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ORIGINAL CONTRIBUTION

The phytoestrogens daidzein and equol inhibit the drug transporter BCRP/ABCG2 in breast cancer cells: potential chemosensitizing effect

Juan Pablo Rigalli[1,2] · Paul Niklas Scholz[1] · Guillermo Nicolás Tocchetti[1,2] · María Laura Ruiz[2] · Johanna Weiss[1]

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Abstract
Purpose The soy isoflavone genistein has been described to up-regulate breast cancer resistance protein (BCRP) and, thus, enhance chemoresistance in breast cancer cells. The aim of this work was to assess the effect of long- and short-term incubation with daidzein, the second most abundant soy isoflavone and its metabolite equol on the expression and activity of P-glycoprotein, multidrug resistance-associated proteins 1 and 2 (MRP1 and MRP2) and BCRP in breast cancer cells.
Methods MCF-7 and MDA-MB-231 cells were treated with phytoestrogen concentrations within the range achieved in individuals with a high isoflavone intake. Transporter expression was evaluated at protein and mRNA level through western blot and qRT-PCR, respectively. Transporter activity was determined using doxorubicin, mitoxantrone and carboxy-dichlorofluorescein as substrates.
Results Daidzein (5 µM) up-regulated MRP2- and down-regulated MRP1 protein expressions in MCF-7 and MDA-MB-231 cells, respectively. Both effects were ER-dependent, as determined using the antagonist ICI 182,780. The decrease in MRP1 mRNA in MDA-MB-231 cells indicates a transcriptional mechanism. On the contrary, MRP2 induction in MCF-7 cells takes place post-transcriptionally. Whereas changes in the transporter expression had a minor effect on the transporter activity, acute incubation with daidzein, R-equol and S-equol led to a strong inhibition of BCRP activity and an increase in the IC_{50} of BCRP substrates.
Conclusions In contrast to previous reports for genistein, daidzein and equol do not provoke a major up-regulation of the transporter expression but instead an inhibition of BCRP activity and sensitization to BCRP substrates.

Keywords Breast cancer · Phytoestrogens · Daidzein · Equol · Breast cancer resistance protein · Chemotherapy

Introduction

Breast cancer constitutes one of the most common and aggressive cancers with more than 1.3 million cases and 500,000 deaths per year worldwide [1, 2]. Chemotherapy with cytostatic agents represents one of the main treatment strategies and, moreover, constitutes the only pharmacological approach available in the triple negative disease. Nevertheless, therapy failure and recurrences appear in at least 25% of the cases and contribute to breast cancer being one of the two most frequent causes of cancer-related death in women [1].

Drug transporters of the ABC (ATP-binding cassette) superfamily are transmembrane proteins mediating the active efflux of a wide range of substrates, including therapeutic drugs. Intrinsic ABC transporter overexpression is a frequent mechanism underlying cancer multidrug resistance, characterized by reduced intracellular accumulation and diminished therapeutic efficacy of multiple cytostatic agents [3]. Similarly, transporter expression can be up-regulated during the course of the disease, also leading to a higher efflux of chemotherapeutic agents [4]. In breast cancer, higher levels of P-glycoprotein (P-gp/ABCB1) [5],

✉ Johanna Weiss
johanna.weiss@med.uni-heidelberg.de

[1] Department of Clinical Pharmacology and Pharmacoepidemiology, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany

[2] Institute of Experimental Physiology (IFISE-CONICET), Suipacha 570, 2000 Rosario, Argentina

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multidrug resistance-associated protein 1 (MRP1/ABCC1) [6], multidrug resistance-associated protein 2 (MRP2/ABCC2) [7] and breast cancer resistance protein (BCRP/ABCG2) [8] were correlated with a reduced efficacy of chemotherapy and poorer outcome in breast cancer patients.

To date, most attempts to counteract multidrug resistance, either by down-regulation or inhibition of ABC transporters, have yielded poor results. For instance, the use of transporter inhibitors, although showing promising results in vitro, has been associated with high toxicity and side effects in vivo, so hindering a potential application in the clinical practice [9, 10]. On the contrary, multidrug resistance induction by xenobiotics up-regulating the transporter expression was reported [11, 12]. Dietary compounds like β-carotene and tangeritin increased P-gp and MRP2 expression and activity in cancer cells [13, 14]. Similarly, treatment of a colon cancer cell line with grapefruit juice resulted in a marked P-gp, MRP1, MRP2 and BCRP up-regulation [15]. Further interactions of dietary compounds with drug transporters and an impact on the chemotherapy efficacy cannot be ruled out.

Phytoestrogens are compounds present mainly in soybeans and red clover used as hormone replacement therapy, for example, to counteract menopause-associated symptoms. The most abundant compounds within this group are genistein and daidzein [16, 17]. Daidzein can be metabolized by the intestinal flora yielding S-equol, a compound with a higher estrogenic activity than the parent substance [18]. Noteworthy, not all the individuals have the capacity to produce S-equol. The frequency of equol producers oscillates between 25–30% in western countries and 50–60% in east Asiatic countries [18]. Although only S-equol can be formed in vivo after daidzein intake, the chemical synthesis process applied in the production of equol supplements leads to a racemic mixture of both R-equol and S-equol. Most of the biological effects of phytoestrogens in mammalians arise as a consequence of their binding to ERα and ERβ [18]. Evidence regarding the risk and benefits of phytoestrogens in relation with breast cancer is highly controversial [16, 19]. Although several studies pointed out a decrease in mortality and recurrence of breast cancer patients consuming soy-rich food or phytoestrogen supplements [20, 21], in vitro evidence has shown both stimulatory and inhibitory effects on breast cancer cell proliferation [16, 22, 23].

In a previous work from our group, we have demonstrated the induction of the expression and activity of BCRP by genistein in the breast cancer cell lines MCF-7 and MDA-MB-231. The increase in BCRP expression correlated with reduced intracellular accumulation and increased resistance to doxorubicin and mitoxantrone. Additionally, our results indicated an acute inhibition of MRP1 activity by genistein [24], an observation also reported in a pancreatic cancer cell line [25]. Currently, there is scarce information concerning the modulatory potential of daidzein, the second most abundant soy isoflavone and its metabolite equol, especially at pharmacologically relevant concentrations, on the expression and activity of ABC transporters in breast cancer cells.

