Aspirin inhibits tumor progression and enhances cisplatin sensitivity in epithelial ovarian cancer

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Background. Ovarian cancer is the most common gynecological malignancy and is difficult to manage due to the emergence of resistance to various chemotherapeutic drugs. New efforts are urgently awaited. Aspirin, traditionally considered a nonsteroidal anti-inflammatory drug (NSAID), has been reported to show potential chemopreventive effects. Therefore, we aimed to investigate the anticancer effect and explore the underlying molecular mechanisms of aspirin in epithelial ovarian cancer (EOC) cells. **Methods.** We conducted wound healing, transwell migration, EdU cell proliferation, colony formation and apoptosis detection assays to observe the effects of aspirin on the migration, proliferation and apoptosis of EOC cells (A2870, Caov-3, SK-OV-3). EOC cells were treated with a combination of aspirin and cisplatin (CDDP) to observe the enhancing effect of aspirin on CDDP sensitivity. Orthotopic xenograft models of ovarian cancer established with A2780-Luciferase-GFP cells were applied to compare the tumor growth inhibition abilities in the control, CDDP and CDDP plus aspirin groups through in vivo imaging, which can be used to continuously monitor tumor growth. The expression and acetylation levels of p53 in EOC cells treated with aspirin were determined by western blotting, and p53 acetylation levels were examined in tumors harvested from the transplanted mice. Quantitative real-time PCR was used to assess the mRNA expression of p53 target genes. **Results.** Aspirin inhibited migration and proliferation and induced apoptosis in EOC cell lines in a concentration-dependent manner. In vitro, aspirin enhanced the sensitivity of EOC cells to CDDP by enhancing its inhibitory effect on proliferation and its inductive effect on apoptosis. In vivo, the differences in the tumor growth inhibition rates among thedifferent CDDP experimental groups were statistically significant (p<0.05). Aspirin did not affect p53 protein expression but increased the p53 acetylation level in a concentration-dependent manner. In addition, the mRNA levels of *p21, bax, foxf1, puma,* and *rad* in EOC cells were significantly increased by aspirin treatment. **Conclusion.** Aspirin can inhibit tumor progression and enhance the CDDP sensitivity of EOC cells. These antitumor effects

of aspirin might be mediated by p53 acetylation and subsequent activation of p53 target genes.

1 **Aspirin inhibits tumor progression and enhances**

2 **cisplatin sensitivity in epithelial ovarian cancer**

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17 **Abstract**

18 **Background.** Ovarian cancer is the most common gynecological malignancy and is difficult to
19 manage due to the emergence of resistance to various chemotherapeutic drugs. New efforts are
20 urgently awaited. Aspirin, which is traditionally considered a nonsteroidal anti-inflammatory
21 drug (NSAID), has been reported to exert potential chemopreventive effects. Therefore, we
22 aimed to investigate the anticancer effect and explore the underlying molecular mechanisms of
23 aspirin on epithelial ovarian cancer (EOC) cells.

24 **Methods.** We conducted wound healing, transwell migration, EdU cell proliferation, colony
25 formation and apoptosis detection assays to observe the effects of aspirin on the migration,
26 proliferation and apoptosis of EOC cells (A2870, Caov-3, and SK-OV-3). EOC cells were
27 treated with a combination of aspirin and cisplatin (CDDP) to observe the effect of aspirin on
28 enhancing CDDP sensitivity. Orthotopic xenograft models of ovarian cancer established with
29 A2780-Luciferase-GFP cells were applied to compare tumor growth inhibition in the control,
30 CDDP and CDDP plus aspirin groups through in vivo imaging, which can be used to
31 continuously monitor tumor growth. The expression and acetylation levels of p53 in EOC cells
32 treated with aspirin were determined using western blotting, and p53 acetylation levels were
33 examined in tumors harvested from the transplanted mice. Quantitative real-time PCR was used
34 to assess the mRNA expression of p53 target genes.

35 **Results.** Aspirin inhibited migration and proliferation and induced apoptosis in EOC cell lines in
36 a concentration-dependent manner. In vitro, aspirin enhanced the sensitivity of EOC cells to
37 CDDP by increasing its inhibitory effect on proliferation and its effect on inducing apoptosis. In
38 vivo, the differences in the tumor growth inhibition rates among the different CDDP
39 experimental groups were statistically significant ($p < 0.05$). Aspirin did not affect p53 protein
40 expression but increased the p53 acetylation level in a concentration-dependent manner. In
41 addition, the mRNA levels of *CDKN1A, BAX, FOXF1, PUMA,* and *RRAD* in EOC cells were
42 significantly increased by the aspirin treatment.

43 **Conclusions.** Aspirin inhibits tumor progression and enhances the CDDP sensitivity of EOC
44 cells. These antitumor effects of aspirin might be mediated by p53 acetylation and subsequent
45 activation of p53 target genes.

46 **Keywords**
47 aspirin, ovarian cancer, cisplatin, p53, acetylation
48

Introduction

49
50   Ovarian cancer is the most common gynecological malignancy, and in 75% of patients,
51 malignant ovarian tumors are diagnosed at an advanced stage due to the absence of symptoms in
52 early stages (Siegel & Miller & Jemal, 2015). Platinum-based combination chemotherapy has
53 been a standard first-line treatment for advanced ovarian cancer, but the development of
54 chemotherapeutic resistance in cancer cells is a major clinical barrier to the successful treatment
55 of cancer (Szakacs et al., 2006). *TP53* mutation is a main mechanism inhibiting propagation of
56 the DNA damage signal to the apoptotic machinery, thus leading to the development of drug
57 resistance (Eckstein, 2011).
58   Aspirin (acetylsalicylic acid) is a nonsteroidal anti-inflammatory drug (NSAID); NSAIDs
59 are widely prescribed analgesic and anti-inflammatory agents. Recently, emerging evidence
60 showing the benefits of aspirin in cancer prevention has ignited renewed interest in aspirin
61 research. Data from numerous studies have shown that prolonged intake of aspirin reduces the
62 risk of and improves survival in patients with several cancers, including colon, breast, lung,
63 prostate, and endometrial cancers (Bardia et al., 2011; Lim et al., 2012; Huang et al., 2014; Cao
64 et al., 2016; Chen, 2017; Takiuchi et al., 2018; Gan et al., 2019; Loomans-Kropp et al 2019).
65 Regular aspirin use is related to a reduction in the ovarian cancer risk (Trabert et al., 2014;
66 Trabert et al., 2019; Hurwitz et al., 2020). Furthermore, Merritt et al. recently assessed the
67 relations between aspirin, other NSAIDs, paracetamol, and ovarian cancer-specific survival after
68 an ovarian cancer diagnosis in approximately 1000 cases. Their results suggested that women
69 who used aspirin or NSAIDs after diagnosis experienced a prolonged ovarian cancer-specific
70 survival compared with never-users (Merritt et al., 2018). Another small cohort study of women
71 with clear cell ovarian cancer (n=77) reported that aspirin use correlated with longer disease-free
72 and overall survival, and it retained independent significance as a positive prognostic factor
73 (Wield et al., 2018).
74   The mechanism underlying the efficacy of aspirin involves the inhibition of cyclooxygenase
75 (COX) enzymes through irreversible acetylation of serine residues (Eckstein, 2011; Cuzick et al.,
76 2009; Robles & Harris, 2010; Silwal-Pandit & Langerød & Børresen-Dale, 2017), leading to
77 inhibited synthesis of prostaglandins (PGs), which cause inflammation, swelling, pain and fever
78 (Alfonso et al., 2014; Lucotti et al., 2019). Moreover, aspirin influences multiple COX-
79 independent biological pathways in tumor progression and metastasis. Aspirin was found to
80 inhibit various signaling pathways, such as the Ras/c-Raf, extracellular signal-regulated kinase
81 (ERK)/MAPK, NF-κB, and mTOR pathways, in different cancer cells (Guo et al., 2016; Wu et
82 al., 2020). Additionally, the chemopreventive mechanism of aspirin might involve the increased
83 number of tumor-infiltrating lymphocytes in the tumor microenvironment. Hence, combination
84 therapy with aspirin might overcome immune resistance and enhance the therapeutic efficacy of
85 chemotherapeutics toward metastatic breast cancer (Liu et al., 2019).
86   Although the molecular mechanisms underlying the anticancer effects of aspirin have been
87 extensively investigated, the involvement of numerous cellular components in ovarian cancer is
88 still incompletely understood. Because of the mutation of *TP53* in platinum-resistant epithelial

