Caffeic Acid 3,4-Dihydroxy-Phenethyl Ester Induces Cancer Cell Senescence by Suppressing Twist Expression

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ABSTRACT

Compared with traditional cytotoxic cancer therapy, therapy-induced cancer cell senescence attracts much interest because it is similarly effective, has fewer side effects, and is more efficiently cleared by immune cells. In this study, we demonstrate that unlike caffeic acid phenethyl ester, caffeic acid 3,4-dihydroxy-phenethyl ester (CADPE), which is isolated from the medicinal plants Sarcandra glabra and Teucrium pilosum, inhibits human cancer cell growth and colony formation by inducing cancer cell senescence, not apoptosis. CADPE induces cell senescence and morphology changes by increasing cellular size and cytoplasmic granularity, enhancing senescence-associated β-galactosidase activity and differentiated embryo-chondrocyte expressed gene 1 expression, and blocking cell-cycle arrest in the G0 phase. To help understand the underlying mechanisms, we show that CADPE significantly suppressed the expression of Twist1 and led to the up-regulation of rat sarcoma, p53, p21WAF1/CIP1, and p16INK4a proteins in a dose-dependent manner, resulting in the hypophosphorylation of retinoblastoma protein. Furthermore, overexpression of Twist1 prevented CADPE-induced cell senescence in tumor cells. Therefore, our studies provide evidence for a novel role of CADPE in cancer cell senescence by targeting the Twist1-dependent senescence signaling pathway.

Introduction

Traditional cancer therapy relies on cytotoxic treatment strategies, which are based on the hypothesis that complete cellular destruction of tumors prolonys the potential for patient survival (Ewald et al., 2010). This viewpoint has limited treatment options to toxic compounds and high-dose radiation (Collado and Serrano, 2010; Ewald et al., 2010). These approaches may produce cancer cell death. However, they can also induce severe side effects and often develop resistance to treatment or even progress to advanced primary and metastatic tumors (Collado and Serrano, 2010; Ewald et al., 2010). An alternative strategy is the induction of cellular senescence, which permanently disables the proliferative capacity of cancer cells (Dimri, 2005; Ewald et al., 2010). Moreover, in contrast to nonsenescent tumor cells, senescent tumor cells are efficiently cleared by immune cells, resulting in efficient tumor regression (Xue et al., 2007; Krizhanovsky et al., 2008). Initial clinical studies using therapy-induced senescence treatments have provided compelling evidence that cellular senescence may be as effective as cytotoxic therapies in preventing tumor growth. This approach of treatment has offered similar or prolonged survival with fewer side effects related to cytotoxicity and could might provide a more realistic goal for the chronic management of some cancers (Dimri, 2005; Ewald et al., 2010).

The senescence phenomenon was first described by Hayflick and Moorhead (1961) in human fibroblasts after extensive culture and replicative exhaustion linked to telomere shortening. This work was supported by the Science and Technology Commission of Shanghai Municipality (Pujiang Program Grant 09PJ1403900); the National Natural Science Foundation of China [Grants 30800653, 81071437, 30800627]; and the Fundamental Research Funds for the Central Universities and 973 Program (Grant 2010 CB929704).

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ABBREVIATIONS: SA-β-gal, senescence-associated β-galactosidase; CAPE, caffeic acid phenethyl ester; CADPE, caffeic acid 3,4-dihydroxy-phenethyl ester; DEC1, differentiated embryo-chondrocyte expressed gene 1; Ras, rat sarcoma; pRB, retinoblastoma tumor suppressor; SRB, sulforhodamine B; TCA, trichloroacetic acid; PI, propidium iodide; PARP, poly(ADP)-ribose polymerase; PCR, polymerase chain reaction; EGFP, enhanced green fluorescence protein; DMEM, Dulbecco’s modified Eagle’s medium; HUVEC, human umbilical vascular endothelial cell; NF-κB, nuclear factor κB; FACS, fluorescence-activated cell sorting; WT, wild type; STAT3, signal transducer and activator of transcription 3.
shortening. More recently, oncogenic signaling, DNA damage, and oxidative stress have been found to result in induced or accelerated senescence (Collado and Serrano, 2010). In contrast to cells undergoing apoptosis or mitotic catastrophe in response to conventional cytotoxic drugs, senescent cells were maintained in the G1 stage of the cell cycle. Cultured in vitro, senescent cells develop a distinct and recognizable flattened and enlarged morphology with a prominent nucleus and increased cytoplasmic granularity. Most notably, these senescent and enlarged morphology with a prominent nucleus and increased cytoplasmic granularity. Most notably, these

Ras proteins comprise a family of signal-transducing GTPases that are frequently mutated in human cancers. In addition to the pathogenic role of oncogenic Ras in cell proliferation and cell survival, induction of senescence is another important function of Ras. Activation of Ras leads to the up-regulation of cell proliferation inhibitors, including p19ARF, p53, p21WAF1/CIP1, and p16INK4a, leading to pRB hypophosphorylation and cell-cycle arrest during cellular senescence.

