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14. ABSTRACT ER-negative human breast cancers are radioresistant due to hypermethylation and hypoacetylation of the ER gene and restoration of ER expression in ER-negative cells by treatment with the demethylating agent (5-Aza-dC) and HDAC inhibitors restores sensitivity to radiation therapy. We used two human ER-negative breast cancer cell lines (MDA-MB-231 and Hs578t) and one ER-positive cell line (MCF-7 cells, positive control) to test the effect of these inhibitors. In addition, MDA-MB-231 cells transfected with either control vector or wild-type human ER-a were used as appropriate controls. Cells were pretreated with HDAC inhibitors or Zebularine and our preliminary data demonstrates that both the drugs can independently restore radiosensitivity to these cells. Both agents reactivated ER-a which could be detected by Western blot. Though preliminary, our data suggests that a combination of both DNA methyl transferase inhibitors and HDAC inhibitors can activate ER protein and sensitize cells to ionizing radiation.					
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INTRODUCTION

Loss of estrogen receptor- α (ER- α) expression in breast cancers is associated with increased tumor grade and proliferation. Moreover, ER negative tumors have been shown to be less responsive to radiotherapy in clinical trials. Increasing evidence suggests that epigenetic alterations play a role in the inactivation of ER gene expression. DNA hypermethylation of CpG islands located within the promoters of ER α is one mechanism for the loss of ER gene transcription in ER-negative tumors and some human breast cancer cell lines. The demethylating agent, 5-aza-2'-deoxycytidine (5-Aza-dC), can restore gene expression leading to re-expression of functional ER protein. Methylated DNA can also serve as a substrate to recruit Histone Deacetylase (HDAC) activity leading to silencing of genes through local deacetylation of histone proteins. We hypothesized that ER-negative human breast cancers are radioresistant due to hypermethylation and hypoacetylation of the ER gene and that restoration of ER expression in ER-negative cells by treatment with the demethylating agent (5-Aza-dC) and HDAC inhibitors would restore sensitivity to radiation therapy. We used two human ER-negative breast cancer cell lines (MDA-MB-231 and Hs578t) and one ER-positive cell line (MCF-7 cells, positive control) to test the effect of these inhibitors. In addition, MDA-MB-231 cells transfected with either control vector or wild-type human ER- α were used as appropriate controls. To investigate the ability of these agents in modulating cellular responses to ionizing radiation, cells were pretreated with the HDAC inhibitor for 24 hr or Zebularine (a relatively non toxic analog of 5-Aza-dC) for 24, 48 and 72hr, followed by radiation at doses of 2, 4 and 6 Gy. Clonogenic cell survival assays were performed and our preliminary data demonstrates that both the drugs can independently restore radiosensitivity to MDA-MB-231 cells. Similar results were obtained with MB-231 cells stably overexpressing ER- α . These cells were found to be relatively more radiosensitive when compared with the cells transfected with the control vector. Both these agents were able to reactivate ER- α leading to expression of detectable protein in MDA-MB-231 and Hs578t cells. The ability of the re-expressed ER protein to mediate an estrogen response is currently being tested using a plasmid containing a luciferase gene under the transcriptional regulation of estrogen responsive element (ERE) driving a thymidine kinase promoter stably transfected into MDA-MB-231 and Hs578t cells. We are also investigating the ability of the drug-induced ER to activate expression of endogenous ER-responsive genes such as progesterone receptor (PR). Though preliminary, our data suggests that a combination of both DNA methyl transferase inhibitors and HDAC inhibitors can activate ER protein which in turn makes the cells responsive to ionizing radiation.

