Molecular and cellular pharmacology

Reversal of P-gp and MRP1-mediated multidrug resistance by H6, a gypenoside aglycon from Gynostemma pentaphyllum, in vincristine-resistant human oral cancer (KB/VCR) cells

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A R T I C L E   I N F O
Article history:
Received 23 April 2012
Received in revised form 21 September 2012
Accepted 21 September 2012
Available online 7 October 2012

Keywords:
Gypenoside aglycon
Gynostemma pentaphyllum
Multidrug resistance (MDR)
KB/VCR cells
P-glycoprotein (P-gp)
Multidrug resistance associated protein 1 (MRP1)

A B S T R A C T

Multidrug resistance (MDR) to anticancer drugs is a major obstacle to successful chemotherapy in the treatment of cancers. Identification of natural compounds capable of circumventing MDR with minimal adverse side effects is an attractive goal. Here, we found that H6, a gypenoside aglycon from Gynostemma pentaphyllum, displayed potent anti-MDR activity. Average resistant fold (RF) of H6 is 1.03 and 1.04 in KB/VCR and MCF-7/ADR cells compared to their parental cells. H6 alone ranging from 2 μmol/l to 40 μmol/l (μM) did not display a significant anti-proliferative effect on KB/VCR cells and other cells, while the compound at these concentrations enhanced the cytotoxicity of vincristine (VCR) to KB/VCR cells. H6 showed a significant synergistic effect in combination with VCR. By quantification of sub-G1 fraction cells, H6 also enhanced the VCR-induced apoptosis in a dose-dependent manner. The short time treatment with H6 increased the intracellular accumulation of rhodamine 123 (Rho123) and 5(6)-carboxyfluorescein diacetate (CFDA) in KB/VCR cells. Further studies showed that H6 treatment resulted in the decrease of the RNA transcript level of P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP). H6 inhibited the function of P-gp by stimulating P-gp ATPase activity and decreased MRP1 expression with a blockade of STAT3 phosphorylation. These findings suggest that H6, a multi-targets reversal agent with no significant toxic effect, may be a potential candidate to circumvent the P-gp and MRP1-mediated MDR.

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1. Introduction

The problem of multidrug resistance (MDR) presents a major obstacle in the chemotherapy of cancer (Wu et al., 2010). For example, the emergence of MDR is often found in cancer cells treated with vincristine (VCR), a cell cycle-specific alkaloid (Ozgen et al., 2000). The typical MDR in tumor cells is mainly associated with a reduced intracellular drug accumulation and an increased cellular drug efflux. Molecular investigations in MDR resulted in the isolation and characterization of genes encoding for several transport proteins including P-glycoprotein (P-gp/MDR1/ABCB1), the multidrug resistance associated protein 1 (MRP1/ABCC1), the breast cancer resistance protein (BCRP/ABCG2) (Sharom, 2008), the lung resistance protein (LRP) and so on (Chen et al., 2011; Slot et al., 2011). They all belong to the ATP binding cassette (ABC) superfamily of transporters.

The overexpression of P-gp and/or MRP1 is a determinant of both intrinsic and acquired drug resistance in many human cancers (Choi, 2005). By inhibiting P-gp and MRP1, it seems that drug resistance could be avoided and tumor cells eliminated (Li et al., 2007). The ongoing search for MDR modulators that can be applied in the clinic is into its third-generation (Bansal et al., 2009; Guns et al., 2001; Kuppens et al., 2007; Morschhauser et al., 2007; Muller et al., 2008; van Zuylen et al., 2002). Although the third-generation modulators are more potent and less toxic than first-generation modulators, some are still prone to adverse effects, poor solubility, and unfavorable changes in pharmacokinetics of the anticancer drugs and limited clinical benefit (Ullah, 2008). These have spurred on efforts to searching for more effective compounds with minimal adverse side effects.

Agents derived from plant origin are being increasingly utilized in drug discovery and drug development programs (Molnar et al., 2010; Palmeira et al., 2012). Identification of natural compounds capable of circumventing MDR with minimal adverse side effects is...
an attractive goal. For example, flavonoids are a group of compounds which have been extensively studied as chemosensitizers in a number of cancer cell lines (Bansal et al., 2009).

