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

Abstract

Because tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) selectively kills tumor cells, it is being tested in cancer patients. Unfortunately, patients develop resistance to the cytokine, therefore, agents that can sensitize cells to TRAIL are urgently needed. In this 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 receptor-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 show that DBA potentiates TRAIL-induced apoptosis through downregulation of cell survival proteins and upregulation of death receptors via activation of ROS and CHOP mediated pathways.

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. Furthermore, although recurrent cancers may respond to chemotherapeutic, cytotoxic, and immunomodulating agents but also may develop resistance to this.

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, 5 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 that 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 this 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 mmol/L 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...
antibodies was obtained from Aldrich-Sigma. Antibodies against bcl-xL, bcl-2, bad, cFLIP, poly (ADP-ribose) polymerase (PARP), c-Jun-NH2-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 μg/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 minutes 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 hours. 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 hours of transfection, cells were treated with DBA for 12 hours and then exposed to TRAIL for 24 hours.

**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).

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 whereas 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). 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 Figure 1C (top) 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, whereas treatment of both showed enhanced cell death (Fig. 1C, bottom).

In addition, we examined the effect of DBA on TRAIL-induced activation of caspases-8, -9, and -3 and on cleavage of PARP. We found that DBA enhanced TRAIL-induced activation of all 3 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 proapoptotic proteins**

In our next set of experiments, we found that DBA cleaved the proapoptotic protein bid and induced the expression of proapoptotic bax (Fig. 2B). These 2 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 potentiates TRAIL-induced apoptosis is through modulation of DR5 and DR4 expression. Treatment of HCT116 cells with various concentrations of DBA for 24 hours resulted in an increased expression of TRAIL-R2/DR5 and TRAIL-R1/DR4 in a dose-dependent manner (Fig. 3A, left). 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 (15 μmol/L) 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). These data suggest that upregulation 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 upregulated 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 DcR 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 μmol/L DBA: KBM-5, U266, MDA-MB-231, PC3, DU145, Caco2, A293 cells, and SCC4 cells for 24 hours. 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
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 hours, cells were treated with 15 μmol/L DBA for 24 hours, and whole-cell extracts were prepared for Western blotting for DR5 and DR4. After 48 hours, cells were pretreated with 15 μmol/L of DBA for 12 hours the media were removed, and then exposed to TRAIL (25 ng/mL) for 24 hours. Cell death was determined by the Live/Dead Assay. Percent dead cells are mentioned below the photo.
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). 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) 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 10 μmol/L (Fig 5B, left).

**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

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Figure 5. Upregulation of death receptors are p53, ERK1/2, and JNK independent. A, HCT116 (p53 parental and p53 knockout) cells (1 × 10⁶/well) were treated with 15 μmol/L DBA for 24 hours. Whole-cell extracts were prepared and analyzed by Western blotting using p53 and DR5 antibodies (left) and p53 antibody (right). 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), and PPARγ (right); 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 hours, cells were treated with 15 μmol/L DBA for 24 hours, and whole-cell extracts were prepared for Western blotting (left). 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). Percent dead cells are mentioned below the photo.
then examined for the phosphorylated ERK and JNK (Fig. 5B, left). 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).

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

It has been shown that the induction of DR 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), 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, left), whereas treatment with control siRNA had no effect. This result suggests that CHOP, in part, plays 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 HT116 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).

Whether DBA-induced induction of TRAIL receptors is also regulated by ROS was examined. As shown in the Figure 6B, pretreatment of HT116 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).

**NAC abrogates the effect of DBA in suppression of antiapoptotic proteins**

Next we examined whether NAC abrogates DBA-induced inhibition of antiapoptotic proteins. The results revealed that pretreatment of NAC effectively abolished the effect 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 Figure 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 with 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 with 7.2% and 2.2% by DBA and TRAIL alone, respectively (Fig. 7B, left). 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).

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 hours. 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 DR 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 antiapoptotic proteins was downregulated by DBA. Numerous studies have shown 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 down-regulates 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 diene and inhibit ubiquitin isopeptidase
Recently it was reported that the synthetic cannabinoid R-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrol-[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 2 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 μmol/L. 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 μmol/L.

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); rotllerin (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 cell line to 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 μmol/L. 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 μmol/L.

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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 shows 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/d (i.p.) 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.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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