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PRECLINICAL STUDY

Rationally derived drug combinations with the novel Mcl-1 inhibitor EU-5346 in breast cancer

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Abstract

Purpose Recent studies have emphasized a key role for the anti-apoptotic Bcl-2 family member Mcl-1 in conferring tumor cell survival and drug resistance in breast cancer (BC). Mcl-1 inhibitors, such as the BH3-mimetic EU-5346, therefore represent an exciting new class of targeting agents and are a current focus of widespread cancer-drug development efforts.

Methods ONCOMINE analysis was utilized to compare expression profiles of Bcl-2 family members across all major BC subgroups. Potential toxicities of EU-5346 were evaluated using iPS-generated cardiomyocytes, blood cells and astrocytes. The anti-BC cell activity of EU-5346-based therapies was evaluated using [^{3}H]-thymidine uptake and spheroid-forming assays as well as immunoblotting and the Chou-Talalay method. Protein level-based activity of EU-5346, the specific anti-Bcl-2 inhibitor ABT-199 and the specific anti-Bcl-x_L inhibitor WEHI-539 was verified in Mcl-$1^{\Delta/null}$ versus Mcl-$1^{wt/wt}$ MEFs.

Results We previously demonstrated significant anti-tumor activity of EU-5346 in all BC subtypes. Our present results go further and suggest that EU-5346 may induce limited adverse events such as cardiotoxicity, hematotoxicity, and neurotoxicity, frequently observed with other BH3 mimetics. As demonstrated by our mathematical scoring model, the prediction of EU-5643-induced IC_{50} not only relies on the protein level of Mcl-1 but also on Bak, Bim, and Noxa. Synergistic anti-BC activity of low-dose EU-5346 with the BH3 mimetics ABT-199 or WEHI-539 was observed only in those BC cells expressing Bcl-2 or Bcl-x_L, respectively. Similarly, when combined with tamoxifen or trastuzumab, low-dose EU-5346 induced significant anti-BC activity in hormone receptor positive or Her2-positive BC cells, respectively. Finally, EU-5346 in combination with paclitaxel induced synergistic anti-BC activity in both paclitaxel-sensitive and paclitaxel-resistant TNBC cells.

Conclusion These data strongly support the further clinical development of EU-5346 to improve BC patient survival.

Keywords Breast cancer · Mcl-1 · BH3 mimetics · Mathematical scoring model · Combination therapies

Abbreviations

BC	Breast cancer
TN	Triple negative

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MOMP	Mitochondrial outer membrane permeabilization
Mcl-1	Myeloid cell leukemia-1
BH3	Bcl-2 homology 3
TCGA	The Cancer Genome Atlas (TCGA)
HR	Hormone receptor
iPS	Induced pluripotent stem cell
MEFs	Murine embryonic fibroblasts
PBMCs	Peripheral blood mononuclear cells

Introduction

Despite significant therapeutic progress during the last two decades, metastatic breast cancer (BC) remains one of the leading causes of cancer-related mortality in women, mainly due to the development of resistance to conventional therapies. Based on gene expression profiling BC is stratified into at least four subgroups luminal A, luminal B, human epidermal growth factor receptor 2 (Her2)-amplified and basal-like (predominantly triple negative, TN) predictive for response and resistance to therapy, occurrence of metastasis and therefore overall survival [1].

Escape of apoptosis is a hallmark of cancer. The upregulation of anti-apoptotic proteins of the Bcl-2 family (e.g., Bcl-2, Bcl-xL and Mcl-1), which typically possess four Bcl-2 homology (BH1-BH4) domains, is the most prominent way for cancer cells to evade cell death [2–4]. They sequester pro-apoptotic "effectors" (Bax and Bak) and/or "activators" (e.g., Bim, Bid) of the Bcl-2 family of proteins via binding to their BH3 domain and therefore prevent high-ordered Bax/Bak-oligomerization, mitochondrial outer membrane permeabilization (MOMP), the release of mitochondrial cytochrome c and SMAC/DIABLO into the cytoplasm, formation of the apoptosome, caspase activation and ultimately cell apoptosis. An additional group of Bcl-2 family proteins, the so-called sensitizers (e.g., Puma, Noxa), bind anti-apoptotic proteins via their single Bcl-2 homology 3 (BH3) only protein and act as their selective antagonists. The development of BH3 mimetics that mimic the action of certain BH3-only proteins (e.g., Bim) and therefore restore apoptosis has evolved as one of the most promising strategies in current cancer therapy [5].

The anti-apoptotic Bcl-2 family member Myeloid cell leukemia-1 (Mcl-1) is characterized by its wide intracellular localization; its short half-life; and its structure. Specifically, Mcl-1 contains two polypeptide sequences enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST), which are responsible for proteasome-dependent Mcl-1 degradation and its localization, thus providing the base for the fine-tuned Mcl-1 protein functions in response to environmental stimuli and the cellular origin [6–8]. The pro-survival function of Mcl-1 is predominantly mediated by its binding to Bak and Bim.

Conversely, release of Bak or Bim from their interaction with Mcl-1 [9] as well as binding to NOXA enhances Mcl-1 degradation followed by induction of apoptosis [10, 11].

To target Bcl-2 and Bcl-x_L up to now BH3 mimetics have predominantly been developed. Although the Bcl-2/ Bcl-x_L/ Bcl-w inhibitor ABT-263 (navitoclax) showed an objective response of 31% in relapsed or refractory CLL patients, grade III-IV thrombocytopenia occurred in 41% of patients. The effects of ABT-263 on platelets are primarily due to inhibition of Bcl-x_L. The most promising Bcl-2 inhibitor to date is the specific Bcl-2 inhibitor ABT-199 (venetoclax/ Venclyxto®). Based on an ORR of 79% it has been approved by the US FDA for the treatment of CLL patients with 17p deletion who have received at least one prior therapy. ABT-199 is currently validated in advanced clinical trials also in AML, NHL, MM and solid tumors. Of note, although the MCL-1 region is one of the most amplified gene regions in the genome of BC and other hematologic and solid malignancies, no Mcl-1 inhibitor has yet entered clinical practice. The clinical development of Mcl-1 inhibitors has especially been challenged by the rigid hydrophobic binding groove relatively flat and large interfaces [12, 13]. However, recent progress has led to the development of promising Mcl-1 inhibitors including A1210477 [14, 15], UMI-77 [16], and S63845 [17]. Another small molecule BH3 mimetic, which disrupts Mcl-1 function with higher affinity when compared to other BH3 mimetics and overcomes resistance to available Bcl-2 family inhibitors [18, 19] is the hydroxyquinoline-derived, small molecule EU-5346 (also known as ML311, Eutropics Pharmaceuticals, Cambridge, MA). EU-5346 is a potent inhibitor of Mcl-1/Bim interaction that shows selective activity against Mcl-1 primed cells [20, 21].

In BC, our own previous and present as well as other investigators' data show consistently high Mcl-1, but variable Bcl-2 and Bcl-x_L protein levels in cell lines of different BC subtypes, indicating that Mcl-1 is the predominant pro-survival protein in BC [22–25] (Fig. 1a). Mcl-1 mediates inherent as well as acquired resistance of tumor cells against widely used anti-BC therapies including tamoxifen, trastuzumab, paclitaxel, vincristine and gemcitabine; but also BH3 mimetics [22, 26–28]. Importantly, due to low affinities ABT-263 and ABT-199 do not block Mcl-1 activity [29–31]. Taken together, preclinical evidence strongly suggests that Mcl-1 is a promising target for BC therapy.

