ROS and CHOP are Critical for Dibenzylideneacetone to Sensitize Tumor Cells to TRAIL Through Induction of Death Receptors and Downregulation of Cell Survival Proteins

Sahdeo Prasad, **Vivek R. Yadav**, **Jayaraj Ravindran**, and **Bharat B. Aggarwal**[*]
Cytokine Research Laboratory, Department of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030.

Abstract

Because TRAIL selectively kills tumor cells, it is being tested in cancer patients. Unfortunately, patients develop resistance to the cytokine, therefore, agents which can sensitize cells to TRAIL are urgently needed. In the present study, we investigated whether dibenzylideneacetone (DBA) can sensitize cancer cells to TRAIL and potentiates TRAIL-induced apoptosis. As indicated by accumulation of the membrane phospholipid phosphatidylserine, DNA breaks, intracellular esterase activity, and activation of caspase-8, -9, and -3, we concluded that DBA potentiated TRAIL-induced apoptosis in colon cancer cells. DBA also converted TRAIL resistant-cells to TRAIL-sensitive. When examined for the mechanism, we found that DBA decreased the expression of antiapoptotic proteins and decoy recptor-2 and increased proapoptotic proteins. DBA also induced both death receptor (DR)-5 and DR4. Knockdown of DR5 and DR4 by small interfering RNA (SiRNA) reduced the sensitizing effect of DBA on TRAIL-induced apoptosis. In addition, DBA increased the expression of CHOP proteins. Knockdown of CHOP by siRNA decreased the induction of DBA-induced DR5 expression and apoptosis. Induction of receptors by DBA, however, was p53-independent, as deletion of p53 had no effect on receptor induction. We observed that DBA-induced induction of DR5 and DR4 was mediated through generation of reactive oxygen species (ROS), as N-acetylcysteine blocked the induction of death receptors and suppression of cell survival proteins by DBA. Overall, our results demonstrate that DBA potentiates TRAIL-induced apoptosis through downregulation of cell survival proteins and upregulation of death receptors via ROS-mediated CHOP activation.

Keywords

DBA; TRAIL; apoptosis; death receptors; ROS

Introduction

Cancer is a major public health problem in the United States and many other parts of the world. In 2009 in the United States, a total of 1,479,350 new cancer cases and 562,340 deaths from cancer are projected to occur (1). Also the incidence of prostate, lung, breast, and colon cancer are higher in Western countries than in Eastern countries (2). Surgical excision and/or radiotherapy typically are the first-line of treatments, but many cancers recur in spite of these. Further, although recurrent cancers may respond to chemotherapeutic, cytotoxic and immunomodulating agents but also may develop resistance to this.

[*]To whom correspondence should be addressed: Bharat B. Aggarwal, aggarwal@mdanderson.org.

The cytokine tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) emerges as one of the most-promising experimental cancer therapeutic drug which is currently being tested in clinical trials (3–5). TRAIL induces apoptosis on binding to its specific receptors called death receptors. To date, five TRAIL receptors have been reported: death receptor (DR) 5, also called TRAIL-R2, TRICK2 or (6–10), DR4, decoy receptor (DcR) 1, DcR2, and osteoprotegerin (11). Only DR4 and DR5 can mediate TRAIL-induced apoptosis. The other receptors play a dominant negative role by competing with DR4 and DR5 for interaction with TRAIL. Some studies showed that repeated administration of soluble TRAIL was not toxic to normal tissues in mice (12) and in non-human primates (13), however, other data suggest that cultured human hepatocytes may be sensitive to the soluble forms of TRAIL (14,15).

Because many human tumor cells are found to develop resistance to TRAIL (16,17), investigators are examining TRAIL pathways for ways to overcome this resistance. The resistance could be due to overexpression of cell survival proteins such as bcl-2, bcl-xl, XIAP, cIAP-1, cIAP-2, and cFLIP or to overexpression of decoy receptors or to limited expression of cell signaling death receptors on the cell surface (18–20). Therefore, agents are needed which can sensitize the cancer cells to TRAIL.

Dibenzylideneacetone (DBA, Fig. 1A) is one such agent that has been shown to induce apoptosis in colon cancer cells through a p53- independent mechanism via inhibition of isopeptidase (21). It inhibits the growth of melanoma in vitro and in vivo through inhibition of N-myristoyltransferase-1, abrogation of mitogen-activated protein kinase, suppression of Akt, downregulation of STAT-3, and inhibition of S6 kinase activation (22). Whether DBA can sensitize tumor cells to TRAIL-induced apoptosis is not known. Our investigation of this question is detailed in the present report. The results are described to show that DBA can potentiate TRAIL-induced apoptosis through downregulation of cell survival proteins, upregulation of death receptors via ROS-mediated and C/EBP homologous transcription factor (CHOP) activation.

Materials and methods

Reagents

A 50 mM solution of DBA (from Aldrich), with purity of 99%, 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, fetal bovine serum and Dichlorodihydrofluorescein diacetate (DCF-DA) were purchased from Invitrogen. Anti–β-actin antibody was obtained from Aldrich-Sigma. Antibodies against bcl-xL, bcl-2, bax, cFLIP, poly (ADP-ribose) polymerase (PARP), c-Jun-NH$_2$-kinase (JNK)-1, CHOP, phospho-Akt1/2, and annexin V staining kit were purchased from Santa Cruz Biotechnology.

Cell lines

HCT116 (human colon adenocarcinoma), HT29 (human colon adenocarcinoma), A293 (human embryonic kidney carcinoma), PC3 and DU145 (human prostate cancer cells), MDA-MB-231 (human breast cancer cells), SCC4 (human squamous cell carcinoma), Caco2 (human colon cancer cells), U266 (human multiple myeloma) and KBM-5 (human chronic leukemic cells) were obtained from American Type Culture Collection. HCT116 variants with deletion of p53 and bax were kindly supplied by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) were cultured in McCoy's 5A medium (Invitrogen). HCT116, A293, MDA-MB-231 and SCC4 were cultured in DMEM, Caco2, PC3, DU145, U266 and HT29 cell lines were cultured in RPMI1640. KBM-5 cells were cultured in

IMDM. All the media were supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin except IMDM contains 15%. The above-mentioned cell lines were procured more than 6 months ago and have not been tested recently for authentication in our laboratory.

Live/dead assay

To measure apoptosis, we used Live/Dead assay kit (Invitrogen). We stained the cells according to the manufacturer's instructions. In principle, 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. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon).

Cytotoxicity assay

The effects of DBA on TRAIL-induced cytotoxicity were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method (23).

Annexin V/PI assay

The early indicator of apoptosis was detected by using annexin V/PI binding kit (Santacruz) and then analyzed with a flow cytometer (FACS Calibur, BD Biosciences).

Flow cytometry for cell-cycle distribution

To determine the effect of DBA on the cell cycle, treated and untreated cells were stained with PI as mentioned earlier (24).

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 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 as described earlier (23).

Transfection with siRNA

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) was added to 50 nmol/L siRNA in a final volume of 100 µL culture medium. After 48 h of transfection, cells were treated with DBA for 12 h and then exposed to TRAIL for 24 h.

Measurement of ROS

Intracellular ROS of cells were detected as described elsewhere (23).

Statistical analysis

All data are expressed as mean ± SE of 3 independent experiments. Statistical significance was determined using unpaired Student's t-test and a P value of less than 0.001 was considered statistically significant.

Results

The objective of this study was to determine whether DBA can potentiate TRAIL-induced apoptosis. The mechanisms by which DBA might enhance the effect of this cytokine, was also investigated in detail. For most experiments, we employed human colorectal cancer cell line HCT116; however, our results were not restricted to this tumor cell line. We also used various other cell lines. HT29 colon cancer cells, which are known to be resistant to TRAIL, are also used to determine whether DBA can also sensitize these cells to TRAIL.

DBA potentiates TRAIL-mediated apoptosis in colon cancer cells

Whether DBA enhances TRAIL-induced apoptotic cell death was investigated by the MTT method. The HCT116 cells were moderately sensitive to either DBA or TRAIL alone. However, pretreatment with DBA significantly ($P<0.001$) enhanced TRAIL-induced cytotoxicity (Fig. 1B, *Left panel*).

