

BH3 mimetics as single agents and with endoxifen in ER α + breast cancer models

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Abstract

Background: Endocrine therapies remain central to estrogen receptor alpha-positive (ERα+) breast cancer treatment, yet their predominantly cytostatic effects highlight the need for combinatorial approaches that promote tumor cell death. We therefore tested BH3 mimetics as single agents and in combination with (Z)-endoxifen (ENDX) across endocrine sensitive and endocrine resistant ERα+ breast cancer models.

Methods: Six BH3 mimetics were tested in MCF7 and T47D cells and matched ENDX-resistant and fulvestrant-resistant derivatives. ENDX combinations were assessed using 2D proliferation and synergy assays, 3D RASTRUM® bioprinted cultures, migration assays, and *in vivo* MCF7 xenograft studies.

Results: BH3 mimetics showed broad anti-proliferative activity as single agents in endocrine sensitive and resistant models. ENDX combinations were predominantly additive in 2D assays, with limited synergy confined to specific dose windows. In 3D culture, selected combinations of an MCL1 inhibitor (MCL1i) or navitoclax with ENDX produced greater suppression than single agents and, in several conditions, reduced growth below baseline, consistent with loss of viability. Combination treatment also enhanced suppression of migration under select concentrations. In MCF7 xenograft studies, ENDX robustly suppressed tumor growth; however, adding MCL1i or navitoclax did not provide additional antitumor benefit at the doses, schedules, and timeframes tested.

Conclusion: BH3 mimetics show activity in ERα+ breast cancer models *in vitro*, including endocrine resistant models. Although ENDX combination benefit did not translate *in vivo* under the conditions tested, these data support further study of optimized BH3 mimetic combinations, including evaluation of dose, ratio, schedule, tumor target engagement, and other clinically advanced agents.

Background

Estrogen receptor alpha-positive (ERα+) breast cancer accounts for nearly 80% of all breast cancer diagnoses making it the most prevalent molecular subtype and a primary contributor to the global burden of cancer.(1) The defining feature of ERα+ breast cancer is its dependence on estrogen signaling for tumor growth and disease progression.(2) As such, patients with ERα+ breast cancer are treated in the adjuvant setting with endocrine therapies such as aromatase inhibitors and tamoxifen,(3) an approach that halves the annual risk for recurrence.(4-10) However, the incidence of late recurrence in ERα+ patients continues to rise even into the fourth decade post treatment cessation and ultimately impacts nearly half of all ERα+ patients.(11)

Tamoxifen remains a cornerstone of endocrine therapy worldwide and has not been associated with the development of activating Estrogen Receptor 1 (ESR1) mutations frequently observed in patients who relapse after aromatase inhibitor therapy.(12, 13) A key limitation of tamoxifen, however, is that it is a prodrug, and its clinical efficacy largely relies on hepatic metabolism by cytochrome P450 enzymes, most notably CYP2D6.(14, 15) CYP2D6 converts tamoxifen into its principal active metabolite, (Z)-4-

hydroxy-N-desmethyl-tamoxifen ((Z)-endoxifen, ENDX).(14) ENDX is both the most abundant and active tamoxifen metabolite, exhibiting substantially higher binding affinity for ER α than tamoxifen itself.(14, 16, 17) Problematically, CYP2D6 is highly polymorphic, rendering it enzymatically deficient in a substantial percentage of patients(18, 19), resulting in lower ENDX plasma concentrations and an increased risk for recurrence in tamoxifen treated patients(19-21). Accordingly, ENDX is being developed as a novel endocrine therapy that bypasses the need for CYP2D6-dependent bioactivation, enabling higher and more predictable steady state drug levels.

Preclinical studies consistently demonstrate that ENDX exerts strong anti-estrogenic and anti-proliferative effects, including in models that are resistant to conventional endocrine therapies.(17, 22-26) Early and ongoing clinical trials have similarly reported meaningful anti-tumor activity in patients with ER α + disease, including advanced-stage cancer, together with favorable safety and tolerability profiles.(27-30) Nevertheless, while endocrine therapies can trigger certain non-cell-intrinsic pro-apoptotic responses, and ENDX has demonstrated direct cytotoxicity at higher concentrations,(22) clinical findings consistently reveal that endocrine therapies are generally cytostatic, primarily suppressing tumor cell proliferation rather than inducing extensive tumor cell death.(31) Thus, strategies that directly engage the apoptotic machinery are required to convert growth arrest into tumor cell killing with the expectation that this will further diminish the prevalence of late recurrence.

Evasion of apoptosis is a hallmark of cancer, and the B-cell lymphoma-2 (BCL2)-family of proteins are central regulators of this process.(32) BCL2 family members consists of both pro-apoptotic (e.g., BAX, BAK, BAD, and BIM) and anti-apoptotic (e.g., BCL2, BCL-XL, and myeloid cell leukemia 1 (MCL1)) proteins, and they have been implicated in promoting tumor growth and reducing sensitivity to anticancer therapy(32-34). Notably, BCL2 is estrogen responsive(35) and is overexpressed in ~85% of ER α + breast cancer,(34, 36, 37) making this pathway an attractive therapeutic vulnerability in ER α + disease.(34) BCL2 homology 3 (BH3) mimetics are small molecules that antagonize the BCL2 family proteins and can restore apoptotic signaling.(38) Early clinical data have shown activity of the BH3 mimetic venetoclax (BCL2 inhibitor) in combination with tamoxifen in ER α + metastatic breast cancer, (39) and additional studies are exploring the targeting of pro-survival dependencies in endocrine resistant disease.(40) However, the therapeutic potential of combining ENDX with BH3 mimetics, in both endocrine sensitive and resistant settings, has not yet been systematically investigated *in vitro* or *in vivo*.

In this study, we evaluated the single agent activity of multiple BH3 mimetics in ER α + breast cancer cell lines and in their matched ENDX-resistant and fulvestrant-resistant derivatives. We then assessed combination responses between ENDX and a panel of BH3 mimetics in 2D and 3D cell culture models and in migration assays. Finally, we tested the *in vivo* efficacy of selected combinations in MCF7 xenograft models. Together, these studies were designed to determine whether BH3 mimetics can enhance early ENDX-mediated antitumor activity and to inform future combination strategies aimed at eliminating persister cell populations and further suppressing risk of recurrence.

Materials and Methods

Cell lines and cell culture

ERα+ breast cancer cell lines MCF7 (ATCC #HTB-22, Manassas, VA) and T47D (ATCC #HTB-133) that were originally obtained from ATCC were used in this study. Cells were maintained at 37°C in a humidified 5% CO₂ incubator in standard growth medium (DMEM/F12, JangoCell #104020, Fitchburg, WI) with 10% fetal bovine serum (FBS, R&D Systems #S11550H, Minneapolis, MN) and 1% antibiotic-antimycotic (Gibco #15240-062, Waltham, MA). ENDX- and fulvestrant (ICI-182780 or ICI)-resistant MCF7 and T47D cells were developed as previously described(23) and continuously maintained in standard growth medium supplemented with 1 μM ENDX or ICI, respectively. In parallel, long-term vehicle (VEH) control cells were established by chronic treatment with an equivalent amount of ethanol (solvent for ENDX and ICI) for the same duration of time. All cell lines were routinely assessed to confirm the absence of mycoplasma contamination and were validated by IDEXX BioAnalytics (Westbrook, ME) at the end of resistance development to maintain their MCF7 and T47D cell line characteristics respectively. Cells were seeded at densities specified for each assay and allowed to adhere overnight prior to treatment.

Determination of drug IC50 values

Cells were seeded in 96-well plates at 1,500 (MCF7) or 2,000 (T47D) cells/well in 100 μL of medium. After 24 hours (h), BH3 mimetics were added in 2-fold serial dilutions ranging from 0.04-10 μM. The BH3 mimetics were sourced from Selleckchem.com (Houston, TX) and their catalogue numbers and targets are as follows: Venetoclax (ABT-199, #S8048; BCL2), Sabutoclax (#S8061; BCL-XL, BCL2, MCL1 and BFL1), Navitoclax (ABT-263, # S1001; BCL2, BCLXL, BCLW), MCL1 inhibitor (i, VU661013, #S8758), BCL-XLi (A-1331852, #S7801) and BTSA1 (#S8650, a BAX activator). DMSO was used as vehicle control. Each treatment was performed in 6-8 replicates. Cultures were maintained for 8-12 days until control wells reached ≥90% confluence. Relative cell number/viability was measured by crystal-violet staining. Briefly, plates were fixed with 25% glutaraldehyde (10 min, RT, Sigma #G6257, Burlington, MA), rinsed with running tap water, and stained with 0.5% crystal violet (Sigma #C6158-50G) prepared in 1:3 methanol (Supelco #MX0488, Bellefonte, PA):water solution (10 min, RT, gentle shaking) followed by excessive washing with running tap water to remove all excess crystal violet. Plates were air-dried and destained with 1:1 200 mM sodium citrate (Sigma #71498):ethanol (Sigma #308934) for 10 min. Absorbance of individual wells was read with a Spectramax M3 plate reader (Molecular Devices, San Jose, CA) at 550 nm excitation using the SoftMax Pro 6.5.1 software (Molecular Devices). IC50 values were calculated from sigmoidal dose-response curves (log[inhibitor] vs. normalized response) using Prism 10.6.1 (GraphPad Software, Inc., La Jolla, CA).

