Piperine Enhances the Efficacy of TRAIL-based Therapy for Triple-negative Breast Cancer Cells

SHERIF ABDELHAMED[1], SATORU YOKOYAMA[1], ALAA REFAAT[1], KEISUKE OGURA[1], HIDEO YAGITA[2], SURESH AWALE[3] and IKUO SAIKI[1]

[1]*Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, Sugitani, Toyama, Japan;*
[2]*Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan;*
[3]*Frontier Research Core for Life Sciences, University of Toyama, Sugitani, Toyama, Japan*

Abstract. *Background/Aim: Triple-negative breast cancer (TNBC) is most the aggressive type of breast cancer and is poorly responsive to endocrine therapeutics; however, one of the most attractive treatments is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-based therapies. To identify compounds that enhance the efficacy of TRAIL-based therapies, we screened 55 compounds from natural products in combination with TRAIL in TNBC cells. Materials and Methods: Human TNBC cells, MDA-MB-468 and MDA-MB-231, and murine TNBC cells, 4T1, were used. Cell viability, apoptotic cells, and cell cycle were quantified by the WST-1 assay, annexin-V/7-amino-actinomycinD (7-AAD) staining and Propidium iodide (PI) staining, respectively. In vivo effects of piperine were evaluated in the orthotopic-inoculated 4T1-luc mouse model. Results: After screening, we identified piperine as the most potent adjuvant at enhancing the efficacy of TRAIL-based therapies in TNBC cells in vitro and in vivo, which might be mediated through inhibition of survivin and p65 phosphorylation. Conclusion: Piperine may enhance TRAIL-based therapeutics for TNBC.*

Breast cancer is the most frequently-diagnosed type of cancer among women and one of the main causes of cancer death (1, 2). Triple-negative breast cancer (TNBC), which is estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and epidermal growth factor receptor type 2 (HER2)-negative, accounts for 15%-25% of all breast cancer cases and is aggressive, poorly-prognosed, and unresponsive to endocrine therapeutics.

Correspondence to: Ikuo Saiki, Ph.D., Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. Tel: +81 764347620, Fax: +81 764345058, e-mail: byosei@inm.u-toyama.ac.jp

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While receptor-targeted therapeutics cannot help in the management of TNBC, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) might be an attractive molecule against TNBC because of its high selectivity for various carcinoma over normal cells (3); however, primary or acquired resistance to TRAIL limits its clinical efficacy in patients with cancer (4, 5). Several anti-apoptotic and pro-survival molecules, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), inhibitors of apoptosis [such as X-linked inhibitor of apoptosis (XIAP) and survivin] and B-cell lymphoma-2 (BCL2) family members [such as myeloid leukemia cell differentiation protein (MCL1)], are involved in acquired or hereditary TRAIL-resistant mechanisms (6-11).

In order to identify compounds that synergistically enhance the efficacy of TRAIL-based therapy, we screened for 55 chemically-defined compounds isolated from natural products in TNBC cells. Among them, we identified piperine as a candidate, which has a potently enhancing effect on TRAIL-induced cytotoxicity in TRAIL-sensitive TNBC cells, MDA-MB-231, and also a sensitizing effect on TRAIL-resistant TNBC cells, MDA-MB-468. Piperine (Figure 1) is the major alkaloid isolated from *Piper nigrum* and *Piper longum* and exhibits a wide range of biological effects, including anti-inflammatory and anti-arthritic effects (12). In cancer, piperine inhibits angiogenesis (13) and the tumor necrosis factor-α (TNF-α)-induced activation of NF-κB (14). It is also known to enhance the effective bioavailability and to inhibit the metabolism of major therapeutics by inhibiting cytochrome *p450* enzymes (15, 16); however, there are no reports showing the potential effect of piperine in combination with TRAIL-based therapy.

In the present study, we investigated the synergistic effects of piperine on TRAIL-induced apoptosis of TNBC cells *in vitro* and *in vivo*. Collectively, aimed to demonstrate that piperine, a commonly used dietary ingredient, is effective in enhancing the TRAIL-based treatment of TNBC cells.

