Subtypes of Triple-negative Breast Cancer Cell Lines React Differently to Eribulin Mesylate

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Abstract. *Background/Aim: Diagnosis of triple-negative breast cancer (TNBC) is associated with adverse prognosis, particularly in cases of chemotherapy resistance. The goal of this analysis was to compare TNBC vs. non-TNBC cell lines and those of distinct TNBC subtypes with regard to sensitivity to eribulin in vitro. Materials and Methods: Breast cancer cell lines were subjected to cell-viability assays, apoptosis analyses, migration and invasion experiments, and quantitative real-time polymerase chain reaction after exposure to eribulin. Results: Eribulin reduced cell viability in TNBC and non-TNBC cell lines in the sub-nanomolar range. Furthermore, exposure to eribulin induced apoptosis and decreased the rate of migration and invasion. Genes known to induce malignant transformation were differentially expressed after eribulin treatment. Conclusion: Eribulin had a strong antiproliferative effect on breast cancer cell lines, although we did not observe a significant difference between TNBC and non-TNBC cell lines with regard to sensitivity to eribulin.*

Breast cancer is a heterogeneous disease comprising of clinically and molecularly distinct subtypes (1, 2). Triple-negative breast cancer (TNBC) represents 10-15% of all breast cancer cases and is defined by the lack of both hormone receptor expression (estrogen and progesterone receptors) and human epidermal growth factor receptor 2 (*HER2*) amplification/overexpression (3, 4). Lehmann *et al*. identified six subtypes within the subgroup of TNBC by cluster analysis displaying unique gene expression and ontologies for two basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal, a mesenchymal stem–like (MSL), and a luminal androgen receptor (LAR) subtype (5). Patients with TNBC suffer from unfavorable prognosis particularly when they respond poorly to anthracycline-taxane chemotherapy (6). Given that limited treatment options exist for patients with TNBC besides classical chemotherapy, novel treatment regimens *e.g*. by development of novel chemotherapeutics, are urgently needed to improve the prognosis of these patients.

The chemotherapeutic agent eribulin affects the formation of the spindle apparatus and induces cell-cycle arrest as well as apoptosis (7, 8). In contrast to taxanes, eribulin does not depolymerize microtubules, thereby causing less toxicity and it prefers another microtubule-binding site. This has rendered eribulin of particular interest for the treatment of taxane-resistant breast cancer (9). A phase II neoadjuvant clinical trial combining carboplatin with eribulin showed promising results despite being a small study comprising of only 30 patients: 43.3% of study subjects achieved a pathological complete remission and 80% had a clinically complete or partial response (10). In a phase III open-labeled randomized study, eribulin therapy led a significant and clinically meaningful improvement in prolonged overall survival compared to treatment of physician's choice (TPC) in women with heavily pretreated metastatic breast cancer. In that study, 19% of all cases were TNBC and eribulin was highly effective, with a 29% decrease in risk of death compared to other chemotherapy (11).

In the present analyses, we investigated the potential impact of eribulin treatment on proliferation, apoptosis, migration of TNBC and non-TNBC cell lines, and regulation of their genes involved in malignant tumor transformation. The aim of this study was to compare (i) TNBC with non-TNBC cell lines, and (ii) cell lines of distinct TNBC subtypes with regard to eribulin sensitivity *in vitro*.

Materials and Methods

Breast cancer cell lines. A total of 17 established breast cancer cell lines comprising both TNBC (two BL1; two BL2; one IM; one mesenchymal; three MSL; two LAR; and one unclassified) and non-TNBC (n=5) phenotypes (purchased from the American Type Cell

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Collection (LGC, Wesel, Germany) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany; Table I) were studied. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Darmstadt, Germany) containing 10% fetal bovine serum, 1% penicillin and 1% streptomycin (all from Life Technologies). Growing cells were split at 80% confluency. Cell numbers were determined *via* live counting using trypan blue in an automated cell counter (TC20; Biorad, Munich, Germany) and plated at an appropriate density in 96-well plates or cell-culture dishes according to the planned experiment.

