Combined treatment with epigenetic, differentiating, and chemotherapeutic agents cooperatively targets tumor-initiating cells in triple negative breast cancer.

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Abstract:

Efforts to induce the differentiation of cancer stem cells through treatment with all-trans retinoic acid (ATRA) have yielded limited success, partially due to the epigenetic silencing of the retinoic acid receptor (RAR)-β. The histone deacetylase inhibitor, entinostat, is emerging as a promising antitumor agent when added to the standard of care treatment for breast cancer. However, the combination of epigenetic, cellular differentiation, and chemotherapeutic approaches against triple negative breast cancer (TNBC) has not been investigated. In this study, we found that combined treatment of TNBC xenografts with entinostat, ATRA, and doxorubicin (EAD) resulted in significant tumor regression and restoration of epigenetically silenced RAR-β expression. Entinostat and doxorubicin treatment inhibited topoisomerase II-β (Topo II-β) and relieved Topo II-β-mediated transcriptional silencing of RAR-β. Notably, EAD was the most effective combination in inducing differentiation of breast tumor-initiating cell in vivo. Furthermore, gene expression analysis revealed that the epithelium-specific ETS transcription factor-1 (ESE-1 or ELF3), known to regulate proliferation and differentiation, enhanced cell differentiation in response to EAD triple therapy. Finally, we demonstrate that patient-derived metastatic cells also responded to treatment with EAD. Collectively, our findings strongly suggest that entinostat potentiates doxorubicin-mediated cytotoxicity and retinoid-driven differentiation to achieve significant tumor regression in TNBC.
Introduction

Triple-negative breast cancers (TNBC) lack expression of estrogen receptor (ER), progesterone receptor (PR) and HER2, and comprise approximately 15-20% of breast cancers. They continue to be a clinical problem because of their relatively poor prognosis, aggressive behavior, and lack of targeted therapies, leaving chemotherapy as the mainstay of treatment (1). Retinoic acid and its products, such as all-trans retinoic acid (ATRA), induce differentiation of various types of stem cells, including those that are present in breast cancer (2,3). However, in clinical trials ATRA has shown limited therapeutic success (4) that may be attributed, in part, to frequent epigenetic silencing of the retinoic acid receptor (RAR)-β (5). We and others have shown that histone deacetylase (HDAC) inhibitors cause re-expression of RAR-β and sensitize the cells to treatment (6,7).

Acetylation of histone proteins controls transcription of genes involved in cell growth, and the expression of histone deacetylases (HDACs) is frequently upregulated in several malignancies (8). Although HDAC inhibitors showed limited effect as single agents in breast cancer, their use in combination with other anticancer agents is currently being evaluated (9). Studies in advanced solid tumors in which HDAC inhibitors were combined either with doxorubicin (10) or with paclitaxel and carboplatin (11) suggested enhanced antitumor activity. The HDAC inhibitor, entinostat, used in combination with retinoic acid in patients with advanced solid tumors was associated with prolonged stable disease (12).

Here, we show that a combination of entinostat, ATRA, and doxorubicin effectively killed tumor cells in culture and decreased tumor size of xenografts of TNBC cell lines, and present pilot data on its effectiveness in metastatic ascites from patients. Further, we provide insights into the mechanisms underlying the enhanced effects observed with the drug combinations.
Materials and Methods

Details are provided in Supplementary Methods online.

Patient samples, Cell Lines, Constructs, and Reagents. Freshly resected breast tissue of women undergoing reduction mammoplasty, primary tumors, and pleural effusion from women undergoing treatment, and also collected through the Rapid Autopsy Tissue Donation Program were provided by the Johns Hopkins Surgical Pathology Department under approved protocols. CD24+ and CD44+ cells were isolated from normal breast tissue as described (13).

Cell lines were recently obtained from the American Type Culture Collection; SUM-149 and SUM-159 cells were obtained from Dr. S. Ethier. The cell lines were not authenticated by us, however early passages (p2-5) of the ATCC authenticated cell lines were used. Sources of other reagents: siRNA to RAR-β (Dharmacon), TopoIIβ and ELF3 (Qiagen), ATRA, doxorubicin, and paclitaxel (Sigma Chemicals), and carboplatin (Johns Hopkins Oncology Pharmacy). Entinostat was provided by Syndax Pharmaceuticals, LLC.

ChIP analysis. ChIP assay was performed essentially as described (14). Antibodies used were acetylated H3 (Millipore), RAR-α (Santa Cruz), and TopoII-β (Santa Cruz).

Flow Cytometry. Cells were stained with CD24-FITC (clone ML5), CD44-PE (clone 515, BD Pharmingen), CD326 (EpCAM)-APC (clone HEA-125, Miltenyi Biotec) and 7AAD (BD Pharmingen), or Annexin V-Alexa fluor 488 and Propidium Iodide (Molecular Probes), for quantification of apoptosis, necrosis, and analysis of the cell cycle.