Synergy Assays

Cells were seeded into three 96-well plates at 2,000 (MCF7) and 3,000 (T47D) cells/well. After 24 h, cells were treated with ENDX and BH3 mimetics at predefined concentration ratios of 3:1, 1:1, 1:3, 1:10, 0.3:10, and 0.1:10, using 3-fold serial dilutions to span a broad concentration range of 4.6 nM-10 μM for ENDX and 0.12-10 μM for BH3 mimetics. Treatments lasted for 8-12 days, depending on cell line growth rates,

and terminated when vehicle (DMSO)-treated wells reached $\geq 90\%$ confluence. Relative cell number/viability was assessed via crystal violet staining as indicated above. Drug interactions were analyzed using Loewe Additivity and Highest Single Agent (HSA) models in SynergyFinder 3.0.(41) Synergy scores were interpreted as follows: >10 (synergy), -10 to 10 (additivity), and <-10 (antagonism).

Rastrum cell printing and 3D culture

Three-dimensional cultures were generated with the RASTRUM® bioprinter (Inventia Life Science, New South Wales, AU) following the manufacturer's protocol. Cells expressing nuclear red fluorescent protein (RFP) were generated by infection with IncuCyte NucLight Red lentiviral particles (Sartorius #4476, Göttingen, DE) at an MOI of 3 and selected for stable integration in medium supplemented with $500\text{ }\mu\text{g/L}$ puromycin (Gibco #A11138). RFP-expressing cancer cells were suspended in cell-specific RASTRUM® hydrogel inks, bioprinted into 96-well RASTRUM® plates with 1.1 kPa matrix stiffness and the following peptide composition: Arg-Gly-Asp, Tyr-Ile-Gly-Ser-Arg, Cys-Asp-Tyr-Tyr-Ser-Asp-Ser and overlaid with $150\text{ }\mu\text{L}$ standard growth medium. After overnight stabilization, cultures were treated with vehicle (DMSO) control or test compounds in a volume of $150\text{ }\mu\text{L}$ standard medium which was refreshed every three days. Plates were imaged at 24 h intervals for nine days using the IncuCyte S3 System (v2025B, Sartorius #4647), and growth kinetics were quantified using the IncuCyte software. All experiments were performed with four technical replicates.

Migration Assays

MCF7 and T47D cells were seeded into 96-well ImageLock plates (Sartorius #BA-04856) at 35,000 and 42,000 cells/well, respectively, to achieve 90-100% confluence within 24 h. Once confluent monolayers formed, a uniform scratch wound was generated using the IncuCyte WoundMaker tool (Sartorius #4563), followed by two gentle rinses with cell culture medium to remove detached cells and debris. Fresh growth medium containing test compounds or controls was added directly to each well. Plates were imaged every 8 h for up to 168h using the IncuCyte S3 System, and wound closure was quantified using the system's analysis software. All experiments were performed with four technical replicates.

In vivo Experiments

All animal studies were approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Eight-week-old female NOD Scid and athymic nude mice were obtained from The Jackson Laboratory (Bar Harbor, ME), acclimated for two days before study initiation, and housed under specific pathogen free conditions with ad libitum access to food and water on a 12 h light/dark cycle. In every cohort purchased, more than 50% of nude mice eventually developed skin hyperkeratosis and were excluded from the study.

Mice underwent bilateral ovariectomy and received 17β estradiol (E2, Sigma #E8875) supplementation in the drinking water at $20\text{ }\mu\text{g/mL}$. Mice were anesthetized with inhaled isoflurane for both ovariectomy

and tumor cell inoculation, using 3-4% isoflurane for induction and 1-2% for maintenance. MCF7 human breast cancer cells were harvested and resuspended in sterile phosphate buffered saline at 1×10^7 cells/mL. One million cells were implanted into the fourth mammary fat pad of anesthetized mouse under aseptic conditions by injecting 0.1 mL of the cell suspension. The approved IACUC protocol permitted a maximum tumor volume of 2,000 mm³. This limit was not exceeded in any animal included in the study.

For the ENDX + navitoclax study, athymic nude mice were randomized 3 months after tumor implantation, when tumors reached approximately 130 mm³. Animals were assigned to treatment groups on the basis of IVIS fluorescence signal using Studylog (Studylog Systems Inc., San Francisco, CA) as follows: vehicle (n = 4), ENDX (n = 4), and ENDX+navitoclax (n = 3). For the subsequent MCL1i plus ENDX study, NOD Scid mice were used to avoid the repeat skin-related issues observed in athymic nude mice during this period. Mice were randomized 5 weeks after tumor implantation, when tumors reached approximately 30 mm³. Animals were assigned to treatment groups on the basis of IVIS fluorescence signal using Studylog as follows: vehicle (n = 12), MCL1i (n = 11), ENDX (n = 11), and ENDX+MCL1i (n = 10).

ENDX was prepared in 0.0153% (w/v) ascorbic acid in Milli Q water, diluted 1:1 with PEG400 (Sigma #25322-68-3), and administered once daily by oral gavage at 25 mg/kg. For combination studies with navitoclax (Selleckchem.com ABT-263 # S1001), the drug was suspended in DMSO (333 mg/mL) and added directly to the ENDX formulation, then co-administered by daily oral gavage at 100 mg/kg. MCL1i (Selleckchem.com #S8383) was prepared in 5% DMSO, 40% PEG300 (MedChemExpress #HY-Y0873, Monmouth Junction, NJ), 5% Tween 80 (Sigma, #P8074), and 50% Milli Q water and administered by intraperitoneal injection once weekly at 10 mg/kg. Appropriate matched vehicle controls were included in each experiment. In the ENDX plus navitoclax study, mice received vehicle formulations matched for volume and composition, but without the active drugs, resulting in a final DMSO concentration of 7.5% v/v. In the MCL1i plus ENDX study, vehicles were administered by both oral gavage and intraperitoneal injection. Body weight and tumor burden were recorded weekly, with tumor burden assessed by both caliper-measured tumor volume and bioluminescence flux using the IVIS Spectrum imaging system (Xenogen, Alameda, CA). At the study endpoint, all mice were euthanized by carbon dioxide inhalation in accordance with the approved IACUC protocol.

Tumor growth inhibition was calculated by normalizing the change in tumor volume in the treatment group to that in the vehicle control group. This value reflects the extent to which treatment reduced tumor growth relative to vehicle and was calculated as follows:

$$\text{Tumor growth inhibition (\%)} = (1 - (T_n - T_0) \div (V_n - V_0)) \times 100$$

In the formula, T_n and V_n stand for tumor volume of treatment group and vehicle control group on day n after treatment, respectively. T_0 and V_0 stand for tumor volume of corresponding groups on the first day of treatment.

Statistical Analyses

Statistical analyses were performed using Prism 10.6.1 (GraphPad Software, Inc., La Jolla, CA). Normality was assessed using the Shapiro Wilk and Kolmogorov Smirnov tests when sample size permitted. For comparisons across multiple groups, two-way ANOVA with Dunnett's multiple comparisons test was used as indicated in the figure legend. For proliferation assays, overall compound effect across concentrations compared to vehicle was assessed by averaging absorption values across all doses within each replicate to generate a single compound value per replicate. Overall values were compared with vehicle using an unpaired two-tailed t-test. P-values < 0.05 were considered statistically significant unless otherwise indicated. All experiments were performed at least three independent times with multiple replicates per treatment as indicated, and representative datasets are shown.

Results

In vitro activity of BH3 mimetics on endocrine-sensitive and -resistant ERα+ breast cancer models

We first determined whether BH3 mimetics have single agent anti-proliferative activity in the commonly used endocrine sensitive ERα+ breast cancer cell lines, MCF7 and T47D. In 2D culture, all BH3 mimetics significantly reduced the proliferation in MCF7 cells compared with vehicle controls, with maximal growth inhibition of approximately 75-95% at the highest concentrations tested (Fig.1a-g). Consistent with these dose response curves, the calculated IC₅₀ values for MCF7 cells fell within low micromolar range (<0.4-5μM). In T47D cells, navitoclax, MCL1i, BCL-XLi and BTSA1 were found to be less inhibitory than what was observed in MCF7 cells, but these BH3 mimetics achieved at least ~75% growth inhibition at the highest concentrations evaluated (Fig.1a-g). T47D cells were more sensitive to venetoclax-induced inhibition (Fig.1a) while both cell lines appear equally sensitive to sabutoclax based on IC₅₀ values (Fig.1b).

