Exploiting off-target effects of estrogen deprivation to sensitize estrogen receptor negative breast cancer to immune killing

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ABSTRACT

Background There are highly effective treatment strategies for estrogen receptor (ER)+, progesterone receptor (PR)+, and HER2+ breast cancers; however, there are limited targeted therapeutic strategies for the 10%–15% of women who are diagnosed with triple-negative breast cancer. Here, we hypothesize that ER targeting drugs induce phenotypic changes to sensitize breast tumor cells to immune-mediated killing regardless of their ER status.

Methods Real-time cell analysis, flow cytometry, qRT-PCR, western blotting, and multiplexed RNA profiling were performed to characterize ER+ and ER− breast cancer cells and to interrogate the phenotypic effects of ER targeting drugs. Sensitization of breast cancer cells to immune cell killing by the tamoxifen metabolite 4-hydroxytamoxifen (4-OHT) and fulvestrant was determined through in vitro health-donor natural killer cell release killing assays. A syngeneic tumor study was performed to validate these findings in vivo.

Results Pretreatment with tamoxifen metabolite 4-OHT or fulvestrant resulted in increased natural killer (NK)–mediated cell lysis of both ER+ and ER− breast cancer cells. Through multiplexed RNA profiling analysis of 4-OHT-treated ER+ and ER− cells, we identified increased activation of apoptotic and death receptor signaling pathways and identified G protein-coupled receptor for estrogen (GPR30) engagement as a putative mechanism for immunogenic modulation. Using the specific GPR30 agonist G-1, we demonstrate that targeted activation of GPR30 signaling resulted in increased NK cell killing. Furthermore, we show that knockdown of GPR30 inhibited 4-OHT and fulvestrant mediated increases to NK cell killing, demonstrating this is dependent on GPR30 expression. Moreover, we demonstrate that this mechanism remains active in a 4-OHT-resistant MCF7 cell line, showing that even in patient populations with ER+ tumors that are resistant to the cytotoxic effects of tamoxifen, 4-OHT treatment sensitizes them to immune-mediated killing. Moreover, we find that fulvestrant pretreatment of tumor cells synergizes with the IL-15 superagonist N-803 treatment of NK cells and sensitizes tumor cells to killing by programmed death-ligand 1 (PD-L1) targeting high-affinity natural killer (t-haNK) cells. Finally, we demonstrate that the combination of fulvestrant and N-803 is effective in triple-negative breast cancer in vivo.

Conclusion Together, these findings demonstrate a novel effect of ER targeting drugs on the interaction of ER+ and, surprisingly, ER− tumors cells with the immune system. This study is the first to demonstrate the potential use of ER targeting drugs as immunomodulatory agents in an ER agnostic manner and may inform novel immunotherapy strategies in breast cancer.

BACKGROUND

Breast cancer is the most frequent female cancer and the second highest cause of cancer mortality in women.1 It is categorized based on genetic and molecular profiles into five tumor subtypes—luminal A, luminal B, basal-like/triple-negative, HER2+, and normal-like—that are most frequently defined on the expression of common receptors.2 Luminal A and B tumors comprise 70%–80% of breast cancer and are characterized by estrogen receptor alpha (ERα) expression. HER2+ tumors are 15%–20% of breast cancers and have amplified expression of HER2. In contrast, basal like/triple-negative breast cancers (TNBCs) are 10%–20% of all diagnoses and are distinct for lack of expression of ERα, ERβ, and HER-2. TNBC is highly aggressive, and patients presenting with it have a dramatically worse prognosis than patients with luminal tumors.2 This is in part due to the limited molecular targeted therapies for patients with TNBC. Luminal A and B tumors are dependent on hormonal signaling and are treated with a variety of estrogen deprivation drugs, most commonly the selective-estrogen receptor modulator (SERM) tamoxifen3 for pre-menopausal women and aromatase inhibitors4 or the selective-estrogen degrader (SERD) fulvestrant5 for post-menopausal women. Similarly, HER-2 patients are treated with the HER-2 blocking small molecule trastuzumab.6 However, the standard of care for TNBC remains surgery, chemotherapy, and radiation.7 Furthermore,
studies have demonstrated the existence of receptor discordance or receptor conversion in breast cancer, wherein a metastatic lesion may express different levels of hormone receptors to the primary tumor. ER conversion has been shown to occur in 26% of patients, and loss of ER expression in a metastatic lesion is associated with worse overall survival.8,9 Harnessing the immune system to target receptor negative primary tumors or metastatic lesions may be an ideal strategy for these patients. Natural killer (NK) cells are members of the innate immune system that are central to cancer immunity. In contrast to T cells, NK anti-tumor killing occurs independent of antigen recognition and is instead based on binding to competing activating and inhibitory receptors.10 This allows for rapid recognition and targeting of tumor cells. NK cells also contribute to the activation of the adaptive immune response through cytokine secretion.11 Interestingly, it has been demonstrated that NK cells may play an important role in targeting breast cancer, recognizing breast cancer stem cells12 and proving necessary for the inhibition of tumorigenesis in certain mouse models in research settings.13 NK-mediated cytoxicity is in part the result of NK activation of apoptotic cell signaling pathways in the tumor cells. Recent studies by our group have found that many standard-of-care agents have off-target immunogenic effects that can be co-opted to enhance immune cell killing. Immunogenic modulation, a process distinct from immunogenic cell death, occurs when sublethal doses of cytolytic therapies result in upregulation of stress molecules, sensitizing tumor cells to immune cell killing by both NK cells and T cells.14,15 Combining standard-of-care therapies known to induce immunogenic modulation with immunotherapy provides an opportunity to sensitize patients’ tumors toward immunotherapy with drugs that are already FDA approved with well-known safety profiles. Here, we sought to investigate the effect of FDA-approved SERM and SERD drugs on human breast cancer cell lines to determine whether they induced immunogenic modulation, and whether their effect was dependent on ER expression. For the first time, we show here how treating both ER+ and ER− breast cancer cell lines with SERM and SERD drugs at sublethal doses sensitizes them to immune cell killing. These studies demonstrate that ER targeting drugs induce immunogenic modulation of breast cancer cells regardless of estrogen receptor expression. SERM and SERD drugs upregulated expression of death receptor and apoptotic signaling molecules, classic signifiers of immunogenic modulation,15,16 and we demonstrated that this occurs through the estrogen-receptor-like molecule GPR30, which is expressed in both ER+ and ER− cell lines. We explored combining fulvestrant with the immunotherapeutic agent N-803, an IL-15/IL-15Rα superagonist that promotes NK cell expansion and cytoxicity,17 and the targeted natural killer cell line programmed death-ligand 1 (PD-L1) targeting high-affinity natural killer (t-haNK) cells.18 Finally, we demonstrate that the combination of fulvestrant and N-803 significantly inhibited growth of the TNBC tumor model EMT-6 in vivo. Collectively, these data support the use of SERM and SERD drugs in ER− patients in an immunotherapy context and identify GPR30 as a potential biomarker for increased patient response.