Tumor Sphere Assay. Tumor sphere assay was performed as previously described (15). Pleural effusion samples from breast cancer patients were plated in serum free MEGM medium containing 10% pleural effusion supernatant and supplements (13).
Xenograft and Limiting Dilution Assay. All animal studies were performed following approval of the Animal Care Committee of the Johns Hopkins School of Medicine. First generation xenografts of MDA-MB-231 cells were established in athymic nude mice by injecting $2 \times 10^6$ tumor cells subcutaneously (s.c.). The mice were treated for 4 weeks with entinostat (2.5 mg/kg) 5 days/week per os (oral); ATRA (5 mg/kg) 5 days/week intraperitoneal (i.p.), doxorubicin (2 mg/kg) once a week intravenous (i. v.), or carboplatin (50 mg/kg) i.p., single dose, three days after the first entinostat treatment. For limiting dilution assays, the tumors were digested with collagenase/ hyaluronidase (13). Single cells were injected at dilutions of $5 \times 10^6$ to $5 \times 10^3$ along with Matrigel into humanized mammary fat pads (16,17).

Transcriptome array. MDA-MB-231 cells were treated for 48h with entinostat (2.5 µM), ATRA (1 µM), and doxorubicin (0.2 µM) singly or in combination. RNA was extracted and Illumina HumanHT12v4 gene expression array was performed. The GEO accession number: GSE63351.

Statistical Analysis. Cell line results were expressed as mean ± standard errors of mean (SEM). Student’s T-tests were performed on pairwise combinations of data to determine statistical significance defined as $p < 0.05$. qRT-PCR data of tumor xenografts, and tumor volume results were analyzed using the median and two-tailed Mann Whitney U Test. For in vivo studies, multilevel mixed-effects models were employed using Tukey’s procedure. Tumor incidence of the secondary transplants following injection of cells at limiting dilutions was used to determine the tumor-initiating frequency by L-Calc software.

Results

A combination of HDAC inhibitor, retinoids, and chemotherapy decreases tumor size.
The contribution of chemotherapeutic agents to the growth inhibitory effects of HDAC inhibitors and retinoids on TNBC cells has not been previously studied. The combination (EAD) of entinostat (E), all-trans retinoic acid (A), and doxorubicin (D) resulted in the strongest growth inhibition of three TNBC cell lines (MDA-MB-231, SUM-149 and SUM-159) in culture (Fig. 1A). Doxorubicin provided the most additive benefit to the triple combination, and EAD was more cytotoxic to TNBC cells in comparison to combinations that included paclitaxel (EAP) or carboplatin (Supplementary Fig. S1A, B).

We next evaluated whether the epigenetic combination therapy causes regression of TNBC xenografts in immunodeficient mice. EAD was most effective in decreasing tumor volume of MDA-MB-231 and SUM-159 xenografts (Fig. 1B and Supplementary Table S1 and S2). Again, the presence of doxorubicin in the triple combination was associated with a higher additive effect on decreasing tumor volume compared to carboplatin (Supplementary Fig. S1C and Supplementary Table S3 and S4).

**EAD therapy induces cell death by apoptosis and necrosis.**

We sought to investigate the mechanisms through which the epigenetic combination therapy induced cell death. Since we found that doxorubicin doses of 200 nM caused cells to arrest at G2 phase (not shown), we determined cell viability following treatment with lower doses of doxorubicin (6.25-50 nM). Addition of entinostat and ATRA to doxorubicin tended to induce cell death even at these lower doses of doxorubicin (Supplementary Fig. S1D). We next treated MDA-MB-231 for 48 h and analyzed effects on apoptosis and necrosis 5 days after removal of drug. E+D treatment doubled the incidence of apoptotic (Annexin V+) and necrotic cells (Annexin V+ and Propidium Iodide+ cells) compared to doxorubicin (12.5 and 25 nM) or entinostat alone (Fig. 1C and Supplementary Fig. S1E). Compared to doxorubicin, EAD
treatment induced slightly more necrosis than E+D (Fig. 1C and Supplementary Fig. S1E). By assessing cells in sub-G1 phase of cell cycle we confirmed in an independent assay, that in comparison to doxorubicin, E+D and EAD increased the number of apoptotic cells (Fig. 1D). Significantly, we observed that epigenetic therapy induced necrosis and apoptosis in vivo. The study pathologist’s quantification of necrosis present in the tumor xenografts of mice treated with the eight different modalities showed that EAD-treated tumors tended to have the highest necrosis score (Fig. 1E and Supplementary Table S5). Further, by immunohistochemistry (IHC), tumor xenografts from E+D and EAD treated mice showed increased cleaved caspase 3 (Fig. 1F), an indicator of increased apoptosis.