The aim of the current work was to assess the effect of daidzein, R-equol and S-equol on the expression and activity of P-gp, MRP1, MRP2 and BCRP in breast cancer cells. Furthermore, we evaluated the acute effect of the phytoestrogens on the transporter activity. Our results suggest an acute inhibition of BCRP activity and a concomitant chemosensitizing effect towards substrates of this transporter by all phytoestrogens tested.

Materials and methods

Materials

Culture media, supplements, MK571, 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA), mitoxantrone, and the β-actin antibody (A2228) were from Sigma-Aldrich (Taufkirchen, Germany). Daidzein, R-equol, S-equol, puromycin and the anti-GAPDH antibody (G-9) were from Santa Cruz Biotechnology (Heidelberg, Germany). Doxorubicin was from Biotrend (Cologne, Germany). Crystal violet and dimethylsulfoxide (DMSO) were from Applichem (Darmstadt, Germany). Fumitremorgin C (FTC) and the anti-P-gp antibody (C-219) were from Merck (Darmstadt, Germany). ICI 182,780 (fulvestrant) was from Tocris (Bristol, UK). The anti-MRP1 antibody (MC-898) was from Kamiya Biomedical Company (Tukwila, USA), the anti-MRP2 (M2-III-6) and the anti-BCRP (BXP-21) antibodies were from Enzo Life Sciences (Lörrach, Germany). All chemicals were of analytical grade quality.

Cell lines and treatments

MCF-7 cells were used as a model of $ER\alpha^+$ positive breast cancer. MDA-MB-231 cells were used as a model of triple negative breast cancer, characterized by the absence of $ER\alpha^+$, human epidermal growth factor receptor 2 and progesterone receptor [26]. Both cell lines express ERβ [27, 28]. Regarding ABC transporter expression, MCF-7 cells express MRP1, MRP2 and BCRP, while MDA-MB-231 express P-gp, MRP1 and MRP2 [29–31]. Cell lines are available at the European Collection of Authenticated Cell Cultures (Salisbury, UK). Cells were cultured as previously described [32]. Treatment medium consisted of phenol-red free Dulbecco's modified Eagle Medium (for MCF-7 cells) or phenol-red free RPMI1640 (for MDA-MB-231 cells) supplemented with charcoal-dextran treated FCS. For expression studies (protein and mRNA) cells were seeded in T-25 flasks (5×10^5 cells/flask). Daidzein, R-equol and S-equol were added at concentrations related to plasma

concentrations reported for individuals consuming a soy-rich diet or phytoestrogen-rich formulations with up to 100 mg of isoflavones (0.05, 0.5 and 5 µM for daidzein; 0.1, 1 and 10 µM for R-equol and S-equol) [18, 33, 34]. Since equol-containing formulations may contain both enantiomers, both R-equol and S-equol were tested. Cells were incubated with the phytoestrogens or the vehicle (DMSO) for 48 h. The participation of the ERs in the regulation of the transporter expression was assessed by coincubation with ICI 182,780, an ERα and ERβ antagonist (1 µM) [35]. To assess whether alterations in the transporter expression by phytoestrogens lead to changes in the transporter activity (i.e. efflux of a substrate), cells were seeded in 6-well plates (3.5×10^5 cells/well) and treated with the phytoestrogen concentrations above mentioned. Then, drug efflux assays were performed (see "Drug efflux assays"). For cell viability assays, MCF-7 or MDA-MB-231 cells were seeded at a density of 1×10^4 cells/well or 3.5×10^3 cells/well, respectively, in 96-well plates. Then medium was replaced by treatment medium containing daidzein (10 µM), R-equol (10 µM), S-equol (10 µM) or DMSO (control cells). After 24 h of incubation, treatment medium was supplemented with doxorubicin (DOX, 0–10 mM) or mitoxantrone (MXR, 0–10 mM). Cells were further incubated for 24 h. Following, crystal violet staining was performed (see "Cell viability assays").

MDA-MB-231 BCRP$^+$ cells were generated by stable transfection of parental MDA-MB-231 cells with the pLZRS-MS-BCRP-IRES-GFP plasmid, constructed as previously described [36]. Transfection was performed by electroporation using a Nucleofector™ 2b device and the Cell Line Nucleofector® Solution V (Lonza, Basel, Switzerland). Transfected cells were selected by addition of puromycin to the culture medium. BCRP overexpression was evaluated at the protein level (see "Protein expression analysis"). Following, the acute effect of the phytoestrogens on MXR efflux and the impact on the cell viability assays were assessed.

Protein expression analysis

Western blot studies were performed as already described [24]. GAPDH and β-actin were used as loading controls. Immunoreactive bands were developed with the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, USA) using a FluorChem Q device (Protein Simple, San Jose, USA). MRP2 expression in plasma membranes of MCF-7 cells was evaluated by western blot as previously described [37].

mRNA expression analysis

mRNA expression was evaluated through qRT-PCR for transporters and treatments for which a significant effect at the protein level was observed. RNA was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Taufkirchen, Germany). cDNA synthesis and qPCR were performed using the Revert Aid H Minus First Strand cDNA Synthesis Kit and the Absolute QPCR SYBR Green Mix (Thermo Fisher Scientific, Waltham, USA), respectively. β2-microglobuline (β2mg) and β-glucuronidase (GU) were used as housekeeping genes in MCF-7 and MDA-MB-231 cells, respectively. Primer sequences and thermal profiles have been described previously [24, 38].

Drug efflux assays

We assessed the efflux of doxorubicin (DOX) and mitoxantrone (MXR), as model drugs used in breast cancer. DOX is transported by P-gp, MRP1, MRP2 and BCRP [3, 39], while MXR is a BCRP and P-gp substrate [3, 40]. DOX efflux was evaluated after a 48-h pre-exposure to the phytoestrogens (conditions leading to changes in transporter expression) or with phytoestrogen addition only during the efflux phase (acute effect, not associated with changes in the transporter expression). Since no effects on BCRP or P-gp expression by phytoestrogens were observed, MXR efflux was evaluated only with phytoestrogen addition during the efflux phase (acute effect).

