Molecular mechanisms underlying N^1, N^{11}-diethylnorspermine-induced apoptosis in a human breast cancer cell line

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Polyamine analogue treatment results in growth inhibition and sometimes in cell death. Therefore, polyamine analogues are considered in the treatment of cancer; however, the cellular properties that govern sensitivity are not known. The objective of this study was to elucidate molecular mechanisms behind apoptosis induced by the polyamine analogue N^1, N^{11}-diethylnorspermine (DENSPM). Four different breast cancer cell lines were treated with DENSPM. Cell death was evaluated with flow cytometry and a caspase 3 assay. The levels of a number of proapoptotic and antiapoptotic proteins in subcellular compartments were evaluated with western blot. In the most sensitive cell line, DENSPM treatment induced the release of cytochrome *c* from mitochondria, resulting in activation of caspase 3 but without decreasing the mitochondrial transmembrane potential. However, in the three other cell lines DENSPM treatment did not induce extensive cell death. This is partly explained by the high levels of antiapoptotic proteins Bcl-2 and Bad and low levels of proapoptotic proteins Bax and procaspase 3 in these three cell lines. The results are also partly explained by the degree of activation of the catabolic enzyme spermidine/spermine-N^1-acetyltransferase and polyamine pool reduction achieved by DENSPM treatment. Our results show that the protein profile of proapoptotic and antiapoptotic proteins may contribute to the outcome to treatment with the polyamine analogue DENSPM. The results also indicate that it should be possible to find molecular markers for sensitivity to DENSPM that could be used in the clinic to predict sensitivity to a polyamine analogue. *Anti-Cancer Drugs* 19:871–883 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Breast cancer is the most common form of female cancer in the Western world. The estimated lifetime risk of obtaining breast cancer is around 10% and about one-third of the afflicted persons will succumb to the metastatic form of the disease. Breast cancer arises from epithelial cells lining the ducts and lobules of the normal breast [1]. For various reasons the epithelial cells have accumulated genetic lesions that drive them into a self-sustaining life with a lost concern for tissue homoeostasis. As the risk of metastasis is very high in breast cancer, affected women usually receive some form of systemic therapy depending on various tumour properties [2]. Despite intensive treatment, it is not always successful and many women die, thus it is obvious that new chemotherapeutic treatment strategies are needed. One group of compounds that belong to a new potential treatment strategy are those that interfere with the cellular polyamine homoeostasis [3–7].

Polyamines are organic compounds that have a wide variety of cellular functions in cell proliferation, cell differentiation, and cell death [8,9]. Cells have an elaborate system for controlling their polyamine pools through biosynthesis, catabolism, and uptake/excretion over the cell membrane. Nonproliferating cells have low polyamine levels and an early event in cell proliferation is an increase in the activity of the biosynthetic enzymes. If this increase is inhibited, or if proliferating cells are depleted of their polyamines, cells cease proliferating and they may even die [8–12]. Obviously, targeting the polyamine pool is a means of combating cancer [3–7].

Cellular polyamine depletion may be achieved by different strategies. One strategy is the use of inhibitors of biosynthetic enzymes [4,5] and another strategy is targeting the polyamine transport system [9]. A very efficient means of depleting cells of their polyamine pool is by treating with different polyamine analogues [4,6]. Polyamine analogues are efficiently taken up by the cellular polyamine transport system and they are accumulated to a high concentration in the cell. The cells recognize them as polyamines by the polyamine homoeostasis system. As polyamine concentrations that are too

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high are also detrimental [7], the cell shuts off biosynthesis and activates catabolism. Only the natural polyamines putrescine, spermidine, and spermine are affected, however [13–17]. Consequently, the pools of natural polyamines are depleted and the cell will instead contain a high level of the analogue. The analogue cannot take over the function of the natural polyamines and cell proliferation ceases. Cell death may also be induced, however, it is not clear under which circumstances cell death occurs.

One polyamine analogue that has been evaluated in the treatment of breast cancer is N^1, N^{11}-diethylnorspermine (DENSPM) [18]. Recently, completed phase II clinical trials with DENSPM in breast cancer have demonstrated that it is a well-tolerated drug [19]. The objective responses observed were minimal, although, it has to be pointed out that the study was small with few patients. It has been proposed that the ability to induce the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) is related to a cytotoxic response [19,20]. DENSPM treatment also reduces polyamine pools by inhibiting the polyamine biosynthetic enzymes ornithine decarboxylase and *S*-adenosylmethionine decarboxylase [6–9]. There may, however, be a number of different tumour factors that regulate responsiveness to DENSPM [21] and may contribute to a favourable treatment combination of DENSPM with other chemotherapeutic drugs [22]. Besides DENSPM, a number of polyamine analogues are presently considered in clinical trials [23].

In this study, we have treated four different human breast cancer cell lines with DENSPM and investigated the effect on apoptosis and apoptosis-related proteins. The four cell lines respond differently to DENSPM-induced polyamine depletion with one of them being highly sensitive and succumbing to apoptotic cell death. The purpose was to extend our knowledge and understanding of the molecular and cellular mechanisms that govern sensitivity to treatment with a polyamine analogue.

Materials and methods

Materials and reagents

Cell culture medium components were purchased from Biochrom, Berlin, Germany. Tissue culture plastics were purchased from Nunc (Roskilde, Denmark). Antibodies against AIF-1 (551429), Bad (610391), Bax (554104), Bcl-2 (556354), Bcl-X_L (551020), cytochrome *c* (556433) and Smac/DIABLO (612245) were purchased from BD Bioscience (San Diego, California, USA). The antibody against caspase 3 (AHZ0052) was purchased from Biosource (Camarillo, California, USA), whereas the antibody against survivin (sc-17779) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). The antibody against glyceraldehyde-3-phosphate dehydrogenase was purchased from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated secondary antibodies used for all proteins for western blot analysis were purchased from DAKO Cytomation Denmark A/S (Glostrup, Denmark). Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane, Advanced ECL detection reagent, Cy3-dCTP and Cy5-dCTP, GFX purification column and the CyScribe GFX Purification kit were purchased from Amersham Biosciences Ltd (Buckinghamshire, UK). Precast NuPage Novex 4–12% Bis–Tris SDS polyacrylamide gels (thickness of 1.0 mm, 12 wells) were purchased from Invitrogen Life Technologies (Stockholm, Sweden). Phosphate-buffered saline (PBS: 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, pH 7.3) was purchased from Oxoid Ltd (Basingstoke, Hampshire, UK). DENSPM was purchased from Tocris Cookson Ltd (Bristol, UK). The water-soluble substance DENSPM was dissolved in PBS to give a stock solution of 2 mmol/l. Stock solution was sterile-filtered and stored at −20°C. The chemical 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbo-amine iodine (JC-1) was purchased from Molecular Probes (Leiden, The Netherlands). A 1 mg/ml stock solution of JC-1 was prepared in dimethyl sulfoxide and stored at −20°C. [^{14}C]Acetyl coenzyme A and ribonuclease A type II (RNase A) were purchased from Sigma Chemicals Co. (St Louis, Missouri, USA). CPP32/caspase 3 colorimetric protease assay kit was purchased from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan). Propidium iodide (PI) was purchased from ICN Biomedicals Inc. (Irvine, California, USA). Protease inhibitor cocktail set III was purchased from Calbiochem (Gibbstown, New Jersey, USA). Trizol, COT-1 DNA and BioPrime Array CGH Genomic Labeling System were purchased from Invitrogen Life Technologies Inc. (Carlsbad, California, USA) and the RNeasy Midi purification kit was purchased from Qiagen (Valencia, California, USA). Pronto! *Plus* Direct System, Pronto! Universal Hybridization Solution and Promega Wizard Genomic DNA Purification kit were purchased from Promega Corporation (Madison, Wisconsin, USA). The MCF-7 (HTB-22), HCC1937 [CRL-2336, and SK-BR-3 (HTB-30)] cell lines were purchased from American Type Culture Collection (Manassas, Virginia, USA). The L56Br-C1 cell line was established in Lund [24].

