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# Capsaicin sensitizes malignant glioma cells to TRAIL-mediated apoptosis via DR5 upregulation and survivin downregulation

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**Capsaicin, a pungent ingredient of red chili peppers, has been reported to possess antitumor activities. Here, we show that subtoxic doses of capsaicin effectively sensitize multiple malignant glioma cell lines to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Although TRAIL alone mediated partial proteolytic processing of procaspase-3 in glioma cells, cotreatment with capsaicin and TRAIL efficiently restored complete activation of caspases. We found that treatment of various gliomas with capsaicin significantly upregulated DR5, a death receptor of TRAIL, and downregulated the caspase inhibitor survivin. The induction of DR5 was mediated by CHOP/GADD153. The reduction in survivin protein level was associated with downregulation of cyclin B and Cdc2 expression, suggesting that inhibition of Cdc2 activity might contribute to capsaicin-induced survivin downregulation. Taken together, these results indicate that the activity of capsaicin toward DR5 and survivin contributes to the amplification of caspase cascades, thereby restoring TRAIL sensitivity in malignant glioma cells. Interestingly, normal astrocytes were resistant to combined treatment with capsaicin and TRAIL. Neither capsaicin-induced DR5 upregulation/survivin downregulation nor the partial processing of procaspase-3 by TRAIL was induced in astrocytes. Thus, a combined regimen using capsaicin and TRAIL may provide a safe and effective strategy for treating malignant gliomas.**

## Introduction

Malignant gliomas are the most common primary brain tumors. They exhibit a relentless malignant progression characterized by widespread invasion throughout the brain, destruction of normal brain tissue, resistance to traditional therapeutic approaches and certain death (1). Therefore, approaches designed to reactivate cell death programs, particularly apoptosis, have important implications for the development of novel therapies for malignant gliomas.

Tumor necrosis factor-related apoptosis-induced ligand (TRAIL) is a potent stimulator of apoptosis, and tumor cells are significantly more sensitive to TRAIL-induced apoptosis than are normal cells (2). Binding of TRAIL to either DR4 or DR5, two death receptors of TRAIL, leads to oligomerization of receptor intracellular death domains, recruitment of the adaptor molecule Fas-associated death domain protein and activation of a caspase-8-mediated cascade of effector caspases including caspase-3 (3). However, several recent studies have shown that many glioma cells remain resistant to TRAIL, even though they express TRAIL receptors (4,5). These findings indicate that other cellular mechanisms may contribute to resistance to TRAIL-mediated cell death and suggest that identifying sensitizing agents capable of overcoming this resistance may be the key to improving TRAIL-based cancer therapies against gliomas.

Capsaicin (8-methyl-*N*-vanillyl-noneamid), enriched in chili peppers and widely used as a food additive (6), has been used to treat pain and inflammation associated with various diseases, including rheumatoid arthritis, diabetic neuropathy, post-mastectomy pain syndrome and herpes zoster (7–9). Although capsaicin is known to act through intracellular binding to the transient receptor potential vanilloid 1 (TRPV1) receptor to exert its physiologic effects in sensory neurons (9,10), TRPV1-independent effects of capsaicin in mammalian cells have also been documented (11,12). Furthermore, recent studies have shown that capsaicin induces the growth inhibition and/or apoptosis of a variety of human cancer cells *in vitro* and *in vivo* (13–16). However, the mechanisms underlying the anticancer effects of capsaicin are not fully understood.

Here, we show that capsaicin is a potent sensitizer of TRAIL-induced apoptosis in multiple malignant glioma cell lines. Moreover, we present clear evidence that subtoxic doses of capsaicin effectively overcome TRAIL resistance in glioma cells via DR5 upregulation and survivin downregulation, thereby contributing to the amplification of TRAIL-mediated apoptotic signaling.

**Abbreviations:** $IC_{50}$, the half maximal inhibitory concentration; mRNA, messenger RNA; PCR, polymerase chain reaction; siRNA, small interfering RNA; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRPV1, transient receptor potential vanilloid 1.

## Materials and methods

### *Chemicals and antibodies*

Recombinant human TRAIL/Apo2 ligand was from KOMA Biotech (Seoul, South Korea). Capsaicin, capsazepine and nonivamide were purchased from Sigma Chemical Corporation (St Louis, MO). Calcein acetoxymethyl ester (calcein-AM) and ethidium homodimer-1 (EthD-1) were from Molecular Probes (Eugene, OR). The following antibodies were used: anti-caspase-8, caspase-3, caspase-6, caspase-7, survivin and XIAP (Stressgen, British Columbia, Canada); anti-caspase-9, caspase-2, DR4, CHOP/growth arrest and damage-inducible gene 153 (GADD153), Bcl-2 and Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA); anti-poly (adenosine diphosphate-ribose) polymerase (Upstate Biotechnology, Lake Placid, NY); anti-Bid, phospho-AKT (Ser 473) and total AKT (Cell Signaling Technology, Beverly, MA); anti-c-FLIP (NF6) (Alexis, San Diego, CA); fluorescein isothiocyanate-conjugated anti-goat IgG (Sigma Chemical Corporation); α-tubulin, anti-DR5 for western blotting (KOMA Biotech); anti-DR5 antibody for fluorescence-activated cell sorting analysis and immunofluorescence (R&D Systems, Minneapolis, MN) and anti-rabbit IgG horseradish peroxidase, mouse IgG and goat IgG (Zymed Laboratories, South San Francisco, CA)

### *Culture of glioma cell lines and normal human astrocytes*

The human malignant glioma cell lines U87MG, U343, T98, SNU-444, U251N and U251MG were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (Gibco BRL). The primary cultures of normal human astrocytes were prepared from 14 week gestation of fetal cerebrum tissues as described previously (17). Human astrocyte cultures were grown in Dulbecco's modified Eagle's medium with high glucose supplemented with 10% fetal bovine serum and 20 Ag/ml gentamicin, subcultured every 2 weeks, and cell culture passage number of $<5$ were used in the present study.

### *Measurement of cellular viability*

Cell viability was assessed by double labeling of cells with 2 μM calcein-AM and 4 μM EthD-1. The calcein-positive cells and EthD-1-positive dead cells were visualized using fluorescence microscope (Axiovert 200M, Carl Zeiss, Jena, Germany).

