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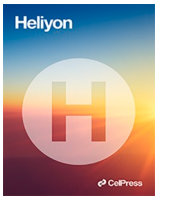

Exposure to anticancer drugs modulates the expression of ACSL4 and ABCG2 proteins in adrenocortical carcinoma cells

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ABSTRACT

Adrenocortical carcinoma (ACC) is a rare and malignant disease, with more than 50 % of patients developing hormone-secreting tumors. These tumors are genetically heterogeneous and potentially lethal, as metastasis is often underway at the time of diagnosis. While chemoresistance can be multifactorial, Acyl CoA synthetase 4 (ACSL4) is known to contribute to the generation of highly aggressive cellular phenotypes, while increased expression and activity of multidrug transporters such as ATP-binding cassette subfamily G member 2 (ABCG2) are known to play a key role. Therefore, the objective of this work was to determine changes in the expression of ACSL4 and ABCG2 in ACC cell lines after exposure to antitumor drugs. Bioinformatics analysis of public database GSE140818 revealed higher ACSL4 and ABCG2 expression in HAC15 cells resistant to mitotane when compared to wild type cells. In addition, our studies revealed an increase in ACSL4 and ABCG2 expression in lowly aggressive H295R cells undergoing early treatment with non-lethal concentrations of mitotane, doxorubicin and cisplatin. Comparable results were obtained in lowly aggressive breast cancer cells MCF-7. The increase in ACSL4 and ABCG2 expression favored tumor cell viability, proliferation and compound efflux, an effect partially offset by ACSL4 and ABCG2 inhibitors. These results provide relevant data on the undesired molecular effects of antitumor drugs and may fuel future studies on patients' early response to antitumor treatment.

1. Introduction

Adrenocortical carcinoma (ACC) is a rare and malignant disease, with an annual incidence between 0.5 and 2 cases per million people [1] and more than 50 % of patients developing hormone-secreting tumors [2]. These tumors are genetically heterogeneous and potentially lethal, as metastasis is often underway at the time of diagnosis [3]. Moreover, unfeasible tumor removal, poor prognosis, and low quality of life of patients with advanced disease curb survival to no more than 5 years [4].

Treatments for patients with metastases are scarce and rather ineffective. Mitotane (or p′-dichlorodiphenyldichloroethylene or p′-DDD), approved and used as standard therapy for ACC, is a cystostatic antineoplastic drug also known to inhibit steroidogenesis [5]. Other types of chemotherapy treatments have been investigated either in combination with mitotane or as single therapy [6]. One of

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the main reasons why pharmacological treatments fail in patients with this type of cancer is cell resistance to drugs [7].

While chemoresistance can be multifactorial, increased expression and activity of multidrug transporters is known to play a key role. In particular, increased expression of the ATP-binding cassette subfamily G member 2 (ABCG2) transporter is associated with drug resistance in bladder, kidney, glioma and glioblastoma cancer cell lines [8–11], and is partly responsible for drug efflux from tumor cells [12,13], ABCG2 is also a prominent cancer stem cell marker in side population cells of different tumor types [14,15]. In turn, Acyl-CoA synthetase 4 (ACSL4) has been recently unveiled as part of chemoresistance mechanisms in breast and prostate cancer [16–18]. ACSL4 is an enzyme taking part in fatty acid metabolism, particularly that of arachidonic acid [19], and its overexpression contributes to the generation of highly aggressive cellular phenotypes in breast, colon, liver, and prostate cancer [20–23]. ACSL4 further boosts the activity and expression of ABCG2 in breast and prostate cancer cells, which is concomitant with an increase in cell resistance to chemotherapy and cell aggressiveness [16,17].

In this context, the objective of this work was to explore variations in the expression of ACSL4 and ABCG2 in lowly aggressive ACC cell lines exposed to antitumor drugs and assess changes in cellular functions associated with chemoresistance. Our studies revealed an increase in the expression of both ACSL4 and ABCG2 in cells exposed to antitumor drugs. In addition, early exposure to antitumor drugs favored cell viability upon later exposure and increased cell proliferation and compound efflux. These results suggest that exposure to antitumor drugs induces alterations in cellular functions which may trigger resistance to future treatments and/or heighten cell aggressiveness along tumor progression.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified Eagle's/F12 medium (DMEM/F12), penicillin-streptomycin sulfate and trypsin-EDTA were purchased Gibco Invitrogen Corporation (Grand Island, NY, USA). HyCloneCosmic Calf Serum was from Cytiva (Marlborough, MA, USA). Fetal calf serum was purchased from PAA laboratories GmbH (Pasching, Austria). 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and Hoechst 33342 were from Sigma Chemical Co. (St. Louis, MO, USA). The polyclonal rabbit anti-human ACSL4 antibody was developed in our laboratory [24], and the monoclonal mouse anti-β-tubulin was purchased from Upstate Group Inc (Temecula, CA, USA). ABCG2 antibody was purchased from Origene Technologies (Rockville, MD, USA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies as well as polyvinylidene fluoride membranes were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Ko143 was from Abcam (Cambridge, UK). The enhanced chemiluminescence kit was acquired from GE Healthcare (Buckinghamshire, UK). Sterile cell culture materials were purchased from Orange Scientific (Braine-l'Alleud, Belgium). The 5-bromo-2′-deoxyuridine (BrdU) ELISA kit was purchased from Roche Diagnostics (Basel, Switzerland). All other reagents were of analytical grade.

2.2. Cell lines

The H295R cell line, obtained from a primary human ACC in the zona glomerulosa of the adrenal gland [25], was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and used for all experiments after 25 passages. The cell line was cultured in DMEM/F-12 1:1 containing 1.1 g/l $NaHCO_3$, 20 mM HEPES, 200 IU/ml penicillin, 200 g/ml streptomycin sulfate, and 5 % HyClone Cosmic Calf Serum at 37 °C and 5 % CO_2.

The MCF-7 cell line, isolated from breast tissue from a patient with adenocarcinoma [26], was provided by from the Lombardi Comprehensive Cancer Center (Georgetown University Medical Center, Washington D.C., USA) and used as control for chemotherapeutic treatment experiments after 30 passages. The cell line was expanded and stored at −80 °C. Cells from these stocks were thawed and cultured in DMEM supplemented with 10 % FBS plus 100 U/ml penicillin and 10 mg/ml streptomycin at 37 °C and 5 % CO_2. MCF-7 cells were validated by the ATCC Cell Line Authentication Service as a 100 % match for the ATCC cell line HTB-22 (MCF-7).

The number of cells seeded was carefully adjusted in control experiments to avoid detachment from the plate surface. Potential mycoplasma contamination was regularly tested through polymerase chain reaction experiments.

2.3. Cell treatment conditions

As it is estimated that the drug volume in cells is in equilibrium with that detected in plasma, in this study we used low concentrations (non-lethal doses) of antineoplastic drugs and exposed cells for 10 days. We considered drug concentration in plasma and half-life, together with an average time to maintain an adequate amount of antitumor drugs in vitro, to emulate the drug volumes that reach the cells after treatment. Non-lethal drug concentrations were chosen on the basis of evidence in the literature and corroborated in control experiments [27–33].

- Mitotane followed by mitotane: H295R cells were treated with mitotane at low concentrations (0.5 or 1 μM) for 10 days in complete medium. Each condition was later treated with mitotane at high concentrations (10 or 20 μM) for 48 h in complete medium.
- Chemotherapeutic drugs followed by chemotherapeutic drugs: H295R cells and MCF-7 cells were treated with doxorubicin (20 nM) or cisplatin (200 nM) for 10 days in complete medium. Each condition was then treated with doxorubicin (1 μM) or cisplatin (5 μM) for 48 h in complete medium.

