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Induction of HEXIM1 activities by HMBA derivative 4a1: functional consequences and mechanism

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Abstract

We have been studying the role of Hexamethylene bisacetamide (HMBA) Induced Protein 1 (HEXIM1) as a tumor suppressor whose expression is decreased in tamoxifen resistant and metastatic breast cancer. HMBA was considered the most potent and specific inducer for HMBA inducible protein 1 (HEXIM1) prior to our studies. Moreover, the ability of HMBA to induce differentiation is advantageous for its therapeutic use when compared to cytotoxic agents. However, HMBA induced HEXIM1 expression required at mM concentrations and induced dose limiting toxicity, thrombocytopenia. Thus we structurally optimized HMBA and identified a more potent inducer of HEXIM1 expression, 4a1. The studies reported herein tested the ability of 4a1 to induce HEXIM1 activities using a combination of biochemical, cell phenotypic, and *in vivo* assays. 4a1 induced breast cell differentiation, including the stem cell fraction in triple negative breast cancer cells. Clinically relevant HEXIM1 activities that are also induced by 4a1 include enhancement of the inhibitory effects of tamoxifen and inhibition of breast tumor metastasis. We also provide mechanistic basis for the phenotypic effects of 4a1. Our results support the potential of an unsymmetrical HMBA derivative, such as 4a1, as lead compound for further drug development.

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Conflict of interest statement

None

Keywords

HMBA; HEXIM1; derivatives; breast cells; differentiation; antiestrogens; metastasis

1. INTRODUCTION

Hexamethylene bis-acetamide (HMBA) is a small molecule that has been investigated by the National Cancer Institute due to its potent anti-cancer and cell differentiation activities [1, 2]. HMBA induces terminal differentiation via upregulation of HMBA inducible protein 1 (HEXIM1) [3]. However HMBA failed at the Phase II clinical trial because of a dose-dependent toxicity, thrombocytopenia, which can be attributed to a very short biological half-life that required infusion of a high dosage [1, 4]. HMBA significantly induces HEXIM1 expression in various cell lines only at millimolar concentrations [3].

HEXIM1 binds to 7SK snRNA, a highly abundant non-coding RNA. Together they act as potent inhibitors of positive transcription elongation b (P-TEFb), a heterodimer between the cyclin-dependent kinase 9 (Cdk9) and its regulatory subunit Cyclin T1 (CycT1) [5-8]. As a result, elongation of RNA Pol II generated transcripts is prevented [5-8]. The regulation of the relative ratio of inactive to active P-TEFb in cells by HEXIM1/7SK snRNP plays a critical role in a wide range of cellular gene expression such as estrogen, androgen and glucocorticoid receptor regulated genes [7-10]. However, there are P-TEFb-independent actions of HEXIM1 as well [11-13].

We have been studying the role of HEXIM1 as a tumor suppressor whose expression is lost during breast [13-16] and prostate tumor progression [10]. Reports from other laboratories are consistent with the role of HEXIM1 as a tumor suppressor [17-19]. We first identified HEXIM1 as a corepressor of the Estrogen Receptor (ER) that is required for the ability of antiestrogens to inhibit the ER [20]. More recently we determined that HEXIM1 is also a corepressor of the Androgen Receptor, and is also required for the inhibitory actions of antiandrogens [10]. Decreased HEXIM1 expression in breast and prostate cancer, results in resistance to antiestrogens and antiandrogens, respectively. Furthermore HEXIM1 functions as an antiproliferative and antiangiogenic factor, resulting in the inhibition of tumor growth and metastasis [13-16]. HEXIM1 also inhibits NF-κB-dependent target genes and in doing so inhibits the transcription of pro-inflammatory cytokine genes through the MAPK pathway [21]. We also demonstrated that HEXIM1 has an important role in heart development and remodeling [12, 22]. Other research suggests that HEXIM1 suppresses Human Immunodeficiency Virus (HIV) replication [14, 23]. Thus, drug candidates that enhance the expression of HEXIM1 will have application in the treatment of cancer, heart disease, and acquired immunodeficiency syndrome.

