

Article

Effects of Eribulin on Epithelial–Mesenchymal Plasticity in Patient-Derived Breast Cancer Cultures and Excised Tissues

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Simple Summary

Eribulin is an approved therapy for the treatment of breast cancer which has been shown to reverse the epithelial-to-mesenchymal transition (EMT) and improve the efficacy of standard chemotherapies in cell lines, animal studies, and clinical specimens. Tumour EMT status has also been linked to eribulin efficacy. Based on this, we evaluated the effects of eribulin in patient-derived breast cancer cultures and a triple-negative breast cancer cell line to assess changes to EMT and therapy response. We further identified the induction of epithelial-like characteristics, including E-cadherin expression in a patient-derived HER2+ primary tissue with a predominantly mesenchymal phenotype following longitudinal eribulin exposure. Additionally, we compared EMT marker expression in breast cancers treated with standard-of-care neoadjuvant docetaxel, Adriamycin and cyclophosphamide (TAC) therapy with that observed in the neoadjuvant eribulin clinical trial.

Abstract

Background: The cytotoxic agent eribulin has been shown to promote EMT reversion, reduce treatment resistance, and potentially enhance responses to a range of therapeutic agents. **Methods:** We examined the effects of eribulin in patient-derived breast cancer tissues and a phenotypically heterogeneous breast cancer cell line to assess EMT and chemotherapy response. **Results:** Nanostring-based analysis of EMT-associated gene expression in breast cancers from patients receiving standard-of-care TAC neoadjuvant chemotherapy compared to a cohort receiving neoadjuvant eribulin (NeoEribulin trial) showed markedly reduced expression of EMT markers in cancers treated with eribulin versus TAC. Through single-cell immunofluorescent imaging and analysis, we identified that HCC38 triple-negative breast cancer (TNBC) cells exhibited a shift towards an epithelial-like state marked by E-cadherin upregulation following acute eribulin exposure, but not with other chemotherapeutic agents. Investigation of primary breast cancer cultures derived from pre-neoadjuvant biopsies also revealed that HER2-enriched primary breast cancer cells displayed heightened chemosensitisation to doxorubicin (Adriamycin),

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which was not recapitulated in TNBC cultures. Conclusions: Our data highlight the presence of contextual parameters which govern the degree of EMT regulation by eribulin.

Keywords: breast cancer; eribulin; EMT; chemoresistance

1. Introduction

The epithelial-to-mesenchymal transition (EMT) is a cellular program in which polarized epithelial cells lose defining characteristics such as a compact morphology and cell-to-cell adhesion to adopt mesenchymal-like properties such as enhanced motility and migratory capacity [1]. Transient activation of a partial, hybrid, or complete EMT is a major driver of cellular plasticity in cancer, contributing to key factors affecting mortality such as oncogenesis, the development of metastases, and treatment resistance—particularly to chemotherapies [2]. The ability to therapeutically modulate epithelial–mesenchymal plasticity (EMP) provides an exciting avenue to improve patient survival and clinical outcomes in a wide range of malignancies.

EMT may also hinder the success of immunotherapy by altering critical immune checkpoint molecules, tumour antigen processing and presentation [3], and expression of immunomodulatory factors [4,5]. In breast cancer, there is established evidence for the efficacy of immunotherapy in TNBC [6], but many tumours in other sub-types, particularly the most common luminal A sub-type, are immunologically cold with respect to current immunotherapies so that patients do not benefit. Therefore, engineered phenotypic shifts along the epithelial–mesenchymal axis could improve the success of cancer immunotherapy strategies. Eribulin has been shown to favourably influence immunosensitivity, including by increasing cytotoxic T-cells and reducing regulatory T-cells in breast cancer patients, changes that correlate with higher response rates [7], making it a suitable candidate for further immunomodulatory research.

Extensive data link tumour hypoxia to aggressive behaviour and enhanced dissemination [8]. Bevacizumab, a treatment that inhibits new blood vessel formation, was found to increase initial response rates to chemotherapy but failed to improve survival, likely due to the resulting tumour hypoxia produced and consequent accelerated progression [9]. Hypoxia is also a well-established driver of EMT, with vascular targeting by bevacizumab also inducing mesenchymal shift [10]. In contrast, eribulin has been shown to increase tumour vessel density and maturation with consequent improved perfusion. This can reverse EMT as well as promote delivery of other therapeutic agents [11]. Eribulin induced maturation of vascular pericytes and promoted vessel normalization in mouse models and eribulin-treated patients, accompanied by improved tumour immune infiltration and enhanced immunotherapy responses and survival in the mouse model [12].

Tumour stroma has been demonstrated to frequently contain fibroblasts with alternative phenotypic features to those in benign stroma, termed cancer-associated fibroblasts (CAFs). Extensive work has demonstrated that CAFs can substantially contribute to cancer progression [13]. Mechanistically, studies across multiple tumour types implicate CAFs in the induction of EMT within the tumour [14]. Again, eribulin has shown the ability to normalize the phenotype of CAFs, causing reversion to a benign stromal expression profile, simulating the impact of inhibition of the classical EMT-driving TGF- β pathway [15].

Eribulin is a potent cytotoxic agent approved for breast cancer and liposarcoma treatment [16,17]. It has been shown to reverse EMT, a process associated with tumour progression, metastasis, and therapy resistance [2,18], introducing the possibility that eribulin could have broader chemosensitising benefits to other agents beyond its direct cytotoxic action. Eribulin can reverse EMT in a broad range of pre-clinical contexts, including TGF β -

and chemotherapy-induced EMT in breast cancer cell lines in vitro and in vivo [19–21], as well as EMT caused by infection of immortalized oral keratinocytes with latent Kaposi's sarcoma-associated herpesvirus and human papillomavirus 31 [22]. In clinical studies, eribulin has also been shown to cause elevated epithelial:mesenchymal protein ratios in recurrent or metastatic breast cancers [23]. This contrasts with most other cytotoxic drug classes that induce EMT [24]. The reversal of EMT by eribulin in clinical trials has been associated with increased overall survival in comparison with other therapies [25], beyond that attributable to direct cytotoxicity and the initial disease control period.

