Epigenetic modulators combination with chemotherapy in breast cancer cells

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Despite the concerning adverse effects on tumour development, epigenetic drugs are very promising in cancer treatment. The aim of this study was to compare the differential effects of standard chemotherapy regimens (FEC: 5-fluorouracil plus epirubicine plus cyclophosphamide) in combination with epigenetic modulators (decitabine, valproic acid): (a) on gene methylation levels of selected tumour biomarkers (LINE-1, uPA, PAI-1, DAPK); (b) their expression status (uPA and PAI-1); (c) differentiation status (5meC and H3K27me3). Furthermore, cell survival as well as changes concerning the invasion capacity were monitored in cell culture models of breast cancer (MCF-7, MDA-MB-231). A significant overall decrease of cell survival was observed in the FEC-containing combination therapies for both cell lines. Methylation results showed a general tendency towards increased demethylation of the uPA and PAI-1 gene promoters for the MCF-7 cells, as well as the proapoptotic DAPK gene in the treatment regimens for both cell lines. The uPA and PAI-1 antigen levels were mainly increased in the supernatant of FEC-only treated MDA-MB-231 cells. DAC-only treatment induced an increase of secreted uPA protein in MCF-7 cell culture, while most of the VPA-containing regimens also induced uPA and PAI-1 expression in MCF-7 cell fractions. Epigenetically active substances can also induce a re-differentiation in tumour cells, as shown by 5meC, H3K27me3 applying ICC.

Significance of the study: Epigenetic modulators especially in the highly undifferentiated and highly malignant MDA-MB-231 tumour cells significantly reduced tumour malignancy thus; further clinical studies applying specific combination therapies with epigenetic modulators may be warranted.

KEYWORDS
breast cancer, DNA-methylation, epigenetic modulators, HDAC-inhibitor

INTRODUCTION

Breast cancer in regard to disease-related mortality and treatment costs represent one of the biggest cancer-related health problems for women. Depending on histomorphologic factors, such as tumour size, grading, estrogen/progesterone receptor status, lymph node metastasis and HER2 status, different treatment regimens are established in clinical guidelines. Nowadays, the treatment of breast cancer by surgical removal of the tumour, radiotherapy, endocrine therapy (eg, tamoxifen, aromatase inhibitors), including immuno-targeted therapies (herceptin, avastin) and multimodal combination chemotherapy are standard of care. Adjuvant therapy with anthracycline-containing regimens (FEC: 5-fluorouracil, epirubicin, cyclophosphamide) proved to be
particularly advantageous for specific subpopulations of patients at high risk of relapse.\(^5\)

In addition to aberrations of the gene sequence (mutations, genomic instability), so-called epigenetic modifications play an important role in cancer formation and evolution.\(^6\) Epigenetic changes can be induced by several factors and it occurs without a change in the DNA sequence.\(^7\) Epigenetics comprises the reversible modification of DNA bases (methylation of cytosine nucleotides), RNA-interference by micro RNAs and the regulation of chromatin status by posttranslational modifications of histone proteins (mainly acetylation and methylation of lysine residues), which represent an additional mechanism of transcriptional control of gene expression.\(^8,9\) DNA methyltransferases (Dnmt1, Dnmt2, Dnmt3a, Dnmt3b) catalyse the covalent modification of the 5\(^{\text{th}}\) position of the cytosine pyrimidine ring in so-called CpG dinucleotide motifs that are frequently found in the promoters of approximately 50% of all known human genes in form of defined CpG motif ‘CpG Islands’.\(^10,11\) Methylation of cytosines in these regions serve as the basis for the gradual assembly of epigenetically active protein complexes, like methylated DNA-binding proteins (MBDS), histone deacetylases (HDAC) and histone methylationtransferases. These protein complexes modify specific lysine residues (deacetylation and methylation) which ultimately leads to a local condensation of chromatin and related transcriptional inactivation of the corresponding genes.\(^12\) With the help of epigenetically active substances, such as the demethylating agent decitabine (5-aza-2\(^{\text{nd}}\)-deoxycytidine, 5-aza-CdR, DAC) or histone deacetylase inhibitors such as valproic acid (VPA), the status of the epigenetic inactivation of gene expression can be reversed.\(^13,14\) Demethylating agents have the ability to reverse the hypermethylation-dependent gene silencing by inhibition of DNA methyltransferases.\(^15\) Decitabine as a nucleotide analogue is incorporated into the DNA during DNA replication and forms irreversible covalent bonds with the active regions of the methyltransferase.\(^16\) In studies of various animal models and cell lines, it has been shown that decitabine induces apoptosis and cell cycle arrest by DNA hypomethylation, and also enhances anti-tumour immunoreactivity and inhibits tumour growth.\(^17,18\) Decitabine therapy in osteosarcoma (OS) cell lines has been shown to induce apoptosis leading to upregulation of pro-apoptotic genes (GADD45A, HSP9A9B, PAWR, PDCD5, NFKBIA and TNFAIP3) with CpG islands in the 5\(^{\text{th}}\) region of their genes.\(^19\) It has also been reported that apoptosis is induced by epigenetic changes such as p53 reactivation and increased expression of p15, p16, PTEN, BRCA1 and BRCA2 tumour suppressor genes in breast cancer cell lines and breast cancer stem cells after low dose decitabine therapy.\(^20\) These results demonstrate the potential of decitabine in the treatment of malignant diseases.\(^21\)

