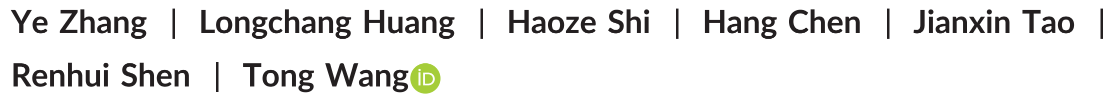
Received: 2 August 2017 | Revised: 9 October 2017 | Accepted: 11 October 2017

DOI: 10.1111/cas.13425

ORIGINAL ARTICLE

WILEY Cancer Science

Ursolic acid enhances the therapeutic effects of oxaliplatin in colorectal cancer by inhibition of drug resistance



Ye Zhang | Longchang Huang | Haoze Shi | Hang Chen | Jianxin Tao | Renhui Shen | Tong Wang

Department of Endoscopy Surgery, Wuxi People's Hospital Affiliated to Nanjing Medical University, Wuxi, China

Correspondence
Tong Wang, Department of Endoscopy Surgery, Wuxi People's Hospital Affiliated to Nanjing Medical University, Wuxi, China.
Email: twang1234@163.com

Funding information
This work was supported by grants from the National Natural Sciences Foundation of China (No. 81371683/H1819), Clinical Medicine Project of Jiangsu Province (BL2014023).

It has been reported that ursolic acid has anti-tumor activity and it enhances the therapeutic effect of oxaliplatin in colorectal cancer (CRC). However, the underlying mechanisms remain unknown. In the present study, the mechanisms of the enhancement of therapeutic effects through use of ursolic acid were investigated. We treated CRC cell lines HCT8 and SW480 with ursolic acid and oxaliplatin and monitored the effects on cell proliferation, apoptosis, reactive oxygen species (ROS) production and drug resistance gene production. We discovered that treatment with a combination of ursolic acid and oxaliplatin resulted in significant inhibition of cell proliferation, significantly increased apoptosis and ROS production, and significant inhibition of drug resistance gene expression. Our study provided evidence that ursolic acid enhances the therapeutic effects of oxaliplatin in colorectal cancer by ROS-mediated inhibition of drug resistance.

KEYWORDS
colorectal cancer, drug resistance, proliferation, reactive oxygen species, ursolic acid

1 | INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer in men and the second most frequently observed cancer in women worldwide.[1,2] It is estimated that the annual number of deaths due to colorectal cancer is more than half a million. CRC accounts for 8% of all cancer deaths, making it the fourth leading cause of death from cancer.[3] In China, it is the fifth most common cancer and also the fifth leading cause of cancer-related deaths. In the year 2012, there were over 150 000 deaths caused by CRC in China and the number is still increasing.[4]

Therapeutic advances have been made in the past few decades. Oxaliplatin is a third generation platinum drug with proven activity against colorectal tumors, having become a standard in the management of this malignancy.[5] It is typically administered together with 5-fluorouracil and folinic acid in a combination known as FOLFOX to treat CRC as palliative or adjuvant chemotherapy.[6] Remarkably, the introduction of oxaliplatin in the year 2000 for the treatment of metastatic colorectal cancer led to an important increase not only in objective response rates, improving the percentage of success of metastasis resection, but also in overall survival (OS). However, a major reason for treatment failure is the existence of tumor intrinsic or acquired resistance to oxaliplatin.[5] For this reason, it is of paramount importance to identify and develop novel drugs that can safely and effectively complement or enhance the therapeutic effects of oxaliplatin. It is also important to elucidate the causes underlying this phenomenon to circumvent it, and to uncover better ways of fighting cancer.

Ursolic acid (3β-hydroxy-urs-12-en-28-oicacid, UA) is a pentacyclic triterpenic acid found in a variety of natural plants, including Chinese medicinal herbs such as *Hedyotic diffusa* and *Prunell avulgaris*.[7] It exhibits a broad range of pharmacological effects, such as anti-inflammatory, antiviral, antioxidant and hepatoprotective effects.[8-10] Ursolic acid has also shown great promise in treating a

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2017 The Authors. *Cancer Science* published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.

number of cancers, such as lung cancer, hepatocellular carcinoma, prostate cancer and breast cancer.[11-14] It is also reported that ursolic acid inhibits the proliferation of certain CRC cell lines,[15] and ursolic acid enhances the therapeutic effects of oxaliplatin in CRC.[16] However, the precise underlying mechanisms remain unknown.

Here we reported that ursolic acid enhanced oxaliplatin-induced inhibition of CRC cell proliferation and induction of apoptosis in CRC cell lines HCT-8 and SW480. The inhibition effect on CRC was also detected in xenograft nude mouse models. The enhanced suppression is correlated to significantly increased reactive oxygen species (ROS) production and decreased expression of drug resistance genes in CRC cells. Thus, our data suggested that ursolic acid enhances the therapeutic effects of oxaliplatin in colorectal cancer by ROS-mediated inhibition of drug resistance.

2 | METHODS AND MATERIALS

2.1 | Cell culture

Human colorectal adenocarcinoma HCT-8 and SW480 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Atlantic Biological, Miami, FL, USA), 2 mmol/L L-glutamine (Gibco, Grand Island, NY, USA) and penicillin/streptomycin (Gibco). The cells were grown in a CO_2 incubator at 37°C.

2.2 | Cell proliferation assay

HCT-8 and SW480 cells were incubated with various concentrations of ursolic acid (final concentrations 10, 20, 30, 40 and 50 μmol/L) or oxaliplatin (final concentrations 0.2, 0.4, 0.6, 0.8 and 1 μmol/L) for 48 hour. Then the treated cells were plated into 96-well plates with 10^4 cell/well. Twenty-four hour later, cells were incubated for an additional 2 hour with CCK-8 reagent (100 μL/mL medium) (Sigma, St. Louis, MO, USA) and the absorbance was read at 450 nm using a microplate reader (BioTek, Vermont, USA). Cell proliferation inhibition rates were calculated according to the following formula: the proliferation inhibition ratio (%) = $1 - [(A1 - A4)/(A2 - A3)] \times 100$, where $A1$ is the optical density (OD) value of the drug experimental group, $A2$ is the OD value of the blank control group, $A3$ is the OD value of the RPMI1640 medium without cells, and $A4$ is the OD value of drugs with the same concentration as $A1$ but without cells. The IC50 (50% inhibitory concentration) value, which represents the concentration of the drug that demonstrates 50% of cell growth inhibition, was calculated by nonlinear regression analysis using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

2.3 | Apoptosis assay

Cells were harvested by trypsinization and then washed twice in ice-cold PBS. Subsequently, cells were resuspended at a density of 1×10^6 cells/mL in 1× binding buffer (Sigma) followed by the addition of 5 μL of annexin V-fluorescein isothiocyanate and 5 μL of propidium iodide (Sigma). Then cells were incubated in the dark at room temperature for 15 minute. Cell death was determined using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and data were analyzed using FlowJo (Flowjo Studio, Carrboro, NC, USA).

