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Regulation of expression and activity of multidrug resistance proteins MRP2 and MDR1 by estrogenic compounds in Caco-2 cells. Role in prevention of xenobiotic-induced cytotoxicity

Agostina Arias[a], Juan Pablo Rigalli[a], Silvina S.M. Villanueva[a], María L. Ruiz[a], Marcelo G. Luquita[a], Virginia G. Perdomo[a], Mary Vore[b], Viviana A. Catania[a], Aldo D. Mottino[a,*]

[a] Instituto de Fisiología Experimental (IFISE-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas (UNR), Rosario, Santa Fe, Argentina
[b] Graduate Center for Toxicology, University of Kentucky, Lexington, KY, USA

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ABSTRACT

ABC transporters including MRP2, MDR1 and BCRP play a major role in tissue defense. Epidemiological and experimental studies suggest a cytoprotective role of estrogens in intestine, though the mechanism remains poorly understood. We evaluated whether pharmacologic concentrations of ethynylestradiol (EE, 0.05 pM to 5 nM), or concentrations of genistein (GNT) associated with soy ingestion (0.1–10 μM), affect the expression and activity of multidrug resistance proteins MRP2, MDR1 and BCRP using Caco-2 cells, an in vitro model of intestinal epithelium. We found that incubation with 5 pM EE and 1 μM GNT for 48 h increased expression and activity of both MRP2 and MDR1. Estrogens did not affect expression of BCRP protein at any concentration studied. Irrespective of the estrogen tested, up-regulation of MDR1 and MRP2 protein was accompanied by increased levels of MDR1 mRNA, whereas MRP2 mRNA remained unchanged. Cytotoxicity assays demonstrated association of MRP2 and MDR1 up-regulation with increased resistance to cell death induced by 1-chloro-2,4-dinitrobenzene, an MRP2 substrate precursor, and by paraquat, an MDR1 substrate. Experiments using an estrogen receptor (ER) antagonist implicate ER participation in MRP2 and MDR1 regulation. GNT but not EE increased the expression of ERβ, the most abundant form in human intestine and in Caco-2 cells, which could lead in turn to increased sensitivity to estrogens. We conclude that specific concentrations of estrogens can confer resistance against cytotoxicity in Caco-2 cells, due in part to positive modulation of ABC transporters involved in extrusion of their toxic substrates. Although extrapolation of these results to the in vivo situation must be cautiously done, the data could explain tentatively the cytoprotective role of estrogens against chemical injury in intestine.

1. Introduction

The major functions of the intestine are to absorb nutrients and to protect the body against xenobiotics including carcinogens. Gastrointestinal absorption of xenobiotics is decreased by the presence of export pumps at the apical membrane of the enterocyte. These transporters belong to the ABC transporter family and actively mediate cellular extrusion, consequently restricting absorption of potentially toxic compounds contributing to the barrier function of the intestine (Dietrich et al., 2003). The most relevant members of these proteins are multidrug resistance-associated protein 2 (MRP2, ABCC2), multidrug resistance protein 1 (MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) (Mottino et al., 2000; Dietrich et al., 2003).

Abbreviations: MRP2, multidrug resistance-associated protein 2; MDR1, multidrug resistance protein 1; BCRP, breast cancer resistance protein; EE, ethynylestradiol; GNT, genistein; ER, estrogen receptor; AP-1, activating protein-1; Sp1, specificity protein-1; PQ, paraquat; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-SG, dinitrophenyl-S-glutathione; DNP-CG, dinitrophenyl-cysteinyl glycine; DMSO, dimethyl sulfoxide; CypA, cyclophilin A; PVDF, polyvinyl difluoride; PBS, phosphate buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretazolium bromide; FBS, fetal bovine serum; PXR, pregnane X receptor; HBSS, Hank's balanced salt solution; PCR, polymerase chain reaction; P, probenecid; V, verapamil.

* Corresponding author at: Instituto de Fisiología Experimental (IFISE-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas (UNR), Suipacha 570, S2002LRL, Rosario, Argentina. Tel.: +54 341 4305799; fax: +54 341 4399473.
E-mail addresses: amottino@unr.edu.ar, aldomottino@yahoo.com.ar (A.D. Mottino).

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MRP2 actively exports amphipathic organic drugs as well as hydrophilic anions in the form of glucuronide, sulfate and glutathione conjugates. It is preferentially expressed in the proximal intestine and the expression gradually decreases from the jejunum to the distal ileum (Mottino et al., 2000). MDR1 has broad substrate specificity for structurally and pharmacologically unrelated hydrophobic compounds and it is highly expressed on the apical surface of ileal and colon enterocytes, with decreasing expression proximally into the jejunum, duodenum and stomach (Takano et al., 2006). BCRP also has a role in limiting oral bioavailability of wide range of compounds with either negative or positive charge. Together with MDR1, BCRP expression is higher in the ileal enterocytes and shows similar distribution in mice and rats along duodenum, jejunum and colon (Enokizono et al., 2007).

Over the last years, epidemiological studies suggest a protective role of estrogens (Di Leo et al., 2001) and phytoestrogens such as genistein (GNT) (Barone et al., 2012) in chemical-induced colon injury and colorectal cancer development. Animal studies offer further evidence supporting the hypothesis that estrogens play a protective role against chemical injury at the intestine level (Guo et al., 2004; Weige et al., 2009). The origin of the estrogen protective effect remains unknown. Intracellular accumulation of xenobiotics and their biotransformation derivatives is minimized by ABC transporters. Considering that intestinal epithelium is constantly exposed to oral xenobiotics and the strategic localization of MRP2, MDR1 and BCRP at the apical membrane of the enterocyte, we postulate that their positive regulation by estrogens could contribute to estrogen protection, e.g. by preventing cellular damage.

We used the Caco-2 cell line, an established in vitro model for intestinal drug transport studies, to evaluate the effect of pharmacological concentrations of ethynylestradiol (EE) and concentrations of GNT associated with soy ingestion, on the expression and activity of MRP2, MDR1 and BCRP. We further determined if changes in expression and activity of these transporters protect against cytotoxicity produced by xenobiotics, substrates of these same transporters, and whether estrogen receptors are implicated as mediators.

2. Materials and methods

2.1. Chemicals

EE, GNT, probenecid, rhodamine 123 (Rh123), verapamil, leupeptin, phenylmethylsulfonyl fluoride, pepstatin A, CDNB, paraquat dichloride (methyl viologen; PQ), NADPH, 2-vinylpyridine, dithionitrobenzoic acid, glutathione reductase and glutathione were obtained from Sigma Chemical Co (St. Louis, MI, USA). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, HE, Germany). All other chemicals were of analytical grade purity and used as supplied. Dinitrophenyl-S-glutathione (DNP-SG) was synthesized with the use of 1-fluoro-2,4-dinitrobenzene (Mottino et al., 2001).

