Different cell cycle kinetic effects of N^1,N^{11}-diethylnorspermine-induced polyamine depletion in four human breast cancer cell lines

Louise Myhre[a], Kersti Alm[a], Cecilia Hegardt[b], Johan Staaf[b], Göran Jönsson[b], Sara Larsson[c] and Stina M. Oredsson[a]

Polyamine analogues are presently undergoing clinical evaluation in the treatment of cancer. To better understand under what circumstances treatment with a polyamine analogue will yield beneficial results, we have investigated the effect of N^1,N^{11}-diethylnorspermine (DENSPM) on cell cycle kinetics of the human breast cancer cell lines SK-BR-3, MCF-7, HCC1937, and L56Br-C1. A bromodeoxyuridine–DNA flow cytometry method was used to evaluate the treatment with 10 μmol/l DENSPM on cell cycle kinetics. A correlation between polyamine pool size after DENSPM treatment and cell cycle kinetic effects was found. The most sensitive cell cycle phase was the S phase, followed by an effect on the G_2+M phase and then the G_1/S transition. The levels of a number of cell cycle regulatory proteins such as cyclin E1, cyclin A2, and cyclin B1 were lowered by DENSPM treatment, which may explain the effects on cell cycle kinetics. The two cell lines that were most sensitive to DENSPM treatment belong to the basal-like subtype of breast cancer and they were deficient with respect to p53, BRCA1, and RB1. *Anti-Cancer Drugs* 19:359–368 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Departments of [a]Cell and Organism Biology, [b]Oncology, Clinical Sciences and [c]Mathematical Statistics, Lund University, Lund, Sweden

Correspondence to Professor Stina M. Oredsson, PhD, Department of Cell and Organism Biology, Lund University, Helgonavägen 3B,223 62 Lund, Sweden
Tel: +46 46 222 93 54; fax: +46 46 222 45 39;
e-mail: stina.oredsson@cob.lu.se

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Introduction

Breast cancer is a complex disease and the mechanisms involved in the development are still an enigma despite intensive research. One major component in breast cancer development is the disturbed regulation of the cell cycle. Proteins and other molecules, which interact in a complex network, regulate normal cell cycle progression. The normal function of the components of this network keeps cell division in balance. The network comprises for example, the cyclins with their cyclin-dependent kinases and inhibitors of cyclin-dependent kinases [1,2]. In addition, oncogenes and tumour suppressor genes as well as a number of checkpoint control proteins including those that have a function in the control of DNA repair are involved in cell cycle regulation [3,4]. In cancer cells, the mechanisms controlling the cell cycle are disrupted owing to the complex interplay of various molecules being disturbed by defectively functioning proteins. The defects comprise nonfunctioning proteins to missing or poor functioning proteins owing to mutation as well as overexpression of proteins.

Another major component in the control of cell cycle progression is the polyamines: putrescine, spermidine, and spermine. Normal cell cycle progression requires certain levels of the polyamines [5,6]. The cellular polyamine pools are carefully controlled by biosynthesis, catabolism, and uptake/excretion [5,7]. It is possible to deplete the cellular polyamine pools by affecting enzymes and proteins involved in these processes [8,9]. Depletion of the intracellular polyamine pools always results in growth inhibition [5–7], and therefore compounds that lower polyamine pools are considered in anticancer treatment [8,9]. One group of compounds that stimulate polyamine catabolism and inhibit polyamine biosynthesis is the polyamine analogues [8]. Polyamine analogues are recognized by the cell as polyamines, and are taken up by the polyamine transport system resulting in intracellular accumulation. Although recognized as polyamines, these compounds cannot take over the natural functions of polyamines in the cell. One such polyamine analogue is N^1,N^{11}-diethylnorspermine (DENSPM). DENSPM has been used in clinical phase I and II studies in the treatment of breast and lung cancer [10–12]. More efficient analogues have been developed [13,14], and are presently being tested in clinical trials.

In this study we have used DENSPM as a model compound to investigate how cell cycle kinetics is

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affected in four different human breast cancer cell lines. The four cell lines SK-BR-3, MCF-7, HCC1937, and L56Br-C1 have different genetic aberrations and they can be classified into different breast cancer groups based on their expression profiles [15,16]. Using bromodeoxyuridine (BrdUrd)–DNA flow cytometry we have studied the rate of G_1/S transition, and the lengths of the S and the $G_2 + M$ phases. Cell cycle kinetics was affected differently in the four cell lines. The effects on cell cycle kinetics were correlated to changes in cell cycle regulatory proteins to understand the mechanism of DENSPM-induced growth inhibition. To achieve increased efficiency in the use of polyamine analogues in the treatment of cancer, the understanding of mechanisms is important. Hopefully specific cancer subgroups suitable for this kind of treatment can be defined.

Materials and methods

Cell culture

The MCF-7 (HTB-22), HCC1937 (CRL-2336), and SK-BR-3 (HTB-30) cell lines were purchased from the American Type Culture Collection (Manassas, Virginia, USA). The L56Br-C1 cell line was established in Lund [17]. The cell lines were cultured as previously described [18]. MCF-7 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (FCS), nonessential amino acids, insulin (10 μg/ml), penicillin (50 U/ml), and streptomycin (50 μg/ml). SK-BR-3 cells were cultured in the same medium as MCF-7 cells, but without the addition of insulin. HCC1937 cells were cultured in Minimum Essential Medium α-medium supplemented with 10% heat-inactivated FCS, nonessential amino acids, gentamycin (0.1 mg/ml), epidermal growth factor (20 ng/ml), and insulin (10 μg/ml). L56Br-C1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, nonessential amino acids, insulin (10 μg/ml), penicillin (50 U/ml), and streptomycin (50 μg/ml).