2.4 | Assessment of reactive oxygen species production

The oxidative conversion of cell-permeable oxidation of 2′,7′-dichlorodihydrofluorescein diacetate to fluorescent 2′,7′-dichlorodihydrofluorescein was used to assess the generation of intracellular ROS. After treatment with ursolic acid or oxaliplatin alone or combination, HCT8 and SW480 cells were removed from the culture medium and washed with PBS twice. The cells were incubated with 10 μmol/L working solution and then cultured at 37°C for 30 minute. The cells were washed with PBS again, and the fluorescence product was assessed by a fluorescence microscope that was connected to an imaging system (Olympus, Tokyo, Japan). The mean fluorescence intensity, as an index of the amount of ROS, from 5 random fields was measured by IMAGEJ software (Sun Microsystems, Bethesda, MD, USA). The experiment was carried out 3 times.

2.5 | Western blot

A total of 20 μg of protein was loaded onto a 12% SDS-PAGE gel. After transfer, membranes were blocked by 5% non-fat milk and incubated with rabbit anti-caspase-3 (Cell Signaling, Danvers, MA, USA), rabbit anti-NAPDH (Abcam, Cambridge, MA, USA), mouse anti-GAPDH (Santa Cruz, Dallas, TX, USA), rabbit anti permeability glycoprotein (P-gp; Abcam), mouse anti-multidrug resistance-associated protein (MRP) and mouse anti-breast cancer resistance protein (BCRP; Santa Cruz) overnight at 4°C. The next day, corresponding HRP-conjugated secondary antibodies were incubated. After washing, the membranes were finally treated with a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce Biotechnology, Waltham, MA, USA). Similarly, in some experiments, p-JNK (MAPK) was monitored. Mouse anti-p-JNK and mouse anti-JNK were purchased from Santa Cruz.

2.6 | siRNA transfection

siRNA against P-gp, MRP and BCRP were purchased from Origene (Rockville, MD, USA). siRNA was transfected into the cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

2.7 | Quantitative real-time PCR (RT-PCR)

RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The QuatiTect Reverse Transcription Kit (Qiagen) was used to synthesize the subsequent cDNA. Real-time PCR was performed using SYBR Green Master Mix (Qiagen). Samples were normalized to internal control GAPDH. Primer sequences used for RT-PCR are listed below: P-gp forward: 5′-CAGGAACCTGTATTGTT TGCCACCAC-3′ reverse: 5′-TGCTTCTGCCCAC CACTCAACTG-3′.

TABLE 1 Inhibition ratio (mean ± SD)

Ursolic acid (μmol/L)	0	10	20	30	40	50
HCT8 of inhibition rate (%)	6.42 ± 0.30	13.11 ± 2.13	45.6 ± 3.02	76.03 ± 6.6	93.81 ± 3.25	98.2 ± 11.1
SW480 of inhibition rate (%)	5.3 ± 0.12	16.0 ± 2.17	45.05 ± 2.3	67.2 ± 5.13	92.5 ± 6.7	99.2 ± 14.2

Statistical analyses were performed by the SPSS test.

TABLE 2 Inhibition ratio (mean ± SD)

Oxaliplatin (μmol/L)	0	0.2	0.4	0.6	0.8	1.0
HCT8 of inhibition rate (%)	5.12 ± 0.4	21.2 ± 1.11	51.18 ± 6.3	79.65 ± 12.3	97.87 ± 9.13	97.15 ± 5.22
SW480 of inhibition rate (%)	3.21 ± 0.57	15.22 ± 1.9	45.2 ± 10.87	65.07 ± 17.8	90 ± 10.52	98.1 ± 12.12

Statistical analyses were performed by the SPSS test.

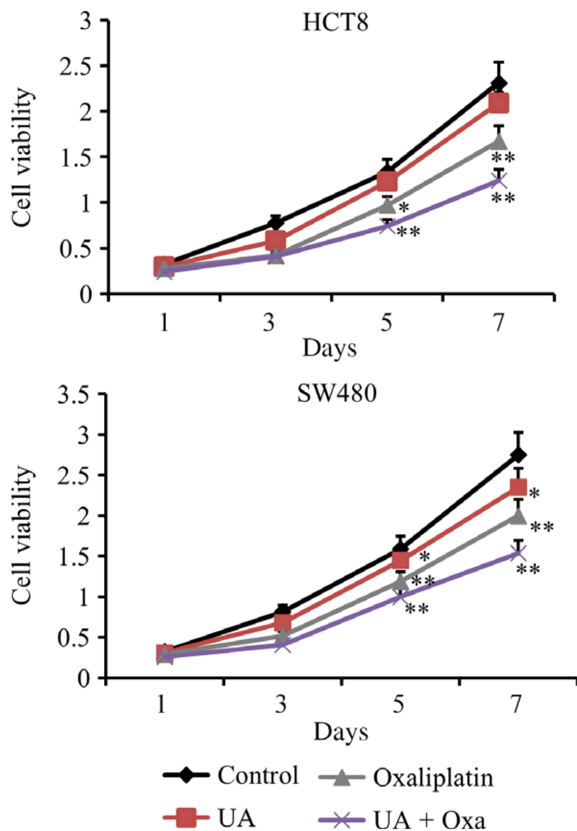


FIGURE 1 Ursolic acid enhanced oxaliplatin-mediated suppression of colorectal cancer cell growth in vitro. After treatment with 20 μmol/L ursolic acid, 0.4 μmol/L oxaliplatin combination (20 μmol/L ursolic acid plus 0.4 μmol/L oxaliplatin) for 48 h, the HCT-8 and SW480 cells were placed in 96-well plates (1×10^4 cells/well) and incubated. Growth curves of ursolic acid and oxaliplatin-treated and non-treated cells were detected using a CCK-8 kit on different days post-treatment (1, 3, 5 and 7 d). Points and range lines represent mean and SD of 3 independent experiments in triplicate. The optical density value was measured at 450 nm and data demonstrated a significant growth decrease by combination of ursolic acid and oxaliplatin (**P < .01, *P < .05)

