Chaetocin enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis by enhancing DR5 stabilization and reactive oxygen species generation in human glioblastoma cells

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Abstract. Chaetocin, a fungal metabolite, exerts notable antiproliferative effects against solid tumors by triggering apoptosis; however, the mechanisms underlying its effects remain unclear. As tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) promotes apoptosis in certain types of tumor, the present study aimed to explore the sensitizing effects of chaetocin in TRAIL-induced apoptosis in human glioblastoma cells and the underlying mechanism. Human glioblastoma cells (U343MG, U87MG, U251MG, and T98G) and embryonic kidney cells (HEK293) were co-treated with chaetocin and TRAIL, followed by assessment of cell viability. The results from viability and apoptosis assays demonstrated a significant increase in caspase-dependent apoptosis in glioblastoma cells, but not in HEK293 cells, upon co-treatment with chaetocin and TRAIL. Additionally, death receptor 5 (DR5) expression analysis demonstrated that co-treatment with chaetocin and TRAIL upregulated DR5 expression in a dose- and time-dependent manner by increasing the stability of DR5 on the cell surface. In glioblastoma cells, small interfering RNA-mediated DR5 knockdown markedly suppressed chaetocin/TRAIL-induced apoptosis. Moreover, chaetocin enhanced reactive oxygen species (ROS) production, which facilitated TRAIL-mediated apoptosis by enhancing DR5 upregulation. Thus, chaetocin sensitized the human glioblastoma cell lines U87MG and T98G to TRAIL-mediated apoptosis by upregulating DR5 expression through ROS-mediated mechanisms. The present findings underscore chaetocin as a potential novel therapeutic agent for glioblastoma.

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Introduction

Glioblastoma is the most common type of malignancy of the central nervous system, accounts for almost 80% of all malignant primary brain tumors and is the second most commonly reported type of brain tumor. It accounts for almost all gliomas in the United States, with a 5-year survival rate of 3% (1). Despite ongoing efforts to develop effective molecular targets and combination therapies, the treatment of glioblastoma remains challenging due to several factors, including high therapeutic resistance, redundancy in aberrantly activated signaling pathways, and difficulties in effective drug delivery (2). Therefore, novel therapeutic strategies and additional research are essential for enhancing the effectiveness and outcome of glioblastoma treatment.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising therapeutic treatment for cancer (3). TRAIL is a cytokine that induces apoptosis by binding to TRAIL-receptor 1 (DR4) and TRAIL-receptor 2 (DR5) and forms an apoptosis-inducing signaling complex by interacting with caspase-8 (4-6). The extrinsic route that initiates TRAIL-induced apoptosis involves proteolytic caspase-8 activation, followed by activation of effector caspases, such as caspase-3 (7). TRAIL induces apoptosis in various types of cancer cells but typically does not affect normal cells (8). However, certain cancer types, such as glioblastoma, colon (9) and breast cancer (10) and hepatocellular carcinoma (11), exhibit resistance to TRAIL-induced apoptosis. Therefore, TRAIL alone may be insufficient for treating certain malignant tumors, highlighting the need to sensitize these cancer cells to TRAIL-induced apoptosis for effective therapy.

Chaetocin is a natural compound produced by members of the fungal genus *Chaetomium*; it inhibits activity of the histone methyltransferase SUV39H1 (12). Chaetocin serves as a potent and selective anti-myeloma agent owing to its ability to induce oxidative stress (13). Additionally, chaetocin modulates SUV39H1 activity in a reactive oxygen species (ROS)-dependent manner, resulting in DR-dependent apoptosis (14). Liu *et al* (15) reported that chaetocin promotes endoplasmic reticulum (ER) stress and increases the expression of ER stress markers, including activating transcription

factor 3 (ATF3) and C/EBP homologous protein (CHOP), ultimately leading to DR5-dependent apoptosis. However, the precise mechanisms underlying chaetocin-induced sensitization of tumors to TRAIL are poorly understood. Therefore, the present study aimed to examine the sensitizing effects and underlying mechanism of chaetocin in TRAIL-induced apoptosis in human glioblastoma cells, in order to evaluate its potential as a therapeutic agent for overcoming treatment resistance in glioblastoma.

Materials and methods

Chemicals. Chaetocin was obtained from Enzo Life Sciences, Inc. (cat. no. BML-GR349). Pan-caspase inhibitor z-VAD-fmk and recombinant human TRAIL were purchased from R&D Systems, Inc. (cat. nos. 375-TL and FMK001, respectively). N-acetylcysteine (NAC; cat. no. 01810), cycloheximide (cat. no. A7250), MG132 (cat. no. M8699), and thapsigargin (TG; ER stress inducer; cat. no. T9033) were purchased from Sigma-Aldrich Dulbecco's modified eagle's medium (DMEM), antibiotics, and fetal bovine serum (FBS; cat. no. PK004-07) were obtained from Welgene, Inc.

Cell culture. Human glioblastoma U343MG, U87MG (glioblastoma of unknown origin, HTB-14), U251MG, and T98G (CRL-1690) and embryonic kidney (HEK293, CRL-1573) cells were purchased from American Type Culture Collection and maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were cultured for 16 h until 60-70% confluency was reached, and then treated with chaetocin (500 nM) or TRAIL (50 ng/ml) for 6 h at 37˚C.

Treatment. U87MG and T98G cells were seeded in 6-well plates at a density of $25x10^4$ cells/well. After 24 h, cells were treated with 10 μM TG for 6 h at 37˚C to induce ER stress.

Small interfering (si)RNA-mediated RNA interference. DR5-targeting and scrambled siRNAs were purchased from Santa Cruz Biotechnology Co., Ltd. (cat. nos. sc-40237 and sc-37007, respectively; sequences not available). U87MG and T98G cells were seeded in 6-well plates at a density of $25x10^4$ cells/well and transfected with 50 nM siRNA with Lipofectamine 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions at 37˚C for 24 h. The medium was replaced with DMEM containing 10% FBS without antibiotics, and the cells were treated as aforementioned. Six hours after treatment, cells were collected for viability assays and western blot analysis.

Cell viability assay and Morphological Analysis. CellTiter 96® AQueous One Solution Cell Proliferation Assay system (cat. no. 3580; Promega Corporation) was used according to the manufacturer's instructions. Cells were cultured in a 37˚C CO_2 incubator in 96-well cell culture plates at a density of $2x10^4$ cells/well for 1 day and treated as aforementioned. Each well was loaded with MTS reagent and incubated at 37˚C for 20 min in a 5% CO_2 environment. Absorbance at 450 nm was measured using a Synergy/HTX spectrophotometer (BioTek Instruments, Inc.). Experiments were carried out in triplicate. For cell morphology analysis, cells were treated with chaetocin (500 nM) and TRAIL (50 ng/ml) for 6 h. Phase contrast images were captured using a Leica light microscope (Leica Microsystems), and image acquisition was performed with Leica LAS X Core software (version 3.6).