Gynostemma pentaphyllum (Thunb.) Makino (Cucurbitaceae), a perennial creeping herb distributed in Japan, Korea, China, and Southeast Asia, was once used as a sweetener in Japan and as a folk medicine in China (Yin et al., 2004). The pharmacological studies of G. pentaphyllum and/or isolated gypenosides have been reported to affect numerous activities resulting in antitumor (Ky et al., 2010; Lin et al., 2011), cholesterol-lowering (Norberg et al., 2004), immunopotentiating (Sun and Zheng, 2005), antioxidant (Zhang et al., 2011), hypoglycemic (Huyen et al., 2010) and anti-ucer (Rujjanawate et al., 2004) effects. Unlike most plants of the Cucurbitaceae family, G. pentaphyllum does not show any significant toxic effect in vivo (Attawish et al., 2004; Choi et al., 2010). The effect of G. pentaphyllum extract on MDR has been reported (Huang et al., 2007), but its active ingredients and reversal mechanism are still unknown. It is generally thought that glycosides cannot be easily absorbed in the intestines (Miura et al., 2002). Because most of traditional medicines are administered orally, the glycosides inevitably interact with the intestinal microflora in the alimentary tract and are metabolized into their aglycons (Park et al., 2007).

This study reports on the chemosensitizing effects of 3\(\beta\),20\(\alpha\),21-trihydroxydammar-24-ene (H6), a gypenoside aglycon from G. pentaphyllum, could selectively restore the cytotoxicity of VCR in VCR-resistant human oral cancer (KB/VCR) cells. The results suggested that H6 is a new class of drug candidates for the treatment of MDR.

2. Materials and methods

2.1. Preparation of H6

H6 was prepared from gypenoside XLIX by alkaloid hydrolysis, and gypenoside XLIX is the main saponin in crude gypenoside Capsule manufactured by Ankang Beiyi Tai Pharmaceutical Co., Ltd. (its content is about 12.6%) (Yin et al., 2004), and the detailed process was as follows (Cao et al., 2005): Na (0.5 g, 21.5 mmol) was added to a stirring solution of gypenoside XLIX (2 g) in n-butyl alcohol (40 ml). Once dissolved, benzoyl peroxide (0.25 g, 1.0 mmol) was added and stirring continued. The reaction was placed under an oxygen atmosphere, and 90 ml of fresh culture medium with 10% FBS. After 24 h incubation, various drug-free medium for 3 days before the experiments.

2.2. Drugs and reagents

VCR, Adriamycin, Verapamil (VPL), rhodamine 123 (Rho123), 5(6)-carboxyfluorescein diacetate (CFDA) and mitoxantrone were purchased from Sigma (Santa Clara, CA, USA). Minimum Essential Medium \(\alpha\) (\(\alpha\)-MEM) and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). P-gp-Glo™ Assay Systems with P-glycoprotein (Catalog #V3601) was obtained from promega. Antibodies for p-STAT3, STAT3, p-ERK1/2, ERK1/2, p-AKT, AKT and GAPDH were obtained from Cell Signaling Technology, Inc. Mouse monoclonal [IU2H10] antibody to MRPI was obtained from Abcam company; Cell Counting Kit-8 was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). All other chemicals used were of reagent grade.

2.3. Cell lines and cell culture

Human cancer cell lines (HepG2, THP-1, K562, HGC-27, SKOV3, Panc-1, SW480, HeLa, A549, and MDA-MB-453) used in this study were procured from American Type Culture Collection. Human Umbilical Vein Endothelial Cells (HUVEC-2C) cell line was procured from Cascade Biologics. Life Technologies Corporation. Resistant cell lines KB/VCR, MCF-7/ADR, and their parental cells were provided by Professor Jian Ding from Shanghai Institute of Materia Medica, Chinese Academy of Sciences. K562, THP-1, and A549 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS); MCF-7, HepG2, HGC-27, SKOV3, Panc-1, SW480, HeLa, and MDA-MB-453 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. HUVEC-2C cells were cultured in Medium 200 (Cascade Biologics) supplemented with Low Serum Growth Supplement (LSGS, Cascade Biologics) in the absence of antibiotics and antmycotics. KB and KB/VCR cells were grown in Minimum Essential Medium Eagle (MEM) \(\alpha\) medium and supplemented with 10% FBS. When KB/VCR cells were cultured in Drug-Free medium for 3 days before the experiments.

