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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Abbreviations: HCC, Hepatocellular carcinoma; AP-1, activator protein 1; LDH, Lactate dehydrogenase; FITC, Fluorescein isothiocyanate; BHA, Butylated hydroxyanisole; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; HRP, horseradish peroxidase; DR5, Death Receptor 5; DR4, Death Receptor 4; CHOP, C/EBP homology protein; zVAD, N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone; H&E, hematoxylin and eosin; PARP, poly (ADP-ribose) polymerase. H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; Rg3, 20(S)-ginsenoside Rg3; Tg, thapsigargin.

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Abstract

The Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) pathway is a potential therapeutic target for anti-cancer 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 death worldwide. Classical chemotherapy is not effective for HCC treatment, and targeted therapy is limited to sorafenib. Isolated from *Panax ginseng* C.A. Meyer, 20(S)-ginsenoside Rg3 is a steroidal saponin with high pharmacological 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 hepatocellular carcinoma (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 CHOP (C/EBP homology protein), an important endoplasmic reticulum (ER) 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 anti-cancer therapeutic agent and supports the further development of Rg3 as a chemosensitizer in combined therapy with TRAIL.
Introduction

Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (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 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 anti-apoptotic 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 death worldwide. Classical chemotherapy is not currently effective for HCC treatment, while 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-2 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.

Isolated from Panax ginseng C.A. Meyer, 20(S)-ginsenoside Rg3 is a steroidal
saponin with high pharmacological activity. Rg₃ has anti-hypertensive and cardio-protective effects (21) and modulates the function of the central and peripheral nervous systems, as well as the immune system (22, 23). Rg₃ may increase the efficacies of cancer chemotherapy, possibly through inhibitory effects on NF-κB and AP-1 (24).

In this study, we found that Rg₃ renders HCC cells more susceptible to TRAIL-induced apoptosis through upregulation of DR5. This upregulation is mediated through upregulation of CHOP (C/EBP homology protein), which acts on the DR5 promoter. This regulation occurs in cancer cells but not in normal cells, allowing for cancer-specific toxicity. Rg₃, in combination with TRAIL, inhibits tumor growth in mouse xenografts, suggesting that Rg₃ may be a sensitizing agent capable of improving sensitivity or overcoming TRAIL resistance and may facilitate the establishment of TRAIL-mediated combination treatment of hepatocellular carcinoma.

Materials and Methods

Reagents.
Recombinant human TRAIL and anti-DR5 antibody were from Koma biotechnologies. Anti-Caspase 3, anti-PARP, anti-phospho-eIF2α, and anti-CHOP antibodies were from Cell signaling. Anti-DR4 antibody was from Rockland. Anti-tubulin antibody was from Abcam and anti-GRP78 antibody was from Stressgen. zVAD was from R&D systems. Anti-actin antibody, thapsigargin, necrostatin-1, N-acetylcysteine (NAC), and BHA were from Sigma. Cycloheximide and actinomycin D were from Calbiochem. Glutathione S-transferase (GST)-TRAIL was described previously (13).
Rg₃ isolation.

1.6 kg of Sun Ginseng (heat treated ginseng) was extracted with 70% MeOH (1.2 L) under reflux for 3 h. 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. 40 g of the fractions were subjected to silica gel column chromatography. Nine fractions were obtained using stepwise gradient elution (EtOAc:MeOH:H₂O=40:1:1→20:1:1→10:1:1) and fraction 8 was chromatographed over silica gel using CHCl₃:MeOH:H₂O mobile phase (200:20:1→150:20:1). The Rg₃-rich fraction which contained the 20(S) and 20(R) forms was obtained and further purified over semi-preparative LC/ELSD analysis using a reverse-phase column (Phenomenex C18, 250 mm x 10 mm) with 40% ACN to isolate the 20(S) form (10 mg).

Cell culture.