- Mitotane followed by ACSL4 inhibitor: H295R cells were treated with mitotane at low concentrations (0.5 or 1 μM) for 10 days in complete medium. Each condition was then treated with PRGL493 (25 μM) for 96 h in complete medium.
- Chemotherapeutic drugs followed by ACSL4 inhibitor: H295R cells and MCF-7 cells were treated with doxorubicin (20 nM) or cisplatin (200 nM) for 10 days in complete medium. Each condition was then treated with PRGL493 (25 μM) for 96 h in complete medium.
- Mitotane followed by receptor inhibitor: H295R cells were treated with mitotane at low concentrations (0.5 or 1 μM) for 10 days in complete medium. Each condition was then treated with ABCG2 inhibitor Ko143 (1 μM) for 12 h in complete medium.
- Chemotherapeutic drugs followed by receptor inhibitors: H295R cells were treated with doxorubicin (20 nM) or cisplatin (200 nM) for 10 days in complete medium. Each condition was then treated with ABCG2 inhibitor Ko143 (1 μM) for 12 h in complete medium.

2.4. Cell viability assays

MTT assays [34] were carried out on cells plated at a density of 1500 cells/well in 96-well plates and submitted to the following experimental conditions: mitotane followed by mitotane and chemotherapeutic drugs followed by chemotherapeutic drugs. Absorbance was measured by means of a Synergy HT multi-detection microplate reader (Biotek, Winooski, VT, USA).

2.5. Cell proliferation assays

BrdU incorporation [20] assays were carried out on cells plated at a density of 1500 cells/well in 96-well plates and submitted to the following experimental conditions: mitotane followed by ACSL4 inhibitor and chemotherapeutic drugs followed by ACSL4 inhibitor. Absorbance was measured by means of a Synergy HT multi-detection microplate reader.

2.6. Intracellular doxorubicin accumulation and efflux assays

Doxorubicin fluorescence assays were carried out following Chen et al. [35] with modifications. Briefly, cells were plated on glass slides at a density of $2x10^4$ per slide and submitted to the following experimental conditions: mitotane plus receptor inhibitor and chemotherapeutic drugs plus receptor inhibitors. Briefly, cells were treated with doxorubicin (10 μM) for 1 h protected from light, washed, and incubated with doxorubicin-free medium for 2 h to analyze doxorubicin efflux. Doxorubicin fluorescence was immediately assessed through epifluorescence microscopy at 480 nm excitation.

2.7. Hoechst exclusion assay

Hoechst 33342 exclusion assays [16] were carried out on cells plated on glass slides at a density of $2x10^4$ per slide and submitted to the following experimental conditions: mitotane followed by receptor inhibitor and chemotherapeutic drugs followed by receptor inhibitors. Briefly, cells were treated with Hoechst 33342 (5 μg/ml) for 10 min at 37 °C in a culture stove protected from light. After Hoechst 33342 treatment, cells were washed and incubated with Hoechst 33342-free medium for 10 min to analyze Hoechst efflux. Hoechst staining was assessed by epifluorescence microscopy at 480 nm excitation.

2.8. Western blot

Western blot analysis was conducted as previously reported by our group [19]. Briefly, cell cultures were rinsed with PBS, lysed in RIPA buffer and centrifuged at 5000×*g* for 10 min. Total protein lysis (40 μg) was resolved on SDS-PAGE, transferred to polyvinylidene fluoride membranes, and incubated with 1 % fat-free powdered milk in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.5 % Tween 20 with gentle shaking for 60 min at room temperature. The membranes were then washed twice in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.5 % Tween 20, and kept overnight at 4 °C in the following primary antibody dilutions: 1:1000 rabbit polyclonal anti-ABCG2, 1:1000 rabbit polyclonal anti-ACSL4 and 1:5000 mouse monoclonal anti-tubulin. Bound antibodies were developed using horseradish peroxidase-conjugated secondary antibodies 1:5000 goat anti-mouse and 1:5000 goat anti-rabbit and detected by chemiluminescence. Immunoblots were quantified by means of Gel Pro Analyzer.

Table 1
Primer sequences for real-time PCR.

ACSL4	Forward	CCTGGAGCAGATACTCTGGA
	Reverse	TCACTCTGCGATTCACTTCA
ABCG2	Forward	TAAAGTGGCAGACTCCAAGG
	Reverse	TTGTTCGTCCCTGCTTAGAC
GAPDH	Forward	TCCCATCACCATCTTCCA
	Reverse	CATCACGCCACAGTTTCC

