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

Anliang Dong, Yuanzhang Fang, Li Zhang, Juan Xie, Xian Wu, Lipeng Zhang, Xiaoyuan Lian, Yihua Chen, Jian Luo, and Mingyao Liu

Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China (A.D., Y.F., L.Z., J.X., X.W., L.Z., X.L., Y.C., J.L., M.L.)

Alkek Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, Texas 77030, USA (M.L.)

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Correspondence should be sent to:

Dr. Mingyao Liu

The Institute of Biomedical Sciences,

East China Normal University.

500 Dongchuan Road

Shanghai 200241, China

Phone: +86-21-5434-5014

Fax: +86-21-5434-4922

Email: mliu@ibt.tamhsc.edu

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624 words in the Introduction,

832 words in the Discussion.

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Abbreviations:

CAPE: Caffeic acid phenethyl ester

CADPE: Caffeic acid 3, 4-dihydroxyphenethyl ester

SA-β-gal: Senescence-associated β-galactosidase

DEC1: Differentiated embryo-chondrocyte expressed gene 1

Ras: Rat sarcoma

RB: Retinoblastoma tumor suppressor

EMT: Epithelial-mesenchymal transition

SRB: Sulforhodamine B

TCA: Trichloroacetic acid

DMSO: Dimethyl sulfoxide

PBS: Phosphate-buffer saline

PI: Propidium iodide

PARP: poly ADP-ribose polymerase

PCR: Polymerase Chain Reaction

EGFP: Enhanced green fluorescence protein

FBS: Fetal bovine serum

DMEM: Dulbecco's modified Eagle's medium

HUVEC: Human umbilical vascular endothelial cells

ECGM: Endothelial cell growth medium

ANOVA: Analysis of Variance

IC_{50}: Half maximal (50%) inhibitory concentration (IC) of a substance

NF-kappaB: Nuclear factor kappaB

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Abstract

Comparing to traditional cytotoxic cancer therapy, therapy-induced cancer cell senescence attracts much interest due to its similar effective, less side effect, and more efficiently cleared by immune cells. In this study, we demonstrate that unlike CAPE (caffeic acid phenethyl ester), CADPE (caffeic acid 3,4-dihydroxy-phenethyl ester), which is isolated from medicinal plant *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-gal activity and DEC1 expression, and blocking cell-cycle arrest in G1 phase. To understand the underlying mechanisms, we show that CADPE significantly suppressed the expression of Twist1, and leads to the up-regulation of Ras, p53, $p21^{WAF1/CIP1}$, and $p16^{INK4a}$ proteins in a dose-dependent manner, and as a result, the hypophosphorylation of pRB. 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 Twist1-dependent senescence signaling pathway.

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Introduction

Traditional cancer therapy relies on cytotoxic treatment strategies, which bases on the hypothesis that complete cellular destruction of tumors prolongs the potential for patient survival (Ewald et al., 2010). This viewpoint has limited the 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). Recently, 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 non-senescent 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 utilizing 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 have offered similar or prolonged survival with fewer and less 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).

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The senescence phenomenon was first described by Hayflick and Moorhead in human fibroblasts after extensive culture and replicative exhaustion linked to telomere shortening (Hayflick and Moorhead, 1961). 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 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 cells can be visualized with a widely accepted and used marker, senescence-associated β-galactosidase (SA-β-gal) staining which stains the perinuclear compartment blue (Dimri et al., 1995a).

Ras proteins comprise a family of signal-transducing GTPases that are frequently mutated in human cancers. Besides 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 upregulation of cell proliferation inhibitors, including $p19^{ARF}$, p53, $p21^{WAF1/CIP1}$, and $p16^{INK4a}$, and as a result, leading to pRB hypophosphorylation and cell cycle arrest during cellular senescence.

Twist proteins are highly conserved basic helix-loop-helix (bHLH)

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transcription factors that have important regulatory functions during embryogenesis and cancer cell epithelial-mesenchymal transition (EMT). Twist1 is overexpressed in numerous carcinomas, sarcomas, gliomas, neuroblastomas, and melanomas (Maestro et al., 1999; Hoek et al., 2004; Valsesia-Wittmann et al., 2004; Elias et al., 2005; Ansieau et al., 2008). Recently, 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 (Ansieau et al., 2008). Moreover, the reduction in Twist1 expression coincided with a specific increase in $p16^{INK4a}$ expression and was accompanied by the induction of senescence-associated SA-β-gal activity (Lee and Bar-Sagi). Therefore, Twist1 could be a new promising target to induce cellular senescence in cancer cells. In this study, we demonstrate that CADPE inhibit 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 both in protein and mRNA levels. The inhibition of Twist1 expression by CADPE leads to the up-regulation of Ras, p53, $p21^{WAF1/CIP1}$, and $p16^{INK4a}$ proteins in a dose-dependent manner, and as a result, the hypophosphorylation of RB and the arrest of cell cycle in G1 stage during senescence.

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Materials and Methods

Materials and cells

CAPE was purchased from Sigma-Aldrich. CADPE was synthesized by Dr. Tang Jie (ECNU) according to previously described (Zhang et al.), and the purity is more than 98%. Antibody against p53, $p21^{WAF1/CIP1}$, Ras, pRb, Rb, caspase-3, PARP, $p16^{INK4a}$, Cyclin E were purchased from Cell Signaling Technology. EGFP-Twist1- FLAG and EGFP-FLAG constructs were kindly gift from Dr. Wang Ping (ECNU). Twist1-luc construct described previously (Qin et al., 2009) is kindly gift from Dr. Xu Jianming (Baylor College of Medicine, USA). p53-luc, p21-luc construct was purchased from Stratagene. Human HCT116, AGS, HGC27,U2OS, H1299 cells obtained from the China Type Culture Collection (Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS). Primary human umbilical vascular endothelial cells (HUVEC) were cultured in endothelial cell growth medium (ECGM):M199 medium (Invitrogen) as described previously (Pang et al., 2009).

Cell viability and colony formation assays

To examine the cell viability assay, we performed SRB assay as previously described (Yang et al., 2010). Briefly, $5X10^3$ cells per well were plated into 96-well plates

treated with different concentrations of CADPE for 72 hours. The SRB assay is terminated by the addition of cold TCA. Cells are fixed *in situ* by the gentle addition of 50 µl of cold 50% TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4°C. The supernatant is discarded, and the plates are washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µl) at 0.4% in 1% acetic acid is added to each well, and plates are incubated for 10 minutes at room temperature. Absorbance was measured with a VERSAmax microplate reader. For colony formation assay, cells were plated into 6 cm dish in triplicate. After treated with different concentrations of CADPE for 21 days, the cells were stained with 0.5% crystal violet (dissolved in 95% ethanol). The number of colonies were photographed and counted to generate the histograms.

Senescence-associated β-galactosidase staining assays

Cells were plated at varying cell densities in 6-well plates. After incubated with CADPE for 72 h, and cells then fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS 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-gal as previously described (Dimri et al., 1995b).

Live and Dead assay

To assess the apoptosis effect of CADPE, we performed Live/Dead assay as previously described (Li et al., 2010). Briefly, $1X10^5$ 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 calcein-AM) at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Leica).

AnnexinV/PI staining assays

To detect whether CADPE induces cell apoptosis, we stained the treated cells with AnnexinV kit (sigma) as previously described . Briefly, $1X10^6$ AGS and HCT116 cells treated with different concentrations of CADPE for 72 hours, and then subjected to AnnexinV/PI staining. The results were analyzed with a flow cytometer (FACSAria; BD Biosciences).