Compounds. Eribulin mesylate (HALAVEN; Eisai GmbH, Frankfurt, Germany) was obtained by the hospital pharmacy (0.44 mg/ml ethanol). Camptothecin was purchased from Sigma-Aldrich (Hamburg, Germany) and diluted in dimethyl sulfoxide (DMSO) at 10 mg/ml.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. Viable cells were determined by the MTT (Sigma-Aldrich) assay. A total of 3×10^3 cells were plated in 100 μl medium in each well of a 96-well plate. After 24 h, eribulin mesylate was diluted to the desired concentrations (1 μM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM) and added in sextuplicate. After 72 h of drug treatment, cell medium was removed and 100 μl medium containing 10% MTT stock solution (5 mg MTT/ml phosphate-buffered saline (PBS) was added. After 4 h of incubation at 37˚C, formazan crystal formation was stopped with 100 μl solubilization solution [10% sodium dodecyl sulfate (SDS) with 50% *N,N*-dimethylformamide, pH 4.7 adjusted with HCl] per well and stored in the dark overnight. The absorption coefficient was determined at 560 nm with a reference wavelength at 650 nm using an Epoch microplate spectrophotometer (BioTek, Bad Friedrichshall, Germany). The MTT assay was repeated at least three times for each cell line to determine the half maximum inhibitory concentration (IC_{50}).

Fluorescence-activated cell sorting (FACS) analysis. To detect apoptosis, we used the fluorescein isothiocyanate (FITC)-Annexin-V Apoptosis Detection Kit-I (BD Biosciences, Heidelberg, Germany) by FACS analyses. Briefly, cells were treated with eribulin ($10 \times IC_{50}$) for 24 h or left untreated. Cells were then harvested, washed, and counted to obtain 10^6 cells in Annexin-V Binding Buffer. After adding 3 μl of FITC-Annexin-V antibody and 3 μl propidium iodide, samples were mixed and incubated in the dark for 15 min. Cells were washed in ice-cold PBS and resolved in 500 μl Annexin-V Binding Buffer. Measurements were performed by Benchtop analyzer LSRII (BD Biosciences). Data were processed by FLOWJO Single Cell Analysis Software (FLOWJO; LCC, Ashland, OR, USA).

Western blot. RIPA buffer (80-100 μl) containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, freshly added 1% protease inhibitor and phosphatase inhibitor 2 (Sigma-Aldrich) was used for protein isolation. Protein concentrations were determined by the BCA Protein Assay (Thermo Scientific, Schwerte, Germany) and diluted to obtain 20 μg per sample. After SDS–polyacrylamide gel electrophoresis, proteins were transferred onto a Hybond-P polyvinylidene difluoride membrane (GE Healthcare, Berlin, Germany). Membranes were blocked at room temperature in a blocking solution (5% non-fat dry milk in Tris-buffered saline, 0.1% Tween 20) for 1 h. The antibodies were diluted in blocking solution at 1:1,000 for poly (ADP-ribose) polymerase (PARP) (New England Biolabs, Frankfurt am Main, Germany) and 1:10,000 for beta-actin (Sigma-Aldrich), and incubated with proteins overnight at 4˚C. The corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies were diluted at 1:4,000 in blocking solution and added to the membranes for 1 h at room temperature. For detection, we used the Immobilon Western Chemiluminescent HRP solution (Merck Millipore, Darmstadt, Germany) and the immunoreaction was visualized on Amersham Hyperfilm ECL (GE Healthcare).

Table I. *Triple-negative breast cancer (TNBC) and non-TNBC cell lines. Classification by Lehmann et al. (5).*

Cell type	Cell line
TNBC	
Basal-like	
BL1	HCC 1937
	MDA-MB-468
BL2	HCC 1806
	HDQ-P1
IM	DU-4475
Mesenchymal-like	
Mesenchymal	BT-549
MSL	HS578T
	MDA-MB-436
	MDA-MB-231
Luminal androgen receptor	MDA-MB-453
	CAL-148
Unclassified	BT-20
Non-TNBC	AU-565
	T-47D
	SKBR-3
	MDA-MB-361
	MCF-7

IM: Immunomodulatory; MSL: mesenchymal stem-like.

