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THE pan-DAC INHIBITOR LBH589 IS A MULTI-FUNCTIONAL AGENT IN BREAST CANCER CELLS: CYTOTOXIC DRUG AND INDUCER OF SODIUM- IODIDE SYMPORTER (NIS).

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ABSTRACT

New drugs with anti-tumour activity, also able to modify the expression of selected molecules, are under evaluation in breast cancer which is becoming resistant to conventional treatment, or in metastatic disease. The sodium-iodide symporter (NIS), which mediates iodide uptake into thyroid cells, and is the molecular basis of radioiodine imaging and therapy in thyroid cancer, is also expressed in a large portion of breast tumours. Since NIS expression in breast cancer is not sufficient for a significant iodide uptake, drugs able to induce its expression and correct function are under evaluation. In the present study, we report for the first time that the pan-deacetylase (DAC) inhibitor LBH589 (panobinostat) significantly induced NIS, both as mRNA and as protein, through the increase of NIS promoter activity, with the final consequence of obtaining a significant up-take of iodide in MCF7, T47D, and MDA-MB231 breast cancer cells. Moreover, we observed that LBH589 causes a significant reduction in cell viability of estrogen-sensitive and –insensitive breast cancer cells within nanomolar range. The anti-tumour effect of LBH589 is sustained by apoptosis induction and cell cycle arrest in G₂/M. In conclusion, our data suggest that LBH589 might be a powerful tool in the management of breast cancer due to its multiple effects and support a potential application of LBH589 in the diagnosis and treatment of this disease.

KEYWORDS

DAC-inhibitors; breast cancer; NIS; targeted therapy; epigenetic

INTRODUCTION

Breast cancer is the most common cancer in women all over the world [1]. Even though its management has become more and more successful over time, owing to the different available therapeutic strategies [2], both metastatic disease and cancer which are becoming resistant to conventional treatment are emerging problems [3, 4]. New drugs with anti-tumour activity and/or able to modify the expression of selected molecules in these situations are under evaluation.

The sodium/iodide symporter (NIS) has been receiving a good deal of attention, both for its function that is requisite for potential clinical application, and for its sensitivity to a number of agents able to induce/increase its cell expression. NIS is a transmembrane glycoprotein mainly expressed in thyrocytes, functioning as iodide carrier [5]. The expression and correct functioning of NIS in differentiated thyroid cancer is the molecular requisite for successful radioiodide therapy in this neoplasm [6]. NIS is also expressed in lactating mammary glands, favouring iodide concentration in milk, and in more than 80 % of human breast carcinomas [7]. Although NIS expression is present and intense in a majority of invasive breast cancers, radioiodide accumulation has been observed in only a small fraction of cases [8, 9, 10]. The achievement of a good level of expression of NIS is regarded as a promising new strategy to enable the use of radioiodide for imaging and treatment of breast cancer [11, 12].

In non neoplastic breast cells, estrogens, oxytocin and prolactin, mainly acting in concert, were reported to induce NIS [7]. In breast cancer cells, many authors reported that retinoic acid is a powerful enhancer of NIS expression especially in association with corticosteroids [12-14]. Furthermore, in one report it was also observed that retinoic acid and dexamethasone treatment determined a significant ^{131}I -induced cytotoxicity in breast cancer cells [15].

NIS expression, in both thyroid and non-thyroid cells, is also regulated by epigenetic phenomena. NIS gene silencing can be related to the level of histone acetylation; in fact,

compounds like Trycostatin A (TSA), sodium butyrate (NaB) and valproic acid (VPA), that belong to the decetylase inhibitor (DAC-inhibitor) group, stimulate NIS expression in thyroid cancer cells [16-18]. DAC-inhibitors [19, 20] could also be used to control NIS expression inducing radioiodine sensitivity in different human extra-thyroid tumours such as breast cancer. **Actually, it was reported that both** TSA and NaB greatly increased NIS mRNA also in breast cancer MCF-7 cell line [21].

The aim of the present work was to evaluate the ability of the powerful pan-DAC inhibitor LBH589 (panobinostat) [22] to induce the expression of functional NIS in breast cancer cells with different estrogen sensitivity.

MATERIALS AND METHODS

Cell lines and reagents

Estrogen receptor-positive MCF-7 and T47D and estrogen receptor-negative MDA-MB 231 cells were purchased from ECACC (Salisbury, UK).

Cells were routinely maintained at 37°C, in 5% CO₂ and 95 % humidity, in RPMI 1640 (Sigma, St Louis, MO, USA) with 100 IU/ml penicillin and 100 µg/ml streptomycin added, supplemented with 10% heat inactivated FCS (Euroclone, Wetherby, West York, UK).

