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Up-regulation of Death Receptor 5/TRAIL-R2 Mediates Apoptosis Induced by *N,N'*-[(3,4-dimethoxyphenyl)methylene] Biscinnamide in Cancer Cells

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Abstract. *Background/Aim: Based on the cytotoxic agent (–)-zampanolide, N,N'-(arylmethylene)bisamides were designed and synthesized as candidate anti-cancer agents. Among them, N,N'-[(3,4-dimethoxyphenyl)methylene]biscinnamide (DPMBC) was identified as the most potent cytotoxic analog against cancer cells. In this study, we investigated the mechanisms underlying DPMBC-induced cell death in HL-60 human promyelocytic leukemia and PC-3 human prostate cancer cells. Materials and Methods: Cell growth was assessed by the WST-8 assay. Induction of apoptosis was assessed by nuclear morphology, DNA ladder formation, and flow cytometry using Annexin V staining. Activation of factors in the apoptotic signaling pathway was assessed by western blot analyses. Knockdown of death receptor 5 (DR5) was performed using siRNA. Results: DPMBC up-regulated expression levels of DR5 protein and induced apoptosis through the extrinsic apoptotic pathway mediated by DR5 and caspases. Conclusion: DPMBC is an extrinsic apoptosis inducer, which has potential as a therapeutic agent for cancer therapy.*

Marine natural products have proved an abundant source of promising candidates in the field of anti-cancer drug discovery (1). (–)-Zampanolide is a unique 20-membered macrolide isolated from the marine sponge *Fasciospongia rimosa* collected in Okinawa (2), and has potent anti-proliferative activity in cancer cells (3-4). The molecule also shows highly potent anti-leukemic effects toward multidrug resistant cells at low nanomolar concentrations (5-6). (–)-Zampanolide binds to the taxane-binding pocket of β-tubulin and stabilizes microtubules (7-8). There are reports in the literature detailing the total synthesis (9-13), synthetic design of analogs (14-15), and biochemical properties (16) of (–)-zampanolide.

Bisamide is a unique functional group that consists of two amides connected with a methylene bridge (17, 18). In pursuit of a chemically stable anti-proliferative molecule, it was reasoned that replacing the chemically unstable *N*-dineoylaminal unit in (–)-zampanolide with a *N,N'*-(arylmethylene)bisamide unit would achieve this goal, and thus, the so called *N,N'*-(arylmethylene)bisamides were synthesized (19). Among these analogs, *N,N'*-[(3,4-dimethoxyphenyl)methylene]biscinnamide (DPMBC) showed the highest anti-proliferative activity in the human promyelocytic leukemia cell line HL-60 and the human leukemic monocytic lymphoma cell line U937 (19). Here, we investigated the mechanism underlying the anti-proliferative activity of DPMBC.

Apoptosis is a mode of programmed cell death associated with activation of a series of caspase proteases leading to chromatin fragmentation, and morphologic changes including nuclear fragmentation (20, 21). Apoptosis can be initiated *via* the intrinsic or extrinsic pathways. The cancer chemotherapeutic agents, such as etoposide or paclitaxel, induce apoptosis *via* the intrinsic pathway, which involves mitochondrial-dependent processes, resulting in cytochrome c release and activation of caspase 9. The extrinsic pathway involves activation of death receptors expressed on the cell surface. Death receptor 5 (DR5) is a receptor for tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and mediates TRAIL-induced apoptosis through the formation of a death-inducing signaling complex containing the death receptor, adapter proteins such as the Fas-associated death domain (FADD), and initiator caspases such as procaspase-8. Several anti-cancer drugs are known to up-regulate DR5 expression (22).

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Key Words: N,N'-(arylmethylene)biscinnamide, apoptosis, caspase, death receptor 5, cancer cells.

