Effects of Natural Polyphenols on the Expression of Drug Efflux Transporter P-Glycoprotein in Human Intestinal Cells

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ABSTRACT: The drug efflux transporter P-glycoprotein, which is encoded by MDRI (ABCB1), plays important roles in drug absorption, distribution, and elimination. We previously reported that dietary polyphenols such as quercetin, curcumin, honokiol, magnolol, caffic acid phenetyl ester (CAPE), xanthohumol, and anacardic acid inhibit P-glycoprotein-mediated drug transport. In the present study, we investigated the effects of polyphenols on the expression of P-glycoprotein using human intestinal epithelial LS174T cells and a reporter plasmid expressing 10.2 kbp of the upstream regulatory region of MDRI. Honokiol, magnolol, CAPE, xanthohumol, and anacardic acid activated the MDRI promoter in LS174T cells, and the cellular uptake of rhodamine 123 and calcein-AM, fluorescent substrates of P-glycoprotein, decreased in polyphenol-treated LS174T cells. These results suggest that dietary natural polyphenols can induce the drug efflux transporter P-glycoprotein and have the potential to promote food–drug interactions.

1. INTRODUCTION

P-glycoprotein, the protein encoded by the MDRI (ABCB1) gene, is a membrane transport protein expressed in the intestine, liver, kidney, placenta, and blood–brain barrier. P-glycoprotein mediates the cellular elimination of a wide variety of chemically unrelated drugs, including verapamil, digoxin, tacrolimus, clarithromycin, fexofenadine, and saquinavir. Therefore, human P-glycoprotein plays important roles in drug absorption, distribution, and elimination, and these effects ultimately determine drug efficacy and toxicity.1–4 For example, P-glycoprotein acts as a biochemical barrier in the apical membrane of enterocytes to restrict the absorption of orally administered drugs. Many clinically significant P-glycoprotein-mediated drug–drug interactions have been reported.1–4 We previously reported that multiple dietary natural polyphenols, including bioflavonoid quercetin, kaempferol, tea catechin epigallocatechin gallate (EGCG), curcumin, capsaicin, [6]-gingerol, resveratrol, sesamin, glycyrrhetinic acid, guggulsterone, and carnosic acid, inhibit the function of P-glycoprotein in P-glycoprotein-overexpressing human carcinoma cells.5–11

Fruits and vegetables are excellent sources of fiber, vitamins, and minerals. They also contain various natural polyphenols that can provide substantial health benefits beyond basic nutrition.12–14 Epidemiological and experimental studies have demonstrated that natural polyphenols can reduce the risk of cancer.12–14 The nuclear factor-κB (NF-κB) transcription factor and the pathways that control NF-κB activation play critical roles in the regulation of inflammation and oncogenesis.15,16 Several polyphenols, including EGCG, curcumin, capsaicin, guggulsterone, honokiol, magnolol, caffeic acid phenethyl ester (CAPE), xanthohumol, anacardic acid, thymoquinone, emodin, aloe-emodin, anethol, and eugenol, block NF-κB activation, thereby acting as chemopreventive agents in cancer.12–14

Multidrug resistance is a phenomenon characterized by the resistance of tumors to a wide spectrum of anticancer drugs, and it represents a significant obstacle to the success of cancer chemotherapy. P-glycoprotein is a transporter overexpressed in the membrane of multidrug-resistant cancer cells.1–3 The overexpression of P-glycoprotein renders cancer cells resistant to a variety of commonly used, pharmacologically unrelated anticancer drugs, including vinblastine, daunorubicin, doxorubicin, imatinib, irinotecan, and paclitaxel, by actively eliminating these drugs. Therefore, natural compounds that inhibit both NF-κB activation and anticancer drug efflux transporters might enhance the efficacy of chemotherapy. We recently examined the effects of natural polyphenols reported to inhibit NF-κB activation on the function of human P-glycoprotein. Several of these natural NF-κB inhibitors, including honokiol, magnolol, CAPE, xanthohumol, and anacardic acid, abolished multidrug resistance by inhibiting P-glycoprotein.17

It is well-established that many drugs and xenobiotics induce the expression of P-glycoprotein.4,16,19 The antibiotic rifampicin enhances the expression of P-glycoprotein in human enterocytes and inhibits the absorption of orally administered digoxin.18 Natural polyphenols can also influence the expression of P-glycoprotein. Ginkgolide B, a terpenoid isolated from Ginkgo biloba extract, enhances the expression of P-glycoprotein, whereas curcumin, a yellow pigment isolated from
turmeric (Curcuma longa), inhibits its expression.20,21 In the present study, we investigated the effects of natural polyphenols known to inhibit both NF-kB activation and P-glycoprotein function, on the expression of P-glycoprotein.

