Amino Acids (2012) 42:899–911
DOI 10.1007/s00726-011-1005-0

ORIGINAL ARTICLE

# A potential estrogen mimetic effect of a bis(ethyl)polyamine analogue on estrogen receptor positive MCF-7 breast cancer cells

**Irina Nayvelt · Shali John · Hui-Chen Hsu · PingAr Yang · Wensheng Liu · Gokul Das · Mervi T. Hyvönen · Leena Alhonen · Tuomo A. Keinänen · Akira Shirahata · Rajesh Patel · Thresia Thomas · T. J. Thomas**

Received: 17 March 2011 / Accepted: 15 June 2011 / Published online: 10 August 2011


**Abstract** BE-3-3-3-3 (1,15-(ethylamino)4,8,12-triazapentadecane) is a bis(ethyl)polyamine analogue under investigation as a therapeutic agent for breast cancer. Since estradiol ($E_2$) is a critical regulatory molecule in the growth of breast cancer, we examined the effect of BE-3-3-3-3 on estrogen receptor α (ERα) positive MCF-7 cells in the presence and absence of $E_2$. In the presence of $E_2$, a concentration-dependent decrease in DNA synthesis was observed using [$^3$H]-thymidine incorporation assay. In the absence of $E_2$, low concentrations (2.5–10 μM) of BE-3-3-3-3 increased [$^3$H]-thymidine incorporation at 24 and 48 h. BE-3-3-3-3 induced the expression of early response genes, *c-myc* and *c-fos*, in the absence of $E_2$, but not in its presence, as determined by real-time quantitative polymerase chain reaction (qPCR). BE-3-3-3-3 had no significant effect on these genes in an ERα-negative cell line, MDA-MB-231. Chromatin immunoprecipitation assay demonstrated enhanced promoter occupation by either $E_2$ or BE-3-3-3-3 of an estrogen-responsive gene *pS2/Tff1* by ERα and its co-activator, steroid receptor co-activator 3 (SRC-3). Confocal microscopy of BE-3-3-3-3-treated cells revealed membrane localization of ERα, similar to that induced by $E_2$. The failure of BE-3-3-3-3 to inhibit cell proliferation was associated with autophagic vacuole formation, and the induction of Beclin 1 and MAP LC3 II. These results indicate a differential effect of BE-3-3-3-3 on MCF-7 cells in the absence and presence of $E_2$, and suggest that pre-clinical and clinical development of polyamine analogues might require special precautions and selection of sensitive subpopulation of patients.

**Keywords** Polyamines · Polyamine analogue · Breast cancer · Estrogen receptor · Autophagy

I. Nayvelt · S. John · T. J. Thomas (✉)
Department of Medicine and Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, NJ 08901, USA
e-mail: thomastj@umdnj.edu

H.-C. Hsu · P. Yang
Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294, USA

W. Liu · G. Das
Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

M. T. Hyvönen · L. Alhonen
Department of Biotechnology and Molecular Medicine, A. I. Virtanen Institute for Molecular Sciences, Biocenter Kuopio, University of Eastern Finland, 70210 Kuopio, Finland

T. A. Keinänen
Department of Biosciences, Laboratory of Chemistry, Biocenter Kuopio, University of Eastern Finland, 70210 Kuopio, Finland

A. Shirahata
Department of Biochemistry and Cellular Physiology, Josai University, Saitama, Japan

R. Patel
Department of Pathology and Laboratory Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, NJ 08903, USA

T. Thomas
Department of Environmental and Community Medicine and Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, NJ 08901, USA

**Abbreviations**

| | |
|---|---|
| BE-3-3-3-3 | 1,15-(ethylamino)4,8,12-triazapentadecane |
| BE-3-3-3 | Bis(ethyl) norspermine |
| APAO | Acetylpolyamine oxidase |
| CHIP | Chromatin immunoprecipitation |
| DAPI | 4′6′-diamidino-2-phenylindole |
| $E_2$ | Estradiol |
| ER | Estrogen receptor |
| ERE | Estrogen response element |
| ER(+) | ER-positive |
| ER(−) | ER-negative |
| GPR30 | G-protein-coupled receptor homologue 30 |
| MAP LC3 | Microtubule-associated protein 1 light chain 3 |
| $N^1$-AcSpd | $N^1$-acetylspermidine |
| NCoR | Nuclear receptor co-repressor |
| ODC | Ornithine decarboxylase |
| Put | Putrescine |
| qPCR | Quantitative polymerase chain reaction |
| SAMDC | S-adenosyl L-methionine decarboxylase |
| STR | Short tandem repeat |
| Spd | Spermidine |
| Spm | Spermine |
| SSAT | Spermidine/spermine-$N^1$-acetyltransferase |
| SMO | Spermine oxidase |
| SRC-3 | Steroid receptor co-activator 3 |

## Introduction

Polyamines, putrescine, spermidine and spermine, are ubiquitous cellular cations involved in cell growth and differentiation (Agostinelli et al. 2010a; Casero and Marton 2007; Gerner and Meyskens 2004; Thomas and Thomas 2001). Polyamines are delicately regulated in the cell by biosynthetic enzymes (ornithine decarboxylase and S-adenosyl L-methionine decarboxylase), catabolic enzymes (spermidine/spermine $N^1$-acetyltransferase and polyamine oxidases) and by uptake/efflux pathways (Casero and Marton 2007; Gerner and Meyskens 2004; Palmer and Wallace 2010; Thomas and Thomas 2001, 2003). Alterations in polyamine levels and metabolism are causatory factors of carcinogenesis in animal models and humans (Agostinelli et al. 2010b; Gerner and Meyskens 2004; Häkkinen et al. 2009; Thomas and Thomas 2003). Our research (Shah et al. 2001; Thomas and Thomas 1994a, b) and other reports (Leveque et al. 2000a, b; Manni et al. 1992), show an essential role of polyamines in the estrogenic pathway of breast cancer cell growth.

Estrogenic action in target cells is mediated through the estrogen receptors (ER), ERα and ERβ (Thomas et al. 2004; Katzenellenbogen and Frasor 2004; O'Malley and Kumar 2009). In the genomic mode of ER action, estradiol ($E_2$) binding to the nuclear receptor induces a conformational change, leading to co-activator recruitment and stimulation of transcription from either the promoter/enhancer site of $E_2$-responsive genes, or through protein–protein interactions with other transcription factors, such as the activating protein 1 (AP-1) and the stimulating protein 1 (SP-1). $E_2$ can also elicit a fast, non-genomic response through cellular membrane-associated ERs (Levin and Pietras 2008). These receptors may interact with G-proteins or other cell membrane growth receptors (EGFR, IGF-1R, etc.), and stimulate events such as calcium flux, phospholipase C activation, production of cyclic adenosine monophosphate (cAMP) and inositol triphosphate (IP3). These second messengers trigger kinase cascades, which can lead to the modulation of multiple genes involved in cell growth and proliferation (Levin and Pietras 2008; Yager and Davidson 2006). Polyamines play a key role in $E_2$-mediated cell growth, which involves the regulation of ER–estrogen response element (ERE) and transcription factor binding, as well as the regulation cell cycle progression (Thomas and Thomas 2001).

Previous studies on polyamine analogues in our laboratory (Shah et al. 2001; Faaland et al. 2000) and others (McCloskey et al. 1995), often used ER-positive [ER(+)] breast cancer cells growing in the presence $E_2$, as these cells represented a major class of ER(+) human breast cancer. In MCF-7 cells, BE-3-3-3-3 inhibited cell proliferation, induced apoptosis, and increased SSAT activity (Faaland et al. 2000). BE-3-3-3-3 was comparable or slightly more effective than the commonly used analogue, bis(ethyl) norspermine (BE-3-3-3, also known as DENSPM), in inhibiting tumor growth in a transgenic mouse model of breast cancer (Shah et al. 1999). Although BE-3-3-3 inhibited cancer cell growth in vitro (Shappell et al. 1992), and tumor growth in animal models (Bernacki et al. 1992; Porter et al. 1993), it failed to produce an objective therapeutic effect in cancer patients during phase I and II clinical trials (Creaven et al. 1997; Streiff and Bender 2001; Wolff et al. 2003). A possible explanation for BE-3-3-3's ineffectiveness as a therapeutic agent could be a growth stimulatory effect of this compound, as pointed out by Minchin et al. (2006) in the case of melanoma cells.

In spite of the extensive research on the action of polyamine analogues, the precise mechanism(s) involved is still not clear. BE-3-3-3 induced the formation of large lysosomal vacuoles in treated cells, indicating a possible involvement of autophagy in the action of these compounds (Dai et al. 1999; Kramer et al. 1998; Porter et al. 1990). Autophagy is a pathway for recycling of long-lived cytoplasmic proteins and organelles, where these cellular components are enclosed into double-membrane vesicles, termed autophagic vacuoles or autophagosomes, and delivered to the lysosome for degradation (Klionsky et al.

2008). Basal levels of autophagy are essential for proper cellular maintenance and tissue remodeling. However, autophagy is upregulated under stressful conditions (i.e. nutrient or growth factor deprivation, oxidative stress), and promotes cell survival through degradation of non-essential cellular components (Levine and Yuan 2005). On the other hand, prolonged activation of autophagy can severely deplete and damage a cell and result in Type II Programmed Cell Death, which is different from apoptosis. Recent studies have demonstrated that autophagy can delay apoptotic cell death, and that an inhibition of autophagy can sensitize cells to apoptosis-inducing drugs, such as doxorubicin (Abedin et al. 2007; Daniel et al. 2006).

