Modulation of ABCC1 and ABCG2 Proteins by Ouabain in Human Breast Cancer Cells

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Abstract. *ABCC1 and ABCG2 are two transporters associated with multi-drug resistance to cancer chemotherapy. Ouabain is a cardiotonic steroid, currently considered as a hormone associated with arterial hypertension. Previous studies have suggested that ouabain can modulate ABCB1 and ABCC1 expression in cancer and renal cell lines. The present study investigated the effects of physiological concentrations of ouabain on the expression and activity of ABCC1 and ABCG2 in two human breast cancer cell lines, MCF7 and MDA-MB-231, the first known to be responsive to estrogens. Cell viability and proliferation assays showed that 1 μM ouabain reduced proliferation of MCF7, but not if MDA-MB-231 cells. On the other hand, 10 nM ouabain increased proliferation of MDA-MB-231, but not of MCF7 cells. Ouabain (10 nM) prevented the cytotoxic effects of doxorubicin in MCF7 cells, but not in MDA-MB-231 cells. Treatment of cells under different ouabain concentrations for 24 h did not cause any significant effects in the expression of ABCG2 or ABCC1 in either cell line. However, the activity of ABCC1 was increased when MCF7 and MDA-MB-231 cells were treated with 10 mM and 1 nM ouabain respectively. These results claim attention to the possibility that breast cancer patients with high levels of endogenous ouabain may have different responses to chemotherapy.*

Cancer is the second cause of death among chronic non-communicable diseases, rated just below cardiovascular diseases, and breast cancer is the most common type of cancer affecting women worldwide according to the World Health Organization.

Although chemotherapy is currently the main treatment for metastatic tumors, the ability of tumor cells to become resistant to different drugs simultaneously, a characteristic known as multi-drug resistance (MDR), is a significant obstacle to its success (1, 2). Among the mechanisms that may lead to MDR phenotype, overexpression of plasma membrane proteins that act as drug efflux pumps stands out. These MDR proteins belong to the ABC (ATP binding cassette) superfamily, using the energy of ATP hydrolysis towards the translocation of various substrates across the cell membrane. The ABC transporters can act either by bringing nutrients and other molecules into the cell in prokaryotes or as efflux pumps for toxins, drugs and other metabolites through the membrane, in both eukaryotes and prokaryotes (3).

Ouabain was firstly recognized as a Na^+-K^+-ATPase specific inhibitor (4-6) and it is still unclear whether there is a receptor for this hormone or if the Na^+-K^+-ATPase pump itself acts as a receptor. Although cardiotonic steroids are widely used in pharmacological treatment of heart failure, it is now known that ouabain is produced endogenously by cells from the adrenal cortex (4). Endogenous ouabain is related to multiple physiological processes such as regulation of salt and water balance, cardiac rhythm and contractility, blood pressure, and cell growth and differentiation (7). Several studies have shown that ouabain and other cardiotonic steroids are cytotoxic against cancer cell lines, and their use has been suggested as adjuvant in cancer chemotherapy (8-10). However, it has been reported that ouabain modulates the expression and activity of ABC family proteins, such as ABCC7 (CFTR), and ABCB1 (P-gp) in lung carcinoma cells (11, 12), in rat cardiomyocytes (13, 14) and in human fibroblasts (15). Therefore, the present study aims to investigate the action of ouabain on the viability of breast cancer cells, as well as its possible

interference on the expression and activity of proteins related to the MDR phenotype.

Materials and Methods

Cells and culture conditions. The human breast adenocarcinoma cell line MCF7 was kindly provided by Dr. Rachel Maia (National Cancer Institute, Rio de Janeiro, Brazil) and the human breast carcinoma cell line MDA-MB-231 was gently provided by Dr. Maria Isabel Doria Rossi (Department of Histology and Embryology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil). All cells were grown in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY, USA), in disposable plastic bottles at 37°C. Cells were sub-cultured using Trypsin 0.05% in 0.53 mM EDTA (GIBCO) every 3-4 days. All experiments were performed 24 h after seeding to ensure uniform attachment of the cells at the onset of the experiments.

Cell counting with trypan blue. Both MCF7 and MDA-MB-231 cell lines were seeded onto 24-microwell plates (TPP, Trasadingen, Switzerland) in densities 2×10^4 and 1×10^5 cells per well. After adhesion, cells were treated with ouabain 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M for 24 h in DMEM without serum in the presence or absence of doxorubicin (DOX). Cells in the supernatant were then collected, transferred to microtubes and centrifuged (1,000 rpm, 6 min.). The remaining cells in the wells were detached with 200 µL of trypsin-EDTA and mixed to the corresponding pellet collected from the supernatant. Total cells were stained with 0.4% trypan blue solution and counted in a hemocytometer.

