Research Article

HDAC Inhibitors (Vorinostat) As A Potential Therapy for Metastatic Breast Cancer

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ABSTRACT

Background. *Histone deacetylase inhibitors (HDACi) are an emerging group of compounds with significant chemotherapeutic potential. The hydroxamic acid and suberanilohydroxamic acid (SAHA) are currently in trials for breast cancer. HDACi, including SAHA, entinostat, panobinostat and butyrate, have previously been shown to induce apoptosis and cell cycle arrest of breast cancer cells, and there are suggestions that HDACi may also inhibit the production of the hypoxia inducible factor (HIF-1α) thereby potentially inhibiting the response of breast cancer cells to hypoxia. The current study aims to establish whether HDACi (SAHA) will inhibit breast cancer cell activity under hypoxic conditions.* ***Methods.*** *MCF7 and MDA-MB-231 Cells were cultured in both normoxia and hypoxia (1% O2) environments in the presence of SAHA (0.5-20mM) for up to 72 hours. Proliferation was then established using the MTS assay.* ***Result.*** *SAHA could inhibit breast cancer cell proliferation under hypoxic (1% O2) conditions in a dose dependent manner. In normoxia, the MDA-MB-231 (high metastatic potential, ER negative) were more sensitive than MCF-7 (low metastatic potential, ER positive) in contrast to hypoxia where MCF-7 were more responsive to SAHA than under normoxia, compared to MDA-MB- 231 which were less responsive compared to normoxia.* ***Conclusion.*** *The obtained data have important therapeutic implications as many current chemotherapies have no effect in hypoxic tumours.*

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INTRODUCTION

Over the past few decades, DNA methylation in particular has been the most intensively studied type of epigenetic modification in human cancers; specifically, its role as a silencer of tumour suppressor genes via induction of hyper-methylation [1]. Recently the process of histone acetylation has received a huge research interest as this process appeared to be directly involved in cancer development and progression [2]. The processes of histone acetylation and de-acetylation are usually held in balance as they are both critically important for the regulation of gene expression. The induction of histone acetylation by histone acetyl transferases (HATs) is crucial for gene transcription, as HAT action leading to acetylation of histones results in histones losing their positive charges thereby preventing interactions with the negatively charged DNA leading to an open chromatin composition thereby enhancing gene transcription [3]. In contrast hypoacetylation i.e. removal of acetyl groups is governed by histone deacetylase (HDAC) which are a group of epigenetic regulators and essential for silencing of genes [2]. Furthermore, HDACs are not only involved in de-acetylation of chromatin proteins but can be also implicated in non-histone protein de-acetylation which plays important regulatory roles in cellular homeostasis [4]. HDAC are known to be overexpressed in cancer and are thought to have a potential role in cancer development and progression, hence HDACs are now among the most interesting and the most promising therapeutic targets for cancer therapy. In addition of HDACs role in cancer is not limited to their impact to histone deacetylation, but also play important role in deacetylation of non-histone proteins which is responsible for DNA stability, cell proliferation, cell

migration, cell death and angiogenesis. HDACs enzymes are more properly known as lysine deacetylases [5,6]. HDAC are known to be involved in breast cancer development. HDACs activity has been reported to be related to the suppression of oestrogen receptor alpha (ER-α) transcriptional regulation [7], subsequently the absence of ER-α in normal breast epithelium has been linked to the progression of breast cancer [8]. In addition to other cancers, there is a growing body of knowledge from several experimental studies demonstrating a link between HDAC overexpression and alteration in acetylation levels which subsequently lead to breast cancer development and invasion. Various studies have shown that overexpression of various classes including class 1 and class 2 HDACs are seen in breast cancer. For example it has been suggested that overexpression of the HDAC1 and HDAC6 is associated with an increase in the invasive ability of breast cancer cells [9]. As a growing body of evidence has now clearly identified that HDAC have a distinct role in cancer, several structurally diverse classes of HDAC inhibitors have been recently designed to target these HDACs [10], in the hopes that they will inhibit cancer growth. Indeed, a number of HDAC inhibitors have been developed during the last decade that induce cell cycle growth arrest, differentiation, and/or increase apoptotic cell death as well as altering the expression of a variety of proteins including the pro-angiogenic factor, vascular endothelial growth factor (VEGF) [11]. Importantly data from breast cancer preclinical research conducted to date supports the exploration of these HDAC inhibitors for breast cancer treatment. To date, beside other HDAC inhibitors, Vorinostat and panobinostat have been approved by FDA for the treatment of cutaneous T-cell lymphoma and multiple myeloma respectively. Following FDA approval, three HDAC inhibitors; vorinostat (SAHA), entinostat, and panobinostat, are currently being evaluated clinically for breast cancer patients either as a single agent or in combination with other standard therapies such as chemotherapies, aromatase inhibitors (exemestane) or SERM (tamoxifen) [12]. Vorinostat (SAHA) is a hydroxamic acid based HDAC inhibitors which have been shown to inhibit the majority of HDACs (class I, class II and class IV) and is therefore known as a pan inhibitor. SAHA was the first HDAC inhibitors approved for clinical use by Food and Drug Administration (FDA), and was approved in 2006 for cutaneous T- cell lymphoma.