Twist proteins are highly conserved basic helix-loop-helix transcription factors that have important regulatory functions during embryogenesis and cancer cell epithelial-mesenchymal transition. Twist1 is overexpressed in numerous carcinomas, sarcomas, gliomas, neuroblastomas, and melanomas (Maestro et al., 1999; Hoek et al., 2004; Vallesia-Wittmann et al., 2004; Elia et al., 2005; Anisieau et al., 2008). It has been shown that induction of Twist1 is sufficient to override oncogene-induced senescence in both murine and human cells by inhibiting the p53-p21 tumor suppressor pathways through suppression of Ras (Anisieau et al., 2008). Moreover, the reduction in Twist1 expression coincided with a specific increase in p16INK4a expression and was accompanied by the induction of senescence-associated SA-β-gal activity (Lee and Bar-Sagi, 2010). Therefore, Twist1 could be a promising target to induce cellular senescence in cancer cells. In this study, we demonstrate that CADPE inhibits human cancer cell growth and colony formation by induction of cellular senescence in a dose-dependent manner. Using Western blot analysis, real-time PCR, and luciferase reporter gene assays, we have shown that CADPE significantly suppressed Twist1 expression at both the protein and mRNA levels. The inhibition of Twist1 expression by CADPE leads to the up-regulation of Ras, p53, p21WAF1/CIP1, and p16INK4a proteins in a dose-dependent manner, resulting in the hypophosphorylation of RB and the arrest of the cell cycle at the G1 stage during senescence.

**Materials and Methods**

**Materials and Cells.** CAPE was purchased from Sigma-Aldrich (St. Louis, MO). CADPE was synthesized by Dr. Tang Jie (East China Normal University, Shanghai, China) as described previously (Zhang et al., 2010), and the purity was more than 98%. Antibodies against p53, p21WAF1/CIP1, Ras, pRB, Rb, caspase-3, PARP, p16INK4a, and cyclin E were purchased from Cell Signaling Technology (Danvers, MA). EGFP-Twist1-FLAG and EGFP-FLAG constructs were gifts from Dr. Wang Ping (East China Normal University). Twist1-luc construct, as described previously (Qin et al., 2009), was a gift from Dr. Xu Jianming (Baylor College of Medicine, Houston, TX). p53-luc and p21-luc constructs were purchased from Stratagene (La Jolla, CA). Human HCT116, AGS, HGC27, U2OS, and H1299 cells obtained from the China Type Culture Collection (Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum. Primary human umbilical vascular endothelial cells (HUVECs) were cultured in endothelial cell growth medium/M199 medium (Invitrogen) as described previously (Pang et al., 2009).

**Cell Viability and Colony Formation Assays.** To examine cell viability, we performed a sulforhodamine B (SRB) assay as described previously (Yang et al., 2010). In brief, 5 × 104 cells per well were plated into 96-well plates treated with different concentrations of CADPE for 72 h. The SRB assay was performed by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 μl of cold 50% TCA (final concentration 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded, and the plates were washed five times with tap water and air-dried. SRB solution (100 μl) at 0.4% in 1% acetic acid was added to each well, and the plates were incubated for 10 min at room temperature. Absorbance was measured with a VERSAmax microplate reader ( Molecular Devices, Sunnyvale, CA). For colony formation assay, cells were plated into 6-cm dishes in triplicate. After being treated with different concentrations of CADPE for 21 days, the cells were stained with 0.5% crystal violet (dissolved in 95% ethanol). The colonies were photographed and counted to generate histograms.

**Senescence-Associated β-Galactosidase Staining Assays.** Cells were plated at varying cell densities in six-well plates. After incubation with CADPE for 72 h, cells were fixed in 2% formaldehyde/0.2% glutaraldehyde in phosphate-buffered saline for 5 min and incubated at 37°C for 12 to 18 h with fresh SA-β-gal staining solution containing 1.0 mg/ml X-galactosidase as described previously (Dimri et al., 1995).