LBH589 was provided by Novartis Pharma AG (Basel, Switzerland), prepared as a 5 mM stock solution in DMSO and stored at -20°C.

Immunoblotting

To evaluate the effect of LBH589 on histone H4 acetylation state, breast cancer cells, after treatment with LBH589 (5-100 nM), were harvested and lysed in the presence of 200 µl RIPA buffer (1% NP40, 0.5% desoxycholate sodium, 0.1% SDS in PBS pH 7.4, with 10 mg/ml PMSF, 30µl/ml aprotinin and 100mM sodium orthovanadate) and incubated on ice for 30 min; cells were then centrifuged for 20 min at 15,000 x g at 4°C, and clear supernatants used. SDS-PAGE was performed on gels, loading 40 µg protein/well. Separated proteins were electro-transferred onto PVDF membrane and probed with anti-acetyl-histone H4 antibody (06-598, 1:1000 dilution, Upstate, Lake Placid, NY, USA). The membrane was then stripped and re-probed with an anti-GAPDH antibody (1:10000 dilution, Sigma, Saint Louis, MI, USA) to check protein loading. Proteins were detected with Pierce Super Signal chemiluminescent substrate following the manufacturer's instructions. Bands were photographed and analyzed using Kodak 1D Image Analysis software.

Cytotoxic activity

To evaluate the LBH589 cytotoxic activity, MCF-7, T47D and MDA-MB 231 cells were seeded at 4×10^3 cells/well in 96-well plates (Corning, New York, NY, USA) in culture medium plus 10% heat-inactivated FCS. After 24 hours, cells were exposed to increasing concentration of LBH589 (1-200 nM). After 72 hours incubation cell viability was assessed using the Cell Proliferation Reagent WST-1 (Roche Applied Science, Penzberg, Germany), following manufacturer's instructions. Four replicate wells were used to determine each data point. Three response parameters, median growth inhibition (GI_{50}), total growth inhibition (TGI) and median lethal concentration (LC_{50}) were calculated for each cell line. The GI_{50} corresponds to the concentration of the compound that inhibits 50% net cell growth; TGI value is the concentration of the compound leading to total growth inhibition; LC_{50} value is the concentration of the compound leading to 50% net cell death.

Apoptosis Detection - Cell Death Detection ELISA

For apoptosis studies, 4×10^3 cells were seeded in 96-well plates and treated with LBH589, using the same schedule as for the viability assay. After treatment, apoptosis was evaluated using Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Penzberg, Germany) following manufacturer's instructions. Apoptosis was expressed as enrichment factor, calculated as a fraction of the absorbance of treated cells versus untreated controls.

Cell cycle analysis

Cells were treated with LBH589 up to 72 hours. At different times (24, 48 and 72 hours), all cells were collected, fixed in 70% ethanol for 30 minutes on ice and incubated in propidium iodide solution (20 μ g/ml propidium iodide, 0.2 mg/ml RNaseA in PBS) for 1 hour at room temperature. The cell population was analyzed by flow cytometry.

Gene expression evaluation with Real Time PCR

Cells (1×10^6) were seeded in 75cm^2 flasks and treated with LBH589 (5-100nM).

Total RNA was extracted using TRIzol Reagent (Invitrogen Ltd, Paisley, UK), as previously described. DNase I was added to remove remaining genomic DNA. 1 μg of total RNA was reverse-transcribed with iScript cDNA Synthesis Kit (BioRad Laboratories, Inc.), following manufacturer's protocol.

Primers (Table 1) were designed using Beacon Designer 5.0 software according to parameters outlined in the BioRad iCycler Manual. Specificity of primers was confirmed by BLAST analysis. Real-time PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories, Inc.) with SYBR green fluorophore. Reactions were performed in a total volume of 25 μl , including 12.5 μl IQ SYBR Green Supermix (BioRad Laboratories, Inc.), 1 μl of each primer at 10 μM concentration, and 5 μl of the previously reverse-transcribed cDNA template. Protocol for primer set was optimised using seven serial 5X dilutions of template cDNA obtained from cells in basal conditions. The protocol used is as follows: denaturation (95°C for 5 min), amplification repeated 40 times (95°C for 15 sec, 60°C for 1 min). A melting curve analysis was performed following every run to ensure a single amplified product for every reaction. All reactions were carried out at least three times for each sample.

Luciferase Transcription Assay

Cells (2.5×10^5) were seeded in 6-well plates. 24 hours after seeding, cells were transfected with 1 μg of NIS-luc plasmid/well using Lipofectin Reagent (Invitrogen Ltd, Paisley, UK). The plasmid, a kind gift of Prof. G. Damante (Dipartimento di Scienze & Tecnologie

Biomediche, Policlinico Universitario di Udine, Udine) contains 2.2 kb of 5' genomic sequence for the NIS promoter and the minimal promoter is linked to the luciferase (LUC) gene as reporter [23]. After 24 hours, cells were treated with LBH589 (10-50 nM) for a further 24 hours. At completion, luciferase activity was assayed with the Luciferase Assay System (Promega Corporation, Madison, WI, USA).