In cell lines rendered resistant (R) to 1 μM ENDX (ENDX-R) or fulvestrant (ICI-R), notable anti-proliferative activity was also observed with many of the compounds (Fig.2). Interestingly, the "vehicle" (VEH) control cells (Fig.2) that were cultured side-by-side during the establishment of ENDX-R and ICI-R cells(23) did not fully recapitulate the results of parental cells shown in Fig.1. MCF7 VEH cells were overall less responsive to all BH3 mimetics, with IC₅₀s not able to be calculated for navitoclax and the BCL-XLi (Fig.2a), whereas T47D VEH cells were quite similar to parental controls (Fig.2b).

In MCF7 ENDX-R cells, all compounds except navitoclax inhibited proliferation by at least about 40% and, based on IC₅₀ values and representative crystal violet images, sabutoclax and MCL1i were the most potent agents in this model (Fig.2a). In T47D ENDX-R cells, all compounds except BTSA1 significantly suppressed growth compared with vehicle, with sabutoclax and MCL1i showing the greatest activity based on IC₅₀ values and crystal violet images (Fig.2b). Notably, whereas navitoclax showed limited activity in MCF7 ENDX-R cells, it remained effective in T47D ENDX-R cells, highlighting cell line-dependent responses to BCL2 family inhibition (Fig.2a-b).

In MCF7 ICI-R cells, only 2 of 6 compounds, sabutoclax and BTSA1, produced significant growth inhibition (Fig.2a). In contrast, T47D ICI-R cells remained sensitive to 4 of 6 BH3 mimetics, including sabutoclax, navitoclax, the MCL1 inhibitor, and the BCL-XL inhibitor (Fig.2b).

These findings were partially consistent with the expression patterns of BCL2 family proteins in MCF7 and T47D parental and resistant cell lines (Fig. S1). Detectable expression of MCL1, BCL-XL, and BAX (Fig. S1) aligned with the relatively broad activity observed for agents targeting these proteins, including the MCL1i, sabutoclax, and BTSA1 (Figs. 1 and 2). However, despite readily detectable BCL-XL expression in both sensitive and resistant cells (Fig. S1), responses to BCL-XLi were marginal in many resistant lines (Fig. 2), indicating that BCL-XL expression alone was insufficient to predict sensitivity to BCL-XL inhibition in these models. Similarly, BCL-2 was detected only in MCF7 VEH cells and not in T47D VEH cells (Fig. S1), yet T47D parental (Fig.1) and VEH (Fig.2) cells showed a greater response to venetoclax.

Impact of ENDX plus BH3 mimetics on cell proliferation

Based on the cell proliferation assays (Fig.1), the most consistently active compounds across models were sabutoclax and the MCL1i which were prioritized for further study. In addition, we included navitoclax because of its broader pro-apoptotic target profile (BCL2, BCL-XL, MCL1), frequent use in the published literature, and its activity in select contexts in our initial screening (Fig.1).

To determine whether BH3 mimetics enhance the anti-proliferative activity of ENDX in endocrine sensitive and resistant settings, we performed combination 2D cell culture proliferation synergy assays in MCF7 and T47D ENDX-sensitive (ENDX-S) and ENDX-resistant (ENDX-R) cell lines (Fig.3; Table 1) with a wide range of concentrations: 4.6 nM-10 µM for ENDX and 0.12-10 µM for BH3 mimetics. Overall, the combination of ENDX plus MCL1i was generally additive across the concentration ranges tested (Fig.3; Table 1). However, regions of synergy (most synergistic score > 10) were observed in MCF7 ENDX-S cells (Fig.3, top row; Table 1). Notably, this effect was largely absent in the corresponding resistant cells (Table 1).

Similarly, ENDX plus navitoclax was overall additive across cell lines and concentration ranges, with the exception of T47D ENDX-R cells, where the interaction was neutral (Fig.3, middle row; Table 1). In ENDX-S cells, the response patterns for MCF7 and T47D were broadly comparable, with MCF7 showing a slightly stronger interaction and a peak synergy score of 13.95, observed at the highest concentrations tested (Fig.3; Table 1).

The combination of ENDX plus sabutoclax showed limited benefit in ENDX-R cells and was predominantly additive in both MCF7 and T47D ENDX-S cells (Fig.3, bottom rows; Table 1). Only small peaks of additivity were observed, including some occurring at lower ENDX concentrations (Fig.3, bottom rows; Table 1).

Table 1. Summary of synergy assays in ERα+ ENDX sensitive (S) and resistant (R) models.

Combination	Model	Highest synergy score	HSA score	Interpretation
ENDX + MCL1i	MCF7 ENDX-S	12.22	6.07	Additive with synergistic peak/s
	MCF7 ENDX-R	2.31	1.05	Additive
	T47D ENDX-S	7.42	5.82	Additive
	T47D ENDX-R	8.84	3.35	Additive
ENDX + Navitoclax	MCF7 ENDX-S	13.95	5.86	Additive with synergistic peak/s
	MCF7 ENDX-R	9.97	3.6	Additive
	T47D ENDX-S	9.14	3.42	Additive
	T47D ENDX-R	0.58	-1.25	Neutral/additive
ENDX + Sabutoclax	MCF7 ENDX-S	4.22	3.14	Additive
	MCF7 ENDX-R	3.64	0.4	Neutral/additive
	T47D ENDX-S	4.82	3.64	Additive
	T47D ENDX-R	7.35	0.14	Neutral/additive

We also tested selected concentrations of these drug combinations for testing in 3D cell culture using the RASTRUM® bioprinting platform, which supports growth in a customizable 3D matrix and provides a physiologically relevant assessment of drug response.(42, 43) Given that most consistent additive interactions in 2D were observed for ENDX plus MCL1i and ENDX plus navitoclax, we prioritized these two combinations for 3D testing. Endocrine-sensitive MCF7 cells were used for the 3D studies, as we were unable to achieve reproducible T47D growth on the RASTRUM® platform across the matrix conditions evaluated (data not shown).

Single agent ENDX, MCL1i, and navitoclax (0.4-7 μ M) each significantly inhibited 3D growth compared with the vehicle (DMSO) control (Fig.4a-b). For the ENDX plus MCL1i combination, the lowest concentrations tested (0.1 μ M ENDX + 0.1 μ M MCL1i) produced a clear additive effect, with

approximately a 4-fold lower viability signal relative to all other treatment groups (Fig.4a). Notably, the downward trajectory of the viability curve at these concentrations over time is consistent with not only growth suppression but a cell killing effect. In contrast, at the higher concentrations tested (1 + 3 μ M and 3 + 3 μ M), the combination treatment response more closely resembled MCL1i alone than ENDX alone, although substantial cell killing effects were observed over time, even with single agents (Fig.4a). This is in contrast to the anti-proliferative effects observed in 2D, highlighting the differences in drug activity that can be observed under more physiologically relevant growth conditions.

For the ENDX plus navitoclax combination, 2 of 3 concentration pairs tested (0.4 μ M + 3.5 μ M and 3 μ M + 7 μ M) produced significantly greater inhibition and/or complete loss of viability compared with both corresponding single agent treatments (Fig.4b). For the concentration pair 1 μ M + 7 μ M, statistical significance was not reached, however the combination effect was numerically lower than that observed for single agents (Fig.4b). Across most of the conditions tested, the overall decline in red fluorescent intensity relative to baseline indicates loss of cell viability rather than a purely cytostatic growth arrest (Fig.4a-b). This is in contrast to the partial cytostatic effects observed in 2D, highlighting the differences in drug activity that can be observed under more physiologically relevant growth conditions.

Effects of navitoclax and MCL1i on cell migration

We next assessed the effects of single agent and combinatorial treatments on MCF7 cell migration. Within 4-5 days of creating scratch wounds, vehicle (DMSO)-treated cells had fully closed the wound area (Fig.5). As expected, ENDX alone inhibited wound closure in a concentration-dependent manner, with greater qualitative inhibition at 3 μ M compared with 1 μ M (Fig.5a-b). At the concentrations tested, inhibition of wound closure was more pronounced with the MCL1i than with navitoclax despite equimolar concentrations of the two drugs (Fig. 5a-b, bottom graphs). Notably, inhibition of wound closure with the MCL1i, either alone or in combination with ENDX, was evident within 24-36 h (Fig. 5b), whereas inhibition with navitoclax (Fig. 5a) became apparent mainly at later time points which are likely more reflective of changes in cell proliferation than actual migration.

For ENDX plus navitoclax, the 1 μ M + 7 μ M condition was the only group with a clearly persistent wound at the experimental endpoint and showed statistically lower overall wound closure compared to other groups (Fig.5a). In contrast, the 3 μ M ENDX + 3 μ M navitoclax combination significantly inhibited wound closure relative to vehicle but was not statistically different from ENDX alone (Fig.5a). For the ENDX plus MCL1i combination, 3 μ M + 3 μ M significantly inhibited wound closure compared with either single agent while the 1 μ M + 3 μ M combination produced inhibition comparable to MCL1i alone (Fig.5b).

