Tepoxalin increases chemotherapy efficacy in drug-resistant breast cancer cells overexpressing the multidrug transporter gene ABCB1

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ABSTRACT

Effective cancer chemotherapy treatment requires both therapy delivery and retention by malignant cells. Cancer cell overexpression of the multidrug transmembrane transporter gene ABCB1 (MDR1, multi-drug resistance protein 1) thwarts therapy retention, leading to a drug-resistant phenotype. We explored the phenotypic impact of ABCB1 overexpression in normal human mammary epithelial cells (HMECs) via acute adenoviral delivery and in breast cancer cell lines with stable integration of inducible ABCB1 expression. One hundred sixty-two genes were differentially expressed between ABCB1-expressing and GFP-expressing HMECs, including the gene encoding the cyclooxygenase-2 protein, PTGS2. Several breast cancer cell lines with inducible ABCB1 expression demonstrated sensitivity to the 5-lipoxygenase, cyclooxygenase-1/2 inhibitor tepoxalin in two-dimensional drug response assays, and combination treatment of tepoxalin either with chemotherapies or with histone deacetylase (HDAC) inhibitors improved therapeutic efficacy in these lines. Moreover, selection for the ABCB1-expressing cell population was reduced in three-dimensional co-cultures of ABCB1-expressing and GFP-expressing cells when chemotherapy was given in combination with tepoxalin. Further study is warranted to ascertain the clinical potential of tepoxalin, an FDA-approved therapeutic for use in domesticated mammals, to restore chemotherapy concentrations in cancer cells overexpressing this harmful xenobiotics and toxic metabolites [9]. Conversely, MDR1 expression has been identified in cancer cells previously exposed to MDR1 substrate chemotherapies [10–12], in which its efflux activity reduces the intracellular drug concentration and the drugs’ cytotoxic effects.

Various attempts to mitigate the chemotherapy efflux activity of MDR1 remain largely unsuccessful. One common approach is to block MDR1 efflux activity with small molecule inhibitors while simultaneously treating with typical chemotherapy regimens, thereby allowing the chemotherapy concentrations in cancer cells overexpressing this transporter to achieve levels comparable to those in cells lacking MDR1 expression [13]. Such efforts to target MDR1 with small molecule inhibitors have long proven successful at increasing intracellular chemotherapy concentrations and cancer cell cytotoxicity in vitro [14–16] but this success has not translated into the clinic. Early clinical trials of MDR1 inhibitors were plagued by poor potency and high toxicity [13], while later clinical trials used more specific MDR1 inhibitors but failed to demonstrate improved clinical response in patients or to solve toxicity

Introduction

Breast cancer is the second-leading cause of cancer-related death among women in the United States [1]. Chemotherapy resistance impedes the successful treatment of breast cancer and can be driven by several mechanisms, including overexpression of genes from the ATP-binding cassette (ABC) transporter family [2]. Genes from this family commonly found overexpressed in treatment-resistant cancers include ABG2, ABC1, and ABCB1, which, respectively, encode the BCRP, MRP1, and MDR1 proteins [3,4].

Multidrug resistance protein 1 (MDR1) is an ATP-dependent transmembrane efflux transporter remarkable in its capacity to transport a wide variety of substrate classes including endogenous substrates such as amyloid-beta peptide, vitamins D and K, and exogenous substrates such as commonly prescribed chemotherapeutic drugs from the vinca alkaloid, taxane, and anthracycline drug classes [5–8]. In the absence of disease, expression of MDR1 is restricted to organs such as the kidney, liver, brain, and digestive tract, where it functions to efflux potentially

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complications due to targeting MDR1 in normal tissues [17,18]. Thus, the current lack of clinically approved MDR1 inhibitors warrants exploration of additional strategies for reversal of the MDR1-driven drug-resistance phenotype [19]. One such approach, employed here, is to study MDR1 overexpression biology to identify acquired sensitivities to other compounds not related to MDR1 inhibitors. Similar approaches have identified that ATP consumption by the MDR1 transporter leads to an energetic demand in multidrug resistant cells and thus could present a targetable vulnerability, i.e., to metabolic inhibitors [20,21].

