

A novel 2-methoxyestradiol analogue is responsible for vesicle disruption and lysosome aggregation in breast cancer cells

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Abstract

Background: 2-Methoxyestradiol (2ME2) is an endogenous metabolite of 17- β -estradiol with anti-proliferative and anti-angiogenic properties. Due to 2ME2's rapid metabolism and low oral bioavailability in *in vivo* settings, 2ME2 analogues have been designed to alleviate these issues. One of these compounds is 2-ethyl-3-*O*-sulphamoyl-estra-1,3,5(10)16-tetraene (ESE-16). Previous work alluded to the ability of ESE-16 to induce autophagic cell death. Therefore, we investigated the mode of action of ESE-16 by studying its effects on autophagy, vesicle formation, and lysosomal organisation.

Summary: Vesicle formation and autophagy induction were analysed by transmission electron microscopy (TEM), monodansylcadaverine (MDC) staining and Lysotracker staining, while autophagosome turnover was analysed using microtubule-associated protein 1A/1B-light chain 3 (LC3 lipidation) analysis. MDC staining of acidic vesicles revealed both an increase in number and size of vesicles after ESE-16 exposure. This was confirmed by TEM. Lysotracker staining indicated an increase in the size of lysosomes, as well as changes in their distribution within the cell. However, autophagy was not induced since LC3 lipidation did not increase after exposure to ESE-16.

Key messages: This study showed that ESE-16 exposure leads to the aggregation of acidic vesicles, identified as lysosomes, not accompanied by an induction of autophagy. Therefore, ESE-16 disrupts normal endocytic vesicle maturation likely through the inhibition of microtubule function.

Keywords: lysosomes, autophagy, cancer, microtubule, cell signalling

Background

2-Methoxyestradiol (2ME2) is an endogenous metabolite of 17- β -estradiol that has been found to exhibit both anti-proliferative and anti-angiogenic properties *in vitro* [1, 2]. Despite initial *in vitro* success, phase II clinical trials showed no clinical effect mostly due to the short half-life of the compound [1]. 2ME2 is rapidly metabolised to 2-methoxyestrone by 17 β -hydroxy steroid dehydrogenase II (SD II), an enzyme that is mainly found in the gastrointestinal tract and the liver [3].

To circumvent the rapid biodegradation and to improve its bioavailability, several 17 β -hydroxy-modified derivatives were synthesized that, in theory, could no longer be metabolised by SD II and could avoid clearance by the kidneys. The methoxy group at carbon 2 was replaced by an ethyl group, while a sulphamoyl group replaced a hydrogen on C3 [4]. Other derivatives such as 2-ethyl-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthrene-3,17-diyl bis sulphamate (EMBS) achieved inhibition of cell growth in the estrogen receptor-positive breast cancer cells (MCF-7), metastatic MDA-MB 231 and non tumorigenic MCF-12A cells at nanomolar concentrations which was correlated with a block in the cell cycle at the G2/M border [4]. All sulphamoylated derivatives synthesised along with ESE-16 were effective at partially inhibiting cancer cell growth [5].

ESE-16 has been shown to inhibit cell growth in many cell lines including human epithelial cervix carcinoma (HeLa), esophageal carcinoma (SNO), MCF-7 cells, as well as MDA MB 231 cells after 24 hours of exposure [2, 5]. This inhibition ultimately leads to the activation of the intrinsic apoptotic pathway [2, 5]. Previous data suggests that autophagy is induced after ESE-16 exposure, but to date no conclusive data has been presented proving that ESE-16 acts via autophagy [6, 7]. 2ME2 and the analogues subsequently synthesised appear to be potent microtubule inhibitors which would lead to blockage of the G2/M border and subsequent apoptosis [5, 8]. Moreover, it is well known that microtubule modulators affect endocytic vesicle formation, maturation, organisation and turnover [5].

Previous reports determining autophagy were limited to flow cytometry and microscopy to visualise autophagosomes. Neither assay provides conclusive evidence of the induction of autophagy or alterations in autophagosome maturation and turnover. Thus, we wanted to clarify whether 2ME2 analogues can induce autophagy and infer this to the inhibition of cell

growth in cancer cells. It would be of importance to fully understand the mechanism when re-designing this class of compounds for use chemotherapeutic.

