Effects of Chemotherapy on Placental Development and Function using *in vitro* Culture of Human Primary Cytotrophoblasts

Authors

Christophe Louis Depoix

Arthur Colson

Mina Mhallem-Gziri

Corinne Hubinont

Frédéric Debiève

Institution

Department of Obstetrics, Institut de Recherche Clinique et Expérimentale (IREC), Université Catholique de Louvain, Bruxelles, Belgium.

Acknowledgments:

This work was fully supported by “Fetus for Life” charity, Belgium. The authors are grateful to Séverine Gonze for her help in trophoblastic cell cultures, real-time PCR, and western blotting experiments.

Summary

Introduction Cancers during pregnancy can be treated with chemotherapy after the first trimester but the treatment is associated with smaller placentas and an increased risk of stillbirth, fetal growth retardation and preterm delivery. We decided to assess the effect of several chemotherapeutic agents on placental development by using *in vitro* culture of human term cytotrophoblasts. *Methods* Cytotrophoblasts isolated from term placentas were cultured for 48 hours and treated for 24 hours with epirubicin, docetaxel, vinblastine, methotrexate, tamoxifen, 4-hydroxytamoxifen, and endoxifen. First, cell viability was assessed. Then, the effect of the treatment on trophoblast differentiation and placental angiogenesis was assessed by quantifying hCG and PlGF mRNA and protein expression. Finally, the expression of two efflux transporters, BCRP and MDR1 was investigated. *Results* Epirubicin only strongly decreased cell viability. Epirubicin, docetaxel, and vinblastine inhibited HCGB and PlGF expression while methotrexate, tamoxifen and its two metabolites increased it. BCRP was essentially expressed in syncytiotrophoblasts and MDR1 in undifferentiated cytotrophoblasts. Their expression was not affected by the drugs but vinblastine increased *BCRP* mRNA expression by 2.8-fold. *Discussion* The most commonly used chemotherapeutic drugs are well supported *in vitro* by syncytiotrophoblasts, except for epirubicin, which was very cytotoxic. Chemotherapy perturbed the expression of genes normally upregulated during placental differentiation and angiogenesis but not the expression of the drug transporters. Further studies looking at the effect of combination therapy and the transporter capacities to reject the drugs will be needed to better define the effects of chemotherapy on placental development and function.

Keywords: Cancer, Chemotherapy, Placenta, Trophoblast, Viability, Gene expression

INTRODUCTION

Cancer in pregnancy is still an uncommon occurrence that concerns 1 to 2 cases per 1000 pregnancies but this number is increasing. Several explanations may be advanced like higher cancer rates in general, a better diagnostic, and the increase of the maternal age at the time of the first pregnancy. Cancers diagnosed during pregnancy are the same as cancers diagnosed in non-pregnant women of the same age. The most common cancers are, in the order of incidence, breast cancer, cervical cancer, Hodgkin and non-Hodgkin lymphoma, melanoma, leukemia, ovarian cancer, and colorectal cancer [1]. Among cancer treatments, chemotherapy can be started depending on the period of gestation. During the first 10 days of gestation corresponding to the period of fertilization and implantation of the embryo, the effect of chemotherapy is either all or nothing: normal development of the embryo or its death [2]. Then, from this period up to 8 weeks, which corresponds to organogenesis, the use of chemotherapeutic drugs is associated with teratogenesis and major congenital malformations. If possible, it is better not to start chemotherapy before the end of the first trimester. After that period, the effects of chemotherapy on pregnancy are still very confused. When the treatments are given during the second and third trimesters, it is relatively well supported by the fetus [3]. The effects appear to be inversely related to the gestational age. However, the risks of stillbirth, fetal growth retardation and preterm delivery are increased [4]. It is still unclear if all these pregnancy outcomes are caused by the chemotherapeutic drugs or the cancer itself.

If chemotherapy is relatively well supported during pregnancy, it may be because of the protective function of the placenta, which forms a blood-placenta barrier. The very important cells of this barrier are the syncytiotrophoblasts. They cover the entire surface of the placental cavity, and are therefore directly in contact with the maternal blood. Interestingly, trophoblasts share common features with cancer cells [5]. They express and secrete the same growth factors, glycoproteins, and their cognate receptors. As being integral part of the placental barrier, trophoblasts express at their apical cell membrane several members of the ATP-binding cassette (ABC) superfamily of transporters, responsible for cancer drug resistance [6]. These efflux transporters are important in preventing xenobiotics present in the maternal blood to enter into the fetal blood circulation. For example, syncytiotrophoblasts express the P-glycoprotein (P-gp), encoded by the *Multidrug resistance 1* (*MDR1*) gene, the multidrug resistance proteins (MRP), and the breast cancer resistant protein (BCRP). All these efflux transporters may play a very important role in the ability of the placenta to prevent placental drug transfer toward the fetal compartment.

Currently, very little is known about the effects of commonly used anti-cancer agents on trophoblast biology. That is why we decided to study the effect of chemotherapy on the

placental development and function by using *in vitro* cultures of human primary cytotrophoblasts treated with a wide panel of chemotherapeutic drugs. Usually, patients receive combination cancer therapies that combine two or more drugs, but for this study, we decided to treat the cells with each drug separately. The drugs chosen were the anthracycline epirubicin and the two antineoplastic agents docetaxel and vinblastine. We also included the derivative of the folic acid analogs, the antimetabolite methotrexate, because it is used to treat not only cancers but also rheumatoid arthritis, lupus, and juvenile arthritis potentially in young women of childbearing age. Therefore, it is important to know if its use during pregnancy may be safe or not, since its effects are unpredictable [7-9]. Finally, we included the selective estrogen receptor modulator tamoxifen and its two most pharmacologically active metabolites 4-hydroxytamoxifen and endoxifen [10,11], because, even if their use in pregnancy is usually contraindicated, their detrimental effects on pregnancy outcomes are still debated [12]. First, we measured the cell viability. Second, we assessed the effects on trophoblast differentiation and placental angiogenesis by quantifying the expression of human chorionic gonadotropin hormone β subunit (hCGβ) and placental growth factor (PlGF). Finally, we also investigated the effect of the chemotherapeutic drugs on the placental barrier by quantifying BCRP and MDR-1 expression.

MATERIAL AND METHODS

Drugs

Epirubicine hydrochloride was purchased from Alfa Aesar (ThermoFisher, Kandel, Germany). Docetaxel was purchased from Acros Organics (New Jersey, US). Vinblastine sulfate salt was purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Methotrexate was purchased from Biochemica (Applichem, Darmstadt, Germany). Tamoxifen (TMX), free base, was purchased from MP Biomedicals (Solon, OH, USA). (Z)-4-Hydroxytamoxifen (4-OH-TMX) was purchased from Enzo Life Sciences (Lausen, Switzerland), and endoxifen (Endo) was purchased from Tocris (Abingdon, UK). All the drugs were prepared in dimethyl sulfoxide (DMSO).

Placenta Collection

The placentas were collected from elective cesarean or vaginal full-term deliveries with the approval of the ethical committee at Université Catholique de Louvain, Brussels, Belgium. Written informed consent was received from all the patients.