For the experiments, a number of replicate cultures consisting of 2×10^6 plateau phase cells seeded into 12 ml medium in Petri dishes (9 cm diameter) in the absence or presence of 10 μmol/l DENSPM were set up. The water-soluble substance DENSPM (Tocris Cookson Ltd, St Louis, Missouri, USA) was dissolved in phosphate-buffered saline (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) to give a stock solution of 2 mmol/l, which was sterile-filtered before addition to the cell cultures.

Cell cycle kinetics

Cells were labelled with 5 μmol/l BrdUrd at 24 and 48 h of treatment as previously described [19]. In short, after a 30-min BrdUrd-labelling period, the cells were further incubated in BrdUrd-free medium. Cells were sampled at 0, 4, 6, 8, 10, and 12 h postlabelling. Owing to extensive apoptosis induced by DENSPM [20,21], L56Br-C1 cells were only investigated at 24 h of treatment.

Flow cytometric analysis of bromodeoxyuridine and DNA contents

The staining procedure of DNA and BrdUrd has previously been described in detail [22]. In short, the cells were incubated with a primary anti-BrdUrd monoclonal antibody (Dakopatts, Glostrup, Denmark). To allow access of the primary antibody to BrdUrd incorporated into DNA, the DNA was partially denatured by incubating the cells in 2 mol/l HCl. A secondary FITC-conjugated antibody (Dakopatts) was used to detect the primary antibody. The double-stranded regions of DNA were then stained with propidium iodide. The cells were analyzed with respect to DNA (red fluorescence) and BrdUrd (green fluorescence) contents in a Cytograph System 50-H flow cytometer (Ohio Instruments, Westwood, Massachusetts, USA). For the computer analysis, Multi2D and MultiCycle software programs (Phoenix Flow Systems, San Diego, California, USA) were used. The data were analysed and calculations made as described previously [19,22].

Western blot analysis

Cells for Western blot analysis were harvested by trypsinization, counted in a hemocytometer, and pelleted at 700*g* for 10 min at 4°C. The cells were diluted in sample buffer (300 μl/10^6 cells, 62.5 mmol/l Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol), sonicated, and heated at 95°C for 6 min and put on ice immediately after. Aliquots containing 50 000 cells were then loaded in the wells of precast SDS–polyacrylamide gels (4–12%). Note that loading was based on cell counting. Electrophoresis was carried out in an Xcell SureLock Mini-Cell electrophoresis system and subsequent blotting by using XCell blot module, from Invitrogen Life Technologies Inc. (Carlsbad, California, USA). The membranes were then blocked with 5% dry milk in 0.05% Tween-20 in phosphate-buffered saline before incubation overnight with the primary antibody. Monoclonal antibodies against p27, E2F1, cyclin D1, RB1, cyclin A2, p53, and cyclin B1 were purchased from BD PharMingen (San Diego, California, USA). Monoclonal antibodies against p21, cyclin E1, and CDK2 and the polyclonal antibody against CDK2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin was used as a secondary antibody (Dakopatts). Advanced ECL protein detection reagent was used according to the manufacturer's protocol (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The ChemiDoc XRS system (Bio-Rad Inc., Hercules, California, USA) was used for imaging and the software Quantity One (Bio-Rad Inc.) was used for the analysis.

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Polyamine analysis

Cells for polyamine analysis were harvested by trypsinization, counted in a hemocytometer, and pelleted at 700*g* for 10 min at 4°C. Quantification of the polyamines was carried out in cell homogenates in 0.2 mol/l perchloric acid using high-performance liquid chromatography (HPLC) (Hewlett Packard 1100; Meadows Instrumentation Inc., Zion, Illinois, USA) [23]. Note that polyamine level determination is based on cell number.

Expression and DNA copy number analysis

Oligonucleotide, complementary DNA (cDNA) and tiling bacterial artificial chromosome (BAC) arrays were produced at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University, Sweden (*http://swegene.onk.lu.se*) [16,24].

RNA was extracted from the cell lines (24 h after seeding) using Trizol (Invitrogen Life Technologies Inc.) followed by the RNeasy Midi purification kit (Qiagen, Valencia, California, USA). 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 were carried out using the Pronto! Plus Direct System (Corning Inc., Acton, Massachusetts, USA; Promega Corporation, Madison, Wisconsin, USA) according to the manufacturers' 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 (Amersham Biosciences, Little Chalfont, UK). Hybridization and washing were carried out as previously described [24].

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 were carried out as described in Jönsson *et al.* [16].

Image and data analysis

Fluorescence signals were recorded using an Agilent G2565AA microarray scanner (Agilent Technologies). Tagged image file format images were analyzed 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 (BASE) [25], where quality and data analysis were carried out. Background correction was carried out by subtracting the 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 [26]. 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 Jönsson *et al.* [16]. Cutoff ratios for gains and losses were set to 1.23 and 0.81, respectively, corresponding to a $\log_2$ ratio of ± 0.3. Oligonucleotide probes and cDNA clones were mapped according to the UniGene database [build 196 (*http://www.ncbi.nlm.nih.gov/UniGene*)]. BAC clones are ordered by position in the genome according to the University of California Santa Cruz Human Genome Assembly, Hg17 (*http://genome.ucsc.edu*).