Flow cytometry. Cells were fixed in 80% ethanol at 4˚C for ≥1 h before determining the DNA content using flow cytometry. The cells were subsequently stained with propidium iodide for 30 min at 4˚C and analyzed using a BD FACSCanto™ II flow cytometer (BD Biosciences). Quantification was performed with BD FACS Diva software version 7.0 (BD Biosciences).

Western blotting. Cells were lysed with RIPA lysis buffer (cat. no. 89900, Thermo Fisher Scientific, Inc.) and supernatant was collected. Bicinchoninic acid was used to measure the protein content. Western blotting was performed as described previously (16). Protein (50 μg/lane) was resolved using 13% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature. Membranes were incubated with primary antibodies: anti-PARP (cat. no. #9542), anti-caspase-8 (cat. no. #9496; Cell Signaling Technology, Inc.), anti-pro-caspase-3 (cat. no. #sc7148; Santa Cruz Biotechnology Co., Ltd.), anti-cleaved caspase-3 (cat. no. #9661S), anti-DR5 (cat. no. #8074S; Cell Signaling Technology, Inc.), anti-DR4 (cat. no. #sc8411), anti-78-kDa glucose-regulated protein (Bip) (cat. no. #sc13968), anti-CHOP (cat. no. #sc7351), anti-p53 (1:1,000; cat. no. #sc126; Santa Cruz Biotechnology Co., Ltd.) and anti-β-actin (ACTB) (1:5,000; cat. no. A5441, Sigma-Aldrich). The membranes were incubated overnight with the primary antibodies at 4˚C. The membranes were then incubated with anti-rabbit (cat. no. 111-035-045) and anti-mouse IgG (both 1:5,000; cat. no. 115-035-062; Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 1 h. Protein bands were identified using Immobilon Western Chemiluminescent HRP Substrate (cat. no. WBKLS0500; MilliporeSigma). The blots were quantified by densitometric analysis using ImageJ v4.0 software (National Institutes of Health) and the relative expression of each target protein was normalized using ACTB.

Caspase-3/7 activity assay. Cells ($5x10^4$/well) were seeded in a 96-well plate and treated as aforementioned. A total of 100 μl Caspase-Glo® 3/7 Assay reagent (cat. no. G8090; Promega Corporation) was added and incubated at 37˚C for 1 h and luminescence was measured using a Synergy/HTX spectrophotometer (BioTek instrument, Inc.).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from cells using TRIzol (cat. no. 15596-026; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using the reverse transcriptase premix (cat. no. EBT-1515; Elpis Biotech, Inc.) at 40˚C for 60 min, followed by enzyme inactivation at 92˚C for 5 min. RT-qPCR was performed using Blend Taq DNA polymerase (cat. no. M8291; Promega Corporation) with primers for DR5 and GAPDH. The sequences were follows: DR5 (forward: 5'-AAGACCCTTGTGCTCGTTGT-3', reverse: 5'-GACACATTCGATGTCACTCCA-3') and GAPDH

(forward: 5'-CGTCTTCACCACCATGGAGA-3', reverse: 5'-CGGCCATCACGCCCAGTTT-3'). The qPCR thermocycling conditions were as follows: 95˚C for 30 sec, followed by 25 cycles of degradation at 94˚C for 30 sec, annealing at 56˚C for 30 sec, extension at 72˚C for 30 sec for DR5. Amplified products were separated on a 2% agarose gel and bands were visualized on a LAS-3000 (FujiFilm Wako Pure chemical corporation). The relative gene expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (17) with normalization to the control.

Cell surface staining for DR5. Cells were washed three times in PBS and suspended in 200 μl 2% FBS/PBS. The primary antibody (anti-DR5, 1:100, cat. no. #8074S; Cell Signaling Technology, Inc.) was added at room temperature for 30 min. The cells were washed twice with PBS, resuspended in 200 μl 2% FBS/PBS, and incubated with fluorescein isothiocyanate-conjugated secondary antibody (Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L), 1:100, cat. no. A11008; Invitrogen, Thermo Fisher Scientific, Inc.) for 30 min at room temperature. The cells were centrifuged at 200 x g for 5 min at room temperature to remove unbound secondary antibodies and resuspended in 500 μl PBS. DR5 expression on the cell surface was measured using flow cytometry as aforementioned.

Cycloheximide (CHX) chase assay. U87MG and T98G cells were seeded in 6-well plates at a density of $25x10^4$ cells/well and cultured for 16 h in a 37˚C CO_2 incubator. The cells were then treated with 500 nM chaetocin for 6 h, followed by treatment with 50 μM CHX or CHX + chaetocin for 1, 3, 5, and 7 h at 37˚C. Protein lysates were prepared using RIPA buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.). A total of 50 μg of lysates were subjected to immunoblotting to assess the stability of DR5. Anti-ACTB was used as a loading control. The blots were quantified by densitometric analysis using ImageJ v4.0 software (National Institutes of Health) and the relative expression level of DR5 was normalized using ACTB.

Proteasome activity assay. Proteasome activity was measured using Suc-LLVY-AMC (Biomol International). Cell lysates were prepared from chaetocin-treated cells using RIPA lysis buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.). A mixture containing 5 μg of protein in 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 2 mM ATP was incubated at 37˚C for 30 min with 50 μM Suc-LLVY-AMC. Chymotrypsin-like proteasome activity was measured using a fluorometric plate reader with excitation and emission wavelengths of 380 nm and 440 nm, respectively. MG132 (10 μM) was used as a positive control to inhibit proteasome activity for 6 h at 37˚C.

ROS production. The fluorescent probe 2',7' dichlorodihydrofluorescein diacetate (H2DCFDA; Sigma-Aldrich; Merck KGaA) was used to measure intracellular ROS production. U87MG and T98G cells were pretreated with 500 nM chaetocin for 6 h at 37˚C, after which 10 μM H_2DCFDA was added for 30 min at room temperature. Following incubation, cells were trypsinized, resuspended in PBS and transferred to Falcon® FACS tubes. H_2DCFDA fluorescence intensity was evaluated with a BD FACS Canto™ II flow cytometer (BD Biosciences). The excitation wavelength was 488 nm and the emission wavelength was 520 nm. Data were analyzed using BD Biosciences FACS Canto™ software (version 6.0).

