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ORIGINAL ARTICLE

Celastrol enhances TRAIL-induced apoptosis in human glioblastoma via the death receptor pathway

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Abstract

Purpose Glioblastoma is the most common, malignant and devastating type of primary brain tumor. Tumor necrosis factor-related apoptosis-induced ligand (TRAIL) is characterized by its lethality to precancerous and cancerous cells. However, many kinds of tumor cells, including most glioma cells, tend to evade TRAIL-induced apoptosis. Celastrol is a pleiotropic compound from a traditional Chinese medicine that has proven to be useful as a sensitizer for TRAIL treatment. However, the underlying mechanism and role of celastrol in the sensitization of glioma cells remain to be elucidated.

Methods The viability of glioma cell lines was examined by the CCK-8 assay. The expression of DR5 was detected by reverse transcriptase quantitative real-time PCR. The protein expression of DR5, cleaved caspase-8, cleaved caspase-3 and PARP were measured by western blot. The apoptosis rates and the sub-G1 population were detected by flow cytometry. The cellular morphological changes were assessed by TUNEL apoptosis and Hoechst 33258 staining assays. The knockdown of DR5 expression was conducted by siRNA.

Results In this study, we observed that celastrol treatment inhibited cell viability in a dose-dependent manner, while glioma and normal human astroglial cell lines were resistant to TRAIL treatment. We also observed that the antiproliferative effects of TRAIL in combination with a noncytotoxic concentration of celastrol were significantly greater than those of celastrol or TRAIL alone. In addition, cell death induced by the combination treatment was apoptotic and occurred through the death receptor pathway via activation of caspase-8, caspase-3, and PARP. Furthermore, celastrol upregulated death receptor 5 (DR5) at the mRNA and protein levels, and siRNA-mediated DR5 knockdown reduced the killing effect of the combination drug treatment on glioma cells and reduced the activation of caspase-3, caspase-8 and PARP.

Conclusions Taken together, the results of our study demonstrate that celastrol sensitizes glioma cells to TRAIL via the death receptor pathway and that DR5 plays an important role in the effects of this cotreatment. The results indicate that this cotreatment is a promising tumor-killing therapeutic strategy with high efficacy and low toxicity.

Keywords Celastrol · TRAIL · U87-MG · DR5 · Apoptosis

Abbreviations

TRAIL	Tumor necrosis factor-related apoptosis-induced ligand
DR5	Death receptor 5
FBS	Fetal bovine serum
PARP	Poly-ADP-ribose polymerase
CNS	Central nervous system
GBM	Glioblastoma multiforme
DR4	Death receptor 4
FADD	Fas-associated death domain
DISC	Death-inducing signaling complex
DMSO	Dimethylsulfoxide
STR	Short tandem repeat
CCK-8	Cell Counting Kit-8
FCM	Flow cytometry
SiRNA	Small interfering RNA
PVDF	Polyvinylidene fluoride
TBST	Tris-buffered saline with Tween
qRT-PCR	Reverse transcriptase quantitative real-time PCR
DcR	Decoy receptors
CDI	Coefficient of drug interaction

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Introduction

The global incidence rate of primary malignant brain tumors and other central nervous system (CNS) tumors is 3.4 per 100,000. Glioblastoma multiforme (GBM) is the most prevalent primary brain malignancy, accounting for approximately 50% of all gliomas. After GBM treatment, including surgery, chemotherapy and radiotherapy, the median overall survival time for all glioblastoma patients is less than 3 years [1]. Therefore, it is necessary to explore novel chemotherapeutic regimens that can improve the effectiveness of glioblastoma treatment.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor superfamily that has been reported to induce cell death in many kinds of tumor cells, whereas it exerts almost no influence on normal cells [2–5]. However, some studies have indicated that a majority of glioma cells are more or less insensitive to TRAIL-induced apoptosis [6, 7]. In addition, correlational studies have observed that some cancer therapeutic agents can sensitize various cancer cells to TRAIL-induced apoptosis by upregulating the death receptors DR4 and DR5 [8–10]. When TRAIL binds to death receptors, it integrates with the intracellular death domain and activates the cytoplasmic adaptor protein Fas-associated death domain (FADD). The recruited FADD in turn interacts with procaspase-8 through its death effector domain to form a death-inducing signaling complex (DISC). Then, the DISC activates caspase-8, which induces apoptosis via the death receptor pathway by activating the effector caspase-3 to cleave PARP [7, 11, 12].

Celastrol is extracted from a traditional Chinese medicine, the *Tripterygium wilfordii*, and has a variety of pharmacological properties, such as anti-inflammatory [13–15], antioxidative [16], and antitumor properties [17–19]. Previous studies have revealed that celastrol has broad-spectrum anticancer activities, including activity against malignant glioma [17–20]. However, some studies have also shown that periods of celastrol injection cause obvious weight loss in mice [21], indicating that the toxicity of celastrol poses a threat to normal cells and tissues. Thus, there is an urgent need to reduce the toxicity of celastrol. Moreover, some studies have shown that the combination of specific chemicals, including celastrol, with TRAIL can enhance the anticancer activity of TRAIL by upregulating DR4 and DR5 [8–10, 22, 23].

