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Ethoxysanguinarine Induces Inhibitory Effects and Downregulates CIP2A in Lung Cancer Cells

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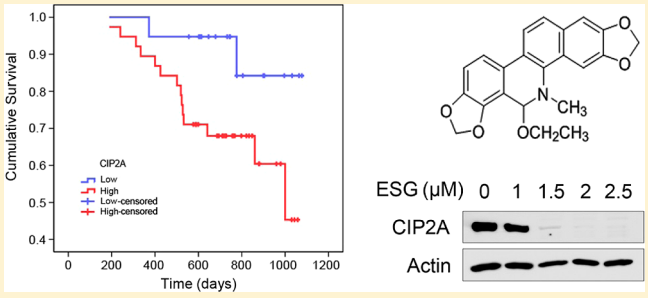
Supporting Information

ABSTRACT: Cancerous inhibitor of protein phosphatase 2A (CIP2A) is an oncoprotein that is able to stabilize c-Myc oncogenic transcription factor and promote proliferation and transformation of cells. CIP2A is overexpressed in many primary tumors, and pharmacological inactivation of CIP2A is an emerging concept for the development of novel anticancer agents. In this study, we demonstrate that overexpression of CIP2A predicts poor prognosis in lung cancer, and a natural compound, ethoxysanguinarine (ESG), effectively downregulates CIP2A protein and its downstream signaling molecules, c-Myc and pAkt, and induces protein phosphatase 2A (PP2A) activity. ESG inhibits proliferation and induces apoptosis of lung cancer cells, and enhances the effects of cisplatin on malignant cells. Taken together, our findings demonstrate that CIP2A is inversely associated with the clinical outcome of lung cancer, and ESG can serve as a lead compound for the development of CIP2A inhibitor for cancer therapies.



KEYWORDS: *Ethoxysanguinarine, CIP2A, lung cancer, cell proliferation, apoptosis*

Lung cancer is the leading cause of cancer deaths worldwide. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for about 85% of this deadly disease.[1] Current treatments for lung cancer include surgery, platinum doublet therapy, radiation therapy, and targeted therapies.[2] For localized cancers, surgical resection remains the single most successful treatment of choice. However, nearly 70% of patients with lung cancer present with locally advanced or metastatic disease by the time it is detected, hence radiation therapy and chemotherapy are usually used alone or in combination.[2] Unfortunately, although there are some improvements in surgical techniques and combined therapies, the disease can hardly be cured and the prognosis is poor. Thus, new drugs based on rational targets remain an agent need for lung cancer.

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a human oncoprotein that is overexpressed in multiple kinds of tumors, such as ovarian cancer, colon cancer, prostate cancer, gastric cancer, and NSCLC.[3−7] CIP2A is required for tumor growth and cell transformation,[8] and its overexpression correlates with poor prognosis in colon cancer,[3] serous ovarian cancer,[9] renal cell carcinomas,[10] and NSCLC.[11,12] In breast cancer, CIP2A-E2F1 positive feedback loop determines cell sensitivity to senescence induction.[13] Proliferation of spermatogonial progenitor cells and spermatogenesis are impaired in a CIP2A deficient mouse model.[14] The expression of CIP2A at diagnosis can consistently predict patients who will progress to blast crisis and may be a novel therapeutic target in chronic myeloid leukemia.[15] Mechanistically, CIP2A modulates c-Myc protein stability through inhibiting protein phosphatase 2A (PP2A) activity and appears to be regulated in a positive feedback loop with c-Myc by promoting each other's expression.[7,8] CIP2A also inhibits Akt-related PP2A activity and activates pAkt, and mediates the anticancer effects of several compounds including bortezomib and erlotinib.[16,17] Thus, targeting CIP2A protein may be an attractive therapeutic strategy for cancer treatment.

In this study, we demonstrated that overexpression of CIP2A predicted poor prognosis in lung cancer patients from southern China, which was the impetus for us to screen for CIP2A-targeting agents. As our laboratory had been engaged in deciphering the mechanisms of action of traditional Chinese medicine for many years, we examined the potential effect of the natural compounds stored in our lab on CIP2A expression.

