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Chemical Therapeutics

Sensitization of TRAIL-Induced Cell Death by 20(S)-Ginsenoside Rg3 via CHOP-Mediated DR5 Upregulation in Human Hepatocellular Carcinoma Cells

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Abstract

The TRAIL pathway is a potential therapeutic target for anticancer drugs due to selective cytotoxicity in cancer cells. Despite considerable promise, TRAIL or TRAIL receptor agonists have been used thus far with limited success in multiple clinical trials, in part due to acquired TRAIL resistance during chemotherapeutic treatment. Hepatocellular carcinoma (HCC) is a common solid tumor and the third leading cause of cancer-related death worldwide. Classical chemotherapy is not effective for HCC treatment and targeted therapy is limited to sorafenib. Isolated from *Panax ginseng* CA Meyer, 20(S)-ginsenoside Rg3 is a steroidal saponin with high pharmacologic activity that has been shown to sensitize cells to some chemotherapeutic agents. We investigated the sensitizing effect of Rg3 on TRAIL-induced cell death in HCC cells. We show Rg3 is capable of promoting TRAIL-induced apoptosis in a number of HCC cell lines, including HepG2, SK-Hep1, Huh-7, and Hep3B, but not in normal HL-7702 hepatocytes, indicating that Rg3 sensitization to TRAIL may be specific to cancer cells. Mechanistically, we found that Rg3 upregulates DR5 expression at the transcriptional level. DR5 upregulation in this case is mediated by C/EBP homology protein (CHOP), an important endoplasmic reticulum stress responsive protein. Furthermore, Rg3 is well tolerated and enhances the therapeutic efficacy of TRAIL in mouse xenograft models, suggesting that chemosensitization also occurs *in vivo*. Taken together, our study identifies Rg3 as a novel anticancer therapeutic agent and supports the further development of Rg3 as a chemosensitizer in combined therapy with TRAIL. *Mol Cancer Ther; 12(3); 274–85. ©2012 AACR.*

Introduction

TRAIL, a member of the TNF family of ligands, is an attractive anticancer agent due to its ability to selectively induce apoptosis in tumor cells but not in normal cells (1–3). Cytotoxicity of TRAIL is mediated by DR4 and DR5 receptors and the formation of the downstream death-inducing signaling complex, which activates apical caspases 8/10 and leads to apoptosis (4, 5). Chemotherapeutic application of TRAIL is hindered by acquired resistance to TRAIL-induced cell death (6–8), conferred by several molecular mechanisms, such as lower expression of DR4 and DR5 and/or higher expression of antiapoptotic molecules, such as antiapoptotic Bcl2 family members (Mcl-1, Bcl-2, and Bcl-xL), c-FLIP, or IAPs, or the homeoprotein Six1 (8–12). Identification of sensitizing agents capable of overcoming resistance may therefore facilitate TRAIL-mediated therapy (13–15).

Hepatocellular carcinoma (HCC) is the sixth most common solid tumor and the third leading cause of cancer-related death worldwide. Classical chemotherapy is not currently effective for HCC treatment, whereas targeted therapy is limited to sorafenib (16, 17). Current therapeutic strategies for HCC, such as surgical resection, percutaneous ethanol injection, arterial embolization, interventional chemotherapy, and radiofrequency ablation, are complicated by recurrence and metastasis following intervention (18). Overall recurrence of HCC can exceed 70% (16, 19), with a 5-year survival rate of stage-II disease of about 50% (18), underscoring the need for novel therapeutic strategies including chemoembolization and neoadjuvant therapy before and after surgery, respectively (18). Some cancer cells, including HCC cells, are resistant to TRAIL (20). Identification of sensitizing agents capable of improving TRAIL sensitivity may permit TRAIL-mediated therapy.

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Isolated from *Panax ginseng* CA Meyer, 20(S)-ginsenoside Rg$_3$ is a steroidal saponin with high pharmacologic activity. Rg$_3$ has antihypertensive and cardio-protective effects (21) and modulates the function of the central and peripheral nervous systems, as well as the immune system (22, 23). Rg$_3$ may increase the efficacies of cancer chemotherapy, possibly through inhibitory effects on NF-κB and AP-1 (24).

In this study, we found that Rg$_3$ renders HCC cells more susceptible to TRAIL-induced apoptosis through upregulation of DR5. This upregulation is mediated through upregulation of C/EBP homology protein (CHOP), which acts on the DR5 promoter. This regulation occurs in cancer cells but not in normal cells, allowing for cancer-specific toxicity. Rg$_3$, in combination with TRAIL, inhibits tumor growth in mouse xenografts, suggesting that Rg$_3$ may be a sensitizing agent capable of improving sensitivity or overcoming TRAIL resistance and may facilitate the establishment of TRAIL-mediated combination treatment of HCC.

Materials and Methods

Reagents

Recombinant human TRAIL and anti-DR5 antibody were from Komabiotech. Anticaspase-3, anti-PARP, anti-phospho-eIF2α, and anti-CHOP antibodies were from Cell Signaling. Anti-DR4 antibody was from Rockland. Antitubulin antibody was from Abcam and anti-GRP78 antibody was from Stressgen. zVAD was from R&D Systems. Antiactin antibody, thapsigargin, necrostatin-1, *N*-acetylcysteine (NAC), and butylated hydroxyanisole (BHA) were from Sigma. Cycloheximide and actinomycin D were from Calbiochem. Glutathione *S*-transferase (GST)-TRAIL was described previously (13).

Rg$_3$ isolation

A total of 1.6 kg of Sun Ginseng (heat-treated ginseng) was extracted with 70% MeOH (1.2 L) under reflux for 3 hours. The solvent was removed *in vacuo* to yield 320 g of 70% MeOH extract, which was suspended in water and extracted with n-BuOH. The n-BuOH fraction was concentrated *in vacuo* to yield 91.5 g of BuOH fraction. Forty grams of the fractions were subjected to silica gel column chromatography. Nine fractions were obtained using stepwise gradient elution (EtOAc:MeOH:H$_2$O = 40:1:1 → 20:1:1 → 10:1:1) and fraction 8 was chromatographed over silica gel using CHCl$_3$:MeOH:H$_2$O mobile phase (200:20:1 → 150:20:1). The Rg$_3$-rich fraction that contained the 20(S) and 20(R) forms was obtained and further purified over semipreparative liquid chromatography/evaporative light scattering detector (LC/ELSD) analysis using a reverse-phase column (Phenomenex C18, 250 mm × 10 mm) with 40% acetonitrile to isolate the 20(S) form (10 mg).

Cell culture

SK-Hep1, HepG2, Hep3B, HT-29, and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium with 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Huh-7 cells were cultured in RPMI-1640 with 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The normal liver cell line, HL-7702, was purchased from Shanghai Institute of Cell Biology (Shanghai, China) and cultured in RPMI-1640 with 20% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. No further authentication of cell lines was done by the authors.

Western blot analysis

Cells were lysed in M2 buffer [20 mmol/L Tris at pH 7, 0.5% NP-40, 250 mmol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L dithiothreitol (DTT), 0.5 mmol/L phenylmethylsulfonylfluoride (PMSF), 20 mmol/L β-glycerol phosphate, 1 mmol/L sodium vanadate, 1 μg/mL leupeptin]. Equal amounts of cell extracts were resolved by 10% or 12% SDS-PAGE, analyzed by immunoblotting and visualized by enhanced chemiluminescence (ECL, Amersham).

