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8-Prenylnaringenin is an inhibitor of multidrug resistance-associated transporters, P-glycoprotein and MRP1

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ABSTRACT

Flavonoids with hydrophobic e.g. prenyl substituents might constitute the promising candidates for multidrug resistance (MDR) reversal agents. The interaction of 8-prenylnaringenin (8-isopentenylnaringenin), a potent phytoestrogen isolated from common hop (*Humulus lupulus*), with two multidrug resistance-associated ABC transporters of cancer cells, P-glycoprotein and MRP1, has been studied for the first time. Functional test based on the transport of fluorescent substrate BCECF revealed that the flavonoid strongly inhibited MRP1 transport activity in human erythrocytes ($IC_{50} = 5.76 \pm 1.80$ μM). Expression of MDR-related transporters in drug-sensitive (LoVo) and doxorubicin-resistant (LoVo/Dx) human colon adenocarcinoma cell lines was characterized by RT-PCR and immunochemical methods and elevated expression of P-glycoprotein in resistant cells was found to be the main difference between these two cell lines. By means of flow cytometry it was shown that 8-prenylnaringenin significantly increased the accumulation of rhodamine 123 in LoVo/Dx cells. Doxorubicin accumulation in both LoVo and LoVo/Dx cells observed by confocal microscopy was also altered in the presence of 8-prenylnaringenin. However, the presence of the studied compound did not increase doxorubicin cytotoxicity to LoVo/Dx cells. It was concluded that 8-prenylnaringenin was not able to modulate MDR in human adenocarcinoma cell line in spite of the ability to inhibit both P-glycoprotein and MRP1 activities. To our best knowledge, this is the first report of 8-prenylnaringenin interaction with clinically important ABC transporters.

1. Introduction

8-Prenylnaringenin (8-isopentenylnaringenin), together with xanthohumol and isoxanthohumol, are members of a large group of prenylated chalcones and flavanones isolated from common hop (*Humulus lupulus*). Their major source in human diet is beer as hop female flowers are used as a flavoring agent and as a preservative in this beverage. 8-Prenylnaringenin has been identified as the most potent plant phytoestrogen, able to bind to both α and β estrogen receptors (Milligan et al., 1999, 2002). Additionally, it was found to be a potent inhibitor of aromatase, a key enzyme in estrogen biosynthesis (Monteiro et al., 2006). Apart from estrogenic activity, 8-prenylnaringenin is also recognized as an inhibitor of inflammation (Paoletti et al., 2009), angiogenesis (Pepper et al., 2004) and cancer cells proliferation (Delmulle et al., 2006; Lee et al., 2007). Moreover, there are some lines of evidence that prenylated flavonoids may modulate different steps of xenobiotics' metabolic activation by inhibiting cytochrome P450 (Henderson et al., 2000) as well as detoxification by affecting the expression of protein transporters associated with multidrug resistance (MDR) (Lee et al., 2007).

Multidrug resistance of cancer cells is either intrinsic or chemotherapy-induced resistance to many anticancer drugs that may be structurally and functionally unrelated. The resistant cells avoid being killed by the drugs because, among other MDR mechanisms, they overexpress proteins belonging to ABC transporters family. These proteins are multispecific transporters that utilize energy of ATP hydrolysis to pump anticancer agents out of the cells, reducing their concentration below therapeutically efficient levels. At least 12 human ABC transporters have been suggested to play a role in MDR (Lage, 2003), among them the most clinically relevant ones are: P-glycoprotein (P-gp, MDR1, ABCB1), Multidrug resistance-associated protein 1 (MRP1, ABCC1) and Breast cancer resistance protein (BCRP, ABCG2) (Sharom, 2008).

Many structurally divergent compounds that are able to minimize MDR by modulating either transport or ATPase activity of ABC transporters have been discovered. Flavonoids were first described to be potent MRP1 inhibitors (Leslie et al., 2001; Versantvoort et al., 1994), subsequently their ability to interact with BCRP (Zhang et al., 2004) and P-gp was discovered (Conseil et al., 1998). It was observed

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that the presence of prenyl groups in the flavonoid structure increased their ability to inhibit P-glycoprotein (Comte et al., 2001).