HDAC inhibitors are currently in clinical development as new drugs in the treatment of cancer.\(^6,8,22\) In many existing studies, the HDAC inhibitor valproic acid provided anti-tumorigenic properties, like cell growth inhibition (Garrett et al, 2016), apoptosis induction (Park et al, 2015), cell cycle arrest and tumour cell re-differentiation induction.\(^23-25\) VPA was first approved for the treatment of epilepsy.\(^26\) It was investigated as a potential anticancer agent with the discovery of HDAC inhibitor activity.\(^27,28\) Clinical trials with glioblastoma patients have shown that VPA has an effect on radiation and chemotherapy susceptibility as in other cancers either.\(^29,30\) As VPA has a good safety profile, clinical use for a new molecular-targeted approach to the treatment of breast cancer is of great interest.\(^31\) Studies have shown that VPA affects proliferation status and gene expression in breast cancer cell lines.\(^32,33\) In many studies, it has been reported that a possible role of epigenetic agents as anti-cancer drugs has been based on their use combined with other drugs (combined therapies with other epigenetic modulators, combinations with cytotoxic chemotherapeutic agents and combinations with immunomodulators).\(^34\) In our previous studies, we analysed the effects of decitabine combined with FEC chemotherapy regimens on breast cancer cells. We observed that the methylation levels of uPA, PAI-1, TMS1, MGMT and DAPK genes promoter were significantly reduced with this type of combination therapy.\(^35,36\)

In the present study, we investigated the epigenetic effects of different combinations of epigenetic modulators, particularly valproic acid, decitabine and their combination with standard chemotherapy (FEC) using cell culture models (MCF-7, MDA-MB-231) on different levels (DNA methylation of selected genes, protein expression, cell survival) and analysed their impact on cell viability and metastasis using cell invasion assays.

### 2 | MATERIALS AND METHODS

#### 2.1 | Chemicals, cell culture

Anticancer drugs [5-fluorouracil (5-FU; EBEWE Pharma, Austria), 4-HC (4-hydroperoxycyclophosphamide, the active metabolite of cyclophosphamide; NIOMECH, Germany) and epirubicine (EBEWE Pharma, Austria)] were obtained from the Klinikum rechts der Isar, Technische Universität München, Germany, representing standard drug regimens normally used for breast cancer treatment. Stock concentrations of drugs used with dilution buffer provided by the pharmaceutical company were prepared (or in PBS-phosphate buffered saline). The concentration of the combination of chemotherapy (FEC) was used as 100% TDC (test drug concentrations). TDC was determined by pharmacoetic/clinical information and clinical evaluation data.\(^37\) The mean plasma drug concentration in standard FEC treatment in cancer patients was defined as 100% TDC.\(^38\) Hereby, 100% TDC values stock solution: were defined as follows: 5-FU: 22.50 μg/mL, epirubicine: 0.50 μg/mL, 4-HC: 3.0 μg/mL. Valproic acid (0.6 μM) and decitabine (10 μM) were obtained from Sigma (St. Louis, Missouri). Drug concentrations of Decitabine and VPA as stated in our previously study which showed non-toxic concentrations were used in cells.\(^35,39\) The stock solution were freshly prepared in H\(_2\)O before each experiment, sterile filtrated and its final test concentration adjusted with cell culture medium.

Human breast cancer cells MCF-7 and MDA-MB-231 were cultured in RPMI 1640 supplemented with penicillin G (100 U/mL),
streptomyein (100 μg/mL), L-glutamine and 10% foetal calf serum (Invitrogen, Paisley, UK) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were harvested with 0.05 EDTA in PBS, centrifuged at 400×g and washed in PBS twice.

2.2 | MTT viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) viability assay was performed as previously described. Cancer cells were seeded in 200 μL culture medium in triplicate at a density of 1 x 10⁴ cells per well in a 96-well plate. After overnight incubation, media were replaced by fresh ones without the drugs (untreated control), single drugs or combination regimes for 48 hours. MTT was prepared as stock solution (5 mg/mL) at pH = 7.2 in PBS buffer and sterile-filtered. After adding 20 μL of MTT solution to each well, samples were incubated for 4 hours at 37°C and 100 μL of solubilizing buffer (10% SDS dissolved in 0.01 N HCl) was added to each well. After overnight incubation at 37°C the absorbance (Abs) was read by an ELISA plate reader at 570 nm to determine cell viability. Cell viability of treated cells was calculated in reference to the untreated control cells using the formula: (%) = 100 × (sample Abs)/(control Abs). All the experiments were repeated twice in technical triplicates.