Cytotoxicity assay

Cell viability was determined using tetrazolium colorimetric tests (MTT test), with absorbance reading at 570 nm. Representative images were taken by phase contrast microscopy. Presented data were from representative experiments of at least 3 independent assays. Cell death was measured by FITC-Annexin V Apoptosis Detection Kit (BD Phamingen), or cell viability was assessed by double labeling of cells with 2 μmol/L calcein-AM and 4 μmol/L EthD-1. Calcein-positive live cells and EthD-1–positive dead cells were visualized using fluorescence microscopy (Axiovert 200M, Zeiss).

Reverse transcription-PCR

RNA was extracted using RNeasy (Qiagen). One microgram of total RNA from each sample was used for cDNA synthesis with reverse transcriptase (Invitrogen). Equal amounts of cDNA product were used in PCR conducted using the Taq DNA polymerase (Takara). PCR amplification was conducted using the following primers:

DR5 sense (5′-AAGACCCTTGTGCTCGTTGTC-3′),
DR5 antisense (5′-GACACATTCGATGTCACTCCA-3′),
β-actin sense (5′-CAGGTCATCACCATTGGCAATGAGC-3′),
β-actin antisense (5′-GATGTCCACGTCACACTTCATGA-3′).

The final PCR products were resolved in 1.5% agarose gel and stained with ethidium bromide.

Measurement of reactive oxygen species

Intracellular reactive oxygen species (ROS) was detected by treating cells with 20 μmol/L H$_2$DCFDA before the end of the indicated treatments (30 minutes) and fluorescence increase was measured by fluorescence-activated cell sorting (FACS).

Transfection

Transfection of HCC cells was conducted with Lipo-fectamine PLUS reagent by following manufacturer's instructions (GIBCO/BRL). Cells were transfected with pDR5-WT and pDR5-mCHOP mutant constructs provided by Dr. T Yoshimori (Osaka University, Osaka, Japan) and Dr. Choi (Ajou University, Suwon, Republic of Korea; ref. 25).

Luciferase assay

The pDR5-WT [containing DR5 promoter sequence ($-605/+3$)] and pDR5-mCHOP (containing point mutation of the CHOP-binding site to the DR5/-605) were transfected into HepG2 cells. After 24 hours, transfected cells were treated with or without Rg_3 for 8 hours, and cell lysates were analyzed for luciferase activity following the maunfacturer's protocol (Promega).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay (Milli-pore) was conducted following the manufacturer's direction with CHOP antibody (Cell Signaling) and control mouse immunoglobulin G (IgG; Santa Cruz). The primers 5′-CCC AAG TGC CTC CCT CAA C-3′ (forward) and 5′-CCA GGC TGA CTT GGG GCG-3′ (reverse) corresponding to a 300-bp fragment of the DR5 promoter were used to PCR amplify immunoprecipitated chromatin.

Lentiviral short hairpin RNA experiments

MISSION short hairpin RNA (shRNA) plasmids targeting the coding region or 3′ untranslated region (UTR) of CHOP mRNA (NM_004083), and nontargeting control sequences (NC: SHC002) were from Sigma-Aldrich. Lentiviral plasmids were transfected into 293TN cells (System Biosciences, LV900A-1) using Lipofectamine 2000 (Invitrogen, 11668019). Pseudoviral particles were collected 2 days after the transfection of plasmids, and infected into HepG2 cells in the presence of polybrene (8 μg/mL). Infected HepG2 cells were selected with puromycin (1 μg/mL) starting 2 days after infection, and CHOP knockdown was confirmed by immunoblotting. CHOP knockdown cells were treated with Rg_3 or thapsigargin for indicated time points and cells lysates were analyzed by Western blot analysis.

Tumor xenograft study

Male nude mice were obtained from Central Lab. Animal Inc., were fed standard rat chow and tap water *ad libitum*, and maintained under 12-hour dark/light cycle at 21°C. Male, 6-week-old nude mice were randomly divided into 4 groups (control, Rg_3, TRAIL, and Rg_3 + TRAIL, $n = 8$/group). Huh-7 cells were mixed with PBS (200 μL/ mouse) and inoculated into 1 flank of each nude mouse (5×10^6 Huh-7 cells). When the tumors had reached a volume of about 50 to 70 mm³, mice were given a daily oral dose of 20 mg/kg Rg_3 or the vehicle (200 μL PBS, control group), and intraperitoneally 3 times per week at dose of 3 mg/kg TRAIL, for 21 days, respectively. Tumor dimen-sions were measured twice a week using a digital caliper and tumor volume was calculated using the formula: $V = $ length $\times$ width$^2 \times 0.5$. The weight of the mice was also measured twice a week as a general measurement of health. At the end of the experiment, the mice were killed and the tumors were excised and weighed. Histopathologic analysis of tumors was conducted by using hematoxylin and eosin (H&E) staining.

Immunohistochemistry and TUNEL staining

Immunostaining was conducted on 8-μm thick sections after deparaffinization. Microwave antigen retrieval was conducted in citrate buffer (pH 6.0) for 10 minutes before peroxidase quenching with 3% H_2O_2 in PBS for 10 minutes. Sections were then washed in water, preblocked with normal goat or horse serum for 10 minutes, and were incubated overnight at 4°C in 1:50 dilutions of antic-aspase-3 (cleaved) antibody. Then sections were incubated with biotinylated secondary antibodies (1:200) for 1 hour. Following a wash with PBS, streptodavidin–horse-radish peroxidase was applied. Sections were developed with diaminobenzidine tetrahydrochloride substrate for 10 minutes, and counterstained with hematoxylin. At least 5 random fields of each section were examined at a magnification of $\times400$ and analyzed by a computer image analysis system, Metaview (Media Cybernetics). The density values represent as mean $\pm$ SD of percentage threshold area. TUNEL was conducted following the manufacturer's protocol (Chemicon).

Statistical analysis

Statistical analysis was conducted using ANOVA and an unpaired 2-tailed Student *t* test. Statistical calculations were conducted using SPSS software for Windows Version 10.0 (SPSS).

Results

Rg_3 sensitizes to TRAIL–induced cell death in human hepatocellular carcinoma cells

Earlier studies suggested that Rg_3 might have antitumor functions, including inhibition of tumor cell proliferation, invasion, and metastasis, as well as induction of differentiation and apoptosis (26, 27). In HepG2 cells, Rg_3 alone at concentrations up to 100 μmol/L did not induce morphologic signs of cell death (Supplementary Fig. S1A), and cell viability was not substantially decreased as measured by MTT assay (Fig. 1A, left). Propidium iodide (PI)– Annexin V staining and Western blotting of caspase-3/ PARP cleavage confirmed that Rg_3 had limited, if any, cytotoxicity in these cells (Supplementary Fig. S1B and S1C). Concentrations of TRAIL more than 25 ng/mL were required for substantial (>20%) cytotoxicity (Fig. 1A, right). However, when the cells were pretreated with Rg_3 for 30 minutes followed by low concentrations of TRAIL (12.5 or 25 ng/mL) for 16 to 18 hours, cells underwent dramatic cell death as evidenced by MTT assay and cell morphology (Fig. 1B). Viability measurements from 2-fold dilution dose

