Zhi Zhen Fang formula reverses Hedgehog pathway mediated multidrug resistance in colorectal cancer

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Abstract. Zhi-Zhen-Fang (ZZR), a Traditional Chinese Medicine (TCM) formula, has been clinically used in China to treat drug-resistant colorectal cancer (CRC) patients as an adjuvant. In this study, the efficacy of ZZR in suppressing multidrug resistance (MDR) on CRC was evaluated *in vitro* and *in vivo*. We observed that ZZR enhanced the sensitivity of chemotherapeutic drugs and induced apoptosis in a dose- and time-dependent mannner in CRC MDR cells. Interestingly, signaling of Hedgehog pathway, particularly Gli1, was also inhibited by ZZR. This effect of ZZR in reversing drug resistance and suppressing Gli1 was attenuated by a Hedgehog activator (SAG). Furthermore, ZZR inhibited MDR CRC tumor growth in a xenograft mouse model as well as down-regulated Gli1 levels. This study provided the first direct evidence demonstrating ZZR can attenuate MDR by repressing Hedgehog signaling in human CRC.

Introduction

Multidrug resistance (MDR) is one of the primary problems to efficient cancer therapy (1). Cancer cells may employ several mechanisms of drug resistance such as apoptosis, autophagy, DNA repair, efflux transporters, and epigenetic regulation to acquire multidrug resistance (2,3).

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In 1980s, Hedgehog (Hh) signal transduction pathway research made remarkable progress in fruit fly (4). The Hedgehog signaling pathway regulates cell proliferation, cell fate and patterning, stem and progenitor cell maintenance, as well as self-renewal and tissue repair (5,6). It is now known that Hh signaling has two distinct facets of action mechanisms: the canonical and non-canonical pathways (7,8). The canonical pathway components include ligands (sonic Hh, Indian Hh, and desert Hh), patched receptors (PTCH1, PTCH2), signal transducer smoothened (SMO), and transcription factors (Gli1, Gli2, Gli3) (9). In brief, it is about binding of Hedgehog ligands secreted from one cell and the PTCH receptor on another cell. The non-canonical pathway initiates Hedgehog signaling, via Shh-mediated ERK activation, Wnt signaling results in expression and activation of Gli proteins, and the atypical mutual interaction of core Hh pathway components (10-12).

Traditional Chinese Medicines (TCMs) have been used as medicines, or health supplements in China and in East Asia for millennia. Traditional Chinese prescriptions and formulae, based on TCM principles, have been considered to target multiple pathways, and have been used to treat cancer, such as breast carcinoma (13), gastric cancer (14) and colorectal cancer (15). Similar efficiency as the chemotherapeutic drugs, traditional Chinese prescriptions and formulae are capable of effectively controling cancer progression, improving quality of life and prolonging survival times.

ZZR, a TCM empirical prescription developed in Shanghai General Hospital, which is a recipe derived from a classic TCM principle (nvigorating spleen and detoxification and opening collateral). Some studies illustrated that a Chinese formula, named JPJD, played an important role in liver cancer cure through upregulating expression levels of ABCC2 and downregulating levels of OATP1B2 (16). Additionally, our *in vivo* study demonstrated that intragastric administration of ZZR remarkedly inhibited P-gp expression levels and increased the sensitivity to chemotherapy drugs in CRC MDR cells. Also, the underlying mechanisms of the anti-metastatic effects associated with this formula remain unknown.

Hh signaling pathway can be considered an actionable target for therapeutic intervention in CRC. The present study explored how Hh signaling pathway regulated ABC transporter-mediated MDR and potential mechanisms of ZZR extract by evaluating antitumor effects of this formula both *in vitro* and *in vivo*.

Materials and methods

Preparation of the extracts for ZZR. ZZR extract was prepared according to our previously published method (17), including Huang-Qi (*Radix Astragali*), Nv-Zhen-Zi (*Fructus Ligustri Lucidi*), Yi-yi-ren (*Semen Coicis*), Shi-Jian-Chuan (*Salvia Chinensis*), Ye-Pu-Tao-Teng (*Vitis quinquangularis Rehd*), Teng-Li-Gen (*Actinidia arguta*), and Zhi-Xiang-Fu (*Cyperus rotundus* L.). In brief, the above mentioned 6 herbs were mixed at a ratio of 6:3:6:6:6:6:2 for a total dry weight of 175 g. The herb concoction was immersed in 55% ethanol (1:10 w/v) for 4 h and refluxed for 1.5 h. After filtration, the residue was again refluxed with 55% ethanol (1:8, w/v) for 1 h and filtered. The two decoctions were dried by lyophilization to obtain the ZZR extract with a yield of dried powder of 24.4%. Simultaneous quantification of five major active constituents in the extract was conducted by high-performance liquid chromatography (HPLC). The extract was stored at 4˚C, and its preparations were standardized, regulated and quality controlled according to the guidelines defined by Chinese State Food and Drug Administration (SFDA).

