OPEN

Selective synergistic anticancer effects of cisplatin and oridonin against human p53-mutant esophageal squamous carcinoma cells

Huiyu Yang[a,b], Jie Wang[b,c], Suliman Khan[d], Yuanying Zhang[a,b,e], Kuicheng Zhu[b,e], Enhui Zhou[b,f], Meiyuan Gong[b,c], Bingrong Liu[a,b,e], Quancheng Kan[g] and Qi Zhang[b,c,*]

Oridonin (ORI) is known to pose anticancer activity against cancer, which could induce the therapeutic impact of chemotherapy drugs. However, such simple combinations have numerous side effects such as higher toxicity to normal cells and tissues. To enhance the therapeutic effects with minimal side effects, here we used ORI in combination with cisplitin (CIS) against different esophageal squamous cell carcinoma (ESCC) cell lines *in vitro*, to investigate the synergistic anticancer effects of the two drugs against ESCC. Calcusyn Graphing Software was used to assess the synergistic effect. Apoptosis, wound healing and cell invasion assay were conducted to further confirm the synergistic effects of ORI and CIS. Intracellular glutathione (GSH) and reactive oxygen species assay, immunofluorescence staining and western blot were used to verify the mechanism of synergistic cytotoxicity. ORI and CIS pose selective synergistic effects on ESCC cells with p53 mutations. Moreover, we found that the synergistic effects of these drugs are mediated by GSH/ROS systems, such that intracellular GSH production was inhibited, whereas the ROS generation was induced following ORI and CIS application. In addition, we noted that DNA damage was induced as in response to ORI and CIS treatment. Overall, these results suggest that ORI can synergistically enhance the effect of CIS, and GSH deficiency and p53 mutation, might be biomarkers for the combinational usage of ORI and CIS. *Anti-Cancer Drugs* 33: e444–e452

Anti-Cancer Drugs 2022, **33**:e444–e452

Keywords: cisplatin, esophageal squamous cell carcinoma cells, oridonin, synergistic effect, GSH, reactive oxygen species

[a]Department of Gastroenterology, The First Affiliated Hospital of Zhengzhou University, [b]State Key Laboratory of Esophageal Cancer Prevention & Treatment, Zhengzhou University, [c]School of Pharmaceutical Sciences, Zhengzhou University, [d]Department of advanced medical Sciences, The Second Affiliated Hospital of Zhengzhou University, [e]Academy of Medical Sciences, Zhengzhou University, [f]BGI College & Henan Institute of Medical and Pharmaceutical Sciences, Zhengzhou University, and [g]Department of Pharmacology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Correspondence to Quancheng Kan, Department of Pharmacology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China
E-mail: kqczzu@126.com

*Qi Zhang is the co-corresponding author.

Received 6 August 2021 **Revised form accepted** 17 August 2021

Introduction

Esophageal cancer is the sixth leading cause of cancer-related death worldwide [1,2]. The 5-year survival rate is less than 20% [3] due to the lack of diagnostic options at earlier stages and promising treatment options in patients with advanced esophageal cancer stage or when surgical resection is inapplicable [4,5]. Moreover, due to the dense submucosal lymphovascular network with direct connections to both the regional lymph nodes and the thoracic duct, the esophageal cancer could frequently have lymph node metastases in the early stage [6]. The targeted therapies, which have been demonstrated effective in other cancers such as lung cancer, breast cancer, showed limited therapeutic effects in the case of esophageal cancer [5]. Considering the necessity of developing effective therapeutic options, combinational drugs therapy can be a suitable option to treat esophageal cancer.

Cisplatin (CIS) is a common first-line chemotherapy drug in treating esophageal cancer [7]; however, its application is limited by drug resistance and dose-related side effects [8,9]. A higher dose, either cumulative dose or doses per treatment, result in irreversible kidney injury [10]. Therefore, combining CIS with another drug can be beneficial, such that the synergistic effects will enhance treatment efficacy, and lower CIS dosage will reduce its side effects.

Oridonin (ORI) is traditional Chinese medicine, which has been tested for antitumor effects against a wide range of cancer [11] either alone or in combination with other clinical chemotherapy drugs [12,13]. It also shows promising therapeutic effects against esophageal cancer [14], and the curative effect can be double enhanced when combined with chemotherapy drugs. However, the mechanism is not clear. Earlier studies demonstrated that ORI could reduce nephrotoxicity induced by CIS [15]. We have previously

0959-4973 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc. DOI: 10.1097/CAD.0000000000001237

reported the selective cytotoxic effect of ORI to esophageal cancer cell lines with p53 mutation [16]. Therefore, in the current study, we have investigated the synergistic effects of ORI and CIS in esophageal cancers.

Materials and methods

Antibodies and reagents

The RAD51 (ab133534) and γH2AX (ab81299) antibodies were purchased from Abcam. Glyceraldehyde-3-phosphate dehydrogenase (BBI D190636) antibody was purchased from Sangon. Cisplatin was purchased from Sigma, Oridonin was purchased from Shanghai Yuanye Biotechnology.

Cell culture

Esophageal cancer cells, kyoto squamous esophageal (KYSE30), KYSE510, TE1, KYSE150, KYSE410 and EC109 are available commercially and through the National Collection of Authenticated Cell Cultures. All cells were cultured at 37 °C, 5% CO_2, in 1640 medium (HyClone, Shanghai, China), all medium containing 10% fetal bovine serum (FBS) (HyClone, Shanghai, China) and 100 units/mL penicillin and 100 mg/mL streptomycin (HyClone, Shanghai, China).

Cell viability assay

The effects of CIS, ORI, CIS plus ORI on cells viability were measured by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2Htetrazo-liumbromide (MTT) assay. All cells (5×10^3 cells/100 μL) were seeded into 96-well plates and treated with a range of concentrations of CIS, ORI alone, or combined respectively for 24 h. MTT reagent was added into each well for 4-h incubation. The formazan crystals were dissolved by 150 μL dimethyl sulfoxide. The absorbance was measured at 570 nm by a microplate reader (Thermo Scientific, USA).

Drug synergy experiment

According to the IC50 value of the drugs in each experimental group, CIS and ORI were given in a constant ratio of drug concentration corresponding to the IC50. Combination index (CI) analysis was performed using Calcusyn Graphing Software (Biosoft, Inc., Missouri, USA). CI greater than 1 indicates antagonistic effect, CI equal to 1 indicates additive effect and CI less than 1 indicates synergistic effect. The smaller the CI, the stronger the synergistic effect.