To date, the xenobiotic transport function of MDR1 has been extensively studied in various cancer types; however, its ability to influence cellular phenotype and gene expression beyond drug transport in breast cancer has remained relatively unexplored. We previously identified strong promoter fusions driving increased ABCB1 expression in treatment-resistant metastatic breast cancer cells isolated from malignant pleural effusion and ascites samples from two of four patients studied, both with ER+/HER2+ breast cancer [11]. Our follow up investigation identified ABCB1 transcriptional fusions in samples from 9 of 33 breast cancer patients studied [12]. In the present study, we investigate the transcriptional consequences of ABCB1 overexpression in normal human breast cells, hypothesizing that changes in ABCB1 expression extend beyond drug metabolism to other cellular pathways and processes that can influence malignant cell signaling. Further, we examine the phenotypic effects of ABCB1 overexpression in various breast cancer cell lines to identify therapies capable of reversing the MDR1-driven multidrug resistance phenotype, and find that the dual 5-lipoxygenase (5-LOX)/cyclooxygenase-1/2 (COX-1/2) inhibitor tepoxalin increases drug response to chemotherapeutics and to histone deacetylase (HDAC) inhibitors in ABCB1-overexpressing breast cancer cells, suggesting it may be a viable candidate for improving treatment response for patients with multi-drug resistant breast cancer.

### Materials and methods

#### Human mammary epithelial cell sample collection

Human mammary epithelial cells (HMECs) were obtained from a noncancer-related breast reduction surgery at the University of Utah and processed as described previously [22]. Protocols were approved by the University of Utah Institutional Review Board. Informed consent was obtained from all patients.

#### Cell culture

Breast cancer cell lines were cultured in RPMI-1640 (Life Technologies, Carlsbad, CA, USA) + 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA). Cells were regularly monitored for mycoplasma contamination using MycoAlert kit (Lonza, Walkersville, MD, USA). Cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and authenticated using the STR profiling of 33 breast cancer patients studied [12]. In the present study, we investigated transcriptional consequences of ABCB1 overexpression in normal human breast cells, hypothesizing that changes in ABCB1 expression extend beyond drug metabolism to other cellular pathways and processes that can influence malignant cell signaling. Further, we examine the phenotypic effects of ABCB1 overexpression in various breast cancer cell lines to identify therapies capable of reversing the MDR1-driven multidrug resistance phenotype, and find that the dual 5-lipoxygenase (5-LOX)/cyclooxygenase-1/2 (COX-1/2) inhibitor tepoxalin increases drug response to chemotherapeutics and to histone deacetylase (HDAC) inhibitors in ABCB1-overexpressing breast cancer cells, suggesting it may be a viable candidate for improving treatment response for patients with multi-drug resistant breast cancer.

#### Adenoviral infection

HMECs were starved in basal MEBM lacking SingleQuot supplements for 36 hours, followed by infection with either GFP (gift from University of Kentucky) or ABCB1 (Applied Biological Materials, Inc., Vancouver, BC, CA) adenoviruses in quadruplicate biological replicates at 50 MOI for 12 hours, at which time RNA and protein were extracted. Adenoviruses were titrated using QuickTiter Immunoassay kit (Cell BioLabs, San Diego, CA, USA) according to manufacturer’s protocol.

### Generation of ABCB1-inducible cell lines

Custom plasmids encoding either eGFP or ABCB1 (transcript NM_000927.4) under the tetracycline-inducible TRE3G promoter and a plasmid encoding the activating rtTA analog Tet3G (cat. #V8180123-101BSq) were purchased from VectorBuilder (Santa Clara, CA, USA). Three separate lentiviruses each containing one of these plasmids were generated by transfection of equal amounts of plasmid containing gene of interest along with three third-generation lentivirus gene plasmids (cat. #12251, #12253, #8454, Addgene, Cambridge, MA, USA) into HEK293T cells using Lipofectamine 3000 reagent (ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer’s protocol. After 2 days, lentivirus was harvested and filtered through a 0.45 µm PES filter, at which time it was applied to breast cancer cell lines with the addition of 10 µg/mL polybrene. Three days following infection, cell lines were selected with either puromycin (TRE3G/GFP and TRE3G:ABCB1 lentiviruses) or hygromycin B (Tet3G lentivirus) at a previously-established selection dose per cell line such that after 72 hours of selection, a flask of non-transduced cells were 100% dead and successfully transduced cells remained alive. Cell lines were infected and selected sequentially, first with either TRE3G/GFP or TRE3G:ABCB1 lentivirus, then with Tet3G lentivirus. For induction of gene expression, doxycycline (Sigma) was applied at 1 µg/mL. For 3D co-culture experiments, cell lines with stable integration of TRE3G:ABCB1 were labeled with constitutively expressed mCherry and cell lines with stable integration of TRE3G:GFP were labeled with constitutively expressed cerulean using lentivirus generated as described above with either the LeGo-C2 (Addgene #27339) or LeGo-Cer2 (Addgene #27338) plasmids. Fluorescence-activated cell sorting was used to positively select a labeled cell population.

