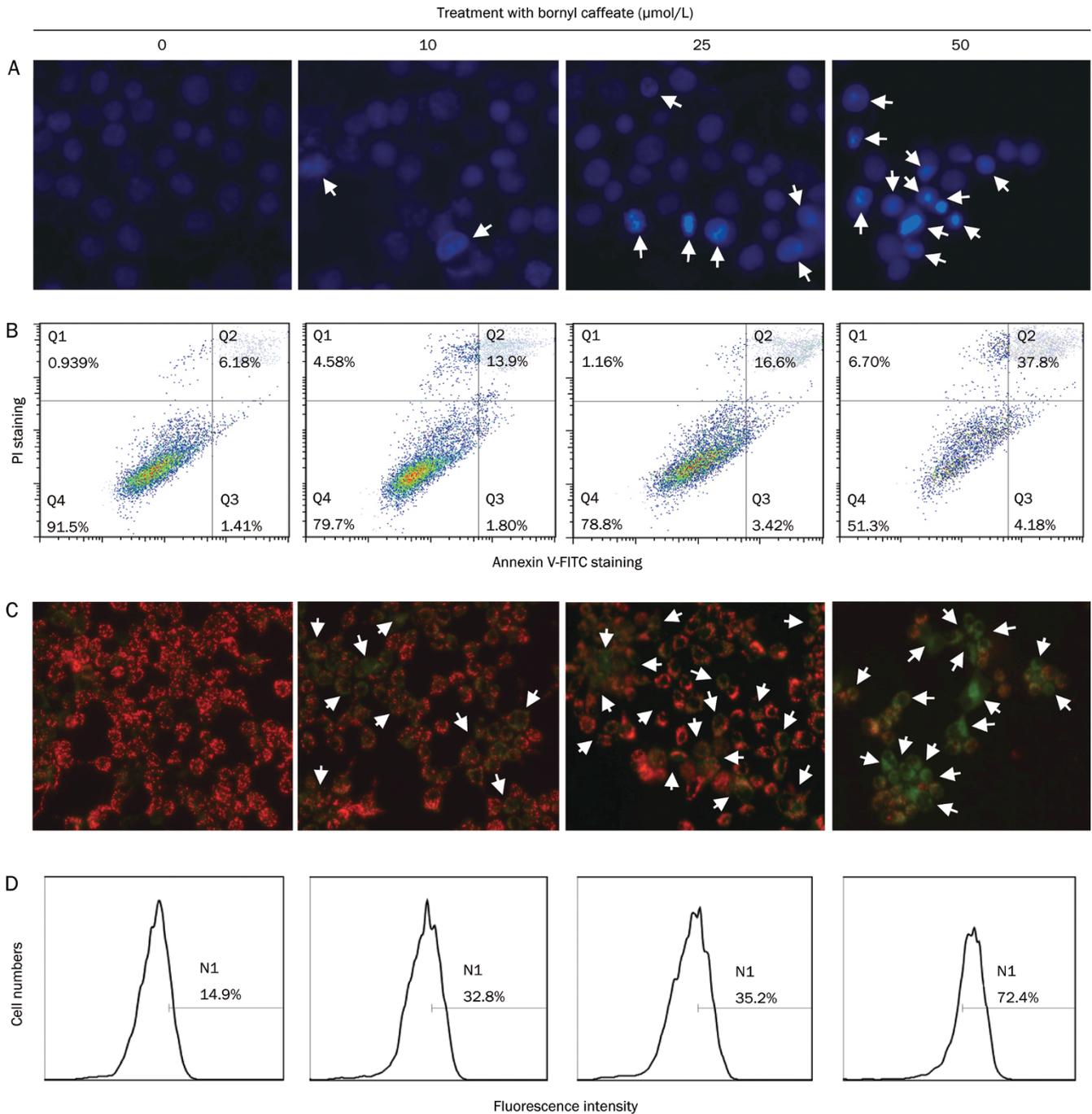
cytosol appears at ~525 nm (green). By measuring such a shift in the fluorescence emission by fluorescence microscopy, mitochondrial polarization was readily detected in bornyl caffeate-treated cells (Figure 3C). Notably, in the cells exposed to 50 μM of bornyl caffeate, a significant amount of the fluorescent dye JC-1 was present in the cytosol rather than the mitochondria (see arrows).