89 ovarian cancer (EOC) and the acetylation effect of aspirin, we hypothesized that aspirin exerts an
90 anticancer effect on EOC through the acetylation of p53. Therefore, in this study, we
91 investigated whether aspirin increases the efficacy of cisplatin (CDDP) in EOC and initially
92 explored its underlying mechanism.
93
94 **Materials & methods**
95 **Cell culture.**
96   The EOC cell lines A2780, Caov-3 and SK-OV-3 were purchased from the China Center
97 for Type Culture Collection (CCTCC). A2780 and Caov-3 are ovarian adenocarcinoma cell lines
98 derived from malignant tumor tissues of untreated patients with ovarian cancer. SK-OV-3 is an
99 ovarian adenocarcinoma cell line derived from malignant ascites. All cell lines were maintained
100 at 37°C in DMEM/F12 (Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal
101 bovine serum (FBS; Gibco, Carlsbad, CA, USA) in a 5% CO_2 atmosphere. Aspirin (Sigma, St.
102 Louis, MO, USA) was added for the indicated times after 12-24 h of cell growth. All assays
103 below were performed in triplicate.
104 **Cell viability assays.**
105   Cells were seeded at a density of 5000 cells/well in 96-well tissue culture plates. Different
106 concentrations of aspirin (0.2 mM, 0.4 mM, 0.8 mM, 1.6 mM, 3.2 mM, or 6.4 mM) were added
107 to the culture media after cell attachment, and five replicate wells for each concentration were
108 established. Forty-eight hours after treatment, 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-
109 diphenyltetrazolium bromide (MTT) were added to the media, and the cells were further
110 incubated for 4 h. Subsequently, the cells were solubilized in 100 μl of solubilization solution for
111 8 h. The optical density (OD) at 570 nm was measured using a microplate reader (Bio-Rad,
112 Berkeley, CA, USA).
113 **Cell proliferation assays.**
114   Cells were seeded in a 96-well tissue culture plate (Corning Costar, Corning, NY, USA) and
115 adjusted to a density of 5000 cells/well. After treatment with different concentrations of aspirin
116 (0 μM, 100 μM, and 1000 μM) and/or CDDP (20 μM), cells were fixed and stained with an EdU
117 cell proliferation kit (RiboBio Co., Guangzhou, China). Cells were immediately observed under
118 a fluorescence microscope (Olympus, Tokyo, Japan), and cells in three random fields per well
119 were counted (100×).
120 **Colony formation assays.**
121   Cells were seeded in 6-well tissue culture plates in triplicate, and the density was adjusted to
122 500 cells/well. Cells were cultured in DMEM/F12 supplemented with 10% FBS and different
123 concentrations of aspirin (0 μM, 25 μM, 100 μM, 500 μM, 1000 μM, and 25 μM) and/or CDDP
124 (5 μM) at 37°C in a 5% CO_2 incubator (Heraeus, Hanau, Germany) for 14 days. Next, cells in the
125 wells were fixed with methanol and stained with 0.1% crystal violet; the cell colonies formed
126 were then visible to the naked eye.
127 **Flow cytometry analysis.**

128   Cells were plated in triplicate in a six-well plate at a density of 1×10^5 cells/well and
129   incubated for 24 h. Then, cells were treated with different concentrations of aspirin (0 μM, 100
130   μM, and 1000 μM) and/or CDDP (20 μM). After a 48-h incubation at 37°C, cells were harvested,
131   trypsinized and washed with cold phosphate-buffered saline (PBS). Subsequently, cells were
132   stained with APC Annexin V and propidium iodide (PI) (BioLegend, Inc., San Diego, CA,
133   USA). The stained cells were immediately analyzed using an LSR flow cytometer (Beckman
134   Coulter, Inc., 250 S. Kraemer Boulevard, Brea, CA, USA), and the data were analyzed using
135   Summit 6.2 software (Beckman Coulter, Inc., 250 S. Kraemer Boulevard, Brea, CA, USA).
136   **Western blot analysis.**
137   After treatment with different concentrations of aspirin (0 μM, 100 μM, and 1000 μM) for
138   24 h, cells were collected in cold Nonidet P40 (NP40) buffer containing a protease inhibitor
139   cocktail for 30 min and centrifuged at 13000 × g for 10 min; subsequently, the supernatants were
140   collected. The protein concentration was measured with a bicinchoninic acid (BCA) assay.
141   Proteins in the samples were separated by 8% sodium dodecyl sulfate-polyacrylamide gel
142   electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF)
143   membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing
144   Tween 20 (TBST) for 1 h at room temperature and incubated with rabbit anti-p53 (1:1500; Cell
145   Signaling Technology, Boston, MA, USA), rabbit anti-acetylated p53 (K382) (1:500; Abcam,
146   Cambridge, UK), and mouse anti-β-actin (1:4000; Cell Signaling Technology, Boston, MA,
147   USA) antibodies at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse
148   secondary antibodies (1:5000; Cell Signaling Technology, Boston, MA, USA) were used for
149   detection as appropriate. Immunoreactions were visualized with an enhanced chemiluminescence
150   kit (Bio-Rad, Berkeley, CA, USA), and the gray values of the bands were quantified with
151   Quantity One Software (Bio-Rad, Berkeley, CA, USA) or a Molecular Imager ChemiDoc™
152   XRS₊ system with Image Lab™ software (Bio-Rad, Berkeley, CA, USA).
153   **Quantitative real-time polymerase chain reaction (qPCR).**
154   After treatment with different concentrations of aspirin (0 μM, 100 μM, and 1000 μM) for
155   24 h, total cellular RNA was extracted from cultured cells with TRIzol reagent (Thermo Fisher,
156   Waltham, MA, USA) according to the manufacturer's protocol. The quality and quantity of total
157   RNA were evaluated with a NanoDrop 2000/2000C spectrophotometer (Thermo Fisher,
158   Waltham, MA, USA), and cDNAs were subsequently synthesized using a reverse transcription
159   kit (Takara, Tokyo, Japan) and the following primers: *CDKN1A*, upstream 5'-
160   CGATGGAACTTCGACTTTGTCA-3' and downstream 5'-
161   GCACAAGGGTACAAGACAGTG-3'; *BAX*, upstream 5'-CCCGAGAGGTCTTTTTCCGAG-
162   3' and downstream 5'-CCCGAGAGGTCTTTTTCCGAG-3'; *FOXF1*, upstream 5'-
163   GCGGCTTCCGAAGGAAAT-3' and downstream 5'-CAAGTGGCCGTTCATCATGC-3';
164   *PUMA*, upstream 5'-CAAGTGGCCGTTCATCATGC-3' and downstream 5'-
165   CAAGTGGCCGTTCATCATGC-3'; *RRAD*, upstream 5'-CAAGTGGCCGTTCATCATGC-3'
166   and downstream 5'-CAAGTGGCCGTTCATCATGC-3'; and *β-actin*, upstream 5'-
167   GCCAACACAGTGCTGTCTGG-3' and downstream 5'-

