Acetaminophen Enhances Cisplatin- and Paclitaxel-mediated Cytotoxicity to SKOV3 Human Ovarian Carcinoma

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Abstract

Background—Ovarian cancer is commonly treated with cisplatin/paclitaxel but many tumors become resistant. Acetaminophen reduced glutathione and enhanced chemotherapy efficacy in treating hepatic cancer. The objective of this study was to examine if acetaminophen enhances the cytotoxicity of cisplatin/paclitaxel in ovarian cancer.

Materials and Methods—SKOV3 human ovarian carcinoma cells *in vitro* and a subcutaneous tumor nude rat model were used and treated with cisplatin/paclitaxel with or without acetaminophen.

Results—*In vitro*, acetaminophen enhanced apoptosis induced by cisplatin and paclitaxel with similar effects on glutathione, reactive oxygen species and mitochondrial membrane potential but different effects on nuclear factor erythroid 2-related factor 2 (NRF2) translocation. *In vivo*, acetaminophen was uniformly distributed in tissue and significantly reduced hepatic glutathione. Acetaminophen enhanced cisplatin chemotherapeutic effect by reducing tumor recurrence

Conclusion—Our results suggest that acetaminophen as a chemoenhancing adjuvant could improve the efficacy of cisplatin and paclitaxel in treating patients with ovarian carcinoma and other tumor types.

Keywords

Acetaminophen; glutathione; chemo-enhancement; cisplatin; ovarian cancer

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Ovarian cancer is the leading cause of death from gynecological disease in the United States. Chemotherapy with a platinum agent (cisplatin, carboplatin) and/or paclitaxel remains the front-line treatment for ovarian cancer (1, 2). However, chemoresistance usually develops, and relapse remains almost inevitable in patients with advanced disease (3, 4). Cisplatin forms both intrastrand and interstrand crosslinks of DNA to induce apoptosis (5), while paclitaxel promotes the assembly of microtubules and inhibits microtubule depolymerization

(6). These differing initial mechanisms cause a cascade of toxic effects in cancer cells, such as a decrease in mitochondrial membrane potential ($\Delta\psi$m) (7), or generation of reactive oxygen species (ROS) (8), converging to cell death. Ovarian cancer often exhibits cross-resistance to platinum agents and paclitaxel (4). Mechanisms to reduce resistance and enhance cytotoxicity should improve chemotherapy efficacy.

A major cause of chemotherapy resistance appears to be inactivation of the drug and activation of drug efflux pumps by endogenous thiols, including glutathione (GSH) and other sulfhydryls such as metallothionein (9, 10). Glutathione-*S*-transferase mRNA expression was found to be inversely correlated to sensitivity to platinum chemotherapy (11), and chemosensitivity may be associated with polymorphisms in the gene encoding this enzyme (2).

Acetaminophen, a commonly used analgesic and antipyretic drug, can alter glutathione levels in different cell types (12–15). The mixed function oxidase system of enzymes, most relevantly cytochrome P450 2E1 (CYP2E1), generates a reactive arylating intermediate that is normally detoxified by reduced GSH (16). Large doses of acetaminophen overwhelm GSH stores allowing the toxic metabolite, *N*-acetyl-*p*-benzoquinonimine, to bind macromolecules and induce cell death (17). High-dose acetaminophen can enhance chemotherapy activity against different cancer cells *in vitro* (15, 18, 19) and *in vivo* (20–22). Kobrinsky *et al.* reported a clinical case of a child with unresectable stage III hepatoblastoma who had failed frontline cisplatin-based therapies (22). This patient was disease-free seven years post surgery after receiving a treatment regimen that combined cisplatin after high-dose acetaminophen followed with *N*-acetylcysteine (NAC) rescue 8 h later to reduce toxicity to normal liver. Acetaminophen may suppress ovulation and affect cell proliferation, angiogenesis, and apoptosis of the ovary epithelium (23). It has also been proposed that acetaminophen inhibits ovarian carcinogenesis through the depletion of GSH leading to apoptosis and necrosis (23).

Regardless of CYP2E1 protein levels, our laboratory previously showed that acetaminophen enhanced cisplatin-induced apoptosis in human hepatocarcinoma and hepatoblastoma cells by reducing GSH concentrations (19). The objective of this study was to further investigate the potential effect of acetaminophen in enhancing cisplatin/paclitaxel-mediated cytotoxicity in ovarian carcinoma. These results may provide clinically relevant information about a potential chemotherapeutic regimen in which acetaminophen is combined with cisplatin/paclitaxel in treating of patients with ovarian cancer.

Materials and Methods

Cell culture, pharmacological agents and antibodies

The SKOV3 human ovarian adenocarcinoma cell line from the American Type Culture Collection (Rockville, MD, USA) was grown in media supplemented with 10% fetal bovine serum (FBS) and antibiotics in a humidified atmosphere of 5% CO_2. Sterile solutions of cisplatin (1 mg/ml) were obtained from the Oregon Health and Sciences University (OHSU) pharmacy. Acetaminophen was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) and sterilely filtered for *in vitro* studies. For the *in vivo* animal studies, commercial baby Tylenol liquid formula containing acetaminophen (100 mg/ml) was purchased from the OHSU pharmacy. Rabbit anti-poly (ADP-ribose) polymerase (PARP) antibody was purchased from Cell Signaling (Danvers, MA, USA), mouse antibody to tubulin was from Sigma-Aldrich, and rabbit antibodies to nuclear factor erythroid 2-related factor 2 (NRF2) and β-actin were from Santa Cruz Biotech (Santa Cruz, CA, USA).

In vitro assays

SKOV3 ovarian carcinoma cells were treated with or without acetaminophen (10 mM) for 2 and 24 h, with or without addition of cisplatin (5 μg/ml) or paclitaxel (100 nM). Cells treated with DMSO alone were used as vehicle control. For in vitro GSH and ROS assays, each treatment group was performed in triplicate in at least three independent experiments. Cellular GSH levels were monitored and analyzed using the Quanticrom Glutathione Assay Kit from BioAssay Systems (Hayward, CA, USA) according to the manufacturer's protocol. The results were normalized to protein concentration measured using a BCA assay kit (Pierce Biotechnology, Rockford, IL, USA). For cell cycle analysis, cells were collected and fixed in 70% ethanol 24 h after treatments. Cells were stained with propidium iodine and subjected to BD Calibur cytometer at the OHSU flow cytometry core and analyzed by BD Modfit software (San Jose, CA, USA). Cellular ROS levels were analyzed by incubating cells with dihydrorhodamine 123 dye, an oxidant-sensitive fluorochrome (Sigma-Aldrich) at 2.5 μg/ml for 30 min at 37°C as previously described (24). Whole cell lysate was then subjected to FLX-800 plate reader with Gen5 software (Biotek, Winooski, VT, USA). The cellular protein level in cells under different treatments was measured by western blot analysis using a chemiluminescence substrate (Pierce Biotechnology, Rockford, IL, USA) as previously described (25). Cellular mitochondrial membrane potential ($\Delta\psi$m) was analyzed using the JC-1 detection reagent (eBioscience, San Diego, CA, USA) at 3 μg/ml for 30 min at 37°C based on the method described by Reers *et al.* (26). Stained cells were viewed using a Zeiss Observer fluorescent microscope and photographs of representative cells were taken by Zeiss AxioCam camera. The percentage of green stained cells was counted in six random fields per group (200–500 cells). For measurement of cellular platinum levels, SKOV3 cells were pre-incubated with or without 10 mM acetaminophen for 2 h prior to a 2 h treatment with or without cisplatin (10 μg/ml). Whole cell lysates of each group were digested in nitrous acid and then subjected to inductively coupled plasma mass spectrometer (ICP-MS) at the OHSU proteomic core shared resource.

