J Korean Soc Appl Biol Chem (2015) 58(1):105–109
DOI 10.1007/s13765-015-0017-y

Online ISSN 2234-344X
Print ISSN 1738-2203

NOTE

# The synthetic compound 2′-hydroxy-2,4,6′-trimethoxychalcone overcomes P-glycoprotein-mediated multi-drug resistance in drug-resistant uterine sarcoma MES-SA/DX5 cells

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Received: 5 November 2014 / Accepted: 13 November 2014 / Published online: 24 January 2015


**Abstract** The antitumor activity of a novel synthetic chalcone derivative, 2′-hydroxy-2,4,6′-trimethoxychalcone (1H3MC), was evaluated using the multi-drug-resistant human uterine sarcoma MES-SA/Dx5 cells. Treatment with 1H3MC reduced P-glycoprotein expression in a time-dependent manner and inhibited MES-SA/Dx5 cell proliferation. Cisplatin alone had no effect on cell viability, but combined treatment with cisplatin and 1H3MC exhibited synergistic cytotoxicity. Furthermore, the combination of cisplatin and 1H3MC synergistically cleaved both caspase-3 and its substrate protein, poly(ADP-ribose) polymerase, which resulted in the fragmentation of genomic DNA, a hallmark of apoptosis. These results suggest that 1H3MC is a promising adjuvant agent for overcoming P-glycoprotein-mediated multi-drug resistance in cancer cells.

**Keywords** Flavonoid · Chalcone · P-glycoprotein · Multi-drug resistance · Cytotoxicity · Apoptosis

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## Introduction

Most cancer cells acquire cross-resistance to a broad spectrum of unrelated compounds upon exposure to bulky hydrophobic agents, such as anthracyclines, epipodophyllotoxins, vinca alkaloids, and taxanes, during chemotherapy. This phenomenon is referred to as multi-drug resistance (MDR) (Gottesman and Pastan 1993). Development of pharmacological agents able to overcome MDR is one of the biggest challenges for successful cancer chemotherapy. P-glycoprotein (P-gp), a member of the ATP-binding cassette transporter superfamily, is an ATP-dependent efflux pump which functions as a biological barrier by pumping out toxic foreign agents (Loo and Clarke 2008). Upregulation of P-gp expression in cancer cells has been proposed to play an important role in the development of the MDR phenotype (Kartner et al. 1983).

Flavonoids are a class of secondary metabolites widely distributed in edible plants such as fruits, vegetables, and tea. They have been shown to have a wide range of biological and pharmacological activities, including anti-allergic, anti-inflammatory, antioxidant, and anti-cancer effects (Ross and Kasum 2002). Based on their chemical structure, flavonoids are classified into several groups, including chalcones, flavones, flavonols, flavanones, anthocyanins, and isoflavonoids (Prasad et al. 2010). Chalcones (1,3-diphenyl-2-propen-1-ones) are open chain flavonoids containing α,β-unsaturated carbonyl group with two phenyl rings (Yadav et al. 2011). It has been well documented that both natural and synthetic chalcones exhibit a wide variety of biological activities (Yadav et al. 2011; Zhang et al. 2013). Previous studies have demonstrated that several chalcone derivatives inhibit P-gp transport activity (Boumendjel et al. 2002; Liu et al. 2008;

Parveen et al. 2014), which suggests that chalcone derivatives could serve as potential P-gp inhibitors.

In the present study, we investigated the effect of a novel synthetic chalcone derivative 2′-hydroxy-2,4,6′-trimethoxychalcone (1H3MC) on drug-resistant cancer cells. Our results show that 1H3MC induces apoptosis and reduces the expression of P-gp protein in multi-drug-resistant MES-SA/Dx5 cells.

## Materials and methods

### Chemicals

The synthesis of (E)-3-(2,4-dimethoxyphenyl)-1-(2-hydroxy-6-methoxyphenyl)prop-2-en-1-one (2′-hydroxy-2,4,6′-trimethoxychalcone; 1H3MC) was described elsewhere (Ruth Devakaram and Kumar 2010). Cisplatin was purchased from Sigma-Aldrich (USA).

### Cells and cell culture

The human uterine sarcoma cell line MES-SA and its multi-drug-resistant variants MES-SA/Dx5 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS; Thermo Scientific Hyclone, Waltham, MA, USA) at 37 °C in a 5 % $CO_2$ atmosphere.

### Flow cytometry for cell death analysis

MES-SA and MES-SA/Dx5 cells were treated with 0.5 μg/ml cisplatin for 24 h, followed by incubation with 2.5 μg/ml propidium iodide (PI) for 5 min at room temperature. For quantification of dead cells, PI-stained cells were analyzed using a FACSCalibur flow cytometer (Becton–Dickinson Immunocytometry Systems, USA).