Our goal in this study was to identify conditions and subsets of breast cancer cells responding to BE-3-3-3-3, and to define the role of autophagic vacuole formation in the mechanism of action of this polyamine analogue. We found that BE-3-3-3-3 induced cell proliferation in the absence of $E_2$, and stimulated promoter binding and expression of $E_2$–modulated genes. Our results show a stimulatory effect of BE-3-3-3-3 on DNA synthesis in ER-positive MCF-7 cells in the absence of $E_2$, and the formation of vacuoles comparable to those found in autophagy.

## Materials and methods

### Materials

MCF-7 and MDA-MB-231 cell lines were obtained from the American Type Culture Collection (Monasses, VA). Cell lines were authenticated by short tandem repeat (STR) analysis, performed at the John Hopkins University (Baltimore, MD), using the Powerplex 1.2 kit from Promega (Madison, WI). Dulbecco's Modified Eagle's Medium (DMEM), phenol red-free DMEM, Minimal Essential Medium (MEM), fetal bovine serum (FBS), anti-$\beta$-actin antibody, spermine tetrahydrochloride, and spermidine trihydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Antibiotics, trypsin, additives for cell culture media, secondary antibody conjugated with Alexa Fluor 488 and Alexa Fluor 546 phalloidin were purchased from Invitrogen (Carlsbad, CA). Anti-Beclin 1 antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-MAP LC3, anti-SRC-3, anti-NCoR, and anti-ERα (rabbit) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). BE-3-3-3-3 was synthesized as described earlier (Musso et al. 1997).

### Cell culture

MCF-7 cells were maintained in DMEM, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamicin, 2 μg/ml insulin, 0.5 mM sodium pyruvate, 50 mM nonessential amino acids, 2 mM L-glutamine, and 10% fetal bovine serum (FBS). MDA-MB-231 cells were maintained in MEM, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamicin, 0.5 mM sodium pyruvate, 2 mM L-glutamine, and 10% fetal bovine serum. Prior to each experiment, MCF-7 cells were grown for 3–4 days in phenol red-free DMEM, containing FBS treated with dextran-coated charcoal (DCC) to remove serum-derived estrogenic compounds (purchased from Sigma or prepared from FBS as described) (Vijayanathan et al. 2006). DCC suspension contained 0.05% dextran, 0.5% charcoal and 25 mM sucrose. Serum was subjected to three 10 min cycles of DCC treatment, centrifugation, and passed through a 0.2 μm membrane filter.

### [$^3$H]-Thymidine incorporation

Cells were seeded at a density of $0.5 \times 10^6$ in 6-well dishes and were treated with BE-3-3-3-3 alone, or together with $E_2$, as indicated in the text. Assay was conducted as described previously (John et al. 2008). Thymidine incorporation was assessed at 24, 48 and 96 h of treatment.

### Quantitative PCR

MCF-7 cells ($1 \times 10^6$) were seeded in 60 mm culture dishes. After 24 h, cells were treated with 0, 5, and 10 μM BE-3-3-3-3 for 0, 2, and 8 h. RNA isolation and qPCR analysis were performed as describe previously (John et al. 2008), using the following primer sequences: 5′-CTCCT CACAGCCCACTGGTC-3′ and 5′-CTTGGCAGCAGGA TAGTCCTTC-3′ for c-myc (101 bp); 5′-CGGGCTTCAA CGCAGACTA-3′ and 5′-GGTCCGTGCAGAAGTCCTG-3′ for c-fos (147 bp); and 5′-CATGAGAAGTATGACAAC AGCCT-3′ and 5′-AGTCCTTCCACGATACCAAAGT-3′ for Gapdh (113 bp). qPCR products were normalized to Gapdh in order to correct for template input variation. Data is presented as fold difference in expression of the indicated gene relative to Gapdh.

### Chromatin immunoprecipitation assay

MCF-7 cells ($8 \times 10^6$) were plated in 10 cm dishes and allowed to attach overnight. Cells were treated with 0, 5, and 10 μM BE-3-3-3-3 in the presence and absence of 4 nM $E_2$. Chromatin immunoprecipitation assay (ChIP) assay was performed as described previously, with minor modifications (John et al. 2008; Konduri et al. 2010). Cells ($8 \times 10^6$) in 10-cm dishes were washed once with PBS and cross-linked with 1.5% formaldehyde at 37°C for 10 min. After washing with PBS, cells were collected in 1.6 ml of

lysis buffer [0.5% SDS, 5.6 mmol/l EDTA, 33 mmol/l Tris–HCl (pH 8.1), 0.5% Triton X-100, 84 mmol/l NaCl] and incubated on ice for 30 min. Cell lysate was sonicated using Sonicator 3000 (Misonix). Samples were diluted 5-fold with dilution buffer [0.01% SDS, 1.2 mmol/l EDTA, 16.7 mmol/l Tris–HCl (pH 8.1), 1.1% Triton X-100, 167 mmol/l NaCl] and then precleared with salmon sperm DNA/Protein A agarose for 2 h at 4°C. Anti-ERα antibody (HC-20, 5 μg), anti-SRC-3, anti-NCoR or rabbit IgG (Santa Cruz) was used to immunoprecipitate from 200 μg of protein. Immunoprecipitated DNA was amplified by PCR using AccuPrime TaqDNA polymerase and visualized with ethidium bromide staining. The following Tff1/pS2 promoter primers were used: 5′-CTAGACGGAATGGGCT TC ATG-3′ (forward) and 5′-TCCTCCAACCTG ACCTT AATCC-3′ (reverse).

### Western blot analysis

MCF-7 cells ($1.5 \times 10^6$) were plated in 60 mm dishes and incubated overnight. After administration of pre-determined dosages, cells were harvested in ice-cold phosphate buffered saline (PBS), centrifuged, and the pellet was stored at −80°C. Western blotting was performed as described previously (John et al. 2008).

### Confocal microscopy

MCF-7 cells ($0.5 \times 10^5$/well) were plated in a Labtek 6-well slide chamber and dosed after 24 h. Cells were labeled and visualized as described previously (Vijayanathan et al. 2006). Alexa Fluor 488 conjugated anti-rabbit IgG (green) was used to detect ERα. Alexa Fluor 546 phalloidin was used for cytoskeletal staining. Nuclear staining was performed with DAPI (4′6′-diamidino-2-phenylindole, 1 nM). No fluorescence was observed in cells treated with fluorescent secondary antibody alone, indicating the absence of non-specific binding.

### Electron microscopy (EM)

MCF-7 cells ($2 \times 10^6$/10 cm dish) were either left untreated (control), or dosed with 4 nM $E_2$, 5 μM BE-3-3-3-3 and 4 nM $E_2$, or 5 μM BE-3-3-3-3 alone. After 24 h, cells were harvested and fixed in a solution containing 2.5% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate (pH 7.4) and 8 mM $CaCl_2$. Cells were post-fixed with 1% osmium tetroxide, dehydrated in graded alcohols, embedded in Epon 812, sectioned with ultra microtome, stained with uranyl acetate and lead citrate. Cellular components were visualized using a JEOL 1200EX electron microscope at 3000×.

### Enzyme activity and polyamine analysis

Cells were plated and allowed to attach overnight. They were treated with 0, 2.5, 5, and 10 μM BE-3-3-3-3 in the presence and absence of 4 nM $E_2$, and incubated for 24 or 48 h. Cells were lysed in a buffer containing 25 mM phosphate solution (pH 7.4), 1 mM EDTA, 0.1% Triton X-100 and 0.1 mM dithiothreitol, and the lysate was aliquoted for two separate experiments. For polyamine concentration determination, samples were mixed in a 9:1 ratio with 50% sulphosalicylic acid and 100 μM diaminoheptane as internal standard. Polyamine concentrations were measured by HPLC as described earlier (Hyvönen et al. 1992). The rest of the lysate was centrifuged at 13,000 rpm for 30 min at 4°C. ODC (Jänne and Williams-Ashman 1971) and SSAT (Bernacki et al. 1995) activities were assayed according to published methods. Spermine oxidase (SMO) and acetylpolyamine oxidase (APAO) activities were assessed using a previously described method (Zhou and Panchuck-Voloshina 1997), with the following modifications. Cell samples (35 μl) were pre-incubated in black fluorescence 96-well plates for 15 min in assay solution containing 50 mM sodium borate buffer (pH 9.0), 1 mM pargyline, 0.1 mM semicarbazide and 0.02 U/ml horseradish peroxidase (Roche Applied Science, IN, USA). Subsequently, a mixture containing 50 μM Ampiflu Red (Sigma) and 250 μM spermine (SMO assay) or $N^1$-acetylspermidine (APAO assay) was added to the samples, to a total volume of 200 μl. Fluorescence was measured for 60 min at 5 min intervals using a Victor 2 counter (PerkinElmer, Waltham, MA, USA), with excitation and emission wavelengths set at 540 and 590 nm, respectively. Standard curve was prepared from a series of $H_2O_2$ dilutions (made immediately before measurement by the addition of fresh 30% stock to cell lysis buffer), and measured simultaneously with samples.

### Statistical analysis

All experiments were repeated at least three times. For thymidine incorporation data, statistical significance of the difference between control and treatment groups was determined by one-way ANOVA followed by Dunnet's post-test using SigmaStat 3.5 program (Systat Software, San Jose, CA). qPCR results were analyzed by GraphPad Prism software (La Jolla, CA), using a *t*-test for comparison between groups. For polyamine levels and enzyme activity data, statistical significance was determined by one-way ANOVA and Tuckey's post-hoc test using SPSS software (SPSS Inc., Chicago, IL). $p < 0.05$ was considered to be statistically significant.