MRP forward: 5′-ACCCTAATCCCTGCCCAGAG-3′ reverse: 5′-CGCATTCCTTCTTCCAGTTC-3′. BCRP forward: 5′-GGTGGAGGCAAATCTTCGTTATTAGA-3′ reverse: 5′-GAGTGCCCATCACAACATCATCTT-3′. GAPDH forward: 5′-AACGGGAAGCTTGTCATCAATGGAAA-3′ reverse: 5′-GCATCAG CAGAGGGGGCAGAG-3′.

TABLE 3 Inhibition ratio (mean ± SD)

Combination of UA + Oxa (μmol/L)	20 μmol/L Ursolic acid 0.4 μmol/L Oxaliplatin
HCT8 of inhibition rate (%)	67.22 ± 0.77
SW480 of inhibition rate (%)	72.07 ± 0.8

Statistical analyses were performed by the SPSS test.

2.8 | Xenograft nude mouse model

1×10^5 HCT8 or SW480 cells were injected subcutaneously into the right flank area of 6-8-week-old female nude mice. Five days after inoculation, animals were divided randomly into 4 groups with 6 mice per group. Animals in the control group (Group 1) were treated with sterile DMSO via intraperitoneal injection for 5 consecutive days. Animals in Group 2 were given 10 mg/kg ursolic acid intraperitoneally for 5 consecutive days. Animals in Group 3 were given a single dose of 10 mg/kg oxaliplatin via intraperitoneal injection and animal in Group 4 were given 10 mg/kg oxaliplatin combined with 10 mg/kg ursolic acid for 5 consecutive days. Mouse survival was monitored on a daily basis. Tumor growth and mouse body weight were assessed twice weekly. Tumor volume (mm^3) was measured using a caliper and was calculated with the following equation: Tumor volume (mm^3) = (length × width^2) × 0.5. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Wuxi People's Hospital Affiliated to Nanjing Medical University.

2.9 | Statistical analysis

Data were presented as mean ± SD and obtained from at least 3 independent experiments. Statistical analysis was performed by 1 or 2-way ANOVA, with P-values < .05 considered as statistically significant.

3 | RESULTS

3.1 | Ursolic acid enhanced oxaliplatin-mediated suppression of colorectal cancer cells growth in vitro

To identify the cytotoxicity of ursolic acid and oxaliplatin, we first examined the inhibition effect of ursolic acid and oxaliplatin on

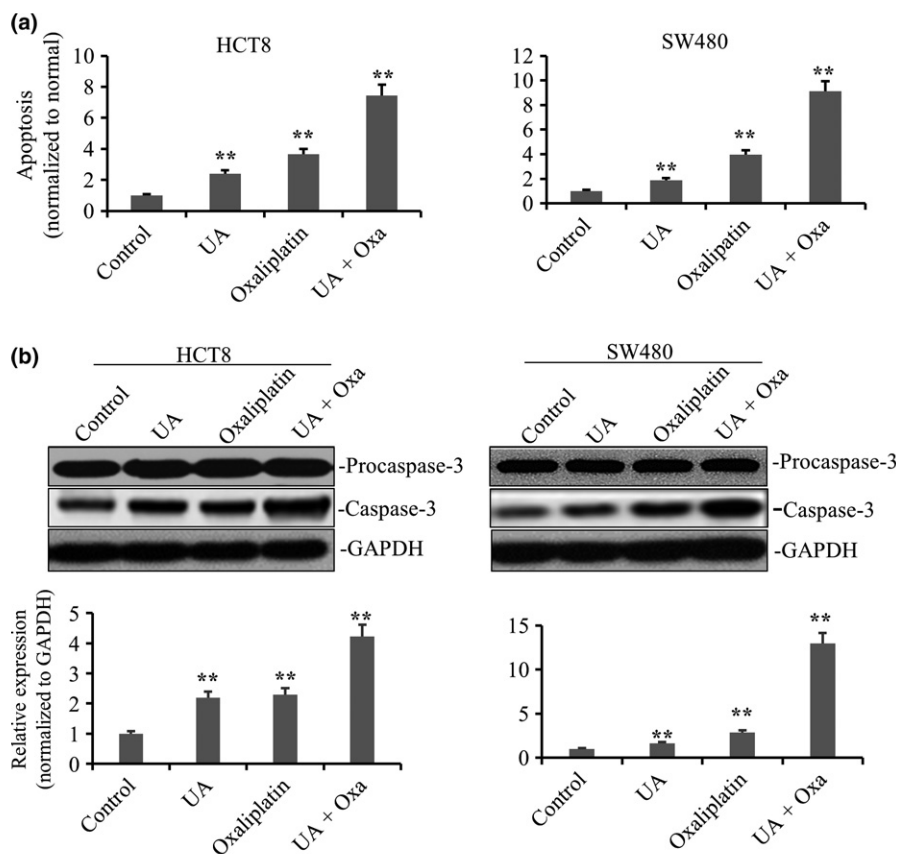


FIGURE 2 Ursolic acid enhanced the apoptosis of colorectal cancer cell lines. A, annexinV/propidium iodide double-staining assay was performed to detect the apoptosis levels of HCT8 and SW480 cells after treatment with 20 μmol/L ursolic acid, 0.4 μmol/L oxaliplatin combination (20 μmol/L ursolic acid plus 0.4 μmol/L oxaliplatin) for 48 h. Relative expression values represent mean and SD from 3 independent experiments (**$P < .01$). The data demonstrated a significant apoptotic increase by combination of ursolic acid and oxaliplatin. B, Procaspase-3 and cleaved caspase-3 expressions of ursolic acid or oxaliplatin-treated and non-treated HCT8 and SW480 cells were analyzed by WB. Relative expression values are presented as means ± SD from 3 independent experiments (**$P < .01$). The data also demonstrated a significant apoptotic increase by combination of ursolic acid and oxaliplatin