Labeling with anti-ABCC1 and anti-ABCG2 antibodies. Cells were seeded onto 24-well plates and treated with different ouabain concentrations as described above. After 24 h incubation with ouabain, cells were incubated with either anti-ABCC1 (MRPm6) or anti-ABCG2 (BXP-21) monoclonal antibodies for 1 h at room temperature. Then, cells were incubated with a secondary antibody (Alexa Fluor 488) for 30 min at room temperature. Cells were then washed with PBS, centrifuged, re-suspended in 200 µL PBS and the analysis was performed in a Beckton-Dickinson flow cytometer (FACSCalibur, New Jersey, NY, USA). Data analysis was performed using Summit software (Dako Inc, Capinteria, CA, USA). A region of auto fluorescence was set in negative control cells, which did not receive treatment or antibody labeling, (Figure 3A). Positively-labeled cells displayed increased fluorescence, leading to a shift to the right (Figure 3B). During the analysis, events located within the increased-fluorescence region were considered positive, while events located within the low-fluorescence region were deemed negative.

ABCC1 activity. Cells were seeded onto 24-well plates and treated with ouabain as described above. Carboxy-fluorescein diacetate (CFDA – Molecular Probes, USA) is a non-fluorescent molecule that is converted by intracellular esterases into carboxy-fluorescein (CF), which is a substrate for ABCC1. Therefore, to observe ABCC1 activity, cells were incubated for 30 min with 500 nM CFDA, washed and incubated for 30 min in dye-free DMEM. Incubations were performed with or without MK571 (an ABCC1 inhibitor). Cells were then harvested with trypsin, washed and re-suspended in PBS. Intracellular fluorescence was measured in a Beckton-Dickinson flow cytometer (FACSCalibur). The ratio between the mean fluorescence intensity (MIF) of the treated cells after extrusion in the presence and absence of MK571 was used as a measure of ABCC1 transport activity. Data analysis was performed using the Summit software (Dako Inc, Carpinteria, CA, USA). For the analysis, histograms obtained after the efflux were divided into two regions, one containing highly fluorescent events, representing cells with a high content of CF, and a low-fluorescence region, representing cells containing low amounts of CF, and therefore exhibiting higher ABCC1 activity (Figures 4A and B). The ratio between fluorescence from cells treated with and without MK571 was used as a mathematical representation of the transport activity.

Immunofluorescence. MCF7 and MDA-MB-231 cells were seeded onto round microscopy cover slips, which were previously placed in 24 well plates, at a density of 10^5 cells per well. After 24 h of incubation at 37°C for cell adhesion, the fluorescence experiment was performed as described in Fonseca *et al*. 2013 (16) using the anti-ABCG2 antibody BXP-21 and Alexa 488 as primary and secondary antibodies. A Nikon Eclipse Ti microscope coupled with a Nikon Digital Sight DS - U3 camera was used for the photographs.
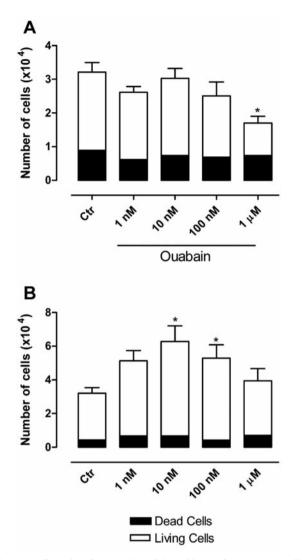
Statistical analysis. Each experiment was repeated from three to seven times. Data were expressed as means±standard error of the mean (S.E.M.) and analyzed by paired one-tailed *t*-test or one-way analysis of variance with Dunnett's post test. Values of $p < 0.05$ were considered statistically significant.

Results

Cell death or proliferation under exposure to ouabain. To observe the effects of ouabain on cell death or proliferation, MCF7 and MDA-MB-231 cells were exposed to different concentrations of ouabain and cell number and viability were measured by the Trypan blue assay. As shown in Figure 1 MCF7 cell growth was not altered by low concentrations of ouabain; however a decrease in total cell number (but not in dead cells) can be observed when treated with 1 µM ouabain. On the other hand, a significant increase in MDA-MB-231 proliferation can be seen when cells were treated with low ouabain concentrations, and no anti-proliferative or toxic effects were observed even for 1 µM ouabain. As 1 µM ouabain was anti-proliferative to MCF7 cells, this concentration was not used in further experiments for this cell line.

