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ORIGINAL ARTICLE

Hyperin Enhances the Sensitivity of HCT8/VCR Colon Cancer Cell Line to Vincristine by Down-Regulating P-Glycoprotein

Li-Min Wang, Ming-Yuan Zhang, Qiu-Shuang Zhu, Chun-Feng Lu, Xue Bai

College of Basic Medicine, Jiamusi University, Jiamusi 154007, China

SUMMARY

Background: **Long-term chemotherapy reduces the sensitivity of colon cancer cells to chemotherapeutics like vincristine (VCR) and lead to drug resistance, which has become a major barrier for colon cancer treatment. Calcium antagonists are used as clinical tumor multidrug resistance reversal agents to regulate the P-glycoprotein (P-gp) level and block efflux pump function now, but they have significant side effects. Hyperin as active component with low toxicity in traditional Chinese medicine has calcium antagonistic effect. Thus, the purpose of this study was to evaluate the inhibitory effect of hyperin on the growth of HCT8/VCR colon cancer cell line (vincristine-resistant) and analyze the enhancing effect of hyperin on the sensitivity of cancer cells to VCR and its relationship to the expression and function of P-gp.**

Methods: **Using the MTT method, we investigated the influence of hyperin, VCR alone, and hyperin plus VCR on the growth of HCT8/VCR cells. Western blot analysis was employed to detect the expression of P-gp, and flow cytometry was used to evaluate P-gp function by detecting the fluorescence intensity of intracellular Rho123.**

Results: **The inhibitory effect of hyperin at the dose of 12.5 μM on HCT8/VCR cell growth was not enhanced as time progressed and no significant inhibitory effect was found for VCR-treated cells at the dose of 2 μM. But the inhibition of cell growth was observed after the combined treatment of hyperin (12.5 μM) and VCR (2 μM). P-gp expression levels in HCT8/VCR cells treated with hyperin plus VCR were markedly lower than the levels in control cells and those treated with VCR. In addition, the intensity of Rho123 fluorescence of HCT8/VCR cells treated with hyperin plus VCR or hyperin alone was significantly higher than intensity observed in control cells and those treated with VCR alone.**

Conclusions: **Hyperin synergistically augments the growth inhibitory effect of vincristine. The underlying mechanism most probably involves down-regulation of P-gp expression and inhibiting the function of the P-gp pump in HCT8/VCR cells.**

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Correspondence:
Li-Min Wang
College of Basic Medicine
Jiamusi University
Jiamusi 154007
China
Phone: +86- 454-8618576
Fax: +86-454-8618322
Email: wlmtong@163.com

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INTRODUCTION

Multidrug resistance (MDR) is a major obstacle limiting the efficacy of chemotherapeutic agents in the treatment of numerous cancer types [1]. The etiology of MDR is complex [2-4]. However, the overexpression of P-gp, which acts as an efflux pump and prevents sufficient in-

tracellular accumulation of several anticancer agents, is a major contributor to MDR. The strategy of reversing MDR has principally focused on the inhibition or modulation of P-gp activity [5-8].

Calcium antagonists, such as verapamil, are used as clinical tumor MDR reversal agents to regulate the P-gp level and block efflux pump function [9,10]. However, they have severe side effects. So, searching for less toxic and powerful reversal agents for MDR is a hot field in regard to cancer drug development.

Hyperin (Figure 1), with the molecular formula $C_{21}H_{20}O_{12}$, is a natural flavonoid compound of low toxicity derived from traditional Chinese herbs such as Hypericum perforatum, Rhododendron ponticum, and so on [11]. Hyperin has a calcium antagonistic effect [12]. Previous reports had shown that hyperin possessed antitumor activity as other flavonoids do, but it is not clear whether it has tumor MDR reversal activity by regulating the expression of P-gp as other calcium antagonists do [13,14].

In this *in vitro* experimental study, we investigated the inhibitory effect of hyperin on the growth of HCT8/VCR colon cancer cell line (vincristine-resistant). We also analyzed the enhancing effect of hyperin on the sensitivity of cancer cells to the chemotherapeutic agent vincristine (VCR) and its relationship to the expression and function of P-gp.

MATERIALS AND METHODS

Chemicals and reagents

VCR was purchased from Merck KGaA (Darmstadt, Germany). Hyperin was obtained from National Institutes for Food and Drug Control (Beijing, China). The purity of this compound was assayed by HPLC, and the purity was 98.7%. 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT), Rhodamine123 (Rho123), and Verapamil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). P-gp and β-actin mouse anti-human monoclonal antibody, and fluorescence labeled goat anti-mouse secondary antibody were obtained from Epitomics (Burlingame, CA, USA).

