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ARTICLE

Chemosensitivity and chemoresistance in endometriosis – differences for ectopic versus eutopic cells

BIOGRAPHY

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KEY MESSAGE

Akt/PKB inhibitor GSK690693, CK2 inhibitor ARC-775, MAPK pathway inhibitor sorafenib, proteasome inhibitor bortezomib, and microtubule-depolymerizing toxin MMAE showed higher cytotoxicity in eutopic cells. In contrast, 10 μmol/l of the anthracycline toxin doxorubicin caused cellular death in ectopic cells more effectively than in eutopic cells, underlining the potential of doxorubicin in endometriosis research.

ABSTRACT

Research question: Endometriosis is a common gynaecological disease defined by the presence of endometrium-like tissue outside the uterus. This complex disease, often accompanied by severe pain and infertility, causes a significant medical and socioeconomic burden; hence, novel strategies are being sought for the treatment of endometriosis. Here, we set out to explore the cytotoxic effects of a panel of compounds to find toxins with different efficiency in eutopic versus ectopic cells, thus highlighting alterations in the corresponding molecular pathways.

Design: The effect on cellular viability of 14 compounds was established in a cohort of paired eutopic and ectopic endometrial stromal cell samples from 11 patients. The biological targets covered by the panel included pro-survival enzymes, cytoskeleton proteins, the proteasome and the cell repair machinery.

Results: Protein kinase inhibitors GSK690693, ARC-775 and sorafenib, proteasome inhibitor bortezomib, and microtubule-depolymerizing toxin monomethyl auristatin E were more effective in eutopic cells. In contrast, 10 μmol/l of the anthracycline toxin doxorubicin caused cellular death in ectopic cells more effectively than in eutopic cells. The large-scale sequencing of mRNA isolated from doxorubicin-treated and control cells indicated different survival strategies in eutopic versus ectopic endometrium.

Conclusions: Overall, the results confirm evidence of large-scale metabolic reprogramming in endometriotic cells, which underlies the observed differences in sensitivity towards toxins. The enhanced efficiency of doxorubicin interfering with redox equilibria and/or DNA repair mechanisms pinpoints key players that can be potentially used to selectively target ectopic lesions in endometriosis.

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KEYWORDS

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INTRODUCTION

Endometriosis is an inflammatory gynaecological disease that manifests itself as a growth of endometrial stromal cells (ESC) and epithelial cells in extrauterine sites. Endometriosis is estimated to affect 2–10% of women in their reproductive years, and as there are still no effective non-surgical treatments, it has a considerable impact on the quality of life of women affected (*Nnoaham et al., 2011*). Endometriosis-associated symptoms such as severe pelvic pain, infertility and impaired psychological and social functioning cause a socioeconomic burden from loss of productivity; furthermore, the risk of developing ovarian cancer is moderately increased in women suffering from endometriosis, being about 1.9% compared with 1.4% in the general population (*Vercellini et al., 2018*). Therefore, the new possibilities in the treatment of endometriosis are being actively explored.

To find potent strategies for treating endometriosis, the mechanisms behind disease initiation need to be understood. The formation of endometriotic lesions presupposes an ability of endometrial cells to attach to peritoneal surfaces, establish neo-angiogenesis and resist apoptosis (*Nasu et al., 2009*). Characteristics such as a high degree of inflammation, an excess of iron and an increase in reactive oxygen species (ROS) have also been described in endometriotic lesions (*Defrere et al., 2008; Lousse et al., 2012; Scutiero et al., 2017*). Furthermore, a comprehensive proteomic study by the current group has shown that extensive metabolic reprogramming (associated with down-regulation of oxidative respiration) and an up-regulation of proteins involved in adhesiveness and motility occur in endometriotic stromal cells (*Kasvandik et al., 2016*), emphasizing the similarities between endometriotic and cancer cells. Therefore toxins affecting various molecular pathways in cancer chemotherapy could find an alternate application in research into – and potentially therapy of – endometriosis. Some such compounds have been briefly explored in the context of endometriosis (*Celik et al., 2008*) yet we are not aware of studies with a focused panel of toxins that would systematically compare the effect of compounds in eutopic and ectopic cells taken from women with endometriosis.

This paper reports on quantification of the cytotoxic effect of 14 compounds (TABLE 1) in a cohort of paired eutopic and ectopic ESC (euESC and ecESC) samples from 11 patients. The biological targets covered by this panel included pro-survival enzymes, cytoskeleton proteins, the proteasome and the cell repair machinery. The rationale behind the choice of compounds took into consideration the high affinity and well-defined selectivity profile of inhibitors in biochemical studies and their applicability in cellular assays. The goal was to find compounds demonstrating different efficiency in eutopic versus ectopic cells from peritoneal lesions, thus highlighting alterations in the corresponding molecular pathways, and to pinpoint compounds that preferentially affect ectopic cells, thus paving the way for future possible therapeutic strategies.

MATERIALS AND METHODS

Chemicals and equipment

Protein kinase inhibitors were obtained from the following sources: SGI-1776 – Axon Medchem (Groningen, Netherlands); H-89 – Biaffin (Kassel, Germany); sorafenib, Y-27632, HA-1077 – Cayman Chemical (Ann Arbor, MI, USA); staurosporine – Cell Guidance Systems (Cambridge, UK); VX-689, CYC116 – Selleckchem (Houston, TX, USA); bortezomib, monomethyl auristatin E (MMAE), doxorubicin – TBD-Biodiscovery (Tartu, Estonia); and GSK690693 – Tocris (Bristol, UK). ARC-775 and ARC-1859 were kindly gifted by Dr Asko Uri (University of Tartu, Tartu, Estonia). The stock solutions of compounds (5–10 mmol/l in dimethylsulphoxide [DMSO]) were stored at –20°C. SYTOX Blue Nucleic Acid

TABLE 1 COMPOUNDS USED IN THE STUDY

Name	Concentrations used (μmol/l)	Major biological target	References
GSK690693	0.4, 2, 10	Akt/PKB 1, 2, 3	(*Levy et al., 2009; Rhodes et al., 2008*)
VX-689 (MK5108)	0.2, 1, 5	Aurora A	(*Chinn et al., 2014; Shimomura et al., 2010*)
CYC116	0.4, 2, 10	Aurora A, B	(*Jayanthan et al., 2014; Wang et al., 2010*)
ARC-775	0.4, 2, 10	CK2	(*Rahnel et al., 2017*)
ARC-1859	0.4, 2, 10	CK2	(*Viht et al., 2015*)
SGI-1776	0.4, 2, 10	PIM 1, 3	(*Chen et al., 2011, 2009*)
H-89	0.4, 2, 10	PKA, PKG1	(*Dabizzi et al., 2003; Yoshino et al., 2003*)
Y-27632	0.4, 2, 10	ROCK 1, 2	(*Grewal et al., 2010; Yotova et al., 2011; Yuge et al., 2007*)
HA-1077 (fasudil)	0.4, 2, 10	ROCK 2	(*Tsuno et al., 2011*)
Sorafenib (BAY 43-9006)	0.4, 2, 10	RAF1, BRAF, KDR (VEGFR2), FLT4 (VEGFR3)	(*Llobet et al., 2010; Moggio et al., 2012*)
Staurosporine	0.2, 1, 5	PKCα, γ, η	(*Izawa et al., 2006; Watanabe et al., 2009*)
Bortezomib (PS-341, Velcade)	0.4, 2, 10	20S proteasome	(*Kao et al., 2014*)
Doxorubicin (adriamycin)	0.4, 2, 10	DNA, topoisomerase-II	(*Byron et al., 2012; Chitcholtan et al., 2012*)
Monomethyl auristatin E (MMAE)	0.04, 0.2, 1	Tubulin	(*Abdollahpour-Alitappeh et al., 2017; Chen et al., 2017*)

Abbreviations: Akt/PKB, protein kinase B; BRAF, V-Raf murine sarcoma viral oncogene homolog B; CK2, casein kinase 2; PIM, proto-oncogene Ser/Thr-protein kinase; PKA, protein kinase A; PKC, protein kinase C; RAF1, V-Raf-1 murine leukemia viral oncogene homolog 1; ROCK, Rho-dependent protein kinase; VEGFR, vascular endothelial growth factor receptor.

