

The anti-obesity drug orlistat promotes sensitivity to TRAIL by two different pathways in hormone-refractory prostate cancer cells

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Abstract. The administration of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is one of the expected cancer therapeutics. However, improvements are required in therapies against TRAIL-resistant tumor cells. We report, here, that the anti-obesity drug orlistat enhances the sensitivity to TRAIL in hormone-refractory prostate cancer (HRPC) cells through two different pathways. The combination of orlistat and TRAIL remarkably induced apoptosis in TRAIL-resistant HRPC, DU145 and PC3 cells. Orlistat induced the expression of death receptor (DR) 5, which is one of the TRAIL receptors, at both the mRNA and protein levels. The suppression of DR5 with siRNA reduced the apoptosis induced by the combination of orlistat and TRAIL, suggesting that the apoptosis was at least partially due to the upregulation of DR5. Although the upregulation by orlistat of CHOP at both mRNA and protein levels was observed in both cell lines, the activation of the DR5 promoter in DU145 cells was CHOP-dependent, but that in PC3 cells was CHOP-independent. Moreover, orlistat induced reactive oxygen species (ROS), and a ROS scavenger diminished the sensitivity to TRAIL through the suppression of CHOP and DR5 expression in both cell lines. These results suggest that there are two pathways of upregulation of DR5 by orlistat, which are the ROS-CHOP pathway and the ROS-direct pathway. In conclusion, orlistat promotes the sensitivity to TRAIL by ROS-mediated pathways in prostate cancer cells, especially in TRAIL-resistant cells. We believe that the combination of orlistat and TRAIL in HRPC is promising as a new chemotherapeutic strategy.

Introduction

It has been reported that several anti-hyperlipidemia medicines unexpectedly exert anti-tumor effects (1-3). Among these drugs, orlistat (XenicalTM) is well known as an anti-obesity agent, which inhibits lipase activity by direct binding, and has been used for more than a decade in the US and Europe. Recently, it was reported that orlistat also binds to fatty acid synthase (FAS) and inhibits its activity (4). The expression of FAS was upregulated in many malignant tumors, and FAS inhibitors, cerulenin, c75 and orlistat, induced apoptosis and cell cycle arrest in prostate and breast cancer cell lines (4-7). However, the precise molecular mechanisms of the anti-tumor effects of orlistat are still unclear.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in various malignant tumor cells *in vitro* and *in vivo*, with little or no toxicity in normal cells and tissues (8,9). Endogenous TRAIL plays a critical role in immune-surveillance against tumor development (10). Death receptor 5 (DR5) is one of the cell surface receptors for TRAIL, and several studies have reported that DR5 is expressed more abundantly in cancer cells than in normal cells (11,12). Therefore, both the administration of TRAIL and the enhancement of TRAIL expression are considered promising strategies for cancer therapy and prevention. However, since some tumors are resistant to TRAIL-induced apoptosis (13), we need to overcome TRAIL-resistance in cancer cells.

Reactive oxygen species (ROS) are known to be important for both carcinogenesis and chemotherapy. In malignant tumor cells, several anti-tumor agents and radiation therapy induce ROS and ER stress, subsequently causing apoptosis (14-16). In the present study, we found that orlistat upregulated DR5 expression by the ROS induction, and subsequently enhanced the sensitivity to TRAIL in hormone-refractory prostate cancer (HRPC) cells but not in normal cells.

Materials and methods

Cell culture. Human prostate cancer cell lines PC3 and DU145 were maintained in RPMI-1640 with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml

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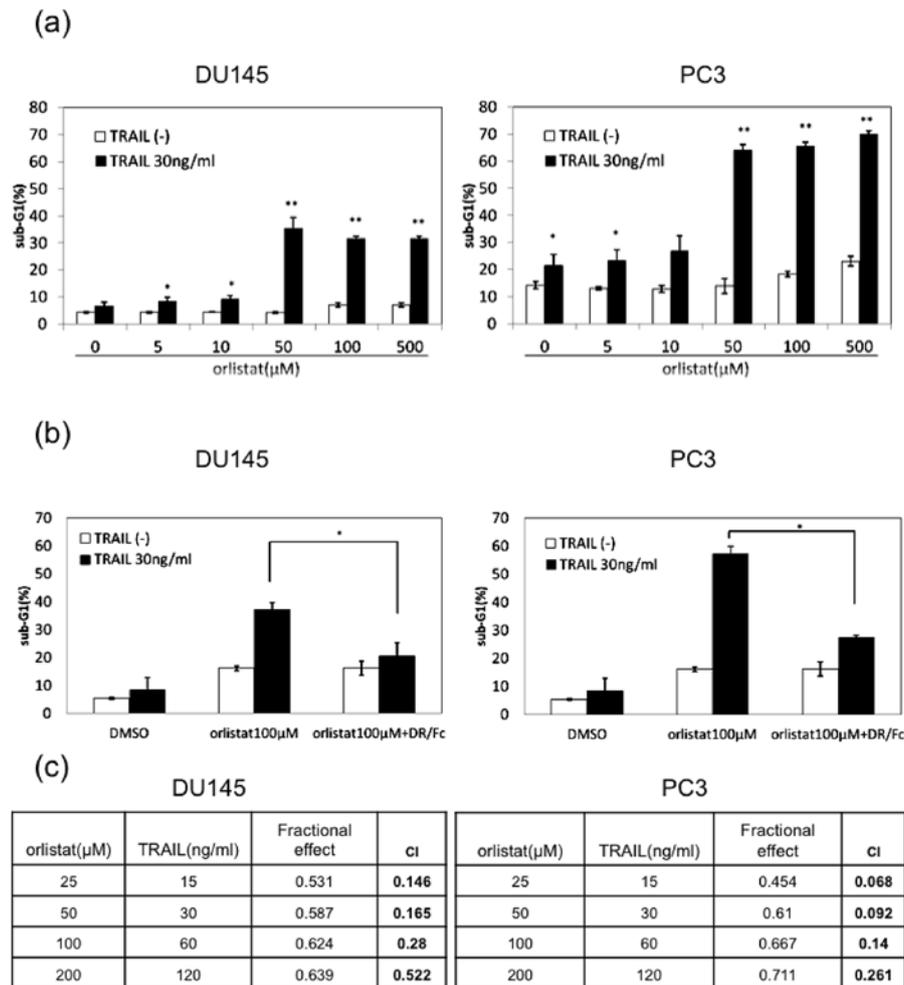


Figure 1. Orlistat promotes TRAIL sensitivity in DU145 and PC3 cells. (a) DU145 or PC3 cells were treated with 30 ng/ml of TRAIL and/or various concentrations of orlistat for 24 h. The population of apoptotic cells was analyzed as described in Materials and Methods. Columns, mean (n=3); bars, SD. *P<0.05, **P<0.01 compared with the TRAIL (-). (b) DU145 or PC3 cells were treated with 30 ng/ml TRAIL and/or 100 μM orlistat with or without 1 μg/ml DR5/Fc chimeric protein for 24 h. The population of apoptotic cells was analyzed. Columns, mean (n=3); bars, SD. *P<0.05 compared with TRAIL (-). (c) The combination index (CI) of TRAIL and orlistat was calculated in DU145 or PC3 cells.

streptomycin. Normal peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque PLUS (GE Healthcare, Waukesha, WI) according to the manufacturer's instructions and maintained in X-VIVO 20 (Cambrex, Charles City, IA) serum-free medium with 0.2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Normal human prostate epithelial cells (PrEC) were obtained from Cell Systems (Kirkland, WA) and maintained in SFM CS-C medium (Cell Systems) according to the instructions of the supplier. All cells were incubated at 37°C, in a humid atmosphere with 5% CO₂.

Reagents. Orlistat and catalase were purchased from Sigma-Aldrich. Soluble recombinant human TRAIL/Apo2L (untagged TRAIL, amino acids 114-281) was purchased from PeproTech (Rocky Hill, NJ). Human recombinant DR5/Fc chimera was purchased from R&D Systems (Minneapolis, MN).

Detection of apoptosis. DNA fragmentation as a marker of apoptosis was quantified with the hypodiploid DNA (sub-G₁) population. In brief, cells were seeded at 1.0x10⁴ per well in 24-well culture plates and incubated for 24 h. The cells were

then exposed to the indicated concentrations of orlistat, and the sub-G₁ population was analyzed using a Guava EasyCyte plus flow cytometer according to the manufacturer's instructions (Millipore, Billerica, MA).

Plasmid preparation. As previously described (17), the digested SacI-NcoI fragment from the DR5 promoter region of human genomic DNA was subcloned into the SacI-NcoI site of the pGVB2 luciferase assay vector (Toyo Ink, Tokyo, Japan) to produce pDR5PF. Deletion and point-mutated mutants of pDR5PF, termed pDR5/-347, pDR5/-252 and pDR5/mtCHOP with a mutation in the CHOP-binding site at -272/-269, were generated as previously reported (18). Full-length CHOP promoter, pCHOP, deletion mutant, pCHOP/150, and point-mutated mutant, pCHOP/-ERSE with mutation in the endoplasmic reticulum stress responsive element (ERSE), were generated as previously reported (19).