2. RESULTS

2.1. Effects of Polyphenols on MDR1 Promoter Activation. Figure 1 shows the chemical structures of several of the polyphenols used in this study. We first tested the effects of polyphenols on MDR1 promoter activation (Figure 2). The promoter construct containing the entire 10.2 kbp MDR1 promoter region (p-10224MDR) was used.22 At a concentration of 10 μM, rifampicin, honokiol, magnolol, CAPE, xanthohumol, anacardic acid, and ginkgolide B activated the MDR1 promoter in LS174T cells. In contrast, 10 μM thymoquinone, capsaicin, and [6]-gingerol had little or no effect on the MDR1 promoter. Rifampicin, honokiol, magnolol, CAPE, xanthohumol, and anacardic acid enhanced MDR1 promoter activation in a concentration-dependent manner (Figure 2).

The plant sterol guggulsterone is an active substance isolated from the guggul tree (Commiphora mukul), and it is used to treat hyperlipidemia, obesity, arthritis, and inflammation.12–14 Brobst et al.23 reported that guggulsterone activates human pregnane X receptor (PXR) but inhibits rifampicin-induced PXR activation, indicating that guggulsterone can function as both an agonist and antagonist of PXR. To investigate the antagonistic activity of polyphenols, LS174T cells were treated with 10 μM polyphenols for 1 h and further incubated with 10 μM rifampicin for 47 h. Honokiol, xanthohumol, and anacardic acid significantly enhanced rifampicin-induced MDR1 promoter activation (Figure 3), indicating that honokiol, magnolol, CAPE, xanthohumol, and anacardic acid exert agonistic, rather than antagonistic effects on the MDR1 promoter.

As honokiol activates retinoid X receptor α (RXRα),24 we examined the effects of PXR or RXRα on polyphenol-induced MDR1 promoter activation. The cells were treated with honokiol, magnolol, or ginkgolide B in the presence or absence of a vector expressing human PXR (pSG5-hPXR) or human RXRα (pCMX-hRXRα).25,26 PXR significantly enhanced honokiol-, magnolol-, and ginkgolide B-induced MDR1 promoter activation (Figure 4).

2.2. Effects of Polyphenols on P-Glycoprotein Substrates in Polyphenol-Treated LS174T Cells. Rhodamine 123 is a fluorescent P-glycoprotein substrate, and calcein-acetoxyethyl ester (calcein-AM), an acetoxyethyl ester of calcein, is a nonfluorescent human P-glycoprotein substrate.1 When calcein-AM crosses the membrane, cytosolic nonspecific esterases immediately convert it into the highly fluorescent compound calcein. The uptake of rhodamine 123 and calcein-AM decreased in cells treated with rifampicin, honokiol, magnolol, CAPE, xanthohumol, and anacardic acid, indicating that these polyphenols induced P-glycoprotein expression (Figure 6).

Figure 1. Chemical structures of dietary polyphenols.

Figure 2. Effects of polyphenols on MDR1 promoter activation. LS174T cells transfected with the MDR1 reporter vector were incubated with 5, 10, or 20 μM polyphenols for 48 h, and luciferase activity was subsequently analyzed. MDR1 luciferase activity was calculated using cells incubated with the vehicle (ethanol) as the control. Open bars: 5 μM, closed bars: 10 μM, and hatched bars: 20 μM. The data shown represent the mean ± SD of nine measurements from three independent experiments. *P < 0.05 and **P < 0.01 compared with the control.

Figure 3. Effects of polyphenols on MDR1 promoter activation in the presence of rifampicin. LS174T cells transfected with the MDR1 reporter vector were incubated with 10 μM polyphenols for 1 h. Then, the cells were incubated with 10 μM rifampicin for 47 h, and luciferase activity was subsequently evaluated. MDR1 luciferase activity was calculated using cells incubated in the medium without polyphenol or rifampicin as the control. The data shown represents the mean ± SD of nine measurements from three independent experiments. **P < 0.01 compared with rifampicin only.

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Figure 4. Effects of polyphenols on P-glycoprotein mRNA and Protein Expression. Figure 5 shows the real-time polymerase chain reaction (PCR) and western blot analyses of P-glycoprotein in the LS174T cells incubated with 10 μM polyphenols for 48 h. Rifampicin and honokiol significantly increased the mRNA and protein expression.