SK-Hep1, HepG2, Hep3B, HT-29, and HeLa cells were cultured in DMEM with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Huh-7 cells were cultured in RPMI 1640 with 10% FBS, 2 mM 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 mM 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 mM Tris at pH 7, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM
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 read 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 µM calcein-AM and 4 µM 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). 1 µg of total RNA from each sample was used for cDNA synthesis with reverse transcriptase (Invitrogen). Equal amounts of cDNA product were used in PCR performed using the Taq DNA polymerase (Takara). PCR amplification was performed using the following primers:

DR5 sense (5'-AAGACCCCTTGTGCTCGTTGTC-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 μM H₂DCFDA before the end of the indicated treatments (30 min) and fluorescence increase was measured by FACS.

Transfection.

Transfection of HCC cells were performed with Lipofectamine PLUS reagent by following manufacturer instructions (GIBCO/BRL). Cells were transfected with pDR5-WT and pDR5-mCHOP mutant constructs provided by Dr. T Yoshimori (Osaka University) and Dr. Choi (Ajou University) (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 h, transfected cells were treated with or without Rg3 for 8 h and cell lysates were analyzed for luciferase activity following the manufacturer’s protocol (Promega).

Chromatin immunoprecipitation assay.

Chromatin immunoprecipitation (ChIP) assay (Millipore) was conducted following the manufacturer’s direction with CHOP antibody (Cell Signaling) and control Mouse 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 shRNA experiments.
MISSION short-hairpin RNA (shRNA) plasmids targeting the coding region or 3’ UTR of CHOP mRNA (NM_004083), and non-targeting 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 Rg3 or Tg 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. (Seoul, Korea), were fed standard rat chow and tap water ad libitum, and maintained under 12 h dark/light cycle at 21 °C. Male, 6-week-old nude mice were randomly divided into four groups (control, Rg3, TRAIL, Rg3+TRAIL, n=8/group). Huh-7 cells were mixed with PBS (200 μL/mouse) and inoculated into one flank of each nude mouse (5 x10^6 Huh-7 cells). When the tumors had reached a volume of about 50-70 mm^3, mice were given a daily oral dose of 20 mg/kg Rg3 or the vehicle (200 μL PBS, control group), and i.p. three times/week at dose of 3 mg/kg TRAIL, for 21 days, respectively. Tumor dimensions were measured twice a week using a digital caliper and tumor volume was calculated using the formula: V = length x width^2 x 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.
Histopathological analysis of tumors was carried out by using hematoxylin and eosin (H&E) staining.

**Immunohistochemistry and TUNEL staining.**

Immunostaining was performed on 8 μm-thick sections after deparaffinization. Microwave antigen retrieval was performed in citrate buffer (pH 6.0) for 10 min prior to peroxidase quenching with 3% H₂O₂ in PBS for 10 min. Sections were then washed in water, preblocked with normal goat or horse serum for 10 min, and were incubated overnight at 4 °C in 1:50 dilutions of anti-caspase-3 (cleaved) antibody. Then sections were incubated with biotinylated secondary antibodies (1:200) for 1 h. Following a wash with PBS, streptavidin-HRP was applied. Sections were developed with diaminobenzidine tetrahydrochloride substrate for 10 min, and counterstained with hematoxylin. At least five random fields of each section were examined at a magnification of ×400 and analyzed by a computer image analysis system, Metaview (Media Cybernetics, Silver Spring, MD). The density values represent as mean ± S.D of % threshold area. TUNEL was performed following the manufacturer’s protocol (Chemicon).

**Statistical analysis.**

Statistical analysis was performed using ANOVA and an unpaired two-tailed Student's t-test. Statistical calculations were performed using SPSS software for Windows Version 10.0 (SPSS, Chicago, IL).