In vivo effects of ENDX in combination with navitoclax or MCL1i

Given the observations of additive effects observed between ENDX and navitoclax/MCL1i *in vitro*, we evaluated whether adding either of these agents could augment the anti-tumor activity of ENDX *in vivo* using MCF7 xenograft models (Figs.6-7). Unfortunately, on multiple occasions for the ENDX+navitoclax studies, mice developed keratosis after tumor inoculation but before treatment initiation. Following

randomization, these mice were deemed unfit for inclusion in the treatment groups and were therefore excluded from the study. Given the resulting reduction in sample size, we did not include a navitoclax only arm.

Mice received daily ENDX with or without navitoclax, or the corresponding controls, by oral gavage for 6 weeks (Fig.6a). Body weights remained stable throughout the study and did not differ between groups, indicating that daily dosing with ENDX and navitoclax was well-tolerated over the 6-week treatment period (Fig.6b). Tumors in the vehicle arm progressed over time, as assessed by IVIS bioluminescence (Fig.6c-d) and caliper-based tumor volume measurements (Fig.6e). Mean vehicle tumor volume increased from $136.63 \pm 52.16 \text{ mm}^3$ at baseline to $983.86 \pm 738.74 \text{ mm}^3$ at week 6 (Fig.6e). Notably, one vehicle mouse (mouse #iv in Fig.6c) showed visibly increased bioluminescence through week 4 followed by reduced signal at week 6, despite continued tumor enlargement by caliper (individual mouse tumor volume not shown). This pattern is consistent with reduced viable tumor signal, potentially due to necrosis with ongoing peripheral growth.

ENDX treatment induced tumor regression relative to baseline (Fig.6c-e). Based on tumor volume data at week 6, the ENDX-treated group achieved a tumor growth inhibition of 102.36% relative to vehicle. However, co-treatment with Navitoclax did not provide additional anti-tumor benefit beyond ENDX alone, with tumor growth trajectories in the combination group completely overlapping those of the ENDX only group (Fig.6c-e).

For the ENDX+MCL1i study, tumor-bearing mice received daily ENDX by oral gavage with or without weekly MCL1i via intraperitoneal injection, alongside the corresponding vehicle controls (Fig.7a) for a total of 4 weeks. Body weights over the treatment period did not differ between groups, indicating that ENDX and MCL1i, alone or in combination, were well-tolerated at the doses and schedules used (Fig.7b). Similar to the navitoclax study (Fig.6), vehicle-treated tumors grew over the 4-week period as measured by IVIS luminescence (Fig.6c-d) and caliper-based tumor volume measurements (Fig.7e). Tumor volume in the vehicle group rose from an average of $34.03 \pm 4.33 \text{ mm}^3$ at baseline to $71.99 \pm 8.23 \text{ mm}^3$ at the experimental endpoint. A nearly identical trend was observed in the MCL1i monotherapy group, which overlapped the vehicle group in both luminescence signal (Fig.7c-d) and tumor volume (Fig.7e).

In contrast, and as expected, ENDX significantly suppressed tumor growth relative to vehicle (Fig.7c-e). Based on tumor volume data following 4 weeks of treatment, ENDX reduced tumor burden by growth inhibition of 132.40% relative to vehicle. However, co-treatment with the MCL1i did not provide additional tumor growth suppression beyond ENDX alone, with the combination group closely tracking and nearly paralleling the ENDX only growth curve as assessed by both IVIS signal (Fig.7c-d) and tumor volume measurements (Fig.7e).

Discussion

ENDX is currently under clinical development for ER α + breast cancer(27-30) and has the potential to offer advantages over tamoxifen and aromatase inhibitors. One potential advantage is that ENDX has

been shown to induce low to moderate levels of cancer cell apoptosis in preclinical models at clinically achievable concentrations.(22) However, the primary activity of ENDX is cytostatic in nature, as is the case for other endocrine agents. Therefore, there remains a need to identify rational combination partners for ENDX that can further tip the balance towards drug-induced cancer cell death. Accordingly, our study evaluated the potential of integrating BH3 mimetics with ENDX in preclinical models.

Evaluation of BH3 mimetics in endocrine-sensitive and endocrine resistant ERα+ breast cancer cell lines demonstrated broad single agent anti-proliferative activity in 2D and 3D settings. Most IC50 values were in the low micromolar range and within clinically achievable exposure levels for several of the drugs. For example, venetoclax, navitoclax and the MCL1i result in peak plasma concentrations (Cmax) of up to ~3.5 µg/mL (~4 µM),(39) ~5 µg/mL (~5 µM) (NCT00445198)(44) and ~4.57 µg/mL (~6 µM) (NCT03465540), respectively. Sensitivity patterns, however, differed between cell models, with MCF7 cells being more responsive to MCL1 inhibition and T47D cells showing the strongest response to sabutoclax, consistent with heterogeneous dependence of ERα+ tumors on pro-survival BCL2 family members.(36, 37, 45) Sabutoclax, which targets four BCL2 family proteins,(46) showed consistent activity in both cell lines, suggesting that broader targeting of the BCL2 family may provide more uniform responses across models overcoming cell type heterogeneity.

We found that many BH3 mimetics retained anti-proliferative activity in endocrine resistant settings, with overall stronger responses in ENDX-resistant derivatives than in fulvestrant-resistant cells. These differences may reflect distinct biology during the adaptation of resistance as we and others have previously reported.(23, 47-49) In addition, we found that long-term vehicle cultured control cells maintained in parallel during resistance selection did not fully reproduce the drug response patterns of the original parental lines, indicating that prolonged culture and repeated passaging can influence baseline drug responsiveness independent of endocrine resistance.(50, 51) Including this matched control was therefore essential for interpretation, as it enabled us to distinguish changes associated with long-term culture from those arising specifically through resistance selection. Interestingly, protein expression of individual BCL2 family members alone was insufficient to predict sensitivity to BH3 mimetics in our models, suggesting that additional regulatory mechanisms, compensatory survival pathways, or undefined targets influence apoptotic dependency.

Despite strong single agent activity, ENDX combinations were predominantly additive rather than broadly synergistic. Notably, navitoclax was only marginally active as a single agent in several settings yet showed greater enhancement with ENDX than sabutoclax, despite sabutoclax being the strongest single agent. This indicates that single agent potency does not necessarily predict combination benefit and suggests navitoclax may target a survival dependency(52) that becomes more relevant under ENDX treatment. When evaluated in the 3D bioprinted RASTRUM® system, combinations of specific ENDX concentrations with either MCL1i or navitoclax produced significantly greater activity than the corresponding single agents, indicating that meaningful combination benefit can emerge under specific conditions. Importantly, these combinations frequently produced declines in signal relative to baseline, consistent with a cell killing effect as opposed to cytostatic growth arrest. These findings highlight the

value of 3D culture using tissue relevant extracellular matrix components for capturing drug effects that do not occur 2D culture experiments.(53)

Our functional scratch wound migration assays showed that combining ENDX with the MCL1i or navitoclax further delayed migration/wound closure. Overall, the effect was more pronounced with MCL1i than with navitoclax, with MCL1i producing early inhibition that was more sustained than either single agent. This effect on migration aligns with reports that the BCL2 family of proteins can influence non-apoptotic processes, including cell migration and invasion.(54-56) However, because MCF7 cells are weakly migratory and scratch wound readouts can be influenced by proliferation, these data are best interpreted as a functional measure integrating both motility and growth.

In contrast to our *in vitro* testing, the *in vivo* studies did not show added benefit of combining ENDX with MCL1 inhibition or navitoclax at the doses and schedules tested. This is largely explained by the fact that ENDX monotherapy robustly suppressed tumor growth, and caused tumor regression below baseline levels, in both xenograft models. This reflects the limited dynamic range for improvement in a setting where ENDX potently drives tumor regression as a single agent. The navitoclax experiment was also limited by reduced sample size and the absence of a navitoclax only arm because of animal exclusions. Moreover, pharmacokinetic and pharmacodynamic analyses were not performed, and therefore tumor exposure, target engagement, and induction of apoptosis by navitoclax or MCL1i could not be confirmed. Therefore, the lack of added benefit under these conditions should not be interpreted as definitive evidence that ENDX does not synergize with BH3 mimetics more broadly, as combination effects are sensitive to dose, drug ratio, duration, and sequencing.(52, 57, 58)

Future work should therefore expand combination testing across dosing regimens and other BH3 mimetic partners, ideally incorporating pharmacokinetic and pharmacodynamic assessments to confirm free drug levels, tissue accumulation, tumor target engagement, and apoptotic response. Such studies should test lower or intermittent ENDX exposure, sequential dosing strategies, resistant or less ENDX sensitive *in vivo* models, and tumor pharmacodynamic markers such as cleaved caspase 3, cleaved PARP, BAX or BAK activation, and BCL2 family remodeling.(32, 33) Venetoclax remains an important candidate given its clinical use and ongoing evaluation in combination with endocrine therapy in ER α + breast cancer.(39, 40) Thus, testing ENDX plus venetoclax across optimized dose and schedule conditions is of interest. More broadly, defining apoptotic dependency biomarkers and matching BH3 mimetic selection to tumor context may be required to identify settings where ENDX combinations provide optimal added benefit.