In the current study, we sought to determine the molecular mechanism underlying the effects of celastrol and TRAIL cotreatment. Our results demonstrate that celastrol is virtually nontoxic to normal cells at low concentrations but upregulates DR5 expression in glioma cells, enhancing TRAIL-triggered apoptosis.

Material and methods

Reagents

TRAIL was purchased from Novoprotein (Shanghai, China), and aliquots of the reconstituted protein were stored at − 80 °C. Celastrol (> 98% purity) was purchased from Solarbio (Beijing, China), dissolved in DMSO (dimethylsulfoxide) and stored at − 20 °C. The pancaspase inhibitor z-VAD-fmk was purchased from Beyotime (Shanghai, China).

Cell lines and culture conditions

The human glioblastoma cell lines U87-MG, U251, and LN229 with short tandem repeat (STR) authentication were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The normal human astroglial cell line HEB was obtained from Bopei Biotech Co. Ltd. (Chongqing, China). U87-MG cells were cultured in MEM (HyClone) supplemented with 10% fetal bovine serum (FBS) (Natocor, Argentina) and a 1% penicillin (100 U/ml) and streptomycin (100 mg/ml) solution. LN229 and U251 cells were cultured in DMEM (HyClone), while HEB cells were maintained in RPMI 1640 medium. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

Detection of cell viability by the Cell Counting Kit-8 (CCK-8) assay

Cells from each cell line were seeded into 96-well plates. After growing for 24 h, the cells were treated with the specified concentrations of TRAIL, celastrol or combinations of TRAIL and celastrol for 24 h. The final concentration of DMSO used in the assay did not exceed 0.1%. Following 24 h of drug treatment, CCK-8 reagent (Bimake, USA) was added into each well. After 2 h of incubation at 37 °C, the absorbance at 450 nm was measured using a microplate reader (Thermo Scientific Varioskan LUX).

The coefficient of drug interaction (CDI) was determined to analyze the interactions between the celastrol and TRAIL, and according to CDI values (Table 1), the interactions were categorized as synergistic, additive or antagonistic. CDI was calculated as follows:

$$\mathrm{CDI} = AB/(A \times B),$$

where, AB is the absorbance value for the mixture of the two active agents/absorbance value for the control A and B are the absorbance value for the single active agent/absorbance value for the control. A CDI value of < 1, $= 1$ or > 1 indicates that the drugs are synergistic, additive or antagonistic,

Table 1 The coefficient of drug interaction (CDI)

Glioma cells	TRAIL (100 ng/ml)	
	0.5 μM celastrol	1 μM celastrol
U87-MG	0.588935	0.147315
U251	0.970534	0.977812
LN229	0.838643	0.711703

CDI = $AB/(A \times B)$. According to the absorbance of each group, AB is the ratio of the combination groups to the control group; A or B is the ratio of the single drug groups to control group. Thus, a coefficient of drug interaction (CDI) value of less than, equal to or greater than 1 indicates that the drugs are synergistic, additive or antagonistic, respectively. A CDI value of less than 0.7 indicate that the drugs are significantly synergistic. The results show that TRAIL and celastrol synergistically influence the viability of U87-MG cells exposed to a fixed concentration of TRAIL (100 ng/ml) and concentrations of celastrol ranging from 0 to 1 μM for 24 h. In addition, the two drugs have a synergistic effect on U251 and LN229 cells

respectively. A CDI value of less than 0.7 indicates that the drugs are significantly synergistic [31, 32].

Detection of apoptosis rates and the sub-G1 population by flow cytometry (FCM)

U87-MG cells were cultured for 24 h and treated with TRAIL, celastrol or both for 24 h. The cells were then washed twice with PBS and collected. The apoptosis rates of the cell samples were analyzed with a flow cytometer (Beckman CytoFLEX, California, USA). The cells were treated as described previously, after which they were resuspended in 400 μl of precooled 75% ethanol. Following 6 h of fixation, the Sub-G1 population in each sample was determined by flow cytometry (FCM).

TUNEL apoptosis assay

U87-MG cells were treated as previously described, after which a TUNEL assay was performed on the cells using the One Step TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) in accordance with the manufacturer's protocol. The condensed or fragmented nuclei of apoptotic cells were observed under a fluorescence microscope (Leica, Germany).

Hoechst 33258 staining assay

U87-MG cells were treated as described above and then were fixed in fixing solution for 10 min and washed twice in PBS. Next, the cells were incubated with Hoechst 33258 staining solution (Beyotime, Shanghai, China) for 5 min and washed twice as before. Subsequently, the nuclei of apoptotic cells were observed using a fluorescence microscope (Leica, Germany).