Cell culture

All cell lines were cultured at 37°C in a humidified incubator with 5% CO_2 in air. The human epithelial breast adenocarcinoma cell line MCF-7 was cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), nonessential amino acids at standard concentrations, insulin (10 μg/ml), penicillin (50 U/ml) and streptomycin (50 μg/ml). The human breast adenocarcinoma cell line SK-BR-3 was cultured in the same medium as MCF-7 cells, but without the addition of insulin. The human ductal carcinoma breast cancer cell

line HCC1937 [25] was cultured in MEM α-medium supplemented with 10% heat-inactivated FCS, nonessential amino acids at standard concentrations, epidermal growth factor (20 ng/ml), insulin (10 μg/ml), penicillin (50 U/ml) and streptomycin (50 μg/ml). The human breast cancer cell line L56Br-C1 [11,24,26] was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, nonessential amino acids at standard concentrations, insulin (10 μg/ml), penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were seeded in the absence or presence of 10 μmol/l DENSPM. Both detached and attached cells were harvested after 24 and 48 h of treatment. In addition, samples of L56Br-C1 cells were harvested at 12 and 36 h of treatment. After counting the cells in a haemocytometer, they were pelleted at 900*g* for 10 min at 4°C and handled for analysis as described below. Only opalescent cells were counted. These cells excluded the vital dye trypan blue.

Flow cytometry and data analysis

Cells were resuspended in ice-cold 70% ethanol and stored at −20°C until analysis. Nuclear DNA was stained with PI-nuclear isolation medium [PBS containing 100 μg/ml PI, 0.60% Nonidet P-40 (NP-40) and 100 μg/ml RNase A] [27]. Flow cytometric (FCM) analysis was performed using a Cytofluorograph System 50-H (Ortho Instruments, Westwood, Massachusetts, USA) as previously described by Fredlund *et al.* [28]. For the computerized analysis of the sub-G_1 region, Multi2D and MultiCycle software programs (Phoenix Flow Systems, California, USA) were used.

Polyamine analysis

Cells were stored at −20°C until analysis. Chromatographic separation and quantitative determination of polyamines in cell extracts in 0.2 mol/l perchloric acid were carried out using high-pressure liquid chromatography (Hewlett Packard 1100; Global Medical Instrumentations Inc., Ramsey, Minnesota, USA) with *O*-phthaldialdehyde as the reagent [29].

Spermidine/spermine N^1-acetyltransferase activity assay

Cells were stored at −80°C until analysis. Cells were sonicated in 50 mmol/l Tris–HCl (pH 7.5) containing 0.25 mol/l sucrose. The activity of SSAT in the sonicate was determined by measuring the amount of [^{14}C]acetylspermidine produced after incubation with [^{14}C]acetyl coenzyme A and spermidine [30].

Caspase 3 activity assay

Cells were resuspended in 50 μl Cell Lysis Buffer (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) and stored at −80°C until analysis. The caspase activity was assessed by measuring the cleavage of the chromophore *p*-nitroanilide (*p*-NA) from a DEVD-*p*NA-labeled substrate according to the instructions for the CPP32/caspase 3 colorimetric protease assay kit. Samples were incubated with 200 μmol/l *p*NA-labeled substrate at 37°C for 2 h before measurement of the emission at 405 nm using a spectrophotometer (SpectraMax M2 microplate reader, Sunnyvale, California, USA).

Mitochondrial transmembrane potential ($\Delta\Psi_m$)

According to manufacturer's instructions, cells were incubated with 10 μg/ml of the fluorescent cationic dye JC-1 in ordinary cell culture medium for 10 min at 37°C in a humidified incubator with 5% CO_2 in air. To interrupt the incubation, 10 ml of ice-cold cell culture medium was added and cells were pelleted at 900*g* for 10 min at 4°C. Pelleted cells were resuspended in 1.5 ml of nonserum containing cell culture medium, and were kept on ice until analysis. After addition of JC-1, the cells were protected from light at all times. FCM analysis was carried out using a Cytofluorograph System 50-H using a laser line at 488 nm for excitation. Green fluorescence was quantified in a wavelength interval of 515–545 nm, defined by a band-pass filter. Red fluorescence was quantified beyond 600 nm limited by a low-pass filter. For the further analysis of the green and red fluorescence signals, Multi2D and MultiCycle software programs were used.

In parallel with the FCM analysis, JC-1 staining was also analysed in the fluorescence microscope. For this analysis, cells were seeded in chamber slides in the absence or presence of 10 μmol/l DENSPM. After 24 h of treatment, cells were incubated with 10 μg/ml of JC-1 for 10 min at 37°C in a humidified incubator with 5% CO_2 in air. In DENSPM-treated cultures, the medium containing floating cells was collected and the cells pelleted. After resuspension in PBS containing 10% FCS, the cell suspension was added to a slide. The adherent cells were rinsed twice with PBS containing 10% FCS. Adherent and floating cells were examined by using a fluorescence microscope (Olympus AX70-U-MPH; Olympus Optical Co. Ltd., Tokyo, Japan) and photographs were taken with a digital camera (Olympus DP-50-CU; Olympus Optical Co. Ltd.). The software Adobe Photoshop 7.0 was used for analysis of the photographs.

Subcellular fractionation

Cytosolic fractions and particulate fractions containing mitochondria were isolated according to a protocol described by Ahn *et al.* [31]. The pelleted cells were washed twice with ice-cold PBS. Cells were then pelleted and resuspended in ice-cold hypotonic extraction buffer [50 mmol/l peperazine-1,4-*bis* (2-ethanesulfonic acid), pH 7.4, 50 mmol/l KCl, 5 mmol/l ethylene glycol-*bis* (b-aminoethyl ether), 2 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol and protease inhibitors (1 : 1000)] containing 250 mmol/l sucrose. After 30 min incubation on ice, cell lysates were resuspended by vigorous swirling. Cell

lysates were then centrifuged at 16 000*g* for 30 min at 4°C, and the cytosolic fractions were removed. The remaining pellet was resuspended in ice-cold hypotonic extraction buffer, containing 0.1% NP-40, and the samples were incubated on ice for 20 min. Then, samples were centrifuged at 16 000*g* for 10 min at 4°C and the supernatants were removed. This fraction was denoted the mitochondrial fraction. The different fractions were stored at −80°C until further analysis.

Western blot analysis

Cytosolic and mitochondrial fractions were diluted in sample buffer (62.5 mmol/l Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol), sonicated and heat-denatured at 95°C for 5 min and then put on ice immediately. Aliquots of cytosolic and mitochondrial fractions equivalent to 10^5 cells were separated by SDS polyacrylamide gel electrophoresis (gradient of 4–12%). The separated proteins were transferred electrophoretically to ECL nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk and 0.05% Tween 20 in PBS for 2.5 h, and then washed 5 × 10 min with 0.05% Tween 20 in PBS, followed by incubation overnight with primary antibody at 4°C. Horseradish peroxidase-conjugated secondary antibodies were used for all proteins and advanced ECL protein detection reagent was used according to the manufacturer's protocol. The ChemiDoc XRS system (Bio-Rad Inc., Hercules, California, USA) and the software Quantity One (Bio-Rad Inc.) were used for imaging.