2.2. Cell culture and treatments

The human Caco-2 cell line was purchased from the American Type Culture Collection (Manassas, USA). Cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, PAA, Pasching, Austria), 1% (v/v) of L-glutamine, 1% (v/v) of a mixture of antibiotics (10,000 units/ml penicillin and 10,000 μg/ml streptomycin), 1% (v/v) of non-essential aminoacids (Cat number 11140050, Invitrogen, Carlsbad, CA, USA) and 6 ng/ml of amphotericin B (PAA, Pasching, Austria). They were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. For studies to quantitate protein and mRNA expression, cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and maintained in culture for 15 days. After that, the medium was changed to phenol red-free DMEM medium supplemented with 10% (v/v) charcoal-dextran treated FBS (Hyclone) (treatment media) and EE (0.05, 0.5, and 5 pM or 0.05, 0.5 and 5 nM) or GNT (0.1, 1, and 10 μM) were added, dissolved in DMSO. Only DMSO was added to control cells (C). The treatment time was 48 h and the treatment medium was renewed every 24 h. The final concentration of DMSO in the culture media was always below 0.1%. Alternatively, cells were pretreated for 30 min with the ER antagonist 7a,17b-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182/780, 1 μM) (Tocris Cookson Inc., Ellisville, MO, USA) followed by treatment with EE or GNT for 48 h. The inhibitor was present during the treatment time. Cell viability after all the treatments was assessed measuring the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretazolium bromide (MTT) to its formazan, which was quantified spectrophotometrically ($\lambda_{measurement}$ = 530 nm, $\lambda_{reference}$ = 630 nm) using a microplate reader LD-400 (Beckman Coulter, Brea, CA, USA). We found that the rate of MTT conversion was not statistically different between cells treated with estrogens and the respective controls (data not shown).

2.3. Western blot studies

At the end of incubations, cells were washed twice with cold PBS and scraped using RIPA buffer (Thermo Scientific, Rockford, IL, USA) supplemented with phenylmethylsulfonyl fluoride (1 mM), leupeptin (5 mg/ml) and pepstatin A (5 μg/ml) as protease inhibitors. Lysates were passed 20 times through a 25-G needle and subjected to protein concentration determination. Expression of MRP2 and MDR1 were additionally determined in total cellular membranes, which were prepared as described (García et al., 2001). Western blotting was performed as previously described (Ruiz et al., 2005) using anti-MRP2 (M2 III-6, Alexis Biochemicals, Carlsbad, CA, USA), anti-MDR1, anti-BCRP, anti-ERα, anti-ERβ, anti-c-FOS, anti-c-JUN and anti-Sp1(H-241, BXP-21, H-184, H-150, H-79, sc-420, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Equal loading and transference of protein was systematically checked by both detection of β-actin using a monoclonal antibody to human β-actin (Sigma–Aldrich, St. Louis, MO, USA), and staining of the membranes with Ponceau S. The immunoreactive bands were quantified with the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

2.4. RNA isolation and quantitative real-time PCR

At the end of incubations, cells were washed twice with cold PBS and scraped using TRIzol® reagent (Invitrogen Carlsbad, CA, USA) and total RNA was isolated according to the manufacturer's instructions. cDNA was produced using the Superscript Preamplification System for the first strand cDNA synthesis using random hexamers, according to the manufacturer's instructions (Invitrogen Carlsbad, CA, USA). Real-Time quantitative PCR was performed on cDNA samples using the Platinum SYBR Green qPCRSuperMix-UDG (Invitrogen Carlsbad, CA, USA) and reactions were carried out on a Stratagene Mx3000P (Stratagene, La Jolla, CA, USA). Sequences of primer pairs and conditions for MRP2, MDR1, Cyclophilin A (CypA) and rRNA 18S were designed to optimally detect the respective mRNAs. For MRP2 mRNA amplification, the forward and reverse primer sequences were, respectively, 5′AGGTTTGCCAGTTATCCGTG3′ and 5′AACAAAGCCAACAGTGTCCC3′. For MDR1 mRNA amplification, the forward and reverse primer sequences were 5′CCAAAGACAACAGCTGAAA3′ and 5′TACTTGGTGGCACATAAAC3′, respectively. Results were normalized to CypA or rRNA 18S as housekeeping genes, using CypA forward and reverse primers, respectively, 5′ GTCAACCCCACCGTGTTCTTC3′ and 5′TTTCTGCTGTCTTTGGGACCTTG3′ and forward and reverse rRNA 18S primers 5′CGCCGCTAGAGGTGAAATTC3′ and 5′TTGGCAAATGCTTTCGCTC3′ respectively. Relative levels of MRP2 and MDR1 mRNAs normalized to CypA or rRNA 18S were calculated based on the $2^{-\Delta\Delta Ct}$ method.

2.5. Transport activities

The activity of MRP2 was determined by the ability of Caco-2 cells to extrude DNP-SG, a model substrate, into the incubation medium. This methodology was previously used to evaluate Mrp2 activity in rat intestinal epithelium (Mottino et al., 2001). Briefly, cells were cultured in 96-well plates and treated with EE or GNT (5 pM or 1 μM, respectively; 48 h), as described above. The treatment medium was replaced with fresh medium containing DNP-SG (100 μM, dissolved in PBS) and cells were incubated at 37 °C for 30 min. At the end of the incubation, the medium was removed, cells were washed twice with PBS and immediately incubated with Hank's balanced salt solution (HBSS) at 37 °C for 30 min, in the presence or absence of probenecid (P, 1 mM), to confirm MRP2 participation (Bakos et al., 2000). Cells were then scraped with HBSS and sonicated. Cell lysates and incubation media were treated with 10% perchloric acid and centrifuged (2 min, 14,000 × g, 4 °C). Supernatants were used for detection of DNP-SG and dinitrophenyl-cysteinyl glycine (DNP-CG) by HPLC (Waters 600; Waters, Milford, MA, USA) as described (Mottino et al., 2001). DNP-CG is the result of γ-glutamyltransferase action on DNP-SG at the apical membrane of Caco-2 cells (Vermeulen et al., 2011). The activity of MRP2 was determined through calculation of the relationship between the amount of DNP-SG + DNP-CG accumulated in the medium after 30 min of incubation and the total amount of DNP-SG available to be extruded. The total amount available to be extruded was estimated as the sum of the amount of DNP-SG remaining in the cells at the end of incubation and that of DNP-SG + DNP-CG extruded into the medium along the 30-min period.

The activity of MDR1 was estimated as described by Rigalli et al. (2011), through assessment of intracellular retention of the model substrate Rh123, which inversely correlates with the ability of the cells to extrude the probe. Briefly, cells were treated with EE or GNT (5 pM or 1 μM respectively; 48 h), and at the end of incubation, treatment medium was replaced with fresh medium containing Rh123 (5 μM, dissolved in DMSO), with or without verapamil (100 μM, dissolved in DMSO), as MDR1 inhibitor (Kota et al., 2010). Cells were incubated for 2 h to allow the probe to enter

