The HSP90 inhibitor 17-N-allylamino-17-demethoxy geldanamycin (17-AAG) synergizes with cisplatin and induces apoptosis in cisplatin-resistant esophageal squamous cell carcinoma cell lines via the Akt/XIAP pathway

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Abstract. Although cisplatin (CDDP) is a key drug in the treatment of esophageal squamous cell carcinoma (ESCC), acquired chemoresistance remains a major problem. Combination therapy may represent one strategy to overcome this resistance. Heat shock protein 90 (HSP90) is known to be overexpressed in several types of cancer cells, and its inhibition by small molecules, either alone or in combination, has shown promise in the treatment of solid malignancies. In the present study, we evaluated the synergistic effects of combining CDDP with the HSP90 inhibitor 17-N-allylamino-17-demethoxy geldanamycin (17-AAG) on two CDDP-resistant human esophageal squamous cancer cell lines, KYSE30 and KYSE150. The results obtained demonstrated the synergistic inhibitory effects of CDDP and 17-AAG on the growth of KYSE30 and KYSE150 cells. Cell growth and cell number were more effectively reduced by the combined treatment with CDDP and 17-AAG than by the treatment with either CDDP or 17-AAG alone. Western blotting revealed that the combined action of CDDP and 17-AAG cleaved poly (ADP-ribose) polymerase (PARP) and caspase-3, which demonstrated that the reduction in both cell growth and cell number was mediated by apoptosis. Time-course experiments showed that reduction in X-linked inhibitor of apoptosis protein (XIAP) and phosphorylated Akt were concomitant with apoptosis. The results of the present study demonstrate that 17-AAG synergizes with CDDP and induces apoptosis in CDDP-resistant ESCC cell lines, and also that modulation of the Akt/XIAP pathway may underlie this synergistic effect. Combination therapy with CDDP and an HSP90 inhibitor may represent a promising strategy to overcome CDDP resistance in ESCC.

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Introduction

Esophageal cancer is one of the most aggressive types of cancer of the gastrointestinal tract. The estimated incidence of esophageal cancer was 3.8% of all cancers and the estimated cancer mortality worldwide was 5.4% in 2008 (1). The incidence of esophageal cancer is particularly high in both males and females in East Asia, with squamous cell carcinoma being the predominant histologic type in this region, accounting for more than 90% of all esophageal cancer cases (1). Multimodality therapy, including surgery, chemotherapy and radiotherapy, is required for the effective management of advanced esophageal cancer. However, the 5-year survival rates of patients treated using surgery, chemotherapy alone, radiotherapy alone and concurrent chemoradiotherapy were reported to be 50.2, 8.6, 15.5 and 26.4%, respectively (2). Therefore, further improvements in outcomes are urgently required (2-5).

Cisplatin (CDDP) is a platinum drug that is widely used to treat esophageal squamous cell carcinoma (ESCC) (6,7). CDDP, either alone or in combination with other agents, has been shown to improve patient outcomes. However, acquired chemoresistance develops during the course of treatment and is often the reason for treatment failure. Therefore, overcoming chemoresistance is essential for improving the outcomes of patients.

Molecular chaperone proteins function to ensure the proper conformation of client proteins when cells experience stress or damage (8). Heat shock protein 90 (HSP90) is a molecular chaperone that participates in stabilizing and activating more than 200 proteins, including serine/threonine kinases (Akt, Raf-1, and Cdk4), and the transcription factors hypoxia-inducible factor 1α (HIF1α) and p53, receptor/non-receptor kinases (HER2, EGFR, and Src family kinases), and steroid hormone receptors (9). Since many of these client proteins have been shown to significantly contribute to tumor growth and survival, abrogating their function with an HSP90 inhibitor is an attractive prospect (10).

In this study, we explored the potential synergistic effect of CDDP and the HSP90 inhibitor, 17-N-allylamino-17-demethoxy geldanamycin (17-AAG), on human ESCC cell lines. We also attempted to identify the molecular mechanism involved in this synergistic effect.