Expression and DNA copy number analysis

Oligonucleotide, cDNA and tiling BAC arrays were produced at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University, Sweden [32–34].

RNA was extracted from the cell lines (24 h after seeding) using Trizol followed by the RNeasy Midi purification kit. The quality of the RNA from each sample was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, California, USA).

For expression analysis of the cell lines, oligonucleotide arrays were used. Labelling, hybridization and washing was performed using the Pronto! *Plus* Direct System according to the manufacturer's instructions. The hybridization was carried out in a MAUI Hybridization Station (BioMicro Systems, Salt Lake City, Utah, USA). Universal Human Reference RNA (Stratagene, La Jolla, California, USA) was used as reference.

cDNA microarrays were used for the expression analysis of DENSPM-treated cells. Untreated control cells were used as reference for the respective cell line. For each hybridization, 25 μg of total RNA from sample and reference were labelled with Cy3 and Cy5, respectively, using anchored oligo(dT) primers and the Cyscribe indirect amino-allyl cDNA synthesis and labeling protocol and GFX purification columns. Hybridization and washing was performed as described previously [32].

Genomic DNA was extracted using the Promega Wizard Genomic DNA Purification kit. Normal male genomic DNA was used as reference (Promega Corporation). Labelling, hybridization and washing was performed as described in the study by Jönsson *et al.* [33].

Image and data analysis

Fluorescence signals were recorded using an Agilent G2565AA microarray scanner (Agilent Technologies). TIFF images were analysed using the GenePix Pro 4.1 software (Axon Instruments Inc., Foster City, California, USA). The quantified data matrix was uploaded to the BioArray Software Environment [35], where quality and data analysis were performed. Background correction was performed by subtracting median background from median foreground signal intensity values, for each channel. For cDNA array data, saturated spots and spots flagged as bad or missing were removed and features with a signal-to-noise ratio less than 2 in either channel were filtered. Data within individual arrays were then normalized using the block-based lowess algorithm [36]. Genes represented by multiple cDNA clones were merged and the average expression value was used. Oligonucleotide and tiling BAC arrays were filtered and normalized as described in the study by Jönsson *et al.* [33]. Cut-off ratios for gains and losses were set to 1.23 and 0.81, respectively, corresponding to log(2)ratio of ± 0.3. Oligonucleotide probes and cDNA clones were mapped according to the UniGene database, build 196, accession number GSE6746 [37] BAC clones are ordered by position in the genome according to the University of California Santa Cruz Human Genome Assembly, Hg18 [38].

Statistical analysis

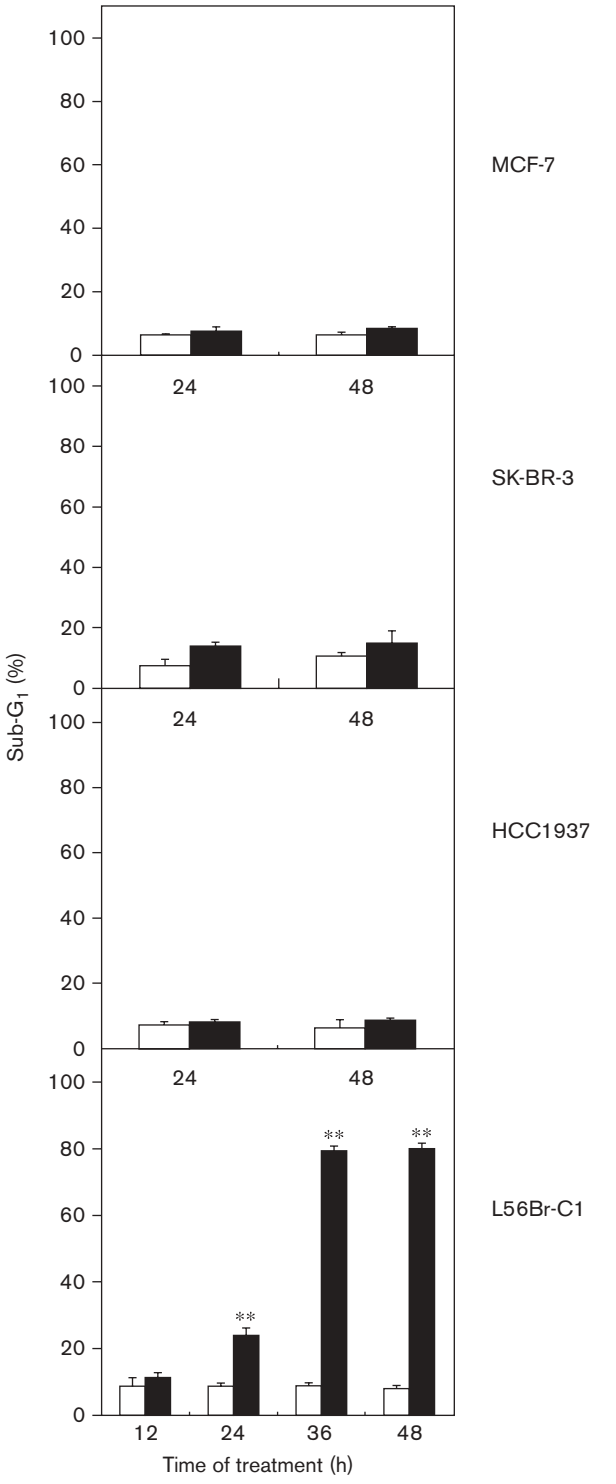
For the statistical evaluation, a two-tailed Student's *t*-test was used.

Results

Percentage of cells in the sub-G_1 region

We started this study by investigating cell death induced by DENSPM treatment in the four cell lines. Cell death was studied by investigating the appearance of cells in the sub-G_1 region of a DNA histogram obtained by FCM of PI-stained cells. The percentage of cells in the sub-G_1 region increased substantially in the L56Br-C1 cell line by DENSPM treatment (Fig. 1). After 36 and 48 h of 10 μmol/l DENSPM treatment, 80% of the cells were found in the sub-G_1 region. An increased sub-G_1 region was, however, seen already after 24 h of treatment.

Fig. 1



Percentage of cells in the sub-G_1 region as a measure of cell death. MCF-7, SK-BR-3, HCC1937 and L56Br-C1 cells were seeded in the absence or presence of 10 μmol/l N^1, N^{11}-diethylnorspermine (DENSPM). At the indicated time points, both detached and attached cells were harvested and pooled. Flow cytometry was used to determine the percentage of cells in the sub-G_1 region in the DNA histogram after staining with propidium iodide. The results are presented as mean values ($n = 6$ independent cultures from two independent experiments) and bars represent ± SD. White bars, control; black bars, 10 μmol/l DENSPM. $^{**}P < 0.01$ compared with control.

No substantial increase in cell death by DENSPM treatment was seen in the other cell lines.