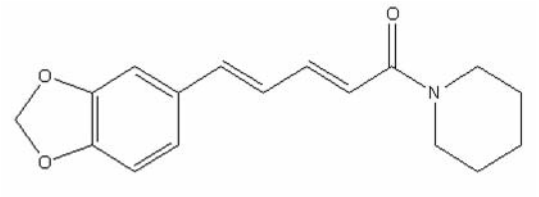

Figure 1. *Structure of piperine.*

Materials and Methods

Reagents. The compounds were isolated and purified at the Institute of Natural Medicine, University of Toyama, Japan and were dissolved in dimethyl sulfoxide (DMSO). Recombinant human TRAIL was purchased from PeproTech (Peprotech, London, UK). Agonistic monoclonal antibody to death receptor 5 (anti-DR5) was prepared as described previously (17).

Cell culture. MDA-MB-231 and MDA-MB-468 human breast cancer cells [American Type Culture Collection (ATCC), Rockville, MD, USA] were cultured in Dulbecco's modified Eagle's medium, and 4T1 murine cells (ATCC) were cultured in RPMI-1640 medium. All media were supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2.

Cell viability assay. Cell viability was determined using the Cell Counting Kit (Dojindo, Kumamoto, Japan), as described previously (6). Briefly, cells were seeded into a 96-well plate (7×10^3/80 μl/well). Thirty minutes before TRAIL addition (50 ng/ml), 10 μl medium containing test compounds (final concentration 50 μM) was added. After 24 h incubation, cells were counted with the Cell Counting Kit. Relative cell viability was calculated by the formula: Relative cell viability=[average absorbance of experimental wells/average absorbance of control wells]. The synergy index was calculated as follows: viability with combination treatment/[viability with TRAIL alone with compound alone].

Apoptosis assay. The cells were treated with piperine and TRAIL, similar to the cell viability assay, and were subjected to the annexin-V/Dead Cell-Kit using a Muse Cell Analyzer (Merck Millipore, Hayward, CA, USA), as described in the manufacturer's instructions.

Cell-cycle analysis. After treatment with piperine (200 μM) for 8 h and 24 h, the cells were collected, washed with cold phosphate-buffered saline (PBS), fixed in cold 70% ethanol and stored at −20˚C overnight. The cells were then subjected to the Muse Cell-Cycle Kit (Merck Millipore).

Western blot analysis. Western blot analysis was performed as previously described (6). Primary antibodies used were to: caspase-3, poly ADP-ribose polymerase (PARP), survivin, XIAP, MCL1, B-cell lymphoma-extra large (BCL-xl) and phosphorylated p65 from Cell Signaling Technology (Danvers, MA, USA); and β-Actin and p65 from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animal model. BALB/c mice (6 weeks old, female) were purchased from Japan SLC Inc. (Hamamatsu, Japan) All experiments were approved (A2012INM-6) and performed according to the guidelines of the Care and Use of Laboratory Animals of University of Toyama.

4T1-luc murine cells were inoculated directly into the mouse mammary fat pad (1×10^5/mouse). The mice were anesthetized with Escain isoflurane (Mylan Seiyaku, Osaka, Japan). The substrate D-luciferin (150 mg/kg) was injected intraperitoneally. The mice were imaged by the *IVIS lumina II* system (Caliper Life Sciences, Hopkinton, MA, USA), and the bioluminescence signals were quantified by Living Image® software (Caliper Life Sciences). We performed imaging every 10 min up to 20 min to obtain the maximum reading with 0.5-1 min exposure time. The primary tumor size was also measured using a caliper square along the longer axis (*a*) and shorter axis (*b*), and tumor volume was calculated by the following formula: tumor volume (mm^3)=$ab^2/2$.

Statistical analysis. Data are presented as the mean±SEM of experiments. Statistical significance for group comparisons was performed using two-way analysis of variance (ANOVA) with the Bonferroni post-tests and Student's *t*-test for single comparisons.