METHODS
Cell lines and culture
MCF7, T47D, BT549, and EMT-6 were obtained from American Type Culture Collection (Manassas, VA). SUM149 was purchased from Asterand Biosciences (Detroit, MI). All cell lines were passaged for fewer than 6 months and cultured at 37°C/5% CO2. MCF7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 1% of HEPES, L-glutamine, non-essential amino acids, sodium pyruvate, and 2.5µg/mL insulin. BT549 and T47D cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 1% of HEPES, L-glutamine, non-essential amino acids, and sodium pyruvate. BT549 cells were supplemented with an additional 10µg/mL human insulin. SUM149 cells were maintained in Ham’s F12 supplemented with 2.5µg/mL insulin and 1µg/mL hydrocortisone. EMT-6 were cultured in Waymouth’s MB 752/a medium with 2mM L-glutamine and 10% fetal bovine serum. PD-L1 t-haNK cells were provided by ImmunityBio through a Cooperative Research and Development Agreement (CRADA) with the National Cancer Institute (NCI), National Institutes of Health (NIH). All cells were regularly tested and determined to be negative for Mycoplasma contamination.

Human healthy donor NK cells
Blood samples were obtained from normal healthy donors on the NCI IRB-approved NIH protocol 99-CC-0168. NK cells were isolated as previously described.19 Research blood donors were provided written informed consent. Donors were non-pregnant adults 18 years or older who met healthy blood donor criteria and tested negative for transfusion-transmissible diseases. Blood was collected by standard phlebotomy and apheresis techniques in ACD-A (Anticoagulant Citrate Dextrose Solution Formula A), and the cells were mixed with the donor’s plasma. All samples were de-identified. Peripheral blood mononuclear cells (PBMCs) were isolated from this apheresis product within 24 hours of donor sample collection using gradient centrifugation with Lymphocyte Separation Medium (Mediatech, Manassas, VA). Cells were washed with PBS (Mediatech) and adjusted to a concentration of 5×107 cells/mL with fetal bovine serum (Atlanta Biologicals, Atlanta, GA) containing 10% DMSO prior to freezing. Cells were cryopreserved using CoolCell LX (Corning, Corning, NY) freezing containers at a cooling rate of 1°C/min in a −80°C freezer for 24 hours, then placed in liquid nitrogen (vapor phase) for long-term storage. PBMC immune cell subsets were identified as previously described.20 NK effector cells were isolated
from PBMCs using the Human NK Cell Isolation (negative selection) Kit 150-092-657 (Miltenyi Biotech, San Diego, CA), according to the manufacturer’s protocol. Median NK cell yield after isolation was typically 0.5–1 × 10⁷ with 94%–98% viability. Purified NK cells were incubated overnight in RPMI-1640 medium (Mediatech) containing 10% fetal bovine serum, glutamine, and antibiotics (Mediatech) prior to use.

**Chemicals and drug preparations**

Tamoxifen, >98% (Z)-4-hydroxytamoxifen (4-OHT), endoxifen, and fulvestrant were all obtained from Millipore Sigma (Darmstadt, Germany). G protein-coupled receptor 30 (GPR30) Agonist G-1 was obtained from Cayman Chemical (Anne Arbor, MI). All agents were suspended in dimethyl sulfoxide. Tamoxifen, >98% (Z)-4-hydroxytamoxifen, and endoxifen were used at 5 µM unless otherwise specified. Fulvestrant was used at 50 nM; G-1 was used at 50 nM. N-803 was obtained from ImmunityBio through a CRADA with the NCI and used at 50 ng/mL.

**Flow cytometry**

Flow cytometry was performed on an Attune NxT flow cytometer (Thermo Fisher Scientific). A Live/Dead Fixable dead cell stain kit (Molecular Probes, Thermo Fisher Scientific, Grand Island, NY) was used to exclude dead cells. EFLIXX-ID Gold Flow Based Multidrug Resistance Assay was used according to the manufacturer’s instructions (Enzo Life Sciences, East Farmingdale, NY). Annexin V/PI staining was performed per the manufacturer’s instructions (Thermo Fisher Scientific). Flow cytometry data were analyzed using FlowJo software (Treestar, Ashland, OR).

**Western blotting**

Cells were incubated with vehicle or 4-OHT as described previously. After 24 hours, the cells were lysed using Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) supplemented with 1 mM phenylmethanesulfonyl fluoride (Cell Signaling Technology) and 10 µL Halt protease/phosphatase inhibitor cocktail (Santa Cruz Biotechnology, Dallas, TX). Protein was added to Bolt 4%–12% Bis-Tris Gels and run with Bolt MES SDS running buffer (Invitrogen, Carlsbad, CA). Samples were transferred to nitrocellulose membranes using the iBlot system (Invitrogen). Western blots were probed with the primary antibody human anti-GRP30 (1:250; Abcam, Cambridge, MA), human anti-GAPDH (1:1000; Cell Signaling Technology), mouse anti-GPR30 (1:500; ThermoFisher), or anti β-actin (1:1000; Cell Signaling Technology) at 4°C for 18 hours. IRDye 800CW or 680RD secondary antibodies (Li-Cor Biosciences, Lincoln, NE) were used at a 1:10,000 dilution at room temperature for 1 hour. Membranes were visualized on a Li-Cor Odyssey Imager (Li-Cor Biosciences).

**Apoptosis protein array**

Array was run using Proteome Profiler Human Apoptosis Array Kit (R&D Systems, Minneapolis, MN). Cells were incubated with vehicle, 4-OHT, fulvestrant, or G-1 as described previously. Protein was isolated and apoptotic array was run per the manufacturer’s instructions.