Migration assay. Scratch assays were executed to compare the migration potential in untreated and eribulin-treated (IC_{50}, 24 h) MDA-MB-231 cells. Therefore, a "scratch" was made on a closed cell monolayer and the interface monitored after 24 h.

Invasion assay. For invasion assays, 0.4 μM pore polycarbonate membrane cell culture inserts in a 24-well plate (Corning Transwell; VWR, Darmstadt, Germany) were coated with 100 μl of Matrigel matrix diluted 1:6 with DMEM (Recon Base Membrane, VWR). Five days after eribulin treatment, 5×10^4 MDA-MB-231 cells were seeded onto the Matrigel in medium without serum in the upper chamber. The lower chamber was filled with 600 μl medium containing 20% fetal calf serum. After 24 h of incubation, cells which passed the membranes were fixed with 3.7% formaldehyde, permeabilized with 100% methanol and stained with Giemsa solution for 15 min. Stained filters were mounted on microscope slides and cells were counted and the means from five randomly selected fields were calculated.

RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (qPCR). RNA of untreated and eribulin-treated (IC_{50} for 24 h) cells was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). After photometric quantification on

NanoDrop 2000c (VWR), 1 μg RNA was reverse transcribed into cDNA using Superscript-II (Life Technologies). qPCR was performed on an Opticon2 cycler (Bio-Rad) using GoTaq qPCR Master Mix (Promega, Mannheim, Germany) and the following primer sequences for analyzing expression of eight genes known to be overexpressed in TNBC and to take part in malignant transformation according to results of analyses of a MD Anderson Cancer Center dataset of 133 patients with breast cancer (12, 13): Gamma-aminobutyric acid type A receptor pi subunit (*GABRP*): forward 5'-GGTGGAGAACCC GTACAGATAG-3', reverse 5'-AGAGTGAAGCTCTTGTTGCCTT-3'; E74 like ETS transcription factor 5 (*ELF5*): forward: 5'-TAGGGAACAAGGAATTTTTCGGG-3', reverse: 5'-GTACACTAA CCTTCGGTCAACC-3'; matrix metalloproteinase 7 (*MMP7*): forward: 5'-ATGAGTGAGCTACAGT GGGAAC-3', reverse: 5'-GCATCTCCTTGAGTTTGGCTT-3'; Y-box binding protein 1 (*YBX1*): forward: 5'-GGGGACAAGAAGGT CATCGC, reverse: 5'-CGAA GGTACTTCCTGGGGTTA-3'; retinoic acid receptor responder 1 (*RARRES1*): forward: 5'-AAA CCCCTTGGAAATAGTCAGC-3', reverse: 5'-GGAAAGCCAAA TCCCAGATGAG-3'; prion protein (*PRNP*): forward: 5'-CACGACTGCGTCAATATCACA-3', reverse: 5'-CTCCATCATCT TAACGTCGGTC-3'; SRY (sex determining region Y)-box 10 (*SOX10*): forward: 5'-AAAGCAAGCCGCA CGTCAAG, reverse: 5'-GCTTGTCACTTTCGTTCAGCA-3'; and epidermal growth factor receptor (*EGFR*): forward: 5'-CAGCAGTGACTTTCTCA GCAAC-3', reverse: 5'-TCAGTTTCTG GCAGTTCTCCT-3'. PCR product specificity was verified by comparative melting-curve analysis. Cycle threshold values of genes of interest were quantified, and normalized to expression of succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) (forward: 5'-TGGGAACA AGAGGGCATCTG-3', reverse: 5'-CCACCACTGCA TCAAA TTCATG-3'; housekeeping gene), and relative expression of genes in eribulin-treated cells were compared to relative expression of genes in untreated cells using the $2^{-\Delta\Delta Ct}$ method (14).