#### Bornyl caffeate stimulates ROS formation

To clarify the effect of bornyl caffeate on the intracellular redox status, we determined the intracellular ROS level by measuring the oxidation of non-fluorescent DCFH-DA to its highly fluorescent derivative 2',7'-dichlorofluorescein (DCF). As shown in Figure 3D, bornyl caffeate stimulated ROS formation in a concentration-dependent manner.

#### Effect of bornyl caffeate on apoptosis biomarkers

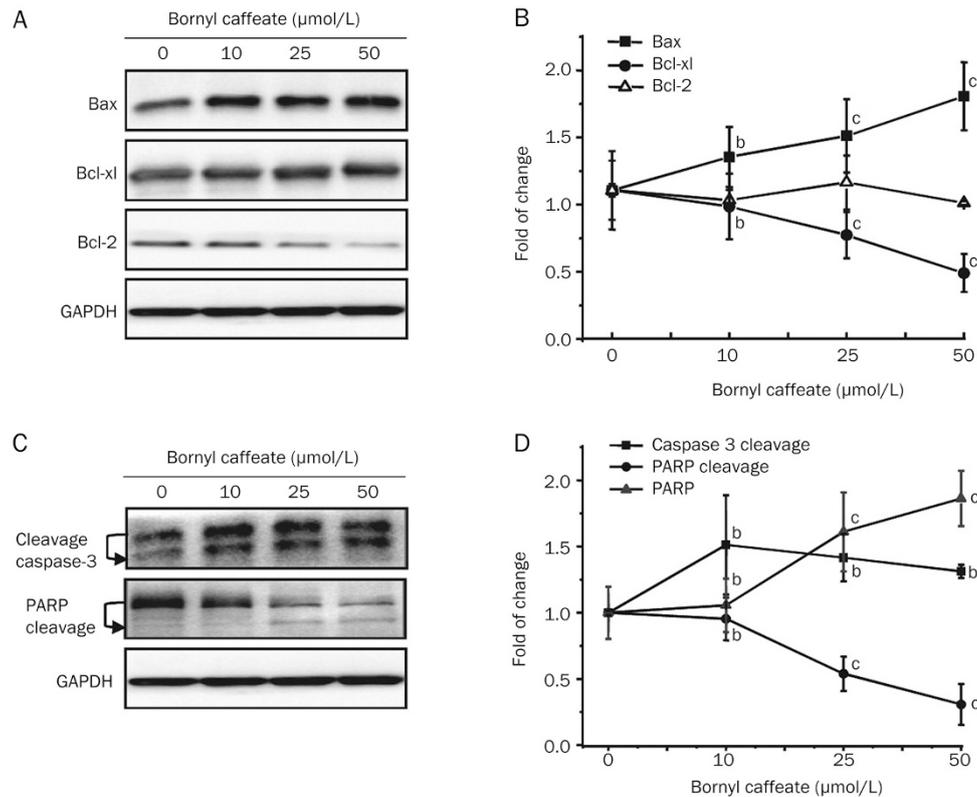
To verify the induction of apoptosis by bornyl caffeate, we investigated the effect of bornyl caffeate on the expression of the pro-apoptotic protein Bax and anti-apoptotic proteins Bcl-2 and Bcl-xl. We found that bornyl caffeate up-regulated the intracellular level of Bax but down-regulated the intracellular level of Bcl-xl in a concentration-dependent manner (Figure 4A and 4B). The expression of Bcl-2 was not dramatically affected even when the drug concentration was increased as high as 50 μmol/L. We further clarified if bornyl caffeate could trigger the activation of the caspase cascade by monitoring the cleav-