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A

Characteristics	Cases, n		Total
	CIP2A high	CIP2A low	
All cases	38	19	57
Gender			
Male	30	11	41
Female	8	8	16
Age			
<65	25	17	42
≥65	13	2	15
Smoking			
Smoker	10	12	22
Nonsmoker	28	7	35
Pathologic stage			
I - II	22	12	34
III - IV	16	7	23
T status			
Pathologic T_{1-2}	31	19	50
Pathologic T_{3-4}	6	0	6
Unknown	1		1
N status			
Pathologic N_0	17	12	29
Pathologic N_{1-2}	20	7	27
Unknown	1		1
Histology			
Squamous-cell carcinoma	18	2	20
Adenocarcinoma	17	16	33
adenosquamouscarcinoma	2	1	3
small cell lung cancer	1	0	1

B

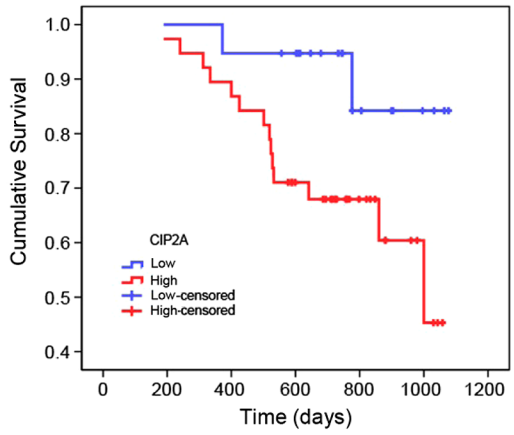

Figure 1. Overexpression of CIP2A is associated with poor prognosis of lung cancer patients. (A) Basic demographic and clinical characteristics of lung cancer patients. (B) Kaplan–Meier survival curve of lung cancer patients with CIP2A expression. The long-rank test, $p < 0.05$.

Among these compounds, ethoxysanguinarine (ESG)[18,19] is a benzophenanthridine alkaloid extracted from plants of the *Papaveraceae* family such as the *Macleaya cordata* (*Willd*) *R. Br.*. ESG is a product converted by sanguinarine upon crystallization with ammoniated ethanol during the isolation process[18,19] and retains the antibacterial activity comparable of sanguinarine.[20] Besides, it is reported that ESG exhibits antiviral activity by causing a significant reduction of porcine reproductive and respiratory syndrome virus (PRRSV)-induced cytopathic effect.[21] However, the effect of ESG on cancer cells remains unclear. We found that ESG was able to significantly downregulate CIP2A at protein and mRNA levels. We further explored the effects of ESG on lung cancer cells, in order to provide evidence supporting the use of ESG as a CIP2A targeting compound for antitumor therapy.

Our previous work showed that CIP2A was overexpressed and associated with cigarette smoking in lung cancer.[5] In this study, to further determine the relationship between CIP2A expression and the survival of lung cancer patients, we performed Kaplan–Meier analysis based on the available follow-up data from 57 lung cancer patients from Guangdong province in southern China. The baseline demographic and clinical characteristics of the patients were shown in Figure 1A. The survival analysis revealed that a survival advantage was identified in patients whose tumors had lower CIP2A expression compared to those with higher CIP2A expression (Figure 1B, long-rank test, $p < 0.05$), indicating that CIP2A overexpression is related with poor clinical outcome in lung cancer.

To determine whether or not ESG (Figure 2A) affects CIP2A protein expression, Western blot analysis was performed after the cells were exposed to ESG at different concentrations. As shown in Figure 2B, the expression of CIP2A protein was reduced in H1975 cells exposed to ESG at 1 μM and became undetectable in cells treated with ESG at 2 μM for 24 h. Also, treatment of A549 cells with ESG at 3.5 μM resulted in an apparent reduction of CIP2A. As the dose increased to 10 μM, CIP2A was eliminated almost completely (Figure 2C). We further showed that ESG caused downregulation of CIP2A in a time-dependent manner (Figure 2D).

In order to investigate the mechanism underlying the downregulation of CIP2A protein, we analyzed the expression of *CIP2A* at mRNA level by real-time quantitative RT-PCR assay. We found that in response to ESG treatment for 24 h, *CIP2A* was decreased drastically (Figure 2E). Since ESG caused a relatively rapid downregulation of CIP2A, we hypothesized that ESG might also affect CIP2A stability. To test this possibility, we used a protein synthesis inhibitor cycloheximide (CHX) to block protein synthesis and found that CIP2A was stable under treatment with CHX for more than 8 h. However, it was downregulated in 4 h in cells coincubated with CHX and ESG (Figure 2F). In addition, exogenous CIP2A was decreased upon ESG treatment (Figure 2G). These data indicate that ESG downregulates *CIP2A* and induces CIP2A proteolysis.