Statistical analysis. Data are presented as the mean ± SD of three independent experiments. Differences were assessed using one-way ANOVA and Tukey's post-hoc test. All statistical analyses were performed with SPSS 11.5 (SPSS, Inc.) software. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Chaetocin enhances TRAIL sensitivity in human glioblastoma cells. To assess the effect of chaetocin on cancer cell sensitivity to TRAIL, the viability of human glioblastoma cell lines U343MG, T98G, U251MG, and U87MG was evaluated after 6 h of treatment with chaetocin. T98G and U87MG cells were the most sensitive to chaetocin (Fig. 1A) and were selected for the subsequent experiments. Chaetocin alone significantly decreased the viability of the U87MG and T98G glioblastoma cell lines (Fig. 1B). In particular, combined treatment with chaetocin and TRAIL significantly decreased cell viability and induced morphological changes, such as cell shrinkage and blebbing, in U87MG and T98G cells (Fig. 1B and C). To investigate the role of caspases in cell death, the present study assessed the impact of the pan-caspase inhibitor z-VAD-fmk on cell viability. z-VAD-fmk inhibited cell death induced by chaetocin + TRAIL (Fig. 1B). Additionally, phase contrast microscopy showed that co-treatment with chaetocin and TRAIL increased the number of apoptotic bodies compared with the untreated group (Fig. 1C). Moreover, chaetocin improved the sensitivity of U343MG and U251MG cells to TRAIL-mediated cell death (Fig. S1A) without affecting normal cells (Fig. S1B). Collectively, these data suggest that chaetocin enhances TRAIL-induced caspase-dependent cell death.

Chaetocin enhances TRAIL-mediated apoptosis in human glioblastoma cells. Since chaetocin promotes TRAIL-mediated cell death through caspase activation, the present study investigated whether the cell death induced by the combination of chaetocin and TRAIL was attributable to apoptosis. U87MG and T98G cells were treated with chaetocin and TRAIL for 6 h and harvested for cell cycle analysis. The combination of chaetocin and TRAIL increased the percentage of U87MG and T98G cells in the sub-G1 phase to 12.5% and 18.4%, respectively, compared with 2.8% and 1.8% in the control group (Fig. 2A). Furthermore, the present study confirmed the effect of chaetocin on TRAIL-mediated apoptosis by examining caspase-3/7 activity. Notably, chaetocin significantly enhanced TRAIL-induced activation of caspase-3/7 both in U87MG and T98G cells (Fig. 2B). However, treatment with pan-caspase inhibitor z-VAD-fmk almost completely blocked apoptosis and caspase-3/7 activity, indicating that apoptosis induced by chaetocin and TRAIL was caspase-dependent (Fig. 2A and B). Additionally, combined treatment with TRAIL significantly induced the cleavage of apoptosis markers PARP, caspase-8, and caspase-3 in U87MG and T98G cells (Fig. 2C). Treatment with z-VAD-fmk inhibited the cleavage of PARP and caspase-3 (Figs. 2C and S2). Collectively, these findings indicate that chaetocin enhances TRAIL-induced apoptosis in glioblastoma cells.

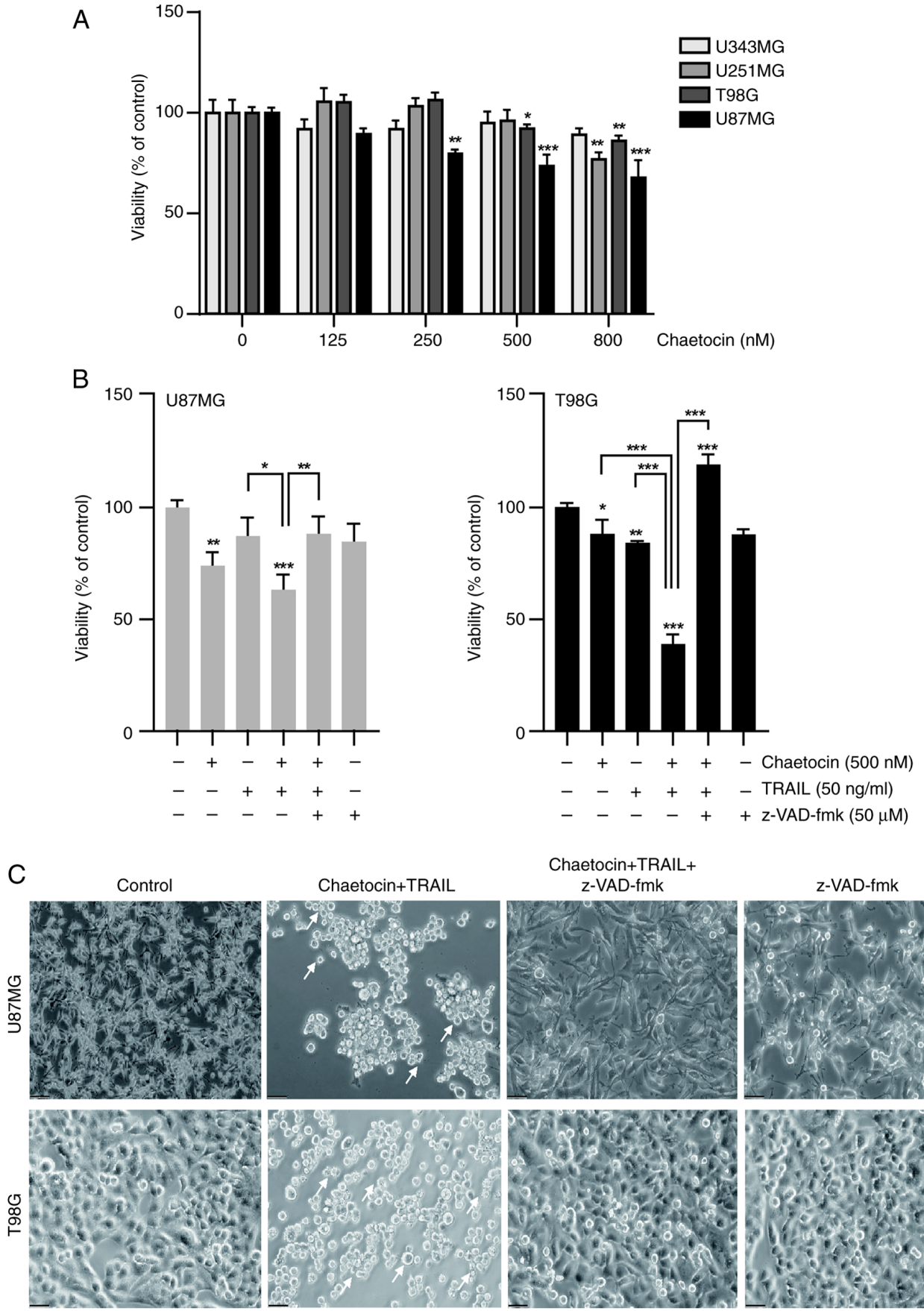

Figure 1. Effects of chaetocin and TRAIL on human glioblastoma cells. (A) Viability of U343MG, T98G, U251MG, and U87MG human glioblastoma cells after 6 h treatment with chaetocin. (B) Viability of U87MG and T98G cells pretreated with 50 μM z-VAD-fmk for 30 min prior to combined treatment with chaetocin and TRAIL for 6 h. (C) Cell morphology was examined using interference light microscopy (magnification, x400). White arrows indicate apoptotic bodies. Scale bar, 50 μm. *P<0.05, **P<0.01, ***P<0.001 vs. control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; z-VAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