168 GCTCAGGAGGAGCAATGATCTTG-3'. β-Actin expression was used for normalization. PCR
169 was performed in an Applied Biosystems StepOnePlus Real-time PCR system (Thermo Fisher,
170 Waltham, MA, USA) using SYBR Green Real-time PCR Master Mix (Takara, Tokyo, Japan).
171 The reactions were performed with a two-step thermal cycling method, 95°C for 30 seconds and
172 40 PCR cycles of 95°C for 5 seconds and 66°C for 30 seconds, followed by a melting curve
173 analysis. The relative expression levels were calculated using the Ct ($2^{-\Delta\Delta Ct}$) method.
174 **Orthotopic xenograft mouse model of human EOC.**
175   The Institutional Animal Care and Use Committee of Tongji Medical College approved this
176 research ([2014] IACUC Number: 399). Six-week-old female BALB/c-nu/nu mice were
177 purchased from Beijing HFK Bioscience Co. and were housed in sterile cages with ad libitum
178 access to food in a specific pathogen-free (SPF) barrier animal room at Tongji Medical College
179 Experimental Animal Center. A2780-Luciferase cells were obtained by transfecting the EOC cell
180 line A2780 with a lentivirus carrying the luciferase gene. A2780-Luciferase cells were
181 suspended in serum-free medium, and the density was adjusted to 1×10^7 cells/ml. A 200-μl
182 aliquot of the cell suspension was subcutaneously injected into the right axilla of nude mice.
183 When the tumor volume reached approximately 1 cm³, mice were anesthetized with isoflurane,
184 and the tumor was harvested. Subsequently, the tumor was cut into fragments with a volume of
185 approximately 1-2 mm³. Eighteen mice were randomly divided into three groups according to a
186 random number table (n=6). Then, six mice per group were inoculated with one tumor fragment
187 in the left ovary. Mice in the CDDP group were treated with CDDP by intraperitoneal injection
188 and with water by gavage, mice in the CDDP plus aspirin group were treated with CDDP by
189 intraperitoneal injection and aspirin by gavage, and mice in the control group were treated with
190 saline by intraperitoneal injection and water by gavage. Only the administering experimenter was
191 aware of the group allocation. The tumor fragments were transplanted on day 0, and after an
192 intraperitoneal injection of XenoLight D-Luciferin Potassium Salt (Perkin Elmer, Waltham, MA,
193 USA), tumor growth was observed with an IVIS Lumina II (Caliper Life Science, Waltham, MA,
194 USA) beginning on day 8 and then once per week thereafter to obtain in vivo bioluminescence
195 images. The acquired optical data were analyzed using Living Image software. Aspirin was
196 initially administered on day 9 at a dose of 20 mg/kg and then every other day thereafter, while
197 CDDP administration was initiated at a dose of 3 mg/kg on day 17 and then every fourth day
198 thereafter. All mice were euthanized via overdose isoflurane inhalation on day 36 and necropsied
199 for the measurement of tumor diameters with Vernier calipers. The tumor volume and relative
200 tumor inhibition rate were calculated using the following formulas: $V= (L\times W^2)/2$ and $R= (V$
201 $_{control\ group} - V_{treatment\ group})/V_{control\ group}$.
202 **Immunohistochemistry (IHC).**
203   Tissue sections (4-μm thick) were prepared from formalin-fixed, paraffin-embedded blocks
204 and were immunohistochemically stained with rabbit anti-p53 (1:100; Abcam, Cambridge, UK),
205 rabbit anti-acetylated p53 (K382) (1:100; Abcam, Cambridge, UK), rabbit anti-Ki67 (1:100;
206 Abcam, Cambridge, UK), rabbit anti-caspase 3 (1:100; Cell Signaling Technology), and rabbit
207 anti-cleaved caspase 3 (1:100; Cell Signaling Technology) antibodies. Localization of the target

208   protein was visualized by incubating sections with a freshly prepared 3,3'-diaminobenzidine
209   (DAB) solution for 3 min. PBS was substituted for the primary antibody as the negative control.
210   Three slides from each tissue were independently evaluated by two observers with an Olympus
211   FV500 optical microscope (Olympus, Tokyo, Japan).
212   **Quantitative analysis of images of immunohistochemical staining.**
213   The expression of target proteins in tumors harvested from transplanted mice was quantified
214   by determining the relative percentage of the positive-stained area to the selected total tissue area
215   and the average staining intensity normalized to the intensity of the selected stroma with an
216   imaging processor to exclude the influence of the tumor volume. Slides were reviewed under a
217   light microscope at 400× magnification, and five regions were randomly selected per slide.
218   ImageJ software was used to analyze the percentage of the positive-stained area and the staining
219   intensity in the tumor tissue from every region, and the overall average value across the slides
220   was considered the level of protein expression.
221   **Statistical analysis.**
222   All statistical analyses were performed using SPSS software version 22.0. Data are
223   presented as the means ± standard deviations (SD, n≥3) values. Student's t-test or one-way
224   ANOVA was adopted to compare group differences, and significance was defined as $p<0.05$.
225
226   **Results**
227   **Aspirin inhibits the growth of human EOC cells in vitro.**
228   A survival curve based on the results of MTT assays showed that the half-maximal
229   inhibitory concentration (IC50) values of aspirin in the EOC cell lines A2780, Caov-3 and SK-
230   OV-3 were 1.27 (95% CI, 0.93-1.72) mM, 2.05 (95% CI, 1.83-2.30) mM, and 1.54 (95% CI,
231   1.30-21.82) mM, respectively (Fig. 1A). Thus, we conducted the functional trial in vitro with
232   doses of 100 and 1000 μM, which were lower than the IC50 in each cell line.
233   The results of EdU cell proliferation and colony formation assays illustrated that the
234   proliferation and growth of the A2780, Caov-3 and SK-OV-3 cell lines were significantly
235   suppressed by aspirin in a concentration-dependent manner (Figs. 1B-E and S1A-D). Moreover,
236   as the aspirin concentration increased, the apoptosis rate in the EOC cell lines increased
237   significantly, and the apoptosis rate differed significantly among the different concentrations
238   (Fig. 1F-G). In addition, the results of wound healing and transwell migration assays showed that
239   the migration of A2780, Caov-3 and SK-OV-3 cells was suppressed by aspirin in a
240   concentration-dependent manner (Fig. S2A-D).
241   **Aspirin enhances the CDDP sensitivity of EOC cells.**
242   A2780 and SK-OV-3 cells were treated with CDDP alone or in combination with aspirin to
243   investigate the role of aspirin in mediating CDDP sensitivity in vitro. Cell proliferation (Figs.
244   2A-B and S3A) and colony formation (Figs. 2C-D and S3B) were significantly inhibited and cell
245   apoptosis (Figs. 2E-F and S3C) was induced by CDDP alone in A2780 and SK-OV-3 cells
246   compared to the corresponding control cells. Moreover, CDDP plus aspirin exerted a
247   significantly stronger effect than CDDP alone.