We tested the expression of CIP2A downstream molecules c-Myc and pAKT and found that ESG treatment downregulated c-Myc and pAKT (but not total AKT) in both H1975 (Figure 3A) and A549 (Figure 3B) cells. We found that siRNA mediated CIP2A silencing induced a decrease of pAKT, while overexpression of CIP2A increased pAKT (Figure 3C, D). CIP2A is an endogenous inhibitor of PP2A and the dephosphorylation of AKT is widely regulated by PP2A, we therefore tested whether ESG could influence PP2A activity. As shown in Figure 3E, PP2A activity was upregulated in cells treated with ESG, suggesting that ESG may affect CIP2A-PP2A-pAKT signaling pathway. Furthermore, inhibition of PP2A by okadaic acid (OA) partially reversed the inhibitory effect of ESG on cell proliferation (Figure 3F).

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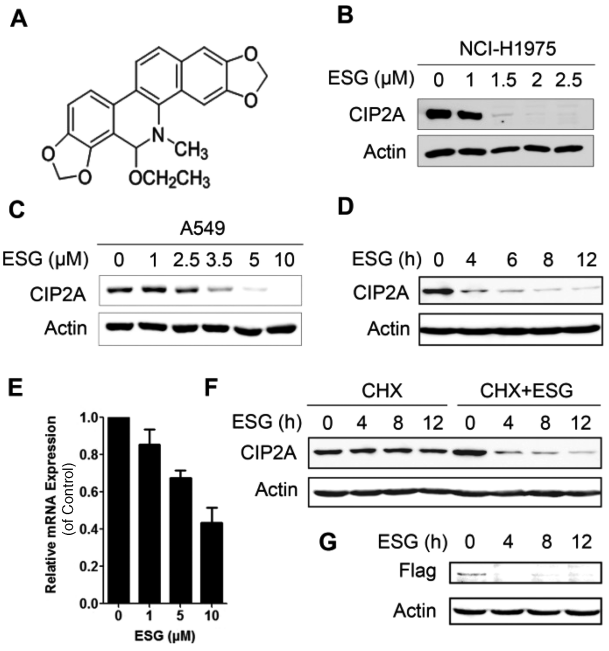

Figure 2. ESG downregulates CIP2A in lung cancer cells. (A) Chemical structure of ESG. H1975 cells (B) or A549 cells (C) were incubated with ESG at indicated concentrations for 24 h, and cell lysates were subjected to Western blot assay using indicated antibodies. (D) A549 cells were treated with 5 μM ESG for indicated time points, and cell lysates were subjected to Western blot assay. (E) The expression of *CIP2A* in cells upon ESG treatment for 24 h was analyzed by real-time quantitative RT-PCR. (F) A549 cells were treated with 100 μg/mL CHX in the absence or presence of 5 μM ESG for indicated time points, and whole-cell lysates were probed for CIP2A. (G) A549 cells were transfected with Flag-CIP2A for 24 h and treated with ESG for indicated time points, and whole-cell lysates were probed for Flag.