**siRNA knockdown**

Control siRNA and siGPR30 were obtained from OriGene (Rockville, MD). Transfection was performed using Lipo- fectamine 3000 (Thermo Fisher Scientific) per the manufacturer’s instructions.

**RNA isolation, RT-PCR, and qRT-PCR**

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using SMARTer PCR cDNA Synthesis kit (Takara Bio, Mountain View, CA) per the manufacturer’s instructions. cDNA (20 ng) was amplified in triplicate using the TaqMan Master Mix in an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific). The following Taqman gene expression primers were used: Human GAPDH (4325792; Applied Biosystems, Foster City CA), ESR1 (Hs00174860_m1; Thermo Fisher Scientific), ESR2 (Hs01100353_m1; Thermo Fisher Scientific), and GPER1 (Hs01922715_s1; Thermo Fisher Scientific).

**Multiplexed RNA profiling analysis**

Total RNA was isolated using RNeasy Mini Kit (Qiagen). NanoString analysis of the isolated RNA was completed with the PanCancer Pathways Panel (NanoString Technologies, Seattle, WA). NanoString was performed by the Genomics Laboratory, Frederick National Laboratory for Cancer Research, Frederick, MD. Raw data (RCC) files were uploaded into nSolver analysis software. 4-OHT-treated samples were compared with DMSO control samples, and a 1.5-fold change cut-off was used to identify initial genes of interest. Further analysis was performed using the Ingenuity Pathway Analysis (IPA) software package (Qiagen).

**IN-111 in vitro killing assay**

Tumor cell lines were treated with DMSO, 4-OHT, fulvestrant, or G-1 as described previously for 24 hours. Cells were then harvested with trypsin, washed, counted, and adjusted to 1×10⁶ cells/mL. Cells were labeled with 40 µL/ mL ^111^In-oxyginoeline (GE Healthcare, Chicago, IL) at 37°C for 30 min. Cells were washed and adjusted to 1.5×10⁶ cells/mL. Cells were plated with healthy donor NK cells at a 20:1 effector-to-target ratio and incubated at 37°C/5% CO₂ for 18 hours. For N-803 experiments, healthy donor NK cells were cultured with N-803 for 24 hours after isolation from PBMCs. NK cells were washed with media prior to being plated with target cells. Maximum release was calculated by lysing labeled cells with 2% Triton X-100. ^111^In-counts were read on a Wizard² gamma counter (PerkinElmer, Shelton, CT). To calculate percent lysis, the following calculation was used:

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\% \text{ Lysis} = \frac{7_{\text{test}} - 7_{\text{spontaneous}}}{\text{Max}_{\text{spontaneous}}} \times 100.
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Real-time cell analysis
Real-time cell analysis was performed using an xCelligence RTCA impedance-based assay (Acea Biosciences, Santa Clara, CA). Cells were plated on E-Plate 96 plates at cell line–dependent densities. After 24 hours, cells were treated with drug of choice as described previously. Data were normalized to time of drug’s addition and analyzed using Excel.

In vivo studies
Balb/c mice were bred and maintained at the NIH (Bethesda, MD). For in vivo studies, female Balb/C mice 8–16 weeks old were inoculated with EMT-6 cells (3×10⁵/mouse, subcutaneous) on the mammary fat pad. For fulvestrant treatment, mice were injected intramuscularly with 5 mg of fulvestrant (AstraZeneca, Wilmington, DE) in 100 µL on days 7 and 14. For N-803 treatment, mice were injected subcutaneously with 1 µg N803 in 100 µL on days 7 and 14. Tumor growth was monitored two times per week. Animal studies were terminated after 21 days for analysis.

Statistical analysis
Statistics were calculated using two-tailed t-test using GraphPad Prism software (GraphPad, San Diego, CA). Differences were considered significant when the p value was <0.05.

RESULTS
Characterization of ER expression and tamoxifen cytotoxicity in breast cancer cell lines
We first investigated the effects of endocrine deprivation in a panel of cell lines and based on previously published studies chose two ER+ (MCF7, T47D) and two ER− (BT549, SUM149) cell lines to study. MCF7 and T47D are both classified as luminal A breast cancers, while BT549 and SUM149 are both representative of the TNBC subtype (figure 1A). We verified the expression of ERα and ERβ by qRT-PCR and found that MCF7 and T47D expressed high levels of ERα and ERβ compared with low or undetectable expression in the BT549 and SUM149 cell lines (figure 1B).

Next, we determined whether ER targeting drugs were capable of killing these cells. The SERM tamoxifen is the most commonly prescribed estrogen deprivation agent and has been in use for over 30 years. Tamoxifen is metabolized by cytochrome P450 into 4-hydroxytamoxifen (4-OHT) and N-desmethyltamoxifen, which is metabolized into endoxifen. Both 4-OHT and endoxifen have been reported to be 30-fold to 100-fold more potent as antiestrogen agents than tamoxifen alone.

As expected, 4-OHT, endoxifen, and tamoxifen induced significant decreases in cell growth starting at approximately 40 hours post-treatment compared with vehicle treatment in the ER+ MCF7 and T47D cell lines (figure 1C). Conversely, we observed no difference in cell growth compared with vehicle in ER− cell lines BT549 and SUM149, indicating their lack of sensitivity to estrogen therapy (figure 1C). It should be noted that we observed a more rapid peak followed by decrease in BT549 cell growth, indicative of nutrient depletion and the variable dynamics of cell growth within the ACEA system. These data demonstrate that our four chosen cell lines reflect the expected susceptibility or lack thereof to ER-targeting agents.

