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Original Articles

# The phytoestrogen genistein enhances multidrug resistance in breast cancer cell lines by translational regulation of ABC transporters

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ABSTRACT

Breast cancer is the most frequent malignancy in women. Multidrug resistance due to overexpression of ABC drug transporters is a common cause of chemotherapy failure and disease recurrence. Genistein (GNT) is a phytoestrogen present in soybeans and hormone supplements. We investigated the effect of GNT on the expression and function of ABC transporters in MCF-7 and MDA-MB-231 breast cancer cell lines. Results demonstrated an induction at the protein level of ABCC1 and ABCG2 and of ABCC1 in MCF-7 and MDA-MB-231, respectively. MCF-7 cells showed a concomitant increase in doxorubicin and mitoxantrone efflux and resistance, dependent on ABCG2 activity. ABCC1 induction by GNT in MDA-MB-231 cells modified neither drug efflux nor chemoresistance due to simultaneous acute inhibition of the transporter activity by GNT. All inductions took place at the translational level, as no increment in mRNA was observed and protein increase was prevented by cycloheximide. miR-181a, already demonstrated to inhibit ABCG2 translation, was down-regulated by GNT, explaining translational induction. Effects were independent of classical estrogen receptors. Results suggest potential nutrient–drug interactions that could threaten chemotherapy efficacy, especially in ABCG2-expressing tumors treated with substrates of this transporter.


## Introduction

Breast cancer is the most frequent malignancy among women, with 1.38 million new cases worldwide every year [1]. Chemotherapy represents a cornerstone of breast cancer treatment. Besides hormonal and immunological therapy, conventional cytostatic drugs remain a choice as neoadjuvant therapy in estrogen receptor (ER) positive tumors. Moreover, they represent the first-line option in triple-negative breast cancer and in metastatic disease. Drugs most frequently used include doxorubicin (DOX), epirubicin, mitoxantrone (MXR), cyclophosphamide, methotrexate, 5-fluorouracil and paclitaxel [2,3]. Chemotherapy schemes vary according to the disease stage and may be combined with surgery and radiotherapy. However, in spite of the advances registered in early detection and treatment, 30% of patients exhibit recurrences that contribute to breast cancer being the second leading cause of death by oncological diseases among women [1].

Multidrug resistance is associated with treatment failure and disease recurrence. It is characterized by overexpression of drug transporters belonging to the ATP-binding cassette (ABC) superfamily that limit intracellular accumulation of cytostatic drugs. Transporters most relevant in breast cancer are the P-glycoprotein (P-gp/ABCB1), the multidrug resistance-associated protein 1 (MRP1/ABCC1) and the breast cancer resistance protein (BCRP/ABCG2). Among drugs used in breast cancer, DOX and epirubicin are substrates of ABCB1, ABCC1 and ABCG2; paclitaxel is transported by ABCB1 and MXR is mainly an ABCB1 and ABCG2 substrate [4–8]. Clinical studies have demonstrated an association between transporter expression and response to treatment and disease outcome. For instance, high ABCB1 levels were related with a poor progression-free survival in advanced breast cancer patients [9]. When response was analyzed against the cytostatic scheme administered, high ABCB1 expressing patients exhibited a worse response to FAC (5-fluorouracil + anthracycline + cyclophosphamide), attributable to anthracycline transport by ABCB1. Such association was not observed with CMF (cyclophosphamide + methotrexate + fluorouracil), where the anthracycline is replaced by the poor ABCB1 substrate methotrexate. In relation, another study demonstrated higher ABCC1 expression in non-responders than responders treated with FAC [10]. Moreover, a negative correlation between ABCG2 expression and disease-free survival of epirubicin-treated patients was also described [11].

ABC overexpression can be intrinsic or acquired through induction; for example, by exposure to therapeutic drugs, environmental

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toxicants and micronutrients present in the diet [7,12]. If such an induction occurs during chemotherapy, lower therapeutic response and worse disease outcome are expected.

Genistein (GNT) is a phytoestrogen present in soybeans and red clover. In addition, it is one of the main active principles of hormonal replacement supplements for menopausal women. During the last decades, beneficial effects of GNT on the cardiovascular system, the bone and in the prevention of menopause-associated complaints were described. Also, a role of phytoestrogens in the prevention of hormone dependent tumors was suggested. However, epidemiological studies failed to provide consistent results in this regard [13]. On the contrary, recent evidence suggested an increase in cell proliferation and proto-oncogene expression by phytoestrogens in breast cancer cells [14,15]. GNT exerts some of its effects through the classical estrogen receptors ERα and ERβ [16]. Additionally, GNT activates the pregnane-X-receptor (PXR) [17], which together with ERs are key regulators of ABC transporter expression [12,18]. Thus, modulation of efflux proteins by GNT is possible and was previously demonstrated in colon and liver cancer cell lines, where GNT up-regulated ABCB1 and ABBC2 and subsequently increased chemoresistance [19,20]. Whether a similar effect occurs in breast cancer has not been investigated yet.

GNT is ingested with diet or with over-the-counter formulations. Thus, its intake and the arising interactions may remain unconsidered in the design of chemotherapeutical strategies. This gains particular importance, taking into account that post-menopausal women, the target group of GNT containing supplements, are also those at higher risk of developing breast cancer. If GNT induced ABC transporters in breast cancer, it could lead to diminished efficacy of chemotherapy and thus to a worse disease outcome. The aim of this work was to assess the effect of GNT on the expression and activity of the most relevant ABC transporters in two human breast cancer cell lines and to evaluate the underlying molecular mechanisms.