In vivo animal studies

Female nude rats (*rnu/rnu*, 200–250 g; from the OHSU Blood Brain Barrier Program in-house colony) were pretreated with cyclophosphamide (100 mg/kg; *i.p.*) 24 h before tumor cell administration. We have shown that cyclophosphamide reduces innate immunity and increases vascular endothelial growth factor production to improve the consistence of xenograft tumor development in nude rats (27). SKOV3 ovarian carcinoma cells (2.5×10^7 in 250 μl) mixed with 250 μl Matrigel (BD Biosciences, Bedford, MA, USA) were injected in the left flank. For the chemotherapeutic study, tumor-bearing rats were untreated (control), treated with cisplatin (4 mg/kg; *i.p.*) alone, or acetaminophen (600 mg/kg; *p.o.*) 2 h before cisplatin treatment on day 7 after inoculation (500–800 mm^3 tumor volume). Tumor growth was measured by dial caliper twice per week. Whole blood (1 ml) was withdrawed through the jugular vein at 7 d and 24 d after treatment when the experiment was terminated. Blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were analyzed on a DRI-CHEM Veterinary Chemistry Analyzer (HESKA, Waukesha, WI, USA). For the pharmacological study, tumor-bearing rats received acetaminophen 0, 300, 600, and 1000 mg/kg *p.o.* (0, 2.1, 4.2 and 7 g/m^2 respectively). Serum and tissues (liver, brain and tumor) were collected 2 h after treatment. Tissue and serum acetaminophen and GSH concentrations were determined using an acetaminophen direct enzyme-linked immunosorbent assay (ELISA) (Immunalysis Corp., Pomona, CA, USA) and Quanticrom Glutathione Assay Kit (BioAssay Systems, Hayward, CA, USA). Tissue data are presented after normalization with protein concentration.

Statistical analysis

Significant differences between the mean values of treated cells/animals and controls were determined by two-sided Student's *t*-test modified by the number of comparisons performed (n) according to Tukey's correction. Two-way analysis of variance was performed by comparing the different arms of treatment for the two variables with Bonferroni post-test to compare means among the different treatments. Significant difference between treatment and control (or vehicle) groups or any two other groups was determined at the 5% level.

Results

Enhancement of chemotherapy toxicity by acetaminophen in ovarian carcinoma cells

Western blotting analysis for cleaved PARP was performed to determine the level of apoptosis in SKOV3 cells. After 24 h incubation, cisplatin (5 µg/ml) in combination with acetaminophen (10 mM) induced significantly more apoptosis in SKOV3 cells than acetaminophen or cisplatin alone (Figure 1A). There was no apoptosis found among any treatment groups at the 2 h time point. Similar results were found when SKOV3 cells were treated with paclitaxel (100 nM) with and without acetaminophen (10 mM) (Figure 1B). For cell cycle analysis, the vehicle control of SKOV3 cells contained only 2.9% $subG_1$ population (apoptotic cells). The $subG_1$ population was elevated to 24.1% in group treated with acetaminophen and cisplatin compared to 10.8% in cisplatin and 14.0% in acetaminophen alone. Additionally, there were no cells found in G_2/M phase in group treated with acetaminophen and cisplatin (Figure 1C).

Mechanisms for chemo-enhancement by acetaminophen in ovarian carcinoma cells

The effects of acetaminophen, cisplatin and paclitaxel on cellular GSH and ROS levels were determined at both 2 and 24 h. Acetaminophen treatment reduced GSH levels in SKOV3 cells at both the 2- and 24-h time points ($p<0.01$, Figure 2A) and increased ROS level significantly only after 24 h ($p<0.05$) not 2 h incubation (Figure 2B). Neither cisplatin nor paclitaxel treatment alone affected cellular GSH or ROS levels. There was no further reduction of GSH or increase in ROS when cells were treated with the combination of acetaminophen and cisplatin or paclitaxel (Figure 2A and B).

Mitochondria play an important role in regulating oxidative stress and apoptosis. Cellular mitochondrial membrane potential ($\Delta\psi m$) was evaluated using the JC-1 stain. Healthy cells are orange/reddish and apoptotic cells with reduced $\Delta\psi m$ are green under fluorescence microscopy. Acetaminophen alone increased the percentage of cells exhibiting mitochondrial toxicity from 1.7±0.3% to 7.9±0.4% (Figure 2C and D). Acetaminophen significantly ($p<0.001$) enhanced the mitochondrial toxic effects of both cisplatin (25.3±1.5%) and paclitaxel (21.6±1.2%). Acetaminophen pretreatment did not alter cellular cisplatin accumulation (51.8±2.8 *vs.* 48.3±2.5 ng/10^6 cells; n=9; Figure 2E).

NRF2 is a nuclear transcription factor that protects against oxidative stress and inflammation by regulating several detoxification and xenobiotic transporter genes. Acetaminophen alone induced nuclear NRF2 translocation in 53.9±6.0% of cells. Cisplatin alone had no effect on NRF2 translocation (3.0±0.2%), while the combination of acetaminophen and cisplatin (13.3±1.0%) significantly ($p<0.01$) reduced nuclear NRF2 localization compared to acetaminophen alone. In contrast, paclitaxel alone induced nuclear translocation of NRF2 (94.6±1.9%), which was not affected by addition of acetaminophen (98.1±1.2%; Figure 3). Our results suggest that activation of the NRF2-mediated pathway is independent of ROS production and acetaminophen-enhanced cisplatin- and paclitaxel-induced cytotoxicity is independent of nuclear NRF2 translocation pathway.