Cell apoptosis detection

KYSE30 cells were incubated with CIS, ORI, CIS plus ORI respectively for 24 h. Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis detection kit was used to detect the cell apoptosis. Cells were harvested and stained with the FITC-labeled annexin V and PI for 15 min under dark conditions. Further procedure for the population of apoptosis was evaluated immediately by flow cytometer (BD Biosciences, St Louis, Missouri, USA).

Wound-healing assay

KYSE30 cells (1×10^5 cells/mL) were seeded in 6-well plates. When the cells reached 85% confluence, each well was manually scratched with 20 μL sterile pipette tips. The cells were then washed with PBS to remove the debris and cultured with a complete medium containing CIS, ORI, or both at the indicated concentrations. Representative images were taken at 0, 8 and 24 h under an inverted microscope.

Trans-well assay

KYSE30 cells were treated with CIS, ORI or both for 24 h. Then, the cells were trypsinized, and 4×10^4 cells in 200 μL serum-free 1640 were transferred into the upper chamber of the transwell (8 μM pore size, BD Biosciences) precoated with or without Matrigel (BD Biosciences). Then, 600 μL of culture medium containing 20% FBS was added to the lower chamber. After 24 h of culture at 37 °C in a 5% CO_2 incubator, the transwell chambers were fixed using 4% paraformaldehyde and then stained with 1% crystal violet. The cells that migrated to the lower surface of the membrane were photographed under a Leica DM2500 microscope. The average numbers of migrated cells were determined by counting three random fields (100×).

Intracellular glutathione and reactive oxygen species assay

Cells were seeded to 6-well plates in triplicates and treated with CIS, ORI, CIS plus ORI for 4 h. Intracellular total glutathione (GSH) content is assayed by GSH Assay Kit (S0053, Beyotime, Shanghai, China) according to the manufacturer's instructions. The total GSH content was calculated by the standard curve.

After cells were treated for 6 h, intracellular reactive oxygen species (ROS) species were detected using the Reactive Oxygen Species Assay Kit (S0033, Beyotime, Shanghai, China). Cells were incubated for 20 min at 37 °C in the dark with 10 μM 2',7'-Dichlorodihydrofluorescein diacetate, collected and analyzed using BD AccuriTMC6 Plus Flow Cytometer and fluorescence microscope respectively.

Immunofluorescence staining

KYSE30 cells were seeded onto glass coverslips in 6-well plates and incubated overnight. Cells were then fixed and analyzed as described previously. Immunofluorescence detection of RAD51 and γH2AX expression in KYSE30 cells were performed. Images were detected and collected by fluorescent microscopy. Staining changes were double-blind read by two pathology physicians.

Western blot analysis

After 24 h of treatment, the total cellular proteins were extracted from KYSE30 cells using radioimmunoprecipitation lysis buffer. The protein concentrations were measured using the bicinchoninic acid Protein Assay Kit. The proteins were then separated using 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes.

After blocking in 5% skim milk for 2 h, the blocked membranes were incubated with primary antibodies at 4 °C overnight. The membranes were then incubated with secondary antibodies for 1 h at room temperature. Finally, the signals were visualized using an enhanced chemiluminescence reagent.

Statistical analysis

All results were confirmed as the mean ± SD of triplicate data. Statistical analysis was conducted by GraphPad Prism (La Jolla, California, USA). *P* values less than 0.05 were considered statistically significant.

Results

Cisplitin and oridonin inhibited esophageal cancer cell proliferation and growth of mutant p53 cells

To evaluate the inhibition of cell growth by ORI and CIS, we treated human esophageal cancer cells with ORI or CIS *in vitro*. Overall, three wild-type p53 (wt-p53) cell lines KYSE150, KYSE410 and EC109 and three mutant p53 (mut-p53) cell lines KYSE30, KYSE510 and TE1 were selected. Cells were cultured with various concentrations (5–80 μM/L) of CIS and ORI respectively for 24 h, followed by cell viability measurement by MTT assay. We found that CIS and ORI inhibited cell proliferation in all six cancer cell lines in dose-dependent manner as shown in Fig. 1a–f.

We then assessed the synergistic effect of CIS and ORI by using a dose range from 1/4 IC50 to 4 IC50 for each drug. Our results indicated that CIS plus ORI successfully inhibited the cell growth of mut-p53 cell lines. However, these combinations of CIS and ORI had no synergistic effect on inhibiting the growth of wt-p53 cells (Fig. 1g–l), indicating that CIS and ORI show synergistic inhibitory effects specifically to mut-p53 esophageal cancer cells

Synergistic effect of cisplitin and oridonin to inhibit intracellular glutathione generation by mutant p53 cell lines

We further investigated the synergistic effects of CIS plus ORI on GSH production in esophageal cancer cells. Our results showed that GSH levels were significantly reduced in response to the CIS plus ORI treatment to mut-p53 cell lines as compared to the same treatment of wild-type cell lines (Fig. 2). To understand the underlying mechanism, we selected KYSE30 cells for further experiments based on their higher sensitivity as compared to the other cell lines. When the actual concentration of CIS was 10 μM and ORI 10 μM, the CI value was 0.73, the actual maximum inhibition rate of KYSE30 was 55%; and when the CI value was 0.75, the concluded concentration of CIS and ORI was 9.6 μM respectively, the maximum inhibition rate of KYSE30 was 50%. Thus, we choose CIS 10 μM and ORI 10 μM in the following experiments.

Synergistic effect of cisplitin and oridonin on apoptosis of KYSE30 cells

To verify the synergistic effect of CIS and ORI, we tested apoptosis in KYSE30 cells treated with CIS, ORI, CIS plus ORI. Our results from flow cytometry revealed that combinational treatment (CIS plus ORI) showed significantly higher apoptotic rate as compared to the treatment of either CIS or ORI alone. Collectively, these data indicate a promising synergistic effect of CIS plus ORI on cell apoptosis, Fig. 3.