## Materials and methods

### *Chemicals*

Culture media, charcoal-dextran fetal calf serum (FCS), buffers, all other supplements, the Gene Elute mammalian total RNA kit, cycloheximide (CHX), GNT and mitoxantrone were from Sigma-Aldrich (Taufkirchen, Germany). Dimethyl sulfoxide (DMSO), crystal violet and doxorubicin were from AppliChem (Darmstadt, Germany). Fumitremorgin C (FTC) was kindly provided by the National Cancer Institute (Rockville, USA). MK571 was purchased from Enzo Life Sciences (Lörrach, Germany). FCS was from Biochrom (Darmstadt, Germany). The RevertAid H Minus first-strand kit was from Fermentas (St-Leon-Rot, Germany). The miRNeasy kit was from Qiagen (Hilden, Germany) and the NCode™ miRNA first-strand kit was from Life Technologies (Darmstadt, Germany). ICI 182,780 was obtained from Tocris (Bristol, UK). The anti-ABCB1 antibody C219 was purchased from Calbiochem (Darmstadt, Germany), the anti-ABCC1 antibody MC-898 from Kamiya Biomedical Company (Tukwila, USA) and the anti-ABCG2 antibody BXP-21 from Enzo Life Sciences. All other chemicals were of analytical grade purity.

### *Cell culture and treatments*

MCF-7 and MDA-MB-231 cell lines, models of ERα positive breast cancer and triple-negative breast cancer (ERα$^-$/human epidermal growth factor receptor 2$^-$/progesterone receptor$^-$), respectively, were acquired from the European Collection of Authenticated Cell Cultures (Salisbury, UK) [21]. Both cell lines are positive for ERβ [22]. As breast tumors *in vivo*, the cell lines used express different ABC proteins. MCF-7 cells express ABCC1 and ABCG2, while MDA-MB-231 cells express ABCB1 and ABCC1 [4,23]. Cell lines were cultured as described [24]. For treatments, cells were seeded in 6-well plates ($3.5 \times 10^5$ cells/well). After 24 h, cells were exposed to GNT concentrations associated with plasma levels after the ingestion of a soy rich diet or hormonal supplements (0.1, 1 and 10 μM) for 48 h [25,26]. Control cells were exposed to the vehicle (DMSO, 0.1% v/v). Treatments were performed in phenol-red free medium with charcoal-dextran treated FCS as described [20].

To assess whether GNT acts at the translational or post-translational level, treatments were repeated in the presence of CHX (translation inhibitor, 100 μM) [20]. ER participation was evaluated using the antagonist ICI 182,780 (1 μM) [19].

### *Western blot*

Western blot studies were performed as described [27]. β-actin was used as a loading control. Detections were performed through chemiluminescence with a FluorChem device (Protein simple, San Jose, USA). The expression of multiple proteins was assessed by stripping the membranes with sodium hydroxide (0.20 M, 10 min) and further reincubation with alternative antibodies.

### *Drug efflux assays*

To determine the functional impact of transporter induction by GNT, we evaluated the efflux of DOX and MXR as model drugs employed in breast cancer treatment [2–4]. The contribution of the respective transporters was confirmed using MK571 (10 μM, ABCC1 inhibitor) and FTC (10 μM, ABCG2 inhibitor) [28].

DOX was quantified as published [29]. Briefly, cells were treated with GNT as described in the section "Cell culture and treatments". Subsequently, medium was removed and fresh medium containing DOX (50 μM) was added for 2 h. Thereafter, cells were rinsed and further incubated with Hank's balanced salt solution for 1 h to allow DOX efflux. Finally, supernatant aliquots were taken and DOX was quantified through spectrofluorometry using a Fluoroskan Ascent device (Thermo Scientific), $\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 538 nm.

MXR was quantified as described [30]. Briefly, after GNT treatment, cells were trypsinized and incubated in medium containing MXR (10 μM, 30 min). Subsequently, a set of cells was rinsed and immediately used for MXR quantification (MXR uptake). Another set of cells was resuspended in RPMI1640 medium supplemented with 2% FCS and incubated for 2 h to allow MXR efflux. MXR was quantified using a BD LSR II flow cytometer with a red laser and APC detection filter. Efflux was calculated as the difference between MXR uptake and remnant intracellular MXR after 2 h efflux period. Cell viability under treatment conditions similar to those used in efflux studies was verified by crystal violet staining (data not shown) [27].

The acute inhibition of DOX and MXR efflux by GNT was evaluated quantifying the extrusion of each drug as above described in the presence of increasing GNT concentrations (0–1000 μM), added only during the efflux phase.

### *Growth inhibition assays*

To evaluate cell viability after exposure to DOX and MXR, cells were seeded in 96-well plates (2500 and 5000 cells/well for MCF-7 and MDA-MB-231 cells, respectively) and treated with GNT concentrations that modified transporter expression. Then, fresh medium with increasing concentrations (0–150 μM) of DOX or MXR was added and cells were further incubated for 16 h. Cell viability was quantified through crystal violet staining [27].