**RAR-β is reactivated by a combination of entinostat, ATRA and doxorubicin.**

Preclinical studies showed that the HDAC inhibitor, trichostatin A, in combination with retinoic acid, reactivated silenced RAR-β and decreased tumor volume more efficiently than the single treatments in ER-positive cells (18). To investigate if chemotherapy potentiates the effect of HDACi and retinoic acid on RAR-β expression, we performed quantitative real time PCR of RAR-β in TNBC cells. We found that EAD treatment was most effective in inducing re-expression of RAR-β (Fig. 2A and Supplementary Fig. S2A) and its downstream genes (Supplementary Fig. S2B) (19-21). Doxorubicin potentiated E+A-induced re-expression of RAR-β (Fig. 2A and Supplementary Fig. S2A), paclitaxel had a small effect (Supplementary Fig. S2C, a), while carboplatin showed no significant effect (Supplementary Fig. S2C, b-d). In the tumors of treated mice, in comparison to single and double treatments, EAD led to higher levels of expression of RAR-β (Fig. 2B) and its target genes (Fig. 2C). These results confirmed that reactivation of the RA pathway occurs in the tumors, and correlates with the effectiveness of EAD.
RAR-β mediates EAD-cytotoxicity and is transcriptionally repressed by topoisomerase II-beta.

Additional evidence for a critical role for RAR-β in EAD-mediated cytotoxicity in TNBC cells was sought by studying their response to treatment following depletion of RAR-β. Knock-down of RAR-β in SUM-149, MDA-MB-231 and SUM-159 cells resulted in a significant decrease in the EAD-mediated induction of RA-target genes (Supplementary Fig. S2D, a, b), and in cell death (Fig. 2D and Supplementary Fig. S2 E, a, b).

We next investigated molecular mechanisms underlying the effect of EAD on expression of RAR-β. Compared to other chemo-agents, doxorubicin exerted a greater effect when combined with entinostat and ATRA on RAR-β re-expression and inhibition of cancer cell growth. Intrigued by this finding, we searched for targets of doxorubicin action. One mechanism of cell death caused by doxorubicin is by stabilizing the topoisomerase II complex after it has cleaved DNA strands for replication, thereby halting cell division. In hepatocellular carcinoma cells, entinostat caused degradation of TopoII-α, but not of TopoII-β (22). Interestingly, we found that in TNBC cell lines MDA-MB-231 and SUM-159, entinostat significantly reduced both TopoII-α and TopoII-β protein (Fig. 2E, a), and mRNA levels (Supplementary Fig. S2F, a, b), in a dose- and time-dependent manner. These results identified TopoII-β as a common target for both doxorubicin and entinostat in TNBC cells. As predicted, the E+D combination resulted in a slightly stronger inhibition of TopoII-β in comparison to treatment with single agents (Fig. 2E, b and Supplementary Fig. S2F, c).

Based on published work in leukemia (23), we hypothesized that ATRA recruits TopoII-β and initiates low levels of RAR-β transcription in breast cancer cells. The E+D effect on degradation of TopoII-β, in addition to entinostat-mediated degradation of HDAC/NCOR, possibly...
potentiates RAR-β expression. To obtain further evidence for TopoII-β occupancy at the RAR-β promoter while avoiding multiple off-target effects of entinostat, we used T47D, a breast cancer cell line model with active RAR-β signaling. ATRA treatment induced binding of TopoII-β to the RAR-β promoter in T47D cells (Supplementary Fig. S2G, a), similar to effects seen in acute promyelocytic leukemia cells where TopoII-β occupies the RAR-β promoter (23). In these cells, early in RA signaling TopoII-β acts as an activator of RAR-β, while at later time points, TopoII-β mediates repression of RAR-β (23). To determine if the inhibitory action of TopoII-β on RAR-β expression was evident in TNBC cells, we used chromatin immunoprecipitation assays to verify RAR-β and TopoII-β occupancy at their respective binding sites (23). RAR-α directly regulates RAR-β transcriptional activity (5). ChIP analysis of three different putative RAR-α binding regions within the RAR-β locus was performed in SUM-159 cells (Fig. 2F). Compared to site 1 and 2, RAR-α was recruited at significantly higher levels to site 3 in SUM-159 cells treated with EAD (Fig. 2F, a and Supplementary Fig. S2G, b). TopoII-β (Supplementary Fig. S2G, c) and NCOR (Fig. 2F, b) levels were significantly decreased at site 3 in the RAR-β promoter upon EAD treatment, while the occupancy of H3K9-Ac (Fig. 2F, c) (a marker of transcriptional activation) was increased. To confirm the specific contribution of TopoII-β in RAR-β expression, we depleted TopoII-β in SUM-159 cells using siRNA (Supplementary Fig. S2H, a). Silencing of TopoII-β resulted in increased expression of RAR-β (Fig. 2G) and its target gene, CYP26A1 (Supplementary Fig. S2H, b) following treatment. Collectively, these results suggested that EAD enhanced RAR-β transcription through: 1) the binding of ATRA on RAR-α/RXR heterodimers, 2) a TopoII-β inhibitory effect of doxorubicin and entinostat, and, 3) HDAC/NCOR inhibition by entinostat at the RAR-β promoter (Fig. 2H).