Data analysis and statistical analysis

For the statistical evaluation of T_S values, two-tailed unpaired Student's *t*-test was used. To determine the length of the $G_2 + M$ phase, regression analysis was used. The Bootstrap technique was used to estimate the standard error and the *P*-values.

Results

HPLC analysis of the polyamine pools showed that the different cell lines have different basic polyamine levels (Fig. 1). HCC1937 cells had the highest total polyamine levels while L56Br-C1 cells had the lowest. The smallest variation in basal pool size was in the spermine pool. In control cells, the spermine levels varied between 2–4 nmol/10^6 cells whereas putrescine and spermidine levels varied between approximately 0.1–2.6 and 0.5–7.6 nmol/10^6 cells, respectively. DENSPM treatment resulted in decreased polyamine levels, however to different degrees in the different cell lines. It is our notion that it is not the decrease in the polyamine level in relation to the control level that has cellular and molecular implications but the absolute level of remaining polyamines after DENSPM treatment. The polyamine pools remaining after DENSPM treatment were highest in SK-BR-3 cells and lowest in L65Br-C1 cells.

Next we studied the effect of DENSPM treatment on cell cycle kinetics. The rate of G_1/S transition was studied by investigating the movement of BrdUrd negative cells from G_1 into S phase (Fig. 2). Figure 2 shows the decreases in the G_1 phase population, which is

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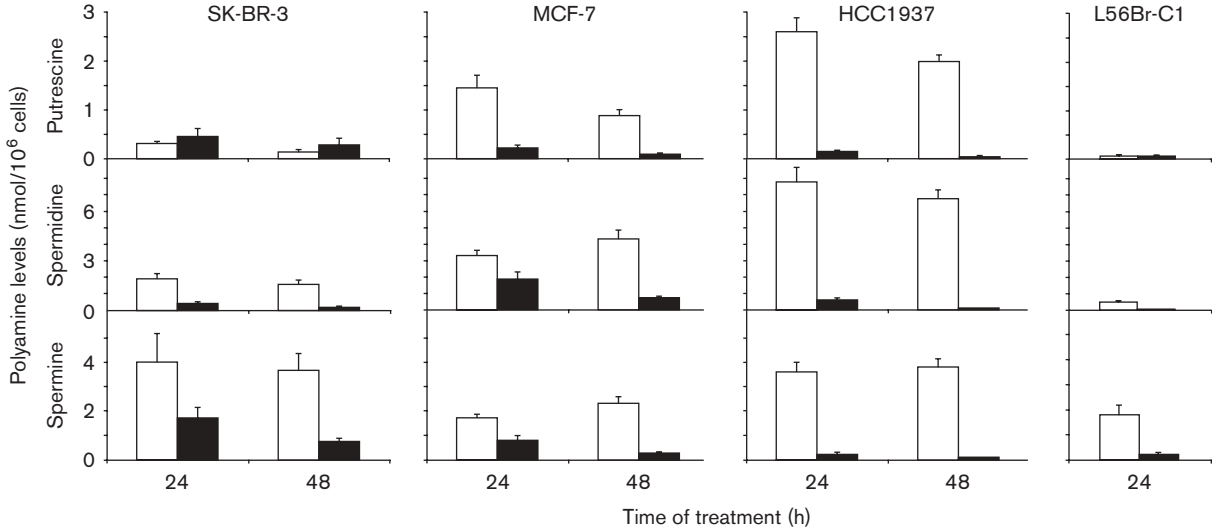
Fig. 1



Effect of N^1,N^{11}-diethylnorspermine (DENSPM) treatment on polyamine levels in SK-BR-3, MCF-7, HCC1937, and L56Br-C1 human breast cancer cell lines. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. After 24 and 48 h of treatment, cells were sampled for polyamine analysis by high-performance liquid chromatography. Control, white columns. DENSPM, black columns. Bars show the mean of three independent samples ± SD.

inversely correlated to the increase in the S phase population. The slopes of the lines indicate the rate of G_1/S transition, that is, the steeper the slope the higher the rate of G_1/S transition. When no cells are entering S phase, the line is horizontal. The rate of G_1/S transition was not affected at 24 h of DENSPM treatment in SK-BR-3 and L56Br-C1 cells while it was substantially decreased in MCF-7 and HCC1937 cells. At 48 h of treatment, the rate of G_1/S transition was still not affected in SK-BR-3 cells. In DENSPM-treated MCF-7 cells, there was a G_1 block. In HCC1937 cells, the rate of G_1/S transition was very low at 48 h of DENSPM treatment.

The S phase was significantly prolonged in all cell lines after 24 h of DENSPM treatment (Table 1); however, the prolongation was different in the different cell lines. The length of the S phase was most increased in L56Br-C1 cells and least in SK-BR-3 cells. At 48 h of treatment the S phase was even further prolonged compared with control.

The progression of BrdUrd-labelled cells from S phase through $G_2 + M$ phase into G_1 phase was followed by determining the percentage of BrdUrd-labelled divided cells found in G_1 in relation to all BrdUrd-labelled cells (Fig. 3). BrdUrd-labelled divided cells appear in G_1 when the minimum time period for $G_2 + M$ phase (T_{G2+M}) has passed. The intercept of the line with the *X*-axis is an approximate measure of the minimum time for cells to spend in $G_2 + M$ phase. When T_{G2+M} is affected, the appearance of cells in G_1 will be delayed as demonstrated by a shift to the right in the intercept of the line with the *X*-axis. The $G_2 + M$ phase was significantly prolonged in HCC1937 cells after 24 h of DENSPM treatment while there was a total block in $G_2 + M$ progression in L56Br-C1 cells (Table 1). The $G_2 + M$ phase was significantly affected in MCF-7 cells after 48 h of treatment. Bootstrap evaluation of the data from SK-BR-3 cells shown in Fig. 3 gave a statistical difference in the intercept with the *X*-axis comparing control and DENSPM-treated cells at both 24 and 48 h of treatment (Table 1). From a biological point of view we, however, do not regard this difference as significant.