Treatment with ER targeting agents sensitizes breast cancer cells to NK cell–mediated killing
To determine whether estrogen deprivation therapy increased the sensitivity of breast cancer cells to NK cell–mediated cell lysis, we treated MCF7 and T47D ER+ cell lines with 4-OHT or vehicle for 24 hours. We chose this time point and dose because we had observed a complete lack of cytotoxicity prior to 40 hours in ER+ cells, indicating it would be likely that any increase in cell death would be due to NK cell cytotoxicity (figure 1C). After 24 hours of treatment, the 4-OHT was washed out and the cells were plated with human healthy donor NK cells isolated from PBMCs at a 20:1 E:T ratio for 18 hours (figure 2A). After treatment with 4-OHT, MCF7 cells exhibited an ~2-fold increase in sensitivity to NK cell killing (NK1, p<0.05; NK2, p<0.0005) and in T47D cells there was an ~1.7-fold increase in cell killing by NK1 (p<0.05) and an ~4-fold increase in cell killing by NK2 (p<0.005) (figure 2B). To determine whether this effect was mediated by the ER, we interrogated ER− cells for the same effect. When TNBC cell lines BT549 and SUM149 were pretreated with 4-OHT, we recapitulated the sensitization to immune cell killing that occurred in ER+ cell lines. BT549 cells exhibited very low killing by NK cells overall, increasing from zero cell death to 8% cell death in 4-OHT-treated cells killing by NK1 (p<0.005), and demonstrating an 8-fold increase in cell killing by NK2 (p<0.05). SUM149 cells demonstrated low-to-no killing when untreated, with 4-OHT resulting in an ~13-fold increase in killing by NK1 (p<0.005) and an ~5-fold increase in killing by NK2 (p<0.005) (figure 2B).

While tamoxifen is commonly prescribed for premenopausal women, fulvestrant is a SERD that is frequently used in postmenopausal women. We next asked whether immune sensitization was specific to tamoxifen or if fulvestrant would also induce increased killing. We pretreated MCF7 and T47D with fulvestrant for 24 hours as previously reported and subjected the cells to 18-hour NK cell killing assays. Similar to 4-OHT, we found that pretreatment with fulvestrant sensitized both MCF7 and T47D to NK cell killing. MCF7 cells demonstrated an ~2-fold increase in killing by NK1 (p<0.05) and NK2 (p<0.005), whereas killing of T47D cells was increased ~2-fold and ~5-fold by NK1 (p<0.0005) and NK3 (p<0.005), respectively (figure 2C). Importantly, when we pretreated ER− cell lines with fulvestrant, we observed similar significant increases in immune cell killing (figure 2C).

Untreated BT549 cells were not killed by NK1, with killing increasing to ~8% following treatment with fulvestrant (p<0.005), whereas NK2 cell killing was increased...
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Figure 1  Characterization of estrogen receptor (ER) expression and tamoxifen cytotoxicity in breast cancer cell lines. (A) Breast cancer cell line phenotypes based on previous studies. (B) Verification of RNA expression levels of ERα and ERβ by qRT-PCR. (C) Breast cancer cells treated with 5 µM tamoxifen and tamoxifen metabolites 4-OHT and endoxifen demonstrate that tamoxifen cytotoxicity is dependent on ER expression.

~8-fold following fulvestrant pretreatment (p<0.005). The fulvestrant-mediated increase in killing of SUM149 cells was dramatic using both NKs, with an increase from no killing to ~26% cell death observed when cultured with NK1 (p<0.0005), and an ~29-fold increase in cell killing by NK2 (p<0.005) (figure 2C). Of note are the differing levels of NK lysis from different donors. This is reflective of the cytotoxicity of each individual donor’s NK cells and is a phenomenon indicating the heterogeneity of primary samples that we have previously observed.18 These data demonstrate that sensitization to NK killing results from multiple modes of endocrine deprivation and is a potential therapeutic target in premenopausal and postmenopausal women.

4-OHT increases immune cell killing in MCF7 4-OHT resistant cells
Twenty to thirty percent of patients with ER+ breast cancer are resistant to tamoxifen.25 As we demonstrated that 4-OHT increases NK cell killing of ER− cell lines, we next generated a 4-OHT resistant cell line to determine whether this strategy would also apply to patients who are tamoxifen resistant.

Parental MCF7 cells were treated with high-dose (20 µM) 4-OHT for 7 days. We then maintained surviving clones in 5 µM 4-OHT for 30 days to allow for regrowth. A stock of resistant cells was maintained indefinitely in 5 µM 4-OHT, which was washed out for 7 days prior to experiments (figure 3A). To verify that these cells were resistant to 4-OHT, we first repeated real-time cell analysis comparing the effects of 5 µM, 10 µM, and 20 µM 4-OHT to the parental and resistant cell lines. We found that 5 µM and 10 µM 4-OHT had a very similar effect on the parental cell line, with a decrease in cell index beginning approximately 60 hours after treatment. When treated with 20 µM 4-OHT, we observed an immediate cytostatic effect compared with vehicle and lower doses, and a
decrease in cell index starting at approximately 48 hours post-treatment. In the 4-OHT resistant cell line, there was no decrease in cell index compared with vehicle treatment at any dose (figure 3B). To determine the mechanism of resistance, we performed a flow-based multidrug resistance (MDR) assay and observed that while there was a trending increase in MDR proteins MRP and BCRP in the 4-OHT resistant cell line, no significant change in MDR-associated molecule expression was associated with 4-OHT resistance (figure 3C).

We next examined ER expression by qPCR and determined that in the 4-OHT resistant cell line, ERα was significantly downregulated (p<0.0005) (figure 3D). These data indicate that loss of ER expression is the likely mechanism for resistance to 4-OHT. Finally, we pretreated MCF7-4OHT resistant cells with 4-OHT for 24 hours before removing the 4-OHT and plating 4-OHT resistant cells with NK cells overnight. We observed ~2-fold increases in NK-mediated cytotoxicity from both NK3 (p<0.05) and NK4 (p<0.005), demonstrating that MCF7 cells resistant to the known cytotoxic activity of 4-OHT remain sensitive to 4-OHT-mediated increased NK killing (figure 3E). These data show that even in patient populations with ER+ tumors that have de novo or acquired resistance to tamoxifen, there remain effects unrelated to tamoxifen cytotoxicity that sensitize them to immune-mediated killing.

