Abstract. Previous studies have demonstrated that *Hedyotis diffusa* Willd (HDW), a traditional Chinese herbal medicine, exhibits potent anticancer activity in models of colorectal cancer (CRC). Aggressive forms of CRC exhibit resistance to widely used chemotherapeutic drugs, including the antimetabolite, 5-fluorouracil (5-FU); however, less is known with regard to the activity of HDW against 5-FU-resistant cancer. In the present study, the mechanism of action and the potency of ethanol extracts of HDW (EEHDW) were investigated on a multidrug-resistant CRC HCT-8/5-FU cell line. Using an MTT cell proliferation assay, EEHDW treatment was shown to significantly reduce the cell viability of HCT-8/5-FU cells in a dose- and time-dependent manner. Furthermore, EEHDW significantly increased the retention of the ATP-binding cassette (ABC) transporter substrate, rhodamine-123, as compared with the untreated controls. To further investigate the molecular mechanisms targeted by EEHDW in the resistant cells, the expression levels of the ABC drug transporter protein, P-glycoprotein (P-gp), and ABC subfamily G member 2 (ABCG2), were analyzed using reverse-transcription polymerase chain reaction and western blot analysis. The mRNA and protein expression levels of P-gp and ABCG2 were reduced in the HCT-8/5-FU cells following EEHDW treatment, indicating that EEHDW inhibits ABCG2-mediated drug resistance by downregulating the expression of ABCG2 and P-gp. Therefore, the potential application of EEHDW as a chemotherapeutic adjuvant represents a promising alternative approach to the treatment of drug-resistant CRC.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer in adults worldwide, with over 600,000 mortalities reported annually. Of the 1.2 million individuals diagnosed with CRC, 40-50% of these patients will relapse or succumb due to the limited efficacy of the current adjuvant chemotherapies (1,2). The development of multiple drug resistance (MDR) in tumors is considered to be the major obstacle in the treatment of CRC. Certain members of the ATP-binding cassette (ABC) family of transporters facilitate drug resistance in numerous types of cancer, and recent clinical data correlates the expression of ABC transporters to the risk of relapse in CRC patients (3).

ABC transporters are plasma membrane-associated, energy-dependent efflux pumps that can effectively translocate a variety of substrates across lipid bilayers (4-6). The ABC transporter superfamily is divided into seven distinct subfamilies (A-G), and proteins are assigned to a subfamily based on amino acid sequence similarities and phylogeny (7). MDR transporters within this superfamily include P-glycoprotein (P-gp) and the breast cancer resistance protein ABC subfamily G member 2 (ABCG2) (8). P-gp and ABCG2 play major roles in the ATP-dependent export of chemotherapeutic drugs and contribute to the MDR phenotype observed in CRC, although...
the expression of these transporters may vary depending on the stage of the disease (3,9). For patients with advanced-stage CRC, ABCG2 is a promising therapeutic target and the focus of recent efforts to identify pharmacophores that effectively inhibit its efflux function (10). In the search for nontoxic sources of ABCG2 inhibitors, natural products have been identified as potential drug candidates to reduce the development of MDR in ABCG2-expressing breast cancer cells (11). Thus, in the continued pursuit of potent pharmacophores to treat drug-resistant CRC, the current study investigated the use of medicinal herbs as potential next-generation therapies.

Traditional Chinese medicines (TCM) have been used as medicinal or health supplements in China for thousands of years. In addition, traditional Chinese prescriptions and formulae, which are based on TCM principles, have been demonstrated to be effective in the treatment of breast carcinoma (12), gastric cancer (13) and CRC (14). The reported benefits of applying TCM as an anticancer therapy have included effectively controlling cancer progression, improving the quality of life and prolonging patient survival (15-22).

_Hedyotis diffusa_ Willd. (HDW), also known as _Oldenlandia diffusa_ Willd, of the Rubiaceae family, is a traditional Chinese herbal medicine that is reported to possess anticancer, antioxidative and anti-inflammatory activities, as well as hepatoprotective and neuroprotective effects. In a previous study, an ethanol extract of HDW (EEHDW) was shown to induce apoptosis via the activation of the mitochondrial-dependent pathway in the human colon carcinoma cell line, HT-29 (23). Furthermore, EEHDW has been shown to inhibit CRC growth in vivo and in vitro via the inhibition of the STAT3 signaling pathway (24), as well as suppressing tumor angiogenesis through the inhibition of the Hedgehog signaling pathway (25,26). Due to the prevalence of MDR in CRC, the potency and precise mechanism of action of EEHDW against cancer cells resistant to standard chemotherapeutic agents is largely unclear. Therefore, to further elucidate the mechanism underlying the tumoricidal activity of HDW, the present study analyzed the effect of EEHDW on the 5-fluorouracil resistant CRC cell line, HCT-8/5-FU, and identified the drug resistance transporters that were susceptible to EEHDW treatment.