Results

Influence of eribulin on cell viability of breast cancer cell lines. The effect of eribulin on cell viability was investigated in 12 TNBC cell lines and five non-TNBC cell lines after 72 h of treatment and the IC_{50} value was determined (Figure 1a). The sensitivity towards eribulin treatment was not significantly different comparing TNBC and non-TNBC cell lines, although there was a tendency for stronger response in TNBC cells (Figure 1b). It is noteworthy that the TNBC cell line DU-4475, representing an immunomodulatory phenotype, responded significantly less to eribulin compared to all other analyzed breast cancer cell lines (Figure 1a, c).

Induction of apoptosis by eribulin treatment. According to FACS analyses (Figure 2a), 10× IC_{50} concentration of eribulin for 24 h induced apoptosis in the TNBC cell line MDA-MB-468 and the non-TNBC cell line MCF-7. Induction of apoptosis by PARP cleavage was shown in western blot analyses for the TNBC, MDA-MB-468, BT-549 and MDA-MB-436 cell lines. After treatment at the IC_{50} concentration of eribulin for 24 h, a cleaved PARP product at 89 kDa appeared in lysates from all three cell lines (Figure 2b).

Decrease of migration and invasion after eribulin treatment. After 24 h cell migration led to reclosing of the monolayer of untreated cells, while the monolayer was still somewhat non-continuous in eribulin-treated cells (Figure 3a). At 24 h after incubation of MDA-MB-231 on Matrigel-coated transwells, considerably fewer cells were able to invade through the membrane after eribulin treatment compared to the control (Figure 3b).

Gene expression after eribulin treatment. After treatment with eribulin (at the IC_{50}) for 24 h, the gene expression of *GABRP* was significantly up-regulated in both BL1 cell lines, HCC1937 and MDA-MB-468, while it was down-regulated in all other subtypes of TNBC, as well as in the non-TNBC cell lines compared to the untreated controls (Figure 4a). *ELF5* was significantly down-regulated in the BL2 cell lines (HCC 1806, HDQ-P1) and down-regulated in the LAR cell line MDA-MB-453, while it was up-regulated in all other tested cell lines (Figure 4b).

In the cell lines HDQ-P1 (BL2), DU-4475 (IM) and MDA-MB-231 (MSL) almost all of the tested genes were down-regulated after treatment with eribulin. In the MDA-MB-468 cell line (BL1), all tested genes with the exception of MMP7 were up-regulated.

Discussion

The present study investigated the effect of eribulin treatment on different subtypes of TNBC and non-TNBC cell lines *in vitro*. Eribulin inhibited the proliferation of all these breast cancer cell lines with IC_{50} values in the sub-nanomolar range (<1 nM) similar to previous studies (8, 15, 16). As an exception, the DU-4475 cell line, representing the IM subtype of TNBC, was inhibited by eribulin at an IC_{50} value of 15.5 nM.

In all examined cell lines, eribulin induced apoptosis as previously described for human histiocytic lymphoma and human prostate cancer cell lines (17). Prolonged mitotic blockage and G_2-M phase blockage leading to apoptosis has also been reported for breast cancer *in vitro* and *in vivo* (8). Eribulin treatment of TNBC cells led to decreased cellular migration and invasiveness capacities, as has been shown before (16).

The primary target of eribulin is tubulin and therefore microtubules (8, 9, 17). However, the interrelation between microtubule dynamics and malignant transformation has received little attention. Through examining the expression of eight genes, characterized by their participation in malignant tumor transformation, we observed a heterogeneous pattern of expression changes in eribulin-treated cells. Worthy of mention is the opposing effect of eribulin on gene expression in the cell line of the TNBC subtype BL1 (seven out of eight genes were up-regulated)