2.3 | DNA methylation assays

Using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), DNA was extracted from the cells according to the manufacturer’s instructions. Bisulfite conversion was performed using the Epitect Bisulfit kit (Qiagen), with 1 μg of DNA as input into the bisulfite conversion using the standard conversion protocol on a PCR thermal cycler and subsequent clean-up of bisulfite converted DNA (bisdNA) according to the manufacturer’s instruction. Quantitative methylation-specific real-time PCR assays have been established previously. For the following genes: LINE-1 (Long interspersed nucleotide element-1), uPA (urokinase-type plasminogen activator), PAI-1 (plasminogen activator inhibitor-I) and DAPK (Death-associated protein kinase). For quantitative PCR the ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems, Inc., Foster City, California) was employed. PCR was performed in a final volume of 20 μL including 10 μL 2x Quantitect Probe mastermix (Qiagen), 2 μL bisulfite-treated DNA, and assay-defined primer and probe concentrations. Cycling conditions were chosen according to manufacturer’s instructions. Evaluation of the methylation signals was performed by relative quantification with the 2exp-ΔΔCT method using an ALU-1 reference for the normalization of the signal on the used amount of bisulfite converted DNA. All groups included a positive control with a known DNA-methylation status and a template-free control. Ssxl-treated and bisulfite converted human chromosomal DNA (Qiagen) was used as a reference for fully methylated bisdNA. Primer and probes were purchased from Metabion (Martinsried, Germany), Applied Biosystems, or Microsynth (Lustenau, Austria). Primer sequences are shown in Table 2.

2.4 | Immunocytochemical (ICC) evaluation

Breast cancer cell lines (MCF-7 and MDA-MB-231) were treated with different treatment combinations (DAC, VPA, FEC, DAC + VPA and DAC + VPA + FEC). Cells (3 x 10⁴) were detached and harvested with 0.05% EDTA in PBS and washed in PBS twice. A cell clot was prepared by adding the following reagents to the cell pellet: 150 μL thrombin (T4648, Sigma), 750 μL casein (C5890, Sigma) and 600 μL fibrinogen (F4883, Sigma). Cells were incubated overnight at 4°C to allow clot formation. Cell clots were fixed in 4% PBS-buffered formalin and subsequently embedded in a paraffin block at the Institute of Pathology, Technical University of Munich. All blocks were cut into 2 μm slides and deparaffinized with xylene followed by a graded series of ethanol (100-70%) and rehydrated in distilled H₂O. After high-pressure cooking with 10 mM EDTA pH 8.0, endogenous peroxidase was blocked with 3% H₂O₂ for 20 minutes at room temperature. After that, avidin-biotin blocking was performed (SP-2001, Biozol, Eching). Slides were incubated with an anti-5-methyl-cytosine-AB (5meC, 33D3, Eurogentec, dilution 1:6500) or an anti-histone 3 lysine 27 trimethylation-AB (H3K27me3, mAbcam 6002, Abcam, dilution 1:700) primary antibody for 14 hours at 4°C. An incubation without primary antibody served as negative control. Subsequently, the detection was employed with LSAB-DAB-kit (Zytomed Systems, Berlin, Germany) according to the manufacturer’s protocol. Sections were washed thoroughly between incubations and cell nuclei were counterstained with Meyer’s haematoxylin for 20 seconds and dehydrated with an ascending row of graded alcohol (70-100%) and 2x Xylene and sealed for ICC analysis.

2.5 | Detection of protein levels of uPA and PAI-1

Protein content of metastasis markers uPA and PAI-1 were measured in MCF-7 and MDA-MB-231 cell culture supernatants by ELISA. After 48 hours incubation with different drugs, three compartments of the cell culture were harvested. Cell culture media containing non-adherent cells were washed twice in PBS and also stored at −80°C until further use. The cell pellets containing non-adherent cells were washed twice in PBS and also stored −80°C at until further use. Finally, adherent cells were detached with 0.05% EDTA in PBS. Protein was separately extracted from both cell containing specimens with Triton X-100 according to standard lab protocols (Schmitt et al, 2007). The uPA and PAI-1 antigen content (ng per million cells) were determined with the FEMTELLE assay kit (American Diagnostica Inc., Stamford, Connecticut) according to manufacturer’s instructions. Results presented are from two independent experiments with tree technical replicates.