Transfection and luciferase assay. A series of DR5 and CHOP reporter plasmids and a vacant vector plasmid (1.0 μg) were transfected into the cells (1.0x10⁵ cells/well) using the DEAE-dextran

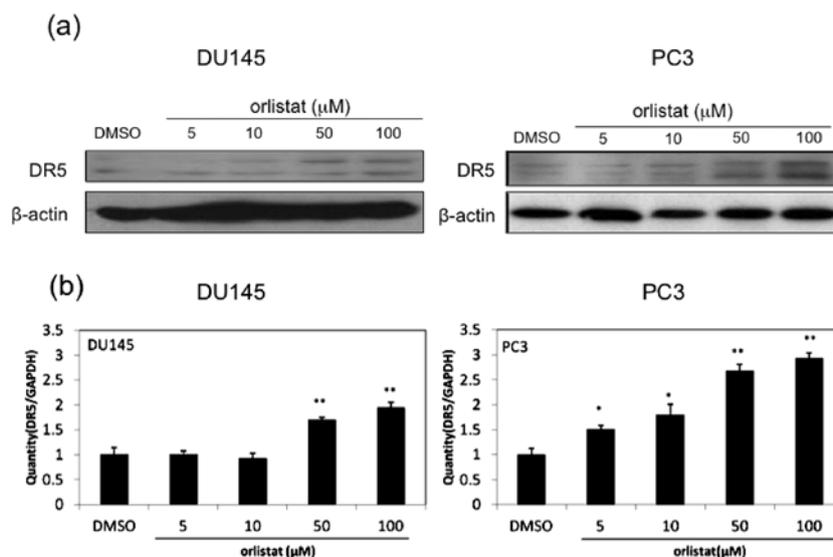


Figure 2. Orlistat induces DR5 protein and mRNA expression. (a) Whole cell lysates of DU145 or PC3 were prepared with various concentrations of orlistat for 24 h. The lysates were analyzed by western blotting for DR5 expression. β -actin was used as a loading control. (b) The induction of DR5 mRNA by orlistat in DU145 or PC3 cells was analyzed by real-time RT-PCR as described in Materials and methods. Columns, mean (n=3); bars, SD. *P<0.05, **P<0.01 compared with the DMSO control. Each sample was standardized by the expression of glyceraldehyde-3-phosphate (GAPDH) as an internal control.

method (GE Healthcare). After 24 h, the cells were treated with or without orlistat (100 μ M) for 24 h and then harvested. Luciferase activity was measured in triplicate, and the experiments were repeated several times.

RNA isolation and quantification analysis by real-time RT-PCR. The DR5 mRNA expression was determined by reverse transcription-PCR (RT-PCR). Total cellular RNA was extracted from cells using the Qiagen-RNeasy Mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 2 mg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI) and amplified by PCR using Taqman probes purchased from Applied Biosystems (Carlsbad, CA). Real-time RT-PCR was performed as previously described (18).

Western blot analysis. Whole cell lysate containing 50 μ g of protein was separated on a 10 or 12.5% SDS-polyacrylamide gel for electrophoresis and blotted onto polyvinylidene difluoride membranes (Millipore). Human DR5 (ProSci, Poway, CA), CHOP (GADD153; Sigma-Aldrich) and β -actin (Sigma-Aldrich) antibodies were used as the primary antibodies. The signal was detected with an enhanced chemiluminescence western blot analysis system (GE Healthcare).

Small interfering RNAs. The DR5, CHOP, and non-targeting siRNA were synthesized by Sigma-Aldrich (18,20). Before transfection, the cells were seeded into the medium without antibiotics at a density of 30-40%. The siRNA was transfected by oligofectamine (Invitrogen, Carlsbad, CA). After 24 h of transfection, the cells were treated with orlistat and/or TRAIL and then harvested.

Measurement of intercellular ROS. Cells were pretreated with 100 μ M orlistat or DMSO for 12 h and then treated with 10 μ M 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) (Molecular Probes, Carlsbad,

CA). After 30 min of incubation with CM-H₂DCFDA, the fluorescence was measured by flow cytometry. The geo-mean fluorescence intensity at 530 nm was calculated.

Statistical analysis. Statistical analysis was performed using a two-tailed, paired Student's t test. Samples were considered significantly different at P<0.05.

Results

Orlistat promotes the TRAIL sensitivity in prostate cancer DU145 and PC3 cells. We investigated the effects of orlistat on TRAIL-induced apoptosis by measuring sub-G₁ population. The administration of orlistat or recombinant human TRAIL alone only weakly induced apoptosis in DU145 and PC3 cells, while the combination with TRAIL at 30 ng/ml and orlistat at 50 μ M or more remarkably induced apoptosis (Fig. 1a). To elucidate whether the enhanced apoptosis was mediated through the death receptor DR5, we employed DR5/Fc chimeric protein binding to TRAIL resulting in prevention of the binding to DR5. As a result, the DR5/Fc chimeric protein blocked the enhanced apoptosis in DU145 and PC3 cells (Fig. 1b). This result suggests that the enhanced apoptosis is mediated through DR5.

We also calculated the combination index (C.I.) to evaluate the combinational effect of TRAIL and orlistat. The C.I. was <1.0 in all of the combinations, indicating that the combinational effect of TRAIL and orlistat is synergistic in DU145 and PC3 cells (Fig. 1c).