**Results**

*Rg₃ sensitizes to TRAIL-induced cell death in human hepatocellular carcinoma cells.*
Earlier studies suggested that Rg₃ might have anti-tumor functions, including inhibition of tumor cell proliferation, invasion, and metastasis, as well as induction of differentiation and apoptosis (26, 27). In HepG2 cells, Rg₃ alone at concentrations up to 100 μM did not induce morphological signs of cell death (Supplemental Figure 1A), and cell viability was not substantially decreased as measured by MTT assay (Figure 1A, left panel). PI-Annexin V staining and western blotting of caspase-3/PARP cleavage confirmed that Rg₃ had limited, if any, cytotoxicity in these cells (Supplemental Figure 1B and 1C). Concentrations of TRAIL well above 25 ng/mL were required for substantial (>20%) cytotoxicity (Figure 1A, right panel). However, when the cells were pretreated with Rg₃ for 30 min followed by low concentrations of TRAIL (12.5 or 25 ng/mL) for 16~18 h, cells underwent dramatic cell death as evidenced by MTT assay and cell morphology (Figure 1B). Viability measurements from 2-fold dilution dose curves demonstrated that 100 μM Rg₃ decreased the IC₅₀ of TRAIL approximately 8 fold (from above 100 ng/mL to about 12.5 ng/mL) while lower doses of Rg₃ had much less affect on the slope of the dose curve (Supplemental Figure 2A). This concentration of Rg₃ (100 μM) was necessary to achieve robust TRAIL-mediated PARP cleavage at 12 h (Supplemental Figure 2B), but had little effect on its own upon PARP cleavage or cell viability (Supplemental Figure 2B and 2C). 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 (Figure 1C). However, human normal liver cells (HL-7702) were not sensitive to Rg₃ and TRAIL, indicating sensitization could be limited to cancer cells (Figure 1D).

**Rg₃ 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 \textsubscript{3} alone did not affect caspase-3 cleavage in HepG2 cells, but Rg \textsubscript{3} pretreatment significantly augmented TRAIL-induced cleavage/activation of caspase-3, as well as PARP cleavage (Figure 2A). Kinetic analysis showed that Rg \textsubscript{3} promotes TRAIL-induced caspase activity, with PARP cleavage occurring about 4 h after treatment (data not shown). Three other hepatocellular carcinoma cell lines, SK-Hep1, Huh-7 and Hep3B were also used to look at Rg \textsubscript{3} sensitization of TRAIL toxicity. Rg \textsubscript{3} had a similar effect on caspase-3 and PARP cleavage in these cancer cells, with Huh-7 cells being especially affected (Figure 2B). Taken together, our data suggest that Rg \textsubscript{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 \textsubscript{3} and TRAIL, since the pancaspase inhibitor, Z-VAD-FMK, inhibited caspase-3, PARP cleavage, and cell death (Figure 2C). Necrostatin-1, an inhibitor of programmed necrosis, was unable to prevent cell death induced by the combined treatment of Rg \textsubscript{3} and TRAIL in HepG2 cells (Figure 2C, right panel), indicating that the death was apoptotic and not necrotic. TRAIL-induced reactive oxygen species (ROS) have been shown to potentiate the activation of caspases and apoptotic cell death in HeLa cells (29). Either Rg \textsubscript{3} or TRAIL alone induced ROS levels, while intracellular ROS were further enhanced by the Rg \textsubscript{3} and TRAIL combination (data not shown). However, although the antioxidants BHA and N-acetylcysteine (NAC) efficiently inhibited ROS generation (data not shown), they were unable to suppress apoptotic cell death induced by Rg \textsubscript{3} plus TRAIL (Figure 2D), suggesting that ROS are not required for Rg \textsubscript{3} sensitization to TRAIL.

**Rg \textsubscript{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). Rg3 markedly induced the expression of DR5 in both HepG2 and SK-Hep1 cells from 4 h onwards (Figure 3A), whereas DR4 expression did not change much (Figure 3B). Consistent with the protein changes, increases in DR5 mRNA were observed from 4 h (Figure 3C). Actinomycin D (ActD), a de novo mRNA synthesis inhibitor, abolished Rg3-induced DR5 mRNA induction (data not shown) and either ActD or the protein synthesis inhibitor cycloheximide (CHX) inhibited Rg3-initiated increases in DR5 protein (Figure 3D). Rg3 failed to further sensitize cells treated with TRAIL in the presence of CHX (data not shown). This suggests that the Rg3-induced sensitization of TRAIL-induced apoptosis may be mediated by upregulation of DR5 solely at the transcriptional level.