Materials and methods

Cell lines and culture. The TE series (TE1, TE4, TE5, TE6, TE8, TE9, TE10, TE11, TE14 and TE15) and EC-GI-10 were obtained from the Riken BioResource Center (Saitama, Japan). The KYSE series (KYSE30, KYSE70, KYSE140, KYSE150, KYSE170, KYSE180, KYSE220 and KYSE270), TT, and TTn were obtained from the Health Science Foundation (Tokyo, Japan). These cell lines were derived from human esophageal squamous cancer. Cells were cultured in a 5% CO_2 atmosphere at 37˚C in RPMI-1640 (R8758; Sigma-Aldrich, St. Louis, MO, USA) complete medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Chemicals and antibodies. CDDP and 17-AAG were purchased from Sigma-Aldrich. CDDP was dissolved in 0.9% sodium chloride solution and 17-AAG was dissolved in dimethyl sulfoxide (DMSO). All drugs were stored in aliquots at -20˚C.

Antibodies to caspase-3 (#9662), PARP (#9542), XIAP(3B6) (#2045), c-IAP1 (#4952), c-IAP2(58C7) (#3130), livin (D61D1) XP (#5471), survivin (71G4B7) (#2808), phospho-Akt (Ser473) (D9E) (#4060), Akt (#9272), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#9101), and p44/p22 MAPK (Erk1/2) (#9102) were from Cell Signaling Technologies (Beverly, MA, USA). The antibody to β-actin was from Sigma-Aldrich. Antibodies to Bcl-2 (#610391), Bcl-xL (#60982), Bid (#61158), Bad (#610391), Bax (#610982), Beclin (#612112), and BAG-1 (#611868) were from BD Biosciences (San Jose, CA, USA).

Drug sensitivity assay. Cells were suspended in RPMI/10% FBS and seeded at between 2,000 and 4,000 cells/well in quintuplicate in 96-well plates. Cells were treated with varying doses of CDDP and 17-AAG 24 h after plating and were allowed to grow for an additional 72 h. Viable cell density was determined by a water-soluble tetrazolium salt (WST-8, Cell Counting kit-8; Dojindo, Japan) according to the manufacturer's instructions using a microplate absorbance reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm.

Determination of IC_{50} and combination index (CI). IC_{50} was calculated using the CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). CI values were calculated for 50% toxicity based on the equation below (11):

$$CI = D_1 / Dx_1 + D_2 / Dx_2 + \alpha \times [(D_1 \times D_2) / (Dx_1 \times Dx_2)]$$

where, Dx_1 = Dose of drug 1 to produce 50% cell kill alone; D_1 = Dose of drug 1 to produce 50% cell kill in combination with D_2; Dx_2 = Dose of drug 2 to produce 50% cell kill alone; D_2 = Dose of drug 2 to produce 50% cell kill in combination with D_1; α=0 for mutually exclusive or 1 for mutually non-exclusive modes of drug action.

Time-dependent cell growth assay. Equal numbers of cells were seeded in quintuplicate in 96-well plates and cell growth was measured using WST-8 Cell Counting kit-8 at 0, 24, 48 and 72 h. The results were expressed as percentages relative to the absorbance at 0 h.

Western blot analysis. KYSE30 and KYSE150 cells were seeded on 100 mm plates and were exposed to CDDP with/without 17-AAG after 24 h. Cells were subsequently cultured for 24, 48 and 72 h; adherent and floating cells were then pelleted, washed with cold PBS and lysed in RIPA buffer [150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 50 mM NaF, 20 μM Na_3VO_4 and protease inhibitor]. Lysates were centrifuged at 15,000 rpm at 4˚C for 10 min and the protein concentration in each sample was determined by the BCA Protein Assay kit (Takara Bio, Inc., Shiga, Japan). Cell lysates or their fractions containing equal amounts of protein (12 μg) were resolved by the Mini-Protean TGX Precast Gel (Bio-Rad Laboratories) and transferred to nitrocellulose membranes. Membranes were probed with the primary antibody, followed by the secondary antibody conjugated to HRP, developed using western blot detection reagents (Amersham Biosciences, Uppsala, Sweden), and then detected by the ChemiDoc SRS image analysis system (Bio-Rad Laboratories).