To further enhance the potential translational impact of our studies, we conducted the first study (to the best of our knowledge) that focused on the lead optimization of HMBA, which resulted in the generation of more potent inducers of HEXIM1 expression such as compound 4a1 [24]. In the reported studies we further tested the therapeutic potential of 4a1. In particular, we report on the ability of 4a1 to induce known HMBA- and HEXIM1-induced activities, including regulation of the expression of HEXIM1 targets, enhancement of

senstivity of resistant breast cancer cells to tamoxifen, and inhibition of metastasis. HMBA was selected for clinical trials because of its ability to induce differentiation, and we now report on the induction of differentiation of triple negative breast cancer cells (TNBC) by 4a1 and the relative role of HEXIM1 in HMBA and 4a1 actions. Finally we provide insight into the mechanism of upregulation of HEXIM1 by HMBA/4a1

2. MATERIAL AND METHODS

2.1. Cell culture and transfections

MCF-7, MDA-MB-231, MDA-MB-468, BT-474, MCF10A, and HBL-100 cells were obtained from the American Tissue Culture Collection, and were maintained as previously described [25, 26]. The tamoxifen resistant cells line (TOTR) was generated by serial passage of parental MCF7 cells in growth media supplemented with 1 uM trans-hydroxytamoxifen.

Construction of expression vectors for control siRNA or HEXIM1 siRNAs were described previously [9]. MCF7 cells were transfected with expression vectors containing either the HEXIM1 miRNA insert or a control miRNA insert as previously described [9, 10]. Following blasticidin selection, cells expressing the highest level of GFP were flow-sorted and expanded. MDA-MB-231 cells were transfected with control or expression vector for Flag-tagged HEXIM1 as previously described [27].

2.2. Western Blot

Cell lysates were analyzed by western blots as previously described [9]. Anti-HEXIM1 was generated in the Montano laboratory [16]. Primary antibodies against p21 (C-19; cat# sc-397), p27 (C-19, cat# sc-528), cyclin D1 (HD11; cat# sc-246), myc (9E10; cat# sc-40), and KDM5B (H-180, cat# sc-67035) were obtained from Santa Cruz Biotechnology. Anti-Nanog was obtained from Cell Signaling Technologies. Anti-GAPDH was obtained from Millipore. Anti-HIF-1α was obtained from Oxy-cell Bioresearch. Anti-PyMT was obtained from Calbiochem.

2.3. Reverse Transcription (RT) PCR Analyses

Cells were subjected to RT-PCR analyses as previously described [9]. Signals from genes of interest were normalized to signals from GAPDH and presented as “relative mRNA expression.”

The following primers were used for RT-PCR:

hCCND1 (forward): 5’-AACAGAAGTGCGAGGAGGAG-3’

hCCND1 (reverse): 5’-CTGGCATTTTGGAGAGGAAG-3’

hMYC (forward): 5’-ATGAAAAGGCCCCCAAGGTAGTTAT-3’

hMYC (reverse): 5’-GCATTTGATCATGCATTTGAAACAA-3’

hHIF1A (forward): 5’-TCCAAGAAGCCCTAACGTGT-3’

hHIF1A (reverse): 5’-TGATCGTCTGGCTGCTGTAA-3’

hKDM5B (forward): 5’-CATCACTGGCATGTTGTTCAAATTC-3’

hKDM5B (reverse) 5’-GAATGTAGTAAGCCACAAGAAGC -3’

hGAPDH (forward): 5’-TCCACTGGCGTCTTCACC-3’

hGADPH (reverse): 5’-GGCAGAGATGATGACCCTTTT-3’

2.4. Chromatin Immunoprecipitation

ChIP assays were carried out as previously described [15]. Anti-CDK9 antibody (D-7; cat# sc-13130) was obtained from Santa Cruz Biotechnology. The primers sequences used were:

hHEXIM1 ORF fwd: ATGGCCGAGCCATTCTTGTCAG

hHEXIM1 ORF rev: GTACGGTTTCCAATGCCGCTT

2.5. Lipid Droplets (Nile Red Staining)

Cells were stained with Nile red for lipid droplets (marker of cell differentiation). Briefly, the stock solution of Nile red 1 mg/ml in acetone was diluted in PBS (1:1000). The fixed cells (4% paraformaldehyde) were incubated with diluted Nile red for 5 minutes at room temperature, rinsed with PBS and observed for the presence of lipid droplets by confocal microscopy. Images were digitally captured using a Leica microscope (Leica Microsystems Inc, IL, USA).

The intensity of Nile red staining and the number stained cells relative to the total number of cells were quantified from five different fields of vision. The product of the two values from HMBA or 4a1 treated cells were normalized to the product of the two values from DMSO treated cells.

2.6. Enrichment of stem cells from TNBC using a NANOG reporter

MDA-MB-231 were obtained that were previously transduced with NANOG promoter GFP lentiviruses [28]. GFP-NANOG transduced cells were flow sorted on a FACsAria flow cytometer, and plated for Western blot analyses and Nile red staining.