The objective of our study is to assess the ability of eribulin to differentially modulate EMP and chemosensitivity in models which closely capture the underlying heterogeneity and clinical presentation of EMT in breast cancer. To accomplish this, we first analysed transcriptomic profiles of tumours from breast cancer patients who had received neoadjuvant eribulin versus neoadjuvant standard chemotherapy to assess differences in canonical epithelial versus mesenchymal gene expression. To resolve acute EMT responses on a cell-by-cell basis, we applied a single cell immunofluorescent imaging workflow to an inherently EMT-heterogeneous breast cancer cell line. We then employed patient-derived breast cancer cultures spanning clinical receptor types to test whether eribulin alters sensitivity to doxorubicin, a key chemotherapy within the standard TAC regime in clinically relevant tissues. Finally, longitudinal immunofluorescent profiling of EMT markers was performed in a HER2+ primary breast cancer culture to characterize temporal changes in EMT-associated proteins and cellular morphology.

Our findings support the ability of eribulin to exert a cytotoxic effect without the induction of EMT, compared to standard anthracycline and taxane cytotoxics, and show the potential for eribulin to suppress constitutive EMT and promote chemosensitisation in some, but not all, breast cancers. Understanding the contextual basis for this will have far-reaching implications for individualizing treatment regimens to enhance breast cancer therapy responses. It is important to understand that this response is not constitutive, and that identification of E/M state biomarkers will be required to guide usage and timing of companion treatments to allow translation.

2. Materials and Methods

2.1. Cell Line Culture

HCC38 cells (triple negative breast cancer cell line), known to exhibit EMP [26] were obtained from the ATCC and maintained in RPMI 1640 (Gibco, ThermoFisher Scientific, MA, USA) supplemented with 10% foetal bovine serum (Gibco, ThermoFisher Scientific, MA, USA) at 37 °C/5% CO₂. All cells were passaged, and their medium replenished every 48–72 h. Cells were routinely screened for mycoplasma using the Translational Research Institute's mycoplasma testing service. To observe and quantify acute changes to EMT following high-dose anti-neoplastic drug treatment, HCC38 cells were exposed to eribulin (1 nM, ERI), doxorubicin (1 µM, DOX), docetaxel (10 µM, DOC), paclitaxel (10 µM), 4-hydroperoxy-cyclophosphamide (50 µM), carboplatin (CPN, 10 µM), cisplatin (10 µM), olaparib (100 µM) or vehicle control (DMSO) for 24 h prior to immunofluorescent staining (Section 2.5) and single-cell image analysis (Section 2.6).

2.2. Patient-Derived Cultures and Treatment

Breast cancer patient-derived cultures were established with human ethics approval (ethics number HREC/2020/QRBW/61294) and informed consent, as previously described [27], with the exception that they were derived from biopsies taken prior to neoadjuvant chemotherapy (NACT). Tumour characteristics, clinical subtypes, and prior treatment status are provided in Table 1. For CBCa51, we also confirmed that estrogen receptor (ER)

expression was retained following long-term culture (Supplementary Figure S1). To determine changes to chemosensitivity following eribulin treatment, patient-derived cultures were subject to dose–response analysis (Section 2.4). To observe acute and sustained changes to EMT in patient culture CBCa50, longitudinal eribulin treatment was also performed for 0, 1, 3, 5 and 7 days prior to immunofluorescent staining (Section 2.5).

Table 1. Patient-derived cultures, clinical subtype and prior treatment status.

Case ID	Clinical Subtype	Prior Treatment
CBCa23	ER−/PR−/HER2−	None
CBCa41	ER−/PR−/HER2−	None
CBCa42	ER−/PR−/HER2−	None
CBCa50	ER−/PR−/HER2+	None
CBCa51	ER+/PR+/HER2+	None

2.3. Comparison of Gene Expression Changes Seen After Neoadjuvant Therapy

We compared gene expression changes from the NeoEribulin trial [28] in which locally advanced breast cancer patients received neoadjuvant eribulin (data kindly provided by SOLTI Breast Cancer Research Group, Barcelona, Spain) and our own cohort of 30 breast cancer patients treated with standard-of-care [29]. These patients were selected from the Medical Oncology Department database at a single institution, Royal Perth Hospital (RPH), Australia. They were all treated between 2001 and 2010 with neoadjuvant anthracycline and taxane and had been similarly consented at initial post-diagnostic assessment for tissue usage. The study was approved by the RPH Human Research Ethics Committee, approval number RGS0000001981 (HREC: 2013-130). Core biopsies and post-treatment surgical specimens were available on all 30 patients. The characteristics of this patient cohort are shown in Table 2. Pathologic complete response (pCR) was achieved in seven patients (23%).

We developed a 250-gene Nanostring assay comprising 130 markers from a range of EMT studies across diverse biological systems and 120 breast cancer contextual markers. This combined probe set was validated using RNA extracted from MDA-MB-468 cells stimulated for EMT by EGF or hypoxia [30]. Pre- and post-TAC NACT formalin-fixed and paraffin-embedded (FFPE) samples were biopsied for RNA extraction and Nanostring analysis as previously described [31,32]. Fifty of these genes profiled were represented in the NeoEribulin trial (SOLTI-1007, NCT01669252) and could be compared to neoadjuvant TAC within the current study. Statistical significance was determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with $Q = 5\%$ between fold change values calculated from each cohort. The complete statistical summary is available in Supplementary Table S1.

Table 2. Clinical characteristics of breast cancer cohort receiving standard chemotherapy.

	N	(%) *
Total	30	
Age		
<50	19	(68)
>50	9	(32)
Unknown	2	-
Histological subtype		
Invasive ductal	23	(82)
Invasive lobular	5	(18)
Unknown	2	-
Histological Grade		

Grade 1	3	(11)
Grade 2	9	(32)
Grade 3	16	(57)
Unknown	2	-
Hormone receptor		
Positive	15	(50)
Negative	15	(50)
HER2 Status		
Positive	9	(30)
Negative	21	(70)
Lymph vascular invasion		
Present	10	(45)
Absent	12	(55)
Unknown	8	-
Lymph Node Involvement		
Positive	13	(46)
Negative	15	(54)
Unknown	2	-

* Calculated from total with known status for variable.