NIS Immunofluorescence microscopy

Cells (5×10^5) were seeded in 6-well plates. 24 hours after seeding, cells were treated with 25 nM LBH589 for 48 hours. After treatment, cells were fixed in acetone/methanol (1:1) at -20°C for 20 minutes, washed with PBS containing 0.5% Triton X-100, 0.05% NaN_3 and 1% Horse Serum and incubated with monoclonal anti-NIS antibody (1:1000) (FP5A, Thermo Fisher Scientific, Fremont CA, USA) in PBS at 4°C overnight. Then cells were washed with PBS containing 0.5% Triton and 0.05% NaN_3 for 10 minutes for three times followed by detection with anti-mouse Cy3-conjugated secondary antibody (1:1000), (GE Healthcare Europe, GmbH, Milano, Italy) in PBS plus 0.5% Triton and 0.05% NaN_3 for 2 hours and nuclei counterstaining with Hoechst 33258 (500 ng/ml in DMSO) in PBS. Cells were washed twice with distilled water and mounted with 50% glycerol-PBS media.

^{125}I uptake by cells

Cells (2×10^4) were seeded into 24-well plates and incubated with LBH589 (5-100nM) for 48 hours. ^{125}I uptake was assayed as reported elsewhere [18]. Briefly, after aspirating drug-containing medium, cells were washed with 1 ml HBSS (Invitrogen, Groningen, The Netherlands). ^{125}I uptake was initiated by adding 0.5 ml HBSS containing 2 μCi carrier-free Na^{125}I (PerkinElmer Life and Analytical Sciences, Inc., Boston, USA) and 30 μM NaI. Incubation was conducted for 30 min at 37°C and terminated by removing the radioactive

medium and washing the cells twice with ice-cold HBSS. Cells were then solubilized with 1 ml of absolute ethanol for 20 min, after which cell-associated iodide was measured in a γ -counter.

Statistical analysis

Data are expressed throughout as means \pm SEM, calculated from at least three different experiments. Comparison between groups was performed with analysis of variance (one-way ANOVA) and the threshold of significance was calculated with the Bonferroni test. Statistical significance was set at $P < 0.05$.

RESULTS

Anti-tumor activity of LBH589

LBH589 acts as deacetylase inhibitor (DAC inhibitor) in breast cancer cells, inducing hyperacetylation of histone H4, as reported in figure 1, and is a powerful cytotoxic agent causing a significant reduction in cell viability of MCF7, T47D, and MDA-MB231 cells (figure 2). Furthermore, in figure 2 we report the calculated values of GI_{50} , TGI, LC_{50} , that are all in nanomolar range.

The mechanisms of action elicited by LBH589 to exert its cytotoxic activity in breast cancer cells are reported in figure 3. Actually, LBH589 was able to induce apoptosis in a dose-dependent manner (panels on the left), and a significant growth arrest in G_2/M (panels on the right). These effects were observed both in ER-positive and ER-negative breast cancer cells, even though higher doses of LBH589 were required in MDA-MB 231.

Effect of LBH589 on NIS Expression and Function

The ability of LBH589 to induce the Na^+/I symporter in breast cancer cells was thoroughly evaluated.

NIS was first studied as mRNA expression. As reported in figure 4 (panels on the left), the expression of NIS mRNA was significantly induced by LBH589 in a dose-dependent manner in all the three breast cancer cell lines. In addition, in figure 4 (panels on the right) we report the effect of LBH589 on the activity of NIS promoter, evaluated with a luciferase assay, demonstrating that LBH589 is effective in inducing NIS promoter activity in the cell lines under study.

The induction of NIS gene transcription has to go together with the presence of NIS protein in cells, and this was clearly demonstrated in MCF7, T47D, and MDA-MB231 breast cancer

cells under LBH589 treatment as shown in figure 5 (upper panels). Moreover, the symporter induced by LBH589 confers the ability of up-taking iodide to breast cancer cells (figure 5, lower panels).

In all these experiments, LBH589 was effective in inducing a functional NIS either in ER-positive and ER-negative breast cancer cells, even though ER-positive cells were shown to be more sensitive to the compound.

DISCUSSION

The present study shows the potent cytotoxic activity of LBH589 in both estrogen-sensitive and –insensitive cells and, especially, highlights its ability to induce the sodium/iodide symporter (NIS) in breast cancer cells that, as a consequence, become able to concentrate radioactive iodide.