Stain and NP40 lysis buffer were from Thermo Fischer Scientific (Rockford, IL, USA); cell culture grade DMSO was from AppliChem (Darmstadt, Germany); resazurin, bovine serum albumin (BSA) and phosphate-buffered saline (PBS) (supplemented with Ca^{2+} and Mg^{2+}; used for the biochemical assays and western blotting) were from Sigma-Aldrich (St Louis, MO, USA). Other solutions, reagents and materials for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were from Thermo Fischer Scientific (Carlsbad, CA, USA).

For the necrosis/late apoptosis and viability assays, the initial number of cells was counted using a TC10 cell counter (Bio-Rad, Hercules, CA, USA), and the cells were seeded onto transparent 96-well, clear, flat-bottom cell culture plates (BioLite 130188; Thermo Fischer Scientific, Rochester, NY, USA). Fluorescence intensity and absorbance measurements were made using Synergy NEO, Cytation 5 (both from Biotek, Winooski, VT, USA) and PHERAstar (BMG Labtech, Ortenberg, Germany) multimode readers.

Patient characteristics and sample collection

The study was approved by the Research Ethics Committee of the University of Tartu (approval 276/M-13) on 18 December 2017 and informed written consent was obtained from the participants. Endometrial tissue samples and peritoneal endometriotic lesions were collected from 11 women endometriosis (TABLE 2) undergoing laparoscopy at the Tartu University Hospital Women's Clinic. Tissue samples were immediately placed into the cryopreservation medium and processed as previously described (*Rekker et al., 2017*). At least one endometriotic lesion sample from each patient was placed into formalin and the diagnosis was confirmed by histopathological examination of the specimens. Disease severity was determined according to the American Society for Reproductive Medicine revised classification system (*American Society for Reproductive Medicine, 1997*). Only women who had not received any hormonal medications for at least 3 months before surgery were enrolled in this study.

Isolation and culturing of cells

Endometriotic and endometrial tissues were treated according to the previously published protocol (*Kasvandik et al., 2016*). Briefly, the tissue was washed twice in 7 ml of fresh medium (a 1:1 mixture of Dulbecco's modified Eagle's medium [DMEM] and Ham's F-12; Sigma-Aldrich, Steinheim, Germany) to remove any debris or excess blood cells. The biopsies were dissociated in 5 ml of DMEM (without phenol red) containing 0.5% collagenase (Sigma-Aldrich) in a shaking incubator rotating at 110 rpm at 37°C until the biopsies had been digested (but not for longer than 1 h). The dispersed cells were filtered through a 50 µm nylon mesh to remove undigested tissue pieces. Next, the cells were resuspended in 10 ml of culture medium in a 15 ml tube; the sealed tubes were placed in an upright position for 10 min to sediment the epithelial glands. The top 8 ml of medium containing stromal cells was then collected and the tube was refilled to 10 ml with fresh medium; the sedimentation process was repeated three times and the collected fractions were pooled. The final purification of stromal cells was achieved by selective adherence of stromal cells to culture dishes for 20–30 min at 37°C in 5% CO_2 in an incubator. Non-adhering epithelial cells were removed by washing the cell layer twice with 5 ml of culture medium.

The isolated ESC were further cultured for 5–6 passages in DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS; Capricorn, Ebsdorfergrund, Germany) and a mixture of penicillin, streptomycin and amphotericin B (Capricorn, Ebsdorfergrund, Germany) at 37°C in 5% CO_2 in an incubator.

Necrosis/late apoptosis assay

euESC and ecESC (passage number 5–6) were seeded onto 96-well plates at a density of 4000–6000 cells per well in DMEM/Ham's F-12 medium supplemented with FBS; euESC and ecESC from the same patient were thawed on the same day, and two plates were prepared for both eutopic and ectopic stromal cells. After incubating the cells for 24 h at 37°C in 5% CO_2 in a humidified incubator, the medium was exchanged and dilution series of compounds in PBS were added (see TABLE 1); the final volume per well was 110 µl, and the concentration of DMSO in the treated wells was ≤0.1% by volume. For each plate, each concentration of each compound to be tested was represented in duplicate; the controls (10% DMSO and 0.1% DMSO) were represented in sextuplicate. The cells were incubated with the compounds for 22 h at 37°C in 5% CO_2 in a humidified incubator; next, the medium was removed

TABLE 2 CHARACTERISTICS OF THE STUDY PARTICIPANTS

Patient ID	Age (years)	BMI (kg/m^2)	Endometriosis stage	Location of lesion	Study
E048	29	19.8	III	Lig. sacrouterina SUP	N, V
E044	32	23.7	III	Excavatio vesicouterina SUP	N, V
E041	39	25.6	I	Fossa ovarica SUP	N, V
E205	36	22.2	I	Lig. latum SUP	N, V
E242	30	20.1	I	Lig. sacrouterina SUP	N, V
E262	40	29.8	II–III	Lig. latum SUP	N, V, V2, WB, seq
E267	25	22.1	I	Pouch of Douglas SUP	N, V, V2, WB
E270	33	21.6	III	Lig sacrouterina SUP	N, V
E278	32	20.8	I	Excavatio vesicouterina SUP	N, V, V2, WB, seq
E279	22	21.4	I	Excavatio vesicouterina SUP	N, V, V2, WB, seq
E310	24	23.5	I	Lig. sacrouterina SUP	N, V

Lig., ligamentum; N, necrosis/late apoptosis assay; seq, mRNA sequencing; SUP, superficial; V, viability assay with large cohort; V2, viability assay with small cohort; WB, western blot.

and 1 µmol/l Sytox Blue solution in PBS (containing Ca^{2+} and Mg^{2+}) was added. The plates were placed into a multimode reader and incubated for 10 min at 37°C, and the fluorescence intensity was measured (excitation 430 nm, emission 480 nm, monochromator, top optics, gain 90; area scan mode 5 × 5, read height 2.5 mm, with lid).

