Icaritin reverses multidrug resistance of HepG2/ADR human hepatoma cells via downregulation of MDR1 and P-glycoprotein expression

LI SUN[1], WEIGANG CHEN[1], LILI QU[1], JIE WU[2] and JIN SI[1]

[1]Department of Laboratory Medicine, The Second Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210011; [2]Department of Laboratory Medicine, Hospital of Jianye District, Nanjing, Jiangsu 210000, P.R. China

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Abstract. Multidrug resistance (MDR) of tumor cells is a serious obstacle encountered in cancer treatment. In the current study a multiple drug-resistant HepG2/adriamycin (HepG2/ADR) cell line was established and its MDR was characterized. Icaritin, an active ingredient isolated from the medical plant *Herba Epimedium*, was observed to reverse MDR in the present model. Icaritin significantly increased the intracellular accumulation of ADR and decreased the expression of the MDR1 gene in HepG2/ADR cells compared with drug-sensitive HepG2 cells. In addition, the present results showed that icaritin may significantly downregulate the expression of P-glycoprotein. These results indicate that icaritin is a novel and potent MDR reversal agent and may be a promising drug for tumor chemotherapy.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of malignant tumor worldwide and the third greatest cause of cancer-related mortality (1-4). HCC is one of the most aggressive human malignancies and previous data showed that the five-year survival rate remained extremely poor (5). HCC is a hypervascular solid cancer characterized by a high degree of drug resistance (6). Multidrug resistance (MDR) against chemotherapeutic agents is key in the failure of HCC therapy (7).

MDR protects cancer cells against a variety of drugs with different structures and functions. MDR of cancer cells is a predominant problem in cancer chemotherapy. A number of mechanisms have been established for the development of MDR, including expression changes of genes involved in apoptosis, increased enzyme activity of the glutathione-mediated detoxification pathways and activation of adenosine triphosphate (ATP) binding cassette transporters (8,9). The classical mechanism of MDR development involves the overexpression of P-glycoprotein (P-gp), a plasma membrane transporter extrudes chemotherapeutic agents from tumor cells (10-12). Significant effort has been aimed at the development of MDR reversers. A significant number of compounds have been identified to function as inhibitors of P-gp and have been tested for their reversal activities on MDR tumor cells (13-16). However, a number of these compounds are currently under clinical evaluation as the majority of tested compounds exhibit unpredictable pharmacokinetic interactions, toxicity or insufficient efficacy (17). Novel therapeutic strategies, including agents that are effective, safe and exhibit low toxicity are required to reverse the MDR of tumor cells.

Flavonoids, a type of plant polyphenol, are well established for analgesic, physiological antipyretic and anti-inflammatory activities and have attracted significant attention due to their antitumor activities and ability to bind with P-gp (18-21). Icaritin, a prenylflavonoid, is a hydrolytic product of icariin, which is derived from the plant *Herba Epimedium* which is commonly used in traditional Chinese medicine. Icaritin exhibits a variety of pharmacological and biological activities, including antirheumatic and antidepressant activities; the stimulation of cardiac and neuronal differentiation (22,23); prevention of steroid-associated osteonecrosis (24); induction of human prostate carcinoma PC-3; breast cancer MCF-7 cell growth inhibition; and cell apoptosis (25,26).

In the present study, the multidrug-resistant HepG2/ADR subclones were developed and treated with icaritin. The reversal effect of icaritin on MDR tumor cells was investigated using the adriamycin (ADR) sensitive HepG2 cells and ADR resistant HepG2/ADR cells.

Materials and methods

Cell culture and icaritin treatment. HepG2 [a human hepatocellular carcinoma (HCC) cell line] was purchased from KeyGen (Nanjing, China). Cells were cultured with

Correspondence to: Dr Jin Si, Department of Laboratory Medicine, The Second Affiliated Hospital of Nanjing Medical University, 121 Jiangjiayuan, Nanjing, Jiangsu 210011, P.R. China
E-mail: sijin_efy@njmu.edu.cn; sijin2004@hotmail.com

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Dulbecco's modified Eagle's medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% newborn calf serum (Gibco-BRL) at 37˚C in a humidified atmosphere containing 5% CO_2. An MDR human HCC cell line, HepG2/ADR, was developed by culturing HepG2 cells in the presence of increasing concentrations of ADR (0.02, 0.05, 0.1 mg/l; Hisun Pharmaceutical Co. Ltd., Zhejiang,China). Resistant cells were selected and resistance was maintained by culturing the cells in medium supplemented with 0.1 mg/l ADR and labeled HepG2/ADR (0.1).