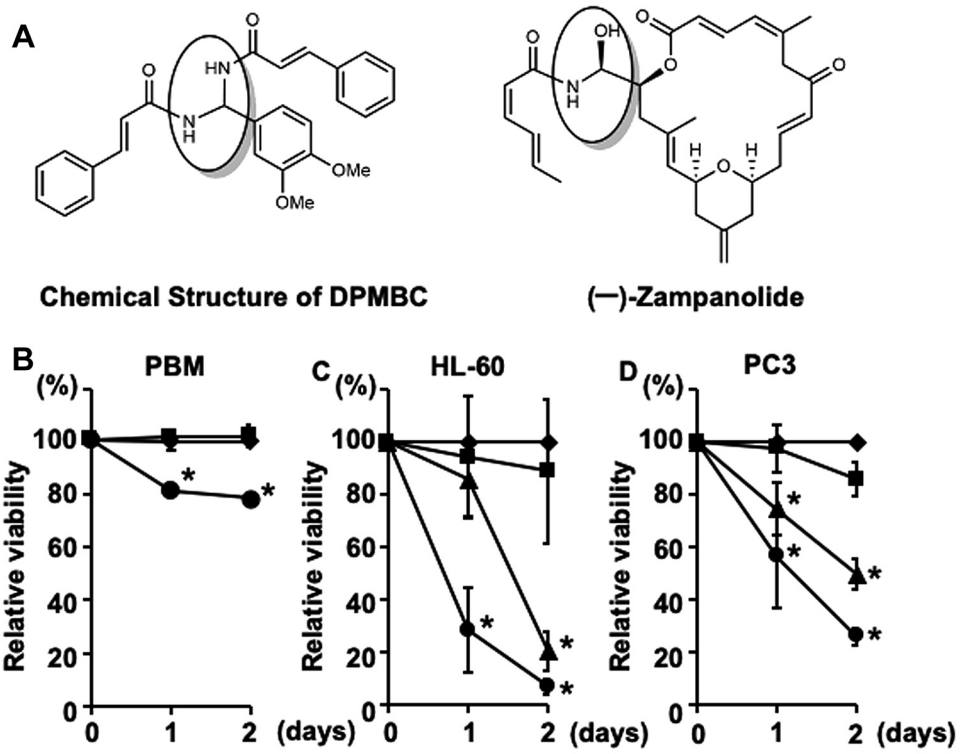

Figure 1. *DPMBC suppresses proliferation of cancer cells. (A) The chemical structures of DPMBC and (–)-zampanolide are shown. (B–D) Cell proliferation assays were performed in cells treated with DPMBC at the indicated concentrations. (B) Normal PBMCs (◆: control, ■: 3 μM, ●: 10 μM), (C) HL-60 cells (◆: control, ■: 4 μM, ▲: 8 μM, ●: 10 μM), and (D) PC-3 cells (◆: control, ■: 5 μM, ▲: 7.5 μM, ●: 10 μM). *p<0.05 vs. control.*

In this study, we show that DPMBC induces apoptosis through the up-regulation of DR5 and activation of caspases, preferentially in cancer cells. Therefore, DPMBC may be a promising candidate for a new anti-cancer drug.

Materials and Methods

Cell culture. Normal peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers using Ficoll-Paque PLUS (GE Healthcare, Chalfont St. Giles, Bucks, UK) gradient centrifugation. The human promyelocytic leukemia cell line HL-60 and the human prostate cancer cell line PC-3 were provided by the RIKEN BioResource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/mL streptomycin, and cultured in a humidified 5% CO_2 atmosphere at 37˚C.

Cell proliferation assay. PBMCs or HL-60 cells were seeded into 96-well plates (5000 cells/well) and DPMBC was added immediately. PC-3 cells (1000 cells/well) were cultured in a 96-well plate at 37˚C for 24 h, and then DPMBC was added and the cells were further incubated for 24-48 h. Cell growth was assessed by counting viable cells with the WST-8 assay using an SF kit (Nacalai Tesque, Kyoto, Japan).

Nuclear staining. HL60 cells were seeded at a density of $1.0x10^6$ cells/mL in a 6 cm dish, and DPMBC was added at a final concentration of 10 μM and incubated for 24 h. As positive control, apoptosis was induced with 1 μM etoposide, and dimethyl sulfoxide (DMSO; 0.1% final concentration) was used as a negative control. The cells were fixed with 3.5% formaldehyde, and nuclei were stained using 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Lonza, Basel, Switzerland) and observed using an Axiovert 200 fluorescence microscope (Zeiss, Jena, Germany).