2.7. Proliferation assay

MCF7 or MCF7/TOTR cells were plated onto 96 well plates and treated with 17β-estradiol ± trans-hydroxytamoxifen in the presence of DMSO (vehicle), HMBA or 4a1 for 7 days. MDA-MB-231, MDA-MB-468, MCF10A, and HBL-100 were treated with DMSO (vehicle) or 4a1 for 7 days. Cell proliferation was assessed using the MTT based Cell Growth Determination Kit from Sigma-Aldrich according to the manufacturer's protocol.

2.8. *In vivo* studies

All animal work reported herein have been approved by the CWRU Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health guide for the care and use of laboratory animals. The maintenance and genotyping of Polyoma Middle-T antigen (PyMT) transgenic mice and production of PLGA ± 4a1 were described

previously [14]. After the appearance of palpable mammary tumors of 100 mm^3 size, 50 ul of PLGA or PLGA-4a1 (50 uM) were injected into tumors every other week.

3. RESULTS

3.1. Lead optimization of HMBA to develop potent HEXIM1 inducers

We conducted the first study, to the best of our knowledge, that is focused on the lead optimization of HMBA to generate more potent HEXIM1 inducers [24]. Our findings have provided unique molecular scaffolds that significantly induced HEXIM1 expression in prostate cancer cells, and have opened a new lead optimization direction for HMBA. We observed increased potency of one of these compounds, 4a1 (Figure 1A), when compared to HMBA in breast cancer MCF7 (Figure 1B), as we have previously observed in prostate cancer cells [24]. 4a1 also induced HEXIM1 expression in other breast cancer cells, MDA-MB-231, MDA-MB-468, and BT474 cells (Figure 1C).

3.2. HEXIM1 is required for HMBA- and 4a1-induced cell differentiation

HMBA was investigated in a Phase II clinical trial due to its potent anti-cancer and cell differentiation activities [1, 4]. Moreover, the roles of HMBA, and potentially 4a1, as differentiating factors are advantageous for their therapeutic use when compared to cytotoxic agents. We examined the relative role of HEXIM1 in HMBA-induced differentiation and regulation of expression of p21 and p27, which are known to promote cellular differentiation [29]. We also examined the ability of 4a1 to induce differentiation and the relative role of HEXIM1.

HBA and 4a1 induced p21 expression, and downregulation of HEXIM1 using HEXIM1 siRNA resulted in inhibition of HMBA- and 4a1-induced p21 expression in MCF7 cells (Figure 2A). We also stained cells with Nile red to detect lipid droplets as a marker of differentiation. Both HMBA and 4a1 induced differentiation of MCF7 cells, and HEXIM1 is required for the induction of cell differentiation (Figure 2B). Expression of Flag-tagged-HEXIM1 or treatment with HMBA or 4a1 of TNBC MDA-MB-231 cells induced expression of p27 (Figure 2C) and differentiation (Figure 2D). Experiments using another TNBC cell line, MDA-MB-468, indicated that HEXIM1 is required for the induction of p27 expression by 4a1 (Figure 2C).

3.3. HMBA and 4a1 enhanced sensitivity of tamoxifen resistant breast cancer cells to tamoxifen, and downregulated proliferation of TNBC but not non-tumorigenic breast epithelial cells

We have reported that HEXIM1 is required for the ability of antiestrogens to inhibit the activity of the Estrogen Receptor, that loss of HEXIM1 results in agonistic activity of tamoxifen, and that recurrence after tamoxifen therapy can be correlated with loss of HEXIM1 expression in human breast tissue samples [20]. Thus we determined if HMBA and 4a1 can enhance sensitivity to tamoxifen of a tamoxifen resistant breast cancer line (MCF7/TOTR). This cell line was developed using long-term exposure to tamoxifen, which resulted in decreased sensitivity to inhibitory effects of tamoxifen (Figure 3B). HEXIM1 protein expression is decreased in MCF7/TOTR when compared to parental MCF7 cells

(Figure 3A). However treatment with either HMBA or 4a1 restored HEXIM1 expression and enhanced the sensitivity of TOTR cells to trans-hydroxytamoxifen (Figure 3B).

Consistent with the induction of differentiation of TNBC by 4a1, 4a1 attenuated proliferation of TNBC cells (Figure 3C). However no downregulation of the proliferation of non-tumorigenic breast epithelial cells, MCF10A and HBL-100, was evident after treatment with 4a1. The high endogenous levels of HEXIM1 in MCF10A and HBL-100 cells resulted in only a modest increase in HEXIM1 expression (Figure 3C), which may not be sufficient to alter proliferation in these cells.

