Eur. J. Biochem. **269**, 1033–1039 (2002) © FEBS 2002

Rapid caspase-dependent cell death in cultured human breast cancer cells induced by the polyamine analogue N^1,N^{11}-diethylnorspermine

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The spermine analogue N^1,N^{11}-diethylnorspermine (DENSPM) efficiently depletes the cellular pools of putrescine, spermidine and spermine by down-regulating the activity of the polyamine biosynthetic enzymes and up-regulating the activity of the catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT). In the breast cancer cell line L56Br-C1, treatment with 10 μM DENSPM induced SSAT activity 60 and 240-fold at 24 and 48 h after seeding, respectively, which resulted in polyamine depletion. Cell proliferation appeared to be totally inhibited and within 48 h of treatment, there was an extensive apoptotic response. Fifty percent of the cells were found in the sub-G_1 region, as determined by flow cytometry, and the presence of apoptotic nuclei was morphologically assessed by fluorescence microscopy. Caspase-3 and caspase-9 activities were significantly elevated 24 h after seeding. At 48 h after seeding, caspase-3 and caspase-9 activities were further elevated and at this time point a significant activation of caspase-8 was also found. The DENSPM-induced cell death was dependent on the activation of the caspases as it was inhibited by the general caspase inhibitor Z-Val-Ala-Asp fluoromethyl ketone. The results are discussed in the light of the L56Br-C1 cells containing mutated *BRCA1* and *p53*, two genes involved in DNA repair.

Keywords: apoptosis; breast cancer cells; caspase; DNA fragmentation; N^1, N^{11}-diethylnorspermine.

The polyamines putrescine, spermidine and spermine are cationic molecules that are essential for cell proliferation and differentiation [1]. A number of studies show that they have a role in apoptosis [2–5] as well. The biosynthesis and catabolism of the polyamines are tightly regulated, which implicates the importance of a balance of polyamine levels in the cell. Careful regulation of the transport of polyamines in and out of the cell also participates in keeping the polyamine pools at an appropriate level for the ongoing cellular activities.

The function of the polyamines has been studied by the use of different biosynthesis inhibitors [1,6]. A disadvantage of using these inhibitors alone is that they usually fail to deplete the cells of all three polyamines. Subsequently, polyamine analogues have been synthesized and some of them have been shown to efficiently deplete all cellular polyamine pools without mimicking the cellular functions of the polyamines [7]. One such analogue of spermine is N^1,N^{11}-diethylnorspermine (DENSPM) which induces a rapid depletion of all polyamines by downregulating the activity of the biosynthetic enzymes and upregulating the activity of the catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) [8]. The effect of DENSPM treatment has been studied extensively in different cell lines and animal tumour models. In two human bladder cancer cell lines, DENSPM showed substantial antiproliferative activity [9]. A number of human solid tumour xenografts were found to be sensitive to DENSPM, as shown by tumour regression, inhibition of tumour growth and sustained antitumour response [10]. Antitumour activity has also been observed in human prostate carcinoma cells both *in vitro* and *in vivo* [11,12]. In MALME-3M human melanoma cells, the growth inhibition induced by DENSPM treatment was subsequently followed by apoptosis [13]. In SK-MEL-28 human melanoma cells, DENSPM treatment appeared to induce growth inhibition and apoptosis concomitantly within 48 h of DENSPM treatment [14].

Apoptosis is induced via distinct signal transduction pathways [15,16]. They involve the activation of a number of caspases that are responsible for many of the morphological features associated with this kind of cell death. Caspases can activate one another through proteolytic cleavage and hence initiate specific caspase cascades [16]. The activation of downstream caspases may serve as an amplification step [17]. The end result is the cleavage of proteins and fragmentation of DNA.