DNA ladder assay. HL-60 cells were treated with DMSO (negative control), etoposide (positive control), or DPMBC for 24 h. DNA was collected using a NaI solution [6 M NaI, 13 mM EDTA, 0.5% sodium *N*-lauroyl sarcosinate, 10 mg/ml glycogen, 26 mM Tris-HCl (pH 8.0)]. The collected DNA was subjected to electrophoresis using 2% agarose gel containing ethidium bromide and visualized under UV light.

Small interfering RNA (siRNA) transfection. PC-3 cells were transfected with LacZ-targeting (as a negative control) or DR5 siRNAs at a final concentration of 50 nM using Lipofectamine RNAiMAX (Invitrogen, Waltham, MA, USA). The siRNA sequences used have been previously described (23).

Antibodies. Anti-poly (ADP-ribose) polymerase (PARP) antibody, anti-lamin A/C antibody, and anti-caspase-3, -7, and -8 antibodies

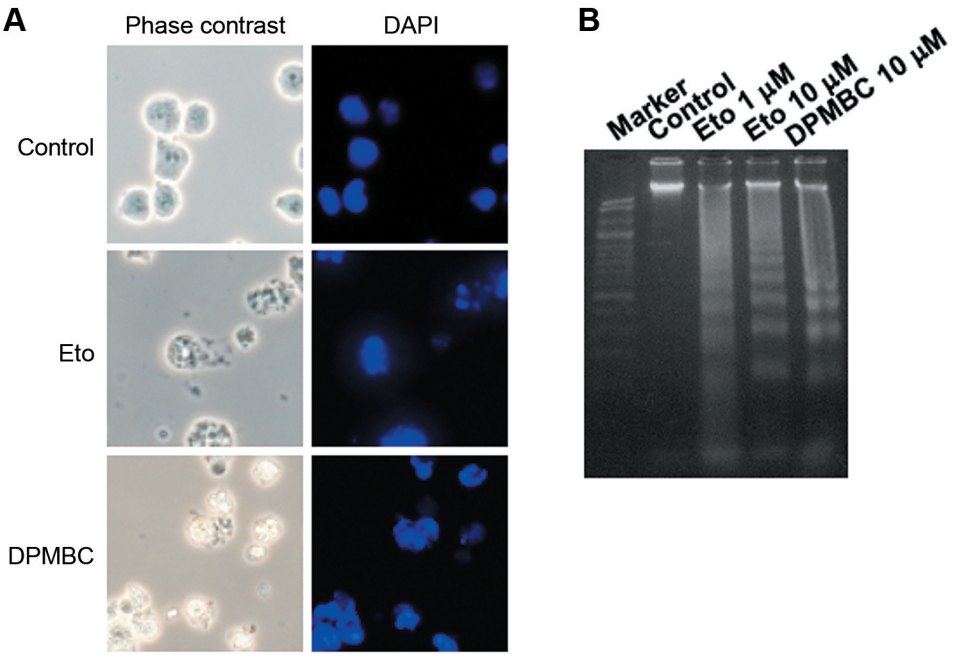

Figure 2. *DPMBC induces nuclear and chromatin fragmentation. (A) Representative phase contrast and fluorescence microcopy images of cells treated with DPMBC and 0.1% DMSO (control) HL-60 cells are shown. Cells were treated with DPMBC or with the apoptosis inducer etoposide (Eto). (B) HL-60 cells were treated with 0.1% DMSO (control) or with DPMBC or Eto, and a DNA ladder assay was performed.*

were purchased from Cell Signaling (Danvers, MA, USA). Purified mouse anti-cytochrome c antibody and anti-DR5 antibody were purchased from BD Bioscience (Franklin Lakes, NJ, USA) or ProSci (Poway, CA, USA). Peroxidase-conjugated affinity purified goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Peroxidase anti-mouse IgG was purchased from Vector Laboratories (Burlingame, CA, USA).