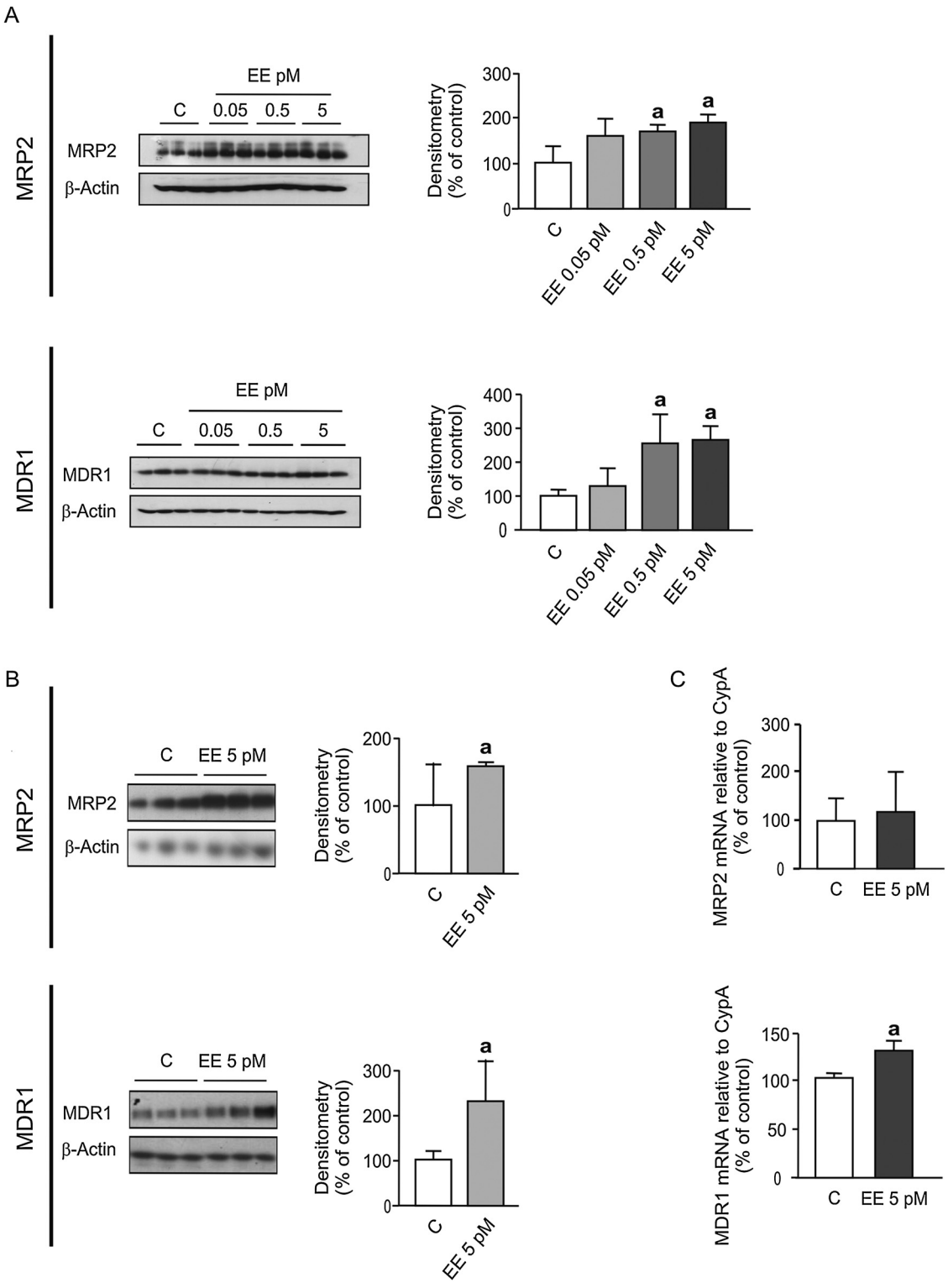


Fig. 1. Effect of EE on expression of MRP2 and MDR1. MRP2 and MDR1 protein expression were detected in total lysates (panel A) or in total cellular membranes (panel B) by western blotting. MRP2 and MDR1 mRNA expression were detected by real time PCR (panel C). Caco-2 total lysates were prepared after 48 h of treatment with EE (0.05, 0.5, 5 pM or 0.05, 0.5, 5 nM) or solvent (control cells, C). For western blotting, equal amounts of total protein (18 μg for MRP2 and 10 μg for MDR1 and 10 μg for MRP2 and 5 μg for MDR1 for total lysates and total cellular membranes, respectively) were loaded in all lanes. Densitometry results were related to β-actin and presented as percentage of controls. Uniformity of loading and transfer from gel to PVDF membranes was also controlled with Ponceau S. MRP2 and MDR1 mRNA levels were detected by real time PCR and presented as % of C. Cyclophilin A was used as a housekeeping gene. Data on western blot and real time PCR studies represent means ± SD ($n = 6$). [a] Significantly different from C, $p < 0.05$.

the cells (Marguerite et al., 2007), then, washed twice with cold PBS, scraped with sucrose 0.3 M and lysed by sonication. The amount of Rh123 in the lysates was determined fluorometrically with a Multimode Detector DTX-880 (Beckman Coulter), $\lambda_{excitation} = 485$ nm, $\lambda_{emission} = 535$ nm.

2.6. Cytotoxicity assays

To test the potential protective role of estrogen treatments against the toxicity produced by CDNB (Diah et al., 1999), Caco-2 cells were seeded in 96-well plates