Cytotrophoblast Isolation and Purification

Cytotrophoblasts were isolated and purified following a modified version of a protocol already published elsewhere [13]. In brief, the placenta was cut into 1-cm^2 pieces that were thoroughly washed with cold 0.9% NaCl solution. The decidua, the amniotic membranes and the large blood vessels were removed. The fragments were minced and incubated at 37 °C under strong stirring for one hour in 1X HBSS (Hank's buffer saline solution, Gibco, Life Technologies, Paisley, UK) containing 2.4 U/ml dispase II (Life Technologies). For the last 15 minutes, DNase I, grade II (Roche, Mannheim, Germany) was added to the solution to a final concentration of 100 U/ml. The solution was then filtered through 200, 100 and 40-µm filters and the cells pelleted at 500 g for 15 min. The cell pellet was resuspended in fetal calf serum (FBS) free-Iscove's Modified Dulbecco Medium (IMDM, Gibco, Life Technologies) containing 50 µg/ml gentamicin (Carl Roth GmbH, Karlsruhe, Germany). The cells were separated by density gradient centrifugation in a 5–70% discontinuous Percoll^{TM} gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The cellular fraction corresponding to a density of 1.048-1.062 g/l was collected. Cells were further washed with FBS-free IMDM containing 50 µg/ml gentamicin. Finally, the cells were incubated for one minute at 37 °C in a 0.05% trypsin solution. The enzymatic reaction was inhibited by addition of pure FBS. The cells were then washed with FBS-free IMDM and counted with a hemocytometer. Cell viability was assessed by Trypan Blue exclusion (SIGMA-ALDRICH). The average yield was 107 x10^6 cytotrophoblasts (±38 x10^6) for 72 gr of placental tissue treated.

Cell culture and treatments

The cells were cultured in IMDM complemented with 10% fetal bovine serum (Life Technologies), and 50 µg/ml gentamicin (Carl Roth GmbH) in a humidified incubator at 37 °C, equilibrated with 95% air (20% oxygen) and 5% CO_2.

Cytotrophoblasts were plated in 96-well plates for cell viability assays or 6-well plates for RNA and protein expression quantification assays at a density of 400,000 cells/cm^2. The cells were cultured for 48 h to allow differentiation prior treatment with the chemotherapeutic drugs or for 4 days to quantify *MDR1* and *BCRP* expression. The cells were treated for 24 h with DMSO as vehicle (control wells), or with the appropriate drugs (see table 1 for the concentrations).

Viability Assays

Cell viability was quantified using CellTiter-Fluor™ cell viability assay kit (Promega, Madison, WI, USA) following the manufacturer protocol. This kit allows the determination of the viable cell number by measurement of protease activities in living cells. The reagent is cell-permeant and generates a fluorescent signal proportional to the number of live cells once it is cleaved by live-cell protease. The fluorescent signal was measured in a SpectaMax i3 multi-mode detection platform (Molecular Devices, Sunnyvale, CA, USA). The background signals (wells without cells) were subtracted from all the results from well-containing wells. The wells with untreated cells (DMSO control wells) were considered as the reference wells, and fixed to 100%. The results were represented as the percentage of viability versus the log of drug concentration, and the median lethal dose (Lethal Dose, 50% or LD 50) was calculated when possible.

RNA Extraction and Purification

The wells were washed three times with 1X PBS and total RNA was extracted and purified using Roti®-Prep RNA Mini kit (Carl Roth GmbH) following the manufacturer protocol. Total RNA concentration was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA). The ratios of absorbance at 260 and 280, and at 260 and 230 were used to estimate RNA quality, sufficient for subsequent qPCR experiments.

Reverse Transcription

Cellular RNA (0.5 µg) was used as a template for the reverse transcription using qScript™ cDNA superMix (Quanta Biosciences, Gaithersburg, MD, USA). cDNA was diluted 10 times with RNase-free water.

Real-Time Quantitative Polymerase Chain Reaction

Five nanograms of cDNAs and 0.1 µmol/L specific primers (see Table 2 for sequences) were added to Takyon ROX SYBR 1X MasterMix dTTP blue (Eurogentec, Seraing, Belgium). The polymerase chain reaction was performed on a StepOne Real-Time PCR System (Applied biosystem, Foster City, CA, USA) following the recommended protocol (Tm at 60 °C). The PCR reactions were performed in duplicate for each cDNA, averaged and normalized to *TBP* and *SDHA* reference transcripts. RNA was quantified using the ΔΔCt method of relative quantification. The specificity of the amplification was verified by doing a melting curve, which generated a single sharp peak for each product.

Western Blotting

Twenty micrograms of total protein extract were loaded onto a 10% Acrylamide/Bisacrylamide Bolt™ Bis-Tris mini gel (Invitrogen by Thermo Fisher Scientific) and separated in 1X Bolt™ MES SDS running buffer for 22 minutes at 200 volts. Prestained Protein Ladder (ab116028, Abcam) was used to check the transfer efficiency and to estimate the molecular weight of the proteins. Proteins were transferred onto an Invitrolon™ PVDF membrane (Invitrogen) for one hour at 100 volts in 1X Bolt™ transfer buffer with 10% methanol. After complete transfer, the membrane was blocked for one hour in 5% non-fat dry milk in 1X PBS-T (50 mM Na/HPO_4, 150 mM NaCl and 0.05% Tween-20). The membrane was then incubated overnight at 4 °C with the primary antibodies (see table 2) diluted in 1X PBS-T containing 5% non-fat dry milk. The membrane was washed three times with 1X PBS-T for 15 min each and incubated for one hour at room temperature with the HRP-conjugated secondary antibodies. Bound antibodies were detected using the SuperSignal™ West Pico PLUS chemiluminescent substrate (BCRP and MDR-1 detection) or Pierce ECL western blotting substrate (GAPDH detection) (Thermo Scientific, Rockford, IL, USA), and Amersham Hyperfilm™ ECL films (GE Healthcare Limited).

Densitometric Analysis

Films were scanned and the intensity of BCRP, MDR-1, and GAPDH specific bands were measured with ImageJ 1.51s [14]. The results were reported as the ratio of BCRP and MDR-1 band relative intensities to GAPDH band relative intensities, and represented as the percentage change over control (0 h or DMSO-treated cells).

Secreted Protein Quantification

Secreted PlGF was quantified in the cell supernatants by ELISA using human PlGF Quantikine ELISA kit (R&D, Minneapolis, MN, USA).

Secreted HCGB was quantified on a B.R.A.H.M.S. Kryptor Compact Plus immune analyzer (Thermo Fisher Scientific) in the department of Laboratory Medicine, Cliniques Universitaires Saint-Luc (Brussels, Belgium).

The total protein quantities were normalized to the percentage of viability determined for each drug, and the results were graphed as the fold change relative to vehicle control.

Statistical analysis

A minimum of 5 placentas was used to isolate and purify cytotrophoblasts. The cell viability assay was done in duplicate. Gene and protein quantification measured by real-time qPCR and ELISA was done in duplicate for each sample. For the western blotting experiments and densitometric analysis, 3 placentas were used. All Results were expressed as the mean ± SEM, and were compared using one sample t-test as indicated in the figure legends. Graphs and statistical analysis were performed using GraphPad Prism® version 8.0.1 for windows (GraphPad software, San Diego, CA, USA).

RESULTS

Cell Viability

Primary cytotrophoblasts were first cultured for 48 h under 21% O_2 prior drug treatment to allow their differentiation into syncytiotrophoblasts. The exact amount of drugs reaching the placental cavity being unknown, we decided to treat with increasing amounts of epirubicin (E), docetaxel (D), vinblastine (V), methotrexate (M), tamoxifen (TMX), 4-OH-tamoxifen (OH-TMX), and endoxifen (Endo). The doses used were higher than the published concentrations measured in the serum of cancer patients in order to study cell toxicity and calculate the median lethal dose. The cells were incubated with the drugs for 24 hours. Because primary cytotrophoblasts do not proliferate *in vitro*, any decrease in the number of live cells will represent a decrease in cell viability but not a decrease in cell proliferation. Therefore, we did not quantify the number of dead cells in our experiments since it is supposed to be inversely proportional to the viability results.