### Cell viability and cell proliferation assays

MES-SA/Dx5 cells ($2 \times 10^3$ cells/sample) were seeded into 96-well plates and treated with 0.5 μg/ml cisplatin in the presence or absence of 20 μM 1H3MC for different time periods (0, 24, or 48 h). Cell viability assays were carried out using a Cell Counting Kit-8™ (Dojindo Molecular Technologies, USA), and a cellular proliferation assay was performed using an ELISA Cell Proliferation Assay Kit (Cell Signaling Technology, USA), according to the manufacturer's instructions. Absorbance was measured using an Emax Endpoint ELISA Microplate Reader (Molecular Devices, USA).

### Immunofluorescence assay

For detection of endogenous P-gp protein, MES-SA and MES-SA/Dx5 cells cultured on coverslips were fixed with 3.7 % paraformaldehyde. The samples were incubated with mouse anti-MDR antibody (Ab-1; Calbiochem, USA) for 90 min, and then stained with Alexa Fluor 555-conjugated secondary antibody (Invitrogen, USA) for 30 min. Nuclear DNA was stained with 0.1 μg/ml Hoechst 33258 (Sigma-Aldrich). Stained cells were observed under an EVOSf1® fluorescence microscope (Advanced Microscopy Group).

### Immunoblot analysis

MES-SA/Dx5 cells were lysed, and immunoblotting was performed as described previously (Shin et al., 2013). Antibodies specific to cleaved caspase-3 and poly(ADP-ribose) polymerase were obtained from Cell Signaling Technology. The antibody against GAPDH was obtained from Santa Cruz Biotechnology (USA). Signals were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, USA).

### Genomic DNA fragmentation assay

MES-SA/Dx5 cells were treated with 0.5 μg/ml cisplatin or 20 μM 1H3MC for 24 h. Genomic DNA was prepared using the Quick Apoptosis DNA Ladder Detection Kit (BioVision Inc., USA), according to the manufacturer's instructions. After precipitation with sodium acetate and ethanol, DNA was dissolved in Tris/EDTA buffer and then electrophoresed on a 1.2 % agarose gel as described previously (Shin et al. 2013).

### Statistical analysis

The statistical significance between control and treated groups was analyzed using the two-sided Mann–Whitney test in GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA; http://www.graphpad.com). A $p$ value of less than 0.05 was considered statistically significant.

## Results and discussion

The human uterine sarcoma cell line MES-SA and its multi-drug-resistant variant, MES-SA/Dx5, are useful model systems for studying MDR phenotypes (Wesolowska et al. 2005). To determine the expression of P-gp in MES-SA and MES-SA/Dx5 cells, immunofluorescence microscopy analysis was carried out. The fluorescence intensity of P-gp was clearly visible on the periphery of