For experiments with phytoestrogen pre-exposure, cells were treated with the corresponding substance for 48 h, washed with PBS and incubated in fresh medium with DOX (10 µM) for 2 h. A set of cells was immediately lysed to assess intracellular DOX accumulation. Another set of cells was rinsed with PBS and incubated with Hank's balanced saline solution (HBSS) for 1 h (efflux medium). The MRP inhibitor MK571 (10 µM) was used to confirm MRP1 and MRP2 participation in DOX efflux [41]. DOX was quantified in cell lysates and efflux media by spectrofluorometry ($\lambda_{excitation} = 485$ nm, $\lambda_{emission} = 538$ nm). In addition, we evaluated the effect of pretreatment with phytoestrogens on the efflux of carboxy-dichlorofluorescein (CDF), a model substrate specific for the MRP family [42]. Cells were incubated with the precursor CDFDA (10 µM, 60 min), which passively diffuses into the cell, where it is hydrolyzed by intracellular esterases to yield the fluorescent CDF [42]. Then, supernatants samples were taken and CDF was measured by spectrofluorometry ($\lambda_{excitation} = 485$ nm, $\lambda_{emission} = 538$ nm).

For acute inhibition experiments, cells were loaded with DOX as described above, washed and incubated in efflux medium supplemented with daidzein, R-equol and S-equol (10 µM, 1 h). Control cells were exposed to DMSO. MK571 and FTC (10 µM) were used as positive controls of MRP and BCRP inhibition, respectively [24, 40]. DOX was quantified as described above. MXR efflux was quantified in MCF-7, MDA-MB-231 and MDA-MB-231 BCRP$^+$ cells as published before [24, 43]. Briefly, cells were trypsinized and incubated for 30 min in medium with MXR (10 µM, 37 °C).

Following, a set of cells was rinsed, resuspended and subjected to quantification of basal intracellular MXR using a BD LSR II flow cytometer (Becton Dickinson, Heidelberg, Germany). Another set of cells was further incubated for 2 h in the presence of daidzein, R-equol and S-equol (10 µM, 1 h). FTC was used as a positive control of BCRP inhibition [24]. Finally, remaining intracellular MXR was measured. MXR efflux was calculated as the difference between basal intracellular MXR and remaining intracellular MXR after 2 h.

Cell viability assays

Cell viability was determined after treatment with the different phytoestrogens and exposure to DOX or MXR using the crystal violet method [24]. Daidzein, R-equol and S-equol per se had no intrinsic effect on the cell growth within the duration of the experiment up to the maximum concentration tested (data not shown).

Statistical analysis

All data are presented as means ± SD. Statistical comparisons were performed using the Student's *t* test or one-way ANOVA with the Tukey's post-hoc test for two or more than two experimental groups, respectively. Experiments were performed at least in triplicates. Cell viability data were fitted to a sigmoidal curve and IC_{50} were calculated using GraphPad Prism 7.02 (GraphPad Software, La Jolla, USA).

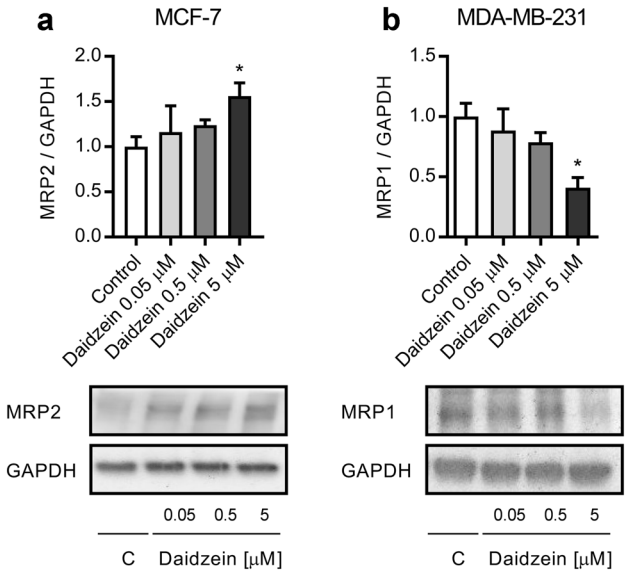

Fig. 1 Effect of daidzein (5 µM, 48 h) on ABC transporter protein expression in MCF-7 and MDA-MB-231 cells. **a** MRP2 protein expression was quantified in MCF-7 cells after treatment with daidzein. **b** MRP1 protein expression was quantified in MDA-MB-231 cells after treatment with daidzein. Quantifications were performed through western blot with GAPDH as a loading control. Representative blots are shown at the bottom. Expression in treated groups was normalized to expression in control cells. *Different from control, $p < 0.05$, $n = 3$

Results

Effect of daidzein, R-equol and S-equol on ABC transporter expression

MCF-7 cells showed a significant induction in MRP2 protein expression (+56%) after exposure to daidzein (5 µM, 48 h) (Fig. 1a). No changes were observed in MRP1 or BCRP expression (data not shown). Likewise, the expression of MRP1, MRP2 and BCRP remained unchanged in R-equol and S-equol treated cells (data not shown). MDA-MB-231 cells exhibited a decrease in MRP1 expression (−59%) by daidzein (5 µM, 48 h) (Fig. 1b). The expression of P-gp and MRP2 showed no changes after treatment with this phytoestrogen. No alterations in P-gp, MRP1 or MRP2 protein expression were observed after exposure to R-equol or S-equol (data not shown). These results indicate a selective effect of daidzein on MRP1 and MRP2 protein expression.

To gain a better insight into the molecular mechanism of MRP2 increase and MRP1 decrease by daidzein, we evaluated the expression of the transporters at the mRNA level under the same conditions that resulted in a modulation of the protein expression. Treatment with daidzein (5 µM, 48 h) resulted in a slight but significant down-regulation of *ABCC2* mRNA (−22%, Fig. 2a) in MCF-7 cells, indicating an opposite modulation at the mRNA and protein levels and suggesting a non-transcriptional mechanism. MDA-MB-231 cells exhibited a down-regulation of *ABCC1* mRNA expression by daidzein (−34%, Fig. 2b), agreeing well with the above described decrease in the protein expression and suggesting a transcriptional down-regulation.

Participation of the ER in ABC transporter modulation

To evaluate whether the effects of daidzein on MRP1 and MRP2 expression are mediated by the classical ERs, cells were incubated with the phytoestrogen (5 µM, 48 h) in the presence of the antagonist ICI 182,780 (1 µM). In fact, coincubation with ICI 182,780 totally prevented MRP2 induction and MRP1 decrease by daidzein in MCF-7 (Fig. 2c) and MDA-MB-231 cells (Fig. 2d), respectively, thus indicating a participation of the classical ERs.