In vivo acetaminophen pharmacology and toxicology studies

To investigate the effect of acetaminophen on tissue distribution of acetaminophen and GSH level, subcutaneous tumor-bearing nude rats received 0, 300, 600 or 1000 mg/kg (~0, 2, 4 and 7 g/m^2) acetaminophen orally (n=3 per treatment). At 2 h after administration, acetaminophen levels in tissues and serum were elevated in a linear correlated dose-dependent manner (Figure 4A). The acetaminophen doses tested in this study did not alter acetaminophen tissue distribution of acetaminophen among the samples (brain, liver, serum and subcutaneous tumor). Rats given 600 mg/kg acetaminophen had a mean acetaminophen tissue level above 250 μg/ml (1.65 mM) in brain, liver, tumor and serum at 2 h. Compared to the control, GSH levels were reduced in the liver of acetaminophen-treated rats in a dose-dependent manner, with significant (p<0.05) difference only being detected at 1000 mg/kg dosage (665.5±61.1 *vs.* 357.9±61.3 μM/g tissue). GSH levels in subcutaneous tumor, serum and brain did not significantly differ between acetaminophen-treated and untreated animals (Figure 4B).

Acetaminophen potentiated the chemotherapeutic effect of cisplatin in a SKOV3 subcutaneous xenograft model

To test if acetaminophen treatment enhances the chemotherapeutic effect of cisplatin in SKOV3 tumor-bearing animals, acetaminophen (600 mg/kg) was given orally 2 h before cisplatin (4 mg/kg) treatment on day 7 after tumor inoculation (500–800 mm^3 tumor volume; n=5 per treatment). Rats given 1000 mg/kg acetaminophen exhibited hypothermia during treatment; therefore we set the maximum dose at 600 mg/kg. Compared to control animals, cisplatin both alone and with acetaminophen significantly (p<0.001) reduced tumor volume starting from 3 d until the end of the study (Figure 5A). There is no significant difference between groups treated with cisplatin alone and those treated with acetaminophen and cisplatin at 3, 7, 10 and 14 d after treatment (Figure 5A). Tumor recurrence was found in animals in the cisplatin-treated group at 17 d after treatment. In contrast, the group treated with acetaminophen and cisplatin retained low tumor volumes of between 35–55% of baseline during this period of time. This resulted in significant differences (p<0.01) between the cisplatin and combined treatment groups. In these animals, serum BUN, creatinine, AST and ALT levels were elevated by the combination of acetaminophen with cisplatin treatment when compared to cisplatin alone (48.2±10.9 *vs.* 24.4±4.1 mg/dl; 0.65±0.06 *vs.* 0.30±0.04 mg/dl; 115.8±26.1 *vs.* 58.0±6.6 IU/l; 30.5±3.4 *vs.* 18.0±1.7 IU/l) at 7 d and then returned back to normal at 24 d after treatment (Figure 5 B–E). Our data demonstrated that acetaminophen combined with cisplatin prevented subcutaneous tumor recurrence in SKOV3 tumor-bearing rats with minimal and managable side-effects.

Discussion

Ovarian carcinoma often exhibits resistance to chemotherapy, limiting the efficacy of chemotherapeutics such as cisplatin or paclitaxel. Circumvention of cancer cell resistance against platinum-based anticancer chemotherapy is of great interest in clinical chemotherapy (28). One of the strategies to overcome resistance to anticancer agents is the application of chemosensitizers or drug-resistance modulators, including multidrug-resistant protein inhibitors and intracellular GSH-depleting agents (29). Buthionine sulfoximine (BSO) and acetaminophen may work to chemosensitize tumor cells by depleting intracellular GSH levels (30, 31). Previous studies showed that acetaminophen could be used as a cancer preventative but not chemo-enhancement agent in ovarian cancer (23). In this study, we have demonstrated that a combined regimen of acetaminophen and cisplatin or paclitaxel enhances cytotoxicity towards SKOV3 cells relative to the use of cisplatin or paclitaxel alone *in vitro* and cisplatin alone *in vivo*.

Acetaminophen alone has demonstrated toxicity in hepatocellular carcinoma (12, 19, 32, 33) and glioma cell lines (34, 35), but had mixed results in breast cancer cell lines (36, 37). In a human endometrioid ovarian carcinoma cell line, acetaminophen significantly increased the percentage of cells at S-phase and reduced cell growth synergistically with carboplatin, but did not affect paclitaxel growth-inhibitory acitivity (18). In contrast, paclitaxel increased the acetaminophen-induced cytotoxicity in rat primary hepatocytes by reducing protein synthesis (38). Regardless of CYP2E1 protein levels, we found that acetaminophen enhanced cisplatin-induced apoptosis in human hepatocarcinoma and hepatoblastoma cells (19). Results of acetaminophen chemoenhancement for other chemotherapeutics are mixed, with increased staurosporine-induced cell death in human neuroblastoma cells (15) but reduced doxorubicin toxicity in hepatocarcinoma cells (12).

Our studies demonstrated that acetaminophen reduces GSH levels and increased ROS, reduced $\Delta\psi$m and increased nuclear NRF2 translocation to enhance the cytotoxicity by cisplatin or paclitaxel of SKOV3 cancer cells *in vitro*. Acetaminophen enhanced apoptosis in both cisplatin- and paclitaxel-treated SKOV3 cells regardless of whether the same (GSH, ROS and mitochondrial pathways) or different (nuclear translocation of NRF2) mechanisms involved. Our results confirm previous studies showing that paclitaxel reduced $\Delta\psi$m without affecting ROS in SKOV3 cells (7) and a human T-cell lymphoblastic leukemia cell line (39). GSH depletion with BSO or acetaminophen increased nuclear NRF2 translocation in mouse hepatocytes (40). A high level of NRF2 in cancer is associated with sensitivity to oxidative stress and chemotherapy (41). Jiang *et al.* (41) found that stable knockdown of NRF2 significantly sensitized human type II endometrial cancer cells to cisplatin and paclitaxel. Our results suggest that activation of the NRF2-mediated pathway is independent of ROS production and that acetaminophen-enhanced cisplatin- and paclitaxel-induced cytotoxicity is independent of nuclear NRF2 translocation pathway. Copple *et al.* (42) demonstrated that NRF2 can be activated either through GSH depletion alone or by direct modification of NRF2 suppressor protein (KEAP1). NRF2 translocation to the nucleus has been shown to be insufficient for the up-regulation of expression of multiple drug resistance ATP-binding cassette transporter proteins (43). Lack of enhanced transporter activity might explain why acetaminophen did not alter cellular cisplatin accumulation *in vitro*. Similarly, BSO treatment reduced cellular GSH but did not affect the intracellular melphalan accumulation in human colorectal HT29 cancer cells (44).

