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Original Article

Hsp90 inhibitor sensitizes TRAIL-mediated apoptosis via chop-dependent DR5 upregulation in colon cancer cells

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Abstract: Heat shock protein 90 (Hsp90), a molecular chaperone, is involved in a variety of physiological and pathological processes. Targeting Hsp90 by small molecules has been developed as an attractive strategy of anticancer therapy. In this study, we investigated the mechanism of Hsp90 inhibitor suppresses CRC growth and potentiates effects of other chemotherapeutic drugs. We found that Hsp90 inhibitor induces chop-dependent DR5 upregulation regardless of p53 status. Furthermore, DR5 is required for Hsp90 inhibitor-induced apoptosis. Hsp90 inhibitor also synergized with TRAIL to induce marked apoptosis via DR5 in CRC. Overall, our results illustrate DR5 play a key role in mediating the anticancer effects of Hsp90 inhibitor in CRC and suggest that DR5 expression can be used as an indicator of Hsp90 inhibitor sensitivity, which has important implications for it clinical applications.

Keywords: Hsp90 inhibitor, TRAIL, DR5, apoptosis, chop

Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed types of cancer in US [1]. Conventional chemotherapy for CRC treatment including DAN damage drugs such as combinations of 5-fluorouracil (5-FU), oxaliplatin, capecitabine and irinotecan (FOLFIRI, CAPOX or FOLFOX) [2]. Right now, targeting therapy has been used to overcome poor therapeutic efficacy of conventional cancer treatment. Heat shock protein 90 (Hsp90) is a key chaperone to keep the integrity of the oncogenic signaling in cancer [3]. There are more than 200 proteins have been identified to be Hsp90 clients, and many clients are oncogenic proteins and play critical roles in cancer cells [4]. Drugs targeting Hsp90 of cancer, including geldanamycin have been under development since 1999 and have become an exciting target in cancer cells [5]. 17-allylamino-17-demethoxygeldanamycin (17-AAG), one of geldanamycin analogs which have low hepatotoxic inhibits the molecular chaperone function of Hsp90 by binding to its ATP/ADP pocket and causing destabilization of its complexes with client proteins [6]. However, the mechanism of cell killing of Hsp90 inhibitor in colon cancer is still not well understood.

Apoptosis plays an important role in the antitumor activities of conventional chemotherapeutic agents and targeted therapies. It is regulated by the intrinsic pathway (mitochondrial pathway), and extrinsic pathway [7, 8]. The mitochondrial-mediated apoptosis pathway is activated through Bcl-2 family members. The stimuli activate BH3-only proteins, which inhibit the pro-survival Bcl-2 proteins, activate the pro-apoptotic effectors BAX/BAK, leading to disruption of the mitochondrial outer membrane [9, 10]. The activation of extrinsic pathway is upon binding of pro-apoptotic ligands to the extended TNF family receptors, and regulated by decoy receptors (DcRs) negatively [11]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, also known as apo2 ligand) is a cytokine well known for its ability to selectively induce apoptosis in cancer cells while it has no effect on most of normal cells [12]. TRAIL binds to cell surface death receptor 5 (DR5, also known TRAIL-R2) or death receptor 4 (DR4, also known TRAIL-R1), which further recruit the adaptor Fas-associated protein with death domain (FADD) and caspase 8, leading to caspase 8 activation [12, 13].

In the present study, our results demonstrate that Hsp90 inhibitor sensitizes TRAIL-mediated

apoptosis via DR5 induction through chop-dependent manners. In addition, our results suggest that DR5 induction is a biomarker of the therapeutic efficacy of Hsp90 inhibitor.

Materials and methods

Cell culture and drug treatment

CRC cell lines including HCT116, Lim1215, Lim2405, SW48, RKO and HT29 were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The anticancer agents and chemicals including 17-AAG, 17-DMAG (Selleckchem) were diluted with DMSO. TRAIL (Sigma) were diluted with water.

Real-time reverse transcriptase PCR (qRT-PCR)

Total RNA was extracted using the TRIzol RNA Kit (Invitrogen, CA) according to the manufacturer's protocol. Briefly, total RNA was used to generate cDNA using SuperScript II reverse transcriptase (Invitrogen). PCR was performed in triplicate using SsoFasr™ Probes Supermix (Bio-Rad) in a final reaction volume of 20 μL with gene-specific primer/probe sets, and a standard thermal cycling procedure (38 cycles) on a Bio-Rad CFX96™ Real-time PCR System. DR5 and β-actin levels were assessed using TaqMangene expression real-time PCR assays. Result was expressed as the threshold cycle (Ct). The relative quantification of the target transcripts was determined by the comparative Ct method (ΔΔCt) according to the manufacturer's protocol. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative changes in gene expression. Control experiments were conducted without reverse transcription to confirm that the total RNA was not contaminated with genomic DNA. β-Actin was used as an internal control.

