

Research Article

Garcinol Potentiates TRAIL-Induced Apoptosis through Modulation of Death Receptors and Antiapoptotic Proteins

Sahdeo Prasad, Jayaraj Ravindran, Bokyung Sung, Manoj K. Pandey, and Bharat B. Aggarwal

Abstract

Whether garcinol, the active component of *Garcinia indica*, can modulate the sensitivity of cancer cells to TRAIL, a cytokine currently in phase II clinical trial, was investigated. We found that garcinol potentiated TRAIL-induced apoptosis of cancer cells as indicated by intracellular esterase activity, DNA strand breaks, accumulation of the membrane phospholipid phosphatidylserine, mitochondrial activity, and activation of caspase-8, -9, and -3. We found that garcinol, independent of the cell type, induced both of the TRAIL receptors, death receptor 4 (DR4) and DR5. Garcinol neither induced the receptors on normal cells nor sensitized them to TRAIL. Deletion of DR5 or DR4 by small interfering RNA significantly reduced the apoptosis induced by TRAIL and garcinol. In addition, garcinol downregulated various cell survival proteins including survivin, bcl-2, XIAP, and cFLIP, and induced bid cleavage, bax, and cytochrome *c* release. Induction of death receptors by garcinol was found to be independent of modulation of CCAAT/enhancer-binding protein-homologous protein, p53, bax, extracellular signal-regulated kinase, or c-Jun-NH₂-kinase. The effect of garcinol was mediated through the generation of reactive oxygen species, in as much as induction of both death receptors, modulation of antiapoptotic and proapoptotic proteins, and potentiation of TRAIL-induced apoptosis were abolished by *N*-acetyl cysteine and glutathione. Interestingly, garcinol also converted TRAIL-resistant cells into TRAIL-sensitive cells. Overall, our results indicate that garcinol can potentiate TRAIL-induced apoptosis through upregulation of death receptors and downregulation of antiapoptotic proteins. *Mol Cancer Ther*; 9(4); 856–68. ©2010 AACR.

Introduction

Natural products have been used as therapeutics for centuries and are thus envisioned as safe. As many as 70% of all drugs approved for cancer treatment between 1981 and 2002 were either natural products or based on natural products (1). The mechanism by which most natural products mediate their effects, however, is less well understood. Garcinol (camboginol), a polyisoprenylated benzophenone derivative (Fig. 1A), derived from dried rind of the fruit *Garcinia indica* (commonly called kokum, Malabar tamarind, or mangosteen), is used as a spice and as a folk medicine to treat diabetes, obesity, and ulcer, and has shown intriguing parallels to this group of products. Garcinol has been shown to exhibit antioxidant (2) and anti-inflammatory (3) activities and to inhibit pro-

tein glycation (2). It exhibits bactericidal activity against *Helicobacter pylori* (4) and can also induce apoptosis in a wide variety of tumor cells, including leukemia (5), colon cancer (6), and gastrointestinal cancer cells (7). In rodents, garcinol has been shown to suppress aberrant colonic crypt foci formation (8) and inhibit tongue carcinogenesis induced by 4-nitroquinoline 1-oxide (9). How this benzophenone exhibits all these effects is not fully understood, but it has been shown to suppress the expression of inducible nitric oxide synthase and cyclooxygenase-2 by inhibiting NF- κ B activation (10), block phosphorylation of cPLA2, decrease inducible nitric oxide synthase protein by inhibiting STAT1 activation (11), repress chromatin transcription and global gene expression through inhibition of histone acetyltransferases (12), and induce apoptosis through the activation of caspase-2, caspase-3, and caspase-9, leading to cleavage of poly (ADP-ribose) polymerase (PARP), D4-GDI, and DFF-45 (5).

TRAIL (tumor necrosis factor–related apoptosis-inducing ligand) is a cytokine known to induce apoptosis in a variety of tumor cells (13) through its action with two distinct receptors, death receptor 4 (DR4) and DR5. These receptors interact with Fas-associated death domain, which leads to sequential activation of initiator caspase-8 and caspase-3. Alternatively, TRAIL can also activate caspase-3 through mitochondrial bid cleavage, cytochrome *c* release, and caspase-9 activation (14). Studies have shown that repeated application of TRAIL induces

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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doi: 10.1158/1535-7163.MCT-09-1113

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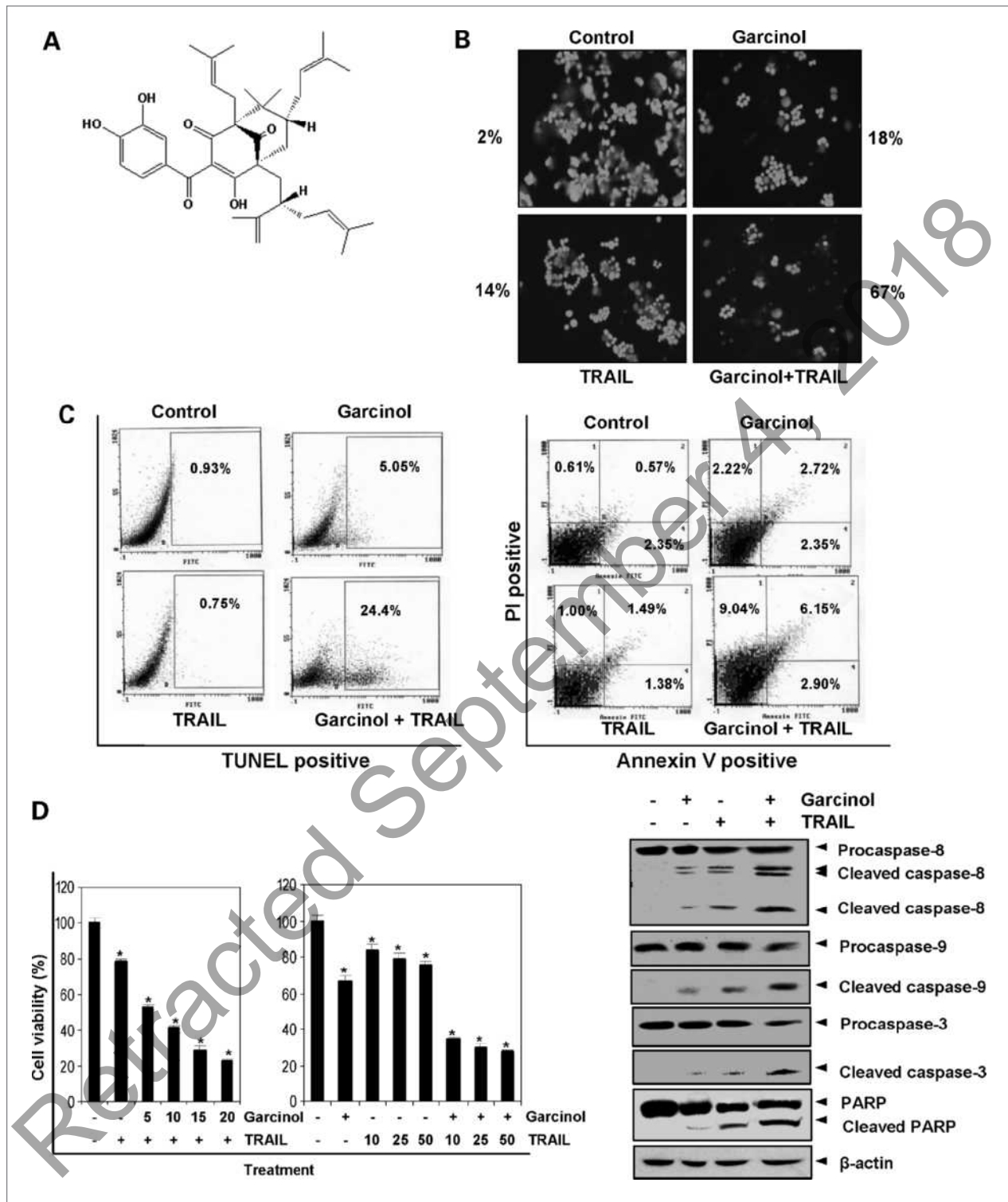