Effect of N^1, N^{11}-diethylnorspermine treatment on polyamine levels

As DENSPM treatment clearly induced cell death in L56Br-C1 cells but not in the other cell lines, we decided to go forward and compare the cell lines regarding various aspects one being polyamine levels. The four cell lines had different basal polyamine levels with MCF-7 and HCC1937 cells containing higher levels than the other two cell lines (Fig. 2). DENSPM treatment resulted in a significant decrease in all polyamine pools in the four cell lines (Fig. 2). The degree of decrease in the polyamine levels in relation to the control levels varied in the cell lines with the lowest relative effect of DENSPM in SK-BR-3 cells. The polyamine levels were not totally depleted in MCF-7, SK-BR-3 and HCC1937 cells after 48 h of treatment. Putrescine and spermidine were, however, lowered to undetectable levels after 24 h of DENSPM treatment in L56Br-C1 cells.

Spermidine/spermine N^1-acetyltransferase activity

As polyamines were depleted in all cell lines, we wished to examine the role of SSAT in this effect. Activation of SSAT did not contribute to the decreased polyamine pools in DENSPM-treated MCF-7 cells, whereas an increase in SSAT activity was found in the other three cell lines treated with DENSPM (Fig. 3). The activity was similar after 24 and 48 h of treatment in SK-BR-3, HCC1937 and L56Br-C1 cells. The degree of DENSPM-induced activation of SSAT, however, varied between these cell lines, where the highest activation was found in L56Br-C1 cells and the lowest in SK-BR-3 cells. In addition, there was a constant low SSAT activity in L56Br-C1 control cells. In L56Br-C1 cells, the SSAT activity was also determined after 12 h of DENSPM treatment. The activity was 1.4 ± 1.1 and 298.8 ± 43.1 ($n = 4$) for control and DENSPM-treated cells, respectively.

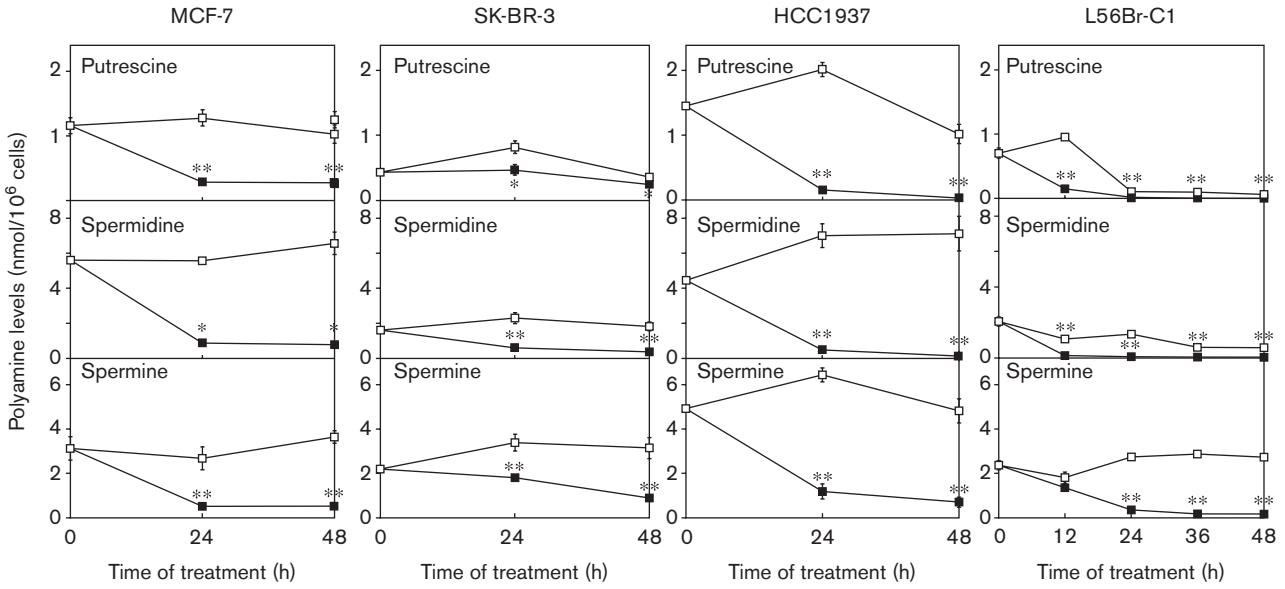
Caspase 3 activity

The appearance of a sub-G_1 peak in the DNA histogram does not indicate the nature of cell death, that is, if it is apoptotic or necrotic. To this end we decided to study caspase 3 activity, which is a marker of apoptosis. No caspase 3 activities were found in MCF-7, SK-BR-3 and HCC1937 cells (not shown). In L56Br-C1 control cells, there was a constant low caspase 3 activity (Fig. 4). A significant stimulation of caspase 3 activity was found after 12 h of DENSPM treatment and the activity increased further at 48 h of treatment.

Mitochondrial transmembrane potential ($\Delta\Psi_m$)

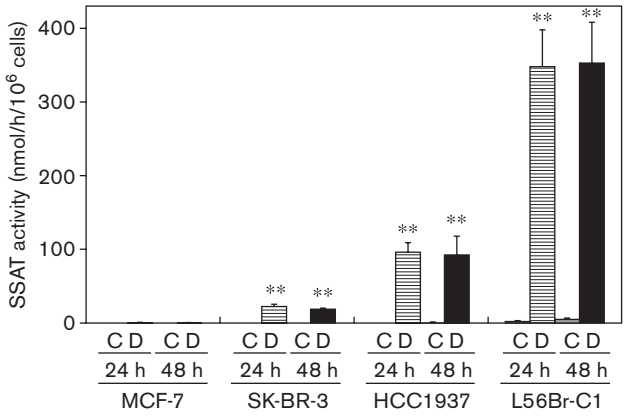
Apoptosis can be induced with or without a change in the $\Delta\Psi_m$. Thus, JC-1 staining was used to investigate the effect of DENSPM treatment on the $\Delta\Psi_m$ in L56Br-C1 cells. The uptake of JC-1 into the mitochondria is driven

Fig. 2



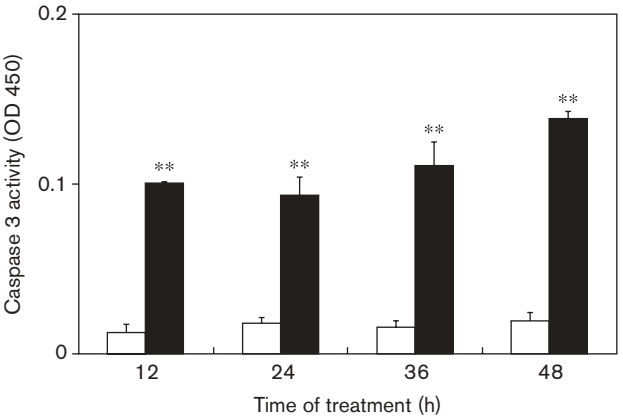
N^1, N^{11}-diethylnorspermine (DENSPM) treatment reduced the polyamine content of MCF-7, SK-BR-3, HCC1937 and L56Br-C1 cells. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. The polyamine content was determined by high-pressure liquid chromatography. The results are presented as mean values (n=3–6 independent cultures from two independent experiments) and bars represent ± SD. When not visible, bars are covered by the symbol. □, control; ■, 10 μmol/l DENSPM. **P<0.01 compared with control; *P<0.05 compared with control.