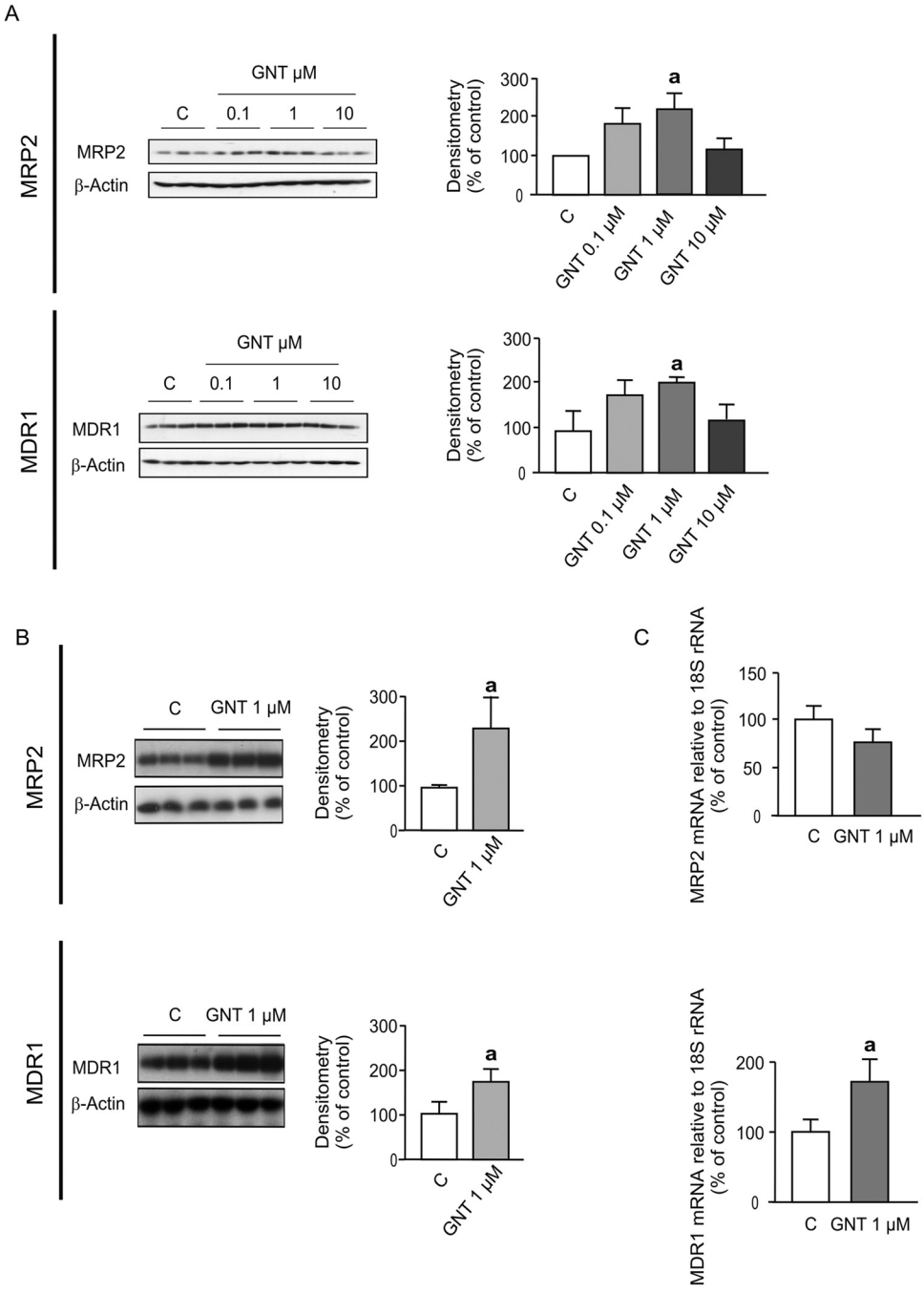


Fig. 2. Effect of GNT on expression of MRP2 and MDR1. MRP2 and MDR1 protein expression were detected in total lysates (panel A) or in total cellular membranes (panel B) by western blotting. MRP2 and MDR1 mRNA expression were detected by real time PCR (panel C). Caco-2 cell total lysates were prepared after 48 h of treatment with GNT (0.1, 1, 10 μM) or solvent (control cells, C). For western blotting, equal amounts of total protein (18 μg for MRP2 and 10 μg for MDR1 and 10 μg for MRP2 and 5 μg for MDR1 for total lysates and total cellular membranes, respectively) were loaded in all lanes. Densitometry results were related to β-actin and presented as percentage of controls. Uniformity of loading and transfer from gel to PVDF membranes was also controlled with Ponceau S. MRP2 and MDR1 mRNA levels were detected by real time PCR and presented as % of C. 18S rRNA was used as an internal control. Data on western blot and real time PCR studies represent means ± SD ($n = 6$). [a] Significantly different from C, $p < 0.05$.

at a density of 8.5×10^3 cells/well and maintained in culture for 15 days. CDNB is detoxified by conjugation with glutathione and thus converted into DNP-SG. Its detoxifying process is completed by extrusion of DNP-SG from the cells, mediated by MRPs (Diah et al., 1999), allowing for continuous, efficient conversion of CDNB into DNP-SG (Townsend et al., 1998). To estimate protection exerted by MDR1, PQ, a toxic and widely used herbicide, substrate of MDR1, was used in the assays (Silva et al., 2011). In both CDNB and PQ cytotoxicity assays, cells were cultured as described above and medium replaced by treatment medium containing estrogens (5 pM EE and 1 μM GNT) or DMSO (controls). The medium was renewed every 24 h, and after 48 h, cells were incubated with a fresh medium containing different concentrations of CDNB (0–75 μM) or PQ (0–175 mM), for an additional 24 h. Cell viability was measured by MTT assay, as described above. The data were best adjusted to a sigmoidal curve using the GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). The goodness of adjustments was confirmed with R^2 values, which were 0.990 or higher in both studies. The IC_{50} value, which represents the concentration of CDNB or PQ resulting in 50% viability, was compared between control and estrogen-treated cells.

We also evaluated intracellular concentration of total and oxidized glutathione in total cell lysates, as an additional component that could explain cytoprotection by estrogens. Cells were lysed by sonication in 5 mM EDTA in PBS, and total and oxidized glutathione were measured as described (Griffith, 1980) followed by calculation of intracellular reduced glutathione (GSH) concentration.

2.7. Statistical analysis

Data are presented as the means ± SD. The Comparison between groups was performed using the Student's *t*-test or one way ANOVA followed by the Newman–Keuls test (when more than two groups were compared). Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Effect of EE and GNT on expression of MRP2, MDR1 and BCRP

Expression of MRP2, MDR1 and BCRP was evaluated by western blotting, followed by real time PCR in case of finding positive results on protein expression. Cells treated with either 0.5 or 5 pM EE for 48 h showed an increase in MRP2 (+75% and +88%) and MDR1 (+158% and +162%) protein expression, with respect to control cells (Fig. 1A); no effects were observed when cells were treated with all other concentrations tested. Additional experiments were performed to determine protein expression of MRP2 and MDR1 in total cellular membranes as well as their mRNA levels in cells treated with 5 pM EE, a concentration producing a maximal effect in total lysates. Fig. 1B shows an increase in MRP2 (+56%) and MDR1 (+128%) protein expression, with respect to control cells. Real time PCR studies demonstrate an increase only for MDR1 mRNA (+32%, Fig. 1C), with no changes for MRP2 mRNA. No changes in BCRP protein expression were observed in total lysates after EE treatments at any of the concentrations tested (data not shown).

Treatment with 1 μM GNT for 48 h increased MRP2 and MDR1 protein expression by 120% and 99%, respectively, when compared to control cells (Fig. 2A). Fig. 2B shows that a similar induction was detected in total cellular membranes after treatment with 1 μM GNT (134% and 71% increases for MRP2 and MDR1 respectively). At this same concentration, GNT produced an increase in MDR1 mRNA (+70%), but did not affect MRP2 mRNA (Fig. 2C). Incubations with 0.1 or 10 μM GNT did not affect protein expression of these same transporters. No changes in BCRP expression were observed at any concentration of GNT tested (data not shown).

3.2. Effect of EE and GNT on transport activity

MRP2 activity was evaluated using DNP-SG as a model substrate. Efflux and intracellular content of DNP-SG were measured in the presence or absence of probenecid as MRP2 inhibitor. The ratio between the amount of DNP-SG extruded by Caco-2 cells (DNP-SG + DNP-CG in the medium) and its total (intracellular + medium) content, assessed at the end of incubations, was considered an estimation of transport activity. Treatment with 5 pM EE and 1 μM GNT increased the ratio by 39% and 99%, respectively, when compared to control cells (Fig. 3A and B). Probenecid decreased the DNP-SG efflux/total DNP-SG ratio in control and treated cells to the extent that former differences between groups were abolished.

MDR1 activity was evaluated using Rh123 as a model substrate. Its intracellular accumulation was measured in the presence or absence of verapamil as MDR1 inhibitor. The intracellular accumulation of Rh123 correlates inversely with MDR1 extrusion activity. Treatment with 5 pM EE, and 1 μM GNT decreased substrate accumulation by 19% and 17%, respectively, when compared to control cells (Fig. 4A and B). Verapamil increased Rh123 accumulation in control and treated cells, resulting in similar activities between groups.

3.3. Effect of EE and GNT on cytotoxicity

The protective effect of EE and GNT against CDNB and PQ cytotoxicity was evaluated through determination of the cell survival

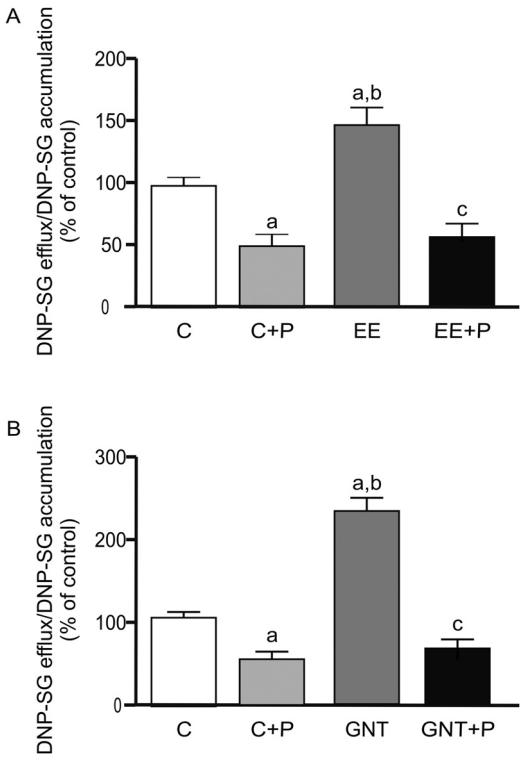


Fig. 3. Effect of EE and GNT on MRP2 transport activity. Caco-2 cells were pretreated with 5 pM EE (Panel A) and 1 μM GNT (Panel B), or with solvent (control cells, C) for 48 h, before transport assay. The activity of MRP2 was determined through calculation of the relationship between the amount of DNP-SG (DNP-SG + DNP-CG) accumulated in the medium after 30 min of incubation and the total amount of DNP-SG available to be extruded, in the presence or absence of probenecid (P, 1 mM). Data are expressed as percentage of C and represent means ± SD ($n = 4$). [a] Significantly different from C, $p < 0.05$. [b] Significantly different from C + P, $p < 0.05$. [c] Significantly different from EE or GNT, $p < 0.05$.