Results

Piperine in combination with TRAIL suppresses cell growth in both TRAIL-sensitive and TRAIL-resistant human TNBC cells. We screened 55 compounds derived from natural products with or without TRAIL for cell growth in human TNBC cells, MDA-MB-231 and MDA-MB-468 cell lines, TRAIL-sensitive and TRAIL-resistant cells, respectively (6). Upon screening, we found that alisol A, curcumin, and piperine showed a synergistic suppression of cell growth in both TNBC cells (synergy index <0.8, Table I). Contrary to TRAIL, piperine treatment alone suppressed growth of MDA-MB-468 but not of MDA-MB-231 cells; therefore, we decided to further focus on piperine.

To confirm the above screening results, both TNBC cell lines were treated with different concentrations of piperine with and without TRAIL (Figure 2a). Consistent with the screening results, piperine suppressed cell growth of MDA-MB-468 cells but not of MDA-MB-231 cells, and furthermore, piperine and TRAIL combination, synergistically inhibited cell growth of both cell lines compared to either treatment alone. We next determined whether the growth suppression of piperine and TRAIL combination is due to induction of the typical apoptotic pathway using annexin-V and 7-Amino-Actinomycin D (7-AAD) staining. Piperine treatment in combination with TRAIL significantly increased apoptotic cells compared to either treatment alone for both cell lines (Figure 2b). Similarly, cleavage of caspase-3 and PARP, both of which are known apoptotic markers, was clearly induced by piperine and TRAIL combination in both cell lines (Figure 2c). Unlike growth suppression, piperine treatment did not induce apoptosis of MDA-MB-468 cells. With regard to cell-cycle status, piperine treatment led to cell-cycle arrest at the G_2/M phase in MDA-MB-468 cells but not in MDA-MB-231 cells (Figure 2d). Collectively, the growth suppression of the piperine and TRAIL