Figure 1. Garcinol enhanced TRAIL-induced HCT116 cell death. **A**, chemical structure of garcinol. **B**, HCT116 cells were treated with 15 μ M garcinol for 12 h and washed with PBS to remove garcinol. Cells were then treated with TRAIL 25 ng/mL for 24 h. Cell death was determined by the Live/Dead assay. **C**, cells were treated with 15 μ M garcinol for 12 h and washed with PBS to remove garcinol. The cells were then treated with TRAIL 25 ng/mL for 24 h. Cells were used for TUNEL assay (left) and propidium iodide (PI)/Annexin V staining (right) and analyzed by FACS. **D**, cells were pretreated with various concentrations of garcinol for 12 h, the media were removed, and the cells were exposed to TRAIL for 24 h. Cell viability was then analyzed by the MTT method as described in Materials and Methods (left). Cells were pretreated with garcinol for 12 h and washed out. After that the cells were treated with TRAIL for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using antibodies against caspase-8, caspase-3, caspase-9, and PARP (right). *, significant ($P < 0.05$) over control.

resistance to TRAIL (15). Irrespective of the pathways, tumor cells are known to develop resistance to TRAIL through multiple mechanisms (15, 16). One potential mechanism involves dysregulation of DR4 and DR5 (17, 18). A second involves defects in the death-inducing signaling complex (DISC; refs. 19, 20). A third mechanism involves defects in effector caspases such as caspase-3. Still a fourth mechanism of TRAIL resistance involves changes in proteins that affect caspase activation, including either inactivation of proapoptotic molecules (bax, bak, bad, bim, or bid) or the overexpression of death inhibitors [FLIP, FAP-1, bcl-2, bcl-xL, or inhibitor of apoptosis (IAP)]; ref. 21]. Whereas bcl-2 and bcl-xL bind to bax and bak and inhibit cytochrome *c* release by pore-forming proteins (bid, bik; ref. 22), IAPs directly bind and inhibit caspase-3, -7, and -9 (23). Two different forms of the proteins FLIP_L and FLIP_S are known to prevent caspase-8 activation (24). Finally, a fifth mechanism of TRAIL resistance involves activation of NF- κ B by PRMT5, a novel TRAIL receptor binding protein (25).

In the present study, we investigated whether garcinol can modulate TRAIL-induced apoptosis in cancer cells, and if so, through what mechanism. The results to be described show that garcinol can enhance TRAIL-induced apoptosis through induction of both DR4 and DR5 and through downregulation of various antiapoptotic proteins.

Materials and Methods

Reagents

A 50 mmol/L solution of garcinol (from Biomol), with purity >95%, was prepared in DMSO, stored as small aliquots at -20°C, and then diluted further in cell culture medium as needed. Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech. Penicillin, streptomycin, RPMI 1640, and fetal bovine serum were purchased from Invitrogen. Anti- β -actin antibody was obtained from Aldrich-Sigma. Antibodies against bcl-xL, bcl-2, bax, cFLIP, PARP, c-Jun-NH₂-kinase (JNK)-1, and Annexin V staining kit were purchased from Santa Cruz Biotechnology. Dichlorodihydrofluorescein diacetate (DCF-DA) was purchased from Invitrogen.

Cell lines

HCT116 (human colon adenocarcinoma), HT29 (human colon adenocarcinoma), A293 (human embryonic kidney carcinoma), PC3 (human prostate cancer cells), MDA-MB-231 and MCF-7 (human breast cancer cells), U266 (human multiple myeloma), SEG-1 (human esophageal epithelial cells), and KBM-5 (human chronic leukemic cells) were obtained from the American Type Culture Collection. MCF-10A (human nontumor breast cells) was supplied by Dr. Kapil Mehta from our Institute. HCT116 variants with deletion of p53 and bax were kindly supplied by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The human colon cancer HCT116 variant cell lines were cultured in McCoy's 5A

medium supplemented with 10% FCS and penicillin/streptomycin (Invitrogen). HCT116, A293, MDA-MB-231, and MCF-7 were cultured in DMEM, and the remaining cell lines were cultured in RPMI-1640 with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin.

Live/dead assay

To measure apoptosis, we also used the Live/Dead assay kit (Invitrogen), which determines intracellular esterase activity and plasma membrane integrity. Calcein-AM, a nonfluorescent polyanionic dye, is retained by live cells, in which it produces intense green fluorescence through enzymatic (esterase) conversion. In addition, the ethidium homodimer enters cells with damaged membranes and binds to nucleic acids, thereby producing a bright red fluorescence in dead cells. Briefly, treated or untreated cells were stained with the Live/Dead reagent (5 μ mol/L ethidium homodimer and 5 μ mol/L calcein-AM) and incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2, Nikon).

Cytotoxicity assay

The effect of garcinol on TRAIL-induced cytotoxicity was determined by the MTT uptake method. Briefly, 5,000 cells were incubated with garcinol in triplicate in a 96-well plate for 12 h and removed, after which they were treated with the TRAIL for 24 h at 37°C. A MTT solution was added to each well and incubated for 2 h at 37°C. An extraction buffer (20% SDS and 50% dimethylformamide) was added, and the cells were incubated overnight at 37°C. Then, the absorbance was measured at 570 nm using a 96-well multiscanner (Dynex Technologies; MRX Revelation).

Annexin V assay

An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface of the membrane to the extracellular surface. This loss of membrane asymmetry can be detected by using the binding properties of Annexin V. To identify apoptosis, we used an Annexin V antibody, which was conjugated with a FITC fluorescence dye. Briefly, 1×10^6 cells were pretreated with garcinol for 12 h and removed, after which they were treated with TRAIL for 24 h at 37°C and subjected to Annexin V staining. The cells were washed in PBS, resuspended in 100 μ L of binding buffer containing a FITC-conjugated anti-Annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur, BD Biosciences).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

To measure the DNA strand breaks during apoptosis, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, which uses the *in situ* cell death detection reagent (Roche), was carried

out. In brief, 1×10^6 cells were preincubated with 15 $\mu\text{mol/L}$ garcinol for 12 h and removed, after which they were treated with TRAIL for 24 h. The cells were washed in PBS and then incubated with a reaction mixture. Cells were analyzed using a flow cytometer (FACSCalibur).

Analysis of cell surface expression of DR4 and DR5

Treated and untreated cells were stained with phycoerythrin-conjugated mouse monoclonal anti-human DR5 or DR4 (R&D Systems) for 45 min at 4°C according to the manufacturer's instructions, resuspended, and analyzed by flow cytometry with phycoerythrin-conjugated mouse IgG2B as an isotype control.