**Figure 3.** Bornyl caffeate induces apoptosis in human breast cancer MCF-7 cells. (A) Effect of bornyl caffeate on the morphology of cell nuclei. MCF-7 cells were treated with 0, 10, 25, or 50  $\mu\text{mol/L}$  of bornyl caffeate for 24 h and subsequently stained with Hoechst 33258, and the images were captured by fluorescence microscopy. The arrows point to the condensed and/or fragmented cell nuclei. (B) Flow cytometric analysis of the cell-surface phosphoserine. MCF-7 cells were sequentially treated with 0, 10, 25, or 50  $\mu\text{mol/L}$  bornyl caffeate for 24 hr, co-stained with Annexin V-FITC and PI and analyzed by flow cytometry (FACSCalibur, BD Biosciences, USA). (C) Effect of bornyl caffeate on the mitochondrial membrane potential. Cells were treated with 0, 10, 25, or 50  $\mu\text{mol/L}$  of bornyl caffeate for 24 h and subsequently stained with a fluorescent dye DJ-1. The cells were visualized for their green and red emission components by using optical filters designed for fluorescein and tetramethylrhodamine fluorescence microscopy. The arrows point to the cells undergoing depolarization of the mitochondrial membrane. (D) Effect of bornyl caffeate on the formation of ROS. Cells were treated with 0, 10, 25, and 50  $\mu\text{mol/L}$  of bornyl caffeate for 24 h. The ROS levels were determined by assaying the fluorescent product 2',7'-dichlorofluorescein (DCF) from 2',7'-dichlorofluorescein diacetate (DCFH-DA) in viable cells on a flow cytometer (FACSCalibur, BD Biosciences, USA).

age of caspase-3 and its action on PARP. As shown in Figure 4C and 4D, bornyl caffeate at concentrations of 10, 25, and

50  $\mu\text{mol/L}$  triggered the cleavage of caspase-3. Importantly, upon activation, caspase-3 subsequently degraded PARP,



**Figure 4.** Effect of bornyl caffeate on the expression and bioprocessing of apoptosis-related biomarkers. (A) Western blot analysis of Bax, Bcl-2, and Bcl-xL. MCF-7 cells were treated with bornyl caffeate at concentrations of 0, 10, 25, or 50 μmol/L for 24 h. Bax, Bcl-xL, and Bcl-2 were detected by specific antibodies, whereas GAPDH was detected as the internal control. (B) Data quantification of Panel A. The Western blots were quantified and expressed as the fold change of signal intensity compared to the untreated sample after normalization to the corresponding GAPDH signals. (C) Western blot analysis of caspase-3 and PARP cleavage. At the end of treatment, the cellular proteins were analyzed for caspase-3 and PARP cleavage by specific antibodies in the same way as described in Panel A. (D) Data quantification of Panel C. The data were analyzed in the same way as described in Panel B, and the results were presented as the means±SD of three independent experiments (<sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs control).

giving rise to a reduction in the intracellular PARP level in a concentration-dependent manner.

#### Bornyl caffeate persistently activates the MAP kinases JNK and p38

To explore the mechanisms modulating the induction of apoptosis, we investigated the effect of bornyl caffeate on different protein kinases. MCF-7 cells were treated with bornyl caffeate (50 μmol/L) for various periods (0, 0.5, 1, 3, 6, and 12 h). At the end of treatment, the cellular proteins were resolved by 10% SDS-PAGE and analyzed by Western blotting. As shown in Figure 5A and 5B, two pro-survival kinases, Akt and ERK, were marginally activated at the early stage (0.5–3 h); however, the phospho-Akt and phospho-ERK signals were gradually reduced to basal levels. In contrast, the phospho-p38 and phospho-JNK signals were gradually increased over a period of 12 h. These results stimulated us to further examine if activation of the MAP kinases p38 and JNK was dependent on the concentration of bornyl caffeate. We found that bornyl caffeate at a concentration of 50 μmol/L induced a dramatic increase in the phospho-p38 and phospho-JNK signals, whereas, at concentrations of 10 and 25 μmol/L, the phospho-