Materials and methods

Materials

Drug synthesis

ESE-16 was *in silico*-designed at the Bioinformatics and Computational Biology Unit, Department of Biochemistry, University of Pretoria, Pretoria South Africa and was subsequently synthesized by iThemba Pharmaceuticals (Pty) Ltd (Modderfontein, Midrand, Gauteng).

Cell line and drug exposure conditions

The MCF-7 (estrogen receptor-positive) cell line is a tumorigenic immortalized adherent breast adenocarcinoma cell line derived from metastatic sites in adenocarcinoma [4]. Cells obtained from American Type Culture Collection (ATCC) (catalogue number HTB-26), were purchased from Highveld Biological (Pty) Ltd. (Sandringham, Gauteng) and were cultured in Dulbecco's Minimum Essential Eagle (DMEM) enriched with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin G, 100 µg/ml streptomycin and 250 µg/l fungizone. Ethical approval was obtained from Faculty of Health Sciences Research Ethics Committee (Protocol Number 291/2013). ESE-16 was dissolved in dimethyl sulfoxide (DMSO) and stored in the freezer at -20°C. The final concentration of DMSO did not exceed 0.05% (v/v) in cell culture [2]. Cells were exposed to 0.18 µM for 24 h as previously reported (Stander *et al.*; Mqoco *et al.*). The concentration of 0.18 µM was selected since it was previously established in our laboratory that 0.18 µM ESE-16 inhibited cell growth by 50% (GI₅₀) after 24 h at 37°C [8].

Reagents

All reagents were supplied by Sigma-Aldrich Co. (St Louis, MO), unless otherwise indicated. FCS, bovine serum albumin (BSA), as well as sterile cell culture flasks and plates were supplied by the Scientific Group (Johannesburg, Gauteng). Trypsin-EDTA, DMEM and buffers were supplied by Highveld Biological (Pty) Ltd. The LC3B antibody was manufactured by BioVision Incorporated (Milpitas, CA).

Cell culture propagation

Cells were propagated as monolayers in growth medium at 37°C in a humidified Forma Scientific water-jacketed incubator (OH, USA) containing 5% carbon dioxide as previously reported (Vorster *et al.* [14], Mqoco *et al.* [2] and Nkandeu *et al.* [19]). Experiments were conducted in 25 cm² culture flasks, 96-well plates or six-well plates as stipulated for a specific technique. For 25 cm² culture flasks, 10⁶ cells were seeded in 5 ml of growth medium for, transmission electron microscopy (TEM) and visualization of MDC-labeling. For 6-well plates, 250 000 cells were seeded per well in a 3 ml growth medium on heat-sterilized cover slips. For 96-well tissue culture plates, 5 000 cells were seeded per well in 200 µl of growth medium. For each experiment, growth medium was renewed prior to cell exposure. Control cells were propagated in growth medium only and the vehicle-treated sample was exposed to 0.05% (v/v) DMSO [2]. This concentration of DMSO was previously shown to have no effect on cell growth and survival [2, 9, 10, 11, 12]. Cells were exposed to 20 µM of tamoxifen for 24 h for autophagy detection as a positive control [2, 13, 14].

Methods

Visualization of acidic vacuoles with monodansylcadaverine

Monodansylcadaverine (MDC) was used to stain for acidic vesicles especially autophagosomes. Briefly, MCF-7 cells (250 000) were seeded on 18 mm x 18 mm coverslips inside a Petri dish filled with 3 ml DMEM. After treatment with 0.02% (v/v) DMSO, tamoxifen or ESE-16 cells were loaded with 0.05 mM MDC in PBS for 10 min at 37°C. Cells were washed four times with PBS and immediately imaged on a Zeiss CFL40 Axiovert inverted fluorescence microscope equipped with a 380-420 nm excitation filter and a 450 nm barrier filter.