Cell viability decreased strongly with epirubicin treatment (Fig. 1a). The calculated LD 50 was 0.31 µg/ml. With docetaxel (Fig. 1b), cell viability slightly decreased when doses were superior to 1 µg/ml to a maximum of 30% with the highest doses. Cell viability also decreased with vinblastine but no plateau was reached even for the higher doses (Fig. 1c). No LD 50 could be calculated but 50% viability was obtained when the cells were treated with 50 µg/ml of vinblastine. Finally, methotrexate, tamoxifen and its 2 metabolites had no real effect on cell viability (Fig 1d, 1e, 1f, and 1g).

Effects of Chemotherapy on HCGB and PlGF Expression.

The effect of chemotherapy treatment on placental development was investigated by quantifying the expression of HCGB, marker of trophoblast differentiation, and PlGF, a placenta-specific pro-angiogenic factor responsible for placental angiogenesis. Cells were treated with a unique dose for 24 h. The doses were chosen based on the viability results, corresponding to no more than 50% decrease in cell viability.

First, to estimate the differentiation status of the cells, we quantified *HCGB* and *PlGF* mRNA expression in untreated cells (Fig. 2a and 2d). *HCGB* mRNA expression increased 492.4 fold (±202.5 SEM) and *PlGF* mRNA expression increased 11.2 fold (±2.7 SEM) compared to undifferentiated cells (0 h), indicating that the cells were effectively differentiated into syncytiotrophoblasts.

Then, we assessed the effect of the chemotherapeutic drugs on HCGB and PlGF expression. For *HCGB* mRNA expression (Fig. 2b), epirubicin and docetaxel decreased its expression by 50%, while vinblastine decreased it by ~85%. Methotrexate slightly increased *HCGB* mRNA

expression. While Tamoxifen had no effect on *HCGB* mRNA expression, both its 2 metabolites, 4-hydroxytamoxifen and endoxifen, increased it by 2.64-fold (±0.26 SEM) and 1.74-fold (±0.17 SEM) over vehicle-treated cells, respectively. HCGB secreted in the supernatants was measured by ELISA (Fig. 2c). The results confirmed our qPCR results. HCGB secretion decreased when the cells were treated with epirubicin, docetaxel, and vinblastin. 4-hydroxytamoxifen and endoxifen increased it. Neither methotrexate nor tamoxifen had an effect on HCGB secretion.

The effect of the drugs on PlGF expression (Fig. 2e and 2f) was the same as the effect on HCGB except for docetaxel. Epirubicin and vinblastine decreased both PlGF mRNA expression and protein secretion while docetaxel had no real effect on PlGF expression. A very weak increase, but not statistically significant, was observed for both mRNA and protein secretion when the cells were treated with methotrexate and tamoxifen. With 4-hydroxytamoxifen and endoxifen, PlGF secretion was increased by 3.4-fold (±0.37 SEM) and 2.3-fold (±0.15 SEM), respectively.

Effect of Chemotherapy on the Placental Barrier

The efflux transporters involved in the protection of the fetus from xenobiotics were shown to be expressed in the placental cells. We decided to study BCRP and MDR-1, products of *ABCG2* and *ABCB1* genes, respectively, and to assess the effect of chemotherapy on their expression.

First, the expression was quantified in a 4-day *in vitro* culture of cytotrophblasts (Fig. 3). *BCRP* mRNA expression increased to a maximum of 6.75-fold (±2.8 SEM) after 72 h (Fig. 3a). *MDR-1* mRNA expression decreased strongly and rapidly after 24 h (0.65-fold ±0.29 SEM), and continued to decrease up to 96 h (0.27-fold ±0.06 SEM) (Fig. 3d). We verified BCRP and MDR-1 protein expression by western blotting using total protein extracts from cells cultured for 96 h (Fig. 3b and 3e). BCRP protein expression increased while MDR-1 protein expression decreased compared to total protein extracts prepared from undifferentiated cells. We then quantified their expression by densitometry. BCRP protein expression increased by 3.3-fold (333% ±104 SEM) compared to 0 h (Fig. 3c). MDR-1 protein expression decreased by 80% (19.66% ±4.4 SEM) compared to 0 h (Fig. 3f).

Finally, we quantified BCRP and MDR-1 expression after a 24-hour treatment with or without the chemotherapeutic drugs. After 72 hours in culture without treatment (DMSO-treated cells), BCRP expression effectively increased (Fig. 4a and 4b), while MDR1 expression decreased (Fig. 4c and 4d). Then, when the cells were treated with the drugs (Fig. 5), we observed a decrease in *BCRP* mRNA expression (Fig. 5a) with epirubicin and a weak increase with docetaxel and methotrexate. Vinblastine treatment increased *BCRP* mRNA expression by 2.8-

fold (±0.22 SEM) above vehicle-treated cells. Tamoxifen, and its 2 metabolites had no effect on *BCRP* mRNA expression. This treatment effect on *BCRP* mRNA expression could not be confirmed by quantification of BCRP protein expression (Fig. 5b), which was decreased with all the drugs previously tested. We did not either observe an increase in BCRP protein expression when the cells were treated with vinblastine.

For MDR1 expression (Fig. 5c and 5d), which was already decreased after syncytialization, epirubicin decreased it further but it was not statistically significant. Docetaxel weakly increased *MDR1* mRNA expression, while 4-hydroxytamoxifen and endoxifen decreased it. The inhibitory effect was stronger with 4-hydroxytamoxifen than with endoxifen, with a ~30% decrease compared with vehicle control. At the protein level, MDR1 expression decreased with all the drugs tested but the difference was not significant, except with 4-hydroxytamoxifen ($p < 0.05$).

DISCUSSION

Cancer during pregnancy is a rare occurrence but when it is diagnosed, it poses a clinical and ethical problem. Should the pregnancy be aborted to allow the use of chemotherapy to treat the mother? Should the pregnancy be allowed to continue and start the same treatment regimens used to treat non-pregnant women, taking the risk to harm the fetus? Should the treatment be started only after the birth of the baby? Actually, studies show that chemotherapy is relatively safe when started after the end of the first trimester, but it increases the risk of stillbirth, fetal growth retardation (FGR), and preterm birth. The placentas in chemotherapy-treated pregnancies are also smaller, indicating that the anti-cancer drugs perturbed the normal placental formation, probably leading to the FGR.

The most common cancer diagnosed during pregnancy is breast cancer. The drugs usually used in combination therapy are anthracyclins like doxorubicin and epirubicin, the latter being considered as less toxic [15], and taxanes (e.g., docetaxel).

We decided to assess the effect of a wide panel of commonly used chemotherapeutic agents on human primary trophoblasts. First, the cells' ability to withstand increasing doses of drugs was determined by measuring the cell viability. We used a wide range of drug concentrations that were much higher than the maximal plasmatic concentrations calculated in patients. The goal was to determine the capacity of the cells to resist to the treatment. Even if they share common features, primary trophoblasts are not cancer cells and therefore are not the target of the molecules used. They do not proliferate *in vitro* and may be more tolerant to anti-cancer agents, which are usually anti-mitotic. Interestingly, epirubicin was the only drug that permitted the calculation of the lethal dose at 50%. Cell viability decreased rapidly with increasing doses, to reach a low plateau representing nearly 100% cell death. With the other drugs, no change in cell viability was observed except with the highest doses, and no LD 50 could be calculated. The exact amount of drugs reaching the placental cavity and the placental villi is not well known but it is expected to be lower than the values measured in non-pregnant women due to the increased blood volume during pregnancy. Based on the values reported in the literature [16], the maximum concentration (Cmax) for epirubicin is 16.6 µM or 9 µg/ml, corresponding in our experiments to the highest doses giving the lowest cell viability. This result indicates that epirubicin is potentially very harmful to the placenta. To the contrary, docetaxel and vinblastine would probably be safer. The Cmax for docetaxel is 5.47 µM. This value corresponds to ~90% viability in our experiments. The Cmax for vinblastine is 0.035 µM, which is even lower than the lowest dose used in our study and would be equivalent to 100% viability. Finally, methotrexate, tamoxifen and its two metabolites did not significantly decreased cell viability, except for the highest doses, which were not physiologically relevant. The absence of toxic

effect on syncytiotrophoblasts may be due to the fact that these drugs act by blocking cell proliferation and division. Yet, syncytiotrophoblasts are differentiated cells that do not proliferate.