To confirm the effect of DBA on TRAIL-induced apoptosis, we measured apoptosis by Live/Dead assay. We found that DBA induced up to 18% cell death and while TRAIL alone showed 10% apoptosis in HCT116 cells. Interestingly, the combination treatment with DBA and TRAIL enhanced apoptosis up to 62% apoptosis (Fig. 1B *Right Panel*).

Next, we examined the effect of DBA on TRAIL-induced apoptosis in HCT116 cells by phosphatidylserine externalization using the annexin V/PI assay. The results shown in Fig. 1C (*Upper panel*) indicate that DBA and TRAIL-induced apoptosis (including early, late and necrosis) about 29% and 23% respectively; and the combination increased the apoptosis to 81%.

To further determine the effect of DBA on TRAIL-induced cytotoxicity, we also investigated the distribution of cells by PI staining. We found that treatment of DBA or TRAIL alone showed moderate apoptosis, while treatment of both showed enhanced cell death (Fig. 1C, *Lower panel*).

In addition, we examined the effect of DBA on TRAIL- induced activation of caspase-8, -9, and -3 and on cleavage of PARP. We found that DBA enhanced TRAIL-induced activation of all three caspases, thus leading to enhanced PARP cleavage (Fig. 1D).

Taken together, these results suggest that DBA enhanced TRAIL-induced apoptosis.

DBA downregulates the expression of cell survival proteins

We next examined the mechanism underlying DBA's enhancement of TRAIL-induced apoptosis. Because various antiapoptotic proteins are known to regulate TRAIL-induced apoptosis, we investigated whether DBA potentiates TRAIL-induced apoptosis through regulation of these proteins. DBA inhibited expression of XIAP, survivin, bcl-2 and both the short and long forms of cFLIP but had no effect on expression of bcl-xL (Fig. 2A). Thus our results suggest that downregulation of cell survival proteins is one of the mechanisms by which DBA potentiates TRAIL-induced apoptosis.

DBA upregulates the expression of pro-apoptotic proteins

In our next set of experiments, we found that DBA cleaved the proapoptotic protein bid and induced the expression of pro-apoptotic bax (Fig. 2B). These two effects are additional ways DBA could enhance the apoptotic effects of TRAIL. However, DBA did not induce DR5 in bax knockout HCT116 cells indicating induction of DR5 by DBA is not dependent on bax (Supplementary fig. 1).

DBA regulates activation of GSK-3β

Glycogen synthase kinase 3 beta has been linked with resistance of cells to TRAIL (25,26). Whether DBA affects TRAIL-induced apoptosis through inhibition of GSK-3β, was examined. We found that DBA downregulated the phosphorylation of GSK-3β (Fig. 2C), thus suggesting that inhibition of activation of this kinase may also contribute to its ability to increase sensitivity of tumor cells to TRAIL.

DBA upregulates expression of death receptor TRAIL-R1/DR4 and TRAIL-R2/DR5

Because TRAIL mediates its activity through the receptors DR4 and DR5; therefore, we investigated whether DBA potentiated TRAIL-induced apoptosis is through modulation of DR5 and DR4 expression. Treatment of HCT116 cells with various concentrations of DBA for 24 h resulted in an increased expression of TRAIL-R2/DR5 and TRAIL-R1/DR4 in a dose-dependent manner (Fig. 3A *Left panel*). The effect on DR5 was more pronounced than on DR4. We also examined whether induction of the TRAIL receptor is time-dependent. For this, cells were treated with DBA (15μM) for different times and then examined for expression of DR5 and DR4 protein. DBA induced both DR5 and DR4 in a time-dependent manner (Fig. 3A *Right panel*). These data suggest that up-regulation of death receptors DR4 and/or DR5 by DBA may be another mechanism by which the agent enhances the proapoptotic effects of TRAIL in colon cancer cells.

Whether DBA enhances the expression of DRs on cell surface was also examined. We found that DBA increased cell surface levels of DR5 and DR4 (Fig. 3B). Collectively, these results indicate that DBA up-regulated the expression of both DRs on the cell surface.

DBA downregulates decoy receptor

Decoy molecules compete with the death receptors for ligand binding and thereby inhibit ligand-induced apoptosis (27,28), so we determined whether DBA modulates decoy receptor expression. We found that indeed DBA decreased the expression of DcR2, but it did not influence the level of DcR1 (Fig. 3C). Therefore inhibition of DcR2 by DBA may potentiate TRAIL-induced apoptosis.

DBA-induced upregulation of death receptors is not cell type specific

Whether upregulation of TRAIL receptors by DBA was specific to HCT116 was investigated. We exposed the following cells to 15 μM DBA: KBM-5, U266, MDA-MB-231, PC3, DU145, Caco2, A293 cells, and SCC4 cells for 24 h. DBA induced the expression of both DR5 and DR4 in almost all of these cell lines (Fig. 3D). Besides HCT116 cells, DR5 and DR4 were also induced in KBM5, U266, SCC4, A293, DU145 and PC3 cell lines. These findings suggest that the upregulation of TRAIL receptors by DBA was not cell-type specific.

DBA-induced death receptors are needed for TRAIL-induced apoptosis

The importance of death receptors to TRAIL-induced apoptosis was investigated using siRNA specific to DR5 and DR4. Transfection of cells with siRNA for DR5 but not with the control siRNA reduced DBA-induced DR5 expression (Fig. 4A). Similarly, transfection of cells with siRNA for DR4 reduced the DBA-induced DR4 expression but not DR5 (Fig. 4A).

We next examined whether the suppression of DR5 or DR4 by siRNA could abrogate the sensitizing effects of DBA on TRAIL-induced apoptosis using Live/Dead Assay. The results reveal that the effect of DBA on TRAIL-induced apoptosis was effectively abolished in cells transfected with either DR5 or DR4 siRNA (Fig. 4B), whereas treatment with control siRNA

had no effect. Silencing of DR5 had a more dramatic effect on DBA's ability to potentiate TRAIL-induced apoptosis than DR4, thus suggesting that both DR5 and DR4 play a major role in TRAIL-induced apoptosis.

DBA-induced upregulation of TRAIL receptors is p53 independent

There are numerous reports that suggest p53 can induce death receptors(10,29). Whether DBA-induced induction of TRAIL receptors is mediated through p53 was examined using HCT116 cell lines that lack p53. DBA induced DR5 and DR4 in p53 parental as well as p53 knockout HCT116 cells in a dose-dependent manner (Fig. 5A; *Left panel*). These results indicate that induction of TRAIL receptors was independent of p53 expression.

Whether DBA affects the expression of p53, was also examined. Interestingly, we found that DBA downregulated p53 at higher doses (Fig. 5A; *Right panel*) further suggesting that induction of DR4/5 is p53-independent.

DBA inhibited activation of Akt

It has been reported that inhibition of Akt1/2 activation are needed to cause TRAIL-induced apoptosis(19,30). Therefore, we determined whether DBA suppressed activation of Akt1/2. We found that DBA suppressed the phosphorylation of Akt1/2, at even its lower dose 10µM (Fig 5B; *Left panel*).

DBA induced upregulation of TRAIL receptors is not mediated through MAPK

Several reports suggest that JNK and ERK could mediate induction of TRAIL receptors (31). To determine whether DBA can activate ERK and JNK, cells were pretreated with DBA and then examined for the phosphorylated ERK and JNK (Fig. 5B; *Left panel*). No activation of either kinase was found. Thus induction of TRAIL receptors by DBA did not require either of the kinases.

DBA regulates activation of PPARγ

There are numerous reports that induction of DR5 is regulated by PPARγ ligands (32,33), so we examined whether DBA affects the expression of PPARγ. We found that DBA upregulated the PPARγ in a dose-dependent manner (Fig. 5B; *Right panel*).