Cell culture and reagents. The human colorectal cancer HCT-116 and HCT-8 parental cell lines were purchased from the Shanghai Cell Collection (Shanghai, China). HCT-116/L-OHP cell line and HCT-8/5-FU cell line were generated and maintained in our laboratory. Cells were maintained in RPMI-1640 containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml) (Invitrogen, Carlsbad, CA, USA) at 37˚C in a 5% CO_2 humidified atmosphere. HCT-116/L-OHP cells were routinely maintained in a medium containing 5,000 ng/ml oxaliplatin (L-OHP), and HCT-8/5-FU cells were in 1,000 ng/ml (5-FU). L-OHP was purchased from Shenzhen Main Luck Pharmaceuticals Co., Ltd. (Shenzhen, China), 5-FU was purchased from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China). Monoclonal antibodies against Ptch1, Gli1, Gli2, and GAPDH were products of Cell Signaling Technology (Beverly, MA, USA). GANT61 and SAG were purchased from Selleck (Houston, TX, USA).

Cell viability asays. Cell proliferation assay was conducted using the cell count kit CCK-8 (Sigma), as previously described (17). Briefly, cells were seeded in 96-well plates at $1x10^4$ cells/well. When the cells reached 60% confluence, the medium was removed and replaced with fresh medium containing varying concentrations of ZZR with or without chemotherapy drugs (L-OHP and 5-FU) and incubated for 48 h. The absorbance was read at 450 nm using a microplate enzyme-linked immunosorbent assay reader (Labsystems Dragon, Wellscan). All experiments were performed with 5 wells per experiment and repeated at least three times. IC_{10}, IC_{20}, IC_{50} (the half maximal inhibitory concentration) was estimated according to the following formula respectively: IC_{50}=lg-1[Xm-i(ΣP-0.5)]; Xm, the maximum concentration; i, logarithm of the concentration; ΣP, sum of growth inhibition rate. IC_{10} and IC_{20} was calculated according to SPSS statistical software.

Apoptosis assay. Cells were plated in 6-well plates ($1x10^6$ cells/well) and after treatment were trypsinized and analyzed using an Annexin V-fluorescein isothiocyanate detection kit (eBioscience Inc., San Diego, CA, USA). Three concentrations of ZZR (obtained from the result of ZZR Cell Viability assays) and L-OHP (10 μM) were added for 24 h. The samples were immediately processed using flow cytometry to detect the relative amount of Annexin V-FITC-positive-PI-negative cells, as previously described (17).

Western blot analysis. Equal protein per lysate was resolved on Tris-glycine gel, transferred onto nitrocellulose membrane, and blocked for 1 h with 5% non-fat dry milk. Membranes were incubated with desired primary antibody: Ptch1, Gli1, and Gli2 with 1:1,000 (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C and then with appropriate secondary antibody as previously reported (18). Equal loading was confirmed with GAPDH (0.1 μg/ml). Densitometric analysis was performed using the Scion Imaging software (Scion Corp.). GAPDH was used as a control for each sample.

Animal experiments. Male athymic nude mice (NCr-nu), 8-12 weeks old, were purchased from Sino-British SIPPR/BK Lab Animal Co., Ltd. (Shanghai, China, license no. SCXK 2010-0012), and maintained under specific-pathogen-free conditions. All animal protocols were approved by the Institutional Animal Use and Care Committee. All the experiments and animal care were approved by Shanghai Medical Experimental Animal Care Commission and in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation.

Mice were subcutaneously injected with $1.0x10^6$ HCT-116/L-OHP cells per animal. When the tumors reach an average size of 100 mm^3, the mice were randomized into 5 groups (n=6 per group) and received intragastric administration of vehicle control, L-OHP and amixture of ZZR and L-OHP. Briefly, L-OHP was given as an intraperitoneal injection every 2 days and the injection dosage (5 mg/kg) was according to half of the maximum tolerated dose (MTD) of oxaliplatin as previously described (19). ZZR was given every day at the doses of 13.27, 26.54 and 53.08 g/kg. In the clinical practice of Chinese herbal medicine, ZZR is usually prescribed at a daily dose of 175 g of herbal materials. When this human dose was converted into an animal dose (a person of 60 kg, and a conversion factor of 9.1 between human and mouse), it was equivalent to the middle dose (26.54 g/kg) used in this study.

The body weight of the animals and the two perpendicular diameters (A and B) of tumor were recorded every 3 days and tumor volume (V) was estimated according to the following formula (17): $V=\pi/6x[(A+B)/2]^3$. The curve of tumor growth was drawn according to tumor volume and time of implantation. Six mice were sacrificed by decapitation in each group on 28th day after treatment.

Immunohistochemical analysis. Hydrated paraffin sections were incubated in a blocking solution (10% donkey