**Live and Dead Assay.** To assess the apoptosis effect of CADPE, we performed a live/dead assay as described previously (Li et al., 2010). In brief, 105 cells were incubated with CADPE after 72 h at 37°C, and then stained with live/dead reagent (5 μM ethidium homodimer and 5 μM calcine-AM) at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Leica, Wetzlar, Germany).

**Annexin V/Propidium Iodide Staining Assays.** To detect whether CADPE induces cell apoptosis, we stained the treated cells with an annexin V kit (Sigma-Aldrich) as described previously. In brief, 106 AGS and HCT116 cells were treated with different concentrations of CADPE for 72 h, and then subjected to annexin V/pro- pidi um iodide (PI) staining. The results were analyzed with a flow cytometer (FACSaria; BD Biosciences, San Jose, CA).

**Cell-Cycle Analysis.** To determine cell-cycle distribution, the cells were treated with 2 mM thymidine (Sigma-Aldrich) for 12 h and released into DMEM for 10 h followed by secondary thymidine block for 12 h, then they were released into DMEM or treated with 10 μM CADPE. The cells were harvested after release at the indicated times, then fixed with 70% cold ethanol and stained with 50 mg/ml PI (Sigma-Aldrich) followed by RNase A (Sigma) treatment for 30 min at room temperature. DNA content was analyzed with a FACscan cell analyzer (BD Biosciences) equipped with CellQuest software (BD Biosciences). The population of cells in each phase was determined by using ModFit LT software (BD Biosciences).

**Luciferase Reporter Gene Assays and Cell Transfection.** A dual-luciferase assay was performed in triplicate as described previ-
ously (Li et al., 2011). In brief, 2 × 10^4 cells were placed on 24-well plates 24 h before transfection. Twist1/p53/p21 luciferase reporters (0.5 μg) and 0.05 μg of the Renilla luciferase assay vector pRL (Promega, Madison, WI) were cotransfected into AGS cells using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Cells were lysed in Reporter Lysis Buffer (Promega), and luciferase and Renilla activity was measured using a luminometer (BMG LUMImat; BMG Labtech GmbH, Offenburg, Germany). Luciferase activity was normalized with Renilla activity in the cell lysate and expressed as an average of three independent experiments.

**Western Blot Assay.** For the Western blot assay, cells were treated with different concentrations of CADPE for the indicated times. Cell lysates were prepared in radioimmunoprecipitation assay buffer (20 mM Tris, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mM NaF, 10 mM Na_2P_2O_7, and 1 mM phenylmethylsulfonyl fluoride). Aliquots of cellular protein were electrophoresed by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). The membrane was blocked with 5% skim milk in phosphate-buffered saline containing 0.1% Tween 20 and then reacted with specific antibodies. Detection of specific proteins was carried out by enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

**Statistical Analysis.** The results are presented as mean ± S.E., and statistical comparisons between groups were done using one-way analysis of variance followed by Student’s t test. P ≤ 0.05 was considered statistically significant.

**Results**

**CADPE Inhibits Human Cancer Cell Growth.** To determine the effects of CADPE, a natural compound isolated from the medicinal plant *Sarcandra glabra* (*Sarcandra*) and *Teucrium pilosum* (Decne) (El-Mousallamy et al., 2000) (Sup-

![Fig. 1. CADPE inhibits human cancer cell proliferation and colony formation.](image-url)
plemental Fig. S1A), on human cancer cell growth, we treated seven different human cancer cell lines, AGS, HGC27 (gastric cancer), H1299, A549 (lung cancer), HCT116 p53 WT and p53(-/-) (colon cancer), and U2OS (osteosarcoma), and a normal human cell, HUVEC, with CADPE, and then measured cell growth by SRB assays. Our data indicated that CADPE significantly inhibited cancer cell growth in a dose-dependent manner, with an IC$_{50}$ of ~10 μM in all of the cancer cell lines (Fig. 1A and Supplemental Fig. S1B). It is noteworthy that the primary cultured normal HUVECs were only slightly affected by CADPE treatment, with an IC$_{50}$ of >50 μM. Similar results were obtained from cell number counts (Fig. 1B and data not shown). To further confirm the inhibitory effect of CADPE on cancer cell growth, we carried out colony formation assays in AGS and HGC27 cells (Fig. 1C). CADPE inhibited cancer cell colony