DBA-induced upregulation of TRAIL receptors is mediated through activation of CHOP

It has been shown that the induction of death receptor by various agents including PPARγ agonists is mediated through activation of CHOP (32,34–36). To determine whether DBA can induce the expression CHOP, we pretreated cells with different concentration of DBA and assayed CHOP expression. We found that DBA increased the expression of CHOP (Fig. 5C).

To determine whether induction of CHOP affects the upregulation of DR, we used siRNA specific to CHOP. The results showed that the upregulation of DR5 but not DR4 by DBA was effectively abolished in cells transfected with CHOP siRNA (Fig. 5D; *Left panel*), whereas treatment with control siRNA had no effect. Thus induction of TRAIL receptors by DBA was correlated with the expression of CHOP.

Next we determined whether suppression of CHOP by siRNA abrogates DBA-induced apoptosis. We found that the effect of DBA on TRAIL-induced apoptosis was abolished in cells transfected with CHOP siRNA (Fig. 5D; *Right panel*), whereas treatment with control siRNA had no effect. This result suggests that CHOP, in part, play a role in TRAIL-induced apoptosis.

DBA induces TRAIL receptors through ROS-dependent mechanism

Whether DBA has ability to generate ROS was examined by treating HCT116 cells with DBA. The level of ROS inside the cells were measured by FACS and found that DBA induced ROS with increasing dose (Fig. 6A; *Left panel*).

Whether DBA-induced induction of TRAIL receptors is also regulated by ROS was examined. As shown in the Fig. 6B, pretreatment of HCT116 cells with the ROS scavenger N-acetylcysteine (NAC) reduced the DBA-induced upregulation of DR5 and DR4 expression in a dose-dependent manner. This suggests of ROS plays a critical role in the induction of TRAIL receptors by DBA (Fig. 6A; *Right panel*).

NAC abrogates the effect of DBA in suppression of antiapoptotic proteins

Next we examined whether NAC abrogates DBA-induced inhibition of inhibition of antiapoptotic proteins. The results revealed that pretreatment of NAC effectively abolished the affect of DBA in suppression of XIAP, survivin, cFLIP and bcl-2 (Fig. 6B). The effect of NAC in inhibiting DBA's effect in XIAP is more prominent than others.

DBA potentiates TRAIL-induced apoptosis through ROS generation

Whether ROS is needed for potentiation of TRAIL-induced apoptosis by DBA was examined. As shown in Fig 6C, pretreatment of cells with NAC markedly inhibited DBA-induced apoptosis enhancement, from 71% to 33%. We also found that NAC reversed the effect of DBA on TRAIL-induced cleavage of procaspases and PARP (Fig. 6D), again suggesting the critical role of ROS in DBA's effects on TRAIL.

DBA sensitizes TRAIL-resistant cells to TRAIL

We also investigated whether DBA sensitizes TRAIL-resistant HT29 cancer cells to TRAIL. HT29 cells were exposed to DBA, treated with TRAIL, and assayed for cell membrane permeability by Live/Dead assay. We found that DBA and TRAIL treatment alone induced 12% and 5% apoptosis, respectively, compared to 3% in control, in HT29 cells. Pretreatment with DBA and TRAIL in combination dramatically enhanced apoptosis, to 44% (Fig. 7A).

FACS analysis revealed that the combination of DBA and TRAIL enhanced apoptosis 21.2% compared to 7.2% and 2.2% by DBA and TRAIL alone, respectively (Fig. 7B, *Left panel*). Next we studied cell cytotoxicity by MTT assay. We found that HT29 cells were moderately sensitive to DBA but resistant to TRAIL alone. However, pretreatment with DBA significantly ($P<0.001$) enhanced TRAIL-induced cytotoxicity (Fig. 7B, *Right panel*).

To determine how DBA sensitizes HT29 to TRAIL-induced apoptosis, we investigated its effect on TRAIL receptors (DR4 and DR5). HT29 cells were treated with DBA and TRAIL separately for 24 h. We found that DBA induced upregulation of DR5 and DR4 but TRAIL failed to (Fig. 7C), suggesting that DBA and TRAIL in combination induced apoptosis of HT29 cells through induction of the death receptor pathway.

Discussion

In this report we describe a novel compound, DBA, that has been shown to induce apoptosis in different tumor cells through novel mechanisms (21,22). We show that DBA enhances TRAIL-induced apoptosis in colon cancer cells through a variety of mechanisms that include downregulation of survivin, cFLIP, XIAP and bcl-2; suppression of expression of decoy receptor-2; induction of bax; and upregulation of death receptors through regulation of ROS-CHOP mediated pathway.

We found that the expression of several anti-apoptotic proteins was downregulated by DBA. Numerous studies have demonstrated that cFLIP overexpression confers resistance to death receptor-mediated apoptosis (19,20). In our study, we showed that DBA decreased the level of cFLIP, which led to association of death inducing complex and apoptosis. How DBA downregulates these proteins, is unclear at present but several possible mechanisms could account for this. Various PPAR-γ agonists such as 15-deoxy-Delta(12,14)-prostaglandin J(2) have been shown to regulate antiapoptotic proteins including survivin (37) and cFLIP(38). They all contain α, β–unsaturated dienone and inhibit ubiquitin isopeptidase (21). Recently it was reported that that the synthetic cannabinoid R-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl)-(1-naphthalenyl) methanone mesylate (WIN 55,212-2) sensitizes human hepatocellular carcinoma cells to apoptosis mediated by TRAIL through downregulation of survival factors survivin, c-inhibitor of apoptosis protein 2, and Bcl-2, and this event seemed to be dependent on the PPAR-γ (39). We did find indeed that DBA induced PPAR-γ.

Our results also show that another potential mechanism by which DBA could enhance the effects of TRAIL is through induction of death receptors. The effect of DBA was more pronounced on DR5 than on DR4. We found silencing the gene of these two receptors abolished the effect of DBA on TRAIL-induced apoptosis, suggesting that induction of these receptors is a critical event in the sensitization of cells to the cytokine. We found that the silencing of DR5 had more pronounced effect on apoptosis than silencing of DR4. These results are consistent to that reported previously (24,40). That DR4 and DR5 can regulate TRAIL-induced apoptosis differentially, has been reported (41–43). How DBA induces these receptors was also investigated in detail. Although several reports suggest that induction of death receptors is mediated through expression of p53 (10,29,44), we found, by treating p53-knock-out cells with DBA that these receptors are induced through a p53-independent mechanism. In addition, DBA did not upregulate but only slightly downregulated the expression of p53 at 20 µM. These results are in agreement with those reported previously (23). Although some have found that induction of JNK or ERK is needed for induction of death receptors(45); however, we found that DBA had no effect on either of the kinases at 20 µM.

When examined for the role of CHOP/GADD153 in induction of death receptors, we found that CHOP plays a critical role in the expression of death receptors induced by DBA. First, we showed that DBA induced the expression of CHOP. Second, silencing of the gene for CHOP abolished the effect of DBA on induction of death receptors. Third, silencing of CHOP also abolished the effect of DBA on apoptosis by TRAIL. Taken together, this evidence indicates that CHOP plays an essential role in the action of DBA. Like DBA, other α, β–unsaturated dienones such as PGJ2(32) and Curcumin (46) induce death receptors through activation of CHOP. In addition induction of TRAIL receptors by endoplasmic reticulum stress (36); rottlerin (35), and WIN (39) is also mediated through CHOP expression.

We also found that induction of ROS is critical for the sensitization of cells to TRAIL by DBA. Our results show that first, DBA induced ROS in a dose-dependent manner; second, quenching of ROS by NAC abolished the DBA-induced expression of death receptors; third, quenching of ROS also abrogated the effect of DBA on TRAIL-induced apoptosis. Thus all these evidence suggest the role of ROS in action of DBA. These results are in agreement with those reported previously on induction of death receptors by Curcumin (46), PGJ2(32), proteasome inhibitors (47), withaferin (34), and zerumbone (24).