Lysotracker staining of lysosomes

MCF-7 cells were stained with the lysosomal stain Lysotracker™ in order to visualize acidic vesicles. This stain accumulates in vesicles and fluoresces only in low pH environments found exclusively in lysosomal vesicles or vesicles that have fused with lysosomes such as mature autophagosomes [15]. To visualize acidic vesicles with Lysotracker™, MCF-7 cells were seeded in growth medium at 30 000 cells in 24-well plates on sterilized coverslips and exposed to ESE-16 and the appropriate controls. After the 24 h incubation period cells were loaded with 75 nM Lysotracker for 1 h at RT before being fixed with 2% paraformaldehyde

for 15 min at room temperature and then washed. Nuclear staining was achieved by incubating coverslips with 300 nM DAPI in PBS (100 µl per well) for 30 min at RT. Samples were mounted on microscope slides with Prolong Anti-fade mounting fluid and imaged with a Zeiss LSM 510 META confocal laser microscope at 63 x magnification.

Transmission electron microscopy

TEM was used to assess the ultrastructural changes associated with the ESE-16 exposure [6]. Cells were seeded at a density of 750 000 per 25 cm² flasks and were allowed to attach overnight. Cells were exposed to 0.18 µM ESE-16 and appropriate controls for 24 h. Cells were trypsinized and fixed in 2.5% glutaraldehyde in phosphate buffer for 1 h. Cells were rinsed thrice with 0.075 M phosphate buffer, fixed in 0.25% aqueous osmium tetroxide for 30 min, dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 90% and 100% x3) and embedded in Quetol resin. Ultra-thin sections were cut using a diamond ultramicrotome and placed on copper grids. Samples were contrasted with 4% uranyl acetate and Reynolds' lead citrate and viewed with a multi-purpose Philips 301 TEM.

LC3 Western blot analysis

Exponentially growing MCF-7 cells were seeded at a density of 250 000 cells in a 6-well plate. After 24 h, the medium was discarded and cells were exposed to 0.18 µM ESE-16 for 24 h. At termination, 150-200 µl 1 x diluted LDS sample buffer containing 2.5% β-ME was heated to 80°C and added to the wells. Cells were scraped collected in microtubes and heated for 5 min at 95°C. After centrifugation, supernatants were loaded onto SDS-PAGE gels and separated under 100 V in MOPS buffer. Proteins were transferred to PVDF. Membranes were blocked in 5 ml PBS with 0.2% tween and 2% BSA for 1 h. Subsequently, membranes were incubated with a 1:1000 dilution of LC3 antibody or a 1:5000 actin antibody for 1 h at RT. The membrane was washed 3 times in PBS-tween 20 and then incubated in 5 ml blocking solution containing 1:10 000 secondary anti-mouse HRP antibody for 1 h at room temperature. Finally, membranes were incubated with Extended Luminescence substrate and viewed using the Bio-Rad Molecular Imager Gel Doc system (Bio-Rad Laboratories Ltd. Rosebank, Johannesburg, South Africa). Bands were quantified using ImageLab software from Bio-Rad (Bio-Rad Laboratories Ltd. Rosebank, Johannesburg, South Africa). Bafilomycin (100 nM) was used as a positive control for autophagosome turnover.

Measurement of autophagosome formation (rabbit polyclonal anti-Atg 4A antibody)

Atg 4 is a conserved cysteine protease involved in Atg 8 cleavage and participates in the processing and attachment of autophagosomes to microtubules [16, 17, 18].

MCF-7 cells were seeded at 500 000 cells per 25 cm² flask. After 24 h of attachment, medium was discarded and cells were exposed to 0.18 µM ESE-16 for 24 h. Cells were washed with ice-cold PBS and pelleted by centrifugation. Cells were fixed with 3 ml 0.01% formaldehyde in PBS for 10 min at 4°C. Samples were permeabilized 3 times for 5 min each with PBS containing 0.1% triton X-100. Cells were washed with PBS and stained with 0.5 ml antibody cocktail (0.05% triton X-100, 1% BSA, 40 µg/ml propidium iodide and 0.5 µg/ml rabbit polyclonal anti-Atg 4A antibody) prepared in PBS for 2 h at 4°C. Cells were washed 4 times for 10 min each with PBS containing 0.05% triton X-100 and 1% BSA and samples were incubated for 90 min with secondary antibody cocktail at room temperature and protected from light. Samples were washed 4 times for 10 min each with PBS and then were analysed by flow cytometry using a Beckman Coulter Cytomics FC500 instrument (Beckman Inc., Fullerton, CA, USA).