proliferation of CRC cell lines HCT8 and SW480. The half minimal inhibitory concentration (IC_{50}) values of ursolic acid in HCT8 and SW480 were around 20 μmol/L (Table 1), while the IC_{50} values of oxaliplatin were 0.4 μmol/L (Table 2) in both cells. Thus, we used these 2 concentrations for the following experiment. After incubation with ursolic acid and oxaliplatin separately or together for 48 hour, the cell proliferation was measured. As shown in Figure 1, ursolic acid or oxaliplatin alone significantly inhibited both HCT8 and SW480 proliferation. The inhibition effects are summarized in Table 3. The combination of ursolic acid and oxaliplatin showed more cytotoxicity to both cell lines when compared to either treatment alone. Thus, our data indicated that ursolic acid enhanced oxaliplatin-mediated inhibition of CRC cell proliferation.

3.2 | Ursolic acid enhanced oxaliplatin induced apoptosis of colorectal cancer cells in vitro

Both ursolic acid and oxaliplatin had been reported to induce apoptosis in cells.[11,17-19] We continued to evaluate the apoptosis of HCT8 and SW480 after treatment with ursolic acid and oxaliplatin separately or together. As shown in Figure 2A, both ursolic acid and oxaliplatin significantly induced apoptosis in HCT8 and SW480 cells. The combination of ursolic acid and oxaliplatin induced significantly more apoptosis when compared to single treatment. Consistent with the apoptosis result, we also identified a significantly increased protein level of cleaved/activated caspase 3, an apoptosis marker/effector, in treated cells (Figure 2B). The combination treatment also resulted in increased protein level of cleaved/activated caspase 3 when compared to single treatment. Thus, our data suggested that ursolic acid enhanced oxaliplatin-induced apoptosis of CRC cells.

3.3 | Ursolic acid enhanced oxaliplatin-induced reactive oxygen species production in colorectal cancer cells

Reactive oxygen species played a role as an important inhibitor of cancer cell proliferation and in induction of apoptosis.[20] As treatment of ursolic acid and oxaliplatin promoted apoptosis and inhibited CRC cell proliferation, we examined the ROS production in treated cells. As shown in Figure 3A, treatment with ursolic acid and oxaliplatin separately or in combination significantly increased ROS production in both HCT8 and SW480 cells. Corresponding to the increased ROS level after treatment, the cells also produced significantly increased NADPH, the resource for ROS production (Figure 3B). This result reflected an increased level of ROS after ursolic acid and oxaliplatin treatment. It has been reported that ROS can activate JNK signaling pathway and promote apoptosis. We also detected the activation JNK signaling pathway in ursolic acid-treated cells (Fig. S1), suggesting the role of JNK signaling pathway in ursolic acid-mediated CRC cell apoptosis. Our data suggested that the enhancement of chemosensitivity by ursolic acid is correlated to the increased ROS level.

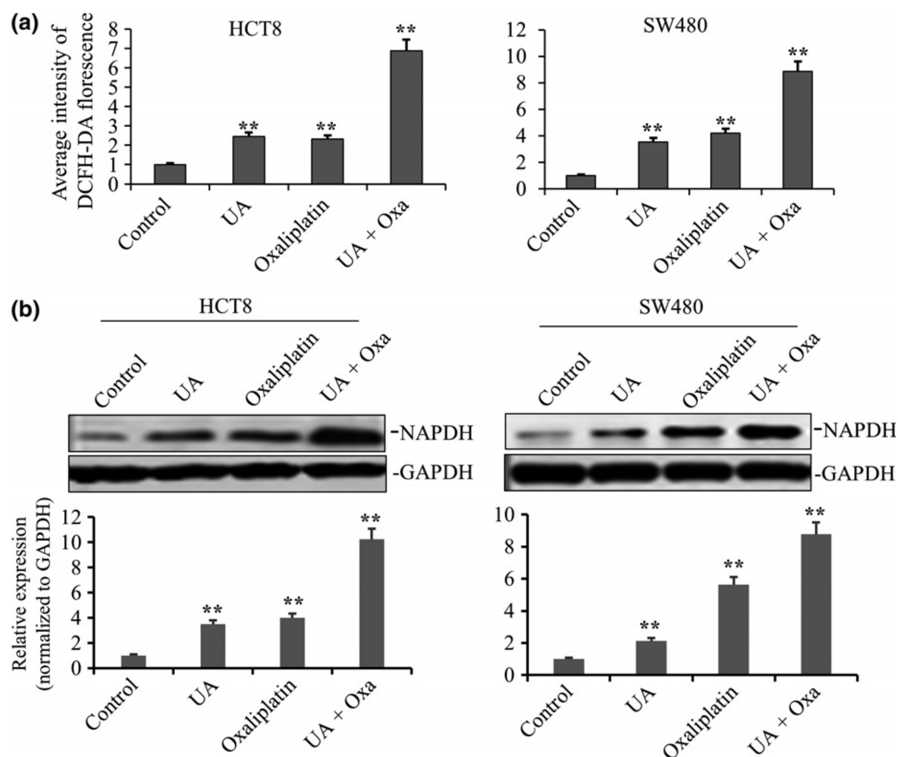


FIGURE 3 Ursolic acid enhanced oxaliplatin-induced reactive oxygen species (ROS) production in colorectal cancer cells. A, The HCT8 and SW480 cells were placed in 6-well plates (1×10^5 cells/well) and incubated with fresh medium as the control group after treatment with 20 μmol/L ursolic acid, 0.4 μmol/L oxaliplatin combination (20 μmol/L Ursolic acid plus 0.4 μmol/L oxaliplatin) for 48 h. ROS level was detected by DCFH-DA. Relative expression values are presented as means ± SD from 3 independent experiments (**$P < .01$). The data demonstrated a significant increase of ROS level by combination of ursolic acid and oxaliplatin. B, NADPH protein expressions were analyzed by WB. Relative expression values are presented as means ± SD from 3 independent experiments (**$P < .01$). The data also demonstrated a significant increase in NADPH level by combination of ursolic acid and oxaliplatin, reflecting an increased level of ROS after treatment