(Schinkel and Jonker, 2003; Rigalli et al., 2011). The MTT data were used to perform the viability curves and the IC_{50} values were further calculated.

IC_{50} values related to CDNB cytotoxicity were higher in cells treated with 5 pM EE, and 1 μM GNT (33.3 ± 0.5 and 29.8 ± 0.1 μM, respectively) than in control cells (25.5 ± 0.5 μM). To confirm an association between these changes and changes in MRP2 expression and activity, this study was repeated in the presence of probenecid (see groups EE + P, GNT + P and C + P in Fig. 5). The MRP2 inhibitor increased the cytotoxic effect of CDNB, as evidenced by increased IC_{50} values detected in either control or estrogen groups. Interestingly, the IC_{50} value was also decreased by probenecid in cells not treated with estrogens, suggesting a role for constitutively expressed MRP2 in cytoprotection.

Similarly, the IC_{50} values related to PQ cytotoxicity were higher in cells treated with 5 pM EE and 1 μM GNT (8.8 ± 0.8 and 11.6 ± 0.5 mM, respectively) vs. control cells (6.8 ± 0.5 mM). To confirm a correlation between the observed increases in IC_{50} values and induction of MDR1 expression and activity, MTT study was also repeated in the presence of verapamil (EE + V, GNT + V and C + V, Fig. 6). We found an increase in the cytotoxic effect of PQ under conditions of MDR1 inhibition in either control or estrogen groups. As in the case of MRP2, decreased value of IC_{50} by verapamil in control cells suggests a cytoprotective role for constitutively expressed MDR1.

In addition to regulation of expression and activity of MDR1 and MRP2 by estrogenic compounds, and considering the potential participation of oxidative stress as a mediator of CDNB and PQ toxic actions, the level of cytosolic GSH was also assessed. The

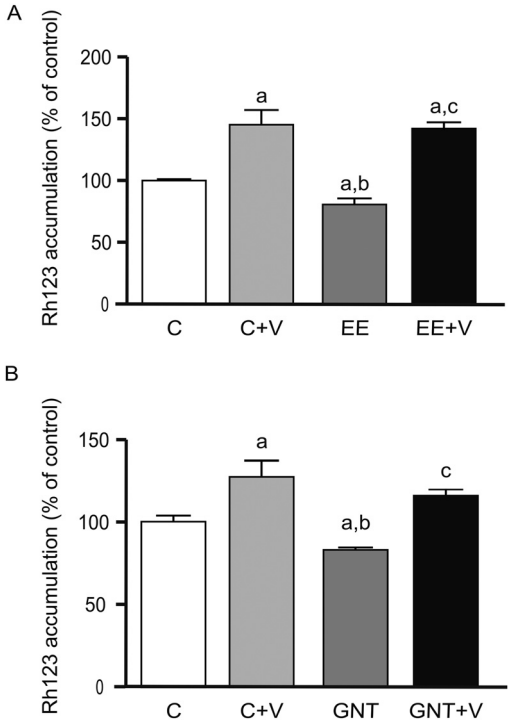
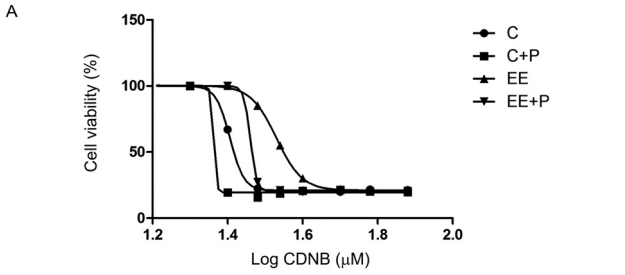
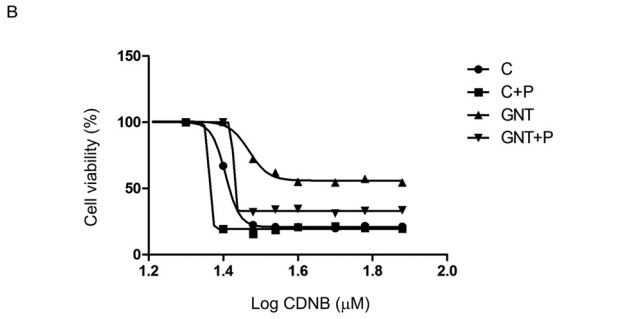


Fig. 4. Effect of EE and GNT on MDR1 transport activity. Caco-2 cells were pretreated with 5 pM EE (Panel A) and 1 μM GNT (Panel B), or with solvent (control cells, C) for 48 h, before transport assay. Rh123 cell accumulation was measured fluorometrically in the presence or absence of verapamil (V, 100 μM), and inversely correlated with MDR1 transport activity. Samples were taken after 2 h of incubation. Data are expressed as percentage of controls and represent means ± SD ($n = 4$). [a] Significantly different from C, $p < 0.05$. [b] Significantly different from C + V, $p < 0.05$. [c] Significantly different from EE or GNT, $p < 0.05$.



	C	C+P	EE	EE+P
IC_{50} (μM)	25.7 ± 0.5	22.5 ± 0.4[a]	33.3 ± 0.5[a,b]	29.0 ± 0.1[a,b,c]



	C	C+P	GNT	GNT+P
IC_{50} (μM)	25.7 ± 0.5	22.5 ± 0.4[a]	29.8 ± 0.1[a,b]	26.3 ± 0.5[b,c]

Fig. 5. Effect of EE and GNT on CDNB-induced cytotoxicity. Caco-2 cells were treated with 5 pM EE (Panel A), and 1 μM GNT (Panel B), or solvent (control cells, C) for 48 h. The medium was then replaced with a medium containing increasing concentrations of CDNB (0, 10, 20, 25, 30, 35, 40, 45, 50, 60 and 75 μM), in the presence or absence of probenecid (P, 1 mM), for additional 24 h. Cell viability was measured by MTT and dose-response curves were fitted using the least squares method. The curves shown represent the average of 3 individual curves per group. Individual IC_{50} values from the different groups were compared by ANOVA followed by the Newman–Keuls test. The data represent means ± SD ($n = 3$). [a] Significantly different from C, $p < 0.05$. [b] Significantly different from C + P, $p < 0.05$. [c] Significantly different from EE or GNT, $p < 0.05$.

data indicate that neither EE (10.0 ± 5.8 μg/μg protein) nor GNT (6.0 ± 2.2 μg/μg protein) induced any change in GSH levels when compared to control cells (6.5 ± 1.9 μg/μg protein) ($n = 4$; $p > 0.05$). This reinforces the relevance of transporters up-regulation as a major mechanism explaining cytoprotection.