Here we investigated the tolerability and the ability of the small molecule EU-5346 in a panel of BC cell lines to increase the anti-BC activity and/or to overcome resistance against conventional BC therapies. The ultimate aim of this study is to aid in the design of Mcl-1 inhibitor-based clinical trials in BC, patient selection and biomarker identification.

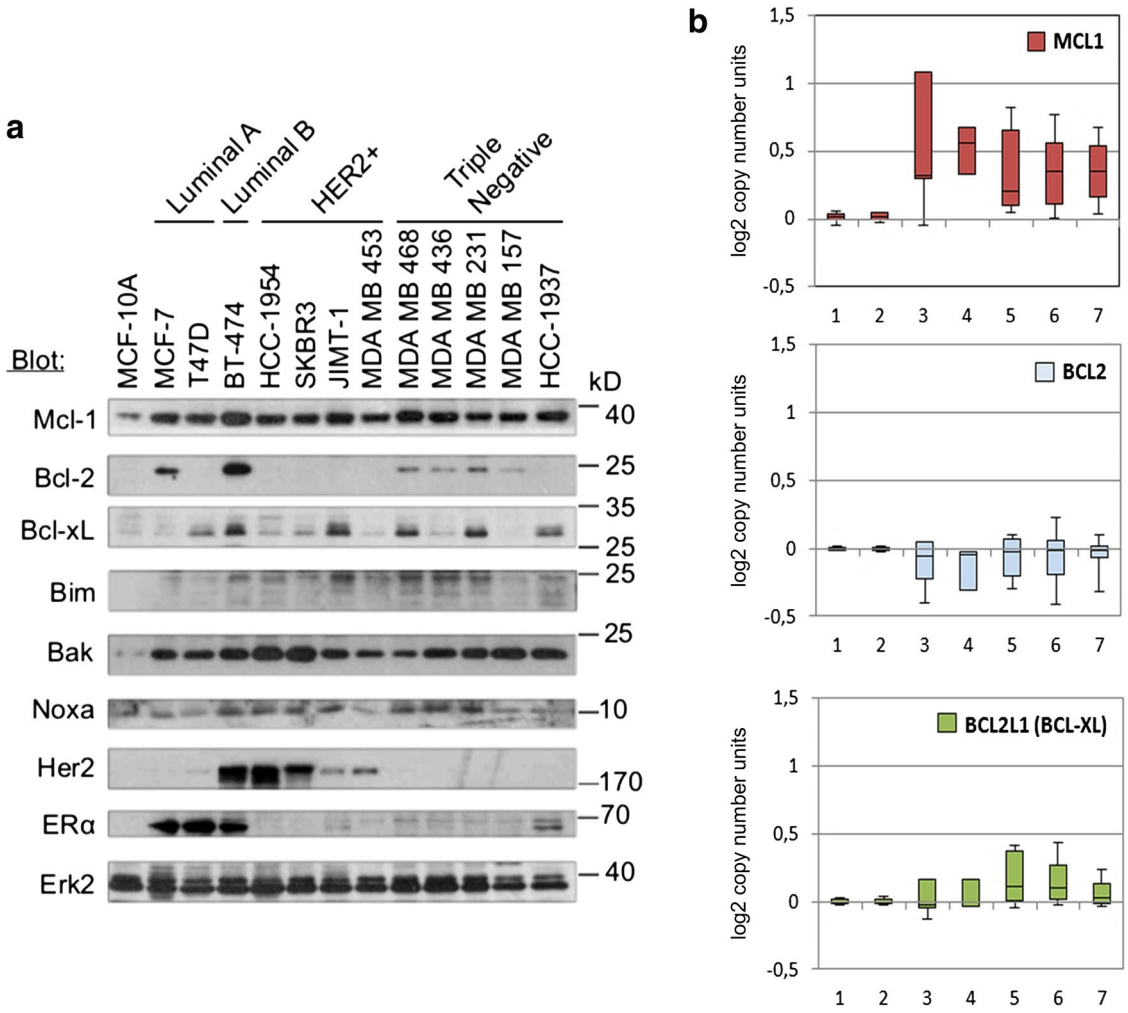

Fig. 1 Expression profile of Bcl-2 family members in the 4 major BC cell subtypes. **a** Bcl-2 family protein levels in BC cell lines representative of luminal A, luminal B, Her2-positive, and triple negative BC. Whole-cell extracts were analyzed by immunoblotting with indicated antibodies. Immunoblotting for Erk2 confirmed equal protein loading. **b** Mcl-1 gene amplification is common in BC. The Cancer Genome Atlas (TCGA) dataset on BC subtypes was grouped and assessed DNA copy number (expressed as a log2 scale) of Mcl-1, Bcl-2, and Bcl-x_L using ONCOMINE database. *1* blood ($n=702$); *2* normal breast tissue ($n=111$); *3* ductal breast carcinoma ($n=5$); *4* invasive ductal and invasive lobular breast carcinoma ($n=3$); *5* invasive ductal and lobular carcinoma ($n=14$); *6* invasive ductal breast carcinoma ($n=639$); *7* invasive lobular breast carcinoma ($n=71$). In the box plot data represent median, first and third quartile. The whiskers indicate the 10th and 90th percentiles

Materials and methods

Materials

WEHI-539, ABT-263 (Navitoclax) and ABT-199 (Venetoclax, Venclyxto®) were purchased from MedchemExpress (Princeton, NJ, USA); tamoxifen from Sigma-Aldrich (Taufkirchen, Germany); and trastuzumab (Herceptin®) from Roche (San Francisco, CA, USA). Antibodies against human Bak (G-23), Bcl-2 (C2), Bcl-x_L (H-5), Bim (H-191), ERK2 (C-14), ERα (HC-20), Mcl-1 (S-19), and NOXA (FL-54) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany); antibodies against Her2/ErbB2 (D8F12), and PARP from Cell Signaling Technology (Boston, MA, USA); and the antibody against murine Mcl-1 from Rockland Immunochemicals (Gilbertsville, PA, USA).

Cell culture and conditions

MCF-7, T47D, HCC-1954, MDA MB 231, MDA MB 468, MDA MB 436, MDA MB 157, HCC-1937 cells purchased all from ATCC were cultured in RPMI 1640 medium (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; PAA Laboratories, Cölbe, Germany), 1% penicillin/streptomycin and 2 mM L-glutamine (all from Gibco, Life Technologies,

Grand Island, NY). MDA MB 453, JIMT-1, U87MG, C8-D1A and CAL27 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin and 2 mM L-glutamine. BT-474, SKBR3 cells were maintained similarly to MDA MB 453 cells, but with the addition of Minimum Essential Medium (MEM) nonessential amino acids (Gibco, Life Technologies, Grand Island, NY). MCF-10A cells were maintained in DMEM/F12 supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 2.5 mg Insulin, 5 mg Hydrocortisone (Sigma), 8 µl Cholera toxin, and 10 µg hEGF (Sigma-Aldrich; Schnelldorf, Germany). MEF cell lines Mcl-$1^{wt/wt}$ and Mcl-$1^{\Delta/null}$ were generated by SV40 large T transformation followed by Tet-Cre-mediated deletion. Single cell clones were selected and then grown in DMEM supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, 2-mercaptoethanol (Sigma-Aldrich; Schnelldorf, Germany) and Minimum Essential Medium (MEM) nonessential amino acids (Gibco, Life Technologies, Grand Island, NY, USA) from early passages. Both Mcl-$1^{wt/wt}$ and Mcl-$1^{\Delta/null}$ were extensively characterized as being hypersensitive to various death stimuli; with restorable resistance upon re-expression of Mcl-1 in Mcl-$1^{\Delta/null}$ cells [32].