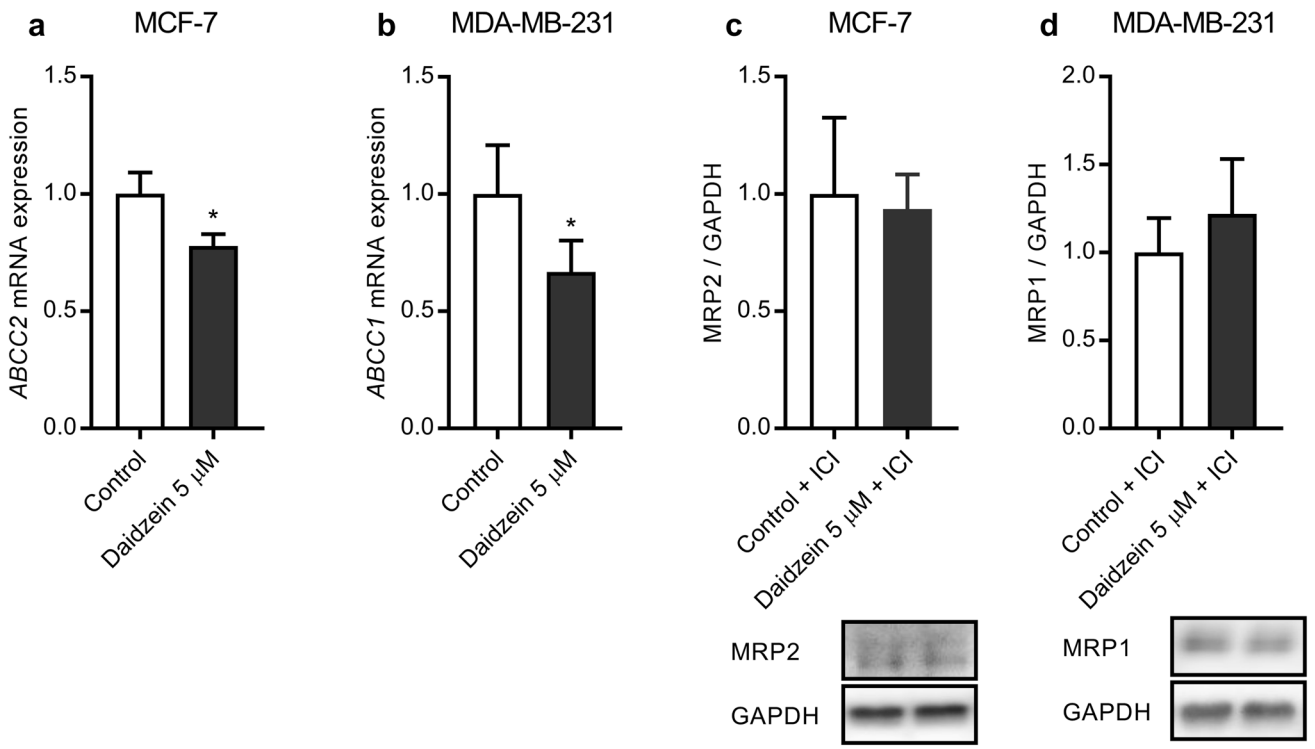

Fig. 2 Molecular mechanisms involved in ABC transporter modulation by daidzein. Cells were treated with daidzein under conditions resulting in transporter modulation at the protein level (5 µM, 48 h). mRNA expression was evaluated for *ABCC2* (MRP2 codifying gene) in MCF-7 cells (**a**) and for *ABCC1* (MRP1 codifying gene) in MDA-MB-231 cells (**b**) through qRT-PCR. The expression of the target genes was normalized to β2mg and GU expression, respectively. Expression in treated groups is expressed relative to the expression in control cells. *Different from control, $p < 0.05$, $n = 4$. To evaluate the participation of the ER, Cells were treated with daidzein and coincubated with ICI 182,780 (1 µM, 48 h), as ER antagonist. Protein expression was evaluated for MRP2 in MCF-7 cells (**c**) and for MRP1 in MDA-MB-231 cells (**d**) through western blot. GAPDH was used as a loading control. Expression in treated groups was normalized to expression in control cells. Representative blots are shown at the bottom. $n = 3$

Effect of daidzein, R-equol and S-equol on drug efflux

Long-term incubation

The functional relevance of MRP1 and MRP2 modulation by daidzein was evaluated assessing the efflux of doxorubicin (DOX), a substrate of MRP1 and MRP2 used in breast cancer chemotherapy. Unexpectedly, neither MRP2 up-regulation nor MRP1 down-regulation by daidzein resulted in a significant alteration of DOX efflux in MCF-7 (Fig. 3a) or MDA-MB-231 cells (Fig. 3b), respectively. MK571 resulted in a significant inhibition of DOX efflux in both cell lines (Fig. 3a, b) thus confirming the specificity of the method to assess MRP activity. To rule out a possible interference by other transporters involved in the efflux of DOX different that MRP1 and MRP2 that may be also regulated by daidzein, we used CDF as a specific MRP model substrate. Daidzein resulted in significant but very slight increase in CDF efflux (+7%), probably lacking biological relevance (Fig. 3c). No changes in CDF efflux by daidzein were observed in MDA-MB-231 cells (Fig. 3d). Total intracellular DOX and CDF were not affected by the phytoestrogen pretreatment in either cell line (data not shown). Unlike results obtained with cell lysates (Fig. 1a), analysis of MRP2 expression in plasma membrane of MCF-7 cells showed a significant decrease by daidzein 5 µM (−40%) compared to control cells (Fig. 3e). These observations may explain the absence of an enhanced DOX and CDF efflux in these cells (Fig. 3a, c).