Effect of ouabain in doxorubicin cytotoxicity. Since certain studies have suggested the use of cardiac glycosides as adjuvant in cancer chemotherapy (17), we tested whether ouabain could interfere with DOX cytotoxicity, a chemotherapic still largely used to treat breast cancer. Figure 2 shows that 10 nM ouabain protected MCF7 cells against DOX toxicity, while no statistically significant effect was observed in MDA-MB-231 cells.

Expression and activity of ABCC1 and ABCG2 in MCF7 and MDA-MB-231 cells treated with ouabain. It has been shown that DOX is transported by the three main ABC
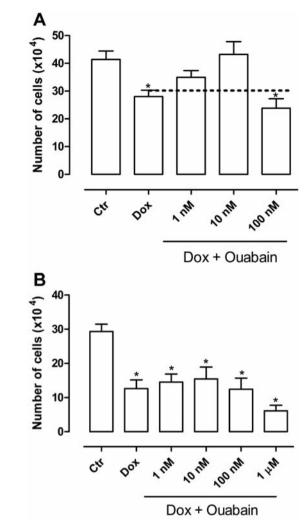
Figure 1. *Effect of ouabain on the viability of human breast cancer cells. (A) MCF7 and (B) MDA-MB-231 cells were seeded onto 24-well plates and ouabain was added 24 h later at various concentrations. After 24-h incubation, the number of live and dead cells was counted by the trypan blue assay. Results are expressed as average±SE (n=3). *p<0,005.*

Figure 2. *Effect of the simultaneous treatment with ouabain and doxorubicin on the viability of breast cancer cells. (A) MCF7 and (B) MDA-MB-231 cells were seeded onto 24-well plates and ouabain was added 24 h later at various concentrations, along with 400 nM of doxorubicin. After a 24-h incubation, the number of live and dead cells was counted by the trypan blue assay. Results are expressed as average±SE (n=3). *p<0,005.*

transporters related to MDR (18, 19), and this could be a possible explanation for the protection observed in Figure 2. Both MCF7 and MDA-MB-231 express two of these transporters, ABCC1 and ABCG2 (20). Therefore, the next step was to evaluate whether ouabain could interfere with the expression and/or activity of these two ABC transporters related to MDR. To do this, the cells were incubated with the respective antibodies, as presented in Materials and Methods, and cell fluorescence was measured by FACS. A region R2 was set in such a way cells in this region are positive for ABCC1 (Figure 3A, C and E) or ABCG2 (Figure 3B, D and F). In Figure 3 it is shown that no significant changes in the

expression of ABCC1 or ABCG2 were seen either in MCF7 or MDA-MD-231 cells when they were pre-incubated with ouabain. However, we observed surprising findings when the activities of these transporters were measured. In Figure 4 it is shown that the two breast cancer cell lines responded differently when the parameter observed was the activity of those transporters. While 10 nM ouabain induced an increase in ABCC1 activity in MCF7 cells, lower concentration (1 nM) was required to increase ABCC1 activity in MDA-MB-231 cells. Moreover, no activity of ABCG2 could be observed for both cell lines, and ouabain did not alter this fact (Figure 5).
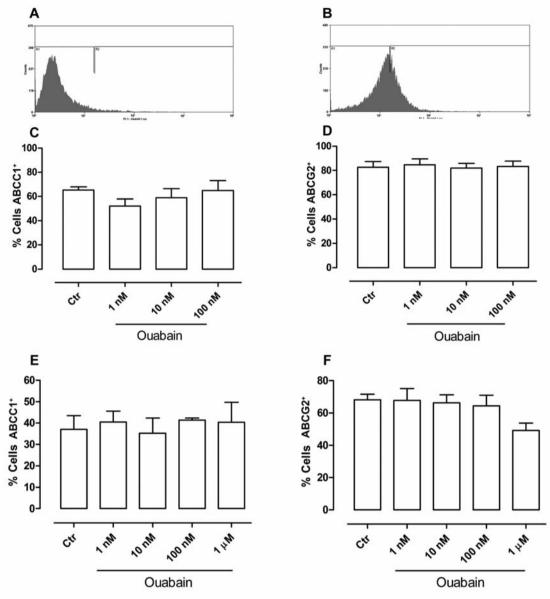
Figure 3. *Effect of ouabain on the expression of ABCC1 and ABCG2. MCF7 and MDA-MB-231 cells were seeded onto 24-well plates and ouabain was added 24 h later in various concentrations. After a 24-h incubation, the cells were then labeled with anti-ABCC1 or anti-ABCG2 antibodies and protein expression was analyzed by flow cytometry. Panels A and B show representative histograms of the low- and high-fluorescence regions used for the analysis. The graphs show the percentage of ABCC1 and ABCG2-positive cells in MCF7 (C and D) and MDA-MB-231 (E and F). Results are expressed as average±SE (n=5).*