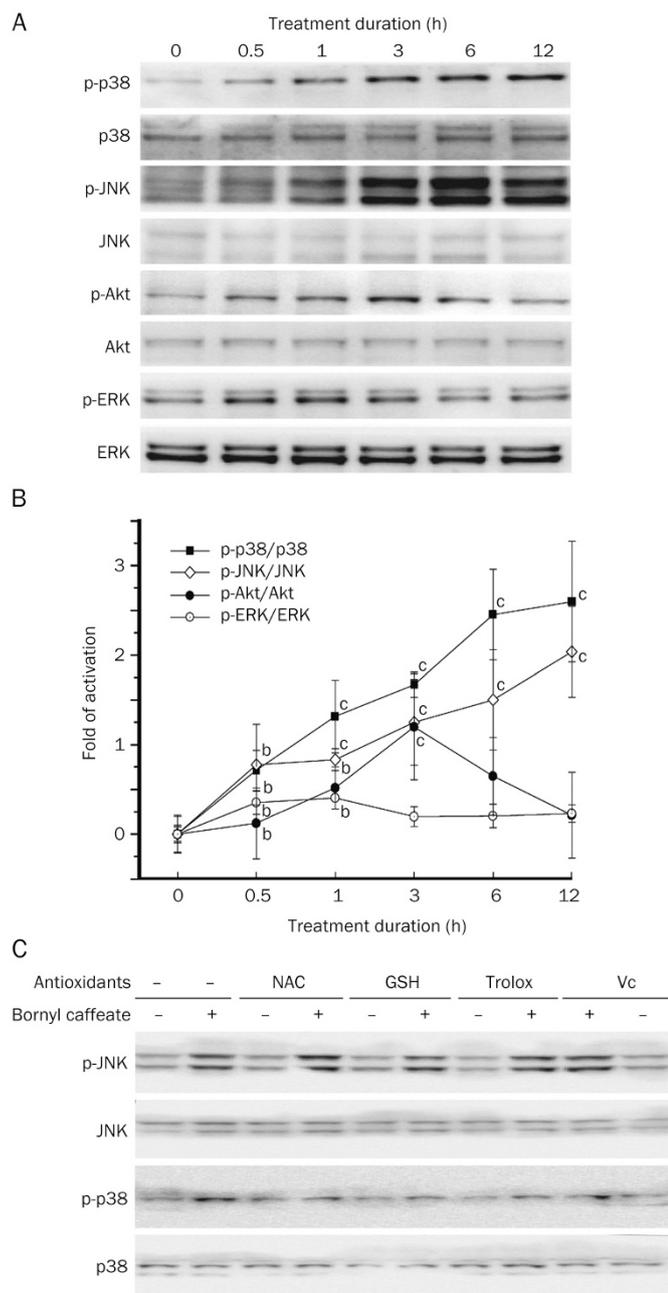
p38 and phospho-JNK signals were barely detectable (data not shown).

#### Antioxidants differentially attenuate the activation of the MAP kinases p38 and JNK

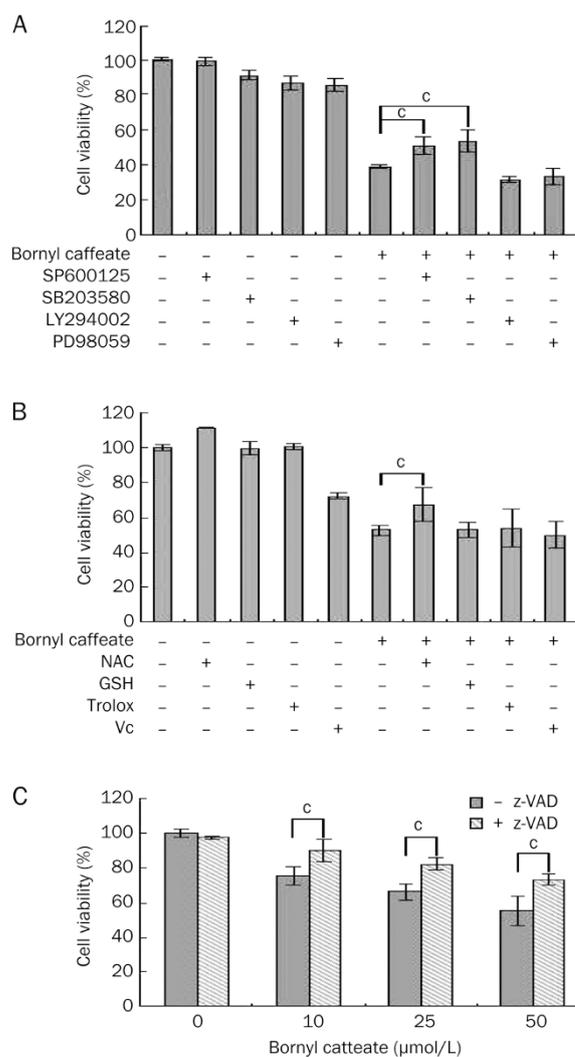
To understand how bornyl caffeate activated the MAP kinases p38 and JNK, we focused on the potential role of intracellular ROS. We employed four common antioxidants, NAC, GSH, vitamin C, and Trolox, to scavenge intracellular ROS. By detecting phospho-p38 and phospho-JNK in cells that were treated with bornyl caffeate and antioxidants, alone or in combination, we found that antioxidants differentially affected the activation of p38 and JNK by bornyl caffeate. In particular, antioxidants effectively antagonized the effect of bornyl caffeate on the activation of p38 but showed little activity against the activation of JNK (Figure 5 C).