Cell culture

HCT8/VCR cells were obtained from Shanghai Bioleaf Biotech Co. (Shanghai, China) and cultured in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Tianjin Biotechnology Co. Ltd., China), 100 μg/mL streptomycin and 100 unit/mL penicillin at 37°C in a 5% CO_2 atmosphere.

Cell growth assay

Cell growth was assessed by MTT assay. HCT8/VCR cells in exponential growth phase were cultured to a density of 1 x 10^6 cells/mL in 96-well plates and treated with hyperin at 12.5, 25, 50 or 100 μM or VCR at 0.5, 1, 2 or 4 μM. After 24, 48, and 72 hours, MTT solution (5 mg/mL in phosphate buffered saline, PBS) was added, and the plates were incubated for another 4 hours at 37°C. The supernatant was then removed and DMSO was added to each well. After 10 minutes, the optical density (OD) value was detected at 570 nm by microplate reader (Bio-Tek, American). The cells without drugs were used as control. The survival of the cells was expressed as percentage of untreated control wells. Assays were performed on three independent experiments.

The culture medium containing hyperin (12.5 μM), VCR (2 μM) or hyperin (12.5 μM) combined with VCR (2 μM) was added to a 96-well plate containing HCT8/VCR cells. After 24, 48, and 72 hours, PBS solution containing 5 mg/mL MTT was added to each well. After incubation for 4 hours, the cells from each well were solubilized with DMSO for optical density determination at 570 nm.

The cell growth inhibition rate was calculated as follows: Inhibition rate (%) = [OD value (control) - OD value (drug)]/OD value (control) x 100%. The interaction of hyperin and VCR was evaluated by using the formula $q = E_{A+B}/(E_A + E_B - EA \times EB)$, where E_{A+B} is the cell growth inhibition rate of combined treatment, E_A is the inhibition rate of drug A only and E_B is the inhibition rate of drug B only. According to the "q" value, the interaction between two drugs can be classified as an antagonistic effect ($q < 0.85$), an additive effect ($0.85 \leq q < 1.15$) or a synergistic effect ($q \geq 1.15$).

Western blot analysis

HCT8/VCR cells were cultured in different media containing hyperin (12.5 μM), VCR (2 μM) or hyperin (12.5 μM) combined with VCR (2 μM) with 5% CO_2 at 37°C for 48 hours. The cells were collected and washed twice with ice-cold PBS, then lysed using cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride). Proteins were separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (Millipore, Billerica, MA, USA). The blots were blocked with 5% skim milk powder in TBST saline (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.1% Tween-20) for 1 hour. Membranes were then incubated overnight at 4°C with primary antibodies. After two washings in PBS, each for 10 seconds, membranes were incubated for 1 hour at room temperature with secondary antibody. Mouse anti-human P-gp monoclonal antibody (dilution 1:1,000) and mouse anti-human β-actin monoclonal antibody (dilution 1:2,000) were used as primary antibody and fluorescence-labeled goat anti-mouse (dilution 1:300) as secondary antibody. The band densities were determined using Chemi Doc Software (BioRad); β-Actin was used as a loading for normalization.

Rho123 accumulation assay

The Rho123 accumulation test was used to estimate intracellular P-gp function. HCT8/VCR cells (1 x 10^6

Table 1. The inhibitory effect of hyperin combined with VCR on HCT8/VCR cell growth (n = 4).

Group	Growth inhibition rate (%)		
	24 hours	48 hours	72 hours
hyperin	15.56 ± 2.64	18.17 ± 1.95	20.59 ± 1.73
VCR	23.62 ± 3.14	28.69 ± 1.62	36.71 ± 2.81
VCR + hyperin	48.27 ± 2.32 [a, b]	59.86 ± 3.19 [a, b]	72.53 ± 3.27 [a, b]
q	1.35	1.44	1.46

[a] VCR + hyperin vs. VCR $p < 0.001$.
[b] VCR + hyperin vs. hyperin $p < 0.001$.

cells/mL) were seeded in six-well plates. After cell adhesion to the wall, hyperin (12.5 μM), VCR (2 μM), hyperin (12.5 μM) combined with VCR (2 μM) or verapamil (5 μM), a classic inhibitor of the P-gp pump, were added to the wells. Verapamil was used as positive control drug, which is known for the reversion of P-gp mediated MDR. The cells were then further cultured in medium supplemented with Rho123 (5μg/mL) for 30 minutes and washed with PBS twice after centrifugation. The fluorescence intensity of intracellular Rho123 was detected by flow cytometry (Partec GmbH, Germany). The mean of the data was considered as final value after three repetitions.