248   Orthotopic xenograft mouse models of EOC were established with A2780-Luciferase cells,
249 and tumor bioluminescence signals were visualized in situ to investigate the role of aspirin in
250 mediating CDDP sensitivity in vivo (Fig. 3A). As shown in Fig. 3B, the tumor growth curve of
251 the CDDP plus aspirin group was shallower than that of the CDDP alone group, and the CDDP
252 alone group had a shallower curve than the control group. Moreover, the difference in the tumor
253 volume among the three groups was consistent with the tumor growth curves (Fig. 3C-D). The
254 tumor inhibition rates in the CDDP group and the CDDP plus aspirin group were 49.3% and
255 78.4%, respectively.
256   In addition, the level of caspase 3 in the tumor tissue was not different among the three
257 groups (control, CDDP, and CDDP plus aspirin); however, the level of cleaved caspase 3 was
258 higher in the CDDP group than in the control group and was higher in the CDDP plus aspirin
259 group than in the CDDP group (Fig. 3E). Ki67 expression was reduced in the CDDP group
260 compared to the control group and was lower in the CDDP plus aspirin group than in the CDDP
261 group (Fig. 3E).
262 **Aspirin acetylates p53 in EOC cells and subsequently activates p53-mediated**
263 **downregulation of target genes.**
264   The expression of p53 was not different among the three groups (control, CDDP, and
265 CDDP plus aspirin), but the acetylation level of p53 in the CDDP plus aspirin group was higher
266 than that in both the control group and the CDDP group (Fig. 4A-B). As shown in Fig. 4C, the
267 level of the p53 protein in the A2780 cell line remained unchanged after treatment with different
268 concentrations of aspirin. However, as the aspirin concentration increased, the acetylation level
269 of p53 gradually increased. Moreover, the mRNA expression levels of the p53 target genes
270 CDKN1A, BAX, FOXF1, PUMA, and RRAD increased significantly in a concentration-
271 dependent manner, as illustrated in Fig. 4D.
272
273 **Discussion**
274   The anticancer action of aspirin has recently received increasing attention. An inverse
275 association between the intake of aspirin and the risk of many types of cancer, including ovarian
276 cancer, has been identified in recent decades (Takiuchi et al., 2018; Gan et al., 2019; Loomans-
277 Kropp et al 2019; Baandrup et al., 2015; Peres et al., 2016; Zhang et al., 2016). In addition to its
278 use as an effective chemopreventive agent, aspirin can also prolong patient survival even after a
279 cancer diagnosis, which suggests the potential for the adjunct use of aspirin in cancer
280 chemotherapy (Merritt et al., 2018). However, despite these observations and reports, the
281 mechanisms underlying the anticancer effects of aspirin are not well understood. In the present
282 study, we demonstrated that both *in vitro* and *in vivo*, aspirin could inhibit tumor cell growth and
283 mobility and enhance their sensitivity to CDDP, which is similar to the previous findings that the
284 combination therapy with aspirin overcome immune resistance and enhance the therapeutic
285 efficacy of chemotherapeutic agent toward metastatic breast cancer. (Liu et al., 2019).
286   Since plasma concentrations of intact aspirin can be up to 100 μM after the administration
287 of a single therapeutic dose (Roberts et al., 1983), this concentration was used to observe the

288 cellular effects of aspirin in the present study. The results of wound healing, transwell migration,
289 EdU cell proliferation and colony formation assays showed that aspirin inhibited the migration,
290 proliferation and growth of A2780, Caov-3 and SK-OV-3 EOC cells, consistent with the results
291 of previous studies (Huang et al., 2016).
292   Animal studies have indicated that aspirin significantly enhances the efficacy of CDDP in
293 reducing the growth of orthotopic xenograft tumors formed by human EOC cells in mice.
294 Notably, because of the gastrointestinal side effects of this drug, as well as the potential for
295 gastroduodenal ulcers and bleeding, we treated mice with a safe dose of 20 mg/kg, which was
296 determined by an equivalent dose conversion from the dosage of 100 mg/day used for oral low-
297 dose aspirin therapy in humans. Moreover, in the present animal study, we established orthotopic
298 xenograft models in mice (Kim et al., 2006) using a luciferase-labeled A2780 cell line and
299 subsequently observed tumor growth using in vivo bioluminescence imaging after an
300 intraperitoneal injection of D-luciferin potassium salt, which interacts with luciferase to generate
301 an optical signal. Unlike previous studies using models established by an intraperitoneal
302 injection of ovarian cancer cells or using subcutaneous tumor models (Huang et al., 2016), we
303 surgically transplanted the tumor tissue into the ovaries of a mice; thus, our model more
304 precisely mimicked the environment of human ovarian cancer. In addition, this approach enabled
305 continual observation of tumor growth deep inside the abdominal cavity via in vivo imaging
306 without the need to sacrifice the mice. Moreover, due to the principle of in vivo bioluminescence
307 imaging, which detects the viable cells in the tumor, our approach provided a more accurate
308 estimate of tumor activity than simply measuring the tumor volume. In summary, the results of
309 our animal experiments were more convincing and reliable than those reported in other studies.
310   Regarding the potential mechanism, we elucidated that aspirin acetylates p53 at lysine 382
311 in EOC cells and subsequently activates p53 target genes, which regulate cell cycle arrest,
312 migration and apoptosis.
313   Aspirin consists of two functional groups, an acetyl group and a salicylate group, both of
314 which have their own distinct targets. The acetyl group of aspirin acetylates COX, thereby
315 inducing its inhibition (Alfonso et al., 2014), and the salicylate group suppresses IκB kinase
316 (IKK) β and prevents NF-κB activation (Kim et al., 2006); thus, both groups contribute to the
317 anti-inflammatory properties of aspirin. Although most hypotheses have focused on the anti-
318 inflammatory activities of aspirin, this drug also interferes with ERK signaling, leading to ERK
319 inhibition by preventing the binding of c-Raf to Ras in vitro (Guo et al., 2016). Moreover, aspirin
320 reduces mTOR signaling by inhibiting the mTOR effectors S6K1 and 4EBP1 in colorectal
321 cancer (Guo et al., 2016) and suppresses esophageal squamous cell carcinoma growth by
322 inhibiting HBXIP and HMGA2 (Wu et al., 2020). In addition, aspirin induces concentration-
323 dependent inhibition of the major oncogenic pathway, the Wnt/β-catenin pathway, in colon
324 cancer (Bos et al., 2006) and acetylates the tumor suppressor protein p53 at lysine 382 in MDA-
325 MB-231 breast cancer cells (Alfonso et al., 2009).
326   TP53 has been recognized as a tumor suppressor gene encoding the p53 protein in human
327 cancers. The p53 protein is a transcription factor that controls the expression of genes and