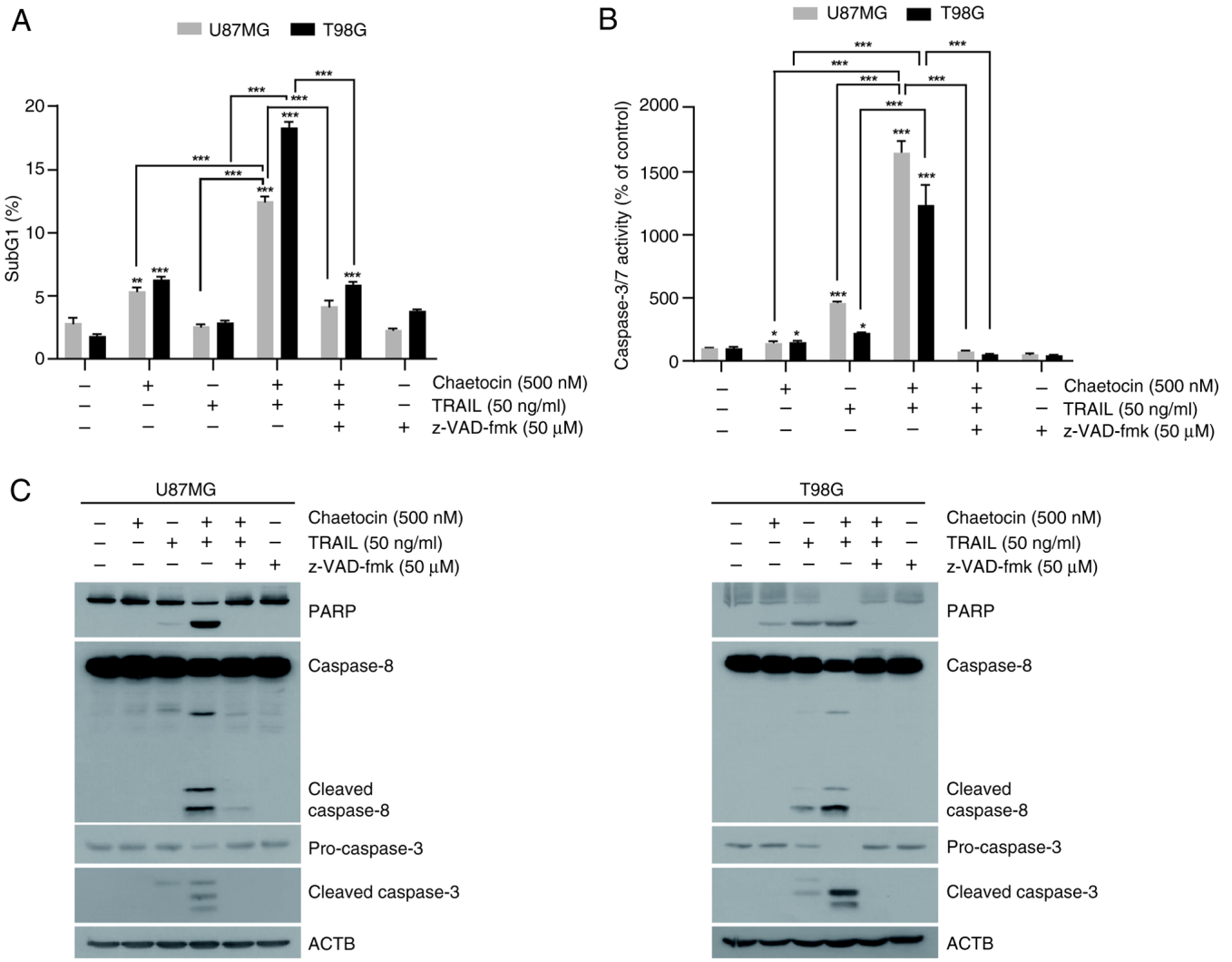

Figure 2. Chaetocin enhances TRAIL-mediated apoptosis in human glioblastoma cell lines. (A) Flow cytometric analysis of U87MF and T98G cells treated with chaetocin alone or in combination with TRAIL and z-VAD-FMK for 6 h to determine the sub-G1 population. (B) Caspase-3/7 activity in U87MG and T98G cells. (C) Western blot analysis of PARP, caspase-8, pro-caspase-3, and cleaved caspase-3 expression in U87MG and T98G cells treated with chaetocin and TRAIL, with or without z-VAD-fmk, for 6 h. *P<0.05, **P<0.01, ***P<0.001 vs. control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; ACTB, β-actin; z-VAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

Chaetocin upregulates DR5 expression in human glioblastoma cells by stabilizing the protein and inhibiting proteasome activity. To clarify the molecular mechanisms of chaetocin-mediated TRAIL sensitization, the present study investigated the expression of the TRAIL receptor DR5. Chaetocin treatment markedly upregulated DR5 expression in a dose- and time-dependent manner (Figs. 3A and B, and S3A and B), whereas DR4 expression remained unchanged (Fig. S3C). Chaetocin did not significantly affect DR5 mRNA expression, suggesting that chaetocin upregulated DR5 expression post-transcriptionally (Fig. 3C). As increased DR5 surface expression is essential for TRAIL-mediated cell death (18), the present study examined whether chaetocin increased DR5 surface expression. Consistent with the western blotting results, chaetocin increased DR5 protein levels on the surface of U87MG and T98G cells (Fig. 3D). Taken together, these results indicate that chaetocin upregulates DR5 expression.