METHODS

Both MCF7 and MDA-Mb-231 cancer cells were grown in RPMI 1640 with 10% FBS and stored at 4°C. Stock solutions (1mM) of SAHA were prepared by dissolving in PBS and the 5 different concentrations were prepared (0.5nM, 1nM, 2nM, 5nM and 10nM). The MTS assay was employed to detect the growth of both breast cancer cell lines under normoxic and hypoxic conditions in multi-well plates. The MTS assays were conducted over three different time points (24, 48 and 72 hrs) under normoxic conditions and two (24 hrs and 48 hrs) for the hypoxic conditions when cells were treated with increasing concentration ranges of SAHA (Vorinostat). For the hypoxia treatment, the cells were seeded, plated and then placed in a MACS-VA 500 hypoxia chamber (Don Whitley Scientific, USA) under 1% of O_2, 5% CO_2 and 194% N_2, for 24 and 48 hrs. Data were analysed using Excel and is shown as mean + standard error of the mean (SEM) in all graphs using a non-parametric Kruskall-Wallis one-way ANOVA to compare medians of data across the groups. The EC_{50} was calculated after analyzing the cell growth percentage against the SAHA dose concentrations, with 100% set as the mean value from the untreated samples.

RESULTS

Inhibition of MCF-7 cell growth in response to SAHA treatment

The inhibitory effect of SAHA was evaluated in MCF-7 cells treated with five increasing concentrations of SAHA for 24, 48 and 72 hours. Following 24 hours treatment, there was a significant ($p=0.020$) dose-dependent inhibition seen in proliferation in response to SAHA treatment, with significance achieved at 10nM SAHA resulting in ~20% inhibition (Figure 1A, 1B). Similarly there was a significant dose-dependent inhibition in proliferation of MCF-7 cells following both 48 hours ($p=0.0165$) and 72 hours ($p<0.0001$) SAHA treatment, with significance achieved at 5nM at both time points. The EC50 for SAHA treatment at 72 hours was 3.4nM whereas it was 5nM for 48 hours of treatment.