328   miRNAs affecting many important cellular processes. The chemotherapy drug CDDP activates
329   the DNA damage response pathway, in which the tumor suppressor p53 controls cell cycle
330   progression and apoptosis. However, studies have reported that the *TP53* gene is mutated and
331   inactivated in more than 50% of all tumors and that these mutations are associated with earlier
332   onset of cancer and the development of drug resistance (Robles & Harris, 2010). A considerably
333   high mutation frequency of 50-100% is reported across all ovarian cancers (Silwal-Pandit &
334   Langerød & Børresen-Dale, 2017). Meanwhile, p53 mutation can prevent the proper folding of
335   the p53 protein or directly disrupt its DNA-binding ability, and thus the tumor may acquire new
336   oncogenic properties such as drug-resistance (Raimundo et al., 2020). Posttranslational
337   modifications (PTMs) of p53 are critical in modulating its tumor suppressor functions (Vousden
338   & Prives, 2009). Acetylation is an important PTM that increases p53 protein stability, its binding
339   to low-affinity promoters and its interactions with other proteins (Reed & Quelle, 2014). For
340   instance, p53 acetylation at K382 promotes the recruitment of its co-activators to the *CDKN1A*
341   promoter and increases histone acetylation following DNA damage (Reed & Quelle, 2014); it
342   also activates pro-apoptotic functions of BAX and PUMA (Yamaguchi et al., 2009). Moreover,
343   RRAD and FOXF1 are also upregulated by p53 activation, which may further inhibit cell
344   migration (Hsiao et al., 2011; Tamura et al.,2013). In our study, aspirin acetylated p53 to
345   subsequently increase the mRNA expression of its target gene *CDKN1A* in A2780 and SK-OV-3
346   EOC cells. Furthermore, aspirin activated other downstream target genes related to cell
347   migration, such as *RRAD* and *FOXF1*, and genes related to apoptosis, i.e., *PUMA* and *BAX*.
348   However, more studies are needed to confirm whether the anticancer efficacy of aspirin is
349   mediated by this pathway.
350   According to previous research, A2780 and SK-OV-3 cells express the wild-type *TP53*
351   gene, while the *TP53* gene is mutated in Caov-3 cells, which is a point mutation that resulted in a
352   chain termination signal likely to truncate the p53 peptide at amino acid 135 (Skilling, 1996;
353   Yaginuma & Westphal,1992). The status of the *TP53* gene in these three EOC cell lines was not
354   validated in our study. Moreover, further research is required to investigate the potential
355   differences in biological function between the acetylation of wild-type p53 versus mutant p53 in
356   ovarian cancer. Beside p53 acetylation, as a multifunctional agent, Aspirin may exhibit
357   anticancer effects through p53-independent pathways, such as inhibition of cyclin dependent
358   kinases by aspirin metabolites and inhibition of nuclear-κB signaling through direct interactions
359   with IκB kinase in colorectal cancers (Sankaranarayanan et al., 2020). The multi-targeting and
360   dose-dependent action manner of aspirin makes its use in the treatment of cancer both promising
361   and puzzling. Although we provide preliminary evidence regarding the potential benefit of
362   adding aspirin to CDDP chemotherapy in ovarian cancer, the interactions between aspirin and
363   traditional chemotherapeutic agents remains largely unclear.
364   In summary, aspirin inhibits tumor progression and enhances the CDDP sensitivity of EOC
365   cells through p53 acetylation and subsequent activation of p53 target genes that regulate tumor
366   migration, proliferation, and chemoresistance. These findings provide a theoretical basis for the
367   use of aspirin in the treatment of ovarian cancer and lay the foundation for future research.

368

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Disclosure Statement

The authors have no relevant conflicts of interest to declare.

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513

514   **Figure legends**
515   **Figure 1. Aspirin inhibits proliferation and growth and induces apoptosis in human EOC**
516   **cell lines in vitro.**
517   (A) MTT cell viability assays were performed to determine the IC50s of aspirin in A2780, Caov-
518   3 and SK-OV-3 cells over a 48-h culture period. (B) Cell proliferation was detected using EdU
519   cell proliferation assays (bar, 50 μm) after treatment with different concentrations of aspirin (0
520   μM, 100 μM, and 1000 μM) in A2780 cells. (C) Quantitative assay of proliferation rate in
521   A2780, Caov-3 and SK-OV-3 cells. (D) Colony formation assays were conducted after cells
522   were cultured with different concentrations of aspirin (0 μM, 25 μM, 100 μM, 500 μM, 1000
523   μM, and 25 μM) for 14 days in A2780 cells. (E) Quantitative assay of colony counts in A2780,
524   Caov-3 and SK-OV-3 cells. (F) Cells were treated with different concentrations of aspirin (0 μM,
525   100 μM, and 1000 μM) for 48 h in A2780 cells, stained with the Annexin V-FITC/PI staining
526   kit, and analyzed using flow cytometry. (G)Quantitative assay of apoptosis rate in A2780, Caov-
527   3 and SK-OV-3 cells. Data are presented as the mean ± SD values. * p<0.05.
528
529   **Figure 2. Aspirin enhances the CDDP sensitivity of EOC cells in vitro.**
530   (A)The cell proliferation rate was determined using EdU cell proliferation assays after the
531   incubation of A2780 cells with CDDP (20 μM) alone or in combination with aspirin (100 μM)
532   (CDDP + aspirin) for 48 h (bar, 50 μm). (B) Quantitative assay of proliferation rate in A2780
533   and SK-OV-3 cells. (C) Colony formation assays were conducted to observe the growth of
534   A2780 cells after an incubation with CDDP (5 μM) alone or with aspirin (25 μM) for 48 h. (D)
535   Quantitative assay of colony counts in A2780 and SK-OV-3 cells. (E) The percentage of
536   apoptotic cells was determined using flow cytometry after the treatment of A2780 cells with
537   CDDP (20 μM) alone or with aspirin (100 μM). (F) Quantitative assay of apoptosis rate in
538   A2780 and SK-OV-3 cells. Data are presented as the mean ± SD values. * p<0.05.
539
540   **Figure 3. Aspirin enhances the CDDP sensitivity of EOC cells in vivo.**
541   Orthotopic xenograft mouse models of EOC were established with A2780-Luciferase cells using
542   surgical orthotopic implantation to observe the effects of aspirin in vivo. Aspirin was initially
543   administered on day 9 at a dose of 20 mg/kg by gavage and then every other day thereafter,
544   while CDDP administration was initiated at a dose of 3 mg/kg by intraperitoneal injection on day
545   17 and then every fourth day thereafter. Tumor growth was observed with an IVIS Lumina II
546   beginning on day 8 and then once per week thereafter to obtain in vivo bioluminescence images.
547   (A) The tumor signals in all mice (control group, CDDP group, and CDDP + aspirin group) at
548   every time point are displayed. (B) The tumor growth curve for each group based on the tumor
549   signals is shown. (C) All mice were euthanized via overdose isoflurane inhalation on day 36 and
550   necropsied for the measurement of tumor diameters with Vernier calipers. (D) Quantitative assay
551   of tumor volume in mice. (E) The expression levels of cleaved caspase 3, caspase 3 and Ki67 in
552   tumor tissues harvested from mice bearing orthotopic EOC xenografts (control group, CDDP
553   group, and CDDP + aspirin group) were assessed using IHC. (F) Quantitative assay for the IHC
554   data of cleaved caspase 3, caspase 3 and Ki67. Data are presented as the mean ± SD values. *
555   p<0.05.