The fact that both cell lines labeled for ABCG2, which is in accordance with the existing literature (21), but none of them presented activity for this protein prompted us to verify the pattern of labeling by immunofluorescence. In Figure 6, a diffuse labeling of ABCG2 in the cytoplasm of MCF7 cells is shown, and this protein is present only in the nucleus of MDA-MB-231. This finding is consistent with the absence of activity observed in Figure 4, since only proteins of the plasma membrane account for the activity measured by flow cytometry.

Discussion

It has been shown that cardiac glycosides have a potent anti-tumor effect on cancer cells, including breast cancer (22). Although we do not know the exact mechanisms responsible for their toxicity, it has been suggested that cardiac glycosides act as estrogen antagonists, often necessary for growth and progression of some types of breast tumors (17). It has been demonstrated that ouabain decreases the viability
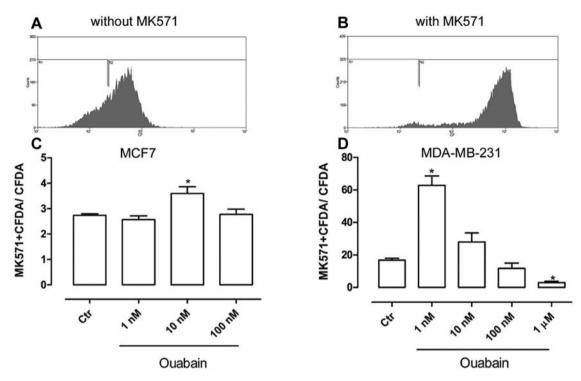
Figure 4. *Effect of ouabain on the transport activity of ABCC1. MCF7 and MDA-MB-231 cells were seeded onto 24-well plates and ouabain was added 24 h later at various concentrations. After another 24-h incubation, ABCC1 activity was measured as described in Materials and Methods. A and B: Representative histograms of the efflux in the absence (A) or presence (B) of the ABCC1 inhibitor MK571. C and D: ABCC1 activity of MCF7 (C) and MDA-MB-231 (D) cells. Results are expressed as average±SE (n=6).*

of certain human tumor cell lines (23). In particular, it was shown that ouabain is anti-proliferative to MCF7 cells (8), and induced apoptosis in MDA-MB-231 by increasing free calcium concentration and activating caspase-3 (24). In the present study, we performed Trypan blue assays (Figure 1), which permits to distinguish between anti-proliferative effects (cell numbers remain unaltered with time, but the cells are not stained) and cell death (cells are stained with the dye). Our results showed that ouabain decreased the proliferation but was not cytotoxic against MCF7 cells, since there was no increase in the number of stained cells in relation to controls (Figure 1A). Literature data correlate growth inhibition with the fact that ouabain inhibits the catalytic activity of topoisomerase II (8, 25). This enzyme has an important role in replication, transcription, and DNA repair processes and its overexpression has been demonstrated in many human tumors, such those of breast (26-28). Contrary to what was expected, MDA-MB-231 cells pre-treated with 10 or 100 nm ouabain had an increase in proliferation (Figure 1B). Indeed, despite many studies relating ouabain-induced decreased proliferation (29), some authors have shown the opposite. For instance, it has been reported that ouabain increased proliferation of human leukemia cells (30) and LLC-PK1 pig kidney cells (29).

Although this finding is not yet understood, it has been suggested that when a tumor tissue goes from benign to malignant, structural changes occur in Na^+-K^+-ATPase, such as changes in the subunits that make up the enzyme, which can modify its binding affinity for ouabain. (10). These alterations in the affinity of cardiac glycosides to Na^+-K^+-ATPase could trigger the activation of several signaling pathways resulting in different cell-cycle regulations.

After verifying the influence of ouabain on the proliferation of tumor cells we investigated whether this glycoside could be acting as an adjuvant to chemotherapy. Surprisingly, the simultaneous treatment of MCF7 cells with doxorubicin and ouabain prevented the toxic effects of the chemotherapic (Figure 2). This result apparently contrasts with literature data, since it has been described that cardiac glycosides would be effective drugs to combat cancer (17), and claim more attention when suggesting ouabain as an adjuvant to chemotherapy.