Cor.4U® cardiomyocytes were derived from transgenic human induced pluripotent stem cells (iPS) cells, which express a puromycin-resistant gene under control of a cardiac-specific promoter (MHC promoter). Cor.4U® cardiomyocytes were cultured in Cor.4U® culture medium at 37 °C, 95% humidity and 7% CO_2 as described in to the handling protocol (Axiogenesis, Cologne, Germany).

Cell lysis and immunoblotting

Treated or untreated cells were washed three times with phosphate-buffered saline and lysed with either lysis buffer (10 mm Tris, 50 mm NaCl, Na-pyrophosphate, 1% Triton, 30 mM sodium pyrophosphate, pH 7.05) or radioimmune precipitation assay (RIPA) lysis buffer (150 mM NaCl, 10 mM Tris pH7.2, 0.1% SDS, 1% Triton X-100, 1% Deoxycholate, 5 mM EDTA) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Pierce, Darmstadt, Germany). Insoluble material was removed by centrifugation (15,000 rpm for 30 min at 4 °C). For Western blotting, cell lysates (10–100 µg/lane) were separated by 8 or 10% SDS-PAGE prior to electrophoretic transfer onto Hybond™-C super nitrocellulose membranes (Amersham, Arlington Heights, IL, USA). After blocking with 5% nonfat milk in phosphate-buffered saline-Tween® 20 buffer at room temperature for 1 h, membranes were sequentially blotted with the indicated specific primary antibodies and then with horseradish peroxidase-conjugated secondary mouse or rabbit antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) and were developed using chemiluminescence (Amersham, Arlington Heights, IL, USA) [33].

Bioinformatics

Copy number variation (CNV) data from tumor tissues for invasive ductal and lobular breast cancer were obtained from The Cancer Genome Atlas (TCGA) dataset (http://cancergenome.nih.gov/). CNV segmentation data were retrieved and processed using the Oncomine database (https://www.oncomine.org/resource/main.html). CNV values > 0 on a log2 scale are defined as amplification, whereas values < 0 on a log2 scale are defined as deletions [34].

iPS-derived cardiomyocyte beating parameters

Induced pluripotent stem cell (iPS)-derived Cor.4U® cardiomyocytes were seeded at 30,000 cells/well on a fibronectin-coated E-plate Cardio in Cor.4U® culture medium. Prior to seeding, a background impedance measurement (plate plus medium, but w/o cells) was taken. EU-5346 was added at indicated concentrations. Treatment was started after approximately 72 h of culture, and standard compound treatment duration was up to 72 h. Data for evaluation of beating parameters were collected at indicated time points. Data on cardiomyocyte beating parameters were generated based on changes in the impedance measured with gold microelectrodes fabricated in the bottom of the wells of a 96 well microtiter plate (E-Plate cardio 96 as part of the xCELLigence® RTCA Cardio instrument). The number of cells, the attachment quality, cell morphology and physiology of the cell membrane all affect the degree of cyclic changes in impedance (Δ Cell index). All measurements were taken at 37 °C in a cell culture incubator, allowing for spontaneous beating of cardiomyocytes at physiologic conditions. Data acquisition was performed using RTCA Cardio software 1.0 using 20 s sweeps at an interplant interval of 12.9 ms frequency. Standard parameters analyzed included total cell index "CI", amplitude of contraction and beating frequency. The amplitude was calculated as the CI value from baseline (negative peak) to peak (positive peak) maximum (ΔAmplitude). The beating frequency was calculated as the number of peaks in a time period (negative peak to negative peak) (Δ Beat rate) [35].

Cell death assays

Cytotoxic effects of EU-5346, ABT-199, and WEHI-539 on PBMC or paclitaxel on MDA MB 231PacR cells were assessed using an MTS® assay, according to manufacturer's instructions (Invitrogen, Darmstadt, Germany).

Proliferation assay

Proliferation was measured by the incorporation of 0.5 µCi/well [^{3}H]-thymidine (Perkin Elmer, Baesweiler, Germany) during the last 10 h of 48 h-experiments. Radioactive labeling was determined by harvesting the cells onto glass-fiber filtermates (Perkin Elmer, Baesweiler, Germany) with an automatic cell harvester (Tomec Harvester 96, Hamden, CT, USA) and counting using the Wallac Trilux Betaplate scintillation counter (Perkin Elmer, Baesweiler, Germany).

Spheroid formation assay

Single multicellular BC spheroids were formed as previously described [36]. Briefly, $4–6 \times 10^3$ BC cells were seeded on agarose (Sigma-Aldrich, Steinheim, Germany)-coated 96-well plates. At the end of experiments, spheroids were stained with Calcein AM (green) (Sigma-Aldrich, Steinheim, Germany) and Propidium Iodide (red) (Sigma-Aldrich, Steinheim, Germany). For image capturing, the Olympus CKX41 microscope using 4x and 10x magnification was connected to an Olympus XC30 digital camera and exported to the cellSens standard software (version 1.4). Based on the automatic measurement of the radius, volumes of the spheroids were calculated ($V = 4/3\pi r^3$).

Isobologram analysis

The combinatorial anti-BC activity of EU-5346 with ABT-199, WEHI-539, tamoxifen or paclitaxel were analyzed using the CompuSyn software program (from CompuSyn, Inc., http://www.combosyn.com/), which is based on the Chou-Talalay method according to the following equation: $CI = (D)1/(Dx)1 + (D)2/(Dx)2 + (D)1(D)2(Dx)1(Dx)2$, where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have x effect when used in combination, and (Dx)1 and (Dx)2 are the doses of drug 1 and 2 that have the same x effect when used alone [37]. Data from [^{3}H]-thymidine uptake assays were expressed as fraction of cells with growth affected (FA) in drug-treated *versus* untreated cells. When combination index (CI) = 1, this equation represents the conservation isobologram and indicates an additive effect. CI < 1 indicates synergism; CI > 1 indicates antagonism.

Statistical analysis

Statistical significance of differences observed in treated *versus* control cultures was determined by means of an unpaired Student *t*-test. Statistical analyses, including multiple linear regression model, were performed using SigmaPlot version 12.5 software. The minimal level of significance was $p < 0.05$.