## Results

### BE-3-3-3-3 stimulates cell growth in the absence of $E_2$

We selected BE-3-3-3-3 for this study because this agent showed growth inhibitory effects on breast cancer cells in the presence of estradiol (Faaland et al. 2000; Shah et al. 1999). We first examined the effect of BE-3-3-3-3 on cell growth in ER(+) MCF-7 and ER-negative [ER(−)] MDA-MB-231 breast cancer cells. Cells were treated with 0, 2.5, 5, and 10 μM BE-3-3-3-3 alone, or in combination with 4 nM $E_2$. [$^3$H]-thymidine incorporation was determined at 24, 48, and 96 h after dosing. At 24 and 48 h, treatment with BE-3-3-3-3, alone, produced a 3- to 5-fold increase in DNA synthesis in MCF-7 cells (Fig. 1A). At the 96 h time point, DNA synthesis was not significantly affected with respect to the untreated control (Fig. 1A). Treatment with $E_2$, alone, generally produced a 3-4-fold increase in thymidine incorporation compared to control (result not shown). In Fig. 1B, the control bars set at 100% represent cells treated with just $E_2$. In the presence of $E_2$, BE-3-3-3-3 inhibited DNA synthesis in MCF-7 cells, starting at 48 h at 10 μM concentration (Fig. 1B). However, at the 96 h time point, DNA synthesis was inhibited at all concentrations of BE-3-3-3-3. Taken together, while the stimulatory effects of BE-3-3-3-3 are not prolonged in the absence of $E_2$, the compound was capable of inhibiting $E_2$-mediated DNA synthesis at all concentrations by the 96 h time point in MCF-7 cells.

The effect of BE-3-3-3-3 on ER(−) MDA-MB-231 cells was studied as a control (Fig. 1C). A modest increase (1.5-fold) in DNA synthesis was observed at 48 h in cells treated with 2.5 μM BE-3-3-3-3. Growth inhibition also occurred at this time point at higher BE-3-3-3-3 concentrations (5 and 10 μM). This result indicates that a possible mechanism for the stimulatory action of BE-3-3-3-3 on MCF-7 cells might be the presence of the ERα, despite lineage and phenotypic differences between MCF-7 and MDA-MB-231 cell lines. For example, MDA-MB-231 cells are invasive while MCF-7 cells are not; MDA-MB-231 cells contain a lot more glycosphingolipids and are Ah receptor positive (Dolfini et al. 2007; Scarlatti et al. 2003; Vogel and Matsumura 2009).

### Effect of BE-3-3-3-3 on the expression of $E_2$-regulated genes

In order to elucidate the mechanism(s) involved in BE-3-3-3-3's growth-promoting effect on MCF-7 cells, we examined the effect of this polyamine analogue on the expression of two well-characterized, $E_2$-responsive genes. Cells were treated with 0, 5, and 10 μM BE-3-3-3-3 in the presence and absence of $E_2$, and the RNA was harvested at

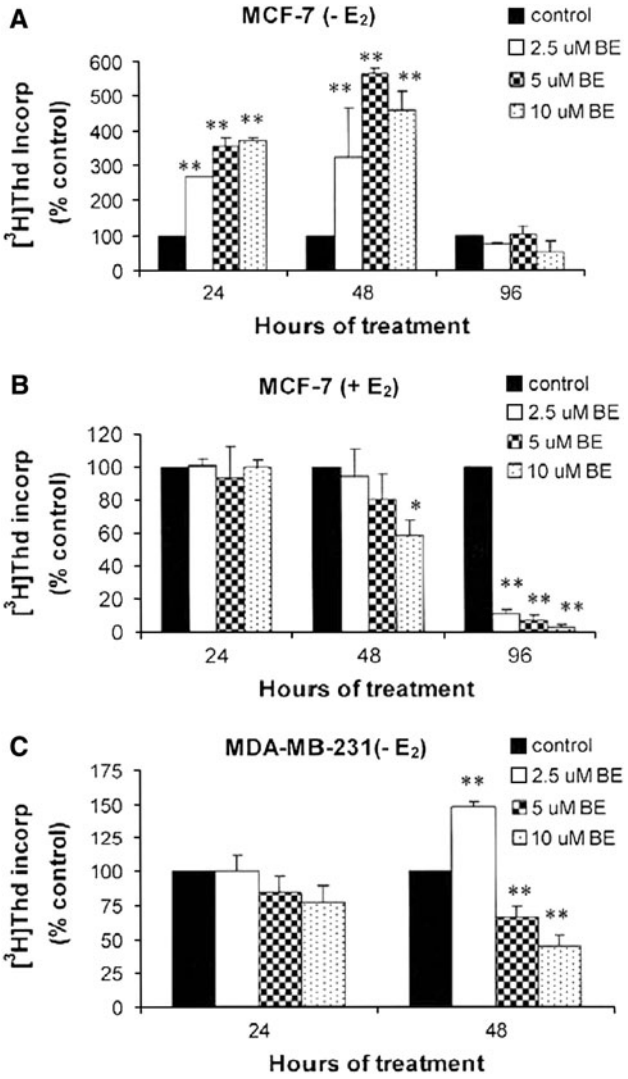


**Fig. 1** Effect of BE-3-3-3-3 on cell growth of ER-positive and ER-negative breast cancer cells in the presence and absence of $E_2$. Cells were treated with 0 (control), 2.5, 5 and 10 μM BE-3-3-3-3, alone, or in combination with 4 nM $E_2$. [$^3$H]-thymidine was added 24, 48, or 96 h after treatment, and cells were harvested for scintillation counting 1 h after its addition. **A** MCF-7 cells treated with BE-3-3-3-3 alone. **B** MCF-7 cells treated with BE-3-3-3-3 and 4 nM $E_2$. **C** MDA-MB-231 cells treated with BE-3-3-3-3. For **A**, 100% [$^3$H]-thymidine incorporation (control) was equivalent to $9.6 \pm 0.4 \times 10^5$ counts per minute (cpm) at the 24 h time point. For **B**, 100% was equivalent to $6.0 \pm 0.3 \times 10^6$ cpm (control with $E_2$), whereas untreated (in the absence of $E_2$) control had $3.4 \pm 0.3 \times 10^6$ cpm (result not shown). For **C**, 100% was equivalent to $15.8 \pm 1.6 \times 10^6$ cpm. Results are the mean ± SEM from 3 experiments. *$p < 0.05$ compared to control; **$p < 0.01$ compared to control

0, 2, and 8 h. Analogue treatment alone stimulated the expression of early response genes, c-myc and c-fos, to a similar degree as treatment with $E_2$ (Fig. 2A). Two hours of treatment with 5 and 10 μM BE-3-3-3-3 alone induced the expression of c-fos [~4.5-fold, Fig. 2A(c)] and c-myc [~2.4-fold, Fig. 2A(a)], respectively, compared to untreated control. However, the stimulation of c-myc was