### RNA and protein extraction

RNA was extracted from HMECs and breast cancer cell lines using RNeasy Plus mini kit with the addition of an on-column DNase I digestion (Qiagen, Germantown, MD, USA) according to manufacturer’s protocol. For protein extraction from HMECs and breast cancer cell lines, cells were washed once in PBS, then lysed on ice in lysis buffer consisting of 5 mM EDTA, 150 mM NaCl, 50 mM Tris, 1% Triton X-100, 0.1% SDS for 15 min. For protein extracted from HMECs, 6X loading buffer with 2-mercaptoethanol (2ME) was added to protein lysate from 200,000 cells per lane and protein was electrophoresed and transferred to membrane as described previously [22] to validate overexpression of MDR1. Blots were incubated with B-actin (Cell Signaling Technologies (CST), Danvers, MA, USA, #3700S) and MDR1 (CST #13342S) antibodies. For protein extracted from breast cancer cell lines, 6X loading buffer with 2ME was added to 20 µg protein per lane and blots were incubated with B-actin antibody as above and MDR1 antibody (CST #13978S).

### RNA sequencing

Transcriptional changes in HMECs infected with either GFP or ABCB1 adenoviruses were captured by RNA sequencing at Fulgent Genetics (Temple City, CA, USA). The cDNA libraries were generated using NEBNext ultra directional RNA prep kit (New England BioLabs, Ipswich, MA, USA) and sequenced using an Illumina NovaSeq 6000 instrument with 150 cycles of paired end reads. Thirty million reads were allotted per sample.

RNA-seq data was processed with the BETSYS system [23]. Briefly, paired-end reads were analyzed with trimmomatic (v0.38) to trim sequencing adapters and low-quality bases. Trimmed reads were aligned to the human reference genome (hg19) with STAR (v2.2.1). Transcript abundance quantified as counts per gene and transcripts per million (TPM) were calculated using HTseq (v0.10.0) and RSEM (v1.3.1),...
respectively. Differential gene expression analysis was performed with DESeq2 (v1.22.2) [24]. Only those genes with adjusted p-value <0.05 and |fold change| > 2 were identified as differentially expressed (DE) genes. Heatmap of DE genes was generated with custom python scripts. Differentially expressed genes were assessed for pathway enrichment for Oncogenic Signatures and Hallmarks (Collections 6 and H) in the Molecular Signatures Database, version 6.2 available at http://software.broadinstitute.org/gsea/msigdb/index.jsp using Gene Set Enrichment Analysis (GSEA, v2.0.4) [25,26]. Pathways were considered significantly enriched at a cutoff of p<0.05 and FDR<0.25 per the GSEA guidelines [27]. This data is accessible at GEO with accession number GSE173411.

Two-dimensional drug response assays

Tariquidar, paclitaxel, doxorubicin, orlistat, JQ1, AS1842856, linsitinib, panobinostat, vorinostat, belinostat, and LMK-235 (all from Selleckchem, Houston, TX, USA) and tepoxalin (Toronto Research Chemicals, North York, ON, CA) were prepared in DMSO. Metformin and 2-deoxy-D-glucose (both from Selleckchem) were prepared in complete culture medium. Prior to plating for drug response assays, breast cancer cell lines transduced with tet-inducible eGFP or ABCB1 lentiviruses were exposed to 1 µg/mL doxycycline for 24 hours to induce gene expression. After 24 hours of doxycycline exposure, 5,000 cells/well were seeded in 30 µL of RPMI + 10% FBS in 384-well microplates (Corning cat. no. 3764, Corning, NY, USA). After 24 hours, 10 µL of 4X drug was added in quadruplicate per dose. Upon 72 hours of drug treatment, 30 µL CellTiterGlo (Promega, Madison, WI, USA) was added and luminescence assessed according to manufacturer’s directions using an Infinite M1000 Pro plate reader (Tecan, Morrisville, NC, USA).