Stock solution of icaritin (purity, >98%; Yousi Biotechnology Inc., Shanghai, China), which was further diluted with cell culture medium before each experiment, was prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mM at -20˚C. The final concentration of DMSO in culture was <0.1%.

Measurement of cellular sensitivity to anticancer drugs. The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT; Sigma-Aldrich, St. Louis, MO, USA] assay was used to determine drug sensitivity. HepG2 and HepG2/ADR cells were seeded into 96-well plates at a concentration of $5x10^3$ cells/200 μl/well. Cells were incubated at 37˚C in a humidified 5% CO_2 incubator. Following 24 h treatment with specific concentrations of the anticancer drugs ADR, vincristine, cisplatin and 5-fluorouracil, plates were returned to standard tissue incubator conditions for an additional 4 h. Medium was removed and cells were solubilized in 150 μl DMSO. The intensity of formazan was measured at 490 nm using an automated microplate spectrophotometer (iMark; Bio-Rad, Hercules, CA, USA). The survival rate was calculated as (OD value of the treated group/OD value of untreated group) x 100%. Assays were performed in triplicate in three independent experiments.

Analysis of MDR reversal activity. Viability of HepG2 and HepG2/ADR cells following treatment with ADR in the presence (1, 15, 30 μM) or absence of icaritin was analyzed by an MTT assay. Following plotting of the dose-response curve, the IC_{50}, the concentration of drug inhibiting 50% of cells, was calculated, from which reversal fold was calculated.

Effect of icaritin on intracellular ADR accumulation. HepG2 and HepG2/ADR cells were incubated with ADR in the presence (1, 15, 30 μM) or absence of icaritin for 4 h. ADR accumulation in HepG2 and HepG2/ADR cells was assessed by fluorescence spectrophotometery. The fluorescence was generated when HepG2 cells were treated with ADR, and the fluorescence intensity was positively associated with ADR accumulation. ADR accumulation in HepG2 cells and HepG2/ADR cells was assessed using a fluorescence spectrophotometer (excitation wavelength, 470 nm; emission wavelength, 590 nm; F-7000, Hitachi, Chiyoda, Japan).

Quantitative polymerase chain reaction (qPCR) assay. Cells were frozen in liquid nitrogen and stored at -80˚C for use in qPCR experiments. MDR1 mRNA expression levels were quantified by qPCR. Total cellular RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Primer sequences used were as follows: sense: 5'-CATCGAGTCACTGCCTAATAAATA-3' and antisense:5'-GCTTCTTGGACAACCTTTTCACT-3' for MDR1; and sense: 5'-CCTCTATGCCAACACAGTGC-3' and antisense: 5'-GTACTCCTGCTTGCTGATCC-3' for β-actin. PCR was performed for 35 cycles, each cycle comprised of denaturation at 95˚C for 45 sec, annealing at 52˚C for 45 sec and extension at 72˚C for 45 sec, prior to a final extension at 72˚C for 10 min. MDR1 mRNA levels were analyzed by one-step qPCR with RNA-direct™ SYBR-Green Realtime PCR Master mix (Toyobo, Osaka, Japan), according to the manufacturer's instructions. The amplification was monitored on an ABI PRISM 7500 real-time PCR apparatus (Applied Biosystems, Carlsbad, CA, USA).

Western blot analysis. Cells were lysed with ice-cold lysis buffer [50 mM Tris-HCl (pH 7.4) 150 mM NaCl, 1 mM $MgCl_2$, 100 μg/ml PMSF and 1% Triton X-100] for 30 min on ice. Total proteins were dissolved in the supernatant following centrifugation at 13,225 x g for 5 min at 4˚C and protein concentrations were measured in the supernatants (Protein Assay Dye; Bio-Rad, Hercules, CA, USA). Equal quantities (40 μg) of lysate proteins were separated on 10% SDS-PAGE gels and electrophoretically transferred onto polyvinylidene fluoride membranes. Following blocking with 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST) buffer [10 mM Tris (pH 7.5) 150 mM NaCl and 0.05% Tween-20] for 2 h at room temperature, membranes were probed with A 1:1,000 dilution of anti-target protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-β-actin antibodies (Sigma-Aldrich) at 4˚C overnight, followed by incubation in a 1:5,000 dilution of horseradish peroxidase-coupled secondary antibody (Sigma-Aldrich). Protein bands were detected using an enhanced chemiluminescence detection system (ChemiDoc; Bio-Rad). Band intensity was quantified by BandScan 5.0 software (Glyko, Hayward, CA, USA). All western blot analyses were performed at least three times.