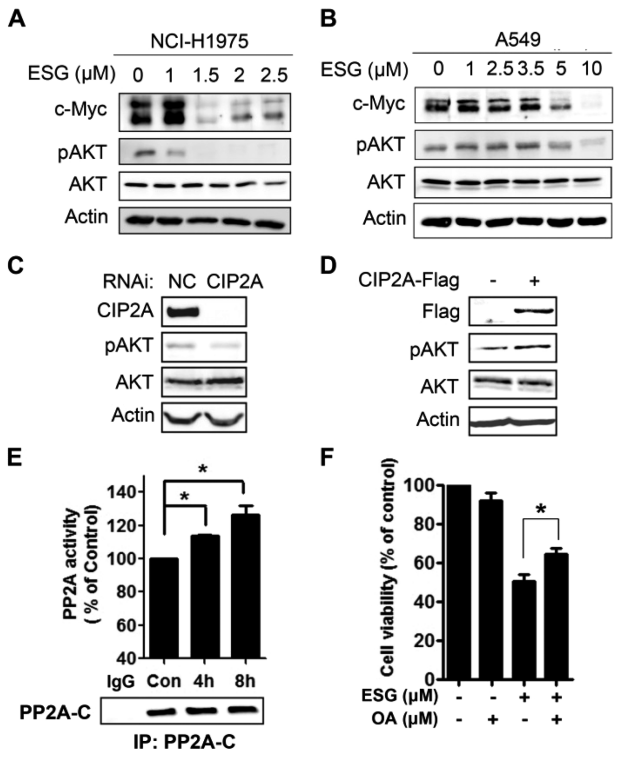

Figure 3. Effects of ESG on CIP2A downstream molecules. H1975 (A) or A549 (B) cells were treated with different concentrations of ESG for 24 h, and the expression of c-Myc, pAKT, and total AKT was detected by Western blot analyses. (C) A549 cells were transfected with 50 nM negative control (NC)- or CIP2A-specific siRNA for 72 h, and cell lysates were subjected to Western blotting. (D) A549 cells were transfected with Flag-CIP2A constructs for 72 h, and cell lysates were probed with indicated antibodies. (E) A549 cells were exposed to 5 μM ESG for indicated time points and lysed, and cell lysates were prepared for detecting PP2A activity as described in Supporting Information. (F) A549 cells were pretreated with OA (25 nM) for 2 h followed by ESG (5 μM) treatment for 24 h and then assessed by MTT assay for cell viability. *, $p < 0.05$.

We then evaluated the antitumor effects of ESG on lung cancer cells. Using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, we showed that treatment with 0.5−10 μM ESG for 48 h significantly inhibited the proliferation of lung cancer cells, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma cell lines (Figure 4A). As shown in Figure 4B, the corresponding IC_{50} values for the cells ranged from 1.0 to 2.74 μM, demonstrating that ESG has potent inhibitory effects on proliferation of lung cancer cells.

Next, we investigated whether ESG triggered apoptosis in lung cancer cells. An annexin V/propidium iodide (PI)-staining and flow cytometry assay showed that treatment of H1975 (Figure 5A, B) or A549 cells (Figure 5C, D) with ESG for 24 h caused apoptosis in a proportion of the cells. We analyzed the expression of poly (ADP-ribose) polymerase (PARP), caspase-8 and caspase-9 in the cells by Western blot assay. ESG treatment resulted in a marked increase in the cleavage of PARP, and a decrease in pro-caspase-8 and pro-caspase-9 in both cell lines (Figure 5E, F), indicative of caspases activation in ESG-caused apoptosis. To prove the activation of caspases as an essential step in the apoptotic pathway induced by ESG, H1975 cells were pretreated with a pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (z-VAD.fmk) at 20 μM for 1 h, followed by ESG incubation for additional 24 h. We showed that ESG-induced PARP cleavage was suppressed by z-VAD.fmk (Figure 5G). The same result was observed in A549 cells (Figure 5H). Z-VAD.fmk also partly suppressed ESG-induced cell proliferation inhibition in H1975 and A549 cells (Figure 5I, J).

AKT is constitutively activated in lung cancer cells and promotes cellular survival and resistance to chemotherapy.[22] Cisplatin is one of the most widely used agents for lung cancer chemotherapy. Given that ESG could reduce CIP2A and pAKT expression, we tested whether ESG could enhance the effect of cisplatin on lung cancer cells. By MTT assay, we found that treatment with 1 μM ESG in combination with 10 μM cisplatin drastically inhibited the cell viability of H1975 cells compared to ESG or cisplatin alone (Figure 6A). Also, enhanced effects were observed in A549 cells cotreated with 3.5 μM ESG and 20 μM cisplatin (Figure 6B). Combined effect of cisplatin and ESG in inducing apoptosis was examined. As shown in Figure 6C, ESG or cisplatin alone did not cause noticeable PARP cleavage in H1975 cells, while combination of the two induced apparent PARP cleavage. Similar results were obtained in A549 cells exposed to ESG and cisplatin (Figure 6D), indicative of enhancement of apoptosis.