#### Characterization of the cytotoxicity of bornyl caffeate

To explore the mechanisms by which bornyl caffeate induced apoptosis, we first investigated the effect of protein kinase inhibitors on the cytotoxicity of bornyl caffeate. As shown in Figure 6A, the p38 inhibitor SB203580 and JNK inhibitor



**Figure 5.** Bornyl caffeate differentially activated MAP kinases in MCF-7 cells. (A) Effect of bornyl caffeate on MAP kinases. MCF-7 cells were treated with 50  $\mu\text{mol/L}$  bornyl caffeate for the indicated times. Total cell lysates were extracted, resolved by 10% SDS-PAGE and transferred onto PVDF membrane. The phosphorylated and total proteins of Akt, ERKs, p38 MAPK, and JNKs were immunodetected. (B) Quantification of Panel A. The Western blots were quantified and expressed as the fold change of the signal intensity value compared to the untreated sample after normalization to the corresponding GAPDH signals. The results are presented as the means $\pm$ SD of three independent experiments, (<sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs control). (C) Effects of antioxidants on the bornyl caffeate-induced activation of MAP kinases. MCF-7 cells were pretreated with 1 mmol/L of different antioxidants, except for vitamin C (100  $\mu\text{mol/L}$ ), for 1 h followed by treatment with 50  $\mu\text{mol/L}$  bornyl caffeate for 24 h. The intracellular proteins were extracted and analyzed by Western blot using specific antibodies.



**Figure 6.** Investigation of potential mechanisms underlying the cytotoxicity of bornyl caffeate. (A) Effect of protein kinase inhibitors on the cytotoxicity of bornyl caffeate. MCF-7 cells were pretreated with different protein kinase inhibitors (PI3 kinase inhibitor LY294002, ERK inhibitor PD98059, p38 MAP kinase inhibitor SP600125 or JNK inhibitor SB203580) for 1 h followed by treatment with 50  $\mu\text{mol/L}$  bornyl caffeate for 24 h. Cell viability was determined using the standard MTT assay. (B) Effect of different antioxidants on the cytotoxicity of bornyl caffeate. MCF-7 cells were pretreated with 1 mmol/L of different antioxidants, except for vitamin C (100  $\mu\text{mol/L}$ ), for 1 h followed by treatment with 50  $\mu\text{mol/L}$  bornyl caffeate for 24 h. The cell viability was determined using the standard MTT assay. (C) Effect of the pan-caspase inhibitor z-VAD-fmk on the cytotoxicity of bornyl caffeate. MCF-7 cells were pretreated with 50  $\mu\text{mol/L}$  z-VAD-fmk for 1 h followed by treatment with 50  $\mu\text{mol/L}$  bornyl caffeate for 24 h. The cell viability was determined using the standard MTT assay (<sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs control).

SP600125 significantly attenuated the cytotoxicity of bornyl caffeate, whereas neither the ERK inhibitor PD98059 nor the PI3K/ Akt inhibitor LY294002 showed any effect. These results suggest that activation of the MAP kinases p38 and JNK plays a role in the cytotoxicity of bornyl caffeate (Martin *et al*, 2004).