Viability assay

The viability assay was performed directly after the necrosis/late apoptosis assay using the same plates. The solution of Sytox Blue was replaced by 50 µmol/l resazurin solution in PBS (containing Ca^{2+} and Mg^{2+}). The plates were placed into the multimode reader, and measurement of absorbance was performed (570 nm and 600 nm, monochromator; kinetic mode with a reading taken every 15 min for 2 h, read height 8.5 mm, with lid). Next, resazurin solution was replaced by fresh sterile DMEM/Ham's F-12 medium supplemented with FBS, and the cells were incubated for 24 h at 37°C in 5% CO_2 in a humidified incubator. Finally, the viability assay was performed again (without the preceding necrosis/late apoptosis assay). In a pilot experiment, it was confirmed that the first application of resazurin for 2 h in PBS did not cause severe cytotoxicity (data not shown).

Western blotting

For the western blot assay, one 6-well plate was prepared for euESC and one plate for ecESC (passage number 5–6). When the confluency of cells was 50% or higher, dilutions of doxorubicin in PBS or DMSO in PBS (control) were added. The final volume per well was 2 ml; the final concentration of doxorubicin was 10 µmol/l, and the final concentration of DMSO was 0.1%. On each plate, both doxorubicin and control incubations were represented in duplicate. The cells were incubated for 48 h at 37°C in 5% CO_2 in a humidified incubator.

After collection and lysis of the cells on ice, the samples for SDS-PAGE were prepared by adding NuPAGE sample loading buffer (ThermoFisher, Carlsbad, CA, USA) to supernatants and heating at 70°C for 15 min. SDS-PAGE was performed on 10% Bis-Tris gels or 4–12% Bis-Tris gradient gel (ThermoFisher, Carlsbad, CA, USA) in MES buffer (ThermoFisher, Carlsbad, CA, USA); samples of treated and untreated euESC and ecESC from the same patients were applied on different lanes of the same gel. Semi-dry transfer followed at 15 V for 60 min using methanol-activated polyvinylidene difluoride (PVDF) membrane and NuPAGE transfer buffer. The membrane was then stained with primary antibody (1,000 × dilution of rabbit anti-procaspase-3; catalogue number 9662 Cell Signaling, RRID: AB_331439) and secondary antibody (5,000 × dilution of goat anti-rabbit conjugated to alkaline phosphatase; T2191 Thermo Fischer Scientific, RRID: AB_11180336) according to the manufacturers' instructions. The same procedure was used for the subsequent staining of the same membrane with mouse antibeta-actin (4,000 × dilution; A1978 Sigma-Aldrich, RRID: AB_476692) and goat anti-mouse conjugated to alkaline phosphatase (5,000 × dilution; T2192 Thermo Fischer Scientific, RRID: AB_11180852).

mRNA isolation and large-scale sequencing

euESC (n = 3) and ecESC (n = 3) were isolated and grown as described in the sections on the isolation and culturing of cells and western blotting, respectively; the cells were isolated from the paired eutopic and ectopic samples that were included in western blot studies. After 24 h incubating the cells with a final concentration of 2 µmol/l doxorubicin or 0.1% DMSO (as a negative control) in growth medium, the medium was removed, the cells were rinsed with PBS and RNA was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNase I treatment was performed using the DNA-free DNA removal kit (Invitrogen, Carlsbad, CA, USA). A 2200 TapeStation system in conjunction with RNA ScreenTape (Agilent Technologies, Palo Alto, CA, USA) was used to determine the quality and quantity of purified RNA. For sequencing library construction, RNA from two technical replicates was pooled together. cDNA was synthesized as previously described (*Teder et al., 2018*), converted to the next-generation sequencing library using a Nextera XT Library Prep kit (Illumina, San Diego, CA, USA) and sequenced with NextSeq 500 high output 75 cycles kit (Illumina, San Diego, CA, USA).

Quantitative real-time PCR

The expression levels of selected genes (*HSPA2*, *PTGS2* and *PTN*) were validated by quantitative real-time PCR (qRT-PCR) using RNA from two technical replicates. cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), and real-time PCR was performed using 1 × HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia). The primer sequences used were as follows: *HSPA2* (F: CTCCACTCGTATCCCCAAGA, R: GTCACGTCGAGTAGCAGCAG), *PTGS2* (F: CCACTTCAAGGGATTTTGGA, R: GAGAAGGCTTCCCAGCTTTT) and *PTN* (F: CAATGCCGAATGCCA GAAGACTGT, R: TCCACAGGTGACA TCTTTTAATCC). *ACTB* (F: TCAAG ATCATTGCTCCTCC and R: ACATCTG CTGGAAGGTGGA) was used as a reference gene.

Statistical analysis

Data are available on request from the authors.

For the necrosis/late apoptosis assay, the mean Sytox Blue fluorescence intensity per well was calculated; the data corresponding to the same concentration of the same compound were pooled and normalized for each plate. For normalization, the signal obtained for incubation with 5 µmol/l staurosporine was considered to be 100% necrosis, and the signal obtained for incubation with 0.1% DMSO as 0% necrosis.

For the viability assay, the ratio of the absorbances at 570 nm and 600 nm was calculated for each well. The data obtained from one plate for the control incubations with 0.1% DMSO or 10% DMSO were pooled and plotted against time, and the linear range of the assay was established. The data corresponding to the same concentration of the same compound were pooled and normalized for each plate. For normalization, data obtained for incubation with 10% DMSO were considered as 0% viability, and data obtained for incubation with 0.1% DMSO as 100% viability.

For western blot data analysis, the membrane was dried and scanned in. The area of bands detected with anti-procaspase-3 and anti-beta–actin was assessed using ImageJ 1.51j8 software (Bethesda, MD, USA), and the ratio of the two values was calculated for each lane; the data were pooled for the lanes where the identically treated samples of the same cells had been applied. Next, data for lanes with samples from euESC and ecESC were normalized separately. For normalization, ratio obtained for incubation with 0.1%

DMSO was considered as 100% to obtain results for one patient; the bottom plateau was fixed at 0%.

In the case of qRT-PCR, the average values of technical replicates were used. The fold change was calculated according to the $2^{-\Delta\Delta CT}$ method (*Livak and Schmittgen, 2001*).

For the final comparison, the results of all the patients were pooled. For the necrosis/late apoptosis and viability assays, the statistical significance for the difference between the inhibitor/toxin-treated cells and the cells treated with 0.1% DMSO was established by ordinary one-way analysis of variance using Dunnett's correction for multiple comparisons ($P \leq 0.05$ was considered significant). For the necrosis/late apoptosis and viability assays as well as the western blotting, the statistical significance of the difference between euESC and ecESC was established by an unpaired t-test with Welch's correction ($P \leq 0.05$ was considered significant). For the qRT-PCR data, the statistical significance of the difference between control and doxorubicin treatment was established using a paired t-test ($P \leq 0.05$ was considered significant), and the statistical significance of the difference between euESC and ecESC was established using an unpaired t-test (again, $P \leq 0.05$ was considered significant). The aforementioned statistical analysis was carried out using GraphPad Prism 6 (San Diego, CA, USA).