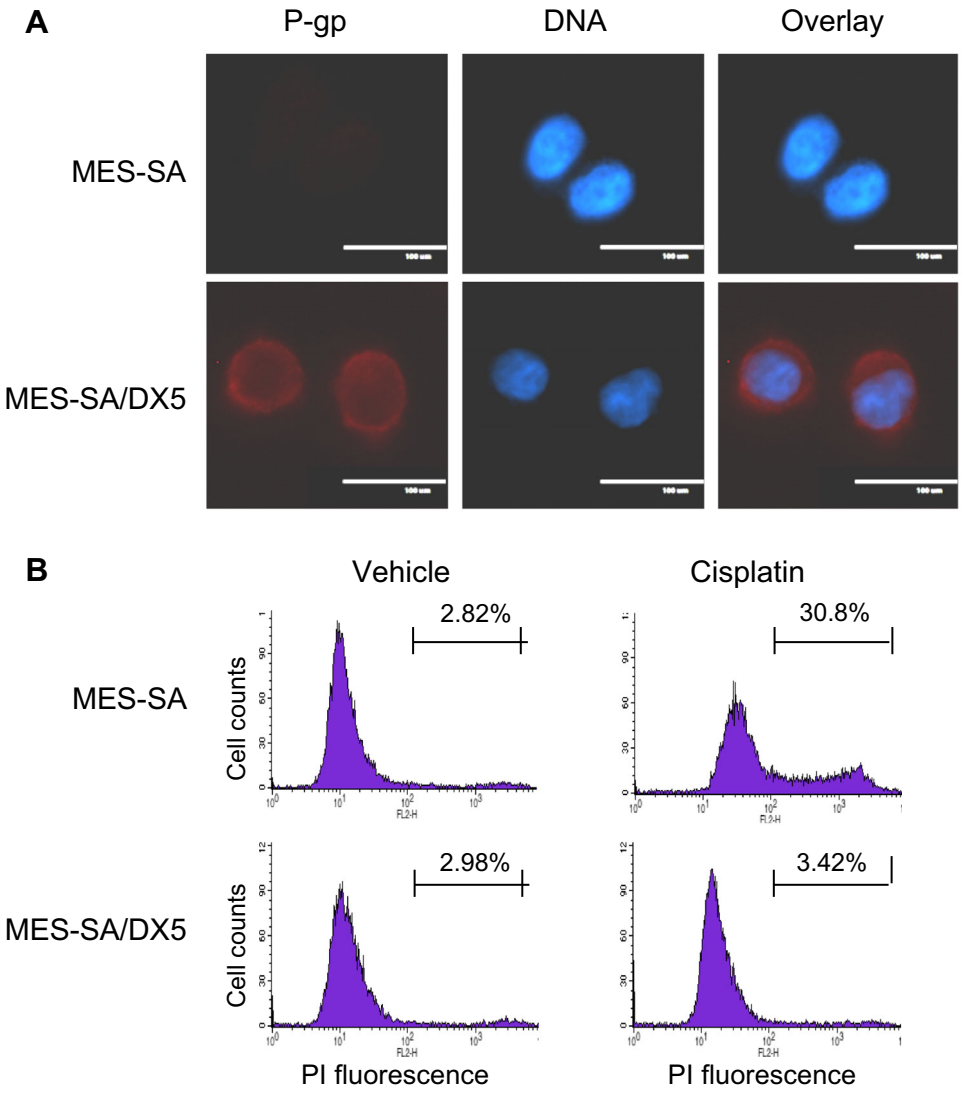


**Fig. 1** Multi-drug-resistant characteristics of MES-SA/Dx5 cells. **a** Immunofluorescence microscopy images of P-gp expression in drug-sensitive MES-SA and drug-resistant MES-SA/Dx5 cells. Cells were fixed and incubated with an anti-Pgp antibody for 90 min, followed by incubation with an AlexaFluor 555-conjugated secondary antibody (*red signal*) for 30 min. Nuclear DNA was stained with 1 μg/ml Hoechst 33258 for 10 min (*blue signal*). **b** Cisplatin resistance in MES-SA/Dx5 cells. MES-SA/Dx5 cells were treated with either vehicle (DMSO) or 0.5 μg/ml cisplatin for 24 h, followed by staining with 2.5 μg/ml PI for 5 min. PI-positive cells (dead cells) were assessed by flow cytometry

drug-resistant MES-SA/Dx5 cells, but not in the drug-sensitive MES-SA cells (Fig. 1a). Cisplatin is a platinum-containing anti-cancer drug that triggers apoptosis by cross-linking DNA. Cisplatin is used to treat a broad spectrum of cancers, including sarcomas, carcinomas, and lymphomas. However, cisplatin has many side effects that can limit its use due to severe nephrotoxicity and neurotoxicity, as well as frequent acquisition of drug resistance. To test whether MES-SA/Dx5 cells are resistant to cisplatin, these cells were treated with 0.5 μg/ml cisplatin for 24 h, and then stained with propidium iodide (PI), an intercalating agent that only stains dead cells. Flow cytometry showed that treatment with cisplatin triggered cell death in drug-sensitive MES-SA cells (from 2.82 to 30.8 %), but had little effect in drug-resistant MES-SA/Dx5 cells (from 2.98 to 3.42 %) (Fig. 1b). These data indicate that MES-SA/Dx5 cells display cisplatin resistance, probably due to enhanced P-gp transporter activity.

We next investigated whether 1H3MC (Fig. 2a) altered the expression of P-gp. Immunoblot analysis showed that 1H3MC reduced the expression of P-gp in a time-dependent manner (Fig. 2b). When cells were cultured in medium containing 5-bromo-2′-deoxyuridine (BrdU), this pyrimidine analog is incorporated into cellular DNA during proliferation. To determine the effect of 1H3MC on the proliferation of MES-SA/Dx5 cells, exponentially growing MES-SA/Dx5 cells were exposed to 0.5 μg/ml cisplatin or 20 μM 1H3MC, and the cellular proliferation rate was measured by the detection of incorporated BrdU. Cisplatin had little effect on the cellular proliferation rate in drug-resistant MES-SA/Dx5 cells (Fig. 2c). However, when MES-SA/Dx5 cells were exposed to 1H3MC, cellular proliferation was strongly reduced, as compared with vehicle-treated controls (Fig. 2c). Cell viability was examined to determine the cytotoxicity of 1H3MC. The cell viability was greater than 90 % in cells treated with