We have treated various human breast cancer cell lines (MCF-7, SK-BR-3, BT-474) with DENSPM and found an initial growth inhibition followed by a delayed apoptotic response (S. M. Oredsson, unpublished results). However, we have established a human breast cancer cell line (L56Br-C1) that shows a similar response to DENSPM treatment as found in human melanoma SK-MEL-28 cells [14]. There was an extensive growth inhibition and apoptotic response within 48 h of DENSPM treatment. This led us to

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Abbreviations: DENSPM, N^1,N^{11}-diethylnorspermine; *p*NA, *p*-nitroanilide; SSAT, spermidine/spermine N^1-acetyltransferase; Z-VAD.FMK, Z-Val-Ala-Asp fluoromethyl ketone.

Enzyme: SSAT, spermidine/spermine N^1-acetyltransferase (EC 2.3.1.57).

(Received 4 October 2001, revised 7 December 2001, accepted 18 December 2001)

investigate the mechanism behind the DENSPM-induced cell death in the L56Br-C1 cells with the further aim of identifying the markers for an apoptotic response to polyamine depletion. The L56Br-C1 cell line was established from malignant tissue of a woman with a germ-line mutation in the breast cancer associated gene *BRCA1*. In addition, the cells also had a mutated *p53* gene. The results are discussed in the light of finding tumour treatment regimens that are tailored to individual tumours.

MATERIALS AND METHODS

Materials

Growth medium components were purchased from Biochrom (Berlin, Germany) and tissue culture plastics from Nunc (Roskilde, Denmark). DENSPM was purchased from Tocris Cookson Ltd. (Bristol, UK) and propidium iodide was obtained from Sigma Chemical Co. (St Louis, MO, USA). [*Acetyl*-1–^{14}C]coenzyme A (60 mCi·mmol^{-1}) was purchased from New England Nuclear, Dupont, Scandinavia AB (Stockholm, Sweden). Caspase-3, -8, -9 Colorimetric Protease Assay Kits and the ICE-family protease/caspase inhibitor Z-Val-Ala-Asp fluoromethyl ketone (Z-VAD.FMK) were purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan).

Cell culture

The cell line LS6Br-C1 was established at the Department of Oncology, the Jubileum Institute, Lund University, Sweden from a patient belonging to a family carrying a known *BRCA1* germ-line mutation (O. T. Johansson, unpublished work). The presence of the germ-line mutation found in the primary tumour, position 1806 C $\rightarrow$ T, was verified in the cell line (personal communication; Å. Borg, Department of Oncology, The Jubileum Institute, Lund University, Lund, Sweden). Sequencing of the *p53* gene revealed a somatic missense mutation in exon 6, position 644 AGT $\rightarrow$ ATT (amino-acid number 215, i.e. serine is changed to isoleucine), which renders p53 nonfunctional [18].

The cell line was maintained in serial passages in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 μg·mL^{-1} insulin, 20 ng·mL^{-1} epidermal growth factor, nonessential amino acids and antibiotics (100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin). The cells were subcultured once weekly and the growth medium was exchanged twice between subcultures. The cultures were incubated at 37 °C in a water-saturated atmosphere containing 5% CO_2 in air. The growth of the cells was monitored at each passage by counting in a haemocytometer and the cells were regularly grown without antibiotics to exclude cryptic infections. Cells were thawed from a frozen stock every 4 months to minimize phenotypic drift. Cells were seeded in the absence or presence of 10 μM DENSPM. DENSPM was made as a 2 mM stock solution in NaCl/P$_i$ (8 g·L^{-1} NaCl, 0.2 g·L^{-1} KCl, 1.15 g·L^{-1} Na_2HPO_4, 0.2 g·L^{-1} KH_2PO_4, pH 7.3). The solution was sterilized by filtration, aliquoted and stored at −20 °C. All treatments were also combined with 10 μM Z-VAD.FMK to ascertain an involvement of caspases where cell death was induced. Both detached (apoptotic cells) and attached cells were harvested at 24 and 48 h after treatment, pelleted at 900 ***g*** for 10 min at 4 °C and handled for analyses as described below.