**Rg3-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). Rg3 treatment of HepG2 cells increased the amount of CHOP protein in both a dose- and time-dependent manner (Figure 4A). Rg3 treatment led to increased CHOP protein both in HepG2 and SK-Hep1 cells, roughly correlating with increases in DR5 expression (Figure 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 Rg3-mediated DR5 upregulation (Figure 4C). TRAIL sensitization was more efficient when cells were pre-treated with Rg3 than if Rg3 was administered after TRAIL (as measured by PARP cleavage and cell viability, Supplemental Figure 3A, 3B, and 3C). This suggests transcription of the DR5 mRNA is required before sensitization occurs.

When HepG2 cells were transfected with DR5 promoter luciferase reporter
constructs (Figure 4D, top left subpanel), Rg₃ 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 (Figure 4D, top right subpanel), indicating that the CHOP binding site is required for Rg₃ transactivation. Chromatin immunoprecipitation verified that CHOP bound to the endogenous DR5 promoter in HepG2 cells upon Rg₃ treatment (Figure 4D, bottom subpanel). Rg₃ therefore induces CHOP-dependent DR5 expression through a direct effect on DR5 transcription.

We investigated whether CHOP upregulation contributes to Rg₃-induced sensitization of TRAIL-mediated apoptosis. Since thapsigargin (Tg) is a well-established CHOP inducer (33), we compared CHOP expression levels upon Tg and Rg₃ treatment in three different HCC cell lines. Rg₃ and Tg both enhanced CHOP expression levels (Figure 5A). Because thapsigargin treatment leads to CHOP upregulation like Rg₃, we would predict that Tg would also sensitize to TRAIL-induced cell death in a similar manner as Rg₃. Indeed, upregulation of CHOP by Tg sensitized to TRAIL-induced cell death to a similar extent as Rg₃ (Supplemental Figure 4A). Though both Rg₃ and Tg caused CHOP upregulation, only Tg caused upregulation of ER stress markers such as p-eIF2α and GRP78 (Figure 5B). CHOP knockdown did not affect thapsigargin-induced upregulation of these markers (Figure 5B). Conversely, Rg₃-induced DR5 upregulation was inhibited by CHOP knockdown, indicating that Rg₃-induced DR5 upregulation requires CHOP (Figure 5C, left subpanel). CHOP knockdown effectively suppressed cell death induced by Rg₃ and TRAIL in HepG2 cells, indicating that CHOP is essential for Rg₃-triggered enhancement of TRAIL-induced apoptosis (Figure 5C, right subpanel, and Supplemental Figure 4B). Thapsigargin-induced sensitization of TRAIL, however, was minimally affected (Supplemental Figure 4B), suggesting that Tg activates other pathways that also sensitize to TRAIL.
Interestingly, Rg₃ treatment did not induce CHOP expression in the normal liver cell line, HL7702 (Figure 5D, left subpanel). This potentially explains why it is unable to sensitize normal cells to TRAIL (Figure 1D and Figure 5D, right subpanel). HL7702 cells were capable of dying a caspase-dependent death in response to TRAIL when sensitized by cycloheximide (Supplemental Figure 5A and B), and western blots indicated that the normal cells did not have higher expression of apoptotic inhibitors such as cFLIP, cIAPS, or Bcl-2 (Supplemental Figure 6), indicating that the normal cells do not lack cellular machinery necessary to undergo TRAIL-mediated cell death and caspase activation. Additionally, Tg was able to induce CHOP expression in normal cells, and to confer some sensitization of the normal cells to TRAIL (Figure 5D). Taken together, Rg₃-induced upregulation of CHOP plays an essential role in Rg₃-induced sensitization of cancer cells to TRAIL-mediated apoptosis.