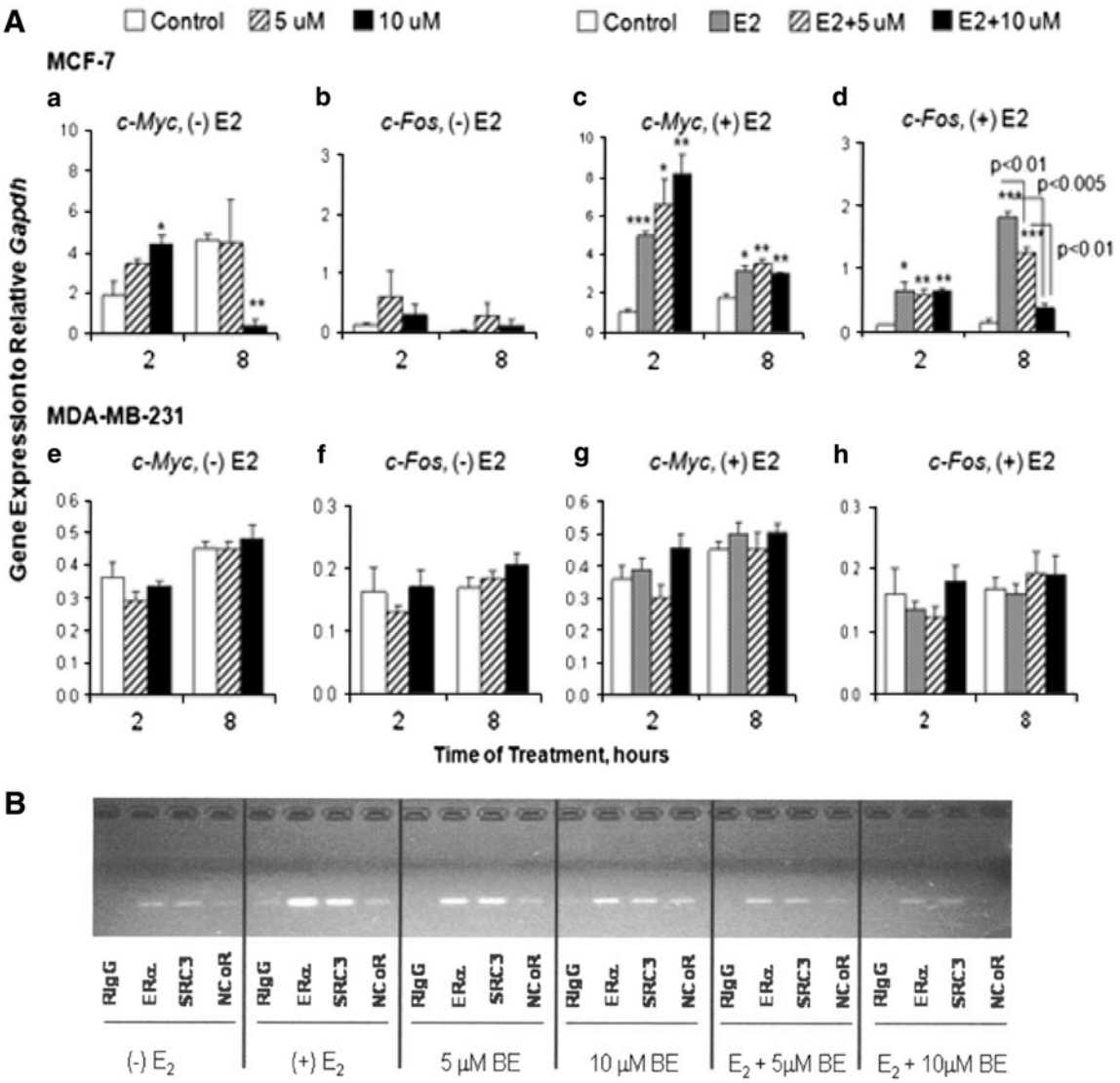


**Fig. 2** **A** qPCR analysis showing the effect of 0, 5, and 10 μM BE-3-3-3-3 on the expression of c-myc and c-fos genes in MCF-7 cells the absence (*a* and *c*) and presence (*b* and *d*) of 4 nM $E_2$. Expression was assessed at 0 (not shown), 2, and 8 h after treatment. Level of expression was normalized to Gapdh. Results are means ± SD. $n = 3$ per time point per group. $^{*}p < 0.05$ versus control; $^{**}p < 0.01$ vs control; $^{***}p < 0.005$ versus control. In the case of MDA-MB-231 cells, there was no significant difference in gene expression levels between the control and any treatment groups. **B** Results of ChIP assay showing the effect of BE-3-3-3-3 on the occupancy of the pS2/Tff1 promoter by ERα, SRC-3 co-activator, and NCoR co-repressor in the presence and absence of 4 nM $E_2$. Promoter occupancy was evaluated in MCF-7 cells, 4 h after treatment with 0, 5, and 10 μM BE-3-3-3-3. Rabbit IgG (RIgG) was used as a negative control. This result is the representative of results from 3 separate experiments

later inhibited by 10 μM BE-3-3-3-3 at the 8 h time point. This experiment shows a possible estrogen-mimetic action of the polyamine analogue at low concentrations.

## Effect of BE-3-3-3-3 on promoter occupancy of pS2/Tff1

We next examined the effect of the polyamine analogue on the recruitment of ERα, steroid receptor co-activator 3 (SRC-3) and nuclear receptor co-repressor (NCoR) to the promoter region of pS2/Tff1, an $E_2$-regulated gene. MCF-7 cells were dosed with 0, 5, and 10 μM BE-3-3-3-3 in the absence and presence of 4 nM $E_2$, and assayed after 4 h of treatment. As shown in Fig. 2b, BE-3-3-3-3 (administered alone) stimulated ERα and SRC-3 binding to the gene promoter, similar to the effect observed in the $E_2$ (alone) group. The increase in binding was more pronounced at 5 μM than 10 μM concentration. In contrast, analogue administration in the presence of $E_2$ resulted in significantly lower promoter occupancy compared to either $E_2$ (alone) or BE-3-3-3-3 (alone) treatment groups.

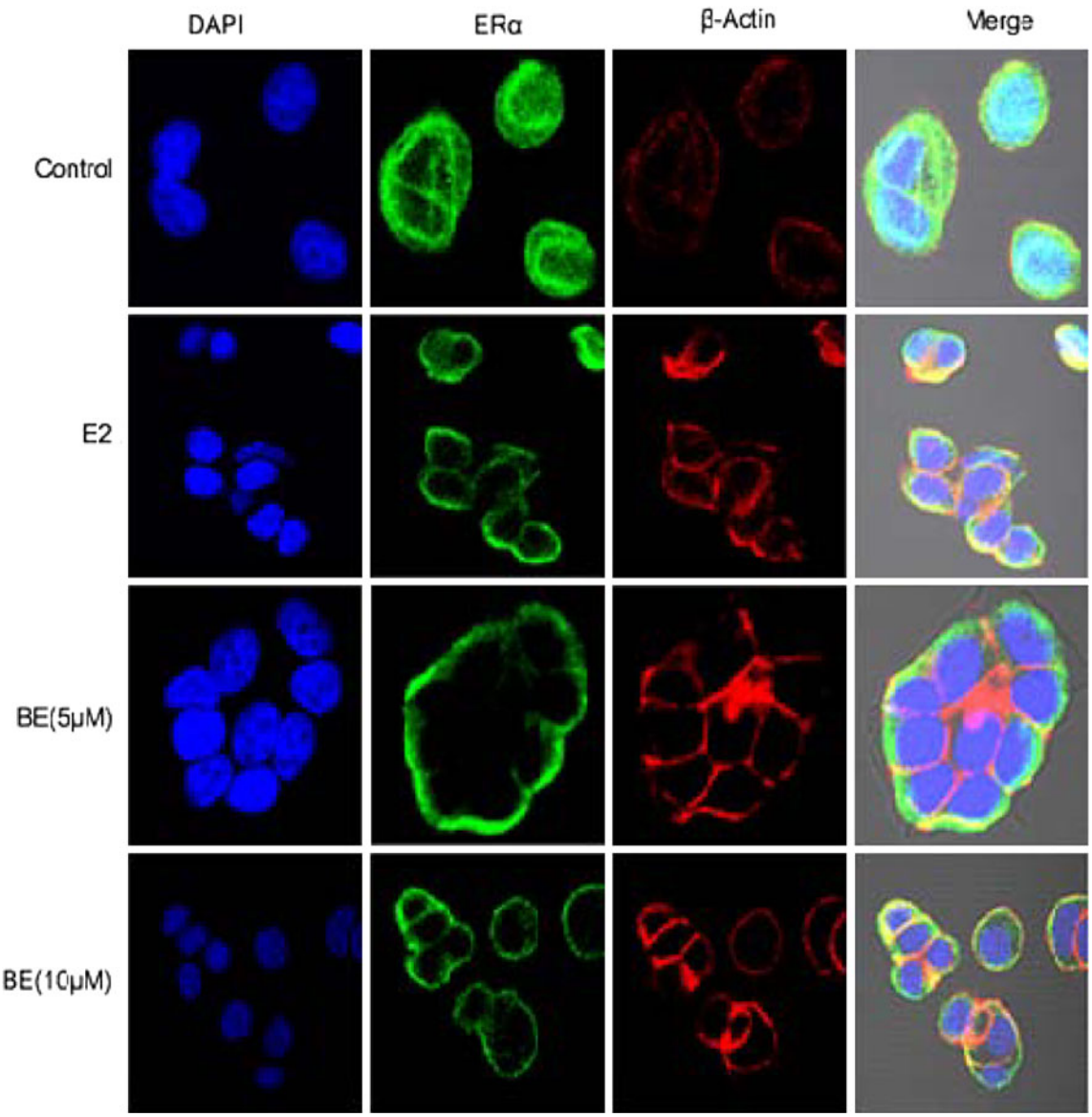


**Fig. 3** Estrogen-mimetic effect of BE-3-3-3-3 on the intracellular distribution of ERα. MCF-7 cells were treated with 0/control, 4 nM $E_2$, 5 μM BE-3-3-3-3, and 10 μM BE-3-3-3-3. At the 24 h time point, cells were subjected to the immunostaining procedure. Images were captured using a Zeiss 510 Laser scanning microscope with a ×60 objective at identical intensity settings for all treatment groups. Nuclei were stained blue using DAPI (4′6′-diamidino-2-phenylindole, 1 nM), and ERα was stained green with rabbit anti-ERα. Filamentous actin component of the cytoskeleton was stained red by Alexa Fluor 546 phalloidin, revealing cell periphery/cytoskeletal margins. Last column represents merged images. Similar results were obtained in 3 separate experiments

### Effect of BE-3-3-3-3 on ERα localization

In order to examine whether BE-3-3-3-3 has a similar effect on subcellular localization of ERα as that of $E_2$, we conducted a series of confocal microscopy experiments. MCF-7 cells were treated with 4 nM $E_2$ or with 0, 5, and 10 μM BE-3-3-3-3, and subjected to a fluorescent antibody staining procedure after 24 h. In Fig. 3, nuclei are stained blue, ERα-green, and cellular membrane/cytoskeletal margins-red. In untreated cells (Fig. 3A), ERα is localized mainly in the nucleus and cytoplasm. The addition of $E_2$ caused a re-localization of the receptor exclusively to the cell membrane area, as demonstrated by the intense yellow color in the merged image [Fig. 3 B(d)]. This change in receptor distribution is in agreement with results from previous studies (John et al. 2008; Vijayanathan et al. 2006). Treatment with 5 and 10 μM BE-3-3-3-3 (Fig. 3C, D, respectively) caused ERα re-localization to the cellular membrane/periphery area, in a manner similar to that observed in the $E_2$–treated group. The addition of $E_2$ and

BE-3-3-3-3 combination caused a redistribution of ERα in a manner similar to that of $E_2$ alone, although membrane localization was less than that of $E_2$ alone (data not shown).

Induction of Beclin 1 and microtubule-associated protein 1 light chain 3 II (MAP LC3 II) expression during the action of BE-3-3-3-3.