Reverse transcriptase quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with the Total RNA Quick Purification Kit (BioTeke, Beijing, China) according to the manufacturer's instructions. The PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Tokyo, Japan) was used to synthesize cDNA, and qRT-PCR was conducted using the 2 × RealStar Green Power Mixture (GenStar, Beijing, China) and the primers listed in Table 2. The relative normalized expression of the target genes was compared with that of the control gene, and the mRNA expression of each gene was calculated with the $2^{-\Delta\Delta Ct}$ method.

Transfection of DR5 siRNA

DR5 and control siRNAs were synthesized by Sangon Biotech (Shanghai, China). The targeting sequences of DR5 siRNA are listed in Table 3. Transfection was performed using complete medium and GP-siRNA-Mate Plus transfection reagent (GenePharma, Shanghai, China) according to the manufacturer's protocol.

Table 3 The targeting sequences of DR5 siRNA

Gene name	Primer sequence (5′–3′)
DR5 siRNA	Sense UCAGAAGACGGUAGAGAUUTT Antisense AAUCUCUACCGUCUUCUGATT
NC siRNA	Sense UUCUCCGAACGUGUCACGUTT Antisense ACGUGACACGUUCGGAGAATT

Table 2 Sequences of the primers used for qRT-PCR

Gene name	Gene number	Primer sequence (5′–3′)	Product length (bp)
DR5	NM_003842.5	Forward GAGCTTGACAAAGTGGTCGT Reverse GATGCAATCTCTACCGTCTTCT	123
GAPDH	NM_002046.7	Forward GTCATCCATGACAACTTTGG Reverse GAGCTTGACAAAGTGGTCGT	185

Western blot analyses

Cells were cultured and treated with drugs as described previously. In addition, total protein was prepared using the whole cell lysis assay (KeyGen, Chongqing, China). Equal amounts of protein were separated by 10% sodium dodecyl sulfate–polyacrylamide (SDS–PA) gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp, USA). The membranes were blocked with 5% skim milk dissolved in 1 × Tris-buffered saline with Tween (TBST) at room temperature for 2 h and then incubated overnight at 4 °C with primary antibodies. The next day, the membranes were washed and incubated with secondary antibodies for 1 h at room temperature. Finally, the membranes were washed thrice, and the protein bands were visualized with a fluorescence detector (ChemiDoc Touch Imaging System, Bio-Rad, USA) using an enhanced chemiluminescence kit (Beyotime Biotech, China). Changes in protein expression were analyzed with ImageJ software (version 1.8.0).

Statistical analysis

All experimental data are presented as the arithmetic mean ± standard deviation (SD). The data were analyzed using one-way analysis of variance (ANOVA). In addition, homogeneity of variance was tested, and post hoc multiple comparisons tests were performed. Data with equal variances were compared using Tukey's post hoc multiple comparisons test and data with unequal variances were compared with Dunnett's T3 test. Two-way ANOVA test was performed for the drug combination assays. The outcomes were considered significant at a p value of < 0.05. The analyses were performed using SPSS version 23.0 (Chicago, IL, USA).

Results

Celastrol significantly sensitizes human glioblastoma cells to TRAIL-induced inhibition of cell proliferation at nontoxic doses

We initially determined the inhibitory effect of celastrol and TRAIL on cellular proliferation in different glioblastoma cell lines and the normal human astroglial cell line HEB via the CCK-8 assay (Fig. 1b–f). The results indicated that within a limited range of doses (within 500 ng/ml), U87-MG, U251, and LN229 cells were relatively TRAIL-resistant (cell death < 30%), whereas HEB cells were almost completely insensitive to TRAIL (cell death < 10%). Celastrol reduced cell viability in the glioma cell lines in a concentration-dependent manner. The 24-h IC_{50} values for celastrol were 1.216, 2.219, 4.576, and 2.188 μM for U87-MG, U251, LN229 and HEB cells, respectively. Among the cell lines, the U87-MG cell line appeared to be the most sensitive to celastrol. We observed that celastrol induced relatively low cell death in HEB cells (< 20%) at concentrations of up to 1 μM. Thus, all glioma cell lines were exposed to a fixed concentration of TRAIL (100 ng/ml) and celastrol concentrations ranging from 0 to 1 μM for 24 h. In addition, because treatment with 0.5 μM celastrol plus 100 ng/ml TRAIL significantly decreased the number of U87-MG cells, these celastrol and TRAIL concentrations were used in all follow-up experiments performed in our study.

Combined application of celastrol and TRAIL induces morphological changes in U87-MG cells

Under a phase contrast microscope, we observed that untreated U87-MG cells appeared healthy. Compared with the control group, the TRAIL-treated group and the combination group exhibited more broken and detached cells (Fig. 1g). As revealed by staining of cell nuclei with Hoechst 33258 (Fig. 1h), the cotreatment group clearly had more chromatin condensation and fragmentation than the TRAIL-treated group, in which cells had become round and were uniformly stained. Furthermore, the TUNEL assay results (Fig. 1i) were consistent with those of Hoechst 33258 staining.