Cell-cycle analysis

To determination of cell-cycle distribution, the cells were then treated with 2 mM thymidine (Sigma) for 12 h, and released into DMEM growth media for 10 hour, then secondary thymidine block for 12 h, finally release cells into DMEM growth medium or treated with 10μM CADPE. The cells were harvested after release in the indicated time, then fixed with 70% cold ethanol and stained with 50 mg/ml propidium iodide

(PI) (Sigma) followed with RNase A (Sigma) treatment for 30 min at room temperature. DNA content was analyzed by FACscan cell analyzer (BD Biosciences) equipped with Cellquest software (BD Biosciences). The population of cells in each phase was determined using ModFit LT software (BD Biosciences).

Real-Time PCR

SYBR green quantitative PCR was performed using GAPDH as an internal control gene. Trans-species primer pairs were 5′-GCAGGACGTGTCCAGCTC-3′ and 5′-CTGGCTCTTCCTCGCTGTT-3′ for Twist1, 5′-GGCGGGGAATAAAACGGAGCGA-3′ and 5′-CCTCACGGGCACAAGTCTGGAA-3′ for DEC1, 5'-CCCACACCTTCCCTAAAATG-3' and 5'-TCCCAAGGCAGTAAACATCC -3' for hRas, 5' -GGAAGACCATGTGGACCTG-3' and 5'-AAGGCAGAAGATGTAGAGCG-3' for $p21^{WAF1/CIP1}$, and 5'-TCTGACTTCAACAGAGACAC -30 and 5'- TCTTCCTCTTGTGCTCTTGC -3′ for GAPDH. The quantitative PCR method was used to analyze genes expression in AGS cell lines is followed the manufacturer's protocol (Mx3005P QPCR System, Stratagene)

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Luciferase Reporter gene assays and cell transfection

A dual-luciferase assay was performed in triplicate according to previously described (Li et al., 2011). Briefly, $2X10^4$ cells were placed on 24-well plates 24 hours before transfection. Twist1/p53/p21 luciferase reporters (0.5 μg) and 0.05 μg of the *Renilla* luciferase assay vector pRL (Promega) 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 luminometer (BMG LUMIstar). Luciferase activity was normalized with the 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 indicated time. Cell lysates were prepared in Radioimmunoprecipitation assay (RIPA) buffer (20 mmol/LTris, 2.5 mmol/L EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mmol/L NaF, 10 mmol/L Na4P2O7, and 1 mmol/L phenylmethylsulfonyl fluoride). Aliquots of cellular protein were electrophoresed on SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk in PBS containing 0.1% Tween 20

and then reacted with specific antibodies. Detection of specific proteins was carried out with an enhanced chemiluminescence (Amersham-Pharmacia).

Statistical analysis

The results are presented as mean ± SE, and statistical comparisons between groups were done using one-way ANOVA followed by Student's t test. $P \leq 0.05$ was considered statistically significant.

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Results

CADPE inhibits human cancer cell growth

To determine the effects of CADPE, a natural compound recently isolated from the medicinal plant *Sarcandra Glabra* (Sarcandra) and *Teucrium pilosum* (Decne) (El-Mousallamy et al., 2000) (Supplemental Fig. S1A), on human cancer cell growth, we treated 7 different human cancer cell lines, including AGS, HGC27 cells (gastric cancer), H1299, A549 cells (lung cancer), HCT116 p53 WT and p53 -/- cells (Colon Cancer), and U2OS cells (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 the cancer cell lines (Fig. 1A and Supplemental Fig. S1B). Interesting, the primary cultured normal HUVEC cells were only slightly affected by CADPE treatment, with an IC_{50} of > 50 μM. Similar results were obtained from cell number count (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 formation in a dose-dependent manner (Fig. 1C), suggesting that CADPE is an effective agent in suppressing human cancer cell growth.

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CADPE has little effect on cancer cell apoptosis

Because CADPE shares 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 figure 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 has no effect to 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. As shown in figure 2B and 2C, CADPE has little effect to induce human cancer cell apoptosis in both anexin-V FACS analysis (Fig. 2B) and live/dead assays (Fig. 2C). However, CAPE can 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 due to the induced cell apoptosis, which is different from the inhibitory effect of CAPE on cell apoptosis.

CADPE induces cellular senescence in human cancer cells

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In our initial observation, most cell phenotypes in the present of CADPE were characterized by enlarged cellular size and nucleus, fattened and vacuolated cellular morphology (Fig. 1B and 2C), which is strongly associated with cell senescence (Ewald et al., 2009). To examine this hypothesis, we examined whether CADPE induces cellular senescence by the SA-β-gal staining, one of the best known markers for detecting senescence. As expected, CADPE can induce SA-β-gal activity (Fig. 3A, 3B, 6A, 6B and Supplemental Fig. S1D) in a dose-dependent manner in all 7 cancer cell lines tested, but CAPE cannot (Fig. 3A, 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 figure 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 CAPDE 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

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morphology, increased senescence-associated SA-β-gal activity, induction of the cellular senescence marker DEC1, and finally, 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 using different approaches in the absence or presence of CADPE. First, we treated three different human cancer cell lines with 10 μM CADPE for indicated time course and subjected to flow cytometric analysis. As shown in figure 4A and 4B, the percentage of G1 phase cells was significantly increased in CADPE treated cells. Correspondingly, the percentage of cells in S or G2/M phase was reduced when compared with the control cells. Second, we tested whether cells accumulate at G1 phase by CADPE using "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 G1 phase more significantly, particularly at the 3 hour time point, accompanying with a decrease in S or G2/M phase compared to the control cells (Fig. 4C and Supplemental Fig. S3). Finally, using Western blot analysis, we examine whether CADPE affected the expression of cyclin E in released synchronized AGS cells, which is essentially for the transition from G1 to S phase and only expressed at the end of G1 phase. Our results indicated that CADPE prolonged cyclin E expression

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after synchronized cancer cells release, suggesting that cells treated with CADPE accumulate at G1 phase compared to the control cells. Together, all our data suggest that CADPE strongly induce G1 phase cell-cycle arrest in human cancer cells, which is consistent with previous observation that inhibition of cell growth by CADPE is due to the induction of cellular senescence and cell-cycle arrest. To further confirm if CADPE can permanently halt the proliferative capacity of cancer cells, we treated AGS cancer cell with CADPE for 72 hours, and removed CADPE or continue treating the cells with CADPE for another 72 hours. Our results indicated that there is no significant difference between the persistent CADPE-retreated cells and the CADPE-withdrew cells (Fig. 3D), suggesting CADPE-induced cell senescence can't be reversed even when the drug is removed.

CADPE induces cellular senescence by regulating 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 to override premature senescence by regulating both Ras-p53 pathway and p16 pathway (Serrano et al., 1997). We first examined the expression levels of Twist1 in five cancer cell lines and

the primary cultured Human Umbilical Vein Endothelial Cell (HUVEC). Consistent with previously report (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 as compared with HUVECs (Fig. 5A). CADPE markedly suppressed Twist1 expression both at the protein level (Fig. 5B) and mRNA level (Fig. 5C) in a concentration-dependent manner. To further confirm CADPE inhibits Twist1 expression, we next assessed the effects of CADPE on Twist1 promoter by luciferase reporter gene assay. As shown in figure 5D, CADPE suppressed Twist1 promoter activity in a dose dependent manner. The IC_{50} for CADPE inhibition of Twist1 expression and promoter activity is approximately10 μM, which is consistent with the IC_{50} of CADPE inhibition of cell growth.