BODY

ER is a ligand-activated nuclear receptor that regulates transcription of estrogen-responsive genes in diverse target cells. ER and its ligand, 17 β -estradiol, not only play a critical role in normal breast development but have also been linked to mammary carcinogenesis, breast tumor progression, and outcome of breast cancer patients. Given the fact that 17 β -estradiol stimulates the growth of ER-positive breast tumors via functional ER, endocrine therapy such as antiestrogen or ovarian ablation has been established as an important part of breast cancer

management. However, up to one-third of breast carcinomas lack ER at the time of diagnosis, and a fraction of cancers that are initially ER positive lose ER during tumor progression. Genetic alterations, such as homozygous deletion, loss of heterozygosity, or *ER* gene mutation, have not been reported to play a major role in loss of ER expression. There is increasing evidence that epigenetic alterations play a role in inactivation of ER gene expression. As demonstrated by Southern and MSP analyses, the ER CpG island is unmethylated in normal breast tissue and most ER-positive tumor cell lines, whereas it is methylated in 50% of unselected primary breast cancers and most ER-negative breast cancer cell lines. The methylation of these CpG cluster sites is associated with either reduced or absent ER expression. There is increasing evidence that epigenetic alterations play a role in the inactivation of ER- α gene expression. Inactive chromatin built on methylated CpG clusters is emerging as an important molecular mechanism to silence a variety of genes. DNA hyper-methylation of CpG islands located within the promoters of ER- α is one mechanism for the loss of ER transcription in ER-negative tumors and some human breast cancer cell lines. The demethylating agent 5-aza-2'-deoxycytidine (5-Aza-dC) (an agent currently in clinical trials for leukemia) can restore gene expression and lead to re-expression of functional ER protein. Methylated DNA can also serve as a substrate to recruit Histone Deacetylase (HDAC) activity leading to silencing of genes through local deacetylation of histone proteins. It has been demonstrated that the combination of 5-Aza-dC and HDAC inhibitor, can induce a 300-400 fold increase in ER- α transcript in ER-negative breast cancer cell lines. Breast tumors expressing estrogen receptor alpha (ER- α) respond well to therapeutic strategies directed at the ER or its ligands whereas those that lack ER do not.

We recently reported the outcome of over 500 patients treated with postmastectomy radiation and chemotherapy and found that negative ER was the most powerful predictor of LRR. This finding was subsequently verified in an independent set of 600 patients treated with neoadjuvant chemotherapy and postmastectomy radiation. These data indicate that ER negativity may be associated with radioresistance, and that this pathway may offer novel therapeutic targets to potentially improve breast cancer cure rates. Our hypothesis in this proposal was that ER-negative human breast cancers are radioresistant due to hypermethylation and hypoacetylation of the ER gene and that restoration of ER expression in ER-negative cells by treatment with the demethylating agent (5-Aza-dC) and the HDAC inhibitor (SAHA, in clinical trials) will restore sensitivity to radiation therapy.

To test our hypothesis we addressed the following specific aims:

1. **Specific Aim # 1. Determine the ability of 5-Aza-dC and SAHA both as single agents and in combination to synergistically enhance radiosensitivity in ER-negative human breast cancer cells *in vitro*.**
2. **Specific Aim # 2. Investigate the mechanisms of *in vitro* activity, especially those related to restoration of radiosensitivity.**

Key Research Accomplishments

As a first test of our hypothesis we obtained MDA-MB-231 cells that were stably transfected with full length estrogen receptor - α (clones designated ER α -3 and ER α -6). MB231 cells transfected with vector backbone were used as controls (designated LxSN2 and LxSN23). The

generation of these stable cell lines has been described in detail by Dr. Nakshatri from whom the cells were obtained

LxSN23 and ER α -6 cells were compared for their radiosensitivity in a clonogenic cell survival assay following exposure to various doses of radiation. As shown in Fig1.A the estrogen receptor expressing ER α -6 clone was more sensitive to increasing doses of radiation when compared with the vector control cells. The survival enhancement ratio was enhanced when the estrogen receptor gene was put back into the cells. Both the cell lines were also compared for the level expression of estrogen receptor- α by western blot analysis.