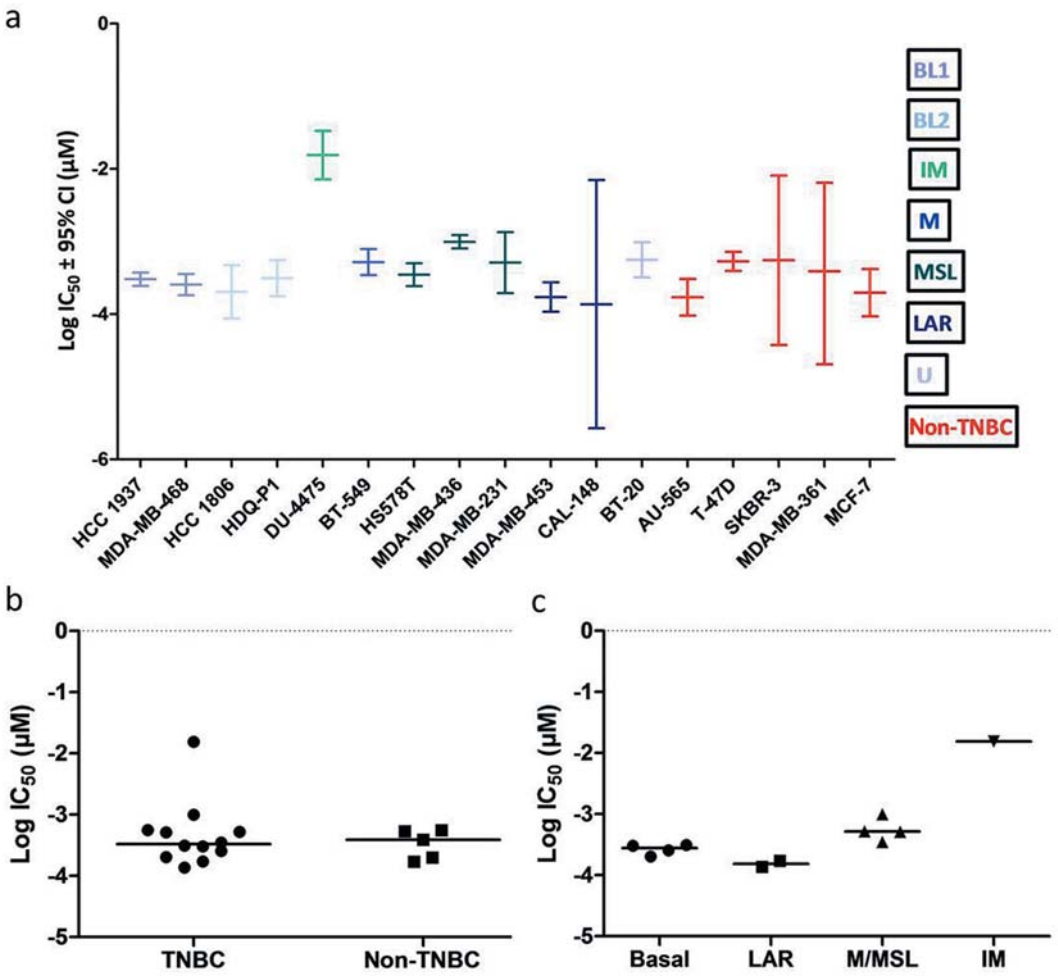

Figure 1. *Determination of the half-maximum inhibitory concentration (IC_{50}) of eribulin in 12 triple-negative breast cancer (TNBC) and five non-TNBC cell lines. a: Boxplot of the log IC_{50} concentration of eribulin for each cell line, with 95% confidence interval (CI). b: Comparison of the IC_{50} of TNBC vs. non-TNBC. c: Comparison of the IC_{50} among each subtype of TNBC. BL1: basal-like 1, BL2: basal-like 2, IM: immunomodulatory, M: mesenchymal, MSL: mesenchymal stem-like, LAR: luminal androgen receptor, U: unclassified.*

compared to the BL2 subtype (seven out of eight genes were down-regulated).