2.4. CCK-8 assay

Cell proliferation was measured using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) (Cell Counting Kit-8). KB and KB/VCR cells (3.5 \(\times\) 10\(^4\)/100 \(\mu\)l/well) were plated into 96-well plates in \(\alpha\)-MEM with 10% FBS. After 24 h incubation, various concentrations of Adriamycin, VCR with or without H6 were diluted in \(\alpha\)-MEM with 10% FBS and added to each well. Cells were cultured for a further 48 h. Then the medium was removed by aspiration, and 90 \(\mu\l\) of fresh culture medium with 10 \(\mu\l\) of Cell Counting Kit-8 solution were added to each well. The plates were
incubated for a further 2 h at 37 °C, and the absorbance at 450 nm was measured using a microplate reader (model 550, Bio-Rad Laboratories, Hercules, CA). The percentage of growth was shown relative to untreated controls. Each experiment was done at least thrice independently.

2.5. Determination of sub-G₁ cells

It is well established that DNA fragmentation during apoptosis may lead to extensive loss of DNA content and result in a distinct sub-G₁ peak when analyzed using flow cytometry (Riccardi and Nicoletti, 2006). The enhancement effect of H₆ for VCR induced apoptosis was also evaluated by the determination of sub-G₁ cells. At the end of various designated treatments, cells were collected and washed in phosphate buffered saline (PBS) twice, and resuspended in PBS containing 0.03% Triton X-100, 200 mg/ml RNase A, and 50 µg/ml PI. The cells were incubated for 15 min at room temperature in the dark. Sub-G₁ analysis was performed by flow cytometry analysis of 20,000 cells in each group. The percentage of cells in the sub-G₁ phase was calculated to reflect the percentage of apoptotic cells.

2.6. Rho123, CFDA and mitoxantrone accumulation assays

KB and KB/VCR cells were seeded into 12-well plates at a density of 1 × 10⁵ per well. The cells were pretreated with H₆ for 3 h and then incubated with 5 µmol/l (µM) Rho123, or 2 µM CFDA or 3 µM mitoxantrone in the dark for another 30 min. After washed twice with ice-cold PBS, the cells were observed and photographed under an Olympus fluorescence microscope (Olympus Optical, Tokyo, Japan). The intracellular fluorescence associated with Rho123, CFDA and mitoxantrone was analyzed by flow cytometry with an excitation wave length of 488 nm. The emitted fluorescence was measured at 530 nm for Rho123, CFDA and at 670 nm for mitoxantrone (Kowalski et al., 2005).

2.7. P-gp ATPase activity assay

The changes of ATPase activity were determined using P-gp-Glo™ assay systems (Promega, USA) as described in the manufacturer’s manual (Tao et al., 2009). Sodium orthovanadate (Na₃VO₄) was used as a P-gp ATPase inhibitor. Various concentrations of H₆ were diluted with assay buffer and incubated with 5 mM Mg ATP and 25 µg recombinant human P-gp membranes at 37 °C for 40 min. After ATP detection buffer added, it was incubated at room temperature for 20 min and transferred to the untreated white opaque 96-well plate (corning, USA). The Luminescence was read on a GloMax® Multi Detection System (Promega, UK). The changes of relative light units (RLU) were determined by comparing Na₃VO₄-treated samples with H₆ treated samples. The experiments were carried out in triplicate for each data point.

2.8. Quantitative real-time PCR

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen) and 1–2 µg of RNA was prepared for reverse transcription using Superscript II reverse transcriptase (Gibco-BRL) and Oligo(dT)₁₅ (Promega) according to the manufacturer’s protocols. Quantification of genes expression was performed using SYBR Green go(dT)₁₅ (Promega) according to the manufacturer’s protocols. Using Superscript II reverse transcriptase (Gibco-BRL) and Oligo(dT)₁₅ (Promega) according to the manufacturer’s protocols. Gene expression levels were normalized to the internal GAPDH levels. The experiments were carried out in triplicate for each data point.

2.9. Western blot analysis

Cells were treated with H₆ for 48 h and lysed in lysis buffer containing 25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM pepstatin for 30 min. Cell lysates were centrifuged and the supernatants were harvested, and protein concentrations were assayed by BCA Protein Assay Kit (Thermo Fisher Scientific). The 150 µg of total protein was subjected to 10% SDS-PAGE, and transferred onto nitrocellulose membrane. The membranes were blocked by 5% (m/v) non-fat dry milk in TBS containing 0.2% Tween 20 at room temperature for 1 h. and then incubated with primary antibodies overnight at 4 °C. After being washed with TBS/Tween buffer, the membrane was incubated with the secondary antibodies for 1 h at room temperature. Blots were visualized with the enhanced chemiluminescence (ECL) system.

