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ORIGINAL ARTICLE

5, 7-Dimethoxyflavone sensitizes TRAIL-induced apoptosis through DR5 upregulation in hepatocellular carcinoma cells

Jian-Feng Yang · Jian-Guo Cao · Li Tian · Fei Liu

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Abstract
Purpose 5, 7-dimethoxyflavone (DMF) has been reported to induce apoptosis in various cancer cells. The aim of this study was to examine whether DMF sensitizes human hepatocellular carcinoma (HCC) cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis and its mechanism.
Methods Human hepatocellular carcinoma cell lines Hep3B, Huh-7, and Hep G2 and human embryo liver L-02 cells were cultured in vitro. The cytotoxic activities were determined using MTT assay. The apoptotic cell death was examined using Flow cytometry using PI staining and DNA agarose gel electrophoresis. The activities of caspase-3, caspase-8, and caspase-9 were measured using ELISA. Intracellular ROS was measured by FCM using the fluorescent probe DCHF-DA, and the expression of DR4, DR5, CHOP, GPR78, and ATF4 proteins was analyzed using Western blot.
Results Our results demonstrated subtoxic concentrations of DMF sensitize HCC cells to TRAIL-induced apoptosis and induce the death receptor 5 (DR5) expression level, accompanying the generation of reactive oxygen species (ROS) and the upregulation of CHOP, GPR78, and ATF4 protein expression. Pretreatment with N-acetylcysteine (NAC) inhibited DMF-induced upregulation of DR5, CHOP, GPR78, and ATF4 protein expression and blocked the cotreatment-induced apoptosis. Furthermore, DMF-mediated sensitization of HCC cells to TRAIL was reduced by administration of a blocking antibody or small interfering RNAs for DR5, salubrinal, an inhibitor of ER stress, and the small interfering RNAs for CHOP. However, DMF could not induce the upregulation of DR5 expression, generation of ROS, and sensitization of TRAIL-induced apoptotic cell death in human embryo liver L-02 cells or normal human peripheral blood mononuclear cells (PBMCs).
Conclusion The present study demonstrates that DMF selectively enhances TRAIL-induced apoptosis by ROS-stimulated ER-stress triggering CHOP-mediated DR5 upregulation in HCC.

Keywords Hepatocellular carcinoma · 5, 7-Dimethoxyflavone · TRAIL · DR5 · Reactive oxygen species

Introduction

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is considered a promising anticancer agent due to its ability to induce apoptosis in a variety of cancer cells but not in normal cells [1–4]. TRAIL induces apoptosis in cancer cells through the death receptor pathway [5]. TRAIL cross-links with the death receptors DR4 or DR5, leading to aggregation of the receptors, recruitment of the adaptor molecule FADD, and activation of the initiator caspase-8 [6]. The activated caspase-8 is released into the cytoplasm and initiates a protease cascade that activates effector caspases, such as caspase-3 [7]. Recent studies have shown that some cancer cells are resistant to the apoptotic effects of TRAIL [8–10]. However, TRAIL-resistant cancer cells can be sensitized by the combined treatment of chemotherapeutic drugs and TRAIL in vitro, indicating that combination therapy may overcome TRAIL resistance in cancer cells. Therefore, both the understanding of the

J.-F. Yang · J.-G. Cao (✉) · L. Tian · F. Liu
Medical College, Hunan Normal University, Changsha 410013, China
e-mail: caojianguo2005@yahoo.com.cn

molecular mechanisms of TRAIL resistance and the development of strategies to sensitize these cancer cells to induce TRAIL-triggered apoptosis are important issues for effective cancer therapy.

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and is the fourth leading cause for cancer deaths worldwide [11]. Surgical resection has been considered the optimal treatment approach, but only a small proportion of patients qualify for surgery, and there is a high rate of recurrence. Approaches to prevent recurrence have included chemoembolization and neoadjuvant therapy before and after surgery, respectively, of which neither has been proven to be beneficial [12]. Therefore, new therapeutic options are eagerly needed for more effective treatment of HCC. Although TRAIL has attracted considerable attention as a novel anticancer agent, recent studies have shown that many cancer cells, including HCC cells, are resistant to the apoptotic effects of TRAIL [13, 14]. Identification of the sensitizing agents capable of overcoming this resistance may facilitate the establishment of TRAIL-based combination regimens for the improved treatment of HCC.

7-dimethoxyflavone (DMF) is a natural flavonoid, which has been isolated from Myrtacea Leptospermum scoparium Forst, Piper methysticum Forst, and other plant sources [15–17]. Recently, many studies have shown that DMF is considered to have anticancer potential and exerts cytotoxic effects in various cancer cells [18–20]. The cytotoxic effects are due to the induction of apoptosis. Moreover, DMF strengthens the toxic effects of chemotherapeutic agents, such as daunorubicin [15], making it a useful candidate agent for cancer treatment.

We show for the first time that DMF is a potent sensitizer for TRAIL-induced apoptosis in HCC. Moreover, we present the first evidence that subtoxic concentrations of DMF upregulate the expression of DR5 via the generation of reactive oxygen species (ROS)-stimulated ER-stress triggering CHOP-mediated DR5 upregulation, leading to the induction of TRAIL-mediated cell death in HCC but not in human embryo liver L-02 cells and normal human peripheral blood mononuclear cells (PBMCs).

Materials and methods

Chemicals

Recombinant human TRAIL (the non-tagged 19-kDa protein, amino acids 114–281) (R&D Systems, Minneapolis, MN) was stored in aliquots at −80°C. 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR). 5, 7-dimethoxyflavone (DMF), N-acetylcysteine (NAC), and propidium iodide (PI) were from Sigma (St. Louis, MO). The ER stress inhibitor salubrinal was purchased from EMD Chemicals, Inc. (San Diego, CA). Mouse monoclonal anti-CHOP (B-3) and rabbit polyclonal ATF4 (CREB-2; C-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-GPR78 antibody was purchased from BD Biosciences (San Jose, CA). The Apoptotic DNA Ladder Detection Kit was from the Bodataike Company, Beijing. The Caspase 3 Activity Detection Kit, Caspase 8 Colorimetric Activity Assay Kit 25, and Caspase 9 Colorimetric Activity Assay Kit were from Millipore (Billerica, MA, U.S.A.). Caspase inhibitors, such as zVADfmk, zDEVD-fmk, zIETD-fmk, and zLEHD-fmk, were purchased from R&D Systems (Minneapolis, MN). The human recombinant DR5/Fc chimera protein was from Enzyme System Products (Livermore, CA).

Cells and cell culture

Human hepatocellular carcinoma cell lines Hep3B, Huh-7, and Hep G2 and human embryo liver L-02 cells were purchased from the China Centre for Type Culture Collection (CCTCC; Wuhan, China). The cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin, and 100 U/mL streptomycin in humidified atmosphere with 5% CO_2 at 37°C. Normal human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density-gradient centrifugation and were cultured in RPMI 1640 medium supplemented with 20% FBS. Peripheral blood mononuclear cells were acquired from a healthy volunteer after obtaining informed consent.