### *Reverse transcription–polymerase chain reaction analysis*

Total RNA was extracted from U251MG cells using the TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription–polymerase chain reaction (PCR) was done, following the manufacturer's protocol (Takara Shuzo Co., Otsu, Shiga, Japan). Conditions for final analysis were chosen when amplification of messenger RNA (mRNA) was in the middle of the exponential amplification phase for all capsaicin concentrations tested. Human DR5 mRNA was amplified using the sense primer 5′-GTCTGCTCTGATCACCCAAC-3′ and the antisense primer 5′-CTGCAAACTGTGACTCCTATG-3′

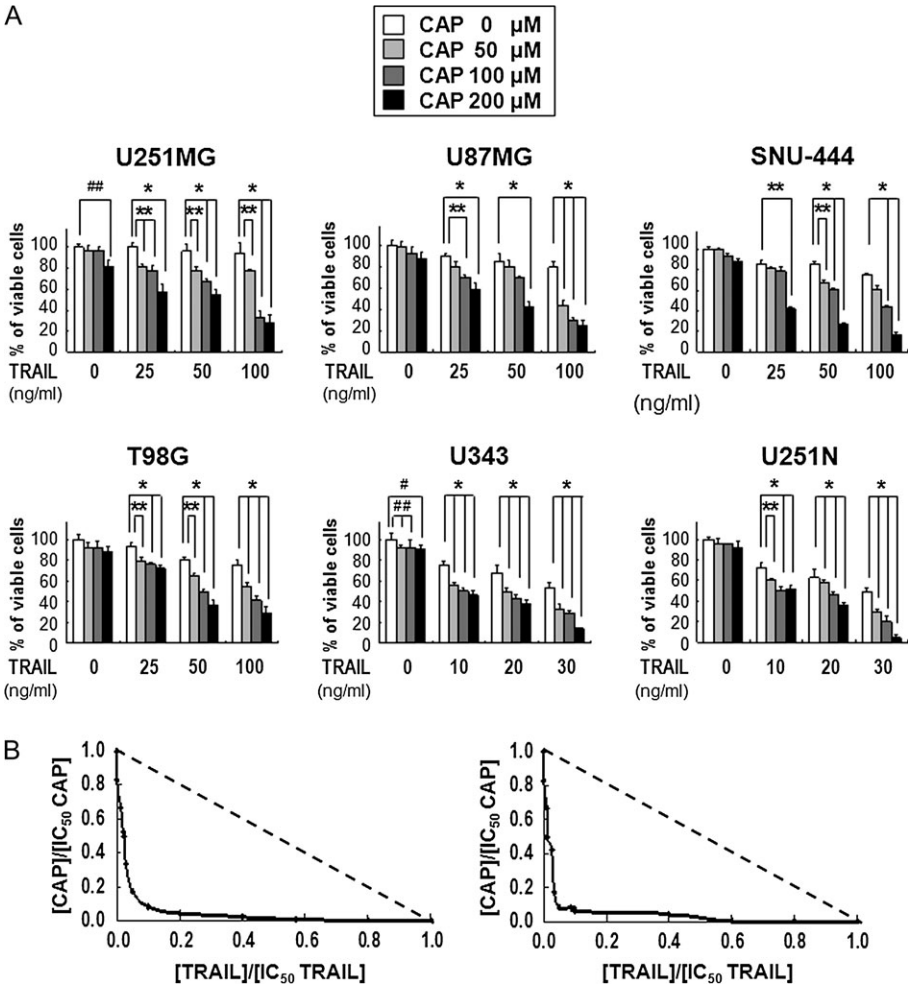


**Fig. 1.** Subtoxic doses of capsaicin (CAP) sensitize various malignant glioma cells to TRAIL-induced apoptosis. (**A**) Six different human glioma cells were untreated or treated with CAP for 30 min and further treated with TRAIL for 16 h at the indicated concentrations. Cellular viability was assessed using calcein-AM and EthD-1. Columns indicate average of three individual experiments; bars represents ± SD; $^{\#}P < 0.001$, compared with untreated cells; $^{\#\#}P < 0.01$, compared with untreated cells; $^{*}P < 0.001$, compared with TRAIL-treated cells and $^{**}P < 0.01$, compared with TRAIL-treated cells. (**B**) Synergistic induction of cell death by CAP and TRAIL. U87MG or U251MG cells were treated for 24 h with increasing concentrations of CAP and TRAIL. Isobologram analysis was performed as described in Materials and Methods. (**C**) U251MG and U87MG cells were treated with 200 μM CAP and/or 100 ng/ml TRAIL for the indicated time points and cell extracts were prepared for western blotting of caspases, poly (adenosine diphosphate-ribose) polymerase and Bid was performed. Western blotting of α-tubulin was used to show equal loading of the protein samples. (**D**) U251MG and U87MG cells were treated with the indicated concentration of CAP for 16 h, and western blotting of various apoptotic regulators and α-tubulin as a loading control was done.

(corresponding to a 424 bp region of DR5). For survivin, the sense primer 5′-CAGATTTGAATCGCGGGACCC-3′ and the antisense primer 5′-CCAGAGTCTGGCTCGTTCTCAG-3′ (corresponding to a 206 bp region of survivin). For glyceraldehyde-3-phosphate dehydrogenase, the sense primer 5′-CGGCCATCACGCCCACAGTTT-3′ and the antisense primer 5′-CGGCCATCACGCCCACAGTTT-3′ were used (corresponding to a 310 bp region of glyceraldehyde-3-phosphate dehydrogenase). The PCR cycling conditions (30 cycles) chosen were as follows: (i) 30 s at 94°C; (ii) 30 s at 68°C for DR5, 30 s at 60°C for glyceraldehyde-3-phosphate dehydrogenase and 60 s at 60°C for survivin and (iii) 1 min 30 s at 72°C, with a subsequent 10 min extension at 72°C. Reaction products were analyzed on 2% agarose gels. The bands were visualized by ethidium bromide.

*Flow cytometry of death receptors*

Cells were analyzed for the surface expression of DR4 and DR5 by indirect staining with primary goat anti-human DR4 and DR5 (R&D Systems) followed by fluorescein isothiocyanate-conjugated rabbit anti-goat IgG. Fluorescence-activated cell sorting analysis from cultured cells performed as described previously (18).

*Small interfering RNA*

Twenty-five nucleotide small interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen and have the following sequences: DR5 (F01), 5′-UUUAGCCACCUUUAUCUCAUUGUCC-3′; DR5 (E11), 5′-UACAAUCACCGACCUUGACCAUCCC-3′ and survivin, 5′-UUUAAGGCUGGGAGCCAGAUGACGC-3′. BLOCK-IT Fluorescent Oligo (Invitrogen) was used as the control. Cells were transfected with siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

*Plasmids, transfection and luciferase assay.*

The pDR5-605 [containing DR5 promoter sequence (−605/+3)] was a gift from Dr T.Sakai (Kyoto Prefectural University of Medicine). Clone representing this point mutation was sequenced to ensure the accuracy of the PCR amplification procedure. Point mutation of the CHOP-binding sites to the DR5-605 promoter was generated by a two-step PCR method using the following primers: mCHOP (5′-CTTGCGGAGGAGGTAGTTGACGA-3′ and 5′-TCGTCAACTACCTCCTCCGCAAG-3′). The clone containing the mutation was sequenced to ensure the accuracy of the PCR amplification procedure and this plasmid was named as pDR5-605-mCHOP. The CHOP promoter-luciferase construct (19) was generously provided by Dr P.Fafournoux (U.R. 238 de Nutrition Cellulaire et Moleculaire, France). Transfection and luciferase assay from cultured cells performed as described previously (5).