Western blot analysis

To determine the levels of protein expression whole-cell extracts were prepared in lysis buffer [20 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 0.01 $\mu\text{g/mL}$ aprotinin, 0.005 $\mu\text{g/mL}$ leupeptin, 0.4 mol/L phenylmethylsulfonyl fluoride, and 4 mmol/L NaVO_4]. Lysates were spun at 14,000 rpm for 10 min to remove insoluble material. To determine the effect of garcinol on cytochrome *c* release and cytosolic bid protein, cytosolic extracts were prepared as described previously (26). In brief, the cells were washed with PBS, resuspended in the buffer containing 0.25 mol/L sucrose, 30 mmol/L Tris-HCl, pH 7.9, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L sodium orthovanadate, 10 mmol/L NaF, 2 $\mu\text{g/mL}$ leupeptin, and 2 $\mu\text{g/mL}$ aprotinin, and then vortexed gently for 10 s. The homogenates were centrifuged at 2,000 rpm for 10 min to remove nuclei, and the supernatants were centrifuged at 14,000 rpm for 30 min to remove mitochondria and other insoluble fragments. The supernatants were again centrifuged as above to ensure complete removal of mitochondria. Supernatants from whole-cell lysate and cytosolic extract were collected and kept at -80°C . Whole-cell lysates and cytosolic extract were resolved by SDS-PAGE. After electrophoresis, the proteins were electro-transferred to nitrocellulose membranes, blotted with the relevant antibody, and detected by enhanced chemiluminescence reagent (GE Healthcare).

Transfection with small interfering RNA

HCT116 cells were plated in each well of 6-well plates and allowed to adhere for 24 h. On the day of transfection, 12 μL Hiperfect transfection reagent (Qiagen) were added to 50 nmol/L small interfering RNA (siRNA) in a final volume of 100 μL culture medium. After 48 h of transfection, cells were treated with garcinol for 12 h and then exposed TRAIL for 24 h.

Measurement of reactive oxygen species

To detect intracellular reactive oxygen species (ROS), cells were preincubated with 20 $\mu\text{mol/L}$ DCF-DA for 15 min at 37°C before being treated with 15 $\mu\text{mol/L}$

garcinol. After 30 min of incubation, the increase in fluorescence resulting from oxidation of DCF-DA to DCF was measured by flow cytometry. The mean fluorescence intensity at 530 nm was calculated. Data were collected from at least 10,000 cells at a flow rate of 250 to 300 cells/s.

Statistical analysis

The data were analyzed for mean values and SE for all treated and vehicle control. Values were compared using the paired Student's *t*-test; $P < 0.05$ was considered significant.

Results

Although TRAIL is in phase II clinical trial for cancer treatment, resistance to TRAIL is one of the major problems with the therapy. The objective of this study was to determine whether garcinol can modulate sensitivity of tumor cells to TRAIL, and if so to delineate the mechanism of sensitivity. For most studies, we employed the human colorectal cancer cell line HCT116; however, our results were not restricted to this tumor cell line only. This cell line was employed because, first, the TRAIL-induced apoptosis in this cell line is well characterized, and second, several variants of the parent cell line that lack p53, p21, and bax are available.

Garcinol potentiates TRAIL-mediated apoptosis in colon cancer cells

Whether garcinol can enhance apoptosis induced by TRAIL was examined by using the Live/Dead assay, which measures cell membrane permeability. We found that garcinol and TRAIL treatment alone induced 18% and 14% apoptosis, respectively, in HCT116 cells. Interestingly, the combination treatment with garcinol and TRAIL enhanced apoptosis to 67% (Fig. 1B). To confirm the effect of garcinol on TRAIL-induced apoptosis, we measured apoptosis by TUNEL assay. It revealed that garcinol potentiated TRAIL-induced apoptosis, from 5% and 0.75% with garcinol and TRAIL alone, respectively, to 24.4% when used in combination (Fig. 1C, left). We also examined cells by phosphatidylserine externalization using the Annexin V assay to determine the effect of garcinol on TRAIL-induced apoptosis in HCT116. The results shown in Fig. 1C (right) indicate that garcinol and TRAIL induced apoptosis 7% and 4% respectively, and the combination increased apoptosis to 18%. Next, we investigated the effects of garcinol on TRAIL-induced cytotoxicity by MTT methods that detect mitochondrial activity. For this colon cancer cells were pretreated with different concentrations of garcinol for 12 hours and then exposed to different concentrations of TRAIL separately for 24 hours. The HCT116 cells were moderately sensitive to either garcinol or TRAIL alone. However, pretreatment with garcinol significantly ($P < 0.05$) enhanced TRAIL-induced cytotoxicity (Fig. 1D, left). Activation of caspases is another hallmark of apoptosis induced by most agents.

Thus, we examined the effect of garcinol on TRAIL-induced activation of caspase-8, -9, and -3, and on cleavage of PARP. We found that garcinol enhanced TRAIL-induced activation of all three caspases, thus leading to enhanced PARP cleavage (Fig. 1D, right). Taken together, all these results suggest that garcinol can enhance TRAIL-induced apoptosis.

Garcinol induces expression of death receptor TRAIL-R1/DR4 and TRAIL-R2/DR5

How garcinol enhances TRAIL-induced apoptosis was investigated in detail. First, we examined the effect of garcinol on the expression of DR5 and/or DR4 on colon cancer cells. Treatment of HCT116 cells with various concentrations of garcinol for 24 hours resulted in an increased expression of TRAIL-R2/DR5 and TRAIL-R1/DR4 in a dose-dependent manner (Fig. 2A, left). Whether induction of the TRAIL receptor is time dependent was also examined. For this, cells were treated with garcinol for different times and then examined for expression of DR5 and DR4 protein. Garcinol induced both DR5 and DR4 in a time-dependent manner (Fig. 2A, right). These data suggest that upregulation of protein for DR4 and/or DR5 by garcinol may be one of the mechanisms by which it enhances the proapoptotic effects of TRAIL in colon cancer HCT116 cells.

Whether garcinol also enhances the expression of death receptors on cell surface was also examined. For this, we analyzed cell surface expression of DR5 and DR4 in cells exposed to garcinol. We found that garcinol increased cell surface levels of DR5 and DR4 (Fig. 2B). The levels of DR4 and DR5 cell surface expression induced by garcinol were comparable. Collectively, these results indicate that garcinol upregulated the expression of both death receptors on the cell surface.

Whether upregulation of TRAIL receptors by garcinol was specific to HCT116 or also occurs in other cell types was investigated. For this we exposed the following cells to 15 $\mu\text{mol/L}$ garcinol for 24 hours: HT29 (human colon adenocarcinoma), A293 (human embryonic kidney carcinoma), PC3 (human prostate cancer cells), MDA-MB-231 and MCF-7 (human breast cancer cells), U266 (human multiple myeloma), SEG-1 (human esophageal epithelial cells), and KBM-5 (human chronic leukemic cells). Garcinol induced the expression of both DR5 and DR4 in all of these lines (Fig. 2C). Besides HCT116 cells, the induction of DR5 and DR4 was also observed in HT29, another colon cancer cell line. Human breast MDA-MB-231 cells showed a very high level of induction of DR4 on exposure to garcinol. These findings suggest that the upregulation of DR5 and DR4 by garcinol was not cell-type specific. Interestingly, no induction of either DR4 or DR5 was observed in nontumorigenic MCF-10A cells by the treatment of garcinol; however, induction of both DR4 and DR5 was observed in MCF-7 breast cancer cells (Fig. 2D). The lack of induction of death receptors by garcinol correlated with lack of cytotoxicity and sensitization to TRAIL in MCF-10A cells (Fig. 2D), thus indi-

ating that induction of death receptors by garcinol mediates sensitization.