At present four different trials are recruiting patients with recurrent or metastatic breast cancer for treatment with LBH589 both in monotherapy and in association with trastuzumab and paclitaxel (www.clinicaltrials.gov), although knowledge concerning the precise mechanism of LBH589 cytotoxicity in breast cancer cells is still incomplete. The primary molecular effect of DAC-inhibitors is to alter the acetylation status of the core histone proteins, facilitating chromatin remodelling and modification in gene expression [24]. It has been reported that 100 and 200 nM LBH589 alone or in association with 4OHTamoxifene were effective in reducing the growth of ER-negative MDA-MB 231 cells [25] and that LBH589 significantly reduced the viability of ER-positive MCF-7 cells [26] and increased the fraction of dying cells after radiation exposure [27]. Here we demonstrate that LBH589 alone, in nanomolar range which is far from toxic range in humans [28], is able to reduce the viability of ER-positive MCF-7 and T47D and ER-negative MDA-MB 231 breast cancer cells. Besides this, we show that the mechanisms underlying its effect on cell viability include both apoptosis induction and cell cycle arrest, as already reported for other DAC-inhibitors [29].

We demonstrate in particular that LBH589 induces and enhances NIS expression in breast cancer cells. The efficiency of NIS is at the basis of successful treatment of differentiated thyroid cancer thus the possibility of increasing NIS expression in thyroid neoplasms which have lost the ability to uptake and concentrate iodide is under extensive evaluation [30, 31].

Similarly, as NIS is expressed in quite a significant percentage of breast cancers [7], NIS-mediated radionuclide imaging and targeted therapy could be useful goals in breast cancer

management. Unfortunately, although NIS expression is present and intense in the majority of invasive breast cancers, only a small fraction of NIS-positive breast cancers are able to concentrate iodide, due to low cell surface NIS levels and cell surface trafficking defects, commonly found in breast tumours [32]. Therefore, research efforts should focus on strategies to induce/increase NIS expression in breast cancer as it has to be adequately expressed in order to get a considerable iodide uptake. A large number of agents with very different mechanisms of action have been utilized to stimulate NIS expression, such as re-differentiating agents [12, 15, 33-35], or compounds that are reported to physiologically regulate NIS in breast cells [7, 36-38]. Retinoid acid, especially in the presence of other compounds such as dexamethasone, induce NIS and iodide uptake in breast cancer cells [12, 15, 33-35] and in animal models [39]. Even though these findings suggest their potential clinical use, as yet no clinical trials have been presently designed to this aim and additional strategies can be foreseen.

Epigenetic modifications, such as histone deacetylation, have been considered possible causes of NIS silencing in cancer cells. Our data clearly demonstrate that the deacetylase-inhibitor LBH589 induced NIS, both as mRNA and as a protein, and determined a significant iodide uptake in breast cancer cell lines. Furthermore, the observation that LBH589 strongly activates the transcription of the construct *luc*-NIS that we had transfected in breast cancer cells both ER-positive (MCF-7 and T47D) and –negative (MDA-MB 231) is consistent with a direct effect of the drug on NIS promoter. Our data suggest that DAC-inhibitor treatment could therefore modify the acetylation state of NIS promoter allowing its transcription and expression as already reported for other DAC-inhibitors [19]. The effect was evident regardless of ER status, even if both ER-positive MCF-7 and T47D cells are more sensitive than ER-negative MDA-MB 231 cells (the increase in NIS expression in ER-positive cells was about three times higher than in ER-negative cells). Thus, our study is the first report

concerning the possibility to induce not only NIS mRNA expression but also iodide accumulation in breast cancer cells by using the pan-DAC inhibitor, LBH589.

In conclusion, the pan-DAC inhibitor LBH589 is a powerful anti-tumour agent and is able to induce NIS and iodide accumulation in breast cancer cells. The effects of LBH589 are independent from estrogen-sensitivity and ER α -status of cells, making the drug of valuable use for diagnostic and therapeutic application in both estrogen-sensitive and –insensitive tumours.

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LEGEND TO FIGURES

Figure 1 - LBH589 effect on histone acetylation. Acetylation of histone H4 in MCF-7, T47D and MDA-MB231 cells incubated for 24 hours with LBH589 (5-100 nM), was assessed by Western blotting. Equal loading and transfer were verified by re-probing the membranes with anti-GAPDH antibody. The figure shows a typical experiment.

Figure 2 – LBH589 effect of cell viability. Breast cancer cells were incubated with LBH589 (1-200 nM) for 72 hours. After treatment, cell viability was determined by the WST-1 method, and expressed as percentage (untreated controls being 100%). Three response parameters, median growth inhibition (GI₅₀), total growth inhibition (TGI) and median lethal concentration (LC₅₀) were calculated for each cell line, and values are reported in the Table.