Acute inhibition

In addition, we evaluated whether the presence of the phytoestrogens during the efflux phase could lead to an acute effect on drug efflux. In fact, daidzein (10 µM), R-equol (10 µM) and S-equol (10 µM) resulted in a significant inhibition of DOX efflux in MCF-7 cells. MK571 and FTC were used as positive controls of MRP and BCRP inhibition, respectively (Fig. 4a). Furthermore, the three phytoestrogens studied also inhibited mitoxantrone (MXR) efflux in the same cell line. Considering that MCF-7 cells do not express detectable levels of P-gp, the other transporter involved in

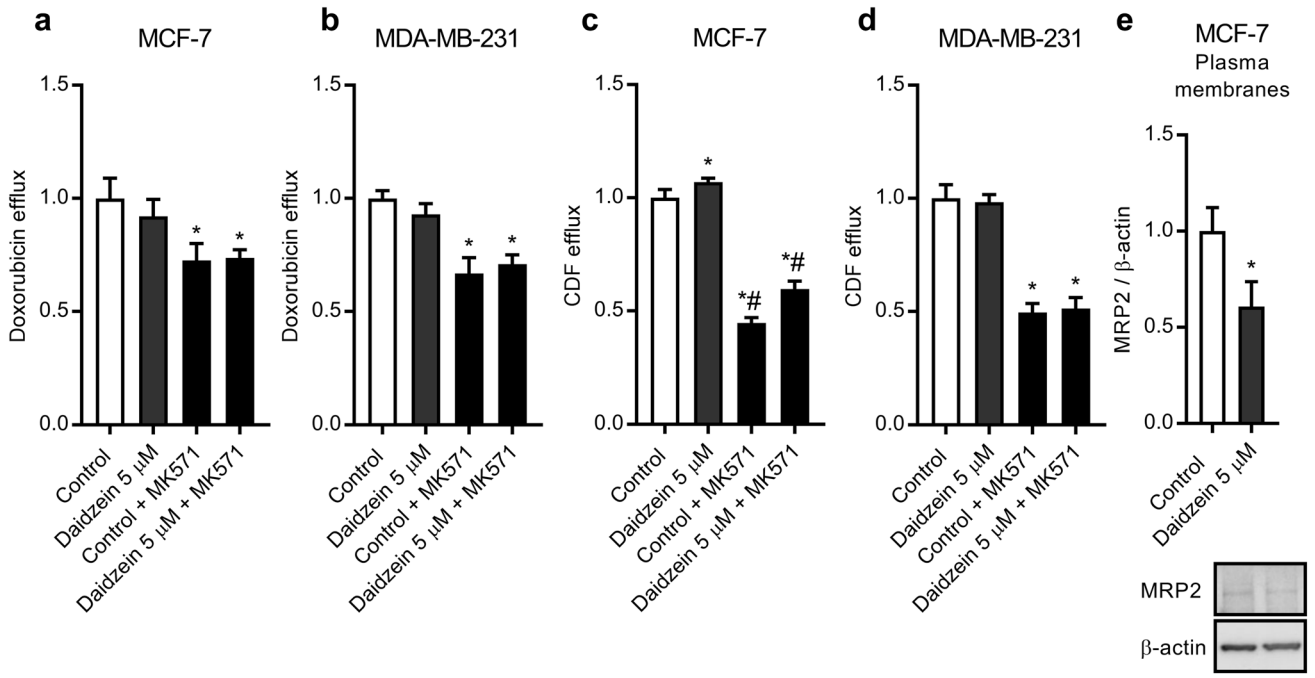

Fig. 3 Effect of transporter induction by daidzein on MRP activity and MRP2 expression in plasma membranes. Cells were treated with daidzein under conditions resulting in transporter modulation at the protein level (5 μM, 48 h). Following the efflux of DOX was quantified by spectrofluorometry in MCF-7 (**a**) and MDA-MB-231 cells (**b**). Similarly, CDF was quantified in MCF-7 (**c**) and MDA-MB-231 cells (**d**). MK571 (10 μM) was used to confirm the participation of the MRPs. Efflux in treated groups was normalized to the efflux in control cells. MRP2 expression was evaluated in plasma membranes of MCF-7 cells through western blot. β-actin was used as a loading control (**e**). *Different from control, $p < 0.05$, $n = 3$–4

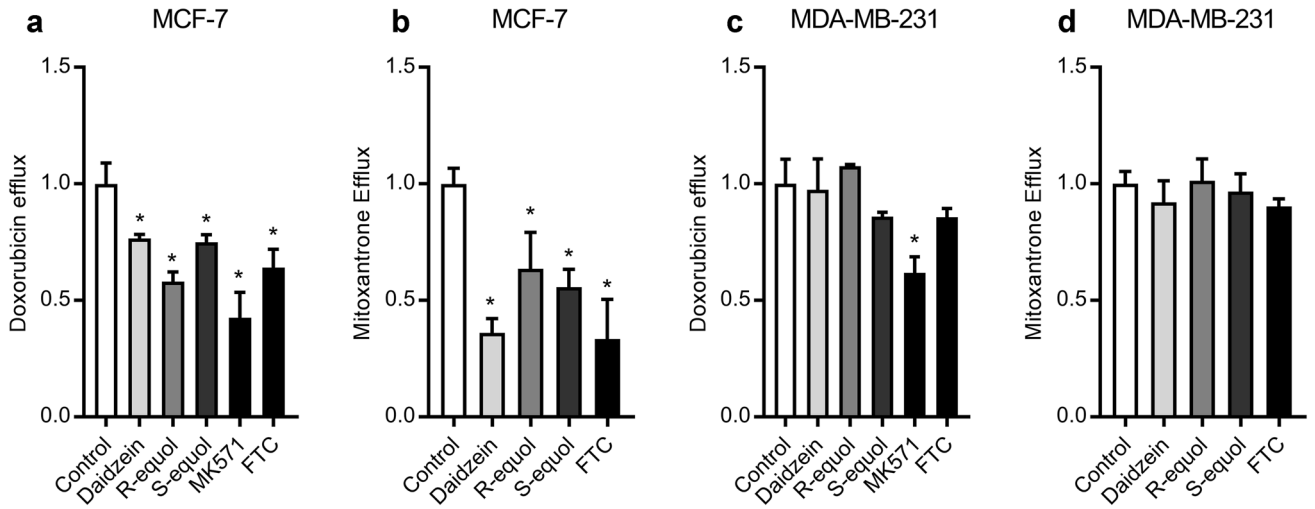

Fig. 4 Acute effect of daidzein, R-equol and S-equol on DOX and MXR efflux. MCF-7 (**a, b**) and MDA-MB-231 cells (**c, d**) were loaded with DOX and MXR, respectively. Following, cells were incubated with daidzein (10 μM), R-equol (10 μM) or S-equol (10 μM). Control cells were exposed to DMSO. MK571 (10 μM) and FTC (10 μM) were used as positive controls of MRP and BCRP inhibition, respectively. Efflux of DOX and MXR were assessed by spectrofluorometry and flow cytometry, respectively. All data were normalized to the efflux in control cells. *Different from control, $p < 0.05$, $n = 4$

MXR efflux [40], the observed decrease in the drug efflux suggests an acute inhibition of BCRP (Fig. 4b).