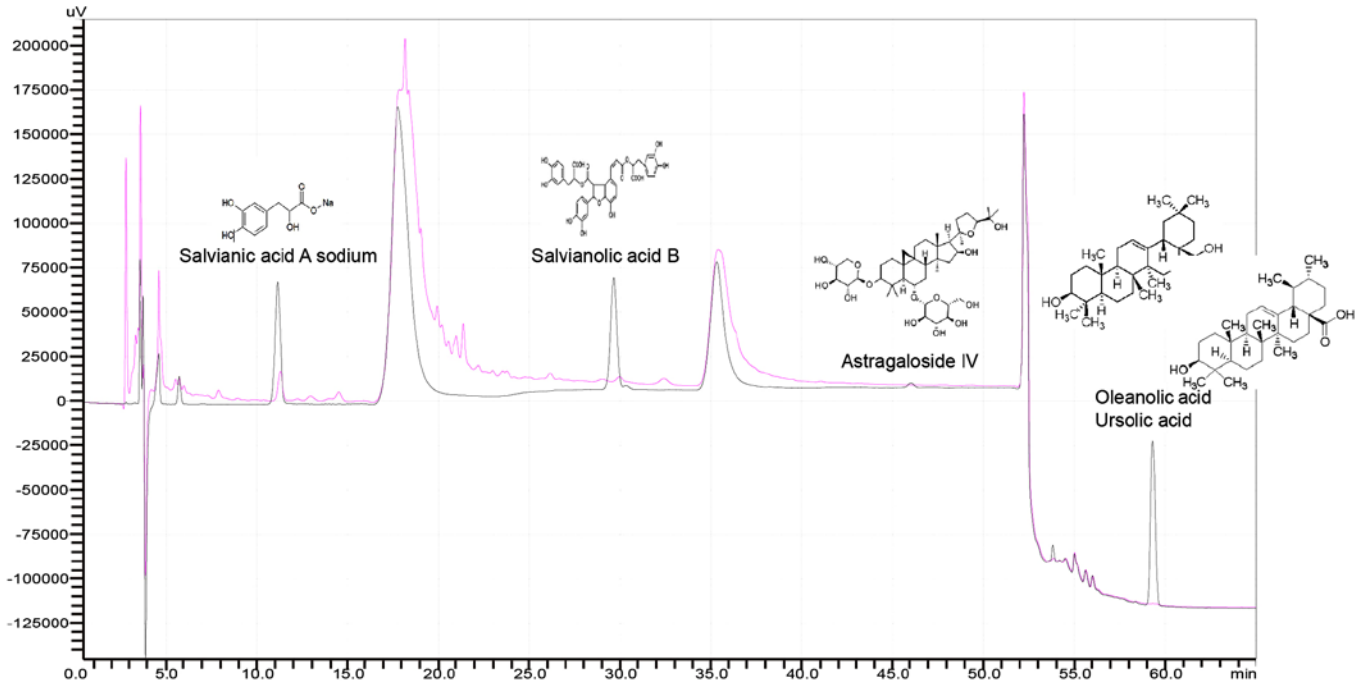

Figure 1. Base peak intensity chromatogram in negative ion modes for ZZR.

serum + 5% non-fat dry milk + 4% BSA + 0.1% Triton X-100) for 10 min, and then incubated at 4˚C overnight with anti-Gli1 (1:500). The analysis was conducted as described previously (19).

Statistical analysis. All quatitative data were expressed as mean ± standard of at least three independent experiments. Statistical analyses were conducted using the Student's t-test. $P<0.05$ or $P<0.01$ were considered statistically significant.

Results

Analysis of active compounds in ZZR. To ensure the quality and stability of ZZR formula, we characterized the active components and their concentration using HPLC. As shown in Fig. 1, compared to standards, the amount of salvianic acid A sodium, salvianolic acid B and astragaloside IV are lower in ZZR ethanol extraction, but higher than that of ZZR water extract (data not shown). Thus, in this study, 55% ethanol was adopted for ZZR extraction. As the ursolic acid and oleanolic acid isomers are inseparable (Fig. 1), salvianic acid A sodium, salvianolic acid B and astragaloside IV were therefore predicted to be the major antitumor components of ZZR solution.

Non-cytotoxic dose of ZZR. To rule out the possibility of cytotoxicity-induced inhibition of cell proliferation, we determined the cytotoxic effect of ZZR on colorectal cancer cells HCT-116 and its MDR derived cells HCT-116/L-OHP. Our data revealed: the doses of IC_{10} and IC_{20} in HCT-116 cells were 103.44 and 212.57 μg/ml, 122.64 and 248.00 μg/ml in HCT-116/L-OHP cells, respectively (Fig. 2A). In any lower doses, little effect was observed on cell survival, thus for the following experiments, cells were treated with ZZR below the dose of 200 μg/ml which was roughly IC_{20}.

ZZR enhances chemo-sensitivity of colorectal cancer cells. To determine if ZZR synergized with chemotherapeutic drugs in treating colorectal cancer cells, HCT-116 and HCT-116/L-OHP cells were treated with ZZR and L-OHP and 5-FU at IC_{50}. As shown in Fig. 2B, ZZR induced significant cytotoxicity in HCT-116/L-OHP cells, decreasing L-OHP IC_{50} from 111.03±20.60 to 20.48±6.19 μM and 16.23±5.31 μM with 100 or 200 μg/ml of ZZR respectively, close to the IC_{50} in the parental cells which was 10.30±1.53 μM. Similarly, the IC_{50} of 5-FU decreased from 165.03±17.97 to 114.65±19.08 μM and 95.03±9.22 μM with 100 or 200 μg/ml of ZZR in a dose-dependent manner (Fig. 2C). These results suggested that ZZR had synergistic effect in combination with chemotherapy in treating human colorectal cancer.