MTT assay

To quantify cytotoxic activities, metabolically active cells were determined using the quantitative colorimetric MTT assay. This assay was completed as described previously [21].

Flow cytometry using PI staining

The cellular DNA was stained by applying 250 μL of propidium iodide (50 μg/mL) for 30 min at room temperature. The stained cells were analyzed by fluorescent-activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content.

DNA agarose gel electrophoresis

Cells were seeded at a density 4×10^6 cells/well in 250 mL culture flasks for 24 h and then treated with

medium containing various concentrations of the test or the control agents and 10% fetal bovine serum for 24 h. The assay was completed as described previously [22].

Analysis of caspase-3, caspase-8, and caspase-9 activities

The activities of caspase-3, caspase-8, and caspase-9 were evaluated using the Caspase 3 Activity Detection Kit, the Caspase 8 Colorimetric Activity Assay Kit 25, and the Caspase 9 Colorimetric Activity Assay Kit, respectively. Briefly, cell lysates were prepared after their respective treatment with experimental agents. The assays were performed in 96-well plates by incubating 20 μg of cell lysates in 100 μL of reaction buffer (1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 10% glycerol) containing 5 μM of the caspase-3 substrate Ac-DEVD-*p*NA, the caspase-8 substrate Ac-IETD-*p*NA, or the caspase-9 substrate Ac-LEHD-*p*NA. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with an enzyme-labeling instrument (ELX-800 type). In the caspase inhibitors assay, cells were pretreated with caspase inhibitors (20 μM zVAD-fmk, zDEVD-fmk, zIETD-fmk, or zLEHD-fmk) for 1 h prior to the addition of the agents tested.

Small interfering RNA

The 25-nucleotide small interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen and had the following sequences: DR5 (E01), UUUAGC CACCUUUAUCUCAUUGUCC; DR5 (E11), AUCAG CAUCGUGUACAAGGUGUCCC, CHOP, 5′-GAGCUCU GAUUGACCGAAUGGUGAA-3′ (470–494); and green fluorescent protein (GFP), AAGACCCGCGCCGAGGU GAAG. Cells were transfected with siRNA oligonucleotides (30 nmol/L) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Measurement of ROS

Intracellular ROS accumulation was measured by FCM using the fluorescent probe DCHF-DA as previously described [22]. Cells were plated at a density of 5×10^5 in 60-mm dishes, allowed to attach for 24 h, and exposed to 10 mmol/L NAC alone, 10 μmol/L DMF alone, or NAC plus DMF for specified time intervals. The cells were stained with 10 μmol/L DCFH-DA for 10 min at 37°C. The fluorescence intensity of dichlorofluorescein in cells was determined using a flow cytometer (American BD Company, FACS 420).

Western blot analysis

Western blot analysis was carried out as previously described [22]. Anti-DR5, anti-DR4 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma) rabbit polyclonal antibodies were used as primary antibodies. The signals were detected using an ECL Advance Western blot analysis system (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA).

Statistical analysis

Data represent the mean ± SD for triplicate experiments and were analyzed using the Student's t test. Differences were considered significant from the controls when $P < 0.05$.

Results

Subtoxic concentrations of DMF sensitize HCC to TRAIL-induced apoptosis

The cytotoxic activity of TRAIL was tested in three HCC cell lines, Hep3B, Huh-7, and Hep G2. Treatment with 50–200 ng/mL TRAIL induced a limited cell viability inhibition (<15%) for 24 h, suggesting that these HCC cells are resistant to the cytotoxic effects of TRAIL. Next, we examined the cytotoxic effects of DMF alone or in combination with TRAIL in these cells. The cellular activity to reduce 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was slightly decreased in the DMF alone group. However, cell viability was significantly reduced by the combined treatment of a fixed TRAIL concentration and a varying concentration of DMF and when holding the concentration of DMF fixed and varying TRAIL (Fig. 1a–c). These results show that the combined treatment with subtoxic concentrations of DMF and TRAIL effectively induces cytotoxicity in the tested HCC cell lines. We then investigated whether apoptotic cell death is induced by a combination of DMF and TRAIL using flow cytometry, which detects the increase in hypodiploid cell populations. Cotreatment of Hep3B cells with 5 μmol/L DMF and TRAIL (25, 50, 100 ng/mL) for 24 h significantly increased the accumulation of sub-G1 phase cells, whereas treatment with DMF or TRAIL alone did not affect this population of cells (Fig. 1d). The increase in the sub-G1 population in Hep 3B cells began at 12 h and peaked at 24 h (Fig. 1e). Furthermore, DNA fragmentation analysis by agarose gel electrophoresis showed a typical ladder pattern of internucleosomal DNA fragmentation in Hep3B cells cotreated with 5 μmol/L DMF and 100 ng/mL TRAIL but not in cells treated with

DMF or TRAIL alone (Fig. 1f). Collectively, these results suggest that DMF stimulates TRAIL-induced apoptosis in HCC.

Activation of caspase-8 is required for the induction of apoptosis by combined treatment with DMF and TRAIL in HCC

We next examined whether caspases were actually activated during the induction of apoptotic cell death by the combined treatment with DMF and TRAIL in HCC cells. Treatment of HCC cells with 5 μmol/L DMF alone for 24 h did not induce any activity of caspase-3, caspase-8, and caspase-9. In response to TRAIL, the activities of caspase-3, caspase-8, and caspase-9 did not change. However, the combined treatment of DMF and TRAIL induced the activation of caspase-3 and caspase-8 and slightly activated caspase-9 (Fig. 2a–c).

We next examined which caspase was specifically activated in response to DMF and TRAIL treatment using caspase inhibitors, including the pancaspase inhibitor zVAD-fmk, the caspase-3 inhibitor zDEVD-fmk, the caspase-8 inhibitor zIETD-fmk, and the caspase-9 inhibitor zLEHD-fmk. As shown in Fig. 2d, zVAD-fmk, zDEVD-fmk, and zIETD-fmk decreased the level of activity of caspase-3 induced by the combined treatment of DMF and TRAIL, but zLEHD-fmk had no effect. zVAD-fmk and zIETD-fmk can simultaneously completely block caspase-8 activation, and zDEVD-fmk partially blocked the activation of caspase-8; however, zLEHD-fmk did not affect