3.4. 4a1 induced differentiation of stem cells in TNBC

Differentiation therapy aimed at favoring differentiation over self-renewal programs in cancer stem cells (CSCs), induced a depletion of the CSC population [30]. We tested the ability of 4a1 to induce differentiation of stem cells in TNBC. We used MDA-MB-231 cells expressing using a green fluorescent protein (GFP) reporter for the promoter of the well established pluripotency gene Nanog [28]. Nanog-GFP1 cells gave rise to cells expressing increased levels of the embryonic stem cell transcription factors and elevated self-renewal and tumor initiation capacities [31]. Nanog-GFP1 cells were enriched using flow cytometry. 4a1 induced increases in HEXIM1 and p27 expression and a decrease in Nanog expression (Figure 4A). Consistent with increased expression of p27 in Nanog-GFP1 cells is the increase in Nile red staining as a result of 4a1 treatment (Figure 4B).

3.5. Regulation of the expression of HEXIM1 targets by HMBA and 4a1

To assess the clinical potential of HMBA and 4a1 we need to determine to what extent the targets of HMBA and 4a1 overlap with that of HEXIM1. These studies should also provide mechanistic basis for the phenotypic effects of 4a1. We focused on direct targets of HEXIM1 that we have identified [9, 10, 13, 27]. 4a1 and HMBA regulated expression of cyclin D1, c-Myc, HIF-1α, and KDM5B, (Figures 5A and 5B), which we have previously determined to be directly regulated by HEXIM1 [9, 13, 32], and play critical roles in mammary tumorigenesis, angiogenesis, and metastasis. HEXIM1 is required for the upregulation of these factors by 4a1 (Figure 5C). In view of the data reported above, we previously reported that KDM5B plays a critical role in the ability of HEXIM1 to inhibit the activity of nuclear hormone receptors in the presence of antihormonal agents [10].

3.6. HMBA derivative 4a1 inhibited metastasis

The Polyoma Middle-T antigen (PyMT) transgenic mouse is a well-characterized model of human metastatic breast cancer used for preclinical testing [33]. We also utilized the PyMT mice to test the effectiveness of 4a1 in inhibiting tumor metastasis, and used the same delivery system and treatment schedule as we used for HMBA [14]. As observed with PLGA-HMBA, PLGA-4a1 (50 uM) also induced an increase in HEXIM1 expression in mammary tumors (Figure 6A). It should be noted that the concentration of 4a1 utilized in these studies is 100-fold lower than the concentration of HMBA used in our published studies [14]. Thrombocytopenia or weight loss were not observed in PLGA-4a1 injected PyMT mice (Figures 6B and 6C). We observed decreases in macrometastatic lesions in PLGA-4a1 treated mice when compared to control mice (Figure 6D). We took advantage of

the mammary-specific expression of PyMT to detect individual early lesions or micrometastasis tumor cells in lung tissue and assessed PyMT expression in the lungs. We observed decreased PyMT levels in the lungs of PLGA-4a1 treated PyMT mice when compared to control mice (Figure 6E).

3.7. Induction of HEXIM1 expression by HMBA and 4a1 through induction of CDK9 recruitment

The induction of HEXIM1 by HMBA appears to involve the release of free P-TEFb from 7SK snRNP, and recruitment of P-TEFb to *HEXIM1* gene [34]. P-TEFb is a heterodimer between the cyclin-dependent kinase 9 (Cdk9) and its regulatory subunit Cyclin T1 (CycT1). P-TEFb phosphorylates the carboxy-terminal repeat domain (CTD) of the largest subunit of RNAP II, a phosphorylation event crucial for effective transition from an abortive to a productive phase of transcriptional elongation [35]. We thus determined if 4a1 could also upregulate the recruitment of CDK9 to the *HEXIM1* coding sequence. Chromatin Immunoprecipitation (ChIP) assays indicate comparative ability of HMBA and 4a1 to induce recruitment of CDK9 to *HEXIM1* gene in MCF7 and MDA-MB-231 cells (Figure 7). The involvement of CDK9 recruitment in the induction of HEXIM1 expression by 4a1 and inhibition of CDK9 activity and transcriptional elongation by HEXIM1 suggests that HEXIM1 may inhibit it own expression.

4. DISCUSSION

HMBA was considered the most potent and specific inducer for HEXIM1 prior to our studies. However, HMBA induced HEXIM1 expression occurs only in cell culture at concentrations of 1-5 mM [2, 3]. It is difficult to reach high concentrations of HMBA in blood circulation due to its highly polar nature and toxicity effects. Thus we used medicinal chemistry approaches to optimize HMBA and improve its ability to induce HEXIM1 expression and identified a more potent inducer of HEXIM1 expression, 4a1. Like HMBA, 4a1 also induced breast cell differentiation, enhanced inhibitory effects of the anti-hormonal agent tamoxifen, regulated the expression of direct targets of HEXIM1, and inhibited breast tumor metastasis. Our results support the potential of unsymmetrical HMBA derivatives, such as 4a1, as lead compounds, and the critical role that HEXIM1 plays in the action of these compounds.