ZZR induces apoptosis without disturbing cell cycle colorectal cancer cells. To explore the mechanisms of antiproliferative effect of ZZR, flow cytometry analysis was performed to determine if apoptosis was induced by L-OHP/ZZR combination. As shown in Fig. 3A, the percentages of apoptotic cells (including the early and late apoptotic cells) induced by 0, 50, 100 and 200 μg/ml of ZZR were 11.41±0.57, 16.01±0.92, 17.45±1.03 and 23.43±0.66%, respectively. This indicates that ZZR augmented L-OHP induced cell apoptosis in a dose-dependent manner. However, the cell cycle analyses obtained from the flow cytometric analysis showed little impact of ZZR (Fig. 3B). These data suggested that the antiproliferative effect of ZZR was mostly via the direct induction of apoptosis.

Effects of ZZR on the Hedgehog pathway in the chemotherapeutic reagent-sensitive, and -insensitive colorectal cancer cells. To further elucidate the action mechanisms of ZZR in MDR colorectal cancer cells, we investigated if ZZR affected

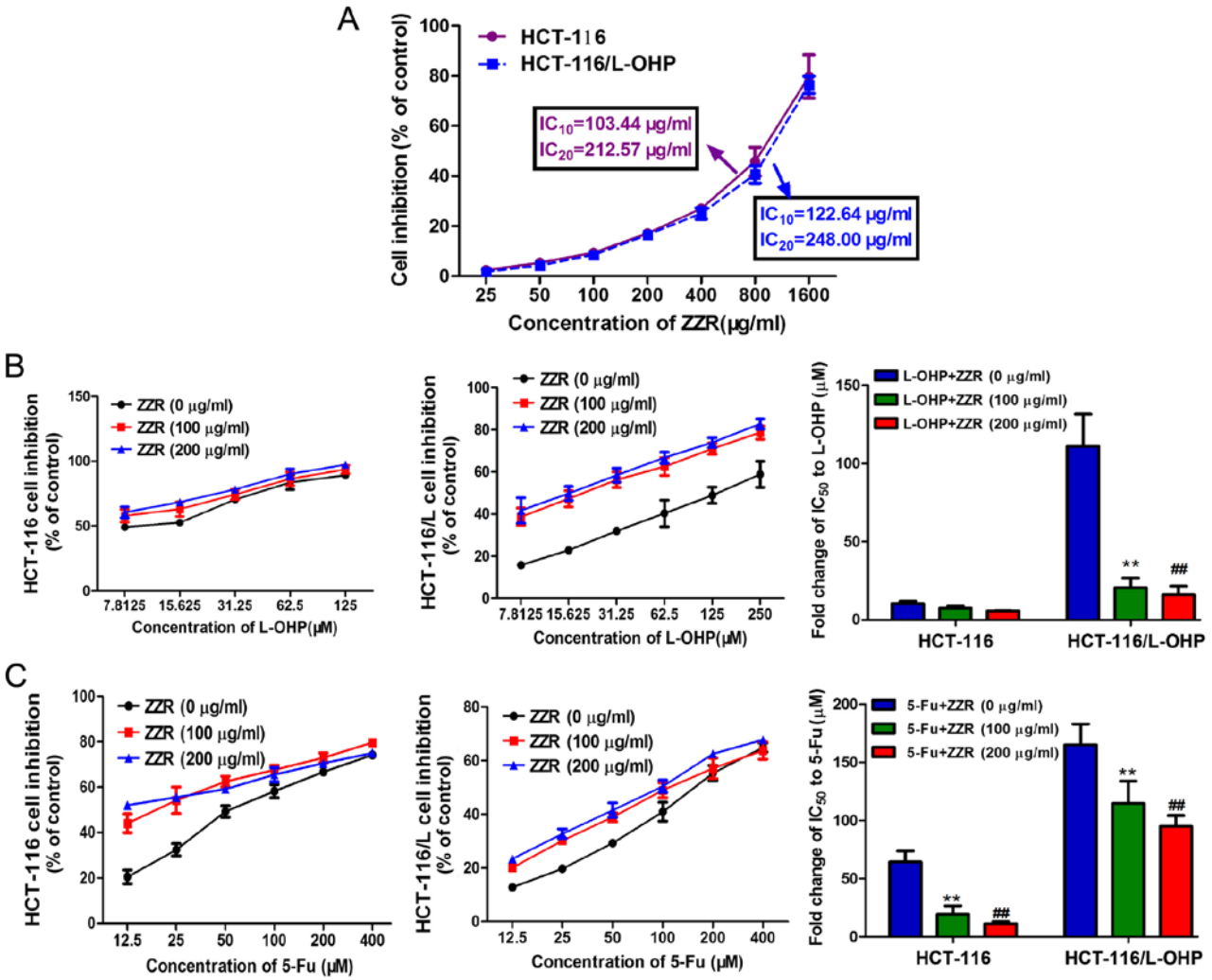

Figure 2. Effect of ZZR on the sensitivity to chemotherapeutic drug in human colorectal cancer cells. (A) Cells were treated with various concentrations of ZZR for 48 h and analysed by CCK-8. (B) IC_{50} values of L-OHP on HCT-116 and HCT-116/L-OHP cells exposed to ZZR at 0, 100 and 200 μg/ml for 48 h. **P<0.01, $^{\#\#}$P<0.01 vs. ZZR (0 μg/ml). (C) IC_{50} values of 5-FU on HCT-116 and HCT-116/L-OHP cells exposed to ZZR at 0, 100 and 200 μg/ml for 48 h. **P<0.01, $^{\#\#}$P<0.01 vs. ZZR (0 μg/ml). All data are presented as mean ± SD of triplicate independent experiments.