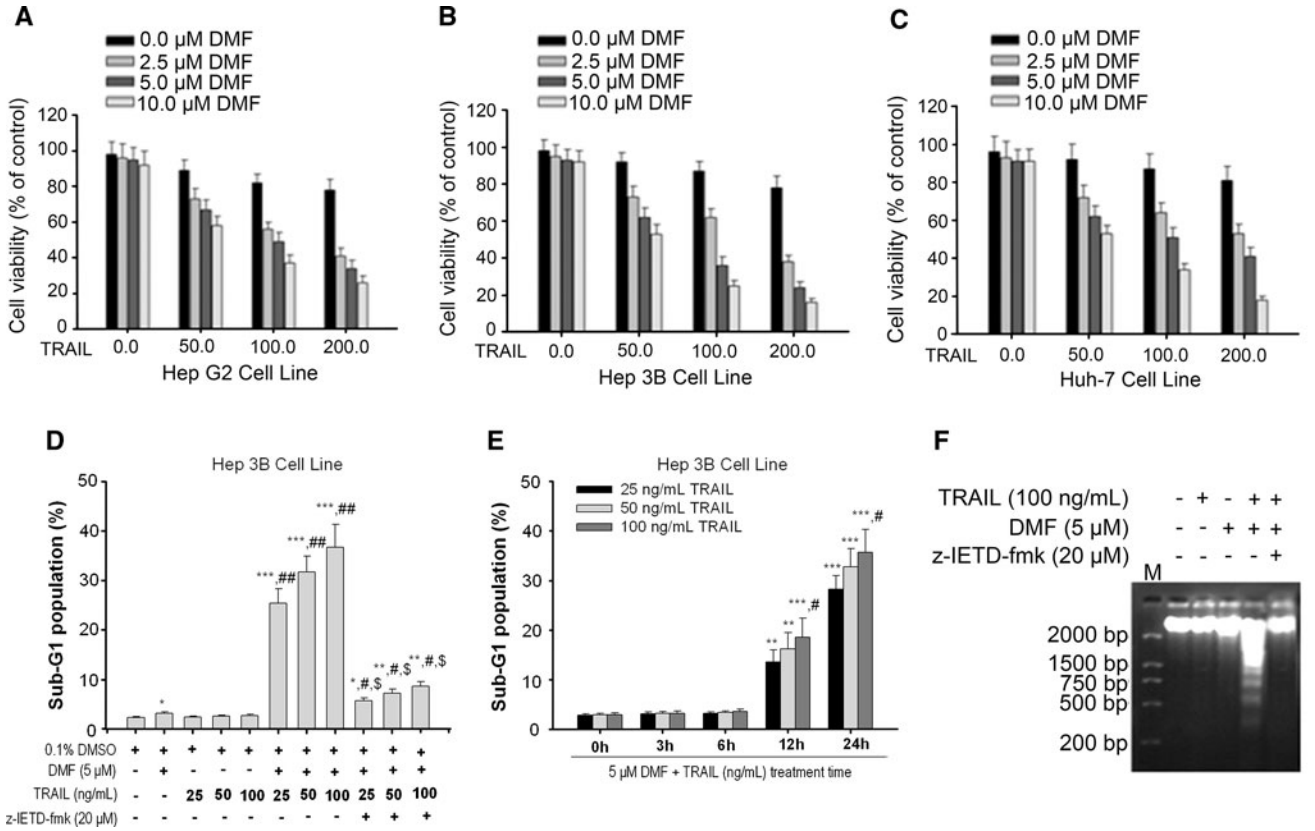

Fig. 1 Effects of DMF and/or TRAIL on hepatocellular carcinoma cell viability. **a–c** Hepatocellular carcinoma cells were treated with 5, 7-dimethoxyflavone (*DMF*) for 30 min and further treated with or without TRAIL for 24 h at the indicated concentrations. Cellular viability was assessed using the MTT assay. *Columns* average of three individual experiments, *bars* SD. **d** Hep 3B cells were treated with 5 μmol/L DMF alone, 25, 50, 100 ng/mL of TRAIL alone for 24 h, or treated with 5 μmol/L DMF for 30 min before treated with 25, 50, 100 ng/mL of TRAIL for 24 h. To examine the effect of the inhibition of caspases, Hep 3B cells were pretreated with 20 μmol/L z-IETD-fmk for 30 min and further treated as described above. The DNA contents of the cells were analyzed by flow cytometry. Data shown are the mean ± SD ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. 0.1% DMSO; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs. treatment with DMF or TRAIL alone; $^{\$}P < 0.05$ vs. treatment with DMF followed by TRAIL. **e** Hep 3B cells were pretreated with 5 μmol/L DMF for 30 min followed by treated with 25, 50, 100 ng/mL of TRAIL for the indicated times. The DNA contents of the cells were analyzed by flow cytometry. *Columns* average of three individual experiments, *bars* SD. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. treatment at 0 and 6 h, respectively; $^{\#}P < 0.05$ vs. treatment with 5 μmol/L DMF followed by 25 ng/mL TRAIL. **f** Hep 3B cells were treated 5 μmol/L DMF alone, 100 ng/mL of TRAIL alone for 24 h, or pretreated with 5 μmol/L DMF for 30 min followed by treated with 100 ng/mL of TRAIL for 24 h.. Fragmented DNA was extracted from the treated cells and analyzed on a 1.8% agarose gel