**Fig. 2.** CAPE can induce cell apoptosis, but CADPE has little effect on apoptosis in different human cancer cells. A, CADPE could not induce PARP and caspase-3 cleavage in AGS cells using CAPE as positive controls. AGS cells were treated with CADPE or positive control (CAPE) in the indicated concentrations for 72 h. Whole-cell extracts were prepared and subjected to Western blot analysis using anti-PARP, anti-caspase3, anti-cleaved caspase3, and β-actin antibodies. B, CADPE could not induce cell apoptosis by annexin V staining in AGS and HCT116 cells using CAPE as positive controls. Cells (8 × 10$^3$ per well) were treated with different concentrations of CADPE or positive control (CAPE) for 72 h, then stained with annexin-V-fluorescein isothiocyanate and analyzed by flow cytometry for early apoptotic effects as described under Materials and Methods. The percentage indicates the ratio of early apoptotic cells/total cells. All results are representative of three independent experiments. C, CADPE could not induce cell death in AGS and HGC27 cells using live/dead assays. Cells (5 × 10$^3$ per well) were treated with the indicated concentrations of CADPE for 72 h. The live (green) or dead (red) cells were stained with the live and dead assay kit as described under Materials and Methods and then analyzed under a fluorescence microscope. The percentage indicates the ratio of dead/live cells. All results are representative of three independent experiments. The scale bars represent 50 μm.
formation in a dose-dependent manner (Fig. 1C), suggesting that CADPE is an effective agent in suppressing human cancer cell growth.

**CADPE Has Little Effect on Cancer Cell Apoptosis.** Because CADPE shares a key chemical structure with CAPE (Supplemental Fig. S1A), a natural compound that has been reported to induce tumor cell apoptosis in different cancer cells (Watabe et al., 2004; Onori et al., 2009), we examined whether CADPE inhibits human cancer cell growth by inducing cell apoptosis. As shown in Fig. 2A and Supplemental Fig. S1C, CAPE significantly inhibited cancer cell growth and increased the accumulation of cleavage PARP and caspase-3 in a concentration-dependent manner (Fig. 2A, right). To our surprise, CADPE did not induce the cleavage of PARP and caspase-3 protein (PARP-CF and Caspase-3-CF), suggesting that CADPE has little effect on cancer cell apoptosis (Fig. 2A). To confirm this observation, annexin V staining and live/dead assays were used to analyze the apoptotic effects of CADPE. CADPE had little effect on inducing cellular senescence in both annexin-V FACS analysis (Fig. 2B) and live/dead assays (Fig. 2C). However, CAPE did induce cancer cell apoptosis by annexin V staining (Fig. 2B). All of the results suggest that CADPE inhibition of human cancer cell growth is not caused by the induced cell apoptosis, which is different from the inhibitory effect of CAPE on cell apoptosis.

**Figure 3.** Induction of cellular senescence by CADPE, but not CAPE in different cancer cell lines. A and B, SA-β-gal staining of AGS, U2OS, and HGC27 cancer cells after treatment with the indicated concentrations of CADPE or CAPE for 72 h. Cells (20 × 10^3 per well) were fixed and stained with fresh SA-β-gal as described under Materials and Methods. Photographed pictures (A) and statistical graphs (B) are shown. The scale bars represent 50 μm. C, CADPE induced senescence marker DEC1 mRNA expression in AGS cells. AGS cells (8 × 10^3 per well) were incubated with the indicated concentrations of CADPE for 72 h. DEC1 transcription levels were assessed by real-time PCR as described under Materials and Methods. Columns, mean; bars, S.D. D, CADPE permanently disabled the proliferative capacity of cancer cells. AGS cancer cells were treated with CADPE for 72 h, and either CADPE was removed or treatment with CADPE continued for another 72 h. Cell viability was quantified by SRB assay as described under Materials and Methods.