As DENSPM treatment caused cell cycle kinetic effects, we decided to study the expression of a number of genes involved in cell cycle regulation. As we are working with cancer cell lines, which are known to have copy number alterations, the obvious starting point was to use tiling BAC array comparative genome hybridization to investigate gene copy number in the four cell lines. Chromosome plots (data not shown) were used for the evaluation of gene copy number. No homozygous deletions, defined as $\log_2$ ratio less than −1.0, or amplifications, defined as $\log_2$ ratio greater than 1.5, of the studied genes were found; however, we observed a number of gains and losses (Fig. 4a, left panel). The mRNA content of the cell lines is shown in Fig. 4a, right panel. No direct correlation between gene copy number and mRNA expression was observed. In a third experimental set up, we investigated the effect of DENSPM on gene expression. Of the

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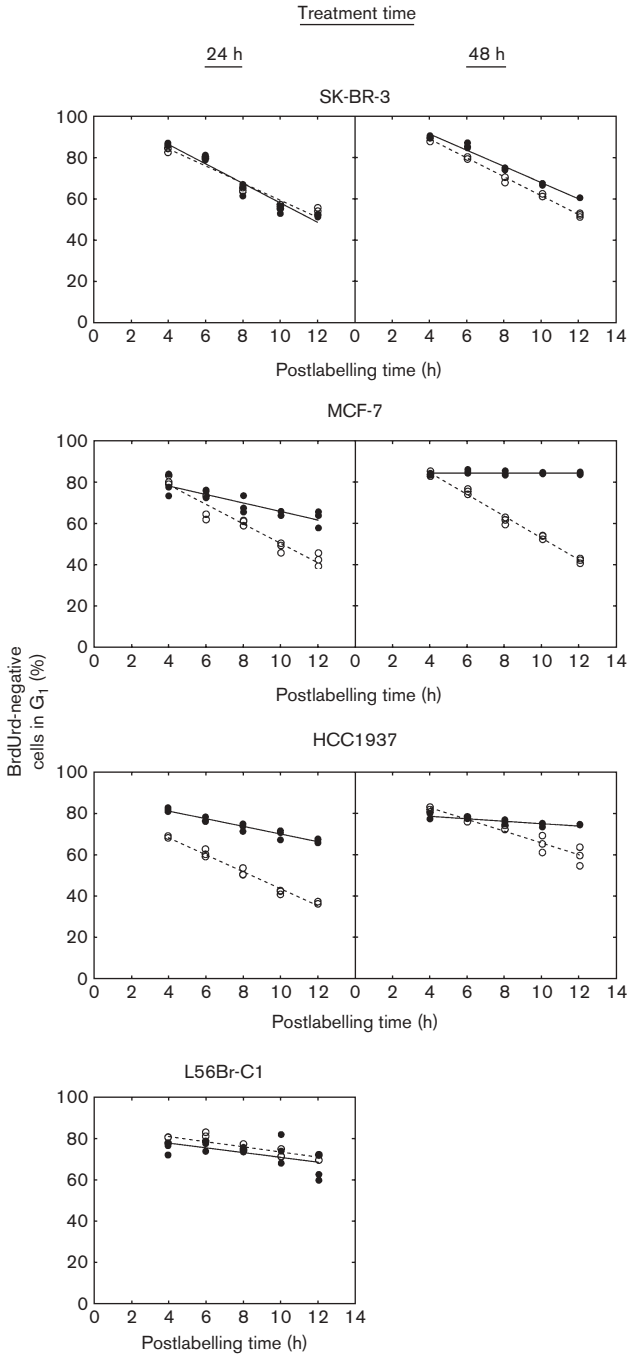
Fig. 2



Effect of N^1,N^{11}-diethylnorspermine (DENSPM) treatment on the rate of G_1/S transition in SK-BR-3, MCF-7, HCC1937, and L56Br-C1 human breast cancer cell lines. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. After 24 h or 48 h of treatment, bromodeoxyuridine (BrdUrd) was added to the culture medium to a final concentration of 5 μmol/l. After a 30-min labelling period, the BrdUrd containing medium was removed and after rinsing, BrdUrd-free medium was added. At 4, 6, 8, 10, and 12 h postlabelling, the cells were harvested by trypsinization, pelleted by centrifugation at 700*g*, resuspended in ice-cold 70% ethanol, and prepared for flow cytometric -mediated determination of DNA and BrdUrd contents. The data were fit by regression analysis. One representative experiment of two is shown. ○, Control cells; ●, DENSPM-treated cells.