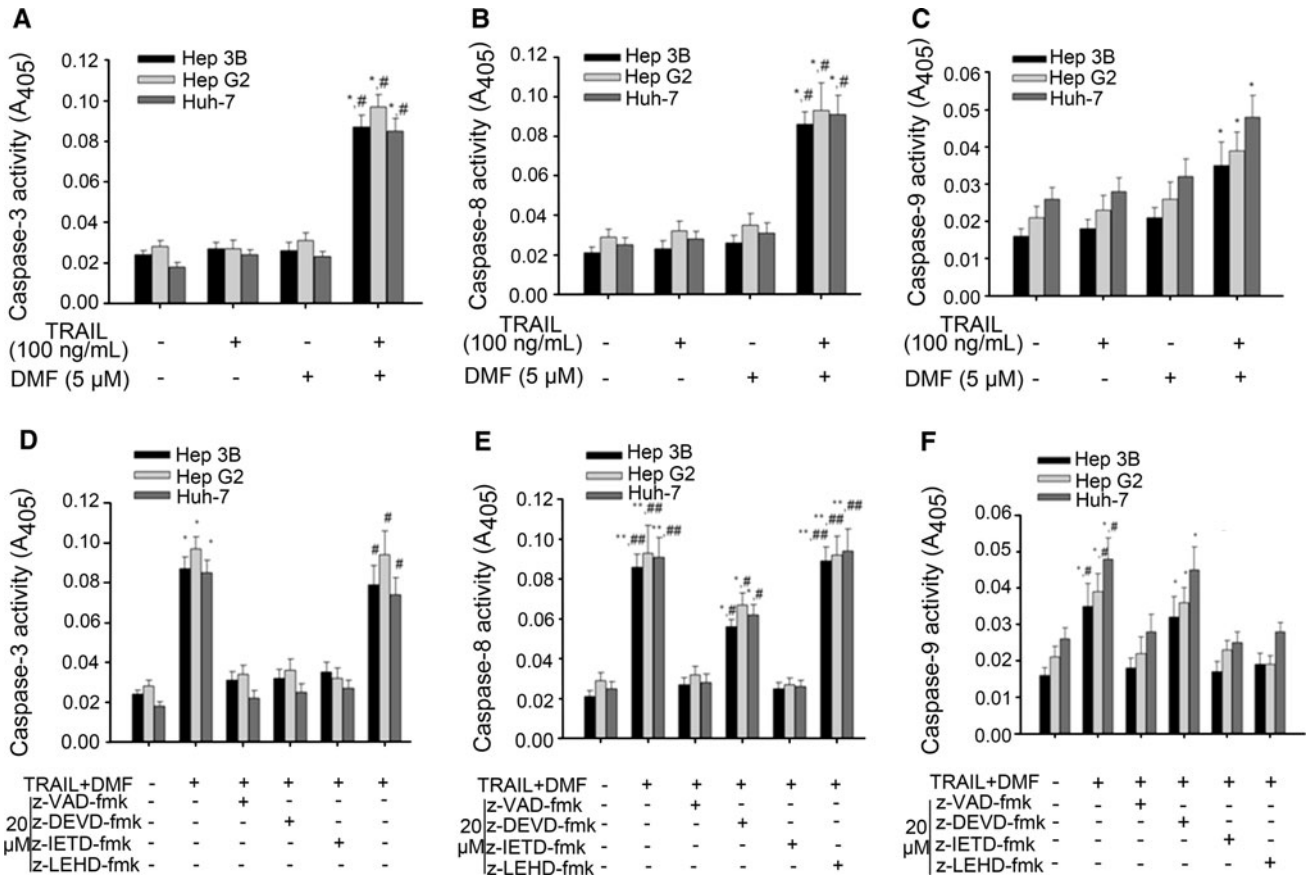

Fig. 2 Effects of DMF and/or TRAIL on the caspase activities of hepatocellular carcinoma cells. **a–c** The enzymatic activities of caspase-3, caspase-8, and caspase-9 were determined by the incubation of 20 μg of total protein with 200 μmol/L chromogenic substrate (Ac-DEVD-*p*NA or Ac-IETD-*p*NA or Ac-LEHD-*p*NA) in 100 μL assay buffer for 2 h at 37°C. The release of chromophore p-nitroanilide (*p*NA) was monitored by a spectrophotometer (405 nm). Data shown are the mean ± SD ($n = 3$). $^{*}P < 0.01$ vs. 0.1% DMSO; $^{\#}P < 0.05$ vs. treatment with DMF and TRAIL alone. **d–f** Hepatocellular carcinoma cells were preincubated with the pancaspase inhibitor zVAD-fmk, the caspase-3 inhibitor zDEVD-fmk, the caspase-8 inhibitor zIETD-fmk, and the caspase-9 inhibitor zLEHD-fmk prior to treatment with 5 μmol/L DMF for 30 min and then treatment with 100 ng/mL TRAIL for other 24 h. Data shown are the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0.1% DMSO; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs. other combined treatment

the activation state of caspase-8 (Fig. 2e). Correspondingly, we found that caspase-9 was slightly activated in cells treated with DMF and TRAIL but not in cells treated with DMF and TRAIL in the presence of z-IETD-fmk, zVAD-fmk, and zLEHD-fmk (Fig. 2f). Collectively, these results suggest that DMF with the addition of TRAIL induces caspase-8 activation upstream of caspase-9 activation.

We further examined the role of caspase-8 activation during apoptosis using the caspase-8 inhibitor z-IETD-fmk. As shown in Fig. 2d, treatment with DMF and TRAIL induced the activation of caspase-3. z-IETD-fmk interrupted the activation of caspase-3 induction by DMF and TRAIL. In addition, we carried out cell apoptotic analysis using z-IETD-fmk in Hep 3B cells. As shown in Fig. 1d and f, z-IETD-fmk rescued the increase of cell apoptotic death by the DMF and TRAIL treatment. These data indicate that the apoptosis induced by treatment with DMF and TRAIL is dependent on caspase-8 activation.

DMF upregulates DR5 in HCC

It is well known that TRAIL interacts with the death receptors DR4 and DR5 to trigger apoptotic signaling, and caspase-8 plays a central role in the apoptosis mediated by death receptors, especially DR5 [23–25]. To assess the molecular mechanisms underlying the synergistic induction of apoptosis by the combined treatment of DMF and TRAIL in HCC cells, we found that the treatment of Hep 3B cells with DMF induced a dose-dependent increase in the protein levels of DR5 (Fig. 3a). However, with the same conditions, the protein level of DR4 did not change (data not shown). The increase in DR5 expression occurred as early as 6 h after treatment and, therefore, preceded the increase of hypodiploid cell

populations after 12 h of treatment in Hep 3B cells (Figs. 2e, 3b). Consistent with this, treatment with 5 μmol/L DMF for 24 h significantly increased DR5 protein levels in other HCC cell lines, such as Hep G2 and Huh-7 (Fig. 3c), showing that upregulation of the DR5 TRAIL death receptor is a common response of HCC cells to DMF treatment.

To clarify the functional role of DR5 in stimulation of TRAIL-induced apoptosis by DMF, we examined the effect of a DR5-specific blocking chimera antibody on DMF/TRAIL-induced apoptosis. Addition of a DR5-specific blocking antibody inhibited the DMF/TRAIL-induced increase in the sub-G1 population in Hep3B cells (Fig. 3d). Furthermore, suppression of DR5 expression by the transfection of Hep3B cells with siRNA for DR5 also effectively inhibited the DMF-stimulated upregulation of DR5 and TRAIL-induced cell death (Fig. 3e, f). These results support the idea that the DMF-induced upregulation of DR5 is critical for the enhancement of TRAIL sensitivity in HCC cells.

