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2′-Hydroxycinnamaldehyde inhibits proliferation and induces apoptosis via signal transducer and activator of transcription 3 inactivation and reactive oxygen species generation

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Inhibition of the signal transducer and activator of transcription 3 (STAT3) signaling pathway is a novel therapeutic strategy to treat human cancers with constitutively active STAT3. During the screening of natural products to find STAT3 inhibitors, we identified 2′-hydroxycinnamaldehyde (HCA) as a STAT3 inhibitor, which was isolated from the stem bark of *Cinnamomum cassia*. In this study, we found that HCA inhibited constitutive and inducible STAT3 activation in STAT3-activated DU145 prostate cancer cells. HCA selectively inhibited the STAT3 activity by direct binding to STAT3, which was confirmed by biochemical methods, including a pull-down assay with biotin-conjugated HCA, a drug affinity responsive target stability (DARTS) experiment and a cellular thermal shift assay (CETSA). HCA inhibited STAT3 phosphorylation at the tyrosine 705 residue, dimer formation, and nuclear translocation in DU145 cells, which led to a downregulation of STAT3 target genes. The downregulation of cell cycle progression and antiapoptosis-related gene expression by HCA induced the accumulation of cells in the G0/G1 phase of the cell cycle and then induced apoptosis. We also found that reactive oxygen species (ROS) were involved in the HCA-induced inhibition of STAT3 activation and cell proliferation because the suppressed p-STAT3 level was rescued by glutathione or N-acetyl-L-cysteine treatment, which are general ROS inhibitors. These results suggest that HCA could be a potent anticancer agent targeting STAT3-activated tumor cells.

KEYWORDS
2-Hydroxycinnamaldehyde, anticancer, antioxidant, ROS, STAT3

1 | INTRODUCTION

Signal transduction and activator of transcription factor 3 (STAT3) is a transcription factor that regulates various cellular process such as proliferation, survival and angiogenesis in cancer cells.[1] STAT3 is persistently activated in many cancers and multiple oncogenic pathways converge on STAT3.[2] STAT3 is constitutively activated in various types of cancer cells, including pancreatic, lung, colorectal,

ovarian, prostate, cervical, breast, brain and renal cancer, as well as melanoma, glioma, lymphoma and leukemia.[3,4] In response to cytokines and growth factors, STAT3 is phosphorylated at the tyrosine 705 residue and forms homodimers, followed by translocation into the nucleus.[1] The activated STAT3 dimers bind to and activate the promoters of STAT3 target genes, which are involved in the regulation of the cell cycle and apoptosis.[5]

As a potential therapeutic target for cancer therapy, natural and synthetic inhibitors targeting the STAT3 pathway have been developed.[1,6] These inhibitors are categorized according to their site of action, including the SH2 domain or dimerization, upstream tyrosine kinases (eg, JAK1/2, SRC), STAT3 decoy oligonucleotides, STAT3-DNA domain binding and negative regulators of STAT3.[1] Numerous studies have confirmed their potential for clinical use; however, only a few compounds, such as OPB-51602, AZD1480, dasatinib, STA-21 and pyrimethamine, have progressed to early-phase clinical trials.[7-9] Thus, novel strategies of STAT3-targeted therapies are being explored to overcome unpredictable pharmacokinetics, limited efficacy and selectivity, and severe toxicity.

Cancer cells exhibit increased reactive oxygen species (ROS) levels compared to normal cells, which can be exploited for the selective killing of cancer cells.[10] In cancer cells, the levels of antioxidant proteins are usually upregulated to remove the ROS as a mechanism to escape apoptosis.[11] Therefore, ROS-targeted anticancer agents effectively kill cancer cells and overcome drug resistance by enhancing ROS generation and/or inhibiting the antioxidant defense.[10,12] The tumor suppressive function of ROS is mediated by apoptosis, autophagy and necroptosis.[12] ROS act as signaling molecules and activate multiple MAP kinase signaling pathways, such as ERK, JNK, p38 MAPK and BMK 1 pathways.[13] The ERK signaling pathway mediates signal transduction involved in cell proliferation, differentiation and migration; however, aberrant ERK activation results in anti-proliferative activities, such as apoptosis, autophagy and senescence.[14] ROS-mediated sustained ERK activation leads to apoptosis characterized by DNA fragmentation, PARP cleavage, caspase activation and depolarization of the mitochondrial membrane.[14,15] In addition, the generation of ROS induces apoptosis by inactivating the STAT3 signaling pathway.[16]

2′-Hydroxycinnamaldehyde (HCA), isolated from stem bark of *Cinnamomum cassia*, is reported as an anticancer agent with inhibitory effects on farnesyl protein transferase.[17] HCA and its derivative 2′-benzoyl-oxycinnamaldehyde (BCA) exhibit anti-tumor activities in various types of cancer cells.[18,19] HCA inhibits cell proliferation and tumor growth by regulating transcription activity and signal transduction.[19] Furthermore, preclinical evaluations of HCA and BCA have been completed, and a phase I clinical trial is currently under way to test BCA as an antitumor drug.

To reveal the mechanism of action of HCA, several molecular targets of HCA have been identified and the underlying mechanisms have been investigated in various cancer cells.[20-22] In colon cancer cells, HCA induces apoptosis by inhibiting the L3-like activity of the proteasome, resulting in the increase of endoplasmic reticulum stress and mitochondrial perturbation.[20] In breast cancer cells, HCA attenuates

cell invasion through stimulating the oxidation of thiols in the extracellular LRP1, LRP1 ligand binding, and extracellular pepsin clearance.[21] Moreover, HCA directly binds to the Pim-1 ATP-binding pocket and inhibits the Pim-1 kinase activity in leukemia and skin cancer cells.[22] Although the proteasome subunits, LRP1 and Pim-1 were identified as direct targets of HCA, different modes of action associated with the binding target of HCA have not been completely understood.

In the present study, we identified STAT3 as a direct target of HCA using various biochemical methods, such as a pull-down assay, a drug affinity responsive target stability (DARTS) experiment and a cellular thermal shift assay (CETSA), and determined its antiproliferative activity in DU145 prostate cancer cells with constitutively active STAT3. HCA suppressed cell proliferation through inhibiting the tyrosine phosphorylation of STAT3 at tyrosine 705, dimer formation and nuclear translocation. The expressions of STAT3-regulated genes associated with cell cycle progression and anti-apoptosis were downregulated by treatment with HCA. In addition to STAT3 inactivation, HCA increased the levels of intercellular ROS and ERK1/2 activation. HCA-induced ROS production and STAT3 inactivation were inhibited by treatment with antioxidants. In conclusion, HCA selectively suppressed cell proliferation and induced cell apoptosis in STAT3-activated DU145 cells through STAT3 inactivation and ROS generation.

2 | MATERIALS AND METHODS

2.1 | Cell culture

All cell lines used in this study were originally obtained from the ATCC (Manassas, VA, USA). DU145 and LNCaP (human prostate cancer), as well as HCT-116 (human colon cancer) cells were maintained in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA). MCF-10A (human mammary epithelial) cells were maintained in DMEM-F12 (Gibco). All culture media were supplemented with 10% heat-inactivated FBS (Gibco), 100 U/mL penicillin and .1 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cell cultures were maintained in a 37°C incubator under a humidified atmosphere with 5% CO_2.

2.2 | Cell proliferation assay

The cells were seeded in 96-well plates in medium containing 10% FBS. After 24 hours, the wells were replenished with fresh complete medium containing either a test compound or .1% DMSO. After incubation for 24-72 hours, the cell proliferation reagent WST-1 (Dojindo Laboratories, Kumamoto, Japan) was added to each well. The amount of WST-1 formazan that was produced was measured at 450 nm using the Multiskan GO system (Thermo Fisher Scientific, Rockford, IL, USA).