Approximately two-thirds of breast tumors express ERα [36], and can be treated with selective ER modulators (SERMs) such as tamoxifen [37]. Tamoxifen functions as an antagonist in breast tissues by competing with estrogen for binding to ERα [38]. While it has been effectively used as an adjuvant therapy for ERα positive patients [39], the efficacy of tamoxifen treatment is limited by the development of resistance in about half of the patients after five years of therapy [40, 41]. More than 70% of tamoxifen-resistant tumors still express functional ER [36]. Our analysis of human breast cancer tissue samples indicated association of lower expression of Hexamethylene bis-acetamide (HMBA) Inducible Protein 1 (HEXIM1) with tumor recurrence in patients who received tamoxifen [20]. Treatment of a tamoxifen resistant cell line with 4a1 enhanced HEXIM1 expression

and responsiveness to tamoxifen. Our studies support the potential of 4a1 in combination with tamoxifen in the treatment of tamoxifen resistant tumors.

The abilities of HMBA and 4a1 to induce differentiation of TNBC cells indicate that their actions are not limited to ER positive breast cancer cells. Moreover, the role of HMBA as a potent differentiating factor is advantageous for its therapeutic use when compared to cytotoxic agents. Because of its important role in induction of p21 and p27, p53 plays a critical role in cell differentiation. Mutations in p53 are common in cancer cells, and are major limiting factors in the use of differentiation agents in cancer therapy. However our findings support a critical role of HEXIM1 in the induction of differentiation by HMBA and 4a1, even in the context of mutant p53 in TNBC. Moreover, 4a1 also induced differentiation of cancer stem cells in TNBC cells, and decreased expression of the pluripotency gene, Nanog. The ability of HMBA and 4a1 to induce differentiation may be through the induction of the expression of p21 and p27 expression [29]. Moreover, loss of p21 results in the acquisition of stem cell properties [29].

Because of the clinical potential of therapeutics aimed at increasing HEXIM1 expression, we set about to optimize HMBA and improve its ability to induce HEXIM1 expression. Along this line we developed polymer-mediated delivery of HMBA to mammary tumors that resulted in increased HEXIM1 expression and inhibited metastasis, without thrombocytopenia, the dose-limiting toxicity associated with HMBA in clinical trials [14]. We present results showing that 4a1 also inhibits breast cancer metastasis. HEXIM1 expression is significantly decreased in invasive or metastatic carcinomas when compared to DCIS [14], and induction of HEXIM1 expression by 4a1 would be a logical approach in the treatment of metastatic breast cancer.

Finally, by regulating the expression of several genes known to be critical in tumorigenesis, HMBA and 4a1 can act directly or indirectly on multiple cell types. By targeting several pathways critical in tumorigenesis and metastasis, HMBA and 4a1 will have therapeutic advantages. The simultaneous targeting of more than one pathway improves the likelihood of sustained inhibition by limiting the cell's ability to bypass the inhibition of any one pathway. Such adaptive or mutational bypass is commonly observed in chemotherapy currently.

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HIGHLIGHTS

- 4a1 induced breast cell differentiation
- 4a1 enhanced inhibitory effects of tamoxifen
- 4a1 inhibited breast tumor metastasis
- Mediators of the phenotypic effects of 4a1 are identified
- 4a1 has potential as lead compound for further drug development

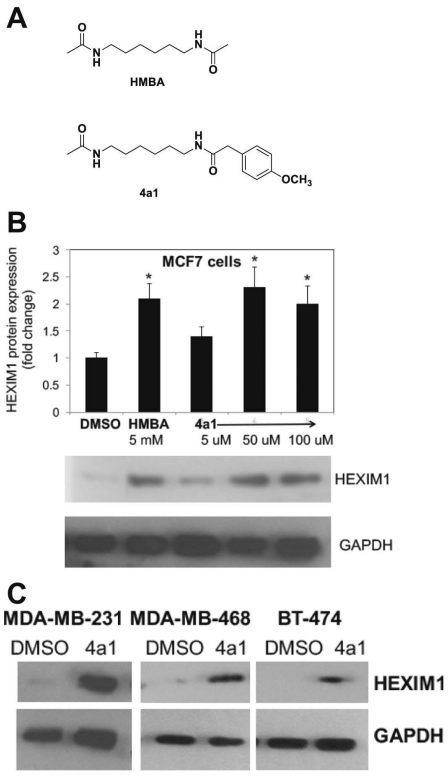

Figure 1. Regulation of the expression of HEXIM1 by HMBA and 4a1

(A) Structures of HMBA and 4a1. **(B)** MCF7 were treated with vehicle (DMSO), HMBA, or 4a1 using indicated concentrations for 8 h. **(C)** MDA-MB-231, MDA-MB-468, or BT-474 cells were treated with vehicle (DMSO), 5 mM HMBA, or 50 uM 4a1 for 8 h. Cells were processed for Western blot analyses of HEXIM1 expression. The figures are representative of 3 experiments. * $p < 0.01$ relative to DMSO treated cells.