It is known that many tumor cell types including HT29 are completely resistant to TRAIL – induced apoptosis (48). Our results revealed that DBA sensitized the TRAIL-resistant HT29

cancer cells and this was accompanied by induction of the TRAIL receptors. Thus DBA has potential to enhance the effects of TRAIL in both TRAIL-resistant and –sensitive tumor cells. We also found that DBA inhibits the activity of GSK-3beta and this could also lead to sensitization of tumors to TRAIL as reported previously (25,26). Liao et al (25) reported that resistance of prostate cancer cells to TRAIL was abolished by inhibition of GSK-3beta. Akt1/2 also play important role in developing resistance to TRAIL (30). In our study, inhibition of activated Akt1/2 by DBA could sensitize TRAIL resistant cells and induce apoptosis. Overall, our study demonstrates that DBA could potentiate the apoptotic effects of TRAIL through activation of multiple mechanisms. Although, animal study have shown that DBA at a dose of 40 mg/kg/day (ip) significantly inhibited the growth of melanoma in mice (22); therefore, furthermore animal studies are warranted in combination of DBA with TRAIL and agonistic antibody to TRAIL receptor. Whether DBA induces TRAIL receptors through the upregulation of ROS and CHOP, as shown here, in the animals models will be determined in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin. 2009; 59:225–249. [PubMed: 19474385]
2. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005; 55:74–108. [PubMed: 15761078]
3. Plummer R, Attard G, Pacey S, et al. Phase 1 and pharmacokinetic study of lexatumumab in patients with advanced cancers. Clin Cancer Res. 2007; 13:6187–6194. [PubMed: 17947486]
4. Hotte SJ, Hirte HW, Chen EX, et al. A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. Clin Cancer Res. 2008; 14:3450–3455. [PubMed: 18519776]
5. Camidge DR, Herbst RS, Gordon MS, et al. A phase I safety and pharmacokinetic study of the death receptor 5 agonistic antibody PRO95780 in patients with advanced malignancies. Clin Cancer Res. 2010; 16:1256–1263. [PubMed: 20145186]
6. Pan G, O'Rourke K, Chinnaiyan AM, et al. The receptor for the cytotoxic ligand TRAIL. Science. 1997; 276:111–113. [PubMed: 9082980]
7. Sheridan JP, Marsters SA, Pitti RM, et al. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science. 1997; 277:818–821. [PubMed: 9242611]
8. Screaton GR, Mongkolsapaya J, Xu XN, Cowper AE, McMichael AJ, Bell JI. TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. Curr Biol. 1997; 7:693–696. [PubMed: 9285725]
9. Walczak H, Degli-Esposti MA, Johnson RS, et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. Embo J. 1997; 16:5386–5397. [PubMed: 9311998]
10. Wu GS, Burns TF, McDonald ER 3rd, et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet. 1997; 17:141–143. [PubMed: 9326928]
11. LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ. 2003; 10:66–75. [PubMed: 12655296]

12. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med. 1999; 5:157–163. [PubMed: 9930862]

13. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest. 1999; 104:155–162. [PubMed: 10411544]

14. Jo M, Kim TH, Seol Seol, et al. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat Med. 2000; 6:564–567. [PubMed: 10802713]

15. Lawrence D, Shahrokh Z, Marsters S, et al. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat Med. 2001; 7:383–385. [PubMed: 11283636]

16. Thorburn A, Behbakht K, Ford H. TRAIL receptor-targeted therapeutics: resistance mechanisms and strategies to avoid them. Drug Resist Updat. 2008; 11:17–24. [PubMed: 18374623]

17. Goncharenko-Khaider N, Lane D, Matte I, Rancourt C, Piche A. The inhibition of Bid expression by Akt leads to resistance to TRAIL-induced apoptosis in ovarian cancer cells. Oncogene. 2010 (in press).

18. Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther. 2005; 12:228–237. [PubMed: 15550937]

19. Nam SY, Jung GA, Hur GC, et al. Upregulation of FLIP(S) by Akt, a possible inhibition mechanism of TRAIL-induced apoptosis in human gastric cancers. Cancer Sci. 2003; 94:1066–1073. [PubMed: 14662022]

20. Irmler M, Thome M, Hahne M, et al. Inhibition of death receptor signals by cellular FLIP. Nature. 1997; 388:190–195. [PubMed: 9217161]

21. Mullally JE, Fitzpatrick FA. Pharmacophore model for novel inhibitors of ubiquitin isopeptidases that induce p53-independent cell death. Mol Pharmacol. 2002; 62:351–358. [PubMed: 12130688]

22. Bhandarkar SS, Bromberg J, Carrillo C, et al. Tris (dibenzylideneacetone) dipalladium, a N-myristoyltransferase-1 inhibitor, is effective against melanoma growth in vitro and in vivo. Clin Cancer Res. 2008; 14:5743–5748. [PubMed: 18794083]

23. 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; 9:856–868. [PubMed: 20371723]

24. Yodkeeree S, Sung B, Limtrakul P, Aggarwal BB. Zerumbone enhances TRAIL-induced apoptosis through the induction of death receptors in human colon cancer cells: Evidence for an essential role of reactive oxygen species. Cancer Res. 2009; 69:6581–6589. [PubMed: 19654295]

25. Liao X, Zhang L, Thrasher JB, Du J, Li B. Glycogen synthase kinase-3beta suppression eliminates tumor necrosis factor-related apoptosis-inducing ligand resistance in prostate cancer. Mol Cancer Ther. 2003; 2:1215–1222. [PubMed: 14617795]

26. Rottmann S, Wang Y, Nasoff M, Deveraux QL, Quon KC. A TRAIL receptor-dependent synthetic lethal relationship between MYC activation and GSK3beta/FBW7 loss of function. Proc Natl Acad Sci U S A. 2005; 102:15195–15200. [PubMed: 16210249]

27. Merino D, Lalaoui N, Morizot A, Schneider P, Solary E, Micheau O. Differential inhibition of TRAIL-mediated DR5-DISC formation by decoy receptors 1 and 2. Mol Cell Biol. 2006; 26:7046–7055. [PubMed: 16980609]

28. Meng RD, McDonald ER 3rd, Sheikh MS, Fornace AJ Jr, El-Deiry WS. The TRAIL decoy receptor TRUNDD (DcR2, TRAIL-R4) is induced by adenovirus-p53 overexpression and can delay TRAIL-, p53-, and KILLER/DR5-dependent colon cancer apoptosis. Mol Ther. 2000; 1:130–144. [PubMed: 10933923]

29. Burns TF, Bernhard EJ, El-Deiry WS. Tissue specific expression of p53 target genes suggests a key role for KILLER/DR5 in p53-dependent apoptosis in vivo. Oncogene. 2001; 20:4601–4612. [PubMed: 11498783]

30. Asakuma J, Sumitomo M, Asano T, Asano T, Hayakawa M. Selective Akt inactivation and tumor necrosis actor-related apoptosis-inducing ligand sensitization of renal cancer cells by low concentrations of paclitaxel. Cancer Res. 2003; 63:1365–1370. [PubMed: 12649200]

31. Lee TJ, Lee JT, Park JW, Kwon TK. Acquired TRAIL resistance in human breast cancer cells are caused by the sustained cFLIP(L) and XIAP protein levels and ERK activation. Biochem Biophys Res Commun. 2006; 351:1024–1030. [PubMed: 17097066]

32. Su RY, Chi KH, Huang DY, Tai MH, Lin WW. 15-deoxy-Delta12,14-prostaglandin J2 up-regulates death receptor 5 gene expression in HCT116 cells: involvement of reactive oxygen species and C/EBP homologous transcription factor gene transcription. Mol Cancer Ther. 2008; 7:3429–3440. [PubMed: 18852146]

33. Zou W, Liu X, Yue P, Khuri FR, Sun SY. PPARgamma ligands enhance TRAIL-induced apoptosis through DR5 upregulation and c-FLIP downregulation in human lung cancer cells. Cancer Biol Ther. 2007; 6:99–106. [PubMed: 17172826]

34. Lee TJ, Um HJ, Min do S, Park JW, Choi KS, Kwon TK. Withaferin A sensitizes TRAIL-induced apoptosis through reactive oxygen species-mediated up-regulation of death receptor 5 and down-regulation of c-FLIP. Free Radic Biol Med. 2009; 46:1639–1649. [PubMed: 19345731]

