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Phenoxodiol Increases Cisplatin Sensitivity in Ovarian Clear Cancer Cells Through XIAP Down-regulation and Autophagy Inhibition

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Abstract. *Background/Aim: To investigate whether XIAP down-regulation and autophagy inhibition sensitize ovarian clear cell cancer cells to cisplatin. Materials and Methods: The ovarian clear cancer cell line KK was used for in vitro analysis. Hydroxychloroquine (HCQ) and phenoxodiol (PXD) or embelin were used as autophagy and XIAP inhibitors, respectively. Non-specific and XIAP-specific siRNAs were transfected using Lipofectamine. Cytotoxicity was assessed by MTT assays. Protein expression was confirmed by western blotting. Results: In KK, down-regulation of XIAP using specific siRNAs together with HCQ treatment enhanced the anti-tumor effect of cisplatin. Although embelin sensitized KK to cisplatin through XIAP down-regulation, it induced autophagy. However, PXD increased cisplatin sensitivity through XIAP down-regulation and autophagy inhibition. Expression of Atg7, Atg12, and Beclin 1 was decreased after PXD treatment. Conclusion: PXD increased cisplatin sensitivity through XIAP down-regulation and autophagy inhibition and could be a new candidate for ovarian clear cell carcinoma treatment.*

Ovarian carcinoma is a major cause of deaths among gynecological malignancies. Incidence of mortality due to epithelial ovarian carcinoma (EOC) has been increasing. The prognosis of patients in the advanced stage is poor despite advances in chemotherapy (1, 2). Among the histological subtypes, treatment for ovarian clear cell carcinomas (CCC) is difficult because of its extremely low response rate to platinum-based chemotherapy compared with that of the more prevalent ovarian serous carcinomas (3-5). This characteristic of CCC is one of the factors of treatment failure and is a problem that needs to be solved urgently.

The X-chromosome-linked inhibitor of apoptosis (XIAP) protein is one of the inhibitors of caspases and apoptosis (6). XIAP is one of the factors of platinum resistance in ovarian serous cancer cells as well as CCC (7, 8). Thus, XIAP could be a potential therapeutic target in CCC and serous subtypes of ovarian cancer. The phase III OVATURE multicenter randomized study demonstrated no evidence of clinical activity against acquired platinum-resistant ovarian cancer with the combination of a XIAP inhibitor (Phenoxodiol: PXD) and carboplatin (9). However, this study did not include CCC in the PXD arm and the anti-tumor activity of PXD in CCC has not been clarified. In addition, embelin, another XIAP inhibitor, has shown anti-cancer activity in experimental models of several cancers (10-13), but experiments targeting ovarian CCCs have not been reported.

On the other hand, high expression of the autophagy protein LC3A correlated with prognosis of patient survival in CCC. Ovarian CCC cell lines underwent apoptosis upon inhibition of autophagy with hydroxychloroquine (HCQ) (14, 15), which suggests that autophagy might be a new therapeutic target in ovarian CCCs. Recently, XIAP protein was reported as one of the regulators of autophagy (16, 17). In brief, down-regulation of XIAP using siRNA and embelin was found to increase autophagy (16); conversely, XIAP gene amplification induced autophagy (17). These results suggest that XIAP regulates autophagy inhibition and activation. The association between the regulation of both XIAP protein and autophagy, and cisplatin sensitivity in ovarian CCC is not known.

Herein, our study investigated the association between XIAP-regulated autophagy and cisplatin sensitivity in ovarian CCC cells and determined whether PXD and

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embelin demonstrate anti-tumor activity in ovarian clear cell cancer, through *in vitro* analysis.

Materials and Methods

Reagents/antibodies. HCQ was purchased from Abcam, Cambridge, UK. Embelin was purchased from Calbiochem, San Diego, CA, USA. Cisplatin was purchased from Bristol Meier's Squib Oncology, Tokyo, Japan. PXD was purchased from Sigma-Aldrich, Tokyo, Japan. Primary antibodies against XIAP, PARP, cleaved PARP, LC3A, and β-actin, secondary antibodies, and XIAP specific and control siRNAs were obtained from Cell Signaling Technology, Beverly, MA, USA.