Orlistat induces expression of DR5 protein and mRNA by enhancing DR5 promoter activity in DU145 and PC3 cells. At first, we examined the effects of orlistat on the expression of DR5 protein in DU145 and PC3 cells. The expression of DR5 protein was upregulated in a dose-dependent manner in DU145 and PC3 cells (Fig. 2a). Next, we examined the effect of orlistat on mRNA expression of DR5 in DU145 and PC3 cells. The

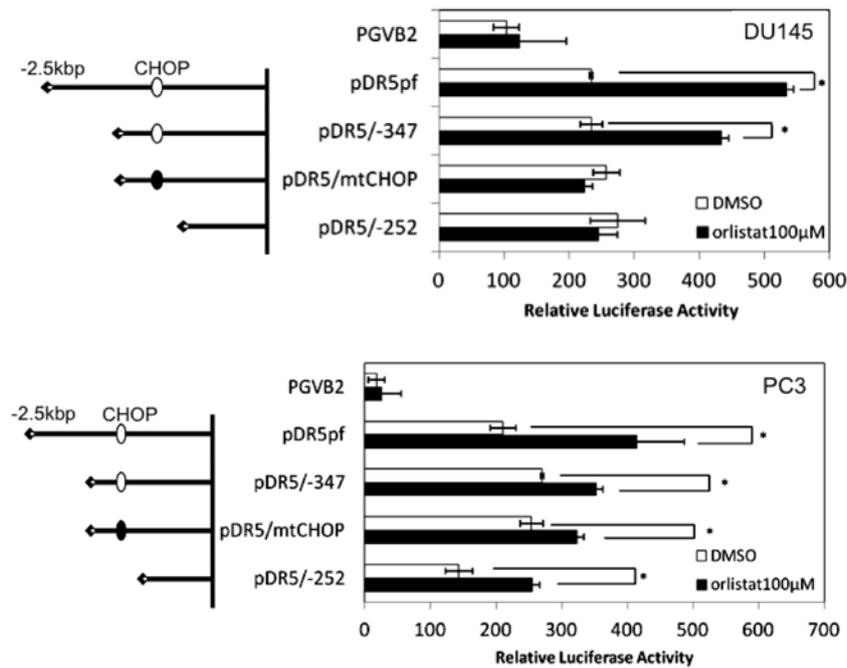


Figure 3. Orlistat enhances the DR5 promoter activity in DU145 and PC3 cells. DU145 or PC3 cells were transiently transfected with the reporter plasmids. After 24 h of transfection, the cells were treated with 100 μ M orlistat or DMSO for 24 h, and cell lysates were harvested for luciferase assays as described in Materials and methods. Columns, mean (n=3); bars, SD. **P<0.05.

DR5 mRNA was also upregulated in a dose-dependent manner in both cell lines (Fig. 2b).

To investigate the mechanism of DR5 induction by orlistat, we carried out a luciferase assay using reporter plasmids containing the DR5 promoter. In both DU145 and PC3 cells, the promoter activity from pDR5PF containing the full-length DR5 promoter was significantly enhanced by orlistat (Fig. 3). Since CHOP upregulates the promoter activity of DR5 through the CHOP binding site (18,21,22), we tried to elucidate whether CHOP was involved in the activation by orlistat. The promoter activity from neither the deleted promoter construct, pDR5/-252, without CHOP binding site, nor pDR5/mtCHOP with a mutation in the CHOP-binding site was stimulated by orlistat in DU145 (Fig. 3). In contrast, the promoter activities from both pDR5/-252 and pDR5/mtCHOP were still enhanced by orlistat in PC3 cells (Fig. 3). The results suggest that the promoter activity of DR5 in both DU145 and PC3 cells is activated by orlistat, but the activation in DU145 cells is CHOP-dependent and that in PC3 cells is CHOP-independent.

Orlistat induces expression of CHOP protein and mRNA by enhancing CHOP promoter activity in DU145 and PC3 cells. We then examined the effects of orlistat on the expression of CHOP in DU145 and PC3 cells. The expression of CHOP protein was upregulated in a dose-dependent manner in both DU145 and PC3 cells (Fig. 4a), and the CHOP mRNA was also upregulated in a dose-dependent manner in both cell lines (Fig. 4b). In DU145 and PC3 cells, the promoter activities from the full-length CHOP promoter construct, pCHOP, and the deleted CHOP promoter construct, pCHOP/150, were significantly enhanced by orlistat (Fig. 4c). Since endoplasmic reticulum (ER) stress regulates the promoter activity of CHOP through the ER stress responsive element (ERSE) (19), we tried to elucidate

whether ER stress was involved in the activation by orlistat. As a result, the promoter activity from pCHOP/ERSE with a mutation in the ERSE site was not stimulated by orlistat in either cell line (Fig. 4c). The results suggest that orlistat induces ER stress subsequently enhancing the CHOP promoter activity and expression of CHOP mRNA and protein.