Statistical analysis. SPSS version 16.0 software was used (SPSS Inc., Chicago, IL, USA). Each assay was performed a minimum of three times. Data are expressed as the mean ± SD; Student's t-test and one-way analysis of variance were used for statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Development of multidrug-resistant HepG2/ADR subclones. MDR was developed in HepG2 cells by treatment with increasing concentrations of ADR. HepG2 cells began to exhibit cell death 24-48 h following treatment with high concentrations of ADR. Therefore, 0.02 mg/l ADR was added to HepG2 cells and the morphological changes were observed in cultured cells. Higher concentrations of ADR were added to the medium once HepG2/ADR (0.02) cell death was not observed and morphological changes became stable. A HepG2/ADR (0.1) cell was produced, which required a minimum of 8-10 weeks culturing (Fig. 1).

Determination of MDR. HepG2/ADR (0.1) cells were investigated for their resistance against other anticancer drugs using

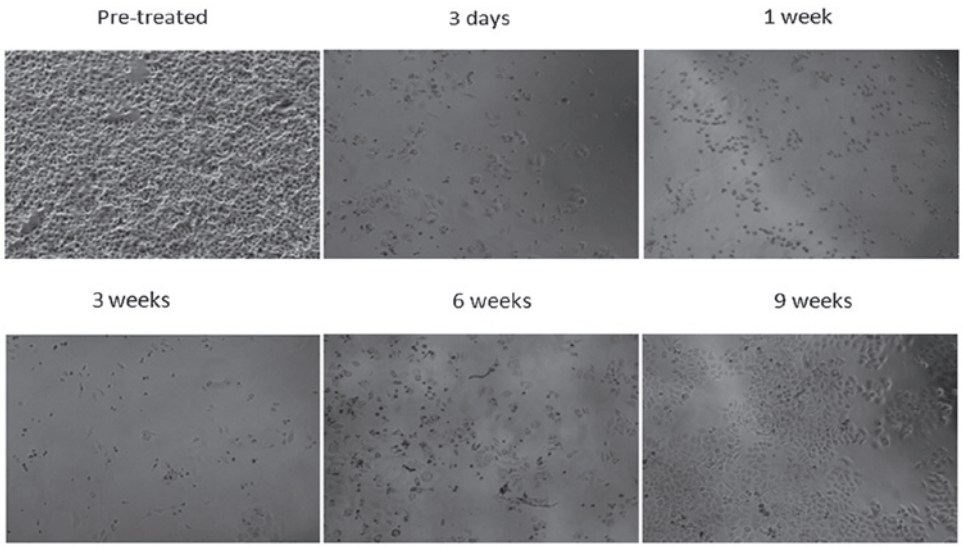

Figure 1. Continuous image record of cell proliferation during the development of HepG2/ADR cells (0.02). ADR was added to HepG2 cells at 0.02 mg/l and resistant cells were selected by removing dead cells. Progress from the parental HepG2 to the resistant HepG2/ADR (0.02) subclone was continuously imaged. Nine weeks was required to confirm a stable phenotype. ADR, adriamycin.

MTT. HepG2/ADR cells were observed to be resistant to ADR and to multiple anticancer drugs, including vincristine, cisplatin and 5-fluorouracil. The IC_{50} of these drugs in HepG2/ADR cells was significantly higer compared with that in non-resistant HepG2 cells (Table I). HepG2/ADR cells were ~25-fold more resistant to ADR in comparison with HepG2 cells.

Icaritin-mediated reversal of HepG2/ADR cell resistance to ADR. MDR modulating activity of the derivatives was evaluated by an MTT assay using human HCC cells, HepG2 and ADR resistant HCC cells (HepG2/ADR). As shown in Table I, the IC_{50} values of ADR on HepG2 and HepG2/ADR cells was 0.024±0.007 mg/l and 0.596±0.063 mg/l, respectively, when treated for 48 h. The MDR of HepG2/ADR cells was 24.83-fold higher compared with the sensitive HepG2 cells. Icaritin was capable of reversing MDR and the sensitivity of the HepG2/ADR cells to ADR was ~1.65, 2.50 and 7.18 fold higher when the cells were treated with 1, 15 and 30 μM icaritin (Table II). The results indicated that icaritin significantly reverses the cytotoxicity of ADR to HepG2/ADR cells in a dose-dependent manner.