Cell lines and culture conditions. The ovarian CCC cell line KK (18) was used for all experiments. These cells were grown as monolayer cultures in RPMI-1640+GlutmaxTM-I (Invitrogen Japan KK, Tokyo, Japan) medium supplemented with 10% fetal bovine serum (Invitrogen Japan KK, Tokyo, Japan), 100U penicillin per ml, and 100 mg streptomycin per ml (Invitrogen, Tokyo, Japan KK) in a humidified atmosphere with 5% CO_2 at 37˚C, and were routinely tested for mycoplasma infection. Protein concentrations were determined using a Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Transient transfection. KK cells cultured in 3.5-cm plates were transfected with 107 nM of XIAP siRNA and control siRNA, using Lipofectamine 2000 (Invitrogen, Tokyo, Japan KK) according to the manufacturer's specifications. Knockdown of XIAP was confirmed by western blot analysis in all experiments.

Cell proliferation and cytotoxicity assays. KK cells were seeded onto 96-well plates at approximately 1×10^4 or 4×10^4 cells cm^{-2} for the cytotoxicity assays. Cell viability was determined by the MTT method using Tetra Color ONE (Seikagaku Corporation, Tokyo, Japan) according to the manufacturer's instructions. Cytotoxicity by PXD, embelin, and HCQ was measured after 5 days of treatment at different concentrations. To determine the effect of cisplatin after pre-treatment with PXD, embelin, and HCQ, or siRNA transfection, KK cells were treated with several reagents at different concentrations for 24 hours. Cisplatin was then added to KK cells at different doses, and cell survival was measured after 5 days of cisplatin treatment.

Preparation of cell lysates for western blot analysis. Protein lysates were extracted in RIPA buffer® according to the manufacturer's instructions (Wako Pure Chemical Industries, Ltd. Osaka, Japan). Cytosolic fractions were prepared (10 μg) and loaded onto a Mini-PROTEIN® TGX™ gel (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, proteins were transferred to PVDF membranes using the Trans-Blot® Turbo™ Transfer System Transfer Pack (Bio-Rad Laboratories, Hercules, CA, USA). Subsequently, the membranes were blocked for 1 hour with 4% BSA in TBS with 0.5% Tween-20 (TBS-T) and incubated overnight at 4˚C with the primary antibody in TBS-T containing 4% BSA. The following antibodies and concentrations were used: 1/2,500 rabbit anti-XIAP, 1/2,500 rabbit LC3A, 1/2,500 rabbit cleaved PARP, 1/2,500 rabbit PARP, 1/2,500 rabbit Atg7, 1/2,500 rabbit Atg12, 1/2,500 rabbit Beclin-1, and 1/5,000 rabbit β-actin. After three washes with TBS-T, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody as appropriate. After three washes with TBS-T, the blots were visualized using an ECL detection system