DR induction by garcinol is needed for TRAIL-induced apoptosis

To determine the role of DR5 and DR4 in TRAIL-induced apoptosis, we used siRNA specific to DR5 and DR4 to downregulate the expression of these receptors. Transfection of cells with siRNA for DR5 but not with the control siRNA reduced garcinol-induced DR5 expression (Fig. 3A). Similarly, transfection of cells with siRNA for DR4 reduced the garcinol-induced DR4 expression (Fig. 3A). We next examined whether the suppression of DR5 or DR4 by siRNA could abrogate the sensitizing effects of garcinol on TRAIL-induced apoptosis using Live/Dead assay. The results reveal that the effect of garcinol on TRAIL-induced apoptosis was effectively abolished in cells transfected with either DR5 or DR4 siRNA (Fig. 3B), whereas treatment with control siRNA had no effect (Fig. 3B). Silencing of DR5 and DR4 both had dramatic effect on TRAIL-induced apoptosis, thus suggesting that DR5 and DR4 both play a major role in TRAIL-induced apoptosis.

Garcinol downregulates the expression of antiapoptotic proteins

Numerous antiapoptotic proteins have been shown to suppress TRAIL-induced apoptosis. Whether garcinol potentiates TRAIL-induced apoptosis through the downregulation of these proteins was investigated. Cells were exposed to different concentrations of garcinol for 24 hours and then examined for expression of XIAP, survivin, bcl-xL, bcl-2, and cFLIP (long and short). Garcinol inhibited expression of the antiapoptotic proteins survivin, bcl-2, XIAP, and both the short and long forms of cFLIP, but had no effect on expression of bcl-xL (Fig. 3C, left). Thus, our results suggest that downregulation of antiapoptotic proteins is another mechanism by which garcinol could potentiate TRAIL-induced apoptosis.

Garcinol regulates expression of apoptotic proteins

Whether garcinol affects the expression of proapoptotic proteins was also examined. Garcinol caused the cleavage of bid protein, enhanced the expression of proapoptotic bax, and increased the release of cytochrome *c* in cytosol (Fig. 3C, right). Induction of bax and release of cytochrome *c* by garcinol suggest that these proteins may disrupt mitochondrial homeostasis, which further would contribute to enhanced apoptosis.

Upregulation of TRAIL receptors by garcinol is p53 and bax independent

There are numerous reports that suggest that p53 can induce death receptors (27, 28). Whether garcinol induces TRAIL receptors through p53 was examined using HCT116 cell lines that lack p53. Garcinol induced DR5 and DR4 in p53 parental as well as p53 knockout HCT116 cells in a dose-dependent manner, although

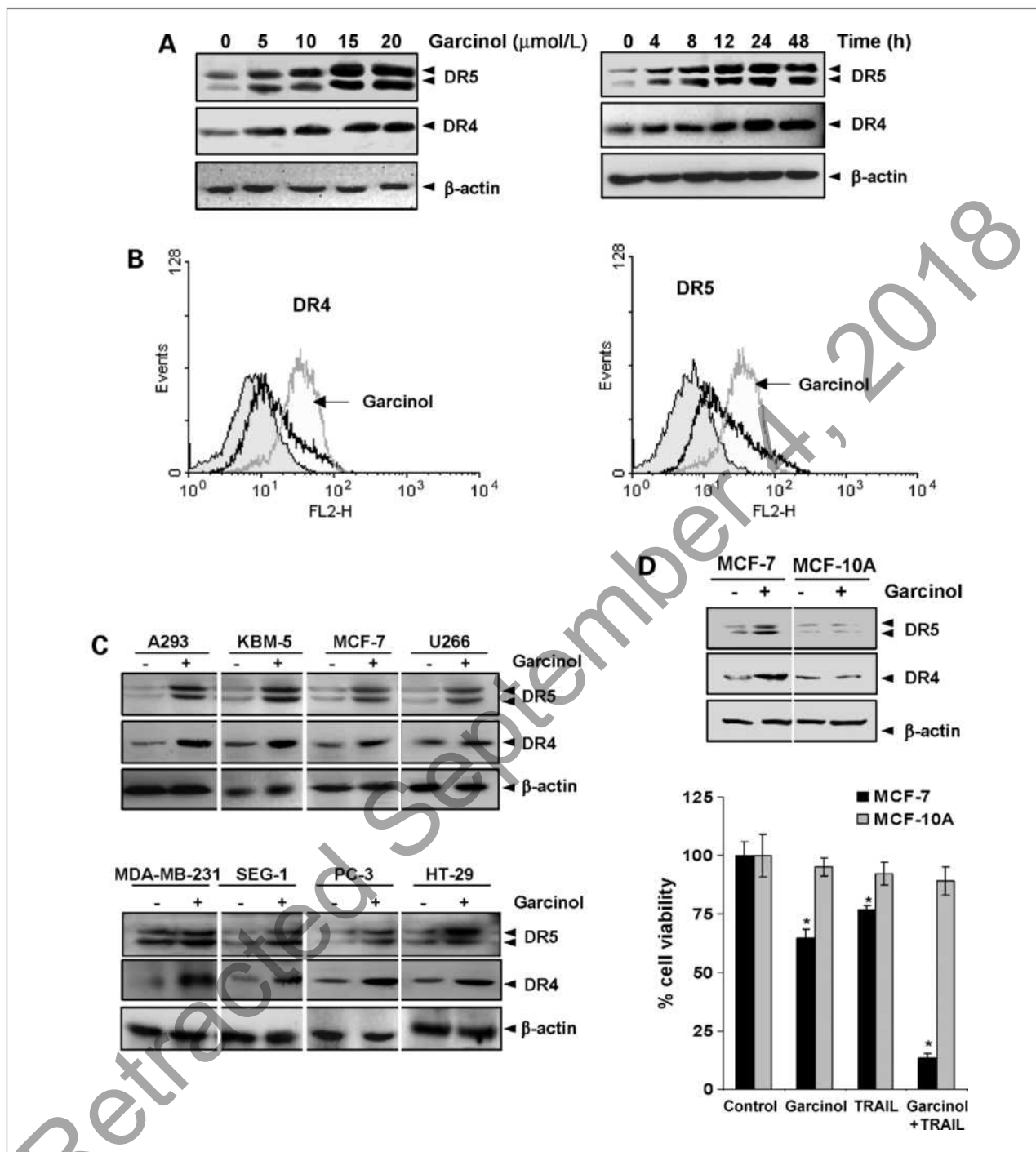


Figure 2. Garcinol induces DR5 and DR4 expression. **A**, HCT116 cells (1×10^6 cells/well) were treated with indicated dose (left) and time (right) of garcinol. Whole-cell extracts were then prepared and analyzed for DR5 and DR4 by Western blotting. β -Actin was used as an internal control to show equal loading of proteins. HCT116 cells were treated with 15 $\mu\text{mol/L}$ garcinol for 24 h and then harvested for analysis of cell surface DR4 and DR5 by immunofluorescent staining and subsequent flow cytometry. Filled grey peaks, cells stained with a matched control phycoerythrin-conjugated IgG isotype antibody. **C**, garcinol upregulated DR5 and DR4 in various types of cancer cells. Cells (1×10^6) were treated with 15 $\mu\text{mol/L}$ garcinol for 24 h, after which whole-cell extracts were prepared and analyzed by Western blotting using antibodies against DR5 and DR4. The same blots were stripped and reprobed with β -actin antibody to verify equal protein loading. **D**, garcinol neither induced the receptors on normal cells nor sensitized them to TRAIL. MCF-7 or MCF-10A cells were pretreated with garcinol (15 $\mu\text{mol/L}$) for 12 h and washed out, and then exposed to TRAIL for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using antibodies against DR5 and DR4 (top). Cells were pretreated with garcinol for 12 h and then media were removed. After that the cell were treated with TRAIL for 24 h. Cell viability was then analyzed by the MTT method (bottom). *, significant ($P < 0.05$) over respective control.