CHOP is responsible for the DR5 upregulation in DU145 cells, but not in PC3 cells. We demonstrated that orlistat upregulated the DR5 expression and sensitized malignant tumor cells to TRAIL-induced apoptosis. To confirm whether the upregulation of DR5 by orlistat is responsible for the sensitization to TRAIL, we performed the knockdown of DR5 expression using siRNA. The treatment with siRNA against DR5 blocked the induction of DR5 expression by orlistat (Fig. 5a). Furthermore, the knockdown of DR5 also prevented the sensitizing effect of orlistat on TRAIL-induced apoptosis in both cell lines (Fig. 5b).

We then investigated whether CHOP was involved in the sensitization to TRAIL using CHOP siRNA. CHOP siRNA blocked the induction of CHOP expression by orlistat in DU145 and PC3 cells. The suppression of CHOP blocked the induction of the DR5 expression in DU145 cells, but not in PC3 cells (Fig. 5c). Furthermore, the knockdown of CHOP prevented the sensitizing effect of orlistat on TRAIL-induced apoptosis in DU145 cells, but not in PC3 cells (Fig. 5d). These results indicate that there are two different pathways, CHOP-dependent and -independent, to induce the DR5 expression by orlistat.

ROS are responsible for the apoptosis induced by the combination of TRAIL and orlistat in DU145 and PC3 cells. It has been reported that ROS production by baicalein causes apoptosis when combined with TRAIL (18). We also found

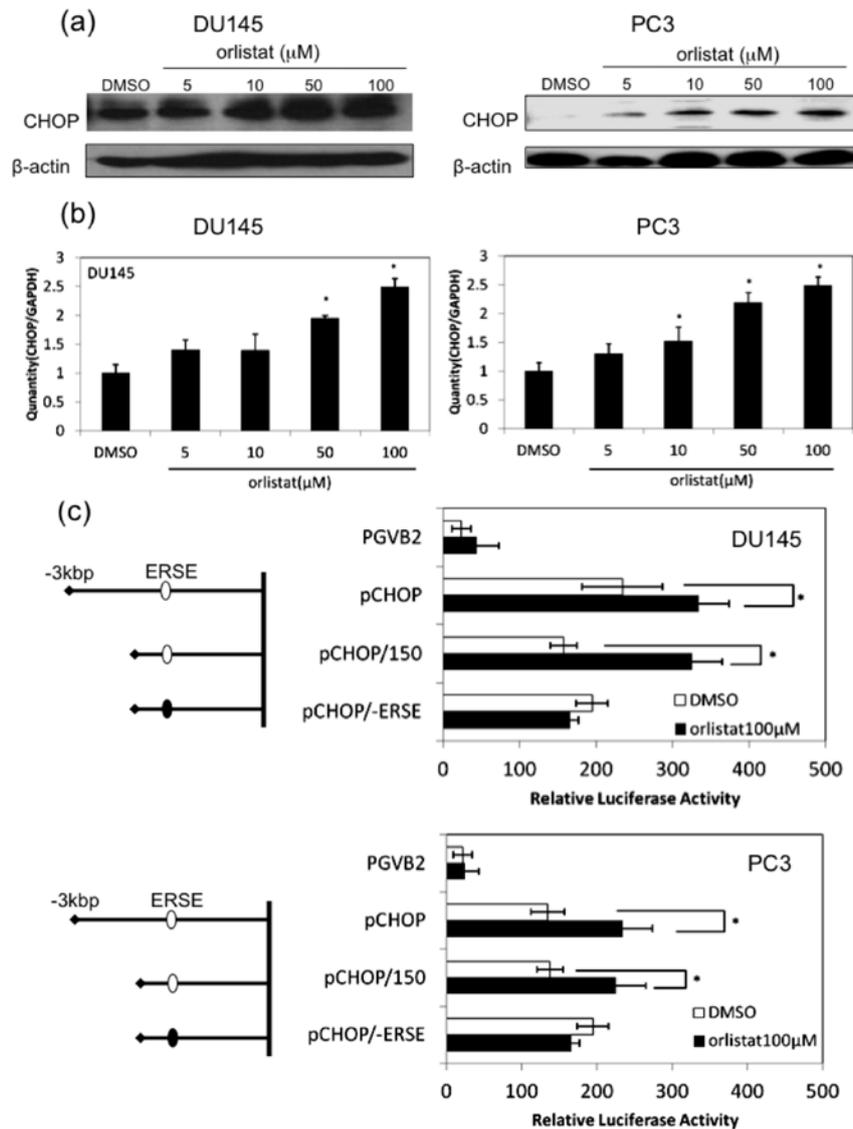


Figure 4. Orlistat induces CHOP protein and mRNA expression through the enhancement of CHOP promoter activity in DU145 and PC3 cells. (a) Whole cell lysates were prepared from DU145 or PC3 cells treated with various concentrations of orlistat for 24 h. The lysates were analyzed by western blotting for CHOP expression. β -actin was used as a loading control. (b) The induction of CHOP mRNA by orlistat in DU145 or PC3 cells was analyzed by real-time RT-PCR as described in Materials and methods. Columns, mean (n=3); bars, SD. *P<0.05 compared with the DMSO control. Each sample was standardized by the expression of GAPDH as an internal control. (c) DU145 or PC3 cells were transiently transfected with the reporter plasmids. After 24 h of transfection, the cells were treated with 100 μ M orlistat or DMSO for 24 h, and cell lysates were harvested for luciferase assays as described in Materials and methods. Columns, mean (n=3); bars, SD. *P<0.05.