3.4 | Ursolic acid and oxaliplatin-induced suppression of drug-resistance genes

Our data demonstrated that ursolic acid and oxaliplatin combination treatment significantly enhanced the inhibitory effect in cancer cells by increasing ROS production. ROS has been shown to correlate to drug resistance.[21] Therefore, we next tested the effect of treatment on drug-resistant genes, including permeability glycoprotein (P-gp), MRP and BCRP. As shown in Figure 4A,B, we tested both the mRNA level and the protein level of these 3 genes and found that both HCT8 and SW480 cells had significantly decreased mRNA and protein levels in all 3 genes after treatment with ursolic acid or oxaliplatin alone, or in combination. The combination treatment resulted in significantly lower mRNA and protein level of all 3 genes when compared to single treatment. Thus, our data suggested that ursolic acid improved chemosensitivity through suppression of drug resistance.

3.5 | Knocking down drug resistance genes resulted in increased apoptosis in colorectal cancer cells

As the ursolic acid and oxaliplatin treatment resulted in inhibition of drug resistance gene expression, we further explored the effect of drug resistance genes using the siRNA knocking down approach. The siRNA treatment significantly decreased the expression of these 3 genes in both HCT-8 and SW480 cells (Figure 5A). Corresponding to the decreased endogenous level, we detected significantly increased apoptosis in all 3 siRNA-treated HCT-8 and SW480 cells (Figure 5B). Our data suggested the essential role of drug resistance genes in cancer cell survival.

3.6 | Antitumor activity of ursolic acid and oxaliplatin in xenograft mouse model

In view of the above in vitro data, we further tested the effects of this combination strategy in vivo. We established HCT-8 and SW480 xenograft mouse models and mice were randomized to 4 groups with different treatment strategies as described in the Materials and Methods. Noticeably, the mice treated with both ursolic acid and oxaliplatin showed the strongest tumor inhibition, indicated by better survival of animals (Figure 6A) and smaller tumors (Figure 6B). Thus, the anti-tumor effect of ursolic acid and oxaliplatin is not only effective in an in vitro model but also in an in vivo model.

4 | DISCUSSION

Oxaliplatin is a member of the family of platinum-containing chemotherapeutic agents that also includes cisplatin and carboplatin. Oxaliplatin is administered intravenously. Pharmacokinetically, it is characterized by a short initial phase of distribution and a long final phase of drug removal, which mainly takes place in the kidneys, 48 hour after drug administration. Once inside the cell, it binds to nucleophilic molecules, mainly DNA but also RNA and proteins. As a DNA interacting agent, it mainly forms intrastrand adducts between 2 adjacent guanine residues or guanine and adenine disrupting DNA replication and transcription.

Unfortunately, intrinsic or acquired resistance to oxaliplatin-based combinations is still the major cause of treatment failure. The drug efflux transporters have a key role in pumping out of tumor

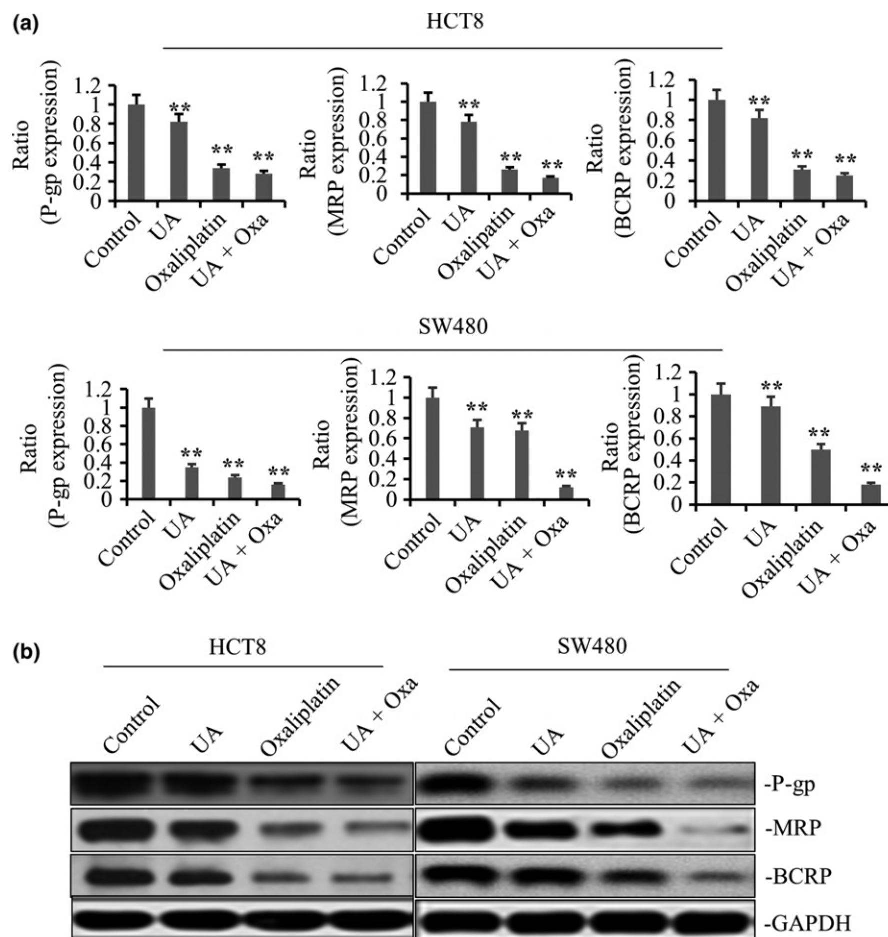


FIGURE 4 Ursolic acid improved chemosensitivity by suppression of the drug resistance of colorectal cancer cell lines. A, The HCT8 and SW480 cells were incubated after treatment with 20 μmol/L ursolic acid, 0.4 μmol/L Oxaliplatin combination (20 μmol/L Ursolic acid plus 0.4 μmol/L Oxaliplatin) for 48 h. mRNA levels of drug resistance associated genes of P-gp, MRP and BCRP were detected by RT-PCR. Relative expression values are presented as means ± SD from 3 independent experiments (**$P < .01$). The data demonstrated a significant decrease of drug resistance genes by combination of ursolic acid and oxaliplatin. B, P-gp, MRP and BCRP protein expression were analyzed by WB. The data demonstrated a significant decrease of drug resistance genes and expressions by combination of ursolic acid and oxaliplatin