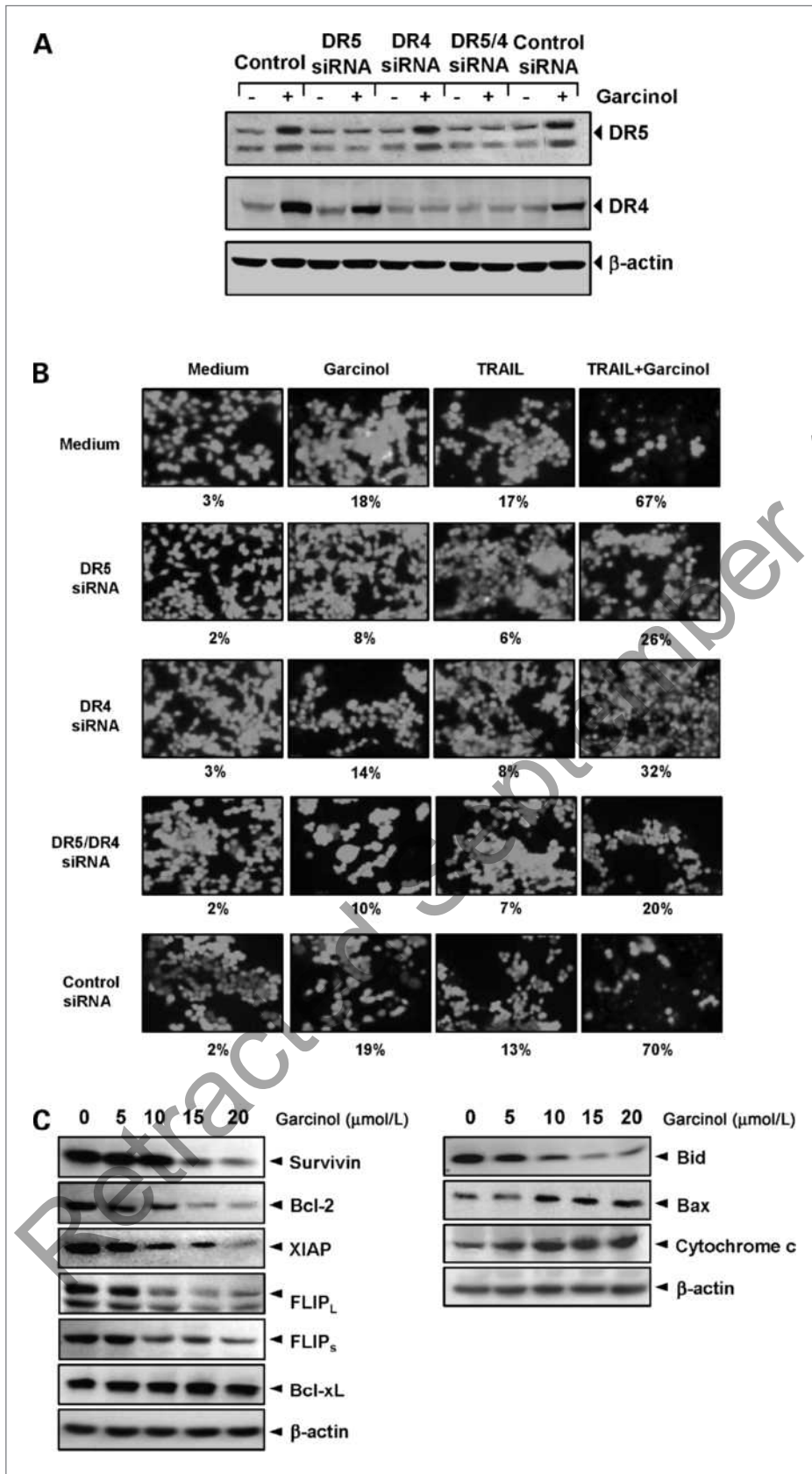


Figure 3. Effects of knockdown of death receptors on garcinol-induced sensitization of TRAIL. **A**, HCT116 cells were transfected with DR5 siRNA, DR4 siRNA, and control siRNA alone or combined. After 48 h, cells were treated with 15 μmol/L garcinol for 24 h, and whole-cell extracts were subjected to Western blotting for DR5 and DR4. **B**, cells were seeded in a chamber slide and transfected with siRNAs. After 48 h, cells were pretreated with 15 μmol/L garcinol for 12 h and then incubated with 25 ng/mL TRAIL for 24 h. Cell death was determined by the Live/Dead assay. **C**, effects of garcinol on antiapoptotic (left) and apoptotic protein expression (right). HCT116 cells were pretreated with indicated dose of garcinol for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using the relevant antibodies. For release of cytochrome c and bid proteins cytosolic extracts were prepared as described in Material and Methods and analyzed by Western blotting using the relevant antibodies. The same blots were stripped and reprobbed with β-actin antibody to verify equal protein loading.

these knockout cells do not express p53 protein (Supplementary Fig. S1A). These results indicate that induction of TRAIL receptors was independent of p53 expression. To determine whether bax is needed for garcinol-induced DR induction, we used bax knockout HCT116 colon cancer cells. Garcinol induced expression of DR5 and DR4 in both bax parental and bax knockout HCT116 cells (Supplementary Fig. S1B). These results indicate that inductions of TRAIL receptors are independent of bax expression.

Garcinol-induced upregulation of TRAIL receptors is not mediated through activation of mitogen-activated protein kinase

Whether garcinol can activate extracellular signal-regulated kinase (ERK) and JNK was examined. For this, cells were pretreated with the indicated concentration of garcinol for 24 hours and then examined for the phosphorylated ERK and JNK (Supplementary Fig. S1C). No activation of either kinase was found. Thus induction of TRAIL receptors by garcinol did not require either of the kinases.

Garcinol-induced upregulation of TRAIL receptors is not mediated through activation of CCAAT/enhancer-binding protein-homologous protein

It has been shown that the induction of death receptor by certain agents is mediated through activation of CCAAT/enhancer-binding protein-homologous protein (CHOP; ref. 29). Whether garcinol can induce the expression CHOP was examined. Cells were pretreated with the indicated concentration of garcinol for 24 hours, and then examined for CHOP expression. We found that garcinol did not increase but decreased the expression of CHOP (Supplementary Fig. S1D). Thus, induction of TRAIL receptors by garcinol did not require the expression of CHOP.

Induction of TRAIL receptors by garcinol is ROS dependent

Whether garcinol has the ability to generate ROS was examined by treating HCT116 cells and using DCF-DA as a probe to measure the increase in ROS levels inside cells. Figure 4A shows that garcinol induced ROS in dose-dependent manner in HCT116 cells. Our results also show that although garcinol induced marked change in the level of ROS in MCF-7 breast cancer cells, the production of ROS was not observed in nontumorigenic MCF-10A cells (Fig. 4B). We also investigated whether garcinol-induced TRAIL receptors is also regulated by ROS. As shown in Fig. 4C, pretreatment of HCT116 cells with the ROS scavenger *N*-acetylcysteine (NAC) reduced the garcinol-induced upregulation of DR5 and DR4 expression in a dose-dependent manner. Glutathione (GSH) also abolished the garcinol-induced induction of both DR5 and DR4 expression in a dose-dependent manner (Fig. 4D). This suggests the critical role of ROS in induction of TRAIL receptors by garcinol.

