Normal-like breast cells, but not breast cancer cells, recovered from treatment with *N′*,*N″*-diethylnorspermine

Louise Myhre[a], Kersti Alm[a], Maria C. Johansson[b] and Stina M. Oredsson[a]

A number of polyamine analogs are currently used in various clinical trials as cancer treatment and it is important to investigate their effects not only on cancer cells but also on normal cells. Treatment with polyamine analogs depletes cells of polyamines and inhibits cell proliferation, but the analogs cannot take over the normal function of the natural polyamines in the cell. In this study, the normal-like breast epithelial cell line MCF-10A was treated with the polyamine analog *N′*,*N″*-diethylnorspermine (DENSPM). The cells were then studied using a bromodeoxyuridine–DNA flow cytometry method as well as western blot. The ability of both normal-like and breast cancer cells to recover from DENSPM treatment was also studied. DENSPM treatment of MCF-10A cells resulted in a prolongation of the S and G_2+M phases, followed by a G_1/S block. The p53/p21/RB1 pathway was involved in the G_1/S block as shown by increased levels of p53 and p21 detected by western blot. Decreased levels of cyclin E1, cyclin A2, and cyclin B1 in DENSPM-treated cells can explain the prolongation of cell cycle phases that occurred before the G_1/S block. We also show that MCF-10A cells rapidly recover from DENSPM-induced growth inhibition in contrast to four human breast cancer cell lines. The goal of cancer treatment is to cause minimal and reversible damage to normal cells, while cancer cells should be eliminated. Altogether, the data show that treatment with polyamine analogs spares normal cells, while negatively affecting the cancer cells. *Anti-Cancer Drugs* 20:230–237 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2009, **20:**230–237

Keywords: cell cycle, flow cytometry, human breast cancer, human breast epithelium, polyamine analog

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

Correspondence to Stina Oredsson, Department of Cell and Organism Biology, Lund University, Helgonavägen 3B, SE-223 62 Lund, Sweden
Tel: +46 46 222 9497; fax: +46 46 222 4539;
e-mail: stina.oredsson@cob.lu.se

Received 21 August 2008 **Revised form accepted** 26 November 2008

Introduction

The cell cycle is a series of events where a cell grows and then divides into two daughter cells. Different opposing forces and points of control participate in cell cycle regulation. The driving force of the cell cycle is the activity of cyclin-dependent kinases (CDKs) [1–3]. The CDKs are activated sequentially during the cell cycle by different cyclins. Together, the cyclin/CDK complexes drive the cell through the cell cycle phases and from one phase to another by phosphorylating different substrates. cyclin-dependent kinase inhibitors (CDKIs) function as opposing forces in the cell cycle by binding to the cyclin/CDK complexes thus inactivating them. The cell cycle checkpoint control systems are surveillance systems that scan the cell for errors [4,5]. When error signals are present, the cell is not allowed to progress through the cell cycle.

Polyamines are essential for normal cell cycle progression [6,7]. The intracellular polyamine pools are well regulated through biosynthesis, catabolism, and transport over the cell membrane. During the cell cycle, polyamine biosynthesis and polyamine levels show cyclic patterns [8]. When polyamine biosynthesis is impaired, cell cycle progression is delayed [6,7].

In cancer, the regulatory system of the cell cycle is defective and the cell continues to progress through the cell cycle despite error signals that would have stopped a normal cell. Polyamine metabolism is deregulated and elevated levels of polyamines are found in many forms of cancer, such as breast cancer [9,10]. Consequently, substances that deplete the intracellular polyamine pools are considered and used as chemotherapeutic agents [10–12]. One such group of agents is the polyamine analogs that deplete the polyamine pools by inhibiting polyamine biosynthesis and stimulating polyamine catabolism [10–12]. As the analogs cannot take over the normal function of the polyamines in the cell, cell proliferation is inhibited. One widely used analog is the spermine analog *N′*,*N″*-diethylnorspermine (DENSPM). DENSPM and other polyamine analogs are currently being evaluated in clinical trials in cancer treatment [10,12–14].

In an earlier study, we have investigated the effect of DENSPM treatment on the four human breast cancer cell lines MCF-7, SK-BR-3, HCC1937, and L56Br-C1 [15]. DENSPM treatment induced different cell cycle kinetic effects that were correlated to the levels of proteins involved in cell cycle regulation. Polyamine analog treatment may also induce cell death [16–18].

0959-4973 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328323fc98

The aim of this study was to investigate the cell cycle effects of DENSPM treatment on the normal-like human breast epithelial cell line MCF-10A and to compare the results with our earlier study on four breast cancer cell lines [15]. We have investigated cell cycle kinetic parameters, such as the rate of G_1/S-transition and the lengths of the G_1, S, and G_2 + M phases, using a bromodeoxyuridine (BrdUrd)–DNA flow cytometry method [19–23]. We further investigated how the normal-like cell line and the breast cancer cell lines MCF-7, SK-BR-3, HCC1937, and L56Br-C1 recovered from DENSPM treatment. Our studies showed that treatment with the polyamine analog DENSPM did not cause irreversible harmful effects on normal-like breast epithelial MCF-10A cells, whereas the breast cancer cell lines did not recover well or even not at all. Polyamine analogs are thus effective chemotherapeutic agents in breast cancer cells with specific types of genetic aberrations. These findings support the use of polyamine analogs as chemotherapeutic agents.

Materials and methods

Cell culture

The MCF-10A (CRL-10317), 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, Sweden [24]. The MCF-7, HCC1937, SK-BR-3, and L56Br-C1 cell lines were cultured as described previously [17]. The MCF-10A cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), nonessential amino acids (1 mmol/l), insulin (10 µg/ml), epidermal growth factor (20 ng/ml), cholera toxin (50 ng/ml), hydrocortisol (250 ng/ml), penicillin (50 U/ml), and streptomycin (50 mg/ml). Medium components were purchased from Biochrom (Berlin, Germany) and Sigma–Aldrich (Sweden AB, Stockholm, Sweden). Tissue culture plastics were purchased from Nunc (Roskilde, Denmark).