The up-regulation of *GABRP, ELF5, YBX1, RARRES1, PRNP, SOX10,* and *EGFR*, which are known to promote malignant transformation, in the BL1 subtype would make it more likely that the BL1 cell lines are more resistant to eribulin than BL2 cell lines. Nevertheless, we detected no difference between the cell lines from these subtypes regarding their sensitivity to eribulin. Both types of basal-like TNBC tumors are proven to overexpress proliferation-activating genes and Ki-67, suggesting that this subtype of patients would preferentially respond to antimitotic agents such as taxanes and eribulin (18-20).

The IM TNBC cell line DU-4475 was less sensitive to eribulin treatment compared to all other cell lines. With regard to the gene expression of *YBX1*, DU-4475 cells reacted similarly to the HDQ-P1 and MDA-MB-231 TNBC cell lines. *YBX1* regulates multiple proliferative pathways, plays a role in invasion and metastasis, and promotes the escape of tumor cells from the immune system (21). Furthermore, *YBX1* is highly expressed in TNBC cell lines (12, 13) and knockdown of YBX1 significantly slowed the growth of TNBC cells (22), consistent with our observation in TNBC cell lines in response to eribulin treatment.

In conclusion, eribulin treatment strongly inhibited cell proliferation, migration and invasion of breast cancer cell lines in the sub-nanomolar range and activated apoptosis. No significant differences with regard to eribulin sensitivity were found within subtypes of TNBC or between TNBC and non-TNBC cell lines. Genes known to promote malignant