To examine the down-stream 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 has been recently 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 increase Ras and p53 expression in a dose-dependent manner (Fig. 5B). Similar results were obtained at the mRNA levels for *Ras* and *p21* expression by Real-time PCR analysis and by promoter luciferase

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reporter gene assays of p53 and p21, suggesting that CADPE can trigger both Ras-p53-$p21^{WAF1/CIP1}$ pathway and $p16^{INK4a}$ pathway. As a result of the two pathways activated by CADPE, the phosphorylation of Rb was strongly suppressed in a concentration-dependent fashion (Fig. 5B). Together, our data suggest that CADPE induce cellular senescence by regulating Twist1-modulated p53 and p16 pathways. To further confirm whether the induction of senescence by CADPE is dependent of p53 or not, we chose two pairs of cell lines, A549 (p53WT) and H1299 (p53-/-), HCT116 (p53WT) and HCT116 (p53-/-), to perform cell senescence staining. As shown in figure 6A and 6B, there is no significant difference between the p53 WT and $p53^{-/-}$ cell lines of the 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 figure 7A and Supplemental Figure 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-

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overexpressed AGS and H1299 cells, respectively (Fig. 7B). These data suggest that overexpression of Twist1 prevents cellular senescence induced by CADPE.

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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 medicine has not been popularly accepted mainly due to their poorly-understood pharmacological mechanisms. CADPE is a natural compound isolated from the medicinal plant *Sarcandra Glabra* (Sarcandra) and *Teucrium pilosum* (Decne), and has been reported in the inhibition of hepatocellular carcinoma growth (Won et al.), 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, different with 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 both at mRNA and protein levels. Furthermore, CADPE regulates the downstream key genes mediated by Twist1, including Ras, p53, $p21^{WAF1/CIP1}$, $p16^{INK4a}$, and pRB protein in a dose-dependent manner (Supplemental Fig. S5).

Previous studies of Twist1 have been convincingly shown to associate with the metastatic dissemination of cancer cells through its ability to induce epithelial-

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mesenchymal transition (EMT) (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 suppress Twist1 expression both in 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-kappaB pathway and inducing cell apoptosis (Watabe et al., 2004; Onori et al., 2009). Our data also confirm that CAPE can induce cleavage of apoptotic pathway marker proteins, such as PARP and caspase-3 using Western blot analysis, and can induce the percentages of apoptotic cells in a dose-dependent manner by annexin V staining. However, as a structural analogue 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 (DEC1), and inducing G1 phase cell-cycle arrest. However, CAPE has little effect in inducing cancer cell senescence, suggesting a different molecular mechanism from

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CADPE. It has been reported that apoptosis was associated with the reduction of NF-κB pathway activity while senescence was associated with the activation of this pathway (Gupta et al.; Rovillain et al.; Zhi et al.). Our previous data (Han et al., 2010) and unpublished studies show that CADPE has little effect on the activity of NF-κB pathway. Twist1 is strongly correlated with cell senescence by regulating both p53-$p21^{WAF1/CIP1}$ pathway and $p16^{INK4a}$ pathway. Our data showed that overexpression of Twist1 prevented CADPE-induced senescence in tumor cells, suggesting CADPE induces cancer cell senescence by suppressing Twist expression. Cheng and Lo groups independently reported that STAT3 can up-regulate Twist1 expression (Lo et al., 2007; Cheng et al., 2008), and 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-$p21^{WAF1/CIP1}$ pathway and $p16^{INK4a}$ pathway induction can lead to the inhibition of pRB phosphorylation by inhibiting CDK2/Cyclin E and CDK4/6/Cylin 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 p53-$p21^{WAF1/CIP1}$ pathway, CADPE has similar effects on growth inhibition and senescence induction in p53WT and $p53^{-/-}$ cells, suggesting that besides

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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 induce cancer cell senescence by regulating 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 our future studies will provide a potential agent for the treatment of human malignant tumors.

Authorship Contributions

Participated in research design: A.D., Y.F., X.L., Y.C., J.L., M.L.

Conducted experiments: A.D., Y.F., L.Z., J.X., X.W., L.Z.,

Performed data analysis: A.D., Y.F., X.L., Y.C., J.L., M.L.

Wrote or contributed to the writing of the manuscript: A.D., J.L., M.L.

Others: J.L., and M.L. acquired funding for the research.

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FOOTNOTES

A.D. and Y.F. have contributed equally to this work. J.L. is the co-correspondence: The Institute of Biomedical Sciences, East China Normal University. 500 Dongchuan Road, Shanghai 200241, China. Phone: +86-21-5434-5016 Fax: +86-21-5434-4922, Email: jluo@bio.ecnu.edu.cn

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Figure Legends

Figure 1. CADPE inhibits human cancer cell proliferation and colony formation.

A, CADPE inhibited human cancer cells proliferation by SRB assay in AGS (gastric cancer), HCT116 (p53WT) and HCT116 (p53-/-) (colon cancer), and H1299, A549 (lung cancer), but not normal cell HUVEC. Cells ($5X10^3$ per well) were treated with different concentrations of CADPE for 72 hour. Cell viability was quantified by SRB assay as described in the Materials and Methods.

B, Inhibition of AGS cell proliferation by CADPE. AGS cells ($5X10^3$ per well) were treated with different concentrations of CADPE. After 72 hours, cells were photographed (left) and the cell number was counted (right). *Columns*, mean; *bars*, SD. The scale bar represents 50 μm.

C, CADPE prevented colony formation in both AGS and HGC27 cell lines. Cells were seeded in 35mm dishes ($1X10^3$ cells per dish) with indicated CADPE concentrations. Medium was change every 2 day. After 21 day, cells were fixed and stained with a 0.5% crystal violet solution. Colonies in wells were imaged (left) and the colony number was counted (right, colonies containing >50 cells scored as positive). *Columns*, mean; *bars*, SD.

Figure 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 indicated concentration for 72 hour. Whole cell extracts were prepared and subjected to Western blot analysis using anti-PARP, anti-caspase3, anti-cleaved caspase3, and beta-actin antibodies, respectively.

B, CADPE could not induce cell apoptosis by annexin V staining in AGS and HCT116 cells using CAPE as positive controls. Cells ($8X10^3$ per well) were treated with different concentrations of CADPE or positive control (CAPE) for 72 hours, then stained with annexin-V-FITC and analyzed by flow cytometry for early apoptotic effects as mentioned in the Materials and Methods. The percentage indicates the ratio of early apoptotic cells/total cells. All of the results shown are representative of 3 independent experiments.

C, CADPE could not induce cell death in AGS and HGC27 cells using Live/Dead assays. Cells ($5X10^3$ per well) were treated with the indicated concentrations of CADPE for 72 hours. The live (green) or dead (red) cells were stained with Live and dead assay kit as mentioned in Materials and Methods and then analyzed under a fluorescence microscope. The percentage indicates the ratio of dead/live cells. All of the results shown are representative of 3 independent experiments. The scale bar

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represents 50 μm.

Figure 3. Induction of cellular senescence by CADPE, but ont 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 hour. Cells ($20X10^3$ per well) were fixed and stained with fresh SA-β-gal as described in the Materials and Methods. Photographed pictures (A) and statistical graphs (B) were shown. The scale bar represents 50 μm.