Western blotting

Western blotting was performed with antibodies for FADD, p65, phospho-p65, cleaved-caspase 3, cleaved-caspase 8, chop (Cell signaling technology, Beverly), DR5, DR4, MZF1, Bip, β-actin (Santa cruz biotechnology, Santa Cruz), FAS, Mcl-1, Bcl-2 and Bcl-X_L (BD, San Jose).

Apoptosis assays

Apoptosis was analyzed by nuclear staining with Hoechst 33258 (Invitrogen) [8, 14, 15]. AnnexinV/propidium iodide (PI) staining was performed using Annexin-Alexa 488 (Invitrogen) and PI. Caspase activity was measured using the SensoLyte Homogeneous AMC Caspase-3/7 Assay Kit (Anaspec).

MTS assay

The indicated cell lines were seeded in 96-well plates at a density of 1×10^4 cells/well. After overnight incubation, cells were treated with 17-AAG for 72 hours. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed using the MTS assay kit (Promega) according to the manufacturer's instructions. Luminescence was measured with a Wallac Victor 1420 Multilabel Counter (Perkin Elmer). Each assay was conducted in triplicate and repeated three times.

Transfection and siRNA/shRNA knockdown

HCT116 cells were transfected with lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Knockdown experiments were performed 24 hours prior to 17-AAG treatment using 300 pmole of siRNA. The control scrambled siRNA and siRNA for human chop (AAGACCCGCGCCGAGGUGAAG) and DR5 (AAGACCCUUGUGCUCGUUGUC) were from Invitrogen. For stable transfection a shRNA-expressing plasmid that containing the FADD-targeting sequence (GCAGUCCUCUUAUUCCUAA), DR5-targeting sequence (AAGACCCUUGUGCUCGUUGUC), or a vector containing a scrambled sequence was transfected into HCT116 cells. Followed transfection, cells were plated in 96-well plates in the presence of 5 μg/mL puromycin. The protein expression of puromycin-resistant clones was then analyzed by western blotting.

Chromatin immunoprecipitation (ChIP)

ChIP with chop antibody (Cell signaling technology, Beverly) was performed using the chromatin immunoprecipitation assay kit (Millipore) according to the manufacturer's instructions. The precipitates were analyzed by PCR using primers 5'-AGGTTAGTTCCGGTCCCTTC-3' and