**EAD therapy induces differentiation of breast tumor-initiating cells in vitro.**
We next investigated whether, besides induction of cell death, induction of differentiation is also part of the EAD’s drug effects and is associated with decrease in tumor volume. Retinoids effectively induce the differentiation of breast tumor-initiating cells (2,3,24). Since we observed higher RAR-β expression following EAD treatment, we hypothesized that epigenetic therapy in combination with chemotherapeutic agents may potentiate the differentiation effects of ATRA on breast tumor-initiating cells. We saw a marked dose response effect of doxorubicin (6.25-50 nM) in potentiating entinostat-mediated (E+D) decrease in CD44+/CD24−/Epcam+ breast tumor-initiating cells (25) in three different TNBC cell lines, MDA-MB-231 (Fig. 3A), SUM-149 (Fig. 3B), and HCC1937 (Supplementary Fig. S3A). Doxorubicin reduced tumor sphere formation in semisolid medium by 32% in comparison to entinostat or ATRA (18%), E+A (34%), E+D (85%), and EAD (90%) (Fig. 3C). Compared to E+D, EAD further decreased (2-fold) the number of tumor-initiating cells as shown in the tumor sphere formation assay (Fig. 3C), suggesting that EAD may institute a program of differentiation in the tumor cells.

To further investigate EAD’s action, we evaluated the expression of several breast differentiation markers by qRT-PCR and western blot analysis. In MDA-MB-231 cells, compared to entinostat alone, EAD increased mRNA expression of basal lineage markers such as cytokeratin 5 and 15 (Fig 3D and Supplementary Fig S3B, b), the luminal markers estrogen- and progesterone-receptors (ER and PR) (Supplementary Fig. S3B, a), and cytokeratin 19 (Fig 3D and Supplementary Fig S3B, b). EAD also increased mRNA levels of epithelial cell-specific genes such as Claudins 1, 3, 4, and 7 (Supplementary Fig S3B, c), Occludin (Supplementary Fig S3B, d) and E-cadherin (CDH1) (Supplementary Fig S3B, e), and decreased the level of the mesenchymal marker, vimentin (Supplementary Fig S3B, d). MDA-MB-231 cells treated with
entinostat alone showed a significant increase in the epithelial proteins, Claudin 1 and Occludin, as well as a decrease in the mesenchymal protein, Zeb1 (Fig 3E and Supplementary Fig S3C).

**EAD therapy induces differentiation of breast tumor-initiating cells in vivo.**

Our observations raised the possibility that EAD treatment also targets breast tumor-initiating cells in vivo. MDA-MB-231 xenografts were excised from mice treated with the different single, double, and triple drug combinations after 4 weeks (Fig. 1B) and digested to single cells (Schema in Fig. 4A). Secondary tumor transplants were significantly smaller in the EAD treated group compared to the other groups (Fig. 4B and Supplementary Fig. S4). EAD treatment significantly decreased tumor-initiating frequencies of MDA-MB-231 tumor cells that were transplanted into secondary hosts at limiting dilutions (Table 1). The frequency of breast tumor-initiating cells in EAD-treated tumor cells was determined to be 1 in 236,570 compared to 1 in 10,818 in vehicle-treated mice (Table 1, week 2.5). The EAD effect on reducing the frequency of breast tumor-initiating cells was also observed in an independent replicate (Supplementary Table S6). Following EAD, E+A was the second most effective treatment for targeting breast tumor-initiating cells in vivo (1 in 150,721) (Table 1). The tumor-initiating cell frequency in EAD treated tumors was significantly lower than in E+A treated tumors (p< 0.001). These data support the efficacy of the EAD combination in targeting tumor initiating cells.