Three-dimensional co-culture drug response assays

After 24 hours of doxycycline exposure, 10,000 cells were seeded per well of a 96-well round bottom ultra-low attachment spheroid microplate (Corning #4520). Wells were seeded with a 50%/50% mix of ABCB1-expressing mCherry-labeled and eGFP-expressing cerulean-labeled cells. Cells were incubated for 24 hours to allow spheroids to form, after which baseline images were taken using the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT, USA). Briefly, 2 × 2 montage images from brightfield, Texas Red (mCherry) and BFP (cerulean) channels were stitched across 4 Z-sections and an integral of the spheroids’ mCherry and cerulean fluorescence intensities was calculated. Following baseline imaging, the spheroids were washed in fresh RPMI containing doxycycline with the addition of the following drugs alone and in combination: doxorubicin (3 µM), tariquidar (3 µM), tepoxalin (10 µM) and vorinostat (3 µM). Spheroids were then imaged approximately every 48 hours for the next 10-12 days (until vehicle-treated control spheroids completely filled the wells and therefore growth could no longer be accurately measured) and RPMI + doxycycline and treatment drugs were refreshed every 72 hours. A ratio of the ABCB1-mCherry:eGFP-cerulean population was plotted for each time point after normalizing to the ratio of cells in vehicle control wells.

NanoString gene expression profiling

Gene expression of inducible breast cancer cell lines BT549, CAMA-1, HCC1428, HS578T, MCF7, T47D, and ZR-75-1 was assessed using the NanoString gene expression panel including ABCB1, which has been described previously [28]. Briefly, sample RNA was hybridized with a custom 345-gene codeset (NanoString Technologies, WA, USA) at 65°C for 16 hours. Hybridized probetarget mixture was then purified and quantified via nCounter MAX Digital Analyzer (NanoString Technologies, WA, USA). The custom 345-gene codeset used has been described previously [29] and includes ABCB1 as a profiling target (Suppl. Table 3). Raw counts were normalized to internal positive control probes and housekeeping genes according to default parameters in nSolver version 4.0 (NanoString Technologies, WA, USA), with background threshold set to 20. Normalized counts data are available at https://github.com/jasminereethmeyer/ABCB1.

Statistics

Statistics analyses were performed in GraphPad Prism version 9. For two-dimensional dose response assays for all drugs except for paclitaxel and carboplatin dose response, curves were modeled with the equation Y=100/(1+10^((LogIC50-X)*HillSlope))), and the extra sum-of-squares F-test was performed with an alpha of 0.05 or less considered significant. For paclitaxel and carboplatin, simple linear regression was performed, and significance determined using the F-test. For three-dimensional co-culture assays, one-way ANOVA was performed on cell population ratios from the final time point, followed by Tukey’s multiple comparisons test, with a significance cutoff of adjusted p-value <0.05. For NanoString ABCB1 gene expression comparison, analysis was performed in nSolver version 4.0—multiple t-tests were performed, and the Holm-Sidak method was used for multiple comparisons adjustment with an alpha of 0.05.

Results

ABCB1 overexpression leads to unique patterns of gene expression changes

To explore the phenotypic impact of ABCB1 overexpression, we overexpressed this gene using adenovirus delivery in normal human mammary epithelial cells (HMECs) to assess its impact in the absence of background oncogenic signaling and to identify potential targetable vulnerabilities of breast cells overexpressing MDR1. Gene expression differences between HMECs overexpressing either ABCB1 or GFP were identified and curated into pathways. One hundred sixty-two genes were differentially expressed between ABCB1 and GFP-overexpressing HMECs (Fig. 1, Suppl. Table 1). Remarkably, all differentially expressed genes were up-regulated in ABCB1-overexpressing HMECs—there were no significant down-regulated genes. Following ABCB1, the most significantly up-regulated genes by FDR-adjusted p-value rank were DDiT3, ILL1, SCML1, and GABBR1. The PTG52 gene encoding the cyclooxygenase-2 protein, a target of numerous FDA-approved compounds, was ranked 26th most overexpressed in ABCB1-overexpressing cells. Gene set enrichment analysis identified 39 pathways from the oncogenic signatures and hallmarks gene set collections from the Molecular Signatures Database [25,26] enriched in HMECs overexpressing ABCB1 and no significant pathways enriched in HMECs overexpressing GFP (Suppl. Table 2). The three top-ranked gene sets were those containing signaling dependent upon RELA, Notch, and HOXA9, implicating a potential NF-kB/inflammation-related phenotype in HMECs over-expressing MDR1.