C, CADPE induced senescence marker *DEC1* mRNA expression in AGS cells. AGS cells ($8X10^3$ per well) were incubated with the indicated concentrations of CADPE for 72 hours. *DEC1* transcription levels were assessed by real-time PCR as mentioned in the Materials and Methods. *Columns*, mean; *bars*, SD.

D, CADPE permanently disabled the proliferative capacity of cancer cells. AGS cancer cells were treated with CADPE for 72 hours, and removed CADPE or continue treating with CADPE for another 72 hours. Cell viability was quantified by SRB assay as described in the Materials and Methods.

Figure 4. CADPE induces G1 phase cell-cycle arrest in human cancer cells.

A, CADPE inhibited the progression of cell cycle through G1 phase arrest in AGS, HCT116, and HGC27 cells. Cancer cells were treated with or without CADPE (10 μM) for 12 hour, 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 hours, and cell cycle distribution was monitored by flow cytometry analysis. The cells in G1 phase were analyzed by FACS analysis in 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 remove thymidine, cells incubated with or without CADPE (10 μM) for 0, 3, 6, 12 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.

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 hour up to 24 hours. Whole cell extracts were collected at the indicated time points and subjected to Western blot analysis using indicated antibodies.

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Figure 5. CADPE induces cellular senescence through regulation 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, $p21^{WAF1/CIP1}$, $p16^{INK4a}$, p-Rb (Ser795). AGS cells were incubated with the indicated concentrations of CADPE for 72 hours. Whole cell extracts were prepared and subjected to Western blot analysis using indicated antibodies. □-actin is used as a loading control.

C, CADPE regulated mRNA expression levels of Twist1, hRas, and $p21^{WAF1/CIP1}$ in AGS cells. The cells were incubated with the indicated concentrations of CADPE for 72 hrs, mRNA levels were then measured by real-time PCR as mentioned in Materials and Methods.

D, CADPE acted as transcriptional regulator of *Twist1*, *p53* and $p21^{WAF1/CIP1}$ genes. AGS cells were transiently transfected with indicated luciferase reporter gene and treated with the indicated concentrations of CADPE for 72 hours. Cell supernatants then were collected and assayed for luciferase activity as described in Materials and Methods. *Columns*, mean; *bars*, SD.

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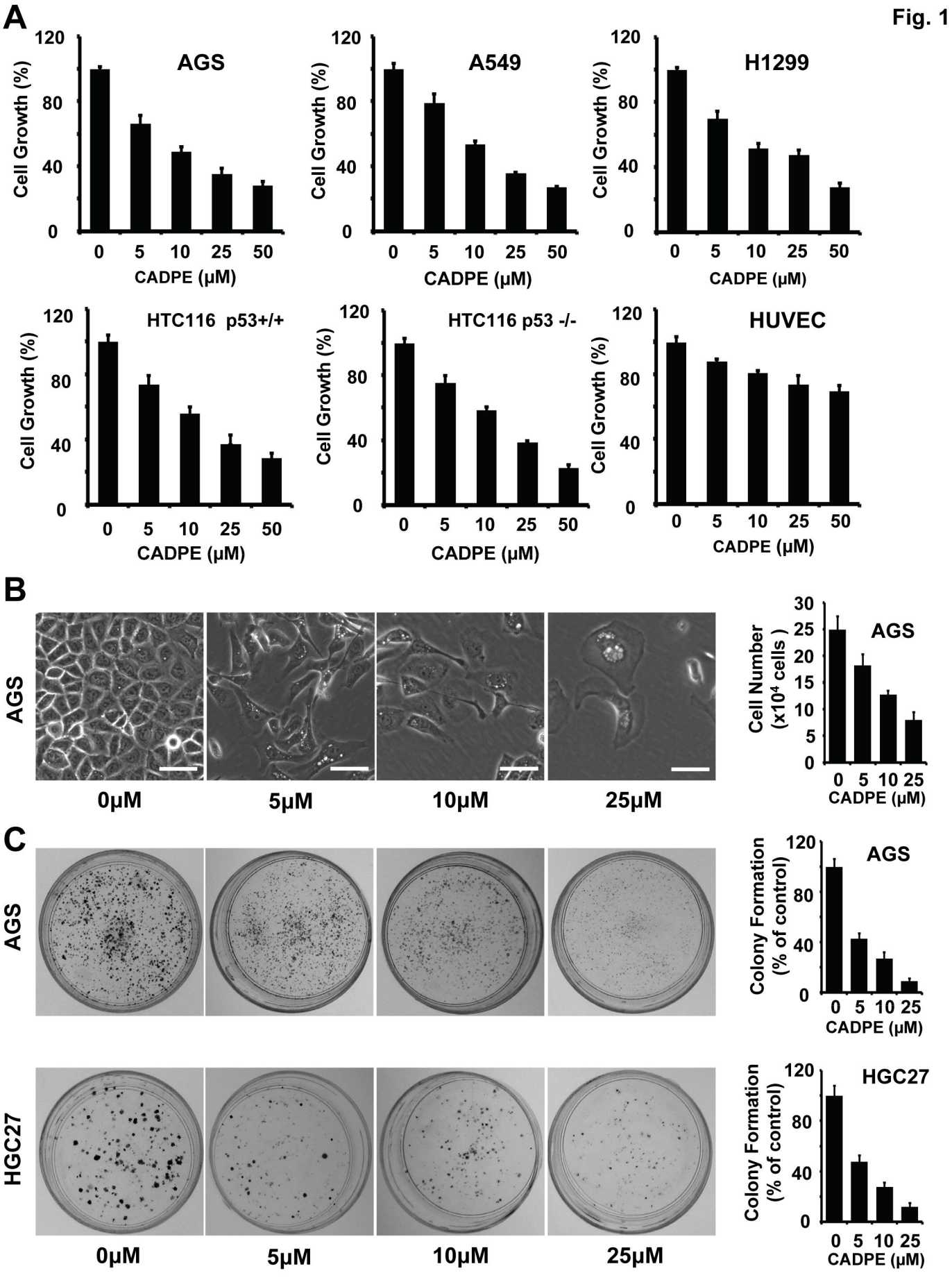
Figure 6. Induction of senescence by CADPE is dependent of p53.