Potential of TRAIL-induced apoptosis by garcinol is ROS dependent

Whether ROS is needed for potentiation of TRAIL-induced apoptosis by garcinol was examined. As shown in Fig. 5A, pretreatment of cells with NAC markedly reduced the effect of garcinol on TRAIL-induced apoptosis, from 69% to 35%. To determine whether NAC

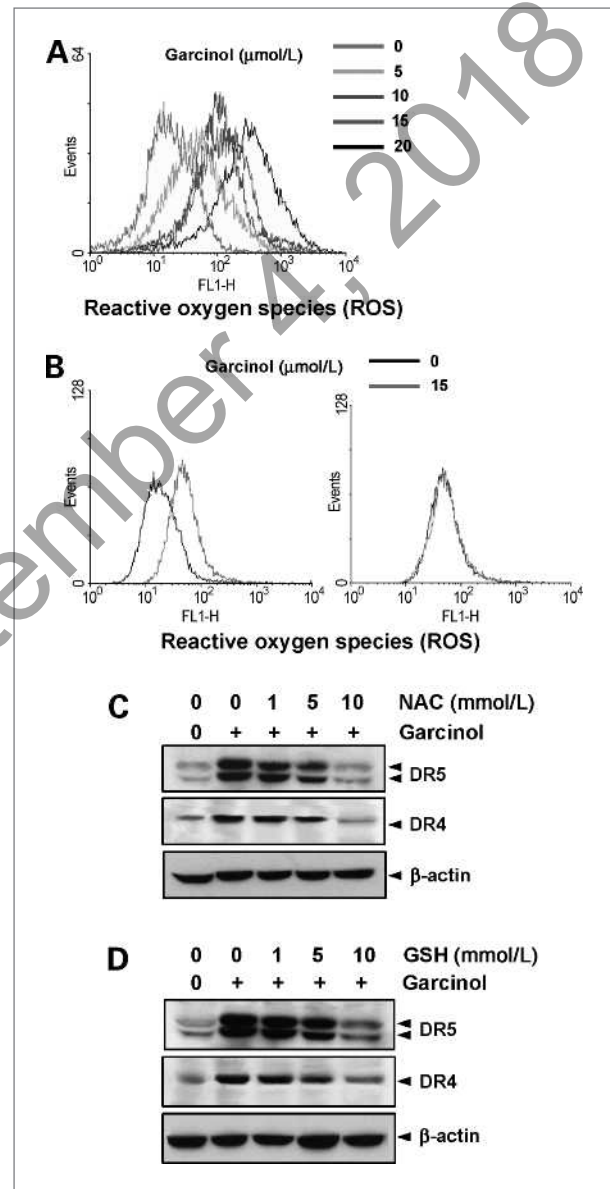


Figure 4. Garcinol induces generation of ROS, and upregulation of DR5 and DR4 by garcinol was mediated by ROS. **A**, HCT116 (1×10^6 cells) were labeled with DCF-DA, treated with indicated concentration of garcinol for 1 h, and examined for ROS production by flow cytometer. **B**, MCF-7 (left) or MCF-10A (right) cells (1×10^6) were labeled with DCF-DA, exposed with garcinol (15 $\mu\text{mol/L}$) for 1 h, and examined for ROS production by flow cytometer. HCT116 cells (1×10^6) were pretreated with various concentrations of NAC (**C**) or GSH (**D**) for 1 h, and then the cells were treated with 15 $\mu\text{mol/L}$ garcinol for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using DR5 and DR4 antibodies.

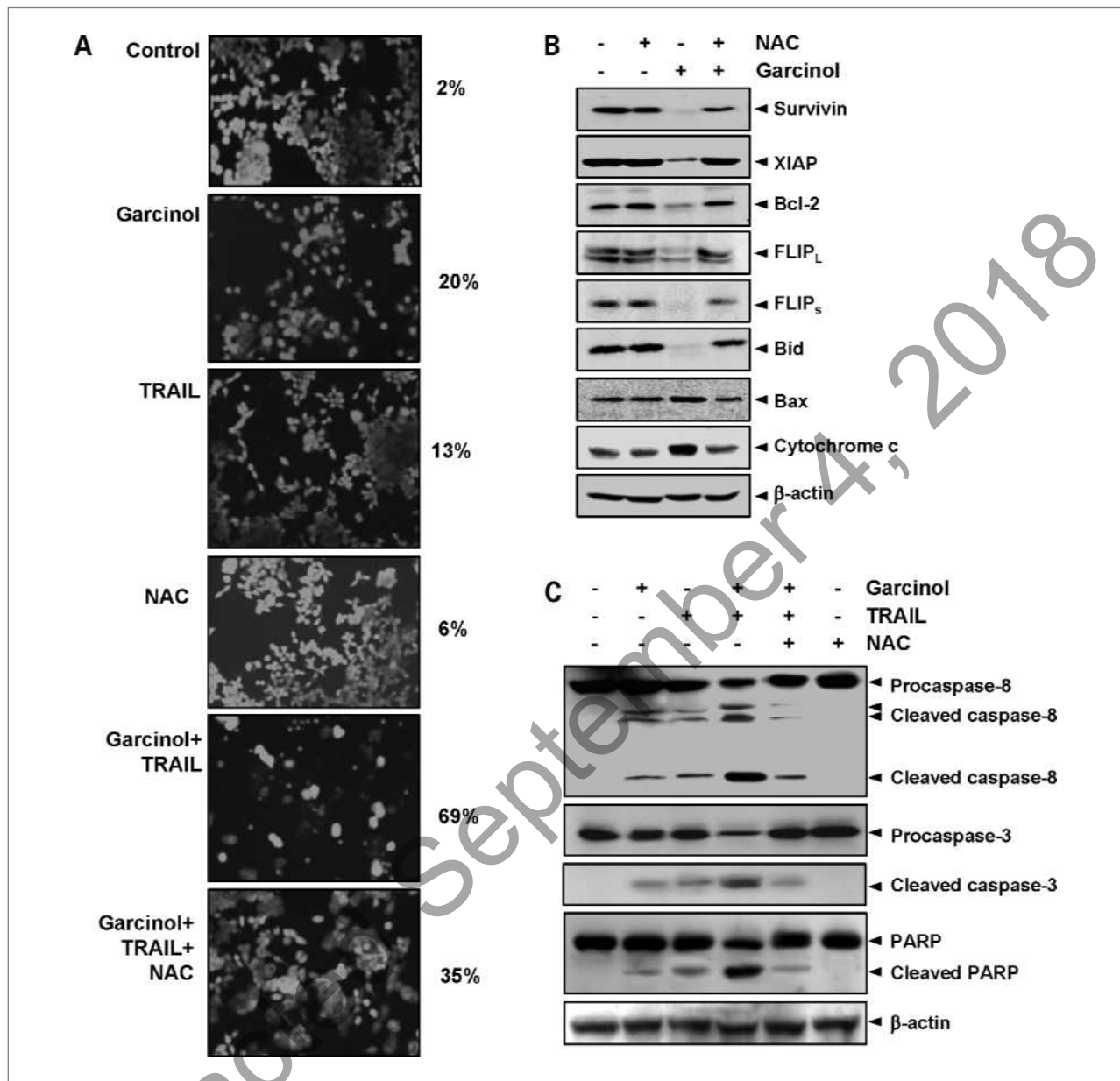


Figure 5. A, NAC reverses cell death induced by the combination of garcinol and TRAIL. HCT116 cells were pretreated with NAC for 1 h and then treated with garcinol for 12 h. Next, cells were washed with PBS and treated with TRAIL for 24 h. Cell death was determined by the Live/Dead assay. B, NAC abrogated the garcinol-induced inhibition of antiapoptotic proteins and induction of apoptotic proteins. HCT116 cells were pretreated with NAC for 1 h and then treated with garcinol for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using the relevant antibodies. β -Actin was used as a loading control. C, NAC suppresses caspase activation and PARP cleavage induced by combination of TRAIL and garcinol. HCT116 cells were pretreated with NAC for 1 h and then treated with garcinol for 12 h. Next, cells were washed with PBS and treated with TRAIL for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using the relevant antibodies. β -Actin was used as a loading control.

can abrogate the garcinol-induced modulation of antiapoptotic proteins, cells were pretreated with NAC and then garcinol for 24 hours and then examined for the expression of proteins. We found that NAC reversed the garcinol-induced suppression of antiapoptotic proteins (Fig. 5B).