Polyamine analysis

Cells were stored at −20 °C until analysis. Chromatographic separation and quantitative determination of the polyamines in cell extracts in 0.2 M perchloric acid were carried out using HPLC (Hewlett Packard 1100), with *O*-phtaldialdehyde as the reagent [19].

SSAT activity analysis

Cells were stored at −80 °C until analysis. The cells were sonicated in 50 mM Tris/HCl (pH 7.5) containing 0.25 M sucrose. The activity of SSAT in the sonicate was determined by measuring the synthesis of [^{14}C]acetylspermidine after incubation with [^{14}C]acetyl coenzyme A and spermidine [20].

Flow cytometry and data analysis

Cells were resuspended in ice-cold 70% ethanol and then stored at −20 °C until analysis. The cellular DNA was stained with propidium iodide-nuclear isolation medium (NaCl/P$_i$ containing 100 μg·mL^{-1} propidium iodide, 0.60% Nonidet P-40 and 100 μg·mL^{-1} RNase A) [21].

Flow cytometric analysis was performed in an Ortho Cytoron Absolute flow cytometer (Ortho Raritan, NJ, USA) as previously described [22].

For the computerized analysis of the sub-G_1 peak, MULTI2D® and MULTICYCLE® software programs (Phoenix Flow Systems, CA, USA) were used. Its percentage of the total DNA histogram was evaluated.

Fluorescence microscopy

Ethanol-fixed cells were stained with propidium iodide-nuclear isolation medium. The stained nuclei were then examined in a fluorescence microscope (Olympus AX70, Tokyo, Japan) and photographs were taken with an Olympus DP50.

Caspase activity assay

Cells were resuspended in 50 μL of cell lysis buffer and stored at −80 °C until analysis. The caspase activity was assayed by measuring the cleavage of the chromophore *p*-nitroanilide (*p*NA) from a *p*NA-labelled substrate according to the manufacturer's instructions. The assay samples were incubated with 200 μM *p*NA-substrate at 37 °C for 2 h before measurement of the absorbance at 405 nm using a spectrophotometer.

Statistical analysis

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

RESULTS

When L56Br-C1 cells were seeded in the presence of 10 μM DENSPM, the cell number started to decrease already at

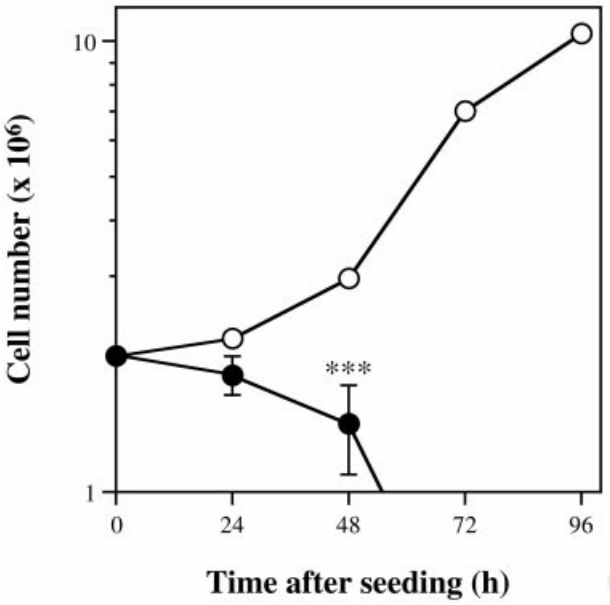

Fig. 1. The effect of DENSPM treatment on the proliferation of L56Br-C1 cells. At time 0, cells were seeded in the absence or presence of 10 μM DENSPM. Results are presented as mean values ($n = 24$ at 24 and 48 h; $n = 3$ at 72 and 96 h). Bars represent ± SEM. When not visible, they are covered by the symbols. ○, Control cells; ●, DENSPM-treated cells. ***, $P < 0.001$.