Figure 1. Rg_3 sensitizes cancer cells to TRAIL. A, HepG2 cells were treated as indicated with Rg_3 (left) or TRAIL (right) for 16 hours and viability was analyzed by MTT assay. B, HepG2 cells were pretreated with 100 μmol/L Rg_3 for 30 minutes followed by treatment with TRAIL (12.5 ng/mL and 25 ng/mL) for 16 to 18 hours and viability was analyzed by MTT assay (left). Representative phase contrast microscopy images are shown (right). C, SK-Hep1 (HCC), HeLa (cervical cancer cells), and HT-29 (colon cancer cells) were treated with Rg_3 (100 μmol/L) plus TRAIL (25 ng/mL) and viability analyzed by MTT assay. D, human normal liver cells (HL-7702) were treated with Rg_3 (100 μmol/L), TRAIL (25 ng/mL), or Rg_3 plus TRAIL for 16 to 18 hours and cell viability was analyzed by MTT assay. All results shown are averages ± SD (##, $P < 0.1$; #, $P < 0.5$; *, $P < 0.01$; **, $P < 0.001$).
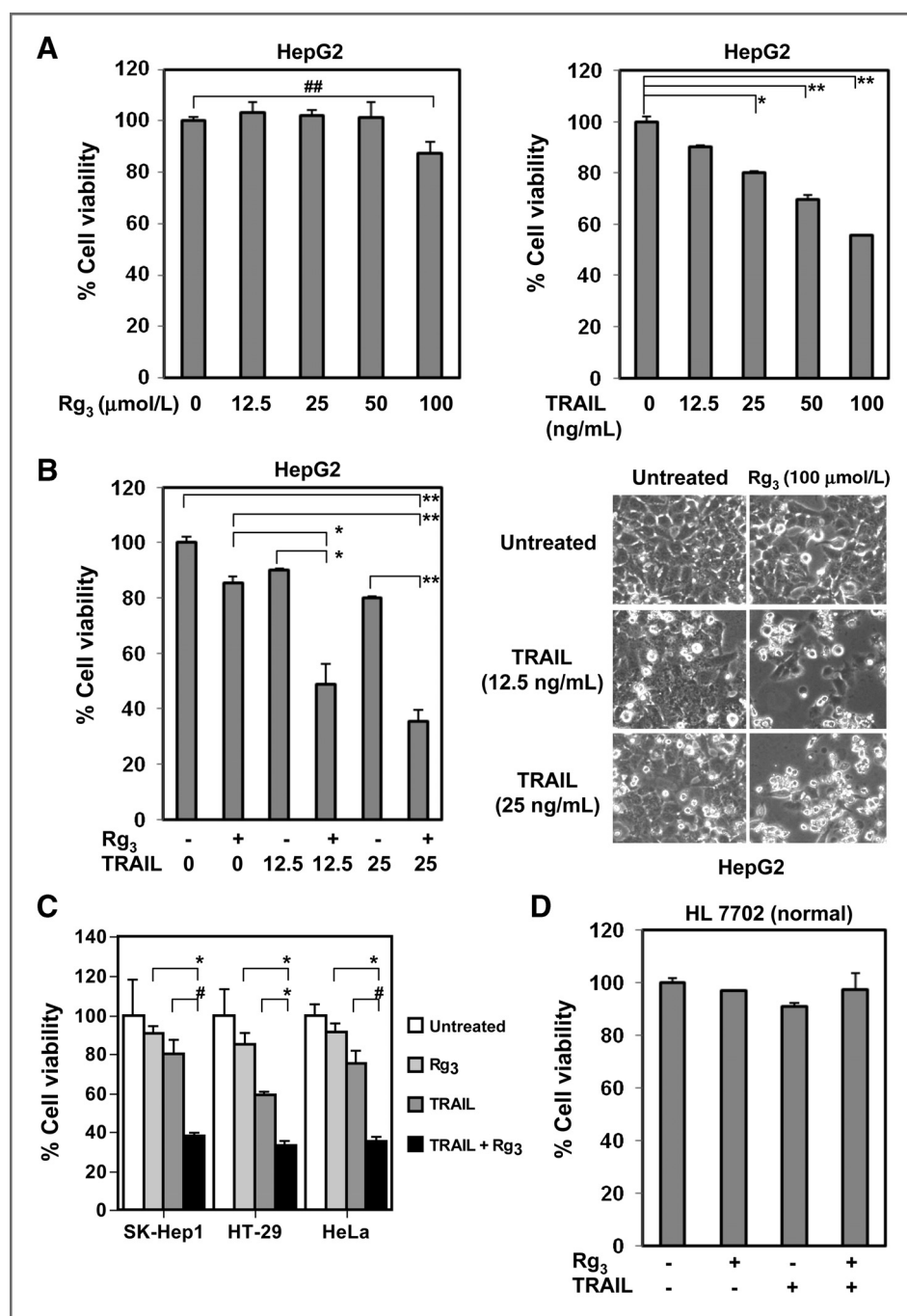
curves showed that 100 μmol/L Rg_3 decreased the IC_{50} of TRAIL approximately 8-fold (from more than 100 ng/mL to about 12.5 ng/mL), whereas lower doses of Rg_3 had much less affect on the slope of the dose curve (Supplementary Fig. S2A). This concentration of Rg_3 (100 μmol/L) was necessary to achieve robust TRAIL-mediated PARP cleavage at 12 hours (Supplementary Fig. S2B), but had little effect on its own upon PARP cleavage or cell viability (Supplementary Fig. S2B and S2C). Similar sensitization was observed in HeLa cervical cancer cells, HT-29 colon

cancer cells, and another HCC cell type, SK-Hep1, indicating that this effect was not confined to HepG2 cells (Fig. 1C). However, human normal liver cells (HL-7702) were not sensitive to Rg_3 and TRAIL, indicating sensitization could be limited to cancer cells (Fig. 1D).

Rg_3 promotes TRAIL-induced caspase-dependent apoptotic cell death

TRAIL-induced apoptosis is executed by the extrinsic cell death pathway, with caspase-3 as executioner caspase

(28). Rg_3 alone did not affect caspase-3 cleavage in HepG2 cells, but Rg_3 pretreatment significantly augmented TRAIL-induced cleavage/activation of caspase-3, as well as PARP cleavage (Fig. 2A). Kinetic analysis showed that Rg_3 promotes TRAIL-induced caspase activity, with PARP cleavage occurring about 4 hours after treatment (data not shown). Three other HCC cell lines, SK-Hep1, Huh-7, and Hep3B were also used to look at Rg_3 sensitization of TRAIL toxicity. Rg_3 had a similar effect on caspase-3 and PARP cleavage in these cancer cells, with Huh-7 cells being especially affected (Fig. 2B). Taken

together, our data suggest that Rg_3 can sensitize TRAIL-induced cell death in different types of HCC cells via promotion of TRAIL-induced caspase activity.