2.3 | Dual-luciferase reporter assay

The reporter assay was performed using the 21pSTAT3-TA-Luc reporter vector that carries the STAT3-binding element for firefly

luciferase activity and the pRL-TK vector for the Renilla luciferase reporter vector (Promega, Madison, WI, USA). HCT-116 cells were transfected with 27 μg of 21pSTAT3-TA-Luc reporter and 9 μg of the pRL-TK vector for Renilla control luciferase activity using the X-tremeGene HP DNA Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA) in serum-free Opti-MEM (Gibco). After 6 hours of transfection, the cells were trypsinized and seeded onto black-bottomed 96-well plates at a density of 2×10^4 cells per well and were then incubated with either HCA or .1% DMSO for 24 hours. After treatment, the cells were washed with 150 μL of PBS, lysed with 25 μL of passive lysis buffer and shaken at 4°C for 15 minutes. The luciferase activity was evaluated using the Dual-Luciferase Reporter Assay kit using the GLOMAX 96 microplate luminometer (Promega). The relative luciferase activity was calculated according to the following formula: relative luciferase activity (%) = [(normalized luciferase activity of sample treated with a test compound)/(normalized luciferase activity of sample treated with .1% DMSO)] × 100.

2.4 | FACS analyses

Cells were trypsinized at specific times after compound treatment and were collected by centrifugation at 250 *g* for 5 minutes at room temperature. The supernatant was discarded, and the precipitated cells were washed twice by repeated suspension in PBS buffer. Precipitated cells were carefully suspended in 500 μL of PBS buffer and were fixed with 4 mL of ice-cold 70% ethanol overnight. Fixed cells were washed twice with PBS. The collected cells were resuspended in PBS and treated with 100 μg/mL RNase A at 37°C for 30 minutes. Propidium iodide was then added at a final concentration of 50 μg/mL for DNA staining. Fixed cells were analyzed on a FACSCalibur flow cytometer until 20 000 cells were counted (BD Biosciences, San Jose, CA, USA). The distribution of the cells across the cycle was analyzed using WinMDI 2.9.

2.5 | Reactive oxygen species measurement

A FACSCalibur flow cytometer (BD Biosciences) was used for the analyses. The excitation wavelength was 488 nm, and the observation wavelength was 530 nm for green fluorescence. The relative change in fluorescence was analyzed with WinMDI software. For the measurement of intracellular ROS, detached cells were incubated with 5 μmol/L CM-H_2DCFDA for 30 minutes at 37°C.

2.6 | Chemical cross-linking assay

Cells were harvested with trypsin/EDTA (Gibco) and washed with PBS twice. The cells were resuspended in 500 μL of PBS and then applied to the chemical cross-linking assays. Specifically, the freshly prepared aqueous cross-linkers, EDC (10 mmol/L) and NHS (5 mmol/L), were added into the cell suspension in PBS and incubated for 1 hour at room temperature. The crosslinking reaction

was quenched by the addition of 50 mmol/L Tris into the reaction mixtures. Finally, the cells were lysed with lysis buffer followed by western blotting.

2.7 | Immunocytochemistry

DU145 cells (1.0×10^5 cells) were plated into 35-mm high-μ dishes (ibidi GmbH, Am Klopferspitz, Germany). The cells were washed once with PBS and treated with DMSO or HCA (20 μmol/L) for 1 or 24 hours. After washing with PBS twice, the attached cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. The fixed cells were permeabilized with .2% Triton X-100 for 10 minutes and blocked with 1.0% BSA in PBS for 1 hour. The cells were incubated with an anti-STAT3 antibody (Cell Signaling, Danvers, MA, USA) followed by goat anti-rabbit IgG-FITC secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The nuclei were counterstained with 2 μg/mL DAPI (Santa Cruz Biotechnology) in PBS for 2 minutes. All images were acquired on a laser scanning confocal microscope (LSM 510 META; Carl Zeiss, St. Cloud, MN, USA) and analyzed with LSM Version 3.2 software (Carl Zeiss).

2.8 | Synthesis of biotin-2′-hydroxycinnamaldehyde

Ninety milligrams of N-biotinylcaproic acid, 72 mg of N,N′-dicyclohexylcarbodiimide (DCC) and 6 mg of N-dimethylaminopyridine (DMAP) were dissolved in DMSO, to which 45 mg of 2′-hydroxycinnamaldehyde was added. The reaction mixture was stirred for 3 hours at room temperature. The reaction solution was concentrated and purified by silica gel column chromatography and HPLC to give 23 mg of 2′-biotinylcaproic-cinnamaldehyde (biotin-HCA). ^{1}H NMR (CDCl$_3$) δ 9.67 (d, J = 7.5 Hz, 1H), 7.65 (d, J = 6.5 Hz, 1H), 7.53 (d, J = 16 Hz, 1H), 7.46 (dt, J = 7.5, 1.1 Hz, 1H), 7.51 (dt, J = 1.1, 7.5 Hz, 1H), 7.15 (d, J = 8.0 Hz, 1H), 6.72 (dd, J = 7.0, 16.0 Hz, 1H), 6.06 (m, 1H), 6.05 (s, 1H), 5.25 (s, 1H), 4.49 (m, 1H), 4.29 (m, 1H), 3.26 (m, 2H), 3.13 (m, 1H), 2.87 (m, 1H), 2.67 (m, 3H), 2.19 (m, 2H), 1.4-1.8 (m, 12). ^{13}C NMR (CDCl$_3$) δ 193.74, 173.17, 171.71, 163.69, 149.39, 145.95, 132.14, 130.21, 128.25, 126.67, 126.48, 123.31, 61.75, 60.12, 55.48, 40.51, 39.15, 35.93, 34.05, 29.25, 28.09, 28.00, 26.34, 25.57, 24.42.

2.9 | Pull-down assay

DU145 cells were washed with PBS and homogenized with a 26-gauge syringe in binding buffer (10 mmol/L Tris-HCl, pH = 7.4, 50 mmol/L KCl, 5 mmol/L MgCl$_2$, 1 mmol/L EDTA and .1 mmol/L Na$_3$VO$_4$). The cell lysate was centrifuged, and the supernatant was collected. The cell lysate was precleared by incubation with NeutrAvidin beads (Thermo Fisher Scientific, 29202) for 1 hour at 4°C. The cleared lysate was incubated with biotin-conjugated HCA (biotin-HCA) overnight at 4°C. Proteins bound to biotin-HCA were precipitated with NeutrAvidin beads. After 3 washes in washing buffer (50 mmol/L HEPES, pH 7.5, 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, .1% Tween-20, 10% (v/v) glycerol, 1 mmol/L NaF, .1 mmol/L Na$_3$VO$_4$ and 1× protease inhibitor cocktail (Roche

Diagnostics), the beads were eluted with 1× sample buffer. The samples were boiled for 10 minutes and separated for Coomassie blue staining or immunoblotting.

2.10 | Drug affinity responsive target stability

The DARTS experiment was conducted as previously described with some modifications.[23] Cells were washed with ice-cold PBS and treated with ice-cold M-PER lysis buffer (Thermo Fisher Scientific) supplemented with a protease inhibitor cocktail, 1 mmol/L Na_3VO_4 and 1 mmol/L NaF. The protein lysates were mixed with 10× TNC buffer (500 mmol/L Tris-HCl, pH = 8.0, 500 mmol/L NaCl and 100 mmol/L $CaCl_2$). The lysates in 1× TNC buffer were incubated with DMSO or HCA for 1 hour at room temperature. Following the incubation, each sample was proteolyzed in various concentrations of pronase (Roche Diagnostics, 10165921001) for 10 minutes at room temperature. After 10 minutes, 2 μL of ice-cold 20× protease inhibitor cocktail was added to stop proteolysis, and the samples were immediately placed on ice. Digestion was further stopped by adding 5× sample loading dye and boiling at 95°C for 10 minutes. An equal portion of each sample was then loaded onto SDS-PAGE gels for western blotting.