For all the experiments with MCF-10A except the recovery experiment (see below), a number of replicate cultures, consisting of 1×10^6 plateau phase cells seeded into 12 ml medium in Petri dishes (9 cm diameter) in the absence or presence of 10 µmol/l DENSPM (Tocris Cookson, St. Louis, Missouri, USA), were set up. DENSPM 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 adding to the cell cultures.

Bromodeoxyuridine labeling

The BrdUrd labeling of cells has been described in detail [19–23]. Briefly, 24 or 48 h after seeding the MCF-10A cells, BrdUrd (Sigma, St. Louis, Missouri, USA) was added to the culture medium to a final concentration of 5 µmol/l. After a 30-min labeling period, the medium was removed and the cultures were rinsed twice with medium containing 0.5% heat-inactivated FCS (37°C). The cultures then received BrdUrd-free culture medium without or with 10 µmol/l DENSPM in control and treated, respectively. At 0, 4, 6, 8, 10, and 12 h post-labeling, cells were harvested by trypsinization, pelleted by centrifugation at $700 \times g$, resuspended in ice-cold 70% ethanol (2×10^6 cells/ml), and stored at −20°C before preparation for flow cytometric determination of DNA and BrdUrd contents.

Flow cytometric analysis of bromodeoxyuridine and DNA contents

The staining of total DNA content and incorporated BrdUrd was performed as described earlier [25–27]. In short, the cells were incubated with primary monoclonal anti-BrdUrd antibodies (M744, Dakopatts, Glostrup, Denmark). To allow access of the primary antibody to the BrdUrd molecules, the DNA was partially denatured using 2 mol/l HCl. Secondary fluorescein isothiocyanate-conjugated antibodies (F313, Dakopatts) were used to detect the primary antibodies. Double-stranded regions of DNA were then stained with propidium iodide (Sigma). The cells were analyzed with respect to DNA (red fluorescence) and BrdUrd (green fluorescence) contents in an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, New Jersey, USA). For the computer analysis, Multi2D and Multicycle software programs (Phoenix Flow systems, San Diego, California, USA) were used. The data were analyzed and calculations were made as described earlier [19–23].

Western blot analysis

MCF-10A cells for western blot analysis were harvested by trypsinization, counted in a hemocytometer, and pelleted at $700 \times g$ 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 then immediately put on ice. Aliquots corresponding to 50 000 cells were then loaded in the wells of precast SDS polyacrylamide gels (4–12%, Invitrogen, Carlsbad, California, USA). Note that loading was based on cell number. Electrophoresis was performed in an XCell SureLock Mini-cell electrophoresis system and subsequent blotting by using XCell blot module, from Invitrogen. The membranes were then blocked with 5% dry milk in 0.05% Tween-20 in phosphate-buffered saline before incubation overnight with the primary antibodies. Monoclonal antibodies against p27 (554069), E2F1 (554213), cyclin D1 (554180), cyclin E1 (14591C), RB1 (554136), cyclin A2 (554176), p53 (554294), and cyclin B1 (554176) were purchased from BD PharMingen (San Diego, California, USA). Monoclonal antibodies against p21 (sc-6246) and rabbit polyclonal antibodies against CDK2 (sc-163) were purchased from Santa Cruz

Biotechnology Inc. (Santa Cruz, California, USA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin was used as secondary antibodies (Dakopatts). Advanced enhanced chemiluminescence 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) and the software Quantity One (Bio-Rad Inc.) were used for imaging.

Polyamine analysis

Cells for polyamine analysis were harvested by trypsinization, counted in a hemocytometer, and pelleted at $700 \times g$ for 10 min at 4°C. Quantification of the polyamines was performed in cell homogenates in 0.2 mol/l perchloric acid using a high-performance liquid chromatography method (Hewlett Packard 1100, GMI, Ramsey, Minnesota, USA) [28]. Note that polyamine level determination was based on cell number.

Assay of spermidine/spermine *N*′-acetyltransferase activity

Cells were stored at −80°C until analysis. The cells were sonicated in 50 mmol/l Tris-HCl (pH 7.5) containing 0.25 mol/l sucrose. The activity of spermidine/spermine *N*′-acetyltransferase (SSAT) in the sonicate was determined by measuring the synthesis of [^{14}C]acetylspermidine after incubation with [^{14}C]acetyl-coenzyme A and spermidine [29].

Recovery experiments with *N*′,*N*″-diethylnorspermine

Recovery experiments were performed with MCF-7, HCC1937, SK-BR-3, L56Br-C1, and MCF-10A cells. 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 presence of 10 μmol/l DENSPM, were set up. After 48 h of DENSPM treatment, the medium was removed and the cultures were rinsed twice with medium, containing 0.5% FCS and then fresh culture medium without DENSPM was added. This procedure was performed using preheated 37°C media. The cells were harvested and counted in a hemocytometer 48 or 96 h after the removal of DENSPM. After 48 h of DENSPM treatment, most of the L56Br-C1 cells were dead and counting of cells in the hemocytometer was not possible. Thus, no recovery data are presented for L56Br-C1 cells.