Statistical analysis. Statistical analyses were performed with IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). P-values of <0.05 were considered to indicate a statistically significant difference. We used Dunnett's test for the time-dependent cell growth assay. Data are presented as means ± standard deviation.

Results

Determination of IC_{50} for CDDP and 17-AAG across a panel of ESCC cell lines. Treating esophageal cell lines with CDDP and 17-AAG resulted in dose-dependent cytotoxicity. Fig. 1 shows IC_{50} values for CDDP (Fig. 1A) and 17-AAG (Fig. 1B) across our panel of ESCC cell lines. IC_{50} values ranged from 0.983 to 14.9 μM for CDDP, and 0.0128 to 2.37 μM for 17-AAG, respectively. Based on these results, we decided to use KYSE30, KYSE150, EC-GI-10 and TE6 as representative CDDP-resistant cell lines in subsequent experiments.

CDDP and 17-AAG exhibit synergistic inhibitory effects on the growth of KYSE30 and KYSE150 esophageal squamous carcinoma cell lines. To evaluate the impact of the combined treatment with CDDP and 17-AAG on CDDP-resistant cell

Table I. Combination index value of the interaction between CDDP with 17-AAG against human esophageal carcinoma cell lines.

Cell line	IC_{50} of 17-AAG (μM)	Concentration of 17-AAG (μM)	CI at IC_{50}	Interpretation
KYSE30	2.37	1.000	0.49	Synergism
		0.500	0.76	Moderate synergism
KYSE150	0.10	0.050	0.71	Moderate synergism
		0.025	0.55	Synergism
EC-GI-10	1.40	1.000	1.80	Antagonism
		0.500	1.78	Antagonism
TE6	0.04	0.050	1.18	Moderate antagonism
		0.025	1.06	Additive effect

Different concentrations of 17-AAG were employed to study the effect on IC_{50} of CDDP. Variable ratios of drug concentrations and mutually non-exclusive equations were used to determine the CI. CI >1.3 indicates antagonism; CI = 1.1 to 1.3, moderate antagonism; CI = 0.9 to 1.1, additive effect; CI = 0.8 to 0.9, slight synergism; CI = 0.6 to 0.8, moderate synergism; CI = 0.4 to 0.6, synergism; and CI = 0.2 to 0.4, strong synergism. CI, combination index; CDDP, cisplatin.