The aim of present work was to study interactions of a phytoestrogen 8-prenylnaringenin with two main MDR-related transporters, P-glycoprotein and MRP1. Human erythrocytes were used as a model system to investigate MRP1 transport activity. We have shown that 8-prenylnaringenin inhibited MRP1-mediated export of fluorescent probe BCECF out of the red blood cells. The flavanone also increased rhodamine 123 accumulation in doxorubicin-resistant human adenocarcinoma cell line LoVo/Dx characterized by elevated P-glycoprotein expression. The studied compound altered doxorubicin accumulation pattern in both sensitive and resistant cell line as observed by confocal microscopy but it was not able to increase doxorubicin toxicity to LoVo/Dx cells. To our best knowledge, this is the first report of 8-prenylnaringenin interaction with clinically important ABC transporters.

2. Materials and methods

8-Prenylnaringenin (8-isopentenylnaringenin), MK-571, novobiocin and fumitremorgin C were purchased from Alexis Biochemicals (Lausen, Switzerland). Doxorubicin, rhodamine 123 and verapamil were the products of Sigma (Poznan, Poland). BCECF-AM (2′,7′-bis-(3-carboxyethyl-5-(and-6)-carboxyfluorescein acetoxymethyl ester) was bought from Molecular Probes (Eugene, OR, USA). Other reagents used were of analytical grade. Rhodamine 123, doxorubicin, verapamil, and novobiocin were dissolved in water. All other compounds were dissolved in DMSO.

2.1. Human erythrocyte preparation

Blood collected from healthy volunteers by venous puncture with the use of EDTA as an anticoagulant was diluted with isoosmotic PBS and centrifuged (2000 × *g*, 5 min, 4 °C). The precipitate was resuspended in PBS and passed through the α-cellulose column to remove leukocytes and platelets. Then, erythrocytes were washed at least three times in PBS and suspended in the transport buffer containing 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose (pH 7.4). Cells were stored at 4 °C and used within 36 h.

2.2. Efflux of BCECF out of erythrocytes

Human erythrocytes constitute a convenient model to study transport activity of MRP1 (Rychlik et al., 2003; Wesolowska et al., 2005, 2007). The functional test is based on measuring the MRP1-mediated efflux of fluorescent probe BCECF out of erythrocytes. Acidic BCECF is formed inside red blood cells by the action of cellular esterases that cleave non-fluorescent, hydrophobic acetoxymethyl ester (BCECF-AM). Shortly, erythrocytes diluted in transport buffer (at 5% hematocrit) were incubated with appropriate concentration of 8-prenylnaringenin for 15 min (room temperature, darkness). Next, the samples were mixed with an equal volume of ice-cold 2 μM BCECF-AM solution in the same buffer and cells were allowed to load for 10 min on ice. Erythrocytes were incubated at 37 °C for 0, 20, 40 and 60 min in the dark. The incubation was finished by rapid cooling the samples on ice and subsequent centrifugation (14,000 × *g*, 3 min, 4 °C). MRP1 transport activity was monitored by measuring BCECF fluorescence in supernatant (excitation and emission wavelengths were 475 nm and 525 nm, respectively). Percentage of MRP1 inhibition was determined by comparing the slopes of fluorescence intensity *versus* time dependencies for control sample and the samples containing 8-prenylnaringenin. It was checked that 8-prenylnaringenin itself did not quench BCECF fluorescence. Maximal DMSO concentration in the samples was 0.5%, according to our previous observations DMSO below this concentration did not influence BCECF transport. Hemolysis level in the samples never exceeded 1%. Experiments were performed in triplicate, for each repetition blood from different donor was used.