Combination of Rg₃ with TRAIL potentiates in vivo anti-hepatocellular 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 Rg₃, TRAIL, or combination of Rg₃ and TRAIL. Tumor volumes in Rg₃, TRAIL, and Rg₃-TRAIL combination groups were about 75, 63, and 42%, respectively, when compared with control (Figure 6A). The combination of Rg₃ and TRAIL suppressed tumor growth not only when compared with the control group, but also when compared with the Rg₃ or TRAIL alone groups. Importantly, no substantial weight loss was observed in the mice in any treatment group during the period of therapy (Figure 6B), indicating that Rg₃ 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 Figure 6C (top panels), there was a greater degree of apoptosis in the Rg3-TRAIL combination group when compared with Rg3 or TRAIL groups in H&E staining. Either Rg3 or TRAIL caused a modest increase in the number of TUNEL-positive cells (brown color) compared with control. However, Rg3 plus TRAIL dramatically increased the number of TUNEL-positive cells compared with either treatment alone (Figure 6C, middle panels). Consistent with these data, caspase-3 cleavage was more pronounced in tumor sections from mice treated with Rg3 plus TRAIL, relative to tumors from mice receiving either Rg3 or TRAIL alone (Figure 6C, bottom panels). 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 to either treatment alone (Figure 6D). Taken together, these data further suggest the combination of Rg3 with TRAIL potentiates in vivo anti-tumor activity.

Discussion

TRAIL is selectively cytotoxic in cancer cells and is therefore a promising anti-cancer 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 anti-cancer agents (15). Here we investigated the ability of Rg3 to modulate TRAIL signaling in cancer cells. We found Rg3 is capable of sensitizing TRAIL-induced apoptosis in hepatocellular carcinoma cells by inducing DR5 expression, which is mediated through CHOP upregulation.

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 ER stress, ROS generation, p53 induction, and NF-κB, and MAPK activation (38, 41-43). ROS did not appear to have a role in mediating the effects of Rg3 on TRAIL-induced apoptosis in our hands. While we saw that Rg3 activated MAP kinases, the inhibition of kinase activity by a pharmacological inhibitor of JNK, SP600125, or 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 Rg3-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 Rg3-induced DR5 expression can be excluded since 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 ER 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 demonstrated upon treatment with various stimuli (45, 46) and CHOP binds to the DR5 promoter and up-regulates the expression of DR5 (41, 47). We found that Rg3 induces CHOP expression in four different hepatocellular carcinoma cell lines and mutation of the CHOP
binding site affects promoter activity of DR5 in response to Rg3 treatment, indicating that CHOP binding site is required for Rg3 transactivation. Since CHOP knockdown completely eliminated Rg3 induced sensitization to TRAIL, DR5 upregulation is likely mediated solely through CHOP induction. Interestingly, unlike Tg, Rg3 was capable of upregulating CHOP without substantially affecting ER stress markers such as p-eIF2α and GRP78. Additionally, Rg3 is unable to induce CHOP expression and TRAIL sensitization in the normal hepatocyte cell line HL7702 though Tg was able to do so to some extent, suggesting that CHOP upregulation by Rg3 is not due to the same ER stress pathway as Tg, or that there may be substantial differences in the magnitude of ER stress in response to these two agents. As discussed above, our data here (and also unpublished data) suggest that many of the candidate pathways for upregulation of CHOP by Rg3, including ER stress, ROS intermediates, and stress-activated MAP kinase pathways are not involved in DR5 upregulation by CHOP. Further investigation into how Rg3 leads to CHOP expression is warranted.

Previous studies have shown that Rg3 has cytotoxic or cytostatic effects in HCC cells on its own (48-50). We observed very little single agent cytotoxic activity of Rg3 in our in vitro experiments, though there was some anti-tumor 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 Rg3 in the previous studies (Figure 7). We used the pure 20(S) enantiomer because of its superior solubility compared to the 20(R) enantiomer, making the 20(S) enantiomer a better choice for pharmaceutical development.

Our study indicates that Rg3 has potential clinical relevance in combination with TRAIL therapy. The combination of Rg3 with TRAIL reduced tumor volume in our in vivo mouse xenograft model, as well as increasing TUNEL-positive cells and cleaved caspase-3-positive 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 Rg₃ may be likely to achieve a clinically efficacious dose. Thus, our study indicates a novel anti-cancer effect of Rg₃ and supports the further development of Rg₃ as a chemosensitizer in combined therapy with TRAIL to increase the efficacy of its anti-tumor activity.