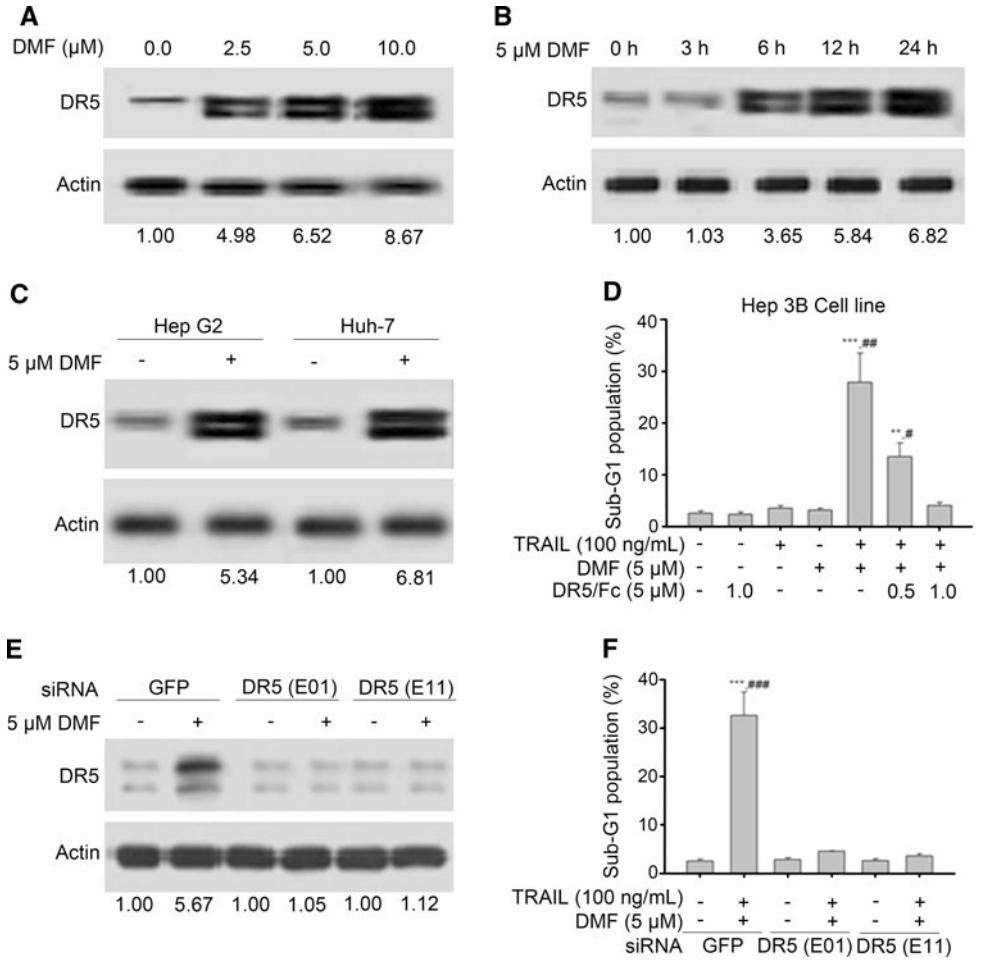

Fig. 3 DMF increases DR5 protein levels in hepatocellular carcinoma cell lines. **a** DMF-induced DR5 upregulation in Hep3B cells. Hep3B cells were treated with the indicated concentrations of DMF for 24 h, and Western blotting of DR5 and the loading control *β*-actin was completed. **b** Hep3B cells were treated with 5 μM DMF for the indicated times, and Western blotting of DR5 and the loading control *β*-actin was completed. **c** The indicated hepatocellular carcinoma cell lines were treated with 5 μM DMF for 24 h, and Western blotting of DR5 and the loading control *β*-actin was completed. **d** The effect of DR5-specific blocking chimera antibody on DMF/TRAIL-induced apoptosis. Hep3B cells were pretreated with or without 5 μmol/L DMF for 30 min followed by treatment with or without 100 ng/mL TRAIL for 24 h in the presence of the indicated concentrations of a DR5-specific blocking chimera antibody. The sub-G1 population was measured using flow cytometry. **e** Hep3B cells were transfected with 30 nmol/L of GFP siRNA or two different siRNA duplexes against DR5 mRNA. Twenty-four hours after the transfection, the cells were treated with 5 μmol/L DMF for 24 h. Western blotting of DR5 was completed to confirm the down regulation of DR5 by siRNA transfection. *β*-actin levels were assessed to show equal gel loading. **f** The suppression of DR5 expression by siRNAs reduces DMF-stimulated TRAIL-induced apoptosis in Hep3B cells. Hep3B cells were transfected with siRNAs, incubated for 24 h, and further treated with or without 5 μmol/L DMF for 30 min followed by 100 ng/mL TRAIL for 24 h. The sub-G1 population was measured using flow cytometry

DMF-induced ROS generation plays a critical role in the upregulation of DR5 and the induction of cell death by cotreatment with DMF and TRAIL

Many antineoplastic agents eliminate tumor cells by inducing apoptosis, and numerous investigations have documented that oxidative stress-mediated cellular changes are frequently induced in cells exposed to cytotoxic drugs, UV or γ-irradiation [26, 27]. Therefore, we examined whether DMF affects the cellular levels of peroxide and/or anion superoxides by measuring the changes in fluorescence using DCFH-DA. DCFH-DA-based FACS detection revealed that intracellular ROS levels increased in Hep3B cells following treatment with 2.5–10 μmol/L DMF for 3 h (Fig. 4a). Increased dichlorofluorescein (DCF) fluorescence was detected as early as 30 min after treatment with 10 μmol/L DMF, which peaked at 3 h and diminished afterward (Fig. 4b). NAC is a widely used thiol-containing antioxidant that is a precursor of reduced glutathione (GSH). Glutathione scavenges ROS in cells by interacting with OH^- and H_2O_2, affecting ROS-mediated signaling pathways. The DMF-induced increases in ROS production were completely blocked by pretreatment with 10 mM NAC (Fig. 4a). Next, we examined the role of DMF-induced ROS generation in the upregulation of DR5. Western blotting showed that pretreatment with NAC caused a significant dose-dependent inhibition of DMF-induced upregulation of the DR5 protein (Fig. 4c). We also tested whether treatment of HCC cells with H_2O_2 could elevate DR5 protein levels. We found that H_2O_2 dose-dependently increased the protein levels of DR5 in Hep 3B cells (Fig. 4d). Collectively, these results clearly show that DMF-induced upregulation of DR5 requires the generation of ROS. We next tested whether scavenging of ROS could attenuate the apoptotic cell death induced by cotreatment with DMF and TRAIL. Pretreatment of Hep 3B cells with NAC dose-dependently blocked cell death induced by the combination of DMF and TRAIL (Fig. 4e), suggesting that ROS generation is required for the induction of cell death. Taken together, these results indicate that DMF-induced ROS generation plays a key role in its ability to upregulate DR5 and, therefore, contributes to its dramatic enhancement of TRAIL-induced apoptosis.

DMF increases CHOP expression leading to DR5 upregulation involves ROS and ER stress

Recently, it has been demonstrated 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 HCC cells to apoptosis mediated by tTRAIL by the induction of DR5 via CCAAT/enhancer binding protein homologous protein(CHOP) [28]. We speculate that CHOP should also contribute to DMF-induced apoptosis in HCC cells. By Western blot analysis, we detected a time-dependent CHOP induction accompanied by DR5 upregulation in cells exposed to DMF (Fig. 5a). To test whether CHOP induction is involved in mediating DMF-induced DR5 upregulation, we silenced CHOP expression via siRNA transfection to block DMF-induced CHOP expression. As presented in Fig. 5b, we detected CHOP induction in cells transfected with the control siRNA, but not or only minimally in CHOP siRNA-transfected cells after exposing to DMF, indicating a successful silencing of CHOP expression. Accordingly, we detected DR5 upregulation only in control siRNA-transfected cells when treated with DMF. Thus, these data clearly indicate that DMF-induced DR5 upregulation is secondary to CHOP induction. Next, we demonstrated the sub-G1 population in CHOP siRNA-transfected cells significantly reduced compared with that in control siRNA-transfected Hep 3B cells using flow cytometry analysis, when exposed to DMF followed by TRAIL (Fig. 5c). These results collectively indicate that blockade of CHOP induction attenuates the cotreatment with DMF- and TRAIL-induced apoptosis. Thus, we conclude that CHOP induction contributes to DMF-stimulated induction of apoptosis by TRAIL.