**Multiplexed RNA profiling analysis of tamoxifen-treated breast cancer cells identified activation apoptotic signaling pathways**

To interrogate the mechanism by which 4-OHT and fulvestrant sensitize cells to NK cell–mediated killing, we treated

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**Figure 2** Treatment with estrogen receptor (ER) targeting agents sensitizes breast cancer cells to NK cell–mediated killing. (A) Experimental schema for 18-hour 111IN labeled NK cell killing assay. (B) 24-hour pretreatment with 5 µM of selective estrogen receptor modulating drug 4-OHT sensitizes both ER+ and ER− breast cancer cells to NK cell–mediated killing. (C) 24-hour pretreatment with 50 mM selective estrogen receptor degrading drug fulvestrant sensitizes both ER+ and ER− breast cancer cells to NK cell–mediated killing. Cell lysis was evaluated through 111IN-release assay at 20:1 effector-to-target cell (E:T) ratio. *p<0.05.
Figure 3  4-OHT-resistant MCF7 cells are still sensitive to 4-OHT-mediated increases in NK cell killing. (A) Experimental schema for generation of 4-OHT-resistant cells. (B) Real-time cell analysis of 4-OHT demonstrates that MCF7 4-OHT-resistant cells are resistant to tamoxifen metabolites. (C) Flow-based multidrug resistance assay indicates no significant difference in expression of common drug-resistance proteins. (D) qRT-PCR reveals decreases in expression of ERα and ERβ in the MCF7 4-OHT-resistant cell line. (E) 4-OHT treatment sensitizes MCF7 4-OHT-resistant cells to NK killing. Cell lysis was evaluated through 111In-release assay at 20:1 E:T ratio. *p<0.05.

The ER+ cell line MCF7 and the ER− cell line SUM149 with 4-OHT for 18 hours, harvested RNA, and performed NanoString multiplexed RNA profiling analysis. After conducting a NanoString multiplexed RNA profiling analysis using Ingenuity Pathway Analysis software, we observed that several of the most highly activated signaling pathways in both MCF7 and SUM149 cell lines were associated with death receptor and apoptosis signaling (Figure 4A). Further investigation revealed that activation of these signaling pathways reflected upregulation of tumor necrosis factor (TNF), TRAIL receptor (TNFRSF10A), and Caspase 9. Increased apoptotic signaling is a key component of immunogenic modulation, and therefore is likely a candidate mechanism driving tumor cell sensitivity to NK cell killing after...
Figure 4  RNA analysis of tamoxifen-treated breast cancer cells identified activation of apoptotic signaling pathways. (A) Summary of 4-OHT-mediated changes in canonical signaling pathways in MCF7 (ER+) and SUM149 (ER−) cell lines. Pathways defined by IPA analysis. (B) Specific genes upregulated by 4-OHT in the death receptor and apoptotic signaling pathways. CASP9, caspase 9; CASP10, caspase 10; FASLG, Fas ligand; GP6, glycoprotein 6; NGF, nerve growth factor; STAT3, signal transducer and activator 3; TNF, tumor necrosis factor; TNFRSF10A, tumor necrosis factor receptor superfamily member 10A (TRAIL receptor 1).

Moreover, TRAIL-mediated killing is a common mechanism of NK-mediated cell lysis. We hypothesized that activation of these pathways by estrogen deprivation sensitizes ER+ and ER− breast cancer cells to NK-mediated killing.

Breast cancer cells express non-canonical estrogen receptor GPR30; specific activation of GPR30 sensitizes breast cancer cells to NK cell killing.

After demonstrating that 4-OHT and fulvestrant sensitize breast cancer cells to NK cell–mediated killing regardless
of ER status (figure 2), and that this likely occurs through immunogenic modulation and activation of apoptotic signaling mechanisms (figure 4), we next examined how this was occurring in cells that do not express ERα. Prior studies have characterized G protein-coupled receptor 30 (GPR30, also known as G protein-coupled estrogen receptor, GPER) and demonstrated that it binds estrogen, as well as ligands such as the estrogen receptor modulating drugs tamoxifen, 4-OHT, and fulvestrant.

GPR30 has been shown to be associated with apoptosis and to activate several pro-apoptotic signaling pathways, including ERK-mediated PI3K/AKT signaling, p53-mediated Bax signaling, and the induction of mitochondrial reactive oxygen species.

We hypothesized that treatment with ER targeting drugs sensitizes both ER+ and ER− breast cancer cells to NK cell-mediated killing through GPR30. To determine the expression of GPR30 and whether it is affected by 4-OHT, we treated cells with 5µM 4-OHT for 24 hours prior to isolating whole cell protein. We observed that GPR30 is expressed in all four cell lines and found no significant difference in GPR30 expression with 4-OHT treatment (figure 5A).

Next, we treated SUM149 ER− breast cancer cells with 4-OHT, fulvestrant, and the small molecule G-1, a selective agonist for GPR30, to interrogate whether all three molecules induced similar apoptotic protein signatures. We treated SUM149 cells with 5µM 4-OHT, 50 nM fulvestrant, or 50 nM G-1 for 24 hours and completed an apoptotic protein array (online supplemental figure S2A), similar to the level of immune cell infiltrate found in patients’ breast tumors, along with the standard 20:1 NK effector cell:target ratio. We found 10 pro-apoptotic and four anti-apoptotic proteins with a fold change >1.5 after treatment (figure 5B). Notably, we observed upregulation in both TRAIL R1, R2 and Fas, which have been previously shown to be associated with immunogenic modulation.

Moreover, the apoptotic array demonstrated increased cytochrome c expression, caspase-3 cleavage, and activation of pro-apoptotic p53 signaling, all of which are promoted by GPR30 signaling in breast cancer.

As there are multiple immune cell types that target apoptotic cells, we next performed a complex coculture killing assay using a 1:1 ratio of tumor cells to immune subset characterized PBMCs (online supplemental figure S2A), similar to the level of immune cell infiltrate found in patients’ breast tumors, along with the standard 20:1 NK effector cell:target ratio. We found that in both BT549 and SUM149 ER− cell lines, the presence of PBMCs did not significantly impact the ability of fulvestrant to sensitize tumor cells to NK cell killing (online supplemental figure S2B). We next examined whether the observed apoptotic signature was indicative of cells fully undergoing apoptosis. We treated SUM149 cells with 50nM fulvestrant for 24 hours and performed Annexin V/PI staining and found that there was no increase in the apoptotic cell subset. These data indicate that while fulvestrant activates apoptotic signaling pathways, it is not inducing cell death in these cases (figure 5C).