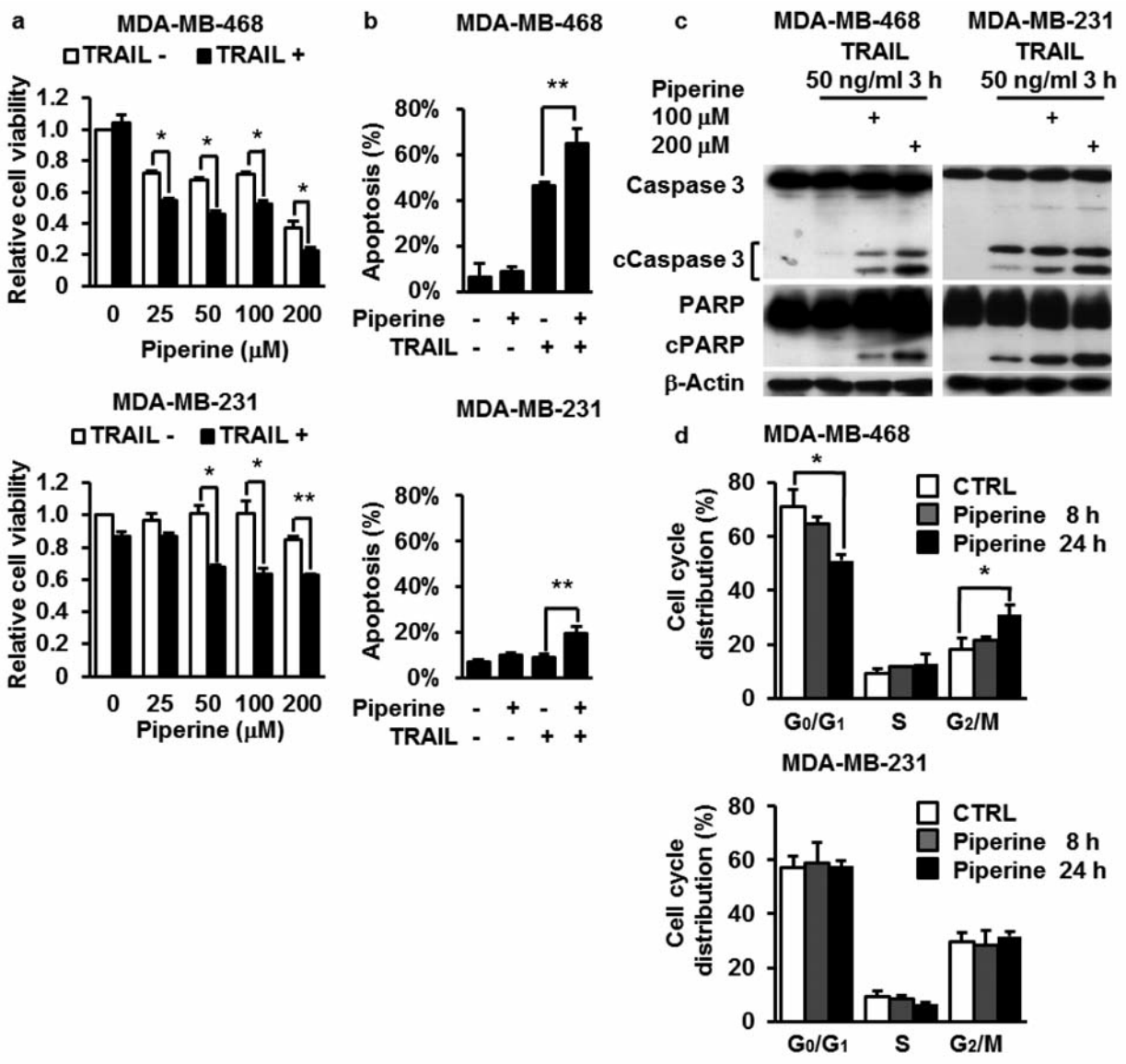

Figure 2. *Piperine potentiated tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of both TRAIL-sensitive and TRAIL-resistant human triple-negative breast cancer (TNBC) cells. a: MDA-MB-468 cells (upper panel) and MDA-MB-231 cells (lower panel) were pre-treated with different concentrations of piperine for 30 min, and then incubated in the presence of TRAIL (50 ng/ml) for 24 h. Relative cell viability was calculated after normalizing to that of the control group. Data are shown as the mean±S.E.M of three experiments and significant differences between groups are shown as *p<0.05 and **p<0.01. b: MDA-MB-468 cells (upper panel) and MDA-MB-231cells (lower panel) were pre-treated with piperine (200 μM) for 30 min, and then TRAIL (50 ng/ml) was added. After 6 h, cells were stained with annexin-V/7-AAD and apoptotic cells were detected using a Muse Cell Analyzer. Data are shown as the mean±S.E.M of three experiments and significant differences between groups are shown as **p<0.01. c: MDA-MB-468 cells (left panel) and MDA-MB-231 cells (right panel) were pre-treated with piperine (200 μM) for 30 min, and then incubated with TRAIL (50 ng/ml) for 3 h. The whole cell lysates were subjected to western blot analysis to detect for the indicated proteins. β-Actin was used as an internal control. d: MDA-MB-468 cells (upper panel) and MDA-MB-231 cells (lower panel) were treated with dimethyl sulfoxide as control (CTRL) or piperine (200 μM) for 8 and 24 h. After fixing with 70% ethanol, cells were stained with the cell-cycle kit. The cell-cycle distribution percentages were analyzed using a Muse Cell Analyzer. PARP: poly ADP-ribose polymerase; cCaspase 3: cleaved caspase 3.*

combination seems to be mediated by the typical apoptotic pathway in both MDA-MB-468 and MDA-MB-231 cells, while cell-cycle arrest by piperine mono-therapy was specifically observed in MDA-MB-468 cells.

