European Journal of Pharmacology 696 (2012) 43–53

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

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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ARTICLE INFO

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)

ABSTRACT

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-G_1 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.

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

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http://dx.doi.org/10.1016/j.ejphar.2012.09.046

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-ucler (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 3β,20(*S*), 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 warmed to 100 °C. After 12 h at this temperature the reaction was cooled and poured into water. The aqueous layer was extracted with EtOAc (3 ml × 40 ml), and the combined organic extracts were washed with distilled water, brine, and then dried over $MgSO_4$. After filtration, the filtrate was concentrated, and the resulting residue was submitted to flash column chromatography ($CHCl_2$/MeOH 100:1–20:1) to give the desired compound, which was determined as 3β,20(*S*),21-trihydroxydammar-24-ene (535 mg) (Fig. 1) by comparison with literature data (Cheung, 1968). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 5.14 (*t*, *J*=6.4 Hz, 1H), 3.53 (d, *J*=3.2 Hz, 2H), 3.22 (dd, *J*=11.2, 5.0 Hz, 1H), 1.70 (s, 3H), 1.65 (s, 3H), 0.99 (s, 3H), 0.98 (s, 3H), 0.90 (s, 3H), 0.87 (s, 3H), 0.79 (s, 3H), 0.75 (dd, *J*=11.4, 2.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 132.2, 124.7, 79.2, 77.2, 67.2, 56.1, 50.8, 50.3, 45.9, 41.6, 40.6, 39.2, 39.1, 37.3, 35.4, 35.0, 31.4, 28.2, 27.6, 27.5, 25.9, 24.4, 22.7, 21.7, 18.5, 17.9, 16.5, 16.4, 15.7, 15.6; HR-ESI-MS: m/z 460.3919 $[M+Na]^+$ ($C_{30}H_{52}O_3Na^+$; calc. 460.3917). The purity of H6 was not less than 95% (Radiochemical Purity, HPLC). The compound was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) for *in vitro* assays.

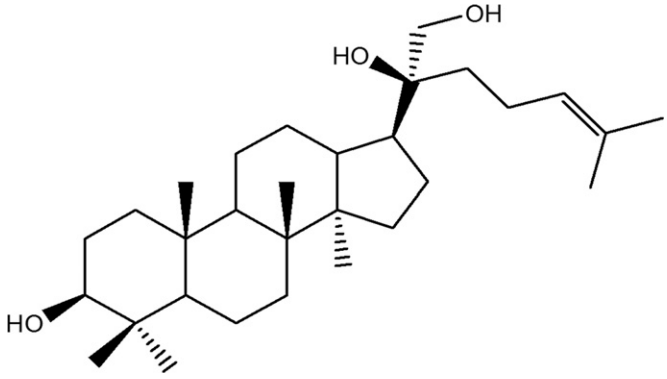

Fig. 1. The chemistry structure of H6.

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 α (α-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. P-gp (D-11), BCRP (5D3) and horseradish peroxidase (HRP)-conjugated secondary antibody were the products of Santa Cruz Biotechnology, Inc. Mouse monoclonal [IU2H10] antibody to MRP1 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 antimycotics. KB and KB/VCR cells were grown in Minimum Essential Medium Eagle (MEM) α medium and supplemented with 10% FBS, 2 mmol/l (mM) Glutamine and 1 mM Sodium pyruvate; MCF-7/ADR cells were cultured in MEM medium and supplemented with 10% FBS, 1 mM Sodium pyruvate and 0.01 mg/ml insulin. All resistant cell lines were incubated in the 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)-2*H*-tetrazolium monosodium salt (WST-8) (Cell Counting Kit-8). KB and KB/VCR cells (3.5×10^3/100 μl/well) were plated into 96-well plates in α-MEM with 10% FBS. After 24 h incubation, various concentrations of Adriamycin, VCR with or without H6 were diluted in α-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 μl of fresh culture medium with 10 μl of Cell Counting Kit-8 solution were added to each well. The plates were