Interestingly, tumor cells isolated from first generation xenografts treated with doxorubicin as a single agent or in combination were arrested at G2 phase of the cell cycle (Fig. 4C). On the other hand, tumor cells from the secondary transplants of ATRA-containing groups alone sustained arrest in the G2 phase (Fig. 4D). This data strongly suggests that ATRA treatment had a long term effect on in vivo induction of tumor cell arrest, providing evidence for ATRA’s beneficial effects and contribution in this combination therapy.
**ELF-3 mediates epigenetic therapy-induced differentiation.**

To gain insight into global changes brought about by the combined therapeutic strategy, we performed gene expression array analysis of MDA-MB-231 cells treated with entinostat, ATRA and doxorubicin singly or in combination. To identify putative candidates that might mediate the EAD-induced differentiation effect, we examined genes that are differentially up-regulated by EAD in comparison to E+D. Among others, we identified ELF-3, IL1β, and DHRS3. It was reported that human embryonic stem cells (hESCs) upregulate ELF-3 and RAR-β upon retinoic acid treatment (26). Treatment of nasopharyngeal squamous cell carcinoma cells (27), and MCF7 breast cancer cells (28) with retinoids also induced expression of ELF-3. qRT-PCR validation of ELF-3, and its inducer IL1β (29) in MDA-MB-231 cells treated with the different drugs showed that their expression was higher in EAD in comparison to E+D-treated cells, and was also induced by ATRA alone and E+A (Fig. 5A and Supplementary Fig. S5A). Also, ELF-3 mRNA was significantly higher in EAD treated MDA-MB-231 xenografts (Fig. 5B, a). Investigating human tissues, we found that in comparison to normal breast epithelial organoids, primary TNBCs had significantly lower expression of ELF-3 mRNA. Further, compared to primary TNBC, distant metastasis showed even lower levels of ELF-3 (Fig. 5B, b). Since metastatic tumors display enhanced cancer stem cell properties (30), and we saw a drastic decrease of ELF-3 in metastasis, we hypothesized that breast tumor-initiating cells will have lower expression of these genes and that our combination therapy might restore their expression. We observed that differentiated CD24+ cells from breast organoids had significantly higher levels of ELF-3 mRNA compared to CD44+ (Fig. 5B, c). Tumor-initiating cells isolated based on CD44+/CD24−/Epcam+ cell surface markers from MDA-MB-231 cells (25), on the other hand, showed 20-fold lower levels of ELF-3 in comparison to CD24+ cells (Fig. 5C, a). Interestingly, E+A and EAD
treatments, which showed more potent differentiation effects in vivo, were also the most effective in restoring ELF-3 in the breast tumor-initiating cell population (Fig. 5C, b).

Finally, to examine the contribution of ELF-3 in epigenetic treatment-mediated induction of differentiation, we studied the effect of silencing and overexpressing ELF-3 in MDA-MB-231 cells. We observed that depleting ELF-3 with siRNA decreased the differentiation effect, abrogating the decrease of CD44+/CD24−/Epcam+ cells in entinostat-containing groups (Fig. 5D and Supplementary Fig. S5A). On the other hand, ELF-3 overexpression enhanced the differentiation effect in entinostat-containing groups (Fig. 5E and Supplementary Fig. S5B), supporting the role of ELF-3 as one of the mediators of the therapy-induced differentiation.

Loss of RAR-β and ELF-3 are associated with aggressive breast cancer.

We next investigated if there is a correlation between RAR-β and ELF-3 gene expression with survival in patients with breast cancer. Higher expression of RAR-β correlated with increased recurrence-free survival in patients with tumors of all subtypes (Fig. 6A). Upon examination of stem cell-specific gene expression data sets, we found that ELF-3 expression was significantly lower in the breast cancer stem cell population (31,32). Interestingly, tumors in patients enriched for the mesenchymal stem-like (MSL) TNBC subtype (33) also expressed significantly lower levels of ELF-3 (Fig. 6B).

EAD targets metastatic cells in patient samples.

Finally, to validate our findings in patient-derived samples, we tested the effect of the novel combination therapy on patient-derived breast metastatic cells. Pleural effusion samples were collected from patients with treatment-refractory breast cancer (Supplementary Table S7), grown as tumor spheres, and treated with the various drugs alone and in combination. EAD treatment tends to be the most effective in decreasing the number of tumor spheres compared to vehicle
control, DMSO (Fig. 6C and Supplementary Fig. S6A). Entinostat treatment showed the strongest long term suppressive effect on sphere formation, and cells exposed to entinostat-containing combinations did not form spheres even at first passage (P1) (Supplementary Fig S6A). Moreover, ELF-3 mRNA expression was induced in metastatic cells treated with E+A and EAD (Fig. 6D and Supplementary Fig. S6B), providing additional evidence from patient-derived tumor cells that ELF-3 could be involved in the killing effect of EAD.

Thus, as shown in the schema in Fig. 6E, our comprehensive molecular and phenotypic analysis of the effect of entinostat, ATRA and doxorubicin as single and combined agents revealed that entinostat and doxorubicin targeted TopoII-β and together with ATRA re-activated RAR-β gene transcription. The addition of ATRA to entinostat and doxorubicin (EAD) induced breast cancer cell death and tumor-initiating cell differentiation, contributing to a significant reduction in tumor size.