2.11 | Cellular thermal shift assay

The CETSA was conducted using cell and tissue lysates as previously described.[24] For CETSA with cell lysates, DU145 cells were lysed with lysis buffer (50 mmol/L Tris-HCl, pH = 7.5, 100 mmol/L NaCl, .2% NP-40, 5% glycerol, 1.5 mmol/L $MgCl_2$, 25 mmol/L NaF, 1 mmol/L Na_3VO_4 and 1× protease inhibitor cocktail). After centrifugation, the lysates were incubated with DMSO or HCA for 1 hour at room temperature. The lysates were aliquoted into .2 mL PCR tubes and heated for 5 minutes at the indicated temperature in a PCR machine (Applied Biosystems). The precipitated proteins were separated from the soluble fraction by centrifugation, and equal portions of the supernatants were loaded onto SDS-PAGE gels for western blotting.

2.12 | Knockdown of target genes

Small interfering RNA (siRNA) against each gene were preincubated for 20 minutes in serum-free Opti-MEM (Gibco) containing the Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA). Scrambled and STAT3 siRNA were purchased from Bioneer (Daejeon, Republic of Korea). RPMI 1640 medium containing 10% FBS was added 6 hours after incubation. After 48 hours, the transfected cells were collected and used for the experiments described.

2.13 | Western blotting

Cell lysates were prepared in RIPA lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics). Proteins (20-50 μg) were resolved by 8%-15% SDS-PAGE and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat dry milk or 5% BSA in TBST and incubated with primary and secondary antibodies according to the manufacturer's protocol. The antibodies used were p-STAT3 (Y705) (Cell Signaling), p-STAT3 (S727) (Cell Signaling), STAT3 (Cell Signaling), p-ERK1/2 (T202/Y204) (Cell Signaling), anti-ERK1/2 (Cell Signaling), Cyclin D1 (Santa Cruz), Cyclin A (Santa Cruz), Bcl-2 (Cell Signaling), Mcl-1 (Santa Cruz), Survivin (Cell Signaling), Bcl-xL (Santa Cruz), PARP (Cell Signaling) and GAPDH (Santa Cruz). The secondary antibodies used were HRP-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The membranes were washed 3 times with TBST and then detected with the Luminata Forte Western HRP substrate (EMD Millipore) using the LAS 4000 mini (GE Healthcare Life Sciences). The densitometric analysis of the bands was performed using the MultiGauge program (Fuji Photo Film, Tokyo, Japan) and the results were normalized to the corresponding GAPDH.

2.14 | In vivo xenograft assay

Animal experiments were performed in accordance with a protocol approved by the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Animal Experimentation Ethics Committee. DU145 cells (9×10^6 cells/mouse) were subcutaneously injected into the right flank of nude mice (6-week-old female BALB/c mice). Tumor growth was measured with calipers using the formula volume = (length × width × height) × .5. Vehicle and HCA (50 mg/kg) was orally administered 5 days per week for 25 days starting on day 1 (the day after cell injection, 6 mice per group). On day 25, the tumor tissue from each mouse was collected and the tumor weight (mg) was examined.

2.15 | Statistical analysis

All experiments were performed at least twice, and multiple samples represent biological (not technological) replicates. All animal experiments were performed using randomly assigned mice without investigator blinding. No statistical methods were used to predetermine the sample size, and no data were excluded. Statistical analyses were performed using Excel. Statistical significance was tested using 2-tailed t tests. Values of $P < .05$, $P < .01$ and $P < .001$ are denoted by *, ** and ***, respectively.

3 | RESULTS

3.1 | 2′-Hydroxycinnamaldehyde suppresses cell proliferation via regulation of signal transducer and activator of transcription 3 activity

To identify STAT3 inhibitors, we screened 650 natural products using the STAT3 reporter assay by luciferase in HCT-116 colon
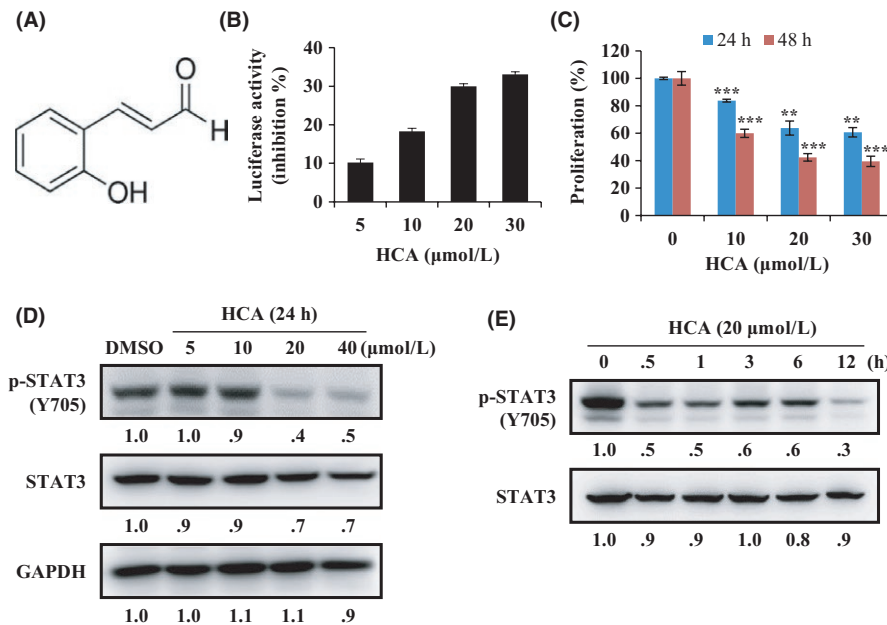
FIGURE 1 2′-Hydroxycinnamaldehyde (HCA) suppresses cell proliferation via regulation of signal transducer and activator of transcription 3 (STAT3) activity in DU145 cells. A, The chemical structure of HCA. B, Luciferase activities were determined in DU145 cells that were co-transfected with the 21pSTAT3-TA-Luc reporter vector and pRL-TK vector for 6 h and subsequently treated with HCA for 24 h (n = 3). C, DU145 cells were treated with HCA for 24 or 48 h. Cell proliferation was measured by WST-1 (n = 3). D, DU145 cells were treated with indicated concentrations of HCA for 24 h, and the expression level of p-STAT (Y705) and STAT3 was analyzed by western blotting (n = 2). E, DU145 cells were treated with HCA (20 μmol/L) for the indicated times, and the expression level of p-STAT (Y705) and STAT3 was analyzed by western blotting (n = 2). The band intensity of each protein was quantified using the MultiGauge program. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data represent mean ± SD

cancer cells.[25] After the dual luciferase assay for the STAT3 activity, we identified HCA as a potent STAT3 inhibitor (Figure 1A). HCA inhibited STAT3 transcription activity in a dose-dependent manner in HCT-116 cells (Figure 1B). DU145 cells exhibit constitutively active STAT3, and they are applied for the validation of STAT3 inhibitor regulating the phosphorylation of STAT3.[26] After 24-48 hours of treatment with HCA, HCA reduced the cell proliferation of DU145 cells with a GI$_{50}$ value of 20 μmol/L (Figure 1C). HCA significantly decreased the phosphorylation of STAT3 at Tyr-705 in a dose-dependent manner (Figure 1D). In a time-course treatment of HCA, a marked decrease of p-STAT3 (Y705) was observed at 30 minutes and maintained for up to 12 hours (Figure 1E). HCA (20 μmol/L) inhibited STAT3 phosphorylation by more than 70%, whereas the total amount of STAT3 protein was not altered by up to 12 hours of treatment (Figure 1E). Our results show that HCA reduces the STAT3 activity and the phosphorylated STAT3 level, resulting in inhibited cell proliferation of DU145 cells.