To confirm that GPR30 was capable of sensitizing breast cancer cells to NK cell killing, we treated MCF7 and SUM149 cells with 50nM G-1 for 24 hours in parallel with cells treated with 4-OHT or fulvestrant. We determined that G-1 recapitulated the effects of 4-OHT and fulvestrant, and that treatment with G-1 increased immune-mediated killing regardless of ER status, with pretreatment by all three drugs resulting in similar ~2-fold increases in cell killing across multiple healthy donor NKs (MCF7, NK5: 4-OHT p<0.05, G-1 p<0.05; NK6: 4-OHT p<0.005, fulvestrant p<0.05, G-1 p<0.05; SUM149, NK5: 4-OHT p<0.05, fulvestrant p<0.0005, G-1 p<0.005; NK6: 4-OHT p<0.05, fulvestrant p<0.0005, G-1 p<0.0005). Finally, to confirm the role of GPR30 in sensitizing breast cancer cells to NK cell killing, we performed a transient shRNA knockdown of GPR30 in ER− SUM149 cells (figure 5E), left panel), followed by treatment with 4-OHT or fulvestrant for 24 hours. As previously demonstrated, both 4-OHT and fulvestrant treatment significantly increased NK cell killing (NK7: 4-OHT p<0.005, fulvestrant p<0.001; NK8: 4-OHT p<0.05, fulvestrant p<0.05). However, this effect was completely abrogated by knockdown of GPR30, demonstrating that increased sensitivity to NK cell killing is dependent on GPR30 (figure 5E). These data support our hypothesis that 4-OHT and fulvestrant binding to GPR30 induces immunogenic modulation.

Fulvestrant sensitization of ER− breast cancer cells increases killing by NK cells treated with N-803 and with PD-L1 t-haNKs

While fulvestrant or 4-OHT treatment sensitizes tumor cells to NK cell killing, the efficacy of this could be restricted by a patient’s innate NK cells. Therefore, we explored the use of two novel immunotherapeutics that have entered clinical trials. N-803 is an interleukin (IL)−15/N72D superagonist complexed with IL-15RSu-shi-Fc fusion protein that promotes the expansion and cytotoxicity of NK cells.

Using ER− BT549 and SUM149 cell lines, we pretreated tumor cells with fulvestrant and pretreated healthy donor NK cells with N-803 for 24 hours. After both drugs were washed out, we plated overnight killing assays. While N-803 alone is highly effective at increasing NK cell killing as previously reported, further synergized with fulvestrant-mediated tumor sensitization to result in high tumor cell killing (figure 6A). In BT549 cells, N-803-treated NK cells resulted in an ~3-fold (NK9; p<0.0005) and ~8-fold (NK10; p<0.0005) increase in cell killing over vehicle-treated BT549 cells, and an ~2-fold (NK9; p<0.0001) and ~5-fold (NK10, p<0.0001) increase in cell killing of fulvestrant pretreated targets. Moreover, BT549 cells that were pretreated with fulvestrant and killed by N-803-treated NK cells had a 1.5-fold (NK9, p<0.0005) and 1.6-fold increase (NK10, p<0.0001) in cell killing over vehicle-treated cells (figure 6A). Similar results were observed when using the SUM149 cell line. Pretreatment with N-803 increased NK killing of vehicle-treated SUM149 ~2-fold using NK9 (p<0.05) and from no killing to ~50% cell lysis in NK10 (p<0.0001). When target SUM149 cells were pretreated with fulvestrant, we observed an ~1.3-fold (NK9, p<0.05) and ~11-fold (NK10, p<0.0001) increase in cell killing by N-803-treated NK cells.
Figure 5  Breast cancer cells express non-canonical estrogen receptor GPR30. Specific activation of GPR30 sensitizes breast cancer cells to NK cell killing. (A) GPR30 protein expression in breast cancer cells is unaffected by tamoxifen treatment. (B) SUM149 cells were treated for 24 hours with 5 µM 4-OHT, 50 nM fulvestrant, or 50 nM G-1 and protein analyzed by apoptotic protein array. (C) SUM149 cells were treated with 50 nM fulvestrant for 24 hours followed by Annexin V/PI staining. (D) Cells were treated for 24 hours with 50 nM of specific GPR30 agonist G-1, followed by co-culture with NK cells. Cell lysis was evaluated through 111 In-release assay at 20:1 E:T ratio. *p<0.05. (E) SUM149 cells were transfected with siCTRL or siGPR30 for 48 hours, followed by 24-hour treatment with 4-OHT or fulvestrant and co-culture with NK cells. Cell lysis was evaluated through 111 In-release assay at 20:1 E:T ratio. *p<0.05 between treatment group and DMSO. cIAP-1, cellular inhibitor of apoptosis 1; FADD, Fas-associated death domain protein; HO-1, heme oxygenase 1; PON2, serum paraoxonase/arylesterase 2; TRAIL R1, tumor necrosis factor–related apoptosis-inducing ligand receptor 1; TRAIL R2, tumor necrosis factor–related apoptosis-inducing ligand receptor 2.

cells compared with untreated. Fulvestrant pretreated SUM149 cells cultured with N-803-treated NK cells had an ~1.6-fold (NK9, p<0.05) and ~1.7-fold increase (NK10, p<0.005) compared with vehicle-treated SUM149 cells cultured with N-803-treated NK cells (figure 6A). These data reveal a significant combinatorial effect between fulvestrant pretreatment of target tumor cells and N-803 treatment of NK cells, demonstrating a potential dual-pronged clinical strategy of both sensitizing tumor cells and boosting the cytotoxicity of innate NK cells.
Figure 6  Fulvestrant sensitization of ER− breast cancer cells increases killing by NK cells treated with N-803 and PD-L1 t-haNKs. (A) 24-hour pretreatment of NK cells with 50 nM N-803 synergizes with 24-hour pretreatment of ER− breast cancer cells with 50 mM selective estrogen receptor degrading drug fulvestrant to increase NK cell–mediated killing over N-803 pretreatment alone. (B) Flow staining demonstrates that 24-hour pretreatment of ER− cells with 50 mM fulvestrant increases PD-L1 protein expression in SUM149 but not BT549 cells (insets). 24-hour pretreatment of ER− cells with 50 mM fulvestrant increases PD-L1 t-haNK-mediated cell killing. Flow cytometry analysis was repeated >3 times with similar results. 24-hour cell lysis was evaluated through $^{111}$In-release assay at 20:1 E:T ratio. *=p<0.05.