In the next set of experiments, we examined whether the inhibition of cell proliferation with BE-3-3-3-3 treatment in the absence of $E_2$ is associated with the induction of autophagy-related proteins in MCF-7 cells. Therefore, we observed the effect of this polyamine analogue on the expression of Beclin 1 and MAP LC3-II proteins, which served as markers of autophagy. MCF-7 cells were treated with 0, 1.25, 2.5, 5, and 10 μM BE-3-3-3-3 (in the presence and absence of $E_2$), harvested after 48 h, and subjected to Western blot analysis using anti-Beclin 1 antibody (Fig. 4a). A dose-dependent increase (1.5-, 2-, 2.3-. and 3-fold, respectively, at 1.25, 2.5, 5, and 10 μM BE-3-3-3-3 compared to untreated control) in the level of Beclin 1 is observed in cells treated with BE-3-3-3-3 alone. The detection of Beclin 1 in untreated (control) MCF-7 cells is consistent with recent reports (Abedin et al. 2007; Scarlatti et al. 2008). By contrast, $E_2$ and $E_2$ + 1.25 μM BE-3-3-3-3 did not significantly change the expression levels of Beclin 1, whereas higher concentrations of BE-3-3-3-3 (in combination with $E_2$) reduced Beclin 1 level by 10–25%, compared to that of control (Fig. 4c). In similar experiments, we examined the induction of MAP LC 3-II in BE-3-3-3-3 treated cells (Fig. 4b). Western blots using anti-MAP LC3 antibody showed a dose-dependent increase in the expression of a 16 kD modified form of LC3 II (lower band in 4B), with maximum stimulation occurring at 5 μM. The induction of Beclin 1 and MAP LC3-II indicated that the growth-stimulatory, pro-survival action of BE-3-3-3-3 might involve the autophagy pathway.

EM of MCF-7 cells treated with BE-3-3-3-3

Our previous experiments demonstrated that treatment with BE-3-3-3-3 elevated the expression levels of MAP LC3-II, which are indicative of increased level of autophagic vacuole formation. In order to visualize the ultra-structural changes in cells treated with BE-3-3-3-3, we conducted an electron microscopic study. Autophagic vacuoles were seldom found in untreated MCF-7 cells [Fig. 4D(a)]. Figure 4D(b) shows a representative $E_2$-treated cell, and demonstrates the absence of autophagy-related cellular alterations. Treatment with 5 μM BE-3-3-3-3 in the presence of $E_2$ did not stimulate autophagy after 24 h, as denoted by the absence of vacuole formation in these cells [Fig. 4D(c)]. In the absence of $E_2$, autophagy was induced in cells treated with 5 μM BE-3-3-3-3 for 24 h [Fig. 4D(d)], as evidenced by the presence of autophagic vacuoles in the cytoplasm. Quantification showed 1, 1, 8 and 6 vacuoles per cell in each of control, $E_2$, 5 μM BE-3-3-3-3 and 10 μM BE-3-3-3-3 treatment groups, respectively. These results suggest that autophagy plays a role in the estrogen-mimetic, growth stimulatory effect of BE-3-3-3-3, delaying the growth inhibitory action of this polyamine analogue.

Cellular polyamine levels and enzyme activities

In the next set of experiments we examined the effect of BE-3-3-3-3 on polyamine metabolism and intracellular polyamine levels during the growth-stimulatory phase of analogue action. MCF-7 cells were treated with 0, 2.5, 5, and 10 μM BE-3-3-3-3 in the presence and absence of 4 nM $E_2$, and polyamine concentrations and enzyme activities were analyzed at 24 and 48 h. Analogue administration, both in the presence and absence of $E_2$, significantly reduced the levels of putrescine, spermidine, and spermine (Table 1). This reduction was associated with intracellular accumulation of BE-3-3-3-3. $N^1$-acetylspermidine ($N^1$-AcSpd) levels rose at 24 h, but at 48 h it became undetectable apparently by the fact that there was not much spermidine available for acetylation and also because $N^1$-AcSpd is rapidly excreted to the culture media. The down-regulation of polyamine levels was also associated with the inhibition of a key polyamine biosynthetic enzyme, ODC (~68-fold at 24 h), and the strong stimulation of a catabolic enzyme, SSAT (~183-fold at 48 h). $E_2$ treatment alone produced a roughly 2-fold increase in ODC activity, which is consistent with previous reports (Vijayanathan et al. 2006). The induction of SSAT by BE-3-3-3-3 was much more pronounced in the absence of $E_2$, especially at the 48 h time point. The strong stimulation of SSAT was not accompanied by the stimulation of oxidative enzymes of the catabolic pathway, such as SMO and APAO.

## Discussion

In this study, we examined the effect of BE-3-3-3-3 on ERα(+) breast cancer cell growth, and found that while it was growth-inhibitory in the presence of $E_2$, it stimulated DNA synthesis in the absence of $E_2$, up to 48 h after treatment. In MCF-7 cells, growth stimulation by this estrogen-mimetic compound was accompanied by the induction of $E_2$-regulated genes, increased promoter recruitment of ERα and SRC-3, and ERα re-localization to the cell membrane. Increased cell proliferation, as measured by [$^3$H]-thymidine incorporation, of MCF-7 cells during treatment with BE-3-3-3-3 was associated with the stimulation of autophagy. Growth inhibitory effects of

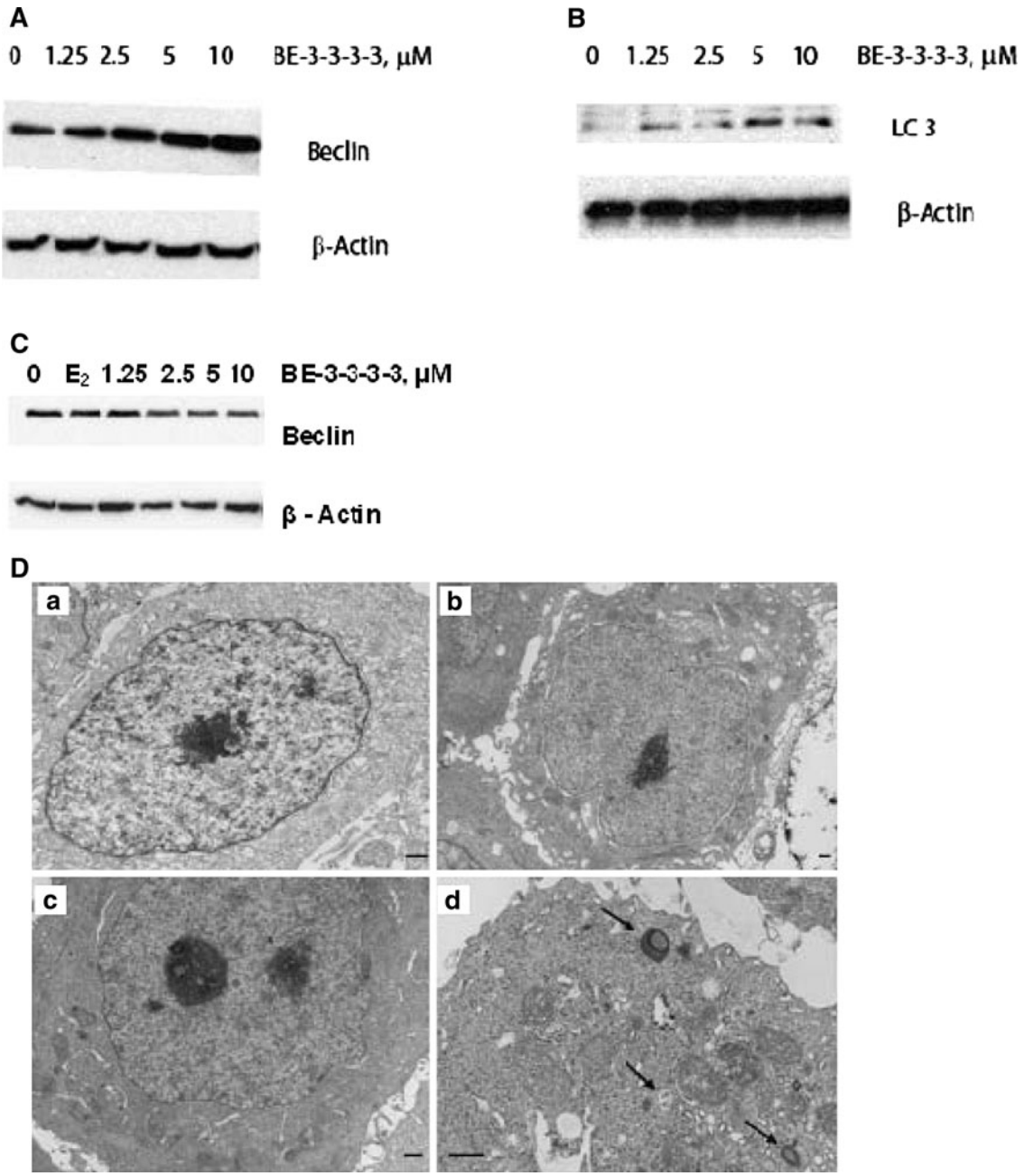


**Fig. 4** Effect of BE-3-3-3-3 on the levels of Beclin 1 and MAP LC3 II in the presence and absence of $E_2$. MCF-7 cells were treated with 0, 1.25, 2.5, 5, and 10 μM BE-3-3-3-3 in the presence and absence of 4 nM $E_2$. After 48 h, cells were harvested and analyzed by Western blots using anti-Beclin (**A** and **C**) and anti-MAP LC3 (**B**) antibodies. For **C**, all lanes (except "0" control) had 4 nM $E_2$. The blots were stripped and then re-probed with an anti-$\beta$-actin antibody. Results are representative of 3 different experiments. D. Ultrastuctural changes in MCF-7 cells treated with BE-3-3-3-3 for 24 h. **a** Control; **b** cells treated with 4 nM $E_2$; **c** cells treated with 4 nM $E_2$ and 5 μM BE-3-3-3-3; **d** cells treated with 5 μM BE-3-3-3-3. *Arrows* indicate autophagosomes. *Bar* represents 500 nm

BE-3-3-3-3 in the presence of $E_2$ might be caused by ER$\alpha$'s ability to antagonize the growth-promoting effects of BE-3-3-3-3. This was evidenced by elevated levels of autophagy-related proteins, Beclin 1 and MAP LC3-II, and autophagosome formation. In addition, BE-3-3-3-3 had a significant impact on the activities of key polyamine regulatory enzymes, ODC and SSAT, and on intracellular polyamine levels.