Hedgehog signaling pathway, protein levels of Ptch1, Gli1, and Gli2 were interrogated. As shown in Fig. 4A, the Ptch1, Gli1, and Gli2 protein levels were higher in colorectal cancer MDR cells than that of the sensitive cells. Next, we found ZZR attenuated levels of Gli1 protein in HCT-116/L-OHP cells, without affecting Ptch1 and Gli2 (Fig. 4B). The data suggested that ZZR did not alter the upstream signaling of Hedgehog pathway, such as Ptch1, in HCT-116/L-OHP cells. To determine whether the ZZR modulated Hedgehog pathway activation, we used a specific agonist SAG in the colorectal cancer sensitive cells. As expected, Hedgehog pathway was activated by SAG, but the Gli1 protein level was decreased by ZZR in a dose-dependent manner, suggesting the activation of Hedgehog pathway was dampened by ZZR (Fig. 4C).

ZZR synergizes with chemotherapeutic agents through down-regulating Gli1 in vivo. The *in vivo* MDR-antagonizing effect of ZZR was evaluated by using colorectal MDR cancer xenograft mice. As shown in Fig. 5A, ZZR and L-OHP combination treatment resulted in a 69.17% reduction in average tumor volume compared with the controls (1025±95 vs. 3352±285 mm^3). These data indicated that ZZR enhanced the inhibitory effect of L-OHP *in vivo*. Immunohistochemistry analyses confirmed the lowered levels of Gli1 in tumors treated by ZZR (Fig. 5B). These findings suggested that ZZR was a MDR inhibitor repressing the activation of Hedgehog pathway incolorectal cancer (Fig. 6).

Discussion

MDR is one of the main factors for colorectal cancer chemotherapy failure (20-22). To overcome MDR, efforts have been made to thwart drug membrane transporters, but broadly applicable inhibitors are to no avail due to severe side effects of the drugs (23,24). In China, Chinese herbal medicines, often prescribed as concoctions, are commonly used as complementary approaches for the prevention and treatment of colorectal cancer (25). Published reports have indicated that adjunctive TCM therapies display remarkable advantages, including maintaining organ functions, improving life quality, ameliorating symptoms, reducing side effects of chemotherapy or radiotherapy, and extending survival time in patients with