Two phase I dose-escalation studies have examined the potential of acetaminophen as a chemotherapeutic adjuvant (20, 21). Due to the significant toxicities experienced at the 20 g/m^2 dose level in these clinical trials, the maximum tolerated dose of acetaminophen was set at 15 g/m^2, yielding a plasma concentration of 250 µg/ml (20). In the current study, we found that oral acetaminophen at 600 mg/kg (~4 g/m^2) gave a mean tissue AAP level >250 µg/ml at 2 h, equivalent to the concentrations achievable in patients (22). This dose level moderately prevented subcutaneous tumor progression when combined with cisplatin in tumor-bearing rats because of the slow growing characteristic of SKOV3 cells. In this study, cisplatin treatment (4 mg/kg; *i.p.*) alone significantly exhibited antitumor efficacy, which might explain why there was no difference between cisplatin and acetaminophen with cisplatin at the early time points after treatment. The *in vivo* results would be more promising and convincing if we could lower the cisplatin dosage and extend the *in vivo* animal study longer. Shaw *et al.* (45) also found that SKOV3 cells were less aggressive and formed tumors more slowly (two to three months) in a xenograft model. In addition, the potential side-effects (increase of serum BUN, creatinine, AST and ALT) by acetaminophen plus cisplatin treatment are minimal and managable. Previous studies from our laboratory have shown that delayed *i.v.* NAC or sodium thiosulfate administration (4–8 h) protected from the side effects without affecting the chemotherapeutic efficacy (46, 47).

There was a dose-dependent reduction of GSH in the liver, the organ most active in acetaminophen metabolism. However, we found no GSH reduction in serum and subcutaneous tumors in rats even at 1000 mg/kg (~ 7 g/m^2) dose. The mechanisms involved in the acetaminophen-enhanced cisplatin chemotherapeutic efficacy of ovarian carcinoma *in vivo* remain unclear. Patients given up to 20 g/m^2 of acetaminophen have similarly shown no reduction in serum GSH levels (20). Rat hepatocytes are found to be far more sensitive to acetaminophen treatment than human hepatocytes (48). In addition, the activity of glutathione-*S*-transferase in rodents is 10 to 20 times higher than in humans (49). These factors exemplify that the major limitations of this study are: a single ovarian cancer cell line was used, although similar studies had been carried out on hepatic tumors (19) and atypical teratoid rhabdoid tumors (50) in our laboratory; the SKOV3 subcutaneous xenograft model with relatively slow growth rate was not ideal or adequately close to the clinical setting; the treatment regimen for maximal efficacy (dosage, timing, route and frequency of acetaminophen and chemotherapy administration) needs to be further defined; and due to the difference in acetaminophen sensitivity among different species, using a rodent model is not ideal for studying the potential of acetaminophen incorporation into a pre-clinical trial.

In conclusion, we found that acetaminophen enhanced the cytotoxic activity of cisplatin/palcitaxel in human ovarian carcinoma *in vitro* and cisplatin treatment *in vivo*. In addition to the potential preventative role of acetaminophen in ovarian cancer incidence (23), incorporation of high-dose acetaminophen, an inexpensive, relatively safe, and widely available drug, into cisplatin- or palcitaxel-containing chemotherapeutic regimens may enhance chemotherapeutic efficacy towards ovarian carcinomas and other malignancy. Potential side-effects could be abrogated by delayed administration of antioxidant chemoprotective agents such as NAC, sodium thiosulfate or other GSH precursors. Therefore, this is a promising approach that merits further clinical trials as the potential treatment benefits could impact on different tumor types.

Acknowledgments

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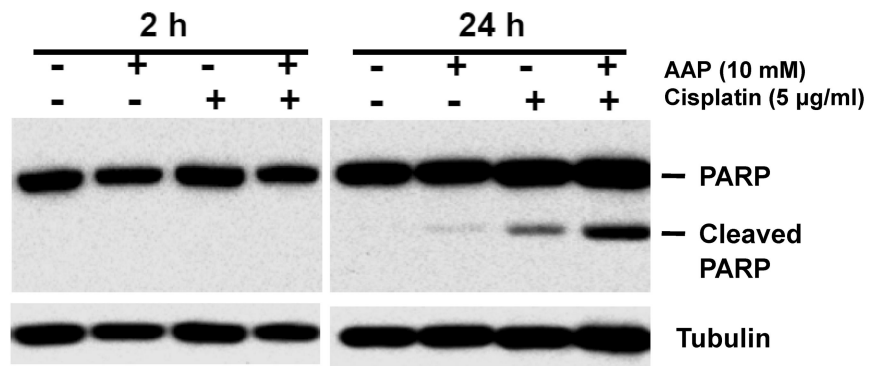
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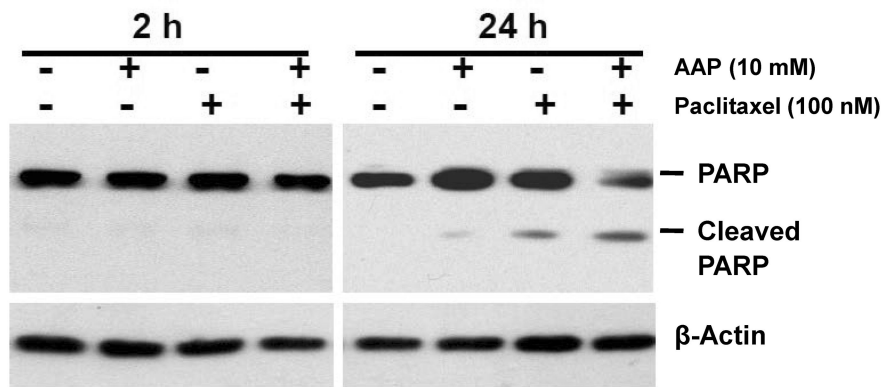
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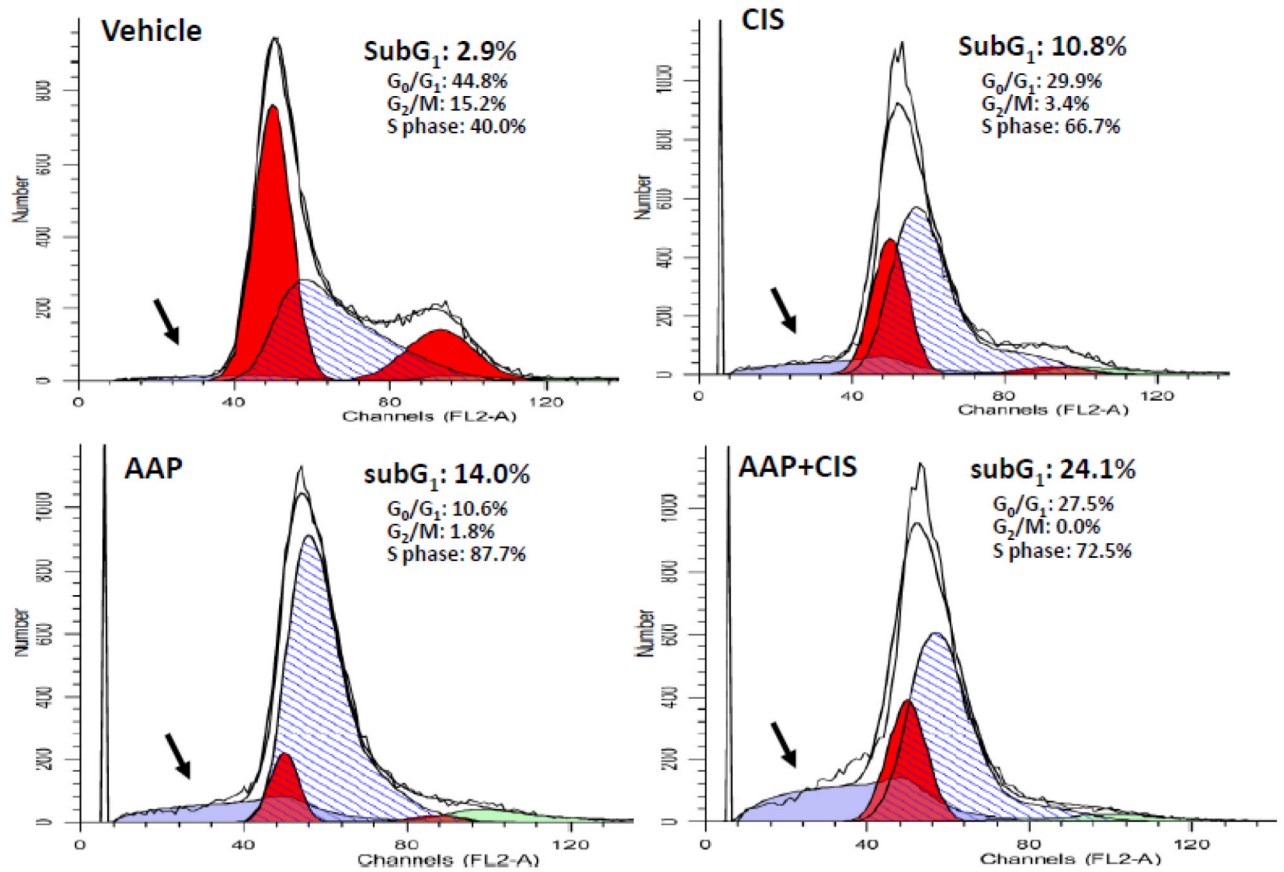
A