cells more than 80% of currently used chemotherapeutic drugs. Specifically, the ABCC subfamily, which comprises the multidrug resistance-associated proteins (MRP), has been shown to be involved in the development of the resistance phenomena associated with platinum drugs. A role of MRP1 and MRP4 has been pointed out in oxaliplatin resistance, as an increased expression and an alteration in N-linked glycosylation of these transporters were associated with a decrease in drug accumulation and an increased oxaliplatin resistance in an ovarian carcinoma in vitro model.[22] The association between oxaliplatin resistance and the MDR1 (P-gp) expression has also been studied. Ekblad and colleagues described an overexpression of MDR1 as a consequence of oxaliplatin resistance acquisition in vitro.[23] In patients with metastatic colorectal cancer undergoing treatment with folic acid, 5-fluorouracil and oxaliplatin (FOLFOX), low BCRP expression was associated with a better response.[24] Thus, it is of paramount importance to identify or develop novel drugs that can enhance the therapeutic effects of oxaliplatin, potentially by targeting drug resistance.

Ursolic acid, 3β-hydroxy-urs-12-en-28-oic-acid, an ursane-type pentacyclic triterpenic acid, belongs to the cyclosqualenoid family and is ubiquitous in the leaves and berries of natural medicinal plants, such as *Arctostaphylos uva-ursi* (L.) *Spreng* (bearberry), *Vaccinium macrocarpon* Ait. (cranberry), *Rhododendron hymenanthes Makino* and in the protective wax-like coatings of apples, pears, prunes and other fruits. Both in vitro and in vivo anticancer effects of ursolic acid have been reported. For example, ursolic acid has been shown to suppress growth and to induce apoptosis in human pancreatic cancer cells.[25] Treatment with ursolic acid (200 mg/kg b.w.) for 6 weeks inhibited the growth of DU145 cells in nude mice without any significant effect on body weight.[26] Shan et al.[16] report that ursolic acid enhances the therapeutic effects of oxaliplantin against CRC both in vitro and in vivo, although the underlying mechanisms are not well elucidated.

In the current study, we first confirmed the anticancer effect of ursolic acid and showed that ursolic acid inhibited proliferation and induced apoptosis in CRC cell lines HCT8 and SW480, and enhanced the cytotoxicity of oxaliplatin. The enhanced inhibition effect was also observed in a mice model. Then, we further elucidated the underlying mechanisms of the inhibitory effect of ursolic acid. We found that the inhibition effect was associated with increased production of ROS. Usually, an increase in ROS is associated with abnormal cancer cell growth and reflects a disruption of redox homeostasis due either to an elevation of ROS production or to a decline of ROS-scavenging capacity, a condition known as oxidative stress.[27] Because the increase of ROS in cancer cells may play an important part in the initiation and progression of cancer, such intrinsic oxidative stress is often viewed as an adverse event. However, as excessive levels of ROS stress can also be toxic to the cells,