2.10. Statistical analysis

Unless stated otherwise, experiments were run in triplicate and data was described as the mean ± S.D. The final results compared in Excel by two-tailed unpaired t-test. In the case of the effects analysis of H₆ on the sub-G₁ distribution, the relative values of Rho123, CFDA and mitoxantrone accumulation and the level of mRNA and protein expression of different treatment groups, P-values below 0.05 were considered as statistically significant.

3. Results

3.1. H₆ circumvents MDR in KB/VCR and MCF-7/ADR cells

The multidrug-resistant KB/VCR and MCF-7/ADR cell lines, in which P-gp is highly expressed compared to their parent-sensitive KB and MCF-7 cell lines, are well-characterized MDR cell model for studying efflux of various anticancer drugs. The effect of H₆ on cell growth was determined with CCK-8 assay. The viability of the cells was evaluated as described in Section 2.

As shown in Fig. 2, KB/VCR cells exhibited 81.9- and 94.4-fold resistance to VCR and ADR, respectively, compared with the parental KB cells (Fig. 2 and Table 1). MCF-7/ADR cells also display resistance to VCR and ADR of the order of 38.1- and 20.9-fold, respectively, compared to their drug-sensitive counterparts (Table 1). These were consistent with previous works reported in literature (Liu et al., 2012a, 2012b) showing that the KB/VCR and MCF-7/ADR cell lines exhibited cross-resistance to a wide range of chemically dissimilar agents, with this being attributed to a P-gp-mediated process. However, as for each of these cell lines, H₆ displayed equal cytotoxicity towards the MDR cells as well as the corresponding parental cells, with resistant fold (RF) values of 0.97 and 0.96 (Table 1).

3.2. H₆ strengthens the potency of VCR in KB/VCR cells

Prior to examining the efficacy of H₆ to reverse MDR in cancer cells, we first assessed the cytotoxicity of H₆ in different cell lines.
using CCK-8 assay. The half maximal inhibitory concentration (IC50) values of H6 for the entire cell lines tested were higher than 50 μM (Table 2). Data in Fig. 2C showed that H6 ranging from 5 μM to 40 μM had no significant inhibitory effects on the growth of KB/VCR and KB cells, while the anti-proliferative effect was observed at higher concentrations (50–80 μM). Actually, cell survival was ~90% in all cell lines under the concentrations used in this experiment to reverse MDR (data not shown). To minimize the effect of H6 itself on the resistant cell growth, we chose lower concentrations (5 μM, 10 μM, 20 μM) of H6 in the reversal experiments.

The modulation of H6 on the sensitivity of VCR against KB/VCR and KB cells was shown in Fig. 3 and further detailed in Table 3. Left shifts in the cytotoxicity profiles of VCR upon the addition of H6 were indicative of reversal of MDR in KB/VCR cells. Dose-response cytotoxicity profiles for VCR were established for both the drug-resistant KB/VCR and drug-sensitive KB cells in the presence or absence of H6 (Fig. 3A and B) as well as VPL (Fig. 3C and D). The treatment of H6 at non-toxic concentrations induced a significant decrease of IC50 values of VCR against KB/VCR cells in a concentration-dependent manner, and the fold resistance (FR) of 10 μM H6 was 7.3 comparable to that of 10 μM VPL (16.6) (Table 3). However, no such activity was found in KB cells. Therefore, significant differences of the IC50 values were seen in cell responses (KB and KB/VCR) and in dose responses (5 μM and 20 μM) to VCR treatment. These findings indicated that H6, similar to VPL, could enhance the potency of VCR against KB/VCR cells, whereas had little effects on KB cells, supporting the notion that H6 could reverse the resistance of KB/VCR cells.