Fig. 3



Differential activation of spermidine/spermine N^1-acetyltransferase (SSAT) by N^1, N^{11}-diethylnorspermine (DENSPM) treatment in MCF-7, SK-BR-3, HCC1937 and L56Br-C1 cells. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. The SSAT activity was determined by a radiometric assay. The results are presented as mean values (n=6 independent cultures from two independent experiments) and bars represent ± SD. C, control; D, 10 μmol/l DENSPM. **P<0.01 compared with control.

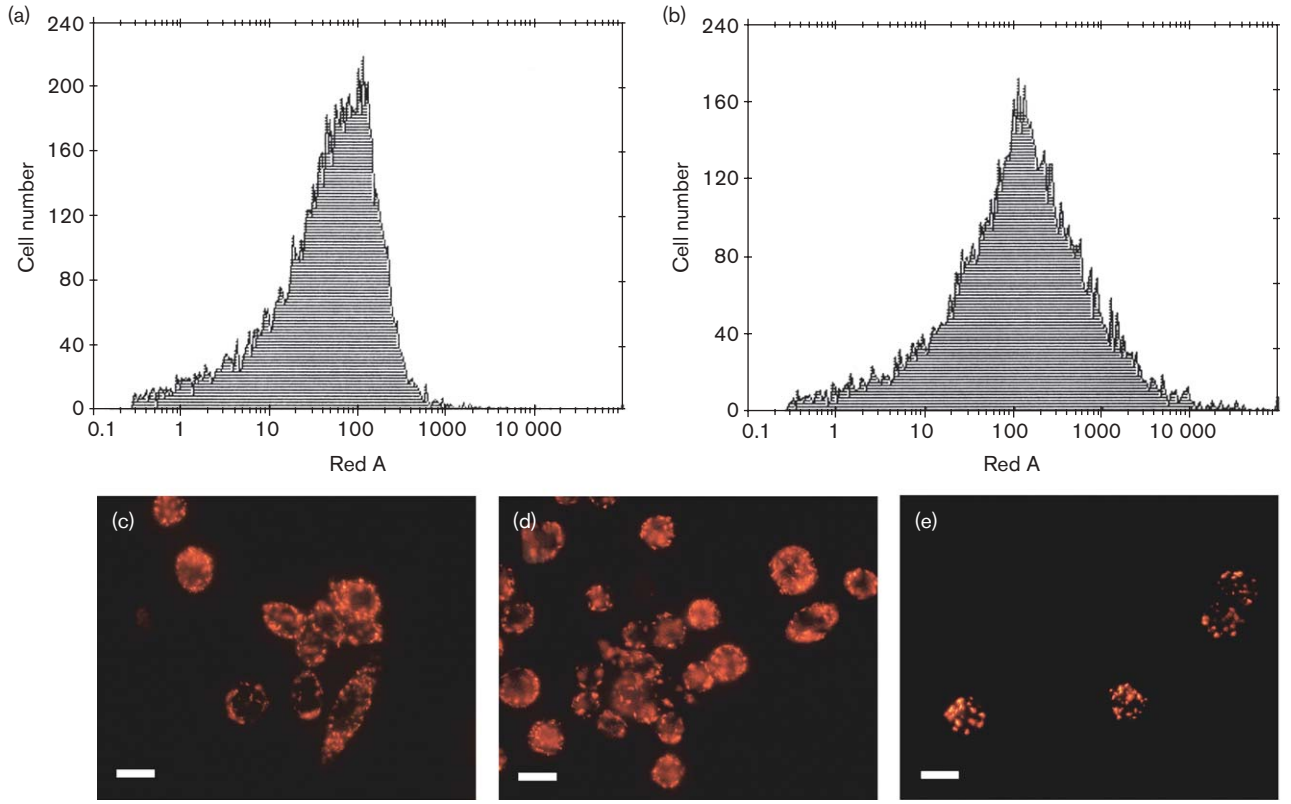
Fig. 4



N^1, N^{11}-diethylnorspermine (DENSPM) treatment induced caspase 3 activity in L56Br-C1 cells. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. The caspase activity per 10^6 cells was assessed using a CPP32/caspase 3 colorimetric protease assay. The results are presented as mean values (n=6 independent cultures from two independent experiments) and bars represent ± SD. White bars, control; black bars, 10 μmol/l DENSPM. **P<0.01 compared with control.

by the $\Delta\Psi_m$ [39]. In the mitochondria, JC-1 molecules aggregate and because of energy transfer red light is emitted. Red signals were observed by FCM and fluorescence microscopy (Fig. 5). Figure 5a and b shows the red fluorescence histograms of control and DENSPM-treated cells, respectively. In DENSPM-treated cells, the histogram was broader than that of control cells owing to the occurrence of cells with higher red fluorescence. These data indicate that DENSPM treatment for 24 h did not disrupt the $\Delta\Psi_m$. If there was a disruption in $\Delta\Psi_m$, the

Fig. 5



N^1, N^{11}-diethylnorspermine (DENSPM) treatment increased the mitochondrial transmembrane potential in L56Br-C1 cells. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. Both flow cytometry and fluorescence microscopy were used in parallel to monitor JC-1-derived red fluorescence. (a) Histogram of red fluorescence in control cells; (b) histogram of red fluorescence in DENSPM-treated cells; (c) fluorescence microscopic analysis of JC-1-stained control cells; (d) DENSPM-treated cells; (e) DENSPM-treated floating cells. Scale bar = 20 μm.

entire histogram should have moved to the left. Instead, the data indicated a higher uptake of JC-1 into mitochondria in DENSPM-treated cells. Figure 5c shows a fluorescence microscopy image of the red JC-1-derived fluorescence in control cells. Figure 5d and e shows the fluorescence microscopy images of the red JC-1-derived fluorescence in adherent and floating cells, respectively, in cultures treated with DENSPM for 24 h. Cells floating in the medium appear in the sub-G_1 region. The red fluorescence images show brightly red-stained mitochondria in control cells and in adherent, but also in floating cells in DENSPM-treated cultures.