Cisplitin plus oridonin synergistically inhibited the migration and invasion of KYSE30 cells

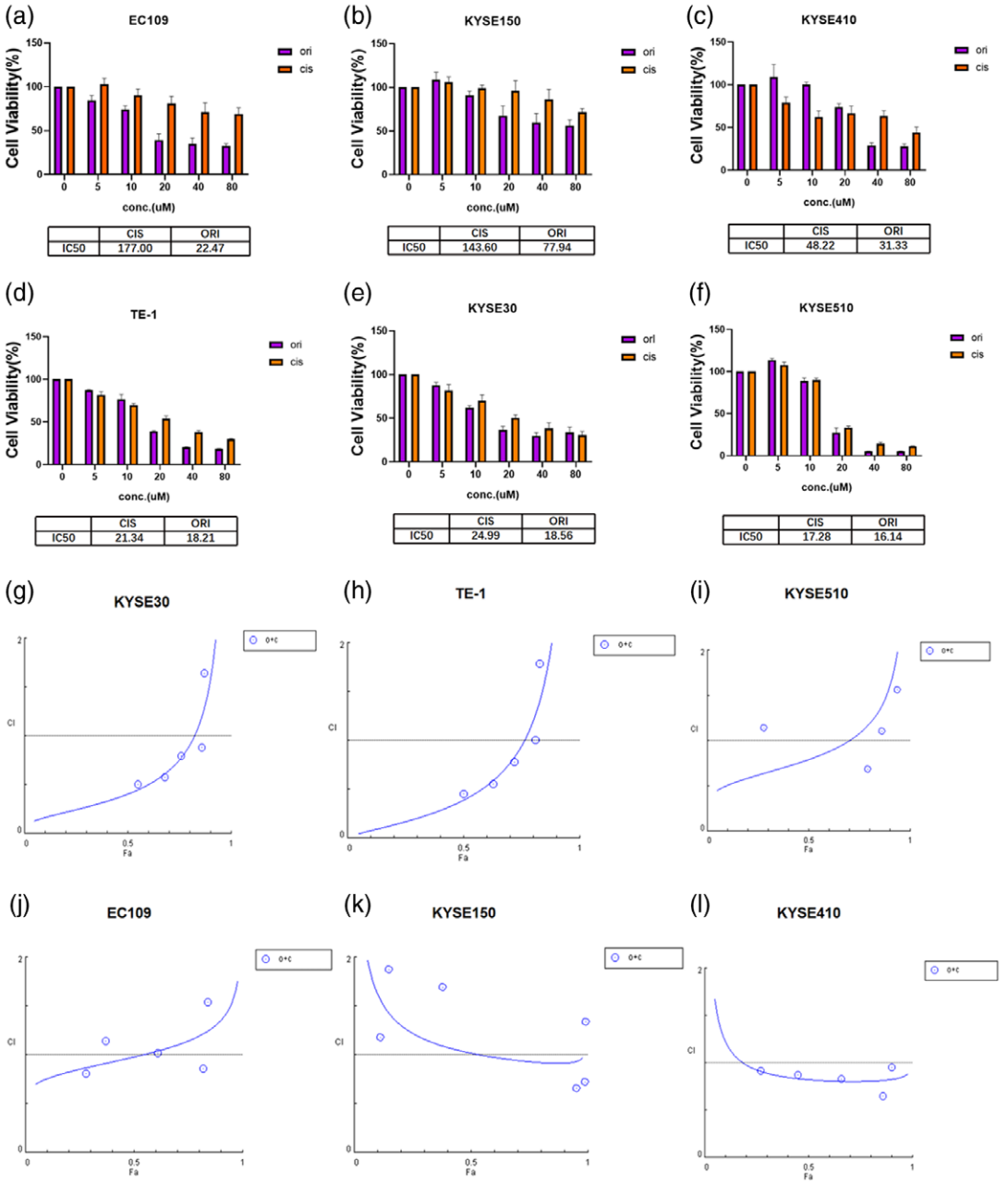
To further elucidate the synergistic effect of CIS plus ORI, we performed wound-healing by applied 10 μM CIS and 10 μM ORI on KYSE30 cells, which showed a higher rate of migration into the scratched wound area than drug-treated cells. Cancer cells treated with CIS plus ORI induced significant inhibition of migration as compared to treatment with CIS or ORI alone (Fig. 4a–c). Furthermore, we performed trans-well assays to determine the effects of migration and invasion. We observed that inhibition was enhanced with the combinational treatment of CIS plus ORI as compared to treatment with CIS or ORI alone(Fig. 4d–g).

Glutathione/reactive oxygen species participates in the synergistic effects of the combined treatment

To confirm that GSH depletion induces cell death during CIS plus ORI treatment, we tested the effects of GSH on cell apoptosis and cell viability. KYSE30 cells were incubated with 1 mM GSH and CIS or ORI, and CIS plus ORI for 24 h. Cell apoptosis and cell viability were determined by flow cytometry and MTT assay respectively. We observed that cell death caused by CIS or ORI, or CIS plus ORI was predominantly inhibited by GSH (Fig. 5a–c).

It is well known that excessive accumulation of ROS leads to oxidative damage and cell death. We measured intracellular ROS levels using flow cytometry and fluorescence microscope. Our results showed that combinational treatment with CIS plus ORI induced a higher intracellular ROS generation as compared to that of CIS or ORI alone. We further used DCFDA and fluorescence microscope to detect the level of ROS in KYSE30 cells treated with CIS or ORI or CIS plus ORI for 6 h. ROS levels were significantly increased in response to the treatment with CIS plus ORI. We also found that ROS levels were decreased in cells treated with *N*-acetyl-L-cysteine (NAC) (5 mM) (Fig. 5d–f). Moreover, NAC, a sulfhydryl-containing antioxidant, can also reverse the cytotoxicity caused by therapeutics(Fig. 5g–i)