Figure 3 – LBH589 effect on apoptosis and cell cycle. *Left Panels:* ELISA detection of DNA-histone complex in MCF-7, T47D and MDA-MB231 cells incubated for 72 hours with LBH589 (1-200 nM). The enrichment factor was calculated as the ratio between the absorbance measurements of treated cells and untreated controls. Results are expressed as means ± SEM; n = 3. Significance vs untreated cells (0): * P< 0.05; ** P< 0.01; *** P< 0.001. *Right Panels:* Cell cycle analysis was performed by flow cytometry after 72 hour treatment with LBH589 (5-100nM). Results are expressed as means ± SEM; n = 3. Significance for G₂/M vs untreated cells (0): *P<0.05; **P< 0.01; ***P< 0.001.

Figure 4 –LBH589 effect on NIS mRNA expression and NIS promoter activity. *Left Panels:* NIS mRNA was evaluated with Real Time PCR in MCF-7, T47D and MDA-MB 231 cells treated with 5-100 nM LBH589. Results were normalized for three different housekeeping genes (β -actin, β 2-microglobulin and L13A) and expressed as relative expression fold *vs* untreated controls (0). Results are expressed as means \pm SEM; n = 3. *Right Panels:* breast cancer cells were transiently transfected with a reporter plasmid carrying the luciferase gene under the control of NIS promoter (NIS-*luc*). The luciferase activity was assayed before and after treatment with 10-50 nM LBH589. Data are expressed as mean \pm SEM; n=3.

Figure 5 – LBH589 effects on NIS protein expression and iodide uptake. *Upper Panels:* immunofluorescence for NIS was performed on breast cancer cells: (A) untreated MCF-7 cells, (B) 25 nM LBH589 treated MCF-7 cells; (C) untreated T47D cells; (D) 25 nM LBH 589 treated T47D cells; (E) untreated MDA-MB 231 cells; (F) 25 nM LBH589 treated MDA-MB 231 cells. *Lower Panel:* ^{125}I uptake in breast cancer cells treated with 5-100 nM LBH589 was evaluated as described; data are expressed as mean \pm SEM; n=3. Significance *vs* untreated cells (0) was: *P<0.05; **P< 0.01.

Table 1. Primers for real time PCR

<i>Gene</i>	<i>Primers</i>
NIS	Sense: 5'- CTG CCC CAG ACC AGT ACA TGC C -3' Antisense: 5'- TGA CGG TGA AGG AGC CCT GAA G -3'
β -Actin	Sense: 5'- GCG AGA AGA TGA CCC AGA TC- 3' Antisense: 5'- GGA TAG CAC AGC CTG GAT AG-3'
β 2-Microglobulin	Sense: 5'-AGA TGA GTA TGC CTG CCG TGT G-3' Antisense: 5'-TCA ACC CTC CAT GAT GCT GCT TAC-3'
L13A	Sense: 5'-GCA AGC GGA TGA ACA CCA ACC-3' Antisense: 5'-TTG AGG GCA GCA GGA ACC AC-3'