Detection of antiapoptotic and proapoptotic proteins using western blot analysis

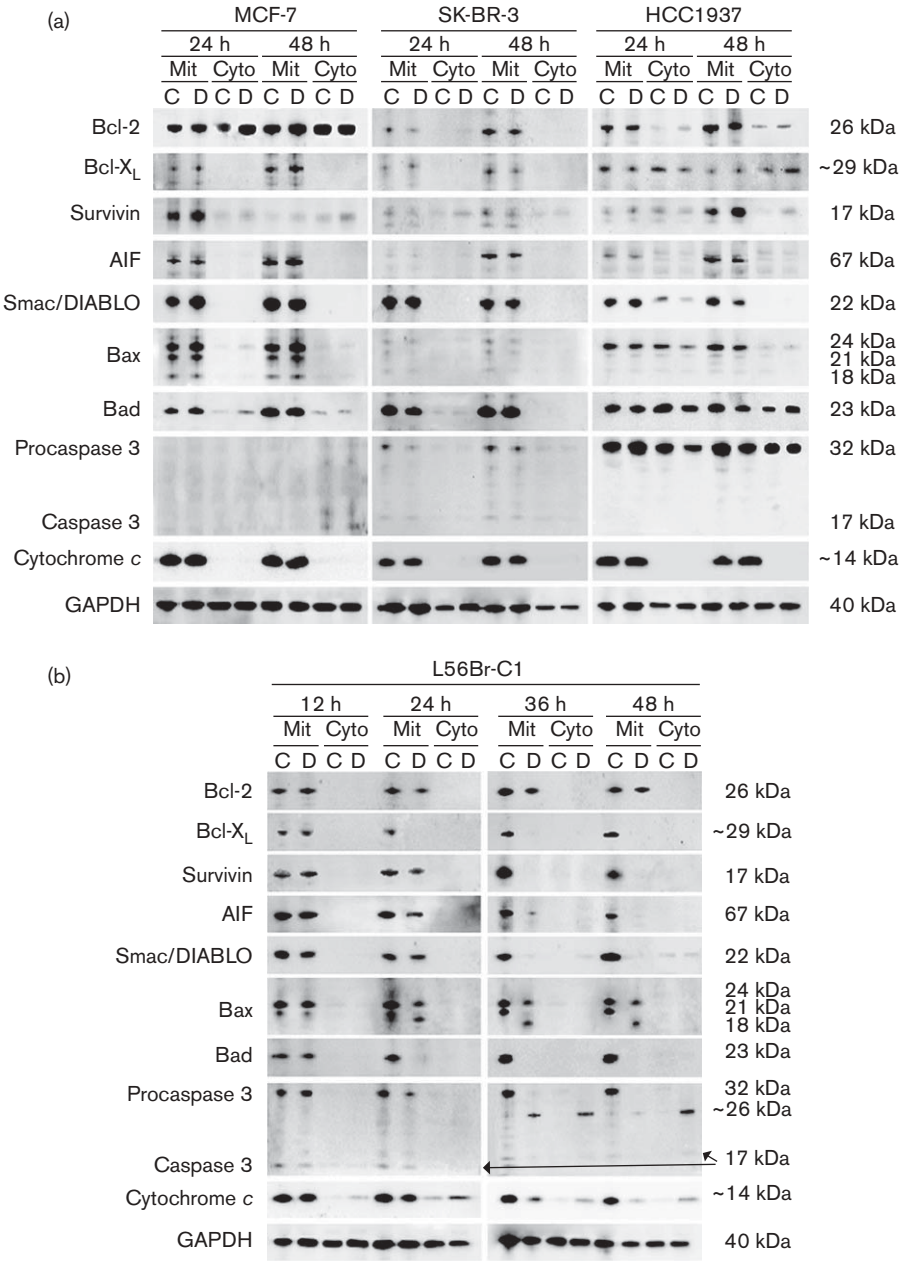
Apoptosis is an extremely complex process involving a number of antiapoptotic and proapoptotic proteins, some of which have been studied more than others. Although apoptosis was not induced in all cell lines, we decided to study the levels of different antiapoptotic and proapoptotic proteins in all cell lines.

The effects of DENSPM treatment on the levels of some antiapoptotic and proapoptotic proteins were investigated by western blot (Fig. 6a and b). When looking at both fractions, the basal levels of the different proteins varied in the four cell lines.

MCF-7 cells had the highest basic level of Bcl-2. A high level of Bcl-2 was found in the cytosol of MCF-7 cells. Some Bcl-2 was also found in the cytosol of HCC1937 cells. Except for the increase in Bcl-2 in the cytosol of DENSPM-treated MCF-7 cells after 24 h, there was no major effect of DENSPM treatment on the Bcl-2 level in any of the cell lines (Fig. 6a and b).

Bcl-X_L was found in the mitochondrial fraction in untreated cells in all cell lines and again at different basal levels (Fig. 6a and b). SK-BR-3 cells had the lowest level. In HCC1937 cells, Bcl-X_L was also found in the cytosol. In L56Br-C1 cells, Bcl-X_L disappeared in the mitochondrial fraction after 24 h of DENSPM treatment, never to appear again.

Fig. 6



Effect of N^1, N^{11}-diethylnorspermine (DENSPM) treatment on proapoptotic and antiapoptotic proteins in MCF-7 (a), SK-BR-3 (a), HCC1937 (a) and L56Br-C1 cells (b). Cells were seeded in the absence or presence of 10 μmol/l DENSPM. Western blot analysis was used to detect the different proteins. Loading was based on cell number: 10^5 cells per lane. In addition, the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. The data are representative of three independent cultures from three independent experiments. C, control; D, 10 mmol/l DENSPM; Cyto, cytoplasmic fraction; Mit, mitochondrial fraction.

Survivin was found in both mitochondrial and cytosolic fractions in MCF-7, SK-BR-3 and HCC1937 cells, but only in the mitochondrial fraction in L56Br-C1 cells (Fig. 6a and b). In L56Br-C1 cells, survivin disappeared from the mitochondrial fraction after 36 h of DENSPM treatment. DENSPM treatment did not affect the survivin level in the other cell lines.

AIF was found in the mitochondrial fraction in all cell lines (Fig. 6a and b). In L56Br-C1 cells, the mitochondrial AIF levels was lowered at 24 h of treatment compared with control, and continued to decrease at 36 h to be absent at 48 h of DENSPM treatment. The AIF level was not affected by DENSPM treatment in the other three cell lines.

Smac/DIABLO was found in the mitochondrial fraction in all cell lines (Fig. 6a and b). Smac/DIABLO was not affected by DENSPM treatment except in L56Br-C1 cells were the protein was absent after 36 h of treatment.

The Bax levels were very low in SK-BR-3 cells (Fig. 6a). In HCC1937 cells, a 24 kDa Bax band was found in both cytosolic and mitochondrial fractions. In MCF-7 cells, three Bax bands of sizes 18, 21 and 24 kDa were found in the mitochondrial fraction. DENSPM treatment did not affect Bax in any of these three cell lines. However, in L56Br-C1 cells, two bands of Bax (21 and 24 kDa) were found in control cells. After 24 h of DENSPM treatment, the 24-kDa band was decreased and the 21-kDa band disappeared whereas a new protein band appeared at 18 kDa (Fig. 6b). This response to DENSPM treatment could also been seen after 36 and 48 h.

In all cell lines, Bad was mainly associated with the mitochondrial fraction, with the exception of HCC1937 cells. In these cells, Bad was also found in the cytosol (Fig. 6a and b). A significant change in Bad was seen in DENSPM-treated L56Br-C1 cells, where Bad disappeared from the mitochondrial fraction (Fig. 6b).