35. Lim JH, Park JW, Choi KS, Park YB, Kwon TK. Rottlerin induces apoptosis via death receptor 5 (DR5) upregulation through CHOP-dependent and PKC delta-independent mechanism in human malignant tumor cells. Carcinogenesis. 2009; 30:729–736. [PubMed: 19037087]

36. Yamaguchi H, Wang HG. CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. J Biol Chem. 2004; 279:45495–45502. [PubMed: 15322075]

37. Ma XM, Yu H, Huai N. Peroxisome proliferator-activated receptor-gamma is essential in the pathogenesis of gastric carcinoma. World J Gastroenterol. 2009; 15:3874–3883. [PubMed: 19701967]

38. Schultze K, Bock B, Eckert A, et al. Troglitazone sensitizes tumor cells to TRAIL-induced apoptosis via down-regulation of FLIP and Survivin. Apoptosis. 2006; 11:1503–1512. [PubMed: 16820965]

39. Pellerito O, Calvaruso G, Portanova P, et al. The synthetic cannabinoid WIN 55,212-2 sensitizes hepatocellular carcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by activating p8/CCAAT/enhancer binding protein homologous protein (CHOP)/death receptor 5 (DR5) axis. Mol Pharmacol. 77:854–863. [PubMed: 20159939]

40. Sung B, Ravindran J, Prasad S, Pandey MK, Aggarwal BB. Gossypol induces death receptor-5 through activation of ROS-ERK-chop pathway and sensitizes colon cancer cells to trail. J Biol Chem. 2010 (in press).

41. Leverkus M, Sprick MR, Wachter T, et al. TRAIL-induced apoptosis and gene induction in HaCaT keratinocytes: differential contribution of TRAIL receptors 1 and 2. J Invest Dermatol. 2003; 121:149–155. [PubMed: 12839575]

42. Chen S, Fu L, Raja SM, Yue P, Khuri FR, Sun SY. Dissecting the roles of DR4, DR5 and c-FLIP in the regulation of geranylgeranyltransferase I inhibition-mediated augmentation of TRAIL-induced apoptosis. Mol Cancer. 2010; 9:23–37. [PubMed: 20113484]

43. Ren YG, Wagner KW, Knee DA, Aza-Blanc P, Nasoff M, Deveraux QL. Differential regulation of the TRAIL death receptors DR4 and DR5 by the signal recognition particle. Mol Biol Cell. 2004; 15:5064–5074. [PubMed: 15356269]

44. Takimoto R, El-Deiry WS. Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. Oncogene. 2000; 19:1735–1743. [PubMed: 10777207]

45. Shenoy K, Wu Y, Pervaiz S. LY303511 enhances TRAIL sensitivity of SHEP-1 neuroblastoma cells via hydrogen peroxide-mediated mitogen-activated protein kinase activation and up-regulation of death receptors. Cancer Res. 2009; 69:1941–1950. [PubMed: 19223550]

46. Jung EM, Park JW, Choi KS, et al. Curcumin sensitizes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis through CHOP-independent DR5 upregulation. Carcinogenesis. 2006; 27:2008–2017. [PubMed: 16613838]

47. Chen JJ, Chou CW, Chang YF, Chen CC. Proteasome inhibitors enhance TRAIL-induced apoptosis through the intronic regulation of DR5: involvement of NF-kappa B and reactive oxygen species-mediated p53 activation. J Immunol. 2008; 180:8030–8039. [PubMed: 18523266]

48. Nawrocki ST, Carew JS, Douglas L, Cleveland JL, Humphreys R, Houghton JA. Histone deacetylase inhibitors enhance lexatumumab-induced apoptosis via a p21Cip1-dependent decrease in survivin levels. Cancer Res. 2007; 67:6987–6994. [PubMed: 17638911]
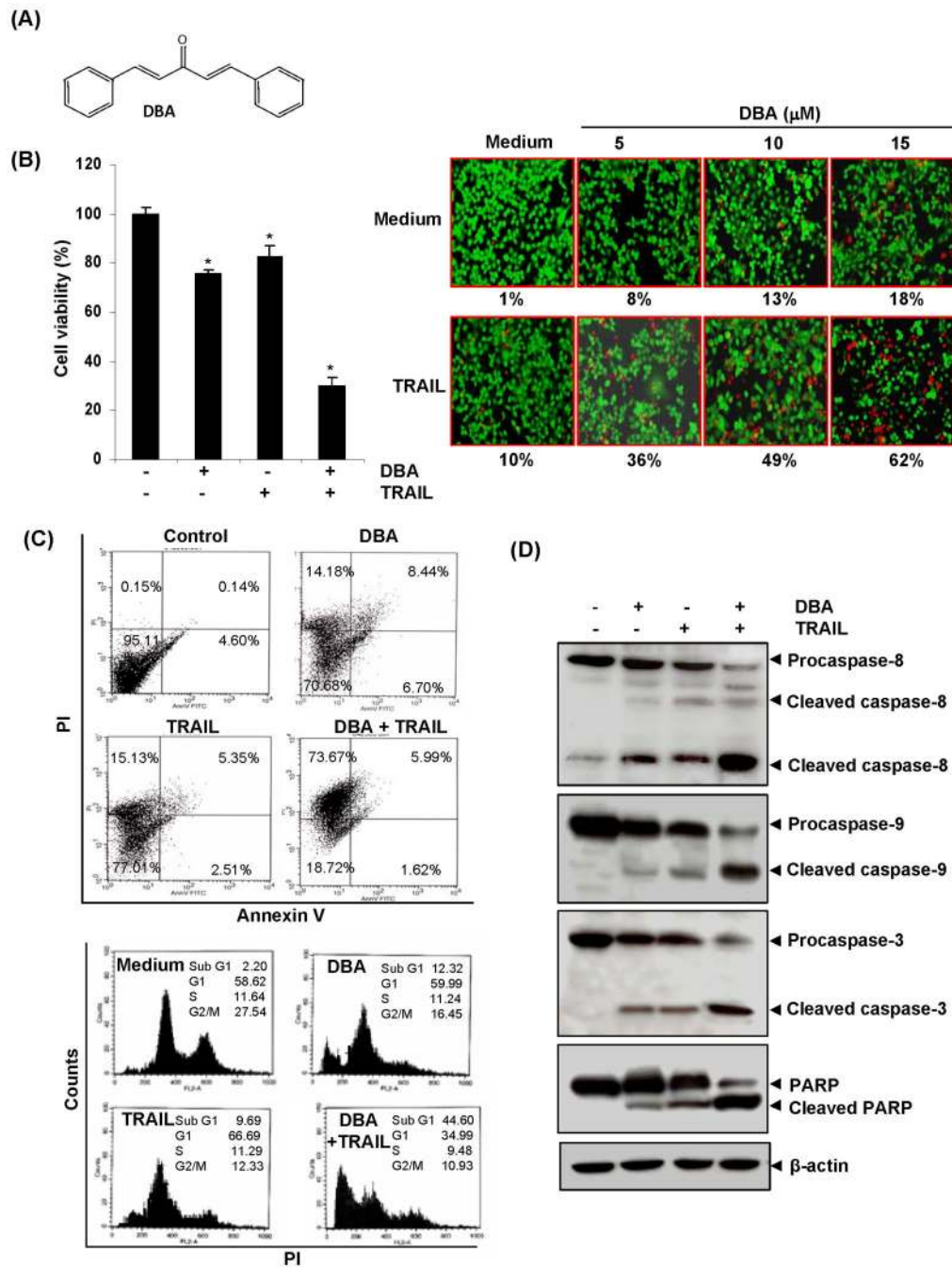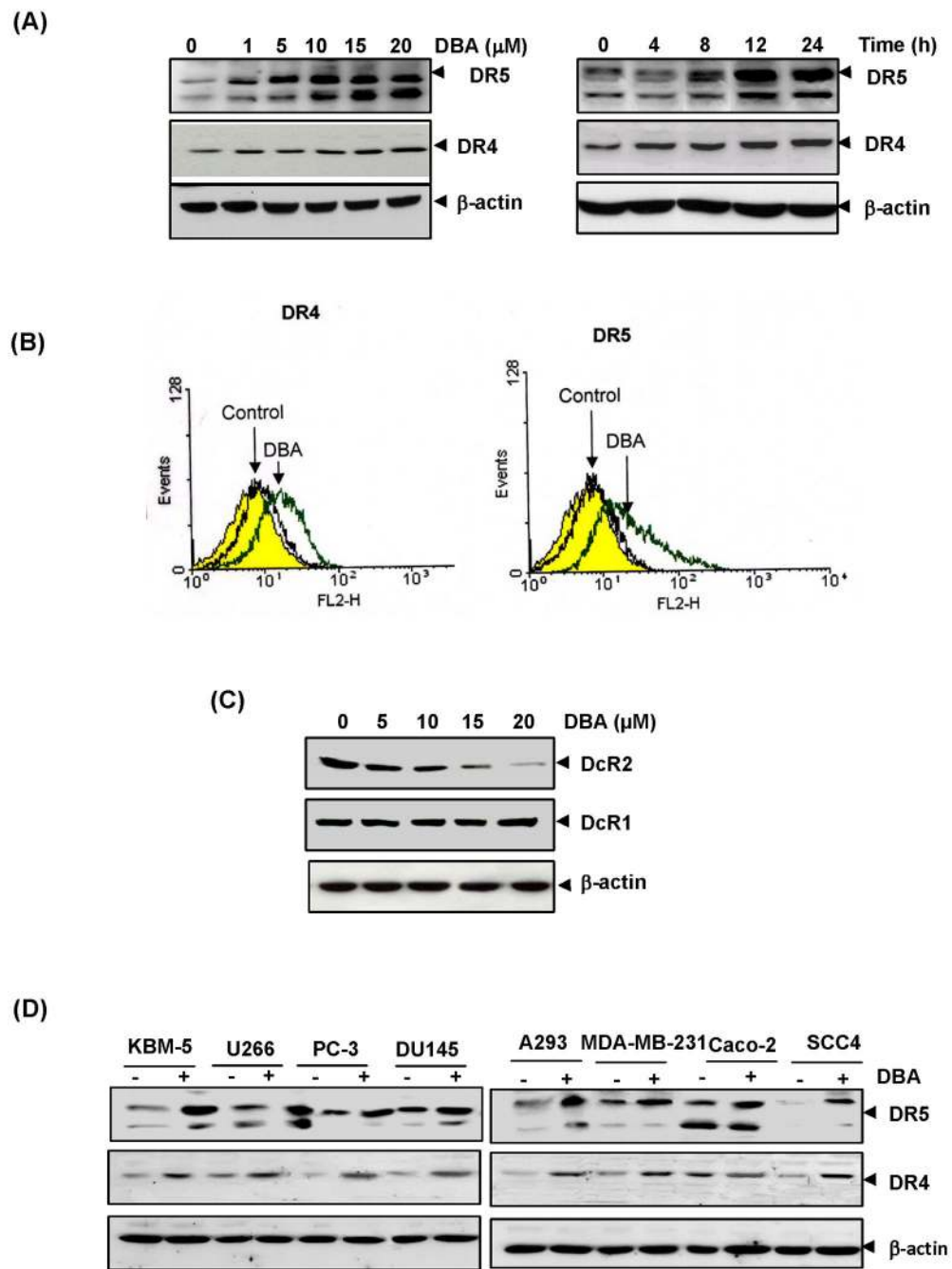
Figure 1.
DBA-potentiated TRAIL induced apoptosis of HCT116 cells. (A) Chemical structure of
DBA. (B) Cells were pretreated with 15 µM of DBA for 12 h the media were removed, and
the cells then exposed TRAIL for 24 h. Cell viability was then analyzed by the MTT method
(*Left panel*) and cell death by the Live/Dead assay (*Right panel*) using indicated
concentration of DBA. Green is live and red is dead cells. Percent dead cells are mentioned
below the photo. * indicates significant over control at P<0.001. (C) Cells were treated with
15 µM of DBA and TRAIL as described above. Cells were stained with PI/Annexin V
(*Upper panel*) and PI alone (*Lower panel*) and then analyzed by FACS. (D) Whole-cell