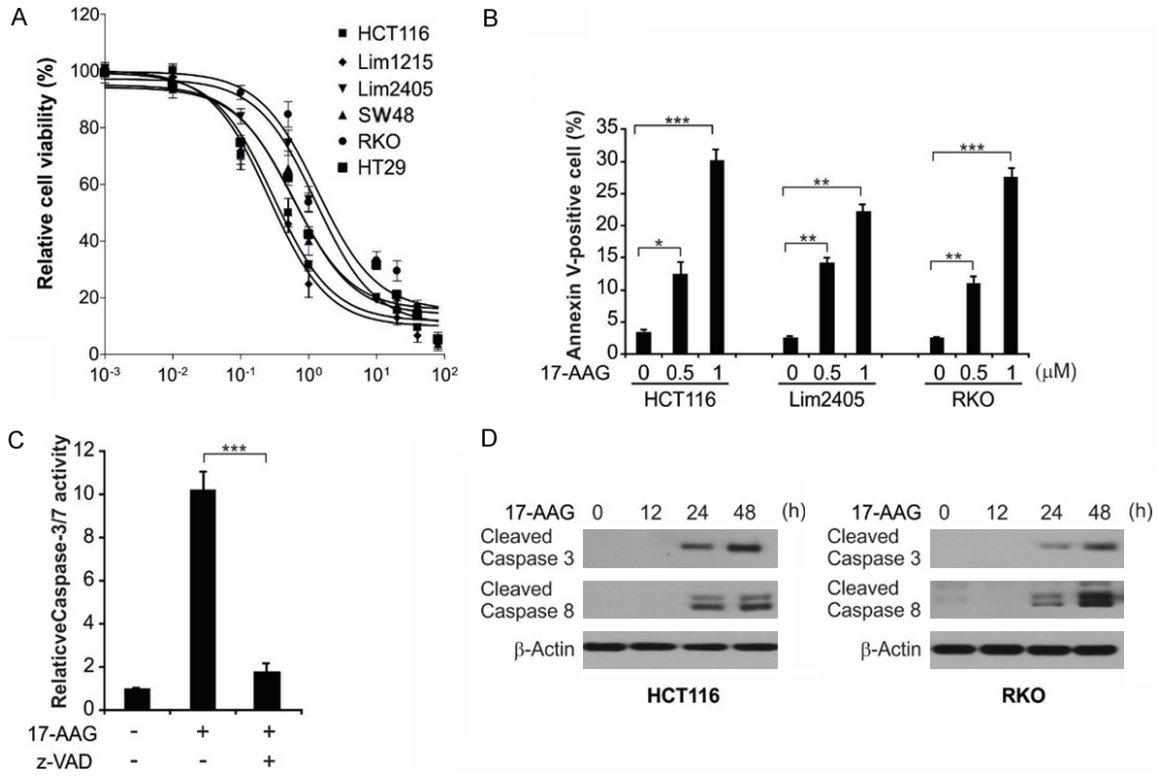

Figure 1. 17-AAG induces apoptosis in CRC. (A) The indicated cell lines were treated with increasing concentrations of 17-AAG for 72 hours. Cell proliferation was determined by MTS assay. (B) The indicated cell lines were treated with 17-AAG for 48 hours at indicated concentrations. Apoptosis was analyzed by Annexin V/PI staining followed by flow cytometry. (C) HCT116 cells were treated with 17-AAG with or without z-VAD-fmk, caspase 3/7 activity was determined by fluorogenic analysis. (D) HCT116 and RKO cells were treated with 1 μmol/L 17-AAG at indicated time point. Cleaved-caspase 3 and 8 were analyzed by western blotting. Results in (A-C) were expressed as means ± SD of three independent experiments. ***, *P*<0.001; **, *P*<0.01; *, *P*<0.05 compared to control cells.

5'-CAACTGCAAATTCCACCACA-3' to amplify a DR5 promoter fragment containing chop binding sites.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism IV software. *P* values were calculated by the student's *t*-test and were considered significant if P<0.05. The means ± standard deviation (SD) is displayed in the figures.

Results

17-AAG decreases proliferation and induces apoptosis in CRC

To determine the effects of Hsp90 inhibitor on CRC, colon cancer cell lines were treated with increasing concentrations of 17-AAG for 72 hours, and cell proliferation was detected by MTS assay. Base on the results, 17-AAG effectively decreased cell proliferation of these cell lines (**Figure 1A**). In apoptotic assays, 17-AAG treatment at 0.5 μM or 1 μM increased the population of Annexin V-positive cells in HCT116, Lim2405 and RKO (**Figure 1B**). Furthermore, our findings showed that 17-AAG induce caspases 3/7 activation in HCT116 cells (**Figure 1C**). The apoptotic response was also found to be attenuated upon pre-treatment with the pan-caspase inhibitor z-VAD-fmk (**Figure 1C**), indicating that the apoptotic response is caspase-dependent. 17-AAG treatment induced caspase 3 and 8 activation in HCT116 and RKO cells (**Figure 1D**). These data indicate that Hsp90 inhibitor decreased cell proliferation and induced caspase-dependent apoptosis in CRC.

FADD and caspase 8 are required for 17-AAG induced apoptosis in CRC

The above data showed that 17-AAG increased caspase 8 activation, which is a well-known ini-