Caspase activity was required for cell death induced by Rg_3 and TRAIL as the pancaspase inhibitor, Z-VAD-FMK, inhibited caspase-3, PARP cleavage, and cell death (Fig. 2C). Necrostatin-1, an inhibitor of programmed necrosis, was unable to prevent cell death induced by the combined treatment of Rg_3 and TRAIL in HepG2 cells (Fig. 2C, right), indicating that the death was apoptotic and not necrotic. TRAIL-induced ROS have been shown to potentiate the
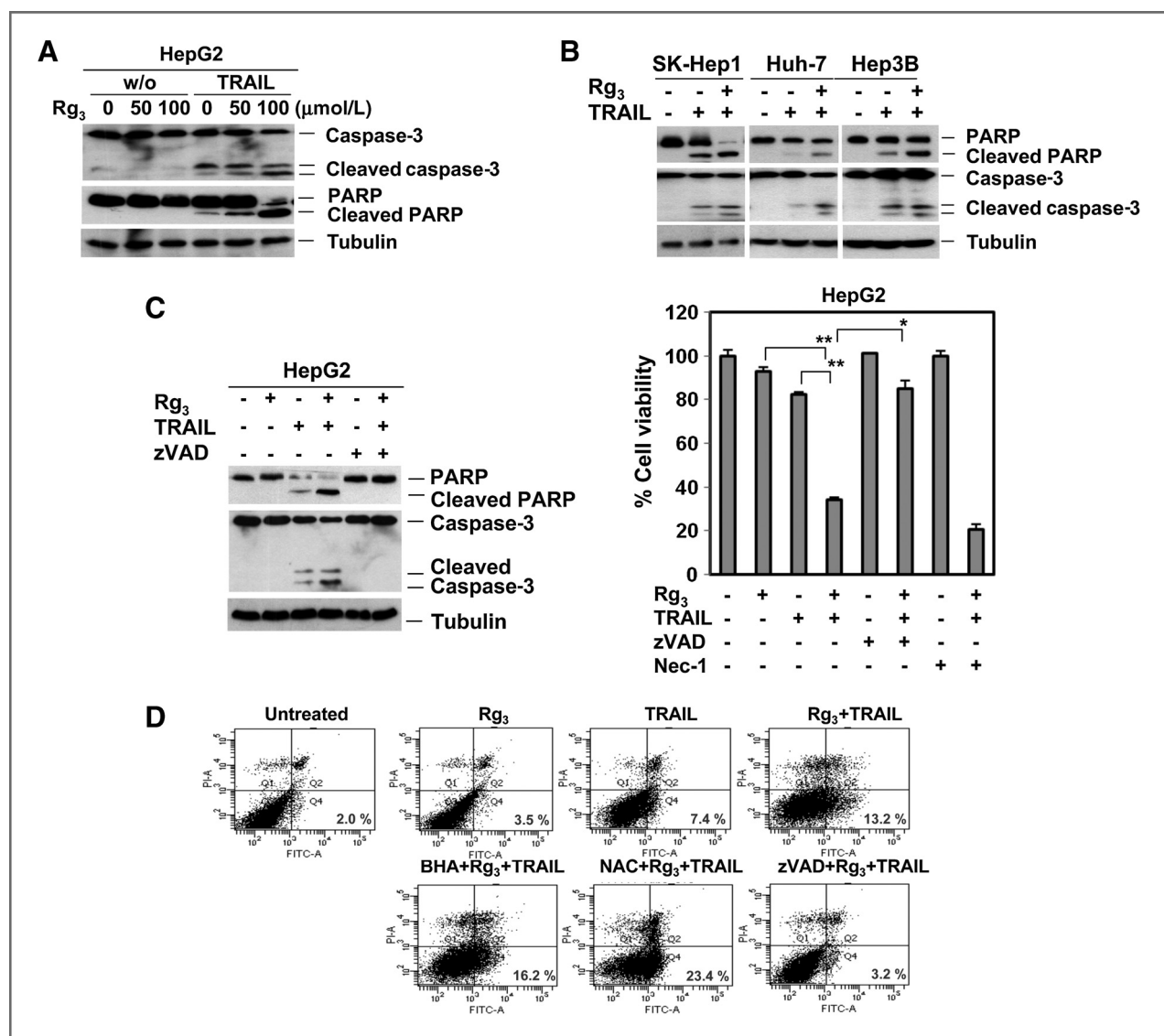

Figure 2. Rg_3 enhances the caspase activity triggered by TRAIL. A, Western blotting of lysates from HepG2 cells pretreated with Rg_3 (50 and 100 μmol/L) for 30 minutes followed by treatment with TRAIL (25 ng/mL) for 12 hours. B, immunoblotting of lysates from SK-Hep1, Huh-7, and Hep3B cells treated with Rg_3 (100 μmol/L) plus TRAIL (25 ng/mL) for 12 hours. C, HepG2 cells were pretreated with zVAD (20 μmol/L) or Nec-1 (40 μmol/L) for 30 minutes followed by treatment with Rg_3 (100 μmol/L) plus TRAIL (25 ng/mL) for 12 hours (left) or 18 hours (right) and subjected to lysis and Western blotting (left) or MTT viability assay (right). D, cells were pretreated with NAC (1 mmol/L) or BHA (100 μmol/L) for 30 minutes followed by treatment with Rg_3 (100 μmol/L) plus TRAIL (25 ng/mL) for 9 hours and cell viability was analyzed by FACS using Annexin–PI staining. All results shown are averages ± SD (*, $P < 0.01$; **, $P < 0.001$). FITC, fluorescein isothiocyanate.

activation of caspases and apoptotic cell death in HeLa cells (29). Either Rg$_3$ or TRAIL alone induced ROS levels, whereas intracellular ROS were further enhanced by the Rg$_3$ and TRAIL combination (data not shown). However, although the antioxidants BHA and NAC efficiently inhibited ROS generation (data not shown), they were unable to suppress apoptotic cell death induced by Rg$_3$ plus TRAIL (Fig. 2D), suggesting that ROS are not required for Rg$_3$ sensitization to TRAIL.

Rg$_3$ sensitizes TRAIL-induced apoptosis via DR5 upregulation

Decreased expression of TRAIL receptors DR4 and DR5 and/or upregulation of the decoy receptors DcR1 and DcR2 account for TRAIL resistance in certain cancer cell lines (30). Rg$_3$ markedly induced the expression of DR5 in both HepG2 and SK-Hep1 cells from 4 hour onwards (Fig. 3A), whereas DR4 expression did not change much (Fig. 3B). Consistent with the protein changes, increases in DR5 mRNA were observed from 4 hours (Fig. 3C). Actinomycin D, a *de novo* mRNA synthesis inhibitor, abolished Rg$_3$-induced DR5 mRNA induction (data not shown) and either actinomycin D or the protein synthesis inhibitor cycloheximide inhibited Rg$_3$-initiated increases in DR5 protein (Fig. 3D). Rg$_3$ failed to further sensitize cells

Figure 3. Rg$_3$ upregulates DR5 transcription. A and B, immunoblotting of lysates from HepG2 and SK-Hep1 cells treated with Rg$_3$ (100 μmol/L) for indicated time periods. C, ethidium bromide agarose gels of the product of reverse transcription-PCR showing the effect of Rg$_3$ on DR5 mRNA level in HepG2 cells treated with Rg$_3$ (100 μmol/L) for indicated time periods. D, immunoblotting of HepG2 cells treated with Rg$_3$ (100 μmol/L), cycloheximide (CHX, 10 μg/mL), actinomycin D (ActD, 1 μg/mL), Rg$_3$ + CHX, or Rg$_3$ + ActD for 12 hours.

treated with TRAIL in the presence of cycloheximide (data not shown). This suggests that the Rg$_3$-induced sensitization of TRAIL-induced apoptosis may be mediated by upregulation of DR5 solely at the transcriptional level.