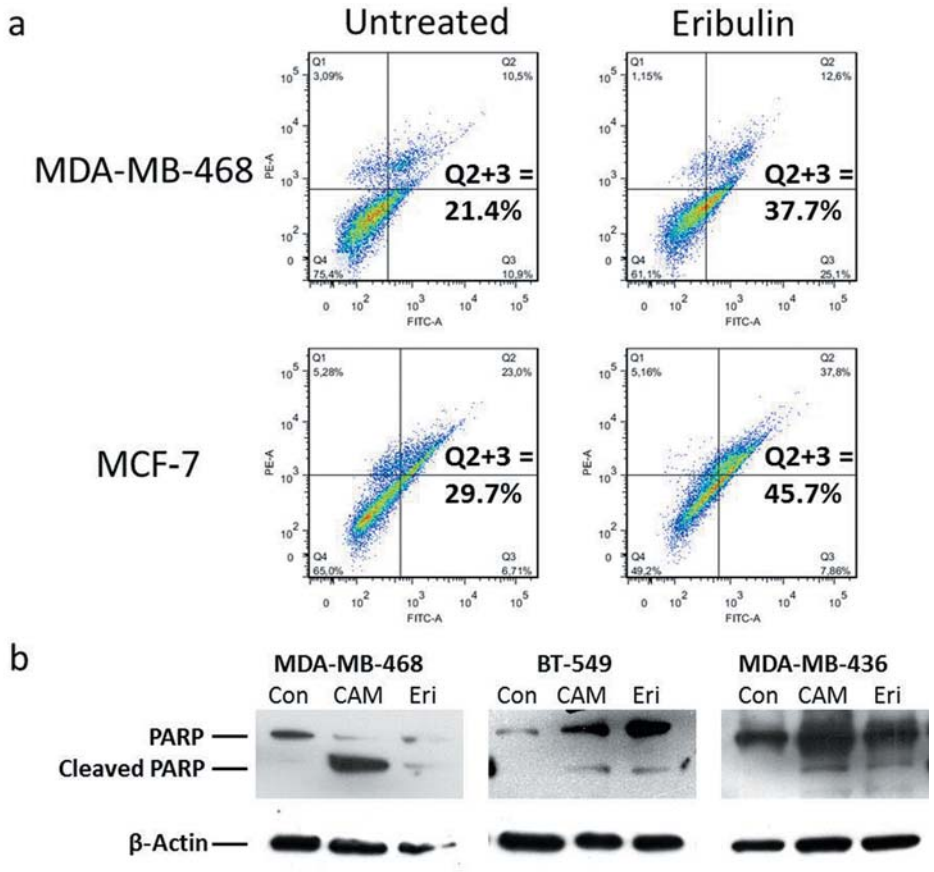

Figure 2. *Induction of apoptosis by eribulin treatment. a: Fluorescence-activated cell sorting (FACS) analysis in MDA-MB-468 triple-negative breast cancer (TNBC) and MCF-7 (non-TNBC) indicated increased apoptosis [early (Q2) and late (Q3)] after treatment with 10× the half-maximum inhibitory concentration (IC_{50}) of eribulin for 24 h. b: Poly (ADP-ribose) polymerase (PARP) cleavage in western blot analyses in TNBC cell lines validated apoptosis induction by eribulin (Eri) (IC_{50} for 24 h), Con: Untreated, CAM: camptothecin treated (positive control).*

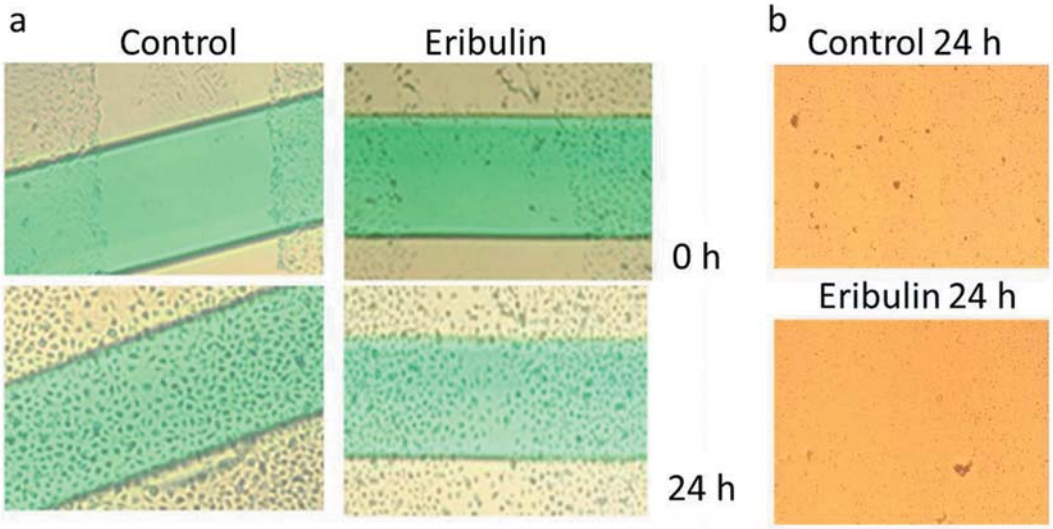

Figure 3. *Decrease of migration and invasion after eribulin treatment. a: A vertical scratch crossing the green line indicates the cell-free area. Cells migrating into the area were observed after 24 h. b: Invasion assays were performed with MDA-MB-231 cells seeded on Matrigel-coated Transwells in 24 well plates. After 24 h incubation, untreated cells and cells treated with the half-maximum inhibitory concentration (IC_{50}) of eribulin that had migrated to bottom surfaces of the membranes were visualized by staining with Giemsa solution.*