PD-L1 t-haNKs are a novel engineered cell line derived from NK-92 and expressing a high-affinity CD16, endoplasmic reticulum-retained IL-2, and a PD-L1 chimeric antigen receptor. As in many cancers, breast tumors express PD-L1, and it has been demonstrated that PD-L1 is upregulated in TNBCs compared...
with ER+. Therefore, PD-L1 specific t-haNKs may be an optimal choice for cell therapy in these models. To examine the expression of PD-L1 in BT549 and SUM149 cells and investigate whether fulvestrant upregulates PD-L1, we performed flow cytometry following 24-hour fulvestrant treatment. While BT549 PD-L1 expression remained unchanged, there was an increase from 11.7% to 26.3% positive in SUM149 (figure 6B inset). Therefore, fulvestrant-treated SUM149 may be especially sensitive to PD-L1 t-haNK killing. To test whether fulvestrant sensitizes breast cancer cells to killing by PD-L1 t-haNK, target cells were pretreated with fulvestrant, and then plated in an overnight killing assay with PD-L1 t-haNK cells. We observed that fulvestrant increased PD-L1 t-haNK killing of BT549 cells ~1.3-fold (p<0.05) and of SUM149 cells ~2.3-fold (p<0.005) (figure 6B). These data demonstrate that fulvestrant pretreatment sensitizes ER− breast cancer cells to killing by PD-L1 t-haNK cells.

**Fulvestrant combined with N-803 decreases TNBC tumor growth in vivo**

Finally, we investigated whether fulvestrant was capable of inducing immunogenic modulation to inhibit TNBC tumor growth in vivo in animals with functional immune systems. To do so, we used the murine TNBC cell line EMT-6. We first performed a western blot to confirm that EMT-6 expresses GPR30 (figure 7A). After verifying GPR30 expression, we treated EMT-6 cells with 50 nM fulvestrant for 24 hours, followed by flow cytometry staining for immunogenic modulation markers we had previously observed to be upregulated through GPR30 (figures 4 and 5). We found that expression of TRAIL R2 increased from 81.2% in vehicle-treated to 97.4% in fulvestrant-treated cells, with an increase in MFI from 13,309 to 17,406. Furthermore, we observed an increase in MHC class 1 from 23.7% to 31.6%, a previously reported characteristic of immunogenic modulation. Similar to the human breast cancer cells examined, we also found that fulvestrant treatment increased expression of PD-L1 from 34.2% to 48.3%. To determine whether the combination of fulvestrant and N-803 inhibited tumor growth, we injected 3×10^6 EMT-6 cells into the mammary fat pad of BALB/c mice, and treated animals with 5 mg/kg fulvestrant, 1 µg N-803, or a combination on days 7 and 14. We found that the combination of fulvestrant +N-803 significantly decreased tumor growth compared with control (p<0.05 at day 21). Future studies will confirm the role of NK cells by in vivo depletion. These data demonstrate that while the TNBC cell line EMT-6 is resistant to fulvestrant monotherapy and N-803 activation of NK cells is insufficient to suppress tumor growth alone, the combination of fulvestrant sensitization of tumor cells and N-803 activation of NK cell activity was sufficient to suppress tumor growth in vivo. Interestingly, we observed that tumors treated with fulvestrant alone grew faster than control tumors. While we found that GPR30 induces an apoptotic cell signature (figure 4), it has also
previously been reported to promote proliferation in TNBC cells.\textsuperscript{44, 45} It is possible the increased tumor growth in fulvestrant-treated tumors is the result of fulvestrant inducing GPR30-mediated proliferation, and in combination with the significant decrease in tumor growth when fulvestrant is combined with N-805 is further evidence for combining fulvestrant treatment with immunotherapy when targeting GPR30.

**DISCUSSION**

Few immunotherapy strategies have materialized for TNBC. While several phase III trials have reported positive results using checkpoint inhibitors,\textsuperscript{46–49} most breast cancer tumors are generally considered immunologically “cold,” with low immune cell infiltration, and are highly difficult to target with immunotherapy.\textsuperscript{48} To effectively generate anti-tumor responses, combinations of immunology agents that target multiple aspects of the immune system are necessary. This includes therapeutics that increase immune infiltrate and induce expansion of cytolytic immune cells, as well as those that enable an anti-tumor response through blockade of immune checkpoint molecules such as PD-L1 or CTLA-4 or by increasing the sensitivity of tumors to immune-mediated killing through immunogenic modulation.

We previously demonstrated that endocrine deprivation agents induce immunogenic modulation in both endocrine receptor-dependent and receptor-independent mechanisms. In prostate cancer, treatment of tumor cells with the androgen deprivation agents enzalutamide and abiraterone sensitized them to immune cell killing through androgen receptor–dependent apoptotic signaling.\textsuperscript{49} Furthermore, when we interrogated androgen receptor–positive and androgen receptor–negative breast cancer cell lines, we observed that both enzalutamide and abiraterone sensitized breast cancer cells to immune cell killing through an androgen receptor agonistic mechanism.\textsuperscript{48} Extending those findings, we hypothesized that estrogen receptor targeting agents might sensitize breast cancer cells to immune cell killing.

Herein, we demonstrated a novel mechanism for immunogenic modulation in breast cancer. When pretreated with tamoxifen metabolite 4-OHT or the SERD fulvestrant for 24 hours, both ER+ cell lines MCF7 and T47D were sensitized to NK-mediated cell killing (figure 2B,C). These effects were recapitulated in the ER− cell lines BT549 and SUM149. The effects of anti-estrogens on non-ERα receptors are underappreciated. Anti-estrogens were not designed with functional activity at any other estrogen receptor than ER, yet the literature demonstrates the specificity of their binding to other ERs, including GPR30, as well as activity via this receptor. Our findings conform and extend a novel effect wherein tamoxifen and fulvestrant bind to the underappreciated and non-canonical estrogen receptor GPR30 and a potential new immunotherapeutic strategy for tamoxifen and fulvestrant in the ER− breast cancer patient population.