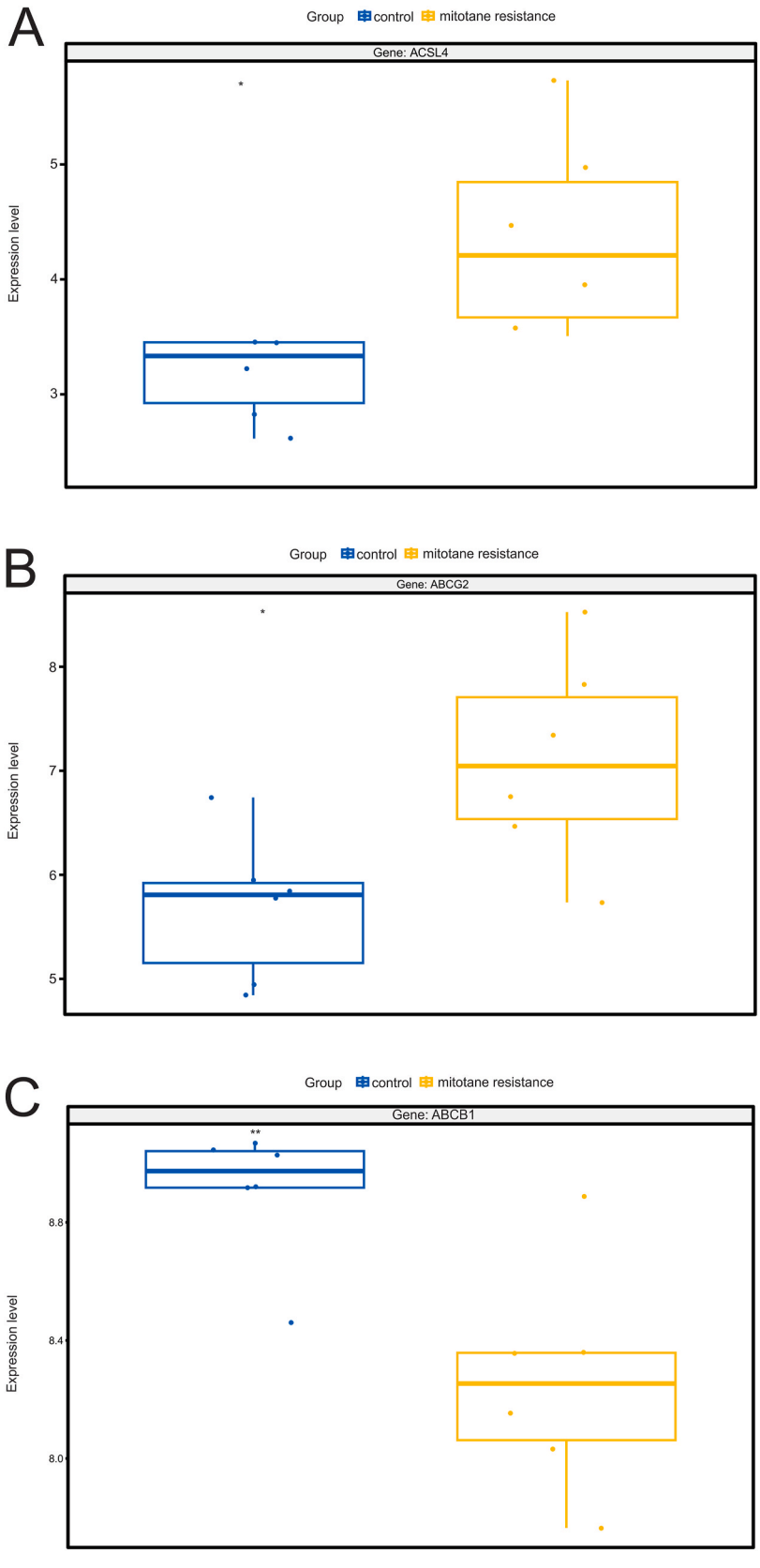

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Fig. 1. ACSL4, ABCG2 and ABCB1 expression in mitotane-resistant ACC cells HAC15. Box plots of mRNA expression levels (y-axis) of A) ACSL4, B) ABCG2 and C) ABCB1 (n = 24, data set GSE140818) in control and mitotane-resistant HAC15 cells. Boxes define the interquartile range; thick center lines represent the median. A) and B) *p < 0.05 control vs. mitotane resistance; C) **p < 0.01 control vs. mitotane resistance.

2.9. Quantitative reverse transcription-PCR (qRT-PCR)

qRT-PCR and real-time PCR assays were carried out as previously reported by our group [16]. Briefly, H295R and MCF-7 total RNA was obtained using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). Residual genomic DNA was removed using RNA with DNase I (Invitrogen, Carlsbad, CA, USA), and two μg of total RNA were reverse-transcribed using random hexamers and M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) following the manufacturer's instructions. For real-time PCR, specific primers for ACSL4, ABCG2 and GAPDH were purchased from Macrogen (Seoul, South Korea) (Table 1), and Mic PCR (Bio molecular Systems, Yatala, Australia) was used. All reactions were run in triplicate. Amplification began with incubation at 95 °C for 3 min, followed by 45 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s mRNA expression was normalized to human GAPDH RNA expression as an endogenous control. Real-time PCR results were analyzed by calculating 2–ΔΔCt value (comparative Ct method) for each experimental sample.

2.10. Data source and analysis of differential expressions

All GEO datasets were obtained from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). Three expression profiling data sets were used in the present study.

1 GSE140818 [36], comparing data from control (70 YM 5–12) and mitotane-resistant (70 YM 5–12 MITOTAN) HAC15 cells (n = 12).
2 GSE28647 [37], comparing data from control (PEO1 1–2, PEA1, PEO14) and cisplatin (PEO4 1–2, PEA2, PEO23) ovarian cancer cells isolated from patients (n = 8).
3 GSE162187 [38], comparing data from doxorubicin-sensitive (Breast_cancer_S) and doxorubicin-resistant (Breast_cancer_R) breast cancer tissue samples obtained from patients (n = 21).

The Wilcoxon test was used to evaluate differential gene expression in tissues and cells. Statistical significance was set at a p-value<0.05. The statistical analysis was carried out using R software (version 4.2.0) and the ggpubr package (version 0.4.0.999). Boxplots were made using the ggplot2 package (version 3.4.2).

2.11. Statistical analysis

Data were evaluated by means of GraphPad InStat Software 3.10 (La Jolla, CA, US). Statistical significance was established using analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests.

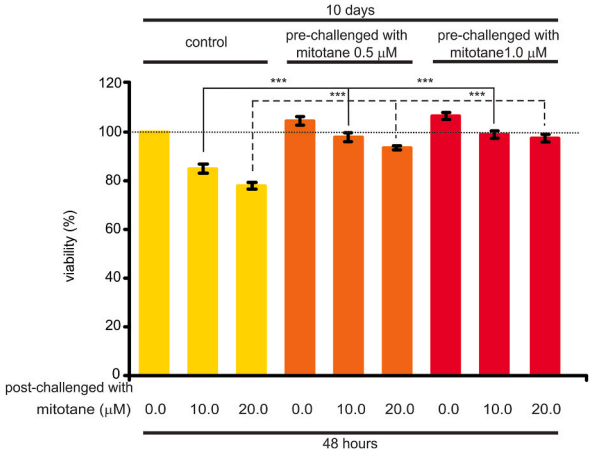
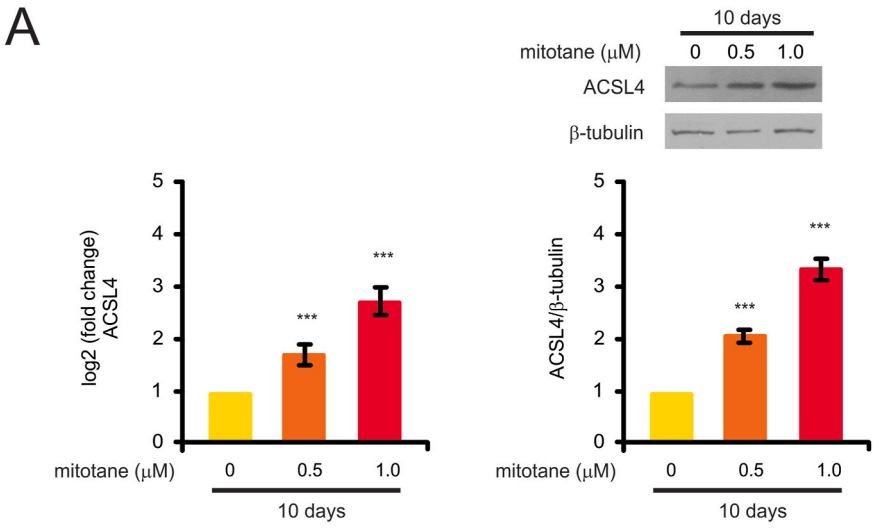
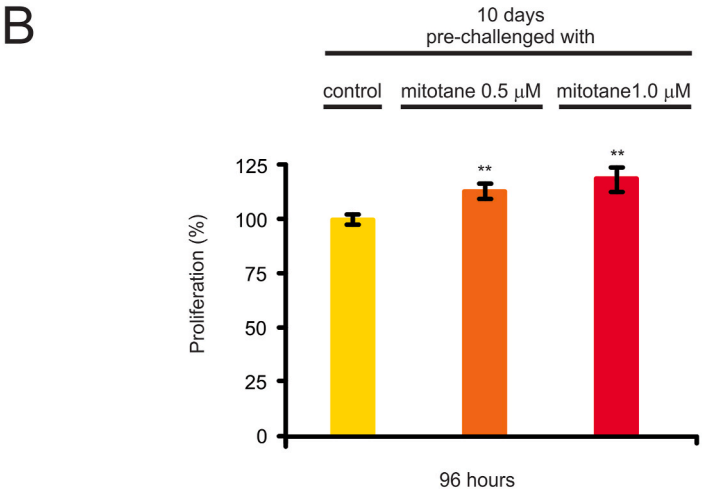


Fig. 2. Mitotane improves viability in H295R cells. After H295R cell pre-exposure to mitotane (0.5 or 1 μM) for 10 days, cells were post-challenged with mitotane (10 or 20 μM) for 48 h, and cell survival was evaluated through MTT assays. Data represent the means ± SD of three independent experiments, ***p < 0.001 pre-post-challenged cells vs. control post-challenged cells.