Cotreatment with celastrol and TRAIL induces cell death in U87-MG cells via apoptosis

To determine whether celastrol combined with TRAIL-induced cell death via apoptosis, we assessed the apoptosis rates (Fig. 1j) and sub-G1 populations (Fig. 1k) by FCM. After the cells were treated with celastrol and TRAIL, Annexin V-FITC and propidium iodide (PI) staining was performed to determine the apoptosis rates. As expected, the population of apoptotic cells in the combination group was clearly greater than those in the monotreatment groups.

Celastrol upregulates DR5 expression to sensitize cells to TRAIL-mediated apoptosis

Previous research has suggested that some TRAIL-resistant human tumor cell lines can be made susceptible to TRAIL by upregulating the expression of TRAIL receptors (DR4 and DR5) with certain chemotherapeutic agents [8–10, 22, 23]. Thus, we determined whether celastrol could induce the expression of DR5 in U87-MG cells at the mRNA and protein levels. As shown in Fig. 2a, celastrol treatment increased DR5 protein expression compared with the control treatment, and a trend toward increasing expression with increasing doses of celastrol was observed. In addition, as shown in Fig. 2b, the qRT-PCR results showed that

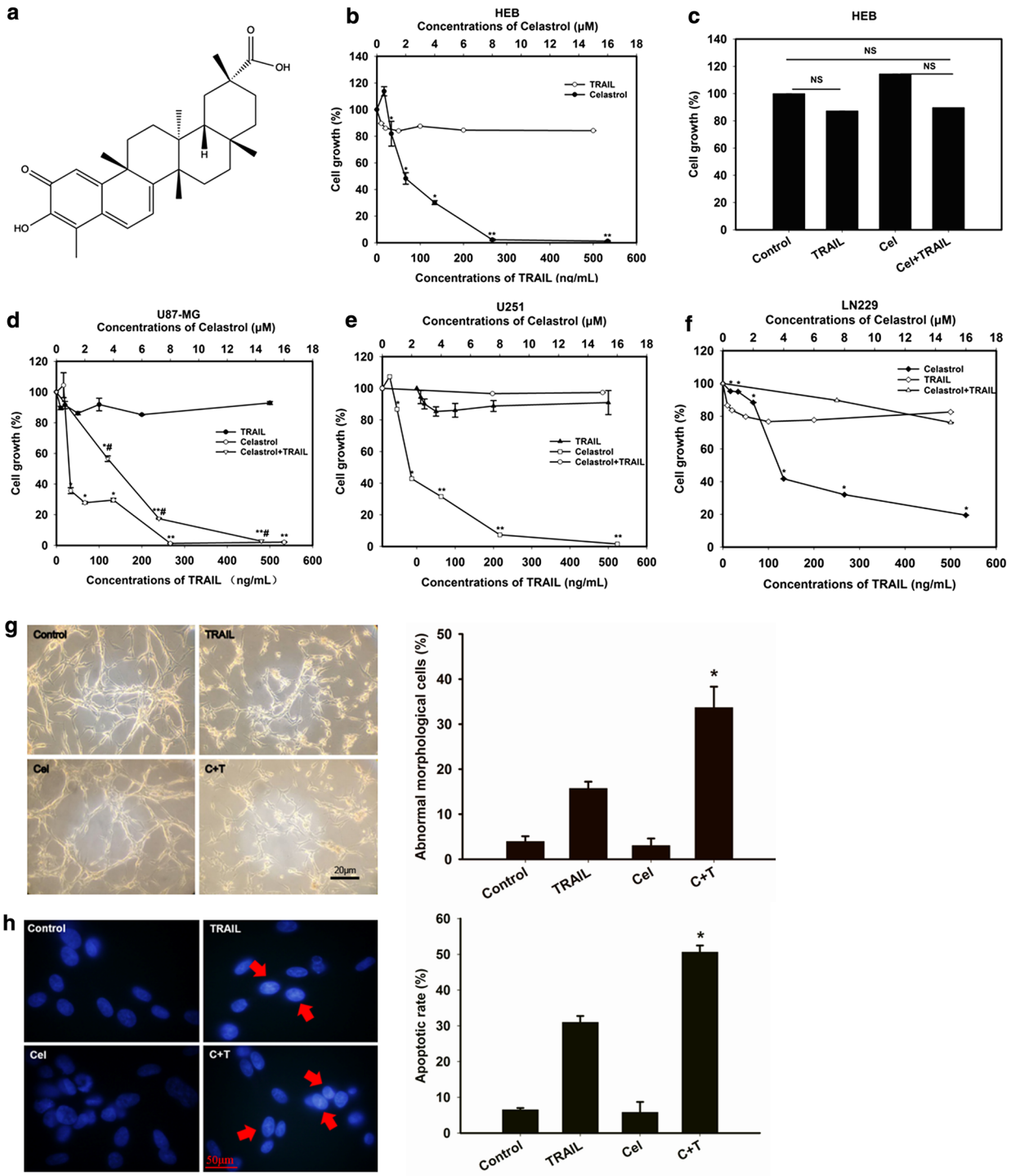