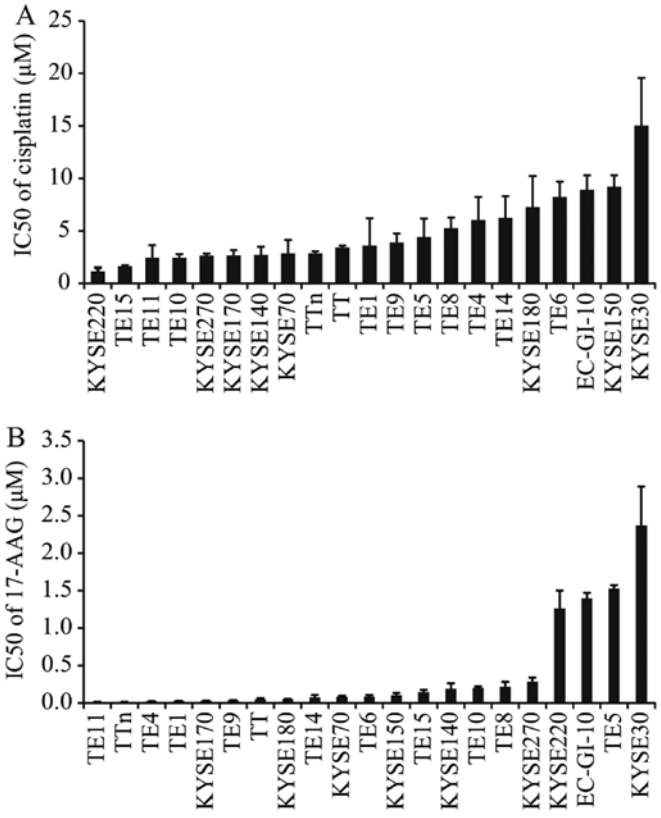

Figure 1. IC_{50} values for cisplatin (A) and 17-AAG (B) across the panel of esophageal squamous cell carcinoma cell lines. Cells were treated with a range of concentrations of cisplatin or 17-AAG for 72 h. Viable cell density was determined by a water-soluble tetrazolium salt and IC_{50} was calculated. The values and error bars represent the mean and standard deviation of at least three experiments performed in quintuplicate.

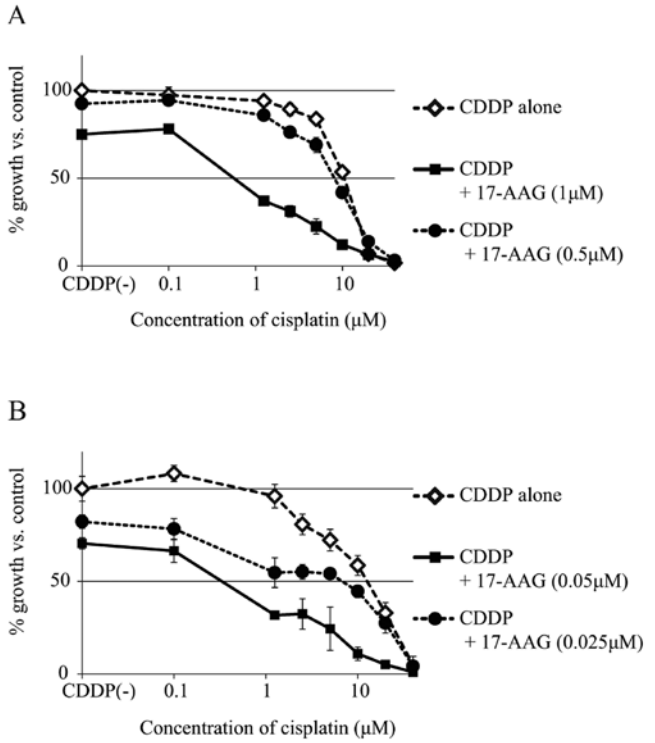

Figure 2. Dose-response curve for cisplatin (CDDP) in the presence of low doses of 17-AAG. KYSE30 and KYSE150 were treated with various doses of CDDP (0.1-40 μM) and 17-AAG (0.5-1 μM for KYSE30; 0.025-0.05 μM for KYSE150) for 72 h, and cytotoxicity was evaluated by a drug sensitivity assay. (A) KYSE30, (B) KYSE150. The experiment was performed twice and representative data are shown. Data are presented as means ± standard deviation of quintuplicate wells.

lines, KYSE30, KYSE150, EC-GI-10 and TE6 were treated with various concentrations of each drug alone or in combination, and were then subjected to a cell viability assay. As shown in Fig. 2, the combination with low-dose 17-AAG shifted the survival curve to the left in KYSE30 and KYSE150. The interaction between CDDP and 17-AAG was determined by calculating the CI. The CI values of KYSE30 and KYSE150 ranged from 0.4 to 0.7 for 50% cell kill, demonstrating synergistic behavior between CDDP and 17-AAG (Table I). In contrast, the CI value of EC-GI-10 and TE6 ranged from 1.0 to 1.8.