**Table 1** Growth inhibition of H6 against MDR cell lines.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>VCR IC50 (nM)</th>
<th>RF</th>
<th>Adriamycin IC50 (nM)</th>
<th>RF</th>
<th>H6 IC50 (μM)</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>8.5 ± 0.8</td>
<td>81.9</td>
<td>26.2 ± 2.7</td>
<td>94.4</td>
<td>58.6 ± 3.47</td>
<td>1.03</td>
</tr>
<tr>
<td>KB/VCR</td>
<td>695.8 ± 18.2</td>
<td>18.2</td>
<td>2473.8 ± 7.2</td>
<td>60.2</td>
<td>2.7 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>30.7 ± 4.3</td>
<td>38.1</td>
<td>3749 ± 24.7</td>
<td>20.9</td>
<td>89.4 ± 4.54</td>
<td>1.04</td>
</tr>
<tr>
<td>MCF-7/ADR</td>
<td>1168.5 ± 49.8</td>
<td>49.8</td>
<td>7819.8 ± 68.5</td>
<td>93.2</td>
<td>6.23</td>
<td></td>
</tr>
</tbody>
</table>

All resistant cell lines are maintained in drug-free medium for 3 days before seeding for growth inhibition assay. Each value represented the mean ± standard deviation (S.D.) of three independent experiments. The resistant fold (RF) was calculated as the ratio of the IC50 value of the multidrug-resistant cells to that of the corresponding sensitive parental cells.

**Table 2** Cell growth inhibition of H6 against various human cancer cell lines.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Cell lines</th>
<th>H6 IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocellular carcinoma</td>
<td>HepG2</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>SMMC-7721</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>Hep3B</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Acute monocytic leukemia</td>
<td>THP-1</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Chronic myelogenous leukemia</td>
<td>K562</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>HGC-27</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>SKOV3</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Pancreatic carcinoma</td>
<td>PANC-1</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>SW480</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>HeLa</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Human umbilical vein endothelial cells</td>
<td>HUVEC-2C</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>A549</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>MDA-MB-453</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Breast adenocarcinoma</td>
<td>MCF-7</td>
<td>89.4 ± 4.54</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>KB</td>
<td>58.6 ± 3.47</td>
</tr>
</tbody>
</table>

The cell viability is expressed as a percentage of the compound-treated viable cells divided by the viable cells of the untreated control. Each value represents the mean ± S.D. of three independent experiments.
3.3. H6 enhances the VCR-induced apoptosis in KB/VCR cells

Cells undergoing apoptosis lose part of their DNA due to DNA fragmentation in late apoptosis, which can be detected as a sub-G1 population with a lower DNA content. We used FACS analysis to quantify the amount of sub-G1 fraction cells, which represents apoptotic cells. The regulation of H6 on the cytotoxicity of VCR in KB/VCR cells was evaluated by quantification of sub-G1 fraction cells. As shown in Fig. 4A, 10 μM H6 treatment resulted in 1% of apoptotic KB/VCR cells, and the percentage of apoptosis still was 1% when exposed to 100 nmol/l (nM) of VCR as well as DMSO control. Interestingly, when VCR was combined with 10 μM and 20 μM H6, the mean apoptotic population of KB/VCR cells increased 10-fold (9.8%) and 15-fold (15.1%), respectively, compared with 100 nm of VCR treatment alone (Fig. 4A and C). H6 enhanced the VCR-induced apoptosis in a dose-dependent manner in KB/VCR cells, however, the significant synergistic effect of H6 in combination with VCR was not detected in KB cells (Fig. 4B and D). These results suggested that the increased inhibitory effect on KB/VCR cells from the combination of H6 with VCR was achieved through the action of H6, which could enhance the VCR-induced apoptosis.

3.4. Effects of H6 on the intracellular accumulation of Rho123, CFDA and mitoxantrone in KB/VCR cells

To understand whether the above-mentioned effects were mediated by P-gp or MRP1 or BCRP, the cellular accumulation of Rho123, CFDA and mitoxantrone were examined in KB and KB/VCR cells, which representing the activity inhibition of P-gp, MRP1 and BCRP, respectively. As shown in Fig. 5B, C, Db and Dd, KB/VCR cells in the absence of H6 exhibited a significant decrease of Rho123 compared to KB cells, while a notable increase was seen in KB/VCR cells in the presence of H6 (10 μM). However, the potent effect of H6 in increasing the accumulation of Rho123 was not seen in KB cells (Fig. 5A and C, Da). Accordingly, we found that H6 also increased the accumulation of CFDA in KB/VCR cells when compared with non treated cells (Fig. 5E and G), while H6 had no significant effects on mitoxantrone accumulation (Fig. 5F and G). These results suggested that H6 may inhibit the activity of P-gp and MRP1 in KB/VCR cells.