556

557 **Figure 4. Aspirin acetylates p53 in A2780 cells and subsequently activates p53-mediated**
558 **downregulation of target genes.**
559 (A) The expression of p53 and acetylation level of p53 in tumor tissues from mice bearing
560 orthotopic EOC xenografts (control group, CDDP group, and CDDP + aspirin group) were
561 assessed using IHC. (B) Quantitative assay of positive rate in IHC. (C) Western blot analysis was
562 conducted to evaluate the level of the p53 protein and its acetylation (p53 K382-ace) after
563 treatment with different concentrations of aspirin (0 μM, 25 μM, 100 μM, 500 μM and 1000
564 μM). (D) Levels of the CDKN1A, BAX, FOXF1, PUMA, and RRAD mRNAs were examined
565 using qPCR after treatment with different concentrations of aspirin (0 μM, 100 μM, and 1000
566 μM). Data are presented as the mean ± SD values. * $p < 0.05$.

567

568 **Supporting figure legends**
569 **Figure S1. Aspirin inhibits proliferation and growth and induces apoptosis in human EOC**
570 **cell lines in vitro.** (A) Cell proliferation was detected using EdU cell proliferation assays (bar,
571 50 μm) after treatment with different concentrations of aspirin (0 μM, 100 μM, and 1000 μM) in
572 Caov-3 and SK-OV-3 cells. (B) Colony formation assays were conducted after cells were
573 cultured with different concentrations of aspirin (0 μM, 25 μM, 100 μM, 500 μM, 1000 μM, and
574 25 μM) for 14 days in Caov-3 and SK-OV-3 cells. (B) Cells were treated with different
575 concentrations of aspirin (0 μM, 100 μM, and 1000 μM) for 48 h in Caov-3 and SK-OV-3 cells,
576 stained with the Annexin V-FITC/PI staining kit, and analyzed using flow cytometry.

577

578 **Figure S2. In vitro evidence that aspirin inhibits the migration of human EOC cell lines.**
579 (A) Wound healing assays were conducted to observe the migration of A2780, Caov-3 and SK-
580 OV-3 cells after treatment with different concentrations of aspirin (0 μM, 100 μM, and 1000 μM;
581 bar, 100 μm) for 24 h. (B) Quantitative assay of migration rate in A2780, Caov-3 and SK-OV-3
582 cells. (C) The cell migration rate was determined by performing transwell migration assays after
583 the incubation of A2780, Caov-3 and SK-OV-3 cells with different concentrations of aspirin (0
584 μM, 100 μM, and 1000 μM; bar, 100 μm) for 48 h. (D) Quantitative assay of migrating cell
585 counts in A2780, Caov-3 and SK-OV-3 cells. Data are presented as the mean ± SD values. *
586 $p < 0.05$.

587

588 **Figure S3. Aspirin enhances the CDDP sensitivity of EOC cells in vitro.** (A)The cell
589 proliferation rate was determined using EdU cell proliferation assays after the incubation of SK-
590 OV-3 cells with CDDP (20 μM) alone or in combination with aspirin (100 μM) (CDDP +
591 aspirin) for 48 h (bar, 50 μm). (B) Colony formation assays were conducted to observe the
592 growth of SK-OV-3 cells after an incubation with CDDP (5 μM) alone or with aspirin (25 μM)
593 for 48 h. (C) The percentage of apoptotic cells was determined using flow cytometry after the
594 treatment of SK-OV-3 cells with CDDP (20 μM) alone or with aspirin (100 μM).

Figure 1

Aspirin inhibits proliferation and growth and induces apoptosis in human EOC cell lines in vitro.

(A) MTT cell viability assays were performed to determine the IC50s of aspirin in A2780, Caov-3 and SK-OV-3 cells over a 48-h culture period. (B) Cell proliferation was detected using EdU cell proliferation assays (bar, 50 μm) after treatment with different concentrations of aspirin (0 μM, 100 μM, and 1000 μM) in A2780 cells. (C) Quantitative assay of proliferation rate in A2780, Caov-3 and SK-OV-3 cells. (D) Colony formation assays were conducted after cells were cultured with different concentrations of aspirin (0 μM, 25 μM, 100 μM, 500 μM, 1000 μM, and 25 μM) for 14 days in A2780 cells. (E) Quantitative assay of colony counts in A2780, Caov-3 and SK-OV-3 cells. (F) Cells were treated with different concentrations of aspirin (0 μM, 100 μM, and 1000 μM) for 48 h in A2780 cells, stained with the Annexin V-FITC/PI staining kit, and analyzed using flow cytometry. (G)Quantitative assay of apoptosis rate in A2780, Caov-3 and SK-OV-3 cells. Data are presented as the mean ± SD values. * p<0.05.

(A)

(B)

(C)

(D)

(E)

(F)