Chemotherapy may also inhibit placental development by interfering with the regulation of genes involved in trophoblast differentiation and placental angiogenesis. We selected hCG because it is considered as the gold standard for trophoblast differentiation [17]. Any changes in hCG expression directly reflect changes in trophoblast differentiation. We also chose to quantify PlGF expression because its expression increases during differentiation and it is an important factor for placental angiogenesis [18]. It would have been interesting to assess the expression of VEGF but we have previously shown that *VEGF* mRNA expression did not increase during the *in vitro* differentiation of cytotrophoblasts, and VEGF protein could not be detected in cell supernatants by ELISA [19]. Epirubicin inhibited both the mRNA and protein expression of HCGB and PlGF. This result is in accordance with the fact that epirubicin intercalates into DNA inhibiting its replication, synthesis, and also RNA synthesis. It is therefore expected that epirubicin will indistinctly inhibit all the genes that are overexpressed during trophoblast differentiation. In our experiments, the cells were treated with 0.3 µg/ml epirubicin for 24 h. This dose was empirically chosen based on the viability experiments showing that cell death occurred rapidly with increasing doses. With 0.3 µg/ml, we still had 57% viable cells but, because this is 55 times less than the Cmax measured in patients, it may explain the relatively small inhibitory effect on *HCGB* and *PlGF* mRNA expression.

Interestingly, docetaxel and vinblastine which both possess antimitotic activities through interaction with microtubules have differential effects on HCGB and PlGF expression. Docetaxel inhibited only HCGB expression while vinblastine strongly inhibited both HCGB and PlGF expression. This confirms the antiangiogenic effect of vinblastine reported in human colon cancer cells showing a decrease in VEGF expression when cells were treated with vinblastine [20].

Methotrexate which acts on DNA synthesis in dividing cells had no effect on PlGF expression, but induced a small increase in HCGB expression. Tamoxifen by itself had no effect on *HCGB* and *PlGF* mRNA expression, but it induced a small increase in HCGB and PlGF protein secretion. On the other hand, its 2 metabolites which possess a higher affinity for the estrogen receptor (ER) [21,22] increased both mRNA and protein expression of HCGB and PlGF. This stimulatory effect of tamoxifen on PlGF expression has been previously shown in human endometrial endothelial cells [23]. Moreover, it has also been found in ERα-positive tumors that *HCGA* gene which encodes for the alpha subunit of HCG was upregulated by estrogen [24]. Interestingly, others found that tamoxifen was responsible for a decrease in *HCGA* and

HCGB mRNA expression in primary trophoblasts isolated from either first trimester placenta or term placenta [25,26]. Actually, to the best of our knowledge, nobody reported the presence of estrogen responsive elements (ERE) in the promoter of these genes. Therefore, transcriptional regulation of the *HCGB* and *PlGF* genes by 4-hydroxytamoxifen and endoxifen may involve another mechanism that still needs to be identified.

Finally, we investigated the role of the placental barrier in the protective function of the placenta toward the chemotherapeutic drugs. The ABC transporters (well reviewed in [6]) are very important in potentially rejecting the xenogenous compounds like the anti-cancer drugs into the maternal blood circulation. We focused our analysis on BCRP and MDR1, and confirmed previous reports [27,28] that MDR1 appears to be essentially expressed in undifferentiated cytotrophoblasts and BCRP in syncytiotrophoblasts. Interestingly, their expression did not change upon drug treatment except with vinblastine which increased *BCRP* mRNA expression by 3-fold. At the protein level, epirubicin was the only drug that appeared to inhibit BCRP production, corroborating the high cytotoxicity of the anthracycline on *in vitro* cultured syncytiotrophoblasts. 4-hydroxytamoxifen was the only drug able to further decrease MDR1 expression. However, since MDR1 expression is already low in syncytiotrophoblasts, this inhibitory effect has probably no physiological consequences. Except for methotrexate, which has a very short half-life, all the other drugs' half-life is between 30 hours and 170 hours [16]. A 24-hour treatment is possibly not long enough to induce an increase in BCRP expression.

These anti-cancer drugs are highly liposoluble, and therefore should easily cross the placental barrier, but their concentrations in the fetal blood measured from animal experiments are relatively low [29]. This low transfer rate may be due to the activity of the efflux transporters. Therefore, assessing the capacity of the trophoblasts to reject the different drugs studied would be very important. In anthracycline-resistant cancer cells, BCRP is characterized by the presence of a threonine or glycine residue at position 482, while the amino acid is arginine (Arg) in normal tissues like the placenta [30]. Because Arg482-BCRP does not appear to be able to transport anthracyclins, it is actually considered as the wild type form. This could contribute to the high cytotoxicity of epirubicin, which cannot be thrown out by the cells, whilst methotrexate, a substrate of the wild type form only did not induce cytotoxicity. It would be very interesting to determine the retention capacities of the transporters for each drug. To realize an exhaustive study of the placental drug transport, the members of the ABCC subfamily, MRPs should also be included [6]. Their expression has been reported in the placenta, with MRP1, MRP2 and MRP3 being the best characterized. Based on their localization and level of expression, MRP2 would probably be the most relevant candidate in playing a role in the placental barrier.

CONCLUSION

Epirubicin is probably the drug that induces the most detrimental effects on isolated trophoblasts *in vitro* while the other drugs were well supported by the cells. Further studies will be needed to characterize the effect of the drugs alone or in combination on placental transporters expression and activity. Even if the *in vitro* culture of human primary term cytotrophoblasts does not exactly reflect what happens in the organism, it is still a great and simple tool to study the direct effect of chemotherapeutic drugs on placental formation. It may be used to determine the potential cytotoxic effects of existing and new chemotherapeutic drugs on placental development in hopes of fine-tuning anti-cancer treatments in pregnant women.

Compliance with Ethical Standards

**Conflict of Interest*

Christophe Louis Depoix declares that he has no conflict of interest. Arthur Colson declares that he has no conflict of interest. Mina Mhallem-Gizri declares that he has no conflict of interest. Corinne Hubinont declares that he has no conflict of interest. Frédéric Debième declares that he has no conflict of interest.

**Funding*

The work was supported by the FETUS FOR LIFE Charity in Brussels, Belgium.