**Materials and methods**

*Materials and reagents*. RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and TRIzol reagent were obtained from Invitrogen Life Technologies (Grand Island, NY, USA). Primary antibodies against P-gp (#13342) and ABCG2 (#4477), and a horseradish peroxidase (HRP)-conjugated secondary antibody (#4967) were provided by Cell Signaling Technology, Inc. (Beverly, MA, USA). The BCA Protein Assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and the PrimeScript RT reagent kit was provided by Takara Biotechnology Co., Ltd. (Dalian, China). All the other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

*Ethanol extraction from HDW and drug formulation*. Authentic plant material was purchased from a commercial supplier (Guo Yi Tang Chinese Herbal Medicines, Fuzhou, China), and the EEHDW was prepared as previously described (21). Stock solutions of EEHDW were prepared by dissolving the EEHDW powder in 40% dimethyl sulfoxide (DMSO) to reach a final concentration of 400 mg/ml. The stock solutions were stored at -20°C. Working concentrations of EEHDW were produced by diluting the stock solution in the culture medium. The final concentration of DMSO in the medium was <0.5%.

*Cell culture*. Human colon carcinoma HCT-8 cells and 5-FU-resistant HCT-8/5-FU cells were purchased from Nanjing KeyGen Biotech. Co. Ltd. (Nanjing, China). Cells were grown in RPMI 1640 medium, supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C in a humidified chamber with 5% CO₂. The HCT-8/5-FU cells were cultured in the supplemented media with the addition of 15 µg/ml 5-FU.

*Evaluation of cell viability using an MTT assay*. Cell viability was determined using an MTT colorimetric assay. Briefly, HCT-8 and HCT-8/5-FU cells were seeded into 96-well plates at a density of 1.0x10⁴ cells/well in 0.1 ml media. After 24 h, the cells were treated with various concentrations of 5-FU or EEHDW for indicated periods of time. Treatment with 0.1% DMSO was included as the vehicle control. At the end of the treatment, 100 µl MTT (0.5 mg/ml) in phosphate-buffered saline (PBS) was added to each well, and the samples were incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 µl DMSO and absorbance was measured at 570 nm using an ELISA reader (EXL800; BioTek Instruments, Inc., Winooski, VT, USA). The resistance index (RI) of the HCT-8/5-FU cells to 5-FU was calculated by dividing the drug concentration required to inhibit growth by 50% (IC₅₀) for HCT-8/5-FU cells by the IC₅₀ value for the parental cells (HCT-8). IC₅₀ values were determined using nonlinear regression analysis.

*Cellular morphology*. HCT-8/5-FU cells were seeded into six-well plates at a density of 2.5x10⁵ cells/well in 2 ml media. The cells were treated with various concentrations of EEHDW for 24 h. Cell morphology was observed with a phase-contrast microscope (Leica Camera AG, Wetzlar, Germany), and images were photographed at a magnification of x200.

*Rhodamine-123 (Rh-123) exclusion assay*. Retention of a P-gp transporter substrate and the subsequent reversal of the drug resistance phenotype were investigated using a Rh-123 exclusion assay. HCT-8/5-FU cells were treated with different concentrations of EEHDW for 24 h. The cells were collected, and a total of 10⁴ cells/ml were incubated with 5 µl Rh-123 (1 mM in PBS) at 37°C for 10 min. The cells were washed twice with chilled PBS and resuspended in 0.5 ml PBS, followed by incubation for an additional 30 min at 37°C. Fluorescence intensity was measured at 488 nm to determine the intracellular content of Rh-123 and quantitated using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