CIP2A is an endogenous inhibitory protein of PP2A, which presents cancer-promoting profile in various types of cancers. PP2A is a tumor suppressor regulating multiple signaling pathways in transformation.[8,23] Our previous work[5] showed

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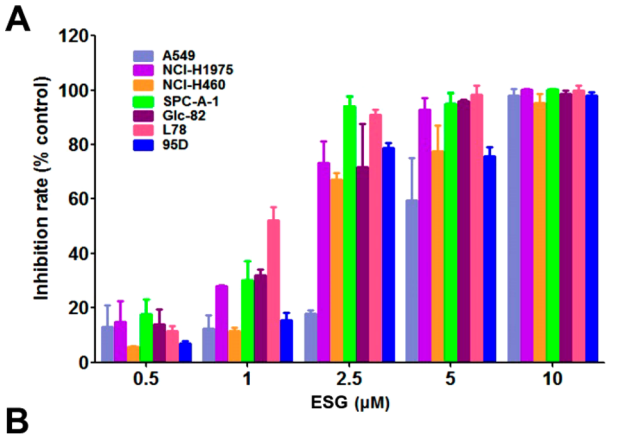

B

Cell lines	A549	NCI-H1975	NCI-H460	SPC-A-1	Glc-82	L78	95D
Histologic type	Ade	Ade	LCC	Ade	Ade	SCC	highly metastatic LCC
IC_{50} (μM)	2.74	1.29	1.56	1.1	1.3	1	2

Ade, adenocarcinoma; LCC, large cell carcinoma; SCC, squamous cell carcinoma

Figure 4. Antiproliferation effects of ESG on lung cancer cells. (A) Lung cancer cells were treated with increasing doses of ESG for 48 h. The cell viability was measured by MTT assay. (B) The IC_{50} values of ESG for lung cancer cells.

that in 60 lung cancer patients, CIP2A was undetectable or very low in paratumor normal tissues but was dramatically elevated in tumor samples in 38 (63.3%) patients. Overexpressed CIP2A was associated with cigarette smoking. In the present study, we reported that CIP2A overexpression was associated with poor prognosis of lung cancer (Figure 1), consistent with previous studies.[11,12] Therefore, targeting CIP2A will be an attractive therapeutic strategy for lung cancer treatment.

We identified a natural compound rabdocoetsin B (RdB), which downregulates *CIP2A* expression in lung cancer cells. Another compound celastrol binds CIP2A, promotes CIP2A-carboxyl terminus of Hsp70-interacting protein (CHIP) interaction and induces proteasomal degradation of CIP2A, and exhibits potent anti-lung cancer activity.[24] Here, we further showed that ESG[18,19] was also able to cause a significant decrease of CIP2A at both mRNA and protein levels at a relatively low concentration compared to RdB (Figure 2). The IC_{50} values of RdB in H1975 and A549 cells were 10.33 and 14.22 μM,[5] higher than that of ESG for the two cell lines (1.29 and 2.74 μM, respectively) (Figure 4B).

CIP2A can stabilize c-Myc and up-regulate pAKT by blocking PP2A activity.[8,25] We tested the expression of these molecules and found that ESG downregulated c-Myc and pAKT (Figure 3A, B) and increased PP2A activity (Figure 3E), indicating that ESG may inhibit CIP2A signaling network, which may partly explain the inhibitory effect of ESG on lung cancer cell growth (Figure 4). In addition, ESG induced apoptosis in lung cancer cells in a caspase-dependent fashion (Figure 5), also contributed to the inhibitory effect of ESG on cell viability.

Cisplatin is one of the most effective and widely used DNA-damaging anticancer agents for the treatment of various human cancers including lung cancer.[26] However, the ability of cancer cells becoming resistant to cisplatin strongly limits its clinical application. Therefore, overcoming cisplatin resistance by novel strategies will benefit patients with lung cancer. AKT is widely overexpressed and associated with cisplatin-resistance in lung