**Ethical approval*

All procedures performed in studies involving our participants were in accordance with the ethical standards of the CUSL and Research Ethics Committee (approval # 2018-23OCT-397) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent*

Informed consent was obtained from all individual participants included in the study.

epirubicin (E)		docetaxel (D)		vinblastine (V)		methotrexate (M)		tamoxifen (TMX)		4-OH-tamoxifen (4-OH-TMX)		endoxifen (ENDO)	
µg/ml	µM	µg/ml	µM	µg/ml	µM	µg/ml	µM	ng/ml	nM	ng/ml	nM	ng/ml	nM
0.10	0.18	0.1	0.12	0.1	0.12	0.1	0.22	5	13.46	0.5	1.29	0.5	1.34
0.20	0.36	0.5	0.62	1.0	1.23	1.0	2.20	10	26.92	1.0	2.58	1.0	2.68
0.30	0.55	1.0	1.24	5.0	6.17	5.0	11.00	25	67.29	2.5	6.45	2.5	6.69
0.40	0.73	5.0	6.19	10.0	12.33	10.0	22.01	50	134.58	5.0	12.90	5.0	13.39
0.50	0.91	10.0	12.38	20.0	24.66	20.0	44.01	75	201.88	10.0	25.81	10.0	26.77
0.75	1.36	20.0	24.75	50.0	61.65	50.0	110.03	100	269.17	25.0	64.52	25.0	66.94
1.00	1.82	40.0	49.51	100.0	123.31	100.0	220.05	250	672.92	50.0	129.03	50.0	133.87
2.50	4.55	60.0	74.26					500	1345.84	100.0	258.06	100.0	267.74
5.00	9.10	80.0	99.01					750	2018.76	500.0	1290.32	500.0	1338.72
7.50	13.65	100.0	123.77					1000	2691.68	1000.0	2580.65	1000.0	2677.45
10.00	18.20	120.0	148.52					1500	4037.52	2000.0	5161.29	2000.0	5354.90

REFERENCE LIST

1. Pavlidis NA (2002) Coexistence of pregnancy and malignancy. Oncologist 7 (4):279-287

2. Ebert U, Loffler H, Kirch W (1997) Cytotoxic therapy and pregnancy. Pharmacol Ther 74 (2):207-220

3. Esposito S, Tenconi R, Preti V, Groppali E, Principi N (2016) Chemotherapy against cancer during pregnancy: A systematic review on neonatal outcomes. Medicine (Baltimore) 95 (38):e4899. doi:10.1097/MD.0000000000004899

4. Van Calsteren K, Heyns L, De Smet F, Van Eycken L, Gziri MM, Van Gemert W, Halaska M, Vergote I, Ottevanger N, Amant F (2010) Cancer during pregnancy: an analysis of 215 patients emphasizing the obstetrical and the neonatal outcomes. J Clin Oncol 28 (4):683-689. doi:10.1200/JCO.2009.23.2801

5. Soundararajan R, Rao AJ (2004) Trophoblast "pseudo-tumorigenesis": significance and contributory factors. Reprod Biol Endocrinol 2:15. doi:10.1186/1477-7827-2-15

6. Joshi AA, Vaidya SS, St-Pierre MV, Mikheev AM, Desino KE, Nyandege AN, Audus KL, Unadkat JD, Gerk PM (2016) Placental ABC Transporters: Biological Impact and Pharmaceutical Significance. Pharm Res 33 (12):2847-2878. doi:10.1007/s11095-016-2028-8

7. Lloyd ME, Carr M, McElhatton P, Hall GM, Hughes RA (1999) The effects of methotrexate on pregnancy, fertility and lactation. QJM 92 (10):551-563

8. Ostensen M, Hartmann H, Salvesen K (2000) Low dose weekly methotrexate in early pregnancy. A case series and review of the literature. J Rheumatol 27 (8):1872-1875

9. Lewden B, Vial T, Elefant E, Nelva A, Carlier P, Descotes J, French Network of Regional Pharmacovigilance C (2004) Low dose methotrexate in the first trimester of pregnancy: results of a French collaborative study. J Rheumatol 31 (12):2360-2365

10. Mourits MJ, Ten Hoor KA, van der Zee AG, Willemse PH, de Vries EG, Hollema H (2002) The effects of tamoxifen on proliferation and steroid receptor expression in postmenopausal endometrium. J Clin Pathol 55 (7):514-519

11. Desta Z, Ward BA, Soukhova NV, Flockhart DA (2004) Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Ther 310 (3):1062-1075. doi:10.1124/jpet.104.065607

12. Schuurman TN, Witteveen PO, van der Wall E, Passier JLM, Huitema ADR, Amant F, Lok CAR (2019) Tamoxifen and pregnancy: an absolute contraindication? Breast Cancer Res Treat. doi:10.1007/s10549-019-05154-7
13. Debieve F, Pampfer S, Thomas K (2000) Inhibin and activin production and subunit expression in human placental cells cultured in vitro. Mol Hum Reprod 6 (8):743-749
14. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9 (7):671-675
15. Kaklamani VG, Gradishar WJ (2003) Epirubicin versus doxorubicin: which is the anthracycline of choice for the treatment of breast cancer? Clin Breast Cancer 4 Suppl 1:S26-33
16. Liston DR, Davis M (2017) Clinically Relevant Concentrations of Anticancer Drugs: A Guide for Nonclinical Studies. Clin Cancer Res 23 (14):3489-3498. doi:10.1158/1078-0432.CCR-16-3083
17. Ho HH, Douglas GC, Qiu QF, Thirkill TL, Overstreet JW, Lasley BL (1997) The relationship between trophoblast differentiation and the production of bioactive hCG. Early Pregnancy 3 (4):291-300
18. Ribatti D (2008) The discovery of the placental growth factor and its role in angiogenesis: a historical review. Angiogenesis
19. Debieve F, Depoix C, Gruson D, Hubinont C (2013) Reversible effects of oxygen partial pressure on genes associated with placental angiogenesis and differentiation in primary-term cytotrophoblast cell culture. Mol Reprod Dev 80 (9):774-784. doi:10.1002/mrd.22209 [doi]
20. Auyeung KK, Law PC, Ko JK (2014) Combined therapeutic effects of vinblastine and Astragalus saponins in human colon cancer cells and tumor xenograft via inhibition of tumor growth and proangiogenic factors. Nutr Cancer 66 (4):662-674. doi:10.1080/01635581.2014.894093
21. Fabian C, Tilzer L, Sternson L (1981) Comparative binding affinities of tamoxifen, 4-hydroxytamoxifen, and desmethyltamoxifen for estrogen receptors isolated from human breast carcinoma: correlation with blood levels in patients with metastatic breast cancer. Biopharm Drug Dispos 2 (4):381-390
22. Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong W, Shi L, Perkins R, Sheehan DM (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol Sci 54 (1):138-153
23. Helmestam M, Andersson H, Stavreus-Evers A, Brittebo E, Olovsson M (2012) Tamoxifen modulates cell migration and expression of angiogenesis-related genes in human endometrial endothelial cells. Am J Pathol 180 (6):2527-2535. doi:10.1016/j.ajpath.2012.02.026
24. Bieche I, Parfait B, Le Doussal V, Olivi M, Rio MC, Lidereau R, Vidaud M (2001) Identification of CGA as a novel estrogen receptor-responsive gene in breast cancer: an outstanding candidate marker to predict the response to endocrine therapy. Cancer Res 61 (4):1652-1658

25. Sharma SC, Purohit P, Rao AJ (1993) Role of oestradiol-17 beta in the regulation of synthesis and secretion of human chorionic gonadotrophin by first trimester human placenta. J Mol Endocrinol 11 (1):91-101

26. Cronier L, Guibourdenche J, Niger C, Malassine A (1999) Oestradiol stimulates morphological and functional differentiation of human villous cytotrophoblast. Placenta 20 (8):669-676. doi:10.1053/plac.1999.0423

27. Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg AC, Schinkel AH, van De Vijver MJ, Scheper RJ, Schellens JH (2001) Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res 61 (8):3458-3464

28. Evseenko DA, Paxton JW, Keelan JA (2006) ABC drug transporter expression and functional activity in trophoblast-like cell lines and differentiating primary trophoblast. Am J Physiol Regul Integr Comp Physiol 290 (5):R1357-1365. doi:10.1152/ajpregu.00630.2005