3.4. Effect of ICI 182/780 on EE- and GNT-induced up-regulation of MRP2 and MDR1

To evaluate whether ER mediates transporter modulation by EE and GNT, we measured MRP2 and MDR1 protein expression after treatment with 5 pM EE (Fig. 7A) and 1 μM GNT (Fig. 7B) for 48 h, in the presence or absence of ICI 182/780. We found that MRP2 and MDR1 protein up-regulation was abolished by the ER antagonist (EE vs. EE + ICI and GNT vs. GNT + ICI in Fig. 7). Interestingly, ICI 182/780 itself increased expression of MRP2 and MDR1 when compared to control cells (C vs. C + ICI in all panels of Fig. 7), suggesting an agonistic action in absence of EE or GNT.

3.5. Effect of EE and GNT on expression of ERα and ERβ

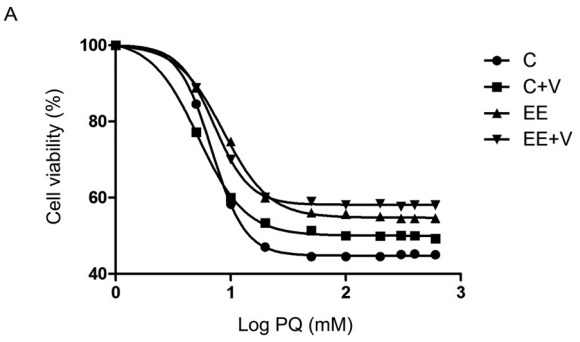
We evaluated protein expression levels of ERα and ERβ after treatment with 5 pM EE and 1 μM GNT for 48 h. We observed that GNT increased ERβ protein expression by +138% over control cells (Fig. 8), and that this effect was abolished by ICI 182/780. GNT treatment did not affect ERα protein expression and neither ERα nor ERβ were modulated by EE (data not shown).

3.6. Effect of EE and GNT on expression of activating protein-1 (AP-1) and specificity protein-1 (Sp1)

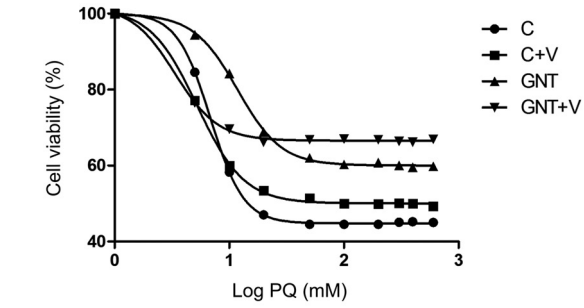
C-Jun and c-Fos are components of dimeric AP-1, and together with Sp1 constitute transcription factors regulating multiplicity of genes. Cells treated with 1 μM GNT for 48 h exhibited a decrease in c-Jun protein expression by 94% with respect to control cells (Fig. 9A). In contrast, this same treatment increased Sp1 levels (+76% Fig. 9B). Incubation of Caco-2 cells with 5 pM EE only affected expression of c-Jun (−19%, Fig. 9A), with no influence on Sp1 levels (Fig. 9B). No changes in c-Fos protein expression were observed in response to either EE or GNT treatment (data not shown).

4. Discussion

Caco-2 cells express the ABC relevant transporters MDR1, MRP2 and BCRP (Englund et al., 2006). These cells have been largely used in pharmacology and toxicology studies, and represent a reliable in vitro model of the human intestinal epithelium (Press and Di Grandi, 2008). In the current study we used this model to investigate if pharmacologic concentrations of a synthetic estrogen, EE, or concentrations of GNT, associated with soy ingestion, affect the



	C	C+V	EE	EE+V
IC_{50} (mM)	6.8 ± 0.5	4.4 ± 0.9 [a]	8.8 ± 0.8 [a,b]	6.2 ± 0.4 [c]



	C	C+V	GNT	GNT+V
IC_{50} (mM)	6.8 ± 0.5	4.4 ± 0.9 [a]	11.6 ± 0.5 [a,b]	5.5 ± 1.9 [c]

Fig. 6. Effect of EE and GNT on PQ-induced cytotoxicity. Caco-2 cells were treated with 5 pM EE (Panel A), and 1 μM GNT (Panel B), or solvent (control cells, C) for 48 h. The medium was then replaced with a medium containing increasing concentrations of PQ (0, 5, 10, 20, 30, 40, 50, 75, 100 and 175 mM), in the presence or absence of verapamil (V, 100 μM), for additional 24 h. Cell viability was measured by MTT and dose-response curves were fitted using the least squares method. The curves shown represent the average of 3 individual curves per group. Individual IC_{50} values from the different groups were compared by ANOVA followed by the Newman–Keuls test. The data represent means ± SD ($n = 3$). [a] Significantly different from C, $p < 0.05$. [b] Significantly different from C + P, $p < 0.05$. [c] Significantly different from EE or GNT, $p < 0.05$.

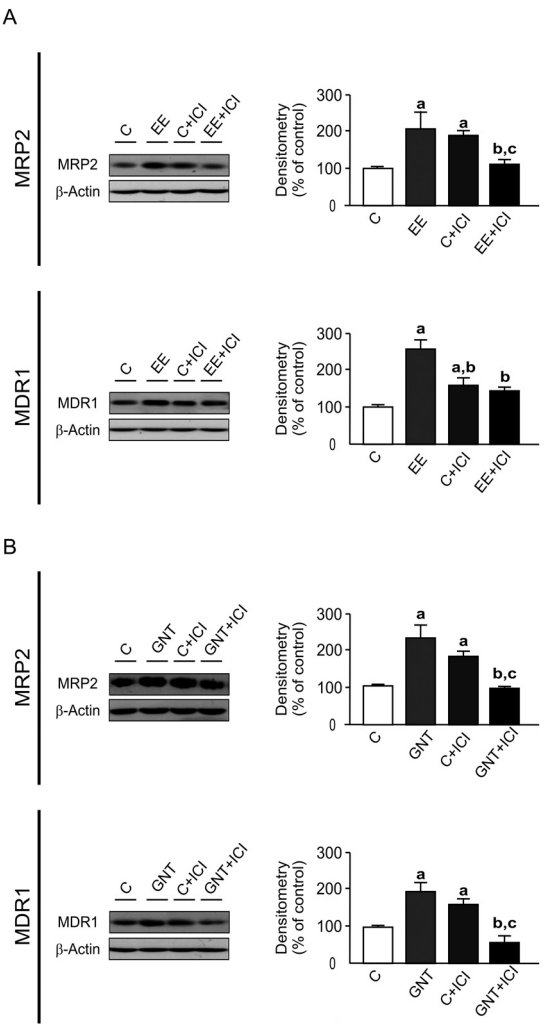


Fig. 7. Mediation of ERs in MRP2 and MDR1 protein up-regulation by EE and GNT. MRP2 and MDR1 were detected by western blotting. Caco-2 cells were treated for 48 h with 5 pM EE (Panel A), 1 μM GNT (Panel B) or solvent (control cells, C) in the presence or absence of ICI 182/780 (ICI, 1 μM). Equal amounts of total protein (18 μg for MRP2 and 10 μg for MDR1) were loaded in all lanes. Densitometry results were related to β-actin and presented as percentage of controls. Uniformity of loading and transfer from gel to PVDF membranes was also controlled with Ponceau S. Data represent means ± SD ($n = 6$). [a] Significantly different from C, $p < 0.05$. [b] Significantly different from EE or GNT, $p < 0.05$. [c] Significantly different from C + ICI, $p < 0.05$.