Second, we investigated the effect of different antioxidants on the cytotoxicity of bornyl caffeate. To our surprise, all of these antioxidants, except for the thiol antioxidant L-NAC, failed to protect the cells against the cytotoxicity of bornyl caffeate (Figure 6). Third, we investigated the effect of the pan-caspase inhibitor z-VAD on the cytotoxicity of bornyl caffeate. As shown in Figure 6C, inhibition of caspases by z-VAD resulted in partial protection of the cells against the cytotoxicity of bornyl caffeate. Taken together, the cytotoxicity of bornyl caffeate can be partially attenuated by scavenging ROS and inhibiting MAP kinases and the caspase cascade.

## Discussion

In the present study, we investigated the anticancer mechanisms of bornyl caffeate in the human breast cancer cell line MCF-7. By assaying the effect of bornyl caffeate on the reduction of MTT in viable cells and the release of lactate dehydrogenase (LDH) from dead cells, we found that bornyl caffeate exhibited strong cytotoxicity in breast cancer MCF-7 cells in a dose- and time-dependent manner (Figure 2). To explore the underlying mechanisms for this process, we further characterized the cytotoxicity of bornyl caffeate by examining the morphological changes of cell nuclei and the presentation of the early apoptotic marker phosphoserine at the cell surface. As shown in Figure 3A and 3B, bornyl caffeate increased the shrinkage of cell nuclei and staining of Annexin V-FITC in a concentration-dependent manner, suggesting an apoptotic mechanism. These apoptotic features were supported by an increase in the protein level of pro-apoptotic Bax and a decrease in the intracellular level of anti-apoptotic Bcl-xL (Figure 4A). In line with the increase in the expression of the mitochondria-damaging protein Bax, bornyl caffeate actually disrupted the integrity of the mitochondrial membrane and induced the activation of caspase-3 (Figure 3C and 4C). These results stimulate us to postulate that the cytotoxicity of bornyl caffeate is mediated by inducing apoptosis in cancer cells.

Induction of apoptosis is a major anticancer mechanism for a variety of cancer therapies<sup>[3]</sup>. Caffeate derivatives such as CAPE and octylcaffeate have been previously demonstrated to induce apoptosis in various cancer cell lines and animal models<sup>[43, 51]</sup>. The common caffeate group in these compounds is presumably the key structural element responsible for inducing apoptosis. Bornyl caffeate is a combination of two well-known, anti-inflammatory chemical elements, caffeate and borneol, which renders this compound a potential anticancer drug candidate. Our results provided experimental evidence that support the potential of bornyl caffeate in inducing apoptosis in the breast cancer cell line MCF-7. We also observed similar cytotoxicity in several other cancer cell lines such as hepatoma HepG2, cervical cancer HeLa and pheochromocytoma PC12 cells (data not shown). Drug-induced apoptosis of cancer cells is ultimately executed by the caspase cascade, especially through caspase 3/6/7<sup>[52, 53]</sup>. However, various anticancer drugs, irradiation and Fas ligand may induce apoptosis through different mechanisms<sup>[4, 54]</sup>.

Aberrant formation of ROS is well recognized as a carcino-

genic factor driving the malignant transformation of normal cells via inducing oxidative DNA damage<sup>[55, 56]</sup>. However, the induction of ROS formation plays an important role in the chemotherapeutic activity of several anticancer drugs and a large number of anticancer compounds<sup>[57, 58]</sup>. Drug-induced ROS mediates the activation of MAP kinases, disrupts MMP and ultimately activates apoptotic caspases in cancer cells<sup>[59-61]</sup>. Following treatment with bornyl caffeate, the intracellular ROS levels were measured using a widely used, permeable probe 2',7'-dichlorofluorescein diacetate (DCFH-DA)<sup>[62]</sup>. After passive loading to the cells, DCFH-DA is hydrolyzed into 2',7'-dichlorodihydrofluorescein (DCFH) by intracellular esterase. DCFH is subsequently oxidized by ROS to become the highly fluorescent compound 2',7'-dichlorofluorescein (DCF). As shown in Figure 3D, bornyl caffeate stimulated ROS formation in a concentration-dependent manner. To determine the effect of bornyl caffeate on the integrity of the mitochondrial membrane, we selected a cationic dye, JC-1, to indicate mitochondrial polarization. Previous studies suggest that JC-1 forms red-fluorescent J-aggregates more specifically in the mitochondria and offers better consistency in its response to drug-induced depolarization than other cationic dyes such as DiOC6(3) and rhodamine 123<sup>[63]</sup>. We separately visualized the green and red emission components by using optical filters designed for fluorescein and tetramethylrhodamine. As a result, we observed that bornyl caffeate caused mitochondrial depolarization in a concentration-dependent manner (Figure 3C). Moreover, bornyl caffeate profoundly upregulated the Bax level and downregulated the Bcl-xL level, thereby causing a dramatic shift in the Bax/Bcl-xL ratio to allow mitochondrial apoptosis to occur<sup>[64]</sup>. As shown in Figure 4C, apoptosis-executing molecules such as caspase-3 and its substrate poly (ADP-ribose) polymerase (PARP) were sequentially activated. Interestingly, pre-treatment with the pan-caspase inhibitor z-VAD-fmk only partially inhibited bornyl caffeate-induced apoptosis, suggesting the involvement of caspase-dependent and -independent mechanisms<sup>[65, 66]</sup>.