Potential estrogen-mimetic behavior of BE-3-3-3-3 was evidenced by its stimulatory effect on the expression of $E_2$-sensitive genes, *c-myc* and *c-fos*. These fast-acting proto-oncogenes are part of the growth-promoting pathway of $E_2$

**Table 1** BE-3-3-3-3 uptake and effect on polyamine levels

| Treatment | Polyamines (pmol/μg DNA) | | | | |
|---|---|---|---|---|---|
| | Put | Spd | Spm | $N^1$-AcSpd | BE-3-3-3-3 |
| 24 h | | | | | |
| 0 μM BE | 156 ± 15[a] | 283 ± 27 | 190 ± 34 | n.d. | |
| 2.5 μM BE | 28 ± 2[a,b] | 63 ± 5[a,b] | 100 ± 12[a,b] | 25 ± 1 | 407 ± 35 |
| 5 μM BE | 28 ± 3[a, b] | 48 ± 9[a,b] | 96 ± 13[a,b] | 27 ± 2 | 390 ± 26 |
| 10 μM BE | 28 ± 3[a, b] | 34 ± 1[a,b] | 86 ± 18[a,b] | 25 ± 2 | 400 ± 39 |
| $E_2$ | 187 ± 8[b] | 293 ± 5 | 190 ± 17 | n.d. | |
| $E_2$ + 2.5 μM BE | 25 ± 0[a, b] | 78 ± 7[a,b] | 66 ± 8[a,b] | n.d. | 425 ± 18 |
| $E_2$ + 5 μM BE | 19 ± 1[a, b] | 57 ± 7[a,b] | 61 ± 6[a,b] | n.d. | 407 ± 4 |
| $E_2$ + 10 μM BE | 23 ± 2[a, b] | 53 ± 11[a,b] | 66 ± 4[a,b] | n.d. | 459 ± 34 |
| 48 h | | | | | |
| 0 μM BE | 109 ± 5[c] | 216 ± 3[c] | 164 ± 7 | n.d. | |
| 2.5 μM BE | 20 ± 3[c, d] | 17 ± 2[c, d] | 40 ± 4[c, d] | n.d. | 294 ± 14 |
| 5 μM BE | 17 ± 2[c, d] | 8 ± 3[c, d] | 33 ± 4[c, d] | n.d. | 314 ± 14 |
| 10 μM BE | 17 ± 2[c, d] | 9 ± 3[c, d] | 27 ± 5[c, d] | n.d. | 316 ± 15 |
| $E_2$ | 199 ± 11[d] | 289 ± 5[d] | 164 ± 6 | n.d. | |
| $E_2$ + 2.5 μM BE | 17 ± 2[c, d] | 25 ± 8[c, d] | 33 ± 3[c, d] | n.d. | 311 ± 11 |
| $E_2$ + 5 μM BE | 11 ± 1[c, d] | 14 ± 2[c, d] | 30 ± 5[c, d] | n.d. | 333 ± 31 |
| $E_2$ + 10 μM BE | 14 ± 3[c, d] | 13 ± 2[c, d] | 26 ± 1[c, d] | n.d. | 314 ± 6 |

MCF-7 cells were treated for 24 and 48 h with 0, 2.5, 5 and 10 μM BE-3-3-3-3 (BE) in the presence or absence of 4 nM $E_2$. Results are means ± SD, $n = 3$

*n.d.* Not detectable. Polyamine abbreviations can be found in the text

[a] $p \leq 0.001$ versus $E_2$ at 24 h; [b] $p \leq 0.001$ versus control (0 BE) at 24 h; [c] $p \leq 0.001$ versus $E_2$ at 48 h; [d] $p \leq 0.001$ versus control (0 BE) at 48 h

action. The stronger induction of mRNA synthesis at 2 h compared to 8 h is consistent with the "early response" profile of these genes. Results of our ChIP experiment show that transcriptional activation by the polyamine analogue involved increased recruitment of ERα and SRC-3 to the promoter of target gene(s). In this report, we have shown ERα and SRC-3 recruitment for a single target gene (PS2/Tff1) only because *c-myc* and *c-fos* respond to many signaling pathways in addition to $E_2$. This finding suggests that transcriptional regulation of $E_2$-responsive genes by BE-3-3-3-3 might result from interactions of these and other transcription factors and co-activators at the promoter/enhancer regions of estrogen-sensitive genes (Thomas and Thomas 1994a, b; Karmakar et al. 2009). Previous studies from our laboratory involving in vitro binding and immunoprecipitation techniques have demonstrated a role for natural polyamines to enhance DNA–protein and protein–protein interactions (Shah et al. 2001). It is possible that BE-3-3-3-3, at low concentrations, interacts with DNA, and facilitates transcription. Since estrogen-deprived cells are poised to respond to $E_2$-directed growth-regulatory pathways, this reaction would involve an increase in DNA synthesis and early gene response. In addition, BE-3-3-3-3 may also modulate gene expression through non-genomic mechanisms, such as activation of the G-protein-coupled receptor homologue 30 (GPR30) (Sasaki et al. 2008).

Previous studies with polyamine analogues in ER(+) breast cancer cells have been conducted in the presence of $E_2$. Under these conditions, various bis(ethyl) polyamine analogues, including BE-3-3-3 and BE-3-3-3-3, inhibited cell growth and induced apoptosis (Faaland et al. 2000). BE-3-3-3 was recently tested in Phase I and II clinical trials and proved to be ineffective as well as toxic in treated patients (Creaven et al. 1997; Wolff et al. 2003). In light of the growth stimulatory effect of this polyamine analogue at low concentrations, which generally correspond to levels attained in serum, pre-clinical and clinical development of these molecules requires special precautions and selection of sensitive subpopulation of patients.

The ability of to BE-3-3-3-3 and related compounds to induce SSAT activity has been demonstrated in several cell lines. Although, in our study, BE-3-3-3-3 increased SSAT activity by approximately 183-fold, this induction was not nearly as potent as that produced by BE-3-3-3 (>1000–fold) (Porter et al. 1993; Maggiolini et al. 2004; Pledgie et al. 2005). Elevation of SSAT activity may result from increased gene expression and/or extended protein half-life (Chen et al. 2001). ODC inhibition demonstrated in our study is also consistent with previously reported results (Maggiolini et al. 2004; Uimari et al. 2009). In contrast to the action of BE-3-3-3, which activates polyamine catabolism by inducing both SSAT and SMO (Casero et al. 2007), BE-3-3-3-3 did not significantly affect SMO activity (Table 2).

Several reports showed that polyamine analogues induced the formation of lysosomal vacuoles in treated cells (Dai et al. 1999; Kramer et al. 1998; Porter et al.

**Table 2** Effect of BE-3-3-3-3 on biosynthetic and catabolic enzymes of polyamine metabolism

| Treatment | Enzyme activities (pmol/h/μg DNA) | | | |
|---|---|---|---|---|
| | ODC | SSAT | SMO | APAO |
| 24 h | | | | |
| 0 μM BE | 13.6 ± 1.3[a] | 23 ± 6 | 292 ± 27[c] | 569 ± 18[a] |
| 2.5 μM BE | 0.2 ± 0.0[a,d] | 1360 ± 52[a,d] | 287 ± 21 | 521 ± 29[a] |
| 5 μM BE | 0.7 ± 0.8[a,d] | 1649 ± 70[a,d] | 305 ± 5[b] | 528 ± 10[a] |
| 10 μM BE | 0.6 ± 0.7[a,d] | 1885 ± 72[a,d] | 277 ± 9 | 527 ± 11[a] |
| $E_2$ | 26.2 ± 1.2[d] | 18 ± 1 | 219 ± 8 | 420 ± 18[d] |
| $E_2$ + 2.5 μM BE | 1.4 ± 1.2[a,d] | 849 ± 42[a,d] | 286 ± 44 | 509 ± 21[a,e] |
| $E_2$ + 5 μM BE | 0.3 ± 0.0[a,d] | 866 ± 13[a,d] | 244 ± 15 | 420 ± 6[d] |
| $E_2$ + 10 μM BE | 2.3 ± 0.8[a,d] | 1198 ± 42[a,d] | 266 ± 53 | 489 ± 18[b,d] |
| 48 h | | | | |
| 0 μM BE | 7.0 ± 0.5[f] | 20 ± 8 | 210 ± 53 | 412 ± 19 |
| 2.5 μM BE | 1.2 ± 0.7[f,g] | 2660 ± 54[f, g] | 189 ± 19 | 372 ± 13 |
| 5 μM BE | 0.3 ± 0.2[f,g] | 3593 ± 415[f,g] | 177 ± 14 | 357 ± 23[h] |
| 10 μM BE | 0.5 ± 0.3[f,g] | 3656 ± 103[f,g] | 180 ± 6 | 362 ± 6 |
| $E_2$ | 16.1 ± 1.5[g] | 14 ± 1 | 168 ± 9 | 381 ± 20 |
| $E_2$ + 2.5 μM BE | 0.5 ± 0.1[f,g] | 1067 ± 63[f,g] | 167 ± 20 | 332 ± 8[g] |
| $E_2$ + 5 μM BE | 0.5 ± 0.0[f,g] | 1162 ± 66[f,g] | 172 ± 4 | 325 ± 17[g] |
| $E_2$ + 10 μM BE | 0.7 ± 0.4[f,g] | 1187 ± 51[f,g] | 184 ± 19 | 334 ± 33[g] |