Conclusion

Our findings show that BH3 mimetics have clinically relevant single agent activity in endocrine sensitive and resistant ER α + breast cancer models, and that combining BH3 mimetics with ENDX most often produced additive interactions that can enhance growth suppression, and likely cancer cell death, *in vitro*. Although additional *in vivo* benefit was not observed with the specific doses and schedules tested

here, these data provide a strong rationale to further explore ENDX and BH3 mimetic combinations using optimized dosing and scheduling strategies and clinically established BH3 mimetics such as venetoclax.

List of abbreviations

BCL2 B-cell lymphoma-2

BCL-XLi BCL-XL inhibitor

BH3 BCL2 homology 3

DMSO Dimethyl sulfoxide

E2 17 β estradiol

ENDX (Z)-Endoxifen

ENDX-R Endoxifen resistant

ER α + Estrogen receptor alpha-positive

ESR1 Estrogen Receptor 1

IACUC Institutional Animal Care and Use Committee

ICI Fulvestrant

ICI-R fulvestrant-resistant

MCL1i MCL1 inhibitor

MOI Multiplicity of Infection

PEG Polyethylene glycol

VEH Vehicle

Declarations

Ethics approval: All animal studies were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Consent for publication: Not applicable.

Data availability: The datasets generated and/or analyzed as part of the current study will be made publicly available upon notice of manuscript acceptance via Figshare.

Competing interests: Atossa Therapeutics, Inc. funded research activities related to this study and provided ENDX. SSH is an employee of Atossa Therapeutics and receives salary from the company. SCQ is Chief Executive Officer of Atossa Therapeutics and holds patents related to ENDX. The authors report no conflicts of interest related to this work other than the funding and disclosures stated above.

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Author contributions: JRH and SCQ conceived the work. JRH and LB contributed to the study design. LB performed all experiments with contributions from XW, ER, GM and AD. LB and JRH analyzed the data. LB generated all figures and drafted the manuscript. LB, XW, ER, GM, AD, CJH, SSH, MPG and JRH contributed to the discussion and interpretation of the results. All authors revised the manuscript, approved the final version and agreed to be accountable for all aspects of the work.

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Figures

Fig.1

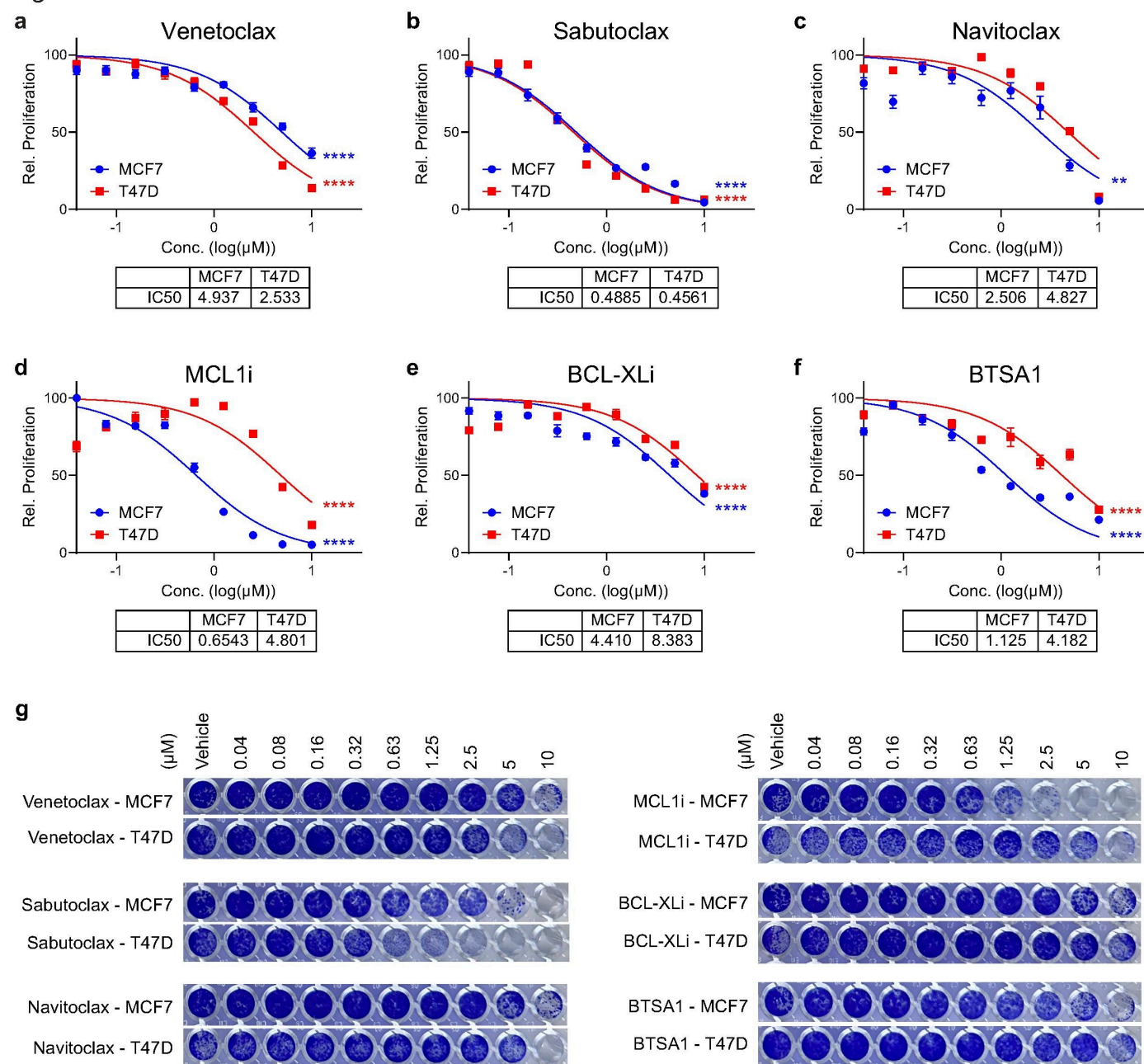


Figure 1

IC50 values and growth inhibition curves for MCF7 and T47D cells treated with BH3 mimetics. a-f: Proliferation relative to vehicle across different drug concentrations was performed to identify inhibitory effects and determine IC50 values. Statistical analyses versus vehicle-treated control (*) were performed using two-tailed t-test. Significance thresholds defined as follows: **** $p < 0.0001$, ** $p < 0.01$. Data are presented as mean $\pm$ SEM. **g:** Representative images of crystal violet staining at each drug concentration evaluated relative to vehicle control.