Piperine enhances TRAIL responsiveness along with suppression of survivin and p65 phosphorylation in TNBC cells. Given that piperine and TRAIL combination induced apoptosis not only in TRAIL-sensitive MDA-MB-231 cells

Table I. *Evaluation of 55 compounds for their apparent cytotoxicity as a single-treatment and additive or synergistic effect with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).*

	MDA-MB-231 TRAIL		Predicted additive effect	Synergy index	MDA-MB-468 TRAIL		Predicted additive effect	Synergy index
	– 1.00	+ 0.76			– 1.00	+ 0.98		
Aconitine	0.91	0.79	0.69	1.14	0.97	0.87	0.95	0.91
Albiflorin	0.89	0.76	0.68	1.13	0.99	1.03	0.97	1.06
Alisol A	0.65	0.17	0.49	0.34	0.42	0.27	0.41	0.64
Alisol B	0.07	0.07	0.05	1.30	0.08	0.08	0.08	1.04
Alkannin	0.08	0.08	0.06	1.33	0.07	0.07	0.07	1.00
Amygdalin	0.95	0.80	0.72	1.11	1.06	1.01	1.04	0.97
Arbutin	0.92	0.86	0.70	1.23	1.02	1.05	1.00	1.04
Artimisine	0.87	0.57	0.66	0.86	0.61	0.65	0.60	1.10
Astragaloside IV	0.77	0.75	0.59	1.27	0.83	1.02	0.81	1.25
Acetylenolide III	0.98	0.75	0.75	1.01	1.09	1.24	1.07	1.16
Aucubin	0.91	0.69	0.69	0.99	1.14	1.13	1.12	1.01
Baicalein	0.90	0.76	0.68	1.12	1.20	1.23	1.18	1.05
Baicalin	0.75	0.68	0.57	1.19	1.21	1.16	1.19	0.98
Barbaloin	0.85	0.76	0.65	1.17	0.99	1.18	0.97	1.21
Bergenin	0.83	0.74	0.63	1.18	0.97	0.94	0.95	0.98
Catalpol	0.80	0.73	0.61	1.20	0.95	1.06	0.93	1.14
E-Cinnamic acid	0.87	0.76	0.66	1.15	1.08	0.98	1.06	0.92
Corydaline	0.98	0.74	0.75	0.99	0.98	0.98	0.96	1.02
Curcumin	0.78	0.30	0.59	0.50	0.52	0.31	0.51	0.61
Dehydrocostuslactone	0.29	0.24	0.22	1.05	0.64	0.57	0.63	0.92
Dimethylesculetin	0.97	0.71	0.74	0.97	1.03	1.03	1.01	1.02
Eleutheroside-B	1.03	0.81	0.79	1.03	1.13	1.10	1.11	0.99
Epihesperidin	0.77	0.72	0.59	1.23	1.06	0.89	1.04	0.86
Ergosterol	1.00	0.88	0.76	1.15	0.98	1.01	0.97	1.05
β-Eudesmol	0.98	0.82	0.75	1.09	0.89	0.96	0.88	1.09
E-Ferulic acid	0.99	0.80	0.76	1.06	1.00	1.02	0.98	1.04
Galangia	1.04	0.89	0.79	1.12	1.15	1.10	1.12	0.98
Galangia 7-glucoside	0.99	0.86	0.76	1.14	1.12	1.13	1.10	1.03
Geniposide	0.98	0.91	0.75	1.21	0.99	1.00	0.97	1.03
Geniposidic acid	0.63	0.87	0.48	1.82	1.03	1.07	1.01	1.06
Gentiopicroside	1.39	1.22	1.06	1.15	1.04	1.07	1.02	1.06
Ginsenoside Rd	0.94	0.81	0.72	1.13	0.96	1.04	0.94	1.11
Glabridin	0.11	0.10	0.08	1.18	0.53	0.45	0.52	0.86
Glycyrrhizic acid	0.99	0.91	0.76	1.20	0.98	1.08	0.96	1.12
Hiokinin	0.95	0.64	0.72	0.89	0.41	0.29	0.41	0.72
Hirsutine	0.91	0.44	0.69	0.64	0.60	0.57	0.59	0.97
Icariin	1.05	1.06	0.80	1.34	0.92	1.04	0.91	1.14
Isofraxidine	1.02	0.95	0.78	1.23	0.97	1.10	0.95	1.15
Ligustilide	1.15	0.90	0.88	1.03	1.18	0.83	1.16	0.72
Loganin	1.04	1.04	0.79	1.31	1.06	1.12	1.04	1.07
Magnolol	0.46	0.27	0.35	0.77	0.62	0.84	0.61	1.38
Mesaconitine	1.10	0.89	0.84	1.06	1.00	1.03	0.98	1.05
Naringin	0.91	0.89	0.69	1.29	1.07	1.13	1.05	1.07
Paeonol	0.92	0.87	0.70	1.24	1.02	1.08	1.00	1.08
Piperine	1.00	0.47	0.76	0.61	0.68	0.46	0.67	0.53
Palmatine chloride	1.08	0.61	0.82	0.74	0.63	0.60	0.62	0.97
S-Perilladelhyde	0.95	0.98	0.72	1.36	1.02	1.09	1.00	1.09
Puerarin	0.94	0.78	0.72	1.09	1.01	1.08	0.99	1.09
Resveratrol	1.03	0.74	0.79	0.94	1.12	1.14	1.10	1.04
Rhynchophylline	1.01	0.95	0.77	1.24	0.94	1.05	0.92	1.13
Saikosaponin a	0.92	0.74	0.70	1.05	0.99	1.04	0.98	1.06
Scutellarin	1.01	0.84	0.77	1.10	1.05	0.99	1.03	0.96
[6]-Shogaol	0.65	0.42	0.50	0.84	0.59	0.63	0.58	1.08
Sinomenine	1.04	0.86	0.80	1.09	0.99	1.10	0.97	1.14