Table 1 Effect of DENSPM treatment on the lengths of the S and G_2 + M phases in SK-BR-3, MCF-7, HCC1937, and L56Br-C1 cells[a]

Cell line	At 24 h of treatment		At 48 h of treatment	
	Control (h)	DENSPM (h)	Control (h)	DENSPM (h)
T_S[b]				
SK-BR-3	12.2 ± 2.5	13.4 ± 1.3*	10.5 ± 0.9	12.7 ± 1.4*
MCF-7	12.7 ± 1.5	15.6 ± 2.4*	13.3 ± 1.9	17.7 ± 2.8*
HCC1937	9.3 ± 1.4	15.0 ± 1.5*	9.9 ± 2.5	14.1 ± 1.2*
L56Br-C1	13.2 ± 2.0	40.6 ± 8.5*	–	–
T_{G2+M}[c]				
SK-BR-3	4.1 ± 0.1	4.5 ± 0.1*	4.6 ± 0.1	4.8 ± 0.1*
MCF-7	3.9 ± 0.1	3.7 ± 0.3	4.0 ± 0.3	4.9 ± 0.4*
HCC1937	5.1 ± 0.1	5.9 ± 0.4*	5.5 ± 0.2	7.4 ± 0.3*
L56Br-C1	5.5 ± 0.3	–	–	–

DENSPM, N^1,N^{11}-diethylnorspermine.
[a]The cells were seeded and treated as described in the text of Figs 2 and 3.
[b]The length of the S phase was calculated as previously described [27,28]. n = 23–30 independent samples from two experiments. The numbers are mean ± SD. For the statistical evaluation Student's unpaired t-test was used. *$P \leq 0.05$.
[c]To determine the length of the G_2 + M phase, regression analysis was used to evaluate the data points shown in Fig. 3. The Bootstrap technique was used to estimate the standard error and the P-values. The experiments were repeated twice with similar results. *$P \leq 0.05$.

various genes investigated in this study, there were no clear uniform DENSPM-induced effects (Fig. 4b). An indication that the expression of p53 was lowered in all cell lines was observed; however, this notion has to be confirmed with another technique than cDNA microarray. Most of the changed mRNA levels were found in L56Br-C1 cells where the expressions of *cyclin A2* (*CCNA2*), *CDK2*, *p53* (*TP53*), and *cyclin E1* (*CCNE1*) were down-regulated. In MCF-7 cells, DENSPM treatment increased the *p21* (*CDKN1A*) mRNA level (Fig. 4b). DENSPM treatment caused an increase in the expression of *SSAT* mRNA to the same level in all cell lines (not shown).

Following analysis of gene expression data, we investigated the protein levels of the studied genes by Western blot (Fig. 5). Our interpretation of the data is based on results from three independent experiments. The basal levels of the cyclins involved in cell cycle regulation were different in the four breast cancer cell lines. Cyclin D1 was higher in MCF-7, HCC1937, and L56Br-C1 than in SK-BR-3 cells. DENSPM treatment only affected the level of cyclin D1 in L56Br-C1 cells where cyclin D1 was almost absent after 24 h of treatment (Fig. 5).

Besides the full length cyclin E1, a number of low molecular bands were found in all cell lines; however, they were most prominent in SK-BR-3 cells and least prominent in MCF-7 cells. DENSPM treatment of L56Br-C1 cells for 24 h resulted in a lowering of the cyclin E1 level compared with control. In MCF-7, SK-BR-3, and HCC1937 cells treated with DENSPM for 48 h, there was also a lower cyclin E1 level than in control cells.

The basal level of cyclin A2 was higher in SK-BR-3 and HCC1937 cells compared with the two other cell lines.

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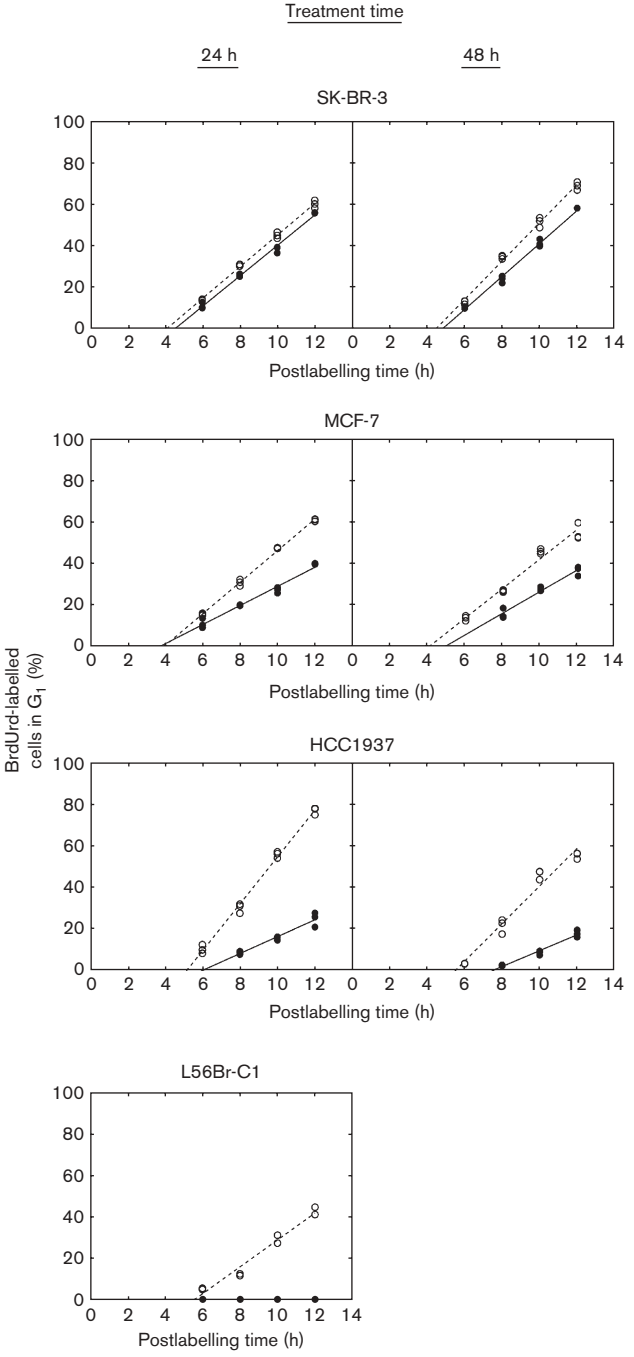
Fig. 3



Effect of N^1,N^{11}-diethylnorspermine (DENSPM) treatment on the length of the G_2 + M phase in SK-BR-3, MCF-7, HCC 1937, and L56Br-C1 human breast cancer cell lines. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. Cells were seeded and treated as described in the text of Fig. 2. The data were fit by regression analysis. One representative experiment of two is shown. ○, Control cells; ●, DENSPM-treated cells.