Fig. 1 Celastrol enhances TRAIL-induced apoptosis. **a** Chemical structure of celastrol. **b–c** One normal human astroglial cell line and **d–f** three human glioma cell lines were treated with serial concentrations of celastrol (0–16 μM) and TRAIL (0–500 ng/ml) separately for 24 h and combinations of a fixed concentration of TRAIL (100 ng/ml) and different concentrations of celastrol ranging from 0 to 2 μM for 24 h. Subsequently, the rates of cell growth were assessed using a CCK-8 assay. **c** The HEB cell line was exposed to 0.5 μM celastrol and 100 ng/ml TRAIL, alone or in combination, for 24 h, and the rates of cell growth were assessed using a CCK-8 assay. **g–k** U87-MG cells were treated with celastrol (0.5 μM) and TRAIL (100 ng/ml), alone or a combination, for 24 h. **g** Cellular morphology assessed by optical microscopy (×original magnification: 40). After fixation, the cells were stained with **h** Hoechst 33258 (×original magnification: 1000) and **i** TUNEL solution (×original magnification: 400). The cells were harvested for FCM detection of **j** apoptosis rates and **k** sub-G1 populations. The blue peak is the apoptotic peak. The results are presented as the mean ± SD. $*p < 0.05$ compared with untreated cells; $**p < 0.01$ compared with untreated cells; $\#p < 0.05$ compared with TRAIL-treated cells; *NS* no significant difference

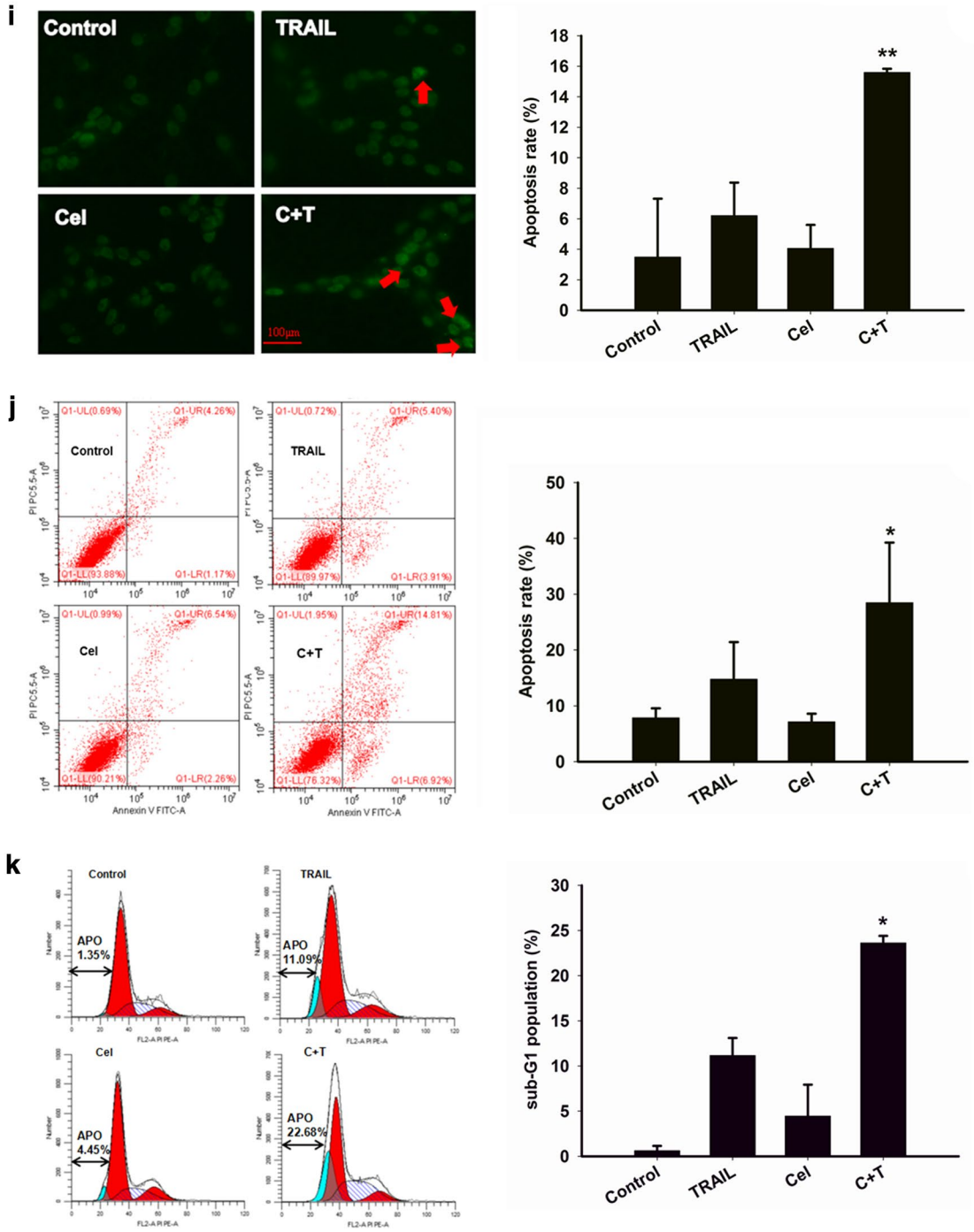

Fig. 1 (continued)