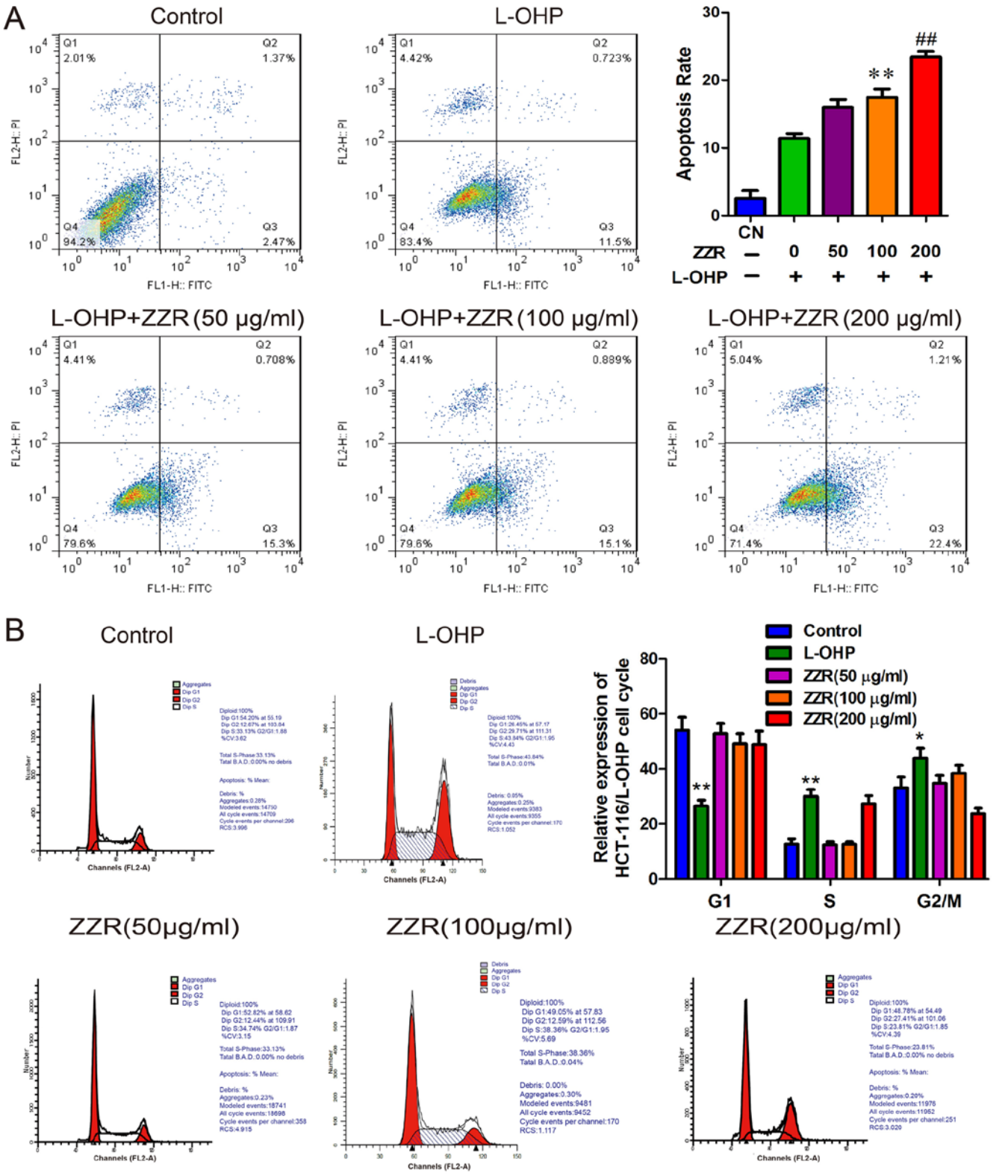

Figure 3. ZZR-induces apoptosis without affecting cell cycle distribution of HCT-116/L-OHP cells. (A) Flow cytometry analysis of cell apoptosis induced by chemotherapeutic agents. Annexin V-FITC/PI binding to HCT-116/L-OHP cells were measured after treatment with L-OHP (10 μM) and L-OHP (10 μM) combination with ZZR at 50, 100 and 200 μg/ml for 48 h. (B) Flow cytometry analysis was used to distinguish cells in different phases of the cell cycle as HCT-116/L-OHP cells were measured after treatment with L-OHP and ZZR at 50, 100 and 200 μg/ml for 48 h. Data are presented as mean ± SD of triplicate independent experiments. **P<0.01, $^{\#\#}$P<0.01.

advanced colorectal cancer (26-28). In our previous study, we found that ZZR, a traditional Chinese herb, has significant anticancer effects in enhancing efficacy of chemotherapeutic drugs. Based on this clinical finding, we aimed to explore the anti-MDR effect of ZZR ethanol extract using *in vitro* and *in vivo* models.

In our study, the herbal components of ZZR extract, including Huang-Qi (*Radix Astragali*), Nv-Zhen-Zi (*Fructus*