*RNA extraction and reverse transcription polymerase chain reaction (PCR) analysis*. HCT-8/5-FU cells (4x10⁵) were
seeded into six-well plates in 2 ml culture media and treated with the indicated concentrations of EEHDW for 24 h. Total RNA from the HCT-8/5-FU cells was isolated using TRIzol reagent (Invitrogen). Oligo(dT)-primed RNA (1 µg) was reverse-transcribed using the PrimeScript RT reagent kit, according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA expression levels of P-gp and ABCG2 by RT-PCR. GAPDH was used as an internal control. The RT-PCR conditions for 30 cycles were performed as follows: Denaturation at 94°C for 40 sec, annealing for 40 sec and extension at 72°C for 45 sec. The primers used for the amplification of ABCG2, P-gp and GAPDH transcripts were as follows: ABCG2 forward, 5'-GCC GTG GAA CTG TTT GTG GTA-3' and reverse, 5'-ACAGCAAGATGTCAATGTTGTG TAGGTAG-3'; P-gp forward, 5'-TGACATTATTTCAAAGTGTAAAGCA-3' and reverse, 5'-TAGACACTTATGCAAACATTTCACA-3'; and GAPDH forward, 5'-GTCATCCATGACAAC TTTGG-3' and reverse, 5'-GACCTTGACAAAGTGTTGCTG-3'.

Western blot analysis. HCT-8/5-FU cells were seeded into 25 cm² flasks at a density of 1.0x10⁶ cells/flask in 5 ml media. The HCT-8/5-FU cells were treated with the various concentrations of EEHDW for 24 h, and lysed with mammalian cell lysis buffer containing different protease inhibitors. The total protein concentration in each sample was determined using a bicinchoninic acid assay. Equal amounts of protein from each cell lysate were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 2 h with 5% nonfat dry milk and the membrane was incubated with primary monoclonal rabbit anti-human antibodies against P-gp (1:1,000), ABCG2 (1:1,000) and β-actin (1:1,000) at 4°C overnight. Then, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5,000) at room temperature. Blots were visualized using an electrochemiluminescence western blotting kit and images were captured and analyzed using ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation for the indicated number of independently performed experiments. The data were analyzed using the SPSS package for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA). Statistical analyses were performed using the Student's t-test and analysis of variance, where P<0.05 was considered to indicate a statistically significant difference.

Results

Resistance of HCT-8/5-FU cells to 5-FU treatment. To verify the 5-FU resistance profiles of the CRC cell lines used in the study, HCT-8 and HCT-8/5-FU cells were exposed to different concentrations of 5-FU for 48 h, and the cell viability was measured using an MTT assay. The viability of the HCT-8 cells was significantly decreased following treatment with 5-FU, whereas the viability of the HCT-8/5-FU cells did not significantly change compared with the parental cells (Fig. 1A and B). The IC₅₀ values of 5-FU in the HCT-8 and HCT-8/5-FU cell lines were 145.16 µM and 4.2668 mM, respectively. In addition, the RI for 5-FU was 29.39. These results support previously obtained data, demonstrating the resistance properties of HCT-8/5-FU cells to 5-FU treatment (27).

EEHDW inhibits the growth of HCT-8/5-FU cells. In order to evaluate the effect of EEHDW on the HCT-8/5-FU cell...
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The viability of the treated cells was determined by an MTT assay. Treatment with 0.5-2 mg/ml EEHDW for 24 or 48 h reduced the cell viability by 13.78-66.61% or 18.02-83.76%, respectively, when compared with the untreated control cells (P<0.05; Fig. 2). Furthermore, the cell viability was reduced to 33.38 and 16.23% at the highest concentration of EEHDW (2 mg/ml) after 24 or 48 h of treatment, respectively. These results indicated that EEHDW inhibited HCT-8/5-FU cell viability in a concentration- and time-dependent manner.

To determine the effects of EEHDW on cell morphology, the appearance of the treated versus untreated HCT-8/5-FU monolayers were compared by phase-contrast microscopy. Untreated HCT-8/5-FU cells appeared as a crowded and disorganized monolayer after 24 h (Fig. 3). However, the cell density was reduced in the confluent monolayers that had been treated with EEHDW, with the attached cells exhibiting a round appearance and condensed nuclei. Therefore, these observations demonstrated that EEHDW inhibited the growth of HCT-8/5-FU cells.

Figure 3. Effect of EEHDW on HCT-8/5-FU cell morphology. HCT-8/5-FU cells were treated with the indicated concentrations of EEHDW for 24 h, and morphological changes were observed using phase-contrast microscopy (magnification, x200). Images are representative of three independent experiments. 5-FU, 5-fluorouracil; EEHDW, ethanol extract of *Hedyotis diffusa* Willd.

Rh-123 accumulation in EEHDW-treated HCT-8/5-FU cells. Rh-123 is a substrate for the ABC transporter, P-gp, and its accumulation and efflux are predictive of the levels of P-gp in the cells exhibiting the MDR-associated phenotype (28,29).