MCF-7 cells were treated for 24 or 48 h with 0, 2.5, 5 and 10 μM BE in the presence or absence of 4 nM $E_2$. Results are means ± SD, $n = 3$. Enzyme abbreviations can be found in the text

[a] $p \leq 0.001$ versus $E_2$ at 24 h; [b] $p \leq 0.01$ versus $E_2$ at 24 h; [c] $p \leq 0.05$ versus $E_2$ at 24 h; [d] $p \leq 0.001$ versus control (0 BE) at 24 h; [e] $p \leq 0.05$ vs. control at 24 h; [f] $p \leq 0.001$ versus $E_2$ at 48 h; [g] $p \leq 0.001$ versus control at 48 h; [h] $p \leq 0.05$ versus control at 48 h

1990; Stanic et al. 2009). Porter et al. (1990) detected rapid vacuole formation in L1210 leukemia cells in response to treatment with 2.5-diamino-3-hexyne, which could be completely resolved by the removal of the polyamine analogue for 12 h. Large cytoplasmic vacuoles were also observed by Kramer et al. (1998) after 24 h of treatment with DENSPM in SAMDC-overexpressing CHO/100 cells. Vacuole formation in these cells was associated with only a modest growth inhibition by BE-3-3-3, even at high intracellular polyamine analogue concentration. The authors suggested that vacuolar sequestration of BE-3-3-3 limited its growth-inhibitory effects. Our results indicate that vacuoles might activate autophagy in response to treatment with BE-3-3-3. Autophagy promotes cell survival and delays growth inhibition by recycling the intracellular components within the observed vacuoles. Dai et al. (1999) also observed vacuole formation after 4 h of treatment with a polyamine oxidase inhibitor MDL-72,527, in hematopoietic cells. In these cells, vacuoles grew in size over time, eventually engulfing key cytoplasmic organelles such as mitochondria. Vacuolar growth could be reversed with the removal of the drug. In our study, evidence for the stimulation of autophagy comes from the polyamine analogue-mediated increase in the expression of autophagy-related proteins, Beclin 1 and MAP LC3 II, in the absence of $E_2$.

Atg6/Beclin 1 was initially identified as a protein mediating autophagy in yeast and, subsequently, in mammalian cells (Eisenberg et al. 2009, Liang et al. 1999). In autophagy, Beclin 1 interacts with class III PI3 kinase (vps34) in the nucleation step of the autophagosomal membrane that engulfs damaged cytoplasmic organelles and long-lived proteins targeted for lysosomal degradation (Zhong et al. 2009). A recent study showed that autophagy delayed apoptotic death of breast cancer cells following DNA damage (Abedin et al. 2007). In another case, silencing Beclin 1, and thereby autophagy, enhanced doxorubicin-induced apoptosis (Daniel et al. 2006). These results raised the possibility that autophagy works against apoptosis-inducing chemotherapeutic agents. In a previous study, we found that Beclin 1 modulated estrogenic signaling by interacting with ERα in Beclin 1-overexpressing breast cancer cells. This interaction promoted anti-estrogen resistance, evidenced by the decreased growth inhibition of breast cancer cells in response to treatment with tamoxifen and raloxifene. Therefore, elevated expression of Beclin 1 in response to BE-3-3-3-3 might be involved in the activation of autophagy, and in the balancing of cell growth and cell death.

MAP LC3 is a mammalian homologue of yeast Atg8, and is essential for autophagosome formation (Noda et al. 2008; Tanida et al. 2004). It is synthesized as pro-LC3, and proteolytically processed and modified during autophagy to produce MAP LC3 II (Kabeya et al. 2000). MAP LC3 II is found on the pre-autophagic membranes and autophagosomes, and is a widely accepted marker for the detection of autophagy (Klionsky et al. 2008; Kadowaki and Karim 2009). Thus, an increase in the levels of this protein in

response to BE-3-3-3-3 treatment further indicates the induction of autophagy.

Taken together, these results demonstrate the differential behavior of BE-3-3-3-3, depending on the presence or absence of $E_2$. It stimulated cell growth in the absence of $E_2$, an effect that was abolished by 96 h after treatment. In contrast, it inhibited cell growth from the earliest stage of response in the presence of $E_2$. Treatment with BE-3-3-3-3 in the absence of $E_2$ enhanced the expression of estrogen-responsive, growth-promoting genes, and increased recruitment of transcription factors and co-activators to the promoter elements of these genes. We also show the activation of autophagy, which promotes cell survival and delayed growth inhibition in response to analogue treatment. It is important to conduct further mechanistic studies in a large number of cell lines with a greater number of polyamine analogues to provide clinical relevance to the novel findings reported herein.

**Acknowledgments** The authors thank Mrs. Tuula Reponen, Mrs. Anne Karppinen and Mrs. Arja Korhonen (University of Eastern Finland) for their skilful technical assistance. This work was supported, in part, by grants from the Foundation of UMDNJ (64-09 and PC28-1), Susan G. Komen 'For The Cure' grant BCTR 0600180, the Academy of Finland, and by UEF Strategic Funding.

## References

Abedin MJ, Wang D, McDonnell MA, Lehmann U, Kelekar A (2007) Autophagy delays apoptotic death in breast cancer cells following DNA damage. Cell Death Differ 14:500–510

Agostinelli E, Marques MP, Calheiros R, Gil FP, Tempera G, Viceconte N, Battaglia V, Grancara S, Toninello A (2010a) Polyamines: fundamental characters in chemistry and biology. Amino Acids 38:393–403

Agostinelli E, Tempera G, Viceconte N, Saccoccio S, Battaglia V, Grancara S, Toninello A, Stevanato R (2010b) Potential anticancer application of polyamine oxidation products formed by amine oxidase: a new therapeutic approach. Amino Acids 38:353–368

Bernacki RJ, Bergeron RJ, Porter CW (1992) Antitumor activity of N, N'-bis(ethyl)spermine homologues against human MALME-3 melanoma xenografts. Cancer Res 52:2424–2430

Bernacki RJ, Oberman EJ, Seweryniak KE, Atwood A, Bergeron RJ, Porter CW (1995) Preclinical antitumor efficacy of the polyamine analogue $N^1$, $N^{11}$-diethylnorspermine administered by multiple injection or continuous infusion. Clin Cancer Res 1:847–857

Casero RA Jr, Marton LJ (2007) Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. Nat Rev Drug Discov 6:373–390

Chen Y, Kramer DL, Diegelman P, Vujcic S, Porter CW (2001) Apoptotic signaling in polyamine analogue- treated SK-MEL-28 human melanoma cells. Cancer Res 61:6437–6444

Creaven PJ, Perez R, Pendyala L, Meropol NJ, Loewen G, Levine E, Berghorn E, Raghavan D (1997) Unusual central nervous system toxicity in a phase I study of $N^1$, $N^{11}$-diethylnorspermine in patients with advanced malignancy. Invest New Drugs 15:227–234

Dai H, Kramer DL, Yang C, Murti KG, Porter CW, Cleveland JL (1999) The polyamine oxidase inhibitor MDL-72, 527 selectively induces apoptosis of transformed hematopoietic cells through lysosomotropic effects. Cancer Res 59:4944–4954

Daniel F, Legrand A, Pessayre D, Vadrot N, Descatoire V, Bernuau D (2006) Partial Beclin 1 silencing aggravates doxorubicin- and Fas-induced apoptosis in HepG2 cells. World J Gastroenterol 12:2895–2900

Dolfini E, Roncoroni L, Dogliotti E, Sala G, Erba E, Sacchi N, Ghidoni R (2007) Resveratrol impairs the formation of MDA-MB-231 multicellular tumor spheroids concomitant with ceramide accumulation. Cancer Lett 249:143–147

Eisenberg T, Knauer H, Schauer A, Buttner S, Ruckenstuhl C, Carmona-Gutierrez D, Ring J, Schroeder S, Magnes C, Antonacci L, Fussi H, Deszcz L et al (2009) Induction of autophagy by spermidine promotes longevity. Nat Cell Biol 11:1305–1314

Faaland CA, Thomas TJ, Balabhadrapathruni S, Langer T, Mian S, Shirahata A, Gallo MA, Thomas T (2000) Molecular correlates of the action of bis(ethyl)polyamines in breast cancer cell growth inhibition and apoptosis. Biochem Cell Biol 78:415–426

Gerner EW, Meyskens FL Jr (2004) Polyamines and cancer: old molecules, new understanding. Nat Rev Cancer 4:781–792

Häkkinen MR, Hyvönen MT, Auriola S, Casero RA Jr, Vepsäläinen J, Khomutov AR, Alhonen L, Keinänen TA (2009) Metabolism of N-alkylated spermine analogues by polyamine and spermine oxidases. Amino Acids 38:369–381

Hyvönen T, Keinänen TA, Khomutov AR, Khomutov RM, Eloranta TO (1992) Monitoring of the uptake and metabolism of aminooxy analogues of polyamines in cultured cells by high-performance liquid chromatography. J Chromatogr 574:17–21