extracts were prepared and analyzed by Western blotting using antibodies against caspase-8, caspase-3, caspase-9 and PARP.

(A)

(B)

(C)
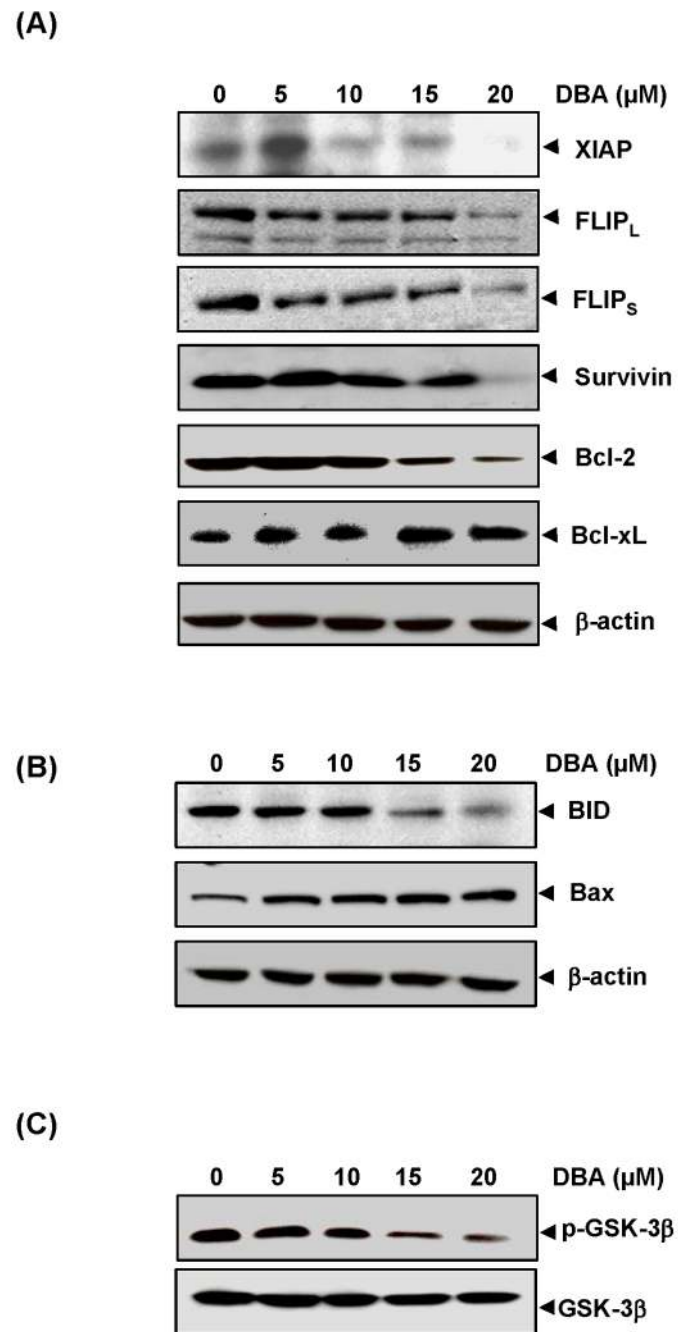
Figure 2.
Effects of DBA on antiapoptotic, pro-apoptotic and GSK-3β expression. HCT116 cells were pretreated with indicated dose of DBA for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using the antibodies against antiapoptotic (A), pro-apoptotic (B) proteins, and GSK-3β (C). The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading.