2.3. Cell culture

Human colorectal adenocarcinoma cell line LoVo and its doxorubicin-resistant subline LoVo/Dx used in the study were a kind gift from Prof. E. Borowski (Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdansk, Poland). The drug resistance of LoVo/Dx cells was induced by prolonged exposure to doxorubicin (Grandi et al., 1986) and was shown to be severely reduced when the cells were cultured for 4 months in the absence of this drug (Sieder et al., 1999). Cells were cultivated in Ham's F12 (Cytogen, Lodz, Poland) medium supplemented with 10% Foetal Bovine Serum (Gibco), L-glutamine and antibiotics (10,000 U penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml) at 37 °C and 5% CO_2. Doxorubicin (at 100 ng/ml concentration) was constantly present in culture medium of LoVo/Dx cells to maintain their resistance. The drug was withdrawn a week before experiments. Cells were detached from the culture flasks by treatment with Non-enzymatic Cell Dissociation Solution (Sigma, Poznan, Poland).

2.4. RT-PCR detection of MDR-related transporters mRNA

Total RNA from 2.5×10^6 cells was isolated using NucleoSpin® RNA II (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. RNA (1 μg, measured photometrically) was transcribed into cDNA using Omniscript RT Kit (Qiagen, Hilden Germany). Shortly, cDNA synthesis was carried out for 60 min at 37 °C in a final volume of 20 μl (containing 0.5 mM of each dNTP, 1 μM of oligo-dT starter, 0.2 U/μl of Omniscript reverse transcriptase and 0.5 U/μl of RNase inhibitor). To perform semi-quantitative PCR the obtained cDNA was diluted (1, 10, 100 and 1000 times). The sequences of specific primers used for PCR amplification are presented in Table 1. The amplification reactions were performed in a total volume of 25 μl containing, apart from cDNA, 2 mM of $MgCl_2$, 0.25 mM of each dNTP, 0.25 μM of each sense and anti-sense primer and 0.1 U/μl of Taq DNA Polymerase (Fermentas, Vilnius, Lithuania). All reactions were performed in a thermal cycler T1 (Biometra, Goettingen, Germany) with 37 cycles for P-gp (58 °C annealing temperature) and MDR3 (60 °C annealing temperature), 36 cycles for BCRP (51 °C annealing temperature), 35 cycles for MRP1 (56 °C annealing temperature), 30 cycles for LRP (56 °C annealing temperature) and 23 cycles for β-actin (55 °C annealing temperature). The amplified fragments were separated in 2% agarose gel by electrophoresis, stained with ethidium bromide and photographed. Gene Ruler 100-bp DNA Ladder (Fermentas, Vilnius, Lithuania) was used as a size marker.

2.5. Immunochemistry

Cells seeded onto microscopy slides and grown for 24 h were fixed by ice-cold acetone:methanol (1:1) treatment (15 min, −20 °C) and stored at −20 °C until use. Before staining slides were hydrated in PBS with 1% Triton X-100. The incubation with primary antibodies diluted in Background Reducing Antibody Diluent (Dako, Gdynia, Poland) or IgG isotypic control Mouse Universal Negative Control (Dako) was held overnight at 4 °C. The following mouse monoclonal antibodies were used: P-gp-specific Mdr (G-1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000, MRP1-specific MRPm6, BCRP-specific BXP-21, MDR3-specific P_3II-26, LRP-specific MVP-37 (all from Alexis Biochemicals, Lausen, Switzerland; diluted 1:20). Subsequently, cells were washed and stained (60 min, room temperature, darkness) with horse anti-mouse IgG antibody conjugated with fluorescein (Vector Laboratories, Burlingame, CA, USA). Secondary antibody was diluted in Background Reducing Antibody Diluent (Dako, Gdynia, Poland) 1:500 in case of experiments with P-gp-specific antibody and 1:200 in all other experiments. Nuclei were stained for 20 min (room temperature, darkness) with 0.1 μg/ml DAPI (Sigma, Poznan, Poland). Fluorescent signals were detected with Eclipse TE2000-E microscope