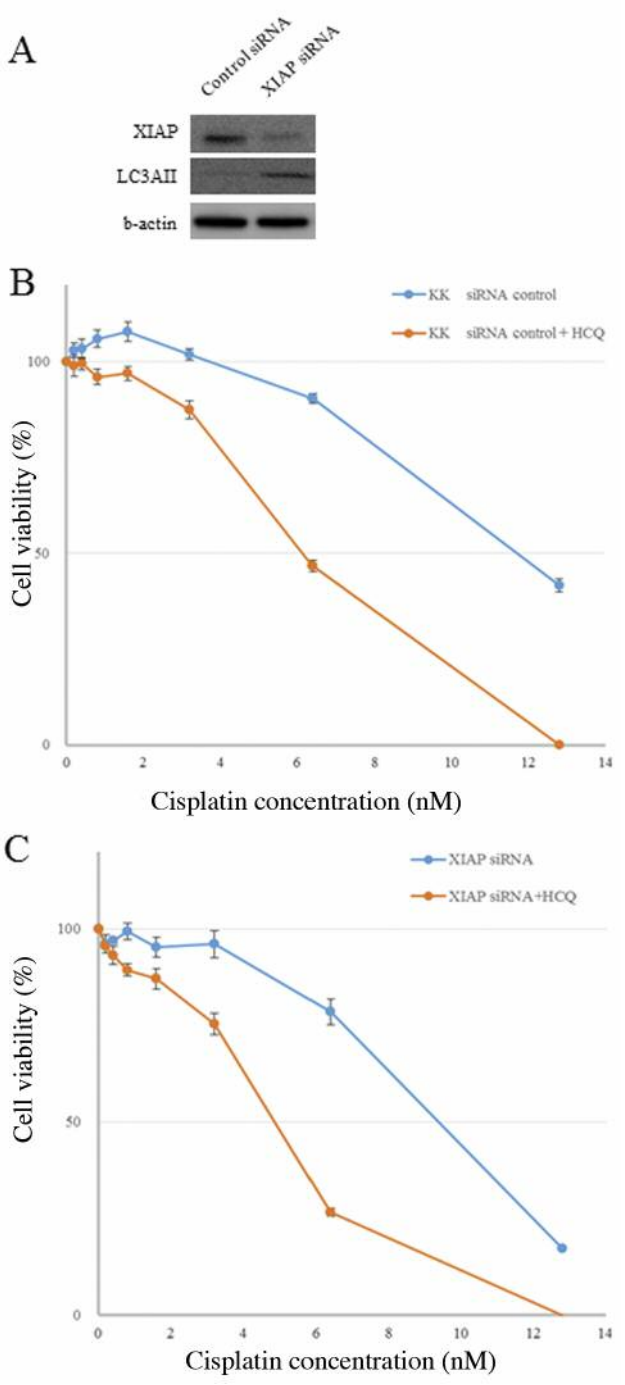

Figure 1. *Hydroxychloroquine (HCQ) treatment after transfection with either control or XIAP siRNA increased cisplatin sensitivity in ovarian clear cell cancer cells. After transfection with control siRNA and XIAP siRNA, western blot analysis revealed XIAP down-regulation and LC3A II up-regulation in KK cells (A). KK cells transfected with control siRNA or XIAP siRNA were treated with HCQ. Cisplatin sensitivity was increased in KK cells transfected with either control (B) or XIAP-specific siRNAs (C). Equivalent amounts (10 μg) of protein were subjected to SDS-PAGE and blotted with anti-XIAP and anti-LC3A antibodies. Cell viability was assessed at 5 days after treatment by an MTT assay. The data represent at least three independent experiments.*