### *Real time RT-PCR*

Real time RT-PCR studies were performed for GNT treatments causing changes in the protein expression. Total RNA was isolated using the Gene Elute mammalian total RNA kit and cDNA was synthesized using the RevertAid H Minus first-strand kit. The most suitable housekeeping gene for normalization of ABC transporter expression was identified as described [27]. Among a panel of 6 genes tested, the ribosomal protein L13 (*RPL13*) proved to be the most stable under the assay conditions.

To gain a deeper insight into transporter translational regulation by GNT, the expression of miR-7 and miR-181a, shown to modulate *ABCC1* and *ABCG2*, respectively [31,32], was assessed. Total RNA was isolated using the miRNeasy kit. cDNA was synthesized using the NCode™ miRNA first-strand kit. Expression of miRNAs was normalized simultaneously to *SNORD61* and *SNORD68*, detected using the miScript system (Qiagen).

Primers and PCR conditions for ABC transporters were described previously [33]. Forward primers for miRNA quantification were: miR-7: 5′-CGTGGAAGACTAGTGA TTTTGTTGT-3′; miR-181a: 5′-GCTGGCAACATTCAACGCTGTC-3′. The universal qPCR reverse primer was provided in the cDNA synthesis kit.

Quantifications were performed in a LightCycler®480 (Roche Applied Science, Mannheim, Germany). Data were evaluated by calibrator-normalized relative quantification with efficiency correction as described [33].

### *Statistical analysis*

Data are presented as means ± SD. Comparisons were performed using the Student's t-test or one-way ANOVA followed by the Tukey's test, for two or more than two experimental groups, respectively. Experiments were performed at least in triplicate.

$IC_{50}$-values for drug efflux inhibition and cell proliferation assays were calculated using a sigmoidal model using GraphPad Prism 5.0 (GraphPad Software, La Jolla, USA).



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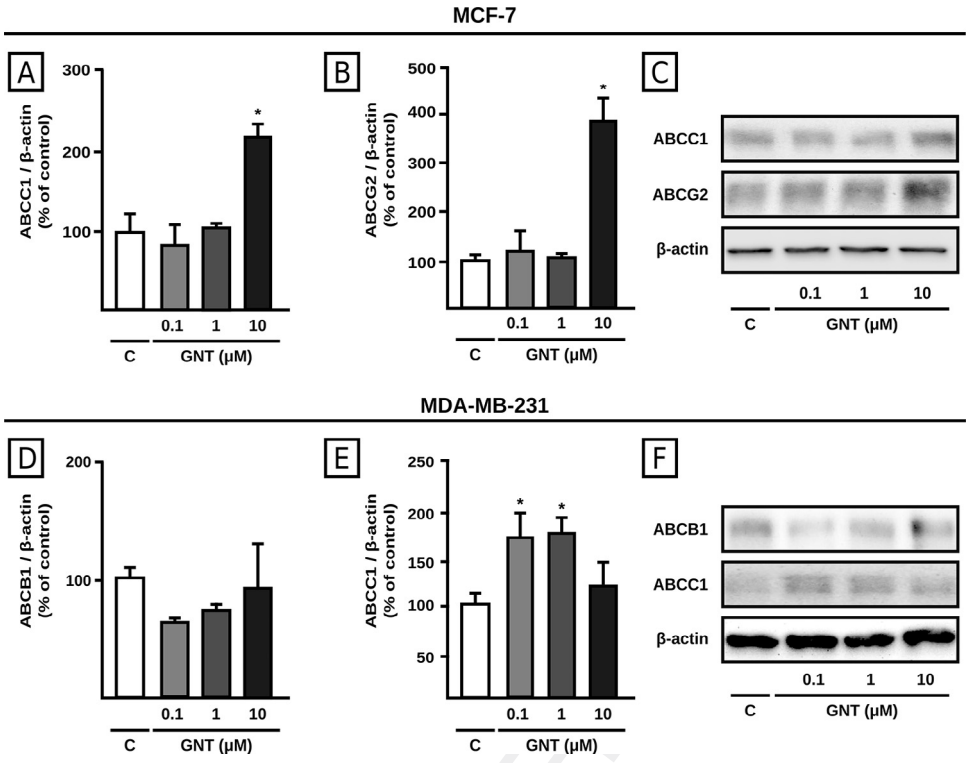


**Fig. 1.** Modulation of ABC transporter protein expression in MCF-7 and MDA-MB-231 cells by GNT. Protein expression after exposure to GNT (0.1, 1 and 10 μM, 48 h) or vehicle (control: C) was assessed through Western blot for ABCC1 (A) and ABCG2 (B) in MCF-7 cells, and for ABCB1 (D) and ABCC1 (E) in MDA-MB-231 cells. β-actin was used as a loading control. Representative blots are shown for each transporter (C and F). *Different from control, $p < 0.05$, $n = 3–4$.

## Results

### *ABC transporter expression in GNT treated breast cancer cell lines*

MCF-7 cells showed an increase both in ABCC1 (+121%) and ABCG2 (+281%) expression after treatment with 10 μM of GNT (Fig. 1A and B, respectively). No changes were observed at lower GNT concentrations. MDA-MB-231 cells showed no significant changes in ABCB1 expression by GNT (Fig. 1D), while ABCC1 was induced at 0.1 and 1 μM GNT (+70% and +74%, respectively) (Fig. 1E).