29. Miyamoto S, Yamada M, Kasai Y, Miyauchi A, Andoh K (2016) Anticancer drugs during pregnancy. Jpn J Clin Oncol 46 (9):795-804. doi:10.1093/jjco/hyw073

30. Mao Q, Unadkat JD (2005) Role of the breast cancer resistance protein (ABCG2) in drug transport. AAPS J 7 (1):E118-133. doi:10.1208/aapsj070112

TABLES AND FIGURES

Table 1 List of drugs and doses used with their equivalence in molarity

epirubicin (E)		docetaxel (D)		vinblastine (V)		methotrexate (M)		tamoxifen (TMX)		4-OH-tamoxifen (4-OH-TMX)		endoxifen (ENDO)	
µg/ml	µM	µg/ml	µM	µg/ml	µM	µg/ml	µM	ng/ml	nM	ng/ml	nM	ng/ml	nM
0.10	0.18	0.1	0.12	0.1	0.12	0.1	0.22	5	13.46	0.5	1.29	0.5	1.34
0.20	0.36	0.5	0.62	1.0	1.23	1.0	2.20	10	26.92	1.0	2.58	1.0	2.68
0.30	0.55	1.0	1.24	5.0	6.17	5.0	11.00	25	67.29	2.5	6.45	2.5	6.69
0.40	0.73	5.0	6.19	10.0	12.33	10.0	22.01	50	134.58	5.0	12.90	5.0	13.39
0.50	0.91	10.0	12.38	20.0	24.66	20.0	44.01	75	201.88	10.0	25.81	10.0	26.77
0.75	1.36	20.0	24.75	50.0	61.65	50.0	110.03	100	269.17	25.0	64.52	25.0	66.94
1.00	1.82	40.0	49.51	100.0	123.31	100.0	220.05	250	672.92	50.0	129.03	50.0	133.87
2.50	4.55	60.0	74.26					500	1345.84	100.0	258.06	100.0	267.74
5.00	9.10	80.0	99.01					750	2018.76	500.0	1290.32	500.0	1338.72
7.50	13.65	100.0	123.77					1000	2691.68	1000.0	2580.65	1000.0	2677.45
10.00	18.20	120.0	148.52					1500	4037.52	2000.0	5161.29	2000.0	5354.90

Table 2 List of primers and antibodies. (BCRP, Breast Cancer Resistance Protein; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HCGB, human Chorionic Gonadotropin Beta; PlGF, Placental Growth factor; MDR-1, Multidrug Resistance Protein 1; SDHA, Succinate Dehydrogenase Complex Flavoprotein Subunit A; TBP, TATA-Binding Protein 1)

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	
BCRP	TGT-ACT-GGC-GAA-GAA-TAT-TTG-GT	GCC-ACG-TGA-TTC-TTC-CAC-AA	
HCGB	GCT-ACT-GCC-CCA-CCA-TGA-CC	ATG-GAC-TCG-AAG-CGC-ACA-TC	
PlGF	CAG-AGG-TGG-AAG-TGG-TAC-CCT-TCC	CGG-ATC-TTT-AGG-AGC-TGC-ATG-GTG-AC	
MDR-1	GAG-CCC-ATC-CTG-TTT-GAC-TG	GCT-GCC-CTC-ACA-ATC-TCT-TC	
SDHA	TGG-GAA-CAA-GAG-GGC-ATC-TG	CCA-CCA-CTG-CAT-CAA-ATT-CAT-G	
TBP	GAA-CAT-CAT-GGA-TCA-GAA-CAA-CA	ATA-GGG-ATT-CCG-GGA-GTC-AT	
Protein	**Dilution**	**Reference**	**Company**
BCRP	1/200	BPX-21, sc-28222	Santa Cruz Biotechnologies, CA, USA
GAPDH	1/5000	VMA00046	Bio-Rad Laboratories, Oxfordshire, UK
MDR-1	1/500	G-1, sc-13131	Santa Cruz Biotechnologies, CA, USA

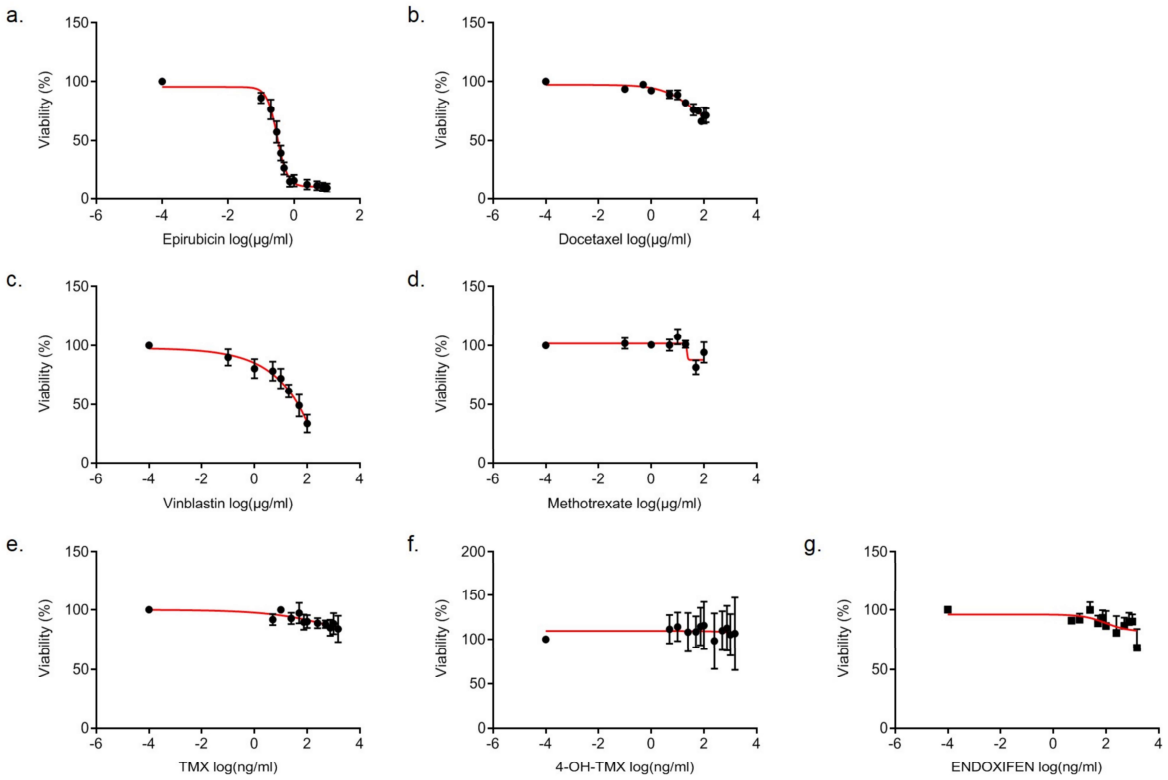

Fig. 1 Effect of Chemotherapeutic Drugs on Trophoblastic Cell Viability. Cells were allowed to differentiate into syncytiotrophoblast for 48 h, before being treated for 24 hours with increasing doses of epirubicin (a), docetaxel (b), vinblastine (c), methotrexate (d), tamoxifen TMX (e), 4-hydroxytamoxifen 4-OH-TMX (f), and endoxifen (g). The viability measured in DMSO-treated cells was fixed at 100%. The results were represented as the mean ± SEM of the percentage of viability versus log-doses. (n=5 placentas)