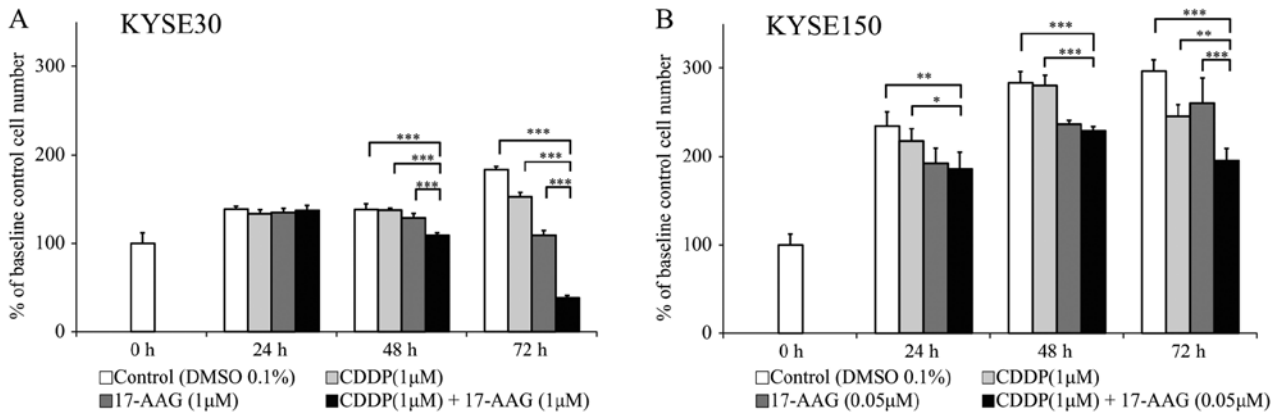

Figure 3. Changes in cell numbers by the treatment with cisplatin (CDDP) and 17-AAG, alone or in combination. (A) KYSE30 and (B) KYSE150 were exposed to 17-AAG alone (1 μM for KYSE30, 0.05 μM for KYSE150), CDDP alone (1 μM for both cell lines), and the combination of CDDP (1 μM for both cell lines) and 17-AAG (1 μM for KYSE30, 0.05 μM for KYSE150). Cell numbers were counted after 24, 48 and 72 h. Data are presented as means and standard deviation of quintuplicate wells. *P<0.05, **P<0.01, ***P<0.001.

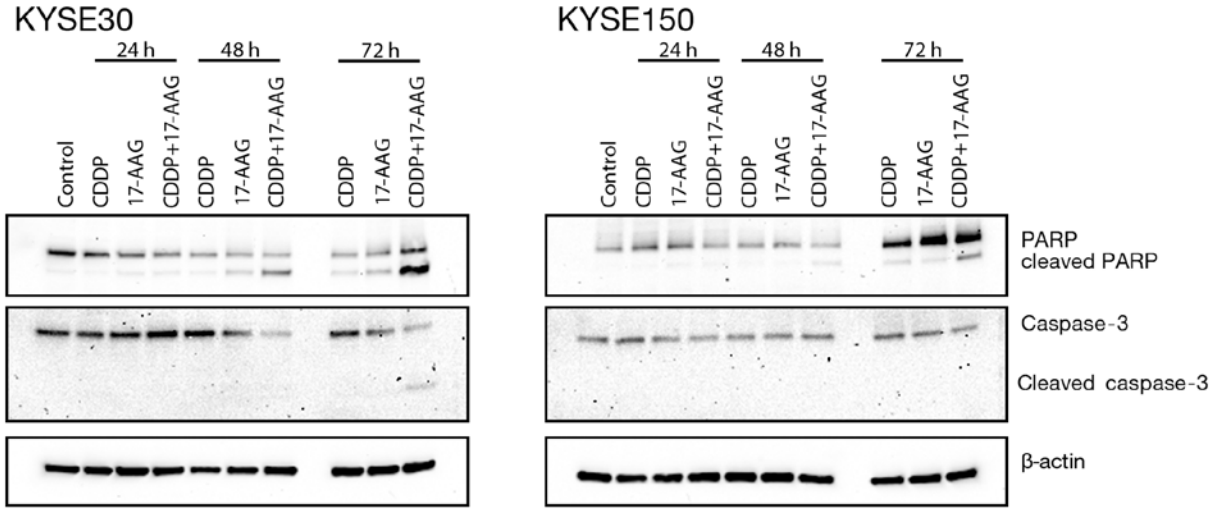

Figure 4. Induction of apoptosis by the combination of cisplatin (CDDP) and 17-AAG. KYSE30 was treated with CDDP (1 μM) and/or 17-AAG (1 μM), while KYSE150 was treated with CDDP (1 μM) and/or 17-AAG (0.05 μM). After the treatment, cell extracts were examined by western blot analysis.