2.4. Dose–Response Analysis

Dose–response analysis was performed on primary patient cultures using the CellTitreGlo ATP-based viability reagent (Promega, WI, USA). Cells were pre-treated for seven days with eribulin (0.5 nM) or vehicle control (DMSO). Eribulin and vehicle was also replenished after 72 h to account for drug degradation in culture. Cells were then exposed to a range of concentrations of doxorubicin, outlined in each relevant figure, for 72 h prior to endpoint using the CellTitreGlo assay. For TNBC cultures, this was also conducted with paclitaxel, docetaxel, and 4-hydroperoxy-cyclophosphamide, an active metabolite of cyclophosphamide, to compare differences in chemosensitivity profiles. Cell viability was then calculated from luminescent readings obtained from the ClarioStar Plus (BMG-labtech, Germany). Dose–response analysis and calculation of IC₅₀ values were determined with GraphPad Prism 10 using the [inhibitor] vs. normalized response (variable slope) non-linear fit model.

2.5. Immunofluorescence Microscopy

Patient-derived CBCa50 cells and the HCC38 cell line were seeded in 96-well optical imaging plates (CellVis P96-1.5H-N) and subject to the experimental conditions outlined in both Sections 2.1 and 2.2. At endpoint, medium was removed and the plate was washed twice with 200 µL PBS (and in-between each step). Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, PA, USA) at room temperature for 15 minutes and then permeabilized with 0.4% Triton X for 10 min (Sigma Aldrich, MA, USA). Cells were blocked in 5% (*w/v*) bovine serum albumin (BSA) for 1 h at room temperature prior to incubation with directly conjugated primary antibodies against: Vimentin (Santa Cruz Biotechnology, Germany; SC-6260); Pan-Cytokeratin (Invitrogen, ThermoFisher Scientific, MA, USA; 41-9003-82); or E-cadherin (BioLegend, CA, USA; 324112) in blocking buffer. Plates were imaged on the Operetta CLS system (Revvity, MA, USA) at the Translational Research Institute (Brisbane, Australia) Microscopy Facility.

2.6. HCC38 Single-Cell Imaging and Analysis

Single-cell imaging analysis was used to quantify EMT on a cell-by-cell basis and to identify cell populations perturbed by each investigated antineoplastic therapy. Images

acquired following immunofluorescent staining of HCC38 cells were subject to a custom image analysis workflow which involves cellular segmentation by Cellpose [33] and subsequent feature extraction of fluorescent signal and morphometric parameters with scikit-image [34]. The resultant feature matrix was processed by principal component analysis and subjected to non-linear dimension reduction by Uniform Manifold Approximation and Projection (UMAP). Unsupervised clustering was accomplished using the Leiden algorithm [35] and data visualization accomplished with the matplotlib and seaborn python libraries [36,37].

3. Results

3.1. Eribulin Is Associated with Muted EMT Induction Compared to Standard-of-Care Chemotherapy

To investigate the clinical relevance of (EMT) in patients treated with eribulin, we compared two Nanostring expression-based datasets derived from patient tumours that had undergone neoadjuvant docetaxel, Adriamycin, and cyclophosphamide (TAC) or neoadjuvant eribulin (NeoEribulin trial [28]). Tumours from patients treated with TAC (Table 2) showed a statistically significant upregulation of canonical EMT markers and transcription factors associated with a mesenchymal cell state, including *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2*, *VIM*, and *SNAIL*, when compared with those treated with eribulin (Figure 1, Supplementary Table S1). In contrast, *CDH1* (E-cadherin) was significantly downregulated following TAC treatment, indicating a loss of epithelial characteristics, whereas eribulin treatment was not associated with *CDH1* downregulation. Notably, other EMT-associated genes, *CAV1*, *CD44*, and *EGFR*, were also among the significantly upregulated genes following TAC treatment. Interestingly, both eribulin and TAC treatment were associated with increased expression of epithelial cytokeratins (*KRT17*, *KRT14*, *KRT5*, and *KRT6B*), as reflected by increased fold change, with no statistically significant difference between the two treatment groups. Collectively, these data indicate that, unlike standard chemotherapy, eribulin is not associated with substantial EMT activation and supports the possibility of an eribulin-mediated mesenchymal-to-epithelial transition (MET).

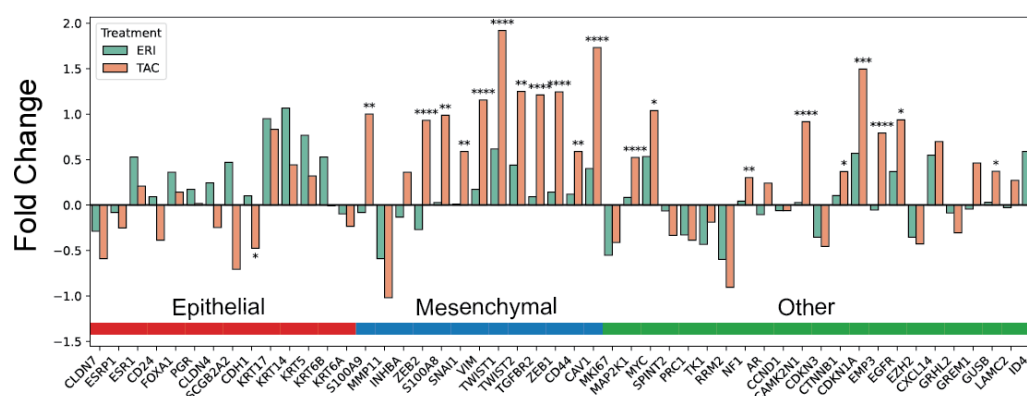


Figure 1. Eribulin is associated with a muted EMT compared to other anti-neoplastic agents. Nanostring-derived gene expression changes following neoadjuvant eribulin (ERI) compared to standard-of-care neoadjuvant docetaxel, Adriamycin and cyclophosphamide (TAC) in patient breast tumours. Statistical significance was determined using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with $Q = 5\%$ between fold change values calculated from each cohort, ns—not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Our team also previously explored whether certain treatments shown to stimulate either EMT or MET [24] correlated with a loss of initial treatment benefit in later disease

based on both pre-clinical (EMT) and clinical (survival data) evidence. Following on from this, we adapted this analysis to include several other cancers and clinical studies not previously shown (Supplementary Table S2). Collectively, therapies with evidence of EMT-inducing capabilities tend to benefit progression-free survival, while therapies with EMT-reverting (or MET-activating) capabilities tend to benefit overall survival (Figure 2). Notably, eribulin was associated with one of the largest progression-free-survival-to-overall-survival benefit ratios compared to other MET-driving therapies in both liposarcoma and, importantly, breast cancer. This supports the idea that EMT-inducing drugs tend to accelerate disease progression in cancers following their initial survival benefit, while EMT-repressing drugs tend to have an inverse relationship.