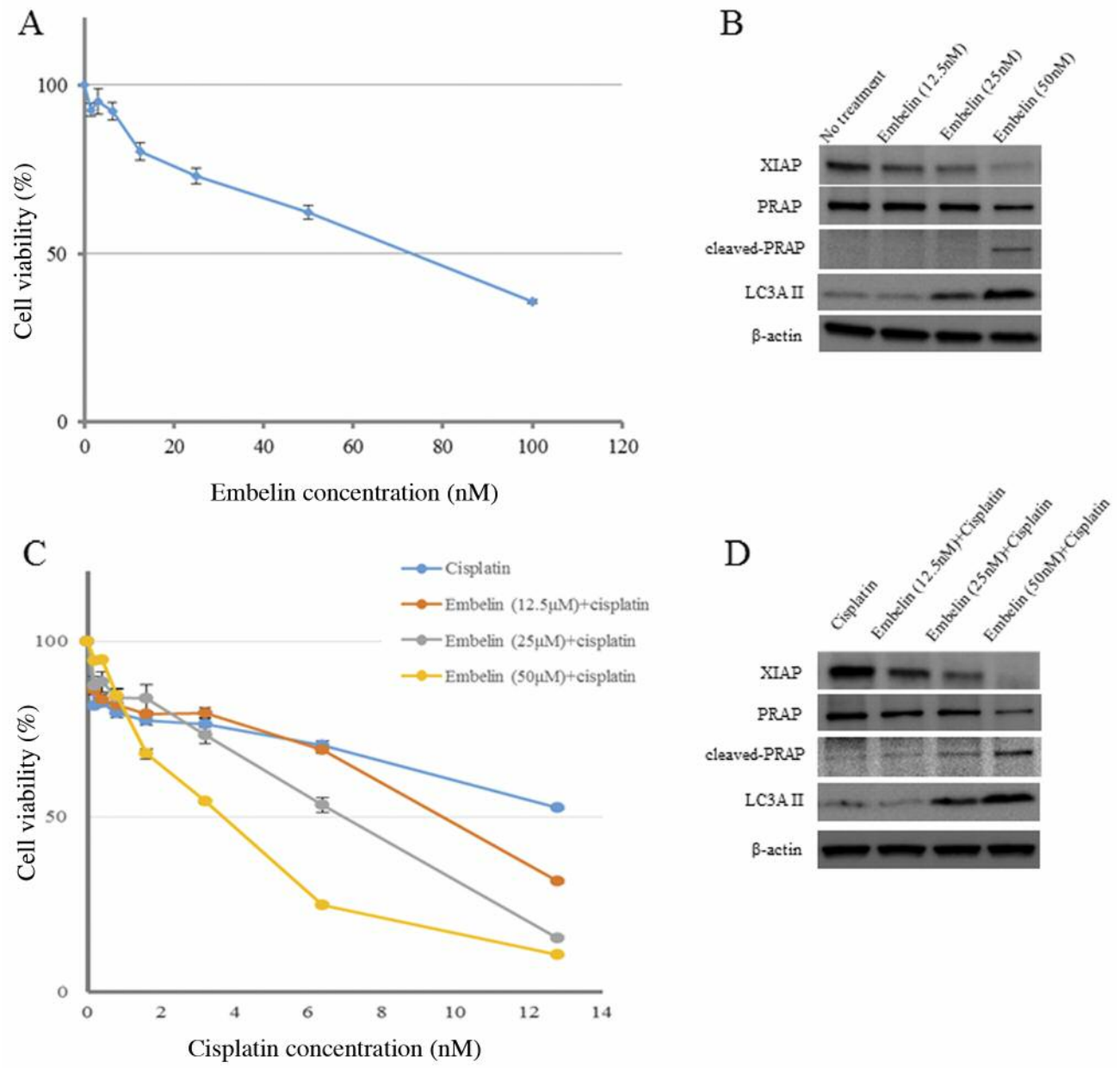

Figure 2. *Embelin increased cisplatin sensitivity in the ovarian clear cell cancer cell line, KK. Embelin showed an anti-tumor effect on KK as a single agent, through down-regulation of XIAP but not LC3A II (A, B). Embelin sensitized KK cells to cisplatin at different concentrations (C). Similarly, the combination of embelin and cisplatin induced down-regulation of XIAP but not LC3A. Equivalent amounts (10 μg) of protein were subjected to SDS-PAGE and blotted with anti-XIAP, anti-PARP, anti-cleaved PARP, anti LC3A, and anti-β-actin antibodies. Cell viability was assessed by an MTT assay. The data represent at least three independent experiments.*

(GE Healthcare UK Ltd, England, UK) on a LAS-3000 (Fujifilm, Minato, Tokyo, Japan). Protein expression was determined densitometrically and normalized against β-actin expression using Multi Gauge version 3.1 (FujiFilm, Tokyo, Japan).

Statistical analysis. The Stat View software ver.5.0 (SAS Institution Inc., NC, USA) was used for statistical analysis. All experiments were repeated independently at least three times. All values are presented as mean±s.d. Statistical significance between two groups was determined by a two-tailed *t*-test or ANOVA. Statistical significance was defined at $p<0.05$.

Results

Transfection with XIAP-specific siRNA induced down-regulation of XIAP and up-regulation of LC3A II (Figure