Jänne J, Williams-Ashman HG (1971) On the purification of L-ornithine decarboxylase from rat prostate and effects of thiol compounds on the enzyme. J Biol Chem 246:1725–1732

John S, Nayvelt I, Hsu HC, Yang P, Liu W, Das GM, Thomas T, Thomas TJ (2008) Regulation of estrogenic effects by beclin 1 in breast cancer cells. Cancer Res 68:7855–7863

Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 19:5720–5728

Kadowaki M, Karim MR (2009) Cytosolic LC3 ratio as a quantitative index of macroautophagy. Methods Enzymol 452:199–213

Karmakar S, Foster EA, Smith CL (2009) Unique roles of p160 coactivators for regulation of breast cancer cell proliferation and estrogen receptor-alpha transcriptional activity. Endocrinology 150:1588–1596

Katzenellenbogen BS, Frasor J (2004) Therapeutic targeting in the estrogen receptor hormonal pathway. Semin Oncol 31:28–38

Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC et al (2008) Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4:151–175

Konduri SD, Medisetty R, Liu W, Kaipparettu BA, Srivastava P, Brauch H, Fritz P, Swetzig WM, Gardner AE, Khan SA, Das GM (2010) Mechanism of estrogen receptor antagonism toward p53 and its implications in breast cancer therapeutic response and stem cell regulation. Proc Natl Acad Sci USA 107:15081–15086

Kramer DL, Black JD, Mett H, Bergeron RJ, Porter CW (1998) Lysosomal sequestration of polyamine analogues in Chinese hamster ovary cells resistant to the S-adenosylmethionine decarboxylase inhibitor, CGP-48664. Cancer Res 58:3883–3890

Leveque J, Foucher F, Bansard JY, Havouis R, Grall JY, Moulinoux JP (2000a) Polyamine profiles in tumor, normal tissue of the

homologous breast, blood, and urine of breast cancer sufferers. Breast Cancer Res Treat 60:99–105

Leveque J, Foucher F, Havouis R, Desury D, Grall JY, Moulinoux JP (2000b) Benefits of complete polyamine deprivation in hormone responsive and hormone resistant MCF-7 human breast adenocarcinoma in vivo. Anticancer Res 20:97–101

Levin ER, Pietras RJ (2008) Estrogen receptors outside the nucleus in breast cancer. Breast Cancer Res Treat 108:351–361

Levine B, Yuan J (2005) Autophagy in cell death: an innocent convict? J Clin Invest 115:2679–2688

Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402:672–676

Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, Pezzi V, Montanaro D, Musti AM, Picard D, Ando S (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. J Biol Chem 279:27008–27016

Manni A, Badger B, Martel J, Demers L (1992) Role of polyamines in the growth of hormone- responsive and—resistant human breast cancer cells in nude mice. Cancer Lett 66:1–9

McCloskey DE, Casero RA Jr, Woster PM, Davidson NE (1995) Induction of programmed cell death in human breast cancer cells by an unsymmetrically alkylated polyamine analogue. Cancer Res 55:3233–3236

Minchin RF, Knight S, Arulpragasam A, Fogel-Petrovic M (2006) Concentration-dependent effects of $N^1$, $N^{11}$-diethylnorspermine on melanoma cell proliferation. Int J Cancer 118:509–512

Musso M, Thomas T, Shirahata A, Sigal LH, Van Dyke MW, Thomas TJ (1997) Effects of chain length modification and bis(ethyl) substitution of spermine analogs on purine–purine-pyrimidine triplex DNA stabilization, aggregation, and conformational transitions. Biochemistry 36:1441–1449

Noda NN, Kumeta H, Nakatogawa H, Satoo K, Adachi W, Ishii J, Fujioka Y, Ohsumi Y, Inagaki F (2008) Structural basis of target recognition by Atg8/LC3 during selective autophagy. Genes Cells 13:1211–1218

O'Malley BW, Kumar R (2009) Nuclear receptor coregulators in cancer biology. Cancer Res 69:8217–8222

Palmer AJ, Wallace HM (2010) The polyamine transport system as a target for anticancer drug development. Amino Acids 38:415–422

Pledgie A, Huang Y, Hacker A, Zhang Z, Woster PM, Davidson NE, Casero RA Jr (2005) Spermine oxidase SMO(PAOh1), Not $N^1$-acetylpolyamine oxidase PAO, is the primary source of cytotoxic $H_2O_2$ in polyamine analogue-treated human breast cancer cell lines. J Biol Chem 280:39843–39851

Porter CW, Stanek J, Black J, Vaughan M, Ganis B, Pleshkewych A (1990) Morphological evidence for an apparent lysosomotropic activity by unsaturated putrescine analogues. Cancer Res 50:1929–1935

Porter CW, Bernacki RJ, Miller J, Bergeron RJ (1993) Antitumor activity of $N^1$, $N^{11}$-bis(ethyl)norspermine against human melanoma xenografts and possible biochemical correlates of drug action. Cancer Res 53:581–586

Sasaki H, Hayakawa J, Terai Y, Kanemura M, Tanabe-Kimura A, Kamegai H, Seino-Noda H, Ezoe S, Matsumura I, Kanakura Y, Sakata M, Tasaka K et al (2008) Difference between genomic actions of estrogen versus raloxifene in human ovarian cancer cell lines. Oncogene 27:2737–2745

Scarlatti F, Sala G, Somenzi G, Signorelli P, Sacchi N, Ghidoni R (2003) Resveratrol induces growth inhibition and apoptosis in metastatic breast cancer cells vid de novo ceramide signaling. FASEB J 17:2339–2341

Scarlatti F, Maffei R, Beau I, Codogno P, Ghidoni R (2008) Role of non-canonical Beclin 1- independent autophagy in cell death induced by resveratrol in human breast cancer cells. Cell Death Differ 15:1318–1329

Shah N, Thomas TJ, Lewis JS, Klinge CM, Shirahata A, Gelinas C, Thomas T (2001) Regulation of estrogenic and nuclear factor kappa B functions by polyamines and their role in polyamine analog-induced apoptosis of breast cancer cells. Oncogene 20:1715–1729

Shah N, Antony T, Haddad S, Amenta P, Shirahata A, Thomas TJ, Thomas T (1999) Antitumor effects of bis(ethyl)polyamine analogs on mammary tumor development in FVB/NTgN (MMTVneu) transgenic mice. Cancer Lett 146:15–23

Shappell NW, Miller JT, Bergeron RJ, Porter CW (1992) Differential effects of the spermine analog, $N^1$, $N^{12}$- bis(ethyl)-spermine, on polyamine metabolism and cell growth in human melanoma cell lines and melanocytes. Anticancer Res 12:1083–1089

Stanic I, Facchini A, Borzi RM, Stefanelli C, Flamigni F (2009) The polyamine analogue $N^1$, $N^{11}$- diethylnorspermine can induce chondrocyte apoptosis independently of its ability to alter metabolism and levels of natural polyamines. J Cell Physiol 219:109–116

Streiff RR, Bender JF (2001) Phase 1 study of $N^1$–$N^{11}$-diethylnorspermine (DENSPM) administered TID for 6 days in patients with advanced malignancies. Invest New Drugs 19:29–39

Tanida I, Ueno T, Kominami E (2004) LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol 36:2503–2518

Thomas T, Gallo MA, Thomas TJ (2004) Estrogen receptors as targets for drug development for breast cancer, osteoporosis and cardiovascular diseases. Curr Cancer Drug Targets 4:483–499

Thomas T, Thomas TJ (1994a) Estradiol control of ornithine decarboxylase mRNA, enzyme activity, and polyamine levels in MCF-7 breast cancer cells: therapeutic implications. Breast Cancer Res Treat 29:189–201

Thomas T, Thomas TJ (1994b) Regulation of cyclin B1 by estradiol and polyamines in MCF-7 breast cancer cells. Cancer Res 54:1077–1084

Thomas T, Thomas TJ (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cell Mol Life Sci 58:244–258

Thomas T, Thomas TJ (2003) Polyamine metabolism and cancer. J Cell Mol Med 7:113–126

Uimari A, Keinänen TA, Karppinen A, Woster P, Uimari P, Jänne J, Alhonen L (2009) Spermine analogue-regulated expression of spermidine/spermine $N^1$-acetyltransferase and its effects on depletion of intracellular polyamine pools in mouse fetal fibroblasts. Biochem J 422:101–109

Vijayanathan V, Venkiteswaran S, Nair SK, Verma A, Thomas TJ, Zhu BT, Thomas T (2006) Physiologic levels of 2-methoxyestradiol interfere with nongenomic signaling of 17beta-estradiol in human breast cancer cells. Clin Cancer Res 12:2038–2048

Vogel CF, Matsumura F (2009) A new cross-talk between the aryl hydrocarbon receptor and RelB, a member of the NF-kappaB family. Biochem Pharmacol 77:734–745

Wolff AC, Armstrong DK, Fetting JH, Carducci MK, Riley CD, Bender JF, Casero RA Jr, Davidson NE (2003) A Phase II study of the polyamine analog $N^1$, $N^{11}$-diethylnorspermine (DENSpm) daily for five days every 21 days in patients with previously treated metastatic breast cancer. Clin Cancer Res 9:5922–5928

Yager JD, Davidson NE (2006) Estrogen carcinogenesis in breast cancer. N Engl J Med 354:270–282

Zhong Y, Wang QJ, Li X, Yan Y, Backer JM, Chait BT, Heintz N, Yue Z (2009) Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. Nat Cell Biol 11:468–476

Zhou M, Panchuck-Voloshina NA (1997) A one-step fluorimetric method for the continuous measurement of monoamine oxidase activity. Anal Biochem 253:169–174