3.2 | 2′-Hydroxycinnamaldehyde downregulates the expression of signal transducer and activator of transcription 3 target genes, which induces G0/G1 arrest and apoptosis

Signal transducer and activator of transcription 3 is a transcription factor and regulates genes that are involved in cell survival and

proliferation.[2] STAT3 target genes include the cell cycle regulatory proteins (cyclin A and cyclin D1), which mediate G1-S phase progression, and antiapoptotic proteins (Bcl-2, Bcl-xL, Mcl-1 and survivin).[25,26] We determined the expressions of STAT3-regulated genes in DU145 cells treated with HCA. After 24 hours of treatment with HCA, HCA inhibited the expression of STAT3 target genes, such as cyclin D1, cyclin A, Bcl-2, Bcl-xL, Mcl-1 and survivin (Figure 2A). HCA showed a more dramatic reduction of STAT3-regulated gene expression at 48 hours of treatment with HCA (Figure 2A). Moreover, we evaluated the effect of HCA on the cell cycle progression of DU145 cells using a FACSCalibur flow cytometer. As shown in Figure 2B, the percentage of cells in the G0/G1 phase began to increase after 6 hours of HCA treatment, whereas the percentage of apoptotic cells increased after 24 hours of HCA treatment. Our results suggest that HCA induces G0/G1 arrest and cell apoptosis by downregulating STAT3 target genes, which are involved in cell cycle regulatory proteins and anti-apoptotic proteins.

3.3 | 2′-Hydroxycinnamaldehyde binds to signal transducer and activator of transcription 3 and inhibits signal transducer and activator of transcription 3 activity

To elucidate the underlying mechanism of STAT3 inactivation by HCA, we analyzed the dimerization and translocation into the
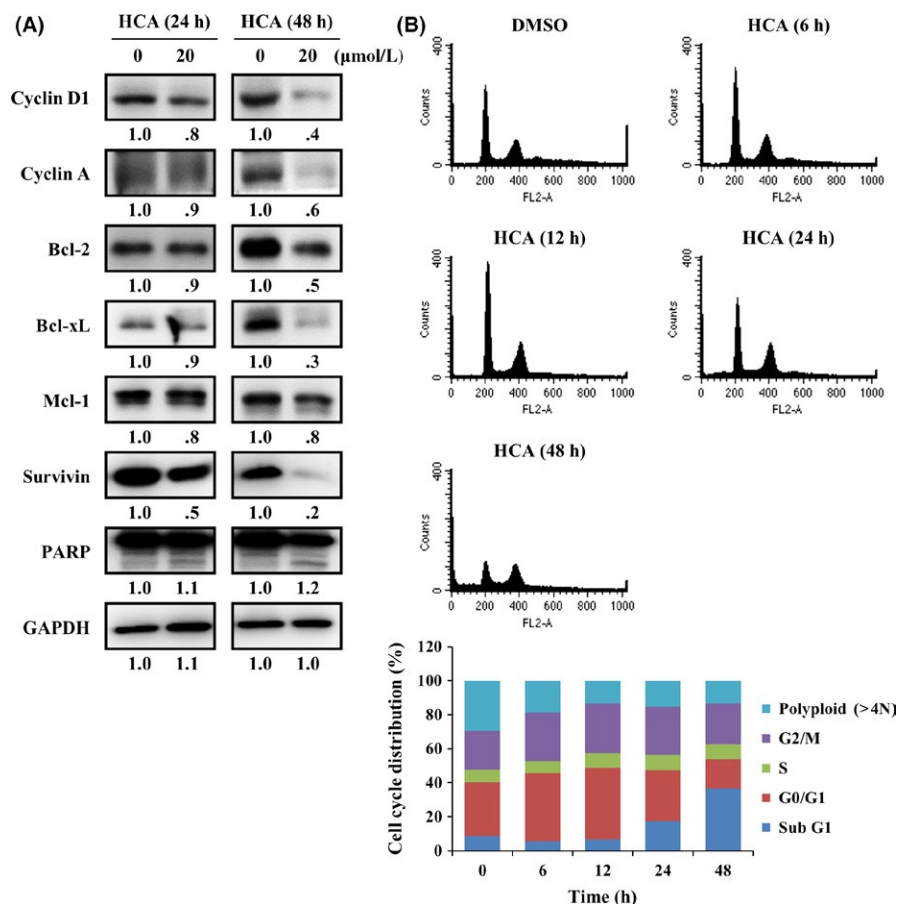
FIGURE 2 2′-Hydroxycinnamaldehyde (HCA) downregulates the expression of signal transducer and activator of transcription 3 (STAT3) target genes that induces G0/G1 arrest and apoptosis. A, DU145 cells were treated with HCA (20 μmol/L) for 24 or 48 h and the expression level of the STAT3 target gene was analyzed by western blotting (n = 2). The band intensity of each protein was quantified using the MultiGauge program. B, DU145 cells were treated with HCA (20 μmol/L) for the indicated times. After treatment, the cell cycle distribution was analyzed using a FACSCalibur flow cytometer (n = 2). The ratios of cells in each phase were analyzed using the WinMDI 2.9 analyzer

nucleus of STAT3 after HCA treatment. We performed a chemical crosslinking assay using EDC/NHS in DU145 cells by treatment with HCA or cryptotanshinone as a positive control.[25] HCA inhibited STAT3 dimerization in a dose-dependent manner and showed a similar reduction at 20 μmol/L HCA as that of cryptotanshinone (7 μmol/L) (Figure 3A). Consistent with the cross-linking data, HCA inhibited the nuclear translocation of STAT3 after 1 and 24 hours of treatment in DU145 cells (Figure 3B). Our data indicate that HCA inhibits the function of STAT3 through inhibiting dimerization and nuclear translocation.

To determine whether HCA inhibits STAT3 activity by directly binding to STAT3, we performed a pull-down assay with biotin-conjugated HCA (biotin-HCA). In our previous study, we synthesized biotin-HCA, showing a similar level of antiproliferation on DU145 cells.[20] STAT3 was detected in the biotin-HCA group, and their binding was competitively reduced by the addition of HCA (Figure 3C). Our data suggest that HCA binds directly to STAT3 and inhibits dimer formation as well as the nuclear translocation of STAT3.

3.4 | 2′-Hydroxycinnamaldehyde specifically binds to signal transducer and activator of transcription 3 in DU145 cells

To further confirm the direct binding and its specificity with STAT3, we performed label-free target validation assays, including DARTS

and CETSA. After the binding of the compound with target proteins, the binding proteins show increased pronase resistance and thermal stability in the DARTS experiment and CETSA assay, respectively.[23,24] DU145 cell lysates were incubated with DMSO or HCA and were then proteolyzed with increasing concentrations of pronase. In the presence of HCA, STAT3 was more resistant to pronase compared to the DMSO group (Figure 4A). In the HCA-treated group, the pronase-resistant STAT3 level was elevated with increasing concentrations of HCA (Figure 4B). Our data suggest that HCA enhances the protein stability of STAT3 against pronase by directly binding to STAT3. When DU145 cell lysates were incubated with HCA, the thermal stability of STAT3 increased with increasing melting temperature (Figure 4C). Moreover, the thermal stability of STAT3 was elevated dose-dependently in HCA-treated cells (Figure 4D). Collectively, HCA specifically binds to STAT3 in DU145 cells, showing the increased pronase resistance and thermal stability of STAT3.