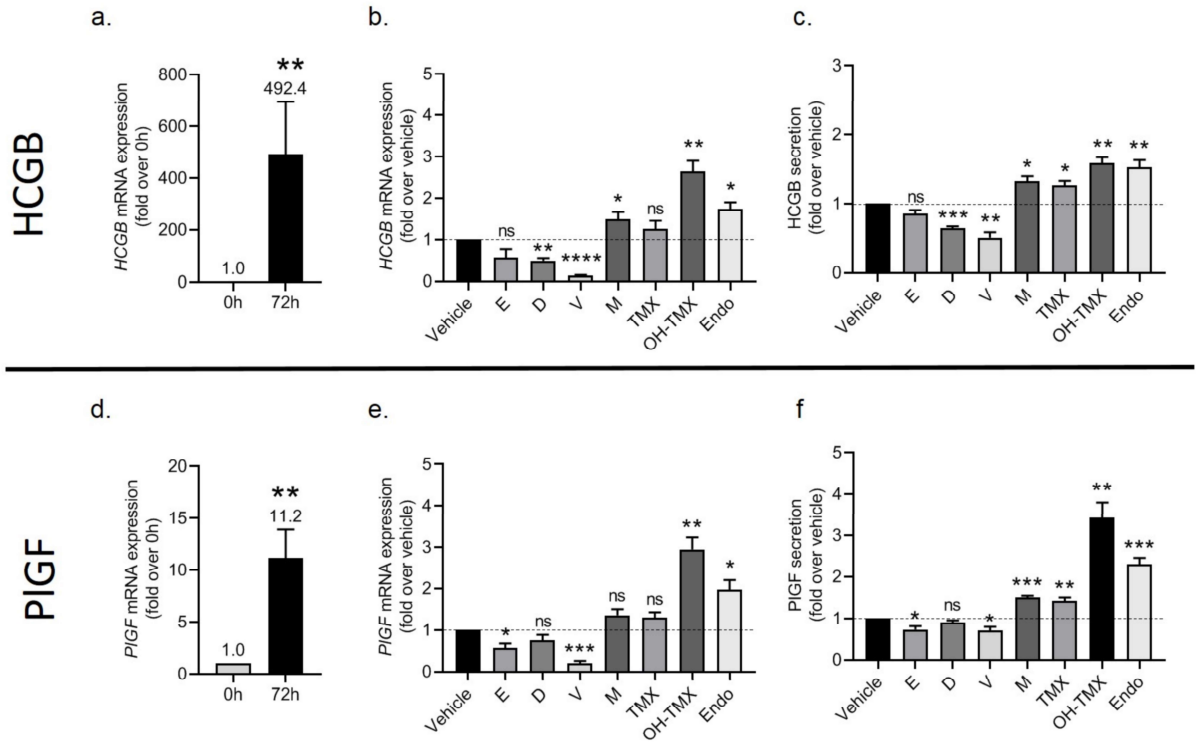

Fig. 2 Effect of the chemotherapeutic drugs on HCGB and PlGF expression, markers of trophoblast differentiation and placental angiogenesis.

Primary syncytiotrophoblasts were treated for 24 hours with 0.3 µg/ml epirubicine (E), 10 µg/ml docetaxel (D), 10 µg/ml vinblastine (V), 100 µg/ml methotrexate (M), 1 µg/ml TMX, 1 µg/ml 4-hydroxytamoxifen (4-OH-TMX), 1 µg/ml endoxifen (Endo), and DMSO (vehicle) as control. HCGB and PlGF expressions were quantified by real-time PCR and ELISA. (a and d) *HCGB* and *PlGF* mRNA expressions in untreated cells (vehicle) versus undifferentiated cells (0 h). (b and e) *HCGB* and *PlGF* mRNA expressions, and (c and f) HCGB and PlGF secretion in treated cells versus DMSO-treated cells (vehicle). The protein quantities per ml were corrected by the percentage of cell viability for each individual drug. DMSO-treated cells were fixed at 100%. The experiments were done in duplicate and repeated a minimum of 5 times (n=5 placentas). Values are mean ±SEM (a-d: Mann-Whitney's test, **<0.01 compared to 0 h) (b-c-e-f: sample t-test, *p ≤0.05, **p ≤0.01, ***p ≤0.005, and ****p ≤0.0001 compared to vehicle)

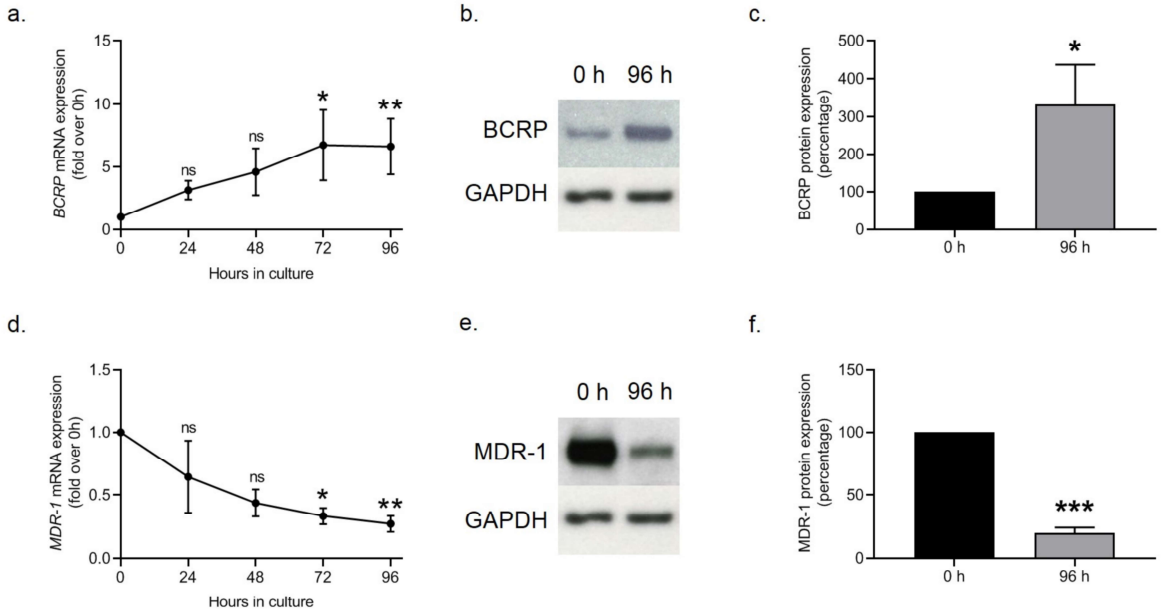

Fig. 3 BCRP and MDR1 Expressions during *in vitro* Differentiation of Human Primary Term Cytotrophoblasts.