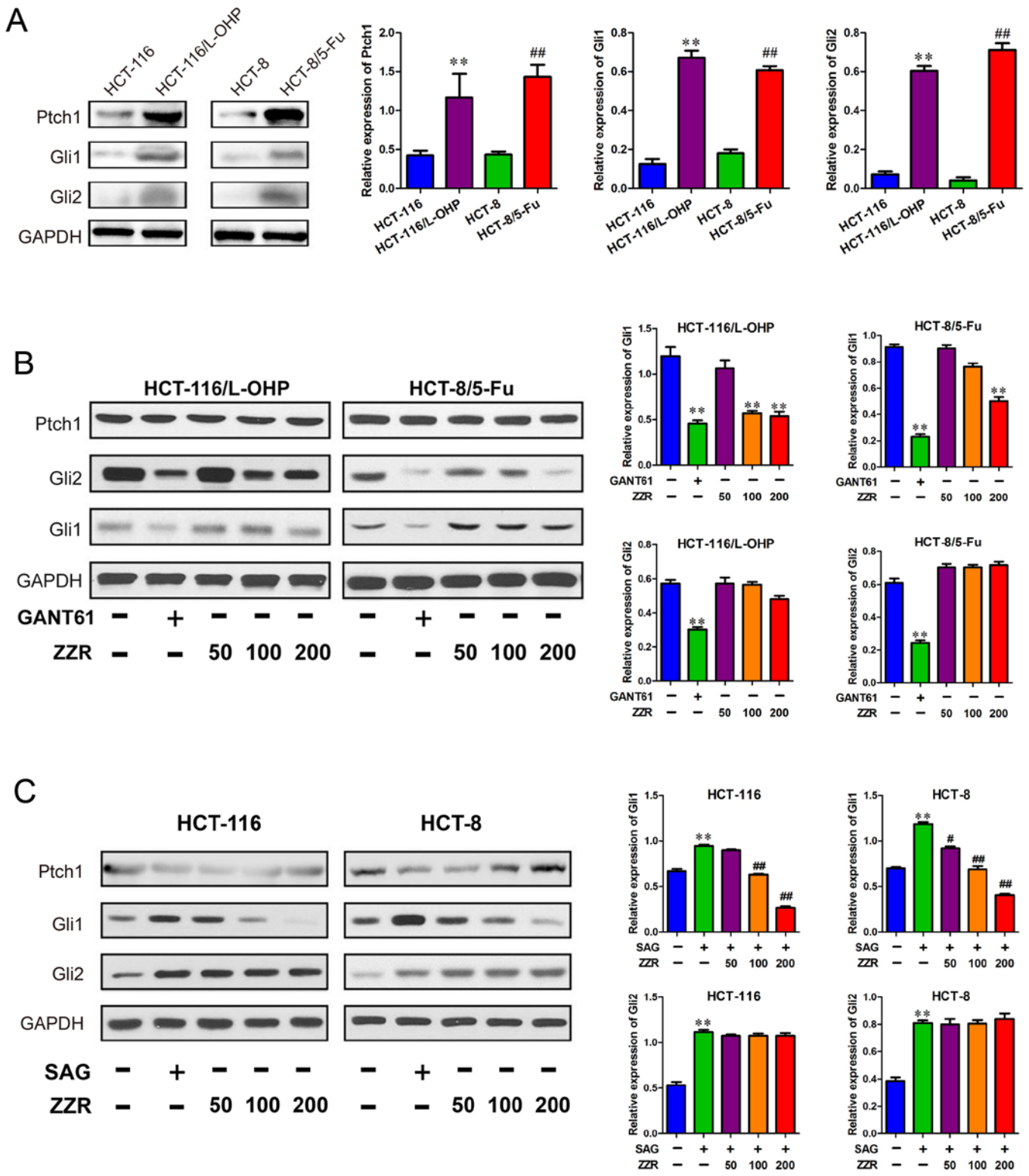

Figure 4. Effects of ZZR on the Hedgehog pathway in sensitive and insensitive colorectal cancer cells. (A) Western blot analysis on the level of Ptch1, Gli1 and Gli2 in sensitive and insensitive colorectal cancer cells. GAPDH was used to ensure equal loading of proteins in each lane. (B) Western blot analysis on the level of Ptch1, Gli1 and Gli2 in colorectal cancer MDR cells treated with ZZR at 50, 100 and 200 μg/ml for 48 h, respectively. GAPDH was used to ensure equal loading of proteins in each lane. (C) Western blot analysis on the level of Ptch1, Gli1 and Gli2 in colorectal cancer cells treated with ZZR at 50, 100 and 200 μg/ml for 48 h, respectively. GAPDH was used to ensure equal loading of proteins in each lane. Data are means ± standard deviation of values from triplicate independent experiments. **P<0.01 vs. control group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. SAG group,

Ligustri Lucidi), Yi-yi-ren (*Semen Coicis*), Shi-Jian-Chuan (*Salvia Chinensis*), Ye-Pu-Tao-Teng (*Vitis quinquangularis Rehd*), Teng-Li-Gen (*Actinidia arguta*), and Zhi-Xiang-Fu (*Cyperus rotundus* L.), were analyzed by HPLC/ESI-MS. To investigate the anti-MDR effects of ZZR on human colorectal cancer, HCT-116 and its MDR counterpart

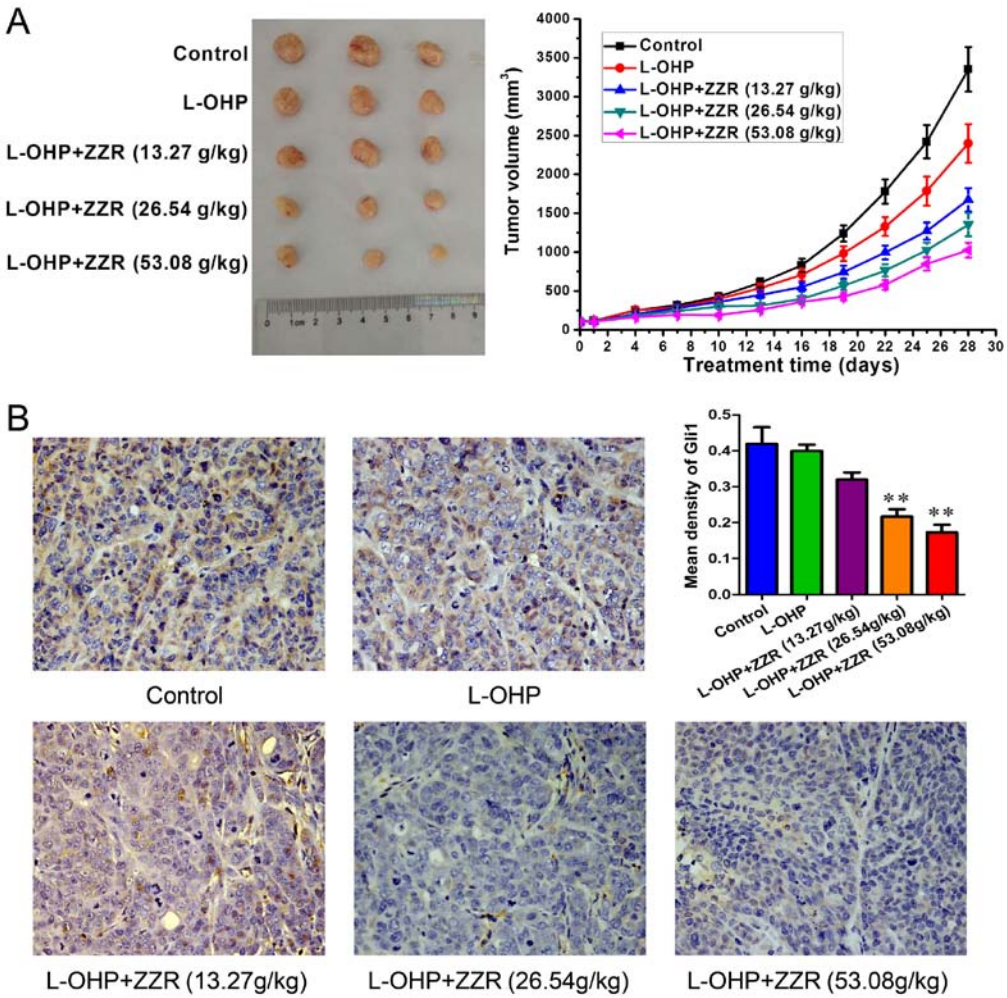

Figure 5. ZZR enhances anticancer efficiency of chemotherapeutic reagent *in vivo*. (A) Tumors removed from nude mice and photographed on the 28th day after administration. Right, tumor volume was quantitated for each sample with ZZR and L-OHP treatment. Data are means ± SEM. (B) Immunohistochemistry for Gli1 on the subcutaneous transplantation tumor samples in mice as described previously. Magnification, x400. Positive cells were stained brown.