Fig 1



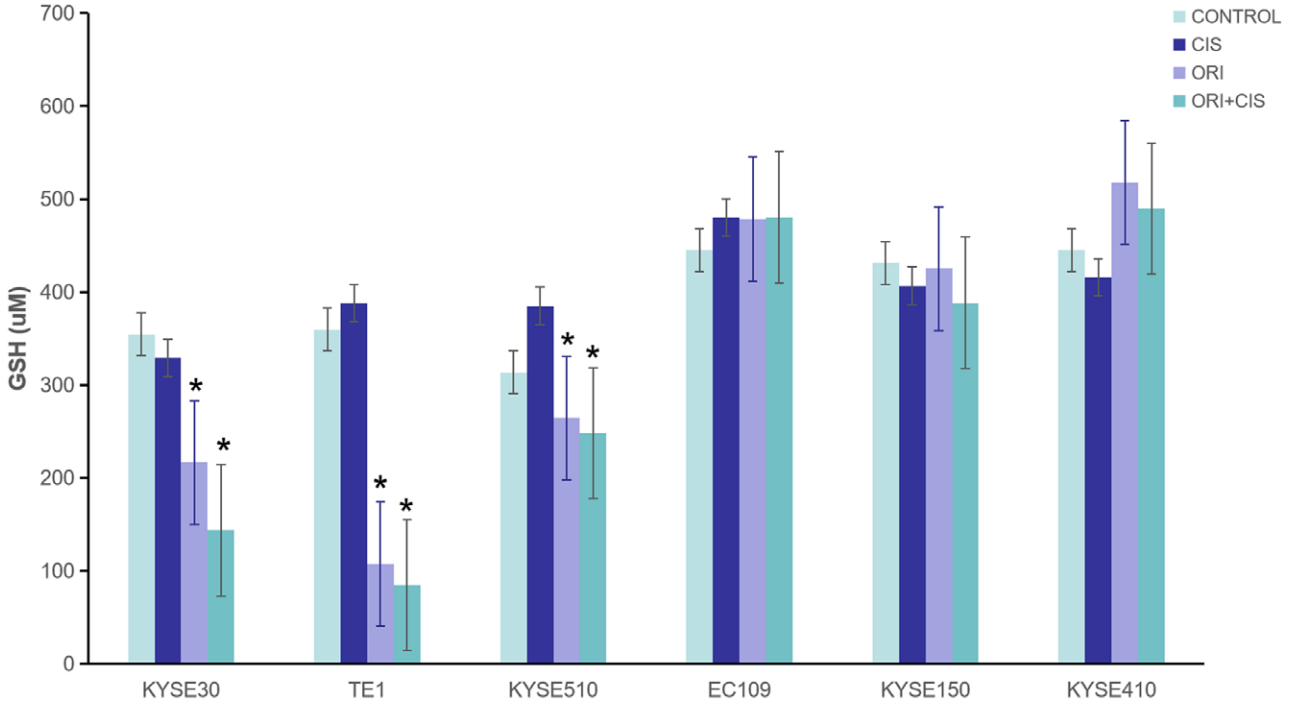
CIS and ORI inhibited cell proliferation and synergistically inhibit the growth of mut-p53 cells. (a–f) Viability of EC109, KYSE150, KYSE410, TE1, KYSE30 and KYSE510 cells after treatment with different concentrations of CIS or ORI for 24 h. CI analysis was performed using Calcusyn Graphing Software. (g) CI=0.403; (h) CI=0.389; (i) CI=0.792; (j) CI=1.004; (k) CI=1.016; (l) CI=1.061; CIS plus ORI had the synergistical effect on the mut-p53 cell lines KYSE30, KYSE510 and TE1. CI, combination index; CIS, cisplitin; KYSE, kyoto squamous esophageal; ORI, oridonin.

Effect of cisplitin and oridonin on DNA damage through glutathione/reactive oxygen species stimulation

To know about the impact of CIS and ORI on DNA, we evaluated the expression of γH2AX using immunofluorescence staining to assess DNA damage. Our results showed that the level of γH2AX was significantly increased in response to the synergistic effect of CIS plus ORI as compared to their individual use (Fig. 6a and b).

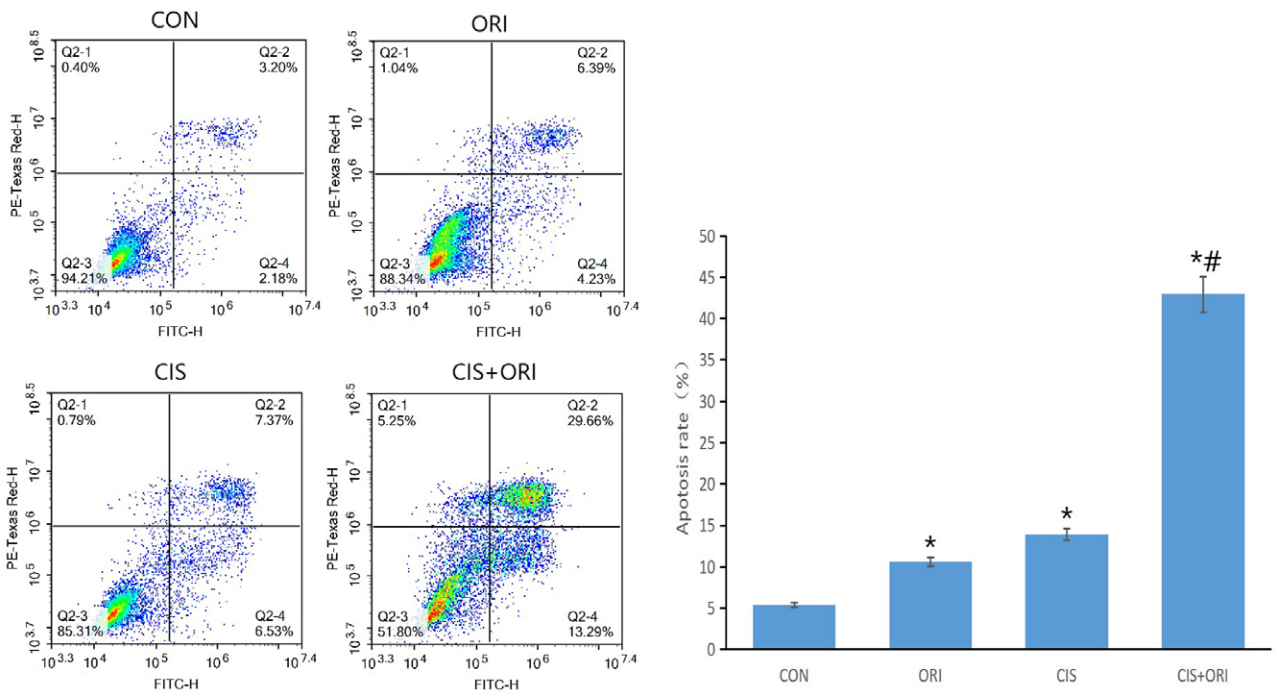
We also perform a western blot to evaluate the expression of RAD51 and γH2AX, which indicated that the levels of RAD51 and γH2AX were significantly reduced and increased respectively following CIS and ORI treatment.