On the contrary, in the BCRP$^-$ MRP1$^+$ MRP2$^+$ MDA-MB-231 cell line, none of the phytoestrogens resulted in an inhibition of DOX (Fig. 4c) or MXR (Fig. 4d) efflux. Likewise, the well-known BCRP inhibitor FTC had no effect on the efflux of MXR in MDA-MB-231 cells (Fig. 4d). These observations further support an inhibition of BCRP-mediated efflux by daidzein, R-equol and S-equol in human breast cancer cells. In addition, our data indicate the absence of a significant inhibition of MRP1 or MRP2, the proteins mediating the efflux of doxorubicin in MDA-MB-231 cells, by daidzein or equol (Fig. 4c).

Effect of daidzein, R-equol and S-equol on resistance to doxorubicin and mitoxantrone

To evaluate whether the above-described inhibition of BCRP by phytoestrogens has an impact on drug resistance, we performed cell viability assays in MCF-7 and MDA-MB-231 treated with DOX and MXR and coincubated with daidzein (10 µM), R-equol (10 µM) and S-equol (10 µM). Phytoestrogen-coincubated MCF-7 cells exhibited a significant chemosensitization towards DOX (IC_{50}: 0.91 ± 0.22, 0.98 ± 0.22 and 0.99 ± 0.21 µM for daidzein, R-equol and S-equol, respectively, vs. control: 1.61 ± 0.23 µM, Fig. 5a). A similar effect was observed for MCF-7 cells exposed to MXR (IC_{50}: 12.12 ± 4.24, 8.43 ± 2.97 and 14.28 ± 0.74 µM for daidzein, R-equol and S-equol, respectively, vs. control: 36.36 ± 7.83 µM, Fig. 5b). On the contrary, no effect on resistance towards DOX or MXR was observed in MDA-MB-231 cells (Fig. 5c, d, respectively).

Effect of daidzein, R-equol and S-equol on mitoxantrone efflux and chemoresistance in BCRP expressing MDA-MB-231 cells

To confirm BCRP inhibition by phytoestrogens, we used MDA-MB-231 BCRP$^+$ cells, stably expressing BCRP (Fig. 6a). BCRP expression restored the inhibition of MXR efflux by daidzein, R-equol and S-equol (Fig. 6b), contrarily to the results previously shown in parental cells (Fig. 4d). Moreover, inhibition by FTC further confirms BCRP mediation in MXR transport (Fig. 6b). In relation with these findings, we observed a chemosensitization of MDA-MB-231 BCRP$^+$ cells towards MXR (IC_{50}: 79.6 ± 13.0, 96.4 ± 6.6 and 86.6 ± 4.0 µM for daidzein, R-equol and S-equol, respectively, vs. control: 117.5 ± 4.6 µM, Fig. 6c). These data indicate a selective interaction potential of daidzein, R-equol and S-equol, depending on the presence of BCRP in each particular cell line.

Discussion

Phytoestrogens are abundant components of the Asiatic diet, also exhibiting increasing propagation in western countries. The risk vs. benefit ratio of phytoestrogens in association with breast cancer is unclear. ABC transporters, in particular P-gp, MRP1, MRP2 and BCRP, have been related to breast cancer chemoresistance [3, 6–8]. The expression of ABC transporters is subject to a positive and negative modulation by several dietary compounds, which may result in an enhanced or decreased chemoresistance [3, 12]. An up-regulation of BCRP by genistein with a simultaneous increase in MXR efflux and chemoresistance has been already described

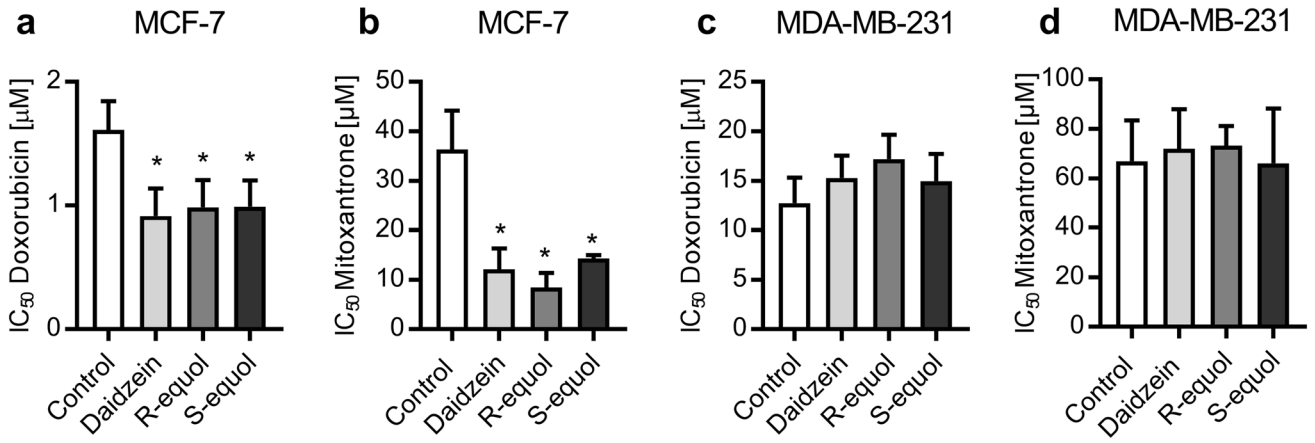

Fig. 5 Effect of daidzein, R-equol and S-equol on the resistance to DOX and MXR. MCF-7 (**a, b**) and MDA-MB-231 cells (**c, d**) were treated with daidzein (10 µM), R-equol (10 µM) or S-equol (10 µM) for 48 h. During the last 24 h of exposition, increasing concentrations of DOX or MXR (0–10 mM) were added to the culture media. Cell viability was determined by staining with crystal violet. Displayed are the IC_{50} for both DOX and MXR, obtained through non-linear regression of the viability vs. concentration curve. *Different from control, $p < 0.05$, $n = 3$