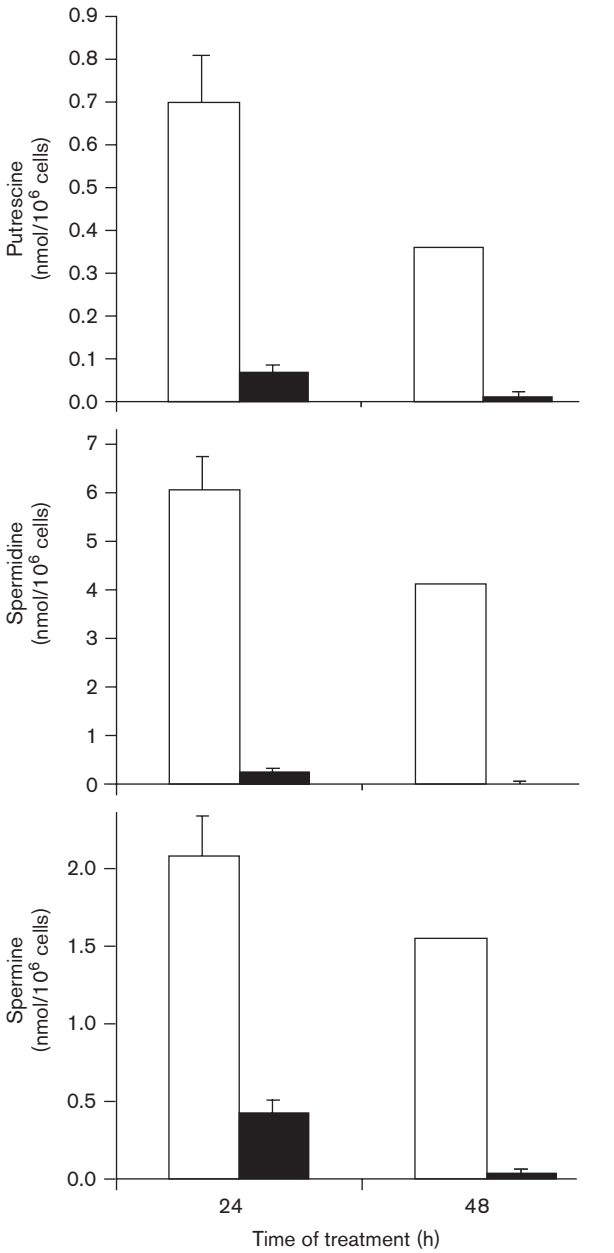
Statistical analysis

For the statistical evaluation of T_S values, two-tailed unpaired Student's *t*-test was used. An unpaired Student's *t*-test was also used to evaluate the recovery of cell proliferation after the removal of DENSPM, comparing MCF-10A cells with the most rapidly recovering cancer cell line MCF-7.

Results

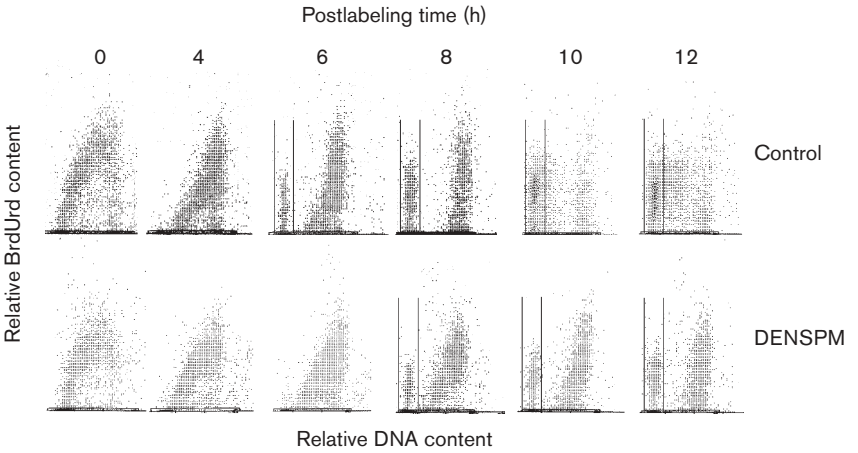
DENSPM treatment of MCF-10A cells resulted in a rapid decrease of the polyamine pools and the pools were almost depleted after 48 h of treatment (Fig. 1). Activation of SSAT contributed to the decrease in the polyamine pools. In control and DENSPM-treated cells,

Fig. 1



Effect of *N*′,*N*″-diethylnorspermine (DENSPM) treatment on polyamine levels in the normal-like breast epithelial cell line MCF-10A. 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. White columns, control; black columns, DENSPM. Bars show the mean of three independent samples ± SD with the exception of control at 48 h after seeding where $n = 2$. The difference between the two samples was less than 20%.

Fig. 2



Cytograms of DNA versus bromodeoxyuridine (BrdUrd) contents showing the effect of *N′*,*N″*-diethylnorspermine (DENSPM) treatment on the length of the G_1 phase in MCF-10A cells. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. After 24 h of treatment, BrdUrd was added to the culture medium to a final concentration of 5 μmol/l. After a 30 min labeling period, the BrdUrd-containing medium was removed and after rinsing, BrdUrd-free medium was added. At 0, 4, 6, 8, 10, and 12 h postlabeling, cells were harvested by trypsinization, pelleted by centrifugation at 700 × *g*, resuspended in ice-cold 70% ethanol, and prepared for flow cytometry-mediated determination of DNA and BrdUrd contents. Representative cytograms from one experiment of two are shown. $n = 3$ for each postlabeling time in each experiment. The figure shows DNA versus BrdUrd cytograms at the various postlabeling times. The lines are set around BrdUrd-labeled divided cells in G_1 phase. In control cells, BrdUrd-labeled divided cells clearly appear in S phase at 10 h postlabeling, that is, cells are found outside the G_1 framing lines in S phase. This information can be used to calculate the length of the G_1 phase. See Table 1.

the SSAT activity was and 1.0 ± 1.3 and 113.1 ± 7.2 pmol/10^6 cells/h (n = 5–6 independent samples from two independent experiments), respectively, at 24 h of treatment. No cell death was observed in DENSPM-treated MCF-10A cells as determined by the absence of a sub-G_1 peak in flow cytometry-derived DNA histograms (not shown).