To determine the mechanism underlying chaetocin-mediated DR5 upregulation, the present study investigated the effect of chaetocin on DR5 protein stability in U87MG and T98G cells. Following chaetocin treatment for 4 h, the cells were exposed to cycloheximide (CHX), a *de novo* protein synthesis inhibitor, in the presence or absence of chaetocin for 1-7 h. DR5 protein levels were determined using western blot analysis. Although CHX resulted in a gradual decrease in DR5 expression, CHX and chaetocin co-treatment sustained DR5 expression (Fig. 4A and B). Additionally, this study investigated the involvement of the proteasome in chaetocin-induced DR5 regulation and found that chaetocin reduced proteasome activity (Fig. 4C). Overall, these results indicated that chaetocin upregulates DR5 expression by increasing the stability of DR5 protein.

Chaetocin enhances TRAIL-induced apoptosis via DR5 upregulation. Glioblastoma cells were transfected with siRNA targeting DR5 to investigate its functional role in chaetocin/TRAIL-induced apoptosis. siRNA-mediated knockdown of DR5 effectively inhibited apoptosis induced by the combined treatment of chaetocin and TRAIL in U87MG

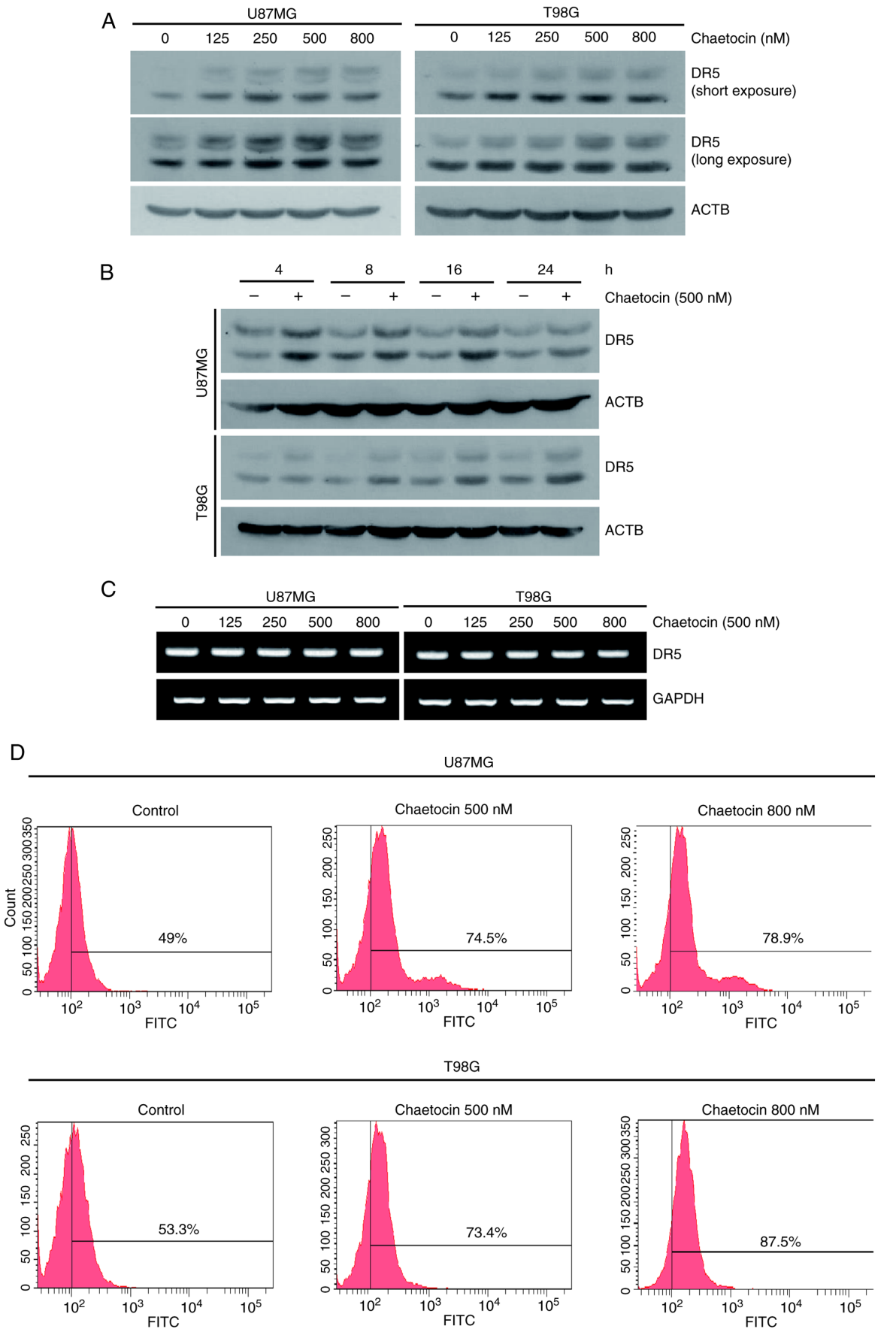

Figure 3. Chaetocin upregulates DR5 expression in U87MG and T98G cells. DR5 expression in U87MG and T98G cells treated with (A) various concentrations of chaetocin for 6 h or (B) 500 nM chaetocin, as determined using western blotting. ACTB was used as the loading control. (C) U87MG and T98G cells were treated with chaetocin for 6 h. DR5 mRNA expression was determined using reverse transcription-quantitative PCR. GAPDH was used as the loading control. (D) Effects of chaetocin on cell surface DR5 expression in U87MG and T98G cells, as determined by flow cytometry after 6 h of treatment. DR, death receptor; ACTB, β-actin.