*Expression of Cdc2, cyclin A or cyclin B by transient transfection*

U87MG cells were plated into 24-well plates at $4 \times 10^4$ cells per well. After 24 h, cells were transfected with the plasmid encoding Cdc2, cyclin A or cyclin B (kindly provided by Dr Paul Robbins, University of Pittsburgh) at the indicated concentrations using Lipofectamine Plus reagent (Gibco BRL)

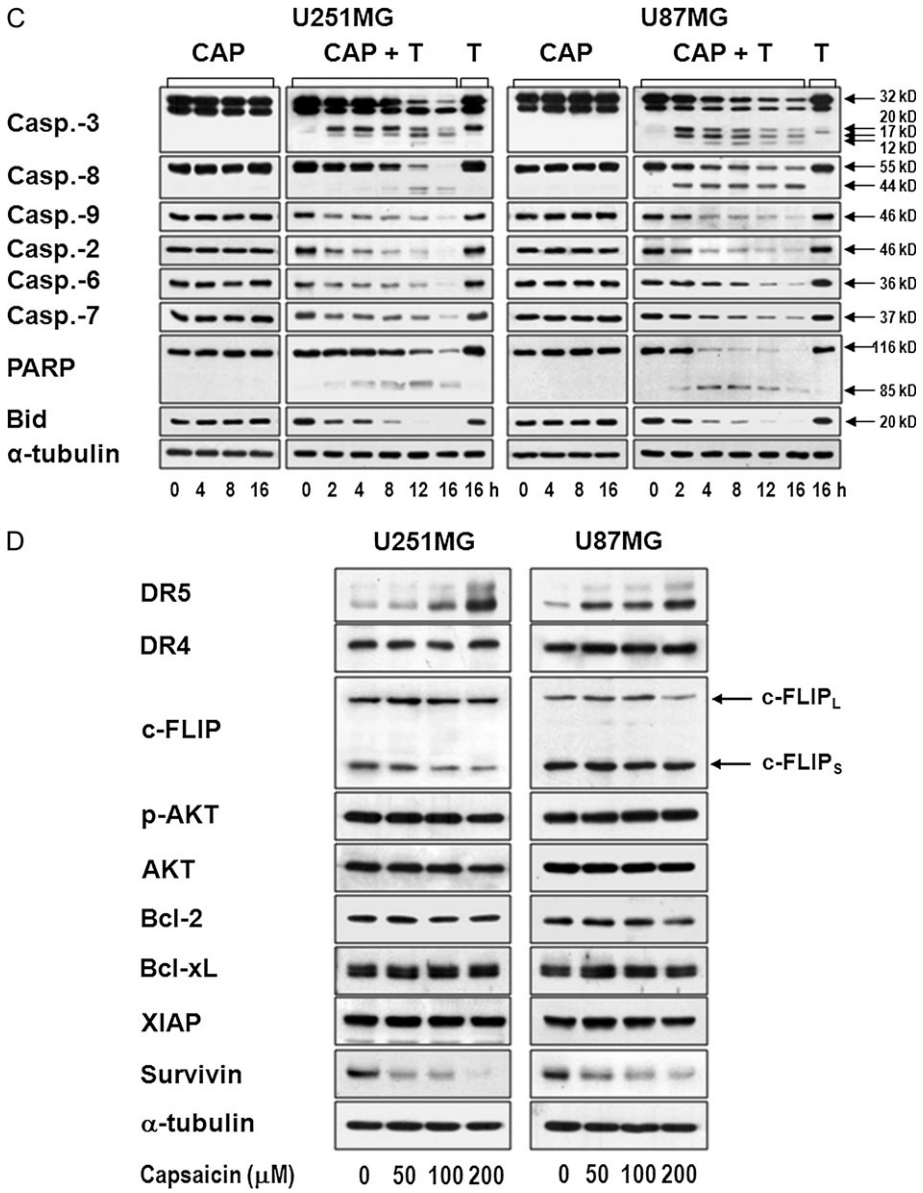


**Fig. 1.** *Continued.*

following the manufacturer's instructions. Transfected cells were incubated for 24 h and then further treated with 200 μM capsaicin and/or 100 ng/ml TRAIL for 16 h. Transfection efficiency, assessed using a green fluorescence protein-encoding plasmid (pEGFP-C1; from Promega, Madison, WI), reached ∼70% in these experiments. Cellular viability was assessed using calcein-AM and EthD-1 as described above. To further confirm the expression of transgenes, western blotting of the transfected proteins was performed.

*Establishment of the stable cell lines overexpressing survivin*

U87MG cells were transfected with a vector containing Flag-tagged survivin. Stable cell lines overexpressing survivin was selected with fresh medium containing 500 μg/ml G418 (Calbiochem, Sandiego, CA). Overexpression of survivin was analyzed by western blotting using anti-Flag (Sigma Chemical Corporation) and anti-survivin (Stressgen) antibody.

*Statistical analysis and determination of synergy*

All data are presented as means ± SDs of at least three independent experiments. The statistical significance of differences was assessed using analysis of variance with Bonferroni or repeated measures analysis of variance followed by Greenhouse–Geisser adjustment. Values of $P < 0.05$ were considered significant. Synergy of capsaicin and TRAIL was evaluated by the isobologram method. The cells were treated with different concentrations of each agent (capsaicin or TRAIL) alone or with the two agents in combination for 16 h. The relative survival was assessed, and the half maximal inhibitory concentration ($IC_{50}$) values for each drug given alone or in combination with a fixed concentration of the second agent were established from the concentration-effect curves. The $IC_{50}$ values of cotreatment were divided by the $IC_{50}$ value of each drug in the absence of the other drug. In a graphical presentation, the straight line connecting the $IC_{50}$ values of the two agents when applied alone corresponds to additivity or independent effects of both agents. Values below this line indicate synergy and values above this line indicate antagonism.

## Results

*Subtoxic doses of capsaicin effectively sensitize human malignant glioma cells to TRAIL-induced apoptosis*

To assess the ability of capsaicin to act as a sensitizer of malignant glioma cells to the apoptotic effects of TRAIL, we first examined the effect of TRAIL with and without capsaicin on the viability of a number of different glioma cell lines. We found that U251MG, U87MG, SNU-444 and T98G cells were relatively resistant to TRAIL over a range of doses, whereas U251N and U343 cells were somewhat sensitive to TRAIL (Figure 1A). Capsaicin alone induced a modest level of glioma cell death (<20%) at concentrations up to 200 μM. In contrast, the viability

of glioma cells was significantly reduced by combined treatment with a fixed concentration of TRAIL and varied concentrations of capsaicin or conversely with a fixed capsaicin concentration and varied concentrations of TRAIL. Furthermore, synergistic effect, as determined by isobologram analysis (20) was observed for capsaicin and TRAIL in U251MG and U87MG cells (Figure 1B). These results demonstrate that the combined treatment of TRAIL-resistant glioma cells with capsaicin and TRAIL very effectively induces cell death.