Statistical analysis

Results were presented as mean ± SD. The statistical evaluation of the results was performed by Student's *t*-test. Significance was established at $p < 0.05$.

RESULTS

Effect of hyperin and VCR on the growth of HCT8/VCR cells

As shown in Figure 2, under the treatment of low-dose hyperin (12.5 μM) for 24, 48, and 72 hours, the cell growth inhibition rates of HCT8/VCR cells were 4.15 ± 1.14%, 4.37 ± 0.43%, and 4.88 ± 1.25%, respectively. The inhibitory effect in cells treated with hyperin for 48 and 72 hours was not enhanced as time progressed at the dose of 12.5 μM, with p-values of 0.3127 and 0.7474, compared with the treatment for 24 hours. When the concentration of hyperin was greater than or equal to 25 μM, the inhibitory effect of hyperin on HCT8/VCR cells was significantly enhanced in a time- and dose-dependent manner (all $p < 0.001$). No significant inhibitory effects were found for VCR-treated cells at doses between 0.5 and 2 μM. On the other hand, the highest VCR dose tested in our study significantly affected HCT8/VCR cell growth ($p < 0.001$), and 40.89 ± 1.44% was determined as the cell growth inhibition rate after treatment with 4 μM VCR for 72 hours (Figure 3).

Effect of combined treatment of hyperin and VCR on the growth of HCT8/VCR cells

As shown in Table 1, the combined treatment of hyperin (12.5 μM) and VCR (2 μM) significantly enhanced the inhibitory effect of VCR on HCT8/VCR cell growth. Following 24, 48, and 72 hours treatment, the growth inhibition rate in cells treated with hyperin plus VCR was significantly higher than that in cells treated with hyperin or VCR alone (all $p < 0.001$). All of the q values were greater than 1.15 after hyperin was combined with VCR treatment for 24, 48, and 72 hours, suggesting that combined treatment results in a synergistic effect.

Effect of hyperin on P-gp expression

As shown in Figure 4, the expression levels of P-gp in cells treated with hyperin plus VCR and hyperin alone were significantly lower than those in cells treated with VCR alone and the control cells. The relative gray-scale values in cells treated with hyperin plus VCR and hyperin alone were significantly lower than those in cells treated with VCR alone and the control cells (all $p < 0.001$). In addition, the relative gray-scale value in cells treated with hyperin plus VCR was significantly lower than that in cells treated with hyperin alone ($p < 0.001$). No statistical significance was noted between cells treated with VCR and control cells in terms of the relative gray-scale value.

Effect of hyperin on the function of the P-gp pump in HCT8/VCR cells

Rho123 is a fluorescence substrate that is applied to investigate P-gp functional activity. When P-gp functional activity declines, the accumulation of the Rho123 substrate within cells increased and vice versa. Our results indicated that the amount of Rho123 accumulation in cells treated with hyperin plus VCR was significantly higher than that in cells treated with VCR alone and control cells and similar to that in cells treated with verapamil (Figure 5), suggesting that hyperin increases intracellular accumulation of Rho123 by inhibiting the function of the P-gp pump in HCT8/VCR cells.

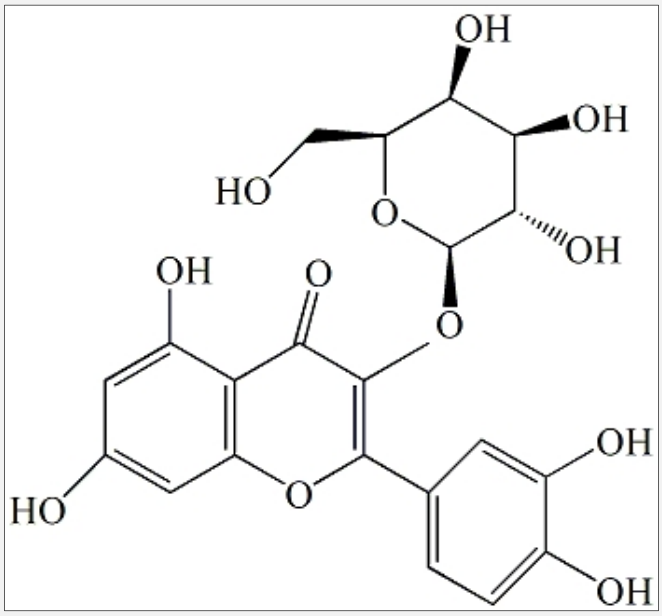

Figure 1. Chemical structure of hyperin.