Given that CHOP is known to be a featured ER stress marker protein involved in ER stress-mediated apoptosis, we further determined whether DMF induces ER stress. DMF elevated the levels of GPR78 and ATF4 (Fig. 5a). Together, these results suggest that DMF induces ER stress. To determine the impact of ER stress on DMF-enhanced apoptosis induced by TRAIL, we compared the effects of the cotreatment with DMF and TRAIL on the induction of apoptosis in the absence and presence of salubrinal, an inhibitor of ER stress-induced apoptosis. Salubrinal (50 μmol/L) significantly protected Hep 3B cells from the cotreatment with DMF- and TRAIL-induced cell death. Thus, it appears that ER stress is involved in the cotreatment with DMF- and TRAIL-induced apoptosis.

Moreover, we examined the role of DMF-induced ROS generation in the induction of ER stress and the expression of CHOP. Western blotting showed that pretreatment with NAC caused inhibition of DMF-induced upregulation of CHOP, GPR78, and ATF4 proteins (Fig. 5d). These data suggest that induction of ER stress and CHOP protein expression seem to be associated with the generation of ROS by DMF treatment.

Combined treatment with DMF and TRAIL did not induce apoptosis in human embryo liver L-02 cells and PBMCs

Because we found that the combined treatment with DMF and TRAIL strongly induced apoptosis in HCC

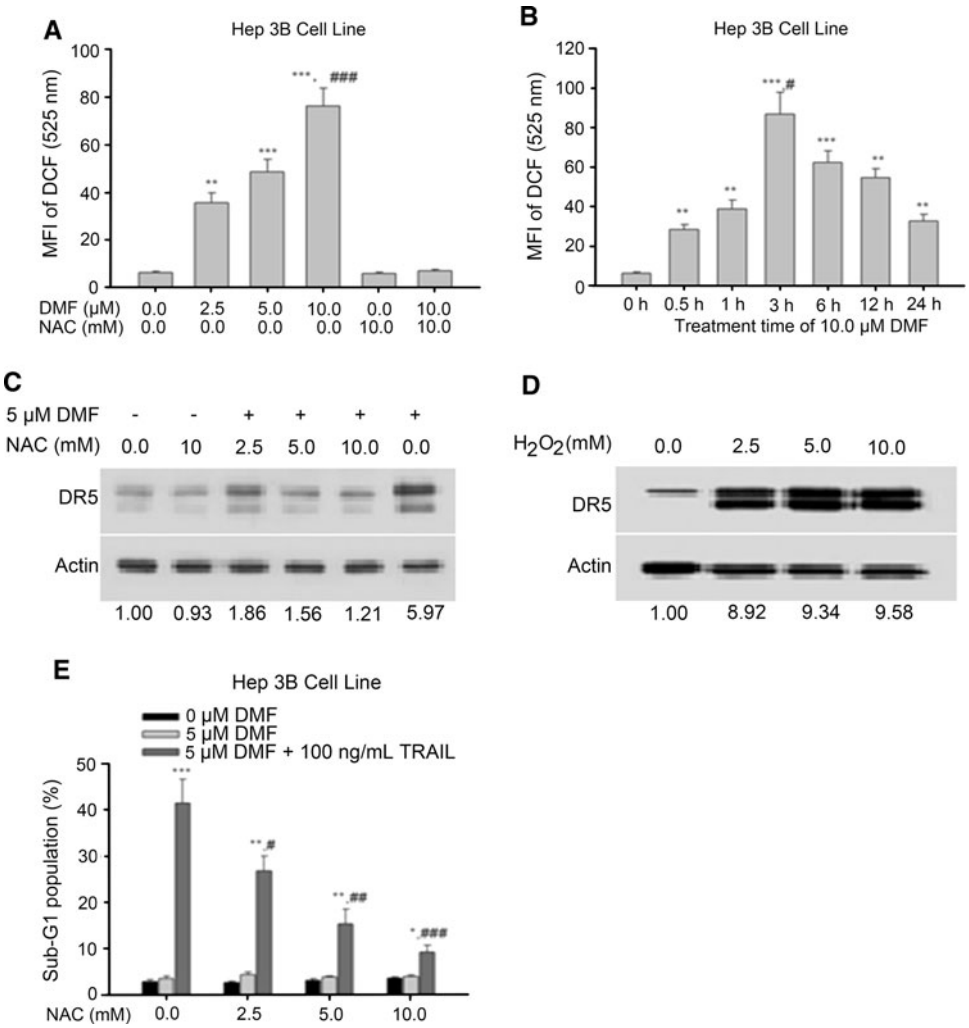

Fig. 4 ROS generation is critical for DMF-induced DR5 upregulation and DMF/TRAIL-induced cell death in hepatocellular carcinoma cells. **a** The mean fluorescence intensity of DCF was measured by FCM using a DCFH-DA fluorescence probe of Hep 3B cells treated with 2.5, 5.0 or 10.0 μM DMF in the presence or absence of 10 mM NAC for 3 h. Data shown here were the results of three independent experiments, expressed as the mean ± SD, $n = 3$. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. 0.1% DMSO; $^{\#\#\#}P < 0.001$ vs. pretreatment with 10 mM NAC followed by cotreatment of 10 mM NAC and 10.0 μM DMF. **b** Hep 3B cells were treated with 10.0 μM DMF at the indicated times, and then ROS generation was tested by FCM using a DCFH-DA fluorescence probe. Data shown here were the results of three independent experiments, expressed as the mean ± SD, $n = 3$. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. treatment at 0 h; $^{\#}P < 0.05$ vs. treatment at other indicated times. **c** NAC treatment blocks DMF-induced increases in DR5 protein levels. Hep3B cells were pretreated with NAC at the indicated concentrations for 30 min and further treated with or without 5 μmol/L DMF for 24 h. Cell extracts were prepared for Western blotting of DR5, and β-actin was used as a loading control. Representative of three independent experiments. **d** Effect of H_2O_2 on DR5 protein levels. Cell extracts were prepared from Hep3B cells treated with the indicated concentrations of H_2O_2 for 24 h, and Western blotting of DR5 and the loading control β-actin was completed. **e** Treatment with NAC blocks the cell death induced by DMF + TRAIL. Hep3B cells were pretreated with NAC at the indicated concentrations for 30 min and further treated with 5 μmol/L DMF for 30 min followed by treated with 100 ng/mL TRAIL for 24 h. The DNA contents of the cells were analyzed by flow cytometry. Data shown are the mean ± SD ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. 0.1% DMSO; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs. treatment with DMF followed by TRAIL

cells, we next examined the effect of the treatment on normal human cells. The recombinant human TRAIL was reported to induce massive apoptosis in normal human liver hepatocytes despite the demonstration that only the polyhistidine tagged or leucine zipper motif containing versions of recombinant human TRAIL with low zinc contents are toxic to the normal cells [29–32]. In addition, the side effects of chemotherapeutic agents on hematopoietic cells are a major problem during clinical treatment. Thus, we used human embryo liver L-02 cells and normal human PBMCs as models. Interestingly, the combination of DMF and TRAIL did not induce apoptosis in L-02 cells and normal human PBMCs (Fig. 6a). Furthermore, in L-02 cells and normal