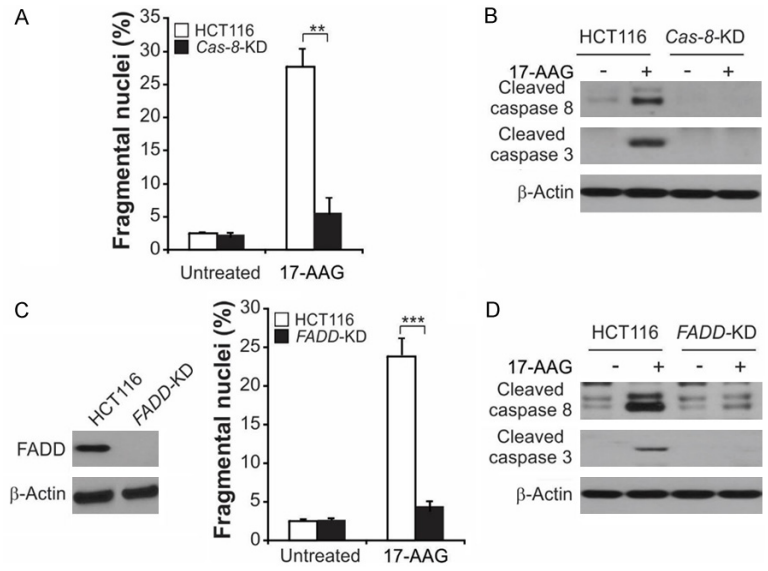

Figure 2. Caspase 8 and FADD are required for Hsp90 inhibitor-induced apoptosis. (A) Parental and *Cas 8*-KD HCT116 cells were treated with 1 μmol/L 17-AAG for 48 hours. Apoptosis was analyzed by a nuclear fragmentation assay. (B) Parental and *Cas 8*-KD HCT116 cells were treated with 1 μmol/L 17-AAG for 48 hours. Cleaved-caspase 3 and 8 were analyzed by western blotting. (C) Parental and *FADD*-KD HCT116 cells were treated with 1 μmol/L 17-AAG for 48 hours. Lift, western blotting confirmed FADD depletion by shRNA. Right, apoptosis was analyzed by a nuclear fragmentation assay. (D) Parental and *FADD*-KD HCT116 cells were treated with 1 μmol/L 17-AAG for 48 hours. Cleaved-caspase 3 and 8 were analyzed by western blotting. Results in (A) and (C) were expressed as means ± SD of three independent experiments. ***, $P<0.001$; **, $P<0.01$ compared to control cells.

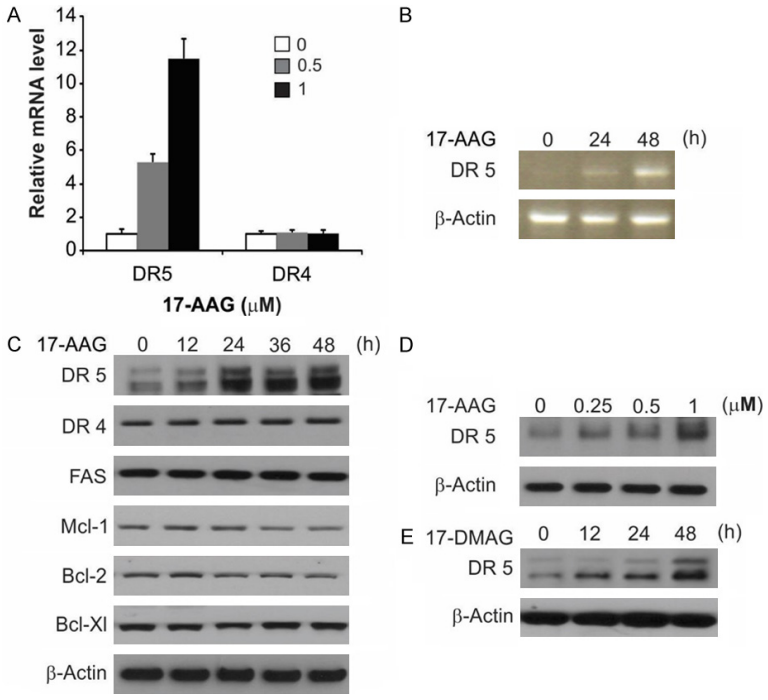

Figure 3. Hsp90 inhibitor induces DR5 induction in CRC. A. HCT116 cells were treated with 17-AAG for 24 hours at indicated concentration. *DR5* mRNA expression was analyzed by RT-PCR. B. HCT116 cells were treated with 1 μmol/L 17-AAG for 24 hours. *DR5* mRNA level was analyzed by gel electrophoresis. β-Actin was used as a control for normalization. C. HCT116 cells were treated with 1 μmol/L 17-AAG at indicated time point. Indicated protein was analyzed by western blotting. D. HCT116 cells were treated with 17-AAG for 24 hours at indicated concentration. DR5 was analyzed by western blotting. E. HCT116 cells were treated with 0.25 μmol/L 17-DMAG at indicated time point. DR5 was analyzed by western blotting.

tiator caspase in the extrinsic apoptotic pathway. So, we examined whether caspases activation, particularly caspase 8, is required for Hsp90 inhibitor-induced apoptosis. Parental and caspase 8 stable knockdown (*Casp 8*-KD) HCT-116 cells were treated with 17-AAG and analysis the apoptosis by nuclei staining. Compared to parental cells, 17-AAG-induced apoptosis was significantly reduced in *Casp 8*-KD cells (**Figure 2A**, **2B**). These results indicated that Hsp90 inhibitor induces caspase 8-dependent apoptosis. Next, we compared the effects of Hsp90 inhibitor on inducing apoptosis in parental and *FADD* stable knockdown (*FADD*-KD) HCT116 cells. Consistent with the results in *Casp-8*-KD cells, 17-AAG-induced significantly apoptosis in HCT116 cells, which was markedly reduced in *FADD*-KD cells (**Figure 2C**, **2D**). The above results demonstrate that deficiency of FADD, an essential component in mediating extrinsic apoptotic signaling, protects cancer cells from Hsp90 inhibitor-inducted apoptosis, suggesting extrinsic apoptotic pathway activation play a role in Hsp90 inhibitor-induced apoptosis.