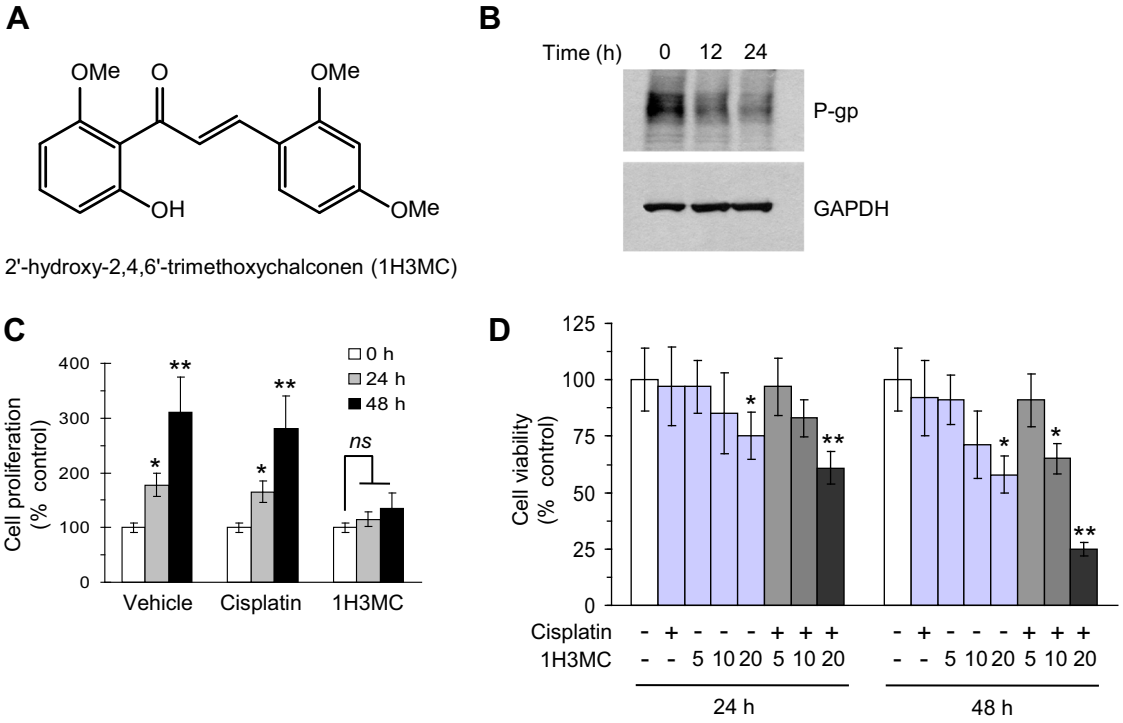


**Fig. 2** Effect of 1H3MC on MDR. **a** Chemical structure of 2′-hydroxy-2,4,6′-trimethoxychalcone (1H3MC). **b** Immunoblot analysis for the detection of P-gp protein levels using a mouse anti-Pgp antibody. **c** Inhibitory effect of 1H3MC on the growth of MES-SA/Dx5 cells. Cells were treated with 0.5 μg/ml cisplatin or 20 μM 1H3MC for 24 or 48 h. Cell growth rate was measured by BrdU incorporation into cellular DNA. The data represent the mean ± SD of one experiment performed in triplicate. $*p < 0.05$; $**p < 0.01$; *ns* not significant, compared with 0 h control. **d** The effect of 1H3MC on cell viability in MES-SA/Dx5 cells. The cells were treated with either 0.5 μg/ml cisplatin or increasing concentrations of 1H3MC, as indicated. Cell viability was determined using the Cell Counting Kit-8 assay kit. The data shown represent the mean ± SD of one experiment performed in triplicate. $*p < 0.05$; $**p < 0.01$, compared with 0 h control

cisplatin alone, whereas a significant decrease in cell viability was observed in cells treated with 1H3MC plus cisplatin, as compared with vehicle-treated control cells (Fig. 2d). Interestingly, combined treatment with cisplatin and 1H3MC exhibited synergistic cytotoxicity. These data suggest that 1H3MC overcomes the drug-resistant phenotype in MES-SA/Dx5 cells, probably due to downregulation of P-gp expression.