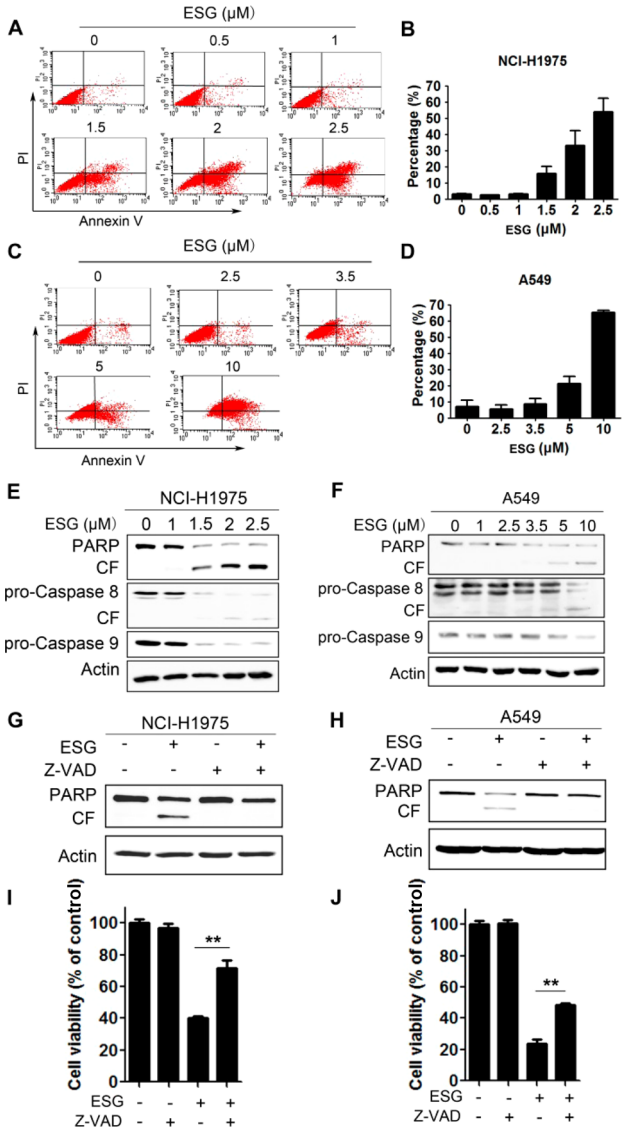

Figure 5. ESG triggers apoptosis of lung cancer cells. H1975 (A, B) or A549 (C, D) cells were treated with various concentrations of ESG for 24 h, and cell apoptosis was assessed by annexin V/PI staining and flow cytometry. H1975 (E) or A549 (F) cells were treated with different concentrations of ESG for 24 h, and cell lysates were subjected to Western blot using indicated antibodies. H1975 (G) and A549 (H) cells were pretreated with z-VAD-fmk (20 μM) for 1 h, followed by treatment with ESG at 1.5 μM (H1975) or 5 μM (A549) for 24 h and lysed, and the lysates were subjected to Western blot using indicated antibodies. CF, cleavage fragment. H1975 (I) and A549 (J) cells were pretreated with z-VAD-fmk (20 μM) for 1 h followed by treatment with ESG at 1.5 μM (H1975) or 5 μM (A549) for 24 h, and cell viability was analyzed by MTT assay. **, $p < 0.01$.

cancer.[22] Our results showed that ESG could trigger CIP2A downregulation and subsequent pAKT inactivation in both H1975 and A549 cells, suggesting that cisplatin-resistance could be overcome by CIP2A-targeting agent. As shown in Figure 6, ESG enhanced the effect of cisplatin on lung cancer cell growth and apoptosis, which confirmed our above speculation.

In summary, we showed that CIP2A is inversely associated with clinical outcome of lung cancer, and CIP2A-targeting agents including ESG and celastrol could serve as lead compounds for the development of CIP2A inhibitors for cancer therapeutics.

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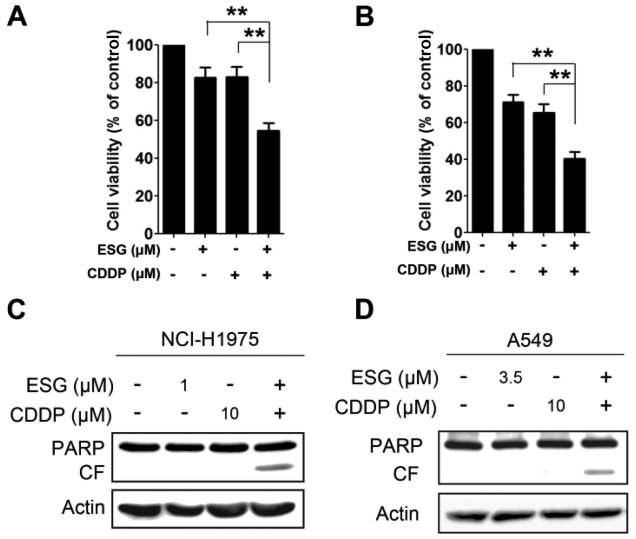

Figure 6. ESG treatment enhances the effects of cisplatin on lung cancer cells. H1975 (A) or A549 (B) cells were treated with ESG and/or cisplatin for 24 h. MTT assay was performed to test cell viability. **, $p < 0.01$. H1975 (C) or A549 cells (D) were cultured with ESG and/or cisplatin for 24 h, and cell lysates were subjected to Western blot analysis using indicated antibodies. CF, cleavage fragment; CDDP, cisplatin.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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