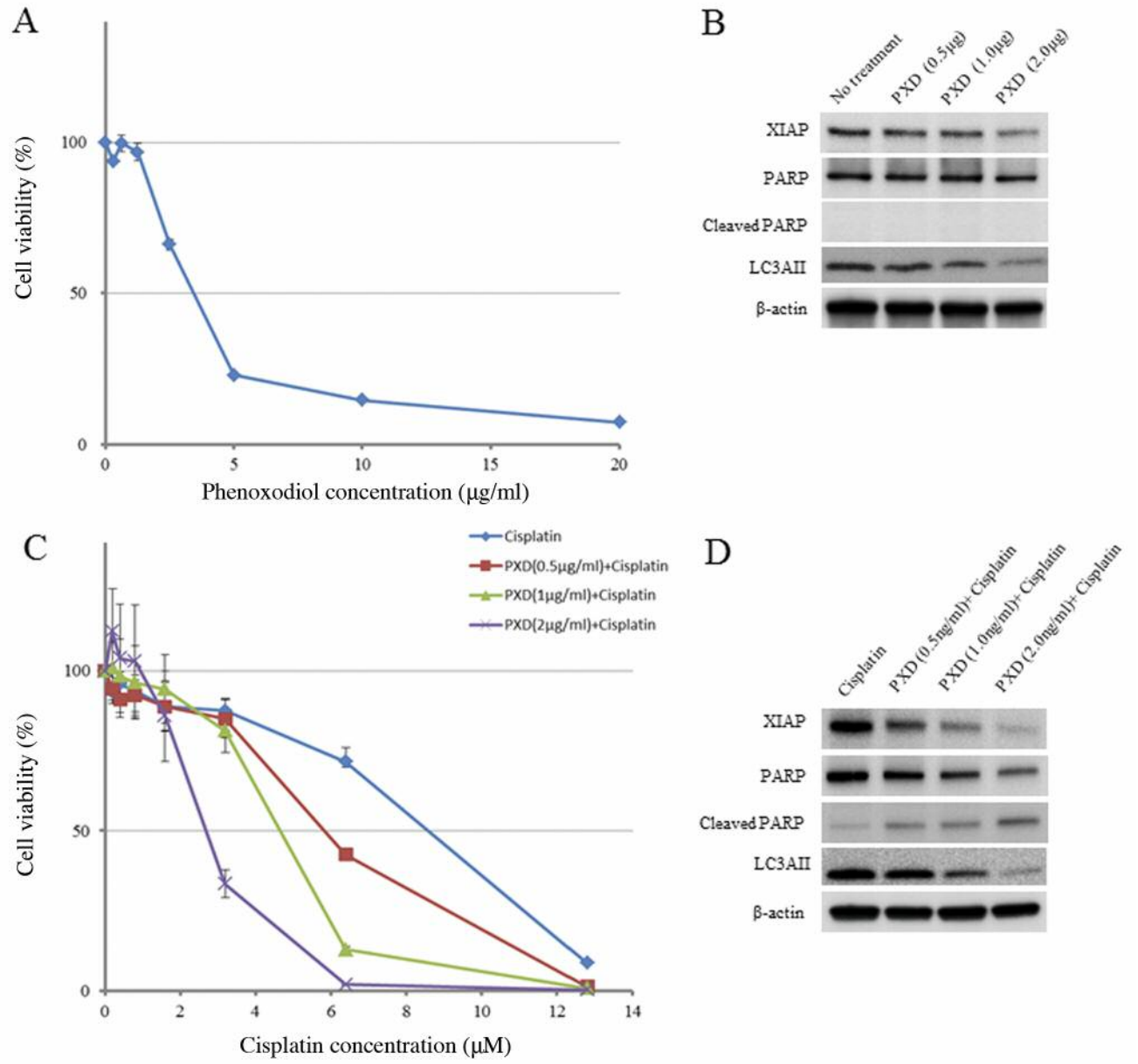

Figure 3. *Phenoxodiol (PXD) sensitized ovarian clear cell carcinoma cells KK through XIAP down-regulation and autophagy inhibition. (A) The anti-tumor activity of PXD as a single agent was determined. (B) Downregulation of XIAP and LC3AII protein was observed after PXD treatment for 24 h. (C) Pre-treatment with PXD increased the sensitivity of KK to cisplatin in a dose-dependent manner. (D) In addition to XIAP and LC3A II protein down-regulation, cleaved PARP was up-regulated after treatment with 10 nM of cisplatin in KK cells pre-treated with PXD. Equivalent amounts (10 μg) of protein were subjected to SDS-PAGE and blotted with anti-XIAP, anti-PARP, anti-cleaved PARP, anti-LC3A, and anti-β-actin antibodies. Cell viability was assessed at 5 days after treatment by an MTT assay. The data represent at least three independent experiments.*

1A). After treatment with 25 μM of HCQ for 24 h in KK cells transfected with either control or XIAP-specific siRNA, an assay for cisplatin cytotoxicity was conducted. HCQ increased the cisplatin sensitivity in both transfected cells (Figure 1B and C). Thus, XIAP down-regulation and autophagy blockade sensitized ovarian CCC cells to cisplatin.

Embelin as a single agent showed anti-tumor activity (Figure 2A). After embelin treatment for 24 h at doses of 12.5 μM, 25 μM, and 50 μM, XIAP protein levels were decreased but LC3A II protein levels increased (Figure 2B). Pre-treatment with embelin for 24 h at several doses increased the sensitivity of KK cells to cisplatin (Figure 2C).