Results

Profiling of Bcl-2 family members in BC subtypes

In order to gain more insight into the pathophysiologic function of Bcl-2 family members in breast cancer (BC), we first determined the expression profiles of the anti-apoptotic guardian proteins Bcl-2, Bcl-x_L and Mcl-1; the pro-apoptotic effector proteins Bax and Bak; and the BH3 only sensor proteins Bim and Noxa in cell lines representative of the major BC subtypes luminal A, luminal B, Her2-positive and triple negative (TN) *versus* the nonmalignant MCF-10A cell line. In agreement with our own and other previous results [21, 23, 24], high Mcl-1 protein levels were consistently observed across all BC subtypes. In contrast, protein levels of Bcl-2 and Bcl-x_L were variable (Fig. 1a). Analysis of The Cancer

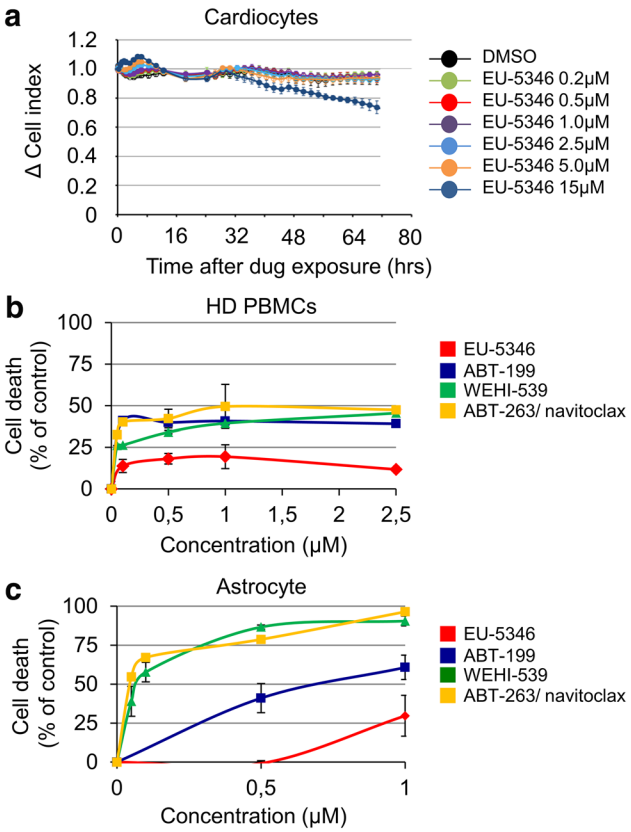

Fig. 2 EU-5346-induced cardiotoxicity, hematotoxicity and neurotoxicity. **a** Contractile property of iPS-derived Cor.4U cardiomyocytes in response to EU-5346 (ΔCell index). Each circle represents the mean of 3 wells (with error bars ± standard deviation) of three independent experiments at individual time points. CI, Cell Index, overall measure of the change in the electrical resistance across the plate. **b** Toxic effect of inhibitors of the Bcl-2 family on PBMCs isolated from a healthy donor (HD PMSCs). **c** Toxic effect of inhibitors of the Bcl-2 family on the astrocyte/ microglial cell line C8D1A. **b, c** EU-5346-, ABT-199-, WEHI-539- and ABT-263-induced apoptosis at indicated doses was assessed after 48 h utilizing an MTS assay. Data represent mean ± standard deviation for triplicate samples. Results shown are representative of 3 independent experiments

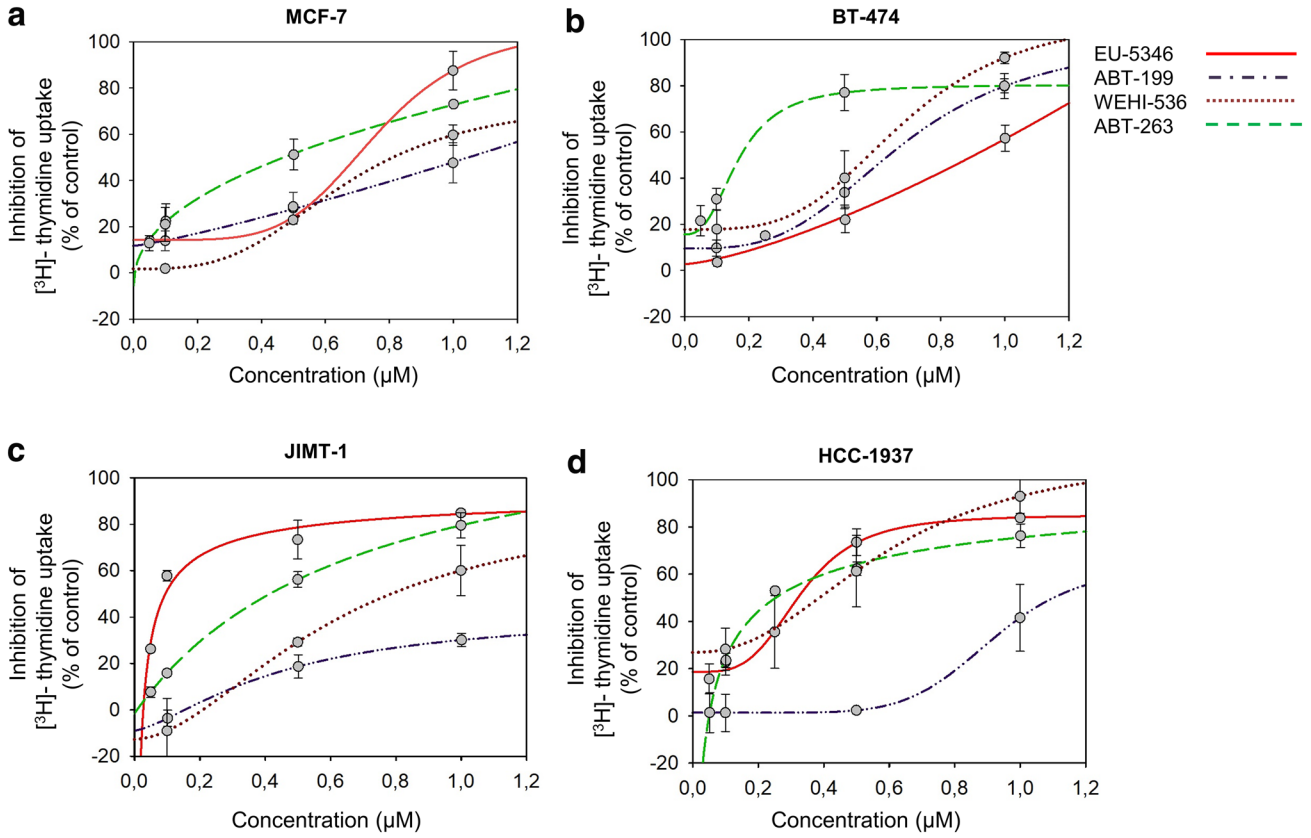

Fig. 3 Protein levels of Bcl-2 and Bcl-x_L predict anti-tumor activity of ABT-199, ABT-263 and WEHI-539 in the major BC subtypes. BC cell lines representative of **a** luminal A (MCF-7), **b** luminal B (BT-474), **c** Her2-positive (JIMT-1) and **d** triple negative BC (HCC-1937) were treated with ABT-199, WEHI-539, ABT-263 or EU-5346. Proliferation was measured using [^{3}H]-thymidine uptake during the last 10 h of 48 h cultures. Data represent mean ± standard deviation for quadruplicate samples. Results shown are representative of 3 independent experiments

Genome Atlas (TCGA) dataset 2 on BC subtypes confirmed Mcl-1 gene amplification across specimens isolated from more than 700 patients with ductal, invasive ductal and lobular BC *versus* low expression in blood as well as breast cells derived from healthy donors (Fig. 1b). In summary, these data indicate a key function for Mcl-1 across all BC subtypes, emphasizing its role as a potential therapeutic target.