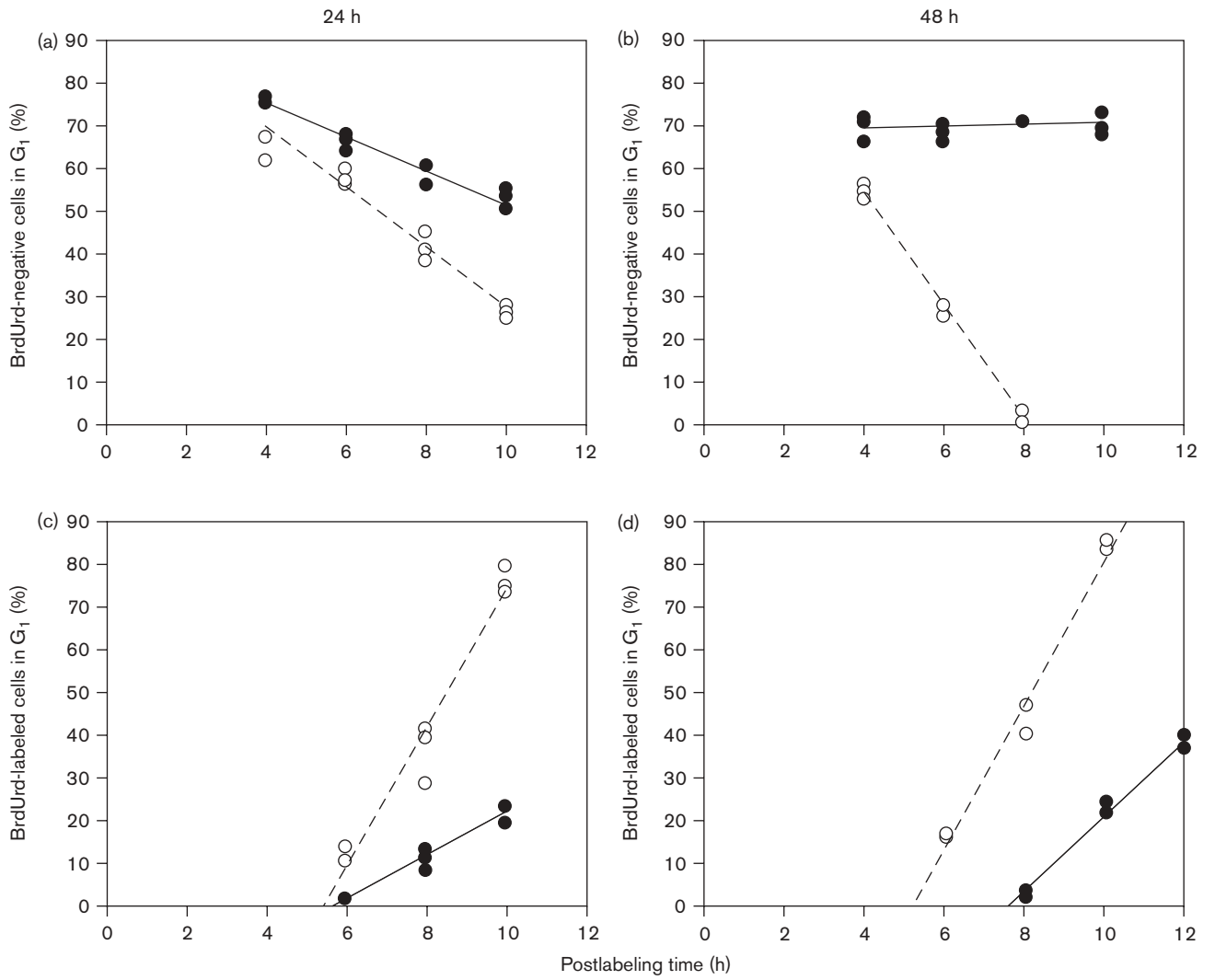
Cell cycle kinetics was markedly affected by DENSPM treatment. After 24 h of DENSPM treatment, the G_1 phase was prolonged as can be seen in Fig. 2, which shows cytograms of DNA versus BrdUrd contents at various times postlabeling. The cytograms at postlabeling time 0 show the distribution of BrdUrd-labeled cells directly after the 30-min labeling period. The BrdUrd-labeled cells are found in S phase directly after labeling. At 6 h postlabeling, there is a clear peak of BrdUrd-labeled cells, which have traversed G_2 and M phases and are found as BrdUrd-labeled divided cells in G_1 phase in the control cultures. A clear peak of BrdUrd-labeled divided cells is visible at 8 h postlabeling in DENSPM-treated cells. At 10 h postlabeling, there is a clear broadening of the peak of BrdUrd-labeled divided cells in control, showing that these cells were passing from G_1 into S phase. Thus, the BrdUrd-labeled divided control cells started to flow into S phase some time between 8 and 10 h postlabeling. This information can be used to calculate an approximate length of the G_1 phase from the following expression: 8 h $< G_1 + G_2 + M <$ 10 h (see below) [23]. Figure 2 shows that none of the BrdUrd-labeled divided cells flowed into S phase during the experimental time period in DENSPM-treated cultures.