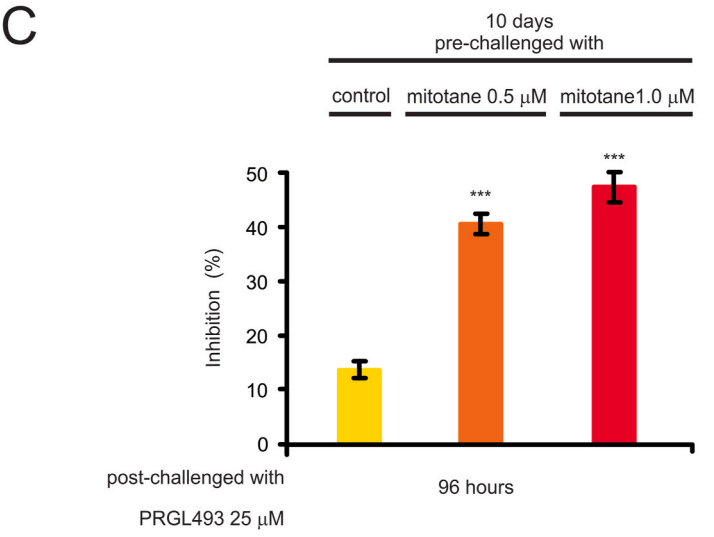


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Fig. 3. Mitotane modulates ACSL4 protein expression and proliferation in H295R cells. After H295R cell pre-exposure to mitotane (0.5 or 1 μM) for 10 days, A) ACSL4 mRNA expression was analyzed through qRT-PCR, and protein levels were analyzed through Western blot in whole cell extracts, and the integrated optical density was normalized to GAPDH and β-tubulin signal, respectively; B) cells were grown for 96 h, and cell proliferation was measured through BrDU incorporation assays; C) cells were post-challenged with ACSL4 inhibitor PRGL493 (25 μM) for 96 h, and the inhibition of cell proliferation was measured through BrDU incorporation assays. Data represent the means ± SD of three independent experiments: A) ***$p < 0.001$ challenged cells vs. non-challenged cells; B) **$p < 0.01$ challenged cells vs. non-challenged cells; C) ***$p < 0.001$ pre-post-challenged cells vs. non-challenged cells.

3. Results

3.1. Association of ACSL4, ABCG2 and ABCB1 with mitotane-resistant ACC cells

Mitotane is a potent inhibitor of tumor growth and enzymes implicated in the synthesis of steroid hormones in adrenal tumors [39]. We used bioinformatics analysis of public database GSE140818, which uses human HAC15 cells clonally isolated from NCI–H295, a ACC cell line [40] and treated with mitotane for a long period to develop resistance. In this database, we evaluated the expression of ACSL4, an enzyme implicated in tumor cell survival [41], proliferation [20,23] and resistance to drugs [16,17]. In addition, we assessed ABCG2 and ATP Binding Cassette Subfamily B Member 1 (ABCB1), both associated with tumor resistance to drugs [42]. Analyses revealed higher ACSL4 and ABCG2 expression in HAC15 cells resistant to mitotane when compared to wild type cells ($p < 0.05$) (Fig. 1A and B). However, lower ABCB1 expression was observed in adrenal tumor cells resistant to mitotane as compared to non-resistant cells ($p < 0.01$) (Fig. 1C). These data show that mitotane resistance in HAC15 cells is associated with increased expression of ACSL4 and ABCG2 but reduced expression of ABCB1.

3.2. Mitotane modulates the viability, expression and activity of ACSL4 and ABCG2 in H295R cell

As described above, prolonged exposure to mitotane triggers resistance and alterations in cell function in HAC15 cells [36]. For these reasons, we analyzed the impact of prolonged exposure to mitotane in H295R cells, which are characterized by a lowly aggressive phenotype [43]. With a view to replicating the low levels of mitotane found in blood at early stages of treatment and the subsequent increase [44], cells were first exposed to either vehicle or low concentrations of mitotane (0.5 and 1 μM) and later treated with higher concentrations of mitotane (10 and 20 μM) (Fig. 2). The viability of cells initially treated with vehicle and later exposed to high mitotane concentrations was 85.03 % ± 2.54 and 78.10 % ± 1.33, respectively. In contrast, cells pre-treated with 0.5 μM of mitotane followed by 10 μM mitotane rendered viability values of 98.22 % ± 2.19, while cells pre-treated with 0.5 μM mitotane followed by 20 μM mitotane showed values of 94.01 % ± 1.08. Furthermore, pre-treatment with 1 μM mitotane followed by 10 or 20 μM mitotane yielded comparable values (Fig. 2). These results indicate that early exposure to low mitotane concentrations reduces cell sensitivity to higher drug concentrations and boosts cell resistance to more aggressive treatments.

On the basis of the results reported in Fig. 1, and given ACSL4 association with cell resistance to drugs [16–18], we further evaluated ACSL4 expression in H295R cells treated with mitotane. Exposure to mitotane induced an increase in ACSL4 mRNA ($p < 0.001$) and protein ($p < 0.001$) expression (Fig. 3A, Suppl. Fig. 3) and, most interestingly, a significant increase in cell proliferation ($p < 0.01$) (Fig. 3B). Using a pharmacological approach, we next assessed the participation of ACSL4 in cell proliferation using ACSL4 inhibitor PRGL493 [18]. Interestingly, exposure to 0.5 μM mitotane followed by 25 μM PRGL493 rendered 40.78 % ± 1.86 inhibition of proliferation, while 1.0 μM mitotane followed by 25 μM PRGL493 produced stronger inhibition (47.48 % ± 3.89). Cell exposure to 25 μM PRGL493 in the absence of mitotane treatment inhibited proliferation only by 14.09 % ± 1.78 (Fig. 3C). In other words, early cell exposure to mitotane and later PRGL493 administration promoted the most significant inhibition of cell proliferation, probably as a result of an increase in ACSL4 expression (Fig. 3A, Suppl. Fig. 3). In sum, this section provides evidence of the impact of mitotane on ACSL4 expression and the role of ACSL4 activity in mitotane-treated H295R cell proliferation.

Even though ACSL4 has been associated with ABCG2 in fostering resistance to drugs in tumor cell systems [16,17], ABCG2 expression had never been addressed in H295R cells so far. For these reasons, and based on the results shown in Fig. 1, we next evaluated ABCG2 mRNA and protein expression following mitotane administration. Strikingly, exposure to mitotane at the concentrations described above induced a marked increase in both ABCG2 mRNA ($p < 0.001$) and protein ($p < 0.001$) (Fig. 4A, Suppl. Fig. 3).

As compound efflux is one of the mechanisms underlying tumoral cell resistance to drugs, we further evaluated accumulation and efflux using doxorubicin, a fluorescent compound which can use different transporters with different degrees of affinity, including ABCG2 [16,45,46]. H295R cells were exposed to low mitotane concentrations and then tracked through fluorescence microscopy [46]. Results showed lower doxorubicin accumulation in cells previously exposed to mitotane as compared to vehicle treatment, which is indicative of higher compound efflux (Fig. 4B). We next examined the role of inhibitor Ko143 in doxorubicin efflux in H295R cells. While Ko143 has been reported to inhibit other transporters, the concentration used in this study is known to particularly inhibit ABCG2 [47,48]. Interestingly, cells pre-treated with mitotane and later treated with Ko143 showed milder compound efflux, as evidenced by higher intracellular doxorubicin levels ($p < 0.001$) (Fig. 4B).

To corroborate this finding, further assays were carried out on transporter activity using Hoechst 33342, a fluorescent compound efficiently transported by ABCG2 [48]. A decrease was observed in intracellular Hoechst 33342 levels in cells pre-treated with mitotane($p < 0.001$) (Fig. 4C). Furthermore, Ko143 treatment offset this effect, increasing intracellular Hoechst 33342 fluorescence, probably as a result of a decrease in ABCG2 activity.