These results indicate that subtoxic doses of capsaicin can sensitize malignant glioma cells to TRAIL-mediated apoptosis. Next, we examined the effect of capsaicin and/or TRAIL on the proteolytic processing of caspases in TRAIL-resistant U251MG and U87MG cells. Treatment with 200 μM capsaicin alone had no effect on the processing of caspase-3, -8, -9, -2, -6 or -7 in these cells (Figure 1C). In contrast, treatment with 100 ng/ml TRAIL alone for 16 h induced partial cleavage of the 32 kDa procaspase-3 to a 20 kDa intermediate form; however, further cleavage to the active p17 and p12 subunits was not detected. Under the same conditions, other caspases were not processed. However, combined treatment with capsaicin and TRAIL induced the complete processing of caspase-3 into p17/p12 and also led to a significant decrease in levels of the precursor forms of caspase-8, -9, -2, -6 and -7. Activation of caspases by combined treatment was further confirmed by western blotting for poly (adenosine diphosphate-ribose) polymerase, a substrate of caspase-3 (21), and Bid, a substrate of caspase-8 (22). Flow cytometric analysis also demonstrated that cotreatment of U251MG cells with 200 μM capsaicin and 100 ng/ml TRAIL significantly increased the accumulation of a sub-$G_1$ cell population, an effect that was almost completely blocked by pretreatment with z-VAD-fmk, a pan-caspase inhibitor (supplementary Figure 1 is available at *Carcinogenesis* Online). Collectively, these results demonstrate that combined treatment with capsaicin and TRAIL induces caspase-mediated apoptosis.

To determine the underlying mechanisms of capsaicin-stimulated TRAIL-mediated apoptosis, we examined the possible involvement of various modulators of death receptor-mediated apoptotic signaling. Neither Akt activity nor protein levels of Bcl-2, Bcl-xL or XIAP were significantly affected by capsaicin treatment (Figure 1D). Protein levels of the short form of cellular FLICE-inhibitory protein but not the long form of cellular FLICE-inhibitory protein were downregulated in U251MG cells following capsaicin treatment, whereas the protein levels of both the long form of cellular FLICE-inhibitory protein and the short form of cellular FLICE-inhibitory protein were essentially unchanged in U87MG cells. Interestingly, we found that capsaicin treatment significantly upregulated protein levels of DR5, but not DR4, in both U251MG and U87MG cells. In addition, survivin protein levels were downregulated in both cell lines. These results indicate that DR5 upregulation and survivin downregulation may have a role in the cell death induced by combined treatment of glioma cells with capsaicin and TRAIL.

*DR5 upregulation is important for capsaicin-mediated sensitization of multiple glioma cell lines to TRAIL-induced apoptosis*

Next, we examined whether capsaicin-mediated DR5 upregulation is a commonly observed phenomenon in different glioma cells. We found that capsaicin treatment dose dependently increased DR5 protein levels in U251N, U343, T98 and SNU-444 cells (Figure 2A). Flow cytometric analysis showed that capsaicin treatment of U251MG cells significantly increased the surface expression of DR5, compared with that of DR4 (Figure 2B). Immunocytochemistry using an anti-DR5 antibody also demonstrated an increase in DR5 expression in capsaicin-treated U251MG cells (Figure 2C). To clarify the functional significance of DR5 in the capsaicin-mediated stimulation of TRAIL-induced apoptosis, we assessed the effects of DR5 knockdown by transfecting U251MG cells with two different siRNAs. siRNA-mediated suppression of DR5 expression effectively inhibited capsaicin-stimulated TRAIL-induced cell death in U251MG cells (Figure 2D). These results demonstrate that capsaicin-induced DR5 upregulation plays a critical role in the capsaicin-mediated enhancement of TRAIL sensitivity in glioma cells.

*CHOP mediates capsaicin-induced DR5 upregulation in glioma cells*

Next, we investigated the underlying mechanism of capsaicin-induced DR5 upregulation in glioma cells. Reverse transcription–PCR analysis demonstrated that DR5 mRNA levels were dose dependently increased by capsaicin treatment in U251MG cells (Figure 3A), suggesting that capsaicin modulates DR5 expression at the transcriptional level. Given our recent demonstration that CHOP/GADD153 is associated with arsenic trioxide- and silibinin-induced DR5 upregulation and resulting sensitization of TRAIL-mediated apoptosis (5,23), we investigated whether CHOP is also involved in capsaicin-induced DR5 upregulation. Capsaicin treatment of U251MG, U343 and U251N cells significantly increased CHOP protein levels in parallel with DR5 expression (Figure 3B). To further examine the transcriptional modulation of DR5 by CHOP, we used the wild-type DR5-reponsive reporter construct pDR5-605, which contains a region (−605/+3) of the DR5 promoter, and a mutant version (pDR5-605-mCHOP) of the promoter-reporter construct in which the potential CHOP-binding site of the DR5 promoter has been mutated (24). Capsaicin (200 μM) treatment increased DR5 promoter activity in U25MG cells transfected with the pDR5-605 plasmid, but not in those transfected with pDR5-605-mCHOP (Figure 3C), indicating that CHOP is responsible for capsaicin-induced upregulation in U251MG cells.

*Capsaicin enhances TRAIL-induced apoptosis by downregulation of survivin*

We next investigated whether downregulation of survivin is critical for capsaicin-mediated sensitization of glioma cells to TRAIL-induced apoptosis. Capsaicin treatment induced a dose-dependent decrease in the levels of survivin protein in U251N, SNU-444, U343 and T98G cells (Figure 4A), indicating that downregulation of survivin may be a common response to capsaicin in glioma cells. We found that cell death induced by capsaicin plus TRAIL was significantly inhibited in U87MG cells stably overexpressing survivin (Figure 4B). In addition, siRNA-mediated knockdown of survivin significantly enhanced TRAIL-induced apoptosis (Figure 4C). Collectively, these results indicate that survivin downregulation is a critical contributor to capsaicin-mediated sensitization of glioma cells to TRAIL-induced apoptosis.

*Capsaicin downregulates survivin by inhibition of cyclin B-associated Cdc2 activity and subsequent proteasome-mediated degradation*

Further investigations of the molecular basis of capsaicin-induced survivin downregulation using reverse transcription–PCR showed that capsaicin treatment did not affect survivin mRNA levels in U251MG cells (Figure 5A). However, we did find that pretreatment with the proteasome inhibitor MG132 attenuated capsaicin-induced reduction of survivin protein levels (Figure 5B), indicating that capsaicin may downregulate survivin via proteasome-mediated degradation. Phosphorylation of survivin by Cdc2 has previously been shown to modulate survivin protein stability (25). Consistent with a role for Cdc2 activity in capsaicin-induced survivin downregulation, we found that the protein levels of cyclin A, cyclin B and Cdc2, key components involved in controlling Cdc2 activity (26), were decreased in glioma cells by capsaicin treatment. This effect of capsaicin was dose dependent and common to U251MG, U87MG, U251N and U343 cells (Figure 5C). Next, we investigated the functional effect of changes in the expression levels of these cell cycle regulators on survivin protein levels in glioma cells. An examination of the effects of exogenous expression of Cdc2, cyclin A and cyclin B, alone or their combination, showed that forced expression of cyclin B alone increased survivin protein levels in U87MG cells to a significantly greater extent than did overexpression of cyclin A or Cdc2 alone (Figure 5D). In addition, overexpression of Cdc2 and cyclin B or cyclin A and cyclin B was more effective in increasing survivin protein levels than was expression of Cdc2 and cyclin A. These results indicate that cyclin B-dependent Cdc2 activity is critical for survivin expression in these cells. Furthermore, we found that forced expression of cyclin B alone, Cdc2 plus cyclin B or cyclin A plus cyclin B significantly attenuated the cell death induced by combined treatment with capsaicin and