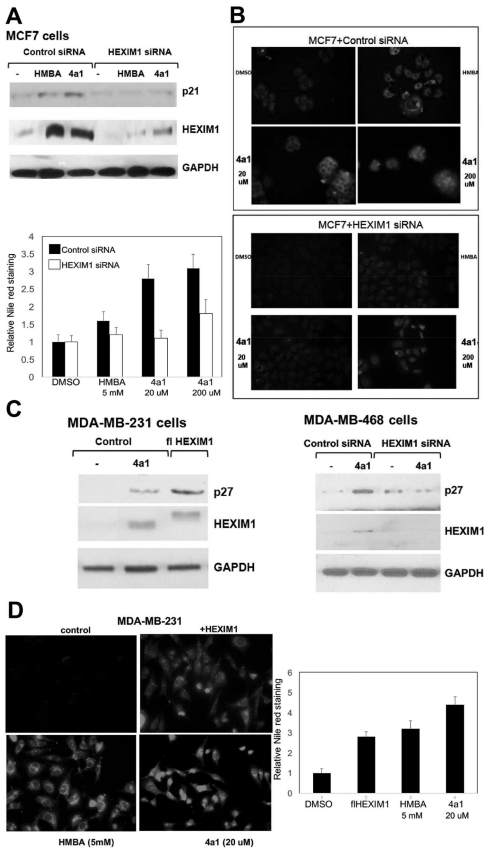

Figure 2. HEXIM1 is required for HMBA- and 4a1- induced p21/p27 expression and differentiation

MCF7 cells transfected with control siRNA or HEXIM1 siRNA were treated with vehicle (DMSO), HMBA (5 mM), or 4a1 (50 uM) for 8, 18 or 72 h. **(A)** Cells were processed for Western blot analyses of HEXIM1 or p21 expression or **(B)** stained with Nile Red to determine lipid vacoule formation (differentiation marker). MDA-MB-231 cells transfected with control or FLAG-tagged HEXIM1 expression vector or MDA-MB-468 cells transfected with control siRNA or HEXIM1 siRNA were treated with vehicle (DMSO), HMBA (5 mM), or 4a1 (50 uM) for 8, 18 or 72 h. **(D)** Cells were processed for Western blot analyses of HEXIM1 or p27 expression or **(B)** stained with Nile Red. All photographs of stained cells were taken at 40x magnification. The figures are representative of 3 experiments.

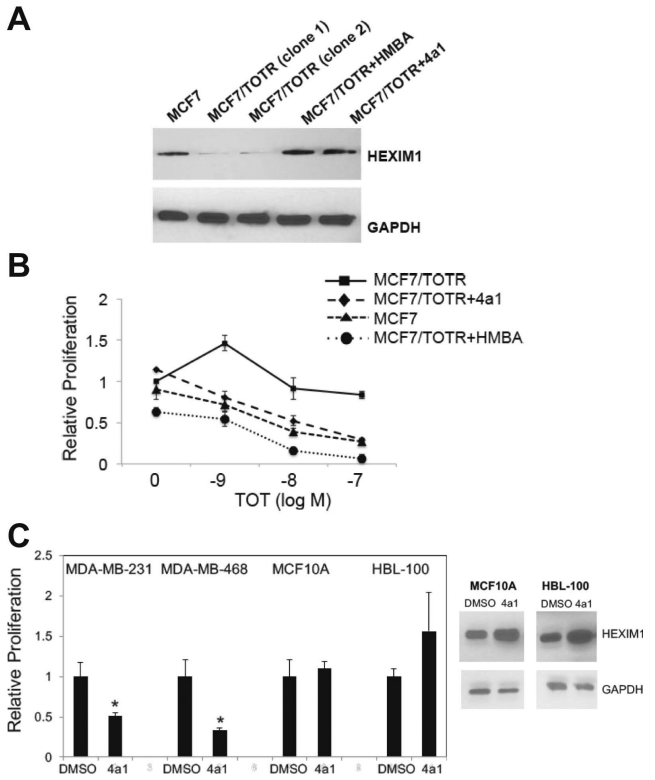

Figure 3. HEXIM1 restored sensitivity to the inhibitory effects of tamoxifen in a tamoxifen resistant cell line

(A) Western blot analyses of HEXIM1 expression in MCF7 and MCF7/TOTR cells. MCF7/TOTR clone 1 cells were also treated with vehicle (DMSO), 5 mM HMBA, or 50 uM 4a1. **(B)** MCF7 or MCF7/TOTR (clone 1) or **(C)** MDA-MB-231, MDA-MB-468, MCF10A, and HBL-100 cells were treated as indicated for 7 days. MTT assays were then performed to assess proliferation. The right panel shows western blot analyses of HEXIM1 expression in MCF10A and HBL-100 cells. The figures are representative of 3 experiments. * $p < 0.01$ relative to DMSO treated cells.