that orlistat could induce intracellular ROS in DU145 and PC3 cells (Fig. 6a and b). A hydrogen peroxide scavenger, catalase, blocked the intracellular ROS induced by orlistat (Fig. 6a and b). Moreover, catalase also blocked the apoptosis induced by the combination of TRAIL and orlistat in both cell lines (Fig. 6c). These findings suggest that the apoptosis induced by the combination of TRAIL and orlistat is dependent on the ROS production by orlistat.

The combination with TRAIL and orlistat does not induce apoptosis in normal peripheral blood mononuclear cells (PBMC) and normal human prostate epithelial cells (PrEC). We investigated whether the combination with TRAIL and orlistat was toxic to normal human cells. We used human PBMC and PrEC as normal models. Neither orlistat nor TRAIL alone induced apoptosis in PBMC and PrEC, and the combination of

TRAIL and orlistat induced hardly any apoptosis in PBMC and PrEC (Fig. 7).

Discussion

Various chemotherapeutic treatments for HRPC have been proposed, but effective treatments are not established so far. We urgently require effective treatment methods with fewer side effects. Since TRAIL induces apoptosis in HRPC cells, but not in normal cells, the administration of TRAIL is considered one of the promising treatments (23,24). However, since HRPC cells are sometimes resistant to TRAIL, we need to develop a novel strategy to overcome this.

In recent years, anti-hyperlipidemia drugs have been reported to have anti-tumor effects against original expectation (1-3). We tried to examine whether such drugs could