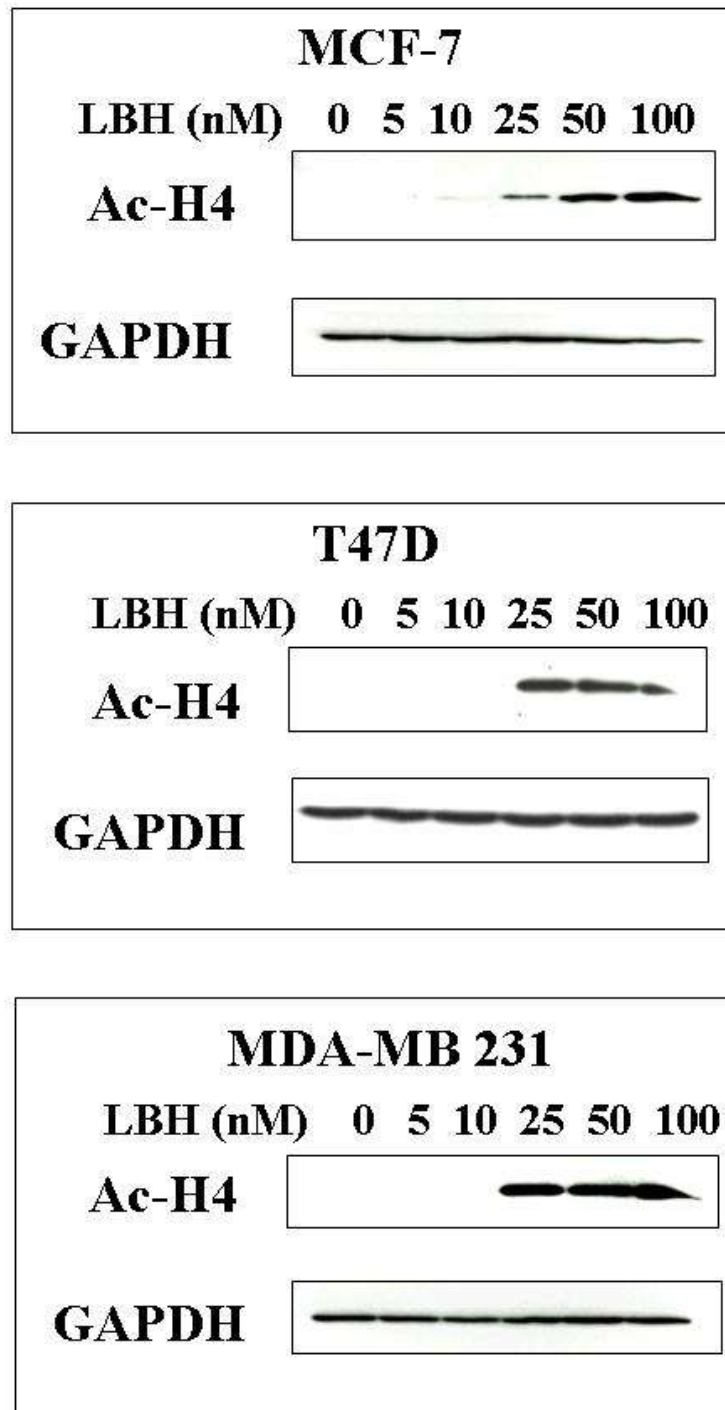
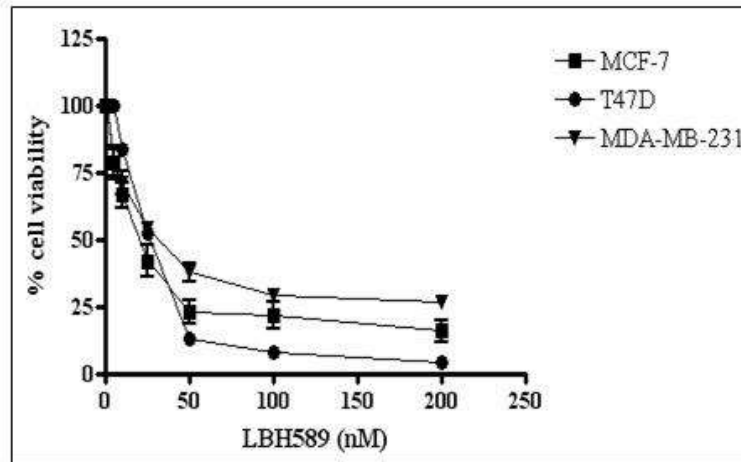


Figure 1



	GI ₅₀ (nM)	TGI (nM)	LC ₅₀ (nM)
MCF-7	11.9 ± 2.6	33.1 ± 1.7	50.9 ± 2.3
T47D	10.9 ± 3.6	28.0 ± 3.7	42.3 ± 3.5
MDA-MB 231	18.1 ± 2.1	44.7 ± 6.8	58.0 ± 2.8