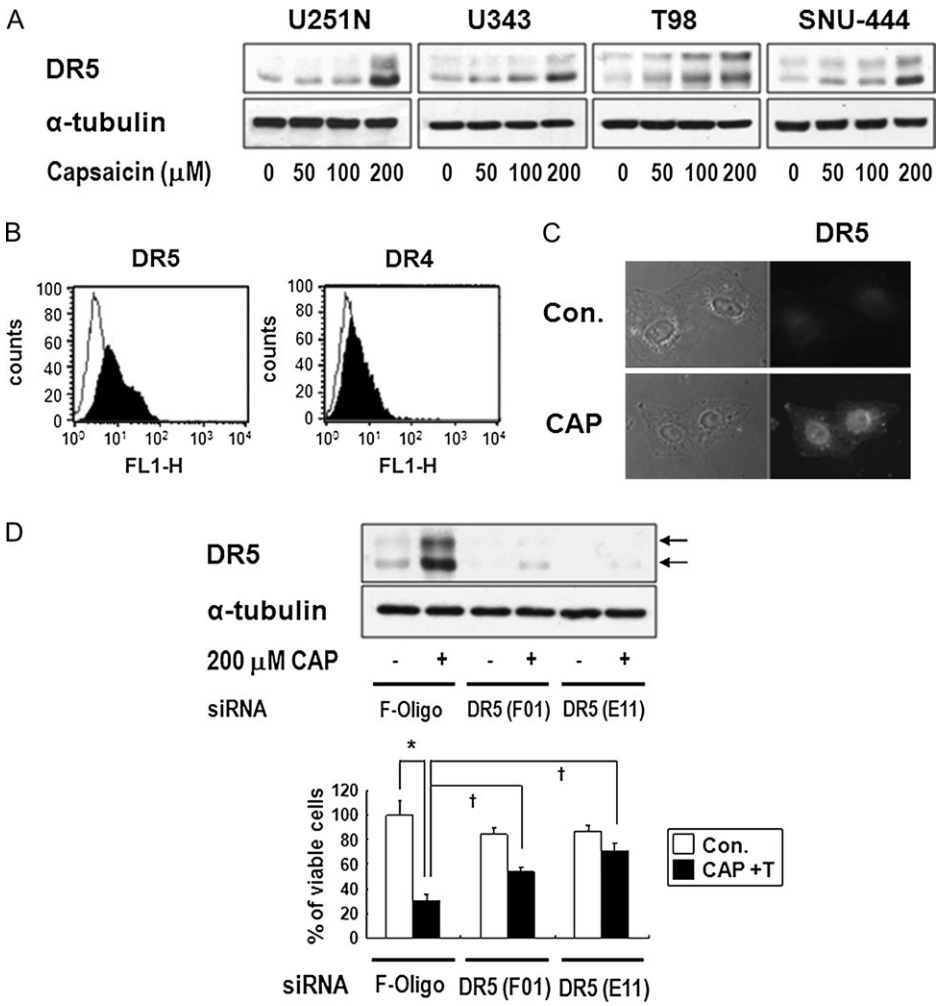


**Fig. 2.** DR5 upregulation is critical for capsaicin (CAP)-sensitized TRAIL-induced apoptosis in glioma cells. (**A**) Cells were treated with the indicated concentrations of CAP for 16 h and cell extracts were prepared for western blotting of DR5 and α-tubulin. (**B**) U251MG cells were incubated with or without 200 μM CAP for 16 h and the surface expression of DR5 and DR4 proteins were analyzed by fluorescence-activated cell sorting analysis. (**C**) U251MG cells were treated or not with 200 μM CAP for 16 h, stained with antibodies against DR5 and processed for the fluorescence microscopy. (**D**) U251MG cells were transfected with fluorescent oligonucleotide (F-Oligo) or two kinds of siRNA duplexes against DR5 mRNA. Twenty-four hours after the transfection, cells were treated with 200 μM CAP for 16 h. Western blotting of DR5 was done to confirm the knockdown of DR5 expression by siRNA transfection. α-Tubulin levels were assessed to show equal gel loading. To examine the effect of DR5 downregulation on CAP/TRAIL-induced apoptosis, U251MG cells were transfected with siRNAs, incubated for 24 h and further treated with or without or 200 μM CAP plus 100 ng/ml TRAIL for 16 h. Cellular viability was determined using calcein-AM and EthD-1. Columns indicate average of three individual experiments; bars represents ± SD; $^{*}P < 0.001$, compared with untreated cells and $^{\dagger}P < 0.001$, compared with the cells transfected with the F-Oligo and further treated with CAP plus TRAIL.

TRAIL (Figure 5E). Taken together, these results demonstrate that survivin downregulation by inhibition of cyclin B-dependent Cdc2 activity contributes to capsaicin-mediated sensitization of glioma cells to TRAIL-induced apoptosis.

*Combined treatment with capsaicin and TRAIL does not affect the expression of DR5, survivin or caspase-3 and does not induce cell death in normal astrocytes*

Finally, we investigated the effects of cotreatment with capsaicin and TRAIL on the viability of normal astrocytes. Astrocytes were very resistant to TRAIL and capsaicin, either alone or in combination (Figure 6A), indicating that the sensitizing regimen of capsaicin plus TRAIL may be preferentially toxic to glioma cells. We then examined whether the resistance of astrocytes to the combined treatment regimen was associated with differential regulation of DR5 and survivin. In contrast to its effects in U251MG cells, capsaicin treatment did not affect CHOP and DR5 protein levels in normal astrocytes (Figure 6B). Survivin protein levels, which were significantly downregulated by capsaicin in U251MG cells, were markedly low in normal astrocytes and were not affected by capsaicin. Furthermore, procaspase-3 processing, which was partially induced in glioma cells by TRAIL alone and was completed in cells cotreated with TRAIL and capsaicin, was not observed in astrocytes (Figure 6C). Taken together, these results indicate that CHOP-mediated DR5 upregulation and survivin downregulation, as well as TRAIL-mediated priming of the proteolytic processing of caspase-3, contribute to the selective enhancement of TRAIL-mediated apoptosis by capsaicin in malignant glioma cells (Figure 6D).