3.5 | 2′-Hydroxycinnamaldehyde induces reactive oxygen species generation by regulating ERK1/2 and signal transducer and activator of transcription 3 activation

2′-Hydroxycinnamaldehyde and its derivative BCA induces apoptosis by ROS generation in human colon and breast cancer cells,
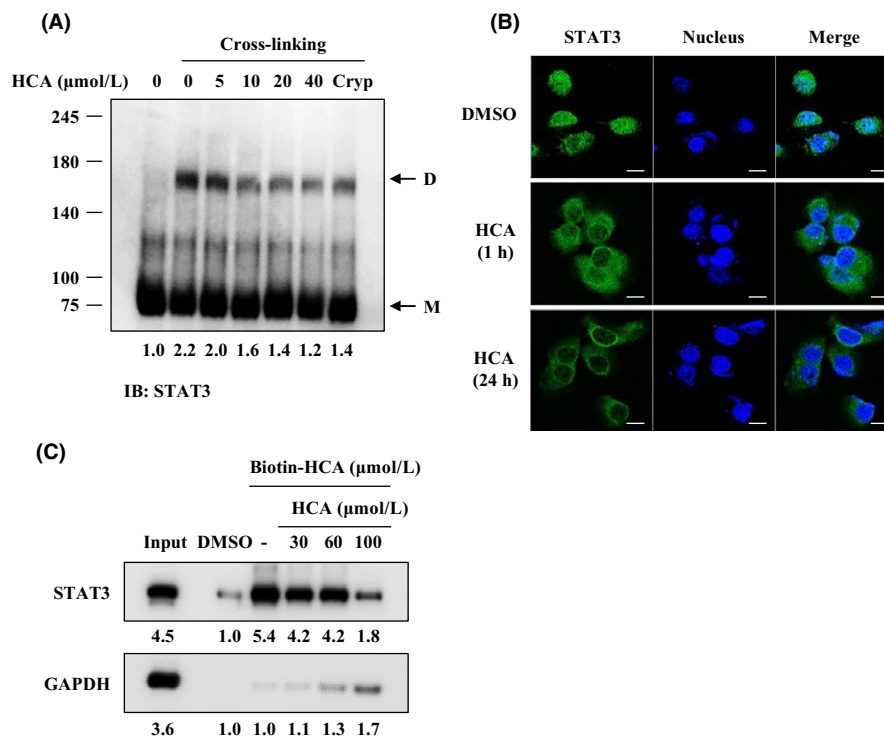
FIGURE 3 2′-Hydroxycinnamaldehyde (HCA) binds to signal transducer and activator of transcription 3 (STAT3) and inhibits STAT3 activity. A, The dimeric formation of STAT3 was analyzed in DU145 cells treated with indicated concentrations of HCA or cryptotanshinone (7 μmol/L) for 24 h using the chemical cross-linking assay with EDC/NHS (n = 2) (D, dimer; M, monomer). B, DU145 cells were treated with HCA (20 μmol/L) for 1 or 24 h. Nuclear localization of STAT3 was analyzed by immunocytochemistry using STAT3 antibody (green). Scale bars, 20 μm. C, Pull-down assay with biotin-conjugated HCA (Biotin-HCA) in DU145 cell lysates (n = 2). DU145 cell lysates were incubated with DMSO or Biotin-HCA in the presence of HCA. Binding proteins were captured by NeutrAvidin Agarose Resin, and the eluted proteins were analyzed by western blotting. The band intensity of each protein was quantified using the MultiGauge program

which are inhibited by ROS scavenger treatment.[27] We evaluated the effect of HCA on ROS generation using FACSCalibur flow cytometer, and the intracellular ROS level was measured by DCF-DA. After time-dependent treatment with HCA, the intracellular ROS level of DU145 cells was greatly increased at 30 minutes and then maintained for up to 6 hours after treatment with HCA (Figure 5A). ROS activates multiple MAP kinase signaling pathways such as the ERK1/2, JNK, p38 MAPK and BMK 1 pathways.[13] In addition, the generation of ROS induces apoptosis by inactivating the STAT3 signaling pathway.[16] Notably, ERK1/2 activation was observed at 1 hour and maintained for up to 12 hours after treatment with HCA in DU145 cells (Figure 5B). Antioxidants abolished HCA-induced STAT3 inactivation and ERK1/2 activation (Figure 5C). Moreover, HCA-induced cell death was abolished by treatment with antioxidants such as N-acetyl-L-cysteine (NAC) and glutathione (GSH) (Figure 5D). Our data show that HCA-induced ROS induces ERK1/2 activation and STAT3 inactivation.

To confirm the effect of ROS on HCA-binding to STAT3, we performed CETSA experiment in DU145 cells treated with antioxidants and HCA. HCA increased the thermally stable STAT3 level in antioxidant-treated cells, similar to those of non-treated cells (Figure 5E). These results suggest that HCA binds to STAT3 regardless of the presence or absence of ROS.

3.6 | 2′-Hydroxycinnamaldehyde suppresses cell proliferation and tumor growth via signal transducer and activator of transcription 3 inactivation and reactive oxygen species generation

To further confirm HCA-induced STAT3 inactivation, we applied DU145 cells with constitutively active STAT3, or LNCaP and MCF-10A cells with nonactive STAT3 (Figure 6A). STAT3 activation is mediated by cytokines such as IL-6[28]; therefore, we investigated IL-6-induced STAT3 activation. HCA inhibited IL-6-induced STAT3 phosphorylation in DU145, LNCaP and MCF-10A cells (Figure 6B). HCA significantly suppressed the proliferation of DU145 cells compared to LNCaP cells (Figure 6C). Moreover, HCA did not affect the proliferation of non-tumorigenic MCF-10A epithelial cells (Figure 6C). These results suggest that HCA selectively and specifically inhibits the proliferation of cancer cells that exhibit constitutively active STAT3.

STAT3 is required for the proliferation and survival of cancer cells; therefore, knockdown of STAT3 expression induces apoptosis and suppresses tumor growth through downregulation of antiapoptotic gene expression.[29,30] When DU145 cells were treated with scrambled and STAT3 siRNA, the protein level of STAT3 and p-STAT3 (Y705) was reduced by 68.4% and 44.7%, respectively, in
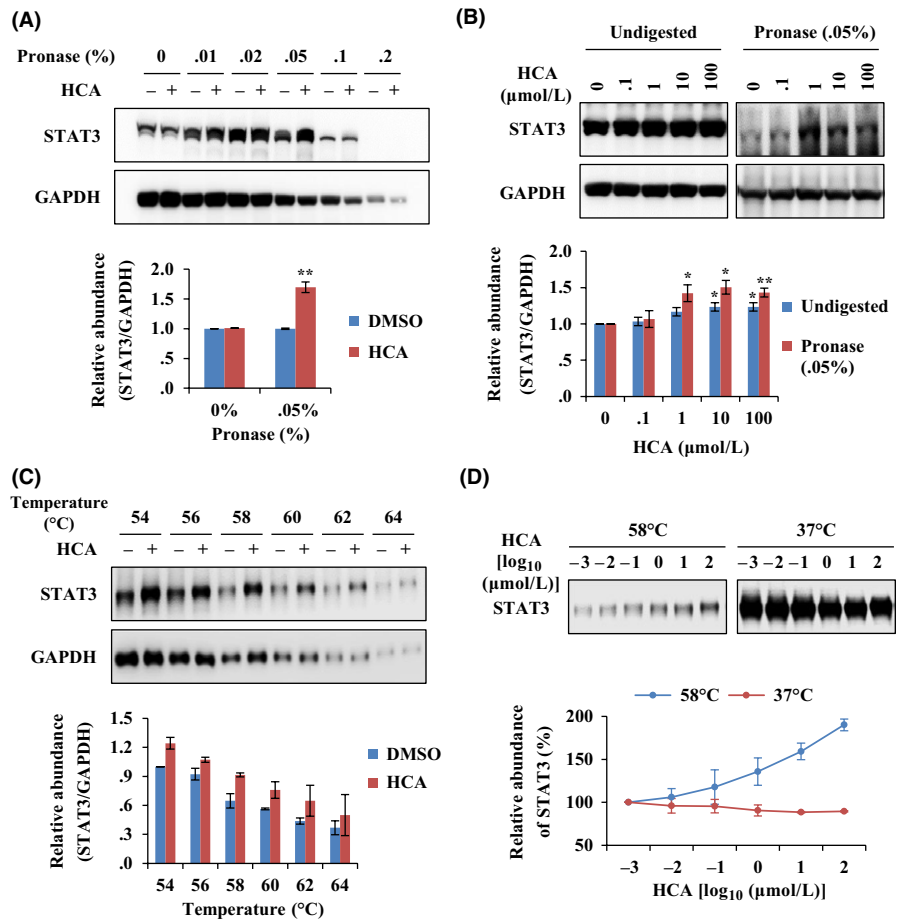
FIGURE 4 2′-Hydroxycinnamaldehyde (HCA) specifically binds to signal transducer and activator of transcription 3 (STAT3) in DU145 cells. A, DU145 cell lysates were incubated with HCA (200 μmol/L) for 1 h at room temperature and then were proteolyzed with increasing concentrations of pronase for 10 min (n = 3). B, DU145 cell lysates were incubated with increasing concentrations of HCA for 1 h at room temperature and then were proteolyzed with pronase for 10 min (n = 3). The pronase-resistant proteins in the presence or absence of HCA were analyzed by western blotting. C,D, CETSA in cell lysates of DU145 cells that were treated with DMSO or HCA (200 μmol/L) for 1 h with increasing melting temperature (54-64°C, interval temperature: 2°C) (C) or drug concentration (D) (n = 2). The band intensity of each protein was quantified using the MultiGauge program. *$P < 0.05$; **$P < 0.01$. Data represent mean ± SD