### *Effect of GNT on DOX and MXR efflux*

In MCF-7 cells, GNT (10 μM, 48 h) increased DOX efflux (+55%, Fig. 2A) consistent with transporter induction by GNT. Although reducing the net efflux of DOX, MK571 did not abolish the increased efflux capacity in GNT pretreated cells, probably due to the concurrently augmented expression of ABCG2 (being a DOX transporter also), not inhibited by MK571. FTC completely abolished the increase in DOX efflux in GNT pretreated cells, suggesting a major role of ABCG2 in the enhanced efflux of DOX (Fig. 2A). GNT (10 μM, 48 h) caused a significant increase of MXR efflux (+136%, Fig. 2B) that was not inhibited by MK571. No GNT-mediated changes in MXR efflux were observed under ABCG2 inhibition with FTC.

DOX efflux in MDA-MB-231 cells was not increased by GNT. On the contrary, cells treated with 1 μM GNT exhibited a decrease in DOX efflux (–38%). MK571 exposed cells did not show differences between groups. However, DOX efflux was generally inhibited by MK571, confirming the ABCC1 inhibitory potency of MK571 (Fig. 3A). MXR efflux remained unchanged in GNT treated cells without inhibitor, whereas under coincubation with MK571, GNT 1 μM inhibited MXR efflux (–43%) with respect to control cells (Fig. 3B).

To test whether an acute inhibition of ABCC1 activity by GNT could account for the unincreased DOX efflux in cells lacking functional BCRP, we quantified the DOX efflux in both cells lines with addition of GNT only during the efflux phase. Results confirmed an acute inhibition of DOX efflux by GNT in both cells lines. A similar effect was also observed for MXR efflux (Table 1).

### *Effect of GNT on chemoresistance toward DOX and MXR*

GNT-treated MCF-7 cells showed a clear increase in resistance to DOX and MXR (Table 2), agreeing well with the increased efflux of both agents. MK571 did not block the increased resistance, probably due to GNT-mediated induction of ABCG2, whose activity is not affected by MK571. FTC completely abolished the enhanced resistance to DOX, attributing ABCG2 the most relevant role in chemoresistance against DOX. A residual increase in the $IC_{50}$ of MXR by GNT in the presence of FTC was still observed. Nevertheless, this observation appears to be independent of MXR accumulation (Fig. 2B) and thus of ABC transporter activity.

In MDA-MB-231 cells, GNT did not modify chemoresistance towards DOX or MXR (Table 3).

**Table 1**
Acute inhibition of drug efflux by GNT.

| | Doxorubicin | Mitoxantrone |
|---|---|---|
| MCF-7 | 9.0 ± 4.7 μM | 32.2 ± 12.1 μM |
| MDA-MB-231 | 42.4 ± 7.3 μM | 3.3 ± 2.1 μM |

Results are expressed as $IC_{50}$-values obtained through non-linear fitting of the concentration-response curves. $n = 3$.

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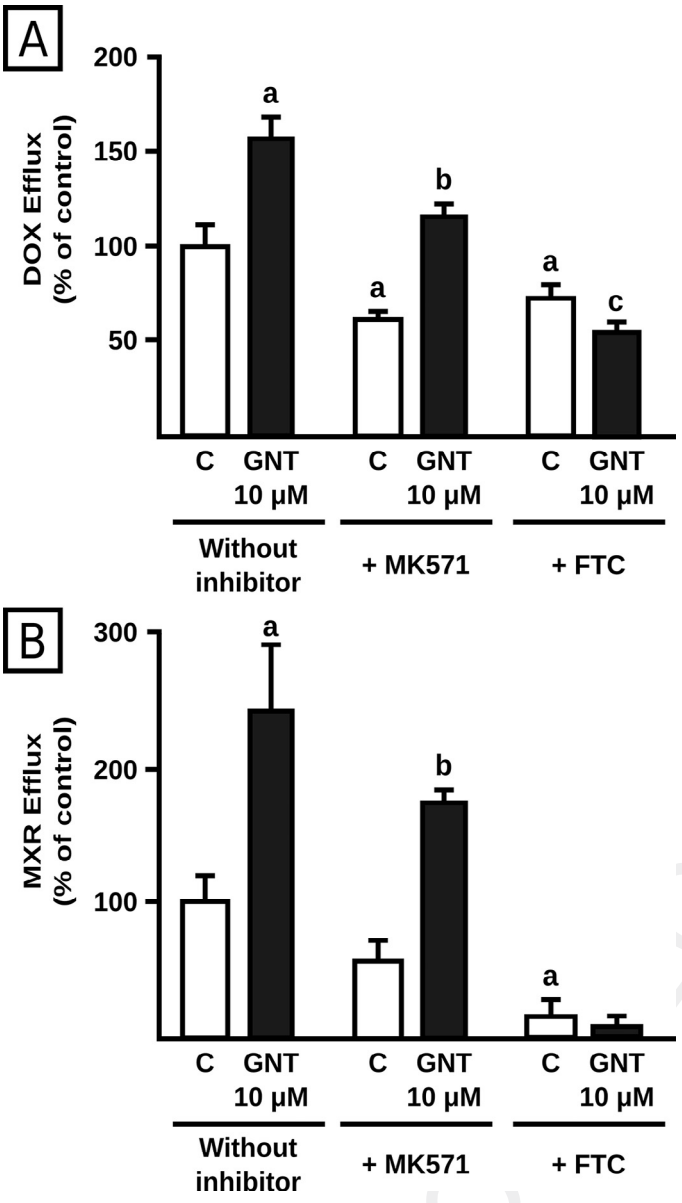


**Fig. 2.** Modulation of cytostatic drug efflux in MCF-7 cells by GNT. DOX (A) and MXR (B) efflux was evaluated through fluorescence measurement in MCF-7 cells. Experiments were performed without transporter inhibitor, with MK571 (10 μM, ABCC1 inhibitor) or with FTC (10 μM, ABCG2 inhibitor). a: different from control without inhibitor, b: different from control + MK571, c: different from control + FTC, $p < 0.05$, $n = 3$.