2 h | 24 h

| - | + | - | + | - | + | - | + | AAP (10 mM) |
| - | - | + | + | - | - | + | + | Cisplatin (5 µg/ml) |

— PARP

— Cleaved PARP

— Tubulin

B

2 h | 24 h

| - | + | - | + | - | + | - | + | AAP (10 mM) |
| - | - | + | + | - | - | + | + | Paclitaxel (100 nM) |

— PARP

— Cleaved PARP

— β-Actin

C

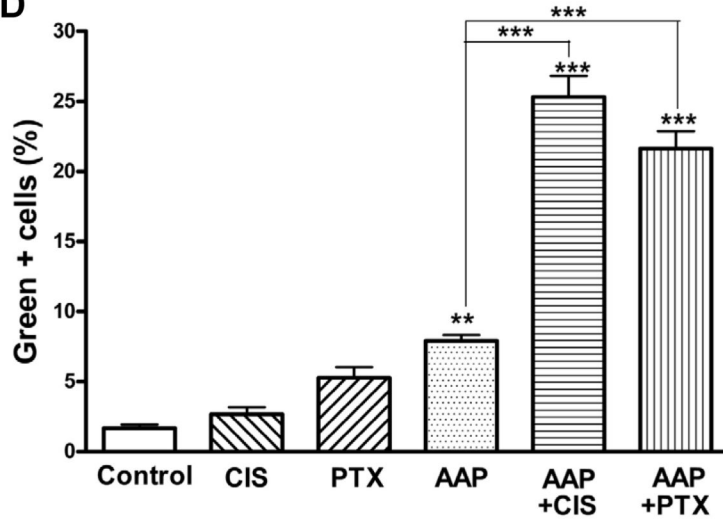**Figure 1. Acetaminophen enhances cisplatin and paclitaxel cytotoxicity**
Human SKOV3 ovarian carcinoma cells were treated with acetaminophen (AAP; 10 mM)
with/without cisplatin (5 µg/ml, panel **A**) or paclitaxel (100 nM, panel **B**). Whole cell
lysates were collected 2 and 24 h after treatments and subjected to immunoblotting analysis.
Tubulin and β actin were used as equal loading controls. **C**: Cell cycle analysis. Cells were
collected and fixed in 70% ethanol 24 h after treated with acetaminophen (AAP; 10 mM)
with/without cisplatin (CIS; 5 µg/ml), then subjected to cytometry after staining with
propidium iodine. The apoptotic subG$_1$ cell population (light blue area) among treatment
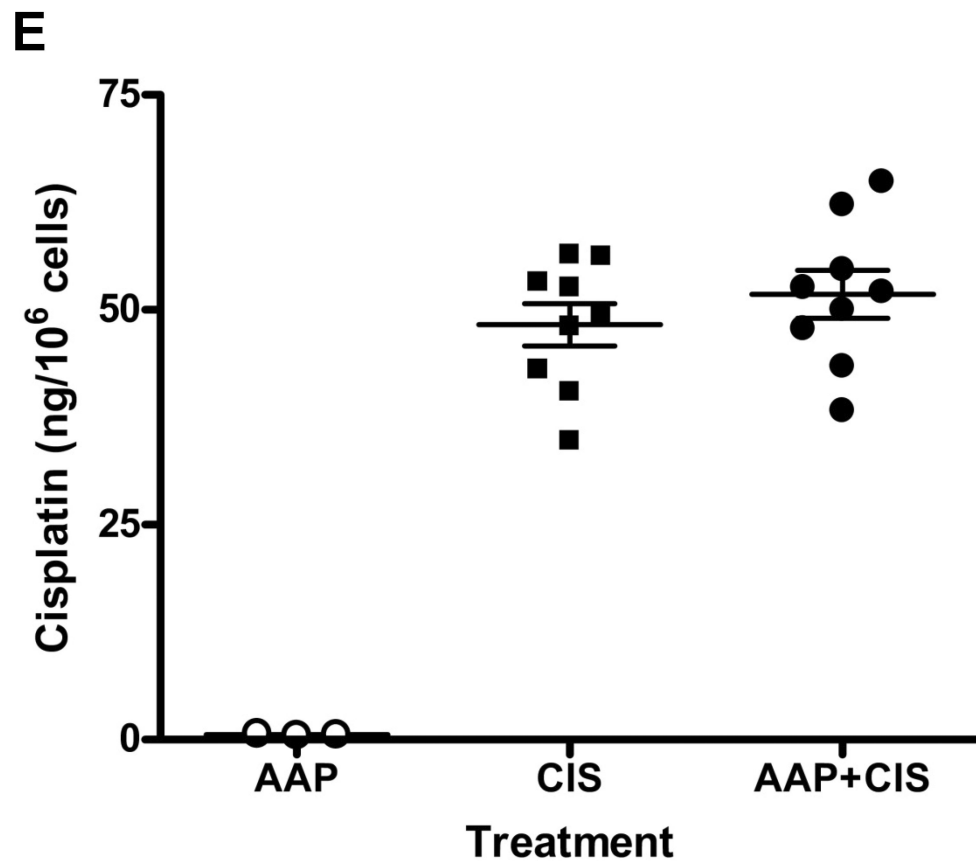groups is indicated by arrows.
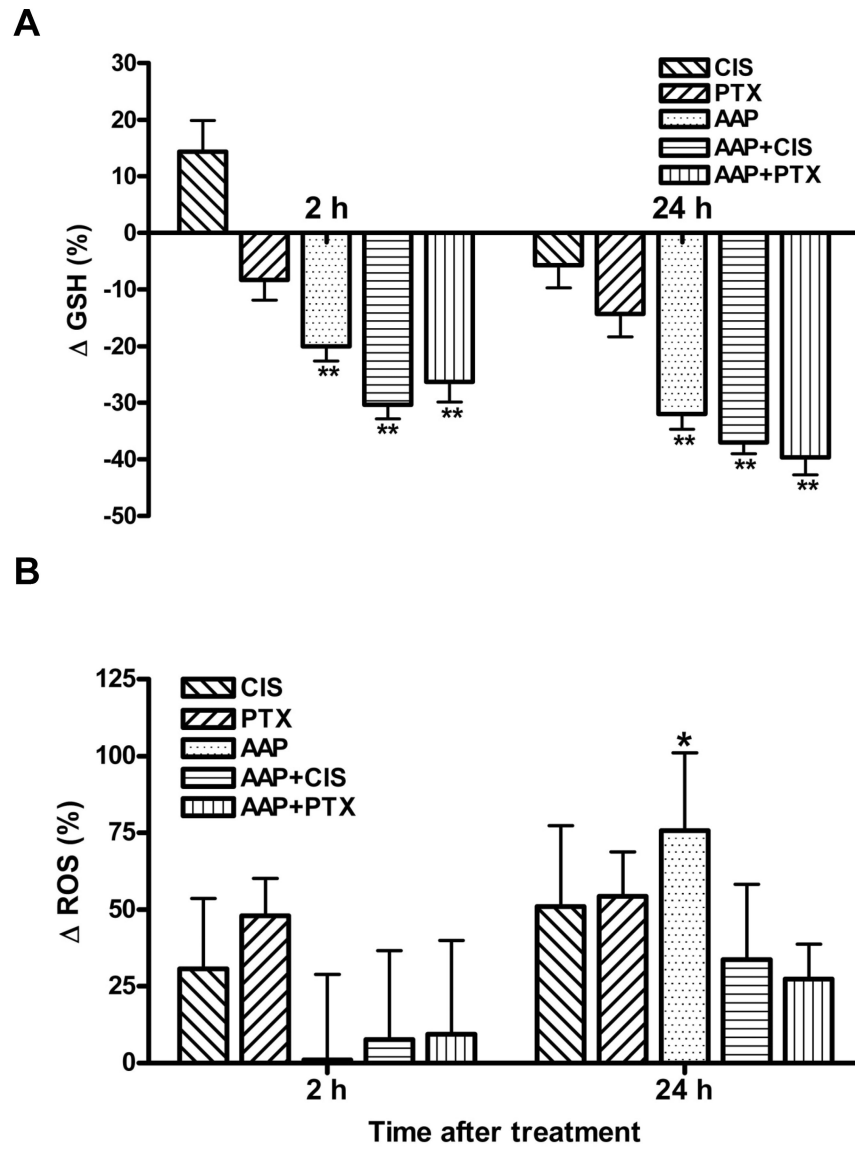
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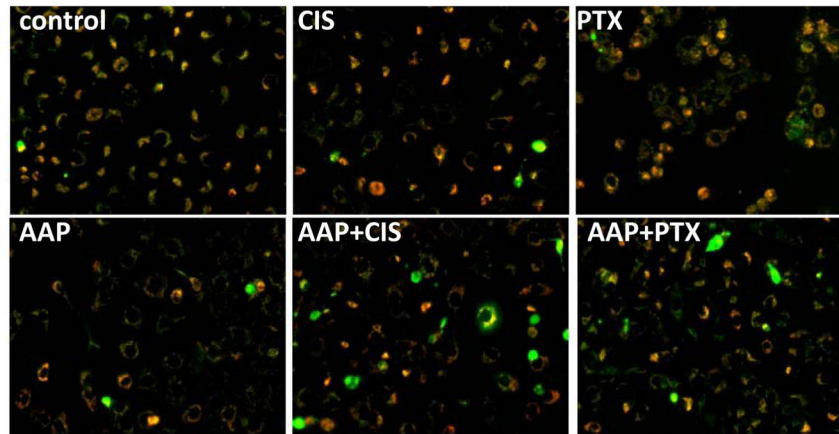
D