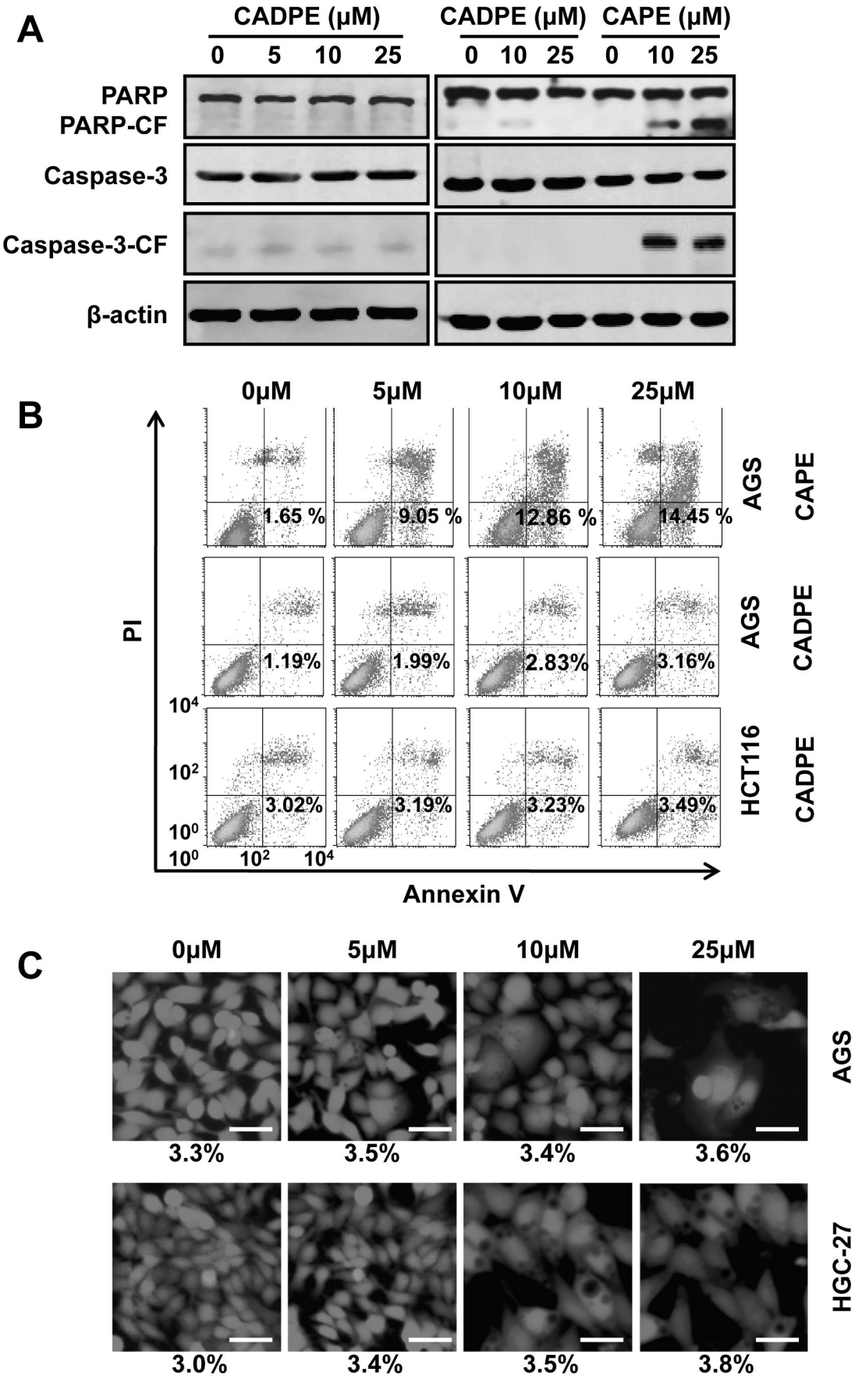
A and B, SA-β-gal staining of two pairs of p53 WT and -/- cell lines, A549 (WT) and H1299 (-/-), HCT116 (WT) and HCT116 (-/-) cancer cells after treatment with the indicated concentrations of CADPE or CAPE for 72 hour. Cells ($20X10^3$ per well) were fixed and stained with fresh SA-β-gal as described in the Materials and Methods. Photographed pictures (A) and statistical graphs (B) were shown. The scale bar represents 50 μm.

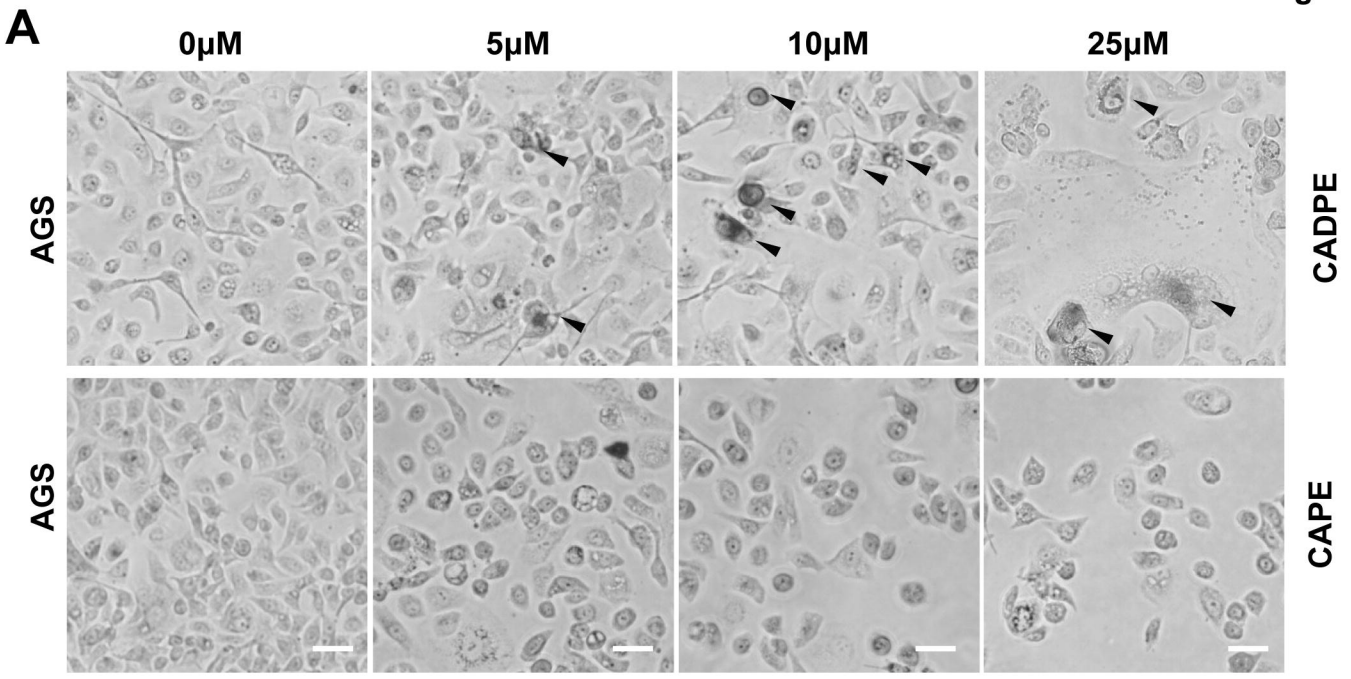
Figure 7. Overexpression of Twist1 prevents CADPE-induced cellular senescence.