Figure 2

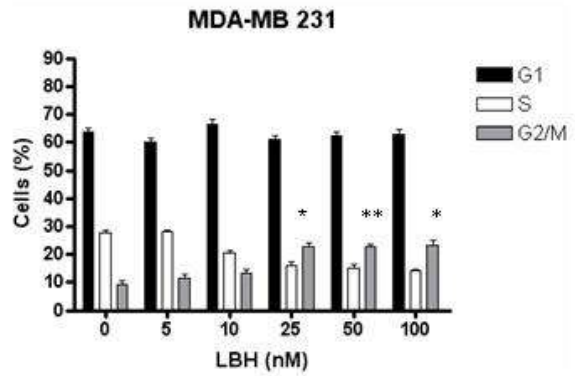
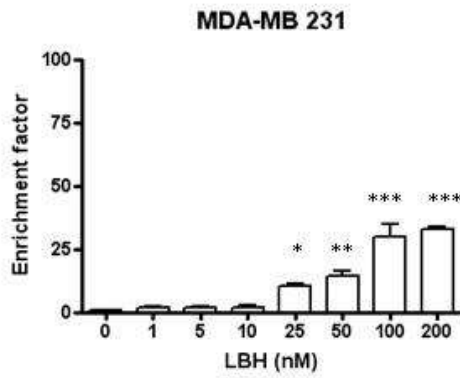
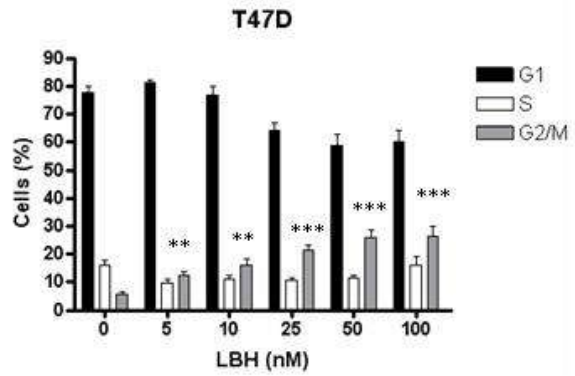
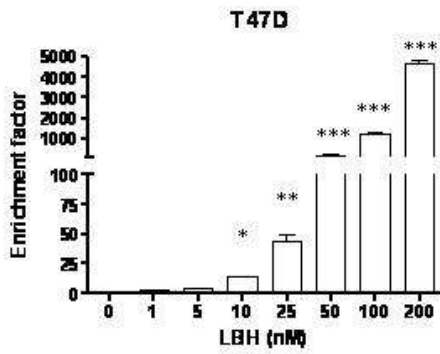
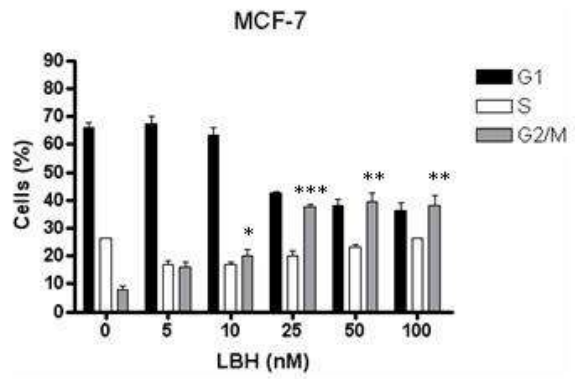
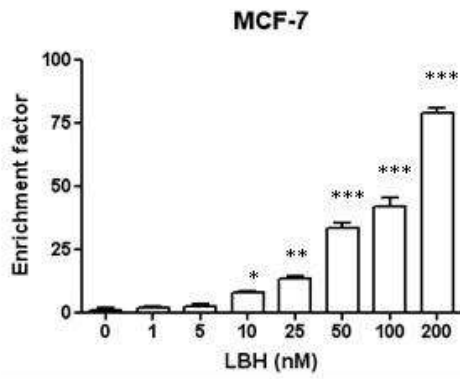