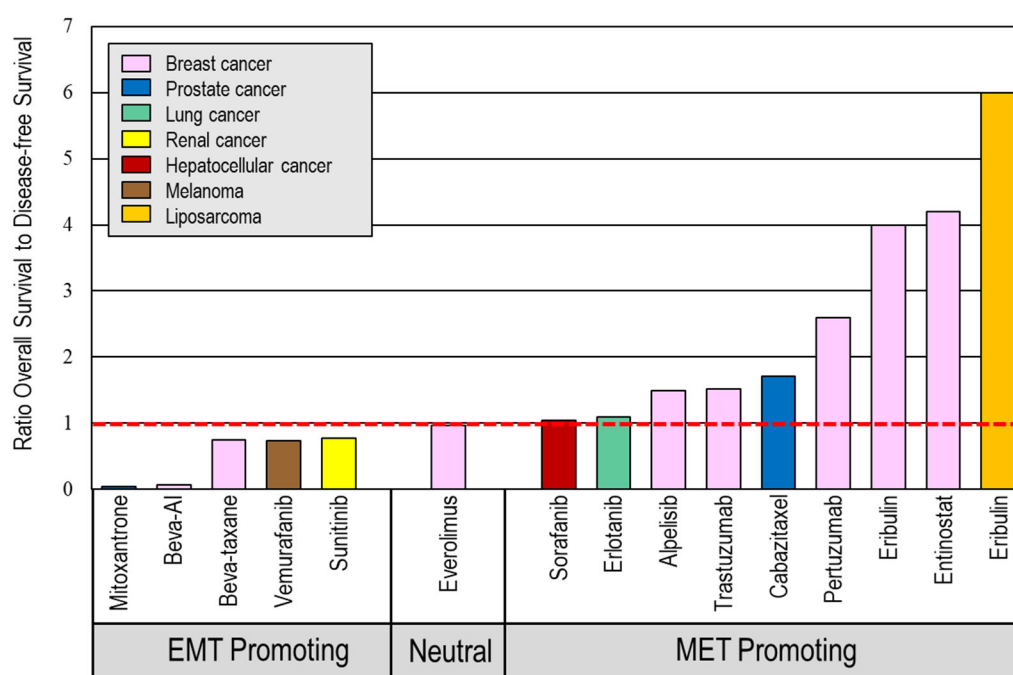


Figure 2. A comparison of overall survival and progression-free survival (disease control) benefits for a range of therapeutic agents in randomized clinical trials according to their impact on EMT. (Beva-bevacizumab, AI-aromatase inhibitor). A ratio below 1.0 indicates that the overall survival benefit is shorter than the increase in disease control period implying accelerated cancer progression after treatment completion. A ratio above 1.0 indicates extended survival beyond that of disease control implying an inhibition of cancer progression after treatment completion.

3.2. Single-Cell Immunofluorescent Imaging Reveals Altered EMT Cell Population Dynamics Following Acute Eribulin Exposure in TNBC

To investigate the short-term effects of eribulin (24 h) compared with other chemotherapies on EMT, HCC38 TNBC cells were exposed to a panel of antineoplastic agents, including eribulin. HCC38 cells were selected due to their intrinsic heterogeneity and the presence of dual cell populations occupying either epithelial or mesenchymal states, as defined by mixed expression of vimentin, pan-cytokeratin, and E-cadherin (Figure 3a). Quantitative, multiparametric single-cell segmentation was performed to extract EMT-related protein expression and morphometric features on a per-cell basis. Whereas most antineoplastic agents induced only minor perturbations in E-cadherin or vimentin expression, eribulin exposure resulted in increased E-cadherin expression within a distinct subpopulation of cells. Notably, a small subset of cells also exhibited increased vimentin signal following eribulin treatment (Figure 3b). Dimensionality reduction using UMAP of

the multiparametric imaging features followed by unsupervised clustering with the Leiden algorithm identified discrete cell populations across the dataset (Figure 3c). A distinct cluster (Cluster 8) was identified, that consisted almost exclusively of eribulin-exposed cells (Figure 3d). However, eribulin-treated cells were not solely confined to Cluster 8 and were also distributed across other clusters, indicating that only a subpopulation of eribulin-exposed cells exhibited this response. Further characterization of Cluster 8 revealed concomitantly high expression of both E-cadherin and vimentin relative to the primary cell cluster (Figure 3e,f).