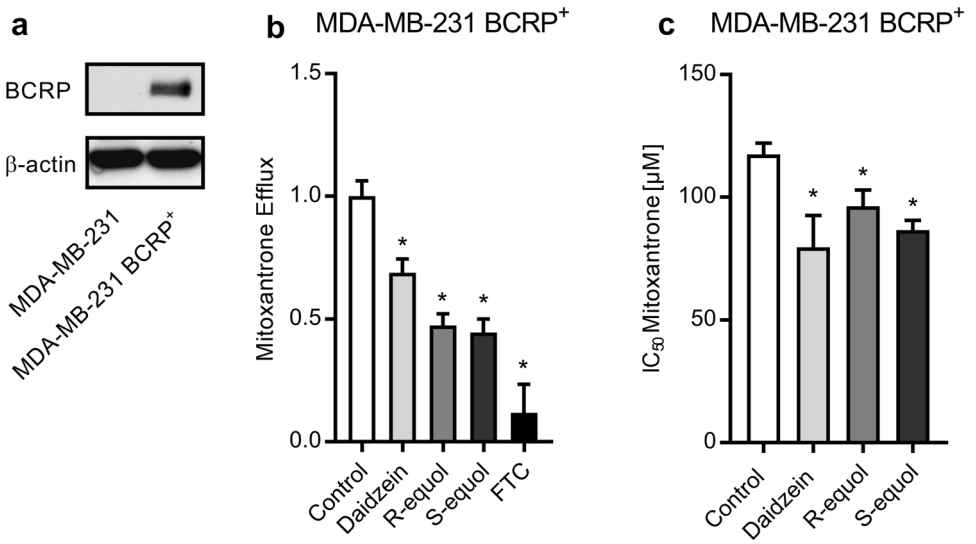

Fig. 6 Effect of daidzein, R-equol and S-equol on MXR efflux and drug resistance in MDA-MB-231 BCRP$^+$ cells. BCRP overexpression was confirmed through western blot. β-actin was used as a loading control (**a**). MXR efflux was determined in the presence of daidzein (10 μM), R-equol (10 μM) and S-equol (10 μM). Control cells were exposed to DMSO. FTC (10 μM) was used as positive control of BCRP inhibition. Efflux in treated groups was normalized to the efflux in control cells. *Different from control, $p<0.05$, $n=3$ (**b**). MDA-MB-231 BCRP$^+$ cells were treated with daidzein (10 μM), R-equol (10 μM) or S-equol (10 μM) for 48 h. During the last 24 h of exposition, MXR (0–10 mM) was added to the culture medium. Cell viability was determined by staining with crystal violet. Displayed are the IC_{50} for MXR. *Different from control, $p<0.05$, $n=3$ (**c**)

in breast cancer cells [24]. Here, we assessed the effect of daidzein, the second most abundant soy phytoestrogen, and its metabolite equol on the expression and activity of the above-mentioned transporters. Our data demonstrated the modulation of the transporter expression by a concentration of daidzein within the plasma range reached in individuals with a high soy intake [34]. In particular, we observed an induction of MRP2 at the protein level in MCF-7 cells (Fig. 1a) with a simultaneous decrease in the *ABCC2* mRNA levels (Fig. 2a). Thus, the induction in MRP2 protein expression is unlikely to result from a direct effect on the MRP2 promoter but instead from a prevailing translational or post-translational modulation by daidzein. As a consequence, higher proteins levels, in spite of decreased mRNA expression, are observed. Noteworthy, dissociation between mRNA and protein expression of ABC transporters after exposure to xenoestrogens has been already described. Treatment with the mycoestrogen zearalenone resulted in an increase in P-gp protein expression and a down-regulation of the mRNA levels in rat testes [44]. Similarly, treatment of a hepatic cell line with genistein (1 μM) resulted in an increase in MRP2 protein expression, without changes in the mRNA levels. The effect was attributed to a down-regulation of the microRNA (miR-379) by genistein [45]. Daidzein was demonstrated to decrease the expression of 11 different microRNAs (miRNAs) in prostate cancer cell lines, which could be associated with an increase in the protein expression of their targets mRNAs [46]. Among the down-regulated miRNAs, miR-136 was pointed out as an *ABCC2* regulating miRNA using the TargetScan platform (http://www.targetscan.org) [47]. In our experimental setting, we observed no changes in miR-136 expression by daidzein in MCF-7 cells, thus ruling out a role of this miRNA as a mediator of MRP2 increase (unpublished results). A down-regulation of other miRNAs or another translational or post-translational process could be postulated as the mechanism underlying the increase in MRP2 protein levels by daidzein. Contrarily to the observations in MCF-7 cells, daidzein repressed MRP1 expression in MDA-MB-231 cells, both at the protein (Fig. 1b) and mRNA level (Fig. 2b), suggesting a transcriptional down-regulation.

Experiments with ICI 182,780 (fulvestrant), a compound reported to prevent the activation of ERα and ERβ [35], showed a total prevention of MRP2 induction and MRP1 down-regulation by daidzein in MCF-7 and MDA-MB-231 cells, respectively (Fig. 2c, d), thus indicating a mediation of the effects by the classical ERs. Since MCF-7 cells express ERα and ERβ, both receptors could be involved in the effects observed. The modulation of several miRNAs by the ERs is well-known [48] and represents a possible explanation for the ER-dependent increase in MRP2 by daidzein at the translational level. In the case of MDA-MB-231 cells, only ERβ is expressed [28]. Thus, the prevention of MRP1 down-regulation by ICI 182,780 suggests mediation by this particular receptor (Fig. 2d). Interestingly, ABC transporter down-regulation by estrogens via ERβ has already been

described. For instance, primary human brain endothelial cells exposed to 5α-androstane-3β,17β-diol exhibited an ERβ-dependent decrease in P-gp protein expression [49]. Likewise, 17β-estradiol and 17α-ethinylestradiol down-regulated BCRP expression at the mRNA and protein levels in rat brain endothelial cells, also through an ERβ-dependent mechanism [50, 51]. Thus, our data agree with previous reports indicating binding of phytoestrogens to ERβ and a modulation of target genes by this isoform [52].