2.6 | Matrigel invasion assay

Invasion was examined applying BD BioCoat Matrigel Invasion Chamber inserts (BD Bioscience, Bedford, Massachusetts)
according to the manufacturer’s protocol. After incubation with the respective drug combination, cells were harvested with 0.05% EDTA in PBS, counted and plated at a density of $2 \times 10^5$ cells per insert in FCS-free medium. In the reservoir volume, medium with standard concentration of FCS was supplied to establish a chemotactic gradient supporting cell invasion. After 48 hours incubation, noninvasive cells and matrigel were removed from the membrane with a cotton-tip applicator. The cells passing through the bottom side of the membrane were fixed and stained with the Diff-Quick Kit (Cat No: 133882, Dade Behring AG, Switzerland). Cells per membrane were counted under a light microscope. Results from two different experiments, including technical triplicates, are shown for each treatment modality. Results were normalized with respect to ongoing cell proliferation during the invasion assay (24 hours in FCS-free medium).

### 2.7 Statistical analyses

The significance of the difference from cell viability values was assayed using one-way analysis of variance (ANOVA). Student's t test was used to analyse the association between the methylation levels of the genes and ELISA results. All statistical analyses were performed by using GraphPad Prism 6.0 statistical software (San Diego, California) for Windows. A value of $P < 0.05$ was considered statistically significant. Results were expressed as mean ± SD (standard deviation).

### 3 RESULTS

#### 3.1 Effect of decitabine, valproic acid and their combination with FEC treatment on breast cancer cell viability

The anti-growth effects of DAC (10 μM), VPA (0.6 μM), FEC (100% TDC) and their combination regimes towards breast cancer cells were investigated applying the MTT cell viability assay. Treatment with epigenetic modulators DAC and VPA as single agents led to no significant reduction of survival in MCF-7 cells (viability >94%, Figure 1A). Combination of DAC and VPA induced a significant but only slight decrease in cell viability (84.4%). A slight reduction in cell viability was observed in MDA-MB-231 for single agent or combination of the two epigenetic modulators (84-75% viability, Figure 1B). A strong reduction in survival was found with the FEC regime for both cell lines, which was further enhanced by combination with epigenetic modulators in MCF-7 in the order DAC + FEC < VPA + FEC < DAC + VPA + FEC (42-28%) as well as in MDA-MB-231 (36-29%).

#### 3.2 Quantification of the methylation status of selected marker genes using methylation-specific real-time PCR assays

LINE-1, as a marker for genome-wide methylation status, displays a moderate overall DNA methylation status in untreated cells (MCF-7:16.6

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**FIGURE 1** Viability of MCF-7 (A), and MDA-MB-231 (B), cells after treatment with decitabine (DAC, 10 μM), valproic acid (VPA, 0.6 μM), combination chemotherapy treatment (FEC, 100% TDC) or their combination for 48 hours was measured by the MTT viability assay and the results are shown as % viability (relative to untreated control). * ($P < 0.001$), denotes statically significant differences in comparison with control. Results from one representative experiment with triplicate measurements are presented.
Treatment of MCF-7 cells with DAC or DAC + VPA led to a significant reduction of global methylation status (LINE-1) compared to the untreated control (DAC: 9.7 ± 2.3; DAC + VPA: 13.6 ± 3.9). MDA-MB-231 cells under treatment with VPA had significantly increased LINE-1 methylation levels (19.4 ± 2.6). Combination treatment of DAC and VPA led to a significant decrease of the LINE-1 signal (13.5 ± 1.9), which was even augmented by combination of both epigenetic modulators with FEC (11.2 ± 2.6). In MCF-7, demethylating effects were found for uPA for both active substances (VPA < DAC < DAC + VPA; 479.2 ± 88.6, 448.2 ± 24.1, 235.6 ± 30.3), which could clearly be enhanced by inclusion of FEC regimens (FEC, DAC + FEC, VPA + FEC, DAC + VPA + FEC; 358.7 ± 54.8, 322.9 ± 39.6, 414.5 ± 102.2, 240.4 ± 38.4). Only a small effect could be seen regarding the PAI-1 methylation status with VPA + FEC < VPA < DAC (101.9 ± 19.0, 86.3 ± 11.3, 66.9 ± 12.9).

DAPK promoter methylation was found to be slightly demethylated with increasing efficiency by the following treatment regimens: DAC < VPA + FEC < FEC < DAC + VPA + FEC < DAC + VPA (652.8 ± 105.1, 627.6 ± 125.5, 604.8 ± 102.0, 470.2 ± 80.4 and 378.4 ± 114.8) (Table 1). In MDA-MB-231, uPA and PAI-1 promoter regions were found to be completely demethylated in the MDA-MB-231 cells and therefore were omitted from the analysis. A highly significant decrease in DAPK promoter methylation status was detected in all treatment regimens (FEC < VPA + FEC < VPA + DAC + FEC < DAC + FEC < DAC + VPA < VPA < DAC; 9.87 ± 2.6, 8.10 ± 2.4, 7.00 ± 1.9, 5.25 ± 2.3, 3.74 ± 3.4, 0.65 ± 0.2, 0.28 ± 0.2) (Table 1).