2.3. Cellular Uptake of Fluorescent P-Glycoprotein Substrates in Polyphenol-Treated LS174T Cells. Rhodamine 123 is a fluorescent P-glycoprotein substrate, and calcein-acetoxyethyl ester (calcein-AM), an acetoxyethyl ester of calcein, is a nonfluorescent human P-glycoprotein substrate.1 When calcein-AM crosses the membrane, cytosolic nonspecific esterases immediately convert it into the highly fluorescent compound calcein. The uptake of rhodamine 123 and calcein-AM decreased in cells treated with rifampicin, honokiol, magnolol, CAPE, xanthohumol, and anacardic acid, indicating that these polyphenols induced P-glycoprotein expression (Figure 6).
1. Closed bars: absence of human PXR or RXR transfecting with a vector expressing human PXR or RXR activity in cells transfected in the absence of a vector expressing human PXR or RXR. Open bars: human RXR incubated in the absence of polyphenols (control, +PXR).

3. DISCUSSION

Honokiol and magnolol are lignans isolated from the stem and bark of Magnolia officinalis and Magnolia obovata that are used in traditional Japanese and Chinese medicines. They are also used as dietary supplements and have been shown to exhibit anticancer, antiarthritic, and antiinflammatory properties.12–14 CAPE, an active component of honey bee hive-derived propolis, possesses anticarcinogenic, antimitogenic, and antiinflammatory properties. Xanthohumol, a prenylated chalcone isolated from hops (Humulus lupulus), is reported to inhibit NF-κB activation and exhibit anticancer properties. Anacardic acid (6-pentadecylsalicylic acid), a compound isolated from cashew nuts (Anacardium occidentale), exhibits anticancer and antiinflammatory properties. Thymoquinone (2-isopropyl-5-methylbenzoquinone), the most active component of black cumin (Nigella sativa) seed oil, exhibits antioxidant, anticancer, and antiinflammatory properties. Capsaicin, a pungent component of hot chili pepper (Capsicum annuum), is a suspected carcinogen or cocarcinogen based on its irritant properties in experimental animal models. However, some reports indicate that capsaicin exerts chemopreventive effects.12–14 [6]-Gingerol, a phenolic substance responsible for the spicy taste of ginger (Zingiber officinale), exhibits antioxidant, anticancer, and antiinflammatory effects. Thymoquinone, capsaicin, and [6]-gingerol had little or no effect on the MDR1 promoter. The accumulation of P-glycoprotein substrates decreased in cells incubated with the natural polyphenols honokiol, magnolol, CAPE, xanthohumol, and anacardic acid (Figure 6).

We previously reported that incubation of 50 μM honokiol, magnolol, CAPE, xanthohumol, and anacardic acid for 1 h increased the accumulation of fluorescent P-glycoprotein substrates in human P-glycoprotein-overexpressing KB/MDR1 cells.17 In contrast, thymoquinone, emodin, aloe-emodin,
honokiol functioned as an RXR glycoprotein function ranked as follows: magnolol > anacardic acid, and the inhibitory effect of polyphenols on P-glycoprotein function ranked as follows: magnolol > anacardic acid > honokiol > CAPE > xanthohumol.\(^\text{17}\) In the present study, we examined the effects of 48 h incubation of natural polyphenols on the MDR1 promoter. The ability of the polyphenols to activate the MDR1 promoter ranked as follows: honokiol > CAPE > xanthohumol > anacardic acid > magnolol (Figure 2). Therefore, there appears to be no correlation between the effects of polyphenols on NF-κB activation and the function and expression of P-glycoprotein, and the mechanisms underlying the effect of polyphenols on NF-κB and P-glycoprotein are different.

The nuclear receptor PXR plays a key role in the regulation of P-glycoprotein expression.\(^\text{4,19,22,27}\) PXR can be activated by a broad range of drugs and xenobiotics, including rifampicin, paclitaxel, dexamethasone, phenobarbital, and ginkgolide B.\(^\text{4,19,20,22,25}\) Activated PXR forms a heterodimer with RXRα and binds to a cluster of DNA response elements in a region between bp −7975 to −7013 in MDR1, thereby inducing gene transcription.\(^\text{22}\) Rifampicin activated the MDR1 promoter and increased the mRNA and protein expression in LS174T cells (Figures 2 and 5), and the uptake of P-glycoprotein substrates decreased in cells incubated with rifampicin for 4 days, indicating that P-glycoprotein was effectively induced (Figure 6). Satsu et al.\(^\text{20}\) demonstrated that ginkgolide B activated the MDR1 promoter and increased the mRNA and protein expression of P-glycoprotein. We confirmed that ginkgolide B activated the MDR1 promoter, and this effect was enhanced by exogenously expressed PXR (Figures 2 and 4). Similar to ginkgolide B, honokiol activated the MDR1 promoter, and this effect was enhanced by exogenously expressed PXR (Figures 2 and 4). Honokiol also increased the mRNA and protein expression, and the uptake of P-glycoprotein substrates decreased in cells incubated with honokiol (Figures 5 and 6). Honokiol and magnolol activated the MDR1 promoter, but the effects on the mRNA and protein expression of magnolol were less than those of honokiol. More studies on the structure−activity relationship are needed.