Fig. 2



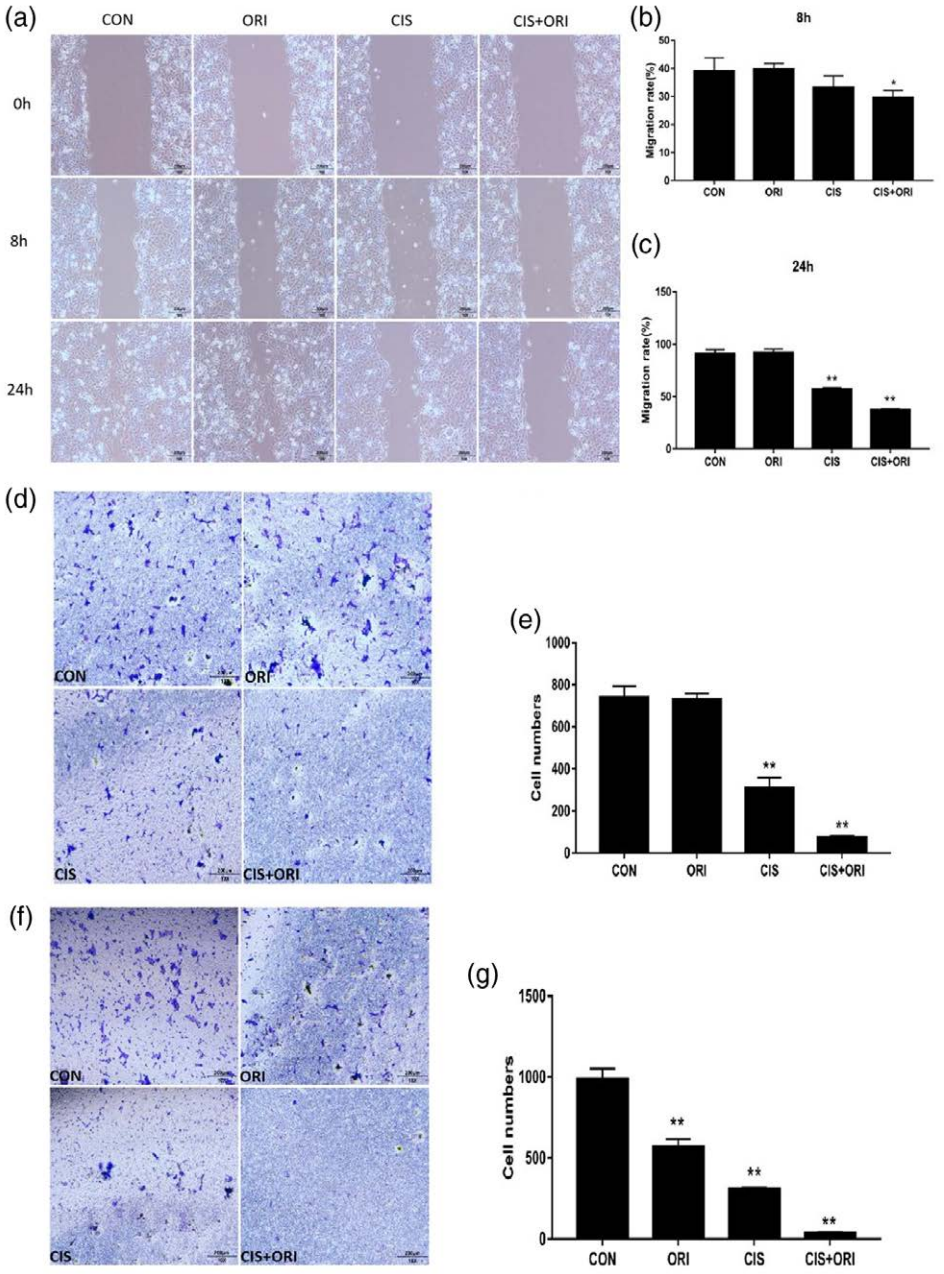
Change of intracellular GSH level among the six cell lines. The level of GSH were reduced significantly in KYSE30, TE1 and KYSE510 cells when treated with CIS plus ORI (*P<0.01), while there were no significant changes among wt-p53 cell lines EC109, KYSE150 and KYSE410. CIS, cisplitin; GSH, glutathione; KYSE, kyoto squamous esophageal; ORI, oridonin.

Fig. 3



Synergistic effect of CIS and ORI on apoptosis of KYSE30 cells. The apoptosis of KYSE30 cells was determined by flow cytometry after staining with annexin V-FITC/PI. * P<0.05, compared with the control group. #P<0.01, compared with the monotherapy group. CIS, cisplitin; KYSE, kyoto squamous esophageal; ORI, oridonin.

Fig. 4



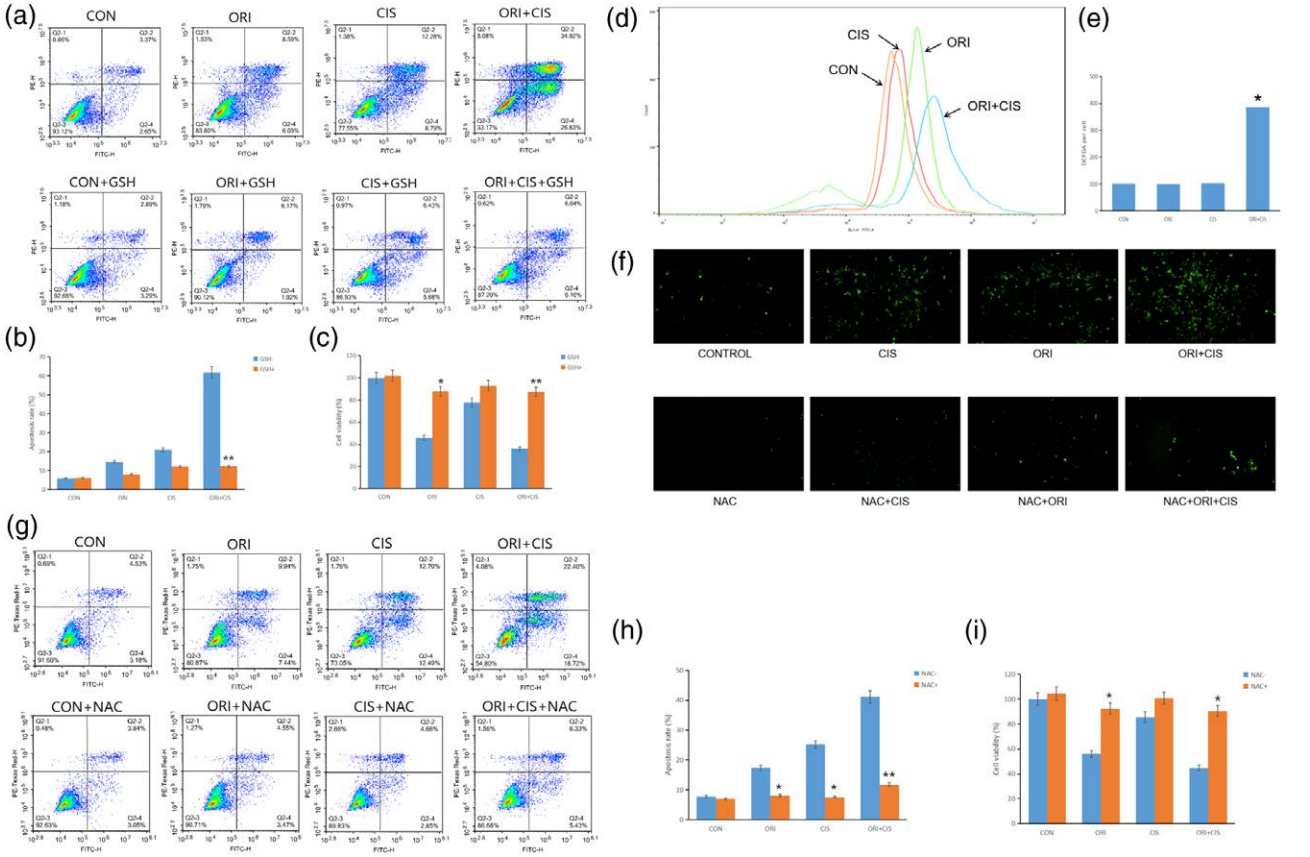
CIS and ORI suppress the migration and invasion capacity of KYSE30 cells. (a–c) Micrographs of wound-healing assays with KYSE30 cells treated with CIS, ORI, CIS plus ORI. Images were obtained at 0, 8, 24 h (100×). (d,e) A trans-well assay was used to detect the invasion of KYSE30 cells. The number of invasive cells was observed and counted by using a light microscope (100×). (f,g) A trans-well assay was used to detect the migration of KYSE30 cells. The cells was observed and counted by using a light microscope (100×).* $P<0.05$, ** $P<0.01$, compared with the monotherapy group. CIS, cisplitin; KYSE, kyoto squamous esophageal; ORI, oridonin.