Cytotrophoblasts were cultured for 96 hours under 21% O_2. *BCRP* and *MDR1* mRNA expressions were quantified by real-time PCR every 24 hours (a and d). The quantification was done in duplicate. The results represent the mean ±SEM of 5 placentas (n=5; Anova Kruskal-Wallis's test followed by Dunn's multiple comparison test, *p≤0.05, and **p≤0.01 compared to undifferentiated cells 0 h). *BCRP* mRNA expression increased during cell differentiation, while *MDR1* mRNA expression decreased. This result was confirmed at the protein level by western blotting (b and e), and protein quantification after densitometric analysis (c and f) using GAPDH as loading control. Values are the mean ±SEM of 4 placentas (n=4; One sample t-test, *p≤0.05, and ***p≤0.005 compared to 0 h)

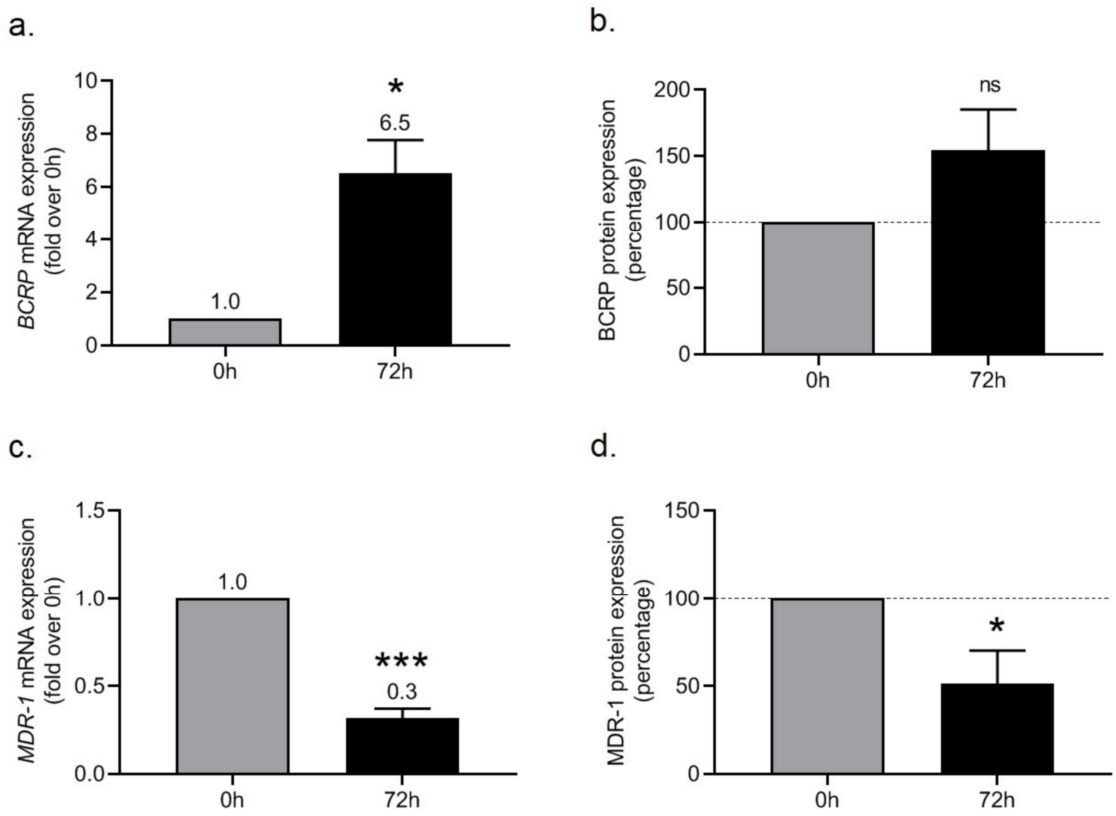

Fig. 4 BCRP and MDR1 expressions in untreated syncytiotrophoblasts after 72 h in culture. Human primary cytotrophoblasts were cultured for 48 hours, then treated for 24 hours with DMSO only (vehicle control). BCRP and MDR1 mRNA (a and c) and protein expressions (b and d) were quantified by qPCR and western blotting followed by densitometric analysis. Cytotrophoblasts were isolated from 5 placentas for mRNA expression and 3 placentas for protein quantification. Values are mean ± SEM and were compared using One sample t test (*p≤0.05, **p≤0.01, and ***p≤0.005 compared to 0 h, ns: not significant).

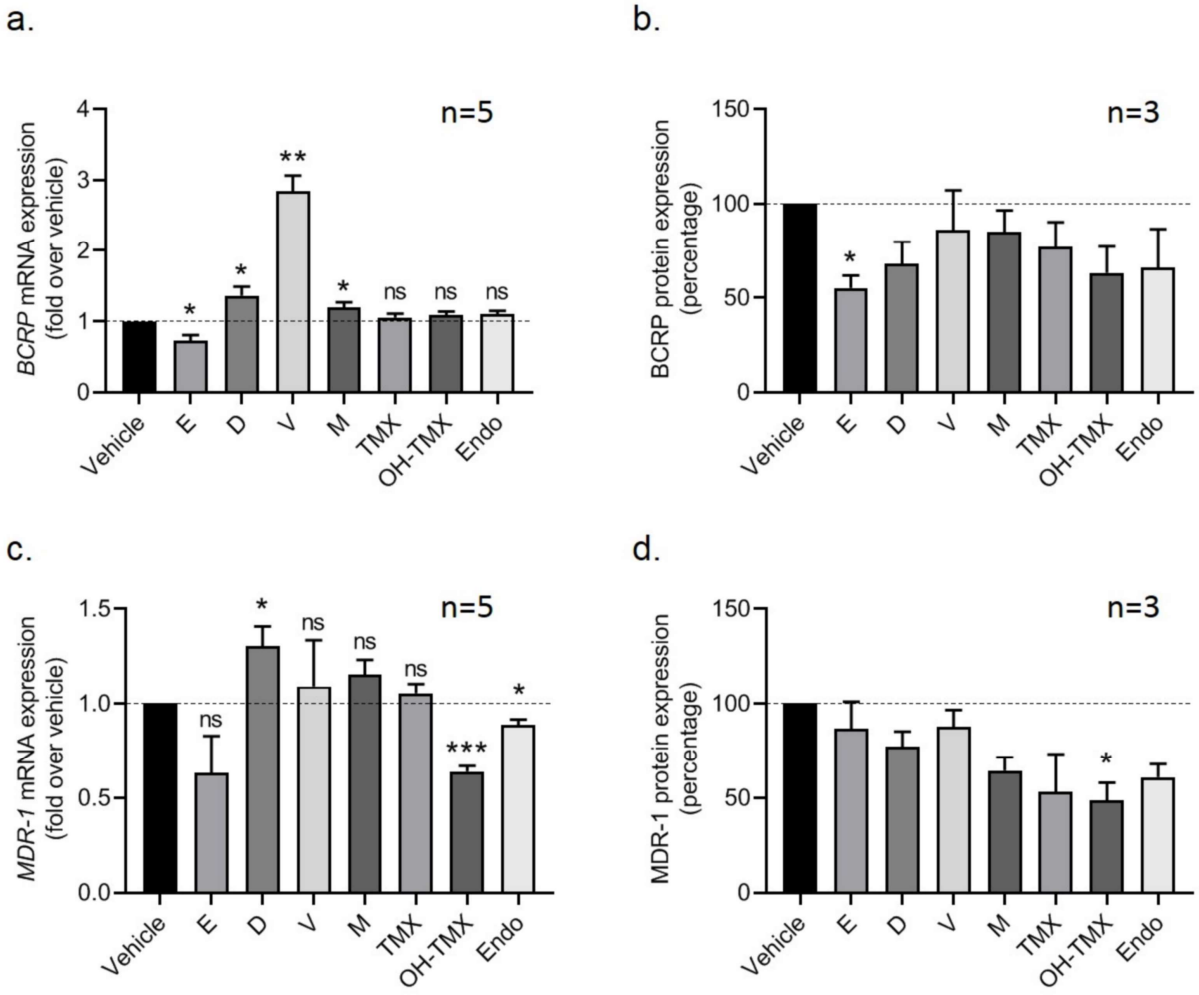

Fig. 5 Effect of chemotherapy on BCRP and MDR1 expression.

BCRP and MDR1 expressions were assessed after a 24 hour-treatment with the chemotherapeutic drugs. (a and c) *BCRP* and *MDR1* mRNA expressions were quantified by real-time PCR (n=5). (b and d) BCRP and MDR1 protein expressions in total protein extracts were assessed by immunoblot (n=3), and their relative quantity estimated by densitometric analysis (*p≤0.05, **p≤0.01, ***p≤0.005, ns: not significant)