### *Effect of GNT on mRNA expression of ABC transporters and evidence for translational regulation*

MCF-7 cells showed no changes in *ABCC1* (Fig. 4A) but down-regulation of *ABCG2* mRNA (–39%, Fig. 4B) by 10 μM GNT. In MDA-MB-231 cells, GNT (0.1 μM) decreased *ABCC1* mRNA (–34%) without changes at 1 μM (Fig. 4C). Dissociation between protein and mRNA expression following GNT treatment suggests either a translational or a post-translational regulation.

CHX was used to discern between the two above mentioned mechanisms. Indeed, coincubation with CHX resulted in no significant changes in ABCC1 (Fig. 5A) or ABCG2 (Fig. 5B) protein expression between control and GNT-treated MCF-7 cells. Similarly, no significant difference was observed between control and GNT-treated MDA-MB-231 cells (Fig. 5C), indicating a translational regulation.

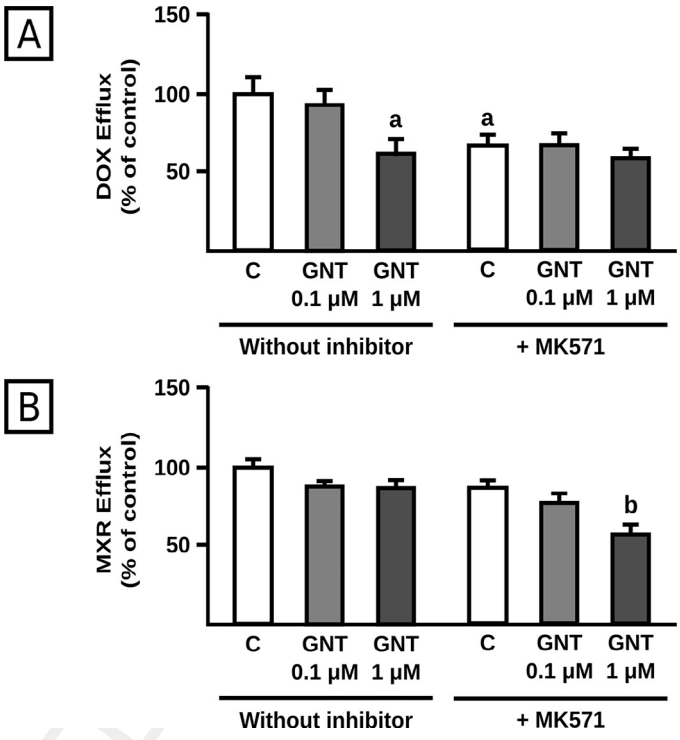


**Fig. 3.** Modulation of cytostatic drug efflux in MDA-MB-231cells by GNT. DOX (A) and MXR (B) efflux was evaluated through fluorescence measurement in MDA-MB-231 cells. Experiments were performed without transporter inhibitor and with MK571 (10 μM, ABCC1 inhibitor). a: different from control without inhibitor, b: different from control + MK571, $p < 0.05$, $n = 3$.

### *Molecular mediators of ABC transporter translational regulation by GNT*

ER participation was evaluated repeating induction experiments in the presence of the inhibitor ICI 182,780. Under these

**Table 2**
Cytotoxicity assays with DOX and MXR in GNT pretreated MCF-7 cells.

| | Without inhibitor | +MK571 | +FTC |
|---|---|---|---|
| A. Doxorubicin | | | |
| Control | 0.35 ± 0.07 | 0.07 ± 0.04 | 0.52 ± 0.09 |
| GNT 10 μM, 48 h | 1.27 ± 0.15* | 1.34 ± 0.41* | 0.36 ± 0.03 |
| B. Mitoxantrone | | | |
| Control | 2.04 ± 0.70 | 1.39 ± 0.22 | 0.54 ± 0.26 |
| GNT 10 μM, 48 h | 3.52 ± 0.80* | 3.85 ± 1.60* | 1.13 ± 0.15* |

Results are expressed as $IC_{50}$ values (μM) obtained through non-linear fitting of viability curves.
* Different from the respective control, $p < 0.05$, $n = 3–4$.

**Table 3**
Cytotoxicity assays with DOX and MXR in GNT pretreated MDA-MB-231 cells.

| | Without inhibitor | +MK571 |
|---|---|---|
| A. Doxorubicin | | |
| Control | 2.59 ± 0.75 | 1.09 ± 0.30* |
| GNT 0.10 μM, 48 h | 2.32 ± 0.80 | 1.82 ± 0.94 |
| GNT 1 μM, 48 h | 1.67 ± 0.27 | 2.17 ± 0.47 |
| B. Mitoxantrone | | |
| Control | 4.00 ± 0.47 | 2.89 ± 0.77 |
| GNT 0.10 μM, 48 h | 3.26 ± 1.42 | 4.07 ± 1.13 |
| GNT 1 μM, 48 h | 3.09 ± 1.08 | 3.38 ± 0.98 |

Results are expressed as $IC_{50}$-values (μM) obtained through non-linear fitting of viability curves.
* Different from control without inhibitor, $p < 0.05$, $n = 3–4$.