but also in TRAIL-resistant MDA-MB-468 cells, we next investigated the expression levels of MCL1, survivin, XIAP, BCL-xL and phosphorylated p65, which are known to correlate with TRAIL sensitivity of cancer cells (6-11). Amongst them, piperine treatment suppressed the expression of survivin and p65 phosphorylation in both cell lines. In addition to human TNBC cells, piperine treatment suppressed survivin expression and the phosphorylation of p65 in murine 4T1 TNBC cells, which are known to be TRAIL-sensitive (Figure 3). These results suggest that piperine likely induces TRAIL sensitivity in both TRAIL-sensitive and TRAIL-resistant cells through the suppression of survivin expression and p65 phosphorylation.

Therapeutic effect of piperine in combination with monoclonal antibody to DR5 in a murine 4T1 breast cancer model. We finally investigated the clinical application of piperine in TRAIL-based therapy. To test the effect of piperine with TRAIL-targeted therapy *in vivo*, mice were orthotopically-inoculated (intra-mammary fat pad) with 4T1-luc cells and treated with piperine and with/without an agonistic monoclonal antibody specific for TRAIL receptor DR5. Combination treatment of piperine and anti-DR5 significantly reduced tumor growth, which was represented as a reduction in both the emitted luminescence fluxes and the tumor volume compared with either treatment alone (Figure 4a-c). Importantly, there was no discernible change in the body weight index of the tested group and no adverse effect on the mice during the experiment (Figure 4d), suggesting no obvious toxicity of piperine treatment at the tested dose.

Discussion

In the present study, we screened the prospective synergistic effects of 55 compounds with TRAIL on human TRAIL-sensitive and TRAIL-resistant TNBC cells (MDA-MB-231 and MDA-MB-468, respectively). After screening, we determined that piperine had synergistic effects with TRAIL in both cell lines, which aligned with the suppression of both the phosphorylation of p65 and the expression of survivin.

Concerning cell-viability suppression, piperine exhibited opposite cell selectivity to TRAIL and its synergistic effect with TRAIL (Figure 2a). While such selective cell growth suppression of piperine in MDA-MB-468 cells might be due to a cytostatic effect through G_2/M arrest (Figure 2d), the synergistic effect with TRAIL seems to be mainly due to a direct cytotoxic effect through apoptosis induction (Figure 2b). The cytostaticity of piperine has been reported in 4T1 murine breast cancer cells as G_2/M arrest (18) and human prostate cancer as G_1/G_0 arrest (19); however, this is the first report, as far as we know, to show that the cytostatic effect of piperine is context-dependent on the cell lines.