We also found that NAC reversed the effect of garcinol on TRAIL-induced apoptosis as indicated by its effects on

the cleavage of procaspases and of PARP (Fig. 5C), again suggesting the critical role of ROS in garcinol's effects on TRAIL.

Garcinol sensitized TRAIL-resistant cells

We also investigated whether garcinol affects TRAIL-resistant HT29 cancer cells. For this HT29 cells were exposed to garcinol for 12 hours and then treated with

TRAIL for 24 hours. We found that HT29 cells were moderately sensitive to garcinol but resistant to TRAIL alone. However, pretreatment with garcinol significantly ($P < 0.05$) enhanced TRAIL-induced apoptosis (Fig. 6A). Further, we studied cell membrane permeability by Live/Dead assay and found that garcinol and TRAIL treatment alone induced 12% and 5% apoptosis, respectively, compared with 2% in control, in HT29 cells. Interestingly, the pretreatment with garcinol enhanced TRAIL-induced apoptosis to 48% (Fig. 6B). Results of fluorescence activated cell sorter (FACS) analysis for apoptosis also revealed that the combination of garcinol and TRAIL enhanced apoptosis from 6% to 19.6%. To determine

how garcinol sensitizes HT29 to TRAIL-induced apoptosis, we investigated its effect on TRAIL receptors (DR4 and DR5). For this, HT29 cells were treated with garcinol for 12 hours, and then TRAIL for 24 hours. We found that garcinol potentiates induction of both DR5 and DR4, suggesting TRAIL-induced apoptosis of HT29 cells is mediated through the induction of death receptors.

Discussion

Among all the apoptosis-inducing cytokines, TRAIL is the only cytokine that is being explored as an anticancer

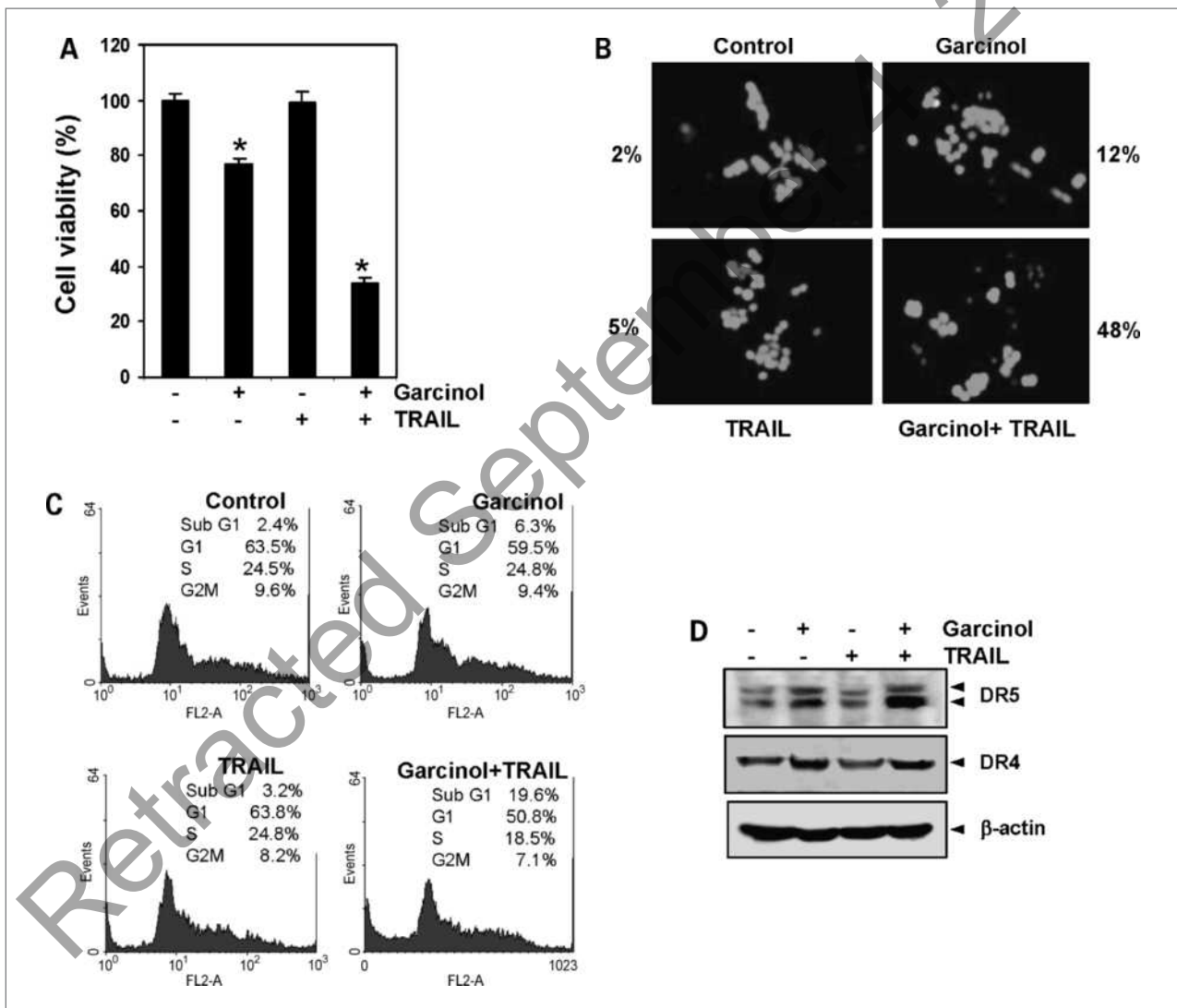


Figure 6. Garcinol sensitizes TRAIL-resistant cells. **A**, HT29 cells were pretreated with garcinol for 12 h and then exposed with TRAIL after removal of media for 24 h. Cell viability was then analyzed by the MTT method. *, significant ($P < 0.05$) over respective control. **B**, cells were treated with 15 $\mu\text{mol/L}$ garcinol for 12 h and washed with PBS to remove garcinol. Cells were then treated with TRAIL 25 ng/mL for 24 h. Cell death was determined by the Live/Dead assay. **C**, HT29 cells were treated with 15 $\mu\text{mol/L}$ garcinol for 12 h and washed with PBS to remove garcinol. The cells were then treated with TRAIL 25 ng/mL for 24 h. Cells were used for FACS analysis for apoptosis. **D**, cells were pretreated with garcinol for 12 h and washed out. After that the cells were treated with TRAIL for 24 h. Whole-cell extracts were prepared and analyzed for DR5 and DR4 expression by Western blotting.

agent. Both TRAIL and the agonistic antibodies against the receptor are currently in phase II clinical trials. TRAIL induces apoptosis by interacting with two different death-inducing receptors, DR4 and DR5. Both receptors engage the same downstream apoptotic mechanism and play crucial roles in cytotoxicity associated with TRAIL and other chemotherapeutic agents (30). Resistance of cancer cells to TRAIL is one of the major roadblocks to the development of this therapy. Thus, agents that can either potentiate the effect of TRAIL or overcome resistance are urgently needed. In the present study, we show that garcinol can potentiate TRAIL-induced apoptosis in cancer cells. The mechanism by which garcinol mediates its effects on TRAIL-induced apoptosis seem to involve the induction of TRAIL receptors and downregulation of antiapoptotic proteins including cFLIP, an inhibitor of caspase-8. Our results also support that DR4 and DR5 have an important role in TRAIL-induced apoptosis. Considerable numbers of cancer cells, however, are resistant to apoptosis induced by TRAIL (15). Although chemotherapeutic agents have been used to overcome the resistance, most of them are highly toxic and thus exhibit major side effects. In contrast, garcinol, which has been shown to be pharmacologically quite safe and used in traditional medicine, was found efficacious in potentiating the effects of TRAIL.