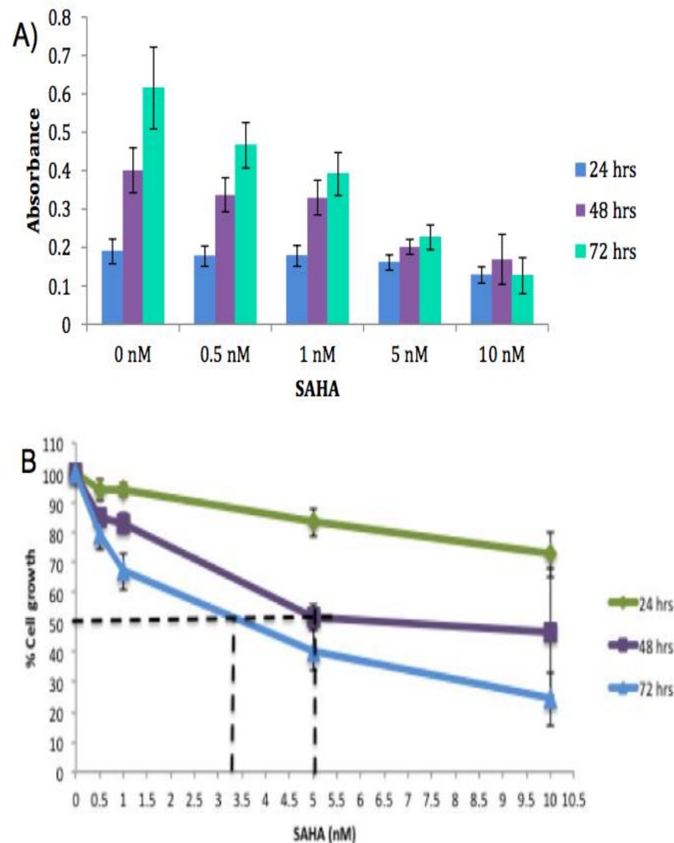

Figure 1: Proliferation of MCF-7 cells in response to SAHA treatment in normoxia. Cell proliferation is inhibited in a dose dependent manner after 48 and 72 hrs of treatment with respect to 0nM control. EC50 values for each time point are marked on the graph (48 hrs; EC50=3.4nM, 72 hrs; EC50=5nM).

Inhibition of MDA-MB-231 cell growth in response to SAHA treatment

Similar to the MCF-7 cells, proliferation of the MDA-MB-231 cells was significantly inhibited after 24 hours (p=0.009), 48 hours (p=0.004) and 72 hours (p=0.001) of treatment with increasing concentrations of SAHA, with significance achieved at 10nM after 24 hours, and 5nM after 48 and 72 hours. However, at the highest dose MDA-MB-231 cells showed a better response to SAHA treatment, compared to MCF-7 cells (Figure 2A, 2B), with the percentage growth compared to controls at 3.7% compared to 22% at the 72-hour time point. This is confirmed by the EC50 data where the EC50 values are lower for the MDA-MB-231 cells.

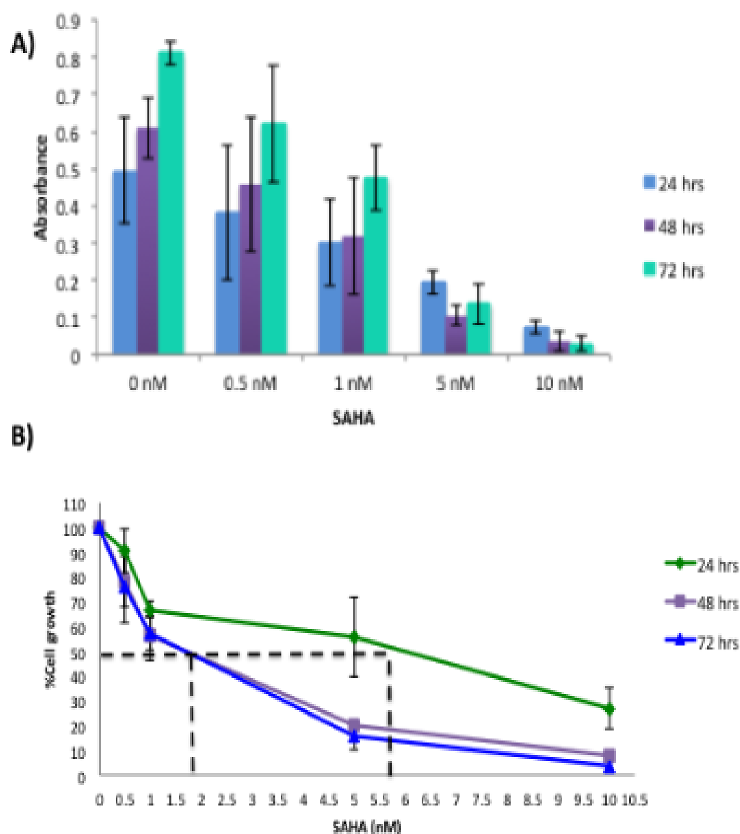

Figure 2: Proliferation of MDA-MB-231 cells in response to SAHA treatment in normoxia. Cell proliferation is inhibited in a dose dependent manner after 24, 48 and 72 hours of treatment with respect to 0nM control. EC50 values for each time point are marked on the graph (24 hrs; EC50=5.7nM, 48 hrs; EC50=1.8nM, 72 hrs; EC50=1.8nM).

Cell Growth in Hypoxia

The growth inhibitory effect under hypoxic conditions (1% O_2), was evaluated using the MTS assay. Only two time points were evaluated under hypoxia due to the fact that at 72 hours we expect a severe drop of cell viability.