**CADPE Induces Cellular Senescence in Human Cancer Cells.** In our initial observation, most cell phenotypes in the presence of CADPE were characterized by enlarged cellular size and nucleus and fattened and vacuolated cellular morphology (Figs. 1B and 2C), which is strongly associated with cell senescence (Ewald et al., 2009). To test this hypothesis, we examined whether CADPE induces cellular senescence by SA-β-gal staining, one of the best known markers for detecting senescence. As expected, CADPE induced SA-β-gal activity (Fig. 3, A and B and see Fig. 6 and Supplemental Fig. S1D) in a dose-dependent manner in all seven cancer cell lines tested, but CAPE did not (Fig. 3, A and B). To confirm the effect of CADPE on cellular senescence, we further investigated the expression level of DEC1, the cellular senescence marker and one of the effectors of p53. As shown in Fig. 3C, similar to SA-β-gal staining results, the mRNA expression level of DEC1 was increased by CADPE in a concentration-dependent fashion, suggesting that CADPE triggered the induction of cellular senescence in human cancer cells. To further clarify the growth inhibition effect of CADPE by another mechanism, we examined whether CADPE induces autophagy in AGS cells. Our data indicate that CADPE has little effect in inducing cancer cell autophagy (Supplemental Fig. S2).
CADPE Causes G₁-Phase Cell-Cycle Arrest in Human Cancer Cells. Cellular senescence is characterized by flattened and vacuolated cell morphology, increased senescence-associated SA-β-gal activity, induction of the cellular senescence marker DEC1, and G₁-phase cell-cycle arrest (Qian et al., 2008). To determine whether CADPE causes G₁-phase cell-cycle arrest in cancer cells, we investigated cell-cycle progression by using different approaches in the absence or presence of CADPE. First, we treated three different human cancer cell lines with 10 μM CADPE for the indicated time courses and subjected them to flow cytometric analysis. As shown in Fig. 4A and B, the percentage of G₁-phase cells was significantly increased in CADPE-treated cells. Correspondingly, the percentage of cells in the S or G₂/M phase was reduced compared with the control cells. Second, we tested whether cells accumulate at the G₁ phase by CADPE by using an “arrest-and-release” strategy in AGS and HCT116 cells. Cells were first synchronized with double-thymidine treatment and treated with CADPE at different time points after withdrawing thymidine in the culture medium. Cells with CADPE treatment were accumulated at the G₁ phase more significantly, particularly at the 3-h time point, accompanied by a decrease in the S or G₂/M phase compared with the control cells (Fig. 4C and Supplemental Fig. S3). Finally, using Western blot analysis, we examined whether CADPE affected the expression of cyclin E in released synchronized AGS cells, which is essentially for the transition from the G₁ to S phase and expressed only at the end of the G₁ phase. Our results indicate that CADPE prolonged cyclin E expression after synchronized cancer cells release, suggesting that cells treated with CADPE accumulate at the G₁ phase compared with the control cells. Together, our data suggest that CADPE strongly induces G₁-phase cell-cycle arrest in human cancer cells, which is consistent with previous observations that inhibition of cell growth by CADPE is caused by the induction of cellular senescence and cell-cycle arrest. To further confirm whether CADPE can permanently halt the proliferative capacity of cancer cells, we investigated whether CADPE can induce G₁-phase cell-cycle arrest in human cancer cells. A, CADPE inhibited the progression of the cell cycle through G₁-phase arrest in AGS, HCT116, and HGC27 cells. Cancer cells were treated with or without CADPE (10 μM) for 12 h, then fixed and stained with PI (25 μg/ml). Cells were then subjected to flow cytometry analysis (cell count versus PI/DNA content). B, AGS cells were incubated with or without CADPE (10 μM) for 0, 3, 6, 12, 18, and 24 h, and cell-cycle distribution was monitored by flow cytometry analysis. The cells in G₁ phase were analyzed by FACS analysis at each time point. *, P < 0.05; **, P < 0.01 versus control. C, CADPE regulates G₁/S-phase transition in synchronized cells. AGS cells were synchronized with double-thymidine block. After thymidine was removed, cells were incubated with or without CADPE (10 μM) for 0, 3, 6, 12 h. The cells then were collected at the indicated time points. Cell-cycle distributions of the cells were analyzed by FACS analysis. Columns, mean; bars, S.D. Thy, thymidine. **, P < 0.01 versus control. D, CADPE prolonged cyclin E expression in released synchronized AGS cells. Cells were synchronized with double-thymidine block, followed by thymidine removal, and then incubated with or without CADPE (10 μM) from 0 to 24 h. Whole-cell extracts were collected at the indicated time points and subjected to Western blot analysis using the indicated antibodies.
cancer cells, we treated AGS cancer cells with CADPE for 72 h and removed CADPE or continued treating the cells with CADPE for another 72 h. Our results indicate that there is no significant difference between the persistent CADPE-retreated cells and the CADPE-withdrawn cells (Fig. 3D), suggesting CADPE-induced cell senescence can not be reversed even when the drug is removed.