While ER+ patients have highly effective targeted agents, 20%–30% of ER patients are resistant or will become resistant to tamoxifen treatment.\textsuperscript{55} Resistance can be either de novo or acquired, with the mechanisms leading to resistance still largely under investigation. It is hypothesized that de novo resistance may be due to loss of P450 expression, resulting in tamoxifen’s inability to be metabolized to the more active 4-OHT and endoxifen forms.\textsuperscript{51} It is also possible that there are modifications to the pathways downstream of ERα, or that additional survival pathways (such as PI3K) rescue the cell from tamoxifen-induced cell death.\textsuperscript{52} To interrogate whether estrogen receptor targeting agents would sensitize tamoxifen-resistant cells to immune cell killing, we generated a 4-OHT-resistant MCF7 cell line. The 4-OHT-resistant cell line exhibited decreased ER expression compared with the parental cell line, rendering it immune to the cytotoxic effects of 4-OHT (figure 4B). However, it maintained the ability to be sensitized to NK cell killing by 4-OHT. Interestingly, we found that the 4-OHT-resistant cell line exhibited decreased NK cell killing compared with the parental cell line, both when treated with the vehicle and with 4-OHT. This is likely due to additional phenotypic changes to the selected resistant clone that resulted in differential expression of immune cell killing or cell death–related signaling pathways. These data suggest that an off-target effect of tamoxifen and fulvestrant induces immunogenic modulation.

To further investigate the effects of 4-OHT on breast cancer cells agnostic of ER status, MCF7 and SUM149 cell lines were treated with 4-OHT for 18 hours and their RNA isolated for multiplexed RNA profiling. We observed that several of the most upregulated canonical pathways between both ER+ MCF7 and ER− SUM149 cell lines were associated with death receptor and apoptotic signaling. Induction of apoptosis is a classic feature of immunogenic modulation, especially upregulation of TRAIL receptors, one of the primary mechanisms for NK-mediated cytotoxicity (figure 3A,B). These data led us to hypothesize that GPR30, a receptor on the cell surface that has been demonstrated to bind estrogen,\textsuperscript{28, 29} tamoxifen,\textsuperscript{30} and fulvestrant\textsuperscript{37} and to be associated with apoptosis,\textsuperscript{33, 34} may be mediating immunogenic modulation in breast cancer cell lines. GPR30 has previously been shown to be expressed in both ER+ and ER− cell lines,\textsuperscript{35} and GPR30 expression is associated with a worse outcome in metachronous contralateral breast cancer\textsuperscript{36} as well as being associated with tamoxifen resistance.\textsuperscript{34, 35} Interestingly, several pathways exhibited different changes in expression in MCF7 and SUM149 cell lines. This is likely due to expression (or lack thereof) of ER in these cell lines, and the interplay between ER-mediated signaling and GPR30-mediated signaling within the cell. Using western blotting, we demonstrated that GPR30 is expressed in both MCF7 and SUM149 at the protein level (figure 5B).

Furthermore, through an apoptosis protein array, we confirmed our multiplexed RNA profiling findings of increased apoptotic signaling (figure 3A,B), and
demonstrated that 4-OHT, fulvestrant, and G-1 induced similar pro-apoptotic signatures (figure 5B). Twenty-four-hour treatment with the selective GPR30 agonist G-1 resulted in sensitization of breast cancer cells to NK-mediated cytotoxicity, indicating that GPR30 is capable of mediating immunogenic modulation (figure 5D). To confirm these data, we knocked down GPR30 and found that this completely abrogated the increased NK cell killing mediated by treatment with 4-OHT and fulvestrant. Finally, we treated EMT-6 inoculated mice with fulvestrant in combination with N-803. We found that while fulvestrant monotherapy did not inhibit tumor growth, the combination of fulvestrant and N-803 did significantly reduce tumor size, demonstrating the necessity of both tumor cell sensitization and immune cell activation for effective tumor therapy.

While several studies have investigated the immunomodulatory effects of tamoxifen in patients with breast cancer, the results have been inconclusive. In 17 patients with single-breast cancer who received tamoxifen for 1 month, researchers observed an increase in NK activity. Conversely, prolonged tamoxifen treatment of 10 patients with bilateral breast cancer resulted in a decrease of NK activity compared with pre-treatment, although both pre-treatment and post-treatment NKs exhibited higher activity than those isolated from healthy controls. Examination of NK cells isolated from patients with breast cancer who had received adjuvant tamoxifen for 1.5–2 years also found decreased NK cell activity in vitro compared with those NK cells isolated from healthy control. While NK cells do express ERs, the mechanisms of these results are unknown and it has been hypothesized they may occur through estrogen-independent mechanisms.

There are currently no published data investigating the impact of fulvestrant treatment on patient immune systems or NK cell function. Interestingly, we observed an increase in PD-L1 expression in ER− SUM149 breast cancer cells after fulvestrant treatment (figure 6B inset). This finding may have important ramifications for future breast cancer immunotherapy and will be further explored in continuing studies. The impact of tamoxifen and fulvestrant on patient immune systems and on immunogenicity of tumor cells themselves indicates the potential necessity of combining standard-of-care with additional immunotherapy agents. Numerous novel immuno-oncology drugs are currently in development that may combine effectively with immunogenic modulation in breast cancer. Previous studies have demonstrated efficacy when combining immunogenic modulation with therapeutic cancer vaccines to expand tumor-specific cytotoxic T cells; however, NK cell populations do not benefit from these strategies. It is clear that either enhancement of innate NK cells or supplementation with an NK adoptive cell transfer strategy may effectively combine with 4-OHT- or fulvestrant-mediated sensitization of tumor cells.

One method of activating innate NK cells is through the use of cytokine superagonists, such as N-803, a recombinant IL-15/IL-15Rα. IL-15 is a cytokine that plays a role in the maintenance of peripheral innate immune cells, T cell memory, and lymphoid development. N-803 has been shown to induce expansion and cytotoxicity of NK and CD8+ T cells and is in phase I and II clinical trials where it has been shown to be well tolerated. In patients with non-small cell lung cancer, N-803 in combination with nivolumab resulted in a 29% objective response rate, and a recent phase II trial of N-803 and BCG in bladder cancer has reported 18/20 subjects having a complete response. We show here that N-803 and the therapeutic chimeric antigen receptor NK cell line PD-L1 t-haNK18 (currently in phase I clinical trials) demonstrate increased NK cell killing in combination with fulvestrant (figure 6); this result warrants further investigation toward a potential clinical path for combination treatment.

CONCLUSIONS

Combining standard-of-care therapies known to induce immunogenic modulation with immunotherapy provides an opportunity to sensitize patients’ tumors toward immunotherapy with drugs that are already FDA approved with well-known safety profiles. These studies provide a rationale for further pre-clinical investigation of ER targeting drugs in an immunotherapy context and in novel ER indications, and for the potential use of ER targeting drugs in clinical trials in combination with immunoncology agents.

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