Fig.2

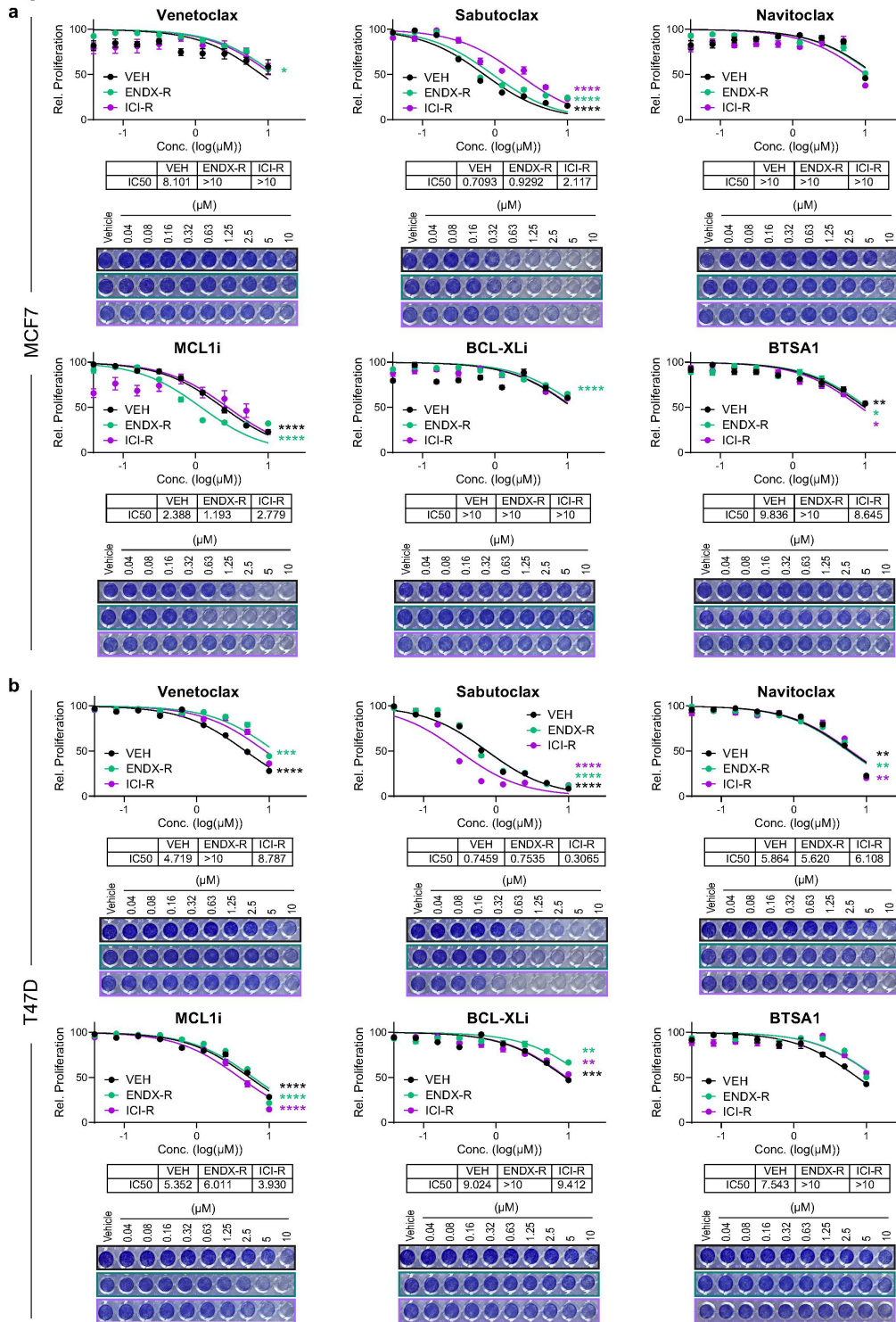


Figure 2

IC₅₀ values and growth inhibition curves for MCF7 and T47D endoxifen- or fulvestrant-resistant cell lines treated with BH3 mimetics. Cells chronically exposed to vehicle (VEH) were cultured side-by-side during the establishment of endoxifen (ENDX) and fulvestrant (ICI) resistance were used as the control line. **a-b:** Proliferation relative to vehicle across different drug concentrations was performed to identify inhibitory effects and determine IC₅₀ values in MCF7 (**a**) and T47D (**b**) resistant and control cells.

Statistical analyses versus vehicle-treated control (*) were performed using two-tailed t-test. Significance thresholds defined as follows: ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. Data are presented as mean ± SEM. Representative images of crystal violet staining at each drug concentration evaluated relative to vehicle control are shown below the IC50 values table and color-coded according to the corresponding group trend line.

Fig.3

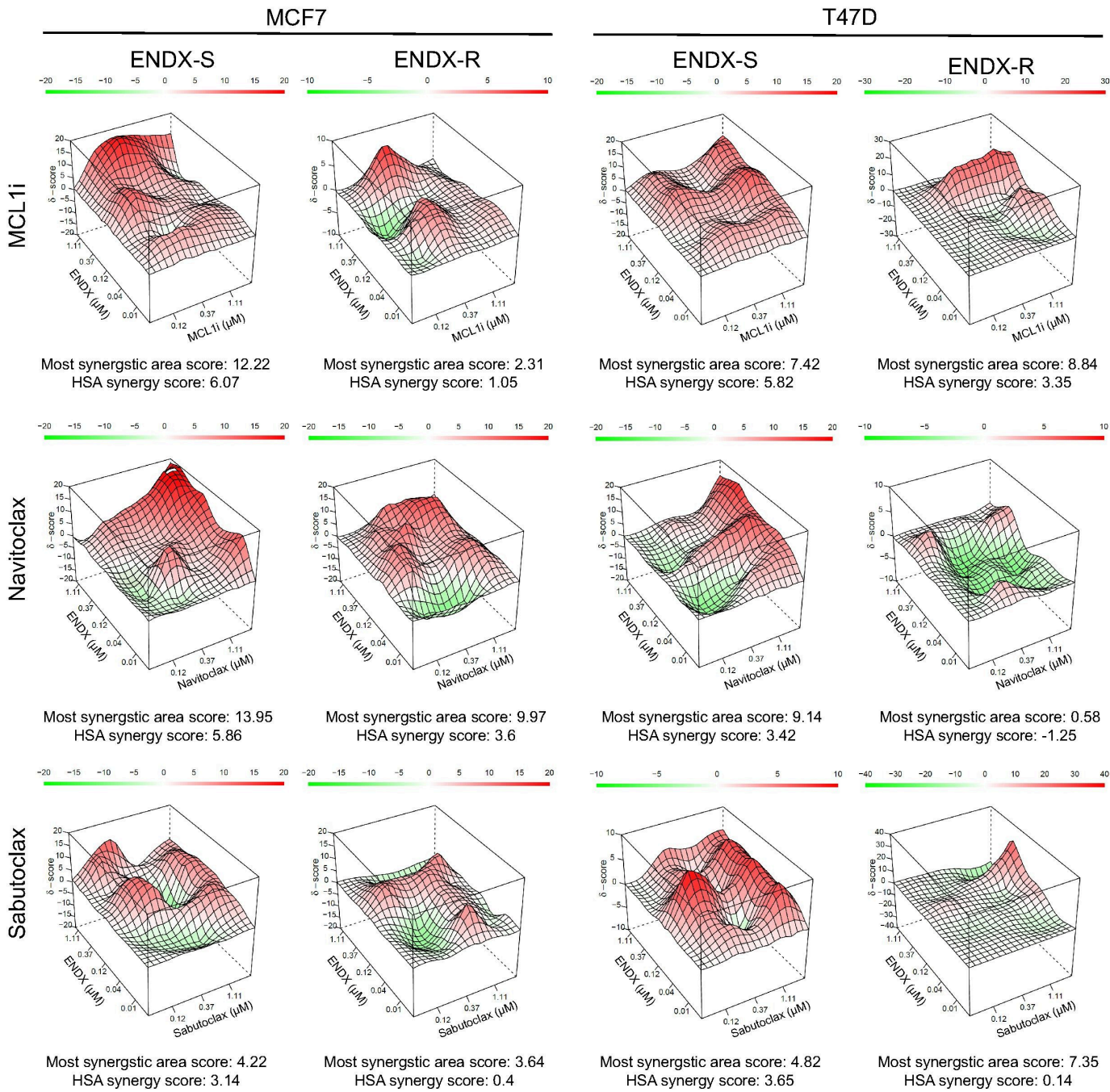


Figure 3

Synergy assays between ENDX and BH3 mimetics in MCF7 and T47D cells with Loewe Additivity plots and Loewe Additivity and Highest Single Agent (HSA) scores indicated. Scores >10 indicate synergy, -10 to 10 indicate additivity, <-10 indicates antagonism across all concentration ranges and ratios tested.