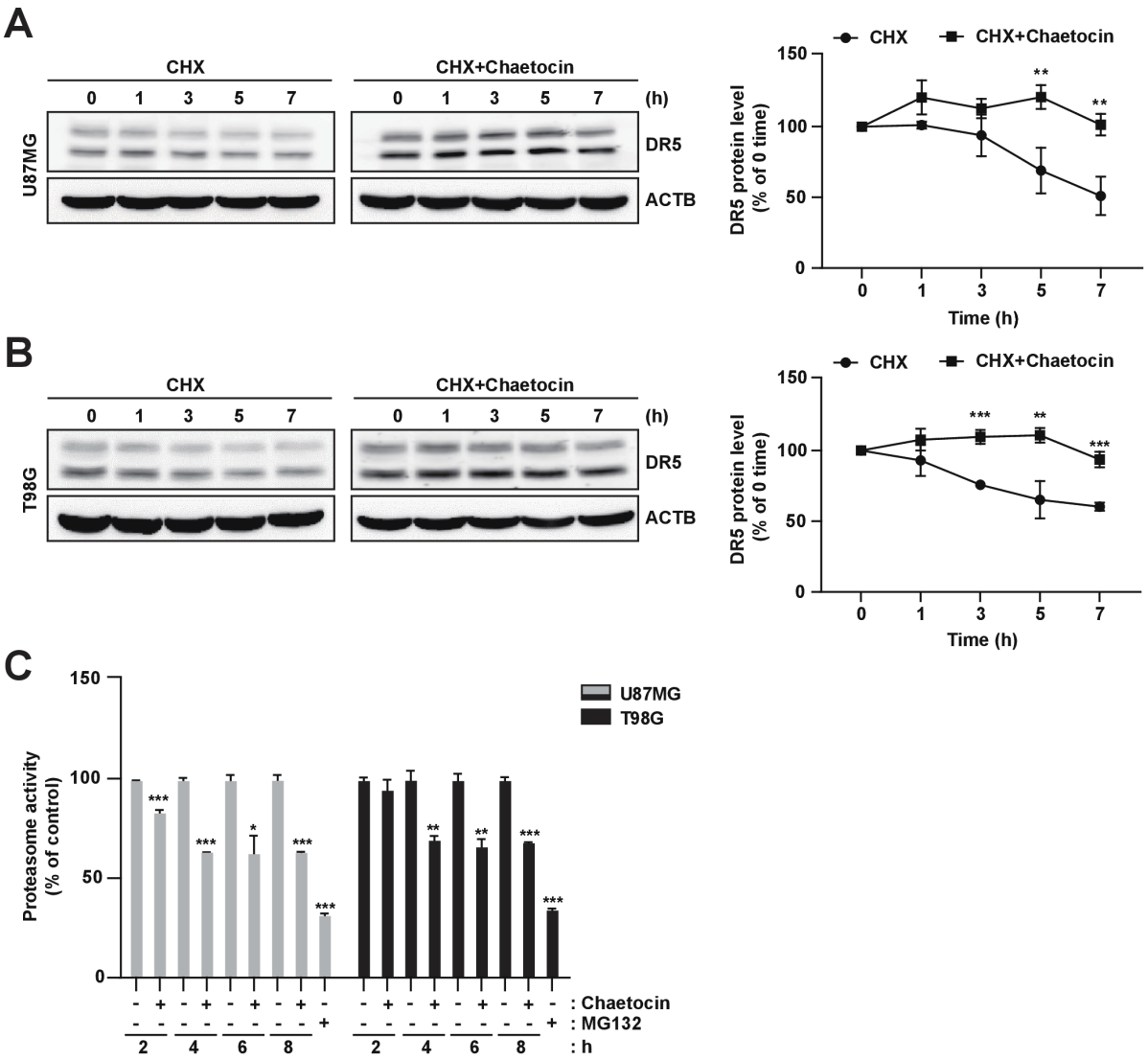

Figure 4. Chaetocin upregulates DR5 expression by stabilizing the protein and decreasing proteasome activity in human glioblastoma cells. (A) U87MG and (B) T98G cells were pre-treated with 500 nM chaetocin for 6 h, washed with PBS, and then treated with 50 μM cycloheximide (CHX) and/or 500 nM chaetocin for the indicated time periods. DR5 expression was determined by western blotting. (C) Proteasome activity in U87MG and T98G cells following treatment with 500 nM chaetocin. MG132 (10 μM, 6 h) was used as a positive control for proteasome inhibition. *P<0.05, **P<0.01, ***P<0.001 vs. control. DR, death receptor; CHX, cycloheximide.

and T98G cells (Fig. 5A). Although chaetocin + TRAIL treatment reduced the viability of cells transfected with scramble by 36%, DR5 knockdown significantly restored cell viability to 87% (Fig. 5B). DR5 knockdown reduced the cleavage of caspase-3 and PARP induced by chaetocin + TRAIL treatment in both U87MG and T98G cells (Figs. 5C and S4). Collectively, these findings indicate that DR5 plays a crucial role in chaetocin/TRAIL-mediated apoptosis in human glioblastoma cells.

Chaetocin enhances TRAIL-mediated apoptosis via ROS generation. ROS are key activators of DR5 upregulation, which leads to TRAIL-mediated apoptosis (19,20). To investigate whether ROS are required for chaetocin/TRAIL-induced apoptosis in U87MG and T98G cells, the present study assessed the impact of chaetocin treatment on ROS production. Chaetocin treatment for 10 min markedly increased H_2DCFDA fluorescence intensity in U87MG cells (Fig. 6A), suggesting increased ROS production. To confirm the role of ROS in chaetocin/TRAIL-induced apoptosis, cells were pretreated with 5 mM NAC, a ROS scavenger, prior to treatment with chaetocin and TRAIL for 6 h. NAC pretreatment significantly decreased DR5 and cleaved caspase-3 expression (Figs. 6B and S5). Treatment with chaetocin + TRAIL promoted cell death, while pretreatment with NAC notably reduced this effect (Fig. 6C). Furthermore, p53 (21,22), CHOP (23,24), and NF-κB (25) may play a role in the upregulation of DR5. Therefore, the present study investigated whether chaetocin upregulated DR5 expression by increasing p53 and CHOP expression. Chaetocin treatment did not significantly affect p53, CHOP, and Bip expression in U87MG and T98G cells (Figs. S6A and B). Collectively, these findings suggest that chaetocin-induced ROS generation was essential for DR5 expression and contributed to sensitizing cells to TRAIL-induced apoptosis.