Figure 3(a and b) shows the effect of DENSPM treatment on the rate of transition of cells from G_1 into S phase. The data are obtained from DNA histograms of BrdUrd negative cells [19–23]. The slope of the lines indicates the rate of G_1/S transition and the steeper the slope, the higher the rate of G_1/S transition. When the line is horizontal no G_1/S transition takes place. After 24 h of DENSPM treatment, the rate of G_1/S transition was reduced and after 48 h there was a block in the G_1/S transition. Figure 3(c and d) shows the effect of DENSPM treatment on the length of the $G_2 + M$ phase [19–23]. The figures show the proportion of BrdUrd-labeled divided cells; that is, cells which have been labeled while in S-phase, and that have then traversed the $G_2 + M$ phase, and now are detected in the G_1 phase. This population of BrdUrd-labeled divided cells is also visible in Fig. 2, at several postlabeling time points. The intercept of the line with the *x*-axis is an approximate measure of the length of the $G_2 + M$ phase as this is the time point when BrdUrd-labeled divided cells first appear in G_1 phase. DENSPM treatment increased the length of the $G_2 + M$ phase and the actual values are found in Table 1.

Using the expression mentioned above (8 h $< G_1 + G_2 + M <$ 10 h) and a $G_2 + M$ phase of approximately 5 h, the G_1 phase length is calculated to be between 3 and 5 h in control MCF-10A cells (Table 1).

The length of the S phase was calculated as described earlier [23]. The S phase was significantly prolonged by DENSPM treatment for 24 and 48 h (Table 1).

Fig. 3



Effect of *N′*,*N″*-diethylnorspermine (DENSPM) treatment on the rate of G_1/S transition (a, b) and on the length of the G_2+M phase (c, d) in the normal-like breast epithelial cell line MCF-10A. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. After 24 (a, c) or 48 h (b, d) of treatment, bromodeoxyuridine (BrdUrd) was added to the culture medium to a final concentration of 5 μmol/l. After a 30-min labeling period, the BrdUrd-containing medium was removed and, after rinsing, BrdUrd-free medium was added. At 4, 6, 8, 10, and 12 h postlabeling, cells were harvested by trypsinization, pelleted by centrifugation at 700 × *g*, resuspended in ice-cold 70% ethanol, and prepared for flow cytometry-mediated, determination of DNA and BrdUrd contents. Data points from one representative experiment of two are shown. $n=3$ at each time point. When all three symbols are not visible, they cover each other. Data were fitted by regression analysis. ○, control cells; ●, DENSPM-treated cells.

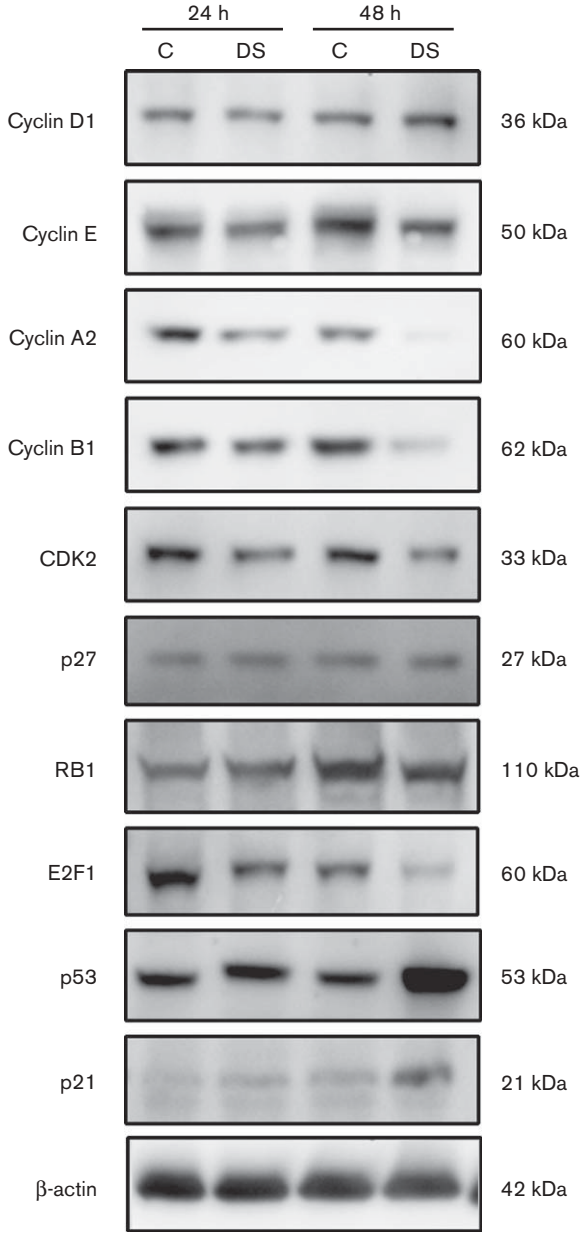
Table 1 Effect of DENSPM treatment[a] on the lengths of the G_1, S, and G_2+M phases in MCF-10A cells

	T_{G_1}[b]	T_S[b]		T_{G_2}[c]	
Time of treatment	24 h	24 h	48 h	24 h	48 h
Control	3–5	6.2 ± 0.6	5.4 ± 0.6	5.4	5.2
DENSPM	–	10.7 ± 0.5*	10.0 ± 1.2*	5.8	7.5

DENSPM, *N′*,*N″* diethylnorspermine.
[a]The cells were seeded and treated as described in the figure captions of Figs 2 and 3.
[b]The lengths (T_{G_1} and T_S) of the G_1 and S phases were calculated as described earlier [18–20,24]. n=9–15 independent samples. The numbers are mean ± SD. For the statistical evaluation, Student's unpaired *t*-test was used.
[c]The length (T_{G_2+M}) of the G_2+M phase was determined from the intercept of the lines with the *x*-axis shown in Fig. 3(c and d).
*$P<0.05$ compared with control.