STAT3 siRNA-treated cells (Figure 6D). Moreover, HCA (20 μmol/L) inhibited STAT3 phosphorylation by 80.6% after 24 hours of treatment (Figure 6D). Notably, the residual p-STAT3 (Y705) of STAT3 knockdown cells was further inhibited by HCA treatment (Figure 6D). Consistent with previous reports, the knockdown of STAT3 using STAT3 siRNA inhibited the cell proliferation by 40.0% compared to scrambled siRNA-treated cells (Figure 6E). Moreover, STAT3 knockdown and HCA treatment synergistically inhibited the cell proliferation of DU145 cells, indicating that STAT3 inactivation is involved in the antiproliferative activity of HCA (Figure 6E). It has been reported that ROS induces apoptosis by inactivating the STAT3 signaling pathway.[16] Interestingly, HCA-induced ROS generation was synergistically increased in STAT3 siRNA-treated cells compared to scrambled siRNA-treated cells (Figure 6F). Moreover, HCA still induced ERK1/2 activation in STAT3 knockdown cells (Figure 6G). Therefore, our data suggest that HCA inhibited cell proliferation by inactivating STAT3 and inducing ROS production, respectively, and STAT3 is also involved in HCA-induced ROS generation.

We treated DU145 cells with the ERK1/2 inhibitor U0126 and analyzed the serine and tyrosine phosphorylation of STAT3 in the presence and absence of HCA. U0126 inhibited STAT3 phosphorylation at Ser727 and increased STAT3 phosphorylation at Tyr705 (Figure 6H). U0126 abrogated HCA-induced STAT3 phosphorylation at Ser727; however, increased STAT3 phosphorylation at Tyr705 by

ERK inhibition was significantly inhibited by HCA treatment, suggesting that HCA-induced STAT3 phosphorylation at Tyr705 is also inhibited by ERK1/2-independent pathway (Figure 6H). Thus, both STAT3 dephosphorylation and ERK1/2 phosphorylation are necessary for anti-cancer activity of HCA.

To elucidate the antiproliferative effects of HCA on in vivo tumors, we examined tumor growth in a DU145 xenograft that was orally administered with either vehicle or HCA (50 mg/kg; Figure 7). HCA significantly suppressed tumor growth, with a 48.4% decrease in the tumor volume and a 49.5% decrease in the tumor weight of mice xenografted with DU145 cells compared with vehicle-treated mice (Figure 7A,B). No significant difference in body weight was observed between the vehicle control and HCA-treated mice (Figure 7C). These results show that HCA inhibits the in vitro cell proliferation and tumor growth of DU145 prostate cancer cells, which suggests the use of HCA as an anticancer agent for STAT3-activated tumor cells.

4 | DISCUSSION

Because of the advantages of herbal medicine-based drugs with low toxicity, several natural STAT3 inhibitors have been developed as potential anticancer therapeutics, including curcumin, butein, capsaicin, celastrol, diosgenin, thymoquinone, resveratrol, piperlongumine
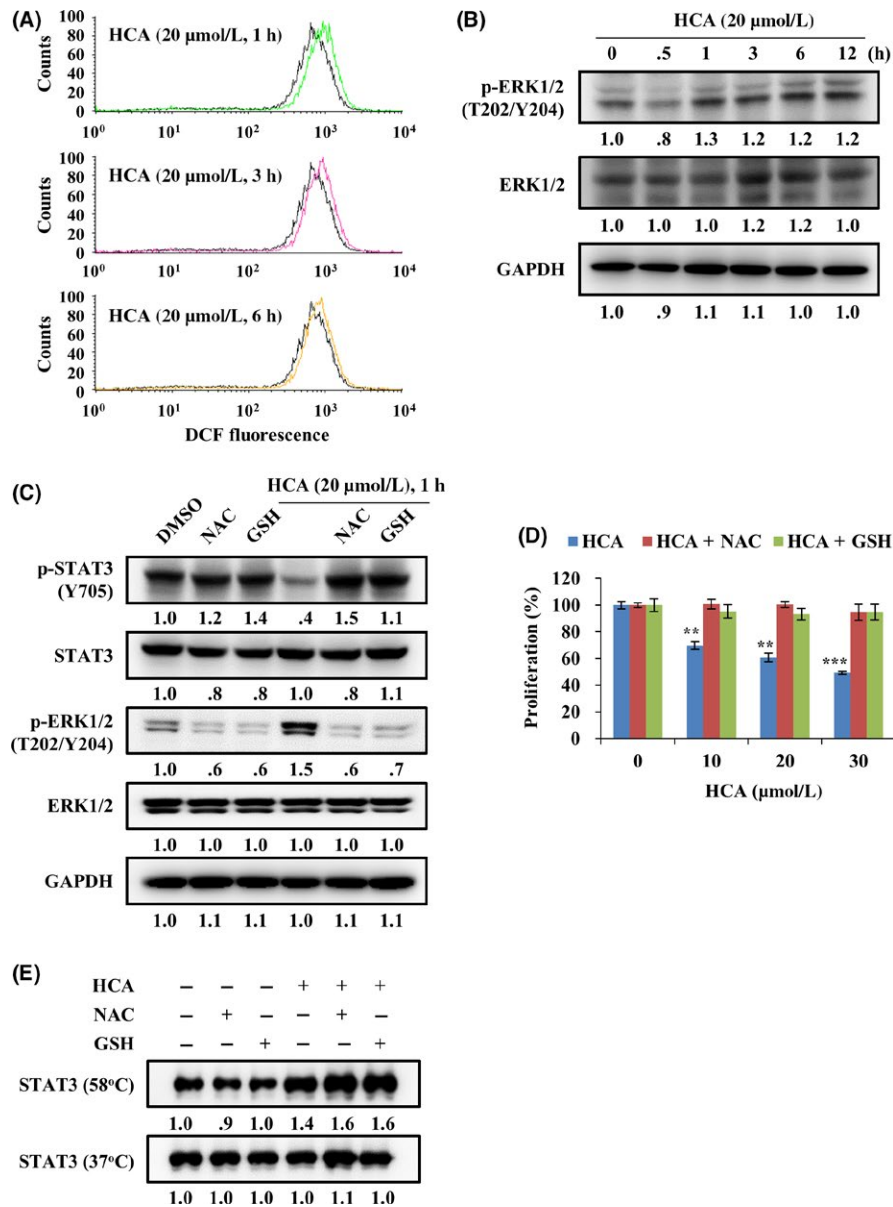
FIGURE 5 2′-Hydroxycinnamaldehyde (HCA) induces reactive oxygen species (ROS) generation by regulating ERK1/2 and signal transducer and activator of transcription 3 (STAT3) activation. A, DU145 cells were treated with HCA (20 μmol/L) for the indicated times. After treatment, intracellular ROS levels were measured by DCF-DA (5 μmol/L) using a FACSCalibur flow cytometer. B, DU145 cells were treated with HCA (20 μmol/L) for the indicated times, and the expression level of p-ERK1/2 (T202/Y204) and ERK1/2 was analyzed by western blotting (n = 2). C,D, After pre-treatment with N-acetyl-L-cysteine (NAC) (2 mmol/L) or glutathione (GSH) (2 mmol/L) for 1 h, DU145 cells were treated with HCA for 1 h (C) or 24 h (D) in the presence or absence of NAC or GSH. The expression level of p-STAT3 (Y705), STAT3, p-ERK1/2 (T202/Y204) and ERK1/2 was analyzed by western blotting (n = 2) (C). Cell proliferation was measured by WST-1 (n = 3) (D). E, CETSA in DU145 cells that were treated with HCA (20 μmol/L) for 1 h in the presence or absence of NAC (2 mmol/L) or GSH (2 mmol/L) at a temperature of 58 or 37°C (n = 2). The band intensity of each protein was quantified using the MultiGauge program. **$P < 0.01$; ***$P < 0.001$. Data represent mean ± SD