These results showed either the additive or antagonistic effects of CDDP and 17-AAG in EC-GI-10 and TE6.

Cell count assay. To confirm the synergistic effect of CDDP and 17-AAG in KYSE30 and KYSE150, we counted actual cell numbers after treatment with the vehicle only (0.1% DMSO), 1 μM CDDP only, 1 μM 17-AAG only, and the combined treatment with 1 μM CDDP and 1 μM 17-AAG for 24, 48 and 72 h.

As shown in Fig. 3, low-dose CDDP alone and low-dose 17-AAG alone modestly suppressed cell growth in KYSE30 and KYSE150 (Fig. 3). However, the reduction in cell growth was significantly greater with the combination of CDDP and 17-AAG than with either drug alone.

Combination of CDDP and 17-AAG induces apoptosis. Using western blot analysis, we determined whether the reduced cell growth caused by the combined treatment with CDDP and 17-AAG occurred due to the induction of apoptosis via the cleavage of poly (ADP-ribose) polymerase (PARP) and activation of caspase-3.

No significant changes were observed in the expression of PARP and cleaved PARP 24 h after treatment with CDDP alone, 17-AAG alone, or CDDP and 17-AAG (Fig. 4). However, a clear increase was observed in cleaved PARP 48 h after the co-treatment with CDDP and 17-AAG in KYSE30 and KYSE150. The increase in cleaved PARP was greater than that with either drug alone. The induction of cleaved PARP was further enhanced at 72 h. Cleaved caspase-3 was detected 72 h after the co-treatment with CDDP and 17-AAG in KYSE30, but the band was weaker than that of cleaved PARP.

Combination of CDDP and 17-AAG reduces the expression of XIAP and phosphorylated Akt. To understand the mechanism underlying the synergy between CDDP and 17-AAG, we investigated the expression of proteins associated with apoptosis (Fig. 5). Under basal conditions, KYSE30 expressed high levels of XIAP, cIAP1, and survivin and low levels of cIAP2. KYSE150 expressed high levels of XIAP and cIAP1, and low levels of cIAP2 and survivin. Livin was not detected in either cell line.

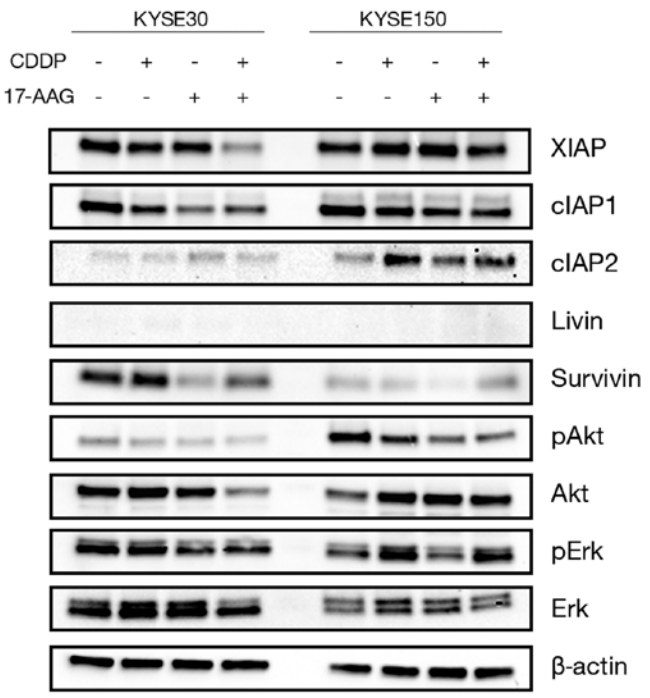

Figure 5. Western blot analysis of the IAP family proteins, the Erk and Akt pathways. KYSE30 and KYSE150 were treated with cisplatin (CDDP; 1 μM), 17-AAG (1 μM for KYSE30, 0.05 μM for KYSE150), and the combination of these drugs for 72 h, and cell lysates were analyzed by western blotting.