**Discussion**

Combining HDAC inhibitors with either doxorubicin (34) or retinoids (12) has been shown to result in increased cancer cell cytotoxicity. HDAC inhibitors also potentiate retinoid effects on retinoic acid receptor beta (RAR-β) expression (18) and thereby could overcome retinoid resistance attributable to an epigenetically silenced receptor. Here, we report that a combination of the HDAC inhibitor, entinostat, a retinoid, ATRA, and doxorubicin (EAD) resulted in the greatest inhibition of xenografts of triple negative breast cancer cells, and provide further insight into the mechanism of their action alone and in combination on tumor regression.

The increase in cancer cell cytotoxicity by EAD was partially mediated by the induction of RAR-β and expression of its target genes. The highest induction of RAR-β expression and
inhibition of cancer cell growth was observed mainly in the presence of doxorubicin in the combination therapy, and less so with other chemotherapeutic agents. Since doxorubicin is an inhibitor of TopoII-β, we addressed the role of TopoII-β in the EAD effect. We found that in TNBC cells, both doxorubicin and entinostat caused degradation of TopoII-β. Previously, a direct binding of TopoII-β to RAR-β promoter was reported in acute promyelocytic leukemia (APL) cells (23). Here we show, for the first time, evidence for the binding of TopoII-β to the RAR-β promoter and inhibition of its transcription in TNBC cells. EAD treatment effectively decreased occupancy of TopoII-β and NCOR, and increased binding of H3K9-Ac to the RAR-β promoter, thereby possibly increasing RAR-β expression.

Conclusions on the effect of retinoids on tumor initiating cells are mainly based on observations in cell culture (2,3). Recently, an HDAC inhibitor, abexinostat, was shown to induce tumor initiating cell differentiation (35). In our study, ATRA and entinostat as single agents had comparable effects on the decrease of TNBC initiating cells in vivo, and were superior to doxorubicin. Most importantly, the combination of E+A was more effective in targeting breast tumor-initiating cells than single agents. Further addition of doxorubicin to E+A (EAD) achieved the optimal combination in targeting breast tumor-initiating cells both in vitro and in vivo. Thus, EAD’s effect on breast tumor initiating cell-differentiation is likely to have profound and lasting effects on decrease of tumor size and recurrence.

In addition to the role of RA/RAR-β in cancer stem cell differentiation (2,5), we identified ELF-3 as a novel mediator of the differentiation effect of epigenetic therapy. E74-like factor 3 (ELF-3) belongs to the E26 transformation-specific family of transcription factors and is known to regulate epithelial cell differentiation (36). Interestingly, we found that ELF-3 level was lower in the progenitor cell populations from normal breast and tumors, and was restored mainly
following E+A and EAD. The increase in ELF-3 levels in the breast tumor-initiating cells together with the functional assays suggest that ELF-3 is, in part, a mediator of EAD’s effects on differentiation.

We observed that high levels of RAR-β expression in tumors correlated with a better outcome in patients with breast cancer, suggesting that they may benefit from the combination therapy. Also, low ELF-3 expression was observed in tumor initiating cells isolated from patients after neoadjuvant chemo- or hormone-therapy (32), and with the Claudin-low breast tumor subtype, known to be enriched in tumor initiating cell features (31). We showed that EAD and entinostat targeted the self-renewal ability of metastatic ascites cells, supporting the possibility of developing alternative treatments to target treatment-refractory disease.

In summary, using a number of culture and xenograft model systems and primary and metastatic samples from patients, we have demonstrated the effectiveness of cooperative targeting of both differentiated and tumor initiating cell populations in breast cancer using a combination of DNA damaging agents, epigenetic and differentiation agents to achieve an optimal therapeutic outcome.

**Author contributions:**

Conception and design: V.M., N.N., and S.S.


Acquisition of data: V.M., N.N., S.A., C.A., P.O., V.S., R.C., A.B., D.P., and S.S.


Writing and/or revision of the manuscript: V.M., N.N., and S.S.
Study supervision: S.S.

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References


Table 1. EAD decreases tumor incidence at limiting dilution.