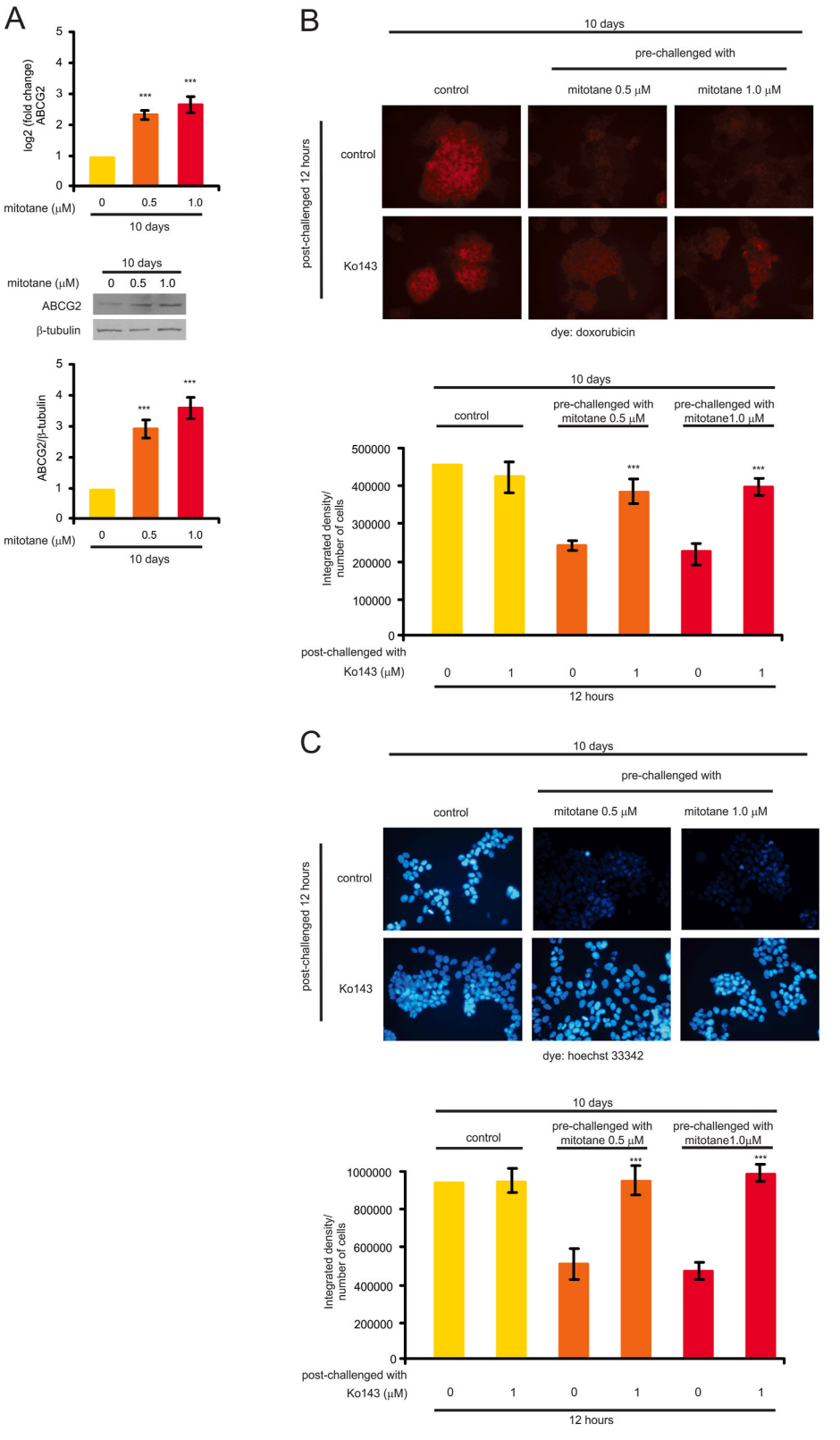

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Fig. 4. Mitotane modulates ABCG2 protein expression and compound efflux in H295R cells. After H295R cell pre-exposure to mitotane (0.5 and 1 μM) for 10 days, A) ABCG2 mRNA expression was analyzed through qRT-PCR, and protein levels were analyzed through Western blot in whole cell extracts, and the integrated optical density was normalized to GAPDH and β-tubulin signal, respectively; B) cells were post-challenged with ABCG2 inhibitor Ko143 (1 μM) for 12 h and then stained with doxorubicin (10 μM) to assess compound efflux through epifluorescence microscopy; C) cells were post-challenged with ABCG2 inhibitor Ko143 (1 μM) for 12 h and then stained with Hoechst 33342 (5 μg/ml) to assess compound efflux through epifluorescence microscopy. Data represent the means ± SD of three independent experiments: A) ***$p < 0.001$ challenged cells vs. non-challenged cells; B) ***$p < 0.001$ pre-post-challenged cells vs. pre- but non-post-challenged cells; C) ***$p < 0.001$ pre-post-challenged cells vs. pre- but non-post-challenged cells.

3.3. Chemotherapeutic drugs modulate the viability, expression and activity of ACSL4 in H295R and MCF-7 cells

Doxorubicin and cisplatin are used in combination therapy for the treatment of ACC [49]. These chemotherapeutic drugs inhibit the cell cycle by interacting with DNA in cells with high proliferative capacity [50]. Once again, to replicate the low levels of cisplatin and doxorubicin detected in blood at early stages of treatment [30,33], H295R cells were exposed to 20 nM doxorubicin or 200 nM cisplatin for 10 days and later treated with 1 μM doxorubicin or 5 μM cisplatin for viability assays (Fig. 5A). Strikingly, viability was most potently affected in cells exposed to higher concentrations of doxorubicin and cisplatin in the absence of previous exposure to low concentrations. Indeed, the viability of H295R cells initially treated with vehicle and later exposed to high concentrations of doxorubicin or cisplatin was 85.87 % ± 0.54 and 80.20 % ± 1.21, respectively. In contrast, cells pre-treated with low concentrations of doxorubicin followed by high concentrations of doxorubicin or cisplatin were 102.58 % ± 2.09 and 100.91 % ± 3.18 viable, respectively. In addition, cells pre-treated with low concentrations of cisplatin followed by high concentrations of doxorubicin or cisplatin were 101.82 % ± 2.12 and 100.91 % ± 1.08 viable, respectively (Fig. 5A). Moreover, viability did not differ between cells undergoing early and late treatments with doxorubicin and cisplatin, and those exposed to the same drug at the two time points and concentrations (Fig. 5A). Interestingly, the same protocol applied to the lowly aggressive cell line MCF-7 [51] rendered viability values comparable to those of H295R cells (Fig. 5B). Overall, our results hint at an improvement in cell survival in lowly aggressive cell lines, as cells lose sensitivity to chemotherapeutic drugs at high concentrations.