Results

ESE-16 exposure leads to an increase in acidic vesicles

To determine whether ESE-16 induce autophagy the number and intensity of MDC positive vesicles can be analysed. MDC fluoresces within acidic vesicles which include autophagosomes that have merged with lysosomes. MCF-7 cells were exposed to DMSO, tamoxifen or ESE-16 for 24 h before being stained with MDC (Fig. 1). Tamoxifen is a potent inducer of autophagy in breast cancer cells. Indeed, MCF-7 cells exposed to tamoxifen displayed an increase in the number of vesicles when visually assessed on the microscope (qualitative analysis), as well as an increase in the intensity of the staining of such vesicles (Fig. 1B). ESE-16 exposure also led to increased fluorescence in MCF-7 cells although the distinct foci seen in tamoxifen-treated cells were absent in ESE-16-exposed cells. Quantification of total MDC emission showed significantly increased fluorescence in both tamoxifen- and ESE-16-treated cells (Fig. 1D). MDC fluorescence was increased in ESE-16 suggesting increased numbers of acidic vesicles were present. However, the pronounced focal staining observed in tamoxifen treated cells was absent in ESE-16-treated cells suggesting

that the vesicles in ESE-16-exposed cells may in fact be distinct from those formed by tamoxifen exposure.

To confirm that ESE-16 exposure leads to an increase in acidic vesicles in MCF-7 cells, cells were exposed to DMSO or ESE-16 for 24 h and stained with LysoTracker™. Thereafter, cells were fixed in paraformaldehyde and stained for nuclei using 4,6-diamidine-2-phenylindole dihydrochloride (DAPI). Cells were visualised on a Zeiss LSM 520 confocal microscope at 63x magnification (Fig.2). DMSO treated cells were mononucleated (Fig. 2B) and contained a small number of lysotracker-positive vesicles in close proximity to the nucleus (Fig. 2A). In contrast, ESE-16-exposed cells displayed increased vesicle numbers of irregular sizes including enlarged vesicles distributed throughout the cytoplasm (Fig. 2D). Moreover, many cells contained multiple nuclei which, in general, were smaller than the nuclei of mononucleated cells (Fig. 2E). Therefore, ESE-16 exposure led to increased number and size of LysoTracker™ positive vesicles and the induction of aberrant nuclear morphology.

Transmission electron microscopy

To gain a better insight into the morphology of the vesicles formed after treatments, MCF-7 cells exposed to either DMSO, tamoxifen or ESE-16 were visualised using transmission electron microscopy (Fig. 3). DMSO-exposed cells displayed a small number of vesicles with some variation in size, distributed throughout the cytoplasm (Fig. 3A). In contrast, tamoxifen-exposed cells contained many tightly packed vesicles of similar sizes close to the nucleus (Fig. 3B). ESE-16-exposed cells displayed increased numbers of vesicles of assorted sizes distributed throughout the cytoplasm. Differences in vesicle morphology and distribution between tamoxifen-and ESE-16 treated cells were observed suggesting formation of distinct vesicle types.

LC3 lipidation indicates no induction of autophagy by ESE-16

During autophagosome maturation, cytoplasmic LC3 is recruited to the membranes of autophagosomes and becomes lipidated through the conjugation to phosphatidylethanolamine (PE). Therefore, LC3 lipidation (LC3-II levels) acts as an important marker of autophagosome formation, maturation and turnover. To determine whether autophagy occurs, levels of LC3 protein accumulation were quantified. MCF-7 cells exposed to DMSO or ESE-16 were analysed by western blot analysis for LC3-II levels. Also, cells were treated with bafilomycin which blocks autophagosome turnover [15]. This was used as a positive control to determine if basal autophagosome formation occurred in the cells (Fig. 4). Western blot

data showed no significant LC3 II levels in DMSO or ESE-16-treated cells (Fig. 4A), (0.02 vs. 0.04, Fig. 4B). Addition of bafilomycin greatly enhanced the level of LC3-II in DMSO- and ESE-16-exposed cells without any significant differences between the two treatments (Fig. 4A), (0.43 vs. 0.48, Fig. 4B). Thus, although MCF-7 cells have basal autophagosome turnover, ESE-16 did not induce autophagosome formation nor affected autophagosome turnover.