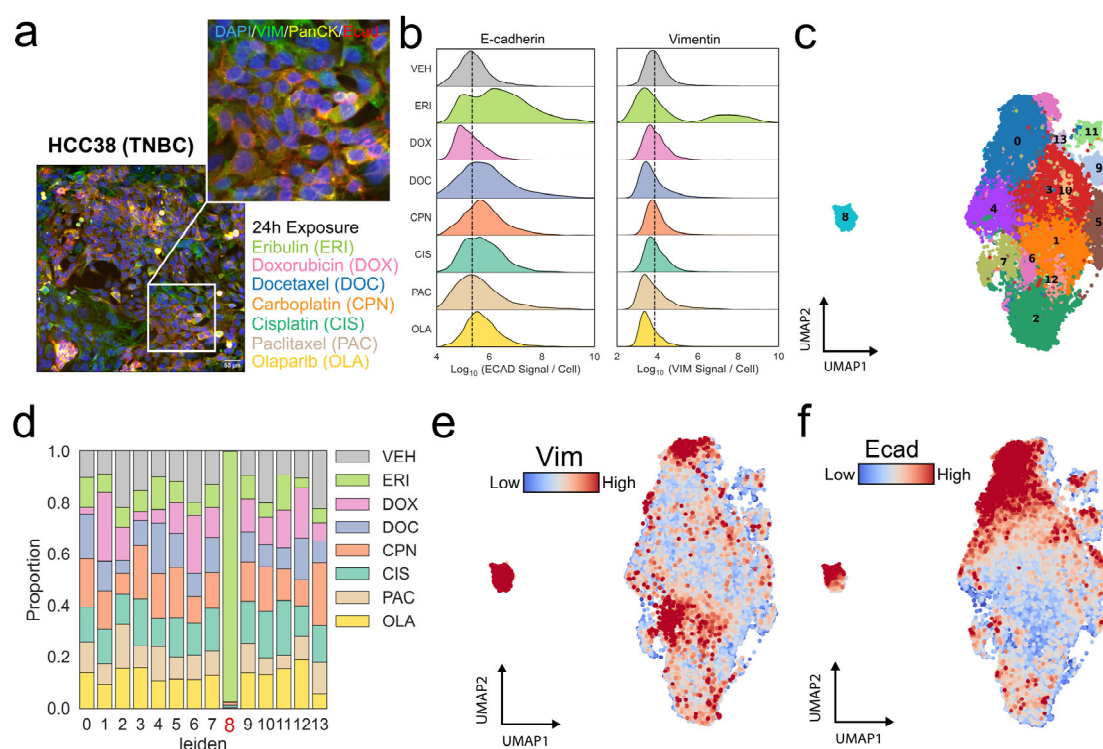


Figure 3. Immunofluorescence profiling and single-cell imaging reveals EMT perturbation following acute exposure to eribulin compared to standard chemotherapies in TNBC. (a) Immunofluorescence staining of vimentin (green), Pan-cytokeratin (yellow) and E-cadherin (red) in the HCC38 TNBC cell line. A magnified region of interest (white box) has been included to show underlying E/M heterogeneity and list of color-coded drugs denoted beneath, scale = 50 µm. (b) Smoothed density plot by kernel density estimation (KDE) of log-transformed immunofluorescent signals per cell of E-cadherin (left) and vimentin (right) across vehicle control (DMSO), standard chemotherapies and olaparib. The dashed-line indicates mean of vehicle control (VEH). (c) Dimension reduction and by UMAP and subsequent Leiden-based unsupervised clustering of HCC38 cells ($n = 27,241$). Cluster identity is overlaid on top of each computed cluster (0–13). (d) Proportion of cells in each cluster annotated by drug which they were exposed to, Cluster 8 (red) predominantly consists of cells treated with Eribulin. Log-transformed immunofluorescent signal of (e) Vimentin (Vim) and (f) E-Cadherin (Ecad) overlaid on previously computed UMAP embeddings.

Eribulin has been previously demonstrated to chemosensitise breast tumours, most likely attributed to its established ability to revert EMT [19,38]. We established multiple primary breast cancer cultures derived from patient biopsies taken prior to neoadjuvant chemotherapy to investigate the effect of eribulin on doxorubicin (Adriamycin) sensitivity, a key therapy employed in standard high-risk adjuvant therapy regimen. Firstly, we compared three primary TNBC, and two HER2-enriched cultures (one triple-positive and one HER2+ only) for stand-alone eribulin sensitivity using a standardized dose (0.5 nM).

We found that while TNBC cultures (CBCa23, 41, 42) and the HER2+ culture (CBCa50) shared similar sensitivity to eribulin exposure, the triple-positive case (CBCa51) was less sensitive, with no significant differences to the vehicle control (Figure 4a). It should also be noted that while CBCa51 had the greatest mean response to eribulin, it was only significantly different from one of the TNBC cases (CBCa41, Supplementary Figure S2). In the same experiment, we exposed each culture to seven days of eribulin (0.5 nM) or vehicle, and then compared their resultant half-maximal inhibitory concentrations (IC_{50}) to doxorubicin. This analysis revealed that the non-TNBC cultures displayed heightened sensitivity to doxorubicin, particularly the ER-/PR-/HER2+ CBCa50 line, while the TNBC cases displayed no change or a minimal reduction in chemoresistance (Figure 4b–f). While no significant changes to chemosensitivity were observed in the TNBC cases, we also compared chemosensitivity profiles of these cases against doxorubicin (DOX), docetaxel (DOC), paclitaxel (PAC) and 4-hydroperoxy-cyclophosphamide (CPA, active mimetic). While eribulin sensitivity was previously identified to be largely conserved amongst all three cultures, unique chemosensitivity profiles were uncovered with respect to other agents for each case (Figure 4g).