expression (protein and mRNA) and activity of MRP2, MDR1 and BCRP. Additionally, we evaluated the impact of these regulations on cell survival upon exposure to toxic xenobiotics. Mediation of ERs in regulation of expression of MDR1 and MRP2 by EE and GNT was also explored, as well as its potential association with expression of the transcription factors AP-1 and Sp1. EE at a concentration well in the range of plasma levels found in women taking contraceptives (5 pM) or even lower (0.5 pM) (Hammond et al., 1982; Goldzieher and Stanczyk, 2008), induced an increase in protein expression and activity of MRP2 and MDR1, whereas BCRP was not affected at any of the concentrations of EE tested. Incubation of the cells with 1 μM GNT, a concentration associated with moderate soy ingestion (Setchell and Cassidy, 1999), induced the expression and transport activity of MRP2 and MDR1. Detection of BCRP expression demonstrated no changes at any GNT concentration. In view of the importance of membrane localization of ABC transporters in their functionality, we further confirmed up-regulation of MRP2

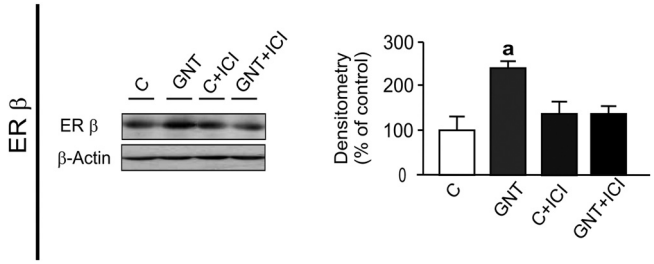


Fig. 8. Effect of GNT on expression of ERβ. ERβ protein was detected by western blotting. Caco-2 cells were treated for 48 h with 1 μM GNT or solvent (control cells, C) in the presence or absence of ICI 182/780 (ICI, 1 μM). Equal amounts of total protein (18 μg for MRP2 and 10 μg for MDR1) were loaded in all lanes. Densitometry results were related to β-actin and presented as percentage of controls. Uniformity of loading and transfer from gel to PVDF membranes was also controlled with Ponceau S. Data represent means ± SD ($n = 6$). [a] Significantly different from C, $p < 0.05$.

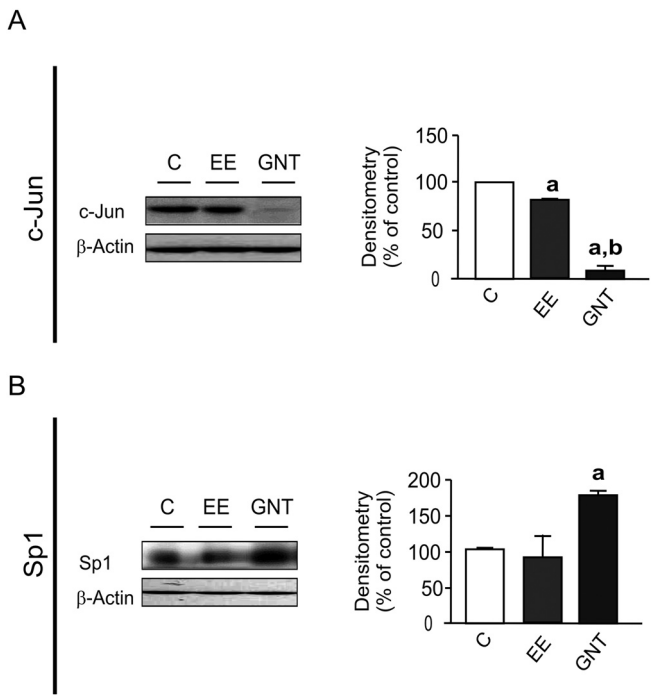


Fig. 9. Effect of EE and GNT on expression of c-Jun and Sp1. C-Jun (panel A) and Sp1 (panel B) protein expression were detected by western blotting. Caco-2 cell were treated with 5 pM EE, 1 μM GNT, or solvent (control cells, C) for 48 h. Equal amounts of total protein (20 μg) were loaded in all lanes. Densitometry results were related to β-actin and presented as percentage of controls. Uniformity of loading and transfer from gel to PVDF membranes was also controlled with Ponceau S. Data represent means ± SD ($n = 4$). [a] Significantly different from C, $p < 0.05$. [b] Significantly different from EE, $p < 0.05$.

and MDR1 using total cellular membranes. The effects observed for EE and GNT clearly suggest increased protection against absorption of MRP2 and MDR1 substrates under relevant pharmacological or nutritional conditions.

In general, biological actions of estrogenic compounds are mediated by ERs (Bjornstrom and Sjoberg, 2005). The binding of estrogens and estrogenic compounds to ERs produces genomic effects (Heldring et al., 2007; Katzenellenbogen et al., 2000) that regulate gene transcription, but may also produce non-genomic effects (Levin, 2005). Our experiments performed in the presence of the ER antagonist ICI 182/780 suggest ER participation in MRP2 and MDR1 up-regulation by EE and GNT. Interestingly, ICI 182/780 itself up-regulated MRP2 and MDR1 in absence of EE or GNT. Its action as agonist of ERs was already described in other studies and likely depended on the gene and cell type analyzed (Robertson, 2001; Zhao et al., 2006). MRP2- and MDR1-up-regulation by ICI 182/780 is probably linked to our current experimental conditions, with use of fetal bovine serum free of endogenous steroids in the incubations. In contrast, when incorporated to the incubation medium together with EE or GNT, ICI 182/780 exhibited antagonist activity, thus confirming ER participation in EE and GNT actions. ER regulates transcription processes through binding to DNA at ER response element (ERE) in the promoter region of target genes (classic way). Two isoforms of ER may be implicated, ERα and ERβ, which can act as ligand-activated transcription factors (Oseni et al., 2008). Caco-2 cells as well as epithelial cells in the digestive tract express predominatly ERβ (Konstantinopoulos et al., 2003). Interestingly, ERβ was the only form to be induced by GNT and this was abolished by ICI 182/780. This suggests self-regulation of expression of this specific form that could lead, in turn, to increased sensitivity to GNT action. Consistent with our findings, it was reported that ERβ presents an ERE in its promoter region (Li et al., 2000), which may be responsible for estrogens regulation of ERβ expression, as demonstrated here for GNT.

A second, non-classical mechanism of ER action involves interaction between the steroid-receptor complex and transcription factors such as AP-1 and Sp1, that in turn bind to their cognate DNA elements to modulate gene transcription (Marino et al., 2006). To gain insight into the mechanisms mediating MDR1 induction by EE and GNT, we performed an in silico analysis using the TFSEARCH database (http://www.cbrc.jp/research/db/TFSEARCH.html). We could not identify any ERE in the MDR1 promoter region. However, the analysis located binding sites for AP-1 and for Sp1, suggesting an indirect action of the ERs involving these transcription factors. We found that EE and GNT decreased c-Jun protein expression, a major component of AP-1. Increased c-Jun expression, activation and subsequent binding to DNA, was associated to down-regulation of MDR1 gene expression in different human cell lines (Miao and Ding, 2003). Also, AP-1 binding sequence in the promoter of mdr-1a negatively regulates its gene expression in mice (Ikeguchi et al., 1991). These reports could explain our findings on up-regulation of MDR1 under GNT and EE treatment conditions. In contrast, up-regulation of Sp1 has been positively linked to expression of MDR1 in human cell lines (Chan et al., 2013; Hua et al., 2003), thus representing an alternative explanation for GNT-induced MDR1 up-regulation, as currently observed. The mechanism involved in regulation of expression of AP-1 and Sp1 by EE or GNT, involving participation of ERs, was not explored in the current study.