ROS regulates cell proliferation, differentiation and apoptosis via the activation of protein kinase C (PKC), ERK, the p38 MAP kinase and JNK<sup>[19, 67, 68]</sup>. In cells exposed to 50- $\mu$ mol/L bornyl caffeate, we detected a gradual increase in phospho-p38 and phospho-JNK MAP kinases over a period of 12 h, whereas phospho-Akt and phospho-ERK1/2 were only detectable during the first 3 hrs (Figure 5A). To determine the mechanism by which bornyl caffeate acts on the p38 MAP kinase and JNK, we applied different antioxidants to scavenge the ROS that were induced by bornyl caffeate. The antioxidants NAC, GSH, Trolox, and vitamin C attenuated the activation of the p38 MAP kinase by bornyl caffeate but showed little effect on the activation of JNK (Figure 5C). These results suggest that bornyl caffeate could induce the activation of the MAP kinases p38 and JNK via ROS-dependent and -independent pathways. Not surprisingly, JNK can be activated by multiple stimuli such as cytokines (eg, tumor necrosis factor and interleukin-1), reactive oxygen species (ROS), pathogens, toxins, drugs, endoplasmic reticulum

stress, free fatty acids and metabolic changes<sup>[69]</sup>. For example, garlic-derived, organosulfur compounds such as diallyl polysulfides activate JNK via direct chemical modification of the cytoskeletal protein tubulin and depends less on intracellular ROS<sup>[70]</sup>. Another study demonstrated that the proinflammatory mediator high-mobility group box-1 protein (HMGB1) induced MUC8 expression in a JNK and PI3K/Akt signaling pathway-dependent manner that was ROS-independent<sup>[71]</sup>. Further investigation is needed to clarify how bornyl caffeate activates JNK in a ROS-independent manner. To explore the mechanisms underlying the cytotoxicity of bornyl caffeate, we employed antioxidants, the JNK inhibitor SP600125, the p38 MAP kinase inhibitor SB203850, the PI3K/Akt inhibitor LY294002 and the pan-caspase inhibitor z-VAD-fmk. As shown in Figure 6, the cytotoxicity of bornyl caffeate was partially attenuated by the JNK inhibitor SP600125, p38 MAP kinase inhibitor SB203850 and antioxidants NAC and pan-caspase z-VAD-fmk. However, none of these reagents could completely rescue the cells from bornyl caffeate-induced injury. These results suggest that the cytotoxicity of bornyl caffeate is mediated by multiple mechanisms.

In conclusion, the present study demonstrated that bornyl caffeate induced cell death in breast cancer MCF-7 cells by inducing apoptosis. We found that bornyl caffeate stimulated the formation of ROS and activated the stress-responsive MAP kinases JNK and p38 in MCF-7 cells. Our results suggest that ROS-dependent pathways and the ROS-independent JNK pathway contribute to the cytotoxicity of bornyl caffeate. Thus, bornyl caffeate may serve as a potential lead for the development of new anticancer drugs.

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### Author contribution

Chuan-bin YANG, Jia ZHAO, and Yuan-yuan CHENG performed the research; Wei-jing PEI and Xiao-hui ZHENG chemically synthesized bornyl caffeate; Jian-hui RONG designed the research and analyzed the data; and Jian-hui RONG and Chuan-bin YANG wrote the paper.

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