The large-scale mRNA sequencing data were acquired using Illumina BaseSpace (San Diego, CA, USA). The reads were quantified with Salmon 0.9.1 (New York, USA / Pittsburgh, PA, USA) in quasi-mapping mode using indexed Ensemble v95 annotation. The quality control of raw sequencing data and statistics on aligned counts was performed with FastQC 0.11.5 (Babraham, UK) and MultiQC 1.7 (Stockholm/Uppsala, Sweden). Based on the quality control, further data transformation was performed by trimming the adapter size with Trimmomatic 0.38 (Jülich, Germany). Quantified transcript read counts were summarized to genes using Bioconductor packages tximport 1.10.1 (Boston, MA, USA / Zurich, Switzerland) and BioMart 2.38.0 (Berkeley, CA, USA / Cambridge, UK). Overall, 175,775 transcripts were identified from all the samples, out of which 28,796 genes with non-zero total counts were summarized. Differential RNA sequencing analysis and ranking was performed with DESeq2 1.22.2 (Heidelberg, Germany). edgeR 3.24.3 (Parkville, Australia) was used in parallel for comparison.

The shortlist of genes with significantly different (*P*-value adjusted for false discovery rate, $P_{adj} < 0.05$) expression in pairwise compared cell types and treatment conditions (control euESC versus control ecESC; control euESC versus toxin-treated euESC; control ecESC versus toxin-treated ecESC; and toxin-treated euESC versus toxin-treated ecESC) was generated as follows. The data for expression of each gene obtained in the same cell type and condition were averaged for three patients, and the binary logarithm of the fold change of averages (log_2FC) was determined. For each pairwise comparison, the latter values were ranked and cut-off values of log_2FC less than or equal to 4 or ≥+4 were applied. The genes showing high variance in expression (for the same cell type and condition between different patients) and the genes for which the number of counts was below 10 in all conditions were eliminated. Finally, after the individual check of the remaining candidates using the GeneCards human gene database (*Weizmann Institute of Science, 2019*) and g:Profiler source (*Reimand et al., 2016*), the pseudogenes and the genes encoding poorly characterized proteins were excluded from the list.

RESULTS

Viability assay

To establish the effect of the compounds (shown in TABLE 1) on the viability of euESC and ecESC, an assay was used that measures the change in absorbance spectrum of the cell membrane-penetrating dye resazurin upon its biochemical reduction in metabolically active cells. TABLE 3 summarizes the results of the viability assay in which a statistically significant reduction of viability ($P \leq 0.01$) was observed after 22 h incubation of cells with the studied compounds and after an additional 24 h incubation in growth medium; the full versions of the data are presented in the Supplemental Tables S1 and S2.

As expectedly, the lowest viability after 22 h of treatment was observed for both euESC and ecESC treated with the well-known apoptosis inducer staurosporine. SGI-1776, a pan-inhibitor of proto-oncogene Ser/Thr-protein kinase Pim family, caused a significant fall in viability at 10 µmol/l concentration in both euESC and ecESC ($P \leq 0.001$); it was also the only compound in the panel demonstrating a large patient-dependent effect: out of 11 patients' samples, low viability of the cells was evident in the samples from three patients, whereas those from four patients were practically insensitive (Supplemental Figure S1A). Other inhibitors of protein kinases did not cause an extended amount of cell death in either euESC or ecESC (the viability of cells remained at 75% or more relative to the 0.1% DMSO control). Interestingly, after 22 h incubation of cells with the Rho-dependent protein kinase inhibitor HA-1077, an apparent increase in viability was observed in both in euESC and ecESC (i.e. cells treated with 10 µmol/l inhibitor had higher values for resazurin reduction than cells treated with 0.1% DMSO). A similar phenomenon was evident in both in euESC and ecESC on treatment with different concentrations of VX-689, and in ecESC on treatment with 10 µmol/l or 2 µmol/l ARC-1859 (see Supplemental Table S1). Chemotherapeutic drugs bortezomib and MMAE were more efficient in eutopic cells, although a significant fall in viability was observed in both euESC and ecESC ($P \leq 0.001$). Conversely, treatment with 10 µmol/l and 2 µmol/l doxorubicin was more efficient in ecESC than euESC, showing a similar effect across all patients (see Supplemental Figure S1A).

The measurement of cell viability after the subsequent 24 h incubation in growth medium demonstrated that the viability of most toxin-treated euESC and ecESC had decreased further, and differences in results between euESC and ecESC had become smaller (TABLE 3). In addition, a significant decrease of viability was now observed for cells treated with the mitogen-activated protein kinase (MAPK) pathway inhibitor sorafenib ($P \leq 0.05$), protein kinase A (PKA) inhibitor H-89 ($P \leq 0.01$) and Aurora A inhibitor VX-689 ($P \leq 0.01$; a fuller version of 22 h + 24 h results from TABLE 3 is presented as Supplemental Table S2). Although sorafenib and H-89 were slightly more active in euESC, the effect of VX-689 was more pronounced in ectopic cells. Notably, after prolonged

TABLE 3 COMPOUNDS INDUCING A SIGNIFICANT DECREASE IN VIABILITY OF EUESC AND/OR ECESC AFTER 22 H AND PROLONGED TREATMENT

Compound	Concentration	Incubation time[a]	% of viability in euESC[b]		% of viability in ecESC[b]		Difference euESC versus ecESC[c]
GSK690693	10 µmol/l	22 h	86 ± 2	***	94 ± 2	ns	* (euESC)
		22 h + 24 h	78 ± 2	***	87 ± 2	***	** (euESC)
	2 µmol/l	22 h	89 ± 2	**	94 ± 2	ns	ns
		22 h + 24 h	85 ± 2	***	89 ± 2	***	ns
CYC116	10 µmol/l	22 h	89 ± 2	***	103 ± 2	ns	*** (euESC)
		22 h + 24 h	87 ± 2	***	93 ± 2	**	* (euESC)
ARC-775	10 µmol/l	22 h	77 ± 2	***	90 ± 2	***	*** (euESC)
		22 h + 24 h	67 ± 2	***	70 ± 2	**	ns
	2 µmol/l	22 h	90 ± 2	***	102 ± 2	ns	*** (euESC)
		22 h + 24 h	92 ± 2	***	91 ± 2	***	ns
SGI-1776	10 µmol/l	22 h	56 ± 5	***	62 ± 5	***	ns
		22 h + 24 h	48 ± 4	***	57 ± 4	***	ns
Staurosporine	5 µmol/l	22 h	15 ± 1	***	6 ± 1	***	*** (ecESC)
		22 h + 24 h	4 ± 1	***	3 ± 1	***	ns
	1 µmol/l	22 h	27 ± 3	***	24 ± 1	***	ns
		22 h + 24 h	16 ± 2	***	15 ± 2	***	ns
	0.2 µmol/l	22 h	41 ± 3	***	50 ± 2	***	** (euESC)
		22 h + 24 h	30 ± 2	***	41 ± 2	***	*** (euESC)
Bortezomib	10 µmol/l	22 h	26 ± 2	***	40 ± 2	***	*** (euESC)
		22 h + 24 h	5 ± 1	***	16 ± 2	***	*** (euESC)
	2 µmol/l	22 h	33 ± 1	***	42 ± 2	***	*** (euESC)
		22 h + 24 h	12 ± 1	***	23 ± 2	***	*** (euESC)
	0.4 µmol/l	22 h	39 ± 2	***	53 ± 2	***	*** (euESC)
		22 h + 24 h	18 ± 1	***	39 ± 2	***	*** (euESC)
Doxorubicin	10 µmol/l	22 h	78 ± 2	***	59 ± 2	***	*** (ecESC)
		22 h + 24 h	38 ± 2	***	22 ± 2	***	*** (ecESC)
	2 µmol/l	22 h	78 ± 2	***	64 ± 2	***	*** (ecESC)
		22 h + 24 h	39 ± 2	***	37 ± 2	***	ns
	0.4 µmol/l	22 h	85 ± 2	***	83 ± 2	***	ns
		22 h + 24 h	67 ± 3	***	68 ± 2	***	ns
Monomethyl auristatin E (MMAE)	1 µmol/l	22 h	60 ± 2	***	65 ± 1	***	* (euESC)
		22 h + 24 h	47 ± 2	***	53 ± 2	***	* (euESC)
	0.2 µmol/l	22 h	60 ± 2	***	66 ± 2	***	* (euESC)
		22 h + 24 h	49 ± 2	***	57 ± 2	***	** (euESC)
	0.04 µmol/l	22 h	61 ± 2	***	64 ± 1	***	ns
		22 h + 24 h	49 ± 2	***	59 ± 2	***	*** (euESC)