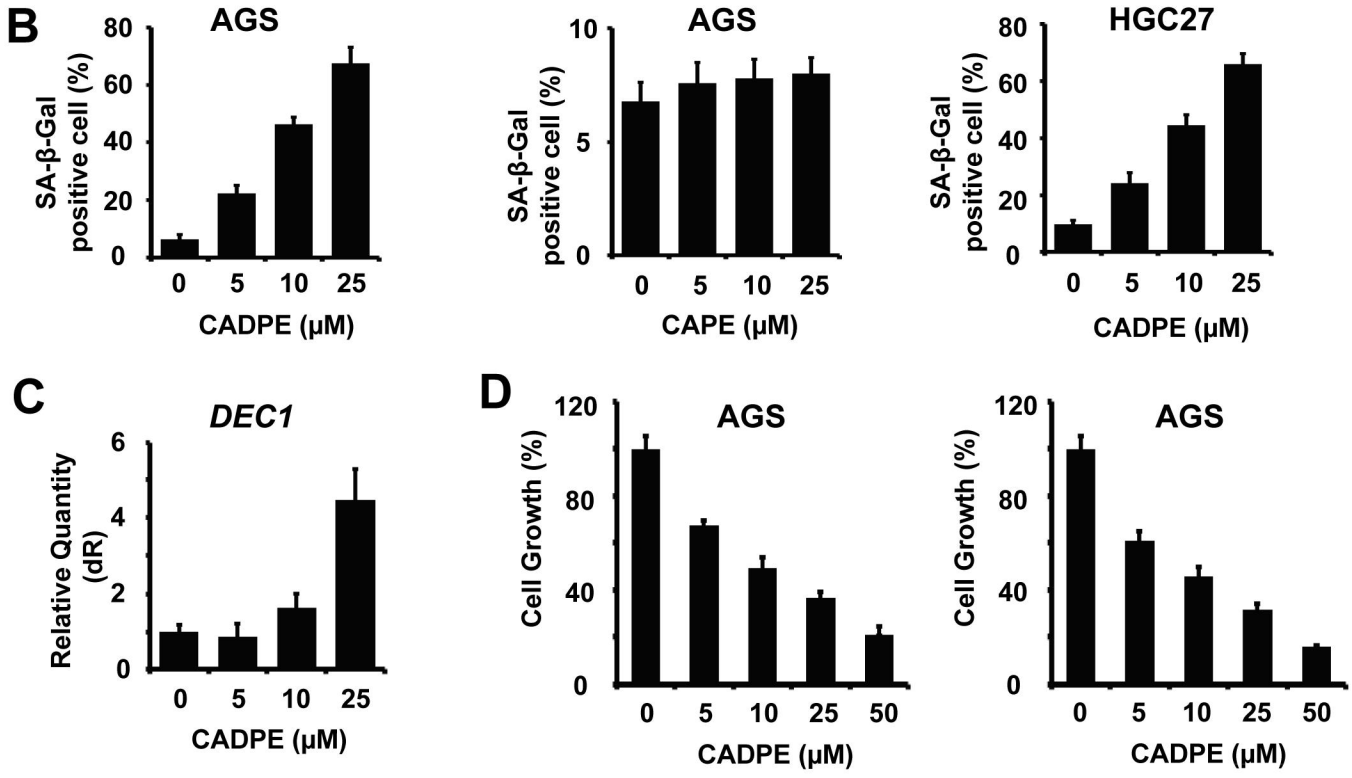
A and B, Overexpression of Twist1 inhibited CADPE-induced cellular senescence in AGS and H1299 cells. Cells were seeded in 24-well plate and transfected with EGFP-Twist1. After 24 hrs, EGFP started to expression, cells then treated with CADPE (10 μM) for 3 days. Cells were fixed and stained with fresh SA-β-gal. Photographed pictures (A) and statistical graphs (B) were shown. White arrows represent EGFP-Twist1 overexpressed cells but SA-β-gal staining negative cells. Black arrows indicate SA-β-gal staining positive but EGFP-Twist1 negative cells (A). *Columns*, mean; *bars*, SD. The scale bar represents 50 μm. ***P<0.001.

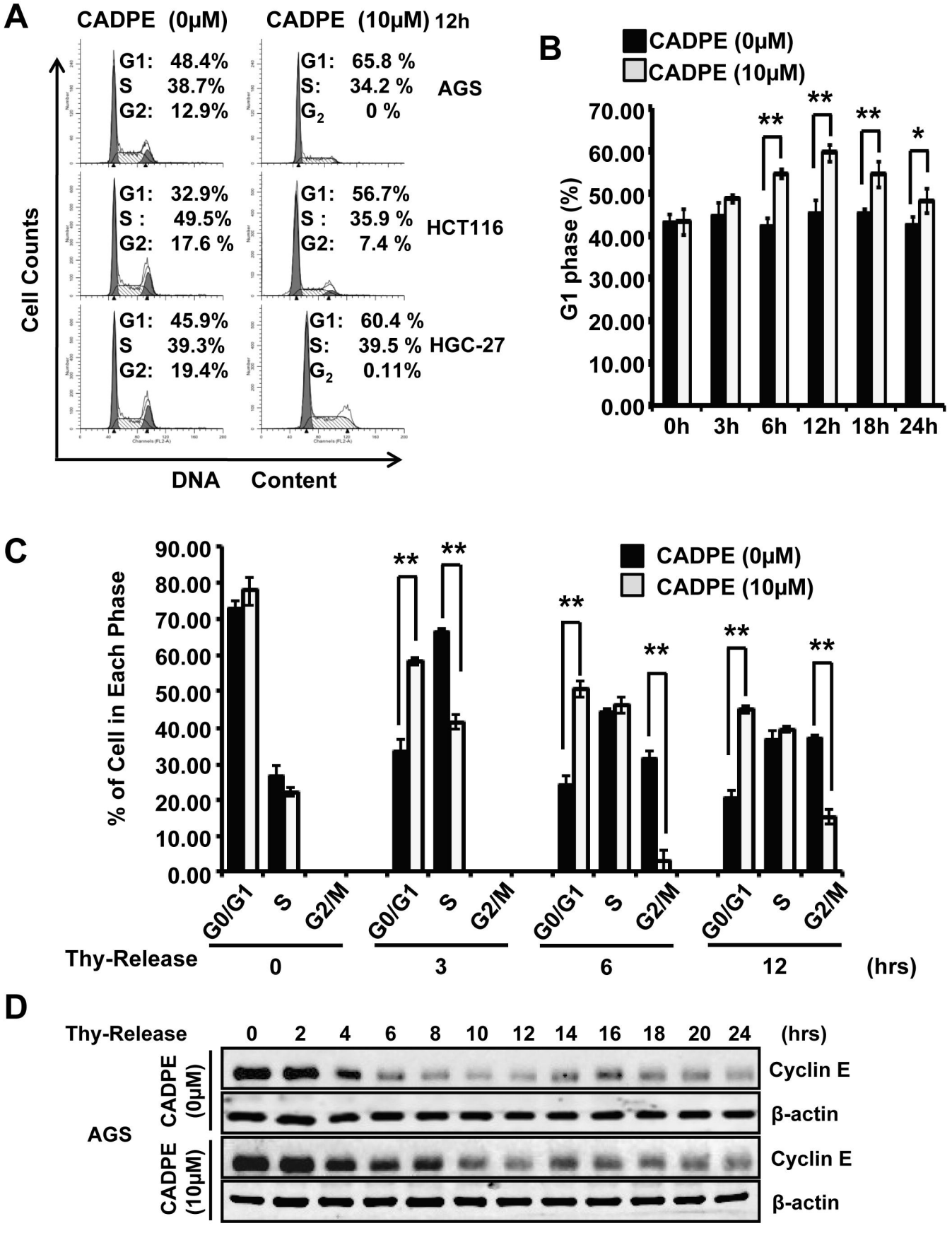
Fig. 1


A
AGS
A549
H1299
HTC116 p53+/+
HTC116 p53 -/-
HUVEC
Cell Growth (%)
120
80
40
0
0 5 10 25 50
CADPE (μM)
B
AGS
0μM
5μM
10μM
25μM
Cell Number (x10⁴ cells)
30
25
20
15
10
5
0
0 5 10 25
CADPE (μM)
C
AGS
HGC27
Colony Formation (% of control)
120
80
40
0
0 5 10 25
CADPE (μM)