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<th>E+D</th>
<th>EAD</th>
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<tr>
<td>SC frequency</td>
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<td>1 in 10,544</td>
<td>1 in 123,342</td>
<td>1 in 23,663</td>
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</table>

Tumor incidence at week 2.5 (top) and week 3.5 (bottom) of secondary transplants of MDA-MB-231 at limiting dilutions. Table shows tumors/numbers of mice/group. The lower row indicates the estimated breast tumor-initiating/stem cell (SC) frequencies.
Figure Legends

**Fig. 1. Entinostat, ATRA, and doxorubicin (EAD) combination therapy induces cell death, by apoptosis and necrosis, and decreases tumor volume.** A) Cell growth inhibition in MDA-MB-231 (a), SUM-149 (b), and SUM-159 (c) cells treated for 48h with entinostat (2.5 µM), ATRA (1 µM), and doxorubicin (0.2 µM) singly and in combinations. B) Mice bearing xenografts of MDA-MB-231 (a) and SUM-159 (b) were treated with entinostat (2.5 mg/kg oral), ATRA (5 mg/kg i.p.), or doxorubicin (2 mg/kg i.v.) singly, or in combinations for 4 weeks. Mean tumor volume of 12-14 tumors per group is reported ± standard errors of mean (SEM). Flow cytometry determination of C) apoptotic (% Annexin V positive and Propidium Iodide (PI) negative, blue) and necrotic cells (% double positive, red) (doxorubicin 25 nM); and D) cells at subG1 following 48h treatment of MDA-MB-231 with the different drugs (doxorubicin 25 and 50 nM) and 5 days of drug withdrawal. E) Quantification of necrosis in xenografts (n= 7-10/group) (score 0-3). F) Cleaved caspase 3 immunohistochemistry and quantification in treated xenografts. Cell viability and flow cytometry results were obtained from triplicate, and after Student’s t-test, results are expressed as mean ± SEM. (Veh), vehicle; Ent (E), entinostat; (A), ATRA; Dox (D), doxorubicin. *p< 0.05; **p<0.01; ***p<0.001.

**Fig. 2. EAD potentiates expression of RAR-β and its depletion decreases drug sensitivity, and direct transcriptional regulation of RAR-β by TopoII-β.** Quantitative RT-PCR of RAR-β expression in MDA-MB-231 A) cells and B) xenografts treated with indicated drugs; and C) RA-target genes in MDA-MB-231 xenografts. D) Cellular viability assay was performed in SUM-149 cells depleted of RAR-β and treated for 48h with vehicle and EAD combination (2.5 µM entinostat, 1 µM ATRA, and 0.2 µM doxorubicin). E) Western blot analysis of a) TopoII-α and -β in MDA-MB-231 (top) or SUM-159 (bottom) for 12, 24 and 48h, following entinostat...
(1.25 and 2.5 µM); and b) TopoII-β following the indicated treatments for 48hs. β-actin: loading control. F) Diagrammatic representation of the putative RAR binding sites (RARE) in the RAR-β promoter (top). ChIP and q-PCR analysis was performed using: a) either an anti-RAR-α antibody or control IgG to Site 1, 2, and 3, b) NCOR, and c) H3K9Ac. G) qRT-PCR analysis of RAR-β in SUM-159 cells following TopoII-β knockdown and treatment. H) Model of the proposed mechanism of RAR-β induced expression by EAD. Student’s t-test was performed and the mean (±SEM) is shown.

**Fig. 3. EAD combination decreases stemness in vitro.** Flow cytometry determination, in quadruplicate, of CD44+/CD24−/Epcam+ population in MDA-MB-231 (A) and SUM-149 (B) cells, treated for 48h with entinostat (2.5 µM), ATRA (1µM), and increasing doses of doxorubicin (6.25- 50 nM) either alone, or in combinations. The percentage of CD44+/CD24− cells is shown on the right. C) Tumor sphere formation, and quantification of MDA-MB-231, treated as above (doxorubicin 25 nM) in duplicate, for 48h in 2D culture, followed by 30 days of drug withdrawal in 3D. p values (*) of each group, in relation to EAD, are shown. D) Quantitative RT-PCR of keratin gene expression in MDA-MB-231 treated for 48 h with the indicated drugs (doxorubicin 12.5 nM). p values (*) of each group, in relation to entinostat, are shown. E) Western blot (left) and quantification (right) of Claudin 1 (CLDN1), Occludin and Zeb1 proteins in MDA-MB-231 treated as above (doxorubicin 12.5 and 200 nM). GAPDH: loading control. Student’s t-test was performed and the mean (±SEM) is shown.

**Fig. 4. EAD combination decreases stemness in vivo.** A) Schematic outline of the tumorigenicity assays. B) Median tumor volume of MDA-MB-231 secondary xenografts established with 5x10⁵ cells (at week 2.5). Mann Whitney U Test was performed, and p values (*) of each group, in relation to EAD, are shown. Flow cytometry determination of cell cycle
distribution in TNBC cells isolated from (C) the first and (D) the second generation xenografts. The p values (*) of each groups in relation to doxorubicin are shown. NS= not-significant.