**CADPE Induces Cellular Senescence by Regulating the Twist1-Modulated Senescence Signaling Pathway.** To determine the mechanism by which CADPE induces cellular senescence in human cancer cells, we focused our attention on the basic helix-loop-helix transcription factor Twist1, a well documented protein that overrides premature senescence by regulating both the Ras-p53 and p16 pathways (Serrano et al., 1997). We first examined the expression levels of Twist1 in five cancer cell lines and the primary cultured HUVECs. Consistent with previous reports (Maestro et al., 1999; Hoek et al., 2004; Valsesia-Wittmann et al., 2004; Elias et al., 2005; Ansieau et al., 2008), the expression levels of Twist1 increased significantly in all cancer cells compared with HUVECs (Fig. 5A). CADPE markedly suppressed Twist1 expression at both the protein level (Fig. 5B) and mRNA level (Fig. 5C) in a concentration-dependent manner. To further confirm that CADPE inhibits Twist1 expression, we next assessed the effects of CADPE on Twist1 promoter by luciferase reporter gene assay. As shown in Fig. 5D, CADPE suppressed Twist1 promoter activity in a dose-dependent manner. The IC_{50} for CADPE inhibition of Twist1

![Fig. 5. CADPE induces cellular senescence through the regulation of the Twist1-mediated signaling pathway. A, the expression of Twist1 in HUVEC and different cancer cell lines (HGC27, AGS, H1299, HCT116, and U2OS). B, CADPE regulated protein expression levels of Twist1, Ras, p53, p21^{RAS-} and p16^{INK4a}, and pRb (Ser795). AGS cells were incubated with the indicated concentrations of CADPE for 72 h. Whole-cell extracts were prepared and subjected to Western blot analysis using the indicated antibodies. β-Actin was used as a loading control. C, CADPE regulated mRNA expression levels of Twist1, hRas, and p21^{WAF1/CIP1} genes. AGS cells were incubated with the indicated concentrations of CADPE for 72 h, and mRNA levels were then measured by real-time PCR as described under Materials and Methods. D, CADPE acted as transcriptional regulator of Twist1, p53, and p21^{WAF1/CIP1} genes. AGS cells were transiently transfected with the indicated luciferase reporter gene and treated with the indicated concentrations of CADPE for 72 h. Cell supernatants then were collected and assayed for luciferase activity as described under Materials and Methods. Columns, mean; bars, S.D.](image-url)
expression and promoter activity was approximately 10 μM, which is consistent with the IC_{50} of CADPE inhibition of cell growth.

To examine the downstream genes mediated by Twist1 in cellular senescence, we further investigated the regulatory effects of CADPE on Ras, p53, p21^{WAF1/CIP1}, p16^{INK4a}, and Rb, which have been identified as essential downstream genes of Twist1 in cellular senescence (te Poele et al., 2002; Narita et al., 2003; Ansieau et al., 2008). Our results indicate that CADPE can dramatically induce the expression levels of p21^{WAF1/CIP1} and p16^{INK4a}, while slightly increasing Ras and p53 expression in a dose-dependent manner (Fig. 5B). Similar results were obtained at the mRNA levels for Ras and p21 by real-time PCR analysis and promoter luciferase reporter gene assays of p53 and p21, suggesting that CADPE can trigger both the Ras-p53-p21^{WAF1/CIP1} pathway and p16^{INK4a} pathway. As a result of the two pathways being activated by CADPE, the phosphorylation of Rb was strongly suppressed in a concentration-dependent fashion (Fig. 5B). Together, our data suggest that CADPE induces cellular senescence by regulating Twist1-modulated p53 and p16 pathways.

To further confirm whether the induction of senescence by CADPE depends on p53 or not, we chose two pairs of cell lines, A549 (p53 WT) and H1299 (p53(-/-)), HCT116 (p53 WT) and HCT116 (p53(-/-)), to perform cell senescence staining. As shown in Fig. 6, there was no significant difference between the p53 WT and p53(-/-) cell lines in SA-β-gal activity, suggesting that CADPE can either regulate Ras-p53-p21^{WAF1/CIP1} pathway or bypass this pathway in p53-null cells.