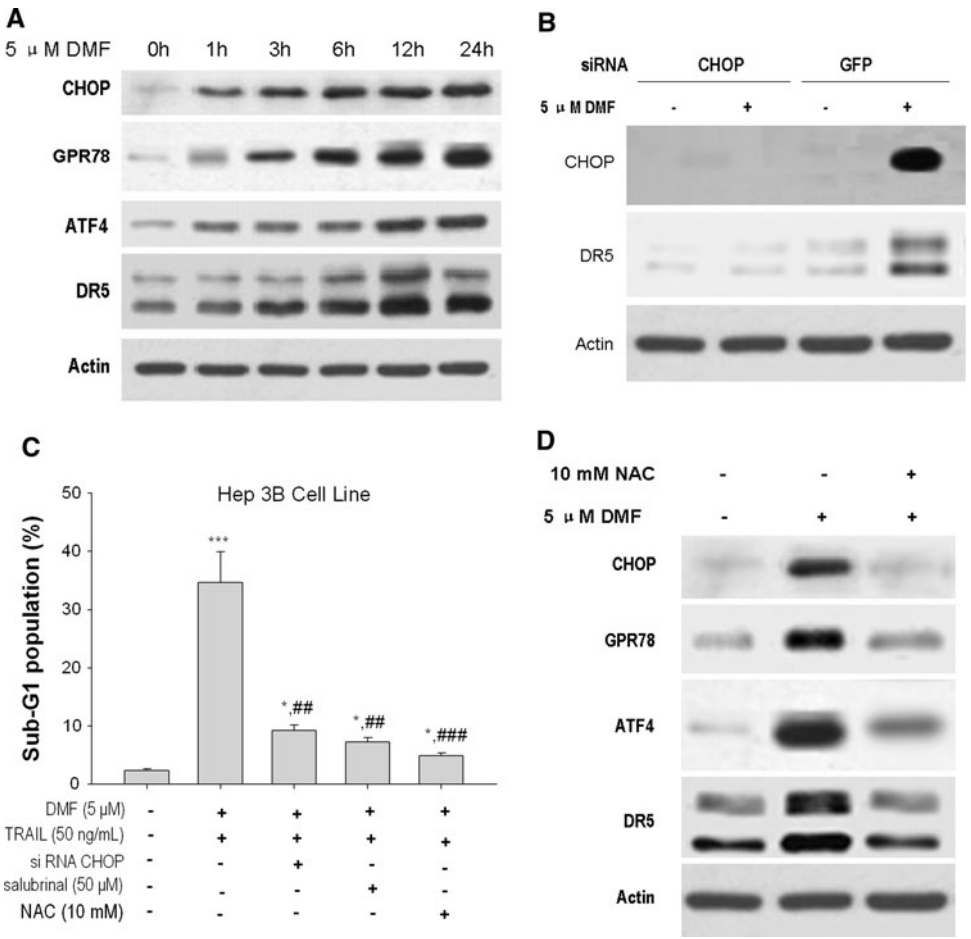

Fig. 5 DMF increases ER stress-related CHOP expression leading to DR5 upregulation involves ROS and ER stress. **a** Hep 3B cells were treated with 5 μmol/L DMF for the indicated time. The expressions of CHOP, GPR78, ATF4, and DR5 proteins were analyzed using Western blotting. β-actin was used as a loading control. **b** Hep3B cells were transfected with 30 nmol/L of GFP siRNA or CHOP siRNA duplexes against CHOP mRNA. Twenty-four hours after the transfection, the cells were treated with 5 μmol/L DMF for 24 h. The expressions of CHOP and DR5 proteins were analyzed using Western blotting. β-actin was used as a loading control. **c** Hep 3B cells were treated with 5 μmol/L DMF followed by 50 ng/mL TRAIL for 24 h at presence or absence of 50 μmol/L salubrinal or 10 mM NAC or 30 nmol/L of CHOP siRNA duplexes. Data shown are the mean ± SD ($n = 3$). $^{*}P < 0.05$; $^{***}P < 0.001$ vs. 0.1% DMSO; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs. treatment with DMF followed by TRAIL. **d** Hep 3B cells were treated with 10 mmol/L NAC for 30 min followed by 5 μmol/L DMF for 24 h. The expressions of CHOP, GPR78, ATF4, and DR5 proteins were analyzed using Western blotting. β-actin was used as a loading control

human PBMCs, DMF did not induce DR5 protein expression and ROS generation (Fig. 6b, c).

Discussion

In this study, we investigated the ability of DMF, a natural flavonoid, to enhance TRAIL-induced apoptosis in HCC. We demonstrate for the first time that subtoxic concentrations of DMF sensitize HCC cells to TRAIL-induced apoptosis (Fig. 1). Furthermore, we examined whether the combined treatment of DMF and TRAIL preferentially induced caspase-8 activity (Figs. 1 and 2). The activation of caspases plays an important role in apoptosis triggered by various proapoptotic signals [33, 34]. It is generally recognized that there are two major apoptotic pathways; one involves death signals transduced through death receptors, and the other relies on a signal from the mitochondria [22, 34]. Both pathways are involved in an ordered activation of a set of caspases, which in turn cleave cellular substrates leading to the morphological and biochemical changes in apoptosis. The activation of caspase-8 and caspase-9 has been documented to play central roles in mediating apoptosis signaled by death receptors and by mitochondria, respectively [22, 34]. However, caspase-8 can activate the caspase-9-mediated apoptotic pathway via activating or cleaving the Bid protein [33, 34]. In the present study, we found that the combined treatment of DMF and TRAIL activated caspase-8. The presence of the caspase-8 inhibitor z-IETD-fmk abrogated the apoptosis

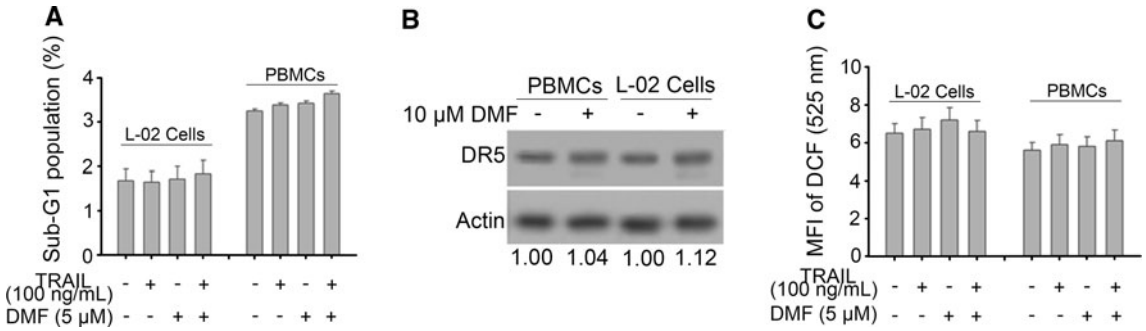

Fig. 6 DMF did not induce the expression of DR5 or generation of ROS, and DMF/TRAIL did not induce apoptosis in human embryo liver L-02 cells and PBMCs. **a** L-02 cells and PBMCs were treated with 5 μmol/L DMF, 100 ng/mL TRAIL or in combination for 24 h and analyzed by flow cytometry. The *columns* represent the mean from three independent experiments, and the bars represent the standard deviation. **b** L-02 cells and PBMCs were treated with 10 μmol/L DMF for 24 h, and Western blotting of DR5 and the loading control β-actin was completed. **c** The mean fluorescence intensity of DCF was measured by FCM using a DCFH-DA fluorescence probe in L-02 cells and PBMCs treated with 5 μmol/L DMF, 100 ng/mL TRAIL or in combination for 3 h. Data shown here were the results of three independent experiments expressed as the mean ± SD, $n = 3$

