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Gomisin A alters substrate interaction and reverses P-glycoprotein-mediated multidrug resistance in HepG2-DR cells

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ARTICLE INFO

Article history:
Received 7 April 2006
Accepted 26 June 2006

Keywords:
Gomisin A
Multidrug resistance
P-glycoprotein
Substrate interaction

Abbreviations:
[^{125}I]IAAP, [^{125}I]iodoarylazidoprazosin
MDR, multidrug resistance
Pgp, P-glycoprotein
PI, propidium iodide
Rh-123, rhodamine-123
SDS-PAGE, sodium doedecyl sulphate-polyacrylamide gel electrophoresis
SRB, sulforhodamine B

ABSTRACT

Through an extensive herbal drug screening program, we found that gomisin A, a dibenzocyclooctadiene compound isolated from *Schisandra chinensis*, reversed multidrug resistance (MDR) in in Pgp-overexpressing HepG2-DR cells. Gomisin A was relatively non-toxic but without altering Pgp expression, it restored the cytotoxic actions of anticancer drugs such as vinblastine and doxorubicin that are Pgp substrates but may act by different mechanisms. Several lines of evidence suggest that gomisin A alters Pgp-substrate interaction but itself is neither a Pgp substrate nor competitive inhibitor. (1) First unlike Pgp substrates gomisin A inhibited the basal Pgp-associated ATPase (Pgp-ATPase) activity. (2) The cytotoxicity of gomisin A was not affected by Pgp competitive inhibitors such as verapamil. (3) Gomisin A acted as an uncompetitive inhibitor for Pgp-ATPase activity stimulated by the transport substrates verapamil and progesterone. (4) On the inhibition of rhodamine-123 efflux the effects of gomisin A and the competitive inhibitor verapamil were additive, so were the effects of gomisin A and the ATPase inhibitor vanadate. (5) Binding of transport substrates with Pgp would result in a Pgp conformational change favoring UIC-2 antibody reactivity but gomisin A impeded UIC-2 binding. (6) Photocrosslinking of Pgp with its transport substrate [^{125}I]iodoarylazidoprazosin was inhibited by gomisin A in a concentration-dependent manner. Taken together our results suggest that gomisin A may bind to Pgp simultaneously with substrates and alters Pgp-substrate interaction.

1. Introduction

Multidrug resistance (MDR) is defined as the simultaneous resistance to various structurally and functionally unrelated drugs and is believed to be one of the major obstacles of successful cancer chemotherapy [1]. MDR can be resulted from molecular alterations in drug targets, apoptotic pathways, drug metabolizing enzymes and expression of drug transporters [2].

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doi:10.1016/j.bcp.2006.06.036

One of the better-understood mechanisms is the over-expression of the membrane P-glycoprotein (Pgp), an ATP-dependent xenobiotic exporter that causes a reduced cellular retention of drugs. Pgp is a 170-kDa ATP binding cassette (ABC) family protein that acts on a wide range of compounds including various chemotherapeutic agents such as anthracyclines, *Vinca* alkaloids, epipodophyllotoxins and tanxanes [1–3]. Pgp up-regulation is often found in patients with cancer relapse after chemotherapy and in cultured cells that become drug resistant after stepwise selection by resistance to chemotherapeutic agents [4].

There is an intense interest in the identification of compounds that specifically block the function of Pgp. Verapamil and cyclosporine A are the typical examples of the first generation MDR modulators that unfortunately have limited clinical usefulness mainly due to their severe side-effects and toxicity. Subsequently, a number of second and third generation modulators such as R-verapamil [5], PSC833 (a cyclosporin analog) [6], GF120918 [7], XR9576 [8,9] and LY335979 [10,11] have been discovered and are being studied for their clinical efficacy.

At the present time the three-dimensional atomic structure of Pgp has not been resolved therefore the identification of new Pgp inhibitors relies mainly on screening. It is generally accepted that there are least three substrate binding sites and one allosteric site on Pgp, which are independent but interacting to each other [12]. A large portion of reported modulators mainly reversibly interact with one or more of the three presumed substrate binding sites and thus are competitive inhibitors. Recently, there has been a report that thioxanthene derivative *cis*-(*Z*)-flupentixol inhibits drug transport by an allosteric mechanism [13].