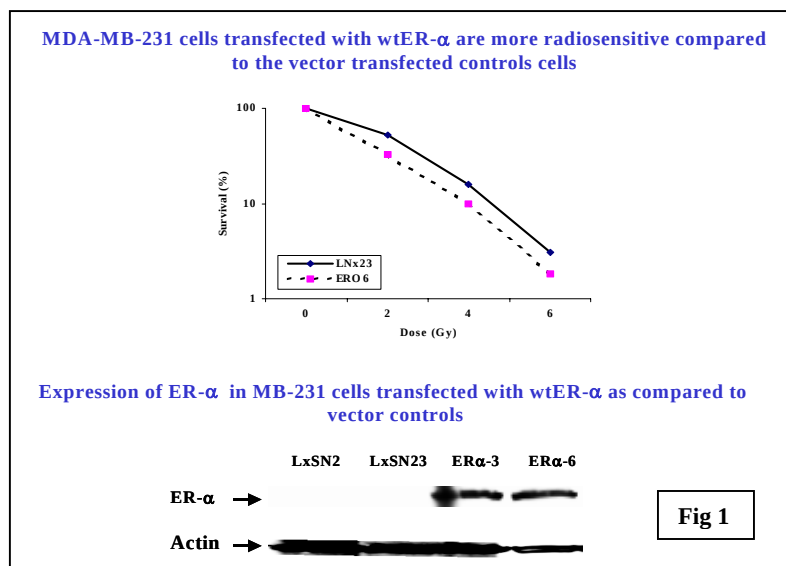
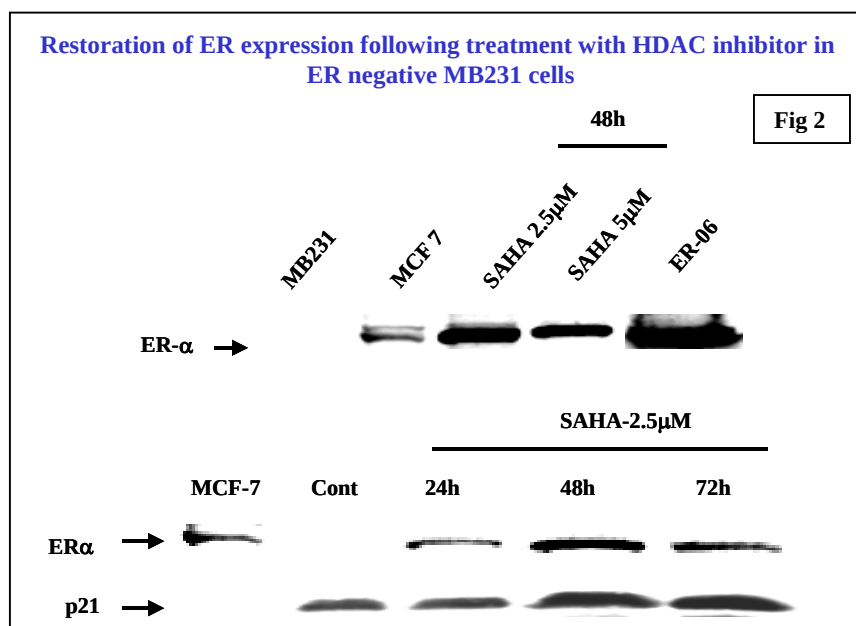


Fig 1A clearly demonstrates the lack of ER- α protein in the vector control cells and a robust expression in the full length ER- α transfected MB231 cells.