Fig.4

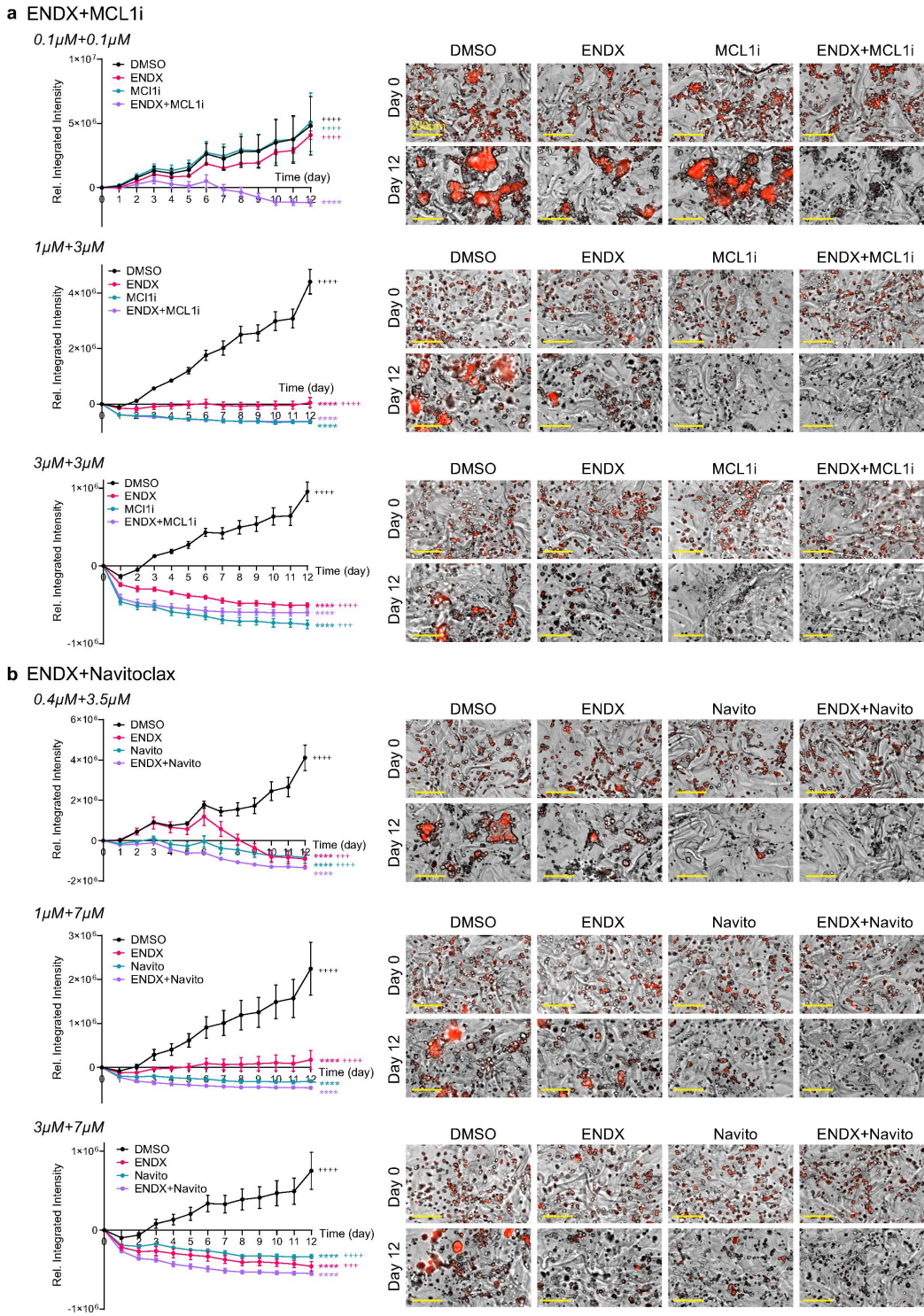
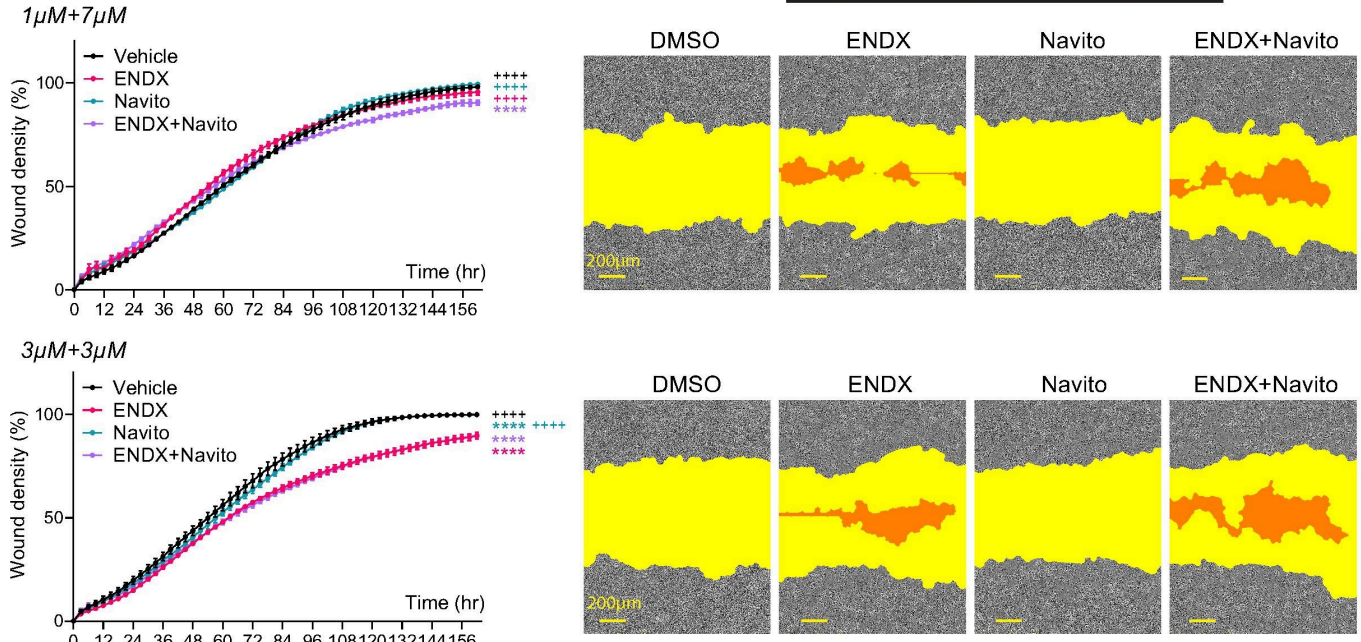


Figure 4

Proliferation and viability kinetics of nuclear RFP-expressing MCF7 cells in 3D culture conditions treated with ENDX plus MCL1i (a) or ENDX plus navitoclax (b) combinations. Representative images of cells treated with ENDX, MCL1i/Navitoclax or vehicle (DMSO) were captured at baseline (Day 0) and at the experimental endpoint (Day 12). Integrated red fluorescent intensity values were determined, and statistical analyses were performed using two-way ANOVA with Dunnett's test (* vs. vehicle, + vs. ENDX+MCL1i/navitoclax). Significant differences are indicated by: ++++/****p<0.0001; +++p<0.001. Symbols are color-coded according to the corresponding treatment group. Data are presented as mean $\pm$ SEM.

Fig.5

a ENDX+Navitoclax



b ENDX+MCL1i

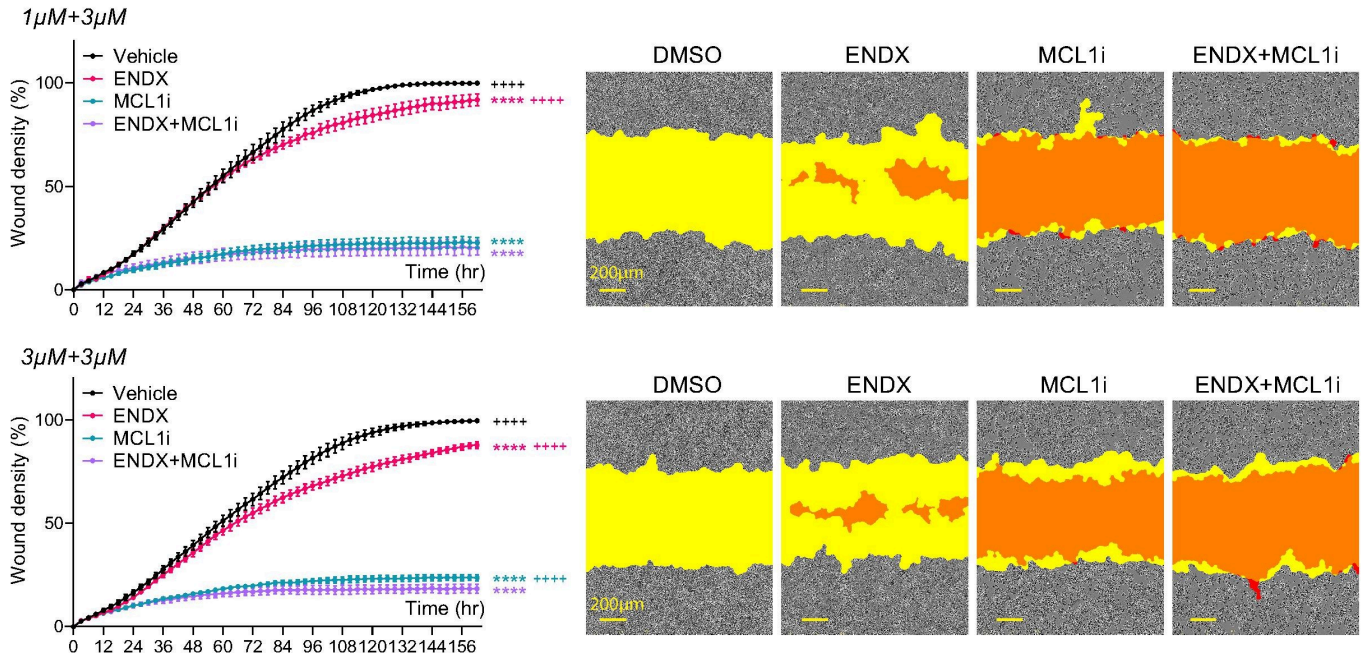


Figure 5

Migration assays of MCF7 cells treated with ENDX plus Navitoclax (a) or ENDX plus MCL1i (b) combinations. Wound density over time was quantified using the IncuCyte software. Representative images of scratch wounds treated with ENDX, navitoclax/MCL1i, or vehicle (DMSO) were captured at treatment initiation (0 h, initial wound area) and at 156 h (residual wound area). Statistical analyses were performed using two-way ANOVA with Dunnett's multiple comparisons test (* vs vehicle; + vs

ENDX+Navitoclax/MCL1i). Significant differences are indicated by: ++++p/****<0.0001. Data are presented as mean ± SEM.