EU-5346 cardiotoxicity, hematotoxicity and neurotoxicity

The hydroxyquinoline-derived, small molecule EU-5346 (also ML311, Eutropics Pharmaceuticals, Cambridge, MA), a potent inhibitor of Mcl-1/ Bim interaction EU-5346 is among the most promising Mcl-1 inhibitors [20, 38]. Our previous results demonstrated anti-BC activity of EU-5346 not only in Her2-positive but also in hormone receptor (HR) positive and TN BC cells [21]. However, whether adverse side effects such as cardiotoxicity, hematotoxicity, neurotoxicity and potential inhibition of mitochondrial respiration may compromise the clinical usability of EU-5346 is

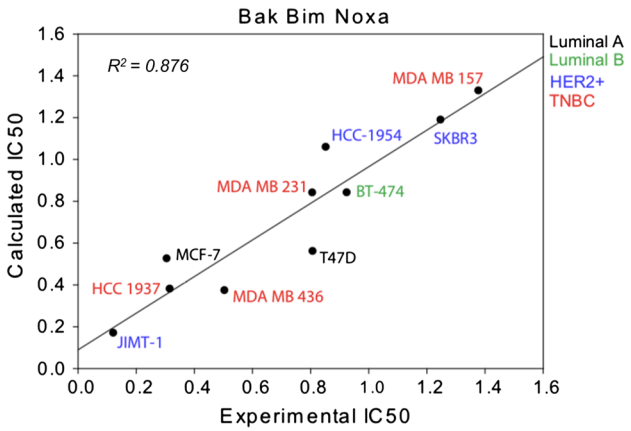

Fig. 4 A combined index of multiple Bcl-2 family proteins predicts sensitivity to EU-5346. Cell proliferation in BC cell line cells representative for Luminal A (black), Luminal B (green), Her2- or TN (red) BC was determined after EU-5346 treatment by [^{3}H]-thymidine uptake and blotted against the multiple linear regression equation $IC50 = -0.880 + (2.278 \times Bak) - (1.940 \times Bim) + (1.200 \times Noxa)$ ($R^2 = 0.676$, $p < 0.001$)

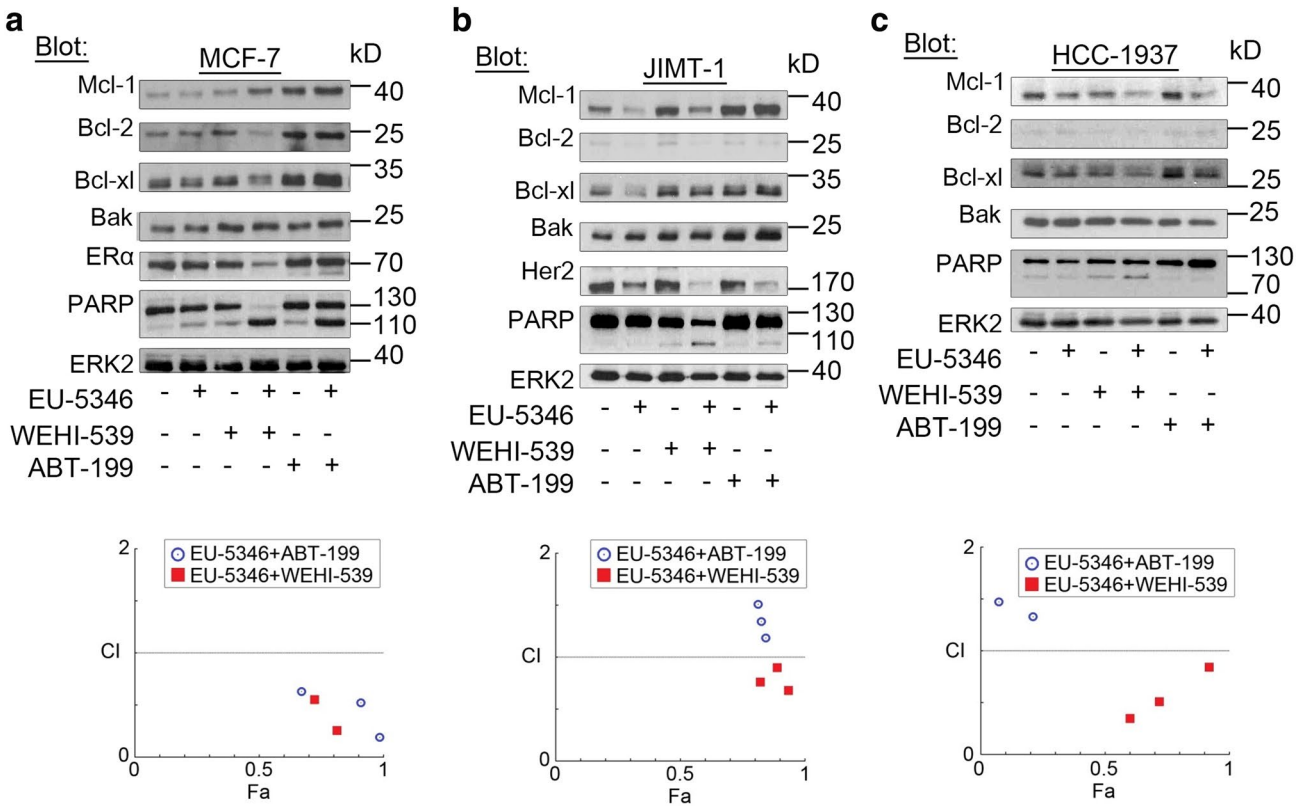

Fig. 5 Anti-BC cell activity of a rationally derived combinations of EU-5346 with WEHI-539 or ABT-199. BC cell lines representative for ER-positive (**a**), Her2- positive (**b**) and TN (**c**) BC subtypes were treated with EU-5346, WEHI-539, and ABT-199 alone or in combination. Whole-cell extracts were analyzed by immunoblotting with indicated antibodies. Immunoblotting for Erk2 confirmed equal protein loading (upper panels); kD kilodalton (**a**–**c**, upper panels). Proliferation was measured using [^{3}H]-thymidine uptake during the last 10 h of 48 h cultures. Fa indicates the fraction of cells with growth affected in drug-treated *versus* untreated cells. CI indicates the combination index. Data represent mean ± standard deviation for quadruplicate samples. Results shown are representative of 3 independent experiments (**a**–**c**, lower panels)

unknown [32, 39–41]. In order to test the potential cardiotoxic effect of EU-5346, we evaluated the effects of EU-5346 on the spontaneous beating characteristics of human induced pluripotent stem cell (iPS)-derived Cor.4U® cardiomyocytes as measured by impedance analysis including cellular index (ΔCell index), cardiomyocyte beating frequency (ΔBeat rate), and amplitude of contraction (ΔAmplitude). With the exception of the highest concentration of EU-5346 (15 µM), we did not observe a concentration-dependent hypertrophic-like response on cardiocytes until up to 72 h of treatment as assessed by determination of the Cell index (CI) (Fig. 2a). Indeed, only highest nonphysiologic doses of EU-5346 immediately slowed the beat rate (Supplemental Fig. 1a) and reduced the amplitude (Supplemental Fig. 1b), while low-dose EU-5346 slowed the beat rate and reduced the amplitude only after a prolonged duration of treatment at a very modest level (Supplemental Fig. 1b). In addition, signals consistent with a blockade of the ether-a-go-go-related gene 1 (hERG), sodium or calcium channel (or trafficking deficits associated with arrhythmias) were not observed upon treatment with EU-5346 (data not shown).