3.5. Effects of H6 on the mRNA and protein levels of P-gp, MRP1 and BCRP in KB/VCR cells

Before studying the mechanism of H6 on MDR in cancer cells, we first examined the mRNA and protein levels of P-gp, MRP1 and BCRP between KB and KB/VCR cells. As shown in Fig. 6A, the mRNA expression levels of P-gp, MRP1, and BCRP in KB/VCR cells, respectively, were 99-, 12- and 14-fold higher than the parental KB cells using semi quantitative RT-PCR. The proteins were all overexpressed in KB/VCR cells (Fig. 6B). To study the mechanism of H6 on MDR, the mRNA and protein levels of P-gp, MRP1 and BCRP were examined too. As expected, when treated with 10 μM

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>KB/VCR IC50 (nM)</th>
<th>FR</th>
<th>KB IC50 (nM)</th>
<th>FR</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCR</td>
<td>695.8 ± 18.2</td>
<td></td>
<td>8.5 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>VCR + H6 5 μM</td>
<td>331.5 ± 9.7</td>
<td>2.1</td>
<td>7.6 ± 0.4</td>
<td>1.1</td>
</tr>
<tr>
<td>VCR + H6 10 μM</td>
<td>95.3 ± 6.4</td>
<td>7.3</td>
<td>9.2 ± 0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>VCR + H6 20 μM</td>
<td>68.1 ± 3.8</td>
<td>10.2</td>
<td>8.2 ± 0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>VCR + VPL 10 μM</td>
<td>41.0 ± 4.3</td>
<td>16.6</td>
<td>7.4 ± 0.3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Cell survival was determined using CCK-8 assay as described in methods. Each value represents the mean ± S.D. of three independent experiments. The fold reversal (FR) of MDR was calculated by dividing the IC50 for cells with the anticancer drug in the absence of H6 by that obtained in the presence of H6.

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H6, the mRNA expression level of P-gp, MRP1, and BCRP were all significantly inhibited in KB/VCR cells (Fig. 6C). Western blot assay revealed that H6 could decrease the protein level of MRP1 in a dose-dependent manner in KB/VCR cells. But no significant changes were observed on the protein levels of P-gp and BCRP (Fig. 6D).

3.6. H6 stimulates P-gp ATPase activity

The aforementioned results proved that H6 could increase the accumulation of Rho123, which is a specific substrate of P-gp, maybe due to the expression or functional inhibition of P-gp. We monitored ATPase activity in cell membrane preparations as a method for identifying whether or not that H6 could interact with the drug efflux transporters. As we known, P-gp exhibits a highly drug-dependent ATP hydrolysis activity, and a variety of P-gp inhibitors and substrates can stimulate ATPase activity. In this assay, a test molecule is considered to interact specifically with P-gp if it significantly modulates ATPase activity, at any concentrations from 0.05 μM to 500 μM. Using this method, we examined the effect of H6 on the function of P-gp. We found that H6 significantly increased the ATPase activity in purified recombinant human P-gp membrane protein in a dose-dependent manner (EC50 = 38 μM) (Fig. 6E). However, the protein level of P-gp was not affected by different concentrations of H6 (up to 20 μM for 48 h; Fig. 6D). The above results suggested that H6 stimulated transporter ATPase activity of P-gp and affected its function indeed.

3.7. H6 decreases MRP1 expression with a blockade of STAT3 phosphorylation.

To understand why H6 could inhibit the activity and protein expression level of MRP1 in KB/VCR cells, KB/VCR cells were treated with various concentrations (5 μM, 10 μM, 20 μM) of H6 for 48 h and several signal pathways involved in the regulation process of MRP1 were detected. As shown in Fig. 6F, similar to the inhibition of MRP1, the phosphorylation level of STAT3 was inhibited by H6 with a dose-dependent manner. However, there were no significant changes within pERK1/2, pAKT and their non-phosphorylation level. These results suggested that Jak/STAT
signal pathway might participate in the reversal process of P-gp and MRP1-mediated multidrug resistance.

4. Discussion

Resistance to drug therapy remains a major challenge in cancer chemotherapy. MDR is often associated with enhanced drug efflux mediated by members of the superfamily of ABC transporters (Nieth and Lage, 2005). The role of ABC transporters in resistance to anticancer drugs has been known for over 30 years. There are not less than 15 members of ABC family can function as drug-efflux pumps, and have been implicated in potentially conferring resistance to chemotherapeutic agents (Sharom, 2008). However, three ABC proteins (P-gp, MRP1 and BCRP) appear to account for most observed MDR in human cancers.