## Discussion

Despite aggressive treatment strategies, the prognosis for patients with malignant gliomas remains poor, highlighting the need for novel treatment approaches (1). Effective killing of cancer cells by current cytotoxic therapies, such as chemotherapy, γ-irradiation, immunotherapy and suicide gene therapy, largely depends on activation of apoptosis programs in cancer cells (27). TRAIL, a member of the cytokine superfamily, is a promising candidate for cancer therapy because it induces apoptosis in numerous cancer cell lines but not in normal cells (2). However, various tumor cells, including

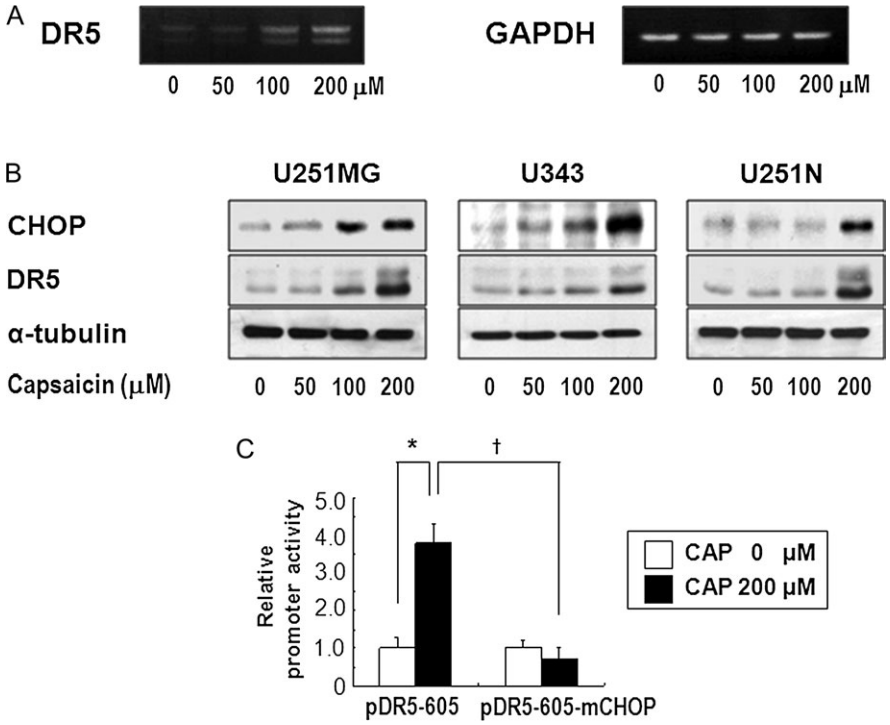


**Fig. 3.** CHOP mediates capsaicin (CAP)-induced DR5 upregulation in glioma cells. (**A**) U251MG cells were treated with CAP at the indicated concentrations and total RNA was prepared for reverse transcription–PCR of DR5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (**B**) Cells were treated with the indicated concentration for 16 h and cell extracts were prepared for western blotting of CHOP, DR5 and α-tubulin. (**C**) U251MG cells were transfected with pDR5-605 or pDR5-605-mCHOP promoter constructs treated with 200 μM CAP, lysed and assayed for luciferase activity. Columns indicate average of three individual experiments; bars represents ± SD; $^{*}P < 0.001$, compared with untreated cells and $^{\dagger}P < 0.001$, compared with cells transfected with pDR5-605 and further treated with CAP.

malignant glioma cells, have defects in components of the cell death signaling pathway, that render them resistant to the apoptotic effects of TRAIL (18,23). Thus, identifying agents that are capable of sensitizing cancer cells to TRAIL-mediated apoptosis is important for overcoming TRAIL resistance in cancer cells and improving TRAIL-based cancer therapy. Several chemotherapeutic agents have been shown to sensitize malignant glioma cells to TRAIL-induced apoptosis (28,29). Recently, we reported that treatment with subtoxic doses of arsenic trioxide or histone deacetylase inhibitors effectively recover TRAIL sensitivity in TRAIL-resistant malignant glioma cells (5,30).

Capsaicin, the pungent active ingredient of hot chili peppers, has been used as an analgesic to treat several painful diseases, including rheumatoid arthritis, diabetic neuropathy, osteoarthritis, human immunodeficiency virus-associated distal sensory polyneuropathy and postherpetic neuralgia (7–9,31). Recent studies have shown that capsaicin induces apoptosis in a number of different cancer cells but not in normal cells (14,15,31), indicating that capsaicin may have therapeutic benefits in clinical settings, either as an anticancer agent or as an adjunct to current cancer therapies.

In the present study, we show for the first time that subtoxic doses of capsaicin effectively sensitize different malignant glioma cell lines to TRAIL-mediated apoptosis. Capsaicin exerts its physiologic function in sensory neurons by intracellular binding to TRPV1 (32). However, recent studies have shown that capsaicin induces apoptosis in a variety of cancer cells via a TRPV1-independent mechanism (13,33–35). In our study, pretreatment of U251MG and U87MG cells with the specific TRPV1 antagonist capsazepine (36) did not block the cell death induced by combined treatment with capsaicin and TRAIL (supplementary Figure 2 is available at *Carcinogenesis* Online). Furthermore, unlike capsaicin, the TRPV1 agonist nonivamide (37) did not stimulate TRAIL-mediated apoptosis up to a concentration of 200 μM (supplementary Figure 2 is available at *Carcinogenesis* Online). These results indicate that capsaicin-mediated sensitization of glioma cells to TRAIL-induced apoptosis does not involve TRPV1, suggesting the existence of other mechanisms that mediate capsaicin-stimulated, TRAIL-mediated apoptosis.

The death receptors DR4 and DR5 are type I transmembrane proteins containing an intracellular death domain that engages the apoptotic machinery upon TRAIL binding (3). The levels of DR4 and/or DR5 expression may have decisive roles in determining the intensity and/or duration of TRAIL-induced death receptor-mediated apoptotic signaling. Recently, we showed that application of DR5 upregulating agents may be a promising strategy for sensitizing malignant cancer cells to TRAIL-induced apoptosis (5,18,23). In the present study, we show for the first time that capsaicin is an effective TRAIL sensitizer that acts via a DR5-upregulation mechanism in multiple glioma cell lines. siRNA-mediated DR5 suppression significantly blocked the cell death induced by combined treatment with capsaicin and TRAIL, demonstrating the functional significance of DR5 in this cell death. We showed that mutating the CHOP-binding site on the DR5 promoter significantly inhibited capsaicin-induced transcriptional activation of DR5 in U251MG cells, indicating that CHOP-mediated DR5 upregulation contributes to capsaicin-stimulated TRAIL-induced apoptosis in these cells. In our study, western blot analysis of DR5 demonstrated two protein bands, p49 and p45 (Figures 1D and 2A), and these two band patterns of DR5 protein are consistent with the previously published results using the specific anti-DR5 antibodies of different sources (38,39). Previously, Screaton *et al.* (40) showed that two isoforms of the DR5 mRNA are generated by alternative pre-mRNA splicing and two protein bands of DR5 are presumed to be their translational products, with or without a 29 amino-acid extension to the extracellular domain (40,41).