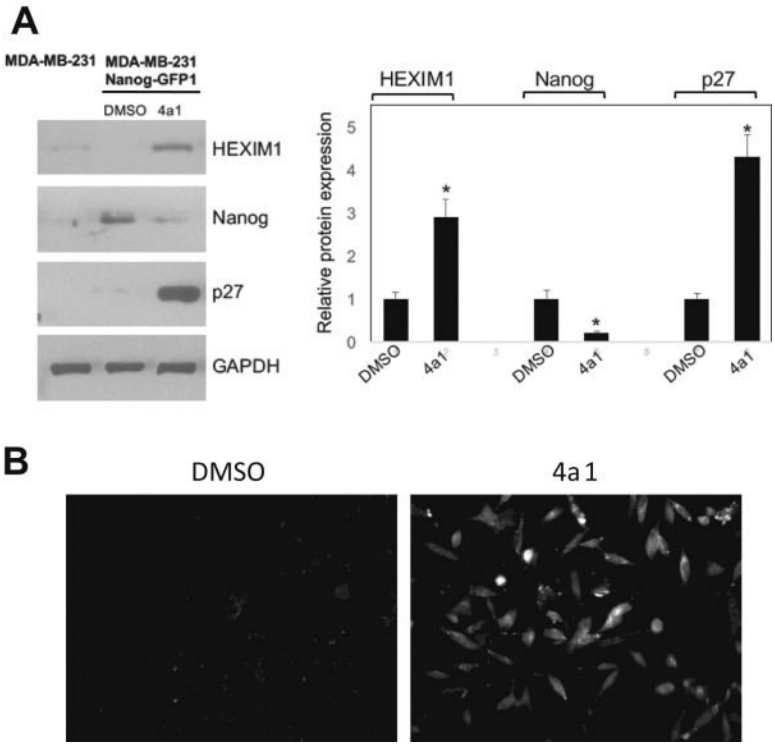

Figure 4. Induction of differentiation of stem cells in TNBC cells by 4a1

(A) MDA-MB-231 and MDA-MB-231-Nanog-GFP1 cells were treated with vehicle (DMSO) or 4a1 for 8 or 18 h and processed for Western blot analyses of indicated proteins. **(B)** MDA-MB-231-Nanog-GFP1 cells were treated with vehicle (DMS0) or 4a1 for 72 h and then stained with Nile Red. All photographs were taken at 40x magnification. The figures are representative of 3 experiments. * $p < 0.01$ relative to DMSO treated cells.

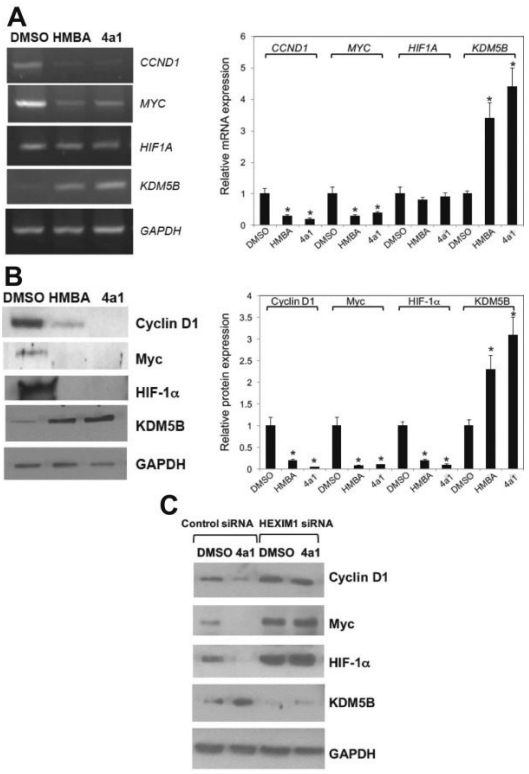

Figure 5. Regulation of the expression of HEXIM1 direct targets by HMBA and 4a1

(A) and **(B)** MCF7 cells were treated with vehicle (DMSO), 5 mM HMBA, or 50 uM 4a1 for 8 h, **(C)** MCF7 cells transfected with control siRNA or HEXIM1 siRNA were treated with vehicle (DMSO) or 50 uM 4a1 for 8 h. Cells were processed for RT-PCR analyses or Western blot analyses of HEXIM1 targets relative to GAPDH. The figures are representative of 3 experiments. * $p < 0.01$ relative to DMSO treated cells.