Considering these findings, we further assessed the expression of ACSL4 mRNA and protein in H295R and MCF-7 cells submitted to low concentrations of chemotherapeutic drugs. qRT-PCR and Western blot analyses demonstrated an increase in ACSL4 mRNA ($p < 0.001$) and protein ($p < 0.001$) expression, respectively, upon early doxorubicin and cisplatin treatment (Fig. 6A, Suppl. Figs. 4 and 5). Moreover, cells pre-treated with chemotherapeutic drugs showed significantly higher proliferation capacity (H295R, $p < 0.01$; MCF-7, $p < 0.05$) (Fig. 6B), an effect attenuated by the inhibitor of ACSL4 activity Specifically, H295R cell exposure to 1 μM doxorubicin followed by 25 μM PRGL493 rendered 47.36±3.69 inhibition of proliferation, while 5 μM cisplatin followed by 25 μM PRGL493 inhibited cell proliferation by 42.72 % ± 3.15. In contrast, single treatment with 25 μM PRGL493 induced only mild inhibition of cell proliferation (14.09 % ± 1.78) (Fig. 6C). These results show that H295R cell exposure to early doxorubicin or cisplatin treatment and later PRGL493 administration promoted the most significant inhibition of cell proliferation, probably as a consequence of an increase in ACSL4. Comparable results were obtained in MCF-7 cells (Fig. 6C). These findings indicate that exposure to chemotherapeutic drugs at low concentrations increases ACSL4 expression, which may have enhanced ACSL4 activity and its impact on cell growth in these lowly aggressive cell lines.

3.4. Chemotherapeutic drugs modulate compound efflux via ABCG2 in H295R cells

Drawing on the findings presented above, we next examined the mRNA and protein expression of ABCG2, expressed in tumor cells which have become resistant to chemotherapeutic drugs [52,53]. ABCG2 mRNA and protein expression increased upon cell treatment with low concentrations of chemotherapeutic drugs ($p < 0.001$; $p < 0.001$) (Fig. 7A, Suppl. Fig. 4).

Cells previously treated with chemotherapeutic drugs exhibited a reduction in fluorescent substrate doxorubicin as compared to untreated cells, which indicates higher drug efflux ($p < 0.001$). Moreover, this effect was offset by Ko143 ($p < 0.001$), which induced a recovery in fluorescence toward control values (Fig. 7B). We further used Hoechst 33342 as a fluorescent substrate. Results showed the activation of, at least, ABCG2 upon cell pre-treatment with doxorubicin or cisplatin −which rendered Hoechst 33342 efflux− and its blockage by later exposure to Ko143 ($p < 0.001$) −which restored control efflux levels (Fig. 7C). These results indicate that H295R cell treatment with low concentrations of chemotherapeutic drugs produces an increase in the expression and activation of ABCG2. These data are in line with results reported by Calcagno et al. in MCF-7 cells [54].

4. Discussion

This manuscript reports an increase in ACSL4 and ABCG2 expression and activity after cell exposure to non-lethal concentrations of antitumor agents for several days and impacted cellular functions associated with the progression to aggressive phenotypes in lowly aggressive ACC cells H295R.

Results showed an increase in ACSL4 expression after H295R cell exposure to mitotane. Most interestingly, recent studies have reported high ACSL4 expression in highly aggressive, metastatic ACC-derived cells as compared to untreated H295R cells [55]. Furthermore, an increase in ACSL4 expression was also observed in breast tumor cells MCF-7 after exposure to chemotherapeutics drugs. This cell line is also known to exhibit lower ACSL4 expression than more aggressive, antitumor drug-resistant breast cancer cell lines [20,56]. Therefore, in being lowly aggressive and having low native ACSL4 expression, H295R and MCF-7 cells constitute suitable

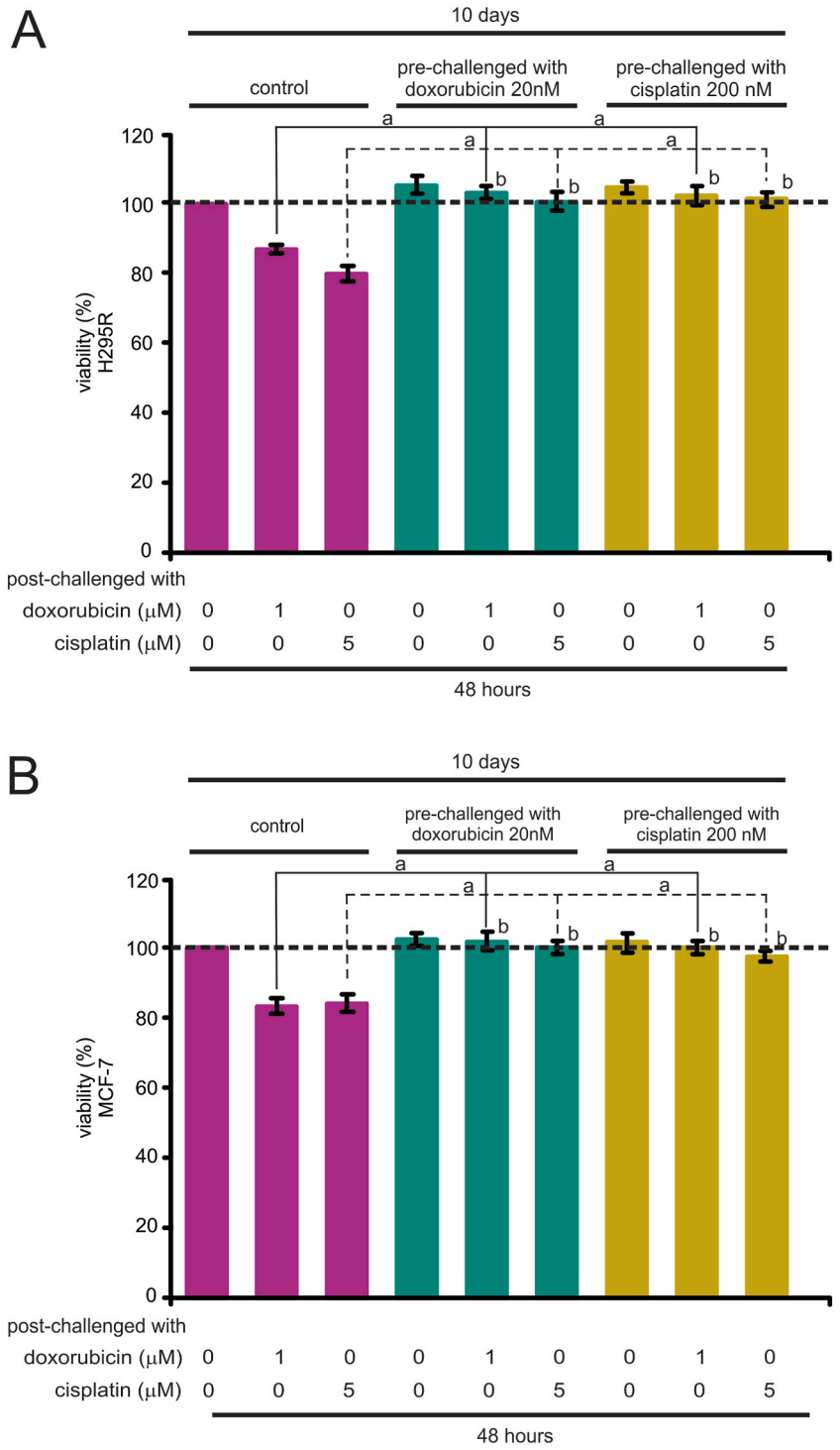


Fig. 5. Doxorubicin and cisplatin change cellular viability in H295R and MCF-7 cells. After H295R and MCF-7 cell pre-exposure to doxorubicin (20 nM) or cisplatin (200 nM) for 10 days, A) H295R cells were post-challenged with doxorubicin (1 μM) and cisplatin (5 μM) for 48 h, and cell survival was evaluated through MTT assays; B) MCF-7 cells were post-challenged with doxorubicin (1 μM) and cisplatin (5 μM) for 48 h, and cell survival was evaluated through MTT assays; Data represent the means ± SD of three independent experiments: A and B) a: ***$p < 0.001$ pre- and post-challenged cells vs. control post-challenged cells; b: not significant, pre-post-challenged cells vs. pre- but non-post-challenged cells.