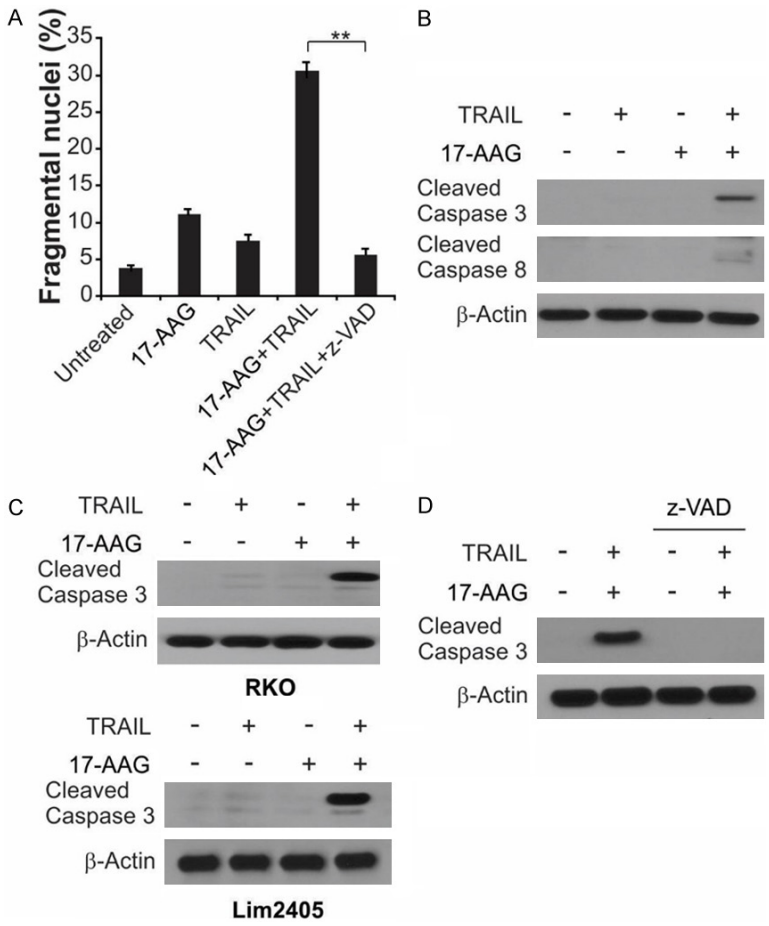

Figure 4. Hsp90 inhibitor sensitizes TRAIL-mediated apoptosis. A. HCT116 cells were treated with 1 μmol/L 17-AAG, 25 ng/mL TRAIL or their combination with or without 10 μmol/L z-VAD-fmk for 24 hours. Apoptosis was analyzed by a nuclear fragmentation assay. Results were expressed as means ± SD of three independent experiments. **, $P<0.01$ compared to control cells. B. HCT116 cells were treated with 1 μmol/L 17-AAG, 25 ng/mL TRAIL or their combination for 24 hours. Cleaved-caspase 3 and 8 were analyzed by western blotting. C. RKO or Lim2405 cells were treated with 1 μmol/L 17-AAG, 25 ng/mL TRAIL or their combination for 24 hours. Cleaved-caspase 3 was analyzed by western blotting. D. HCT116 cells were treated with the combination of 1 μmol/L 17-AAG and 25 ng/mL TRAIL with or without 10 μmol/Lz-VAD-fmk for 24 hours. Cleaved-caspase 3 was analyzed by western blotting.

DR5 is induced by Hsp90 inhibitor in CRC

We then investigated the mechanism of Hsp90 inhibitor activates extrinsic apoptosis. Both of DR4 and DR5 are important cell surface death receptors that activate extrinsic apoptotic signaling through recruitment of FADD. DR4 and DR5 expression were detected in HCT116 cells treated with 17-AAG. DR5, but not DR4, protein and mRNA levels were induced in a dose- and time-dependent manner upon 17-AAG treatment in HCT116 cells (**Figure 3A-D**). Furthermore, 17-DMAG, a 17-AAG analog, markedly increased DR5 expression in a dose-dependent manner (**Figure 3E**). The above results indicate Hsp90 inhibitor strongly increases DR5, but not DR4, expression in CRC.

17-AAG synergizes TRAIL to induce DR5-induced apoptosis

Next, we investigated whether Hsp90 inhibitor sensitizes TRAIL-mediated apoptosis in CRC. The combination of 17-AAG and TRAIL induced more apoptosis compared to single agent alone treatment (**Figure 4A**). In addition, the apoptotic response was also found to be attenuated upon pre-treatment with the pan-caspase inhibitor z-VAD-fmk (**Figure 4A**). In agreement, combination of 17-AAG and TRAIL was also much more effective than either agent alone treatment in increasing caspase 3 and 8 activation in HCT116 cells, as well as increasing cleaved caspase 3 in RKO and Lim-2405 cell lines (**Figure 4B**, **4C**). Pre-treated with pan-caspase inhibitor z-VAD-fmk, the ability of 17-AAG and TRAIL combination to induced caspase 3 activation was inhibited (**Figure 4D**), indicating that the combination of Hsp90 inhibitor and TRAIL induced caspase-dependent apoptosis in CRC.