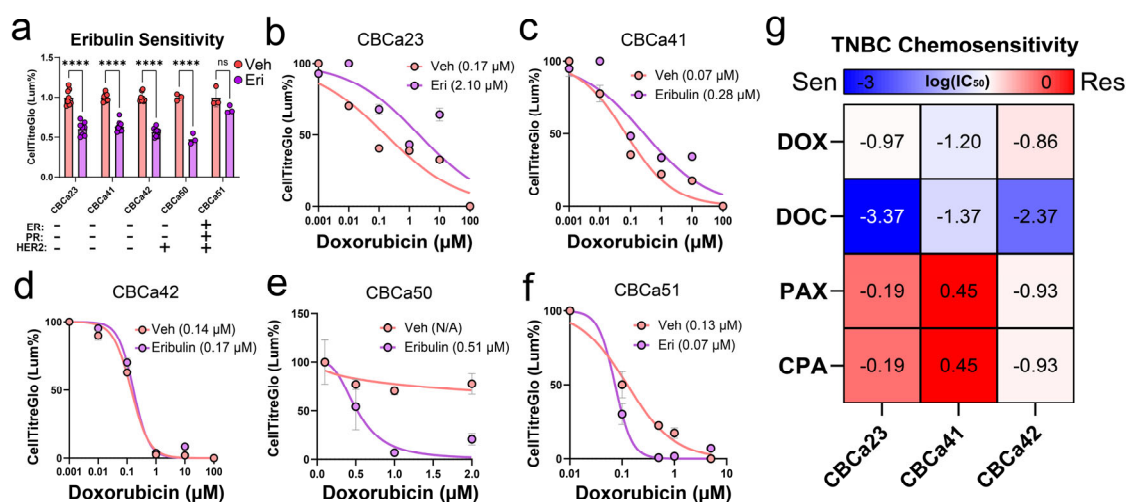


Figure 4. Eribulin and chemosensitivity profiles of primary breast cancer cultures. (a) Comparison of patient-derived primary culture sensitivity to eribulin at 0.5 nM determined by ATP-based viability (CellTiterGlo), technical replicates range between 3 and 8 for each sample due to variable amount of material obtainable from primary samples. (b–f) ATP-based viability (CellTiterGlo) dose–response profiles of CBCa23, 41, 42 (ER-/PR-/HER2-), CBCa50 (ER-/PR-/HER2+) and CBCa51 (ER+/PR+/HER2+) to doxorubicin following 7 days of pre-exposure to vehicle control (Veh) or 0.5 nM eribulin mesylate (Eri). Half-maximal inhibitory concentrations $\log_{10}(IC_{50})$ are shown adjacent to the figure legend text in brackets. (g) Heatmap detailing differences in $\log_{10}(IC_{50})$ derived chemosensitivity profiles of TNBC primary cells against doxorubicin (DOX), docetaxel (DOC), paclitaxel (PAX) and 4-hydroperoxy cyclophosphamide (CPA). Statistical significance determined by two-way ANOVA with Sidak’s multiple comparisons test, ns—not significant, **** $p < 0.0001$.

Given our observation of chemosensitisation of HER2+ primary breast cancer cells (CBCa50) to doxorubicin, we next investigated whether longitudinal exposure to eribulin was associated with alterations in EMP. Immunofluorescence analysis of untreated CBCa50 cells demonstrated a pronounced mesenchymal phenotype at baseline, characterized by an elongated cellular morphology and minimal E-cadherin expression (Figure 5). Following 24 h of eribulin exposure, marked morphological changes were observed, including a transition from an elongated, mesenchymal-like morphology to a more cuboidal, epithelial-like appearance. Consistent with these morphological alterations, E-

cadherin expression became detectable after 24 h of treatment and was maintained at 3, 5, and 7 days of continuous exposure. In contrast, vimentin expression was not reduced over the course of the experiment. However, a clear change in vimentin subcellular organization was evident, with a shift from a structured, aligned filamentous pattern in untreated cells to a more disrupted and dispersed distribution following eribulin treatment.

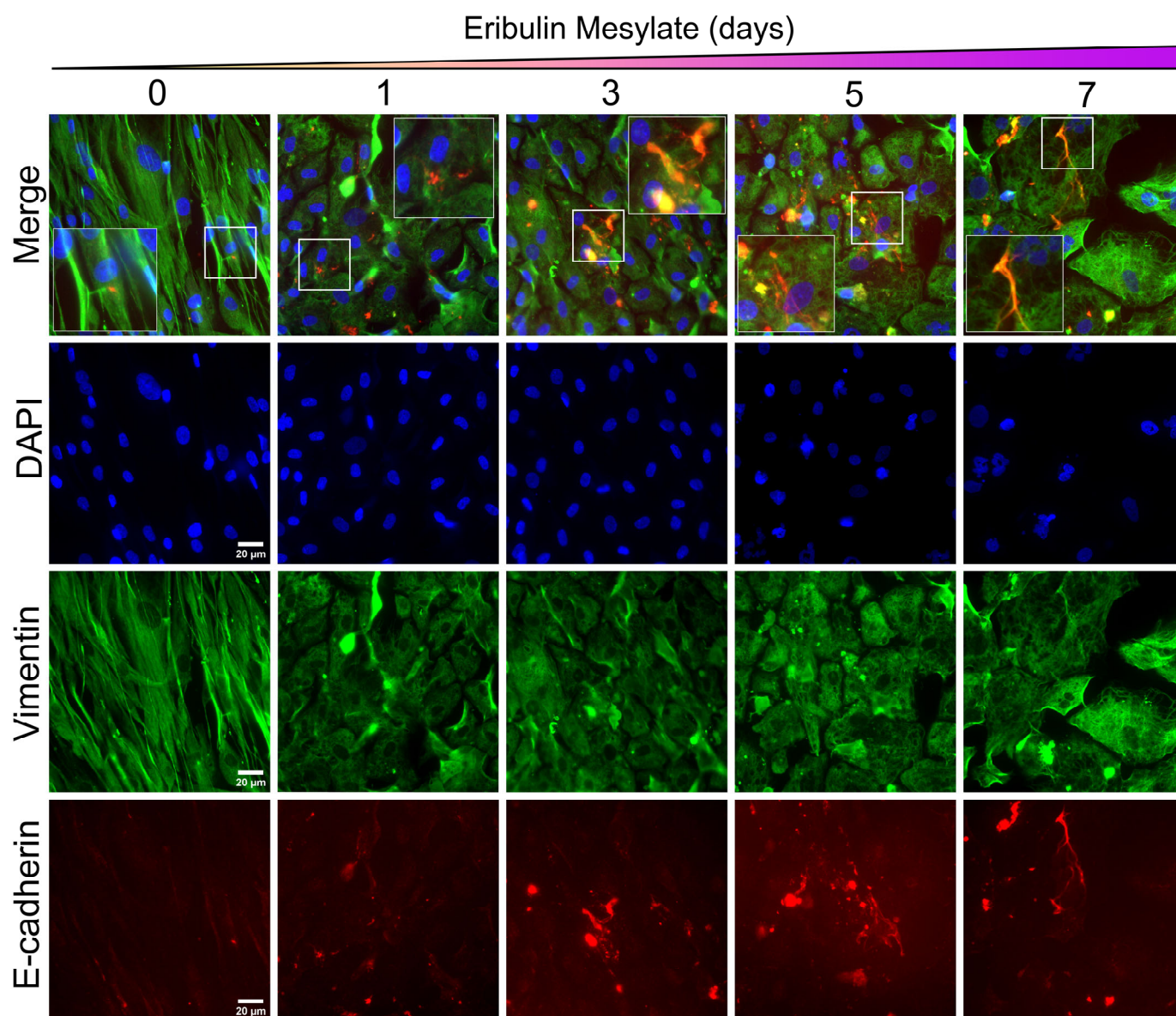


Figure 5. Eribulin induces E-cadherin expression and disrupts intermediate filament organization in primary HER2+ breast cancer cells. Immunofluorescent staining of vimentin (green) and E-cadherin (red) in primary patient-derived breast cancer cells (Case #CBCa50, ER-/PR-/HER2+) following 1, 3, 5 and 7 days of exposure to eribulin (0.5 nM), compared to vehicle control (DMSO, day 0). Induction of E-cadherin expression is observable as early as day 1 and remains sustained throughout the treatment period. Magnified region of interest indicated by white box, scale = 20 μ m.