The x-axis represents the fluorescent intensity of Rh-123 in the HCT-8/5-FU cells. The y-axis represents the mean fluorescence intensity of Rh-123 in the cell population. (B) Statistical analysis of the intracellular fluorescence intensities of Rh-123 in the various EEHDW treatment groups. Data are representative of at least three independent experiments, and are presented as the mean ± standard deviation. *P<0.05, vs. untreated control cells. 5-FU, 5-fluorouracil; EEHDW, ethanol extract of *Hedyotis diffusa* Willd; Rh-123, rhodamine-123; FITC, fluorescein isothiocyanate.

Figure 4. Effect of EEHDW on intracellular Rh-123 accumulation in HCT-8/5-FU cells. Flow cytometry was used to detect intracellular Rh-123 accumulation in HCT-8/5-FU cells that had been treated with the indicated concentrations of EEHDW for 24 h. (A) Mean fluorescence intensity of Rh-123 in the HCT-8/5-FU cells. The x-axis represents the fluorescent intensity of Rh-123. (B) Statistical analysis of the intracellular fluorescence intensities of Rh-123 in the various EEHDW treatment groups. Data are representative of at least three independent experiments, and are presented as the mean ± standard deviation. *P<0.05, vs. untreated control cells. 5-FU, 5-fluorouracil; EEHDW, ethanol extract of *Hedyotis diffusa* Willd; Rh-123, rhodamine-123; FITC, fluorescein isothiocyanate.
To determine whether EEHDW affects the activity of P-gp transporters, the intracellular accumulation of Rh-123 was measured in EEHDW-treated HCT-8/5-FU cells. Compared with the untreated controls, there was a significant increase in the accumulation of intracellular Rh-123 in the HCT-8/5-FU cells treated with EEHDW for 24 h (Fig. 4). These results indicated that EEHDW may inhibit the efflux activity of P-gp.

**EEHDW inhibits the expression of P-gp and ABCG2 in HCT-8/5-FU cells.** To further investigate which drug resistance mechanisms are targeted by EEHDW, the mRNA and protein expression levels of P-gp and ABCG2 in EEHDW-treated HCT-8/5-FU cells were analyzed. EEHDW treatment was shown to significantly reduce the mRNA and protein expression levels of P-gp and ABCG2 in the HCT-8/5-FU cells in a concentration-dependent manner (Fig. 5A and B). These observations indicated that EEHDW may modulate the MDR phenotype in HCT-8/5-FU cells via the regulation of P-gp and ABCG2 expression.

**Discussion**

Currently, the standard treatment regimens for CRC are based on combining various chemotherapeutic agents, including 5-FU/leucovorin calcium, capecitabine, irinotecan, oxaliplatin, bevacizumab, cetuximab and panitumumab (30). However, the development of drug resistance during carcinogenesis is a critical problem that has decreased the number of CRC patients who remain in long-term remission. Increasing evidence supports the hypothesis that ABCG2 plays an important role in cancer drug resistance (31-33); thus, the modulation of ABCG2 may be regarded as a therapeutic approach to overcome drug resistance. Numerous studies have attempted to develop reversing agents that target ABC transporters (34,35); however, poor solubility and reduced oral bioavailability have limited the clinical application of these agents, including the P-gp/ABCG2 dual inhibitor, Elacridar (36). Faced with limited methods to reduce the side-effects and lower the risk of developing drug-resistant CRC, the use of alternative remedies, such as traditional medicines and herbs, is gaining support among patients and clinicians alike (37).

TCM is regarded as an ideal source of multidrug reversal agents for the treatment of cancer, due in large to the high bioavailability and low toxicity of the prescribed compounds. Compared with modern medicine, the combination of several herbs using traditional methods may enhance the therapeutic outcomes in cancer treatment by targeting multiple molecular pathways simultaneously. HWD, a traditional Chinese herbal medicine, has been shown to exhibit potent antitumor effects (23-26), although the mechanism of action underlying the ability of EEHDW to overcome drug resistance remains unknown.

In conclusion, the results of the present study demonstrated that EEHDW inhibits P-gp- and ABCG2-mediated drug resistance by downregulating the expression of ABCG2 and inhibiting the efflux activity of P-gp in HCT-8/5-FU cells. Therefore, the observations support further research investigating EEHDW as a potent inhibitor of drug-resistant CRC.
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