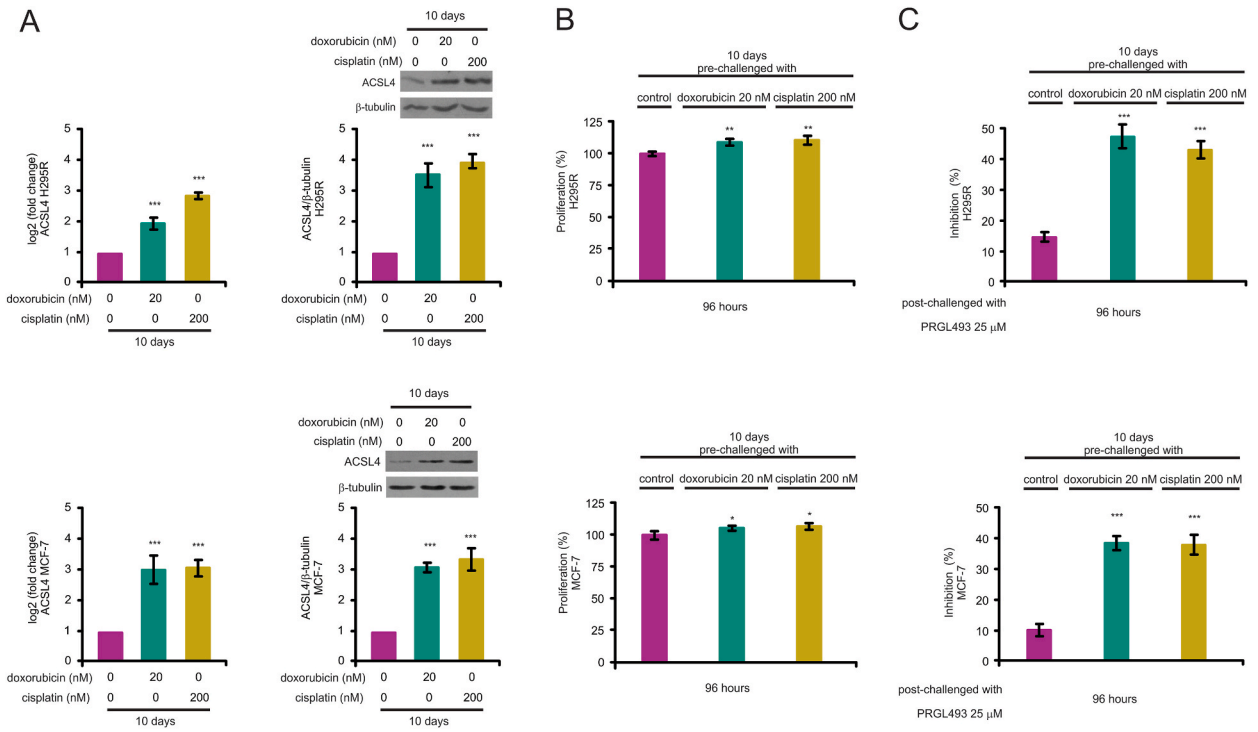


Fig. 6. Doxorubicin and cisplatin modulates ACSL4 protein expression and proliferation in H295R and MCF-7 cells. After H295R and MCF-7 cell pre-exposure to doxorubicin (20 nM) or cisplatin (200 nM) for 10 days, A) ACSL4 mRNA expression was analyzed through qRT-PCR, and protein level was analyzed through Western blot in whole cell extracts, and the integrated optical density was normalized to GAPDH and β-tubulin signal, respectively; B) cells were grown for 96 h, and the cell proliferation was measured through BrDU incorporation assays; C) cells were post-challenged with ACSL4 inhibitor PRGL493 (25 μM) for 96 h, and the inhibition of cell proliferation was measured through BrDU incorporation assays. Data represent the means ± SD of three independent experiments: A) ***$p < 0.001$ challenged cells vs. non-challenged cells; B) H295R **$p < 0.01$, MCF-7 *$p < 0.05$ challenged cells vs. non-challenged cells; C) ***$p < 0.001$ pre-post-challenged cells vs. non-challenged cells.

models to study changes in ACSL4 and cell response to antitumor treatment.

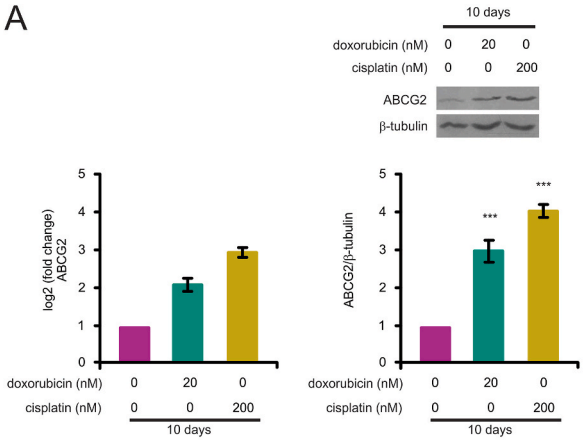
Regarding ABCG2, evidence consistent with our results has been reported by other authors in different cell lines. For instance, low mitotane concentrations increase ABCG2 expression in human colon adenocarcinoma cells LS180 [57]. Mitotane also generates the activation of signaling pathways associated with drug elimination, including increased expression of ABCG2 mRNA, in LS180 cells [57].

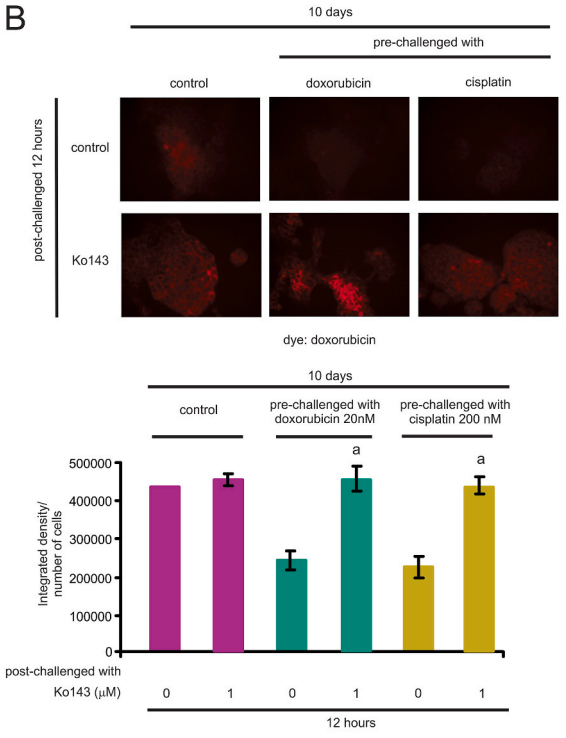
Studies carried out in biological tumor samples treated with chemotherapeutic drugs are in line with the results reported here. For instance, public database GSE28647, which comprises data on ovarian cancer cells of patients, has shown an increasing tendency in ACSL4 and ABCG2 expression in cells treated with cisplatin (Suppl. Fig. 1). A comparable tendency was observed in database GSE162187, where breast cancer tissue samples resistant to doxorubicin treatment showed higher ACSL4 and ABCG2 expression than tissue samples sensitive to treatment (Suppl. Fig. 2). In addition, single treatment with low concentrations of doxorubicin in breast (MCF-7), ovary (IGROV-1) and colon (S-1) tumor cells induces epigenetic changes which increase the expression of ABCG2 and resistance to doxorubicin and mitoxantrone [54]. In lung cancer cells NCI–H446, low cisplatin concentrations upregulate proteins associated with drug resistance, including the ABCG2 transporter [58]. Furthermore, reports have revealed considerably higher levels of ABCG2 in cyclophosphamide-treated xenograft tumor adrenocortical carcinoma cells SW-13 as compared to untreated tumor cells [59].