### 3.3 Immunocytochemical analysis

Next, immunocytochemical analysis of the epigenetic markers 5-methylcytosine (5meC, Eurogentec) and histone 3 lysine 27 trimethylation (H3K27me3, Abcam) were performed. 5meC correlates with the global methylation status of the tumour cell. H3K27me3 is a marker for a repressive chromatin status. Figure 2 shows an overview of treated MCF-7 cells. Depending on the treatment regimens, differences in overall staining of the nuclei and in the distribution of staining, especially in the case of the H3K27me3 marker, were observed which often displayed a nuclear membrane staining (DAC, VPA, DAC + VPA + FEC). The strongest 5meC staining was observed following treatment with DAC and DAC + VPA + FEC.

Figure 3 gives an overview of the treatment of MDA-MB-231 cells. Depending on the treatment regimens, differences in overall staining of the nuclei and in the distribution of the staining particularly in the case of the H3K27me3 marker was observed, which often displayed a nuclear membrane staining (DAC, VPA, DAC + VPA + FEC). 5meC staining was, compared to the untreated MCF-7 cells, very weak and only showed in the combination therapy (DAC + VPA + FEC) a noticeable increase in overall methylation. MDA-MB-231, generally has a more undifferentiated phenotype with a high invasive potential and thus shows generally a very low genome methylation status with 5meC. Nevertheless, slight increases in the methylation signal in the (DAC + VPA + FEC) group were observed. H3K27me3

### Table 1

<table>
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<tr>
<th>Gene</th>
<th>Control</th>
<th>DAC</th>
<th>VPA</th>
<th>FEC</th>
<th>DAC + VPA</th>
<th>DAC + FEC</th>
<th>VPA + FEC</th>
<th>DAC + VPA + FEC</th>
<th>Sss1</th>
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<tr>
<td>LINE-1 MCF-7 (PMR)</td>
<td>16.6 ± 4.0</td>
<td>9.7 ± 2.3</td>
<td>11.8 ± 2.3</td>
<td>178 ± 3.0</td>
<td>116 ± 3.9</td>
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<td>uPA</td>
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<td>479.2 ± 88.6</td>
<td>358.7 ± 54.8</td>
<td>235.6 ± 30.3</td>
<td>322.9 ± 39.6</td>
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<td>240.4 ± 38.4</td>
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<td>PAI-1</td>
<td>184.5 ± 13.0</td>
<td>66.9 ± 12.9</td>
<td>69.2 ± 5.8</td>
<td>109.4 ± 21.2</td>
<td>52.8 ± 4.3</td>
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<td>DAPK</td>
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<td>60.4 ± 102.0</td>
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<td>100.0 ± 19.5</td>
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Note: *P < 0.05; **P < 0.01; ***P < 0.001. Denotes statistically significant differences in comparison with control. Data are presented as mean ± SD (n = 2). PMR: Percentage methylated reference (Sss1); Sss1: Ss1 methyltransferase.
TABLE 2  Primer and probe sequences (MethyLight systems)

<table>
<thead>
<tr>
<th>Gene primer and probe</th>
<th>Sequence 5'-3'</th>
<th>Reference Sequence</th>
<th>Size of PCR product (bp)</th>
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<tr>
<td>Probe</td>
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<td>Probe</td>
<td>VIC-CCTACCTAAACTTCCTCCC - MGB</td>
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Note: Primer and probe concentrations were as follows: uPA, 600 nmol/L primer/200 nmol/L probe; PAI-1, 600 nmol/L primer/200 nmol/L probe; LINE-1, 300 nmol/L primer/100 nmol/L probe; DAPK, 600 nmol/L primer/200 nmol/L probe; and Alu1, 300 nmol/L primer/100 nmol/L probe.

Abbreviations: BHQ, Black Hole Quencher; I, Inosine binding to cytosine or uracil; MGB, Minor groove binder.

FIGURE 2  Immunocytochemical staining of 5-methylcytosine (5meC) and histone 3 lysine 27 trimethylation (H3K7me3) in MCF-7 cells after treatment with decitabine (DAC, 10 μM), valproic acid (VPA, 0.6 μM), combination chemotherapy treatment (FEC, 100% TDC) or their combination (DAC + VPA + FEC) for 48 hours. Control: Omission of the primary antibody. The scale bar represents 10 μm

FIGURE 3  Immunocytochemical staining of 5-methylcytosine (5meC) and histone 3 lysine 27 trimethylation (H3K7me3) in MDA-MB-231 cells after treatment with decitabine (DAC, 10 μM), valproic acid (VPA, 0.6 μM), combination chemotherapy treatment (FEC, 100% TDC) or their combination (DAC + VPA + FEC) for 48 hours. Control: Omission of the primary antibody. The scale bar represents 10 μm
staining showed increased values in all treatment regimens, indicating an increase in the proportion of heterochromatin, which suggests a higher degree of differentiation in treated MDA-MB-231 cells.