To evaluate EU-5346- versus ABT-199-, WEHI-539- and ABT-263-induced hematotoxicity, peripheral blood mononuclear cells (PBMCs) from a healthy donor were exposed to different doses of these inhibitors, and cell death was determined using an MTS assay. Compared to the Bcl-2 specific inhibitor ABT-199, the Bcl-x_L specific inhibitor WEHI-539, and the Bcl-2/ Bcl-x_L/Bcl-w specific inhibitor ABT-263, EU-5346 induced significantly less cell death (Fig. 2b). Finally, in order to investigate the potential neurotoxicity of these compounds the astrocyte cell line C8D1A was exposed to ABT-199, WEHI-539, ABT-263 and EU-5346. Similarly to hematotoxicity, EU-5346 induced significantly less cell death when compared to ABT-199, WEHI-539 and ABT-263 (Fig. 2c). Taken together these data suggest that EU-5346 may have tolerable cardio and neurotoxicity.

Anti-tumor activity of ABT-199, ABT-263, and WEHI-539 correlates with protein levels of Bcl-2 and Bcl-x_L

The optimal use of BH3 mimetics requires the accurate prediction, on which anti-apoptotic Bcl-2 proteins tumor cell survival depends. We therefore next sought to verify whether protein levels of Mcl-1, Bcl-2 and Bcl-x_L correlate with responses to EU-5346, as well as to ABT-199, ABT-263, and WEHI-539. In contrast to Mcl-1, Bcl-2 as well as Bcl-x_L are expressed in both Mcl1$^{\Delta/null}$ as well as Mcl-1$^{wt/wt}$ Murine Embryonic Fibroblasts (MEFs). Based on protein levels, ABT-199 as well as WEHI-539, but not the Mcl-1 inhibitor EU-5346, was active in both Mcl1$^{\Delta/null}$ and Mcl-1$^{wt/wt}$ MEFs, as expected. Correlating with higher Bcl-2 protein levels, the selective Bcl-2 inhibitor ABT-199 had a slightly higher anti-proliferative activity in Mcl-1$^{wt/wt}$ versus Mcl1$^{\Delta/null}$ MEFs. In contrast, correlating with lower Bcl-x_L protein levels, the selective Bcl-x_L inhibitor WEHI-536 had a slightly lower anti-proliferative activity in Mcl-1$^{wt/wt}$ versus Mcl1$^{\Delta/null}$ MEFs (Supplemental Fig. 2a).

We next treated various BC cell lines including MCF-7 (Fig. 3a), BT-474 (Fig. 3b), JIMT-1 (Fig. 3c) and HCC1937 (Fig. 3d) with ABT-199, ABT-263, WEHI-539 and EU-5346. Anti-tumor activity of ABT-199, ABT-263 and WEHI-539 was observed in correlation with protein levels of Bcl-2 and/ or Bcl-x_L. Specifically, low anti-tumor activity of ABT-199 but high anti-tumor activity of WEHI-539 was observed in JIMT-1 and HCC-1937 cells (Fig. 3c, d), in which Bcl-2 protein levels are low, and Bcl-x_L protein levels are high. The higher efficacy of ABT-263 compared to ABT-199 or WEHI-539 treatment is most likely due to its dual simultaneous inhibition of Bcl-x_L and Bcl-2. Taken together, these data suggest that the protein expression of Bcl-2 family members may, at least in part, predict the drug response of corresponding BH3 mimetics within BC cells.

Development of a therapeutically relevant mathematical scoring system, which predicts IC50 of EU-5346 across all BC subtypes

Despite high protein levels of Mcl-1 across all cell lines used in this study, we observed a wide range of sensitivity to EU-5346 (Fig. 3). Bcl-2 family members work in concert with each other to induce apoptosis; consequently, expression levels of single Bcl-2 family members only weakly correlate with the anti-apoptotic effect of Mcl-1 inhibitors [42, 43]. We therefore next sought to correlate and predict the response to EU-5346 in BC cells based on a more holistic approach by also including levels of Bcl-2 family proteins other than Mcl-1 [44]. Specifically, protein levels of Bcl-2, Bcl-xL, Mcl-1, Noxa, Bim and Bak in BC cell lines were analyzed by immunoblotting and quantification by densitometric analysis (Fig. 1a). Using data obtained from seven BC cell lines, we then generated an equation by using a multiple linear regression model [$y = b + m1 \times (P1) + m2 * (P2) + m3 \times (P3)$], where [y] is the IC50 after treatment with EU-5346, and (P1-3) are the protein levels normalized to tubulin from western blot analyses. A least-squares-fit of this equation was used to obtain the constant factors *b* and m 1–3. The predictive ability of the resulting equation [IC50 $= -0.880 + (2.278 \times Bak) - (1.940 \times Bim) + (1.200 \times Noxa)$] was confirmed by plotting experimental and calculated IC50 of EU-5346 in six additional BC cell lines. The pro-apoptotic Bcl-2 protein Bak was chosen due to its key role to depolarize mitochondria after Mcl-1 antagonization; Bim and Noxa were chosen due to their pivotal role of a general and Mcl-1-specific antagonizing BH3 only protein, respectively. The resulting fit had a coefficient of determination

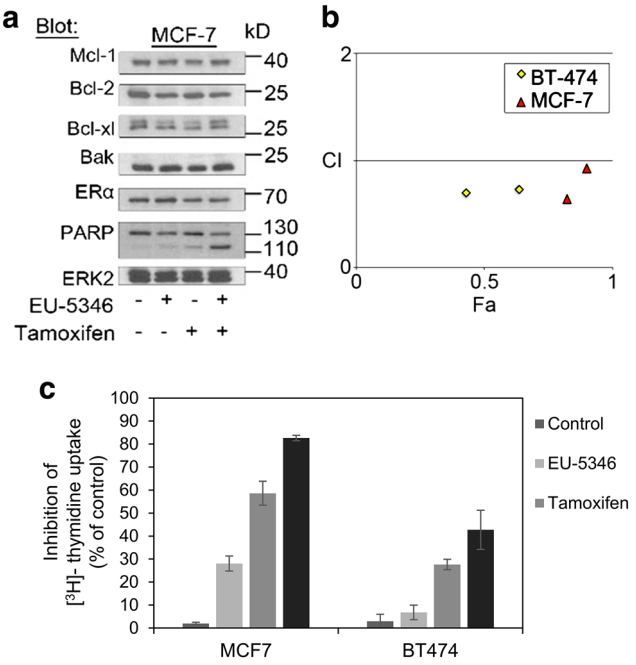

Fig. 6 Synergistic anti-BC cell activity of EU-5346 in combination with tamoxifen. **a** ER-positive MCF-7 cell line cells were treated with EU-5346 alone or in combination with tamoxifen. Whole-cell extracts were analyzed by immunoblotting with indicated antibodies. Immunoblotting for Erk2 confirmed equal protein loading. **b** Synergistic activity of EU-5346 and tamoxifen in MCF-7 or BT-474 cells. MCF-7 or BT-474 cells treated with EU-5346 or left untreated were exposed to tamoxifen. Proliferation was measured using [^{3}H]-thymidine uptake during the last 10 h of 48-h cultures. Fa indicates the fraction of cells with growth affected in drug-treated versus untreated cells. CI indicates the combination index. Data represent mean ± standard deviation for quadruplicate samples. Results shown are representative of 3 independent experiments (right panels). **c** ER-positive MCF-7 and BT-474 BC cell lines were treated with EU-5346 0.1 µM and/or tamoxifen 0.5 µM. Proliferation was measured using [^{3}H]-thymidine uptake during the last 10 h of 48 h cultures. Data represent mean ± standard deviation for quadruplicate samples. Results shown are representative of 3 independent experiments