Overexpression of ABCB1 sensitizes breast cancer cell lines to tepoxalin

We next examined the phenotypic impact of ABCB1 expression on drug response in breast cancer cell lines. To create a gene expression system for multi-day drug screen experiments, we generated stable cell lines capable of induction of either ABCB1 or GFP gene expression under the control of the tetracycline-inducible promoter TRE3G. We validated ABCB1 gene expression in seven breast cancer cell lines after either 4, 24, or 72 hours of induction with 1 µg/mL doxycycline using a custom NanoString gene expression panel including ABCB1 (Suppl. Fig. 1). Apart from ABCB1, no additional genes measured in the panel were significant. We also validated protein overexpression of MDR1 in these breast cancer cell lines via western blot at each of the three time points (Suppl. Fig. 2).

To examine the impact of ABCB1 expression on drug response, we screened the inducible ABCB1-overexpressing breast cancer cell lines
with various chemotherapies and small molecule inhibitors. We first validated resistance of the \(\text{ABCB1}\)-overexpressing cell lines to MDR1 substrates doxorubicin and paclitaxel, as well as the canonical reversal of resistance when given in combination with the MDR1 inhibitor tariquidar (doxorubicin: all cell lines \(p<0.001\), paclitaxel: all cell lines \(p<0.001\) except for ZR-75-1 \(p=0.0167\), Fig. 2). Similarly, we demonstrated that these cells did not exhibit marked resistance to the non-MDR1 substrate carboplatin (Suppl. Fig. 3). We next investigated the response to metabolic inhibitors based on the presumed energetic demands of the ATP consumption coupled to MDR1 transmembrane transport activity [13]. However, in testing response to metabolic inhibitors, we did not identify consistent sensitivity in breast cancer cells overexpressing \(\text{ABCB1}\) to the following: 1) the glucose analog 2-deoxy-D-glucose (2-DOG, Suppl. Fig. 4A), 2) the BET family bromodomain inhibitor JQ1 (Suppl. Fig. 4B), 3) the lipase inhibitor orlistat (Suppl. Fig. 5A), 4) the FOXO1 inhibitor AS1842856 (Suppl. Fig. 5B), 5) the IGFIR inhibitor linsitinib (Suppl. Fig. 6A), or 6) the mitochondrial respiratory chain inhibitor metformin (Suppl. Fig. 6B). In some rare instances, the \(\text{ABCB1}\)-expressing cell lines even demonstrated resistance, rather than sensitivity, to these metabolic inhibitors (e.g., T47D response to JQ1 and 2-DOG, \(p<0.001\)). While we did not identify increased sensitivity of \(\text{ABCB1}\)-overexpressing cells to metabolism-targeting drugs, the relatively consistent response between the \(\text{ABCB1}\) and \(\text{GFP}\)-overexpressing cell lines suggests that their mechanism of action and/or their accumulation in cancer cells can still be achieved despite MDR1 overexpression.

Next, based on the increased expression of the COX-2 protein-encoding gene \(\text{PTGS2}\) identified in HMECs overexpressing \(\text{ABCB1}\) in the above genomic experiments and a recent demonstration of tepoxalin’s efficacy in \(\text{ABCB1}\)-expressing cancer cell lines [30], we also tested response to the 5-LOX, COX-1/2 inhibitor tepoxalin. Remarkably, some \(\text{ABCB1}\)-overexpressing cell lines demonstrated increased sensitivity to tepoxalin at the micromolar dose range (BT549: \(p<0.001\), CAMA-1: \(p<0.001\), T47D: \(p=0.0025\), MCF7 and ZR-75-1: ns, Fig. 3). Those cell lines demonstrating sensitivity to tepoxalin typically did not also demonstrate sensitivity to the COX-2 inhibitor celecoxib. The exception, cell line T47D, demonstrated mild sensitivity to celecoxib, but its response to tepoxalin was more pronounced (T47D celecoxib \(p=0.0142\)). Interestingly, increased sensitivity to tepoxalin was reversed when co-treating with tepoxalin and the MDR1 inhibitor, tariquidar, suggesting a potential antagonistic drug relationship also previously seen by Corsello and colleagues (Suppl. Fig. 7) [30].