These expressions were reversed with NAC application (Fig. 6c and d).

Discussion

Currently practiced chemotherapy for esophageal squamous cancer has several limitations, therefore, finding new therapeutic options is inevitable. CIS is one of the first-line choices chemotherapeutic drugs for esophageal squamous cell carcinoma (ESCC), which shows the promising effect; however, it is a cell cycle nonspecific drug, has no clear target tissue and poses several adverse effects. On the other hand, ORI, a major diterpenoid

Fig. 5



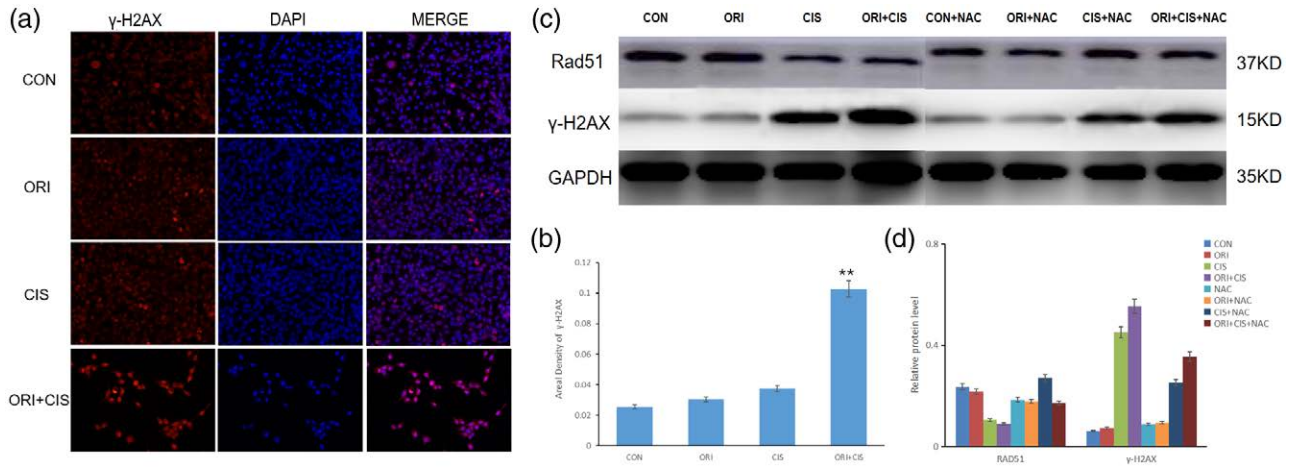
GSH/ROS participates in the synergistic effects of the combined treatment. (a–c) The effect of GSH on reverse of cell viability of CIS and ORI treatment. (a,b) The apoptosis of KYSE30 cells was determined by flow cytometry after staining with annexin V-FITC/PI. (c) The viability of KYSE30 cells when added to GSH. * $P<0.05$, ** $P<0.01$, compared with the group without adding GSH. (d–f) Synergistic effect of CIS and ORI on promoting intracellular reactive oxygen species (ROS) accumulation. (d,e) The generation of intracellular ROS of KYSE30 cells was determined by flow cytometry. * $P<0.05$, compared with the monotherapy group. (f) The ROS level was measured by fluorescence microscope when added with *N*-acetyl-L-cysteine (NAC). (g–i) The effect of NAC on reverse of cell viability of CIS and ORI treatment. (g,h) The apoptosis of KYSE30 cells was determined by flow cytometry after staining with annexin V-FITC/PI. (i) The viability of KYSE30 cells when added to NAC. * $P<0.05$, ** $P<0.01$, compared with the group without adding NAC.. CIS, cisplitin; GSH, glutathione; KYSE, kyoto squamous esophageal; ORI, oridonin.

component of leaf extracts from Rabdosia rubescens, has been demonstrated to be effective to a number of cancers [17], such as esophagus [18], leukemia [19], lung [20], pancreatic [21], prostate [22], breast [23] and colon[24] both *in vivo*/*in vitro*. In the current study, we report for the first time a synergistic antitumor effect of CIS and ORI on human ESCC cells, the main finding is that using CIS plus ORI has a selective synergistically effect to p53-mutant ESCC.

We verified the cytotoxicity of ORI and CIS on ESCC *in vitro*. The results showed that the cytotoxicity of these drugs on mut-p53 cell lines was significantly higher than wt-p53 cell lines, this is consistent with the recent research which showed that cancer cells with p53 mutations might retain residual function in relation to cell death activity and respond better to anticancer therapy than p53 null mutations [25]. However, p53 mutation is part of the cause of CIS resistance [26], and p53 gene mutations occur in more than 80% of ESCC, thus, find a concomitant medication to reduce the toxicity of CIS and improve its efficacy is particularly important. Fortunately, in the current study, CIS plus ORI showed a promising synergistic effect on mut-p53 cell lines when compared with their effect on wt-p53 cell lines.