(A)

(B)

(C)

(D)

Figure 3.
DBA induces expression of death receptors and decoy receptors. (A) HCT116 cells (1×10^6 cells/well) were treated with indicated dose (*Left panel*) of DBA and time (*Right panel*). Whole-cell extracts were then prepared and analyzed by Western blotting. (B) HCT116 cells were treated with 15 µM DBA for 24 h and analyzed for cell surface DR4 and DR5 by immunofluorescent staining and subsequent flow cytometry. *Filled yellow peaks*, cells stained with a matched control phycoerythrin-conjugated IgG isotype antibody. (B) HCT116 cells were pretreated with indicated dose of DBA for 24 h. Whole-cell extracts were prepared and subjected for Western blotting. (D) DBA upregulated DR5 and DR4 in various types of cancer cells. Cells (1×10^6 cells) were treated with 15 µM DBA for 24 h, after which
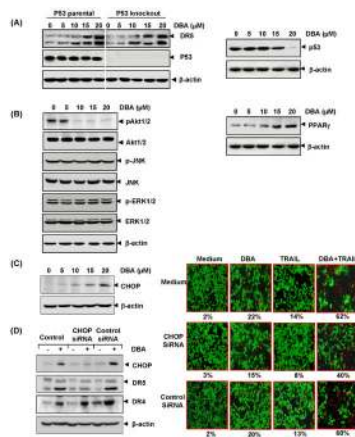
whole-cell extracts were prepared and analyzed by Western blotting. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading.
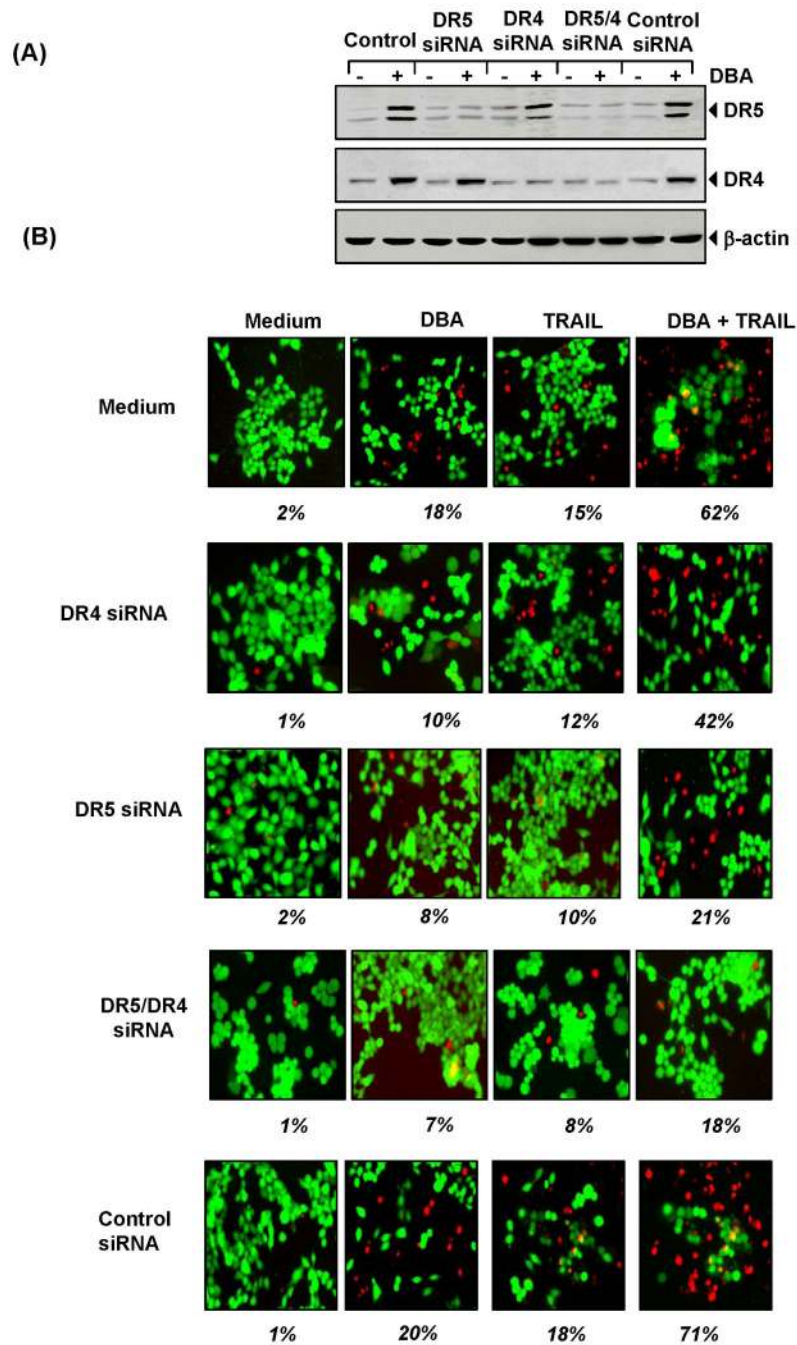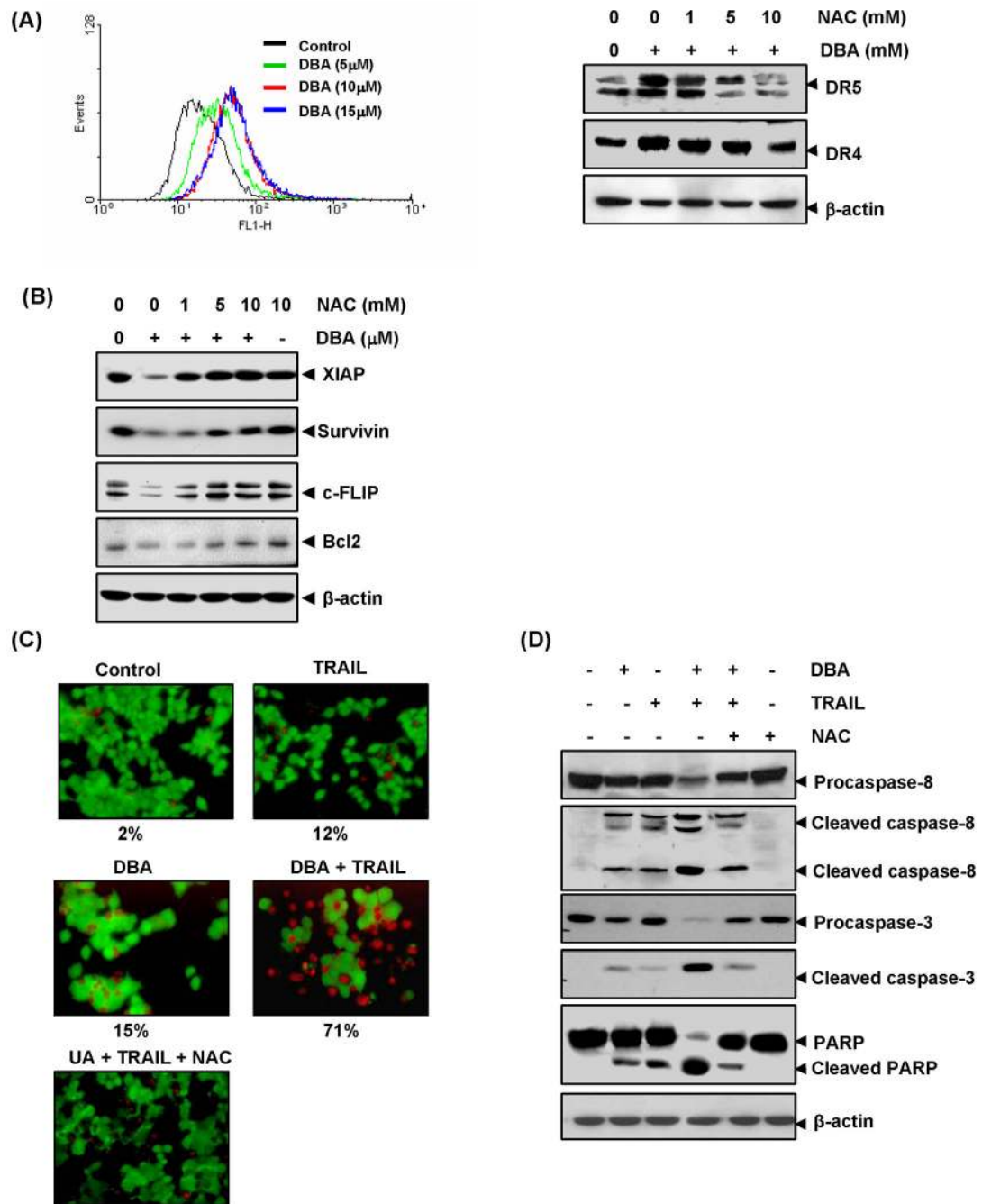
Figure 4.
Involvement of DRs on DBA-induced sensitization of TRAIL. (A) HCT116 cells were transfected with DR5 siRNA, DR4 siRNA alone or combined and control siRNA. After 48 h, cells were treated with 15 µM DBA for 24 h, and whole- cell extracts were prepared for Western blotting for DR5 and DR4. (B) Cells were seeded in a chamber slide and transfected with siRNAs. After 48 h, cells were pretreated with 15 µM of DBA for 12 h the media were removed, and then exposed to TRAIL (25 ng/mL) for 24 h. Cell death was determined by the Live/Dead Assay. Green is live and red is dead cells. Percent dead cells are mentioned below the photo.
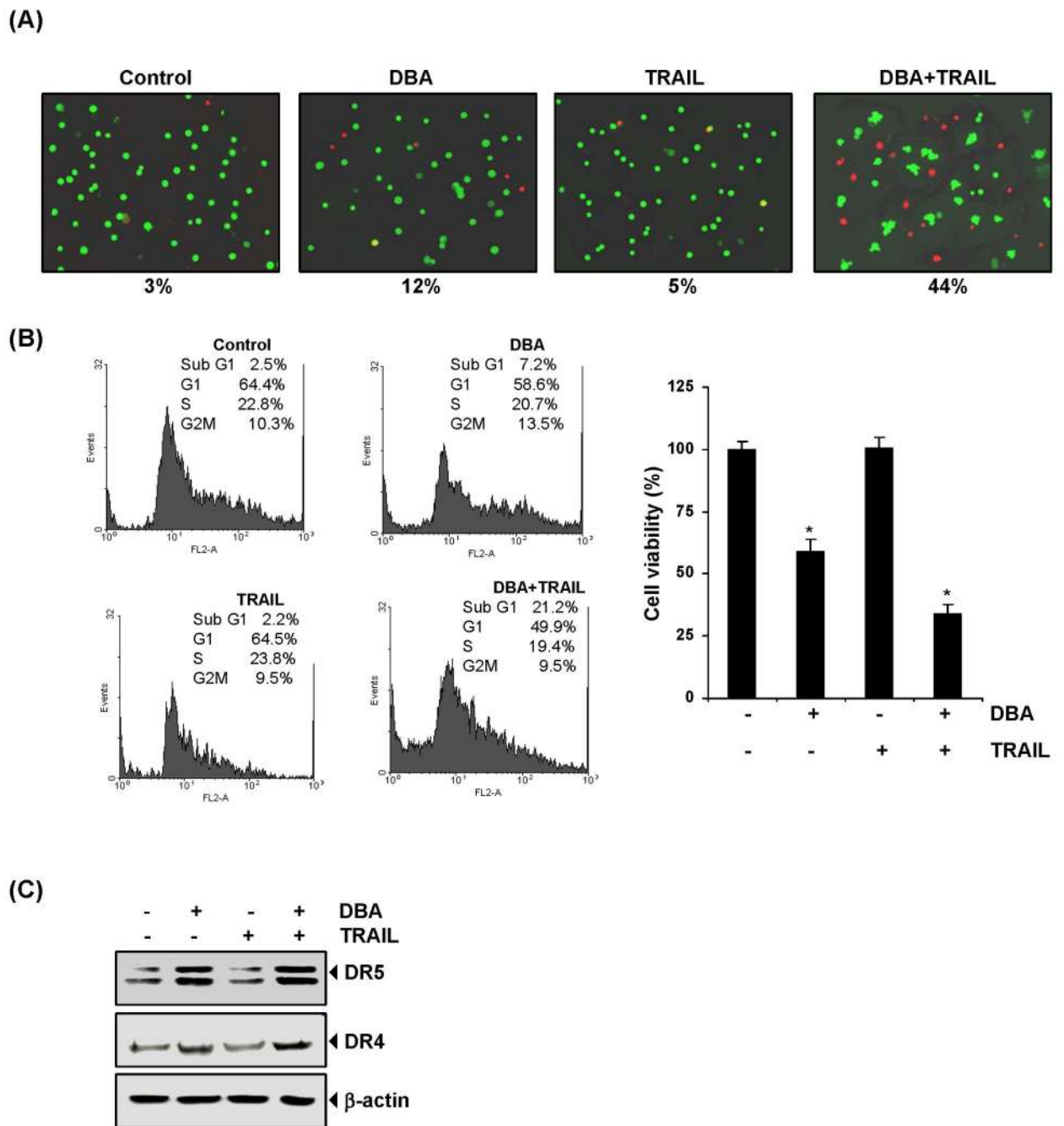
Figure 5.
Upregulation of death receptors are p53, ERK1/2 and JNK independent. (A) HCT116 (p53 parental and p53 knockout) cells (1×10^6/well) were treated with 15 µM DBA for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using p53 and DR5 antibodies (*Left panel*) and p53 antibody (*Right panel*). The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. (B) HCT116 cells were treated as indicated above and subjected to Western blotting for phosphorylated Akt1/2, ERK1/2 and JNK (*Left panel*) and PPARγ (*Right panel*), (C) CHOP. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. (D) HCT116 cells were transfected with CHOP siRNA and control siRNA. After 48 h, cells were treated with 15 µM DBA for 24 h, and whole- cell extracts were prepared for Western blotting (*Left panel*). Cells were seeded in a chamber slide and transfected with CHOP siRNAs and treated with DBA and TRAIL as indicated above. Cell death was determined by the Live/Dead Assay (*Right panel*). Green is live and red is dead cells. Percent dead cells are mentioned below the photo.