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Figure legends

Figure 1. Rg3 sensitizes cancer cells to TRAIL.

A. HepG2 cells were treated as indicated with Rg3 (left panel) or TRAIL (right panel) for 16 h and viability was analyzed by MTT assay.  B. HepG2 cells were pretreated with 100 μM Rg3 for 30 min followed by treatment with TRAIL (12.5 ng/mL and 25 ng/mL) for 16~18 h and viability was analyzed by MTT assay (left panel). Representative phase-contrast microscopy images are shown (right panel).  C. SK-Hep1 (hepatocellular carcinoma), HeLa (cervical cancer cells) and HT-29 (colon cancer cells) were treated with Rg3 (100 μM) plus TRAIL (25 ng/mL) and viability analyzed by MTT assay.  D. Human normal liver cells (HL-7702) were treated with Rg3 (100 μM), TRAIL (25 ng/mL), or Rg3 plus TRAIL for 16~18 h and cell viability was analyzed by MTT assay.

All results shown are averages +/- S.D. (##P<0.1, #P<0.5, *P<0.01, **P<0.001)

Figure 2. Rg3 enhances the caspase activity triggered by TRAIL.

A. Western blotting of lysates from HepG2 cells pretreated with Rg3 (50 μM and 100 μM) for 30 min followed by treatment with TRAIL (25 ng/mL) for 12 h.  B. Immunoblotting of lysates from SK-Hep1, Huh-7, and Hep3B cells treated with Rg3 (100 μM) plus TRAIL (25 ng/mL) for 12 h.  C. HepG2 cells were pretreated with zVAD (20 μM) or Nec-1 (40 μM) for 30 min followed by treatment with Rg3 (100 μM) plus TRAIL (25 ng/mL) for 12 h (left panel) or 18 h (right panel) and subjected to lysis and western blotting or (left panel) or MTT viability assay (right panel).  D. Cells were pretreated with NAC (1 mM) or BHA (100 μM) for 30 min followed by treatment with Rg3 (100 μM) plus TRAIL (25 ng/mL) for 9 h and cell
viability was analyzed by FACS using Annexin-PI staining.

All results shown are averages +/- S.D. (*P<0.01, **P<0.001)

**Figure 3. Rg3 up-regulates DR5 transcription.**

A & B. Immunoblotting of lysates from HepG2 and SK-Hep1 cells treated with Rg3 (100 μM) for indicated time periods.  
C. Ethidium bromide agarose gels of the product of reverse transcription-PCR showing the effect of Rg3 on DR5 mRNA level in HepG2 cells treated with Rg3 (100 μM) for indicated time periods.  
D. Immunoblotting of HepG2 cells treated with Rg3 (100 μM), cycloheximide (CHX, 10 μg/mL), actinomycin D (ActD, 1 μg/mL), Rg3 + CHX, or Rg3 + ActD for 12 h.

**Figure 4. Rg3-induced upregulation of CHOP is involved in increased DR5 expression.**

A. Western blotting of HepG2 lysates from cells treated with different concentrations of Rg3 for 12 h (left panel), or with 100 μM Rg3 for different time periods (right panel).  
B & C. Immunoblotting of SK-Hep1 and HepG2 cells treated with Rg3 (100 μM) for indicated time points.  
D. Schematic diagram of the DR5 promoter constructs used for the luciferase activity assay (top left subpanel). HepG2 cells were transfected with pDR5-605-WT or pDR5-605-mCHOP promoter constructs and β-gal plasmid and then treated with Rg3 (100 μM) for 8 h and cells were lysed for luciferase assay (top right subpanel). Chromatin immunoprecipitation from HepG2 cell lysates treated with Rg3 (100 μM) for 8 h 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 subpanel). All
results shown are averages +/- S.D. (*P<0.01)