Inhibition of MCF-7 cell growth in response to SAHA treatment in hypoxia

For the MCF-7 cells (Figure 3), growth rate was reduced to 47.9% at 24 hours and 32.6% at 48 hours with the highest concentrations of SAHA. This contrasts to the normoxia treatment where the growth rate was reduced to 72.7% and 46.6% after 24 and 48 hours respectively. In contrast to normoxia SAHA significantly inhibited MCF-7 proliferation after both 24 hours ($p=0.0011$) and 48 hours ($p=0.0005$) achieving significance at 5nM concentration in both cases. The EC50 was 5nM and 3.4nM at 24 and 48 hours respectively.

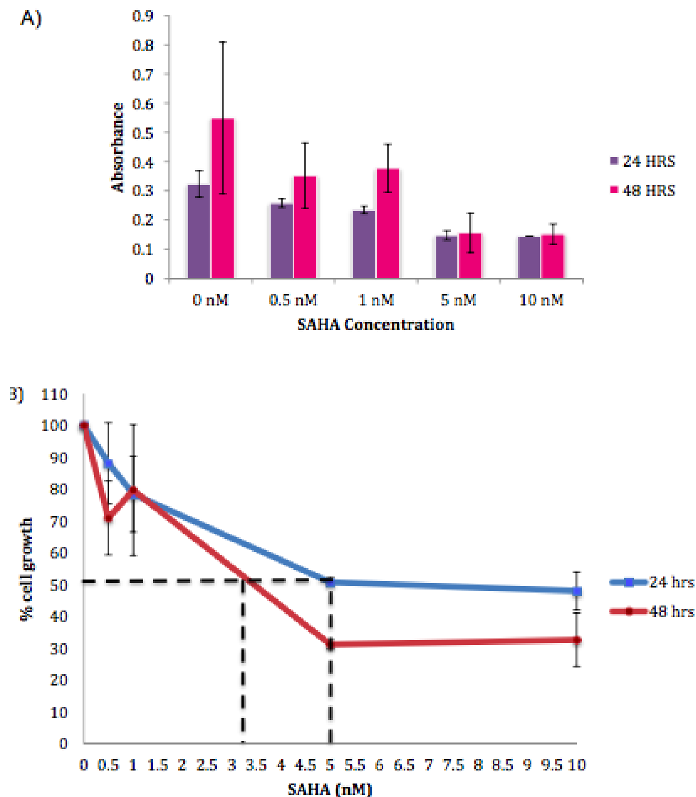

Figure 3: Proliferation of MCF-7 cells in response to SAHA treatment in hypoxia. Cell proliferation is inhibited in a dose dependent manner after 24 and 48 hrs of treatment with respect to 0nM control (24hrs; p=0.0011, 48 hrs: p=0.0005). EC50 values for each time point are marked on the graph (24 hrs; EC50=5nM, 48 hrs; EC50=3.4nM).

Inhibition of MDA-MB-231 cell growth in response to SAHA treatment in hypoxia

Similar to normoxia, SAHA treatment resulted in a greater inhibitory effect on MDA-MB-231 cells compared to MCF-7 cells with growth rate reduced to 33.8% at 24 hours and 24.95% at 48 hours with the highest concentrations of SAHA (figure 4). Similar to MCF-7 cells SAHA significantly inhibited the growth rate of MDA-MB-231 cells under hypoxia after 24 hours (p=0.0008) and 48 hours (p=0.0031) treatment, achieving significance at 5nM at both time points. However, the growth inhibition seen under hypoxia was less than that seen in normoxia for the MDA-MB-231 cells; the EC50 was 6.5nM at 24 hours, and 3.4nM at 48 hours under hypoxia compared with 5.7nM and 1.8nM under normoxia.