Western blot analysis. Whole cell lysates were solubilized in 1% sodium dodecyl sulfate (SDS) lysis buffer with protease inhibitor cocktail (Nacalai Tesque). The cytosol fraction was isolated for cytochrome c detection as follows: cells were collected and washed in ice-cold phosphate-buffer saline (PBS), resuspended in S-100 buffer (20 mM HEPES, 10 mM KCL, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, and 1% protease inhibitor cocktail), and incubated on ice for 15 min. Cells were centrifuged at 10,000xg for 15 min at 4˚C. Supernatants were further centrifuged at 100,000xg for 1 h at 4˚C. Samples were mixed with 3x Laemmli sample buffer, heated for 5 min at 95˚C, and subjected to 7–15% SDS-polyacrylamide gel electrophoresis at 150 V followed by electroblotting onto nitrocellulose for 30 min at 25 V using a Trans-Blot Turbo Transfer System (Biorad, Hercules, CA, USA). After blocking with 5% fat-free milk in PBS-Tween, membranes were probed for 1 h with primary antibodies in 3% bovine serum albumin/PBS-Tween, and then washed and incubated with secondary antibodies. The bound antibodies were detected using Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions.

Apoptosis detection by Annexin V-propidium iodide (PI) double-staining analysis. HL60 cells were seeded at a density of 1.0×10^5 cells/ml in a 6-well plate and DPMBC was added at a final concentration of 8 μM with or without 50 μM caspase inhibitors (ZVAD-FMK, pan-caspase inhibitor; ZLED-FMK, caspase-9 inhibitor; ZIETD-FMK, caspase-8 inhibitor; or ZDEVD-FMK, caspase-3 inhibitor) and incubated for 24 h. Apoptotic cells were detected using a MEBCYTO Apoptosis Kit (MBL, Nagoya, Japan). Annexin V-positive and PI-negative early apoptotic cells were assessed by flow cytometry using a BD LSRFortessa X-20 cell analyzer (BD Bioscience, Franklin Lakes, NJ, USA). At least 10,000 cells per sample were analyzed.

Statistical analyses. The statistical significance of differences between groups was assessed by two-tailed Student's *t*-test using Microsoft Excel software. A *p*-value of <0.05 was considered significant.

Results

DPMBC shows anti-proliferative activity in cancer cells. The structure of DPMBC is shown in Figure 1A. We first examined whether DPMBC at various concentrations (3-10 μM) showed anti-proliferative activity in normal PBMCs, HL-60 cells, or PC-3 cells. When we compared the viability of cells treated with DPMBC at a concentration of 10 μM, an anti-proliferative effect was more evident in the malignant cells than in the normal PBMCs (Figure 1A-C). The IC_{50} of DPMBC in HL-60 and in PC-3 cells was 4.6±0.8 μM and 7.9±0.6 μM, respectively, whereas treatment of normal PBMCs with 10 μM DPMBC for 2 days did not reach 50% inhibition.