3.4 | Determination of protein biomarkers uPA and PAI-1

To investigate the effects of treatment through epigenetically induced demethylation and accompanying transcriptional activation of relevant genes for tumour metastasis, uPA and PAI-1 as known biomarkers for tumour progression and metastasis in breast cancer were analysed with the FEMTELLE ELISA assay (American Diagnostica Inc.). The protein content of uPA and PAI-1 from three different fractions in the cell culture experiment, supernatant (Figure 4), adherent and non-adherent cancer cells (Figure 5) was determined. Untreated control MDA-MB-231 cells showed generally higher PAI-1 protein levels than MCF-7 cell cultures (20 vs 0.015 ng/million cells, Figure 5C vs D), which was also seen for the uPA-fraction (Figure 5A vs B). Antigen content in the cell culture supernatant was about 100-fold elevated in MDA-MB-231 for uPA compared to MCF-7 (Figure 5B vs A) but similar for PAI-1 antigen levels.

The uPA protein content seen in MCF-7 cell supernatants compared to the untreated control for all three subfractions increased after DAC treatment (Figure 4A and 5A). The non-adherent cell fraction showed an increase of uPA antigen levels under DAC, VPA and DAC + VPA treatment (Figure 5A), while adherent cell fractions showed a trend towards higher antigen levels in DAC, FEC, DAC + VPA, DAC + FEC and VPA + FEC (Figure 5A). In general, the cell supernatants, with the exception of the DAC and DAC + VPA fractions contained lower protein levels of uPA than the untreated control (Figure 4A). In case of MDA-MB-231 cells, only FEC treatment alone showed a

![Graphs showing protein content of uPA and PAI-1](image-url)
significant increase of uPA in supernatants as well as non-adherent and adherent cell fractions compared to control (Figure 4B and 5B). All others showed no significant changes in the overall antigen levels. Overall, there was a trend towards increased uPA antigen levels in the epigenetic modulator groups observed in the cell culture supernatant. Regimens including treatment with FEC showed a reduced expression compared to the epigenetic modulator group alone (Figure 4A, B).

Regarding PAI-1 antigen levels, the culture supernatants represented the group with the highest antigen content (Figure 4C, D and 5C, D). PAI-1 protein levels in the supernatants of MCF-7 cells were increased in the VPA + FEC fraction. DAC-containing regimens as well as FEC alone showed a trend to lower PAI levels (Figure 4C). MDA-MB-231 cell supernatants contained increased antigen levels compared to the epigenetic modulator group alone (Figure 4A, B).

Under treatment, for MDA-MB-231 there was a concordant increase of the uPA and PAI-1 content in all three investigated samples (supernatant, non-adherent cells and adherent cells) by treatment with FEC alone (Figure 4B, D and 5B, D). uPA showed no significant elevation in any other treatment regimen (Figure 4B and 5B), whereas PAI-1 was elevated in VPA-containing regimens in the supernatant (Figure 4C) as well as for DAC + VPA in non-adherent cells (Figure 5B). In MCF-7, increased uPA levels were found in the DAC-only treated fraction (Figure 4A). Also, uPA was in general elevated in almost all treated cell-containing fractions (Figure 5A) except for the

**FIGURE 5** Analysis of the antigen content of the metastasis markers uPA and PAI-1 in MCF-7 and MDA-MB-231 cells (adherent and non-adherent cell subpopulations) by ELISA. Cells were treated with decitabine (DAC, 10 μM), valproic acid (VPA, 0.6 μM), combination chemotherapy treatment (FEC, 100% TDC) or their combination for 48 hours. * (P < 0.05); ** (P < 0.01); *** (P < 0.001), denotes statistically significant differences in comparison with control. Data are presented as mean ± SD (n = 2)
treatment with DAC + VPA + FEC. PAI-1 was elevated in all three investigated samples in the VPA + FEC fraction (Figure 4C and 5C). Moreover, there was a general trend of increased levels of PAI-1 in VPA-containing regimens in the non-adherent cell fraction (Figure 5C).

Since uPA and PAI-1 protein expression is strongly associated with a higher metastatic potential and invasive malignancy of the tumour cells, the above-mentioned results show a first insight into differential aspects related to the tumour progression by both standard chemotherapies, as well as combination therapies with epigenetic modulators.