24 h after seeding and the cell number was significantly ($P < 0.001$) decreased at 48 h after treatment (Fig. 1). At 48 h after seeding, all DENSPM-treated cells were in fact detached and the cells were difficult to discern due to fragmentation. At 72 and 96 h after treatment, it was not possible to detect any intact cells. All cells were also detached after 48 h of treatment with 10 μM DENSPM when the drug was added 24 h after seeding (results not shown).

To confirm the effect of DENSPM on polyamine homeostasis, polyamine levels and SSAT activity were measured. As expected, treatment with 10 μM DENSPM resulted in decreased polyamine pools compared to control (Fig. 2). Putrescine was depleted at 48 h after seeding. Spermidine was significantly ($P < 0.01$) decreased at 24 h after treatment and spermine was significantly decreased at both 24 ($P < 0.001$) and 48 h ($P < 0.01$). The activity of SSAT was markedly induced with DENSPM treatment (Fig. 3). A 60-fold increase in activity could be observed at 24 h, and at 48 h the increase was almost 240-fold compared to control.

Using various methods, we investigated the nature of the rapid cell death found in DENSPM-treated L56Br-C1 cells. Using flow cytometry, we examined if DENSPM treatment induced a sub-G_1 peak and if that could be reversed by adding the general caspase inhibitor Z-VAD.FMK. The percentage of cells in the sub-G_1 region was significantly ($P < 0.001$) increased at 24 h with DENSPM treatment, and at 48 h approximately 50% of the cells were found in this region (Fig. 4). When treating the cells with 1 μM DENSPM, fragmentation of the DNA could also be observed, but the percentage of cells in the sub-G_1 region was lower than when treating the cells with 10 μM DENSPM (results not shown). Addition of Z-VAD.FMK to DENSPM-treated cells decreased the percentage of cells in the sub-G_1 region to