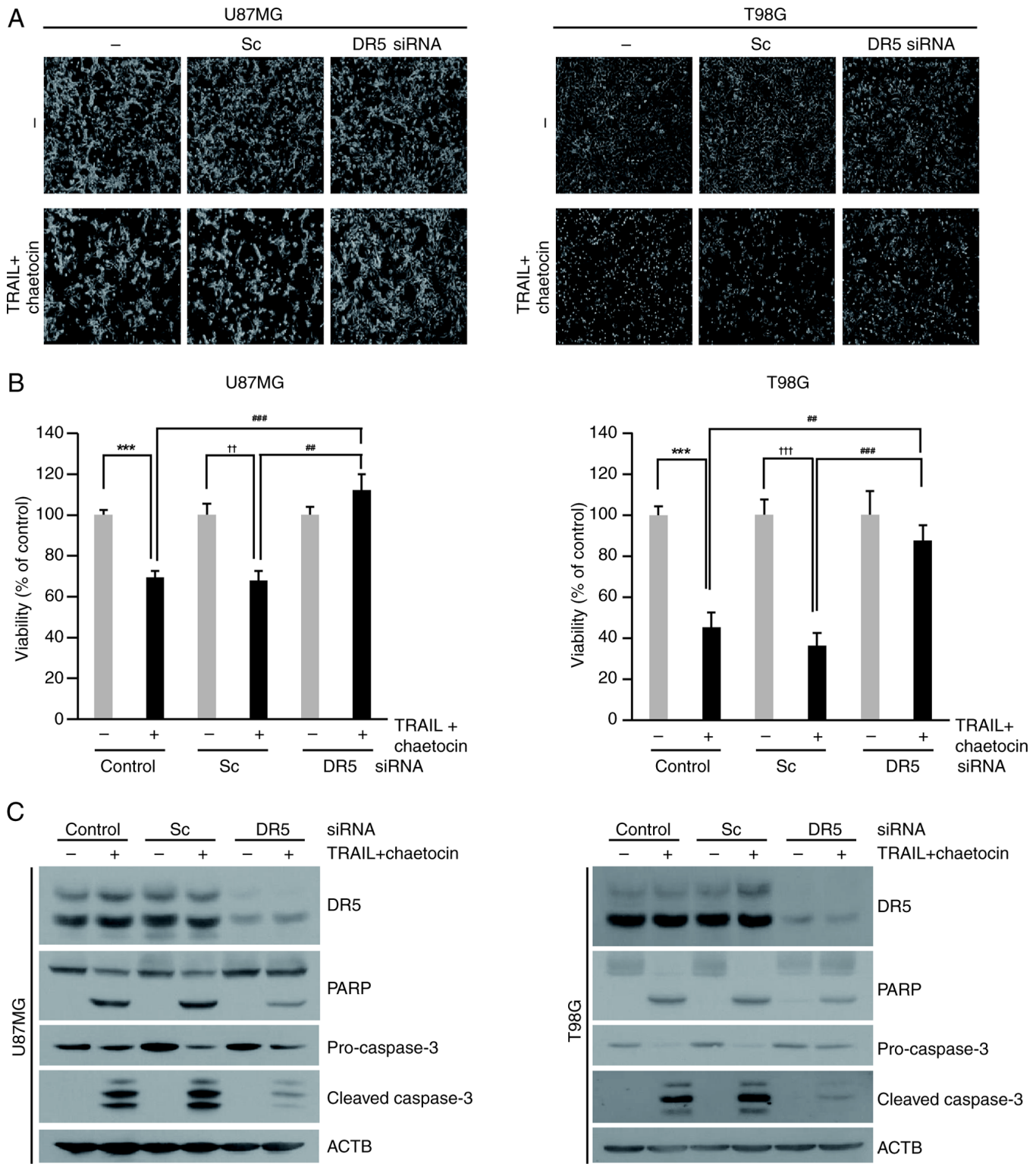

Figure 5. Blocking DR5 expression inhibits the effect of chaetocin on TRAIL-mediated apoptosis. (A-C) Cells were transfected with DR5 or Sc siRNA (50 nM) for 24 h and then treated with chaetocin (500 nM) and TRAIL (50 ng/ml) for 6 h. (A) Cell morphology was examined using interference light microscopy (magnification, x200). (B) U87MG and T98G cell viability. (C) Western blot analysis was performed to determine the expression of DR5 and apoptotic markers (PARP, cleaved caspase-3, and pro-caspase-3). ***P<0.001 vs. control, ††P<0.05, †††P<0.001 vs. Sc, $^{\#\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. TRAIL + chaetocin. DR, death receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Sc, scrambled control; si, small interfering; ACTB, β-actin.

Discussion

Our data demonstrate that the co-treatment of chaetocin and TRAIL induces apoptosis in U87MG and T98G human glioblastoma cells via chaetocin-induced ROS-dependent upregulation of DR5. Notably, the present findings also highlight that chaetocin plays a pivotal role in sustaining DR5 stability in these cells.

Chaetocin, a fungal metabolite derived from species of the genus *Chaetomium*, has a thiodioxopiperazine structure (26).