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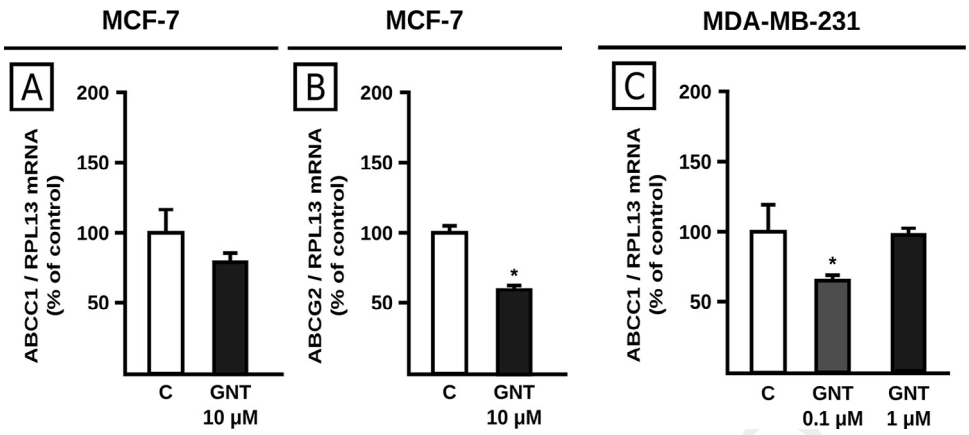


**Fig. 4.** Modulation of ABC transporter mRNA expression in MCF-7 and MDA-MB-231 cells by GNT. mRNA levels of *ABCC1* (A), *ABCG2* (B) in MCF-7 cells and *ABCC1* (C) in MDA-MB-231 cells were assessed through real time RT-PCR after treatment with GNT concentrations previously shown to modify transporter expression at the protein level. Target gene expression was normalized to *RPL13* expression. *Different from all other groups, $p < 0.05$, $n = 4$.

conditions, GNT still up-regulated ABCC1 and ABCG2 in MCF-7 cells (Fig. 6A and B) and ABCC1 in MDA-MB-231 cells (Fig. 6C), arguing against a major role of classical ERs for the transporter induction by GNT.

In addition, the effect of GNT on the expression of miR-7 and miR-181a was tested as possible modulators of ABCC1 and ABCG2 translation, respectively. In MCF-7 cells, miR-7 expression was slightly increased by GNT 10 μM (133 ± 19% vs. control: 100 ± 12%, $p < 0.05$,

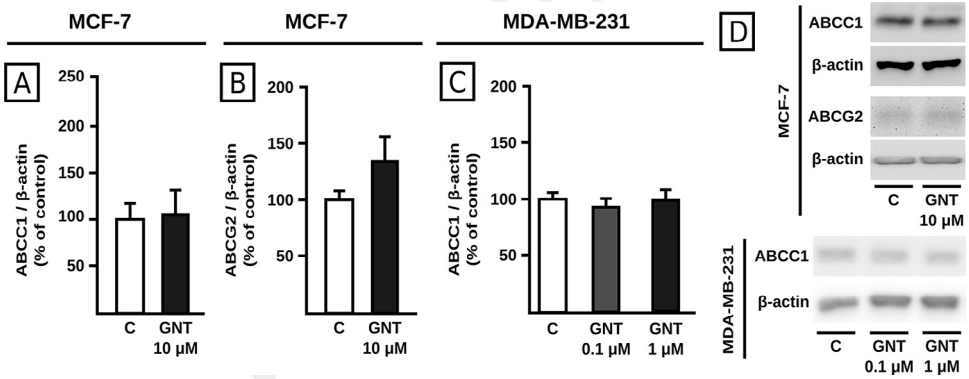


**Fig. 5.** Effect of CHX on ABC transporter protein induction in MCF-7 and MDA-MB-231 cells by GNT. Cells were exposed to GNT concentrations previously shown to modify transporter expression at the protein level (10 μM for MCF-7 cells and 0.1 and 1 μM for MDA-MB-231 cells, 48 h) in the presence of CHX (100 μM). Protein expression was assessed through Western blot for ABCC1 (A) and ABCG2 (B) in MCF-7 cells, and for ABCC1 in MDA-MB-231 cells (C). β-actin was used as a loading control. Representative blots are shown for each transporter (D). $n = 3$.