The mechanism explaining the up-regulation of MRP2 protein by EE and GNT is less known. Because mRNA levels were not affected, a post-transcriptional regulation is likely involved. Dissociation between MRP2 protein and mRNA has been well documented (Gerk and Vore, 2002). Different hypothesis have been postulated to explain such dissociation involving participation of a nuclear receptor (Johnson and Klaassen, 2002). For example, it was described a negative regulation of MRP2 protein synthesis by micro-RNAs (miRNAs) (Haenisch et al., 2011). Regulation of expression of miRNAs, in turn, can be mediated by nuclear receptors. ERβ has been reported to down-regulate several clusters of miRNA in the colon in association with cancer prevention (Edvardsson et al., 2013). Other forms of protein modulation without concomitant changes in mRNA may implicate modifications in translation efficiency, protein degradation, etc. Further studies are necessary to provide direct evidence on the mechanism/s selectively affecting MRP2 protein expression by EE and GNT.

Modification of expression of MDR1 and MRP2 by the estrogenic compounds was currently evidenced at specific concentrations, and not at lower or higher concentrations. Estrogens are able to act as xenobiotic and interact with other type of nuclear receptors like pregnane X receptor (PXR). Both EE and GNT are activators of human PXR (Jacobs et al., 2005; Mnif et al., 2007). Because the affinities of xenobiotics for PXR are much lower than that of estrogens for ERs, estrogenic derivatives such as EE or GNT could exhibit a biphasic response, with selective estrogenic activities at low concentrations, but more complex, estrogenic and xenobiotic activities, at high concentrations (Mnif et al., 2007). This kind of response has been described for EE and GNT. For example, in the human estrogen receptor (ER)-positive MCF-7 breast cancer cell line, GNT has been shown to exert both proliferative (estrogenic) and antiproliferative (antiestrogenic) effects (Wang et al., 1996). At low concentrations, GNT stimulates the expression of pS2 mRNA, a specific marker of ER-mediated estrogen-like activity (Wang et al., 1996), whereas the antiproliferative effects, also evidenced in ER-negative cell lines, are not likely mediated by ER (Wang et al., 1996), but rather by PXR (Jacobs et al., 2005). A similar behavior could be predicted for EE, since the up-regulation of MRP2 and MDR1 currently observed at EE concentrations in the nM range contrasts with their down-regulation when EE was administered to rats at a

repeated, high cholestatic dose (Arias et al., 2009), or when Caco-2 cells were incubated with 100 μM EE for 48 h (unpublished results). Alternatively, high concentrations of estrogenic compounds such as GNT and EE may result in ER inactivation through preferential recruitment of co-repressors vs. co-activators (Sane et al., 2008), or may induce receptor degradation (Tateishi et al., 2004; Tiong et al., 2012).

In contrast to MRP2 and MDR1, expression of BCRP was not affected by any of the estrogen treatments. Gutmann et al. (2005) found no significant differences in expression of BCRP along the intestine between adult women and men, suggesting that sexual hormones probably exert no regulation on this particular transporter. Our results also agree with those from Ebert et al., who reported that GNT has no effect on BCRP mRNA expression levels in Caco-2 cells when incubated at either 1 or 10 μM (Ebert et al., 2007). There are no previous reports on the effects of EE on human intestinal BCRP, but physiologic levels of estradiol decrease BCRP expression in ERα-positive human breast cancer MCF-7 cells, whereas no changes were detected in the lung cancer cell line A549 (Imai et al., 2005). This selective response to estradiol could be linked to the observation that MCF-7 cells are ERα-positive, whereas A549 are ERα-negative but ERβ-positive, so that estrogen-mediated BCRP down-regulation would depend on signaling pathways downstream of ERα (Imai et al., 2005). Absence of regulatory action of EE and GNT on BCRP expression in intestine and intestinal cell lines, as also demonstrated in our study, could be tentatively linked to preferential expression of ERβ over ERα.

The intestinal mucosa is daily exposed to food contaminants and pollutants. The abundant food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, benzo[a]pyrene, 7,12-dimethylbenz[a]anthracene, mycotoxins, among others, are examples of contaminants, substrates of MRP2 and MDR1 (Dietrich et al., 2003; Leslie et al., 2007; Penny and Campbell, 1994; Videmann et al., 2007). We postulate that up-regulation of expression and activity of MRP2 and MDR1 should confer protection against xenobiotic-induced cytotoxicity. CDNB is a highly toxic hydrophobic compound, and its conjugation leads to formation of DNP-SG, a less toxic derivative and established substrate of MRP2 (Chan et al., 2004), which is further extruded from the cells in order to complete the overall detoxification process (Townsend et al., 1998). CDNB was chosen as a model toxin to evaluate detoxification processes involving MRP2 participation. Cytotoxicity induced by CDNB was characterized by an immediate, marked, and persistent decrease in the cellular concentration of reduced glutathione with formation of oxidized glutathione (Minhas and Thornalley, 1995). We evaluated the cytotoxic effect of CDNB in cells exposed to 5 pM EE or 1 μM GNT for 48 h. All estrogen treatments resulted in decreased CDNB cytotoxicity as shown by the increase in the IC_{50} values relative to control cells. We also used probenecid, an MRP2 inhibitor, to confirm the association between MRP2 induction and the reduction in CDNB-induced cytotoxicity. The protective effects exerted by EE and GNT were only partially abolished by probenecid, suggesting that other participants, apart from MRP2, contribute to protection against CDNB toxicity. PQ is the third most extensively used herbicide in the world, causing thousands of deaths due to accidental or intentional ingestion (Silva et al., 2011). Studies performed by Nagao et al. suggested that PQ is absorbed through a specialized mechanism associated with a carrier-mediated transport system for choline on the brush border membrane of the enterocytes (Nagao et al., 1993). This carrier-mediated transport system for choline is present in Caco-2 cells (Kamath et al., 2003), allowing PQ to accumulate inside the cells (Silva et al., 2011). We used PQ as a model MDR1 substrate to perform cytotoxic assays. The protective effects mediated by estrogenic compounds were studied after treatment of the cells with 5 pM EE or 1 μM GNT for 48 h. Both treatments significant decreased PQ cytotoxicity as shown by the increase in the IC_{50} values. The MDR1 inhibitor, verapamil, was used to investigate whether estrogen-mediated MDR1 induction is associated with reduction in PQ-induce cytotoxicity. The analysis of IC_{50} values in the presence of verapamil, resulting in values similar to those seen in controls, confirmed that EE and GNT protective effects were indeed associated with induction of MDR1. Further studies are necessary to demonstrate a similar protective effect of these specific concentrations of estrogens in the in vivo situation.

In summary, we demonstrated that the estrogens tested at relevant concentrations up-regulate the expression and activity of MRP2 and MDR1, concomitantly protecting Caco-2 cells against toxicity induced by CDNB and PQ. ERs are likely implicated. Although any extrapolation to the human being must be cautiously done, the data suggest that estrogen-induction of two major apical ABC transporters exerts a cytoprotective action against chemical injury in the intestine.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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