DR5 up-regulation contributes to Hsp90 inhibitor-induced apoptosis

Next, we determined the role of DR5 in Hsp90 inhibitor-induced apoptosis in CRC. 17-AAG induced DR5 expression and apoptosis were suppressed in *DR5*-KD cells (**Figure 5A**, **5B**). Moreover, 17-AAG-induced caspase 3 activation was blocked in *DR5*-KD cells (**Figure 5C**).

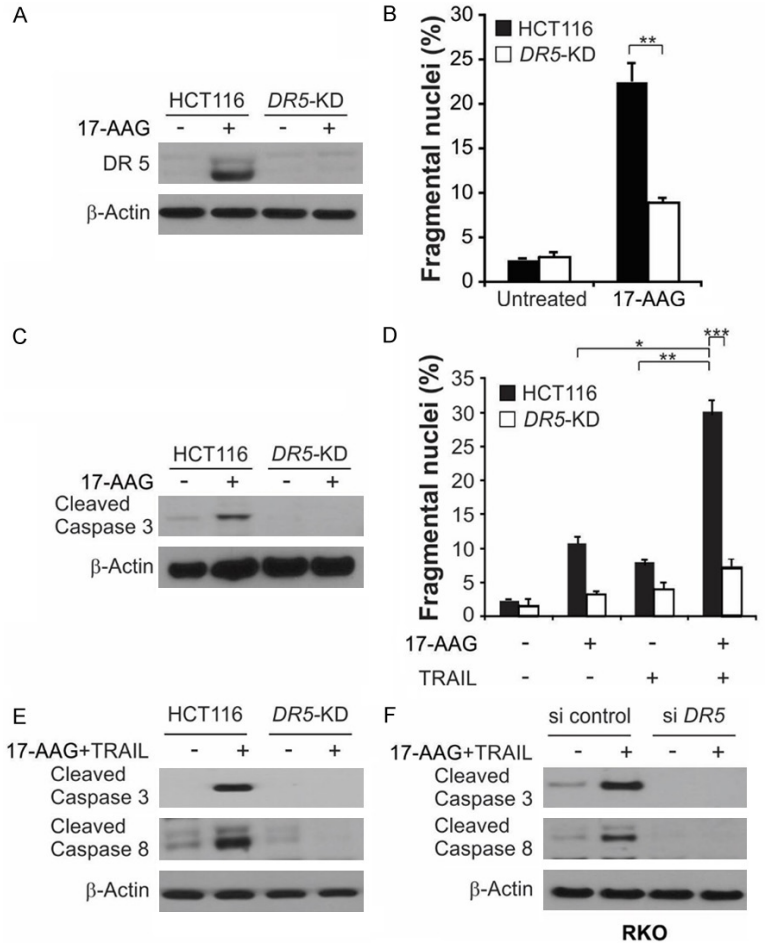

Figure 5. DR5 is required for Hsp90 inhibitor-induced apoptosis. (A) Parental and *DR5*-KD HCT116 cells were treated with 1 μmol/L 17-AAG for 24 hours. DR5 was analyzed by western blotting. (B) Parental and *DR5*-K DHCT116 cells were treated with 1 μmol/L 17-AAG for 48 hours. Apoptosis was analyzed by a nuclear fragmentation assay. (C) Parental and *DR5*-KD HCT116 cells were treated with 1 μmol/L 17-AAG for 24 hours. Cleaved-caspase 3 was analyzed by western blotting. (D) Parental and *DR5*-KD HCT116 cells were treated with 1 μmol/L 17-AAG, 25 ng/mL TRAIL or their combination for 24 hours. Apoptosis was analyzed by a nuclear fragmentation assay. (E) Parental and *DR5*-KD HCT116 cells were treated with the combination of 1 μmol/L 17-AAG and 25 ng/mL TRAIL with or without 10 μmol/Lz-VAD-fmk for 24 hours. Cleaved-caspase 3 and 8 were analyzed by western blotting. (F) RKO cells were transfect with sicontrol or si*DR5* for 24 hours, and then treated with the combination of 1 μmol/L 17-AAG and 25 ng/mL TRAIL with or without 10 μmol/L z-VAD-fmk for 24 hours. Cleaved-caspase 3 and 8 were analyzed by western blotting. Results in (B) and (D) were expressed as means ± SD of three independent experiments. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$ compared to control cells.