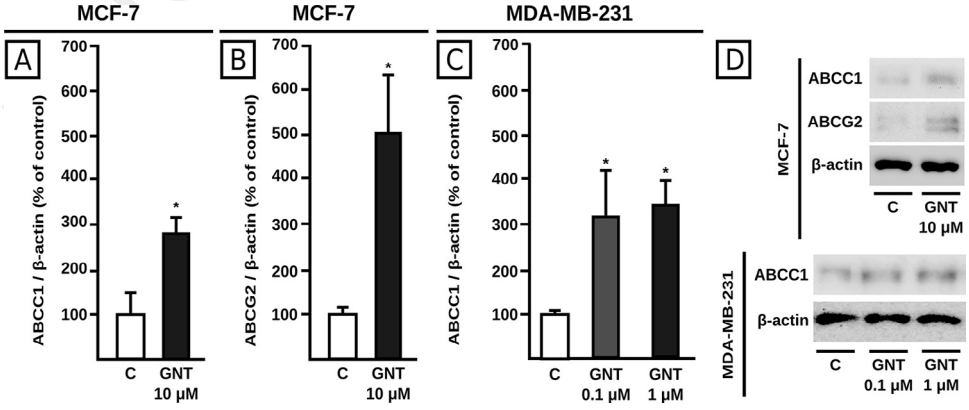


**Fig. 6.** Effect of ICI 182,780 on ABC transporter induction in MCF-7 and MDA-MB-231 cells by GNT. Cells were exposed to GNT concentrations previously shown to modify transporter expression at the protein level (10 μM for MCF-7 cells and 0.1 and 1 μM for MDA-MB-231 cells, 48 h) in the presence of ICI 182,780 (1 μM). Protein expression was assessed through Western blot for ABCC1 (A) and ABCG2 (B) in MCF-7 cells, and for ABCC1 in MDA-MB-231 cells (C). β-actin was used as a loading control. Representative blots are shown for each transporter (D). *Different from control, $p < 0.05$, $n = 3$.

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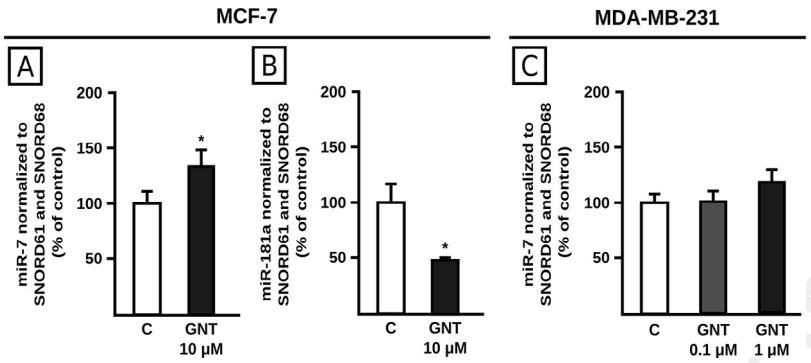


**Fig. 7.** Modulation of miR-7 and miR-181a expression in MCF-7 and MDA-MB-231 cells by GNT. Expression levels of miR-7 (A), miR-181a (B) in MCF-7 cells, and miR-7 (C) in MDA-MB-231 cells were assessed through real time RT-PCR after treatment with GNT concentrations previously shown to modify transporter expression at the protein level. miRNA expression was simultaneously normalized to *SNORD61 and SNORD68* expression. *Different from control, $p < 0.05$, $n = 3$.

$n = 3$) (Fig. 7A). On the contrary, miR-181a was significantly down-regulated by GNT 10 μM in MCF-7 cells (49 ± 1% vs. control: 100 ± 17%, $p < 0.05$, $n = 3$) (Fig. 7B). No changes were observed in miR-7 in MDA-MB-231 cells at GNT 0.1 μM or 1 μM (101 ± 10% and 117 ± 12%, respectively) vs. control cells (100 ± 8%) (Fig. 7C).

## Discussion

ABCB1, ABCC1 and ABCG2 are drug transporters playing a key role in the efflux of cytostatic agents used in breast cancer chemotherapy. Clinical evidence points to an association between transporter expression and disease prognosis [9–11]. To date, several cases of transporter modulation by natural compounds and therapeutic drugs have been described, representing one of the molecular bases underlying diet–drug and drug–drug interactions, respectively [7,12,34]. GNT is a phytoestrogen present in soy and red clover shown to induce the expression of drug transporters and to cause chemoresistance in cancer models [19,20]. A similar transporter induction was observed *in vivo* in liver and intestine of soybean fed rats [35]. Although experimental evidence in this field is highly contradictory, GNT supplements are frequently consumed by women due to their assumed effects alleviating menopausal disorders and, even, preventing breast cancer [13]. In the present work, we described the effects of GNT on drug transporter expression, function and their molecular regulation. A clear GNT-mediated protein induction of ABCC1 and ABCG2 in MCF-7 cells and of ABCC1 in MDA-MB-231 cells was observed. On the contrary, GNT did not modify ABCB1 expression, agreeing well with a previous report by Limtrakul et al. [36], showing no changes in ABCB1 expression in cervical cancer cell lines incubated with GNT 10 μM. Since mRNA expression exhibited a clear discrepancy with protein levels, transcriptional or post-transcriptional mechanisms can vastly be ruled out. In contrast, regulation of drug transporter expression appears to take place at the translational level. Protein inductions described are clearly dependent on GNT concentrations. Only the highest GNT concentration elicited an effect in MCF-7 cells. On the other hand, MDA-MB-231 showed ABCC1 induction only at the lower concentrations. Such an effect had been observed in Caco-2 cells treated with GNT and with ethynylestradiol [19]. Results can be attributed to simultaneous activation of opposite regulatory mechanisms with a differential dependence of estrogen concentration. In fact, estrogens modulate ABC transporters through interaction with diverse signaling pathways comprising classical ERs, the G protein-coupled estrogen receptor GPR30, PXR and several tyrosine kinases (TKs) including their downstream transcriptions factors [16–18,37,38]. Thus, the occurrence of opposite effects resulting from concomitant regulatory events seems feasible. Noteworthy, GNT plasma levels achieved with a soy rich diet differ from those after hormonal supplement intake. For instance, Verkasalo et al. [39] reported GNT plasma $C_{max}$ values of 0.52 μM in subjects drinking 284 mL or more of soy milk daily and consuming solid soy food between 2 and 4 times per week, whereas after the intake of a soy-based supplement (100 mg soy isoflavone) GNT $C_{max}$ reached 6.08 μM [40]. In this regard, the recommended daily intake of soy-based supplements represents up to 200 mg of soy isoflavone, thus even higher $C_{max}$ values may be expected. If a similar dose-dependence was observed *in vivo*, it could imply a differential effect of GNT according to the source of the phytoestrogen.