the gene expression of DR5 was significantly higher in the cells treated with 0.5 μM celastrol than in the untreated cells. We further examined whether the upregulation of DR5 was indispensable for the celastrol-stimulated sensitization to TRAIL-induced apoptosis by transfecting cells with DR5 siRNA to reduce the basal expression levels of DR5 at the gene (Fig. 2c) and protein levels (Fig. 2d). We observed that cell viability in the cotreatment group was effectively reduced in NC siRNA (the control)-transfected cells, while the cell viability in DR5 siRNA-transfected cells was significantly increased compared with that observed in the NC siRNA-transfected groups (Fig. 2e). Furthermore,

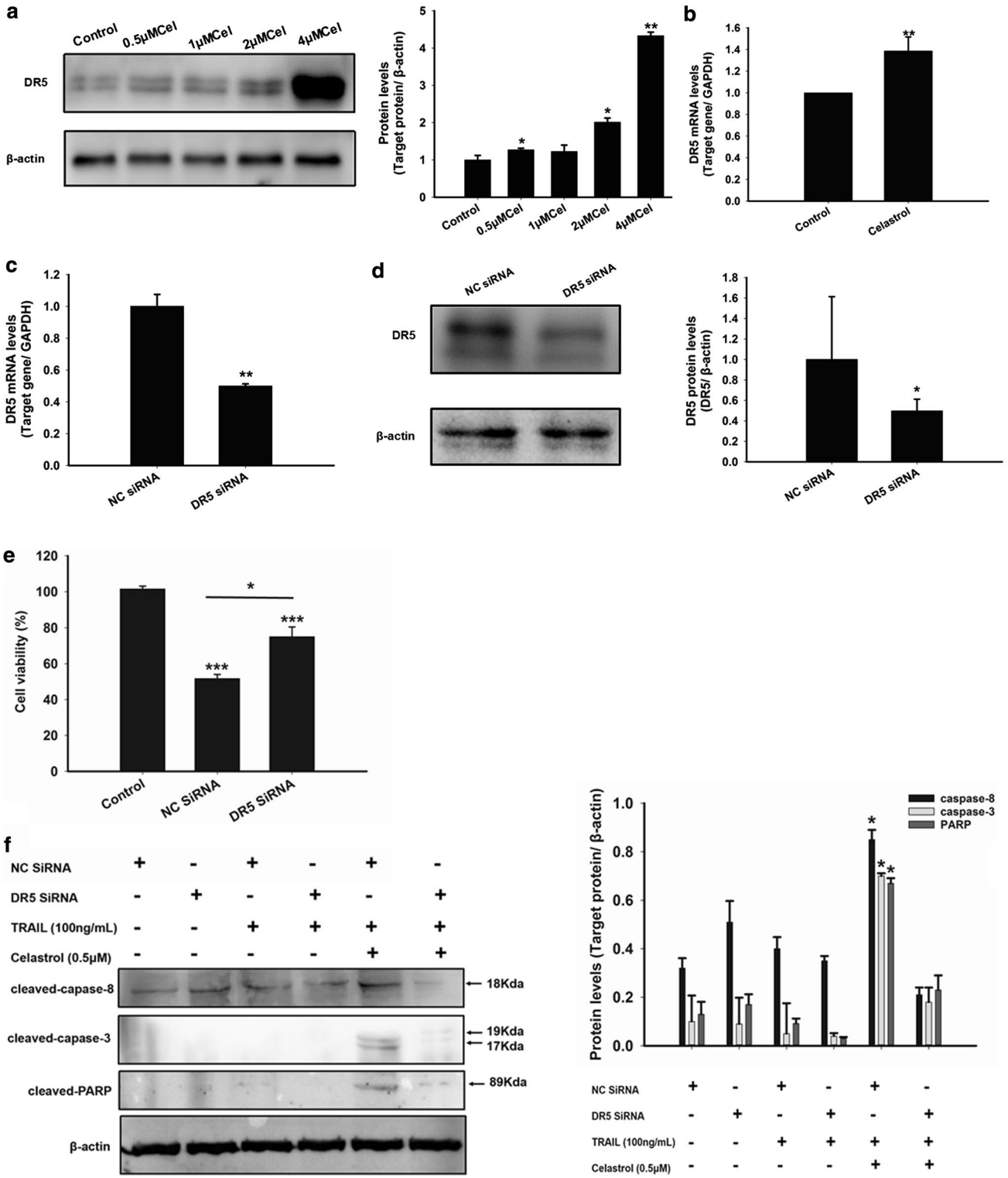

Fig. 2 Celastrol upregulates the expression of DR5. **a** U87-MG cells were treated with serial concentrations of celastrol for 24 h. Then, total protein was extracted and analyzed for DR5 expression by Western blot analysis. **b** The levels of DR5 in U87-MG cells (untreated or treated with 0.5 μM celastrol for 24 h) were analyzed by qRT-PCR. **c** U87-MG cells were transfected with siRNA, after which the level of DR5 gene expression in the cells was analyzed by