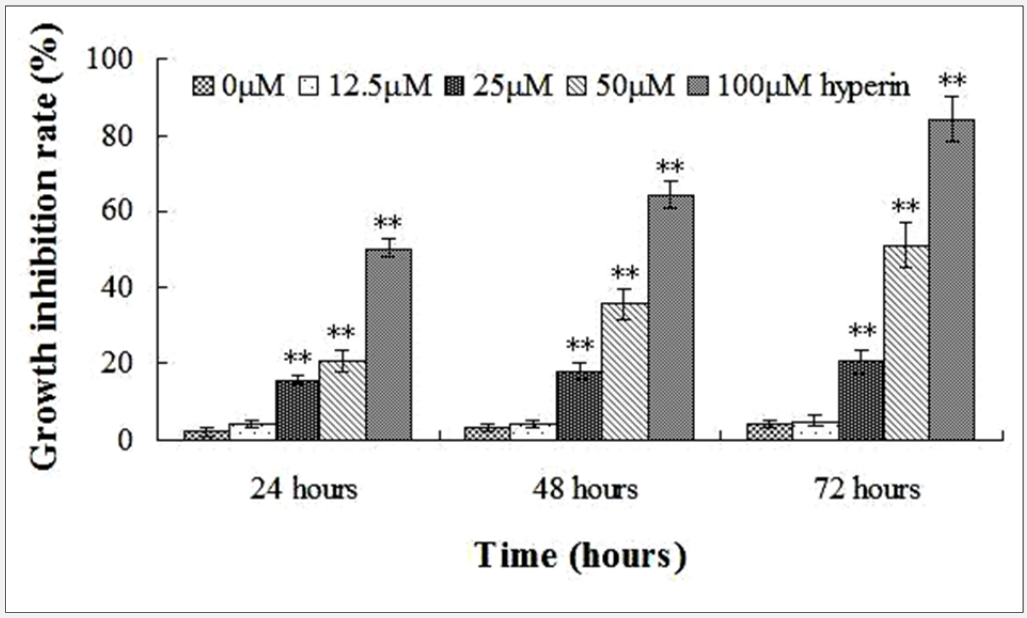

Figure 2. Detection of the inhibitory effect of hyperin on HCT8/VCR cell growth via the MTT method.

The HCT8/VCR cells were treated with different concentrations of hyperin for 24, 48, or 72 hours. The growth inhibition rate increased in a dose-dependent manner. Data from three repeated experiments are presented as mean ± SD. ** $p < 0.001$compared with the control group (0 μM). No significant difference was noted at 24, 48, or 72 hours of hyperin treatment at the dose of 12.5 μM. When the concentration was greater than or equal to 25 μM; however, hyperin significantly enhanced the inhibition rate as the treatment time progressed (all $p < 0.001$).

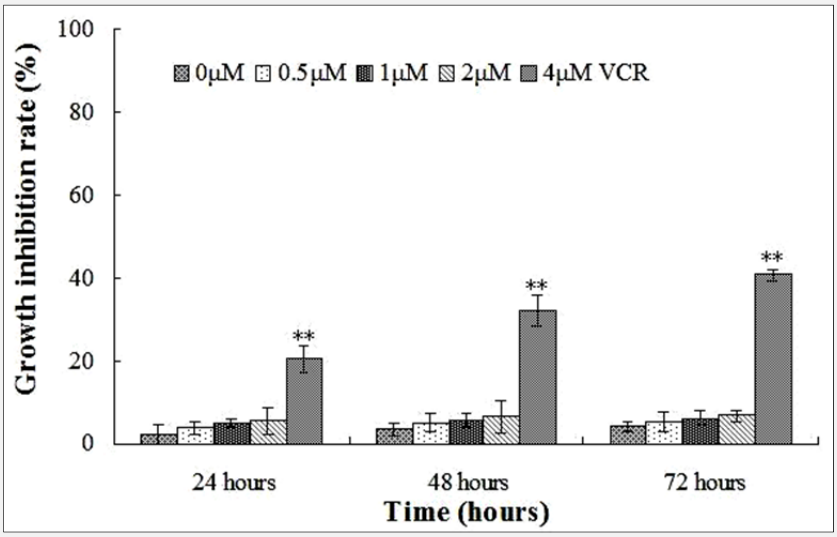

Figure 3. Detection of the inhibitory effect of VCR on HCT8/VCR cell growth via the MTT method.