We investigated the effect of DENSPM treatment on a number of cell cycle regulatory proteins in MCF-10A cells by western blot analysis (Fig. 4). The level of the G_1 cyclin D1 was slightly increased by DENSPM treatment at 48 h of treatment. The level of cyclin E1, which is involved in the G_1/S transition, was decreased after 24 and 48 h of DENSPM treatment. The level of cyclin A2, which is involved in S phase progression, was decreased after 24 h of DENSPM treatment and was almost absent after 48 h of treatment. The level of cyclin B1, which is active in mitosis, was decreased after 24 h of DENSPM treatment and was almost absent after 48 h of treatment.

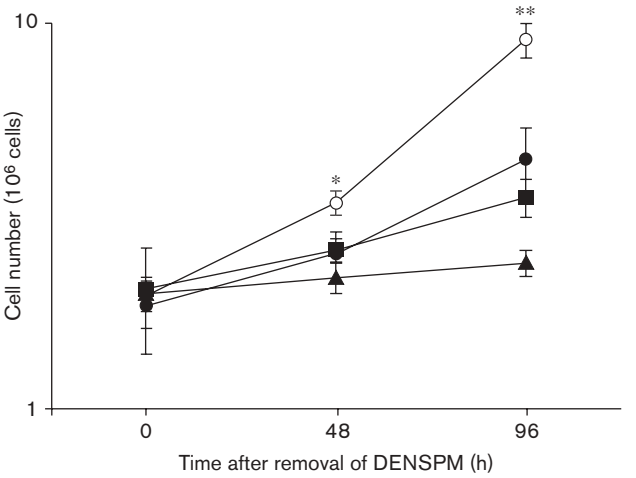
Fig. 4



Effect of *N′*,*N″*-diethylnorspermine (DENSPM) treatment on the level of various cell cycle regulatory proteins in the normal-like breast epithelial cell line MCF-10A evaluated by western blot. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. After 24 and 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 and DS, DENSPM-treated cells.

The levels of other cell cycle regulatory proteins, besides the cyclins, were also affected by DENSPM treatment (Fig. 4). The level of CDK2, which first acts together with cyclin E1 at the G_1/S transition and then switches partner to cyclin A2 in the S phase, was decreased after 24 and 48 h of DENSPM treatment. No differences were observed between control and treated cells regarding the level of p27. The level of RB1 was slightly decreased by DENSPM treatment for 48 h. The gels used in the electrophoresis do not permit an exact evaluation of the phosphorylation status of RB1. The level of the transcription factor E2F1 was decreased by DENSPM treatment for 24 h and E2F1 was almost absent after 48 h. There was a slight increase in the p53 level after 24 h of treatment and a marked increase after 48 h of treatment. MCF-10A cells contain a very low level of p21; however, DENSPM treatment for 48 h resulted in an increased p21 level.

Fig. 5



Recovery of *N′*,*N″*-diethylnorspermine (DENSPM)-induced inhibition of cell proliferation in MCF-7, SK-BR-3, HCC1937, and MCF-10A cells. Cells were seeded in the presence of 10 μmol/l DENSPM. After 48 h of treatment, the DENSPM-containing medium was removed, the cells were rinsed twice and regular growth medium was added. This was defined as time 0 shown in the figure. The cells were then counted at 48 and 96 h after the removal of DENSPM and addition of fresh medium. Data are from three experiments with $n=2$ in each experiment. Bars show ± SD. ○, MCF-10A; ●, MCF-7; ■,SK-BR-3; ▲, HCC1937. The cell numbers of MCF-10A and MCF-7 cultures were compared using an unpaired Student's *t*-test and were found to be significantly different: $*P< 0.05$; $**P<0.001$.

We further investigated how MCF-10A cells recovered from 48 h of DENSPM treatment compared with the four human breast cancer cell lines MCF-7, SK-BR-3, L56Br-C1, and HCC1937 (Fig. 5). The two cell lines most sensitive to DENSPM treatment were HCC1937 and L56Br-C1, both of which have mutated BRCA1 and p53 and they also lack RB1 [15]. The majority of L56Br-C1 cells had died by mitochondrial apoptosis after 48 h of DENSPM treatment [15–17] and no live cells were detected in the hemocytometer and therefore there are no data from L56Br-C1 cells in Fig. 5. HCC1937 cells did not resume cell proliferation within 96 h after the removal of DENSPM. MCF-10A cells recovered excellently from DENSPM treatment. SK-BR-3 and MCF-7 cells recovered slowly after the removal of DENSPM; however, the wild-type p53 containing MCF-7 cell line recovered faster than

the SK-BR-3 cell line, which contains mutated p53. The cell numbers of the normal-like MCF-10A cell line were compared with those of the cancer cell line showing best recovery, that is, MCF-7, using an unpaired Student's *t*-test. The difference was statistically significant at 48 and 96 h after the removal of DENSPM. The significance increased with time pointing to a more rapid recovery of the MCF-10A cells (Fig. 5).

Discussion

The MCF-10A cell line was isolated from fibrocystic tissue of the breast and represents normal-like human breast epithelial cells although there are homozygous deletions of the two cyclin-dependent kinase inhibitors, p15 and p16 [30]. Our cell cycle kinetic study shows that the MCF-10A cells have a short cell cycle time with G_1, S, and $G_2 + M$ phase lengths of approximately 3–5, 6, and 5 h, respectively, resulting in a cell cycle time around 15 h. This is in stark contrast to the cell cycle times of the breast cancer cell lines MCF-7, SK-BR-3, HCC1937, and L56Br-C1, which have considerably longer G_1 and S phase lengths [15]. In the breast cancer cell lines, the G_1 phase was longer than 12 h and the S phase was between 9 and 13 h. The $G_2 + M$ phase was 4–5 h, that is, a length resembling that of MCF-10A cells. Thus, it seems that most of the genetic alterations in the cancer cell lines had a greater impact on the length of the G_1 and S phases than on the length of the $G_2 + M$ phase. This may have implications for cancer treatment as it implies that drugs affecting the $G_2 + M$ phase will not show enough selective toxicity for cancer cells compared with normal cells.