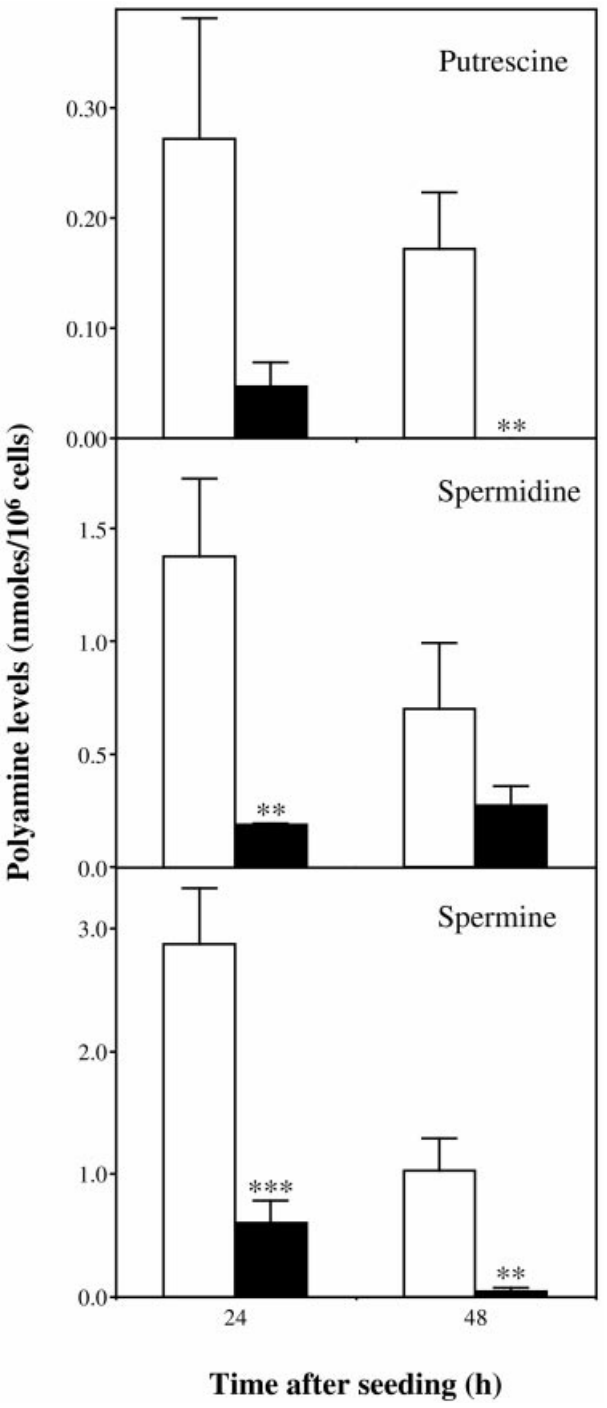

Fig. 2. The effect of DENSPM treatment on the polyamine content of L56Br-C1 cells. Cells were seeded in the absence or presence of 10 μM DENSPM. The results are presented as mean values ($n = 6$) and bars represent ± SEM. White bars, control cells; black bars, DENSPM-treated cells. **, $P < 0.01$; ***, $P < 0.001$.

control values (Fig. 4). When studying the propidium iodide-stained nuclei of DENSPM-treated cells in the fluorescence microscope, apoptotic bodies could clearly be seen (Fig. 5). The appearance of apoptotic bodies was prevented with the addition of Z-VAD.FMK.

Caspase-3, -8 and -9 were activated in L56Br-C1 cells treated with DENSPM (Fig. 6). A significant ($P < 0.05$)

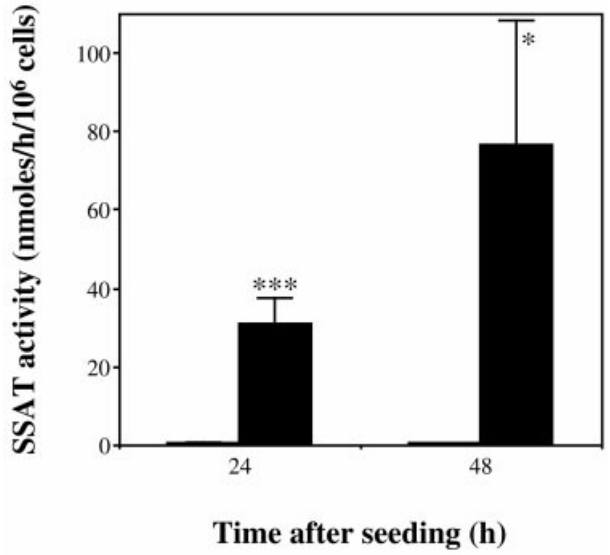

Fig. 3. The effect of DENSPM treatment on the activity of spermidine/spermine N^1-acetyltransferase (SSAT) in L56Br-C1 cells. Cells were seeded in the absence or presence of 10 μM DENSPM. The results are presented as mean values ($n = 6$) and bars represent ± SEM. White bars, control cells; black bars, DENSPM-treated cells. *, $P < 0.05$; ***, $P < 0.001$.