Data are mean normalized viability ± SEM.

[a] Incubation with inhibitors was performed for 22 h, followed by the addition of growth medium for 24 h.

[b] $n = 11$ for the 22 h measurement and $n = 10$ for the 22 h + 24 h measurement. Data obtained for incubation with 10% dimethylsulphoxide (DMSO) were considered to show 0% viability, and data obtained for incubation with 0.1% DMSO were considered to show 100% viability. Significance of effect difference relative to the negative control (treated with 0.1% DMSO): ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; ns, $P > 0.05$.

[c] Significance of effect difference between euESC and ecESC; the cell type with the lowest viability is shown in brackets. ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; ns, $P > 0.05$. ecESC, ectopic endometrial stromal cell; euESC, eutopic endometrial stromal cell.

incubation, 10 µmol/l doxorubicin still affected ecESC more than euESC. The increased sensitivity of ecESC towards high concentrations of doxorubicin was confirmed in a repeated assay with samples representing four patients from the same cohort (Supplemental Figure S2).

All in all, based on the results of the viability assay, characteristic differences in the viability fingerprint between euESC and ecESC could be formulated (FIGURE 1).

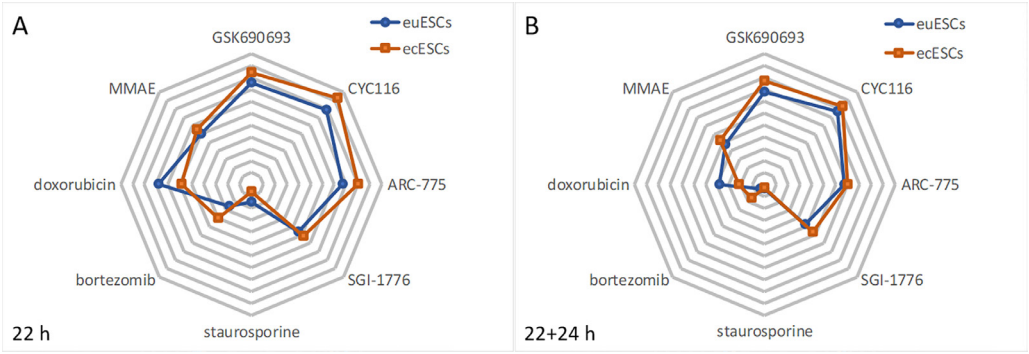

FIGURE 1 Viability fingerprint of euESC versus ecESC (blue and orange lines, respectively) after 22 h (A) or 22 h + 24 h (B) of treatment with various compounds. The compounds were chosen based on *Table 3*. Mean data corresponding to treatment with the highest concentrations of compounds was plotted. The axis scale ranges from 0% (centre of the plot) to 110% (outer line) with a grid interval of 10%. ecESC, ectopic endometrial stromal cell; euESC, eutopic endometrial stromal cell.

Necrosis/late apoptosis assay

To confirm the trends observed in viability studies, we applied an additional assay by using cell membrane-impermeable Sytox Blue dye after 22 h incubation of euESC and ecESC with the different compounds. The increase in fluorescence of Sytox Blue resulting from intercalation of dye into the DNA is only possible in cells with compromised membrane structure, indicating an elevated extent of necrosis/late apoptosis.

The results of the assay are presented in the Supplemental Table S3. The highest effect in euESC as well as in ecESC was observed for 5 µmol/l staurosporine, a generic protein kinase inhibitor, which was therefore chosen as the standardizing condition setting the maximal threshold for normalization of the data. ecESC seemed overall less prone to necrosis/late apoptosis than euESC; however, high levels of cell death in both euESC and ecESC were also observed upon treatment with 10 µmol/l SGI-1776 (which targets PIM family protein kinases) and 10 µmol/l ARC-775 (which targets casein kinase 2 [CK2]). The Akt/protein kinase B (PKB) inhibitor GSK690693 in the 10 µmol/l concentration induced more necrosis/late apoptosis in eutopic cells; furthermore, the toxins bortezomib and MMAE were more effective in euESC than ecESC at all concentrations. Other compounds showed no effect even at the highest concentrations used (5–10 µmol/l).

The data for doxorubicin were not included as here we observed a characteristic fall in Sytox Blue signal below the value observed for the negative control (cells treated with 0.1% DMSO), which occurred in both euESC and ecESC from all patients. We propose that such behaviour is related to the mode of action of doxorubicin, which intercalates into DNA; in this way, doxorubicin competes with Sytox Blue for the binding sites, and necrosis or apoptosis assays based on dyes that gain fluorescence upon binding to DNA are incompatible with doxorubicin studies.

Western blotting

To gain further independent evidence related to the elevated efficiency of doxorubicin in ecESC versus euESC, we proceeded with an alternative assay. Due to the strong autofluorescence of doxorubicin (*Wang et al., 2016*), most of the 'classical' techniques such as imaging or fluorescence-activated cell sorter (FACS) using immunostaining or BrdU detection can be highly prone to artefacts; therefore, we chose western blotting to quantify the fall in procaspase-3 concentrations in doxorubicin-treated samples of euESC and ecESC from four patients (the same samples used for the repeated viability assay; see above). The ratio of signals corresponding to procaspase-3 and beta-actin was quantified for each treatment condition, and the data were normalized separately for euESC and ecESC from each patient according to the corresponding negative control (0.1% DMSO; FIGURE 2A).

The results confirmed that 48 h of treatment with 10 µmol/l doxorubicin caused a statistically significant ($P \leq 0.05$) difference in apoptosis in ectopic versus eutopic cells, with mean normalized procaspase-3 content reduced to 39% (±8% SEM) in ecESC and 60% (±4% SEM) in euESC relative to the corresponding negative controls (0.1% DMSO) (FIGURE 2B).

mRNA sequencing

Finally, to obtain detailed insight into the signalling pathways affected by doxorubicin in euESC and ecESC, large-scale mRNA sequencing was performed after 24 h incubation of cells from three patients with 2 µmol/l doxorubicin or 0.1% DMSO control. The concentration of doxorubicin was chosen based on the results of the viability assay, in order to see a significant difference between euESC and ecESC while still yielding a sufficient population of surviving cells for mRNA isolation.