**Figure 5. Inhibition of CHOP expression by shRNA inhibits TRAIL sensitization by Rg3.**

A. Immunoblotting of HCC lysates treated with Rg3 (100 μM) or thapsigargin (Tg, 1 μg/mL) for indicated times.  
B. Immunoblotting of lysates from HepG2 cells stably expressing CHOP shRNA or non-silencing hairpin showing expression of CHOP and ER stress markers upon treatment with Rg3 (100 μM) or thapsigargin (1 μg/mL) for 12 h.  
C. Cells from (B) were treated with Rg3 (100 μM) for 12 h and cell lysates were analyzed by western blotting (left subpanel) or were treated with Rg3 (100 μM), TRAIL (25 ng/mL), or Rg3 plus TRAIL for 16~18 h and viability was analyzed by MTT assay (right subpanel).  
D. Western blots of lysates from HepG2 and normal liver cells (HL7702) treated with Rg3 (100 μM) or Tg (1 μg/mL) for 12 h (left subpanel). MTT assay of HL7702 cells treated with Rg3 (100 μM), TRAIL (25 ng/mL), Rg3 plus TRAIL, Tg (1 μg/mL), or Tg plus TRAIL for 16~18 h (right subpanel).

All viability results are averages +/- S.D. (§P>0.1 #P<0.05, *P<0.01)

**Figure 6. The Combination of Rg3 plus TRAIL inhibits hepatocellular carcinoma growth in mouse xenografts.**

A & 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) was monitored. Results shown are averages +/- S.E.M. T-test P Values for 21 day comparisons: Rg3 + TRAIL vs. control (P ≤ 0.01), Rg3 + TRAIL vs. Rg3, (P ≤ 0.05) Rg3 + TRAIL vs. TRAIL (P ≤ 0.10).  
C. In situ detection of cell death in xenografted HCC tumors from (A&B) was carried out by using hematoxylin and eosin (H&E), TUNEL, and cleaved caspase-3 staining.  
D. Quantitations of the TUNEL and cleaved caspase-3

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staining are shown in the bottom panels.

Results shown in (D) are averages +/- S.E.M. (\#P \leq 0.05, *P \leq 0.01, **P \leq 0.001)

**Figure 7. Structure of 20(S)-Ginsenoside Rg3.**

Shown is the chemical structure of 20(S)-Ginsenoside Rg3 compared with the 20(R) enantiomer.
Figure 1.

A.

B.

C.

D.
Figure 2.

A.

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B.

SK-Hep1  
Huh-7  
Hep3B

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C.

HepG2

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PARP  
Cleaved PARP  
Caspase 3  
Cleaved Caspase 3  
tubulin

D.

Untreated  
Rg₃  
TRAIL  
Rg₃+TRAIL

BHA+Rg₃+TRAIL  
NAC+Rg₃+TRAIL  
zVAD+Rg₃+TRAIL

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Figure 3.

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![Diagram showing the relationship between CHOP, Luc, pDR5-WT, pDR5-mCHOP, and the relative luciferase activity](image)

- IP: IgG, CHOP
- Input: +, -
Figure 5.

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% Cell viability

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Figure 6.

A. Tumor volume (mm$^3$) vs. Days (1-21) for different treatments: Control, Rg3 20mg/kg, TRAIL 3mg/kg, Rg3 + TRAIL.

B. Body weight vs. Days (1-21) for different treatments: Control, Rg3 20mg/kg, TRAIL 3mg/kg, Rg3 + TRAIL.

C. H&E staining and TUNEL analysis for different treatments: Control, Rg3, TRAIL, Rg3 + TRAIL. Cleaved Caspase-3 expression images are also shown.

D. TUNEL positive cells (%) and Cleaved caspase 3 expression (arbitrary units) for different treatments: Control, Rg3, TRAIL, Rg3 + TRAIL.
Figure 7.

20(S)-Ginsenoside Rg₃

20(R)-Ginsenoside Rg₃
Molecular Cancer Therapeutics

Sensitization of TRAIL-induced cell death by 20S-Ginsenoside Rg3 via CHOP-mediated DR5 upregulation in human hepatocellular carcinoma cells


Mol Cancer Ther Published OnlineFirst October 10, 2012.

Updated version
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Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/10/15/1535-7163.MCT-12-0054.DC1

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