Measurement of autophagosome formation (rabbit polyclonal anti-Atg 4A antibody)

The measurement of the number of autophagosomes formed after treatment of MCF-7 cells with 0.18 μ M ESE-16 for 24 h showed a 1.4-fold increase in the ratio of Atg 4A-positive MCF-7 cells relative to cells propagated in medium only (Fig. 5).

Discussion

Previous studies suggested that ESE-16 exposure in certain cancer cell lines leads to increased autophagy [2, 9, 10]. This conclusion was drawn from MDC staining of cells and autophagy-regulating protease 4A (Atg 4A) expression levels (Fig. 2 and Fig. 5).

While suggestive, these methods cannot conclusively show that autophagy increases after ESE-16 exposure. Here, we set out to obtain conclusive data to allow us to identify the origin of the vesicles formed after ESE-16 exposure. Firstly, we compared vesicle size and distribution using MDC staining between cells exposed to ESE-16 and cells exposed to the known autophagy inducer tamoxifen [9, 10]. Our data shows that tamoxifen does indeed induce the propagation of distinct, bright MDC positive foci. However, ESE-16 exposure, while also resulting in increased MDC staining, did not induce the same distinct foci, but rather a more general fluorescence in each cell. This suggests that MDC may not be specifically fluorescent in autophagosomes, but can fluoresce in other subcellular structures as well. ESE-16-induced MDC positive structures are therefore possibly different from the tamoxifen-induced autophagosomes.

To confirm this finding, cells were also stained with the lysosomal stain LysoTracker™. Staining with LysoTracker™ revealed that ESE-16 exposure leads to an accumulation of acidic vesicles of different sizes with some positive vesicles being very large. Moreover, they are no longer present around the nucleus as seen in control cells, but distributed

throughout the cytoplasm. This was further confirmed by TEM. While tamoxifen-treated cells appeared to display small, uniformly sized vesicles mostly close to nucleus, ESE-16-treated cells contained vesicles of different sizes with a distinct lack of organisation. The appearance and distribution of the vesicles in ESE-16-treated cells bears similarities to those appearing in cells treated with microtubule poisons such as nocodazole or taxol [19]. Nocodazole-treated cells display a scattered distribution of lysosomes along with enlargement as lysosomes fail to turnover. This is similar to what was observed in ESE-16-exposed cells suggesting that the compound interferes with microtubule dynamics leading to the disorganisation of lysosomal targeting and turnover. Indeed, western blot analysis for LC3 lipidation showed that there was no increase in LC3-II levels suggesting that autophagy was not induced by ESE-16. Indeed, the use of bafilomycin supports this as evidenced by its ability to induce autophagy. Therefore, the increase in vesicles is most likely not due to increased autophagosome formation [20, 21]. This leads us to suggest that ESE-16 acts on the lysosomal pathway through its previously described effect on microtubule dynamics [22]. The formation of multinuclei in ESE-16-treated cells further underscores our finding. Multinucleated cells, as seen here, are the result of improper cell division which, in many cases, is caused by defective microtubule spindle formation [23].

Taken together, our data shows that ESE-16 does induce the accumulation of acidic vesicles, but these are not autophagosomes. Therefore, ESE-16 does not induce autophagy, but rather disrupts microtubule function to induce the accumulation of acidic endocytic vesicles and lysosomes. The data presented here conclusively shows that ESE-16 exposure leads to an accumulation of vesicles within the cells. These vesicles are acidic as confirmed by both MDC and lysotracker staining. However, microscopic analysis and most importantly, LC3 II protein lipidation analysis show that there is no increase in autophagosome formation or a blockage in autophagosome turnover. Our data instead suggest that, likely through an effect on microtubule dynamics, endocytic vesicle formation, maturation and turnover are affected.