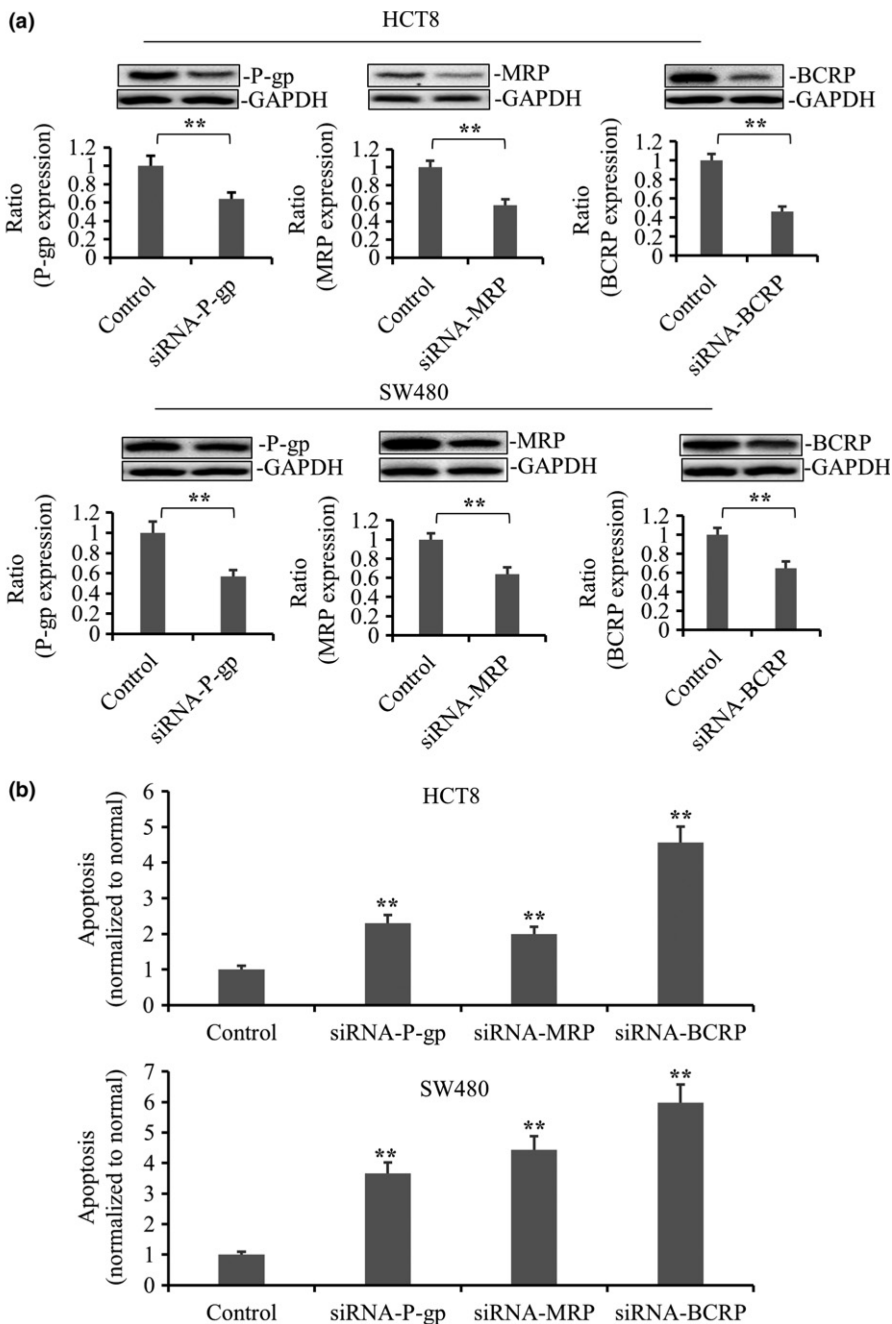


FIGURE 5 Knocking down the drug resistance genes promotes apoptosis. A, Knocking down efficiency of P-gp, MRP and BCRP after siRNA transfection in HCT8 and SW480 cells was detected by real-time PCR and western blotting. Relative expression values are presented as means ± SD from 3 independent experiments (***P* < .01). B, Annexin-V/propidium iodide double-staining assay was performed to detect the apoptosis levels of HCT8 and SW480 cells after the drug resistance genes were knocked down. Relative expression values are presented as means ± SD from 3 independent experiments (***P* < .01)

cancer cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous agents.[28] Thus, ursolic acid enhanced the cytotoxicity of oxaliplatin by promoting the ROS production in CRC cells, resulting in more apoptosis in CRC cells.

The relationship between ROS and cancer drug resistance has been reported.[21,29] It has been shown that ROS could also work as a negative regulator to downregulate drug resistance gene P-gp expression.[30,31] In this study, we demonstrated that the treatment of ursolic acid and oxaliplatin in combination significantly suppressed both mRNA and protein levels of drug resistance genes P-gp, MRP and BCRP, possibly through enhanced production of ROS. Knocking down the endogenous protein level of these 3 proteins by siRNA transfection also resulted in enhanced apoptosis in CRC cell lines, indicating a significant role of drug resistance genes in CRC development. Thus, our data suggested that targeting drug resistance gene expression could be a potential approach to enhance the therapeutic effects of oxaliplatin.

Our study showed that ursolic acid enhanced the therapeutic effects of oxaliplatin. Ursolic acid and oxaliplatin treatment resulted in increased inhibition of proliferation and increased apoptosis in CRC cells. These pheromones were correlated with significantly increased ROS production and decreased expression of drug resistance genes P-gp, MRP and BCRP. Thus, our study demonstrated that ursolic acid enhances the therapeutic effects of oxaliplatin in colorectal cancer by ROS-mediated inhibition of drug resistance,

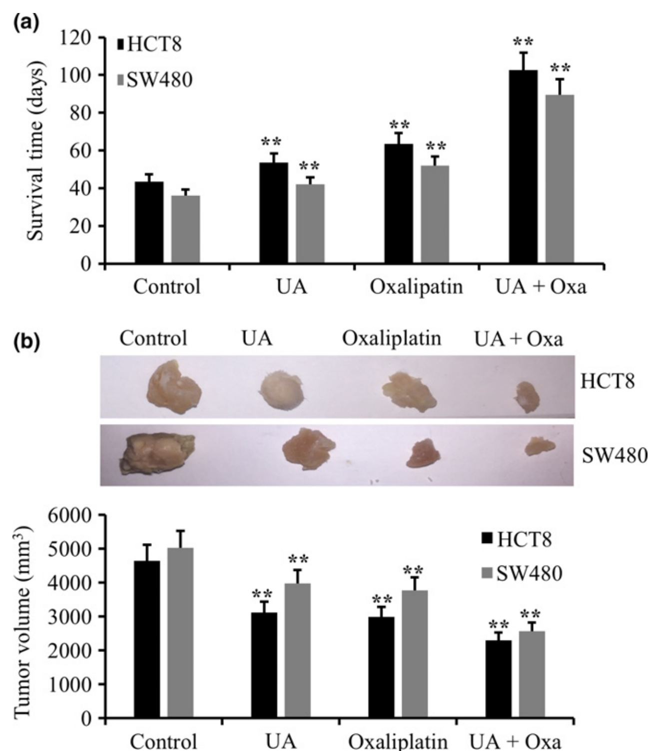


FIGURE 6 Ursolic acid promoted animal survival and suppressed the development of colorectal tumor in vivo. A, The xenograft mouse models were established after injecting 1 × 10^5 colorectal cancer cells with treatment of 10 mg/kg ursolic acid and 10 mg/kg oxaliplatin or a combination of them, respectively. The survival times were calculated after each mouse was dead (n = 6, $^{**}P < .01$). B, Tumor growth in xenografts inoculated with treatment of 10 mg/kg ursolic acid and 10 mg/kg oxaliplatin or combination after 35 d while all the mice were still alive but sick. Data is presented as means ± SD from at least 3 independent experiments in duplicate ($^{**}P < .01$)

suggesting a possible therapeutic effect of ursolic acid in treating cancer. However, currently, ursolic acid itself is used in certain local district. To enable worldwide usage of ursolic acid, more studies need to be followed up to analyze its stability and activity, and biological effect mechanisms.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ORCID