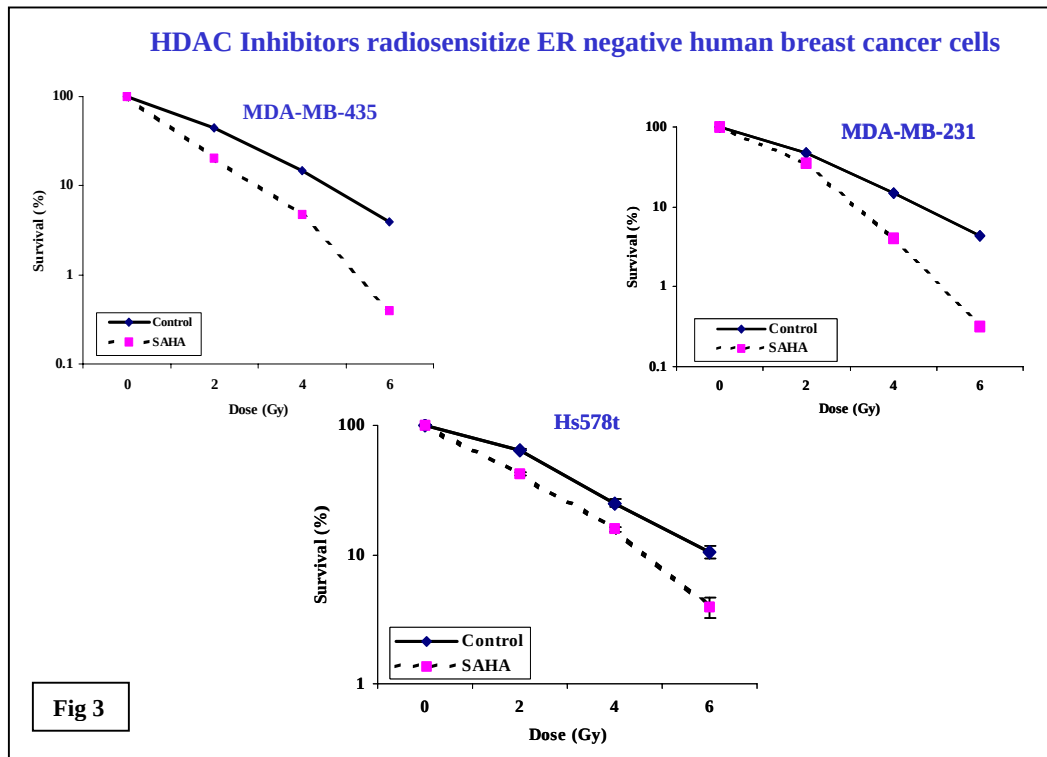
Following these experiments, we investigated the ability of the histone deacetylase inhibitor to restore estrogen receptor – α expression. MB231 cells were exposed to increasing concentrations of the drug for 48 h. The MCF-7 cells which are positive for ER- α and the full length ER transfectants were used as positive control.

Expression of ER- α in MB231 cells could be demonstrated following treatment for 48hrs with 2.5 μ M and 5 μ M SAHA. Since maximum expression of ER- α was evident at the 2.5 μ M dose of SAHA we carried out a time course experiment where MB231 cells were exposed to 2.5 μ M SAHA for 24, 48 and 72 hrs. As we could detect ER- α protein even with a 24 hr exposure to 2.5 μ M SAHA, this dose and time point was picked for all following experiments.



We next set up clonogenic assays to determine if restoration of ER- α with SAHA restored radiosensitivity to the ER-negative human breast cancer cell lines. In addition to MB231 we also evaluated the effect of SAHA on MDA-MB-435 and Hs578t. All the cell lines were treated with 2.5 μ M SAHA 24 h and then irradiated with a high dose-rate ^{137}Cs unit (4.5 Gy/min) at room