**Overexpression of Twist1 Prevents CADPE-Induced Cellular Senescence.** To confirm that CADPE induces cellular senescence by suppressing Twist1 expression, we investigated whether overexpression of Twist1 in human cancer cells (AGS and H1299) can prevent the induction of cellular senescence by CADPE. As shown in Fig. 7A and Supplemental Fig. S4, CADPE induces cellular senescence at 25% ± 1.9 and 28% ± 2.5% in AGS and H1299 cells, respectively. However, CADPE can only induce cellular senescence at 3% ± 1.1 and 4% ± 1.5% in Twist1-overexpressed AGS and H1299 cells.
cells, respectively (Fig. 7B). These data suggest that overexpression of Twist1 prevents cellular senescence induced by CADPE.

Discussion

It has been reported that during the last 30 years at least 70% of all drugs approved by the Food and Drug Administration for cancer have been derived from traditional medicine or natural sources (Yang et al., 2010). However, traditional medicines have not been popularly accepted mainly because of their poorly understood pharmacological mechanisms. CADPE is a natural compound isolated from the medicinal plants *S. glabra* (Sarcandra) and *T. pilosum* (Decne) and has been reported in the inhibition of hepatocellular carcinoma growth (Won et al., 2010), tumor angiogenesis (Jung et al., 2007), and gastric cancer metastasis (Han et al., 2010). However, its molecular mechanism remains largely unknown. Here, we report that, unlike CAPE, CADPE can inhibit human cancer cell growth and colony formation by inducing cellular senescence, not cell apoptosis in a dose-dependent manner. Using Western blot analysis, real-time PCR, and luciferase reporter gene assays, we demonstrated that CADPE significantly suppressed Twist1 expression at both the mRNA and protein levels. Furthermore, CADPE regulates the downstream key genes mediated by Twist1, including Ras, p53, p21<sup>WAF1/CIP1</sup>, p16<sup>INK4a</sup>, and pRB protein in a dose-dependent manner (Supplemental Fig. S5).

Previous studies of Twist1 have convincingly shown it associates with the metastatic dissemination of cancer cells through its ability to induce epithelial-mesenchymal transition (Yang et al., 2004). It has been reported that CADPE can inhibit gastric carcinoma cell invasion and migration (Han et al., 2010). In this study, we demonstrate that CADPE supresses Twist1 expression at both the protein and mRNA levels in gastric cancer cells, suggesting that CADPE inhibition of cell invasion and migration in gastric cancer cells is through the suppression of Twist1-mediated signaling pathways.

CAPE, which lacks two hydroxyl groups, has a wide spectrum of antitumor effects by inhibiting NF-κB pathway and inducing cell apoptosis (Watabe et al., 2004; Onori et al., 2009). Our data from Western blot analysis also confirm that CAPE can induce cleavage of apoptotic pathway marker proteins, such as PARP and caspase-3 and can induce the percentages of apoptotic cells in a dose-dependent manner by annexin V staining. However, as a structural analog of CAPE, CADPE has little effect on the induction of cell apoptosis and marker protein cleavage in different apoptosis assays. To further investigate the different effects of CAPE and CADPE on cancer cells, we compared the effects of cell senescence by CAPE or CADPE treatment. Our data show that CADPE can significantly induce cancer cell senescence by enhancing SA-β-gal activity, increasing marker gene expression (DE1), and inducing G1-phase cell-cycle arrest. However, CAPE has little effect in inducing cancer cell senescence, suggesting a different molecular mechanism from CADPE. It has been reported that apoptosis was associated with the reduction of NF-κB pathway activity, whereas senescence was associated with the activation of this pathway (Gupta et al., 2010; Rovillain et al., 2011; Zhi et al., 2011). Our previous data (Han et al., 2010) show that CADPE has little effect on the activity of the NF-κB pathway. Twist1 is strongly correlated with cell senescence by regulating both the p53-p21<sup>WAF1/CIP1</sup> pathway and p16<sup>INK4a</sup> pathway. Our data show that overexpression of Twist1 prevented CADPE-induced senescence in tumor cells, suggesting CADPE induces cancer cell senescence by suppressing Twist expression. Lo et al. (2007) and Cheng et al. (2008) independently reported that STAT3 can up-regulate Twist1 expression. CADPE can block STAT3 activation in cancer cells (Jung et al., 2007). Therefore, it is reasonable to suggest that CADPE can down-regulate Twist1 expression by suppressing STAT3 activation.