P-gp is a 170-kDa transmembrane protein, which causes the efflux of antineoplastic agents from tumor cells via an ATP-dependent process. P-gp is intrinsically overexpressed in many neoplasms, including the majority of carcinomas arising in the colon, rectum, pancreas, liver, and kidneys (Patel and Rothenberg, 1994). Expression of P-gp confers resistance to a broad spectrum of the most commonly used chemotherapeutic agents including the anthracyclines, taxanes, epipodophyllotoxins, vinca alkaloids, dactinomycin and many other drugs (Sharom, 2011).
MRP1, like P-gp, is a 190-kDa membrane transport protein. MRP1 protein seems to be expressed in epithelial cells of most normal tissues, including hematopoietic cells, the digestive, urogenital, and respiratory tracts, and endocrine glands (Flens et al., 1996; Jian et al., 1997). Elevated levels of MRP1 have been observed in relapsed acute myelogenous leukemia, chronic lymphatic leukemia, small-cell and non-small-cell lung cancer, and neuroblastoma. MRP1 also confers resistance to a broad spectrum of chemotherapeutic agents such as Anthracyclines, Vinca alkaloids, Epipodophyllotoxins, and mitoxantrone, but probably not taxanes, by causing the efflux of glutathione conjugated natural product agents (Keppler, 2010).

Inhibiting drug transporter and modulating MDR are one of the most important strategies in the field of cancer chemotherapy. First-generation inhibitors of P-gp such as VPL and cyclosporine were combined with a range of chemotherapy regimens for many cancers, but the results were not convincing. The second-generation inhibitors of P-gp such as PSC 833 and VX-710 were attempted in subsequent clinical trials, but the results of these trials were largely negative, failing in some cases because of pharmacokinetic interaction between the chemotherapeutic agents and the P-gp inhibitors (Dean et al., 2005). The third-generation inhibitors of P-gp and MRP1 such as tariquidar (XR9576), Zosuquidar (LY335979), Laniquidar (R102933), ONT-093 (OC144-193) and...
GF120918 may be more effective in certain patients with MDR (Bansal et al., 2009; Guns et al., 2001; Kuppers et al., 2007; Morschhauser et al., 2007; Muller et al., 2008; van Zuylen et al., 2002). However, none of them was approved for clinical use due to their unwanted side effects. Therefore, the screening of more potent chemosensitizers with desirable pharmacology is of paramount clinical importance.

In this study, we evaluated the efficacy of H6, a gypenoside aglycon from *G. pentaphyllum*, as a potent reversal agent to overcoming resistance of KB/VCR cells. The results showed that H6 alone ranging from 2 μM to 40 μM did not display a significant antiproliferative effect on KB/VCR cells, while the compound at these concentrations enhanced the cytotoxicity of VCR toward KB/VCR cells. Moreover, the increased sensitivity of KB/VCR cells to VCR was also confirmed by the enhanced VCR-induced apoptosis in the presence of H6 (Fig. 3 and Table 2). Additionally, IC50 values of H6 for the entire cell lines tested were higher than 50 μM (Table 2). Regarding the facts that *G. pentaphyllum* once used as a sweetener in Japan and has been used as a folk medicine in China (Yin et al., 2004) and it does not show any significant toxic effect in vivo (Attawish et al., 2004; Choi et al., 2010), we concluded that H6 would be safer than other reversal agents.

Rho123 is a specific substrate for P-glycoprotein. The uptake of Rho123 is resulted from passive inward diffusion, while the efflux is known to be P-glycoprotein-dependent (Xu et al., 2009). The alteration of Rho123 fluorescence intensities in MDR cells responded to the alteration of the P-gp function (Li et al., 2011). Herein, Rho123 was used to assess the modulating ability of H6 in drug-transport function of P-glycoprotein. As shown in Fig. 5B–D, the treatment of 10 μM H6 resulted in a remarkable increment of the fluorescent intensity from Rho123 in KB/VCR cells, indicating that H6 elevated accumulation of VCR in KB/VCR cells by suppressing the drug-transport activity of P-glycoprotein. CFDA is a specific substrate for MRPI (Pec et al., 2002) and CFDA uptake assays are the best choices to probe MRPI activity (Dogan et al., 2004). Using the CFDA assay, we found that H6 could increase the accumulation of CFDA in KB/VCR cells (Fig. 5E and G). Mitoxantrone is also a specific substrate for BCRP (Kowalski et al., 2005), however, Fig. 5F and G showed that H6 had not obviously affected the function of BCRP. The aforementioned results concluded that H6 enhanced VCR-induced cytotoxicity toward KB/VCR was probably mainly due to its inhibition of P-gp and MRPI.