Medicinal herbs are rich sources of compounds with diverse chemical structures and are abundantly used in treating human diseases. In recent years, herbs are being used increasingly in conjunction with chemotherapeutic agents, particularly in terminally ill patients, with beneficial outcomes. We have been screening Chinese medicinal herbs traditionally used in treating cancers for their complementarity to chemotherapeutic agents. In particular, we are interested in the identification of compounds that may modulate Pgp-mediated multidrug resistance (MDR) and in agents that induce cell differentiation. Successful examples are the isolation of alisol B 23-acetate from *Alisma orientale* as a Pgp inhibitor [14] and honokial from *Magolia officinalis* as an inhibitor of NF-κB and inducer of cancer cell differentiation [15,16].

Gomisin A is a small molecular weight lignan present in the Chinese medicine Fructus Schisandrae, the dried seed of *Schisandra chinensis* that is widely used as a health food product. The LD_{50} of gomisin A in ICR mice by p.o. and s.c. administrations were 878 and 855 mg/kg, respectively [17], indicated that gomisin A is relatively non-toxic. Previous studies have shown that gomisin A protected CCl_4- and acetaminophen-induced hepatotoxicity and glutamate-induced oxidative neuronal damage [18,19]. Gomisin A has also been shown to inhibit TPA-induced tumor formation in animals [17]. In this report, we demonstrate that gomisin A reverses Pgp mediated MDR by altering Pgp-substrate interaction.

2. Materials and methods

2.1. Chemicals and antibodies

Doxorubicin, vinblastine, taxol, 5-fluoruracil, verapamil, sulforhodamine B (SRB), propidium iodide (PI), rhodamine-123 (Rh-123) and cell culture grade agarose were purchased from Sigma Chemicals, U.S. Materials for cell culture were purchased from Invitrogen. Anti-Pgp antibodies clone c-494 and UIC-2 were purchased from Calbiochem and Immunotech, respectively. Anti-β-tubulin, Anti-mouse IgG_{2a}-PE and normal IgG_{2a} were obtained from Santa Cruz Biotechnology.

2.2. Cell cultures

Human HepG2 hepatic carcinoma and its multidrug resistant, Pgp overexpressing subline HepG2-DR were kindly provided by Prof. K.P. Fung. The Chinese University of Hong Kong. Cells were maintained at 37 °C in 5% CO_2 in RPMI-1640 medium containing antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), 10% fetal bovine serum and 1.2 μM doxorubicin.

2.3. Bio-assay guided isolation of gomisin A

Biological activity of the plant extracts, fractions and purified compounds were monitored by in vitro growth inhibition assay and drug retention assay. Powdered Fructus Schisandrae (the dried fruit of *S. chinensis*) was extracted with 95% ethanol twice at room temperature. The extracts were combined and ethanol was removed by a rotary evaporator under reduced pressure. The residue was first separated by solvent extraction using petroleum ether and water and the petroleum ether portion was separated on a silica gel column eluted with a discontinuous gradient of petroleum ether (PE):ethyl acetate (EA) = 10:0, 10:1 and 2:1. The bioactivity was found in the PE:EA = 2:1 fraction. This fraction was further purified on a silica gel column eluted with a continuous gradient of PE/EA. Fractions were checked by HPLC and combined if they contained the same compounds. Gomisin A was isolated from one of three combined fractions by reverse phase HPLC. The chemical structure of gomisin A was verified by LC-MS and NMR analysis (Fig. 1A) and its purity was determined by HPLC (Fig. 1B). The solid form of gomisin A was dissolved in 50% DMSO and 50% ethanol to make a 10 mg/ml (24 mM) stock that was freshly diluted in culture medium in all experiments described below.

2.4. In vitro growth inhibition assay

Cell number was estimated by sulforhodamine B (SRB) protein assay and cell growth under chronic toxicity was assessed by soft-agar colony formation assay. In SRB assay, 5×10^5 cells were seeded in 96-well plates. After 18 h various concentrations of chemotherapeutic agents were added together with gomisin A. The plates were further incubated for 72 h and cell number estimated as described. In soft-agar colony formation assay, tests were performed in 35-mm dishes containing 2 ml underlayer (0.6% agar in RPMI-1640 medium and 10% FBS). Cell suspensions (1×10^4 cells per 1.5 ml) in drug-containing 0.3%