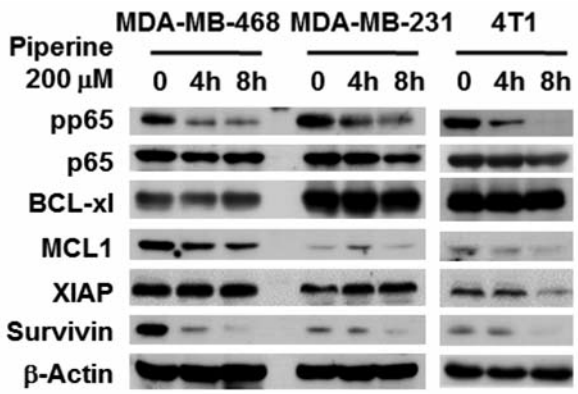

Figure 3. *Piperine consistently suppresses survivin and phospho-p65 on human and murine triple-negative breast cancer (TNBC) cells. MDA-MB-468 cells (left panel), MDA-MB-231 cells (middle panel) and 4T1 cells (right panel) were treated with piperine (200 μM) for 4 and 8 h. The whole cell lysates were subjected to western blot analysis to detect for the indicated proteins. β-Actin was used as an internal control. BCL-xl: B-cell lymphoma-extra large; MCL1: myeloid leukemia cell differentiation protein; XIAP: X-linked inhibitor of apoptosis; pp65: phosphorylated p65.*

Considering this, it is less likely that the higher sensitivity of MDA-MB-468 cells to piperine is due to common mechanisms in G_2/M arrest-inducing compounds, because a histone deacetylase inhibitor, panobinostat, also induces G_2/M arrest with less sensitivity to MDA-MB-468 than MDA-MB-231 cells (20). The synergistic effect of piperine with TRAIL is likely expressed through suppression of p65 and survivin (Figure 3), both of which are related to TRAIL responsiveness (6-11). In addition, it is known that cell cycle-arrested cells exhibit increased sensitivity to TRAIL (21), implying that piperine-induced G_2/M arrest could be related not only to the cytostatic effect but also to the synergistic effect with TRAIL in MDA-MB-468 cells. We also cannot exclude non-apoptotic cell death in MDA-MB-468 cells. Strikingly, an autophagic marker, LC3 cleavage, was increased in piperine-treated MDA-MB-468 cells but not in MDA-MB-231 cells (data not shown). This suggests that piperine-suppression of cell viability in TNBC cells could occur through multiple mechanisms.

As shown in Figure 4, combination treatment of piperine and anti-DR5 significantly inhibited the growth of orthotopically-implanted tumors. This combination therapy also apparently reduced lung metastasis, as well as primary tumor growth by ectopic (sub-cutaneous) implantation of the 4T1 cells (data not shown). Additionally, significant prolongation of survival rates was observed in mice after receiving piperine co-treated with anti-DR5 mAb (data not shown). In conclusion, the present study provides valuable information that piperine is a promising adjuvant for potentiating efficacy of TRAIL-based therapy in patients with TNBC.