Both p53-p21<sup>WAF1/CIP1</sup> and p16<sup>INK4a</sup> pathway induction can lead to the inhibition of pRB phosphorylation by inhibiting CDK2/cyclin E and CDK4/6/cyclin D, respectively.
p21\(^{WAF1/CIP1}\) and p16\(^{INK4a}\) are likely to cooperate to keep pRB in a hypophosphorylated form during senescence. In our study, although CADPE correlated with the p53-p21\(^{WAF1/CIP1}\) pathway, CADPE had similar effects on inhibition of senescence and induction in p53 WT and p53\(^{K/-}\) cells, suggesting that in addition to the p53-p21\(^{WAF1/CIP1}\) pathway, CADPE can induce cellular senescence by triggering the p16\(^{INK4a}\) pathway.

In summary, several lines of evidence support a proposal that CADPE induces cancer cell senescence by regulating the Twist1-modulated p53-p21\(^{WAF1/CIP1}\) and p16\(^{INK4a}\) signaling pathways. Furthermore, our data show that CADPE has little effect on Twist1 low expression normal cells, suggesting that CADPE could be a useful therapeutic agent by targeting Twist1 expression. However, one must carefully evaluate the metabolism, dynamics, absorption, and toxicity of CADPE. The identification of CADPE property in future studies will provide a potential agent for the treatment of human malignant tumors.

Authorship Contributions

**Participated in research design:** Dong, Fang, Liu, Chen, Luo, and Liu.

**Conducted experiments:** Dong, Fang, Li, Zhang, Xie, Wu, and Lip. Zhang.

**Performed data analysis:** Dong, Fang, Liu, Chen, Luo, and Liu.

**Wrote or contributed to the writing of the manuscript:** Dong, Luo, and Liu.

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Caffeic acid 3,4-dihydroxy-phenethyl ester (CADPE) induces cancer cell senescence by suppressing Twist expression*

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Supplementary Data Figure Legend:

**Fig. S1.** A, Chemical structure of CADPE and CAPE. B, CADPE inhibited human cancer cells proliferation by SRB assay in HGC27 (gastric cancer) and U2OS (osteosarcoma). C, CAPE inhibited human cancer cells proliferation by SRB assay in AGS (gastric cancer), HCT116 (p53WT), HCT116 (p53-/-) (colon cancer), and H1299, A549 (lung cancer). D, CADPE induces U2OS cell senescence by SA-β-gal staining.

**Fig. S2.** CADPE can’t significantly induce cell autophagy. Cells (8X10^3 per well) were treated with different concentrations of CADPE for 72 hours, then fixed and stained with acridine orange. Photographed pictures (top) and statistical graphs (bottom) were shown. The scale bar represents 50 μm.

**Fig. S3.** CADPE regulates G1/S phase transition in synchronized HCT116 cells. HCT116 cells were synchronized with double-thymidine block. After remove thymidine, cells incubated with or without CADPE (10 μM) for 0, 3, 6 hours, respectively. The cells then were collected at the
indicated time points. Cell-cycle distributions of the cells were analyzed by FACS analysis.

*Columns*, mean; *bars*, SD. Thy: thymidine. *P*<0.01, **P**<0.001 versus control.

**Fig. S4.** EGFP transfection has no significant effect on human cancer cell senescence. Cells were seeded in 24-well plate and transfected with EGFP construct. After 24 hours, EGFP started to expression, cells then treated with CADPE (10 μM) for 3 days. Cells were fixed and stained with fresh SA-β-gal. Photographed pictures (left) and statistical graphs (right) were shown.

**Fig. S5.** Schematic diagram of mechanism by which CADPE induces human cancer cell senescence by modulating Twist1-mediated signaling pathway.
CADPE induces cancer cell senescence, Dong et al. Supplementary Data Fig. S1

A

Caffeic acid 3,4-dihydroxyphenethyl ester (CADPE)

Caffeic acid phenethyl ester (CAPE)

B

Cell Growth (%) vs. CADPE (µM)

C

Cell Growth (%) vs. CAPE (µM)

D

Cell Growth (%) vs. CAPE (µM)

HCT116+/+ vs. HCT116-/- vs. U2OS

SA-β-Gal positive cell (%) vs. CADPE (µM)
CADPE induces cancer cell senescence, Dong et al. Supplementary Data Fig. S2
CADPE induces cancer cell senescence, Dong et al. Supplementary Data Fig. S3
CADPE induces cancer cell senescence, Dong et al. Supplementary Data Fig. S4
CADPE induces cancer cell senescence, Dong et al. Supplementary Data Fig. S5

Diagram:
- CADPE
- Twist 1
- Ras
- p53
- p21
- p16
- p-Rb
- Senescence