(G)
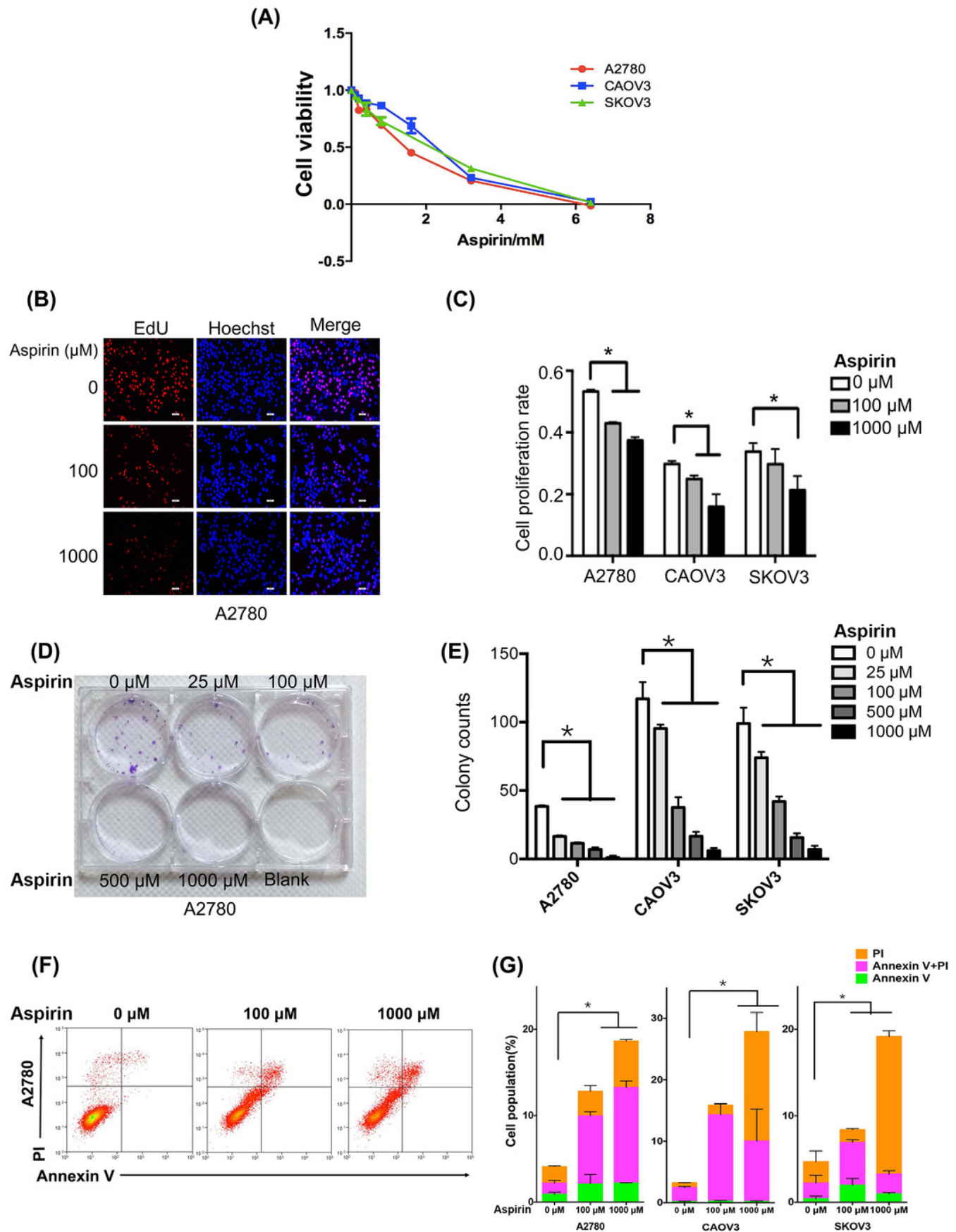
Figure 2

Aspirin enhances the CDDP sensitivity of EOC cells in vitro.

(A)The cell proliferation rate was determined using EdU cell proliferation assays after the incubation of A2780 cells with CDDP (20 μM) alone or in combination with aspirin (100 μM) (CDDP + aspirin) for 48 h (bar, 50 μm). (B) Quantitative assay of proliferation rate in A2780 and SK-OV-3 cells. (C) Colony formation assays were conducted to observe the growth of A2780 cells after an incubation with CDDP (5 μM) alone or with aspirin (25 μM) for 48 h. (D) Quantitative assay of colony counts in A2780 and SK-OV-3 cells. (E) The percentage of apoptotic cells was determined using flow cytometry after the treatment of A2780 cells with CDDP (20 μM) alone or with aspirin (100 μM). (F) Quantitative assay of apoptosis rate in A2780 and SK-OV-3 cells. Data are presented as the mean ± SD values. * p<0.05.

(A)

	EdU	Hoechst	Merge
Control			
CDDP			
CDDP+Aspirin			

A2780

(B)

(C)

Control	CDDP	CDDP+Aspirin

A2780

(D)

(E)

Control	CDDP	CDDP+Aspirin

(F)
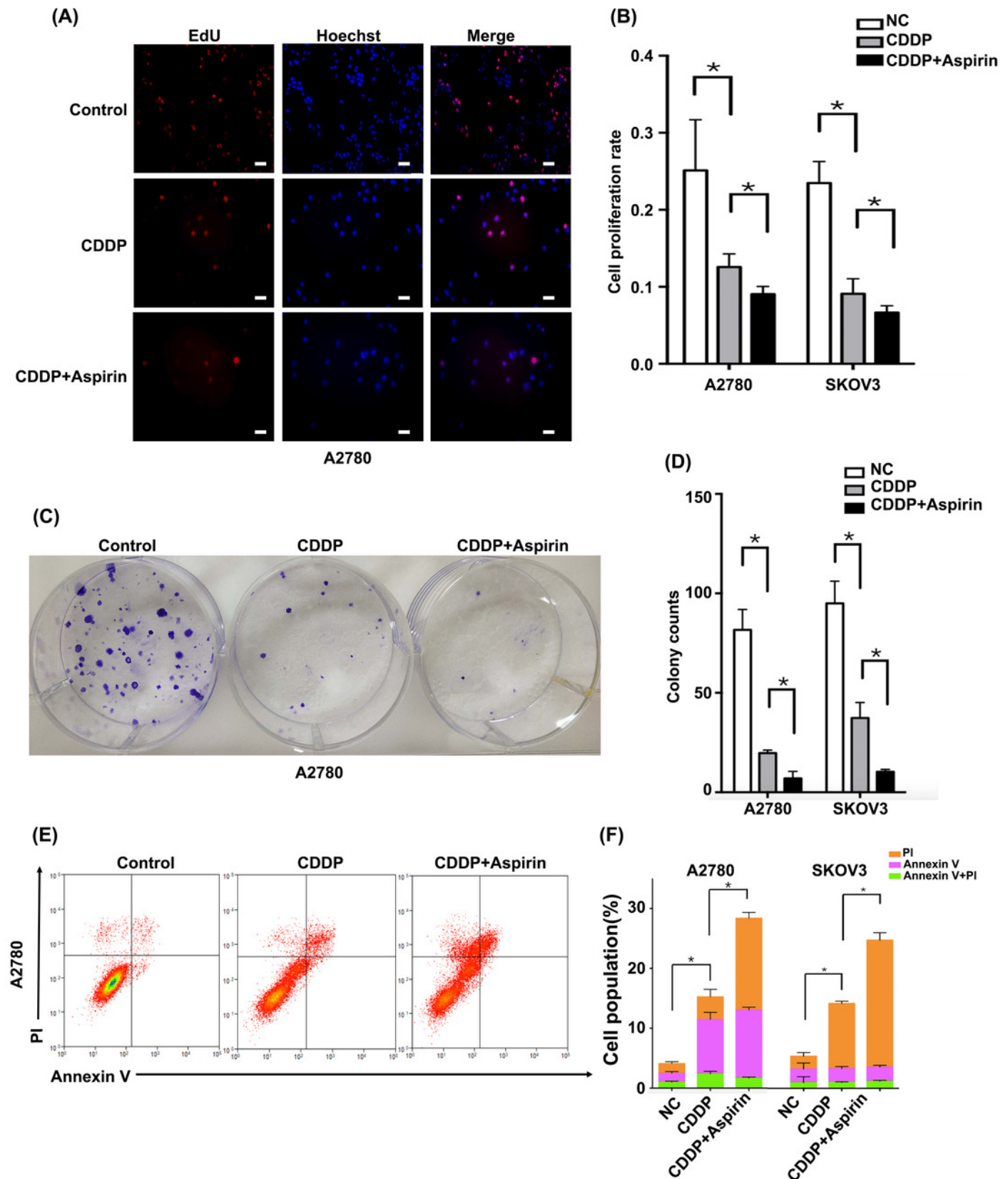
Figure 3

Aspirin enhances the CDDP sensitivity of EOC cells in vivo.