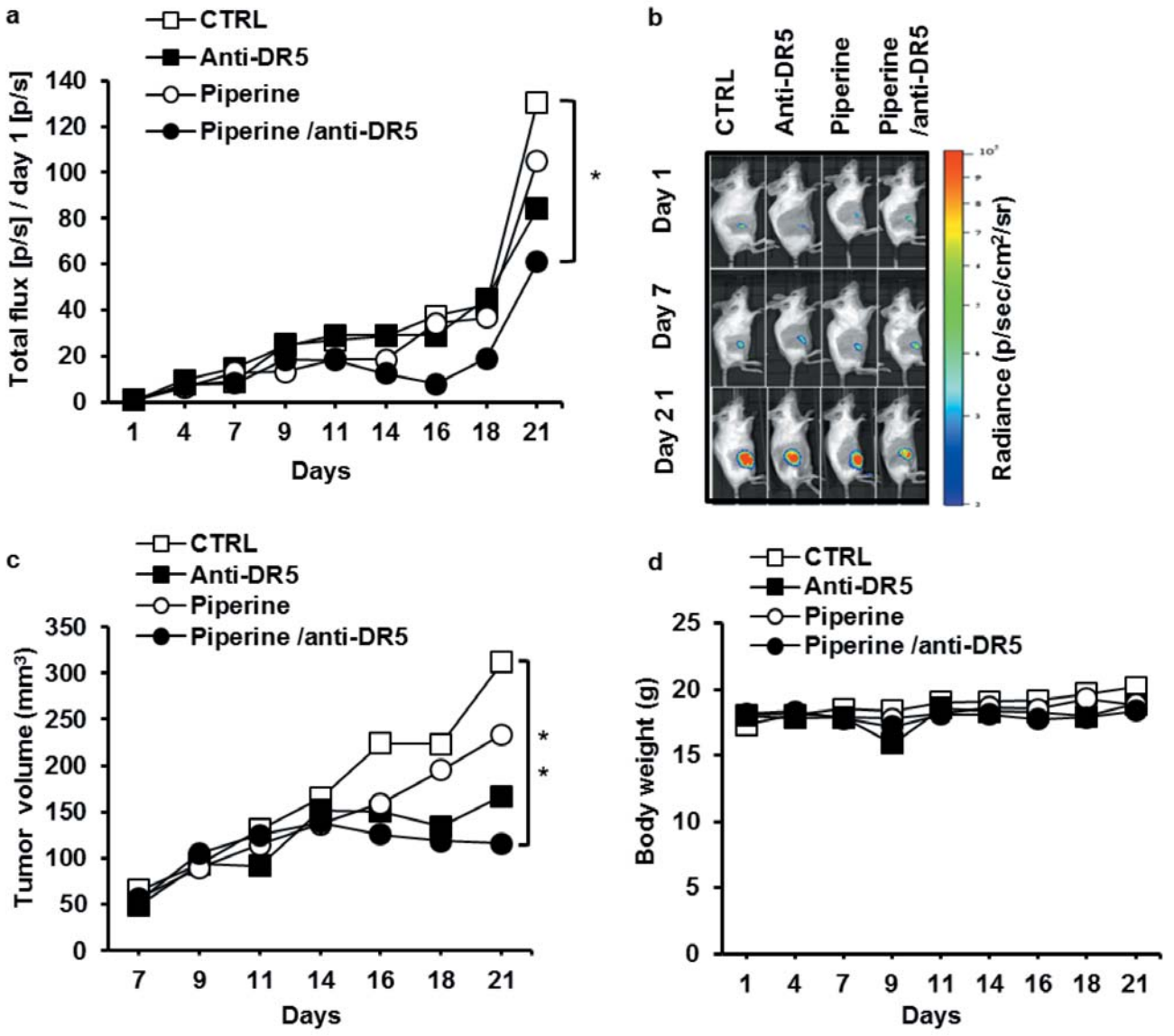

Figure 4. *Piperine enhances the inhibitory effect of monoclonal antibody to death receptor 5 (anti-DR5) on tumor growth in vivo. 4T1-luc cells were orthotopically-inoculated into syngeneic BALB/c female mice that received piperine (50 mg/kg/day) or vehicle through oral administration daily from day 7. Designated mouse groups received agonistic anti-DR5 days 14 and 16 after tumor inoculation. a: Total bioluminescence refluxes from localized tumors relative to that on day 1 were detected twice-a-week via the in vivo imaging system. b: Mouse images showing tumor progression at days 1, 7 and 21. c: Tumor volumes were measured using a caliper square. d: Body weights of the tested mice. Data are expressed as mean±SEM (n=6). Significant differences between groups are shown as *p<0.05 and **p<0.01.*

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Competing Interests

The Authors declare that they have no competing interests.

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