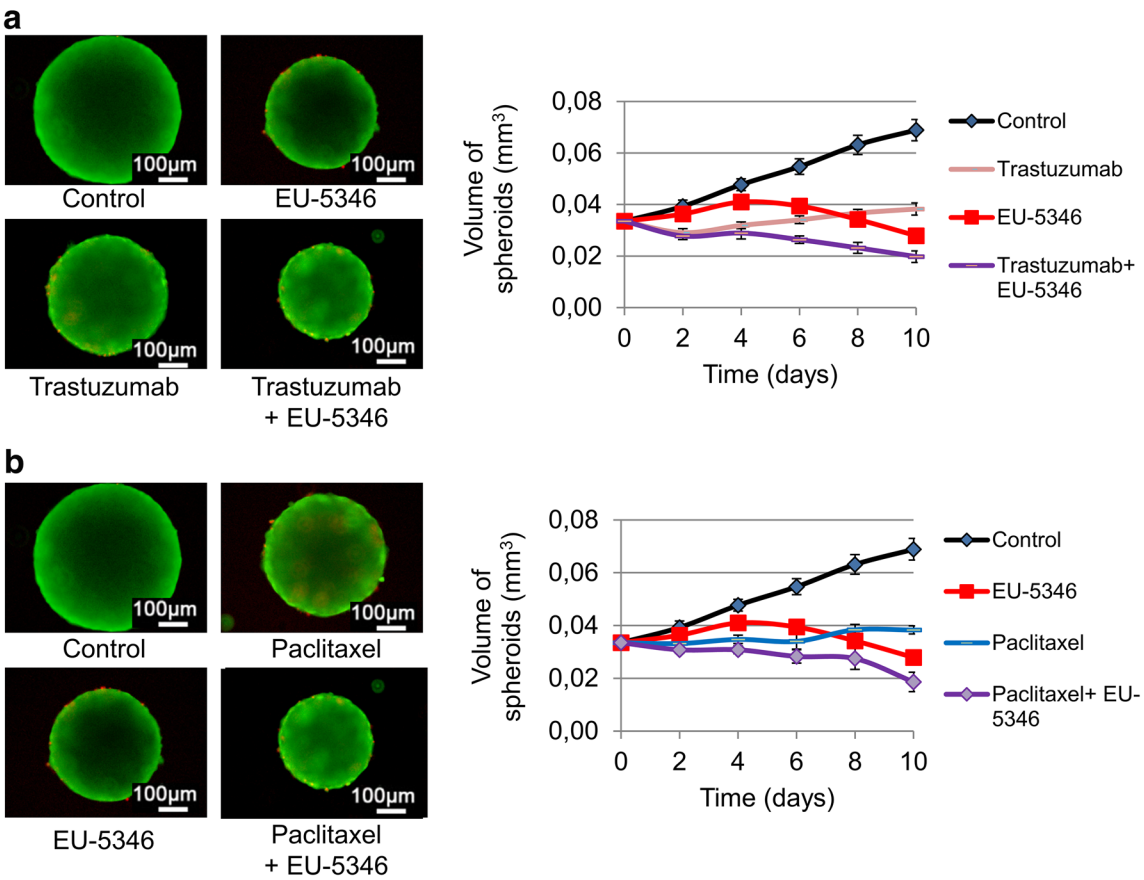

Fig. 7 Anti-BC cell activity of EU-5346 in combination with trastuzumab or paclitaxel. **a**, **b** Anti-BC activity of trastuzumab (**a**) or paclitaxel (**b**) in combination with EU-5346 in BT-474 cell line cells. 5.5×10^3 BT-474 cells were seeded per well in agar-coated 96-well plates prior to EU-5346 treatment. Spheroid formation was assessed in EU-5346- and/or trastuzumab (**a**)- and/or paclitaxel (**b**)-treated and control cells using an inverted fluorescence light microscope at indicated time points. Photographs (×10 magnification) of spheroid formation are representative of each group and three independent experiments (left). Spheroid volumes were calculated as described in "Material and Methods". Data represent mean ± standard deviation for triplicate samples. Results shown are representative of 3 independent experiments (right)

R^2 of 0.876 ($p < 0.05$) (Fig. 4). Importantly, the inclusion of Bcl-x_L or Mcl-1 had no significant effect on the correlation (data not shown). Removing Bak and Bim had the largest effect on R^2, followed by Noxa as the least significant contributor. The anti-proliferative effect of EU-5346 was independent of the BC subtype (Fig. 4).

Anti-BC cell activity of a rationally derived combinations of EU-5346 with WEHI-539 or ABT-199

Since Mcl-1 mediates resistance against widely used BC therapies (e.g., tamoxifen, paclitaxel, gemcitabine, vincristin) as well as specific BH-3 mimetics [26, 45, 46], treatment with an Mcl-1 inhibitor may re-sensitize tumor cells in general and BC cells in particular, to BH3 mimetics. Moreover, due to their functional redundancy, the combination of Mcl-1 with other Bcl-2/ Bcl-x_L inhibitors or pan-active Bcl-2 inhibitors with at least some activity against Mcl-1 are likely to achieve higher response rates than targeting one individual member of the Bcl-2 subfamily.

We therefore first evaluated the anti-BC activity of rationally derived combinations of low-dose EU-5346 (dose < IC50) with Bcl-2 and/or Bcl-x_L inhibitors in three BC cell lines. While the combination of EU-5346 and WEHI-539 induced synergistic anti-tumor activity in MCF-7 (Fig. 5a), JIMT-1 (Fig. 5b) and HCC-1937 (Fig. 5c) cells, indicating that these cell lines depend on both Mcl-1 and Bcl-x_L; the combination of EU-5346 and ABT-199 induced synergistic anti-tumor activity only in MCF-7, but not in the two other cell lines characterized by absent/ very low protein levels of Bcl-2 (Fig. 5a-c).

Anti-BC cell activity of EU-5346 in combination with conventional BC therapies (tamoxifen, trastuzumab, paclitaxel)

The anti-BC activity of EU-5346 in combination with tamoxifen, the anti-Her2 monoclonal antibody trastuzumab and the taxane paclitaxel was investigated next. In an ER-positive BC cell line, low-dose EU-5346 induced synergistic anti-BC activity when used in combination with tamoxifen (Fig. 6). In contrast, no synergistic anti-BC activity was observed in an ER-negative BC cell line (data not shown), as expected. Moreover, addition of EU-5346 to trastuzumab (Fig. 7a) or to paclitaxel (Fig. 7b) increased anti-BC activity of each agent alone, as evidenced by inhibition of spheroid growth.