The comparison of treated versus control cells yielded 4009 significantly differentially expressed genes in the case of euESC, yet only 249 significantly differentially expressed genes for ecESC (using a base mean cut-off value of >10 and a P_{adj} cut-off value of <0.05). To shortlist genes showing a significantly different expression in different cells and treatment conditions (TABLE 4), we sorted the sequencing data as described in the section on statistical analysis, above. Overall, several genes that had a higher expression in control euESC relative to control ecESC (*MMP1/3/10*, *PENK*, *PTN* and *GRP*) or in control ecESC relative to control euESC (*ESM1*, *IL33* and *PTX3*) also showed greater expression in the same cell type following treatment with doxorubicin. Furthermore, treatment with doxorubicin resulted in a reduced expression of several genes in euESC (e.g. *DUSP1/10* and *BARD1*) as well as in ecESC (e.g. *DKK1*, *HAS2*) relative to the control cells of the same type. On the other hand, although the expression of some genes (such as histone cluster 1 and 2 family members *HIST1H2AE*, *HIST1H2BK* and *HIST2H2AA4*) in euESC increased upon treatment with

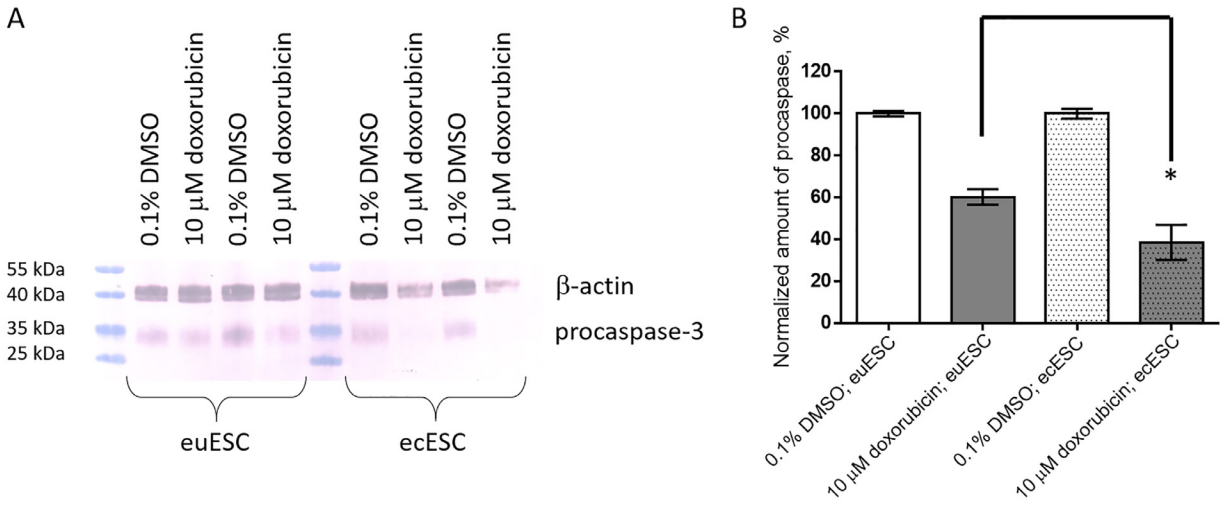

FIGURE 2 Effect of doxorubicin on procaspase-3 concentrations in euESC and ecESC. (A) Representative example of western blot membrane with euESC and ecESC from one patient; different lanes represent independent incubations. (B) Pooled normalized western blot data of euESC and ecESC from four patients (mean ± SEM). Significance of effect difference between euESC and ecESCs: *$P \leq 0.05$. DMSO, dimethylsulphoxide; ecESC, ectopic endometrial stromal cell; euESC, eutopic endometrial stromal cell.

doxorubicin relative to control cells, there was no significant increase in gene expression in toxin-treated ecESC relative to the control treatment.

For technical validation of the results of large-scale mRNA sequencing, qRT-PCR analysis of *PTN* and *HSPA2* was carried out, as examples of genes considerably highly expressed in eutopic cells, with *PTN* expression elevated in both control and toxin-treated euESC relative to the correspondingly treated ecESC (TABLE 4). Also validated was the expression of *PTGS2*, which, according to large-scale mRNA sequencing data, possessed higher expression in ectopic relative to eutopic cells after doxorubicin treatment; however, the statistical significance of this difference was slightly higher than the classical cut-off P_{adj} value of 0.05 (Supplemental Table S4). qRT-PCR confirmed the general trends observed in large-scale transcriptomic analysis, indicating significantly higher expression of *PTN* in both control and doxorubicin-treated euESC versus the corresponding ecESC (both $P < 0.05$), and significantly higher expression of *HSPA2* in control euESC versus ecESC ($P = 0.05$). In addition, doxorubicin treatment elevated the level of *PTN* and *HSPA2* in eutopic and ectopic stromal cells, respectively (both $P < 0.05$). Furthermore, qRT-PCR showed significantly higher expression of *PTGS2* in control ecESC versus euESC as well as doxorubicin-treated ecESC versus euESC (both $P < 0.05$), confirming that *PTGS2* can indeed serve as an important target in endometriosis.

TABLE 4 GENES SHOWING SIGNIFICANTLY DIFFERENT EXPRESSION IN CONTROL AND TOXIN-TREATED EUESC AND ECESC

Comparison[a]		Gene names and log_2FC values[b,c]
euESC control versus ecESC control	Higher expression in euESC	*MMP12* (8.4), ***MMP10*** (8.0), ***MMP3*** (8.0), *TFAP2C* (7.4), ***RGCC*** (6.8), ***HTR2B*** (6.4), ***GRP*** (6.4), ***DIO2*** (5.7), ***MMP1*** (5.5), *RBP1* (4.9), ***CARD16*** (4.8), *LEPR* (4.8), *PRDM1* (4.7), *CTSK* (4.6), *HSPA2* (4.6), *NID1* (4.6), *GCNT4* (4.5), *PLAU* (4.5), ***PENK*** (4.5), *PTN* (4.4), *IFI6* (4.2), *SEMA5A* (4.1), *AREG* (4.0), *NPY1R* (4.0)
	Higher expression in ecESC	*GIPC2* (–9.7), ***PTX3*** (–9.0), *EFEMP1* (–6.1), ***IL33*** (–6.0), *SFRP4* (–4.5), *PPP1R3C* (–4.3), ***ESM1*** (–4.0)
euESC control versus euESC + toxin	Higher expression in control treatment	***HTR2B*** (8.0), *CCDC107* (7.0), *ING3* (6.4), ***BARD1*** (6.2), *CARNMT1* (5.9), *KRT19* (5.8), *TUBA1A* (5.3), ***DIO2*** (5.2), *PAN3* (5.1), *DUSP1* (4.9), *PKIG* (4.9), *PBK* (4.9), *UTP18* (4.8), *CEMIP* (4.7), *SLC5A3* (4.5), *CITED2* (4.5), *CTGF*(4.4), *SASS6* (4.1), *DUSP10* (4.1), *NOP10* (4.1)
	Higher expression in toxin treatment	*HIST1H2AE* (–7.0), *INSYN2* (–6.7), *TMEFF2* (–6.0), *HIST1H2BPS2* (–5.2), *HIST1H2BK* (–5.0), *HIST2H2AA4* (–4.8), *CXCL3* (–4.7)
ecESC control versus ecESC + toxin	Higher expression in control treatment	*HAS2* (6.9), *MRPL14* (5.0), ***CARD16*** (4.5), *DKK1* (4.0)
euESC + toxin versus ecESC + toxin	Higher expression in euESC	***GRP*** (7.3), ***MMP3*** (7.1), ***MMP10*** (6.1), *PTN* (5.1), ***RGCC*** (4.7), *IFITM1* (4.5), *SOX11* (4.3), ***MMP1*** (4.2), *PENK* (4.1)
	Higher expression in ecESC	***ESM1*** (–6.2), *TFPI2* (–5.3), ***PTX3*** (–4.9), *IL33* (–4.4), ***BARD1*** (–4.1)