Rg$_3$-induced DR5 upregulation is mediated through induction of CHOP

CHOP/GADD153 contributes to MG132- and tunicamycin-mediated upregulation of DR5, leading to sensitization of TRAIL-mediated cell death (31, 32). Rg$_3$ treatment of HepG2 cells increased the amount of CHOP protein in both a dose- and time-dependent manner (Fig. 4A). Rg$_3$ treatment led to increased CHOP protein both in HepG2 and SK-Hep1 cells, roughly correlating with increases in DR5 expression (Fig. 4B). Analysis of short-term temporal expression patterns indicated that increases in CHOP protein were detected before DR5 increases, supporting a possible role for CHOP in Rg$_3$-mediated DR5 upregulation (Fig. 4C). TRAIL sensitization was more efficient when cells were pretreated with Rg$_3$ than if Rg$_3$ was administered after TRAIL (as measured by PARP cleavage and cell viability, Supplementary Fig. S3A–S3C). This suggests transcription of the DR5 mRNA is required before sensitization occurs.

When HepG2 cells were transfected with DR5 promoter luciferase reporter constructs (Fig. 4D, top left), Rg$_3$ markedly increased the activity of the wild-type promoter but had no effect on a promoter with a mutation in the potential CHOP binding site (−281 to −261) as measured by luciferase activity (Fig. 4D, top right), indicating that the CHOP binding site is required for Rg$_3$ transactivation. ChIP verified that CHOP bound to the endogenous DR5 promoter in HepG2 cells upon Rg$_3$ treatment (Fig. 4D, bottom). Rg$_3$ therefore induces CHOP-dependent DR5 expression through a direct effect on DR5 transcription.

We investigated whether CHOP upregulation contributes to Rg$_3$-induced sensitization of TRAIL-mediated apoptosis. Because thapsigargin is a well-established CHOP inducer (33), we compared CHOP expression levels upon thapsigargin and Rg$_3$ treatment in 3 different HCC cell lines. Rg$_3$ and thapsigargin both enhanced CHOP expression levels (Fig. 5A). Because thapsigargin treatment leads to CHOP upregulation such as Rg$_3$, we would predict that thapsigargin would also sensitize to TRAIL-induced cell death in a similar manner as Rg$_3$. Indeed, upregulation of CHOP by thapsigargin sensitized to TRAIL-induced cell death to a similar extent as Rg$_3$ (Supplementary Fig. S4A). Although both Rg$_3$ and thapsigargin caused CHOP upregulation, only thapsigargin caused upregulation of endoplasmic reticulum stress markers, such as p-eIF2α and GRP78 (Fig. 5B). CHOP knockdown did not affect thapsigargin-induced upregulation of these markers (Fig. 5B). Conversely, Rg$_3$-induced DR5 upregulation was inhibited by CHOP knockdown, indicating that Rg$_3$-induced DR5 upregulation requires CHOP (Fig. 5C, left). CHOP knockdown effectively suppressed cell death induced by Rg$_3$ and TRAIL in HepG2 cells, indicating that CHOP is essential for Rg$_3$-triggered
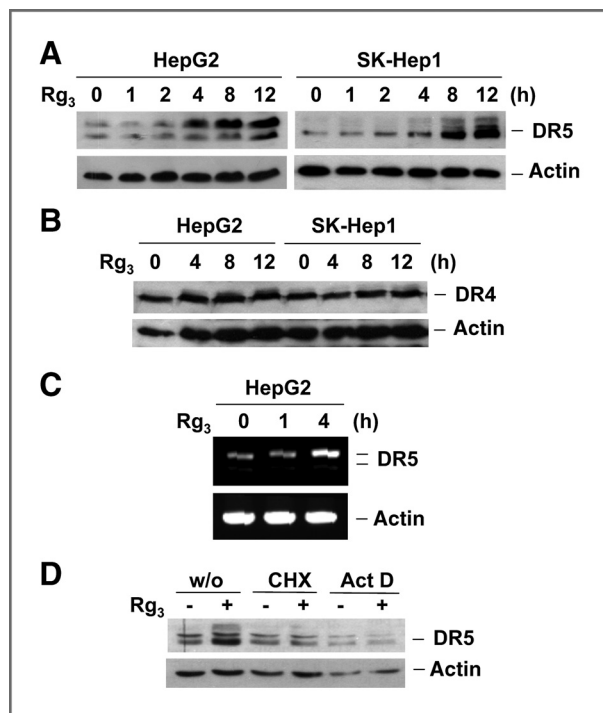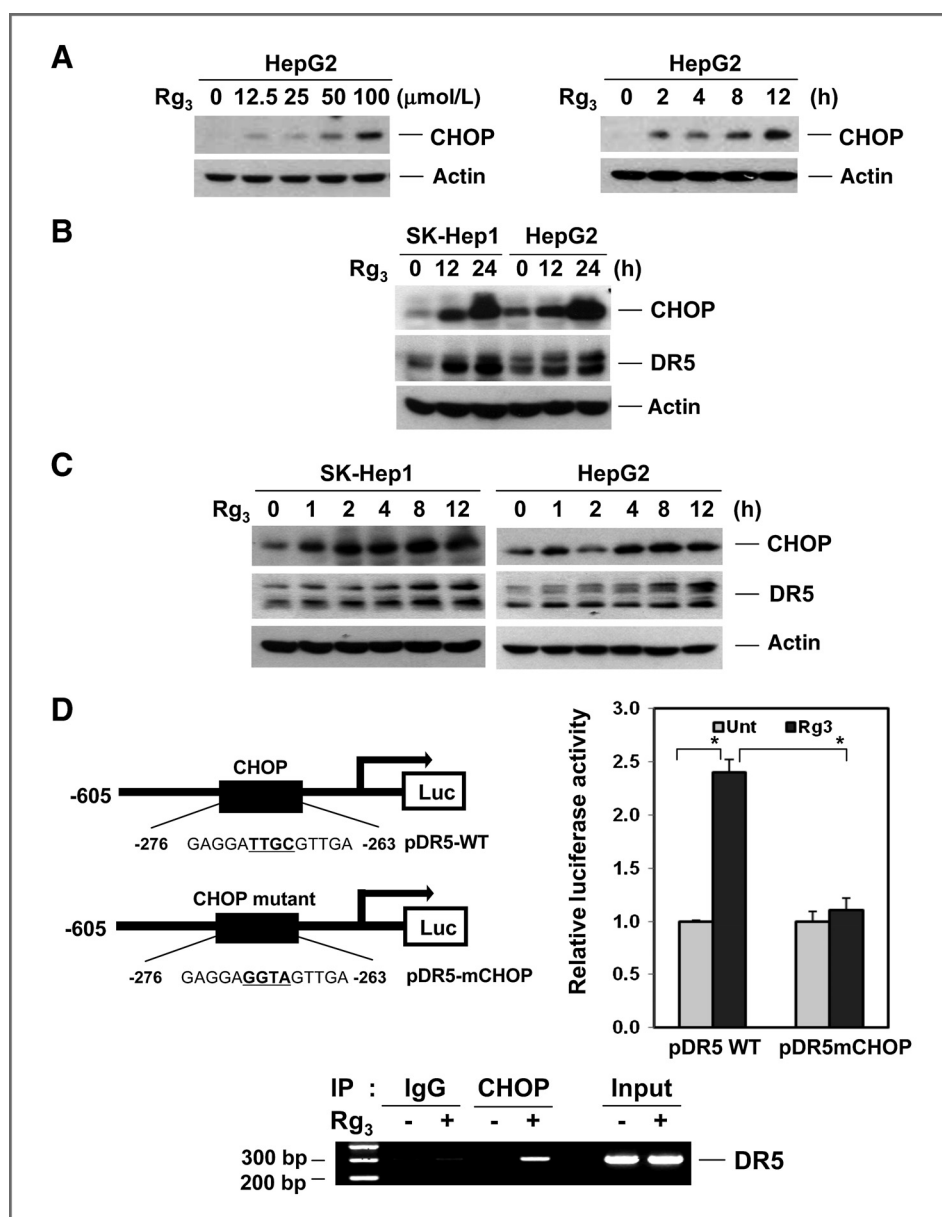
Figure 4. Rg$_3$-induced upregulation of CHOP is involved in increased DR5 expression. A, Western blotting of HepG2 lysates from cells treated with different concentrations of Rg$_3$ for 12 hours (left) or with 100 μmol/L Rg$_3$ for different time periods (right). B and C, immunoblotting of SK-Hep1 and HepG2 cells treated with Rg$_3$ (100 μmol/L) for indicated time points. D, schematic diagram of the DR5 promoter constructs used for the luciferase activity assay (top left). HepG2 cells were transfected with pDR5-605-WT or pDR5-605-mCHOP promoter constructs and β-gal plasmid and then treated with Rg$_3$ (100 μmol/L) for 8 hours and cells were lysed for luciferase assay (top right). ChIP from HepG2 cell lysates treated with Rg3 (100 μmol/L) for 8 hours using an anti-CHOP antibody or mouse IgG isotype control followed by PCR amplification of a 300-bp fragment of the DR5 promoter containing the CHOP-binding site (bottom). All results shown are averages ± SD ($*$, $P < 0.01$).