qRT-PCR, and the levels of DR5 protein were analyzed by Western blot (**d**). **e** Cell viability in U87-MG cells transfected with DR5 siRNA or NC siRNA (controls) was determined using a CCK-8 assay. **f** After transfection, U87-MG cells were exposed to TRAIL, celastrol or TRAIL plus celastrol at the indicated concentrations for 24 h, and the protein levels were determined by Western blot analysis. Error bars, SD. $*p<0.05$, $**p<0.01$ and $***p<0.001$ compared with celastrol-treated or transfected cells

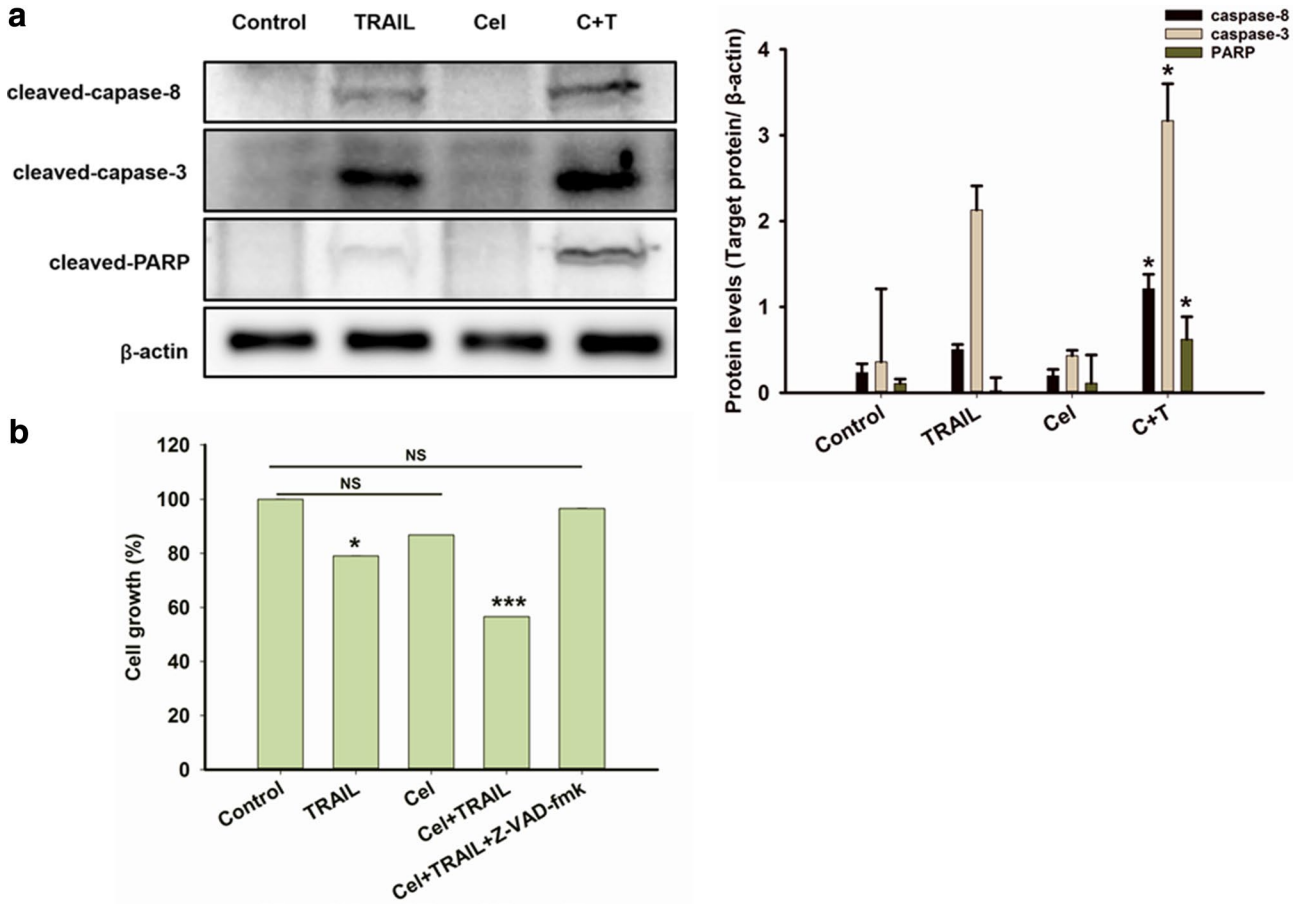

Fig. 3 Celastrol promotes TRAIL-induced caspase activation. **a** Total protein was extracted from cells after treatment with the specified drugs for 24 h and subjected to Western blot analysis using specific antibodies for cleaved caspase-8, cleaved caspase-3 and cleaved PARP. **b** Cells were treated with celastrol + TRAIL or celastrol + TRAIL + 25 μM z-VAD-fmk for 24 h, and cell viability was assessed using a CCK-8 assay. Mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the C + T group. *C* celastrol, *T* TRAIL

knockdown of DR5 expression markedly blocked the cleavage of caspase-3, caspase-8 and PARP in the combined treatment group (Fig. 2f).