The HCT8/VCR cells were treated with different concentrations of VCR for 24, 48, or 72 hours. Data from three repeated experiments are presented as mean ± SD. ** $p < 0.001$compared with the control group (0 μM). No significant inhibitory effects were found for VCR-treated cells at doses between 0.5 and 2 μM. On the other hand, the highest VCR dose tested in our study significantly affected HCT8/VCR cell growth ($p < 0.001$).

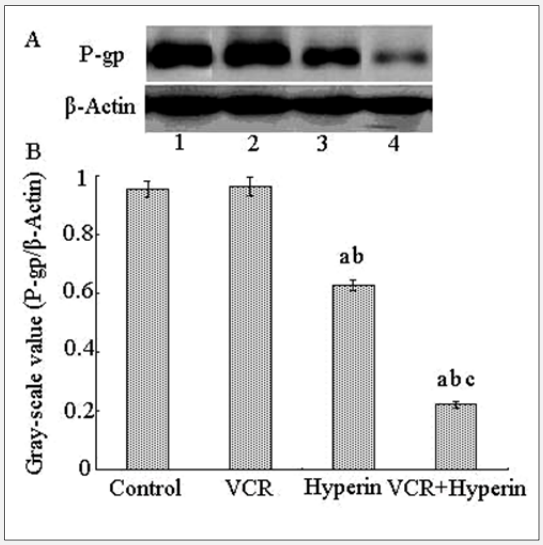

Figure 4. The effect of hyperin on P-gp expression in HCT8/VCR cells.

(A) Total protein in control and study cells was collected. SDS-PAGE was employed to separate protein, transmembrane, and fluorescence coloring following the antigen-antibody reaction. β-Actin was used as the internal reference for the detection of P-gp expression. Lane 1: Control cells; Lane 2: VCR-treated cells; Lane 3: Hyperin-treated cells; Lane 4: Hyperin plus VCR treated cells. (B) The relative gray-scale values in the P-gp expression. Data from three repeated experiments are presented as mean ± SD. [a] $p < 0.001$ compared with the control group. [b] $p < 0.01$compared with the VCR group. [c] $p < 0.001$ compared with the hyperin group.