enhancement of TRAIL-induced apoptosis (Fig. 5C, right; Supplementary Fig. S4B). Thapsigargin-induced sensitization of TRAIL, however, was minimally affected (Supplementary Fig. S4B), suggesting that thapsigargin activates other pathways that also sensitize to TRAIL.

Interestingly, Rg$_3$ treatment did not induce CHOP expression in the normal liver cell line, HL7702 (Fig. 5D, left). This potentially explains why it is unable to sensitize normal cells to TRAIL (Figs. 1D and 5D, right). HL7702 cells were capable of dying a caspase-dependent death in response to TRAIL when sensitized by cycloheximide (Supplementary Fig. S5A and S5B), and Western blot analyses indicated that the normal cells did not have higher expression of apoptotic inhibitors, such as cFLIP, cIAPS, or Bcl-2 (Supplementary Fig. S6), indicating that

the normal cells do not lack cellular machinery necessary to undergo TRAIL-mediated cell death and caspase activation. In addition, thapsigargin was able to induce CHOP expression in normal cells, and to confer some sensitization of the normal cells to TRAIL (Fig. 5D). Taken together, Rg$_3$-induced upregulation of CHOP plays an essential role in Rg$_3$-induced sensitization of cancer cells to TRAIL-mediated apoptosis.

Combination of Rg$_3$ with TRAIL potentiates *in vivo* antihepatocellular carcinoma activity

Huh-7 cells were inoculated into the flanks of nude mice, and when tumors were measurable, mice were matched for tumor volumes and assigned to Rg$_3$, TRAIL, or combination of Rg$_3$ and TRAIL. Tumor volumes in Rg$_3$,

Figure 5. Inhibition of CHOP expression by shRNA inhibits TRAIL sensitization by Rg$_3$. A, immunoblotting of HCC lysates treated with Rg$_3$ (100 μmol/L) or thapsigargin (Tg, 1 μg/mL) for indicated times. B, immunoblotting of lysates from HepG2 cells stably expressing CHOP shRNA or nonsilencing hairpin showing expression of CHOP and endoplasmic reticulum stress markers upon treatment with Rg$_3$ (100 μmol/L) or thapsigargin (1 μg/mL) for 12 hours. C, cells from B were treated with Rg$_3$ (100 μmol/L) for 12 hours and cell lysates were analyzed by Western blotting (left) or were treated with Rg$_3$ (100 μmol/L), TRAIL (25 ng/mL), or Rg$_3$ plus TRAIL for 16 to 18 hours and viability was analyzed by MTT assay (right). D, Western blot analysis of lysates from HepG2 and normal liver cells (HL7702) treated with Rg$_3$ (100 μmol/L) or thapsigargin (1 μg/mL) for 12 hours (left). MTT assay of HL7702 cells treated with Rg$_3$ (100 μmol/L), TRAIL (25 ng/mL), Rg$_3$ plus TRAIL, thapsigargin (1 μg/mL), or thapsigargin plus TRAIL for 16 to 18 hours (right). All viability results are averages ± SD (§, $P > 0.1$; #, $P < 0.05$, *, $P < 0.01$).
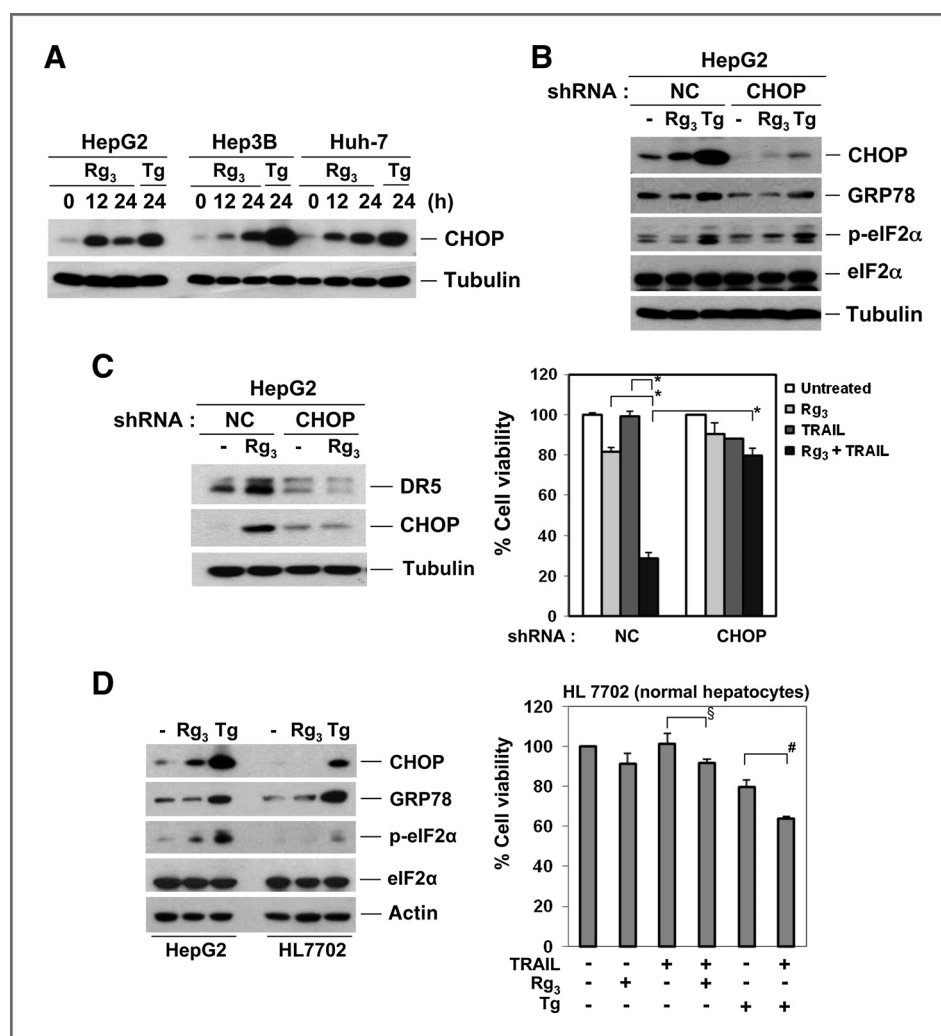
TRAIL, and Rg$_3$–TRAIL combination groups were about 75%, 63%, and 42%, respectively, when compared with control (Fig. 6A). The combination of Rg$_3$ and TRAIL suppressed tumor growth not only when compared with the control group, but also when compared with the Rg$_3$ or TRAIL alone groups. Importantly, no substantial weight loss was observed in the mice in any treatment group during the period of therapy (Fig. 6B), indicating that Rg$_3$ is generally well tolerated *in vivo*.