Effect of icaritin on intracellular ADR accumulation. To investigate the mechanism of the MDR reversal activity of icaritin, the intracellular ADR accumulation was examined. As shown in Fig. 2, the intracellular ADR accumulation in HepG2/ADR cells was 50% lower compared with that in HepG2 cells. The treatment of icaritin increased the intracellular ADR accumulation in HepG2/ADR cells at a specific range. However, it was observed that 0-30 μM icaritin did not trigger a significant effect in drug sensitive HepG2 cells. It was hypothesized that the mechanism of the MDR reversal activity of icaritin may have an association with the increase of intracellular ADR accumulation.

mRNA expression of the MDR1 gene. To determine whether icaritin altered the expression of the MDR1 gene, mRNA expression of the MDR1 gene was investigated (Fig. 3). A higher level of MDR1 expression was detected in HepG2/ADR compared with HepG2 cells. However, when treated with icaritin, the MDR1 level was significantly decreased in HepG2/ADR cells.

Downregulation of P-gp protein expression by icaritin in HepG2/ADR cells. The present study showed that icaritin inhibits the expression of the MDR1 gene in HepG2/ADR cells (Fig. 3). To investigate whether the mechanism of icaritin on HepG2/ADR cells was responsible for the expression of

Table I. Determination of IC_{50} of various anticancer drugs.

Anticancer drugs	HepG2	HepG2/ADR	Resistant fold
Adriamycin	0.024±0.007	0.596±0.063	24.83
Vincristine	0.012±0.003	0.267±0.034	22.25
Cisplatin	0.035±0.42	1.285±0.125	36.71
5-Fluorouracil	0.812±0.102	63.092±2.174	77.70

ADR, adriamycin.

Table II. Effects of icaritin on the cytotoxicity of adriamycin to HepG2/ADR cells.

Concentration, μM	HepG2/ADR (IC_{50})	Reversal fold
0	0.596±0.063	1
1	0.362±0.112	1.65
15	0.238±0.085	2.50
30	0.083±0.011	7.18

ADR, adriamycin.

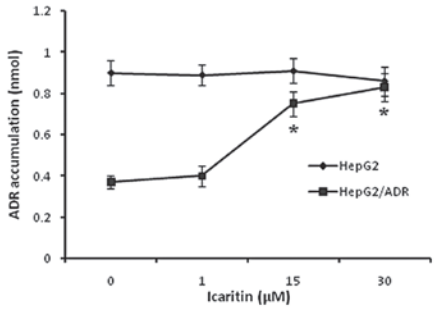

Figure 2. Effect of icaritin on intracelluar ADR accumulation in HepG2 and HepG2/ADR (0.1) cells. ADR accumulation in HepG2 cells and HepG2/ADR (0.1) cells was assessed following 4 h treatment with 0-30 μM icaritin using fluorescence spectrophotometery. Results of six independent experiments were averaged and are presented as the mean ± SEM. *P<0.05, vs. the HepG2/ADR cell (0 μM icaritin) group. ADR, adriamycin.

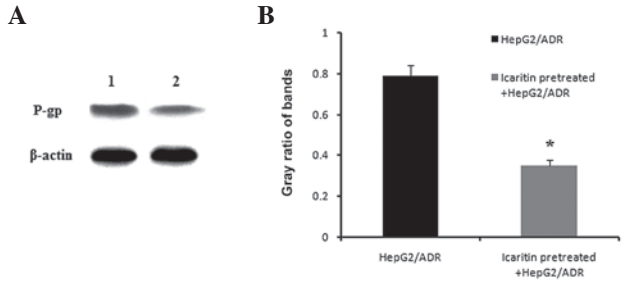

Figure 4. Effects of icaritin on the expression of P-gp protein in HepG2/ADR cells. HepG2/ADR cells were plated for 24 h, 10 μM icaritin was added to the designated HepG2/ADR cells for a subsequent 24 h. (A) P-gp proteins were detected in HepG2/ADR cells and icaritin pretreated HepG2/ADR cells were assayed by western blot analysis. Lane 1, HepG2/ADR cells; and lane 2, icaritin pretreated HepG2/ADR cells. (B) Gray analysis of protein bands. *P<0.05, vs. HepG2/ADR cells. P-gp, P-glycoprotein.