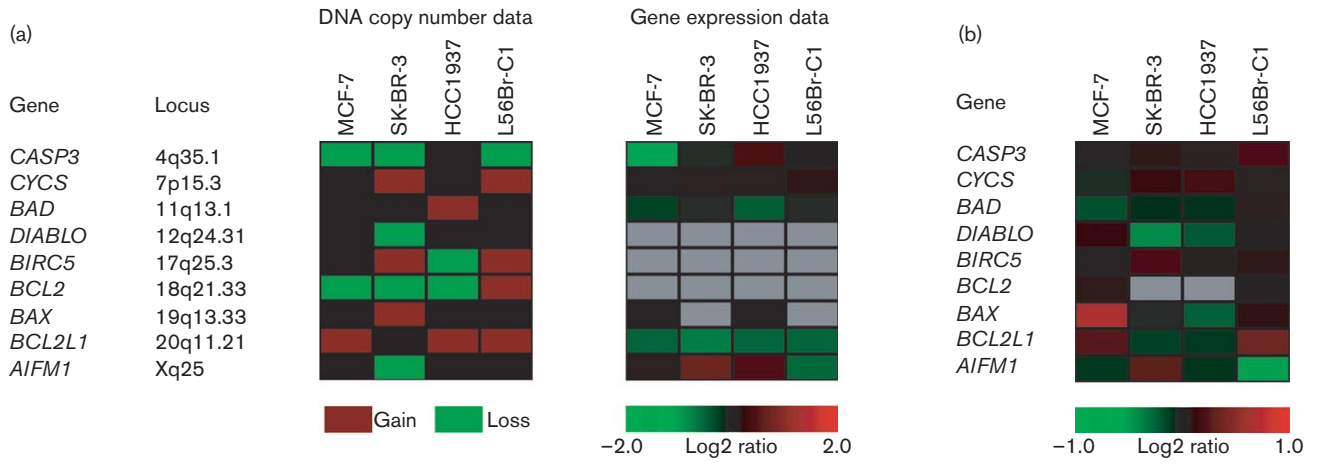
The level of procaspase 3 varied significantly between the four different cell lines (Fig. 6a and b). A high level of procaspase 3 was found in HCC1937 cells in both mitochondrial and cytosolic fractions. Procaspase 3 was absent in MCF-7 cells as expected [40] and SK-BR-3 cells had a low procaspase 3 level. Procaspase 3 was found in L56Br-C1 cells where a faint 17-kDa band representing active caspase 3 was also found. DENSPM treatment only affected procaspase 3 in L56Br-C1 cells (Fig. 6a and b). After 24 h of DENSPM treatment, the level of procaspase 3 decreased in the mitochondrial fraction in L56Br-C1 cells. However, there was no increase in caspase 3. In L56Br-C1 cells, treated with DENSPM for 36 and 48 h, a new band of approximately 26 kDa appeared in the mitochondrial fraction. This band was also found in the cytosolic fraction.

Cytochrome *c* was found in the mitochondrial fractions (Fig. 6a and b). In L56Br-C1 cells, there was also a low level of cytochrome *c* in the cytosol. The cytosolic levels of cytochrome *c* increased while the mitochondrial levels decreased when the cells were treated with DENSPM. A tendency to an increased cytosolic cytochrome *c* content was evident already after 12 h of treatment. At 24 h of DENSPM treatment, the cytosolic fraction of cytochrome *c* had clearly increased compared with control in L56Br-C1 cells. DENSPM treatment had no effect on cytochrome *c* in MCF-7, SK-BR-3,and HCC1937 cells (Fig. 6a).

DNA copy number and gene expression

Figure 7a shows the DNA copy number analysis of the genes investigated. Chromosome plots were used to interpret the data (not shown). A number of copy number alterations were seen, both gains and losses. Homozygote deletions or high level amplifications were, however, not found for the studied genes. When looking at gene expression data, there were clear differences between the cell lines (Fig. 7a). None of the genes had the same basic

Fig. 7



(a) DNA copy number and gene expression data for the selected genes in the four different breast cancer cell lines. (b) Gene expression 24 h after N^1, N^{11}-diethylnorspermine (DENSPM) treatment compared with respective untreated cells. Results are the mean from two or three experiments. The colour scales indicate $\log_2$ ratios where green represents losses or lower expression and red represents gains/amplifications or increased expression. Grey areas denote undetectable values. In the figure, the name of the gene is always written in capital letters while the name of the protein may be written with mixed letters, that is, *CASP3* alias procaspase 3; *CYCS* alias cytochrome c; *BAD* alias Bad; *DIABLO* alias Smac/DIABLO; *BIRC5* alias survivin; *BCL2* alias Bcl-2; *BAX* alias Bax; *BCL2L1* alias Bcl-X_L; *AIFM1* alias AIF, mitochondrion-associated 1.

expression in all cell lines. DENSPM treatment did not have a large effect on the expression of the studied genes in the different cell lines (Fig. 7b). Bax mRNA expression increased in MCF-7 cells, and AIF mRNA expression decreased in L56Br-C1 cells with DENSPM treatment, however, comparative changes in the protein levels were not seen. DENSPM treatment increased the SSAT activity most extensively in L56Br-C1 cells, whereas the SSAT mRNA levels increased to similar levels in all cell lines (not shown).

Discussion

There is a great need for new chemotherapeutic drugs in the treatment of breast cancer. As intracellular polyamines have critical roles in cell proliferation and death pathways, the polyamine metabolic pathway represents a potential target for cancer intervention. The polyamine metabolic pathway can be manipulated in several different ways, involving inhibition of biosynthesis and uptake as well as stimulation of catabolism. As for chemotherapeutic treatment in general, treatment with drugs that affect polyamine metabolism has different effects in different cell lines. Although cell proliferation is always inhibited in cells where polyamine depletion is taking place, the rate with which this takes place differs between different cell lines. We have performed an extensive study of the effect of DENSPM treatment on cell cycle kinetics in the four breast cancer cell lines used in this study [41]. The most interesting aspect of polyamine depletion from a cancer treatment point of view is the induction of apoptosis. Polyamine analogues have been shown to induce apoptosis; however, it is not a consistent phenomenon as growth inhibition is. In this study, we have compared the molecular mechanism regarding mitochondrial apoptosis-related proteins in the four human breast cancer cell lines MCF-7, SK-BR-3, HCC1937 and L56Br-C1. The reason for choosing the analysed proteins was the observation that the cell line L56Br-C1 was extremely sensitive to DENSPM treatment and the fact that the mitochondrial death pathway was induced [10]. DENSPM-induced cell death was shown to be caspase dependent as it was blocked by Z.VAD.FMK, a general caspase inhibitor. In addition, using different methods to study cytotoxicity, we found that DENSPM treatment, and also treatment with other polyamine analogues, affected the mitochondria in all cell lines presumably by increasing the rate of respiration [11,26].

As has been seen in a number of studies, the activation of SSAT in DENSPM-treated cells varied in the different cell lines with the highest activation in L56Br-C1. Others have pointed out that the cytotoxicity of DENSPM is related to the degree of activation of SSAT [21], and our data partially support that notion. It is believed that the cytotoxicity is related to the production of H_2O_2 and 3-acetamidopropanal in the polyamine oxidase (PAO)-catalysed oxidation of the products of the SSAT reaction [42]. In HCC1937, the increase in SSAT activity after DENSPM treatment was almost 1/3 of that in L56Br-C1 cells. However, there was no increase in cell death in the former cell line. The oxidation of the acetylated SSAT products takes place in the peroxisomes [43]. The peroxisomes contain the enzyme catalase, which catalyses the degradation of H_2O_2. Thus, HCC1937 cells may have enough catalase in their peroxisomes to neutralize the H_2O_2 produced. The activity of PAO can be inhibited by treatment with the PAO inhibitor MDL72527 as a means to investigate the role of H_2O_2 in the cytotoxic response to DENSPM treatment [9,44,45]. We have made the observation that MDL72527 treatment did not significantly decrease the cytotoxicity of DENSPM in L56Br-C1 cells [46], thus, pointing to polyamine depletion *per se* having an important role in DENSPM-induced apoptosis. In HCC1937 cells, polyamines were not depleted to the same levels as in L56Br-C1 cells, which may also partly explain the absence of cell death in the former cells. However, there is compelling evidence that reactive oxygen species produced through SSAT activity, as well as activation of the polyamine catabolic enzyme spermine oxidase, do play a significant role in the cytotoxic response to DENSPM treatment [47–49]. The relative roles of polyamine depletion and reactive oxygen species production in cytotoxic responses to polyamine analogue treatment have to be investigated further.