We have previously reported that ORI selectively affects mut-p53 by decreasing GSH during esophageal cancer treatment. Thus, we hypothesized that GSH/ROS mechanism may be involved in the synergistic effect of CIS plus ORI. GSH content of three wt-p53 esophageal cancer cell lines and three mut-p53 esophageal cancer cell lines was determined. Treatment with ORI plus CIS significantly reduced GSH content of mut-p53 cell lines

Fig. 6



The expression of RAD51 and γH2AX. (a,b) The expression of γH2AX was evaluated by immunofluorescence staining (200×), $^{**}P<0.01$, compared with the monotherapy group. (c,d) The levels of RAD51 and γH2AX were detected by western blot. When add *N*-acetyl-L-cysteine (NAC), the expression of the two proteins were obviously reversed. $^{*}P<0.01$, compared with the group without NAC.

as compared to treatment with CIS or ORI alone. On the other hand, these treatments showed similar effects when applied on wt-p53 cell lines in the aspects of GSH production. These effects were further verified by testing ROS generation.

Our earlier study on GSH production showed that the SLC7A11 gene is a key modulatory gene involved in GSH production [16]. SLC7A11 is in turn regulated by mut-p53 such that mut-p53 protein when combined with Nrf2, can inhibit the expression of SLC7A11, which induces cysteine entry into the cell, and as a result, GSH synthesis is depleted. Therefore, ORI successfully reduced GSH content in the mut-p53 cell line.

CIS is a commonly used metal coordination compound that was approved for the clinical treatment of various cancers. Previously studies indicated that CIS can generate ROS directly or indirectly [27]. Moreover, it strongly induces oxidative stress leading to lipid peroxidation, increased MDA production, reduced GSH activity and DNA damage in acute promyelocytic leukemia cells [28]. Upon CIS administration, the platinum atom binds to endogenous thiols GSH and metallothioneins (MTs) forming a complex that is effluxed from the cell in an ATP-dependent fashion by the glutathione transporter family [29]. Prolonged exposure to CIS leads to overexpression of MT, GSH, and other cellular thiols, leading to cell resistance to CIS [30].

Rad51 is a protein that plays a vital role in DNA double-strand break repair and maintenance of telomeres, which overexpresses in ESCC [31]. Overexpression of Rad51 is correlated with more aggressive tumor behavior and poor prognoses thus can be a predictive factor for the efficacy of neoadjuvant chemoradiotherapy in ESCC [32]. Its overexpression confers resistance to DNA cross-linking agents, such as CIS. Therefore, a combination of chemotherapeutic drugs with CIS can be useful. In this study, we chose ORI as a synergistic agent in combination with CIS as it has been previously reported to suppress the growth of malignant cells. Our findings indicate that the combination of CIS and ORI can synergistically enhance the antitumor activity against KYSE30 cells by inhibiting the expression RAD51.

γH2AX is a hallmark of DNA damage, which has been reported to play a key role in DNA damage [33]. Our results revealed that cells treated with CIS express a higher level of γH2AX, whereas ORI treatment did not alter γH2AX expression. However, the expression of γH2AX was considerably increased in cells treated with the combination of CIS and ORI as compared with the treatment of cells with CIS or ORI alone. To verify this effect, we added NAC, which reversed the expression of γH2AX and RAD51, suggesting that the synergistic effect of CIS and ORI on ESCC might be due to GSH/ROS stimulated DNA damage.

Taken together, these results indicate that the synergistic effect of CIS and ORI is likely to be driven by inhibition of GSH and increased generation of ROS, which ultimately increased apoptosis and DNA damage. According to previous reports, the high content of GSH is easy to cause resistance to CIS, and GSH mitigation induces the sensitivity of CIS. Therefore, we further hypothesize that the combination of the two drugs can reduce CIS resistance and improve its curative effect. It is also reported that ORI can reduce the hepatotoxicity and nephrotoxicity of CIS, therefore, we believe that ORI plus CIS can be a suitable option for the treatment of esophageal cancer.

Conclusion

The results of the present study indicated that ORI and CIS can synergistically inhibit mut-p53 ESCC cell lines proliferation and promote apoptosis through GSH/ROS stimulated DNA damage. Therefore, ORI plus CIS can be a good choice for the treatment of esophageal cancer, especially for esophageal cancer with p53 mutation.

Acknowledgements

The experimental work was conducted at the State Key Laboratory of Esophageal Cancer Prevention & Treatment, Zhengzhou University.

This work was supported by grants from Henan Medical Science and Technology Research Program (SBGJ202002063, SBGJ202002120, SBGJ2018041), and the Key R&D Program of Henan Province (202102310777).

Study design, Q.Z.; data collection, H.Y. and J.W.; data analysis: H.Y., Y.Z., K.Z., and M.G.; data interpretation, H.Y., E.Z.; writing-original draft: H.Y.; writing-review and editing, S.K; administrative support: Q.K., B.L. All authors have read and agreed to the submitted version of the manuscript.

Conflicts of interest

There are no conflicts of interest.