Further results showed that H6 stimulated ATPase activity in a dose-dependent manner, which is required for the proper function of P-gp (Higgins, 2007). Although ATPase activity is closely associated with the function of P-gp, an increase in ATPase activity does not necessarily lead to an enhancement of P-gp function (Susa et al., 2010). For example, VPL and Gefitinib can stimulate the ATPase activity of P-gp and also inhibits P-gp function (Garrigues et al., 2002; Kitazaki et al., 2005). 5,7,30,40,50-pentamethoxyflavone (PMF), a MDR inhibitor, also stimulates the ATPase activity in a dose-dependent manner (Choi et al., 2004). Our results showed that H6 inhibits the efflux of P-gp substrates such as VCR without affecting the expression level of P-gp, suggested that H6 is capable of reversing MDR in cancer cells by directly inhibiting its function rather than content.

To study the mechanism of H6 on MDR, the MDR related genes were examined. As shown in Fig. 6C, the mRNA expression level of P-gp, MRPI, and BCRP were all significantly inhibited in KB/VCR cells when treated with 10 μM H6. H6 also decreased the protein level of MRPI in a dose-dependent manner, whereas it had not obviously affect the protein levels of P-gp and BCRP. As we known, among modulators of MDR, some are specific for a single transporter, for example, valsapar and XR9576 etc. are inhibitors for P-gp only, MK571 is for MRPI only, FTC is a specific inhibitor of BCRP. However, VPL, cyclosporine A, MS-209 and biricodar etc. inhibit more than one ABC drug transporter (Dai et al., 2009). In this study, we conclude that H6 also modulates more than one ABC transporter. H6 could target both P-gp and MRPI.

Some previous studies have demonstrated that there was a correlation between P-gp function (as measured by the accumulation of Rho123) and P-gp expression (Chaudhary et al., 1992; De Lucia et al., 1995). However, other studies showed there was no significant relationship between the expression and functional ability of P-gp (Bailly et al., 1995; Leith et al., 1995). When induced by representative P-gp inducers in vivo, Kageyama et al. (2006) found that there was a significant correlation between the induction levels of P-gp in the liver or small intestine, but not in the kidney. Our inhibition studies also showed the lack of correlation between P-gp function and P-gp expression in KB/VCR cells (Fig. 5B and Fig. 6D). As for MRPI, the correlation between function and expression in KB/VCR cells was confirmed (Figs. 5E and 6D).

There are several signal pathways involved in the regulatory process of MRPI. Activation of Jak/STAT pathways, Erk/MAPK or p38/MAPK resulted in an up-regulation of MRPI expression and activity (Dreuw et al., 2005). A possible p53-dependent mechanism of MRPI regulation demonstrated that WT p53 represses transcription of MRPI promoter, whereas mutated p53 enhances it (Sampath et al., 2001; Sullivan et al., 2000). The PI3K/Akt signal transduction pathway also could regulate MRPI expression in prostate cancer cells and AML cells (Tazzari et al., 2007). To understand why H6 could inhibit the protein level of MRPI in KB/VCR cells, we further detected the phosphorylation of ERK1/2, STAT3 and AKT under the treatment of H6. Western blot assay revealed that H6 could inhibited the phosphorylation of STAT3 with a dose-dependent manner, which suggested that Jak/STAT signal pathway might participate in the reversal process of P-gp and MRPI-mediated resistance treated with H6. However, there were no obvious changes within pERK1/2, pAKT and their non-phosphorylation level (Fig. 6F). However, the effects of H6 on STAT3 pathway and the precise mechanism of the regulation of MDR remain unknown. Elucidations of this mechanism will likely further our understanding of ABC transporter function and regulation. Additionally, the reversal effect study of H6 on different resistant cancer cell lines and its in vivo pharmacokinetics are in progress.

5. Conclusion

H6, a gypenoside aglycon from *G. Pentaphyllum*, could be a potential multidrug resistance reversal agent by inhibiting the function of P-gp and MRPI. Understanding the mechanism of H6 will increase our knowledge about the MDR reversal agents and help us to design other better MDR reversal compounds.

Acknowledgments

This work was supported by China Postdoctoral Science Foundation (grant 2011MS00066) and the National Natural Science Foundation of China (grant 30925040).

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