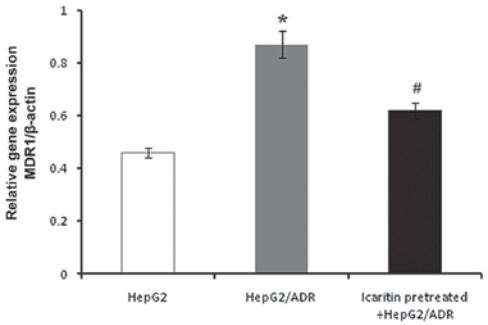

Figure 3. Effects of icaritin on the relative gene expression of MDR1/β-actin in HepG2 and HepG2/ADR cells. MDR1 genes were analyzed by qPCR. mRNA levels of MDR1 were quantified relative to β-actin mRNA. A higher level of MDR1 expression was detected in HepG2/ADR cells compared with HepG2 cells. However, when treated with icaritin, the MDR1 level was significantly decreased in HepG2/ADR cells. *P<0.05, vs. HepG2 cells. $^{\#}$P<0.05, vs. HepG2/ADR cells. qPCR, quantitative polymerase chain reaction.

P-gp, western blot analysis was performed. Results in Fig. 4 showed that the expression of P-gp was significantly repressed by icaritin in HepG2/ADR cells.

Discussion

MDR of cancer cells is a significant problem in cancer chemotherapy. P-gp is a plasma membrane transporter, which extrudes chemotherapeutic drugs from cells using ATP hydrolysis as an energy source. Overexpression of P-gp has been observed in numerous cancer cells with MDR (27,28). The aim of the present study was to identify an effective MDR reversing agent from Chinese traditional medicine and to gain an insight into its reversal effect and the molecular mechanisms of that effect.

A number of tumor cells were observed to develop MDR when cells were treated with drugs over a period of time (29,30). ADR is a chemotherapeutic drug, principally used for the treatment of solid tumors, including HCC (31). It functions via specific mechanisms; however, resistance to ADR develops in a broad range of cell lines and results in the loss of therapeutic efficacy (32,33). Thus, ADR was selected to develop the current drug resistant HCC cell subclones by culturing the cells in the presence of increasing concentrations of ADR. Although the HepG2/ADR cell line was developed by treating the cells with ADR only, it was observed that multiple drug resistance was achieved. The IC_{50} of specific anticancer drugs to the HepG2/ADR (0.1) subclone was higher compared with the parental HepG2; 24.83-fold for ADR, 22.25-fold for vincristine, 36.71-fold for cisplatin and 77.70-fold for 5-fluorouracil. The results showed that the acquired MDR of the HepG2/ADR (0.1) subclone was stable.

Icaritin is an active ingredient derivative from the traditional Chinese medical plant *Herba Epimedium*. Icaritin exhibits a variety of pharmacological and biological activities, including antitumor activity and also induces cell apoptosis in a number of types of tumor cells (34). However, the effect of icaritin on the reversal of MDR and its molecular mechanism was unclear. Therefore, the reversal activity of icaritin on MDR and the molecular mechanisms involved in this process were investigated. As shown in Table II, icaritin treatment resulted in a significant reversal of resistance to ADR at a concentration of 1, 15 and 30 μM. In addition, the icaritin-mediated reversal of HepG2/ADR cell resistance to ADR was observed in a dose-dependent manner. Furthermore, treatment of icaritin increased the intracellular ADR accumulation in HepG2/ADR cells at a specific range. It was hypothesized that the mechanism of the MDR reversal activity of icaritin may be associated with the increase of intracellular ADR accumulation.

The MDR resistance was due, in part, to the appearance of P-gp, which exerts an efficient pumping action to extrude the drugs from tumor cells. The mechanism of P-gp is cell- or tissue-specific, including the excretion of chemicals into the bile duct or kidney tubules, protection against toxic xenobiotics by blocking absorption by the intestine, efflux of steroid hormones and cholesterol from feces and prevention of chemicals that enter into the brain through the blood-brain barrier (35,36). The current results showed that icaritin significantly increased the intracellular accumulation of ADR and decreased the expression of the MDR1 level in HepG2/ADR cells compared with drug sensitive HepG2 cells. It was observed that icaritin may significantly downregulate the expression of P-gp. Reduction of P-gp expression may be proposed as a mechanism for specific agents or modulators to reverse MDR phenotype.

In conclusion, HepG2/ADR multidrug-resistant HCC cell subclones were successfully established. The current observations indicate that icaritin effectively reverses MDR, via the increase of intracellular ADR accumulation, and downregulation

of the MDR1 gene and P-gp protein expression. The present results indicate that icaritin is a novel and potent MDR reversal agent, and may be a promising drug for tumor chemotherapy.

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