and vitamin E.[5] Piperlongumine (PL), a natural product isolated from the *Piper longum* Linn., induces apoptosis by inhibiting STAT3 activity and increasing the intracellular level of ROS.[31,32] PL inhibits the phosphorylation and nuclear translocation of STAT3 by inhibiting its direct binding to the phosphotyrosyl peptide ligand.[32] Curcumin, an active component of turmeric (*Curcuma longa*), has antitumor activity by targeting the SH2 domain of STAT3.[33,34] Although curcumin

has been reported to be safe at doses as high as 8 g/d in humans, its low bioavailability in serum leads to the development of curcumin with modifications.[35] In addition, cryptotanshinone from *Salvia miltiorrhiza* Bunge (Danshen) is a potent STAT3 inhibitor that targets the SH2 domain of STAT3.[25] Our data show that HCA also inhibits the STAT3 activity by directly binding to STAT3 (Figures 3C and 4). Furthermore, preclinical evaluations of HCA show that HCA is a safe
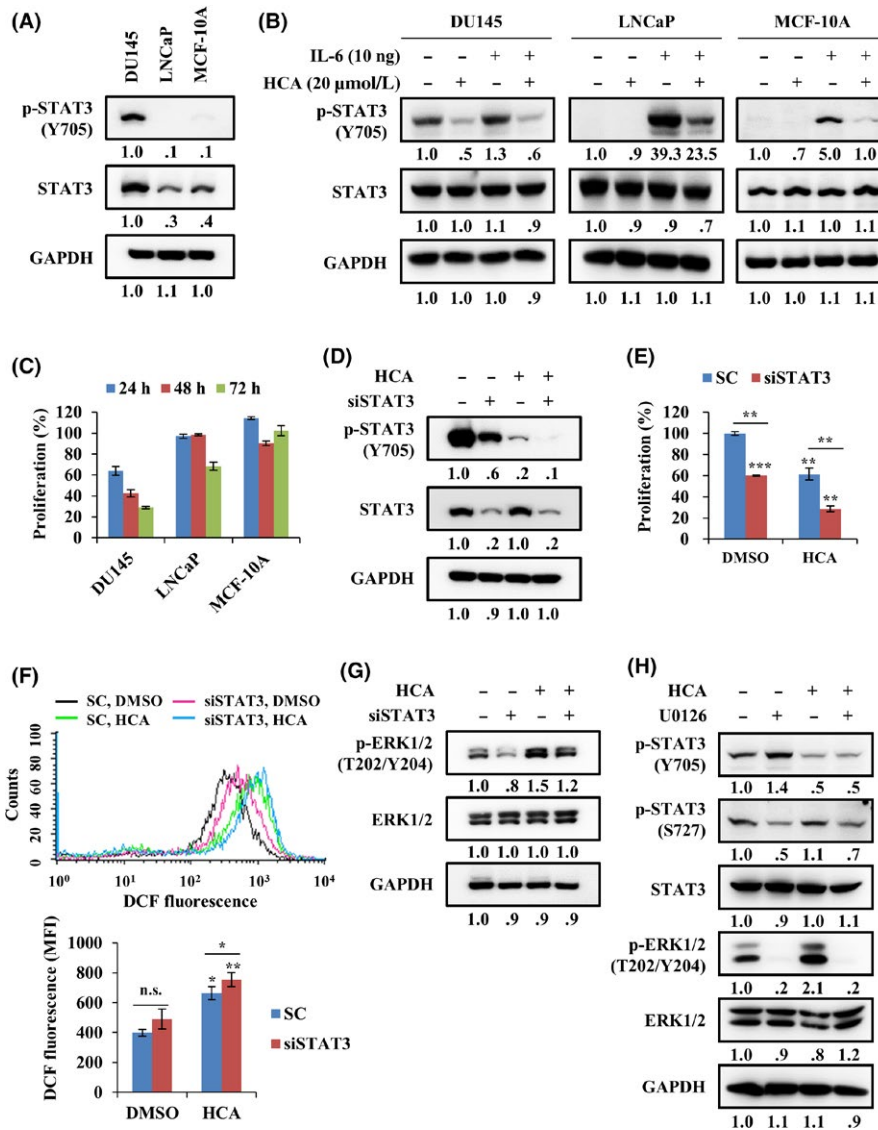
FIGURE 6 2′-Hydroxycinnamaldehyde (HCA) suppresses cell proliferation via signal transducer and activator of transcription 3 (STAT3) inactivation and reactive oxygen species (ROS) generation. A, The protein expression level of p-STAT3 (Y705) and STAT3 in DU145, LNCaP and MCF-10A cells (n = 2). B, DU145, LNCaP and MCF-10A cells were stimulated with IL-6 (10 ng/mL) for 1 h in the presence or absence of HCA (20 μmol/L). The expression level of p-STAT3 (Y705) and STAT3 was analyzed by western blotting (n = 2). C, DU145, LNCaP and MCF-10A cells were treated with HCA (20 μmol/L) for 24, 48 or 72 h. Cell proliferation was measured by WST-1 (n = 3). D,E, DU145 cells were transfected with scrambled siRNA (SC) or STAT3 siRNA (siSTAT3) for 48 h. After STAT3 siRNA transfection, DU145 cells were treated with HCA (20 μmol/L) for 24 h. The expression level of each protein was analyzed by western blotting (D) and cell viability was measured by WST-1 (n = 3) (E). F, Representative FACS plot and mean fluorescence intensity (MFI) showing ROS level detected by DCF-DA staining in or STAT3 siRNA (siSTAT3)-transfected DU145 cells after 24-h treatment with HCA (20 μmol/L) (n = 3). G, DU145 cells transfected with scrambled siRNA or siSTAT3 were treated with HCA (20 μmol/L) for 1 h. The expression level of p-ERK1/2 (T202/Y204) and ERK1/2 was analyzed by western blotting (n = 2). H, DU145 cells were treated with HCA (20 μmol/L) for 1 h in the presence of U0126 (20 μmol/L) and analyzed by western blotting (n = 2). The band intensity of each protein was quantified using the MultiGauge program. *$P < 0.01$; **$P < 0.05$; ***$P < 0.001$; n.s., not significant. Data represent mean ± SD

and potent anticancer agent.[19] Therefore, our results suggest that HCA could be a potent anticancer agent targeting STAT3-activated tumor cells.

Current targeted therapeutics targeting a single key factor lead to the acquisition of drug resistance and relapse due to highly redundant and bypassable signaling pathways.[36] Multitarget agents have attracted attention as potential therapeutic solutions to diseases

of complex disorders and drug resistance.[37,38] Natural products are a historically rich source of biologically active compounds for cancer treatment.[39] Therefore, the potential of natural products is discussed as a starting point for multitarget drug discovery. As multitarget drugs, compounds have multiple but concrete molecular targets while maintaining their beneficial broad target profile.[37] For multitarget drug development, the concrete molecular targets

should be elucidated to minimize side effects and adverse compensatory mechanisms. For example, natural products, such as curcumin, EGCG, resveratrol, salicylate and quercetin, are considered to be valuable leads for multitarget drugs.[37] In this study, we suggest STAT3 as one of the multiple molecular targets of HCA as well as the proteasome subunits, LRP1 and Pim-1 kinase.[20-22] Further elaborate

elucidation of molecular targets and systemic understanding of their interactions will help us to design more effective and safe multitarget drugs for cancer treatment.