**Fig. 5. ELF-3 is increased in differentiated stem cell and mediates differentiation effect.** qRT-PCR of A) ELF-3 mRNA levels in MDA-MB-231 treated with entinostat (2.5 µM), ATRA (1µM), and doxorubicin (12.5 and 200 nM) combinations; B) ELF-3 in: a) tumor xenografts treated as indicated; b) normal breast ORG, primary and metastatic samples from TNBCs patients; and c) differentiated (CD24+) and stem cells (CD44+) isolated from normal breast organoids; and C) ELF-3 in the differentiated (CD44+/CD24−) and breast tumor-initiating (CD44+/CD24−) cells isolated from MDA-MB-231 cells, a) following DMSO treatment, and b) the fold change (in comparison to DMSO) following the different treatments (doxorubicin 25 nM). Flow cytometry determination of CD44+/CD24−/Epcam+ breast tumor-initiating cells in MDA-MB-231 cells: D) transfected with scramble or ELF-3 siRNA, or E) infected with lentivirus containing H163-vector or H163-ELF-3 and treated with the different drugs (doxorubicin, 25 nM). Mann Whitney U Test was performed, and the median of ELF-3 expression in xenograft and primary samples are shown. Student’s t-test was performed and the mean (±SEM) is shown.

**Fig. 6. RAR-β and ELF-3 are associated with better outcome of breast cancer patients, and EAD targets metastatic samples.** A) Overall survival Kaplan-Meier curves of 633 patients from TCGA breast cancer cohort (all subtypes), separated by median RAR-β expression. B) ELF-3 log2 expression in Lehmann et al (33) patients (n= 225) of different TNBC subtypes, including 2 basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem–like (MSL), and a luminal androgen receptor (LAR) subtype. Welch's modified t-test was used. C) Pleural ascites cells from 6 metastatic breast cancer patients (TNBC) a) grown in 3D
low adhesion cultures, for 15 days, with combination treatments of entinostat (2.5 µM), ATRA (1.0 µM), and doxorubicin (25 nM), and b) quantification. P0 denote cell passage as mammospheres. Not all treatments could be tested in 2/6 patient samples. For PE#6, a composite figure, all at the same magnification, is shown since tumor spheres were scattered. D) qRT-PCR of ELF-3 expression in the metastatic patient samples following combination therapies. E) Summary of findings on the effect of EAD combination therapy in TNBCs. Student’s t-test was performed and the mean (±SEM) is shown.
Fig. 1

A. a. MDA-MB-231 viability (%)

B. a. MDA-MB-231 tumor volume (mm$^3$)

C. Cytometry images showing Annexin V and PI staining for different treatments

D. Sub-G1 distribution (%) for MDA-MB-231

E. Necrosis score

F. Cleaved Caspase 3 positive cells

Legend:
- Veh
- Ent
- ATRA
- Dox
- E + A
- E + D
- EAD

Significance levels:
- **p < 0.01
- ***p < 0.001
- NS = not significant
- *p < 0.05
- **p < 0.01
- ***p < 0.001
Fig. 2

A. MDA-MB-231, RAR-β

B. Xenograft, RAR-β

C. Xenograft, RA-targets

D. SUM-149

E. a. b. c.

F. RARB promoter

G. SUM-159, RAR-β

H. ATRA

Dox

Ent

NCOR

RARβ silenced

Low RARβ expression

High RARβ expression
Fig. 3

A. MDA-MB-231

% CD44+CD24−/EpCAM+

Dox (nM) A+D E+D EAD

# vs Veh, * vs Dox, & vs Ent, @ vs E+A

B. SUM-149

% CD44+CD24−/EpCAM+

Dox (nM) A+D E+D EAD

# vs Veh, * vs Dox, & vs Ent, @ vs E+A

C. MDA-MB-231

Tumor sphere (%)

D. Relative expression

KRT14 KRT19 KRT15 KRT5

E. CLDN1 Occludin Zeb1 GAPDH

12.5 200 nM Veh ATRA A+D Dox 12.5 E+D EAD
Fig. 4

A. Single cells
Primary xenograft
Treatment
Secondary xenograft
Limiting Dilution

B. MDA-MB-231
(Secondary)
Tumor volume (mm³)

C. Primary Xenograft

D. Secondary Xenograft

Cell cycle distribution (%)

Sub-G1
G1
S
G2/M
Poly

Dox A+D E+A E+D EAD

Cell cycle distribution (%)

Sub-G1
G1
S
G2/M
Poly

Dox A+D E+A E+D EAD
Fig. 5

A. MDA-MB-231, ELF3

B. Xenograft, ELF3

C. Breast ca., ELF3

D. MDA-MB-231 sorted, ELF3

E. ELF3 knockdown

% CD44+/CD24-/Epcam+

Veh E+A E+D EAD

% CD44+/CD24-/Epcam+

Veh Ent ATRA E+A Dox A+D E+D EAD
Combined treatment with epigenetic, differentiating, and chemotherapeutic agents cooperatively targets tumor-initiating cells in triple negative breast cancer


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