On the other hand, ouabain did not alter doxorubicin cytotoxicity in MDA-MB-231. A possible explanation for this difference could be that MDA-MB-231 is an estrogen receptor-negative cell line while MCF7 is responsive to estrogen. Cardiotonics such as ouabain, have a steroidal nucleus, and therefore, a structural similarity with estrogen,
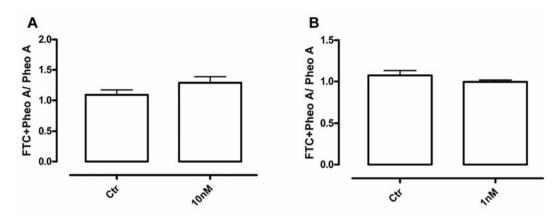
Figure 5. *Analysis of ABCG2 activity after treatment of MCF7 (A) and MDA-MB-231 (B) cells with ouabain by flow cytometry. 2×10^5 cells were seeded onto 24-well and 24 h after initial incubation plates were treated with ouabain for 24 h (n=3).*
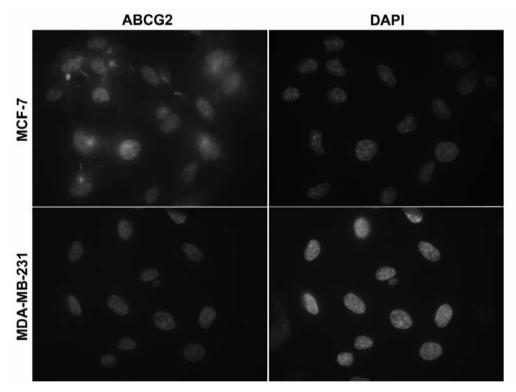

Figure 6. *Immunofluorescence of ABCG2 (left) in MCF7 and MDA-MB-231 cells. The cell nuclei were stained with DAPI (right).*

acting as estrogen receptor antagonists (mainly in the form associated with the membrane), hampering the receptor-dependent signaling. This structural similarity may explain the anti-estrogenic properties of glycosides (17) and could be an explanation for the differences in response of MCF7 and MDA-MB-231 cells to the effects of ouabain in doxorubicin cytotoxicity. However, a much better explanation for the protection against doxorubicin toxicity in

MCF7 cells could be an alteration in the expression or activity of the MDR proteins. It has been reported that ouabain can modulate the expression and activity of several ABC proteins, such as ABCC7 (CFTR), and ABCB1 (P-gp) in lung carcinoma cells (11, 12), in rat cardiomyocytes (13, 14) and in human fibroblasts (15). Furthermore, our group showed that ouabain decreases ABCC1 expression and changes the cellular localization of ABCC1 in embryonic

kidney cells (31). The results obtained in the present study showed that, although pre-treatment of cells with ouabain did not alter the expression of ABCC1 and ABCG2 proteins in either breast cancer cell line (Figure 3), low concentrations of ouabain increased the activity of ABCC1 in both cells lines (Figure 4).

It is noteworthy that the same concentration of ouabain that increased resistance of MCF7 cells to DOX also increased ABCC1 activity in this cell line, suggesting that, at least in MCF7 cells, this resistance could be associated with an increased efflux of drugs by the carrier. This result becomes even more interesting since the glycoside concentrations that resulted in this resistance have been observed in humans with increased levels of endogenous ouabain (32). Therefore, more studies are needed to explain this effect.

Although several studies have attempted to verify alterations in ABCG2 expression in tumor cell lines, little is known regarding its localization in wild-type cell lines. The majority of the studies showing plasma membrane labeling of ABCG2 were performed in cells overexpressing this protein (33) but there are some reports showing that folate deprivation, oxidative stress or increased xanthine contents could lead to internalization of this protein (34-36). Since the cell lines used in the present study are wild-type and the expression of both ABCC1 and ABCG2 are constitutive, it is possible that the pattern of labeling observed is normal for constitutive expression.

Finally, since it has been shown that changes in plasma levels of endogenous ouabain are related to hypertension (37-39), the results presented here highlight a potential problem in the treatment of hypertensive patients presenting elevated levels of endogenous ouabain and undergoing chemotherapy. Our results become particularly important when considering the fact that in some treatment protocols, glycosides are administered in combination with chemotherapy to prevent heart damage (11). Our work brings-up concerns about a possible increased resistance to chemotherapy with doxorubicin (and perhaps other chemotherapics) in such patients.

Conflicts of Interest

The Authors declare they have no conflicts of interest.

Acknowledgements

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