We next investigated the effect of the treatments on apoptosis *in vivo* by examining H&E staining, as well as TUNEL and cleaved caspase-3 staining of paraffin-embedded sections of the xenografted tumors. As shown in Fig. 6C (top), there was a greater degree of apoptosis in the Rg$_3$–TRAIL combination group when compared with Rg$_3$ or TRAIL groups in H&E staining. Either Rg$_3$ or TRAIL caused a modest increase in the number of TUNEL-positive cells (brown color) compared with control. However, Rg$_3$ plus TRAIL dramatically increased the number of TUNEL-positive cells compared with either treatment alone (Fig. 6C, middle). Consistent with these data, cas-

pase-3 cleavage was more pronounced in tumor sections from mice treated with Rg$_3$ plus TRAIL, relative to tumors from mice receiving either Rg$_3$ or TRAIL alone (Fig. 6C, bottom). Quantitation of TUNEL and cleaved caspase-3 staining of tumor sections indicated a significant increase in cell death in the tumors of the combination treatment compared with either treatment alone (Fig. 6D). Taken together, these data further suggest the combination of Rg$_3$ with TRAIL potentiates *in vivo* antitumor activity.

Discussion

TRAIL is selectively cytotoxic in cancer cells and is therefore a promising anticancer therapeutic agent (34, 35). However, acquisition of TRAIL resistance presents a major obstacle in therapy. One strategy to overcome this obstacle is to combine TRAIL with other anticancer agents (15). Here, we investigated the ability of Rg$_3$ to modulate TRAIL signaling in cancer cells. We found Rg$_3$ is capable of sensitizing TRAIL-induced apoptosis in HCC cells by inducing DR5 expression, which is mediated through CHOP upregulation.
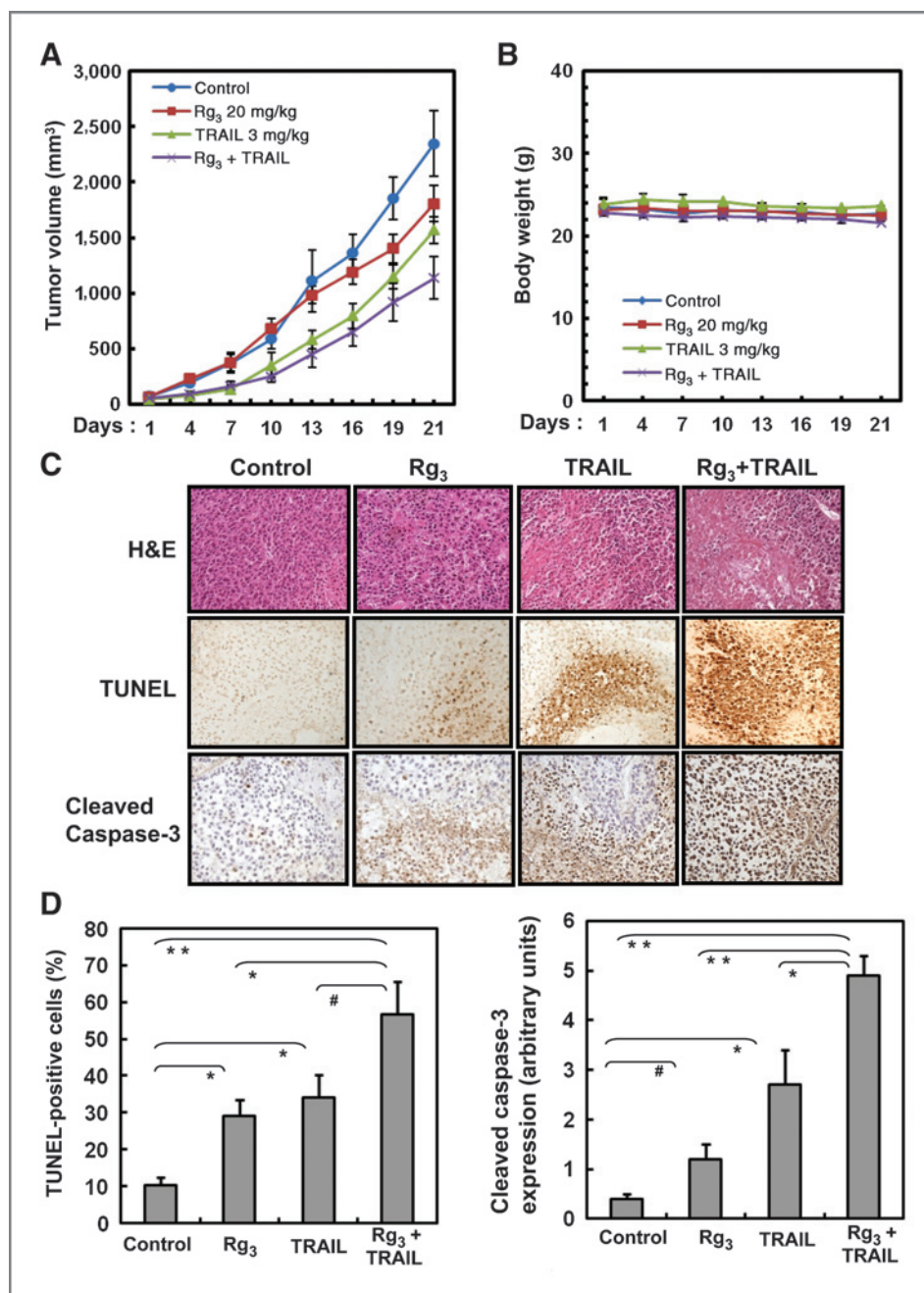
Figure 6. The combination of Rg3 plus TRAIL inhibits HCC growth in mouse xenografts. A and B, Huh-7 tumors established subcutaneously in athymic BALB/c nude mice were treated with Rg3 (20 mg/kg), TRAIL (3 mg/mL), or the combination for 21 days. Tumor growth (A) and body weight (B) were monitored. Results shown are averages ± SEM. Student t test P values for 21-day comparisons: Rg3 + TRAIL versus control ($P \leq 0.01$), Rg3 + TRAIL versus Rg3 ($P \leq 0.05$), Rg3 + TRAIL versus TRAIL ($P \leq 0.10$). C, *in situ* detection of cell death in xenografted HCC tumors from A and B was carried out by using H&E, TUNEL, and cleaved caspase-3 staining. D, quantitations of the TUNEL and cleaved caspase-3 staining are shown in the bottom. Results shown in D are averages ± SEM (#, $P \leq 0.05$; *, $P \leq 0.01$; **, $P \leq 0.001$).