Figure 2. *In vitro* **mechanism of acetaminophen chemoenhancement**
Human SKOV3 ovarian carcinoma cells were treated with acetaminophen (AAP,10 mM)
with/without cisplatin (CIS, 5 µg/ml) or paclitaxel (PTX, 100 nM) for 2 and 24 h. Cells
treated with dimethyl sulfoxide (DMSO) alone were used as control. Whole cell lysates
were collected 2 and 24 hr after treatments and subjected to analysis for glutathione (GSH;
A), reactive oxygen species (ROS; **B**) and mitochondrial membrane potential (Δψm; **C, D**).
A, B) Data are presented as the mean±SD of percentage change in GSH and ROS compared
with the control (n=3 per treatment group). **C**: Representative micrographs of each treatment
group at 2 h. Healthy cells are orange and cells with reduced Δψm are green under
fluorescence microscopy. **D**: Quantification and analysis of the percentage of green cells in
six random fields. **E**: SKOV3 ovarian carcinoma cells were treated with or without
acetaminophen (10 mM) for 2 h, cisplatin (10 µg/ml) was then added and cells incubated for
an additional 2 h. Whole cell lysates were subjected to inductively coupled plasma mass
spectrometry for platinum measurement (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).
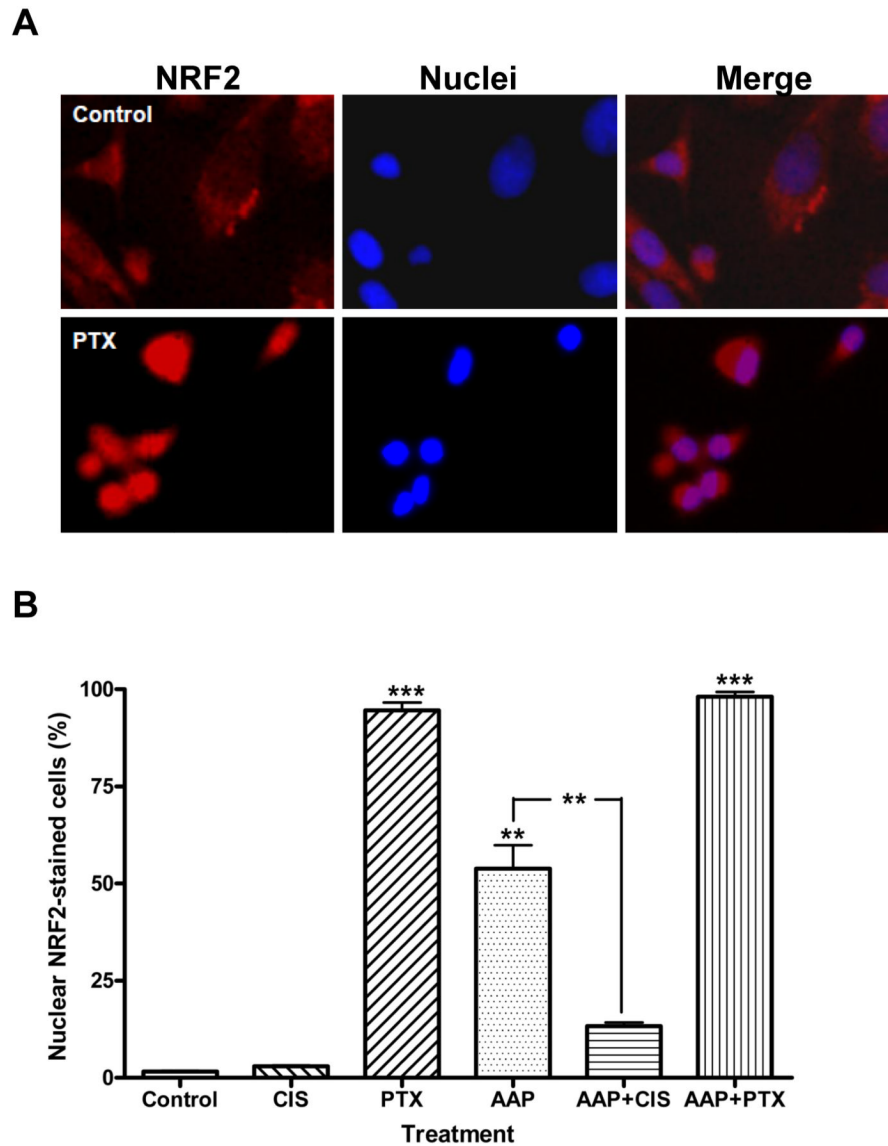