induced by the combined treatment of DMF and TRAIL, indicating that caspase-8 activation is required for the apoptosis induced by DMF and TRAIL in HCC cells (Figs. 1 and 2). Moreover, DMF upregulated DR5 expression, and silencing of DR5 expression using a DR5 siRNA abrogated the combined treatment of DMF and TRAIL-induced apoptosis, which shows that DR5 upregulation is required for DMF and TRAIL-induced apoptosis (Fig. 3). Therefore, we conclude that the combined treatment with DMF and TRAIL induces DR5-mediated caspase-8 activation and the induction of apoptosis in HCC cells. Thus, our results, for the first time, highlight a novel death receptor-mediated mechanism by which the combined treatment with DMF and TRAIL induces apoptosis in HCC cells.

Recently, it has been suggested that oxidative stress plays a role as a common mediator of apoptosis [34]. We and other groups reported that some flavonoids generate reactive oxygen intermediates in cancer cells [22, 35]. The accumulation of intercellular ROS leads to the disruption of the mitochondrial membrane potential, the release of cytochrome c into the cytosol with subsequent activation of the caspase cascade, and ultimately to apoptosis [36]. In this study, we demonstrate that exposure of Hep 3B cells to 2.5, 5.0, or 10 μmol/L DMF induces ROS generation. DMF-induced ROS generation was evident as early as 30 min and peaked at 3 h after treatment with DMF, whereas a significant increase in protein levels of DR5 was observed only after 6 h. Pretreatment with NAC blocked the DMF-induced increase in DR5 expression. In addition, treatment with H_2O_2 significantly enhanced DR5 protein levels. Finally, pretreatment of Hep 3B cells with NAC blocked the induction of cell death by the combined treatment of DMF and TRAIL. These results collectively show that ROS act as upstream signaling molecules for the initiation of DMF-induced DR5 expression and are critical for the sensitization of the cells to TRAIL-induced apoptosis.

In addition, several papers have reported that ER stress-mediated C/EBP homologous protein (CHOP) is a potential transcription factor for DR5 [28, 37]. Here, we provide evidence that the CHOP transcription factor is critical for DMF-induced DR5 upregulation. First, DMF induces CHOP expression (Fig. 5a). Second, siRNA-mediated CHOP knockdown significantly blocks DMF-induced DR5 upregulation (Fig. 5b). Third, siRNA-mediated CHOP knockdown significantly attenuated the induction of apoptosis by combined treatment of DMF and TRAIL (Fig. 5c). In our study, DMF also induces GPR78 and ATF4 proteins (Fig. 5a), and salubrinal apparently protects Hep 3B cells from the cotreated with DMF and TRAIL-induced apoptosis (Fig. 5c). Thus, DMF upregulates DR5 via ER stress-related CHOP induction in HCC cells. The study by Eom et al. shown that berberine induces apoptosis via ER stress through the elevation of ROS in human glioblastoma T98G cells [38]. Here, our results also revealed that NAC caused inhibition of DMF-induced upregulation of CHOP, GPR78, and ATF4 proteins (Fig. 5d). These data suggest that generation of ROS seems to be necessary for the induction of ER stress and CHOP protein expression by DMF treatment. However, the mechanism underlying DMF-induced ROS-dependent CHOP upregulation in ER stress needs further investigation.

Clinically, resistance to apoptosis is a major obstacle in the chemotherapeutic treatments of cancers. The apoptosis-inducing ability of the combined treatment of DMF and TRAIL, in conjunction with its nontoxic nature, could make it a potentially effective preventive and/or

therapeutic tool against tumor progression. Moreover, the combination of DMF and TRAIL demonstrated considerable cytotoxicity not only in human hepatoma cells with wild-type p53 (Hep G2 cells; Ref. [38]) but also in those with mutant p53 [Huh-7 cells, Hep3B (p53 deleted)] commonly caused by DR5 upregulation. These results suggest that this combined treatment of DMF and TRAIL could be useful for HCC cells whose p53 function has been compromised by aflatoxin B-mediated mutations or by binding of the X protein of the hepatitis B virus [36, 39]. Thus, in terms of a clinical perspective, the combination of DMF and TRAIL may be a novel strategy for the treatment of a variety of human cancers that are resistant to chemotherapy. However, the potential clinical implications of our studies will depend on whether DMF can be given safely to humans at high enough doses to achieve pharmacologically active levels. Clinical trials of oral flavonoids cannot be utilized because of poor bioavailability due to their rapid metabolism in liver and intestinal wall. However, Walle et al. [37, 40] reported that DMF was clearly detected in plasma with a peak concentration of 2.5 ± 0.8 μM (mean ± SEM) at 1 h after gavage (5 mg/kg each) in a rat in vivo model. The area under the plasma concentration–time curve was 58.8 μg mL^{-1} min. DMF was also clearly detected in liver, lung, and kidney tissues, with concentrations in the liver exceeding those in the plasma by as much as sevenfold, i.e., 16.5 ± 5 μM at 1 h after the dose. The liver, which showed the highest accumulation of DMF, is predicted to be a site where DMF might exert its greatest activity. Therefore, additional in vivo studies are needed to evaluate the applicability of DMF as a chemopreventive and/or therapeutic agent for cancer.

The study by Walle et al. [37, 40] demonstrated that DMF was a 10 times more potent inhibitor of cell proliferation (IC_{50} values 5–8 microM) than the corresponding unmethylated analogs of chrysin. Importantly, DMF inhibited the proliferation of the cancer cell lines, including oral squamous cell carcinoma SCC-9 cells and breast cancer MCF-7 cells, and had very little effect on two immortalized normal cell lines, virus-transformed normal human esophageal epithelial HET-1A cells, and virus-transformed normal human lung epithelial BEAS-2B cells. In the present study, we showed that the combined treatment of DMF and TRAIL was specific to the induction of apoptosis in human HCC cells but not in L-02 cells and PBMCs (Figs. 1, 5), although the mechanism of the selective induction of apoptosis needs to be investigated. Our findings suggest that the combined treatment of DMF and TRAIL may be an effective low toxic therapeutic strategy for specific cancers.

In conclusion, DMF selectively enhances TRAIL-induced apoptosis associated with ROS-triggered ER stress-related CHOP-mediated DR5 upregulation in HCC cells, and the use of TRAIL in combination with subtoxic doses of DMF may provide an effective therapeutic strategy for safely treating hepatocellular carcinoma.

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