Despite the observed increase in MRP2 and decrease in MRP1 expression by daidzein in MCF-7 and MDA-MB-231 cells, respectively, our results do not show a consequent alteration in the efflux of DOX, substrate of both MRP1 and MRP2 (Fig. 3). The absence of a significant effect could be attributed to other transporters not affected by daidzein also contributing to the efflux of DOX. Noteworthy, P-gp and BCRP have been reported as DOX transporters [3, 29]. To rule out possible interferences by other transporters we measured CDF as a specific MRP substrate, also without observing notable changes (Fig. 3). The absence of a markedly enhanced MRP activity in MCF-7 cells could be attributed, for instance, to an improper localization of the up-regulated MRP2 in the plasma membrane. In this regard, the transporter allocation constitutes a dynamic process characterized by the exocytic insertion of newly synthesized transporter molecules or molecules from subapical reservoirs and the retrieval of apically expressed transporters to subapical compartments [53]. Indeed, we observed a significant decrease in MRP2 expression in the plasma membrane of daidzein-treated MCF-7 cells (Fig. 5e), probably accounting for the majorly unchanged efflux of DOX and CDF, in spite of the higher transporter expression observed in total lysates. These observations highlight the importance of evaluating the expression of drug transporters at different levels as well as the efflux activity. Additionally, MRP inhibition by daidzein (or its metabolites) as observed for genistein in MCF-7 cells [24] cannot be ruled out. In MDA-MB-231 cells, the remaining MRP1 after daidzein treatment may be enough to transport DOX and CDF efficiently, thus not leading to a significant decrease in their efflux. In contrast to the well-known inducing potential on ABC transporter expression and activity by genistein [24, 44], our data indicate a negligible effect of daidzein and equol as long-term inducers of the transport activity and thus the absence of a negative impact on chemotherapy efficacy. These findings highlight the importance of analyzing the individual components of the nutrition (i.e. individual isoflavones in soy and soy-derivatives) as perpetrators in pharmacological interactions.

Besides a long-term modulation, phytoestrogens can modulate transporter activity in an acute way independent of changes in the transporter expression. In the present work we observed a significant decrease in DOX and MXR efflux by daidzein, R-equol and S-equol in MCF-7 cells (Fig. 4a, b), while no changes were observed in MDA-MB-231 cells (Fig. 4c, d). Moreover, the decreased DOX and MXR efflux (i.e. higher intracellular accumulation) was associated with an enhanced sensitivity towards both chemotherapeutic agents (Fig. 5a, b). Breast cancer cells exhibit a differential expression pattern of ABC transporters, with MCF-7 cells expressing MRP1, MRP2 and BCRP and MDA-MB-231 cells expressing P-gp, MRP1 and MRP2 [29–31]. In fact, DOX is a substrate of P-gp, MRP1, MRP2 and BCRP. Taking into consideration that the inhibition by phytoestrogens takes place only in MCF-7 cells ($BCRP^+$ $P\text{-}gp^-$) and not in MDA-MB-231 cells ($BCRP^-$ $P\text{-}gp^+$), an acute inhibition of BCRP could be suggested. Moreover, MXR is a BCRP and P-gp substrate. The inhibition of MXR efflux by all phytoestrogens in MCF-7 cells ($BCRP^+$ $P\text{-}gp^-$) and the absence of an inhibitory effect in MDA-MB-231 cells ($BCRP^-$ $P\text{-}gp^+$) further support the hypothesis of BCRP inhibition. Finally, the inhibition of MXR efflux (Fig. 6b) and the chemosensitization (Fig. 6c) by daidzein and equol after expression of BCRP in MDA-MB-231 cells confirm this transporter as the main target of daidzein and equol. The inhibition of BCRP activity could be attributed to a competitive mechanism, since phytoestrogens and their conjugates can be transported by this protein [17].

Previous reports described an inhibition of BCRP by daidzein in breast cancer cells, although at concentrations between 30 and 50 μM, notably higher than those achieved in individuals with a high isoflavone intake [17, 34, 54]. BCRP inhibition by suprapharmacological concentrations of equol has also been described [55], albeit in a model consisting of kidney cells overexpressing bovine and caprine BCRP. Our data support an inhibition of human BCRP in breast cancer cells at biologically relevant concentrations and thus highlight a potential clinical application of these findings. To date, advances in the development of inhibitors of ABC transporters exhibiting simultaneously efficient transport inhibition and a high safety profile are limited [56]. In our study we have not observed a stimulation of cell proliferation by the phytoestrogen treatment (data not shown). Noteworthy, most studies showing stimulation on breast cancer cell growth by soy isoflavones were performed using genistein [57]. The antiestrogen tamoxifen is usually administered as part of long-term treatments and is also the only drug approved by the FDA for breast cancer prevention [58]. BCRP plays a key role in the efflux of its active metabolite 4-hydroxy-tamoxifen, thus affecting its therapeutic efficacy [58]. Similarly, MXR, DOX and 5-fluorouracil, all used in breast cancer chemotherapy, are also substrates of BCRP [3, 29, 59]. Our results demonstrating an increased drug accumulation suggest a beneficial effect of daidzein or equol coadministration on chemotherapy efficacy. In support of this hypothesis, daidzein intake was associated with a longer time until recurrence in breast cancer patients treated with

tamoxifen [20]; however, the causes leading to this observation were not investigated. If verified in vivo, an inhibition in the transporter activity by daidzein and equol and the concomitant increase in the intracellular concentration of chemotherapeutic agents could allow reducing the doses required and thus the adverse effects frequently related to cancer chemotherapy.

S-equol represents the only form synthesized by equol producers and represents the most frequent enantiomer found in nutritional supplements [18]. Although an antiproliferative role of ERβ in breast cancer has already been reported, ERα activation has been more predominantly associated with increased proliferation [57, 60]. S-equol is characterized by a high affinity for ERβ, while R-equol displays higher affinity for ERα [52]. To date the modulatory effect of R-equol alone on ABC transporters was unknown. Our data indicating a similar chemosensitizing effect by R-equol and S-equol suggest a negligible benefit of R-equol-only or racemic formulations and points to the advantage of daidzein and S-equol-containing preparations in the case of a potential coadministration with chemotherapy.

In conclusion, we have demonstrated a minor effect of long-term exposure to daidzein and equol on the expression and activity of multidrug resistance-related ABC transporters in breast cancer cells. However, our results indicate a strong inhibition of BCRP and a chemosensitizing effect by daidzein, R-equol and S-equol during an acute treatment. Although the occurrence and the magnitude of these findings must be validated in more complex models (e.g. animal models, clinical trials) and further studies should be performed to confirm the safety of these particular phytoestrogens, our data support a potential benefit of combining daidzein and S-equol with chemotherapeutic agents substrates of BCRP.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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