Combination therapy of tepoxalin with chemotherapies doxorubicin and paclitaxel significantly improved drug response in \(\text{ABCB1}\)-overexpressing cells (doxorubicin: all cell lines \(p<0.001\), paclitaxel: all cell lines \(p<0.001\), Fig. 4). This increase in treatment efficacy was also seen when combining tepoxalin with HDAC inhibitors, a drug class demonstrated to reduce \(\text{ABCB1}\) transcription in drug-resistant cancer cells [31]. \(\text{ABCB1}\)-expressing lines demonstrated a significant increase in response to the pan-HDAC inhibitors vorinostat, panobinostat, belinostat, and the HDAC 4/5 inhibitor LMK-235 when combined with tepoxalin (vorinostat all cell lines \(p<0.001\) except for ZR-75-1, \(p=0.0074\), panobinostat, belinostat, LMK-235 \(p<0.001\) for all cell lines, Fig. 5, Suppl. Fig. 8). Remarkably, this effect was most pronounced at low doses of HDAC inhibitors.

Treatment with tepoxalin prevents emergence of a dominant \(\text{ABCB1}\)-overexpressing population in a three-dimensional co-culture model

To assess the potential of tepoxalin to prevent chemotherapy-induced selection of \(\text{ABCB1}\)-overexpressing, chemotherapy-resistant cells, we leveraged co-culture of mCherry-labeled inducible \(\text{ABCB1}\)-expressing and cerulean-labeled inducible \(\text{GFP}\)-expressing breast cancer cells in a three-dimensional spheroid model system with imaging to

![Fig. 1. \(\text{ABCB1}\) overexpression leads to gene expression changes in human mammary epithelial cells (HMECs). A) Hierarchical clustering and heatmap of genes differentially expressed between HMECs infected with either GFP or \(\text{ABCB1}\) adenoviruses at 50 MOI for 12 hours. B) Validation of MDR1 protein overexpression in HMECs infected with \(\text{ABCB1}\) adenovirus. Molecular weight markers in kDa notated at right.](image-url)
monitor changes in each cell population, as described previously [32]. We measured the ratio of \( \text{ABCB1} \)-expressing cells to \( \text{GFP} \)-expressing cells in treated conditions as compared to a vehicle-control condition, across four breast cancer cell lines over a series of 10 to 12 days. As expected, treatment with doxorubicin led to the presence of a significant \( \text{ABC} \) -expressing majority, with this population representing at least twice that of the \( \text{GFP} \) population in each of the four tested cell lines at the study end point (Fig 6, \( p < 0.001 \) for all cell lines at end point). This outcome was prevented by combination treatment of doxorubicin plus the canonical \( \text{MDR1} \) inhibitor tariquidar (Fig. 6, \( p < 0.001 \)). Remarkably, treatment with tepoxalin alone resulted in a majority of \( \text{GFP} \)-expressing, chemotherapy-sensitive cells in the CAMA-1, MCF7, and T47D cell lines at the study end point, and led to relatively balanced \( \text{ABCB1} \) and \( \text{GFP} \) populations in the BT549 cell line. Indeed, in the MCF7 and T47D cell lines, tepoxalin alone was more efficacious at controlling the \( \text{ABC} \) -expressing population than was doxorubicin plus the \( \text{MDR1} \) inhibitor tariquidar (MCF7 and T47D \( p < 0.001 \), BT549 and CAMA-1: ns). Interestingly, the potential antagonistic effect of tepoxalin and tariquidar.