Cyclin A2 was almost absent in L56Br-C1 cells treated with DENSPM for 24 h. The cyclin A2 level was decreased by 48 h of DENSPM treatment in SK-BR-3, MCF-7, and HCC1937 cells.

The basal cyclin B1 level was also different in the different cell lines with L56Br-C1 cells having the lowest level. Cyclin B1 was almost absent in L56Br-C1 cells treated with DENSPM for 24 h. In SK-BR-3 cells, the level of cyclin B1 was lower in cells treated with DENSPM for 24 and 48 h compared with control. In MCF-7 and HCC1937 cells, a lowering of the cyclin B1 level compared with control was apparent at 48 h of treatment.

Apart from the cyclins, other proteins involved in cell cycle regulation also showed variations in the basal level in the four different cell lines. The basal level of p27 was higher in MCF-7 cells than the other cell lines (Fig. 5). In L56Br-C1 cells the level of p27 decreased after 24 h of DENSPM treatment. DENSPM treatment for 48 h lowered the p27 level in SK-BR-3 and MCF-7 cells.

RB1 was found in SK-BR-3 and MCF-7 cells whereas it was absent in HCC1937 and L56Br-C1 cells (Fig. 5). The RB1 level was not affected by DENSPM treatment. We have not specifically investigated the different phosphorylated forms of RB1.

The highest level of E2F1 was found in SK-BR-3 cells, whereas MCF-7 cells had the lowest level of the protein. At 24 h of DENSPM treatment, E2F1 was absent in L56Br-C1 cells. DENSPM treatment did not affect E2F1 in the other cell lines.

p53 protein was found in SK-BR-3, MCF-7, and L56Br-C1 cells (Fig. 5). p53 is a transcription factor for p21 and this protein was only found in MCF-7 cells, which contain wild-type p53. DENSPM treatment increased the level of p53 in MCF-7 cells at both 24 and 48 h of treatment. At 48 h of DENSPM treatment there was an increase in the p21 level in MCF-7 cells.

Discussion

In this study, we have investigated the effect of the polyamine analogue DENSPM on cell cycle kinetics in four human breast cancer cell lines. DENSPM treatment induced growth inhibition in all four cell lines and in addition apoptosis is induced in the L56Br-C1 cell line [20]. We have earlier carried out a careful study of a number of proteins involved in apoptosis and their possible roles together with the polyamines in determining the fate of the cell in relation to apoptosis when treated with DENSPM (Holst CM, Staaf J, Jönsson G, Hegardt C, Oredsson SM, submitted article). With this study, we have extended our knowledge of the reaction of these four cell lines with respect to the effect of DENSPM on cell cycle kinetics.

DENSPM treatment had the lowest effect on the polyamine pools in SK-BR-3 cells. In SK-BR-3 cells, the