Tumor cytotoxicity of antitubulin chemotherapeutics such as paclitaxel is, at least in part, mediated *via* posttranslational Mcl-1 degradation. Consequently, restoration of Mcl-1 activity promotes resistance against paclitaxel [26]. In order to test whether Mcl-1 inhibition by EU-5346 is able to overcome resistance of BC cells to paclitaxel we generated a paclitaxel-resistant MDA MB231 (MDA MB231PacR) cell line by continuous treatment for 8 weeks. We observed a significant upregulation of Mcl-1 in MDA MB231PacR cells *versus* the parental MDA MB231 (MDA MB231Par) (Fig. 8a). Consequently, MDA MB231PacR cells were more sensitive to EU-5346 than MDA MB231Par cells (data not shown). Moreover, low-dose EU-5346 re-sensitized resistant tumor cells to paclitaxel (Fig. 8b). Based on high protein levels of Bcl-x_L, paclitaxel was also combined with WEHI-539 and similarly resulted in synergistic activity. Taken together, these data demonstrate that EU-5346 increases the anti-BC activity of conventional therapies including tamoxifen in ER-positive, trastuzumab in Her-2-positive and paclitaxel in TNBC cells.

Discussion

Our own and other studies demonstrate a crucial role for the anti-apoptotic Bcl-2 family member Mcl-1 in BC cell survival and drug resistance. Indeed, high Mcl-1 protein levels at diagnosis are associated with adverse BC patient outcome. Mcl-1 inhibitors such as the BH3 mimetic EU-5346 therefore represent an exciting new class of targeting agents in BC therapy [15]. Here we investigated the specificity of EU-5346 against BC cells as well as the potential therapeutic impact of its rationally derived combination with other BH3 mimetics and conventional BC therapies. Our in vitro results suggest that EU-5346 may be associated with a low risk of adverse events including cardiotoxicity, hematotoxicity, and neurotoxicity, frequently reported for other BH3 mimetics. Additional in vivo studies need to confirm the therapeutic window of EU-5346 alone and in combination regimens. We also provide evidence for the feasibility to predict the anti-proliferative effect of selective inhibitors of Bcl-2 family members based on their protein levels in BC cells. In contrast to variable protein levels of Bcl-2 and Bcl-x_L, high Mcl-1 protein levels have consistently been observed across all BC subtypes indicative for its impact on tumor cell survival [21, 23, 24, 47, 48]. A key role of Mcl-1 in mediating BC cell survival is further supported by its very short half-life when compared to Bcl-2 and Bcl-x_L. However, despite high Mcl-1 protein levels across all BC cell subtypes a wide range of sensitivity to EU-5346 indicates that the anti-apoptotic function of Mcl-1 is the result of its complex interaction and cross talk with other Bcl-2 family members, Bcl-2, Bcl-x_L and BH3 only proteins in particular; as well as of the extent of unbound apoptotic proteins. Indeed, the predictive index generated in this report emphasizes a role for Bak, Bim and NOXA as the relevant individual predictors of EU-5346 sensitivity in BC cells. Similar multi-protein indexes have been generated before to predict response rates to Mcl-1 inhibitors in other entities including lung cancer and leukemia [43, 49, 50]. Whether our in vitro results translate into

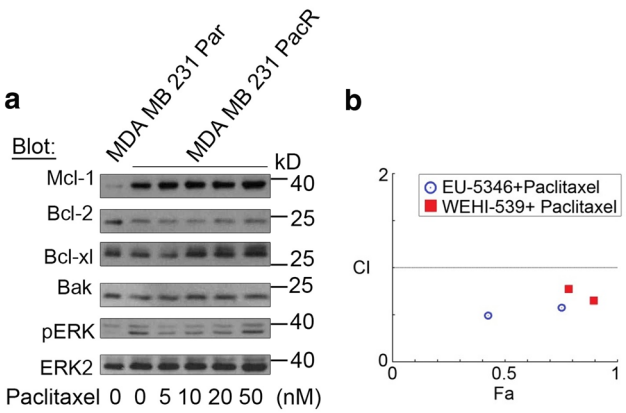

Fig. 8 Anti-BC cell activity of EU-5346 in combination with trastuzumab or paclitaxel. **a** Bcl-2 family protein levels in the parental *versus* paclitaxel-resistant TNBC cell line MDA MB 231. Whole-cell extracts were analyzed by immunoblotting with indicated antibodies. Immunoblotting for Erk2 confirmed equal protein loading. MDA MB 231 Par, parental MDA MB 231 cell line cells; MDA MB 231 PacR, paclitaxel-resistant MDA MB 231 cell line cells. **b** Synergistic activity of EU-5346 and paclitaxel or WEHI539 in MDA MB 231 PacR cells. Paclitaxel-resistant MDA MB231PacR cells treated with low-dose EU-5346 or left untreated were exposed to paclitaxel or WEHI-539. Proliferation was measured using [^{3}H]-thymidine uptake during the last 10 h of 48 h cultures. Fa indicates the fraction of cells with growth affected in drug-treated *versus* untreated cells. CI indicates the combination index. Data represent mean ± standard deviation for quadruplicate samples. Results shown are representative of 3 independent experiments (right panels)

the *clinical* setting which also reflects the impact of the tumor microenvironment on tumor cell survival remains to be investigated. Based on the pivotal role of Mcl-1 in BC cell survival, we hypothesized that EU-5346 is particularly effective when used in combination with other anti-BC agents. Indeed, our data demonstrate the clinical relevance of rationally derived combinations of low-dose EU-5346 with other BH3 mimetics (ABT-199, WEHI-539), as well as tamoxifen, paclitaxel and trastuzumab. Moreover, high Mcl-1 expression is linked to acquired as well as inherent resistance against widely used but also investigational BC therapeutics [24–26, 51]. Of note, our data demonstrate that EU-5346 is able to re-sensitize paclitaxel-resistant BC cells to therapy. Similar data have been recently reported for the Mcl-1 inhibitor S63845 [25].

In summary, the present study demonstrates the safety and therapeutic potential of the Mcl-1 inhibitor EU-5346 alone and in combination with other anti-BC therapies and strongly supports the clinical development of EU-5346 to improve BC patient survival.

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Author contributions SV conceived of the study, designed experiments, analyzed data, and wrote the manuscript. FF and SM performed experiments and participated in data analysis and interpretation. MS, JTO and MHC conceived of the study and participated in data analysis and interpretation. MP, AS and DJ made substantial contributions to the acquisition and interpretation of data. KP conceived of the study, designed and coordinated experiments, analyzed and interpreted data and wrote the manuscript. All authors were involved in revising the manuscript critically for important intellectual content. All authors approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest KP received speaker honorarium from Celgene, Janssen, and Amgen. MP and KP received research support from Roche Pharmaceuticals. JTO received consultant honorarium and research support from AbbVie. DJ received consultant honorarium from Bayer, Amgen, MSD, CureVac, Roche, BMS. MHC is the co-funder, president and CEO of Eutropics, Inc. The remaining authors declare no conflict of interest.

Ethical approval This study complied with current laws of Germany, Austria and USA. The collection and use of primary cells has been approved by the Ethics committee of the Medical Faculty, University of Heidelberg (Approval Number 022/2013).

Informed consent Informed consent was obtained in accordance with the Declaration of Helsinki. This article does not contain any studies with animals performed by any of the authors.

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