Honokiol, xanthohumol, and anacardic acid enhanced rifampicin-induced activation of the MDR1 promoter, and none of the polyphenols examined inhibited rifampicin-induced promoter activation (Figure 3). Kotani et al.\(^\text{24}\) reported that the methanol extract of Magnolia obovata activated RXRα and that honokiol functioned as an RXRα agonist. We evaluated the effect of PXR or RXRα on honokiol- and magnolol-induced activation of the MDR1 promoter (Figure 4). Human PXR, but not human RXRα, significantly enhanced honokiol- and magnolol-induced activation of MDR1, indicating that the activation of the MDR1 promoter by these polyphenols is primarily mediated by PXR.

Xu et al.\(^\text{28}\) and Han and Van Anh\(^\text{29}\) reported that honokiol decreased P-glycoprotein expression in P-glycoprotein-overexpressing multidrug-resistant ovarian cancer MCF-7/ADR (designated NCI/ADR-RES) cells. These results could be associated with PXR-independent and/or cell-type-specific mechanisms. Further studies are needed to clarify the precise mechanism of P-glycoprotein expression in different tissues.

Geick et al.\(^\text{25}\) reported that 10 μM rifampicin activates the MDR1 promoter in LS174T cells, and Satsu et al.\(^\text{20}\) demonstrated that 100 μM rifampicin and 10, 50, and 100 μM ginkgolide B induced P-glycoprotein expression in LS180 cells. On the basis of these findings, we selected 10 μM as the concentration of polyphenols to use in the study. The concentration of some polyphenols is higher in dietary supplements than in commonly consumed foods. For example, magnolia bark extract capsules with 8 mg of honokiol (30.04 μmol) are available as dietary supplements.

In conclusion, the MDR1 promoter was activated by natural polyphenols, honokiol, magnolol, CAPE, xanthohumol, and anacardic acid in human intestinal LS174T cells. Human PXR enhanced honokiol-induced MDR1 promoter activation. Honokiol increased the mRNA and protein expression of P-glycoprotein, and the uptake of P-glycoprotein substrates decreased in honokiol-treated LS174T cells. Together, these results suggest that natural polyphenol honokiol can induce the drug efflux transporter P-glycoprotein and have the potential to modulate the pharmacokinetics of drugs.

4. METHODS

4.1. Materials. Eagle’s minimum essential medium (EMEM), rhodamine 123, honokiol, magnolol, and [6]-gingerol were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Life Technologies (Carlsbad, CA, USA). Xanthohumol and ginkgolide B were obtained from Tokiwa Phytochemical Co., Ltd. (Chiba, Japan). Calcium-AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Anacardic acid was purchased from Enzo Life Sciences (Lausen, Switzerland). CAPE, thymoquinone, and rifampicin were obtained from Sigma-Aldrich (St. Louis, MO, USA). All of the other chemicals used in the study were of the highest purity available.

The promoter construct containing the entire 10.2 kb MDR1 promoter region (p-10224MDR) was kindly provided by Dr. Oliver Burk (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany).\(^\text{22}\) The vectors expressing human PXR (encoded by NR1I2), pSG5-hPXR, and human RXRα (encoded by RXRA), pCMX-hRXRα, were kindly provided by Prof. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX).\(^\text{23,24}\)

4.2. Cell Culture. LS174T cells (CL-188) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in EMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 ng/mL streptomycin. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air.