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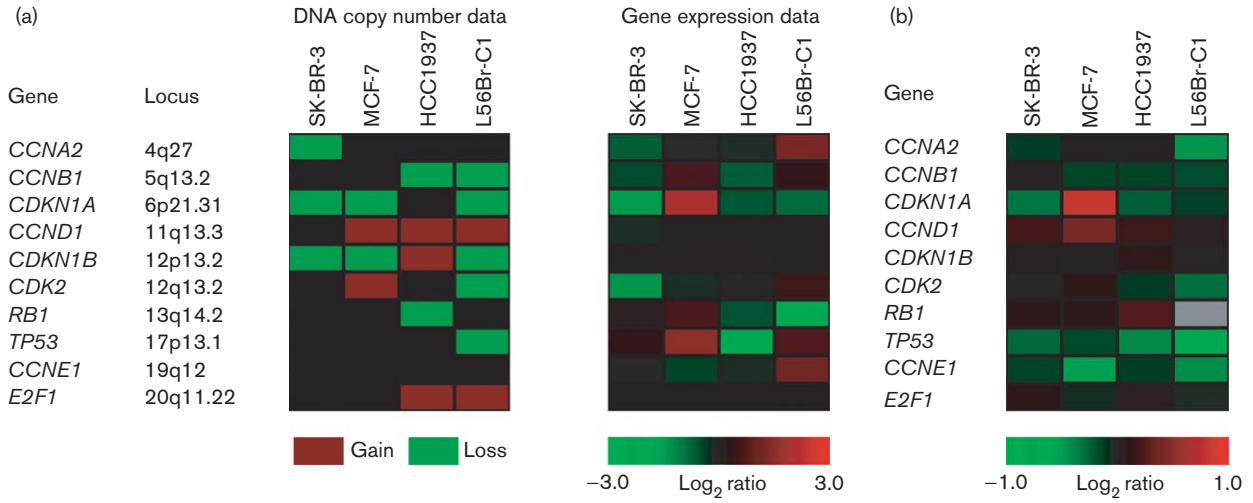
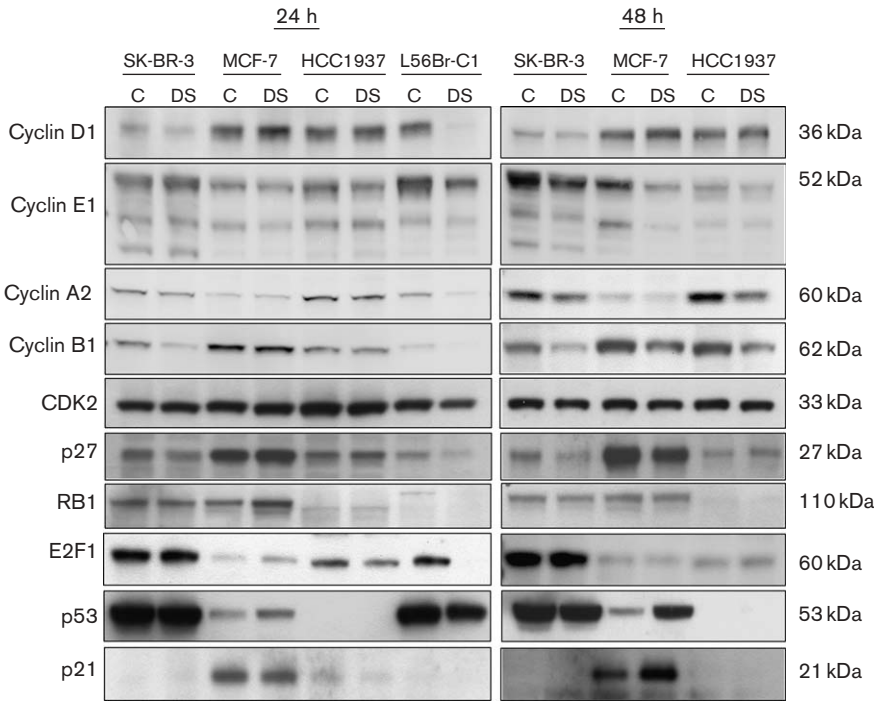
Fig. 4



DNA copy number and gene expression data in SK-BR-3, MCF-7, HCC1937, and L56Br-C1 human breast cancer cell lines evaluated by microarray-based methods. (a) DNA copy number and gene expression data for the selected genes. (b) Effect of treatment with 10 μmol/l N^1,N^{11}-diethylnorspermine for 24 h on gene expression (n=2–3). The colour scales indicate $\log_2$ ratios where green represents losses or lower expression and red represents gains or increased expression. Grey areas denote missing values.

Fig. 5



Effect of N^1,N^{11}-diethylnorspermine (DENSPM) treatment on the levels of various cell cycle regulatory proteins in the human breast cancer cell lines SK-BR-3, MCF-7, HCC1937, and L56Br-C1 evaluated by Western blot. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. After 24 or 48 h of treatment cells were harvested by trypsinization, counted in a hemocytometer, and pelleted. Cells were then sonicated in sample buffer (10^6 cells/300 μl) and subjected to Western blot analysis. In each well, an aliquot equal to 50 000 cells was added; thus, loading was based on cell counting. C, control cells; DS, DENSPM-treated cells.

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major cell cycle effect after 24 h of treatment was a prolongation of the S phase. In MCF-7 cells, the S phase was prolonged and the rate of G_1/S transition was lowered at 24 h of DENSPM treatment; however, there was no effect on the $G_2 + M$ phase. In this cell line, DENSPM treatment had a higher polyamine-depleting effect than in SK-BR-3 cells. In HCC1937 and L56Br-C1 cells, DENSPM treatment resulted in even lower polyamine pools than in the other two cell lines. All the studied cell cycle kinetic parameters were affected in the former cell line whereas the G_1/S transition was not affected in the latter. From all these results it is tempting to speculate that the polyamines have their most important role in regulating S phase progression since this cell cycle phase was affected in all cell lines already 24 h after seeding in the presence of DENSPM. We have previously shown that the S phase is affected early by polyamine pool manipulation in Chinese hamster ovary cells cells [22,27–29], and now we have extended this to human breast cancer cell lines. By studying DNA replication domain organization, we have suggested that it is the elongation of DNA replication that is affected rather than initiation [30]. The importance of polyamines in the S phase and also in the $G_2 + M$ phase is reflected in peaks in polyamine biosynthesis in conjunction with these phases [31]. The sensitivity of the $G_2 + M$ phase to low polyamine levels was evident in HCC1937 and L56Br-C1 cells, where this cell cycle phase was prolonged at 24 h of DENSPM treatment. Interestingly, there was no obvious correlation between polyamine pool size and the rate of G_1/S transition. This was most obvious in L56Br-C1 cells where there was no effect on the G_1/S transition despite very low polyamine pools in DENSPM-treated cells.