Statistical analysis

Quantitative data was obtained from three independent biological repeats with graphs representing the average of repeats and error bars denoting the standard error of the mean. Student's t test was performed to determine significance and *P*-values lower than 0.05 were denoted with an asterisk. Densitometry was performed on the LC3-II and actin bands to

calculate ratios of LC3-II to actin and was averaged over three independent experiments to quantify LC3-II levels.

Abbreviations

2ME2: 2-Methoxyestradiol; ESE-16: 2-Ethyl-3-*O*-sulphamoyl-estra-1,3,5(10)16-tetraene; MCF-7: Estrogen receptor positive breast cancer cells; LC3: Microtubule associated-protein 1 light chain 3; MDC: monodansylcadaverine; TEM: Transmission electron microscopy; DMSO: Dimethyl sulphoxide; DMEM: Dulbecco's modified Eagle's minimal essential medium; FCS: Fetal calf serum; BSA: Bovine serum albumin; PBS: Phosphate-buffered saline.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SDN conducted all experiments, analyzed the data and drafted the manuscript. MJC and DvP assisted with the design of the study. IvdB assisted with conducting of experiments, data analysis, and editing of manuscript. AJ designed the study and helped with data analysis and the drafting of the manuscript. All the authors read and approved the final manuscript.

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Figure legends

Figure 1 ESE-16 exposure leads to increased MDC staining. MCF-7 cells were exposed to DMSO (0.05%) (A), tamoxifen 20 μ M (B) and ESE-16 (0.18 μ M) (C) for 24 h. Subsequently, cells were loaded with 0.05 mM MDC for 10 min at 37°C and visualised on a fluorescence microscope. Average MDC fluorescence intensity was quantified by measuring total fluorescence divided by the number of cells per field imaged (D). Scale bars represent 5 μ m. Arrows indicate acidic vesicles.

Figure 2 ESE-16 exposure leads to an accumulation of lysotracker positive vesicles and multinucleated cells. MCF-7 cells were treated with 0.18 μ M ESE-16 for 24 hours. Afterwards cells were loaded with Lysotracker™ (A, D) for 2 h before being fixed and stained for nuclei using DAPI (B, E). Scale bar represents 5 μ m. Arrows indicate lysotracker™ positive vesicles.

Figure 3 Transmission electron microscopy confirms the formation of distinct vesicles after ESE-16 exposure. Vehicle-treated cells (A), tamoxifen-treated cells (B) and ESE-16-treated samples (C) were processed for TEM after 24 h treatment. Vesicles are indicated with an arrow.

Figure 4 ESE-16 exposure does not lead to LC3 lipidation and increased autophagy. Western blot analysis of LC3 and actin was performed on cells treated with DMSO and 0.18 μ M ESE-16 for 24 h (A). To block autophagosome turnover cells were also incubated with 100 nM bafilomycin. The ratios of LC3-II to actin and was averaged over three independent experiments to quantify LC3-II levels (B). The bars represent the mean \pm standard deviation from three independent experiments ($P < 0.05$ is significant *).

Figure 5 Ratio of Atg 4A expression levels in MCF-7 cells relative to cells propagated in medium only (normalized to 1). A 1.4-fold increase in Atg 4A-positive cells was observed when compared to the vehicle-treated control. An asterisk (*) indicated a P -value < 0.05 when compared to the vehicle control.