In addition to DR5 upregulation by capsaicin, we found that survivin downregulation was a common feature of capsaicin-facilitated TRAIL-induced apoptosis among different glioma cells. Survivin,

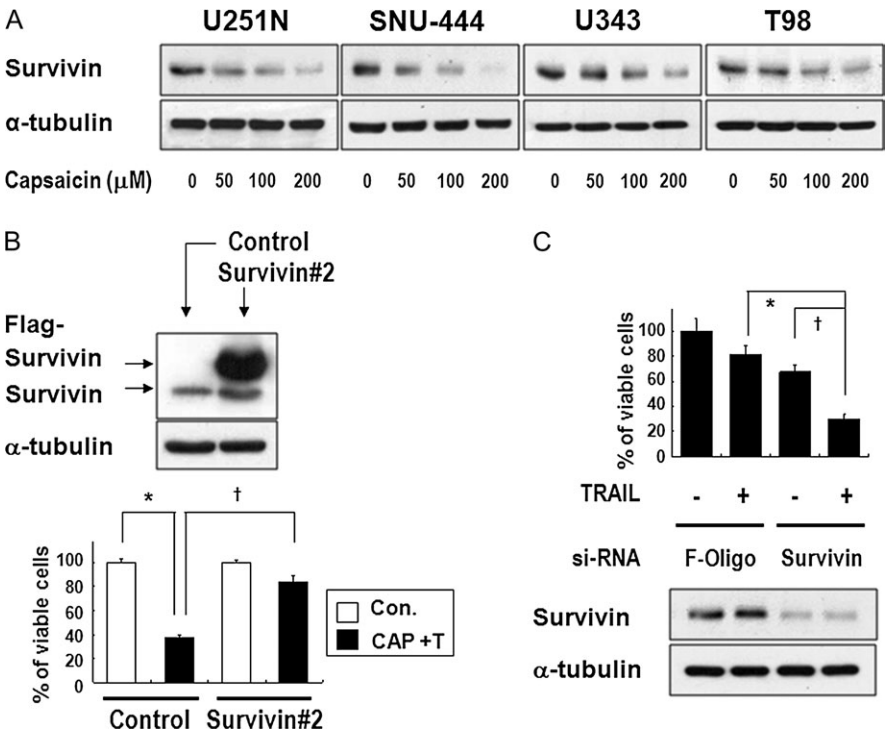


**Fig. 4.** Capsaicin (CAP)-induced survivin downregulation critically contributes to CAP-stimulated TRAIL-induced apoptosis. (**A**) Cells were treated with the indicated concentrations of CAP for 16 h and western blotting of survivin and α-tubulin was performed. (**B**) The protein levels of survivin were examined by western blotting using anti-survivin antibody in U87MG cells stably transfected with the control vector or the plasmid encoding Flag-tagged survivin. Control cells (Con.) and the cells stably overexpressing survivin were treated with 200 μM CAP and 100 ng/ml TRAIL for 16 h. Cell viability was assessed using calcein-AM and EthD-1. Columns indicate average of three individual experiments; bars represents ± SD; $^{*}P < 0.001$, compared with untreated cells and $^{\dagger}P < 0.001$, compared with the control cells treated with CAP plus TRAIL. (**C**) To examine the effect of survivin downregulation on CAP/TRAIL-induced apoptosis, U251MG cells were transfected with the control fluorescent oligonucleotide (F-Oligo) or survivin siRNA, incubated for 24 h and further treated with or without or 100 ng/ml TRAIL for 16 h. Cellular viability was determined using calcein-AM and EthD-1. Columns indicate average of three individual experiments; bars represents ± SD; $^{*}P < 0.001$, compared with the cells transfected with the F-Oligo and further treated with TRAIL; $^{\dagger}P < 0.001$, compared with the cells transfected with survivin siRNA but untreated with TRAIL. Western blotting of survivin was done to confirm the downregulation of survivin by siRNA transfection. α-Tubulin levels were assessed to show equal gel loading.

a member of the inhibitor of apoptosis family, has been implicated in the control of mitotic progression (42). It also directly binds to caspase-3 and -7, inhibiting their activities (43). Survivin is overexpressed in many glioma cells, and high expression levels of survivin are associated with poor prognosis (44). Survivin blocks apoptosis at the effector phase, a stage at which multiple signaling pathways converge, therefore, strategies that target survivin may be particularly effective for overcoming the resistance of glioma cells to various anticancer therapies. In our study, survivin overexpression in U87MG cells significantly inhibited capsaicin-stimulated TRAIL-induced apoptosis. In addition, siRNA-mediated suppression of survivin expression in U251MG cells markedly stimulated TRAIL-induced apoptosis. Furthermore, survivin downregulation by capsaicin seems to be more closely related to its TRAIL sensitization in Figure 1A than does its DR5 enhancement (Figures 1D, 2A and 4A), indicating that survivin downregulation plays a critical role in capsaicin-mediated sensitization of glioma cells to TRAIL-induced apoptosis. Recently, Bhutani *et al.* (45) showed that capsaicin downregulates survivin expression in multiple myeloma cells, possibly by blocking signal transducer and activator of transcription 3 activity. In their study, the expression of the signal transducer and activator of transcription 3-regulated gene products, Bcl-2, Bcl-xL, cyclin D1 and vascular endothelial growth factor were also downregulated by capsaicin. However, in our study, Bcl-2 and Bcl-xL protein levels in glioma cells were not affected by capsaicin. Furthermore, survivin mRNA levels were not affected by capsaicin but pretreatment with MG132 significantly inhibited capsaicin-induced survivin downregulation. These results indicate that capsaicin-induced downregulation of survivin may occur via proteasome-mediated degradation of survivin rather than through the transcriptional control. Akt is a critical factor in conferring cancer cells TRAIL resistance (46,47) and inhibition of Akt activity by kaempferol was shown to be important for the reduction of survivin protein levels, contributing to sensitization of glioma cells to TRAIL-induced apoptosis (48). However, in our study, the activity or expression level of Akt was not altered by capsaicin treatment, suggesting that Akt is not involved in this process. Survivin in interphase cells has been reported to be polyubiquitinated and degraded via the proteasome pathway (49), whereas mitotic phosphorylation of survivin on Thr34 by Cdc2 is associated with increased protein stability at metaphase (25). Previously, we reported that inhibition of Cdc2 activity by roscovitine treatment or expression of a dominant-negative Cdc2 mutant increased TRAIL-mediated apoptosis in glioma cells by downregulating survivin protein levels (50). In the present study, we found that capsaicin significantly downregulated levels of Cdc2, cyclin B and cyclin A, all of which are cell cycle regulators important for Cdc2 activity, in various glioma cells. An analysis of survivin protein levels in U87MG cells after overexpression of Cdc2, cyclin A and cyclin B, alone or in combination, demonstrated that increased cyclin B expression levels were critically involved in the increase in survivin protein levels, possibly through an associated increase in cyclin B-dependent Cdc2 activity. Presumably, capsaicin-induced inhibition of Cdc2, due to downregulation of Cdc2, cyclin A and cyclin B, may result in the dephosphorylation of survivin and subsequently lead to its proteasome-mediated destruction. Interestingly, the 32 kDa procaspase-3 was partially cleaved into a 20 kDa procaspase-3 in TRAIL-resistant glioma cells following TRAIL treatment, but further cleavage into the active p17 subunit did not occur. Increased expression levels of survivin in glioma cells may contribute to TRAIL resistance in these cells by blocking the completion of TRAIL-induced processing of