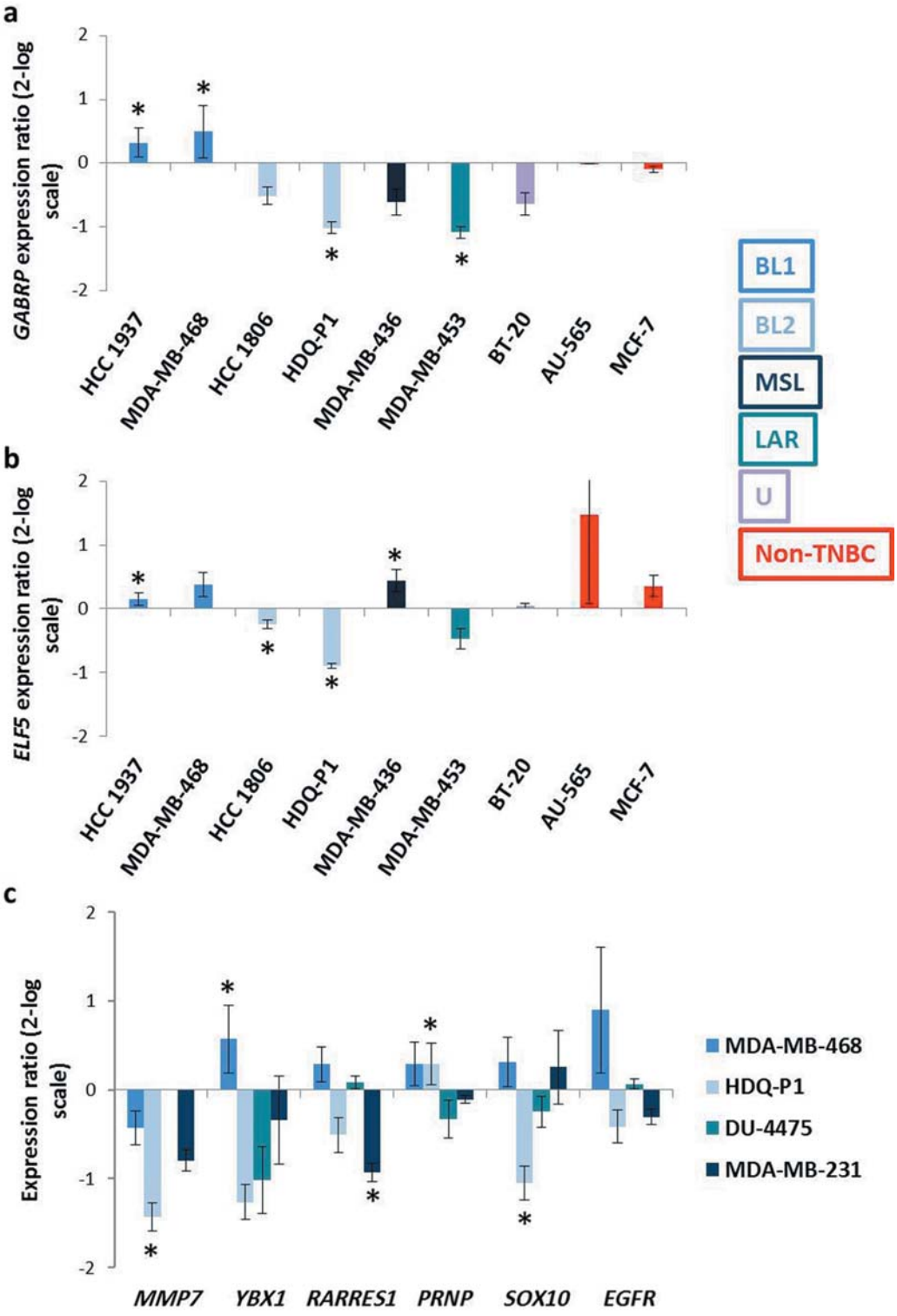

Figure 4. *Gene expression changes after treatment with eribulin. The expression of gamma-aminobutyric acid type A receptor pi subunit (GABRP) (a) and (E74-like ETS transcription factor 5) ELF5 (b) after treatment with eribulin at the half-maximum inhibitory concentration (IC_{50}) for 24 h compared to untreated controls in cell lines representing all triple-negative breast cancer (TNBC) subtypes and non-TNBC. c: The expression of tumor-related genes after eribulin treatment in four cell lines representing the TNBC subtypes basal-like 1 (BL1) (MDA-MB-468), BL2 (HDQ-P1), immunomodulatory (IM) (DU-4475) and mesenchymal stem-like (MSL) (MDA-MB-231). Expression ratios are shown as 2-log scale values. LAR: Luminal androgen receptor, U: unclassified. *Significantly different from the control at $p<0.05$.*

transformation were strongly deregulated after treatment with eribulin, with opposing effects between the cell lines representing BL1 and BL2 subtypes. Further investigation is needed with more cell lines of different TNBC subtypes to verify a diverse response to eribulin in the TNBC subtypes and in comparison to non-TNBC.

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