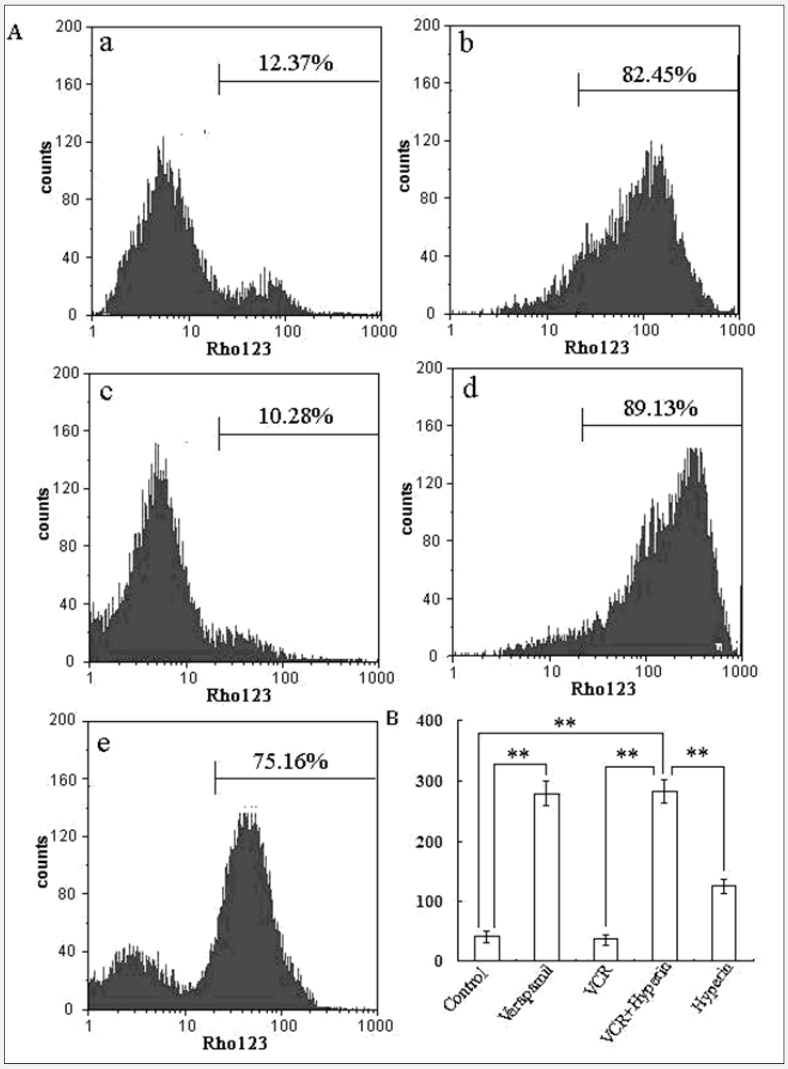

Figure 5. The effect of hyperin on P-gp pump function in HCT8/VCR cells.

(A) The amount of Rho123 accumulated in HCT8/VCR cells was detected by flow cytometry to reflect P-gp function. (a) Control cells; (b) Verapamil-treated cells (5 μM); (c) VCR-treated cells (2 μM); (d) Hyperin + VCR treated cells (12.5 μM + 2 μM, respectively); (e) Hyperin-treated cells (12.5 μM). (B) The fluorescence intensity of Rho123 in the five treatment groups was 40.73 ± 8.96, 278.65 ± 20.23, 35.81 ± 7.47, 282.17 ± 18.82, 124.96 ± 12.27, respectively. The fluorescence intensity in hyperin + VCR treated cells was significantly higher than those of VCR alone and control cells ($p < 0.001$), and similar to verapamil-treated cells ($p = 0.2207$).**

DISCUSSION

Chemotherapy serves as one of the important treatments for colon cancer [15]. However, long-term chemotherapy reduces the sensitivity of cancer cells to chemotherapeutics and leads to drug resistance, which has become a major barrier for cancer treatment. One of the lessons learned from cancer research in recent years is that combinatorial strategies in cancer therapy can provide dramatic improvement in safety and efficacy over monotherapy regimens, especially if the drugs differ in their mode of action [16].

Hyperin is an active component in traditional Chinese medicine and has recently shown significant promise in the treatment of some types of cancer by inducing G_0/G_1 arrest in the cell cycle involving the transforming growth factor (TGF)-β signaling pathway [17]. The trials of reversal effects in combination with conventional chemotherapy are being undertaken. We were therefore interested to evaluate the *in vivo* inhibitory effect of this compound on the growth of HCT8/VCR colon cancer cell line (vincristine-resistant) and the en-

hancing effect on the sensitivity of cancer cells to the chemotherapeutic agent vincristine (VCR). Since hyperin was not cytotoxic for HCT8/VCR cells at the concentration of 12.5 μM, hyperin at 12.5 μM was chosen to modulate MDR. In addition, vincristine had no significant inhibitory effect on HCT8/VCR cells at concentrations up to 2 μM. When hyperin (12.5 μM) was combined with low dose vincristine (2 μM), a remarkable improvement in antitumor activity was observed compared with either drug alone. Hyperin significantly restored the sensitivity of HCT8/VCR to VCR. In addition, the combination of hyperin and VCR results in a synergistic effect. We propose that this relatively non-toxic drug combination using hyperin with low-dose vincristine could advantageously be used in the treatment of tumors.

The mechanism of MDR reversal has been widely explored. MDR modulators are likely to reverse MDR through multiple pathways, such as by inhibiting P-gp function and/or expression.

The western blotting results indicated that the expression level in HCT8/VCR cells treated with hyperin plus VCR was significantly decreased compared to that in untreated cells, whereas intercellular accumulation of Rho123 determined by flow cytometry was significantly increased compared to that in the untreated counterparts, which provided indirect evidence that the restoration of drug sensitivity was at least partly attributed to the reduction of drug efflux effect of the resistant cells.

CONCLUSION

Our findings indicate that hyperin synergistically augments the growth inhibitory effect of vincristine. The underlying mechanism most probably involves down-regulation of P-gp expression and inhibiting the function of the P-gp pump in HCT8/VCR cells.

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Declaration of Interest:
The authors declare no conflict of interest.

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