CD104 (integrin β 4; ITGB4) has been identified as a marker of hybrid epithelial–mesenchymal (hybrid E/M) cell populations and has been associated with cancer cell stem-like behaviour [39,40]. Baseline immunofluorescence analysis revealed moderate CD104 expression in CBCa50 cells, consistent with a partial EMT or hybrid-E/M phenotype, in agreement with our prior observations of high vimentin and low E-cadherin expression.

Using the same experimental conditions described above, CBCa50 cells exhibited a marked reduction in CD104 expression following 24 h of eribulin exposure. This loss of CD104 was maintained with continued treatment at 3, 5, and 7 days (Figure 6). Notably, the reduction in CD104 coincided temporally with the emergence of epithelial-like morphological features and induction of E-cadherin expression, suggesting a coordinated shift in epithelial–mesenchymal marker expression following eribulin treatment.

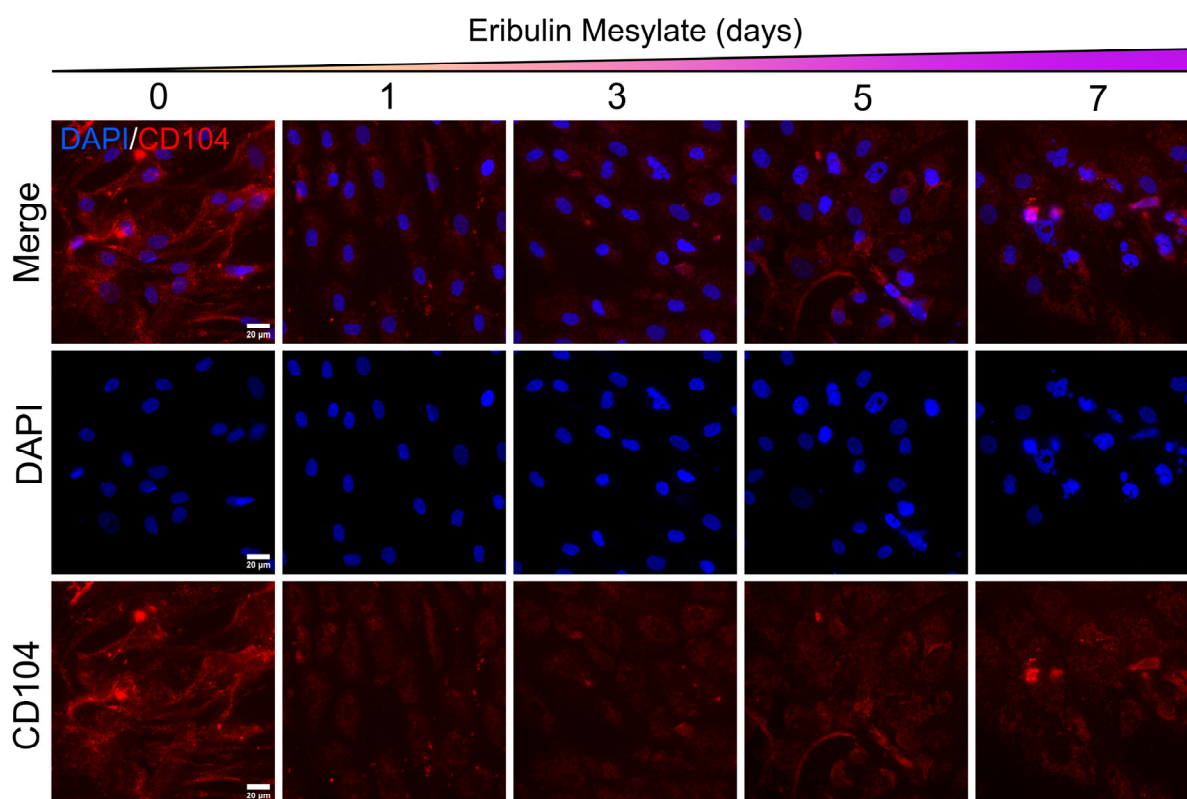


Figure 6. Eribulin reduces CD104 (ITGB4) expression in primary HER2+ breast cancer cells. Immunofluorescent staining of CD104 (ITGB4, red) in primary patient-derived breast cancer cells (Case #CBCa50, ER-/PR-/HER2+) following 1, 3, 5 and 7 days of exposure with eribulin (0.5 nM), compared to vehicle control (DMSO, day 0). Reduction in CD104 expression is observable as early as day 1 and remains sustained throughout the treatment duration. Scale = 20 μ m.

4. Discussion

There are several studies showing the effects of eribulin, primarily in its ability to induce epithelial phenotypes [2,19,38]. In our study, we highlight a reduction in EMT-related features across clinical specimens and cell lines. We saw evidence of this in the HCC38 TNBC cell line, which exhibited significant *de novo* epithelial–mesenchymal plasticity [26], as evident in Figure 3a, and responded strongly both morphologically and in epithelial gene expression to eribulin. Similarly, the HER2+ CBCa50 patient-derived culture showed a prominent increase in E-cadherin expression and adopted cobblestone cell morphology (Figure 5). Although the levels of expression of vimentin remained high, the protein lost filamentous form, which would be expected to affect the functionality or regulation of the protein in migration and invasion, important mechanisms during EMT [41–43].

Furthermore, Bagheri and colleagues observed a consistent reduction in the EMT score in breast tumours after neoadjuvant treatment with eribulin compared to TAC. This was based on the multiplexed immunohistochemistry for Snail1, Zeb1, Keratin-8, Keratin-14, vimentin and E-cadherin, compared to the parallel study of TAC NACT where the EMT score remained high [38]. The attenuation of EMT features was largely due to a

decrease in cells expressing vimentin and an increase in cells expressing Keratin-8 (an established luminal phenotype marker) or Keratin-8 plus E-cadherin, rather than proportional changes in E/M-hybrid phenotype of cellular expressing E-cadherin and vimentin. The SOLITI group that undertook the NeoEribulin trial also reported clinical shifts from basal to normal-like subtypes, which would be consistent with reduced EMT, importantly, in addition to the shift from luminal-B to luminal-A also reported in their studies [28].