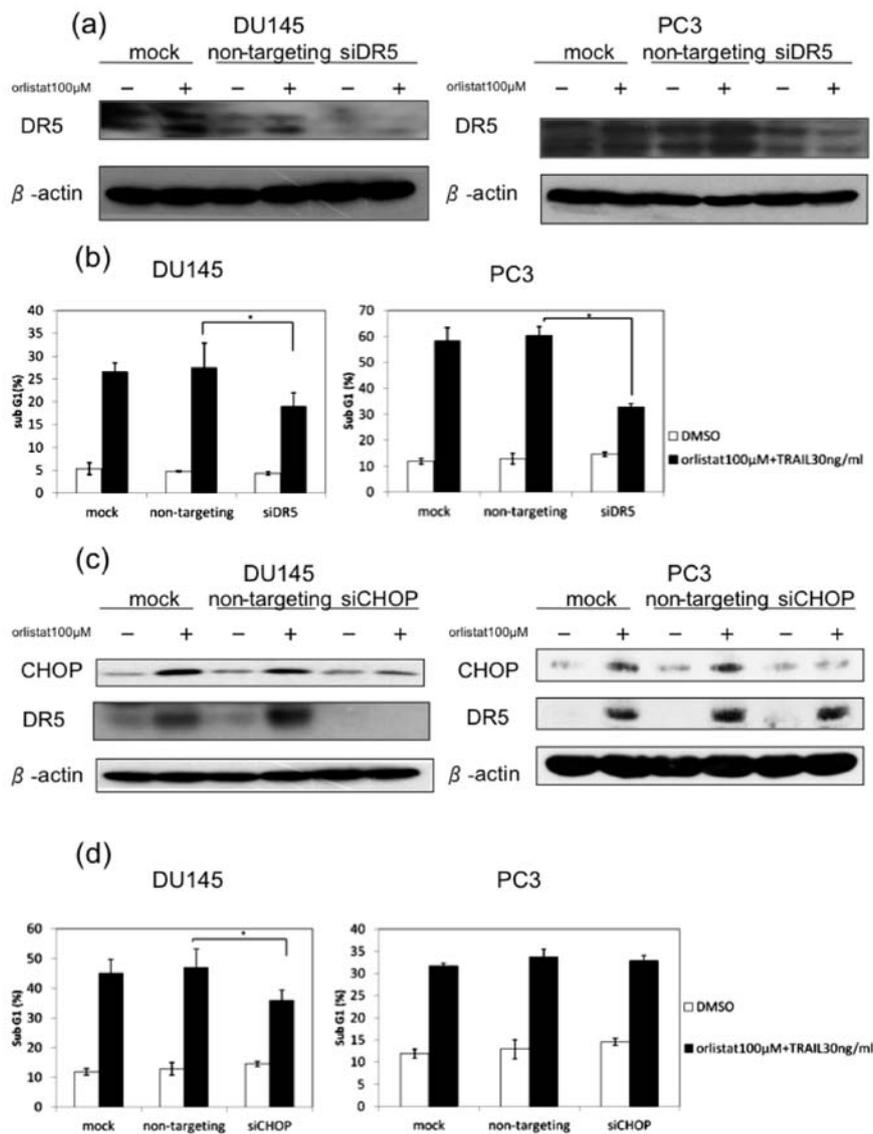


Figure 5. CHOP is induced by orlistat in both DU145 and PC3 cells, however, it is responsible for the DR5 upregulation in DU145 cells, but not in PC3 cells. (a) DU145 or PC3 cells were treated with 100 nM non-targeting or DR5 siRNA. After 24 h of transfection, cells were treated with 100 μ M orlistat or DMSO. Cell lysates were analyzed by western blotting for DR5 expression. (b) After 24 h of transfection, cells were treated with 40 μ M orlistat and/or 30 ng/ml of TRAIL. The population of apoptotic cells was analyzed. Columns, means (n=3); bars, SD. *P<0.05. (c) DU145 or PC3 cells were treated with 100 nM non-targeting or CHOP siRNA. After 24 h of transfection, cells were treated with 100 μ M orlistat or DMSO. Cell lysates were analyzed by western blotting for CHOP and DR5 expression. (d) After 24 h of transfection, cells were treated with 40 μ M orlistat and/or 30 ng/ml of TRAIL. The population of apoptotic cells was analyzed. Columns, means (n=3); bars, SD. *P<0.05.

increase the sensitivity to TRAIL in HRPC cells. First, we chose orlistat because it has already been used for many years as an anti-obesity drug and its safety has been validated. As demonstrated in the Results, we found a synergistic effect of TRAIL and orlistat in HRPC cells. The combination with orlistat apparently reinforced the apoptosis induced by TRAIL suggesting that orlistat may be useful as a TRAIL sensitizer. Although we have reported on TRAIL sensitivity enhancers (18-21,25,26) other than orlistat, we consider that orlistat is of great interest because it has been proved to be safe for patients.

In addition, we elucidated that the upregulation of DR5 was mediated by enhancement of the promoter activity of DR5 by orlistat. CHOP is a representative transcription factor of DR5 (22), and we examined whether CHOP was involved in the

enhancement of the promoter activity by orlistat. The results showed that the activation of the DR5 promoter by orlistat in DU145 cells was CHOP-dependent and that in PC3 cells was CHOP-independent. These results suggest that there are different ways of upregulating DR5 in DU145 cells and PC3 cells, respectively. Orlistat induced the expression of protein and mRNA of CHOP and also enhanced the promoter activity of CHOP in both cell lines. However, the knockdown of CHOP prevented the sensitizing effect of orlistat on TRAIL-induced apoptosis in DU145 cells, but not in PC3 cells. The results also indicate that there are two different pathways, CHOP-dependent and -independent, to induce TRAIL sensitivity by orlistat. Next, we tried to elucidate which molecule was responsible for the induction of CHOP and DR5. It has been reported that ER stress is induced by metabolism medicines (27-29), and we