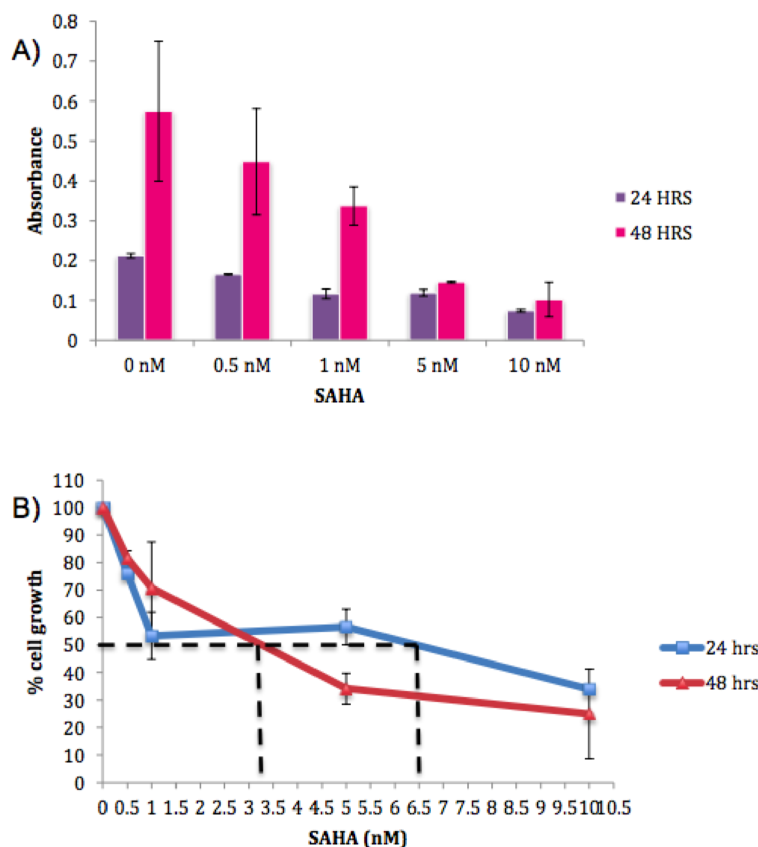

Figure 4: Proliferation of MDA-MB-231 cells in response to SAHA treatment in hypoxia. Cell proliferation is inhibited in a dose dependent manner, predominantly after 48 hours of treatment with respect to 0nM control (24hrs; p=0.0003, 48 hrs: p=0.0031). EC50 values for each time point are marked on the graph (24 hrs; EC50=6.4nM, 48 hrs; EC50=3.4nM).

DISCUSSION

Breast cancer studies revealed that Vorinostat could inhibit the clonogenic growth by inducing G1 and G2/M cell cycle arrest and subsequent apoptosis in both ER- positive and negative BC cell lines [13]. However, several in vitro experiments revealed that the cell cycle arrest is concentration dependent, and while cells accumulate mainly at G1 when exposed to low vorinostat concentrations, higher concentrations can result in arrest at G2/M [14]. Further research showed that Vorinostat arrested the growth and differentiation of a wide spectrum of human cancer cells including breast cancer cells in vitro [12,15]. In vivo, vorinostat has been shown to decrease tumour incidence by 40% in NMU-induced rat mammary tumorigenesis [16]. Based on the success of treating mammary tumours in vivo, vorinostat has now progressed to clinical trials for breast cancer. Results from the trials so far are in favour of a potent anticancer effect against a wide spectrum of breast cancer subtypes in both pre- and postmenopausal women. Results from on-going trials in combination with either standard or conventional chemotherapeutics are all encouraging. The effect of combining vorinostat with radiation therapy for breast cancer has not been looked at before. Therefore, this could provide a novel insight to evaluate this effect.

This study aimed to investigate the effect of SAHA on MCF-7 and MDA-MB-231 breast cancer cell lines in response to normoxia and hypoxia environments. The data presented show that SAHA inhibits cell proliferation in response to increasing concentrations of SAHA in both normoxia and hypoxia in both breast cancer cell lines, suggesting that SAHA could be used in combination with therapeutic agents that induce hypoxia such as anti-vascular therapies. However, further

work is required to assess whether or not the apoptosis pathway is involved in this response. It is well known that breast cancer cell lines demonstrate diversity in their morphology, molecular profiles and response to treatment [17]. The EC50 values of SAHA in MDA-MB-231 (ER negative) and MCF-7 (ER positive) cells were determined by MTS cell proliferation assays. MDA-MB-231 cells were more susceptible to SAHA treatment compared to MCF-7 cells, reflected by both the EC50 values, and the reduction in percentage growth at 72 hours. This is in contrast to previous studies which have demonstrated the EC50 of MCF-7 cells to be lower than that of MDA-MB-231 cells [18] or the same in both cases using the MTT assay, although these studies both used higher concentrations of SAHA (0.2-5μM). Several studies treating different breast cancer cell lines, reported variations in proliferation and cytotoxic sensitivity among them. Two studies, supporting the present findings, demonstrated that MDA-MB-231 cells were more susceptible compared to MDA-MB-486, BT474 [19], MCF-7, MDA- MB-435 and normal breast epithelial cells [20]. The data presented in this study demonstrate a dose-dependent effect at all concentrations of SAHA, achieving significance in most cases at 5nM. Previous data supports this finding, suggesting that SAHA has a wide therapeutic effect and an EC50 at 1-10 nanomolar concentrations (<86nM) [21], with a dose-dependent proportional response, with a mean of 3.8nM in several cancer cell lines [22].