4.3. Luciferase Assay. LS174T cells (1 × 10⁵ cells/well) were seeded in 24-well plates and cultured for 24 h. Then, the cells were transiently transfected with 300 ng of MDR1 promoter firefly luciferase plasmid (p-10224MDR) and 200 ng of the control HSV-TK Renilla luciferase plasmid (pGL4.74) (Promega, Madison, WI, USA) in the presence or absence of 50 ng of the vector expressing human PXR or RXRα (pSG5-hPXR or pCMX-hRXRα, respectively) using FuGENE HD transfection reagent (Promega) for 24 h. Then, the cells were incubated with 5, 10, or 20 μM polyphenols for 48 h. The cells were lysed using Promega Reporter Lysis Buffer. Firefly and Renilla luciferase activity in cell lysates was measured using a Dual-Glo Luciferase Assay System (Promega) and GloMax-20/
20 luminometer according to the manufacturer’s instructions. Firefly luciferase activity was normalized to *Renilla* luciferase activity. The *MDR1* luciferase activity was calculated using cells incubated with the vehicle (ethanol) as the control.

### 4.4. Real-Time PCR and Western Blot Analysis

Reverse transcription real-time quantitative PCR assay was performed using a CellAmp Direct SYBR RT-qPCR Kit and a Thermal Cycler Dice Real Time System TP-800 (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. The specific primer pairs used for human *MDR1* and β-actin (*ACTB*) were as follows: *MDR1*, 5'-ATGTCACCATGATGAGATTGAG-3' and 5'-TGGCGATCCTGCTTCTGT-3'; *ACTB*, 5'-ACCGAGCCGCGCTAACA-3' and 5'-CAGCCGTGACCATCTTCT-3'. The threshold cycle (CT) value for each mRNA was determined using the crossing point method. The relative mRNA levels of *MDR1* were normalized to *ACTB* as follows: CT(*MDR1*) − CT(*ACTB*) = ΔCT. Then, the relative mRNA levels of *MDR1* after polyphenol treatment were calculated using the ΔΔCT method: ΔΔCT = ΔCT(polyphenol) − ΔCT(vehicle). The fold changes in mRNA levels of *MDR1* upon polyphenol treatment were expressed as 2−ΔΔCT.

Cell lysates were prepared using Læmmli sample buffer without 2-mercaptoethanol and bromophenol blue. Protein concentrations were measured using a DC Protein Assay kit (Bio-Rad). Then, 2-mercaptoethanol (final concentration 5% (v/v)) and bromophenol blue (final concentration 0.005% (w/v)) were added to the sample. The protein samples (3 μg protein/lane) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting using 4–20% Mini-POLYANNE TX gels (Bio-Rad) and Can Get Signal immunoreaction enhancer solution (Toyobo, Osaka, Japan). Antibodies used were as follows: mouse monoclonal C219 (Enzo Life Sciences) against human P-glycoprotein (1:100), horseradish peroxidase (HRP)-conjugated goat polyclonal antimouse IgG (1:5,000, Jackson Immunoresearch Inc., West Grove, PA, USA), and HRP-conjugated mouse monoclonal AC-15 (ab49900, Abcam plc., Cambridge, UK) against human β-actin (1:200 000). Immunostain Zeta (Wako Pure Chemical Industries Ltd.) was used for chemiluminescence detection of proteins with ImageQuant LAS 4000 (GE Healthcare UK Ltd., Little Chalfont, UK). Protein levels of P-glycoprotein were determined using the ImageQuant TL software (GE Healthcare UK Ltd.) and normalized to β-actin.

### 4.5. Measuring the Total Cellular Uptake of Fluorescent P-Glycoprotein Substrates

LS174T cells were seeded in 24-well plates incubated with 10 μM polyphenols for 96 h. After 96 h incubation, the medium was aspirated and washed to remove the remaining polyphenol. Polyphenol-treated cells were incubated with 20 μM rhodamine 123 or 1 μM calcine-AM for 1 h at 37 °C, and the medium was subsequently aspirated. Then, the cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with 0.1% Triton X-100 in PBS. Fluorescence intensity of rhodamine 123 and calcine-AM-derived calcine in the cells was measured using a DTX 880 microplate fluorimeter (Beckman Coulter, Inc., Indianapolis, IN, USA) with excitation and emission wavelengths of 485 and 535 nm, respectively. Protein concentrations were measured using the detergent-compatible bicinchoninic acid (BCA) method and a TaKaRa BCA protein assay kit (Takara Bio Inc.). Bovine serum albumin was used as the standard. Fluorescence intensities were normalized to protein concentrations. The uptake was calculated as the percent of the vehicle (ethanol) control.

### 4.6. Statistical Analysis

The data are expressed as the mean ± standard deviation (SD). The statistical significance of differences was determined using one-way analysis of variance followed by Dunnett’s test. P values < 0.05 were considered statistically significant.

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