3.5 | Effect of decitabine, valproic acid and their combination with FEC on the invasive capacity of tumour cells

Treatment of both cells with different drug combinations led to gene-promoter-demethylation. In line with this, the increased transcriptional activity of the metastasis and invasion promoted genes uPA and PAI-1 was observed, which also ultimately had an impact on protein expression. At the same time therapy regimens were found to decrease the survival rate in the treated groups. In order to investigate to which extent these two effects have an impact on the malignant tumour cells on invasion, Matrigel invasion experiments were performed. Cells were treated for 48 hours, harvested and subsequently added to serum-free medium. Then the cells were placed onto Matrigel for 24 hours in the presence of an FCS serum gradient suspended, which served as a stimulus for cell migration. Figure 6 shows the results of the invasion experiments. Treatment with decitabine resulted in an approximately 50% increase of invasive cells in MCF-7 and in opposite a 75% decrease of the immigrant cells in MDA-MB-231. With regard to the treatment with other combinations, no significant statement for MCF-7 could be made. For MDA-MB-231, however, a significant decrease in invasion ability was observed for all treatment regimens (Figure 6).

4 | DISCUSSION

Epigenetic modifications play an important role in cancer development. DNA methylation and histone acetylation have been suggested to be relatively stable and may potentially represent markers for the transport of epigenetic information through cell division. These changes affect the chromatin structure, leading to the active or repressed state of a gene to initiate or inhibit gene expression. DNA methylation-mediated gene inactivation was found in various cellular signalling pathways which influence transcription of tumour suppressor genes involved in DNA repair, hormonal signal transduction, cell adhesion, drug degradation and apoptosis. Moreover, it has been shown, that epigenetic markers can be of clinical relevance in breast cancer.

Epigenetically active agents show great potential in the individual treatment of chemotherapy-resistant breast cancers. Furthermore, extensive studies on the efficacy of decitabine and valproic acid combination therapy are reported in the literature. We examined the effects of decitabine, valproic acid, standard chemotherapy (FEC) and combinations thereof on MDA-MB-231 and MCF-7 breast cancer cells. Treatment of both cells with FEC, decitabine and valproic acid increased cell death observed with the MTT cell viability assay. We observed that cell viability was reduced by combination of the FEC...
regimen with epigenetic modulators in a synergistic or additive way. It was already shown in the literature, that the combination of decitabine or valproic acid with chemotherapeutics exhibit synergistic/additive effects against cancer cells.\textsuperscript{56,57} Also, in clinical phase studies, promising results of decitabine combination with valproic acid have been observed in patients with acute myeloid leukaemia.\textsuperscript{52,58} Furthermore, it has been shown that a combination of decitabine and valproic acid inhibits proliferation and migration in clear renal cell carcinoma and breast cancer cells.\textsuperscript{14,59}

We also investigated the role of DNA methylation of selected marker genes, relevant for tumorigenesis. LINE-1 methylation level indicates the overall genome-wide methylation by analysis of long interspersed non-coding repeats; DAPK is involved in apoptosis-relevant pathways with overexpression inducing apoptosis, whereas loss of function leads to protection against apoptosis. DAPK therefore may function as a suppressor of metastasis by inducing apoptosis.\textsuperscript{60} For example; It is known that high level of DAPK methylation is associated with invasion and metastasis in breast, testicular, bladder and lung cancer patients.\textsuperscript{61-64} DAPK has been shown to inhibit cell motility and thus metastasis by affecting integrin-mediated cell adhesion signalling pathways, especially in triple negative breast cancer.\textsuperscript{65} uPA/PAI-1 play an important role in metastasis and cell invasion and represents a predictive marker for prognosis in breast cancer on the protein level.\textsuperscript{66,67} PAI-1 in its active form binds to cell adhesion molecules and inhibits cell adhesion, changing the fate of cell migration.\textsuperscript{68,69} In addition, uPA/PAI-1 expression generally increases gradually during the progression of breast cancer. Therefore, highly invasive cells such as MDA-MB-231 used in breast cancer in-vitro models appear to have higher uPA/PAI-1 expression compared to less invasive cells such as MCF-7.\textsuperscript{70} Thus, promotor methylation levels of these genes may also be usable as tumour biomarkers or even as a therapeutic target for breast cancer using demethylating agents or histone modifiers. Treatment of MCF-7 cells with sole or combined decitabine, valproic acid and FEC regimens led to demethylation of the uPA, PAI-1 and DAPK promoter and to a demethylation of DAPK in MDA-MB-231 cells, which may lead to increased pro-apoptotic stimuli by increased DAPK expression, which was already seen in our cell viability assays. On the other side, the impact of these treatments on uPA and PAI-1 gene activity has to be further elucidated.