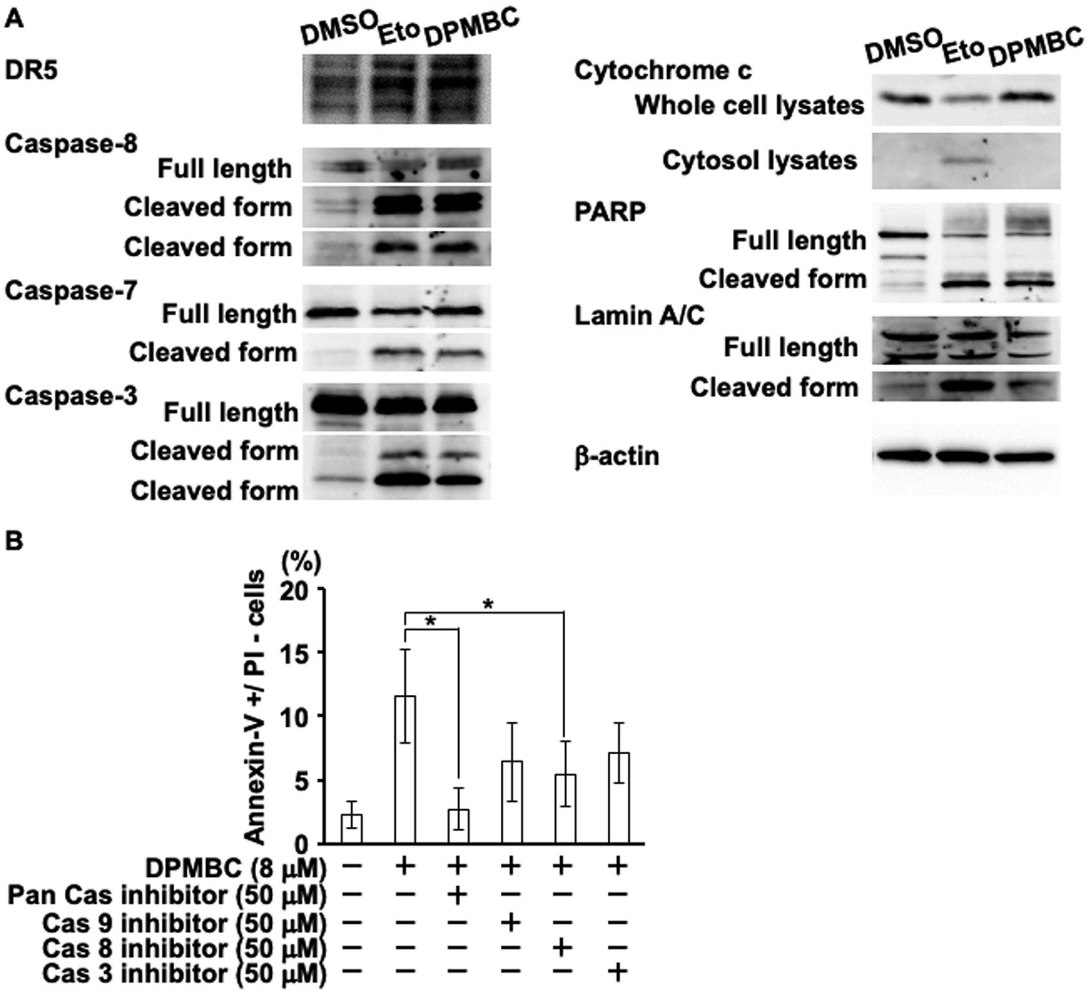

Figure 3. *DPMBC induces DR5 expression and the extrinsic apoptotic pathway. (A) HL-60 cells were treated with 0.1% DMSO as a negative control, etoposide (Eto; 1 μM), or DPMBC (10 μM) for 24 h. After treatment, proteins were extracted and analyzed by western blotting. (B) Quantitative analysis of the proportion of Annexin V-positive and PI-negative apoptotic HL-60 cells treated with DPMBC and/or caspase inhibitors. *p<0.05 vs. DPMBC (8 μM).*

DPMBC induces nuclear and chromatin fragmentation. Using HL-60 cells, we next investigated the cellular phenotype induced by DPMBC. Fluorescence microscopy of DAPI-stained cells showed that DPMBC induced nuclear fragmentation, which was identical to the morphological changes induced by etoposide, a known apoptosis inducer (Figure 2A). Both DPMBC and etoposide treatment also induced chromatin fragmentation, as shown by DNA laddering, in HL-60 cells (Figure 2B).

DPMBC induces DR5 expression and activates the extrinsic apoptotic pathway. Next, we explored mechanisms underlying the anti-proliferative effects of DPMBC. Using western blot analysis, we found that DPMBC up-regulated DR5 expression in HL-60 cells (Figure 3A). The level of DR5 protein was also increased by treatment with etoposide, a known inducer of DR5 and apoptosis. DPMBC induced the cleavage of PARP, caspase-3, -7, and -8, and lamin A/C proteins in HL-60 cells. Whereas etoposide induced release of cytochrome c from mitochondria to the cytoplasm, no cytochrome c release due to DPMBC treatment was observed (Figure 3A). These results suggest that DPMBC mainly induces the extrinsic apoptotic signaling pathway, whereas etoposide induces apoptosis *via* both the mitochondria-dependent intrinsic and extrinsic pathways. We also confirmed that the pan-caspase inhibitor ZVAD-FMK efficiently blocked induction of apoptosis and the specific caspase-8 inhibitor significantly attenuated apoptosis induced by DPMBC (Figure 3B).