We found that the induction of death receptors by garcinol was not cell type specific. Rather, it was observed in a wide variety of cell types, including colon, breast, prostate, kidney, leukemic, and esophageal cancer cells. Induction of TRAIL receptors in some cells, however, was much more pronounced than in other cell types. Thus, garcinol is likely to potentiate the effect of TRAIL in a wide variety of cells. It has been suggested that oxidative stress plays a major role as a common mediator of cell death (31). ROS generation has been proposed to be involved in DR5 upregulation by cancer chemopreventive agents, including curcumin and sulforaphane (32, 33). In the present study, our data show that the mechanism by which garcinol induces death receptor upregulation is through production of ROS. The antioxidant GSH and NAC abolished the upregulation of DR by garcinol. These antioxidants also reversed the garcinol-induced downmodulation of antiapoptotic and proapoptotic proteins. The effect of garcinol on TRAIL-induced apoptosis was also neutralized by the antioxidants. This reversal was apparently due to inhibition of induction of TRAIL receptors. Apoptosis induced by TRAIL alone is also known to be regulated through generation of ROS in colon cancer cells (34).

Several studies provide evidence that death receptor upregulation may be a promising strategy for sensitizing tumor cells to TRAIL-induced apoptosis (32, 33). The upregulation of death receptors is known to be regulated by either a p53-dependent or a p53-independent mechanism (35, 36). Garcinol induced the expression of DR5 in a colon cancer cell line, regardless of p53 status (parental p53 and knockout p53 HCT116 cells), indicating that garcinol upregulates DR4 and DR5 expression

via a p53-independent mechanism. This result is supported by the effect of another compound, baicalein, which overcomes TRAIL resistance in colon cancer cells through DR5 upregulation in a p53-independent manner (37). In addition, our result also show that garcinol-induced apoptosis mediated through expression of death receptors is independent of bax expression. Thus, it is possible that the role of p53 and bax on induction of DR5 and DR4 depends on the nature of the stimulus and the cell type.

Garcinol was found to be ineffective in activation of ERK1/2, mitogen-activated protein kinase (MAPK), and JNK. Although ROS can lead to induction of MAPK (38), in our study garcinol induced TRAIL receptors independent of MAPK. In another study quercetin augmented TRAIL-induced apoptosis through the ERK-mediated downregulation of the survivin signal transduction pathway (39). In our study, however, garcinol induced apoptosis through downregulation of survivin but independent of ERK activation.

It is well documented that the death receptor-mediated apoptotic signaling pathway requires recruitment of Fas-associated death domain and caspase-8, which results in caspase-8 activation and subsequent activation of its downstream caspase cascades and apoptosis (40). In addition, for efficient apoptosis, the activation of intrinsic apoptosis pathway is critical. Our results show that garcinol induced activation and cleavage of caspase-8, resulting in the decrease of cytosolic bid (truncation bid; tbid), a BH3-only proapoptotic protein (41). The resulting tbid plays a role in the generation of conformational changes of bax and subsequent translocation to mitochondria (42), leading to the formation of mitochondrial pores, which is critical for mitochondria-mediated apoptosis. Besides induction of death receptors, downregulation of cFLIP by garcinol may also lead to enhancement of TRAIL-induced apoptosis. Recently it has been shown that Withaferin A and Rosiglitazone also enhance TRAIL-induced apoptosis through downregulation of cFLIP (29, 43).

There are reports indicating that overexpression of bax enhances cytochrome *c* release from mitochondria to the cytosol (44). Our results also establish that garcinol induces release of cytochrome *c* in cytosol and upregulation of bax and downregulation of survivin, bcl-2, and bcl-xL, but not XIAP protein. In mammalian cells, the release of cytochrome *c* from the mitochondria has been proposed as a critical event for cells to initiate the apoptotic cascade. In cytosol, cytochrome *c* plays a key role in the formation of apoptosome complex by activating the binding of procaspase-9 (45). The formation of the apoptosome then causes cleavage of caspase-9, which propagates the death signal by activating caspase-3 and causing cleavage of PARP. Activation and cleavage of PARP is the hallmark of apoptosis that in turn causes DNA fragmentation and cell death. The above results show that TRAIL alone induced apoptosis to some extent in HCT116 cells. However, resistance to TRAIL can be due to several mechanisms,

including expression of death receptors. In our study, garcinol induced DR4 and DR5 in TRAIL-resistant HT29 cells and sensitized them to TRAIL.

Taken together, our results provide the first mechanistic evidence that garcinol treatment results in ROS-mediated upregulation of DR4 and DR5 and downregulation of c-FLIP and other antiapoptotic proteins, thus rendering cancer cells more sensitive to the cytotoxic activities of TRAIL. In addition, our studies also show that the combined treatment with garcinol and TRAIL induces apoptosis in TRAIL-resistant colon cancer cells. Considering that garcinol alone is highly safe and exhibits anticancer activities *in vitro* (5–7, 12) and *in vivo* (8, 9) against a wide variety of tumors, its potential use in combination with TRAIL should be explored. Thus, these studies suggest that TRAIL can be given in combination with garcinol, a component of Malabar tamarind, especially for those tumors that develop resistance to TRAIL.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Walter Pagel for carefully editing the manuscript and providing valuable comments.

Grant Support

Clayton Foundation for Research (B.B. Aggarwal), a core grant from the National Institutes of Health (CA-16 672), a program project grant from National Institutes of Health (NIH CA-124787-01A2), and grant from Center for Targeted Therapy of M.D. Anderson Cancer Center.

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Received 12/01/2009; revised 01/25/2010; accepted 01/25/2010; published OnlineFirst 04/06/2010.

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Retraction: Garcinol Potentiates TRAIL-Induced Apoptosis through Modulation of Death Receptors and Antiapoptotic Proteins



This article (1) has been retracted at the request of the editors. Following an institutional review of the figures, it was determined that data for some of the figures could not be supported. Specifically, in Fig. 1B, the same microscopy image was used to represent two different experimental conditions: garcinol and garcinol + TRAIL. In addition, in Fig. 6B, a part of the microscopy image representing garcinol was duplicated within the image itself. The original research records provided during the institutional review did not sufficiently address these concerns.

A copy of this retraction notice was sent to the last known email addresses for all five authors. Four authors (S. Prasad, B. Sung, M.K. Pandey, and B.B. Aggarwal) did not agree to the retraction; the remaining author (J. Ravindran) did not respond.

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1. Prasad S, Ravindran J, Sung B, Pandey MK, Aggarwal BB. Garcinol potentiates TRAIL-induced apoptosis through modulation of death receptors and antiapoptotic proteins. *Mol Cancer Ther* 2010;9:856–68.

Published first September 4, 2018.

doi: 10.1158/1535-7163.MCT-18-0870

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Molecular Cancer Therapeutics

Garcinol Potentiates TRAIL-Induced Apoptosis through Modulation of Death Receptors and Antiapoptotic Proteins

Sahdeo Prasad, Jayaraj Ravindran, Bokyung Sung, et al.

Mol Cancer Ther 2010;9:856-868. Published OnlineFirst April 6, 2010.

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