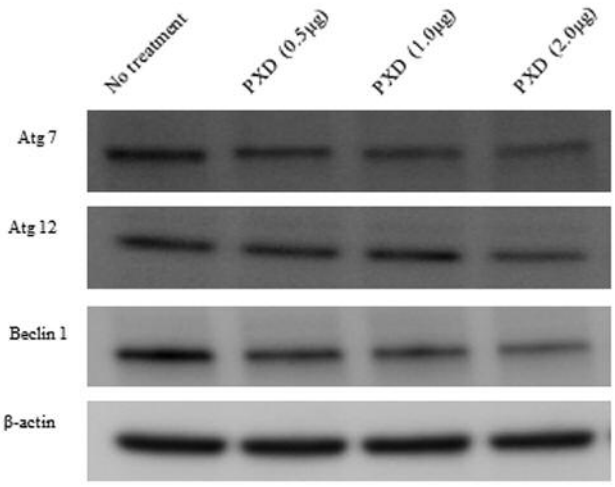

Figure 4. *The cascade of autophagy blockade in KK cells treated with phenoxodiol. Cell lysates were prepared after treatment with phenoxodiol for 24 h. Equivalent amounts (10 μg) of protein were subjected to SDS-PAGE and blotted with anti-Atg7, anti-Atg12, anti-Beclin-1, and anti-β-actin antibodies. Atg7, Atg12, and Beclin1 protein levels were found to be downregulated. The data represent at least three independent experiments.*

After embelin treatment at several doses for 24 h followed by cisplatin treatment for 24 hours at a dose of 10 μM, XIAP protein levels decreased and both cleaved-PARP and LC3A II protein levels were increased (Figure 2D). Thus, embelin increased sensitivity to cisplatin through down-regulation XIAP and induction of autophagy.

PXD showed anti-tumor activity as a single agent (Figure 3A). After PXD treatment for 24 h at a dose of 0.5 μg/ml, 1.0 μg/ml, and 2.0 μg/ml, XIAP and LC3A II protein levels were decreased (Figure 3B). Pre-treatment with PXD at several doses for 24 hours increased the sensitivity of KK cells to cisplatin (Figure 3C). After PXD treatment at several doses for 24 h followed by cisplatin treatment at a dose of 10 μM for 24 h, XIAP and LC3A II protein levels were found to be decreased, whereas cleaved-PARP protein levels were increased (Figure 3D). Thus, PXD increased cisplatin sensitivity through down-regulation of XIAP and autophagy blockade. After PXD treatment for 24 h, western blot analysis was performed and Atg7, Atg12, and Beclin-1 protein levels were found to be down-regulated (Figure 4).

Discussion

XIAP protein regulates the induction and inhibition of autophagy (16, 17). Autophagy activation was associated with poor prognosis in CCC and inhibition of autophagy was found to induce apoptosis in ovarian CCC cell lines (14, 15). In our study, XIAP down-regulation using siRNA induced activation of autophagy. Even in this condition, XIAP protein down-regulation enhanced cisplatin sensitivity in KK cells. Furthermore, HCQ treatment in addition to XIAP down-regulation increased the sensitivity of ovarian CCC cells to cisplatin more effectively. Embelin demonstrated an anti-tumor effect as a single agent and enhanced cisplatin sensitivity but also activated autophagy. Conversely, PXD increased the sensitivity to cisplatin through XIAP down-regulation as well as inhibition of autophagy. PXD-induced XIAP down-regulation inhibited autophagy through Beclin-1. Although it was unclear whether PXD and embelin possess clinical activity, PXD might be more suitable for treating CCC considering the underlying mechanism.

PXD, an isoflavone derivative, has demonstrated anti-tumor effects in several human cancer cell lines, including ovarian serous cancer cell lines (19). PXD caused apoptosis in cancer cells by downregulating XIAP, as well as by disrupting FLICE inhibitory protein (FLIP) expression (20), anti-angiogenesis activity (20), causing cell cycle arrest at G1 (21), inhibiting topoisomerase II (22), and upregulating Bax (23). In addition to these effects, the present study demonstrates that PXD inhibits autophagy. Previous studies have demonstrated XIAP protein (8), autophagy (14, 15), and anti-angiogenesis (24) as candidates of future targeted therapies against CCC. PXD inhibits these factors as a single agent. Therefore, future studies could confirm whether PXD shows clinical activity in CCC treatment.

XIAP-targeted therapy might be a promising treatment for CCC. In colon cancer, PIK3CA-mutated colorectal cancer cells treated with XIAP-targeting drugs underwent receptor-induced apoptosis (25). As *PIK3CA* mutations are more frequently observed in CCC, at a frequency of 33% (26), it would be interesting to investigate the association between *PIK3CA* mutation and responses to XIAP inhibitors.

In conclusion, XIAP down-regulation and inhibition of autophagy increased cisplatin sensitivity in ovarian CCC cell lines. PXD sensitized ovarian CCC cell lines to cisplatin through down-regulation of XIAP and inhibition of autophagy.

Conflicts of Interest

The Authors declare no potential conflicts of interest.

Acknowledgements

None.

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