The high efficiency of DENSPM treatment, and the fact that it does not only function through SSAT activation, is apparent in the polyamine pool data. Polyamine pool depletion results in inhibition of cell proliferation in all four-cell lines but with different kinds of kinetics [41]. The most extensive decrease in the polyamine pools was, however, found in L56Br-C1 cells where apoptosis is induced.

The mitochondrial involvement was clear through the change in the level of various proapoptotic and anti-apoptotic proteins. An ordered sequence of events in L56Br-C1 cells regarding the fate of several mitochondrial intermembrane space proteins was found. Already at 12 h after seeding in the presence of DENSPM, cytochrome *c* was found in the cytosol. When cytochrome *c* is released into the cytosol, it can activate downstream effector caspases, that is, caspase 3 [39]. According to the measurement of the caspase 3 activity, caspase 3 was activated at 12 h of DENSPM treatment. At this time point there were no changes in the other proteins investigated. Thus, in L56Br-C1 cells, the initial step in the activation of apoptosis is the release of cytochrome *c* from the mitochondria. Others have also reported that DENSPM treatment induces release of cytochrome *c* from the mitochondria [42]. In L56Br-C1 cells, the

release of cytochrome *c* took place without a change in $\Delta\Psi_m$. Instead, $\Delta\Psi_m$ seemed to be increased as determined by an increased uptake of JC-1. The uptake of JC-1 is dependent on the mitochondrial transmembrane potential [39]. We have seen the same phenomenon using another mitochondrial stain [50]. Our data point to a polyamine analogue-induced increase in mitochondrial respiration, thus supporting the notion that $\Delta\Psi_m$ is increased in DENSPM-treated cells [11,26]. In contrast, it was found that $\Delta\Psi_m$ decreased in DENSPM-treated SK-MEL-28 human melanoma cells in conjunction with cytochrome *c* release [42].

After the initial release of cytochrome *c* observed after 12 h of DENSPM treatment in L56Br-C1 cells, a number of changes in the level of various mitochondrial-associated proteins were shown. A decrease in Bcl-X_L also points to active caspase 3 as it is a caspase substrate [51]. Bcl-X_L is an antiapoptotic protein involved in the inhibition of pore formation in the outer mitochondrial membrane [52,53]. Thus, degradation of Bcl-X_L sustains the apoptotic process. Two of the proteins that are inhibited by Bcl-X_L are the proapoptotic proteins Bax and Bad. In DENSPM-treated L56Br-C1 cells, Bax was cleaved into a more proapoptotic fragment of 18 kDa [54,55]. Cytochrome *c* was not released in MCF-7 cells even though the western blot showed the presence of the highly apoptotic 18 kDa Bax band in both control and DENSPM-treated cells. Presumably the high level of Bcl-2 prevented the possibility of Bax to form pores in the outer mitochondrial membrane [56]. We have shown that overexpression of Bcl-2 in L56Br-C1 cells delayed the apoptotic response [57], pointing to a negative role of Bcl-2 in the outcome to DENSPM treatment.

The level of Bad decreased in DENSPM-treated L56Br-C1 cells. Bad is an interesting member of the BH3 only family and it has different functions depending on the phosphorylation status [58]. Phosphorylated Bad acts as a survival factor and is bound to 14-3-3 proteins in the cytoplasm [59]. If Bad is present in the cell in its unphosphorylated form, it is proapoptotic and is supposedly found in association with the mitochondria. We have not investigated the phosphorylation status of Bad in L56Br-C1 cells. We conclude that the protein is found in the fraction containing mitochondria in L56BrC1 cells and it is not found free in the cytosol. It is interesting to note that HCC1937 cells had high levels of Bad in both the mitochondrial and cytosolic fractions, and it may be hypothesized that the cytosolic Bad contributed to the fact that these cells did not react to DENSPM treatment with cell death despite a significant activation of SSAT and polyamine pool depletion.

AIF and survivin also disappeared from the mitochondrial fraction in DENSPM-treated cells. When released from the mitochondria, AIF is known to rapidly translocate to the nucleus, where it induces DNA condensation and fragmentation in a caspase-independent manner [60–62]. Survivin belongs to the group of inhibitors of apoptosis and is supposed to act as an antiapoptotic factor when released from the mitochondrial intermembrane space [63]. In L56Br-C1 cells, survivin released from the mitochondria was presumable degraded, as it was not found in the cytosol. DENSPM treatment did not affect the level of survivin (*BIRC5*) mRNA. DENSPM-induced proteasomal-mediated degradation of survivin has been shown in several human melanoma cell lines [21], and in this study it was also shown that this took place without an effect on the survivin mRNA. Thus, our study in human breast cancer cells supports their finding in human melanoma cells, pointing to a common principle of DENSPM-induced cell death. It must, however, be remembered that the decrease in survivin took place after the release of cytochrome *c*. In the study by Chen *et al.* [21], a decrease in the mitochondrial Smac/DIABLO level was also found. In L56Br-C1 cells, Smac/DIABLO disappeared and we can only speculate that it also was subjected to degradation. The main role of Smac/DIABLO is to bind to inhibitor of apoptosis proteins, thus relieving the inhibition of caspase activation and letting the apoptotic pathway proceed [61,62].

Although, as already mentioned above, we could not find a significant caspase 3 band in the western blots, the procaspase 3 band disappeared and a 26-kDa band appeared in DENSPM-treated L56Br-C1 cells. We have not found any reports of a similar finding and have so far no explanation for this observation.

It should be pointed out that all the DENSPM-induced responses in L56Br-C1 cells are p53 and pRB independent [41]. This is favourable from a cancer treatment point of view as the nonfunctional forms of these proteins have been associated with resistance to chemotherapeutic intervention [64]. The least sensitive cell MCF-7 has wild-type *p53* and this may implicate that normal cells that have wild-type *p53* may not be severely injured by DENSPM treatment. We have shown that MCF-7 cells have a wild-type *p53*-dependent response with a G_1 accumulation in the cell cycle distribution as an effect of polyamine analogue treatment [41]. Similar results have previously been described by Kramer *et al.* [65], in which they treated wild-type *p53* containing MALME-3M cells with DENSPM.

DENSPM treatment of MCF-7, SK-BR-3 and HCC1937 did not affect the level of any of the proteins that were significantly affected in DENSPM-treated L56Br-C1 cells. Most striking is the high variability in the levels of the different proteins in the four cell lines. It is tempting to speculate that a protein pattern similar to

that of L56Br-C1 cells may indicate that DENSPM treatment will induce cell death. In L56Br-C1 cells, all the studied proteins were present in roughly similar amounts according to western blot analysis while there was a large variability among the other three cell lines. DENSPM treatment did not have any uniform effect on the mRNA levels of any of the apoptosis-related proteins studied. The only uniform effect was on SSAT mRNA where an increase was found in all cell lines (not shown). This is a known effect of DENSPM treatment [43].

In conclusion, the four breast cancer cell lines MCF-7, SK-BR-3, HCC1937 and L56Br-C1 showed different sensitivity to DENSPM treatment with respect to cell death. A similar spectrum of sensitivity is expected when treating patients with DENSPM. Our data indicate that it should be possible to define molecular and cellular characteristics of a tumour to predict sensitivity to a polyamine analogue and that the L56Br-C1 cell line could be used for that purpose. We suggest that the levels of proapoptotic proteins such as Bax and procaspase 3 as well as antiapoptotic proteins such Bcl-2 and Bad can be used to partly predict the outcome to DENSPM treatment.

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