The treatment with CDDP alone, 17-AAG alone, or combined treatment with CDDP and 17-AAG induced variable changes in the levels of cIAP1, cIAP2 and survivin; however, a correlation was not observed between these changes and cell growth inhibition or the induction of apoptosis by the treatment. XIAP levels were, in contrast, unchanged by either agent alone, but were significantly reduced by the combined treatment. We also examined the expression of Bcl-2 family members, including Bcl-2, Bcl-xL, Bid, Bad, Bax, Bim, Beclin and BAG-1; however, no significant changes were observed by the treatment with CDDP alone, 17-AAG alone, or combined treatment with CDDP and 17-AAG (data not shown). These results demonstrated that the combination of CDDP and 17-AAG mainly induced apoptosis by inhibiting XIAP.

To identify the mechanism for the reduction in XIAP, the expression of the Akt and the Erk pathways was examined. As shown in Fig. 5, phosphorylated Akt levels were slightly reduced by either CDDP or 17-AAG alone in both cell lines. However, the combination of CDDP and 17-AAG clearly diminished phosphorylated Akt levels (Fig. 5). Phosphorylated Erk levels remained unchanged by the treatment in KYSE30. Phosphorylated Erk levels were modestly increased by CDDP alone and the combined treatment with CDDP and 17-AAG in KYSE150; however, no correlation was observed between these changes and the inhibition of cell growth or induction of apoptosis.

Time-dependent changes in the expression of phosphorylated Akt, total Akt, and XIAP. In order to determine time-dependent changes in phosphorylated Akt, total Akt and XIAP, we examined the expression of these proteins after the treatment with CDDP and/or 17-AAG, either alone or in combination (Fig. 6). Phosphorylated Akt levels were modestly reduced by 17-AAG alone and by the combination of CDDP and 17-AAG after 24 h. Further reductions occurred at 72 h, especially with the combination of CDDP and 17-AAG in KYSE30. The expression of phosphorylated Akt was not significantly changed by CDDP alone. The expression of XIAP was reduced by 17-AAG alone and by the combination of CDDP and 17-AAG, similar to that for phosphorylated Akt.

Discussion

In the present study, we demonstrated that the combined treatment with CDDP and 17-AAG had synergistic inhibitory effects on cell growth in CDDP-resistant ESCCs. The synergistic interaction between CDDP and 17-AAG resulted in significant increases in the cytotoxicity of CDDP; a strong cytotoxic effect was obtained in the presence of low-dose 17-AAG, which hardly has a cytotoxic effect by itself, in combination with a low concentration of CDDP. This cytotoxic effect occurred via induction of apoptosis, as demonstrated by the cleavage of PARP and caspase-3.