Fig. 2. \( \text{ABCB1} \)-overexpressing cells are resistant to the \( \text{MDR1} \) substrates doxorubicin and paclitaxel. Plots of drug response normalized to DMSO vehicle control in \( \text{ABCB1} \) and \( \text{GFP} \)-overexpressing cell lines following 72 h chemotherapy treatment with and without the \( \text{MDR1} \) inhibitor tariquidar. Error bars represent standard deviation of 4 replicates.
Fig. 3. ABCB1- and GFP-expressing cells demonstrate sensitivity to the 5-LOX, COX-1/2 inhibitor tepoxalin but not to the COX-2 inhibitor celecoxib. Plots of drug response at 72 h normalized to DMSO vehicle control. Presence of single red curve indicates calculated regression equation is shared between ABCB1 and GFP lines. Error bars represent standard deviation of 4 replicates.
identified in the two-dimensional drug assays was even more pronounced in the three-dimensional assays—the proportion of ABCB1-expressing cells at the study end point was significantly more in the tepoxalin plus tarquidar condition than in tepoxalin treatment alone in the MCF7 and T47D lines (MCF7: p = 0.0285, T47D: p < 0.001), with borderline significance seen in the BT549 line (p = 0.0804). Finally, tepoxalin also significantly reduced the proportion of ABCB1-expressing cells when combined with the HDAC inhibitor vorinostat by the study end point in the BT549 and MCF7 cell lines (Suppl. Fig. 9, BT549 and MCF7 p < 0.001, CAMA-1 and T47D: ns).

Discussion

To date, the influence of ABCB1 overexpression on downstream gene
expression in breast cancer remains largely unexplored. Indeed, few studies have examined the isolated effects of \textit{ABCB1} overexpression in any cancer type—instead, previous studies have typically employed chemotherapy dose escalation to generate drug resistant cell lines harboring \textit{ABCB1} overexpression [e.g., 33, 34] in addition to gene expression changes in numerous additional ABC family members. These studies therefore cannot link specific effects to the sole overexpression of \textit{MDR1}. While this experimental strategy more closely models the clinical development of drug resistance, it complicates the study of \textit{MDR1}-specific effects. To our knowledge, only one previous study has leveraged overexpression of \textit{ABCB1} in cancer to examine its downstream phenotypic effects [35]. This study employed retroviral delivery of \textit{ABCB1} to the lymphoblast cell line TK6 and found transcriptional changes in 61 genes related to apoptosis, metabolism, calcium signaling, and transcription-related genes via microarray. Notably, two genes overlapped between this study and our experiment in HMECs—\textit{GEM} and \textit{CCNL1}.

Our gene expression profiling studies identified multiple
transcriptional changes in HMECs overexpressing ABCB1. Notably, genes related to stemness and invasiveness (GREM1, MMP1, FOXQ1) were identified as upregulated in ABCB1-overexpressing HMECs. GREM1 has recently been linked to migration and invasiveness in murine models of breast cancer [36,37], and overexpression of MMP1 has been linked to drug resistance and invasiveness phenotypes [34,38]. Ectopic FOXQ1 expression has been shown to increase migration and invasion in the human mammary epithelial cell line HMLE [39] and has recently been linked to transcription of VEGFA in breast cancer cells [40], a gene we also identified as upregulated. In addition to FOXQ1, several other transcription factors were overexpressed including FOSB, JUN, SP8, KLF4, MAFF, NR0B1, DDI13, and ATF3, among others. In addition to VEGFA, growth factors BMP2, FGF2, FGF18, VGF, CTNF, and GDNF were also overexpressed. We also identified increased expression of ABCC2 (MRP2), suggesting that transcriptional mechanisms of regulation of this additional ABC family member in drug resistance could be linked.

Long chain fatty acid binding protein gene FABP3 was overexpressed downstream of ABCB1 overexpression, as was the carnitine palmitoyltransferase gene CPT1B. Together, these genes are involved in the transport and metabolism of long-chain fatty acids. Their elevation could indicate an increased energetic need in ABCB1-overexpressing cells, and interestingly, elevation of fatty acid binding protein genes is associated with poor outcomes in non-small cell lung cancer as is CPT1B expression in prostate cancer [41,42]. However, sensitivity to the metabolic inhibitors tested in the present study did not appear to be influenced by ABCB1 expression in our inducible cell lines, except for some resistance to these inhibitors in T47D cells. Future assays of drug response to carnitine palmitoyltransferase inhibitors such as oxefinecin and perhexiline, as well as to inhibitors of additional upregulated targets identified in the ABCB1-expressing HMECs may prove illuminating. Because we examined gene expression changes at an early 12 hour time point to identify primary changes and to avoid capturing the effects of feedback loops, additional experiments studying the implications of long-term ABCB1 overexpression, a model more closely resembling the multidrug resistance phenotype in patients, are warranted.