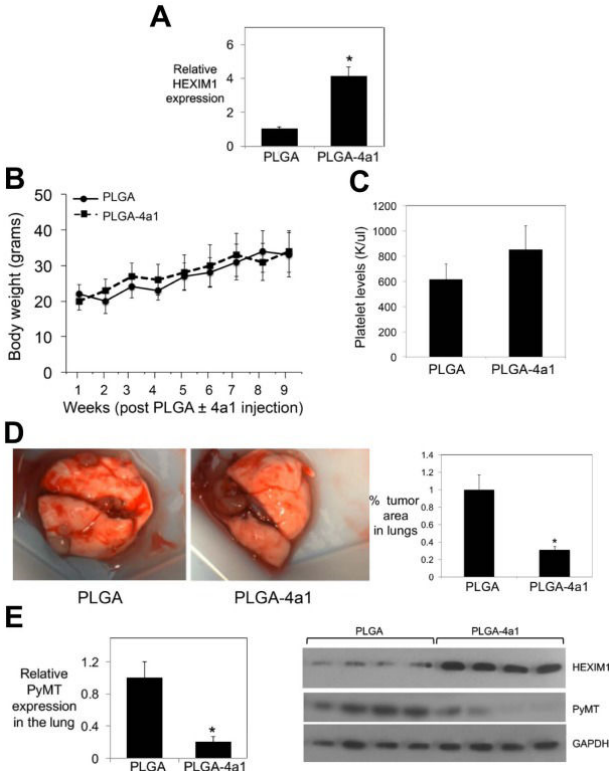

Figure 6. Injection of PLGA-4a1 resulted in increased HEXIM1 expression and decreased metastasis

After the appearance of palpable mammary tumors in PyMT mice, PLGA or PLGA-4a1 (50 uM, 50 ul volume) were injected into the tumors every other week. Mammary glands, blood, and lungs were then collected. **(A)** HEXIM1 expression in mammary tumors of PLGA ± 4a1 treated mice were determined by Western blot analyses. **(B)** Body weights were monitored weekly as indicated. **(C)** Platelet levels were determined using the HEMAVET 950FS Multi-species Hematology System. **(D)** Left panel shows lungs from PLGA or PLGA-4a1 treated mice. Right panel shows quantification of tumor area in H&E stained lung tissue sections. **(E)** Western blot analyses of PyMT expression in the lungs of PLGA or PLGA-4a1 treated mice. In **(A)** and **(E),** expression of HEXIM1 and PyMT, respectively, are expressed relative to expression of GAPDH, a loading control. Panels represent 5 mice per group (PLGA ± 4a1). * $p < 0.01$ relative to PLGA treated cells.

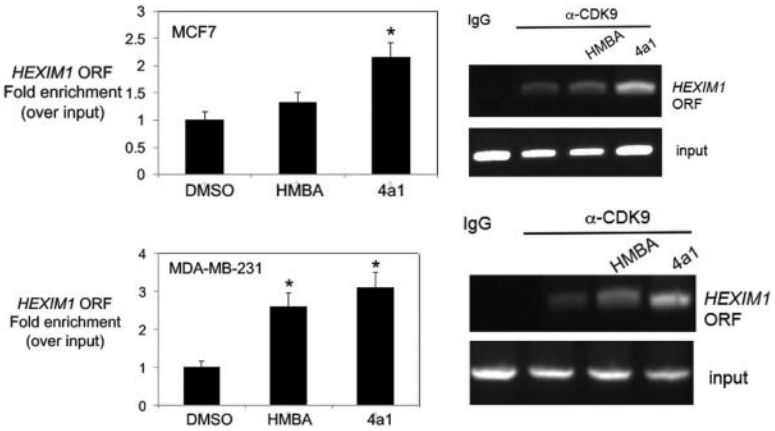

Figure 7. HMBA and 4a1 induced CDK9 recruitment to the HEXIM1 promoter

MCF7 and MDA-MB-231 cells were treated with vehicle (DMSO), 5 mM HMBA, or 50 uM 4a1 for 90 min. ChIP assays were performed with CDK9 antibodies. PCR primers were for the coding region of *HEXIM1*. The figures are representative of 3 experiments. * $p < 0.01$ relative to DMSO treated cells.