We further determined whether Hsp90 inhibitor enhance TRAIL-induced apoptosis via upregulation of DR5. The combination of 17-AAG and TRAIL induced apoptosis and caspase 3 and 8 activation were blocked in *DR5*-KD cells (**Figure 5D**, **5E**). Knockdown of DR5 by siRNA significantly suppressed 17-AAG-induced caspase 3 and 8 activation in RKO cells (**Figure 5F**). These finding suggest that Hsp90 inhibitor upregulates DR5 expression, leading to enhancement of TRAIL-induced apoptosis.

Hsp90 inhibitor induces chop-mediated upregulation of DR5

We then analyzed the mechanism of p53-independent DR5 induction by Hsp90 inhibitor in CRC. Several transcription factors that can mediate DR5 upregulation were examined. P65 is not involved because of unchanged phosphorylation following 17-AAG treatment. MZF1 were also ruled out because of lack of induction after 17-AAG treatment (**Figure 6A**). Several studies demonstrated that chop is involved in DR5 upregulation and contributed to the sensitization of TRAIL-mediated apoptosis [16-18]. Therefore, we investigated whether chop is involved Hsp90 inhibitor-induced DR5 upregulation in CRC. Chop knockdown by siRNA attenuated 17-AAG-induced DR5 induction, as well as apoptosis and caspase 3 activation (**Figure 6B**, **6C**). Next, we determined the effects of Hsp90 inhibitor on the expression of chop. Treating HCT116 cells with 17-AAG markedly induced DR5 protein and mRNA expression in a time-dependent manner (**Figure 6D**, **6E**). Furthermore, 17-DMAG markedly induced chop expression in a time-dependent manner (**Figure 6F**). Next, we investigated whether chop can directly binding to *DR5* promoter. The recruitment of chop to the promoter of *DR5* was found following 17-AAG treatment by chromatin immunoprecipitation (ChIP) (**Figure 6G**). These results indicate that chop directly binds to *DR5* promoter region to drive its transcrip-