A
CADPE (μM)
0 5 10 25
CADPE (μM)
0 10 25
CAPE (μM)
0 10 25
PARP
PARP-CF
Caspase-3
Caspase-3-CF
β-actin
B
0μM
5μM
10μM
25μM
AGS
CAPE
1.65 %
9.05 %
12.86 %
14.45 %
AGS
CADPE
1.19%
1.99%
2.83%
3.16%
HCT116
CADPE
3.02%
3.19%
3.23%
3.49%
PI
Annexin V
10^4
10^2
10^0
10^0
10^2
10^4
C
0μM
5μM
10μM
25μM
AGS
3.3%
3.5%
3.4%
3.6%
HGC-27
3.0%
3.4%
3.5%
3.8%


A
0μM
5μM
10μM
25μM
AGS
AGS
CADPE
CAPE


B
AGS
SA-β-Gal positive cell (%)
CADPE (μM)
AGS
SA-β-Gal positive cell (%)
CAPE (μM)
HGC27
SA-β-Gal positive cell (%)
CADPE (μM)
C
DEC1
Relative Quantity (dR)
CADPE (μM)
D
AGS
Cell Growth (%)
CADPE (μM)
AGS
Cell Growth (%)
CADPE (μM)


A
CADPE (0μM)
CADPE (10μM) 12h
G1: 48.4%
S 38.7%
G2: 12.9%
G1: 65.8 %
S: 34.2 %
G2 0 %
AGS
G1: 32.9%
S : 49.5%
G2: 17.6 %
G1: 56.7%
S : 35.9 %
G2: 7.4 %
HCT116
G1: 45.9%
S 39.3%
G2: 19.4%
G1: 60.4 %
S: 39.5 %
G2 0.11%
HGC-27
Cell Counts
DNA Content
B
CADPE (0μM)
CADPE (10μM)
G1 phase (%)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0h 3h 6h 12h 18h 24h
C
CADPE (0μM)
CADPE (10μM)
% of Cell in Each Phase
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
G0/G1 S G2/M
Thy-Release
0 3 6 12 (hrs)
D
Thy-Release
0 2 4 6 8 10 12 14 16 18 20 24 (hrs)
AGS
CADPE (0μM)
CADPE (10μM)
Cyclin E
β-actin