In an attempt to further elucidate the role of the polyamines in cell cycle regulation we have studied a number of proteins involved in the process. One of the questions was if the effect of polyamine depletion on cell cycle kinetics was because the levels of these proteins were affected. The western blot analyses clearly show that the cell lines had different basal levels of the investigated proteins, which may reflect that they represent different breast cancer subtypes [15,16]. HCC1937 and L56Br-C1 cell lines were of basal-like subtype. SK-BR-3 had an expression signature close to luminal B type and MCF-7 to luminal A type. The most obvious changes in protein levels induced by DENSPM treatment was found in L56Br-C1 cells. In this cell line there is a rapid DENSPM-induced activation of the mitochondrial apoptotic pathway with release of cytochrome *c* from the mitochondria followed by caspase 3 activation [18,20,32]. The rapid disappearance of some proteins found in L56Br-C1 cells may partly be owing to the action of caspases [33]. Thus, for L56Br-C1 cells, it is difficult to discern between effects that only occur on cell cycle kinetics and secondary effects on cell cycle kinetics because of the induction of apoptosis. L56Br-C1, however, is a model cell line to find markers for high sensitivity for efficient polyamine analogue treatment in the clinic.

Of the cell lines studied, only MCF-7 has wild-type p53. DENSPM treatment resulted in increased p53 level, which resulted in increased mRNA level of p21 and consequently an increased p21 protein level. Similar results have been obtained in MALME-3M melanoma cells [34]. It is interesting to note that the levels of p53 protein did not increase in the cell lines with mutated p53. Thus, one may speculate about the signal that increases the translation of p53 message and why it does not function in the cell lines with mutated p53. p53 is induced by various forms of cellular stress for example, signals from damaged DNA [35]. Obviously a prolongation of S phase in all cell lines presumably caused by the same mechanism was observed, but p53 is only increased in one of the cell lines.

RB1 is a protein with an important checkpoint control function in G_1 [36]. Despite the presence of message, RB1 protein was missing in the two BRCA1-deficient cell lines HCC1937 and L56Br-C1. The cause for this has to be further investigated. The activity of E2F1 is normally under the control of RB1 [36]. E2F1 is a transcription factor for proteins required for the G_1/S transition and S phase progression. To maintain proper cell cycle phase expression of these proteins, E2F1 is sequestered by RB1 during the G_1 phase. During G_1 phase, RB1 is increasingly phosphorylated by cyclin D–cyclin-dependent kinase complexes and towards the end of G_1, phosphorylated RB1 is released from E2F1, which then is free to exert its function as a transcription factor for genes required for the G_1/S transition and S phase progression. The cell then proceeds from G_1 into the rest of the cell cycle. Apparently this control mechanism does not function in HCC1937 and L56Br-C1 cells.

The cyclin E1–CDK2 complex is important for the G_1/S transition [36]. The only cell line in which there was a lowering of the cyclin E1 level at 24 h of DENSPM treatment was L56Br-C1 where also the CDK2 level decreased; however, this did not affect the rate of G_1/S transition. At 48 h of treatment, the cyclin E1 level had decreased in DENSPM-treated MCF-7, SK-BR-3, and HCC1937 cells whereas there was no change in the CDK2 level. The lowering of cyclin E1 at 48 h of DENSPM treatment may partly explain the decreased rate of G_1/S transition in these three cell lines.

The lengthening of the S phase has been discussed above in conjunction with polyamine pools. Cyclin A2 is important for S phase progression. At the G_1/S transition

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CDK2 switches partner from cyclin E1 to cyclin A2 and this is important for proper S phase progression [37]. In our study we found that DENSPM treatment for 24 h only lowered the cyclin A2 level in L56Br-C1 cells. At 48 h of treatment, the cyclin A2 level was also lowered in SK-BR-3 and HCC1937 cells. Thus, the prolongation of S phase in DENSPM-treated cells at 48 h can partly depend on a decreased level of cyclin A2.

Cyclin B1 is required for efficient $G_2 + M$ progression [38]. Cyclin B1 was lowered in SK-BR-3 and L56Br-C1 cells at 24 h of DENSPM treatment and in all cell lines at 48 h of treatment. In SK-BR-3 cells, however, DENSPM treatment did not prolong the $G_2 + M$ phase substantially. SK-BR-3 cells may be able to proceed almost normally through the $G_2 + M$ phase despite a lowered cyclin B1 level because the putrescine level is sufficiently high. Putrescine is produced by the action of the enzyme ornithine decarboxylase (ODC) on the amino acid ornithine [5–9]. ODC is activated during the $G_2 + M$ phase and ODC mRNA has been demonstrated to have an internal ribosomal entry site required for translation during this cell cycle phase [39]. In a study of the changes of polyamine levels during the cell cycle, we found that a doubling of the putrescine content mainly took place in the G_2 phase of the cell cycle [31]. Thus, much data indicate a role for putrescine in the $G_2 + M$ phase in addition to cyclin B1.

Above we have discussed DENSPM-induced changes in the levels of a number of proteins. It is not clear whether DENSPM-induced polyamine depletion initially affects cell cycle kinetics which then results in lower levels of the proteins investigated or if DENSPM-induced polyamine depletion lowers the protein levels and/or function, which then results in cell cycle kinetic effects. It is our notion that it is a combination of both these alternatives that give the observed cell cycle kinetic effects by DENSPM treatment.

In conclusion, we have shown that there is an ordered effect on cell cycle kinetics as cells are depleted of their polyamines by DENSPM treatment. The S phase is the most sensitive, followed by the $G_2 + M$ phase and the G_1 phase. We have found that the levels of a number of cell cycle regulatory proteins are affected by DENSPM treatment. Presently we cannot say if this is a result of the delay in cell cycle progression or if it causes the delay in cell cycle progression. Presumably it is a combination of both. From a cancer treatment point of view, the two BRCA1- and RB1-deficient cell lines belonging to the basal-like subtype are interesting. These cell lines were the most sensitive to DENSPM treatment indicating that BRCA1 and RB1 deficiencies act as markers for the prediction of successive treatment with polyamine analogues.

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