Natural products, such as curcumin, sugiol, EGCG, PL and resveratrol, produce ROS and induce apoptotic cell death, resulting in the activation of caspase and PARP degradation.[27,31,40,41] Curcumin
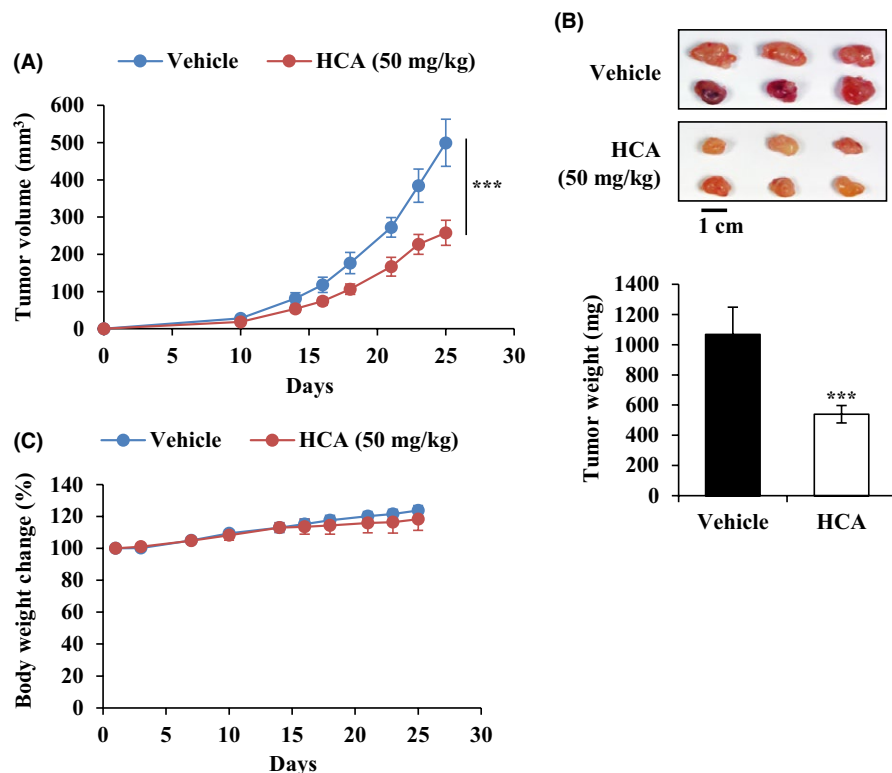

FIGURE 7 2′-Hydroxycinnamaldehyde (HCA) suppresses tumor growth of DU145 prostate cancer cells in vivo. A, Evaluation of the tumor volume (mm³) of each mouse (n = 6 per group) at the indicated time points. DU145 cells were injected subcutaneously into nude mice, and the mice were orally treated with vehicle or HCA (50 mg/kg) 5 times per week for 25 d. B, Representative images of tumor tissue and quantification of the tumor weight of each mouse (n = 6 per group) on day 25. C, Evaluation of the body weight of each mouse (n = 6 per group) with implantation of DU145 cells at the indicated time points. ***$P < 0.001$. Data represent mean ± SD
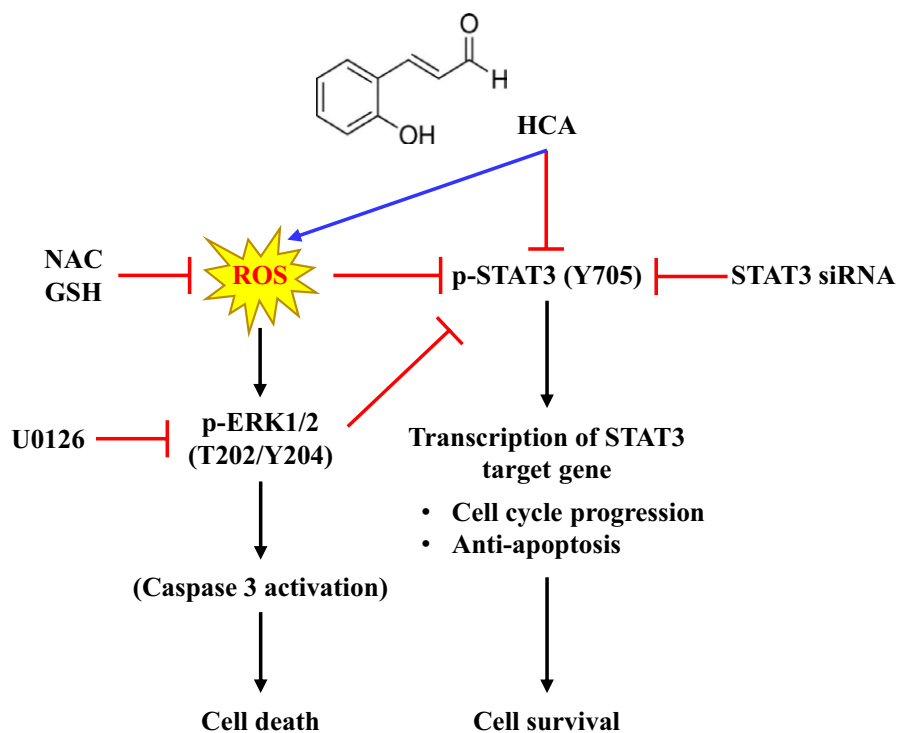

FIGURE 8 Proposed mechanism for anti-tumor activity of 2′-Hydroxycinnamaldehyde (HCA) via reactive oxygen species (ROS) generation and STAT3 inactivation

or PL-induced apoptosis is mediated by ROS and the activation of ERK1/2, which is blocked by antioxidant treatment.[31,40] Sugiol isolated from the roots of salvia has antitumor activity through the inhibition of transketolase, which leads to ROS production and MEG2 activation.[41] As a derivative of HCA, BCA produce ROS, and BCA-induced apoptosis is abolished by the pretreatment of cells with antioxidants.[27] Similarly, ROS generation and ERK1/2 activation were observed 1 hour after treatment with HCA, which was blocked by pretreatment with NAC or GSH (Figure 5).

Reactive oxygen species activate MAP kinase signaling pathways and are involved in the regulation of a variety of biological processes.[13] In addition to ROS-mediated MAPK activation, ROS generation leads to the inactivation of STAT3 signaling.[16,41] Sugiol induces STAT3 dephosphorylation at Tyr705, which is abrogated by treatment with antioxidants or the ERK inhibitor U0126.[41] HCA-induced STAT3 dephosphorylation was rescued by pretreatment with antioxidants, NAC or GSH (Figure 5C), showing STAT3 inactivation by ROS as well as direct binding to STAT3.

To further validate STAT3 as a binding target of HCA, we treated STAT3 knockdown cells with HCA. Consistent with previous reports on STAT3 knockdown cancer cells,[29,42] STAT3-downregulated DU145 cells exhibited decreased cell proliferation and increased intracellular ROS levels (Figure 6). Moreover, STAT3 knockdown and HCA treatment synergistically inhibited cell proliferation and increased ROS generation (Figure 6E,F). Similar results are observed in the tumor sphere formation of NCI-H460 lung cancer cells treated with curcumin and STAT3 siRNA.[43] Taken together, our data suggest that HCA inhibits cell proliferation through STAT3-dependent and ROS-dependent pathways.

In this study, we suggest that HCA functions as a STAT3 inhibitor through direct targeting of the STAT3 proteins and inhibiting dimerization as well as translocation into the nucleus, resulting in selectively suppressed STAT3-activated cancer cell proliferation. In addition to direct inhibition by interaction with STAT3, ROS-mediated ERK1/2 activation and STAT3 inactivation are responsible for the antiproliferative effect of HCA in DU145 prostate cancer cells (Figure 8). An unmet need for antitumor drug development is to develop a new, safe and multitargeting drug candidate because single target therapeutics frequently induce drug resistance and relapse within a couple of months. HCA, which has a relatively low toxicity and is a multitargeting agent, may be a more promising candidate for antitumor drug development.

CONFLICT OF INTEREST

The authors declare no conflict of interests for this article.

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