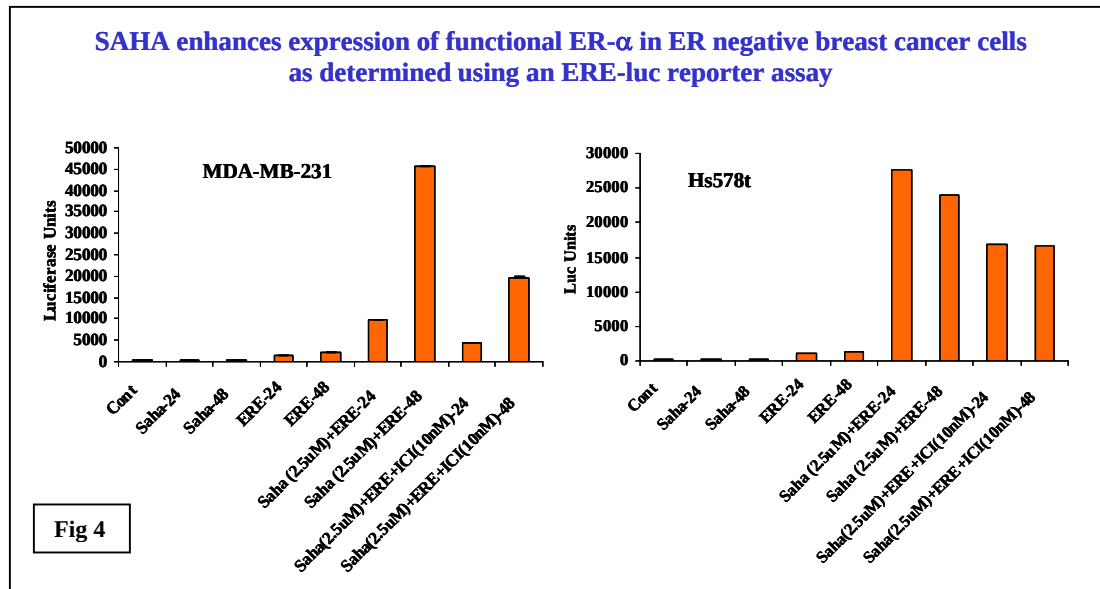
temperature. Following treatment, cells were trypsinized and counted. Known numbers were then re-plated in 100 mm tissue culture dishes and returned to the incubator to allow macroscopic colony development. Colonies were counted after about 14 days, and the percent plating efficiency and fraction surviving a given treatment was calculated based on the survival of non-irradiated cells treated with the vehicle or SAHA. Our results are depicted in Fig. 3. which shows that SAHA suppressed the clonogenic survival of the three breast cancer cell lines – MDA-MB 231, MDA-MB 435 and Hs578t. Survival at 2 Gy (SF2) was reduced from 47.5% in the control cells to 35% in the SAHA-treated MDA-MB 231 cells (Fig. 3). Similar results were obtained upon exposure of Hs578t cells to SAHA with SF2 being reduced from 65% in the control cells to 42% in SAHA-treated Hs578t cells respectively (Fig. 3).



We next tested if the SAHA induced ER in MB-231 and the Hs578t cells was functional i.e. whether the activated receptor could mediate an estrogen response. For this purpose we used a previously reported model system in which the cells were transfected with a plasmid that contains a firefly luciferase gene under the transcriptional control regulation of two Estrogen responsive elements (ERE) driving a thymidine kinase promoter. This plasmid, designated as ERE₂-tk-luciferase/SV-neo plasmid (ERE-luc) was provided by Dr. K Keyomarsi (UT MDACC). MDA-MB-231 cells were transfected with the plasmid using Fugene reagent (Roche Biochemicals). The luciferase activities were determined by the

The MB-231 and the Hs578t cell lines were treated with 2.5 μ M SAHA for 24 and 48 hrs and simultaneously transfected with the ERE-luc reporter construct using Fugene reagent (Roche Biochemicals). Lysates from these cells were analyzed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI), and the activities were measured on a luminometer. As shown in Fig 4. treatment of MB-231 and Hs578t cells with SAHA led to an

increase in luciferase activity. The kinetics of induction of luciferase activity however, were different in the two cell lines with MB231 cells having maximum induction of luciferase activity at 48hrs whereas the Hs578t cells had maximum induction of luciferase activity at 24 hrs. To confirm that the SAHA-induced reporter activity was a specific estrogen effect mediated through the action of the activated ER, the ER antagonist, ICI 182,780 was used to block the effect of estrogen in culture. As expected, ICI 182,780 suppressed SAHA-induced ERE-mediated reporter activity, suggesting that the increased reporter activity is indeed ER-mediated.



Studies are currently ongoing to gain an insight into the mechanism of radiosensitization. We are examining in detail the biochemical and molecular pathways activated/repressed by addition of these inhibitors. Since binding of estrogen to the ER is known to activate transcription of numerous factors, the effect of ER re-expression on its target genes is being evaluated.

Conclusions

1. Treatment with demethylation agents and histone deacetylase inhibitors restores functional ER- α in ER negative human breast cancer cells.
2. Induction of ER- α expression restores radiosensitization to ER-negative human breast cancer cells.

In summary, our study suggests that HDAC inhibitors can activate ER expression in ER-negative human breast cancer cell lines. The drawback of this approach however is the lack of specific effects of SAHA as it may reactivate other epigenetically regulated genes which may also play a role in the radiosensitization process.