Figure 3. Effect of acetaminophen and chemotherapy on nuclear translocation of nuclear factor erythroid 2-related factor 2 (NRF2)
Human SKOV3 ovarian carcinoma cells were treated with acetaminophen (AAP, 10 mM) with/without cisplatin (CIS, 5 µg/ml) or paclitaxel (PTX, 100 nM) for 2 h. Cells treated with dimethyl sulfoxide (DMSO) alone were used as control. Cells were fixed and stained for immunofluorescence with rabbit anti-NRF2 with 4', 6-diamidino-2-phenylindole (DAPI) nuclear counterstain. **A**: Representative micrographs of nuclear NRF2-negative (vehicle control; top panel) and -positive (100 nM paclitaxel; bottom panel) stained SKOV3 cells. **B**: Quantification and analysis of the percentage of nuclear NRF2-positively stained cells in four random fields (** $p<0.01$; *** $p<0.001$).
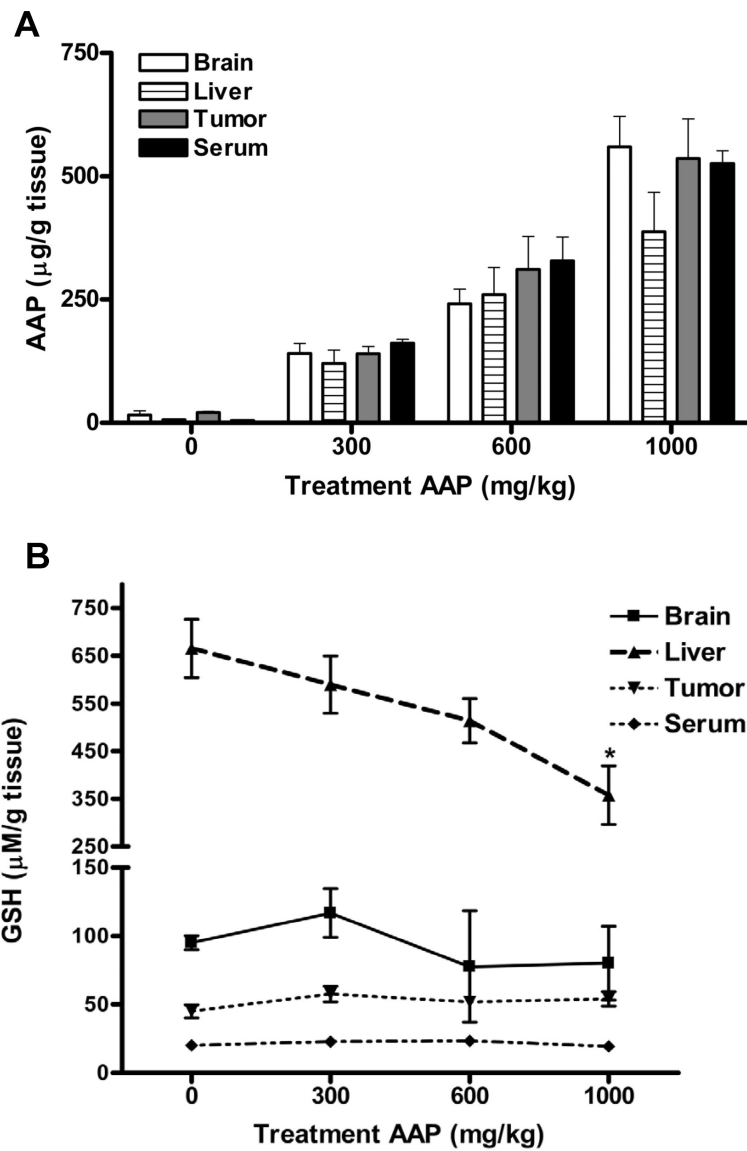
Figure 4. *In vivo* acetaminophen distribution and glutathione levels
Female nude rats with subcutaneous SKOV3 xenografts were treated with acetaminophen
(AAP; 0, 300, 600 and 1000 mg/kg; n=3 per treatment) on d 7 after tumor inoculation.
Serum and tissues were collected and analyzed for levels of acetaminophen (**A**) and
glutathione (**B**) 2 h after treatment. Tissue data (except serum) are presented after
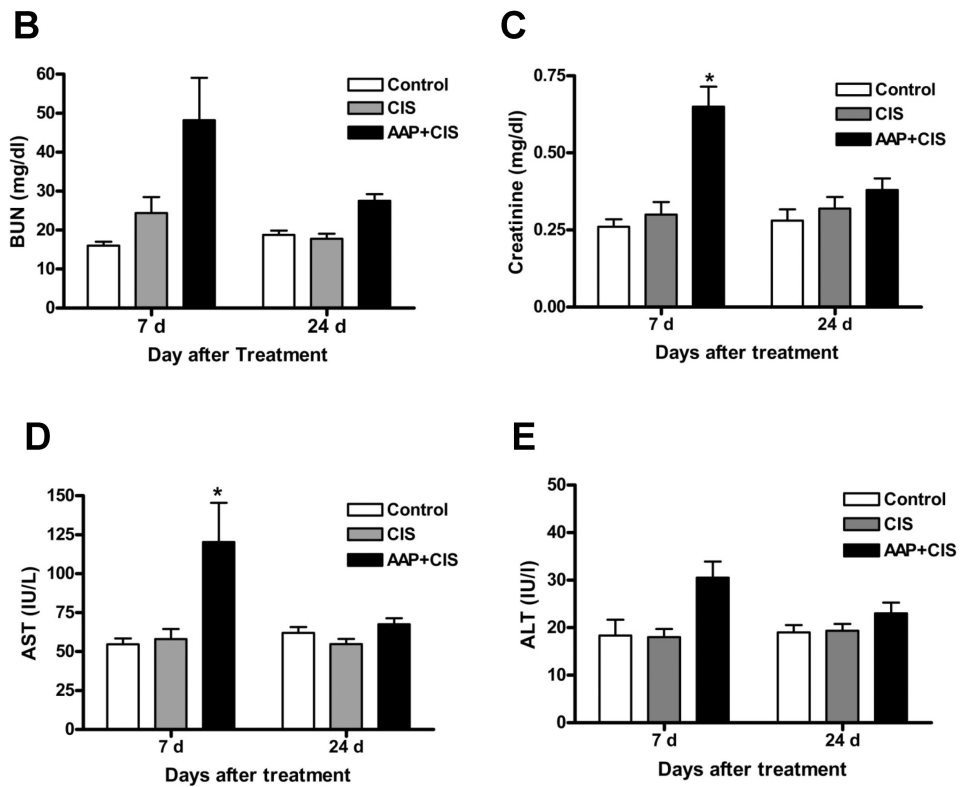normalization with protein concentration (* $p<0.05$).