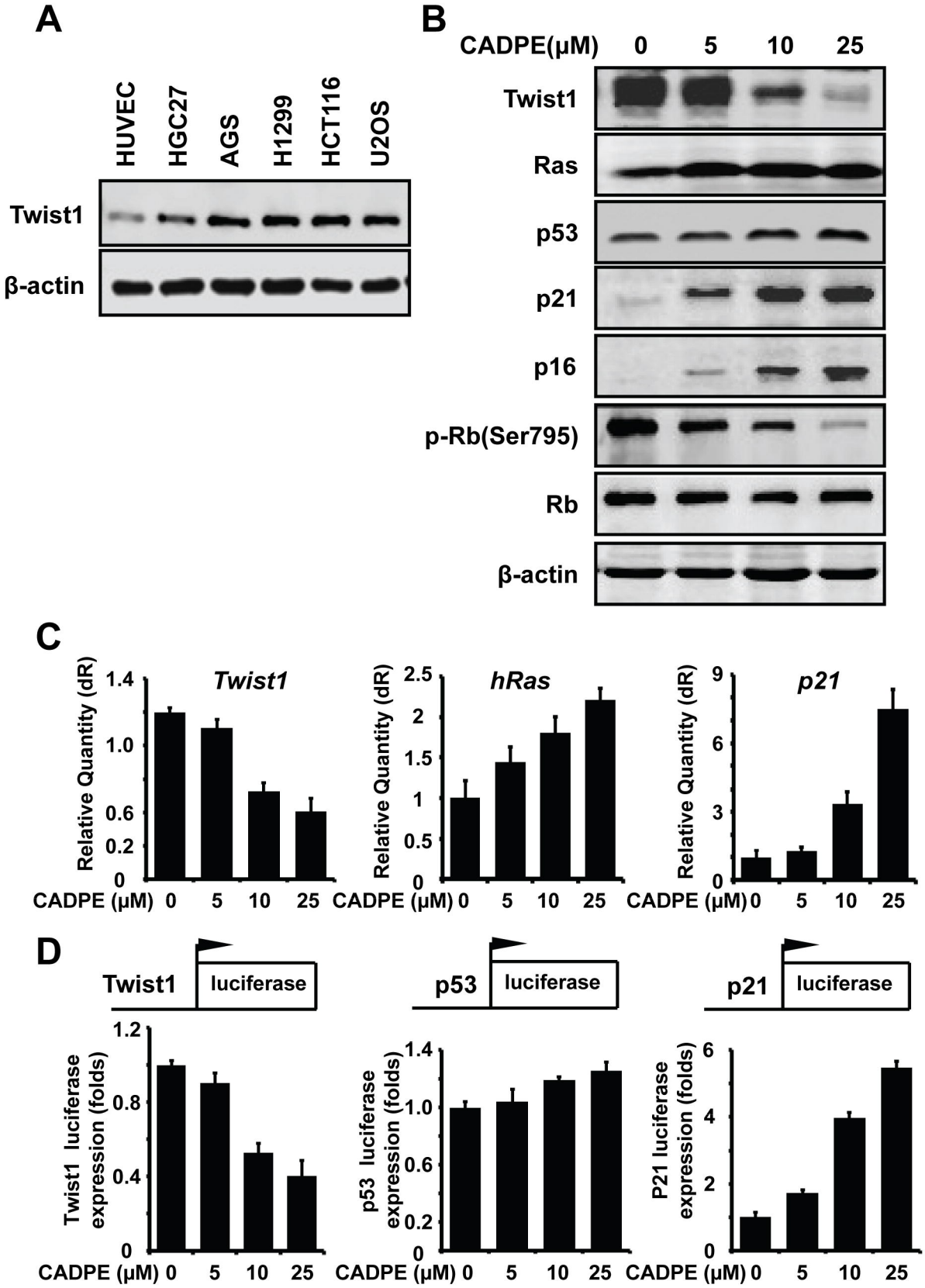
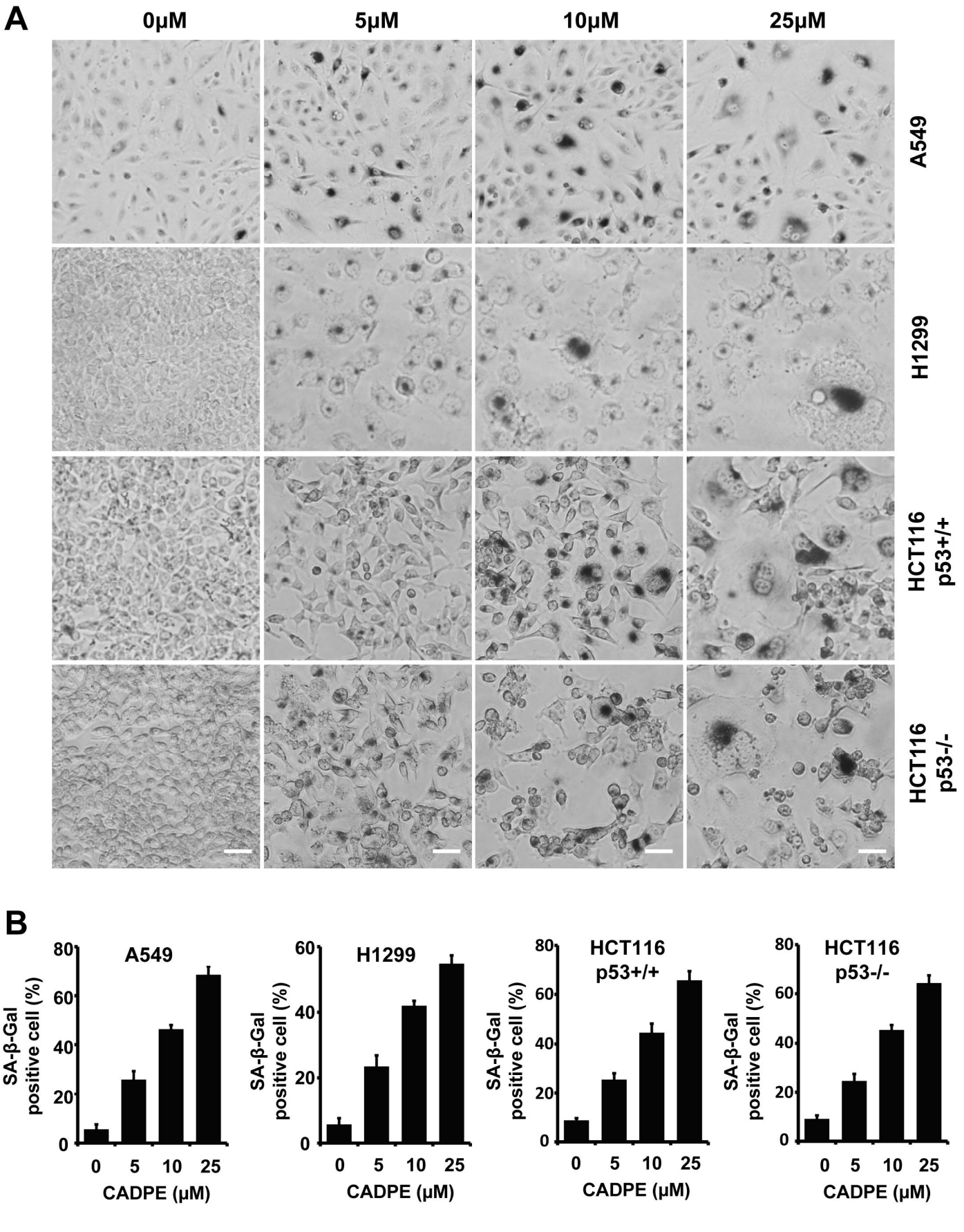
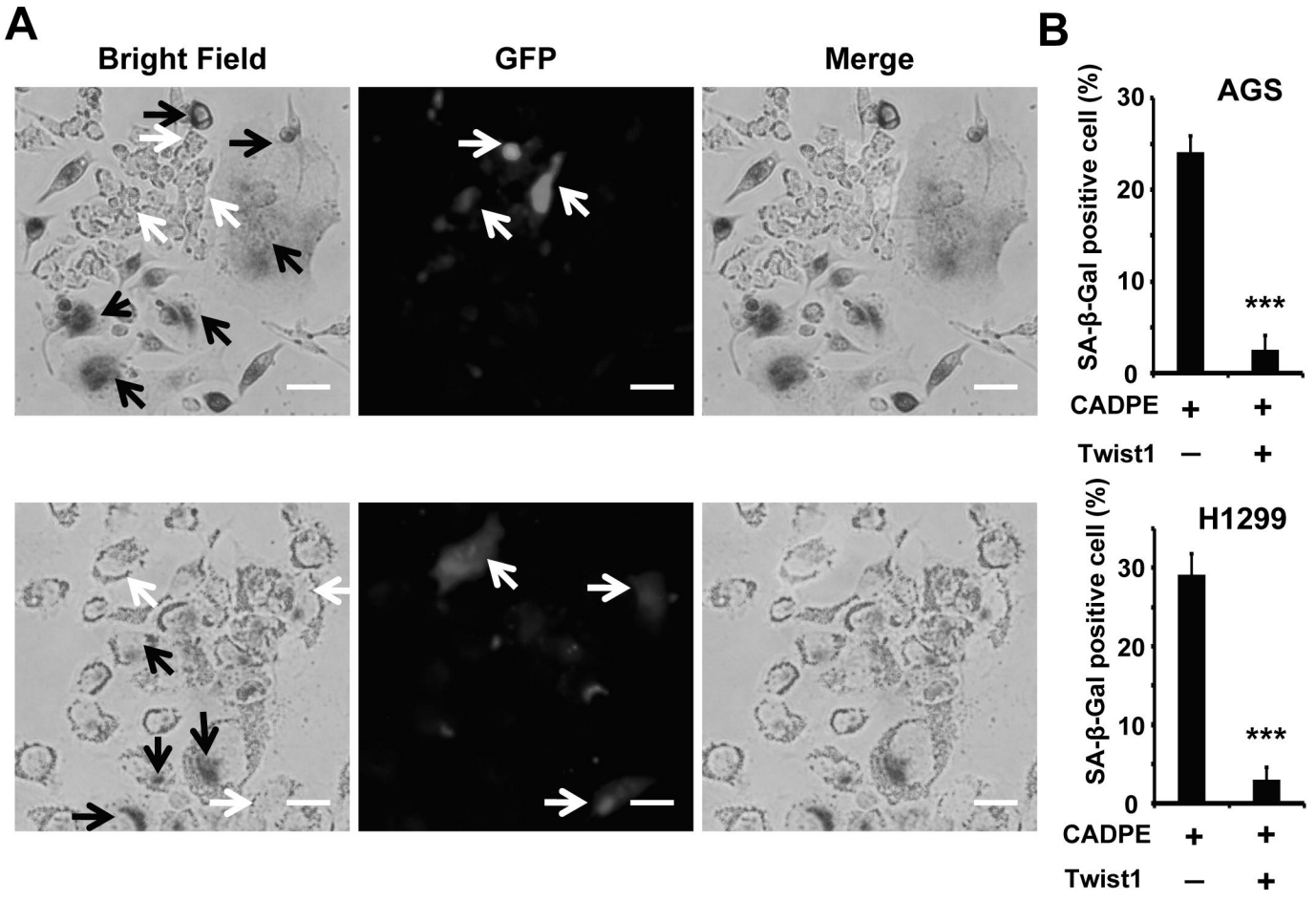

A
HUVEC
HGC27
AGS
H1299
HCT116
U2OS
Twist1
β-actin
B
CADPE(μM) 0 5 10 25
Twist1
Ras
p53
p21
p16
p-Rb(Ser795)
Rb
β-actin
C
Twist1
hRas
p21
Relative Quantity (dR)
CADPE (μM) 0 5 10 25
D
Twist1 luciferase
p53 luciferase
p21 luciferase
Twist1 luciferase expression (folds)
p53 luciferase expression (folds)
P21 luciferase expression (folds)
CADPE (μM) 0 5 10 25

Fig. 6


A
0μM
5μM
10μM
25μM
A549
H1299
HCT116 p53+/+
HCT116 p53-/-
B
A549
H1299
HCT116 p53+/+
HCT116 p53-/-
SA-β-Gal positive cell (%)
CADPE (μM)
0
5
10
25
20
40
60
80


A
Bright Field
GFP
Merge
B
AGS
SA-β-Gal positive cell (%)
30
20
10
0
***
CADPE + +
Twist1 – +
H1299
SA-β-Gal positive cell (%)
30
20
10
0
***
CADPE + +
Twist1 – +