Although most cancer cells express DR4 and DR5, the expression level of receptors plays a critical role in determining cell fate in response to TRAIL (36). Numerous studies have shown convincing data that the upregulation of DR4 and DR5 can sensitize to TRAIL-induced apoptosis (37, 38). Several studies have shown that chemotherapy often sensitizes cancer cells to TRAIL by heightening the activation of the mitochondria-dependent caspase activation cascade (39, 40). In our study, Rg3-induced sensitization on TRAIL-induced cell death is mainly executed via modulation of DR5. Numerous mechanisms have been described for induction of the DR5, including endo-

plasmic reticulum stress, ROS generation, p53 induction, and NF-κB and mitogen-activated protein kinase (MAPK) activation (38, 41–43). ROS did not seem to have a role in mediating the effects of Rg3 on TRAIL-induced apoptosis in our hands. While we saw that Rg3 activated MAPKs, the inhibition of kinase activity by a pharmacologic inhibitor of c-*jun*-NH2-kinase (JNK), SP600125, or extracellular signal–regulated kinase (ERK) inhibitors, PD98059 and U0126 did not attenuate DR5 upregulation upon Rg3 treatment (data not shown). Rg3 did not induce IκBα degradation or upregulation of the NF-κB target gene Bcl-xL (data not shown), suggesting that Rg3-induced

DR5 upregulation is NF-κB–independent. Therefore, Rg$_3$-induced upregulation of DR5 and/or CHOP is likely independent of both NF-κB and MAPK activation.

Although DR5 is a target of p53 (43), the involvement of p53 in Rg$_3$-induced DR5 expression can be excluded as p53 status varies among the HCC cells used: HepG2 and SK-Hep1 are p53 wild-type, Huh-7 are p53 mutant, and Hep3B are p53 null.

CHOP/GADD153 is transcription factor of the C/EBP family that is involved in endoplasmic reticulum stress, including the unfolded protein response. CHOP can bind to members of the C/EBP family to regulate their transcriptional activity and can enhance AP-1–mediated transcription by binding to the AP-1 complex (44). CHOP-dependent DR5 induction has been shown upon treatment with various stimuli (45, 46), and CHOP binds to the DR5 promoter and upregulates the expression of DR5 (41, 47). We found that Rg$_3$ induces CHOP expression in 4 different HCC cell lines and mutation of the CHOP-binding site affects promoter activity of DR5 in response to Rg$_3$ treatment, indicating that CHOP-binding site is required for Rg$_3$ transactivation. Because CHOP knockdown completely eliminated Rg$_3$-induced sensitization to TRAIL, DR5 upregulation is likely mediated solely through CHOP induction. Interestingly, unlike thapsigargin, Rg$_3$ was capable of upregulating CHOP without substantially affecting endoplasmic reticulum stress markers, such as p-eIF2α and GRP78. In addition, Rg$_3$ is unable to induce CHOP expression and TRAIL sensitization in the normal hepatocyte cell line HL7702, though thapsigargin was able to do so to some extent, suggesting that CHOP upregulation by Rg$_3$ is not due to the same endoplasmic reticulum stress pathway as thapsigargin, or that there may be substantial differences in the magnitude of endoplasmic reticulum stress in response to these 2 agents. As discussed earlier, our data here (and also unpublished data) suggest that many of the candidate pathways for upregulation of CHOP by Rg$_3$, including endoplasmic reticulum stress, ROS intermediates, and stress-activated MAPK pathways are not involved in DR5 upregulation by CHOP. Further investigation into how Rg$_3$ leads to CHOP expression is warranted.

Previous studies have shown that Rg$_3$ has cytotoxic or cytostatic effects in HCC cells on its own (48–50). We observed very little single agent cytotoxic activity of Rg$_3$ in our *in vitro* experiments, though there was some antitumor activity *in vivo*. The discrepancies with previous studies may be due to differences in time courses or to purity of the compound or the use of the 20(R) enantiomer of Rg$_3$ in the previous studies (Fig. 7). We used the pure 20(S) enantiomer because of its superior solubility compared with the 20(R) enantiomer, making the 20(S) enantiomer a better choice for pharmaceutical development.

Our study indicates that Rg$_3$ has potential clinical relevance in combination with TRAIL therapy. The combination of Rg$_3$ with TRAIL reduced tumor volume in our *in vivo* mouse xenograft model, as well as increasing TUNEL-positive cells and cleaved caspase-3–positive
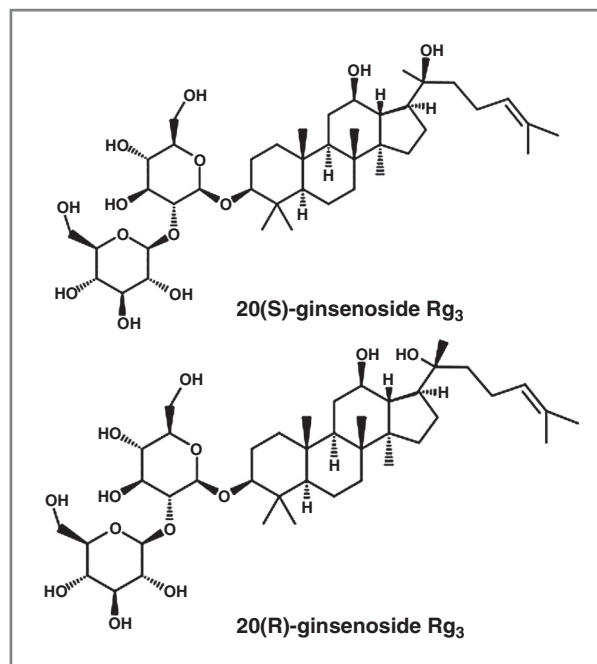

Figure 7. Structure of 20(S)-ginsenoside Rg$_3$. Shown is the chemical structure of 20(S)-ginsenoside Rg$_3$ compared with the 20(R) enantiomer.

cells in tumor sections. While we do not currently have any pharmacodynamic or pharmacokinetic information, the dose of the drug that we used (20 mg/kg) sensitizes HCC cells to TRAIL *in vivo* without cytotoxicity, indicating that Rg$_3$ may be likely to achieve a clinically efficacious dose. Thus, our study indicates a novel anticancer effect of Rg$_3$ and supports the further development of Rg$_3$ as a chemosensitizer in combined therapy with TRAIL to increase the efficacy of its antitumor activity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.W. Kwon, Y.-S. Kim
Development of methodology: Y.-S. Kim
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.-Y. Lee, S.W. Kwon, Y.-S. Kim
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.H. Jung, Y.-R. Kang, G.-B. Koo, S.-S. Hong, S.W. Kwon, Y.-S. Kim
Writing, review, and/or revision of the manuscript: M.J. Morgan, S.W. Kwon, Y.-S. Kim
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-S. Lee, Y.-S. Kim
Study supervision: S.W. Kwon, Y.-S. Kim

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