Figure 6.
DBA induces generation of ROS and DBA-induced up-regulation of DR5 and DR4 was mediated by ROS. (A) HCT116 (1×10^6 cells) cells were labeled with DCF-DA, treated with indicated concentration of DBA for 1 h and examined for ROS production by flow cytometer (*Left panel*). HCT116 cells (1×10^6 cells) were pretreated with various concentrations of NAC for 1 h and then the cells were treated with 15 µM DBA for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting (*Right panel*). (B) NAC reverses the DBA-induced inhibition of antiapoptotic proteins. HCT116 cells were pretreated with NAC for 1 h and then treated with 15 µM DBA for 24 h. Whole-cell extracts were prepared and subjected to Western blotting. The same blots were stripped and reprobed

with β-actin antibody to verify equal protein loading. (C) NAC reverses cell death induced by combination of DBA and TRAIL. HCT116 cells were pretreated with NAC (10 mM) for 1 h and then treated with 15 µM DBA for 12 h. After washing with PBS cells were treated with TRAIL (25 ng/mL) for 24 h. Cell death was determined by the Live/Dead assay. Percent dead cells are mentioned below the photo. (D) NAC inhibited caspase activation and PARP cleavage induced by combination of TRAIL and DBA. HCT116 cells were treated with NAC, DBA and TRAIL as indicated above. Whole-cell extracts were prepared and analyzed by Western blotting using the relevant antibodies. β-actin was used as a loading control.

(A)

(B)

(C)

Figure 7.
DBA sensitizes TRAIL resistance cells and induces apoptosis. HT29 cells were pretreated
with of DBA (15 µM) for 12 h. After removal of the media cells were exposed to TRAIL for
24 h. (A) Cell death was analyzed by the Live/Dead assay. Green is live and red is dead
cells. Percent dead cells are mentioned below the photo. * indicates significant over control
at P<0.001. (B) Cells were stained with PI for FACS analysis (*Left panel*) and cell viability
was determined by MTT assay (*Right panel*). (C) Whole cell extract were prepared and
subjected for western blotting using relevant antibodies.