When MCF-10A cells were treated with DENSPM, there was a rapid decrease in polyamine pools. Initially this resulted in a decreased rate of G_1/S transition and a prolongation of the S phase. The increased length of the cell cycle phases may be explained by the increased amounts of p53 found in the cells treated with DENSPM for 24 h. p53 is known to exert transcriptional repression of a number of cyclins and other genes [31], something which may contribute to the decreased cyclin levels found in DENSPM-treated cells. At 48 h of treatment, the function of transcriptional activation of p53 was noted as an increased level of p21. The G_1/S block noted at 48 h of DENSPM treatment is most likely because of the activation of the p53/p21/RB1 pathway. DENSPM treatment has been shown to activate this pathway in a melanoma cell line containing wild-type p53 [32]. The p53/p21/RB1-induced G_1 block presumably protected MCF-10A cells from cell death despite the extensive polyamine depletion induced by DENSPM treatment.

One of the goals in cancer research is to develop chemotherapeutic agents that kill malignant cells without irreversibly disrupting the functions of normal cells. Studies of drug sensitivity and recovery are necessary to understand what causes different responses to the same treatment and to find out treatments that suit specific types of genetic aberrations in breast cancer. We performed studies on normal-like MCF-10A cells as well as on four breast cancer cell lines to determine their potential for recovery. The MCF-10A cells recovered rapidly from DENSPM-induced inhibition of cell proliferation, whereas L56Br-C1 breast cancer cells underwent extensive cell death. The breast cancer cell line MCF-7 containing wild-type p53 also recovered from DENSPM treatment, although less well than the MCF-10A cells. In MCF-7 cells, DENSPM treatment also induced a G_1/S block dependent on the p53/p21/RB1 pathway, something that presumably protected the cells from the detrimental effects of polyamine depletion [15]. MCF-7 cells are of the luminal A breast cancer subtype [30]. SK-BR-3 cells, which contain mutated p53 also recovered to some extent. Our previous study of cell cycle kinetics showed that the SK-BR-3 cell line was least affected of the four cancer cell lines after 48 h of DENSPM treatment [15]. This time is equivalent to the time point when DENSPM was removed in this study. However, SK-BR-3 cells being least affected apparently did not mean that they had the most rapid recovery of the four cancer cell lines as the MCF-7 cells recovered faster.

SK-BR-3 cells are of luminal B breast cancer subtype [30]. Besides the L56Br-C1 cell line, HCC1937 cells were most sensitive to DENSPM treatment and these cells did not show any recovery within the experimental period. These two cell lines both belong to the basal-like subtype group of breast cancer [30], indicating that this subgroup may be most responsive to DENSPM treatment. Thus, it seems as if both the subtype groups and the p53 status are important determinants for the recovery from DENSPM treatment.

We have shown that rapid polyamine depletion results in mitochondrial apoptosis in L56Br-C1 breast cancer cells [16,17]. Importantly, this shows a difference in response to polyamine analog-induced polyamine deficiency between a normal cell and a cancer cell, which should be exploited in the treatment of cancer. Our studies as well as other studies, however, show that not all cancer cell lines react with the rapid cell death that is found in L56Br-C1 cells [33]. Thus, it is important to find biomarkers for sensitivity to polyamine analog treatment. In conclusion, our data show that DENSPM-induced polyamine deficiency results in a prolongation of the cell cycle phases in MCF-10A cells, which is presumably caused by decreased levels of cyclins and other proteins.

We also show that the normal-like human breast epithelial cell line MCF-10A recovers rapidly from DENSPM-induced cell cycle effects. The breast cancer cell lines

do not recover to the same extent, and breast cancer cell lines of the basal-like subtype seem to be most susceptible to DENSPM treatment.

Acknowledgements

The authors thank Ewa Dahlberg for expert technical assistance and Lena Thiman for help with the polyamine analysis. The authors wish to thank Prof. Bo Baldetorp for the use of the flow cytometer at the Department of Oncology, Clinical Sciences, Lund University. Support sources are the Gunnar Nilsson Cancer Foundation, the Royal Physiographical Society in Lund, the Mrs. Berta Kamprad Cancer Foundation, the Crafoord Foundation, the University Hospital of Lund Foundation, and the Per-Eric and Ulla Schybergs Foundation.