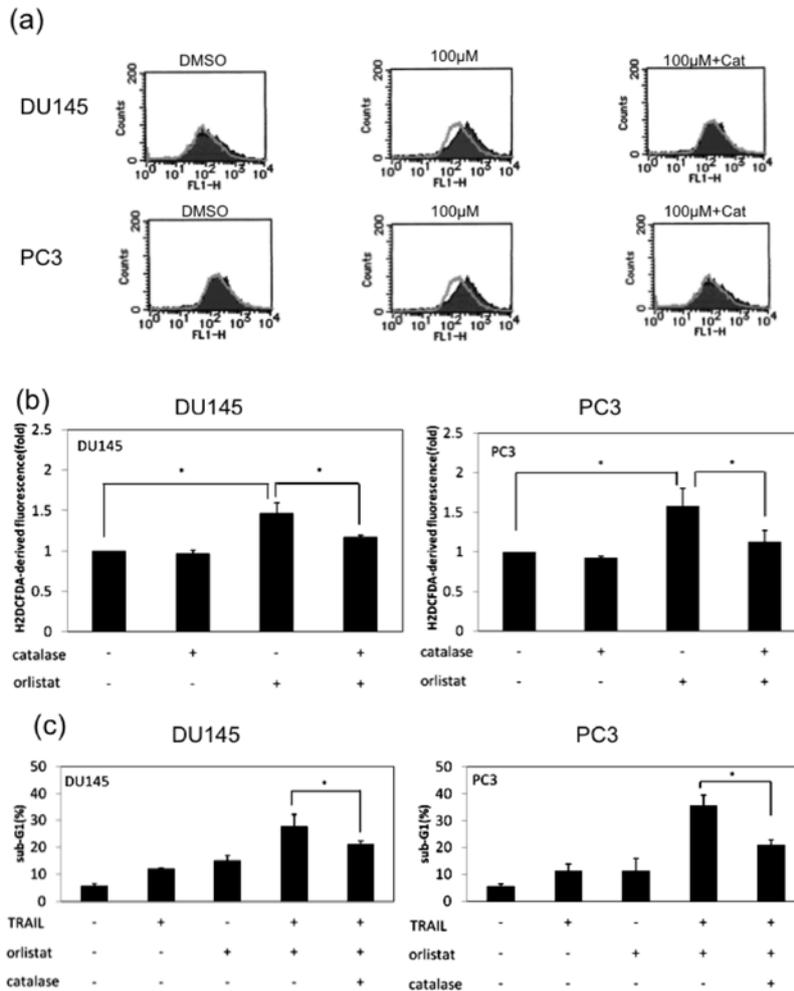


Figure 6. ROS are responsible for the apoptosis induced by the combination of TRAIL and orlistat in both DU145 and PC3 cells. (a and b) DU145 or PC3 cells were treated with DMSO or 100 μM orlistat with or without 1000 U/ml of catalase. After 24 h of treatment, ROS levels were analyzed using CM-H₂DCFDA as described in Materials and methods. Columns, means (n=3); bars, SD. *P<0.05. (c) DU145 or PC3 cells were treated with 100 μM orlistat and/or 30 ng/ml TRAIL with or without 1000 U/ml catalase. The population of apoptotic cells was analyzed. Columns, mean (n=3); bars, SD. *P<0.05.

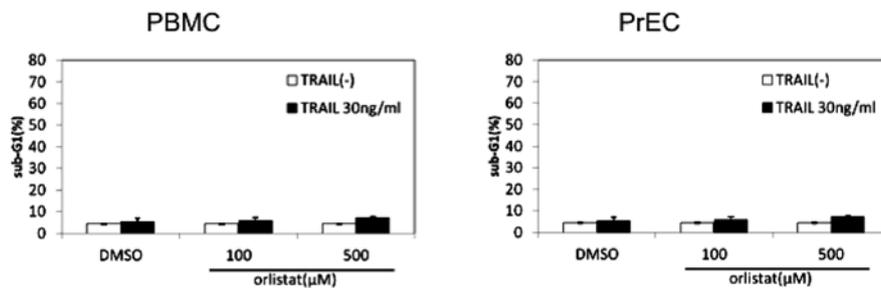


Figure 7. The combination of TRAIL and orlistat does not induce apoptosis to normal PBMC and prostate epithelial cells. PBMC or PrEC were treated with 30 ng/ml of TRAIL and/or 100 μM orlistat for 24 h. The population of apoptotic cells was analyzed. Columns, mean (n=3); bars, SD.

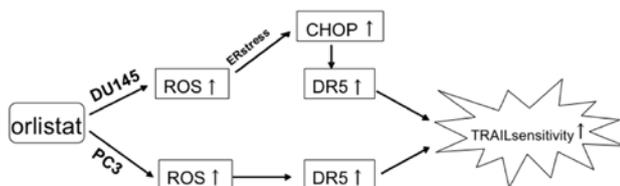


Figure 8. Orlistat has two different pathways to enhance the sensitivity to TRAIL through the upregulation of DR5 in DU145 and PC3 cells.

have also found that orlistat enhanced the promoter activity of CHOP through the ER stress response element. This suggests that orlistat induced ER stress, subsequently inducing CHOP expression. Since it has been reported that ROS induce ER stress (30-32), we investigated whether ROS were involved in the induction of DR5 by orlistat. Orlistat induced intracellular ROS in both DU145 and PC3 cells, and catalase blocked the apoptosis induced by the combination in both cell lines. These

findings suggest that the apoptosis induced by the combination is dependent on the ROS-producing by orlistat. These results indicate that orlistat acts as a ROS productive reagent.

Recently, it has been reported that several compounds, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 , withaferin A, celastrol, gossypol, dibenzylideneacetone, 5, 7-dimethoxyflavone and cardamonin, induced DR5 expression through the CHOP induction accompanied by ROS generation (33-39). Our data showed that the induction of DR5 by orlistat was also ROS-dependent in both DU145 and PC3 cells. Interestingly, even though the induction in DU145 cells was CHOP-dependent, that in PC3 cells was exceptionally CHOP-independent. The results suggest that there are two different pathways of the upregulation of DR5 by orlistat, which are ROS-CHOP pathway and ROS-direct pathway (Fig. 8).

In conclusion, the administration of TRAIL and orlistat is considered a promising strategy for cancer therapy in HRPC. Moreover, since endogenous TRAIL plays a critical role in immune-surveillance against tumor development, the administration of orlistat may enhance the potency of TRAIL against carcinogenesis, and may become a promising strategy for cancer prevention.

Acknowledgements

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