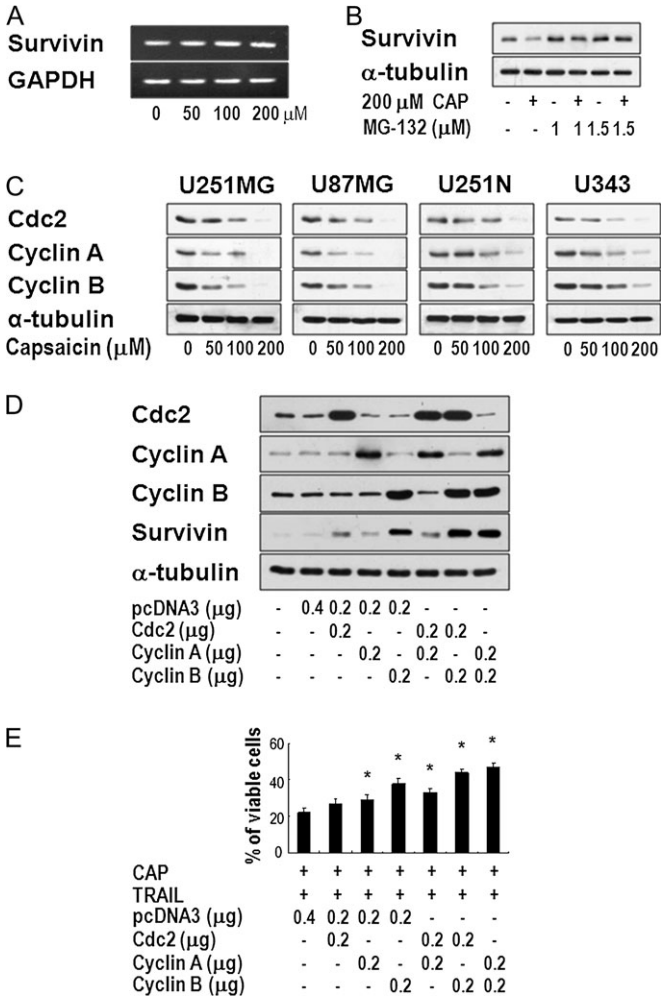


**Fig. 5.** Inhibition of Cdc2 activity may be responsible for capsaicin (CAP)-induced downregulation of survivin. (**A**) U251MG cells were treated with CAP for the indicated concentrations and total RNAs were prepared for reverse transcription–PCR of survivin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (**B**) U251MG cells were treated with 200 μM CAP with or without MG132 at the indicated concentrations for 16 h and the protein levels of survivin and α-tubulin were analyzed by western blotting. (**C**) Cells were treated with CAP at the indicated concentrations for 16 h, and western blotting of the indicated cell cycle regulators and α-tubulin was performed. (**D**) U87MG cells were transiently transfected with the plasmid encoding Cdc2, cyclin A and cyclin B alone or cotransfected with plasmids encoding Cdc2 and cyclin A, Cdc2 and cyclin B or cyclin A and cyclin B at the indicated concentrations. Expression of the transfected cyclin A, cyclin B or Cdc2 was confirmed by western blotting. α-Tubulin protein levels served as the control for protein loading. (**E**) U87MG cells were transiently transfected with the plasmid encoding Cdc2, cyclin A and cyclin B alone or cotransfected with plasmids encoding Cdc2 and cyclin A, Cdc2 and cyclin B or cyclin A and cyclin B at the indicated concentrations. Transfected cells were then treated with 200 μM CAP and 100 ng/ml TRAIL for 16 h. Cellular viability was measured using calcein-AM and EthD-1. Columns indicate average of three individual experiments; bars represents ± SD; $^{*}P < 0.001$, compared with the cells transfected with pcDNA3.

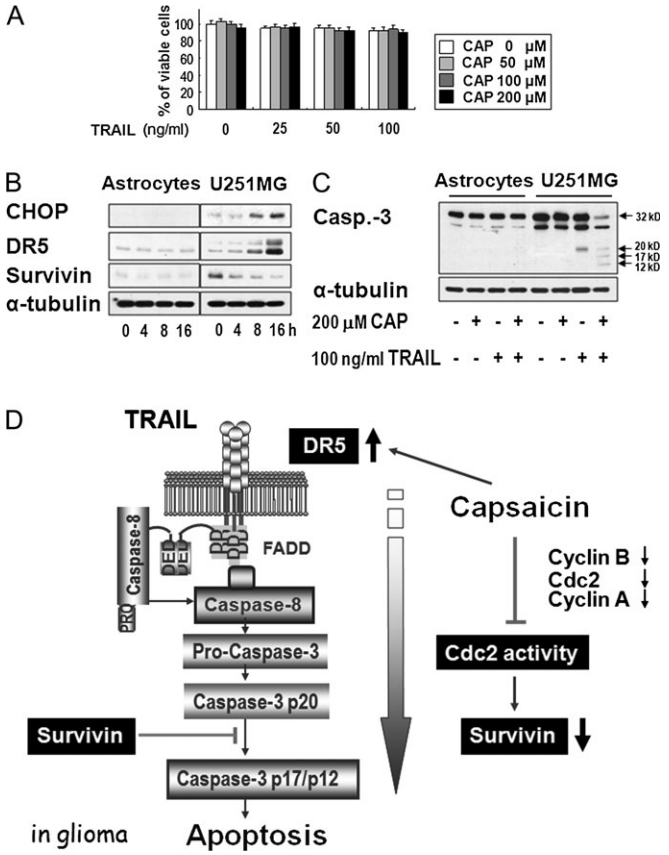


**Fig. 6.** Astrocytes are resistant to the combined treatment with capsaicin (CAP) and TRAIL. (**A**) Human astrocytes were treated with or without CAP for 30 min and further treated with TRAIL for 16 h at the indicated concentrations. Cellular viability was assessed using calcein-AM and EthD-1. (**B**) Following treatment of astrocytes or U251MG cells with 200 μM CAP for the indicated times, western blotting of DR5, survivin and α-tubulin was done. (**C**) Astrocytes or U251MG cells were treated with 200 μM CAP alone, 100 ng/ml TRAIL alone or a combination with CAP and TRAIL for 16 h. Western blotting of caspase-3 and α-tubulin was performed. (**D**) Schematic diagram of the apoptotic pathway induced by the combined treatment with CAP and TRAIL.

procaspase-3 into the active p17 subunit. Therefore, capsaicin-induced survivin downregulation ultimately contributes to the recovery of TRAIL-sensitivity in glioma cells, leading to removal of the caspase-activation barrier.

Interestingly, we found that normal astrocytes were protected from the apoptotic effects of combined capsaicin and TRAIL treatment. Neither capsaicin-induced DR5 upregulation/survivin downregulation nor TRAIL-mediated priming of procaspase-3 processing occurred in astrocytes. Therefore, the combined regimen of capsaicin and TRAIL may act through activation of glioma-selective pro-apoptotic signals to provide a safe and effective strategy for treating resistant glioma cells.

## Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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