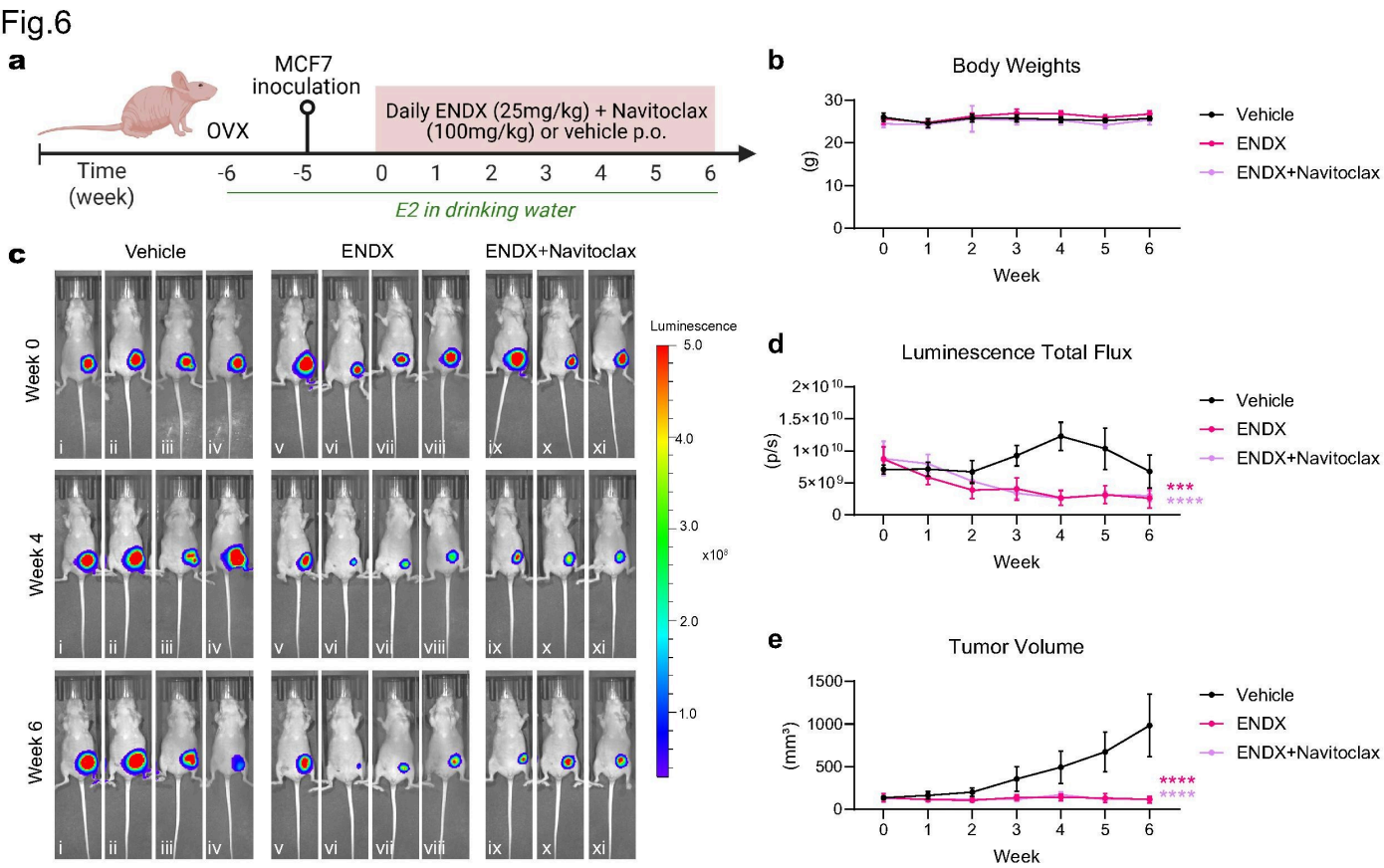


Figure 6

Efficacy of ENDX and Navitoclax in an MCF7 xenograft mouse model. **a:** Schematic of experimental design. **b:** Weekly body weights of mice in each treatment group over time. **c:** IVIS images showing representative tumor luminescence in mice from each group at baseline and after 4 or 6 weeks of treatment. **d, e:** Luminescence total flux (**d**) and tumor volume (**e**) across treatment groups. Statistical analyses were performed using two-way ANOVA with each treatment group compared with vehicle. ***p<0.001, ****p<0.0001. Symbols are color-coded by treatment groups. Data are presented as mean ± SEM. Vehicle (n = 4), ENDX (n = 4), ENDX+navitoclax (n = 3).

Fig.7

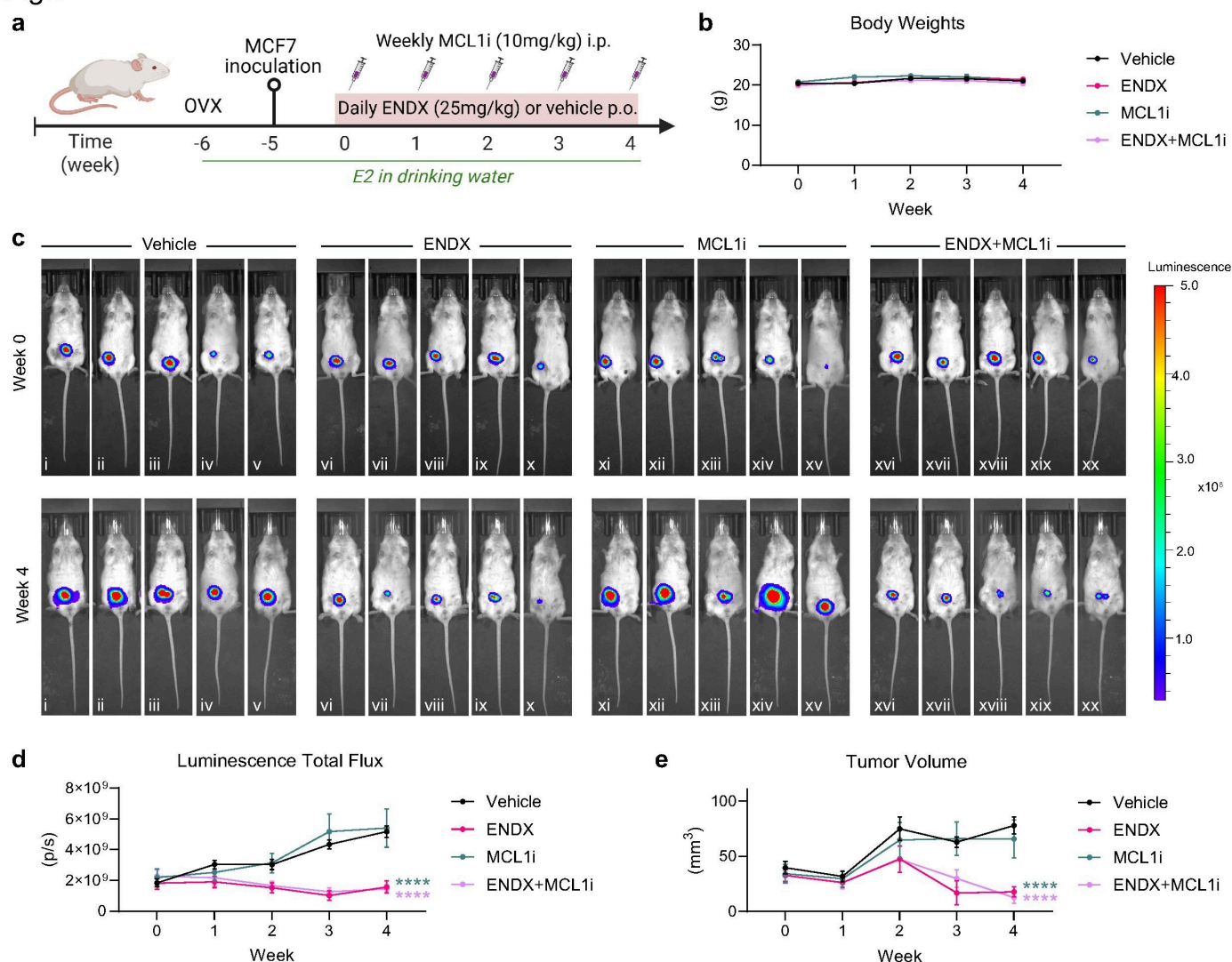


Figure 7

Efficacy of ENDX and MCL1i in an MCF7 xenograft mouse model. **a:** Schematic of experimental design. **b:** Weekly body weights of mice in each treatment group over time. **c:** IVIS images showing representative tumor luminescence in mice from each group at baseline and after 4 weeks of treatment. **d,e:** Luminescence total flux (**d**) and tumor volume (**e**) across treatment groups. Statistical analyses were performed using two-way ANOVA with each treatment group compared with vehicle. ****p<0.0001. Symbols are color-coded by treatment groups. Data are presented as mean $\pm$ SEM. Vehicle (n = 12), ENDX (n = 11), MCL1i (n = 11), ENDX+MCL1i (n = 10).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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