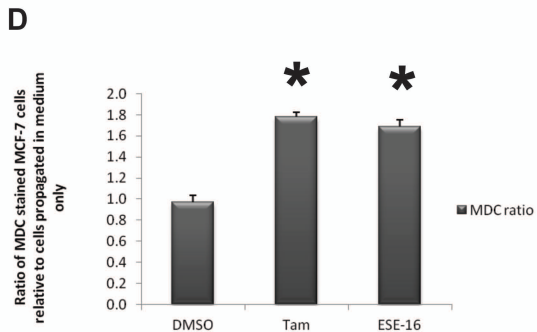
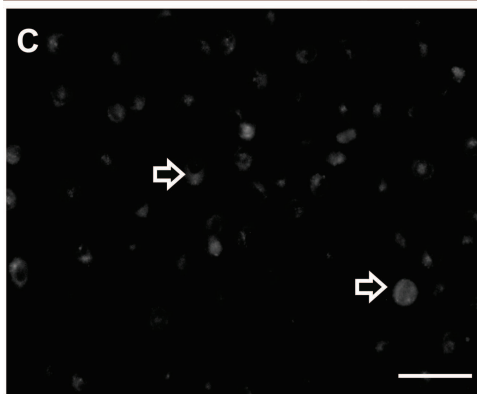
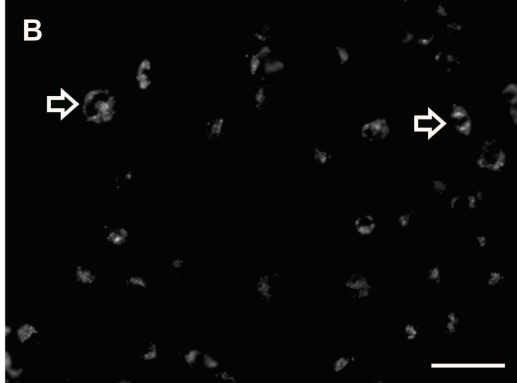
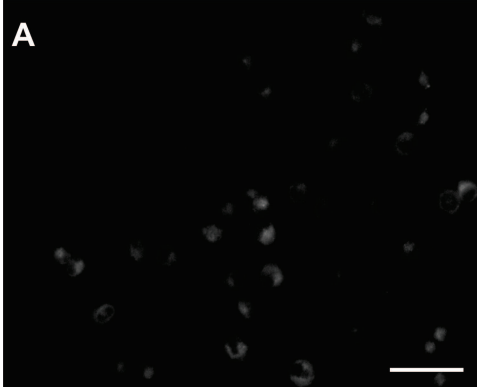


Figure 1

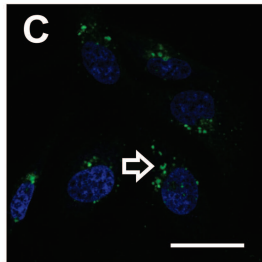
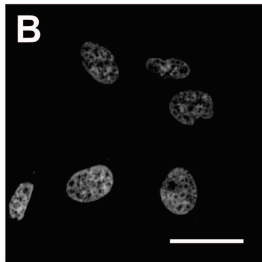
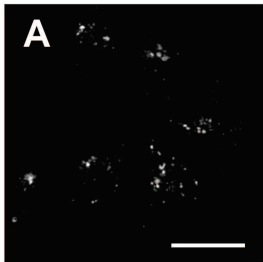
Figure 2

Lysotracker

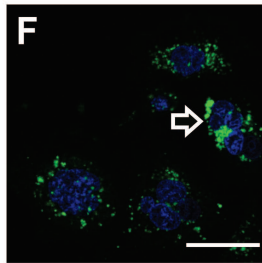
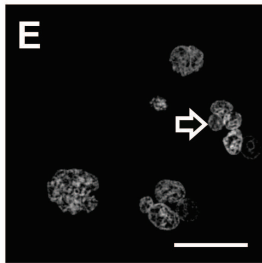
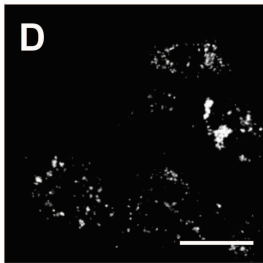
DAPI