[a] Control treatment: 24 h incubation in growth medium containing 0.1% dimethylsulphoxide; toxin treatment: 24 h incubation in growth medium containing 2 µmol/l doxorubicin.

[b] The binary logarithm of the fold change of averages is shown in brackets; $n = 3$. Negative values indicate higher expression in ectopic cells (for euESC versus ecESC comparisons) or in doxorubicin-treated cells (for treatment comparisons).

[c] Genes that are listed under more than one comparison in the table are shown in bold.

ecESC, ectopic endometrial stromal cell; euESC, eutopic endometrial stromal cell; log_2FC, binary logarithm of fold change of averages.

DISCUSSION

Although the molecular players behind the onset and progression of endometriosis are still unclear, several pathways have been closely inspected, with a special focus on inflammation processes, cell migration and adhesion, abnormal proliferation and resistance to apoptosis. The current study explored the differences in cell viability of euESC and ecESC on treatment with selective compounds inhibiting a focused number of molecular players, as well as compounds with a wide profile of biological targets. Methodologically, this study has two major limitations: first, the focus was only on stromal cells, yet the physiological milieu contains epithelial cells that may be involved in unique patterns of signalling and cellular interactions. Second, as only ESCs isolated from superficial peritoneal lesions were investigated, the observed results may not necessarily reflect the effects of toxins in other types of lesions.

Phosphorylation of proteins serves an example of a signalling mechanism that on one hand is ubiquitous, yet on the other can be dissected with a high degree of precision by selective targeting of the catalysing machinery – protein kinases. The human kinome includes 538 protein kinases, most of which have been termed as potentially druggable by virtue of the incorporation of a narrow solvent-hidden pocket (ATP-binding site) that can be selectively targeted by low molecular weight inhibitors. The panel that was used here for screening included 11 inhibitors of protein kinases, 10 of which possessed focused selectivity profiles, while staurosporine was selected as a widely used apoptosis inducer (see TABLE 1 and Supplemental Figure S3). Among the protein kinases targeted by the selective inhibitors were enzymes for which up-regulation in endometriotic cells has been reported: MAPKs (*Ngô et al., 2010; Yotova et al., 2011*), Akt/PKB (*Cinar et al., 2009; Shoji et al., 2009*), PIM1 (*Hu et al., 2006; Jiménez-García et al., 2017*) and CK2 (*Feng et al., 2012; Llobet et al., 2008*). In this study, inhibitors of MAPK (sorafenib), Akt/PKB (GSK690693) and CK2 (ARC-775) were more effective in euESC than ecESC, whereas the PIM inhibitor (SGI-1776) showed a cell type-independent effect: in patients whose euESC were affected, ecESC were also affected (see Supplemental Figure S1B and C). Overall, although the overexpression of certain pro-survival protein kinases in cancer cells can lead to the degeneration of other anti-apoptotic pathways and the establishment of the so-called oncogene addiction (*Ruzzene and Pinna, 2010; Sharma and Settleman, 2007*), this does not seem to be the case for ectopic endometriotic cells.

Surprisingly, CK2 inhibitor ARC-1859, despite featuring a structural design highly similar to that of ARC-775, did not reduce cell viability. Whereas in biochemical assays with recombinant CK2, the affinity of the unmasked counterpart of ARC-775 was indeed higher than that of the unmasked counterpart of ARC-1859 (*Rahnel et al., 2017; Viht et al., 2015*), this is hardly likely to be the only reason underlying the lack of potency of ARC-1859 in assays with ESC. Instead, it is likely that a more hydrophobic ATP-site targeting a fragment of ARC-1859 (the tetrabromobenzimidazole moiety) contributes to the accumulation of inhibitor in membranes, where it is not accessible by either esterases or the cytosolic CK2.

The effect of some compounds included in the panel in this study has previously been explored in the context of endometriosis. The generic protein kinase inhibitor staurosporine has been reported to demonstrate a greater apoptotic effect in euESC from patients without endometriosis than in ecESC from patients with endometriosis (*Watanabe et al., 2009*). In the current study, the sensitivity of eutopic versus ectopic cells to staurosporine depended on its concentration: whereas 5 µmol/l staurosporine caused greater cellular death in ecESC, 0.2 µmol/l staurosporine was more effective in euESC (TABLE 3). The proteasome-targeting compound bortezomib had been shown to reduce the size of endometriotic implants in rats (*Celik et al., 2008*), yet no studies of bortezomib in euESC from women with endometriosis have been reported; in this study study, treatment with bortezomib was significantly more efficient in euESC than in ecESC even after prolonged incubation ($P \leq 0.001$).

The ROCK-targeting inhibitors Y-27632 and HA-1077 have been used to reduce the contractility of ecESC; whereas Y-27632 had demonstrated no cytotoxicity, 0.1–10 µmol/l HA-1077 had caused significant apoptosis of ecESC – albeit after 48 h incubation (*Yotova et al., 2011; Yuge et al., 2007*). In the current study, no reduction in viability was observed even after prolonged incubation of euESC and ecESC with either Y-27632 or HA-1077. In principle, it is possible that the effect of ROCK-targeting inhibitors is only evident in cell motility assays, although it had been hoped that the altered dynamics of cytoskeleton might manifest itself as a retardation of proliferation. The latter was true for the microtubule-depolymerizing compound MMAE; this showed a characteristic concentration-independent profile of effect on cell viability connected to the mode of action of this compound, which serves as an antimitotic agent than an apoptosis inducer (*Abdollahpour-Alitappeh et al., 2017; Chen et al., 2017*).

Furthermore, 22 h treatment of cells with some of the chosen compounds (including inhibitors targeting ROCK, Aurora family kinases or PKA) caused an apparent increase in viability (see Supplemental Table S1), which was alleviated after a subsequent 24 h incubation in medium. This abnormal temporary phenomenon might be triggered by several factors. On one hand, ROCK inhibitors can interfere with the apoptotic caspase 3-ROCK signalling pathway (*Song and Gao, 2011*), and consequently increase the number of viable cells. However, a more likely explanation is that, as a response to treatment with toxins within a certain time window, cell metabolism tends to increase, which manifests itself as enhanced reduction of resazurin.