Our inducible ABCB1 overexpressing breast cancer cell lines exhibited increased resistance to the known MDR1 substrate chemotherapies doxorubicin and paclitaxel, which was mitigated by treatment with the MDR1 inhibitor tariquidar. Following identification of upregulation of the COX-2 encoding PTGS2 gene in HMECs, we treated these inducible cell lines with the 5-LOX, COX-1/2 inhibitor tepoxalin, and remarkably, ABCB1-expressing cells demonstrated sensitivity to this compound in both two- and three-dimensional assays, an effect which was diminished upon co-treatment with tariquidar. Previous work by Corsello and colleagues [30] demonstrated tepoxalin’s efficacy across a wide variety of cancer cell lines with ABCB1 expression in two-dimensional culture, and also identified an antagonistic relationship between tepoxalin and tariquidar. We also found that response to tepoxalin in ABCB1-overexpressing cells was more pronounced than to the COX-2 inhibitor celecoxib, suggesting that tepoxalin may elicit its effect via either its COX-1 or 5-LOX activities or by an additional mechanism yet to be determined. Treatment with the COX-1/2 and 5-LOX substrate arachidonic acid has been shown to decrease the activity of MDR1 in porcine brain endothelial cells and both gene expression and efflux activity in human colon cancer cells, suggesting accumulation of arachidonic acid as a potential mechanism of tepoxalin action [43,44]. Inhibition of either COX-1/2 or 5-LOX alone may fail to accumulate sufficient arachidonic acid for MDR1 inhibition, potentially explaining the inefficacy of a single COX-2 inhibitor as celecoxib. Conversely, several studies have demonstrated tepoxalin NF-xB inhibitory activity, raising an additional potential mechanism of its action in ABCB1-expressing cells [45,46].

Further, tepoxalin treatment was capable of controlling the proportion of ABCB1-expressing cells in our three-dimensional co-culture model, maintaining a population of chemotherapy-sensitive GFP-expressing cells up to 10 days in culture both when given alone or in combination with the MDR1 substrate chemotherapy doxorubicin. Recent advances in mathematical modeling of tumor subclone dynamics suggest superiority of a multidrug adaptive therapy approach, whereby different therapies are temporally spaced to minimize selection and maintain the presence of therapy sensitive subclones, rather than promote evolution of therapy resistance [47]. Future work is required to determine if a temporally spaced combination treatment of tepoxalin plus chemotherapy can maintain treatment sensitivity in tumors.

The sensitivity of ABCB1-expressing cells to tepoxalin warrants...
further investigation into its clinical use. Tepoxalin is a non-steroidal anti-inflammatory drug (NSAID), a class of drugs commonly prescribed for pain and fever control whose use has been linked to cancer prevention [48,49]. Despite FDA-approval for animal use, tepoxalin was briefly used by veterinarians for pain management in domesticated mammals before being removed from the market [50,51], and few studies of its use in humans are published in the scientific literature. One study monitored its effects in 20 healthy young males and reported five participants experienced minor adverse effects including diarrhea, abdominal discomfort, and lightheadedness [52]; however, a Phase II study of tepoxalin’s effect in 14 patients undergoing knee replacement surgery reported no adverse effects [53]. The oral availability and prevention [48,49]. Despite FDA-approval for animal use, tepoxalin was used without a required prescription, it is possible tepoxalin would not be any more harmful to patients than ibuprofen or naproxen, and may be used by oncologists in refractory cancer.

MDR1 expression in patient breast tumors associates with shorter disease free and overall survival [54]. Clinical trials of MDR1 inhibitors have largely been abandoned, necessitating additional in vitro research to identify compounds to which ABCB1-expressing cells demonstrate collateral sensitivity, and to improve clinical outcomes for patients with drug resistant tumors. Tepoxalin could be such a compound—further research is warranted to fully characterize tepoxalin’s effects in humans and to examine the outcomes of combination tepoxalin and chemotherapy treatment in patients with tumors bearing high MDR1 expression.

Declaration of Competing Interest

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

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Supplementary material

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