Sensitization of cells to TRAIL-induced apoptosis by celastrol is dependent on caspase activation

To further assess the activation of downstream apoptotic signaling molecules upon increased expression of DR5, we examined the protein expression of cleaved caspase-8, cleaved caspase-3 and PARP. The results presented in Fig. 3a show that treatment of cells with celastrol alone did not affect the expression of cleaved caspase-8, cleaved caspase-3 or cleaved PARP. However, compared to the TRAIL treatment alone, the combined treatment with TRAIL and celastrol caused an approximately twofold increase in the activation of these factors. To further investigate whether the synergistic increases in caspase activation caused by celastrol and TRAIL lead to apoptosis, we pretreated cells with 25 μM z-VAD-fmk to inhibit caspase activation (Fig. 3b) and observed that this treatment significantly inhibited the cotreatment-induced apoptosis.

Discussion

Reduction in chemotherapeutic sensitivity is a hallmark of recurrent glioblastoma [24]. TRAIL is an ideal anticancer chemotherapy drug that kills tumor cells while leaving most normal cells intact. The selective cell-killing effects of TRAIL are attributed to a higher expression of decoy receptors (DcR1 and DcR2) and a lower expression of DR4 and DR5 on the surface of normal cells compared to tumor cells [3–5]. However, most glioma cells are resistant to TRAIL-induced apoptosis [6, 7]. Therefore, the identification of a way to reduce such drug resistance is of crucial importance. Notably, previous studies have shown that combining TRAIL with several other anticancer agents can overcome the resistance of some tumor cells to TRAIL [7–10, 21–23, 25, 26].

Celastrol is a kind of triterpene that has many pharmacological properties, the most of important of which are its anticancer and anti-inflammatory activities [13–15, 17–20]. However, the inevitable toxicity of celastrol is the greatest disadvantage of its application. Previous studies have demonstrated that reducing the dose of celastrol by combining it with other cancer therapeutic agents can effectively reduce its cytotoxicity and related side effects [27]. Based on these findings, we first examined the toxicity of celastrol and TRAIL separately and in combination on normal human astroglial HEB cells using a CCK-8 assay. Intriguingly, we observed that 0.5 μM celastrol plus 100 ng/ml TRAIL had almost no impact on HEB cells, while the growth of U87-MG cells was effectively inhibited by this cotreatment regimen. Thus, we discovered that the lowest evaluated dose of celastrol (0.5 μM) effectively sensitized U87-MG cells to TRAIL-induced cell death. In addition, the viability of glioma cells was reduced with increasing concentrations of celastrol in combination with the same concentration of TRAIL (100 ng/μl). Numerous studies have demonstrated that several chemotherapeutic agents can attenuate TRAIL resistance in glioma cells by increasing the expression of DR4 and DR5 [8–10, 22, 23]. Moreover, recent studies have indicated that DR5, rather than DR4, preferentially induces apoptosis in transformed or malignant cells, demonstrating the potential of DR5 as a tumor-selective apoptosis-inducing cytokine for cancer therapy [28]. Furthermore, glioblastoma primarily express DR5, and the prolonged lifespan of glioma patients is positively correlated with high DR5 expression, whereas there is no obvious correlation between prolonged lifespan and DR4 expression [28–30]. Thus, we assessed only DR5 expression in our study. Although a previous study reported that the sensitivity of malignant glioma cells to TRAIL is unrelated to the expression of DR4 and DR5 [31], our data demonstrate that celastrol treatment markedly increased the expression of DR5 in U87-MG cells in a dose-dependent manner. Furthermore, knockdown of DR5 significantly attenuated the apoptosis elicited by cotreatment with celastrol and TRAIL, indicating that DR5 plays an important role in this apoptotic pathway. The TRAIL-induced cytotoxicity enhanced by celastrol was effectively inhibited by the addition of the pancaspase inhibitor z-VAD-fmk, suggesting that the cotreatment-induced cell death is dependent on caspases and proceeds through the death receptor pathway.

In summary, for the first time, we showed that a noncytotoxic dose of celastrol significantly sensitizes human glioblastoma cells to TRAIL-induced cell death, and the underlying mechanisms have been preliminarily elucidated. We demonstrated that the combination of celastrol and TRAIL not only increases the anticancer effects of TRAIL but also alleviates the toxicity of celastrol, making this cotreatment regimen a promising tumor-killing strategy. Unfortunately, although we could not further investigate this synergistic effect in vivo, we expect to establish an orthotopic implantation model of human glioma for future in vivo analyses.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. The manuscript does not contain clinical studies or patient data.

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