Higher ABC transporter expression usually leads to enhanced efflux of respective substrates. Our results in MCF-7 cells indeed demonstrate that increased ABCG2 expression results in higher extrusion and enhanced chemoresistance to DOX and MXR. Additionally, our observations point out a malfunction of ABCC1 under GNT treatment. This can be clearly noted for DOX efflux under ABCG2 inhibition by FTC. A similar effect was observed in MDA-MB-231 cells, not expressing ABCG2. Although DOX is also an ABCC1 substrate [7], GNT did not increase its efflux as expected from transporter induction. Instead, reduced DOX transport was observed. Competitive inhibition of ABCC subfamily members by GNT was already reported [41,42]. In line with this observation, we here report an inhibition of ABCC1 activity by intracellular GNT remnant in MCF-7 and MDA-MB-231 cells, ruling out a major role of induction of this particular transporter by GNT in cancer chemoresistance. Conversely, a major role of ABCG2 in enhanced resistance to DOX and MXR is suggested, agreeing well with a recent clinical study showing poor prognosis of anthracycline treated breast cancer patients exhibiting high activity of ABCG2 [43]. If GNT exerted a similar increase of ABCG2 activity *in vivo,* a detrimental effect on both the efficacy of chemotherapeutic drugs and on disease prognosis could be expected. Furthermore, adjuvant endocrine therapy with tamoxifen could also be affected since ABCG2 transports and thus influences tamoxifen efficacy [44]. Thus, our results suggest also a detrimental effect of soy (and other GNT containing products) on the efficacy of breast cancer endocrine therapy. This might additionally result in a higher risk of disease recurrence given the side population of ABCG2 overexpressing breast cancer stem cells [45]. Together, these data reinforce the necessity of avoiding soy consumption during treatment.

ABC transporters expression is regulated at different levels. We here reported a translational regulation of ABCC1 and ABCG2 by GNT. Although most cases of induction by xenobiotics take place at the transcriptional level [7,12], translational effects are common among estrogenic compounds *in vitro* and *in vivo* [19,20,46,47]. miRNAs mediate several effects of estrogens in breast cancer, exhibiting a



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prognostic role in some cases [48,49]. miR-7 and miR-181a are validated as negative modulators of ABCC1 and ABCG2, respectively [31,32]. Consequently, down-regulation of these miRNAs leads to increased transporter expression. Using an online available algorithm [50], promoter analysis of these two miRNAs revealed *cis* elements that bind transcription factors like c-jun, c-fos, junB and junD (unpublished results), which are regulated by tyrosine kinases (TK) [51,52]. Thus, general inhibition of TK activity by GNT can result in the inhibition of transcription factor activity (e.g. due to reduced nuclear translocation or increased proteasomal degradation) and thus in a diminished miRNA expression [53]. In this regard, c-jun down-regulation by GNT was already reported in Caco-2 cells [19]. Unexpectedly, no decrease in miR-7 was observed, suggesting a minor role of this miRNA in ABCC1 up-regulation. On the contrary, miR-181a was indeed down-regulated by GNT in MCF-7 cells, probably due to TK inhibition and transcription factor down-regulation by GNT. Noteworthy, ABC transporters exhibit single-nucleotide polymorphisms (SNPs) modifying their susceptibility to miRNAs [54]. Further analysis of ABCG2 SNPs could help to predict the risk of miR-181a associated translational regulation, and thus of GNT triggered transporter induction.

Regulation of ABC transporters by estrogens can occur either through ER-dependent or ER-independent mechanisms [55]. Our results show a similar transporter induction in MCF-7 and MDA-MB-231, even though both cell lines differ in their ER expression pattern, suggesting a minor role of ER. This hypothesis was confirmed by using the ER antagonist ICI 182,780, which failed to prevent transporter induction. A previous work also demonstrated an ER independent effect as GNT potently induced ABCB1 and ABCC2 even in ER$^-$ HepG2 cells [20]. These observations suggest that GNT-chemotherapy interactions may take place even under treatment with ER antagonists like tamoxifen.

In conclusion, we describe an induction of ABC transporters by GNT in breast cancer cell lines, with ABCG2 induction exhibiting the highest pharmacological relevance in terms of increased *in vitro* chemoresistance. Induction is at least in part mediated by suppression of miR-181a, a negative regulator of drug transporter expression. Our results here suggest a higher risk of adverse GNT-chemotherapy interactions in patients under treatment with substrates of ABCG2 such as anthracyclines, MXR or tamoxifen.

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