Merge

DMSO



ESE-16



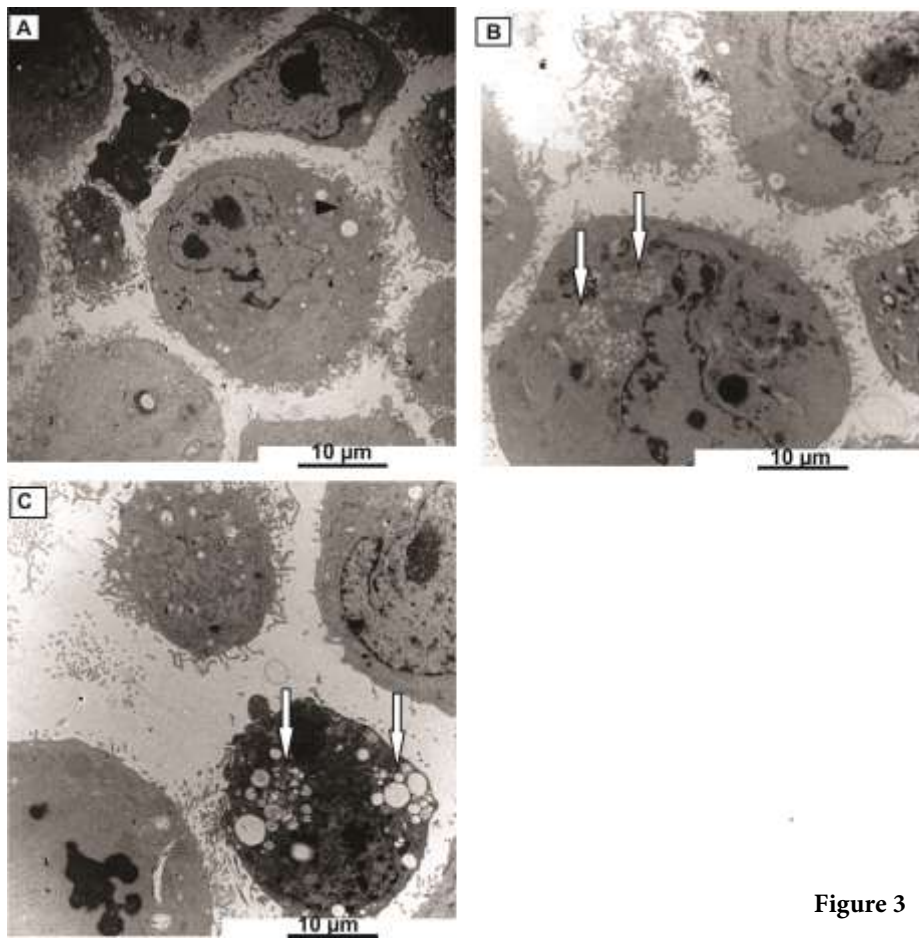


Figure 3

Figure 4

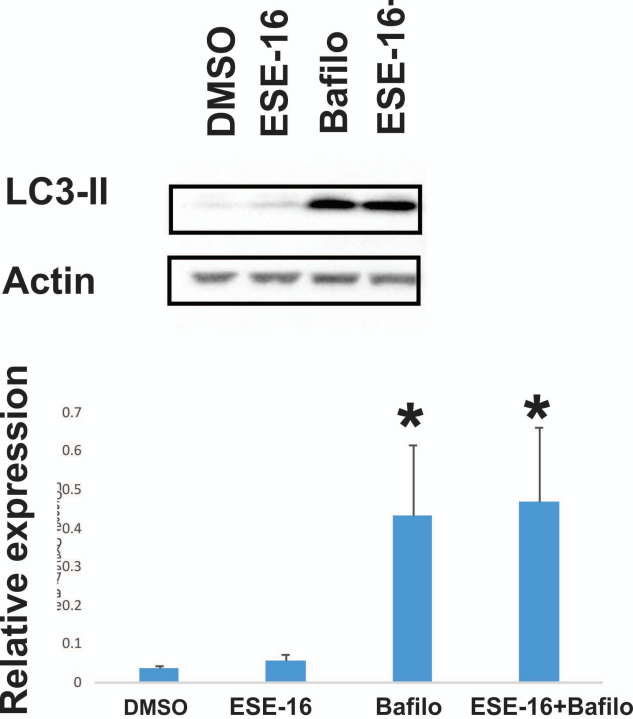


Figure 5