These phenotypic changes may underpin the improved clinical benefit of eribulin, evidenced by the prolongation of overall survival to a greater extent than the progression-free survival period seen on treatment (Figure 2) [44,45]. A reduction in new metastases has been observed, rather than effects on established metastases [25], consistent with an EMT-reversal action suppressing metastatic spread. As shown above (Figure 2), other cancer therapies that categorically reverse EMT (i.e., cause MET, e.g., eribulin, cetuximab and entinostat) also show extended survival benefits in clinical trials beyond on-treatment disease control periods. This contrasts with the majority of current chemotherapies, hormonal therapies, and targeted agents that cause EMT [24], where overall survival benefits are often disappointingly short despite initially encouraging on-treatment control periods, suggestive of accelerated progression following treatment cessation.

It is interesting to consider the observations seen clinically—that mesenchymal positioning on the EMT axis has been associated with better eribulin response. Mesenchymal differentiation of the primary tumour, indicated by reduced E-cadherin or increased vimentin immunostaining, was associated with eribulin response in metastatic breast cancer cases [46]. Chan et al., in 2024, further showed that ZEB1 overexpression in metastatic samples had shorter times to recurrence, while high expression levels for SNAIL1 or TWIST1 in the primary BC were associated with significantly longer survival in patients who received eribulin [47]. They also showed that high vimentin levels were associated with a clinically relevant trend toward shorter survival after CDK4/6 inhibition. Although we did not assess this quantitatively, we did see rather consistent potent responses of our different patient-derived cultures to eribulin at 0.5 nM (established as typical IC_{50}), with the exception of CBCa51 (Figure 4f). Importantly, sublethal concentrations of eribulin in CBCa51 were sufficient to chemosensitise them to doxorubicin.

Although we saw appreciable chemosensitisation after eribulin pre-treatment in CBCa50 and CBCa51 cells, this was not universally seen in the other patient-derived cultures. It was somewhat surprising that the chemosensitisation by eribulin was evident in the ER and/or HER2 positive lines compared to the TNBC samples CBCa23, CBCa41 and CBCa42. Enhancement of therapy response to other agents has been seen with in vitro synergism between eribulin and 5-fluoruracil (5-FU) in TNBC models in vitro and in vivo [20,48], and in mouse (PB3) and human (MDA-MB-231) mammary cancer cells [38]. Eribulin also sensitized oral squamous carcinoma cells to cetuximab therapy via induction of MET [21]. Similarly, the combination of the PI3K inhibitor alpelisib with eribulin more effectively suppressed the growth of paclitaxel-resistant cells in vitro and in vivo than alpelisib alone [49]. In a pre-clinical model of ovarian carcinosarcoma, where the sarcomatous component is characterized by EMT [50], eribulin produced more potent growth inhibition than standard-of-care platinum-based chemotherapy and caused reduced mesenchymal characteristics in patient-derived xenografts. An important consideration is the nature of our patient-derived cultures being treatment-naïve (pre NACT). Even prior to the current advent of the Keynote 522 regimen of Pembrolizumab in addition to TAC [51], the TAC regimen provided important clinical benefits. In particular, chemo-naïve TNBCs in the clinic are initially highly responsive to doxorubicin, and this was reflected in the strong responses seen amongst the patient-derived cultures. It is possible that chemosensitisation was not seen in the TNBC cultures because they are already exquisitely sensitive to doxorubicin. Further studies will seek to treat the cells initially with doxorubicin and

then treat with eribulin versus vehicle after the onset of doxorubicin resistance, followed by re-challenge.

On the other hand, there is potential for EMT to be associated with resistance to HER2-targeted therapies, which may be reflected in the strong partial or E/M-hybrid phenotype present in the HER2+ CBCa50 cells and chemosensitisation seen with both CBCa50, and minimally in the triple positive sample (ER+/PR+/HER2+) CBCa51. Little has been reported on EMT in HER2-positive breast cancer, however EMT has been seen in trastuzumab resistance [52–54]. Furthermore, HER2 has also been shown to induce TGF β signalling and subsequently promote EMT, which may explain the eribulin-induced chemosensitisation and epithelial reprogramming observed in the study [55]. Regarding eribulin response, in the SOLTI NeoEribulin trial which was conducted in HER2-negative breast cancers, patients with a HER2-enriched molecular profile appeared to benefit the most from eribulin [28]. Further work on HER2+ breast cancer cultures may provide some explanation.

5. Conclusions

In summary, our findings indicate that eribulin can induce clinically relevant phenotypic changes by promoting the acquisition of epithelial features and reducing mesenchymal characteristics across breast cancer patient samples and cell lines, which may limit metastatic dissemination and create a tumour state more sensitive to subsequent therapies. The capacity for tumour cells to respond to eribulin appears to be dependent on their epithelial–mesenchymal status and breast cancer subtype, with more evident effects in HER2-positive and hormone-receptor-positive patient-derived cultures in the present study. These findings support the clinical relevance of incorporating EMT biomarkers in tumour profiling to identify patients who can benefit from eribulin and other treatments that target the EMT/MET processes.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/cancers18040598/s1>, Figure S1: Evidence of Estrogen Receptor and Proliferative Marker Ki67 Expression in CBCa51 Triple-Positive Breast Cancer Primary Cell Culture; Figure S2: Statistical Comparison of Primary Patient-Derived Cell Responses to Eribulin Treatment Denoted by Subtype; Table S1: Statistical Summary of Nanostring Gene Expression Analysis; Table S2: A comparison of overall survival and progression-free survival (disease control) benefits for a range of therapeutic agents in randomized clinical trials according to their impact on EMT [20,30,45,56–84].

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Metro North Health and Health Services and Queensland University of Technology for studies involving humans (HREC/2020/QRBW/61294, QUT Project ID: #3179, Approved: 5th December 2024).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data associated with this study is included within both the manuscript and its Supplementary Materials or was uploaded during submission. Any additional data is available from the corresponding author upon reasonable request.

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