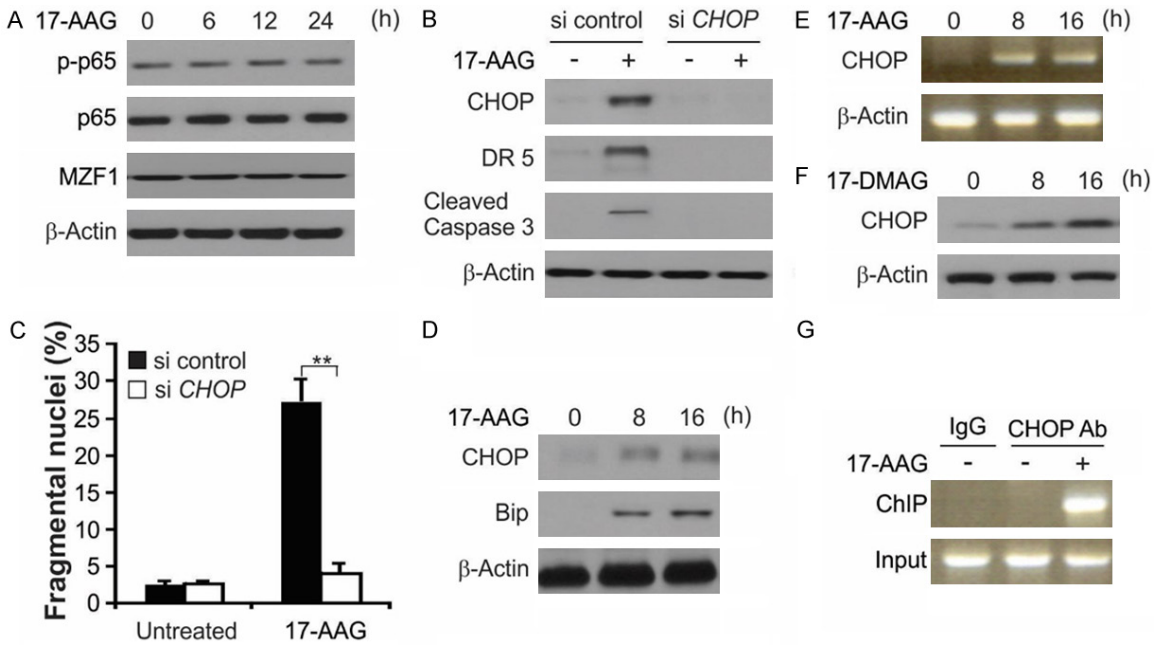

Figure 6. Hsp90 inhibitor induces chop-mediated DR5 expression. A. HCT116 cells were treated with 1 μmol/L 17-AAG at indicated time point, phospho-p65, p65 and MZF1 were analyzed by western blotting. B. HCT116 cells were transfect with si control or si*chop* for 24 hours, and then treated with 1 μmol/L 17-AAG for 48 hours. Chop, DR5 and cleaved-caspase 3 were analyzed by western blotting. C. HCT116 cells were transfected with si control or si*chop* for 24 hours, and then treated with 1 μmol/L 17-AAG for 48 hours. Apoptosis was analyzed by a nuclear fragmentation assay. Results were expressed as means ± SD of three independent experiments. **, $P<0.01$ compared to control cells. D. HCT116 cells were treated with 1 μmol/L 17-AAG at indicated time point. Chop and Bip were analyzed by western blotting. E. HCT116 cells were treated with 1 μmol/L 17-AAG at indicated time point. ChopmRNA level was analyzed by gel electrophoresis. β-Actin was used as a control for normalization. F. HCT116 cells were treated with 0.25 μmol/L 17-DMAG at indicated time point. Chop was analyzed by western blotting. G. Chromatin immunoprecipitation (ChIP) was performed using anti-chop antibody on HCT116 cells following 17-AAG treatment for 12 hours. ChIP with the control IgG was used as a control. PCR was carried out using primers surrounding the chop binding sites in the *DR5* promoter.

tional activation in response to Hsp90 inhibitor treatment.

Discussion

In the present study, we have shown that Hsp90 inhibitor effectively decreases the cell proliferation and induces apoptosis in colon cancer cell lines. Furthermore, our results demonstrated that Hsp90 inhibitor-induced extrinsic apoptotic pathway due to deficiency of FADD or caspase 8 protected cells from Hsp90 inhibitor-induced cell death and cleavage of caspases (**Figure 2**). Hsp90 inhibitor-induced chop-mediated DR5 upregulation is required for the anticancer effects of Hsp90 inhibitor. Next, our results showed that Hsp90 inhibitor sensitizes TRAIL-mediated apoptosis, as evidenced by the synergistic induction of DR5-dependent apoptosis by the Hsp90 inhibitor and TRAIL combination. This is the first report to demonstrate that Hsp90 inhibitor induces extrinsic apoptosis in CRC.

TRAIL is an alternative anticancer agent, which induces apoptosis in various cancer cell types without cytotoxic effects on normal cells, representing a promising novel target for anticancer therapeutics [19]. However, many studies suggested that a variety of cancer types are resistant to the anticancer effects of TRAIL [19-21]. So, it is important to develop agents that sensitize cancer cells to TRAIL-mediated anticancer effects to improve the therapeutic impact of TRAIL. TRAIL mediates apoptosis by binding to cell surface receptors, DR4 and DR5, which lead to form the death-inducing signaling complex (DISC) with subsequent binding of caspase 8 [22, 23]. In addition, DR4 and DR5 expression is also critical for sensitization of cells to TRAIL [24, 25]. In this study, we found that the DR5 expression was upregulated following Hsp90

inhibitor treatment in CRC. So, this study primarily focused on demonstrating the role of DR5 in mediating Hsp90 inhibitor-induced apoptosis and enhancement of TRAIL-induced apoptosis and the mechanism accounting for Hsp90 inhibitor-induced DR5 upregulation.

Chop is upregulated by endoplasmic reticulum (ER) stress and involved in ER-mediated apoptosis [26]. Chop is a transcription factor of the C/EBP family responsible for DR5 upregulation [26, 27]. Here, our results showed Hsp90 inhibitor-mediated chop upregulation is a key regulator of DR5 expression and that chop is essential for Hsp90 inhibitor-induced apoptosis. Also, chop is known as an apoptotic inducer through ER stress and that depletion of chop prevents apoptosis against various anticancer drugs [28]. ER stress also accelerated chop expression leading to promotion of the expression of ER stress-mediated apoptosis molecules, such as DR5 [28, 29].

In conclusion, our results demonstrated that Hsp90 inhibitor exerts potent inhibitory effect against human CRC and similarly cytotoxic activity regardless of p53 status. In addition, Hsp90 inhibitor sensitizes TRAIL-mediated apoptosis through the chop-dependent upregulation of DR5. Our results provide a novel therapeutic approach of overcome TRAIL-resistant cancer therapy.

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Disclosure of conflict of interest

None.

Authors' contribution

ZY, AC, XL and ZZ conceived and performed the experiments. ZY and XJ analyzed the data and wrote the manuscript.

Abbreviations

17-AAG, 17-allylamino-17-demethoxygeldanamycin; ChIP, Chromatin immunoprecipitation; Chop, C/EBP homologous protein; CRC, Colorectal cancer; DcRs, Decoy receptors; DISC, Death-inducing signaling complex; DR4, Death Receptor 4; DR5, Death Receptor 5; ER, Endoplasmic Reticulum; FADD, Fas-associated protein with death domain; FBS, Fetal bovine serum; Hsp90, Heat shock protein 90; 5-FU, 5-fluorouracil; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand.

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