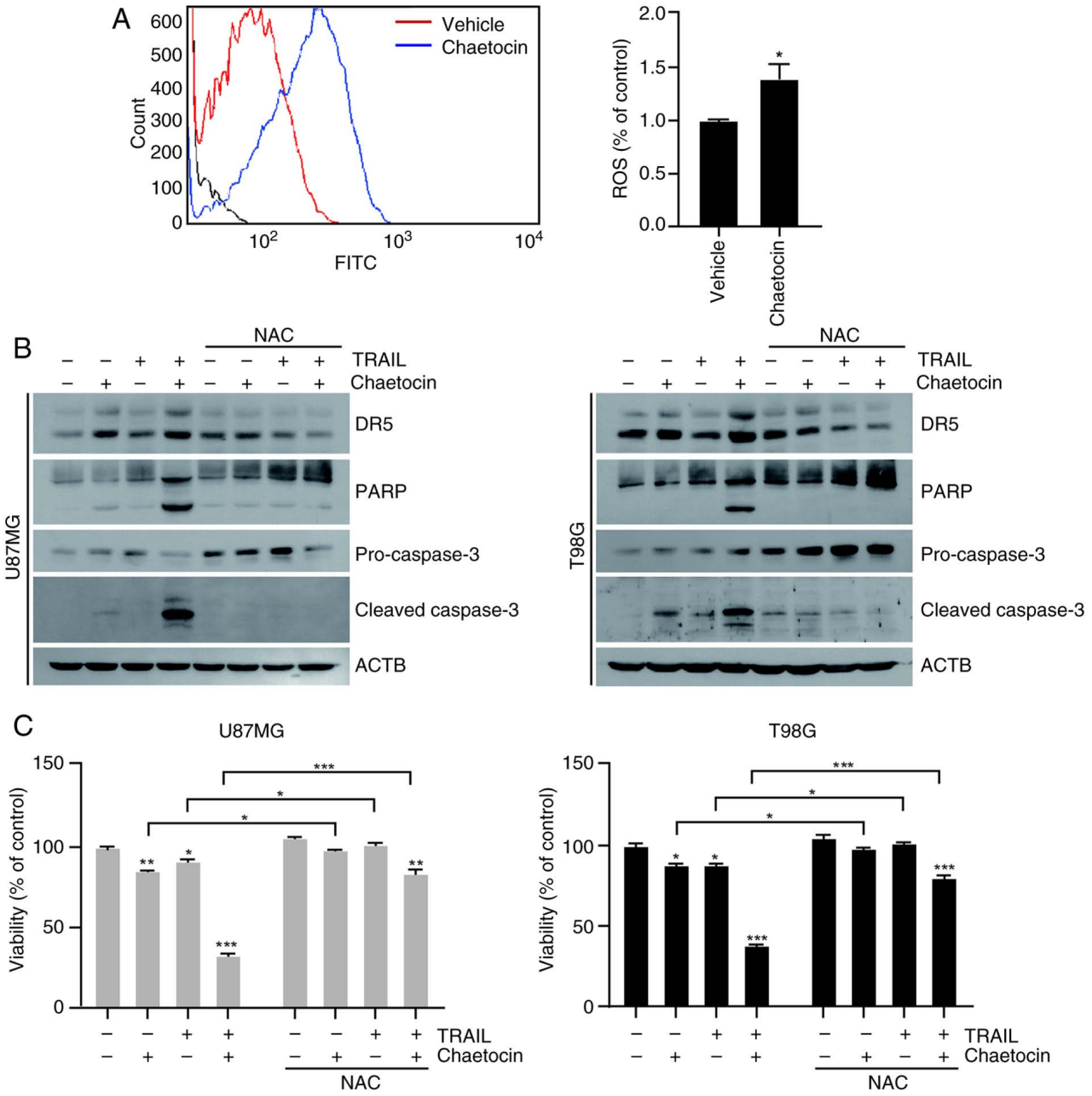

Figure 6. Chaetocin-induced DR5 upregulation is mediated by ROS generation in human glioblastoma cells. (A) ROS production in cells treated with chaetocin for 10 min, as determined by flow cytometry. Fluorescence intensity is directly proportional to the amount of ROS. (B) DR5 expression in U87MG and T98G cells treated with chaetocin (500 nM) and TRAIL (50 ng/ml) in the presence or absence of 5 mM NAC, assessed using western blotting. (C) Cell viability. *P<0.05, **P<0.01 and ***P<0.001 vs. control. DR, death receptor; ROS, reactive oxygen species; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; NAC, N-acetylcysteine.

It exhibits potent antiproliferative activity against solid tumors via apoptosis induction. Chaetocin has been shown to induce ROS production, JNK/c-Jun pathway activation and macrophage phagocytosis to trigger caspase-dependent apoptosis in colorectal cancer cells (27). Additionally, chaetocin increases apoptosis by enhancing ROS levels in OVCAR-3 ovarian cancer cells (28). Han *et al* (13) reported that chaetocin induces ROS-mediated apoptosis in human melanoma cells and suppresses the growth of melanoma tumors in nude mice. Moreover, chaetocin triggers not only apoptosis but also autophagy; it exerts anticancer effects in hepatoma cell lines by inducing autophagy (29). Despite these findings, the anticancer effects of chaetocin in human glioblastoma and the underlying mechanisms have not been fully elucidated.

TRAIL, a member of the TNF family, induces apoptosis in various types of cancer cell but not in normal cells (3,30). Notably, although TRAIL is regarded as a promising anticancer agent due to its ability to selectively target cancer cells, several cancer types - including colon, breast, and hepatocellular carcinoma - exhibit resistance to TRAIL-induced apoptosis (9-11). Therefore, novel therapeutic strategies are required to sensitize cancer cells to TRAIL-induced apoptosis. TRAIL functions by binding to cell surface DRs, primarily DR4 and DR5, to initiate apoptotic signaling (31). Additionally, the strength and duration of TRAIL-induced apoptotic signaling are influenced by expression

of DRs (32). Notably, DR5 is upregulated by silibinin (33), mitoxantrone (34), and lovastatin (35) during TRAIL-mediated apoptosis in glioblastoma cells. In the present study, chaetocin effectively upregulated DR5 expression and the knockdown of DR5 significantly inhibited chaetocin/TRAIL-induced apoptosis in both U87MG and T98G cells.

The roles of DR4 and DR5 are critical for the cytotoxic effects of TRAIL and other chemotherapeutic agents (20,33,36,37). In the present study, chaetocin treatment led to DR5 upregulation in a dose- and time-dependent manner and increased DR5 surface expression on cells. Overall, these results suggest that chaetocin-induced upregulation of DR5 may play an essential role in chaetocin- and TRAIL-induced apoptosis. Although a previous study demonstrated that chaetocin serves as an apoptosis-inducing agent and induces DR5-mediated cell death in TRAIL-treated glioblastoma cells (38), the present study found that chaetocin regulated expression and stability of DR5 in U87MG and T98G cells. Additionally, further experiments using protein synthesis inhibitor CHX showed that chaetocin did not affect the mRNA expression of DR5 but affected its protein levels.

Chaetocin regulates SUV39H1 activity in an ROS-dependent manner, influencing the expression of DR-related genes and leading to DR-dependent apoptosis (14). Additionally, chaetocin promotes ROS generation and upregulates antioxidant genes, such as heme oxygenase 1, NAD(P)H quinone dehydrogenase 1, glutamate-cysteine ligase modifier subunit and thioredoxin reductase 1 (39,40). In the present study, chaetocin induced ROS generation, whereas NAC pretreatment inhibited chaetocin-induced ROS production. Collectively, these results revealed the key role of ROS in chaetocin-induced DR5 expression. Furthermore, the present data emphasize the interaction between TRAIL-mediated signaling and oxidative stress response, with previous studies demonstrating that ROS production enhances DR5 expression in human carcinoma cell lines (41-43). Notably, compounds such as baicalein (42) and vitisin A (43) sensitize prostate cancer cells to TRAIL by promoting ROS generation and upregulating DR5.

The combination of chaetocin and TRAIL has clinical relevance for glioblastoma treatment by addressing key challenges in current therapeutic strategies. This approach selectively induces apoptosis in glioblastoma cells while sparing normal cells, highlighting its potential safety and specificity. Mechanistically, chaetocin enhances TRAIL sensitivity through ROS-mediated DR5 stabilization, offering a basis for personalized therapy using biomarkers including ROS or DR5. In future, *in vivo* experiments will be essential to validate the findings of *in vitro* study and assess the effects of chaetocin and TRAIL co-treatment on glioblastoma tumor growth to determine whether chaetocin enhances TRAIL-mediated apoptosis and tumor suppression in glioblastoma, further highlighting its potential as a therapeutic agent for glioblastoma treatment. Its integration with standard treatments such as radiotherapy and temozolomide may provide synergistic benefits, particularly in treatment-resistant cases. Although further preclinical studies are needed, the dual role of chaetocin as an epigenetic modifier and apoptosis sensitizer positions it as a promising candidate for clinical trials. In conclusion, chaetocin enhances TRAIL-induced apoptosis in human glioblastoma cells by increasing DR5 stability, promoting ROS production and upregulating DR5 expression. Overall, the present study clarified the mechanism by which chaetocin enhances cancer cell sensitivity to TRAIL and offers potential therapeutic strategies for cancer treatment.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HJJ wrote the manuscript, performed experiments and analyzed data. JKK wrote the manuscript and performed experiments. SIS performed the literature review. WKB conceived and supervised the study. HJJ and WKB confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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