The prevalence of hypoxia within several solid tumours has previously been demonstrated. Around 40% of breast cancers present hypoxic regions, where several transcriptional pathways, like angiogenesis, glucose metabolism and altered apoptosis are activated, by induction of the main regulator of the hypoxic response HIF-1α. As a consequence, cancer cells develop mechanisms of resistance to current chemotherapy and systemic drug treatment [23]. To overcome this, new therapeutic approaches have been attempted; i.e., HIF-1α inhibitors [24]; PARP inhibition and synthetic lethality [25], pH modulation, glucose/lactate regulation, vascular disruptive agents [26] and HDAC inhibitors [27].

Different techniques have been used to study the effects of drugs in an environment similar to the actual tumour hypoxic microenvironment. Some of them use hypoxic incubators with constant O_2, N_2 and CO_2 concentrations; inducing chemical hypoxia by using cobalt chloride (CoCl2) or glucose oxidase and catalase that deplete the generation of oxygen. Afterwards, all of them assess cell viability to look for the toxicity drug effect under hypoxia. To achieve this, clonogenic and non-clonogenic colorimetric assays like MTT, trypan blue and sulforhodamine have been used. In terms of the HDACi only a limited number of these approaches have been performed in a few cell lines [28]. Some of the reasons of the research scarcity in hypoxia may be related to the costs of the gases and the requirement for a specific style of incubator [29]. The study presented in here used a hypoxic culture chamber (MACS VA500 workstation, Don Whitley Scientific, West Yorkshire, UK) with 1% O_2 and in order to avoid any other chemical that may alter the results. To prevent any transient re-oxygenation effects caused by addition of the MTS reagent, the MTS reagent was added under anoxia.

As in the normoxia studies the EC50 values of SAHA in MDA-MB-231 (ER negative) and MCF-7 (ER positive) cells were determined by MTS cell proliferation assays. In contrast to normoxia, the EC50 values after 48 hours were almost the same with each cell line. These data demonstrate that SAHA was more efficacious on MCF-7 under hypoxia and less on MDA-MB-231 cells under hypoxia.

There are no publications investigating the effect of SAHA treatment in breast cancer cell lines in hypoxia and only one recent study by Saelen et al., 2012 [29] has investigated the effect in colorectal carcinoma. This study found that SAHA has a role as a radiosensitizer under hypoxic conditions in vitro and in a xenograft model. They also described a positive combination effect with capecitabine. However, a precise effect on proliferation and tumour growth prevention has not been assessed under hypoxic conditions [29]. The experiments performed under hypoxia and normoxia, demonstrated that SAHA was efficacious in preventing proliferation in both breast cancer cell lines, mainly in the hypoxic environment where other therapies are less or no effective [26]. However, although the MTS assay seems to be a good approach, because it relies on mitochondrial activity that can be easily affected by acidic and enzymatic effects rather than actual cell viability, it is suggested to use this assay with extreme care or to use another assay to confirm the data [30].

CONCLUSION

This study demonstrated for the first time that SAHA could inhibit breast cancer cell proliferation under hypoxic (1% O_2) conditions in a dose dependent manner. Differences could be seen in the response under normoxia and hypoxia between the

cell lines, where in normoxia, the MDA-MB-231 (high metastatic potential, ER negative) were more sensitive than MCF-7 (low metastatic potential, ER positive) in contrast to hypoxia where MCF-7 were more responsive to SAHA than under normoxia, compared to MDA-MB- 231 which were less responsive compared to normoxia. There are no previous reports looking at the effect of SAHA under hypoxic conditions, so the data presented here have important therapeutic implications as many current chemotherapies have no effect in hypoxic tumours. Therefore, it is essential to confirm this finding, establish the mechanisms of action via looking at cell cycle, apoptosis and HIF-1α expression, before assessing the potential in in vivo models of hypoxic tumours prior to clinical trials.

Disclaimer

The article has not been previously presented or published, and is not part of a thesis project.

Conflict of Interest

There are no financial, personal, or professional conflicts of interest to declare.

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