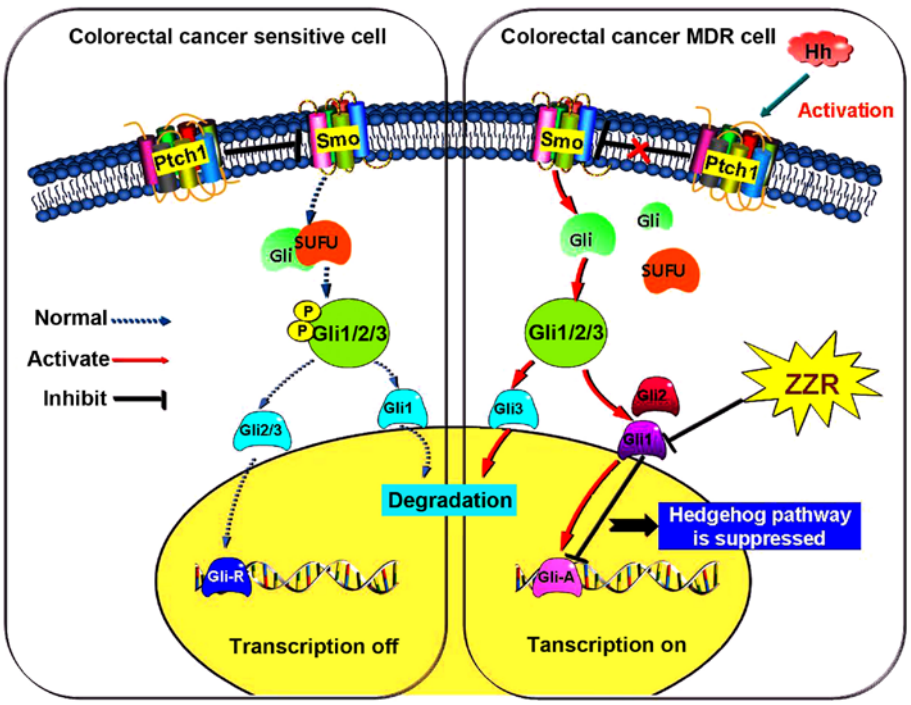

Figure 6. Proposed working model of ZZR on the Hedgehog signaling pathway-mediated MDR in colorectal cancer cells.

HCT-116/L-OHP cells were treated with ZZR, and the non-toxic dose of ZZR was determined. The result suggested that concentrations of ZZR <200 μg/ml are safe in experiments *in vitro*. Furthermore, cell viability assays were performed in HCT-116 and HCT-116/L-OHP cells to determine their relative sensitivity in increasing concentrations of chemotherapy drugs, L-OHP and 5-FU. Data showed that HCT-116/L-OHP cells are much more sensitive to death induced by chemotherapeutics after ZZR treament, when compared with its parental cells.

In our study, we found ZZR synergized with chemotherapeutic drugs to enhance cell apoptosis in a time-dependent manner, consistent with what several previous reports have shown with traditional Chinese prescriptions and formulae (29,30). However, in our study with ZZR, we did not observe any disturbance on cell cycle. One possible explanation is because of the interaction between different herb constituents of this formula which could lead to complicated effects. These findings are in accordance with several reports showing opposite effects for some antitumor drugs, causing apoptosis but not cell cycle alterations (17,31).

Earlier study from our laboratory revealed an association between Hedgehog pathway and P-gp in MDR colorectal cancer cells (32). Here we extended that study by using another two MDR colorectal cancer cell lines, and found ZZR perturbed the expression levels of Ptch1, Gli1 and Gli2. We uncovered that expression levels of Ptch1, Gli1 and Gli2 were higher in resistant HCT-116/L-OHP and HCT-8/5-FU cells than that of their parental sensitive cells. It was reported that Hedgehog signaling pathway was activated in MDR cancer cells (33). In this study, in HCT-116/L-OHP and HCT-8/5-FU cells, we observed elevated protein levels of Ptch1, Gli1, and Gli2, among which ZZR treatment specifically repressed the levels of Gli1.

We next investigated the *in vivo* therapeutic potential of ZZR. Indeed, animal experiment results showed a smilar synergistic anticancer effect of ZZR with chemotherapy drugs on resistant colorectal cancer cell xenografts. Our data further confirmed that ZZR markedly inhibited Gli1 protein level in the xenograft tumors of MDR colorectal cancer cells.

In conclusion, the present study for the first time demonstrated that ZZR suppresssed Gli1 protein levels in colorectal cancer through inhibiting Hedgehog signaling pathway. The traditional Chinese prescription and formula ZZR, used alone or incombination with chemotherapeutic agents, may provide promising approach for the treatment of drug-resistant colorectal cancer.

Acknowledgements

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