References

1 Sanchez I, Dynlacht BD. New insights into cyclins, CDKs, and cell cycle control. *Semin Cell Dev Biol* 2005; **16**:311–321.
2 Sherr CJ. Cancer cell cycles. *Science* 1996; **274**:1672–1677.
3 Caldon CE, Daly RJ, Sutherland RL, Musgrove EA. Cell cycle control in breast cancer cells. *J Cell Biochem* 2006; **97**:261–274.
4 Nakayama K, Nakayama K. Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development. *Bioessays* 1998; **20**:1020–1029.
5 Sherr CJ. The INK4a/ARF network in tumour suppression. *Nat Rev Mol Cell Biol* 2001; **2**:731–737.
6 Thomas T, Thomas TJ. Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell Mol Life Sci* 2001; **58**:244–258.
7 Oredsson SM. Polyamine dependence of normal cell-cycle progression. *Biochem Soc Trans* 2003; **31**:366–370.
8 Fredlund JO, Johansson MC, Dahlberg E, Oredsson SM. Ornithine decarboxylase and *S*-adenosylmethionine decarboxylase expression during the cell cycle of Chinese hamster ovary cells. *Exp Cell Res* 1995; **216**:86–92.
9 Pegg AE. Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. *Cancer Res* 1988; **48**:759–774.
10 Casero RA Jr, Marton LJ. Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. *Nat Rev Drug Discov* 2007; **6**:373–390.
11 Seiler N. Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 2. Structural analogues and derivatives. *Curr Drug Targets* 2003; **4**:565–585.
12 Seiler N. Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 1. Selective enzyme inhibitors. *Curr Drug Targets* 2003; **4**:537–564.
13 Wolff AC, Armstrong DK, Fetting JH, Carducci MK, Riley CD, Bender JF, *et al*. A Phase II study of the polyamine analog *N′*,*N″*-diethylnorspermine (DENSpm) daily for five days every 21 days in patients with previously treated metastatic breast cancer. *Clin Cancer Res* 2003; **9**:5922–5928.
14 Hahm HA, Ettinger DS, Bowling K, Hoker B, Chen TL, Zabelina Y, *et al*. Phase I study of *N′*,*N″*-diethylnorspermine in patients with non-small cell lung cancer. *Clin Cancer Res* 2002; **8**:684–690.
15 Myhre L, Alm K, Hegardt C, Staff J, Jönsson G, Larsson S, *et al*. Different cell cycle kinetic effects of *N′*,*N″*-diethylnorspermine-induced polyamine depletion in four human breast cancer cell lines. *Anti-Cancer Drugs* 2008; **19**:359–368.
16 Hegardt C, Johannsson OT, Oredsson SM. Rapid caspase-dependent cell death in cultured human breast cancer cells induced by the polyamine analogue *N′*,*N″*-diethylnorspermine. *Eur J Biochem* 2002; **269**: 1033–1039.
17 Holst CM, Oredsson SM. Comparison of three cytotoxicity tests in the evaluation of the cytotoxicity of a spermine analogue on human breast cancer cell lines. *Toxicol In vitro* 2005; **19**:379–387.
18 Holst CM, Frydman B, Marton LJ, Oredsson SM. Differential polyamine analogue effects in four human breast cancer cell lines. *Toxicology* 2006; **223**:71–81.
19 Begg AC, McNally NJ, Shrieve DC, Karcher H. A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. *Cytometry* 1985; **6**:620–626.
20 Fredlund JO, Oredsson SM. Normal G_1/S transition and prolonged S phase within one cell cycle after seeding cells in the presence of an ornithine decarboxylase inhibitor. *Cell Prolif* 1996; **29**:457–466.
21 Fredlund JO, Oredsson SM. Ordered cell cycle phase perturbations in Chinese hamster ovary cells treated with an *S*-adenosylmethionine decarboxylase inhibitor. *Eur J Biochem* 1997; **249**:232–238.
22 Alm K, Berntsson PSH, Kramer DL, Porter CW, Oredsson SM. Treatment of cells with the polyamine analog *N′*,*N″*-diethylnorspermine retards S phase progression within one cell cycle. *Eur J Biochem* 2000; **267**:4157–4164.
23 Nasizadeh S, Myhre L, Thiman L, Alm K, Oredsson S, Persson L. Importance of polyamines in cell cycle kinetics as studied in a transgenic system. *Exp Cell Res* 2005; **308**:254–264.
24 Johannsson OT, Staff S, Vallon-Christersson J, Kytola S, Gudjonsson T, Rennstam K, *et al*. Characterization of a novel breast carcinoma xenograft and cell line derived from a BRCA1 germ-line mutation carrier. *Lab Invest* 2003; **83**:387–396.
25 Dolbeare F, Gratzner H, Pallavicini MG, Gray JW. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc Natl Acad Sci U S A* 1983; **80**:5573–5577.
26 Schutte B, Reynders MM, van Assche CL, Hupperets PS, Bosman FT, Blijham GH. An improved method for the immunocytochemical detection of bromodeoxyuridine labeled nuclei using flow cytometry. *Cytometry* 1987; **8**:372–376.
27 Van Erp PE, Brons PP, Boezeman JB, de Jongh GJ, Bauer FW. A rapid flow cytometric method for bivariate bromodeoxyuridine/DNA analysis using simultaneous proteolytic enzyme digestion and acid denaturation. *Cytometry* 1988; **9**:627–630.
28 Seiler N, Knodgen B. Determination of amino acids by separation of their ion pairs with dodecyl sulphate. *J Chromatogr* 1985; **341**:11–21.
29 Matsui I, Wiegand L, Pegg AE. Properties of spermidine *N*-acetyltransferase from livers of rats treated with carbon tetrachloride and its role in the conversion of spermidine to putrescine. *J Biol Chem* 1981; **256**:2454–2459.
30 Jonsson G, Staaf J, Olsson E, Heidenblad M, Vallon-Christersson J, Osoegawa K, *et al*. High-resolution genomic profiles of breast cancer cell lines assessed by tiling BAC array comparative genomic hybridization. *Genes Chromosomes Cancer* 2007; **46**:543–558.
31 Spurgers KB, Gold DL, Coombes KR, Bohnenstiehl NL, Mullins B, Meyn RE, *et al*. Identification of cell cycle regulatory genes as principal targets of p53-mediated transcriptional repression. *J Biol Chem* 2006; **281**:25134–25142.
32 Kramer DL, Vujcic S, Diegelman P, Alderfer J, Miller JT, Black JD, *et al*. Polyamine analogue induction of the p53-p21WAF1/CIP1-Rb pathway and G1 arrest in human melanoma cells. *Cancer Res* 1999; **59**:1278–1286.
33 Oredsson SM, Alm K, Dahlberg E, Holst CM, Johansson VM, Myhre L, *et al*. Inhibition of cell proliferation and induction of apoptosis by *N′*,*N″*-diethylnorspermine-induced polyamine pool reduction. *Biochem Soc Trans* 2007; **35**:405–409.