Activation of caspases is critical for the induction and progression of apoptosis (Danial and Korsmeyer 2004). To determine whether 1H3MC has the potential to sensitize MES-SA/Dx5 cells to cisplatin, MES-SA/Dx5 cells were treated with 0.5 μg/ml cisplatin and 20 μM 1H3MC for 24 h. Immunoblot analysis showed that cisplatin alone had no effect on the cleavage of caspase-3; however, combined treatment with cisplatin and 1H3MC synergistically increased cleavage of both caspase-3 and its substrate protein, poly(ADP-ribose) polymerase (PARP) (Fig. 3a). DNA agarose gel electrophoresis also showed that combined treatment with cisplatin and 1H3MC synergistically induced the fragmentation of genomic DNA, a hallmark of apoptosis, in MES-SA/Dx5 cells (Fig. 3b). These results suggest that 1H3MC reduced P-gp expression, thereby restoring the cytotoxic activity of cisplatin in drug-resistant MES-SA/Dx5 cells.

One of the major clinical problems in cancer chemotherapy is the frequent occurrence of MDR. It is generally thought that the overexpression of drug efflux pump proteins, such as P-gp, is a predominant cause of MDR development. P-gp is a product of the *MDR1* gene. The human *MDR1* promoter contains a nuclear factor (NF)-κB binding site, which is involved in carcinogen-induced *MDR1* gene expression (Kuo et al. 2002). Previously, we have demonstrated that 2-hydroxy-3′,5,5′-trimethoxychalcone inhibits lipopolysaccharide-induced NF-κB activation in BV2 microglial cells (Lee et al. 2012). Thus, it is possible that 1H3MC reduces *MDR1* gene promoter activity through the inhibition of NF-κB activity. Further analysis of the drug target involved in the regulation of P-gp expression may be necessary for the rational design of novel cancer therapeutics.

In summary, 1H3MC reduces P-gp expression and triggers apoptosis against drug-resistant MES-SA/Dx5 human uterine sarcoma cells. Combined treatment with

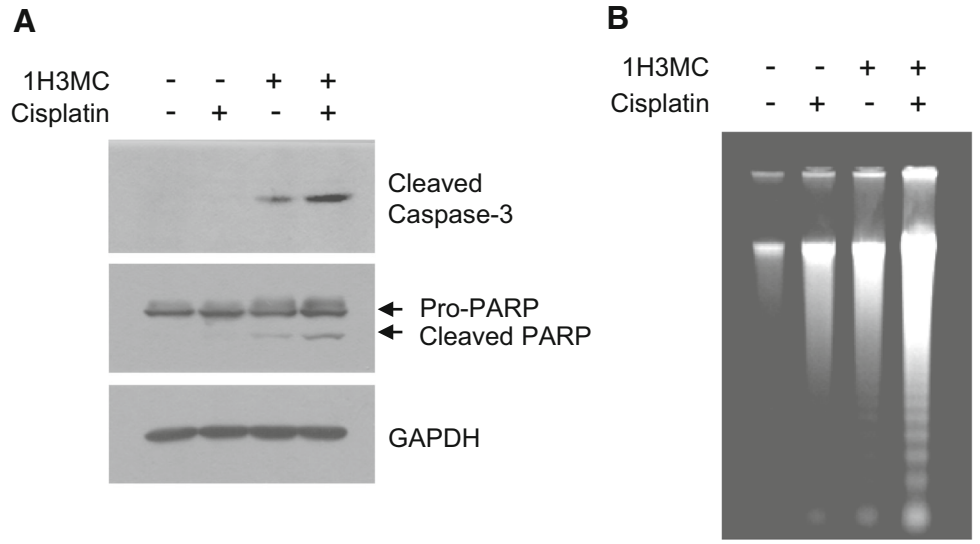


**Fig. 3** Effect of 1H3MC on the induction of apoptosis. **a** MES-SA/Dx5 cells were treated with either 0.5 μg/ml cisplatin or 20 μM 1H3MC, or combined as indicated. Whole-cell lysates were subjected to immunoblotting using antibodies against the indicated proteins. GAPDH antibody was used as an internal control to demonstrate equal protein loading. **b** MES-SA/Dx5 cells were treated with 0.5 μg/ml cisplatin or 20 μM 1H3MC for 24 h, and genomic DNA was extracted. DNA laddering was visualized by UV light illumination after 1.2 % agarose gel electrophoresis

1H3MC and cisplatin synergistically activated caspase-3, suggesting that 1H3MC has the potential to overcome MDR probably by reducing P-gp protein levels. Our findings suggest that methoxychalcone may be an attractive template for further development of chemotherapeutic adjuvants that overcome P-gp-mediated MDR in cancer cells.

**Acknowledgments** This paper resulted from the Konkuk University research support program.

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