References

1 He F, Wang J, Liu L, Qin X, Wan Z, Li W, *et al*. Esophageal cancer: trends in incidence and mortality in China from 2005 to 2015. *Cancer Med* 2021; **10**:1839–1847.
2 Fan J, Liu Z, Mao X, Tong X, Zhang T, Suo C, *et al*. Global trends in the incidence and mortality of esophageal cancer from 1990 to 2017. *Cancer Med* 2020; **9**:6875–6887.
3 Lambert R, Hainaut P. The multidisciplinary management of gastrointestinal cancer. Epidemiology of oesophagogastric cancer. *Best Pract Res Clin Gastroenterol* 2007; **21**:921–945.
4 Schena M, Battaglia AF, Munoz F. Esophageal cancer developed in a radiated field: can we reduce the risk of a poor prognosis cancer? *J Thorac Dis* 2017; **9**:1767–1771.
5 Xiong Z, He J, Chen XL. New strategies in esophageal carcinoma: promises and problems. *J Thorac Dis* 2016; **8**:E1501–E1504.
6 Liu BR, Ahmed MR, He D, Zhao L, Yang W, Zhang X, *et al*. Endoscopic mediastinal lymph node identification and resection using carbon nanoparticles in a porcine model. *Gastroenterology* 2019; **156**:1250–1252.e1.
7 Abe T, Higaki E, Hosoi T, Nagao T, Bando H, Kadowaki S, *et al*. Long-term outcome of patients with locally advanced clinically unresectable esophageal cancer undergoing conversion surgery after induction chemotherapy with docetaxel plus cisplatin and 5-fluorouracil. *Ann Surg Oncol* 2021; **28**:712–721.
8 Kawakami H, Takeno A, Endo S, Makari Y, Kawada J, Taniguchi H, *et al*. Randomized, open-label phase ii study comparing capecitabine-cisplatin every 3 weeks with S-1-Cisplatin every 5 weeks in chemotherapy-naïve patients with HER2-negative advanced gastric cancer: OGSG1105, HERBIS-4A trial. *Oncologist* 2018; **23**:1411–e147.
9 Wang J, Xu R, Li J, Bai Y, Liu T, Jiao S, *et al*. Randomized multicenter phase III study of a modified docetaxel and cisplatin plus fluorouracil regimen compared with cisplatin and fluorouracil as first-line therapy for advanced or locally recurrent gastric cancer. *Gastric Cancer* 2016; **19**:234–244.
10 Miyoshi T, Uoi M, Omura F, Tsumagari K, Maesaki S, Yokota C. Risk factors for cisplatin-induced nephrotoxicity: a multicenter retrospective study. *Oncology* 2021; **99**:105–113.
11 Owona BA, Schluesener HJ. Molecular insight in the multifunctional effects of oridonin. *Drugs R D* 2015; **15**:233–244.
12 Zhao Y, Xia H. Oridonin elevates sensitivity of ovarian carcinoma cells to cisplatin via suppressing cisplatin-mediated autophagy. *Life Sci* 2019; **233**:116709.
13 Dong X, Liu F, Li M. Inhibition of nuclear factor κB transcription activity drives a synergistic effect of cisplatin and oridonin on HepG2 human hepatocellular carcinoma cells. *Anticancer Drugs* 2016; **27**:286–299.
14 Pi J, Jin H, Jiang J, Yang F, Cai H, Yang P, *et al*. Single molecule force spectroscopy for in-situ probing oridonin inhibited ROS-mediated EGF-EGFR interactions in living KYSE-150 cells. *Pharmacol Res* 2017; **119**:479–489.
15 Yang H, Gao Y, Fan X, Liu X, Peng L, Ci X. Oridonin sensitizes cisplatin-induced apoptosis via AMPK/Akt/mTOR-dependent autophagosome accumulation in A549 cells. *Front Oncol* 2019; **9**:769.
16 Li Y, Li N, Shi J, Ahmed T, Liu H, Guo J, *et al*. Involvement of glutathione depletion in selective cytotoxicity of oridonin to p53-mutant esophageal squamous carcinoma cells. *Front Oncol* 2019; **9**:1525.
17 Dong Y, Zhang T, Li J, Deng H, Song Y, Zhai D, *et al*. Oridonin inhibits tumor growth and metastasis through anti-angiogenesis by blocking the Notch signaling. *PLoS One* 2014; **9**:e113830.
18 Jiang JH, Pi J, Jin H, Cai JY. Oridonin-induced mitochondria-dependent apoptosis in esophageal cancer cells by inhibiting PI3K/AKT/mTOR and Ras/Raf pathways. *J Cell Biochem* 2019; **120**:3736–3746.
19 Zhou GB, Chen SJ, Wang ZY, Chen Z. Back to the future of oridonin: again, compound from medicinal herb shows potent antileukemia efficacies *in vitro* and in vivo. *Cell Res* 2007; **17**:274–276.
20 Xu L, Bi Y, Xu Y, Zhang Z, Xu W, Zhang S, *et al*. Oridonin inhibits the migration and epithelial-to-mesenchymal transition of small cell lung cancer cells by suppressing FAK-ERK1/2 signalling pathway. *J Cell Mol Med* 2020; **24**:4480–4493.
21 Liu QQ, Chen K, Ye Q, Jiang XH, Sun YW. Oridonin inhibits pancreatic cancer cell migration and epithelial-mesenchymal transition by suppressing Wnt/β-catenin signaling pathway. *Cancer Cell Int* 2016; **16**:57.
22 Lu J, Chen X, Qu S, Yao B, Xu Y, Wu J, *et al*. Oridonin induces G2/M cell cycle arrest and apoptosis via the PI3K/Akt signaling pathway in hormone-independent prostate cancer cells. *Oncol Lett* 2017; **13**:2838–2846.
23 Li J, Wu Y, Wang D, Zou L, Fu C, Zhang J, *et al*. Oridonin synergistically enhances the anti-tumor efficacy of doxorubicin against aggressive breast cancer via pro-apoptotic and anti-angiogenic effects. *Pharmacol Res* 2019; **146**:104313.
24 Zhou J, Li Y, Shi X, Hao S, Zhang F, Guo Z, *et al*. Oridonin inhibits tumor angiogenesis and induces vessel normalization in experimental colon cancer. *J Cancer* 2021; **12**:3257–3264.
25 Eichelmann AK, Mayne GC, Chiam K, Due SL, Bastian I, Butz F, *et al*. Mutant p53 mediates sensitivity to cancer treatment agents in oesophageal adenocarcinoma associated with MicroRNA and SLC7A11 expression. *Int J Mol Sci* 2021; **22**:5547.
26 Tonino SH, Mulkens CE, van Laar J, Derks IA, Suo G, Croon-de Boer F, *et al*. Induction of TAp73 by platinum-based compounds to overcome drug resistance in p53 dysfunctional chronic lymphocytic leukemia. *Leuk Lymphoma* 2015; **56**:2439–2447.
27 Lieberthal W, Triaca V, Levine J. Mechanisms of death induced by cisplatin in proximal tubular epithelial cells: apoptosis vs. necrosis. *Am J Physiol* 1996; **270**:F700–F708.
28 Kumar S, Tchounwou PB. Molecular mechanisms of cisplatin cytotoxicity in acute promyelocytic leukemia cells. *Oncotarget* 2015; **6**:40734–40746.
29 Ishikawa T, Ali-Osman F. Glutathione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. *J Biol Chem* 1993; **268**:20116–20125.
30 Hagrman D, Goodisman J, Dabrowiak JC, Souid AK. Kinetic study on the reaction of cisplatin with metallothionein. *Drug Metab Dispos* 2003; **31**:916–923.
31 Chiu WC, Fang PT, Lee YC, Wang YY, Su YH, Hu SC, *et al*. DNA repair protein Rad51 induces tumor growth and metastasis in esophageal squamous cell carcinoma via a p38/Akt-dependent pathway. *Ann Surg Oncol* 2020; **27**:2090–2101.
32 Nakanoko T, Saeki H, Morita M, Nakashima Y, Ando K, Oki E, *et al*. Rad51 expression is a useful predictive factor for the efficacy of neoadjuvant chemoradiotherapy in squamous cell carcinoma of the esophagus. *Ann Surg Oncol* 2014; **21**:597–604.
33 Xu E, Gong Y, Gu J, Jie L, Ajani JA, Wu X. Risk assessment of esophageal adenocarcinoma using γ-H2AX assay. *Cancer Epidemiol Biomarkers Prev* 2013; **22**:1797–1804.