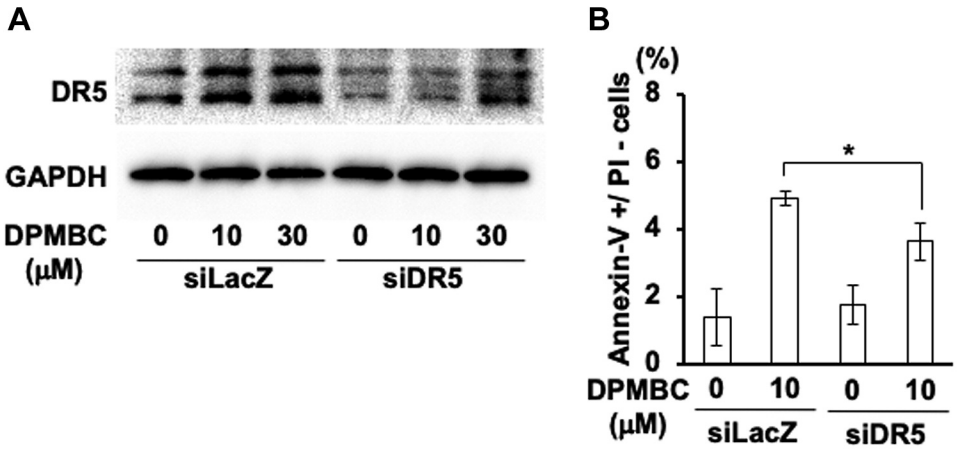

Figure 4. *Knockdown of DR5 reduces apoptosis induced by DPMBC. (A) Western blot analysis of DR5 in PC-3 cells treated with or without DPMBC with DR5 knockdown (siDR5) or without (siLacZ). (B) Quantitative analysis of the proportion of Annexin V-positive and PI-negative apoptotic HL-60 cells treated with or without DPMBC with DR5 knockdown or without. *p<0.05 vs. DPMBC (10 μM).*

Up-regulated DR5 mediates apoptosis induced by DPMBC. Finally, we performed knockdown of DR5 in PC-3 cells to examine whether DPMBC treatment induced apoptosis through DR5. We found that treatment with siRNA targeting DR5 efficiently blocked the up-regulation of DR5 seen with 10 μM DPMBC (Figure 4A), and confirmed that the DR5 knockdown partially but significantly reduced apoptotic induction by DPMBC in PC-3 cells (Figure 4B). These results indicate that DR5, at least in part, mediates the activation of the extrinsic apoptotic signaling pathway by DPMBC treatment.

Discussion

In the present study, we report for the first time that DPMBC induces apoptosis in HL-60 and PC-3 human malignant cells *via* DR5 up-regulation. While etoposide activates both the extrinsic and intrinsic apoptotic signaling pathways, DPMBC mainly activates the extrinsic apoptotic pathway. Our knockdown experiments demonstrated the significance of the DR5 up-regulation, which involves apoptosis induction as a mechanism of action for DPMBC.

DR5 was originally identified as a p53 downstream target gene (23). In HL-60 and PC-3 cells, however, the p53 gene is defective, thus suggesting that DPMBC up-regulates DR5 in a p53-independent manner. Indeed, in addition to p53 activation, several mechanisms of DR5 modulation have been reported, including regulation by the transcription factors SP1, CHOP, YY1, and NF-κB (24) and by epigenetic regulation (25, 26). It has been reported that DR5 can be up-regulated by a number of therapeutic agents including conventional chemotherapeutic drugs. Although the precise mechanisms underlying DR5 up-regulation by DPMBC remain to be elucidated, we observed DNA damage as detected by histone H2AX phosphorylation (data not shown). Given that (–)-zampanolide binds to the taxane-binding pocket of β-tubulin and stabilizes microtubules (7, 8), it is assumed that DPMBC induces microtubule stabilization and DNA damage *via* aberrant mitosis. Further investigation is now required to fully elucidate the mechanism.

Since the ligand of DR5, TRAIL, is induced specifically in cancer cells, DR5 agonists are now being tested as potential cancer therapeutics in clinical settings (27, 28). Although the efficacy of the strategy is currently limited due to resistance to the therapy of cancer cells, up-regulation of the target molecule DR5 by DPMBC could be beneficial for sensitization to DR5 agonist therapies.

In summary, we report that DPMBC is a novel DR5 and apoptosis inducer in cancer cells. DPMBC may be useful to potentiate cancer therapeutics using DR5 agonists to induce apoptotic cell death in cancer cells.

Conflicts of Interest

The Authors declare no conflicts of interest pertaining to the present study.

Authors' Contributions

HI performed experiments and drafted the manuscript. JU designed experiments. SN designed, supervised the study, and wrote the manuscript.

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