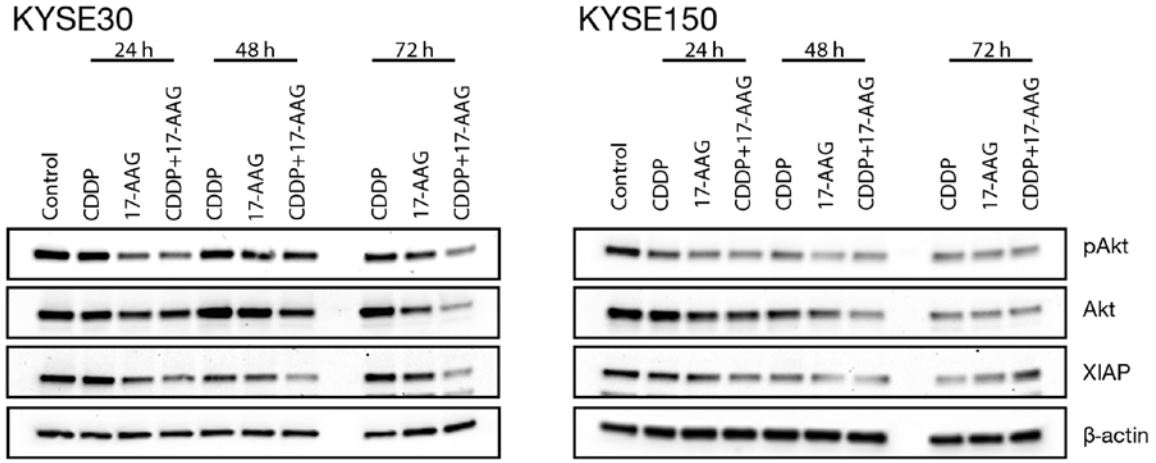

Figure 6. Time-dependent changes in the expression of phosphorylated Akt, Akt and XIAP. KYSE30 and KYSE150 were treated with cisplatin (CDDP; 1 μM), 17-AAG (1 μM for KYSE30, 0.05 μM for KYSE150), and the combination of these drugs for the indicated times, and cell lysates were analyzed by western blotting.

The synergy between CDDP and an HSP90 inhibitor has been reported in previous studies; for example, McCollum *et al* reported that CDDP was synergistic with geldanamycin or 17-AAG and combined treatment with the two drugs increased apoptosis in the lung cancer cell line A549 (12). This synergy was attributed in part to the CDDP-induced abrogation of heat shock factor-1 activity. Weng *et al* also showed that 17-AAG enhanced CDDP cytotoxicity in the non-small cell lung cancer cell lines, A549 and H1650 (13). This synergistic effect was reported to be mediated by the downregulation of thymidine phosphorylase and Akt activation. We have yet to examine whether similar mechanisms occur in our cell lines. Nevertheless, our study provides the first evidence for the synergy of CDDP and 17-AAG in CDDP-resistant esophageal squamous cell lines.

We also revealed that, among the major regulators of apoptosis, the Akt/XIAP pathway mediated the synergistic effect of CDDP and 17-AAG; the expression patterns of phosphorylated Akt and XIAP levels closely correlated with their inhibitory effect on cell growth, induction of PARP cleavage, and apoptosis, which occurred by the combined treatment with CDDP and 17-AAG. Moreover, time-course experiments demonstrated that the reduction in phosphorylated Akt and XIAP levels were concomitant with the induction of PARP cleavage and apoptosis.

Previous studies indicated the Akt/XIAP pathway as the main regulator of apoptosis by chemotherapy in some cancer cell lines, including carcinomas of the breast, ovary, uterine cervix and melanoma (14-17). Akt phosphorylates and stabilizes XIAP, and the deactivation or knockdown of Akt destabilizes XIAP, leading to apoptosis (18). In addition, specific inhibition of XIAP expression was shown to induce apoptosis and increase caspase-3 activity in prostate cancer cells (19). These findings support our conclusion that the synergistic effect of CDDP and 17-AAG is mediated by the induction of apoptosis via the Akt and XIAP pathways.

One limitation of our study is that we lack data showing the synergy of CDDP and 17-AAG *in vivo*, and such data will be required to extrapolate the current results to clinical situations. We also cannot explain why synergy occurred in KYSE30 and KYSE150, but not in EC-GI-10 or TE6. A recent study indicated phosphatase and tensin homolog (PTEN) as a potential predictive marker for HSP90 inhibitor sensitivity among four ESCC cell lines (20). However, biomarkers that predict the HSP90 response or synergy of an HSP90 inhibitor and CDDP have not yet been established (21). The identification of such biomarkers is crucial in clinical settings.

In conclusion, we showed that the combination of low concentrations of CDDP with low-dose 17-AAG exerted synergistic effects on CDDP-resistant ESCC cell lines. The mechanism of the synergy was attributed to apoptosis mediated by downregulation of the Akt/XIAP pathway. Our results indicate that the co-administration of low-dose 17-AAG and CDDP overcomes CDDP chemoresistance and may improve the outcomes of patients with ESCC.

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