For this, our study also showed that in the cell culture supernatant and protein extracts from treated cells the uPA and PAI-1 protein levels increased in the FEC only treated group in MDA-MB-231 cells, whereas combination treatment with epigenetic modulators resulted in a reduction of the uPA antigen levels. For MCF-7, significant upregulation of uPA expression was associated with DAC-only treatment. Since uPA and PAI-1 protein expression is strongly associated with higher metastatic potential and invasive malignancy of the tumour cells, the above-mentioned results show a first insight into differential aspects related to the tumour progression by using standard chemotherapies alone, as well as combination therapies with epigenetic modulators. This may even be dependent on the choice of epigenetic modulator for treatment since VPA as histone deacetylase inhibitor and decitabine as demethylating agent had different impact on uPA and PAI-1 expression in the two cell lines investigated. According to the Matrigel invasion test results, which represents more or less the overall impact of all treatment-induced cell changes in these cell lines regrading invasive capability, migration and cell survival, the invasive capacity of MCF-7 cells was stimulated by decitabine, which may in part be associated with the elevation of uPA antigen levels in the DAC-treated cell lines. In contrast, in MDA-MB-231 cells, invasive capacities were strongly reduced with any treatment combination of decitabine, FEC and valproic acid, stating that (a) FEC-treatment and (b) epigenetic modulators can decrease overall invasive capacity of MDA-MB-231 cells and (c) low invasion capacity of MCF-7 cells.

For this reason, decitabine may transform MCF-7 cells into a more metastatic phenotype, but not other drug combinations. On the other side combination of FEC, decitabine and valproic acid may reduce the invasive capacity of MDA-MB-231 cells. These results show, that treatment of cells with different combinations of epigenetic modulators and/or chemotherapy can have distinctive impact on tumour properties by changing gene specific methylation and subsequent changed transcription and translation. These effects were specific on the tumour subtype (oestrogen receptor positive MCF-7 and triple-negative MDA-MB-231 cells) as was already seen in former publications of the authors group.\textsuperscript{35} Sharma et al. already stated that epigenetic mechanisms may induce drug tolerance states in cell line subpopulations, therefore treatment of resistant tumour cells with combination treatment including epigenetic modulators may warrant future clinical applications, for example, recurrent breast cancer treatment.\textsuperscript{71}

Immunocytochemical investigation of two epigenetic markers 5meC and trimethylated histone 3 lysine 27 (H3K27me3) particularly pointed out in the highly undifferentiated MDA-MB-231 cells that VPA and decitabine regimens can re-establish a more differentiated cell status.

Overall, we could see partly conflicting effects by the different treatment settings on the induction of tumour progression genes vs pro-apoptotic genes, cell survival and cell migration, for FEC treatment alone and in distinctive combinations with epigenetic modulators.

Drugs like decitabine and valproic acid may act globally as modulators of gene expression patterns, inducing or even silencing hundreds of genes depending on cell specific regulation networks. It has been shown that these drugs exerts different effects on signal transduction pathways associated with angiogenesis, apoptosis and cell survival, which still have to be elucidated in more detail.\textsuperscript{72-74} For MCF-7, no precise conclusions could be drawn (Figure 6A), however in the end, the number of invasive cells was significantly upregulated by treatment with decitabine alone, but not with other treatment regimens. In the MDA-MB-231 cell line, however, all tested therapeutic regimens reduced significantly the invasive potential (Figure 6B). As MCF-7 cells showed in general much less invasive capacity in Matrigel invasion assays, the overall effect of combination treatment may still be beneficial in a clinical setting regarding manageable invasive capacity of cancer cells. On the other side we observed an increased invasion inhibition in the highly invasive MDA-MB-231 cells, which may
warrant further investigations into clinical applications. Moreover, cell survival assays showed in most cases (FEC-containing regimens for MCF-7 and all treatment regimens for MDA-MB-231) a significantly reduced survival of the cells. Therefore, combination treatment of chemotherapy and epigenetic modulators may have the potential for clinical application for specific breast cancer subsets. One weakness of this study is that not global gene expression pattern analysis was available for these experiments which may show more insight. Detailed genomic and proteomic analyses are required to better explain the changes that will occur as a result of combinations of these epigenetic agents with chemotherapy regimens such as FEC.

In summary, our results have shown that valproic acid and/or decitabine in combination with standard chemotherapy (FEC) inhibit growth of MCF-7 and MDA-MB-231 cells and show a significant reduction of the invasive potential for most treatment settings, especially for MDA-MB-231 cells. Upon combination therapy, reduction of uPA, PAI-1 protein levels and immunocytochemical findings are in line with the reduced invasive potential. In addition, decrease in DAPK hypermethylation levels indicates that apoptosis is induced.

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CONFLICT OF INTEREST
Rudolf Napieralski and Viktor Magdolen is an equity owner of Therawis Diagnostics GmbH. Rudolf Napieralski is since 2016 affiliated with Therawis Diagnostics GmbH. All other authors have no conflict of interest.

DATA AVAILABILITY STATEMENT
All data included in this study are available upon request by contact with the corresponding author.

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REFERENCES