Orthotopic xenograft mouse models of EOC were established with A2780-Luciferase cells using surgical orthotopic implantation to observe the effects of aspirin in vivo. Aspirin was initially administered on day 9 at a dose of 20 mg/kg by gavage and then every other day thereafter, while CDDP administration was initiated at a dose of 3 mg/kg by intraperitoneal injection on day 17 and then every fourth day thereafter. Tumor growth was observed with an IVIS Lumina II beginning on day 8 and then once per week thereafter to obtain in vivo bioluminescence images. (A) The tumor signals in all mice (control group, CDDP group, and CDDP + aspirin group) at every time point are displayed. (B) The tumor growth curve for each group based on the tumor signals is shown. (C) All mice were euthanized via overdose isoflurane inhalation on day 36 and necropsied for the measurement of tumor diameters with Vernier calipers. (D) Quantitative assay of tumor volume in mice. (E) The expression levels of cleaved caspase 3, caspase 3 and Ki67 in tumor tissues harvested from mice bearing orthotopic EOC xenografts (control group, CDDP group, and CDDP + aspirin group) were assessed using IHC. (F) Quantitative assay for the IHC data of cleaved caspase 3, caspase 3 and Ki67. Data are presented as the mean ± SD values. * p<0.05.
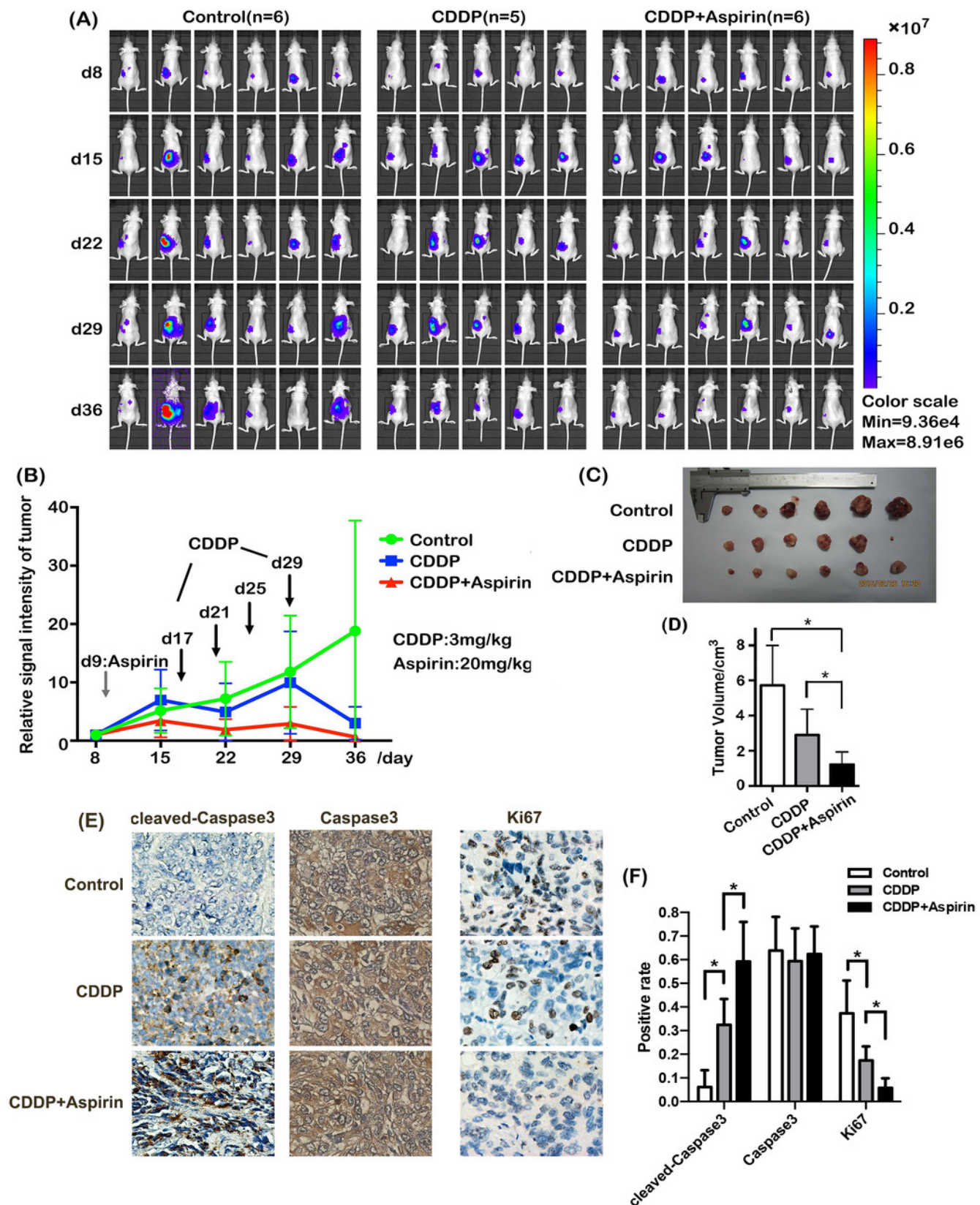
Figure 4

Aspirin acetylates p53 in A2780 cells and subsequently activates p53-mediated downregulation of target genes.

(A) The expression of p53 and acetylation level of p53 in tumor tissues from mice bearing orthotopic EOC xenografts (control group, CDDP group, and CDDP + aspirin group) were assessed using IHC. (B) Quantitative assay of positive rate in IHC. (C) Western blot analysis was conducted to evaluate the level of the p53 protein and its acetylation (p53 K382-ace) after treatment with different concentrations of aspirin (0 μM, 25 μM, 100 μM, 500 μM and 1000 μM). (D) Levels of the p21, bax, foxf1, puma, and rad mRNAs were examined using qPCR after treatment with different concentrations of aspirin (0 μM, 100 μM, and 1000 μM). Data are presented as the mean ± SD values. * $p<0.05$.

(A)

Control   CDDP   CDDP+Aspirin

p53 k382-ace

p53

(B)

□ Control
▨ CDDP
■ CDDP+Aspirin

Positive rate

p53   p53 k382-ace

(C)

A2780

Aspirin (µm)   0   25   100   500   1000

p53 k382-ace   53 kD

p53   53 kD

β-actin   42 kD

(D)

A2780

Aspirin
□ 0 µM
▨ 100 µM
■ 1000 µM

Relative expression level of mRNA

CDKN1A   BAX   PUMA   RRAD   FOXF1