A
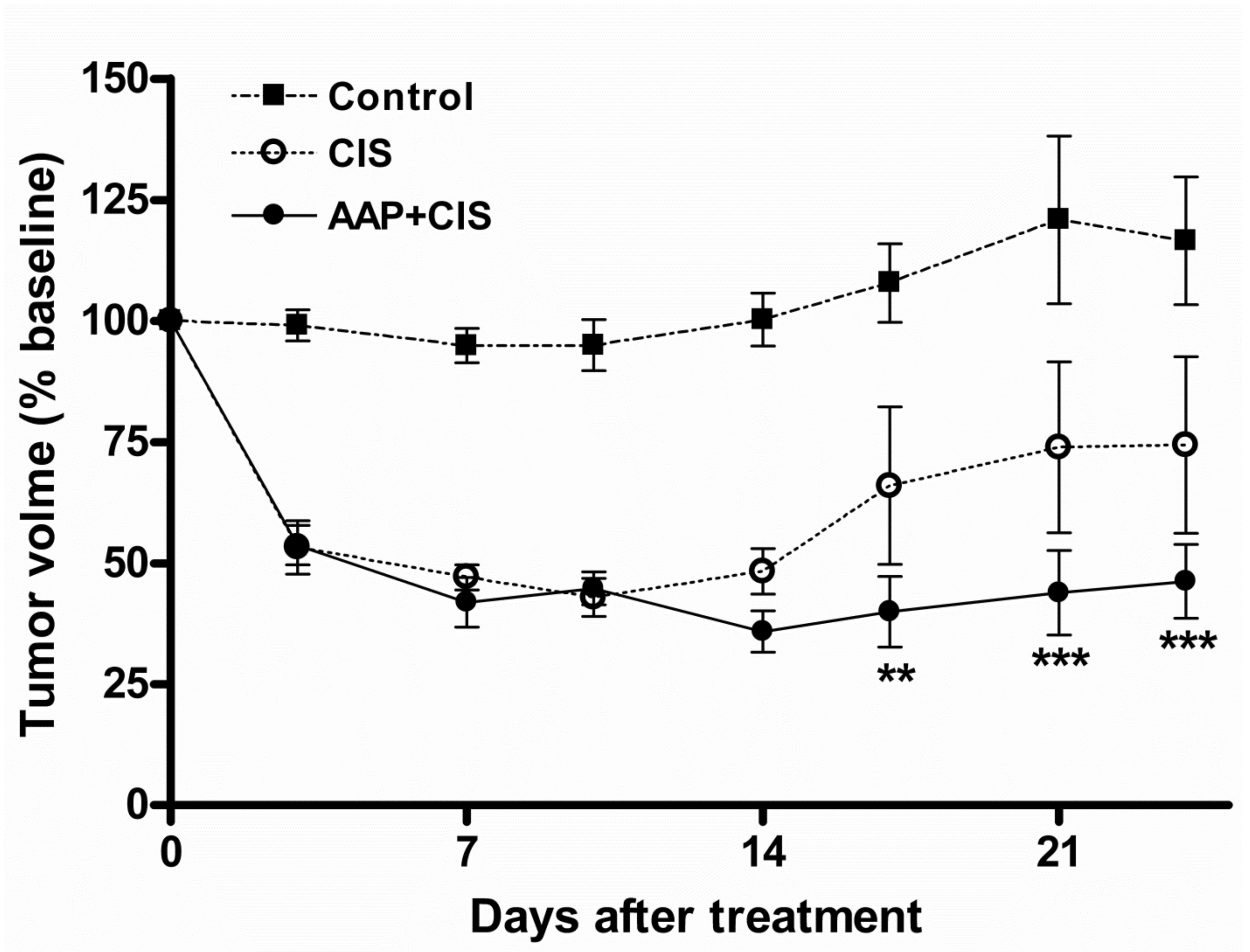
Figure 5. Acetaminophen enhances cisplatin chemotherapeutic efficacy in ovarian subcutaneous xenografts
Female nude rats with subcutaneous SKOV3 xenografts were treated with acetaminophen (AAP; 600 mg/kg) or saline orally 2 h before cisplatin treatment (4 mg/kg; *i.p.*) on d 7 after inoculation. **A**: Subcutaneous tumor growth and tumor volume among the treatment groups was measured by dial caliper twice per week. Tumor volume at each time point was normalized to that at pretreatment baseline. The statistical significance between cisplatin and combination groups is indicated by ** $p<0.01$ and *** $p<0.001$. Blood urea nitrogen (BUN; **B**), creatinine (**C**), aspartate aminotransferase (AST; **D**) and alanine aminotransferase (ALT; **E**) levels in serum 7 and 24 d after treatment. The statistical significance between control and treatment group is indicated by * $p<0.05$.