Although mitotane has been reported to reduce cell viability and boost apoptosis in non-resistant H295R cells treated with lethal high concentrations (62 μM) [60], in the current work, non-lethal drug concentrations generated an increase in H295R of cellular viability, proliferation, and compound efflux. These results resemble those obtained in mitotane-resistant ACC cell lines [36], which again suggests that pre-treatment with low mitotane concentrations triggered events associated with resistance.

Pre-treated H295R cells developed resistance to later treatment with higher drug concentrations. Worth highlighting, this cell response differs from that of previously untreated H295R cells and is actually comparable to the response observed in more resistant, metastatic ACC-derived cell lines exposed to different treatments [61,62]. Moreover, H295R cells are more sensitive to mitotane than metastatic ACC-derived cell line [63].

As H295R cells have a low rate of proliferation (doubling every 48 h), modifications in the expression of ACSL4 may have yielded a gain of function which was abolished by inhibitor PRGL493. Of note, ACSL4 inhibition by PRGL493 increases sensitivity to chemotherapeutic treatment in highly aggressive breast and prostate cancer cells [18]. The inhibitory action of PRGL493 on ACSL4





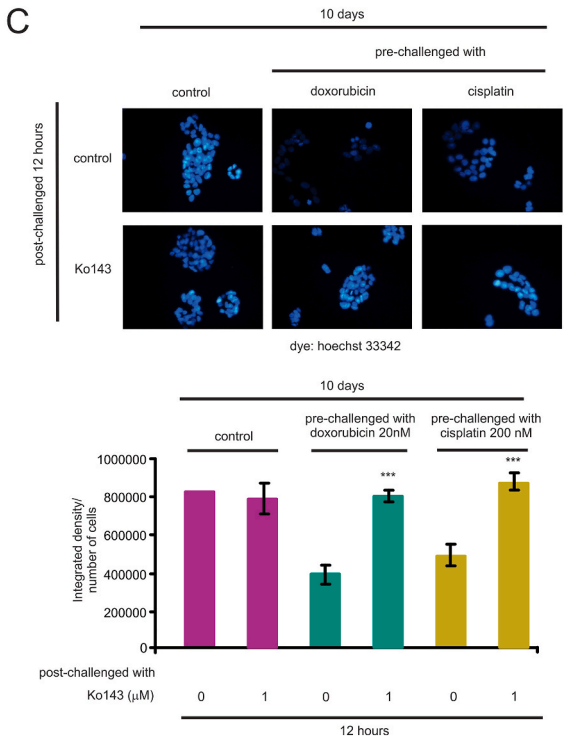


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Fig. 7. Doxorubicin and cisplatin modulate ABCG2 protein expression and compound efflux in H295R cells. After H295R cell pre-exposure to doxorubicin (20 nM) or cisplatin (200 nM) for 10 days, A) ABCG2 mRNA expression was analyzed through qRT-PCR, and protein level was analyzed through Western blot in whole cell extracts, and the integrated optical density was normalized to GAPDH and β-tubulin signal, respectively; B) cells were post-challenged with ABCG2 inhibitor Ko143 (1 μM), and then stained with doxorubicin (10 μM) to assess compound efflux through epifluorescence microscopy; C) cells were post-challenged with ABCG2 inhibitor Ko143 (1 μM) and then stained with Hoechst 33342 (5 μg/μl) to assess compound efflux through epifluorescence microscopy. Data represent the means ± SD of three independent experiments: A) ***$p < 0.001$ challenged cells vs. non-challenged cells; B) a: ***$p < 0.001$, pre-post-challenged cells vs. pre- but non-post-challenged cells; C) ***$p < 0.001$ pre-post-challenged cells vs. pre- but non-post challenged cells.

enzymatic activity has been previously described. Moreover, ACSL4 inhibition −either by doxycycline or PRGL493− in MCF-7 Tet-Off/ACSL4, have rendered a strong inhibition of cell proliferation [18]. In contrast, PRGL493 has only mild inhibitory effects on control MCF-7 cells, as they have low endogenous ACSL4 expression [18].

Worth pointing out, the participation of other transporters cannot be ruled out. However, ABCB1 expression decreases in mitotane-resistant ACC tumor cells, although its native expression is known to be high in cancer cell lines and adrenocortical tumors [64]. At a pharmacological level, the inhibition of ABCB1 has been shown to increase sensitivity to antitumor treatment with doxorubicin and etoposide in non-resistant adrenal tumor cells [65]. Furthermore, the participation of ABCB1 in compound efflux in the current work might be disregarded, as the Ko143 concentration used is known to preferentially inhibit ABCG2 [47]. Differences between ABCG2 and ABCB1 expression have been reported in triple-negative breast tumors, with metastatic tumors showing high ABCB1 expression [66].

An increase in cell viability and proliferation was also observed in lowly aggressive breast tumor cells MCF-7 after exposure to doxorubicin or cisplatin. It should be pointed out that, in MCF-7 cells, mitotane has been shown to mimic 17β-estradiol, binding to and activating estrogen receptor alpha (ERα) and thus boosting cell viability and proliferation [67]. 17β-estradiol is also known to induce ACSL4 via ERα in ERα-positive breast tumor cell lines [68].

Tumor cells pre-treated with mitotane, cisplatin and doxorubicin also provided evidence indicative of cross-resistance to new treatments. Furthermore, pre-challenge with antitumor drugs increased doxorubicin efflux in H295R cells, at least in part through the action of ABCG2. In agreement with our findings, SK-BR-3 and MCF-7 breast tumor cells resistant to chemotherapeutic agents of the taxane family have shown cross-resistance to doxorubicin, with ABC transporters playing a crucial role [52]. Several ovarian cancer cell lines exposed to cancer-specific drugs in first- and second-line chemotherapy generated cross-resistance between treatments and effects associated with the expression and activity of ABCG2 and ABCB1 [34]. Cross-resistance is linked to epigenetic modifications, regardless of drug targets. Studies in MCF-7 cells resistant to doxorubicin and cisplatin have revealed several common epigenetic alterations such as the loss of global DNA methylation, changes in histone methylation and phosphorylation, and the expression of transferases [69]. In contrast, ACC HAC-15 cell clones stably resistant to mitotane are sensitive to doxorubicin [36]. Moreover, as mitotane, cisplatin and doxorubicin are used as first-line treatment in patients with ACC, it has been suggested that cross-resistance should be checked to other agents used as second-line treatment, for instance, temozolomide [70]. This evidence may hint at a connection between the degree of resistance to treatments and the origin of the cell type.

In this scenario, our studies at a cellular level unveil events associated with increased native expression of these proteins in tumor cells with lowly aggressive phenotypes. In this context, with ABCG2 as a marker of tumor-initiating and chemoresistant cells [71] and ACSL4 as a marker of cell and tumor aggressiveness [72], our results shed light on the impact of these proteins at stages in which cells are only lowly aggressive and non-resistant. These results call for new strategies which contemplate overall drug effects, their cellular target, and their interaction with transporting proteins, and which may thus help prevent unwanted cellular changes and the progression to more aggressive stages. More specifically, new strategies may benefit from the analysis of ACSL4 and ABCG2 expression and aid decision making in patient therapy.

Author contribution statement

Mayra Agustina Ríos Medrano: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

María Mercedes Bigi: Conceived and designed the experiments; Analyzed and interpreted the data.

Paloma Martínez Ponce: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Ernesto Jorge Podestá: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ulises Daniel Orlando: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Funding statement

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to publish or preparation of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20769.

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