Tong Wang http://orcid.org/0000-0002-2111-7086

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015;136:E359-E386.
2. Demeyer D, Honikel K, De Smet S. The World Cancer Research Fund report 2007: a challenge for the meat processing industry. *Meat Sci*. 2008;80:953-959.
3. Sreevalsan S, Safe S. Reactive oxygen species and colorectal cancer. *Curr Colorectal Cancer Rep*. 2013;9:350-357.
4. Chen W, Zheng R, Zeng H, Zhang S, He J. Annual report on status of cancer in China, 2011. *Chin J Cancer Res*. 2015;27:2-12.
5. Martinez-Balibrea E, Martinez-Cardus A, Gines A, et al. Tumor-related molecular mechanisms of oxaliplatin resistance. *Mol Cancer Ther*. 2015;14:1767-1776.
6. Andre T, Boni C, Navarro M, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol*. 2009;27:3109-3116.
7. Wozniak L, Skapska S, Marszalek K. Ursolic acid–a pentacyclic triterpenoid with a wide spectrum of pharmacological activities. *Molecules*. 2015;20:20614-20641.
8. Saravanan R, Viswanathan P, Pugalendi KV. Protective effect of ursolic acid on ethanol-mediated experimental liver damage in rats. *Life Sci*. 2006;78:713-718.
9. Checker R, Sandur SK, Sharma D, et al. Potent anti-inflammatory activity of ursolic acid, a triterpenoid antioxidant, is mediated through suppression of NF-kappaB, AP-1 and NF-AT. *PLoS ONE*. 2012;7:e31318.
10. Li S, Liao X, Meng F, et al. Therapeutic role of ursolic acid on ameliorating hepatic steatosis and improving metabolic disorders in high-fat diet-induced non-alcoholic fatty liver disease rats. *PLoS ONE*. 2014;9:e86724.
11. Gao N, Cheng S, Budhraja A, et al. Ursolic acid induces apoptosis in human leukaemia cells and exhibits anti-leukaemic activity in nude mice through the PKB pathway. *Br J Pharmacol*. 2012;165:1813-1826.
12. Shanmugam MK, Dai X, Kumar AP, Tan BK, Sethi G, Bishayee A. Ursolic acid in cancer prevention and treatment: molecular targets, pharmacokinetics and clinical studies. *Biochem Pharmacol*. 2013;85:1579-1587.
13. Shishodia S, Majumdar S, Banerjee S, Aggarwal BB. Ursolic acid inhibits nuclear factor-kappa B activation induced by carcinogenic agents through suppression of IkappaBalpha kinase and p65 phosphorylation: correlation with down-regulation of cyclooxygenase 2, matrix metalloproteinase 9, and cyclin D1. *Cancer Res*. 2003;63:4375-4383.
14. Xiang L, Chi T, Tang Q, et al. A pentacyclic triterpene natural product, ursolic acid and its prodrug US597 inhibit targets within cell adhesion pathway and prevent cancer metastasis. *Oncotarget*. 2015;6:9295-9312.
15. Shan JZ, Xuan YY, Zheng S, Dong Q, Zhang SZ. Ursolic acid inhibits proliferation and induces apoptosis of HT-29 colon cancer cells by inhibiting the EGFR/MAPK pathway. *J Zhejiang Univ Sci B*. 2009;10:668-674.
16. Shan J, Xuan Y, Zhang Q, Zhu C, Liu Z, Zhang S. Ursolic acid synergistically enhances the therapeutic effects of oxaliplatin in colorectal cancer. *Protein Cell*. 2016;7:571-585.
17. Manu KA, Kuttan G. Ursolic acid induces apoptosis by activating p53 and caspase-3 gene expressions and suppressing NF-kappaB mediated activation of bcl-2 in B16F-10 melanoma cells. *Int Immunopharmacol*. 2008;8:974-981.
18. Marchetti P, Galla DA, Russo FP, et al. Apoptosis induced by oxaliplatin in human colon cancer HCT15 cell line. *Anticancer Res*. 2004;24:219-226.
19. William-Faltaos S, Rouillard D, Lechat P, Bastian G. Cell cycle arrest and apoptosis induced by oxaliplatin (L-OHP) on four human cancer cell lines. *Anticancer Res*. 2006;26:2093-2099.
20. Jeong JB, Choi J, Baek SJ, Lee SH. Reactive oxygen species mediate tolfenamic acid-induced apoptosis in human colorectal cancer cells. *Arch Biochem Biophys*. 2013;537:168-175.

21. Okon IS, Zou MH. Mitochondrial ROS and cancer drug resistance: implications for therapy. *Pharmacol Res*. 2015;100:170-174.
22. Beretta GL, Benedetti V, Cossa G, et al. Increased levels and defective glycosylation of MRPs in ovarian carcinoma cells resistant to oxaliplatin. *Biochem Pharmacol*. 2010;79:1108-1117.
23. Ekblad L, Kjellstrom J, Johnsson A. Reduced drug accumulation is more important in acquired resistance against oxaliplatin than against cisplatin in isogenic colon cancer cells. *Anticancer Drugs*. 2010;21:523-531.
24. Nakanishi T, Ross DD. Breast cancer resistance protein (BCRP/ABCG2): its role in multidrug resistance and regulation of its gene expression. *Chin J Cancer*. 2012;31:73-99.
25. Prasad S, Yadav VR, Sung B, Gupta SC, Tyagi AK, Aggarwal BB. Ursolic acid inhibits the growth of human pancreatic cancer and enhances the antitumor potential of gemcitabine in an orthotopic mouse model through suppression of the inflammatory microenvironment. *Oncotarget*. 2016;7:13182-13196.
26. Shanmugam MK, Rajendran P, Li F, et al. Ursolic acid inhibits multiple cell survival pathways leading to suppression of growth of prostate cancer xenograft in nude mice. *J Mol Med (Berl)*. 2011;89:713-727.
27. Toyokuni S, Okamoto K, Yodoi J, Hiai H. Persistent oxidative stress in cancer. *FEBS Lett*. 1995;358:1-3.
28. Barrera G. Oxidative stress and lipid peroxidation products in cancer progression and therapy. *ISRN Oncol*. 2012;2012:137289.
29. Dharmaraja AT. Role of reactive oxygen species (ROS) in therapeutics and drug resistance in cancer and bacteria. *J Med Chem*. 2017;60:3221-3240.
30. Wartenberg M, Ling FC, Schallenberg M, et al. Down-regulation of intrinsic P-glycoprotein expression in multicellular prostate tumor spheroids by reactive oxygen species. *J Biol Chem*. 2001;276:17420-17428.
31. Wartenberg M, Hoffmann E, Schwindt H, et al. Reactive oxygen species-linked regulation of the multidrug resistance transporter P-glycoprotein in Nox-1 overexpressing prostate tumor spheroids. *FEBS Lett*. 2005;579:4541-4549.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Zhang Y, Huang L, Shi H, et al. Ursolic acid enhances the therapeutic effects of oxaliplatin in colorectal cancer by inhibition of drug resistance. *Cancer Sci*. 2018;109:94–102. https://doi.org/10.1111/cas.13425