FIGURE 3

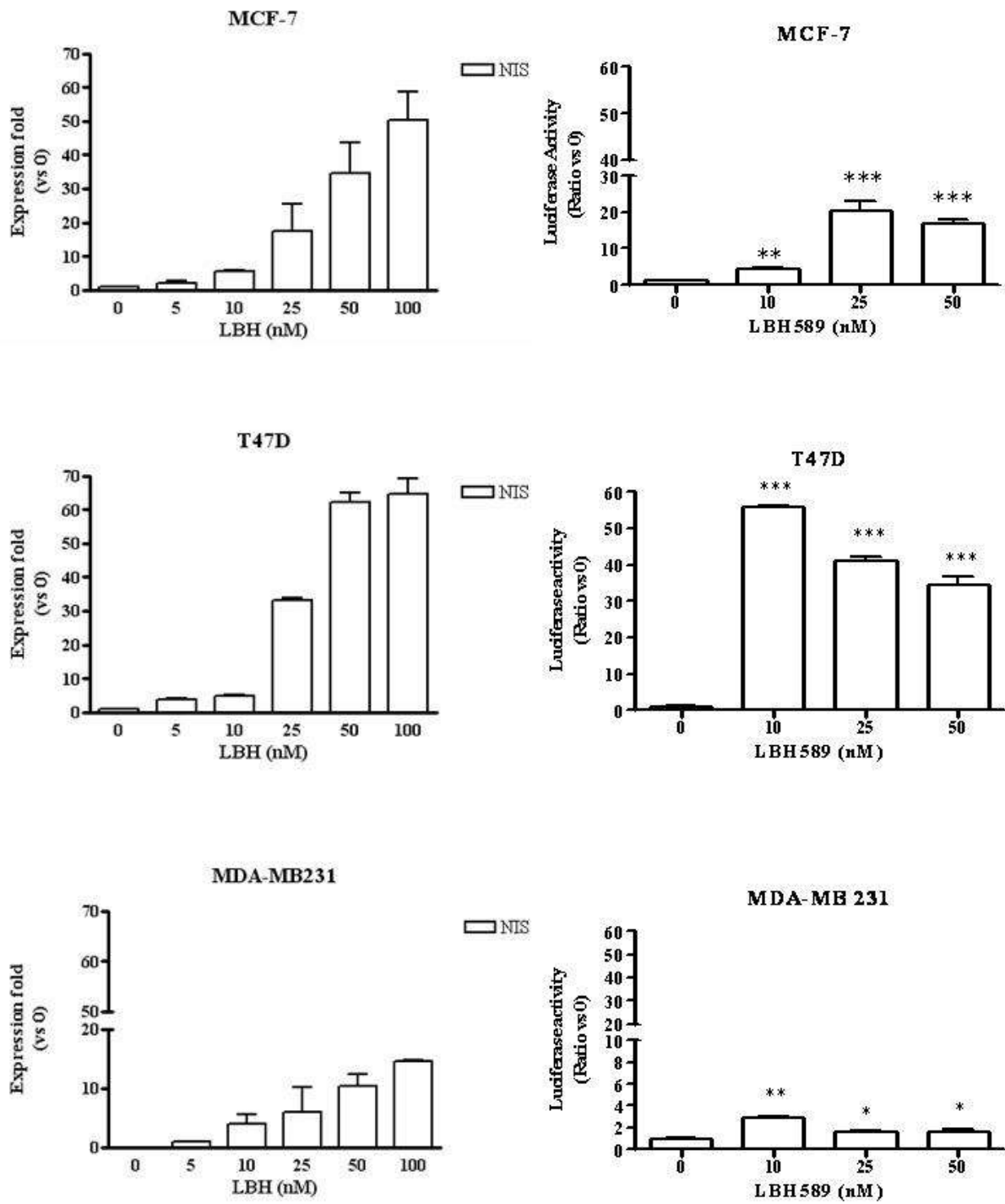


FIGURE 4

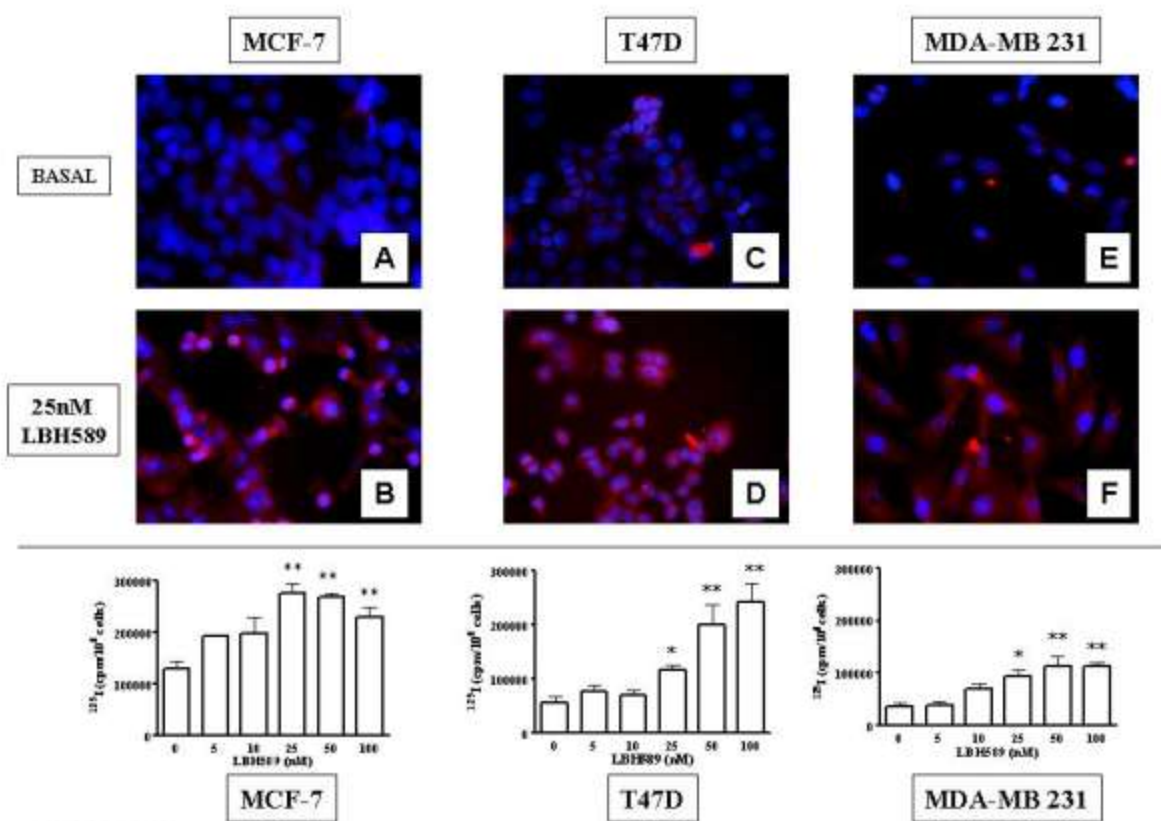


FIGURE 5