Overall, the compounds that significantly affected the viability of cells after 22 h of treatment also caused a significant amount of cellular death according to the necrosis/late apoptosis assay (as illustrated by GSK690693, ARC-775, SGI-1776, staurosporine, bortezomib and MMAE; $P \leq 0.05$). The only exception was CYC116, which did not trigger necrosis/late apoptosis yet remarkably reduced viability in euESC at the 10 µmol/l concentration. It is possible that AURORA B-targeting CYC116 acts as an antimitotic substance and hence slows the proliferation of cells rather than triggering cellular death, yet it is not as efficient or as quick as the toxin MMAE, which has a similar mode of action.

Differently from other compounds used in the panel, doxorubicin demonstrated

an enhanced effect on viability in ectopic versus eutopic cells after 22 h as well as 22 h + 24 h of incubation at the 10 µmol/l concentration in the resazurin assay (FIGURE 1), and after 48 h of incubation in the western blot assay (FIGURE 2). Several mechanisms of action have been reported for doxorubicin. It accumulates in cell nuclei, intercalating into DNA and preventing its repair by topoisomerase II (*Thorn et al., 2011*). In addition, doxorubicin can be reversibly oxidized into an unstable semiquinone metabolite, which releases ROS upon spontaneous re-formation of doxorubicin (*Finn et al., 2011*); the liberated ROS attack cellular components, triggering cellular death. In the context of altered redox equilibria in ectopic versus eutopic endometrial cells (*Kasvandik et al., 2016; Scutiero et al., 2017*), enhanced efficiency of doxorubicin in ecESC might be explained by its redox properties.

In this way, although doxorubicin has been used in the treatment of endometrial cancer (*Byron et al., 2012; Chitcholtan et al., 2012*), this compound might also be of remarkable interest for endometriosis studies. Unfortunately, the application of anthracyclines in chemotherapy has revealed high cardiotoxicity for this class of compounds, which complicates their use in model organisms. However, several pharmacokinetic and pharmacodynamic strategies have been actively suggested to prevent anthracycline-induced cardiotoxicity (*Menna and Salvatorelli, 2017*). Furthermore, specifically in the context of doxorubicin, the development of novel derivatives with reduced side effects (*Shaul et al., 2013*) and methods for targeted delivery (*Tran et al., 2017*) have been intensely pursued.

The large-scale transcriptome analysis revealed sets of genes that featured significantly higher expression in eutopic relative to ectopic ESC or in ectopic relative to eutopic ESC, irrespective of the treatment conditions ($P_{adj} < 0.05$, log_2FC of –4 or less or ≥+4; TABLE 4). It was postulated that these sets might reflect variations in survival strategies in eutopic and ectopic endometrium, because it is likely that, after 24 h of treatment of cells with 2 µmol/l doxorubicin, the isolated mRNA profile was characteristic of the population of survivors.

Interestingly, the comparison of treated versus control cells yielded in excess of 10 times more significantly differentially expressed genes in the case of euESC than ecESC ($P_{adj} < 0.05$). Given the fact that the majority of candidate genes in the comparison of control versus doxorubicin-treated ecESC were eliminated on the basis of the P_{adj} cut-off, this difference originates primarily from the large interpatient variation of gene expression in the ecESC group. The latter can in turn be explained by the characteristic heterogeneity of lesions, especially taking into consideration differences in location of the lesions in the three patients whose samples were used for mRNA sequencing (see TABLE 2).

In euESC, among other genes, this set included genes encoding several members of the matrix metalloproteinase (*MMP*) family, and a precursor for the endogenous opioid peptides, preproenkephalin (*PENK*). Another gene with a significantly higher expression in both control and doxorubicin-treated euESC versus ecESC encodes a growth factor, pleiotrophin (*PTN*; $P_{adj} < 0.05$, $log_2FC > +4$); interestingly, doxorubicin treatment further elevated *PTN* expression in drug-treated eutopic but not ectopic cells. Importantly, *MMP*, *PENK* and *PTN* have previously been linked to endometriosis, showing significantly higher expression in eutopic endometrium from women with endometriosis relative to healthy controls, or lower expression in ectopic than eutopic tissue (*Burney et al., 2007; Chung et al., 2002; Kobayashi et al., 2012*), thus pointing to their possible role in initiating peritoneal invasion. Furthermore, PTN has been reported to promote chemoresistance to doxorubicin in several cancers, including osteosarcoma and breast cancer (*Huang et al., 2018; Wu et al., 2017*). Therefore, it can be suggested that the lower expression of *PTN* in untreated ectopic cells is one of the factors responsible for the higher chemosensitivity of this cell type to doxorubicin – although it should be considered that the viability of euESC was still significantly affected by doxorubicin treatment ($P \leq 0.001$; TABLE 3).

A similar effect on cell viability, may be mediated by *HSPA2*, which was, according to sequencing data, more highly expressed in eutopic than ectopic cells. Heat shock-related 70 kDa protein 2 (HSPA2) protects cells from the cytotoxic and growth-inhibiting effects of doxorubicin by several mechanisms, including binding misfolded or damaged proteins and enabling these proteins to acquire proper folding, and controlling the duration of cell cycle arrest (*Karlseder et al., 1996*). According to the qRT-PCR data, the drug treatment enhanced the expression of *HSPA2* in ecESC (average fold change 4.5), suggesting a response to the toxic effect; however, as the initial expression of *HSPA2* in untreated cells was much lower in ectopic then eutopic cells (average fold change –11.8), the expression was still less than that of the eutopic cells.

In ecESC, the set of interest defined by the large-scale transcriptome analysis and qRT-PCR data included genes tightly connected with immune system functioning: these genes encoding interleukin-33 (*IL33*), cyclooxygenase 2 (*PTGS2*) and genes whose expression is regulated by cytokines – pentraxin 3 (*PTX3*) and endothelial cell-specific molecule 1 (*ESM1*). The proteins encoded by all of the aforementioned genes have been reported to be connected with endometriosis (*Cobellis et al., 2004; Fagotti et al., 2004; Kobayashi et al., 2012; Miller et al., 2017; Pelch et al., 2010*), showing a correlation with endometriosis-associated inflammation and angiogenesis; inhibitors of PTGS2 have also been explored in the context of management of endometriosis-related pain (*Cobellis et al., 2004*). Furthermore, IL33 and PTGS2 have been shown to protect cells against doxorubicin-induced apoptosis, albeit in the context of tissues other than endometrium (*Puhlmann et al., 2005; Singh et al., 2008; Yao et al., 2017*). The latter observation indirectly confirms the hypothesis that the mRNA profile identified for doxorubicin-treated euESC and ecESC reflects the corresponding cellular survival strategies. The fact that the viability of ecESC was severely affected by doxorubicin treatment indicates that the major chemoresistance-ensuring players that contribute to the survival of ectopic cells under DNA damage and ROS-triggered conditions of stress might be less efficient than those in eutopic tissue.

The mRNA sequencing results thus underline the interplay of factors that contributing to development and sustainment of endometriosis, and necessitate the application of more complex models, for example enabling the presence of epithelial cells and/or involving immune system components.

Overall, the results of this study seem to have pinpointed a set of clues for future research into endometriosis, both from the aspect of showing a resistance of endometriotic lesions to possible therapeutic candidates, and in terms of providing candidate biomarkers and targets for the succeeding exploration.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2019.05.019.

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