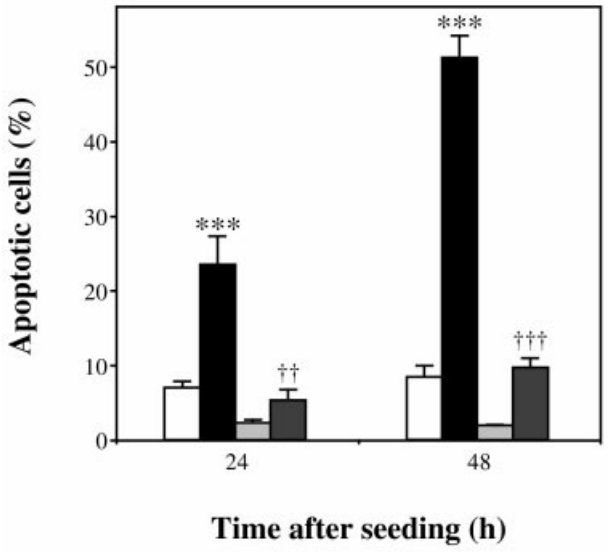

Fig. 4. The percentage of cells in the sub-G_1 region as a measure of apoptotic cells. The L56Br-C1 cells were seeded in the absence or presence of 10 μM DENSPM with or without the addition of the general caspase inhibitor Z-VAD.FMK. The results are presented as mean values ($n = 10$ for control or DENSPM-treated cells; $n = 3$ for Z-VAD.FMK treatment; $n = 7$ for DENSPM + Z-VAD.FMK treatment) and bars represent ± SEM. White bars, control cells; black bars, DENSPM-treated cells; light grey bars, Z-VAD.FMK-treated cells; dark grey bars, DENSPM- and Z-VAD.FMK-treated cells. ***, $P < 0.001$ compared to control cells. ††, $P < 0.01$; †††, $P < 0.001$ compared to DENSPM-treated cells.

increase in caspase-3 activity could be observed at 24 h compared to control, and at 48 h the increase in activity was even higher. A significant ($P < 0.001$) increase in caspase-8 activity was observed but not until 48 h after treatment. Caspase-9 activity was significantly higher in DENSPM-treated cells at both 24 ($P < 0.05$) and 48 h ($P < 0.001$) after seeding even though the activity was low at 24 h.

DISCUSSION

In most cell lines and animal tumour models, the effect of DENSPM treatment is growth inhibition. Cytotoxic effects have mostly been seen with chronic exposure of the drug. Rapid and extensive induction of cell death (within 48 h of treatment) has been observed in SK-MEL-28 cells [14], a human melanoma cell line that contains a mutated *p53* gene. In the present work, DENSPM was also found to rapidly and extensively induce cell death in the human breast cancer cell line L56Br-C1. This cell line carries a germ-line mutation (1806 C → T) in the *BRCA1* tumour

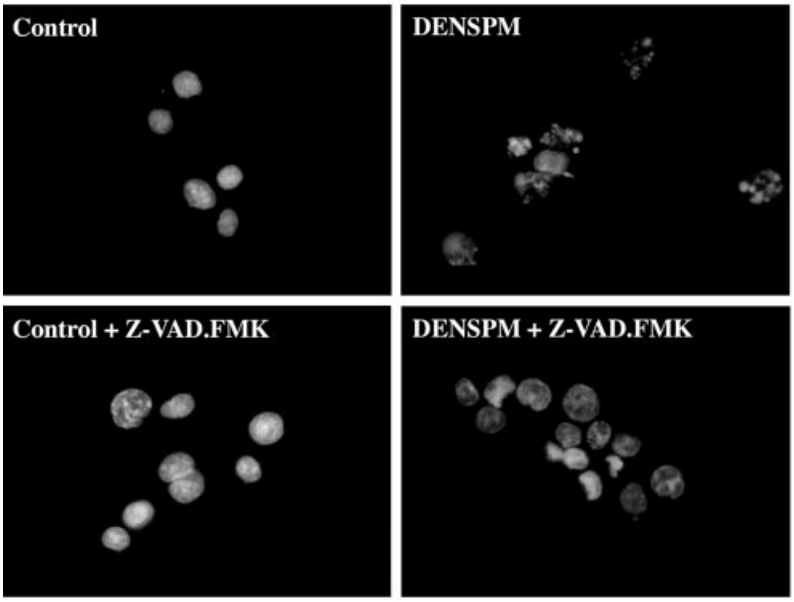

Fig. 5. Propidium iodide-stained nuclei of L56Br-C1 cells. Cells were seeded in the absence or presence of 10 μM DENSPM with or without the addition of Z-VAD.FMK. The diameter of an intact nucleus is 20–25 μm. Results presented are from one representative experiment.

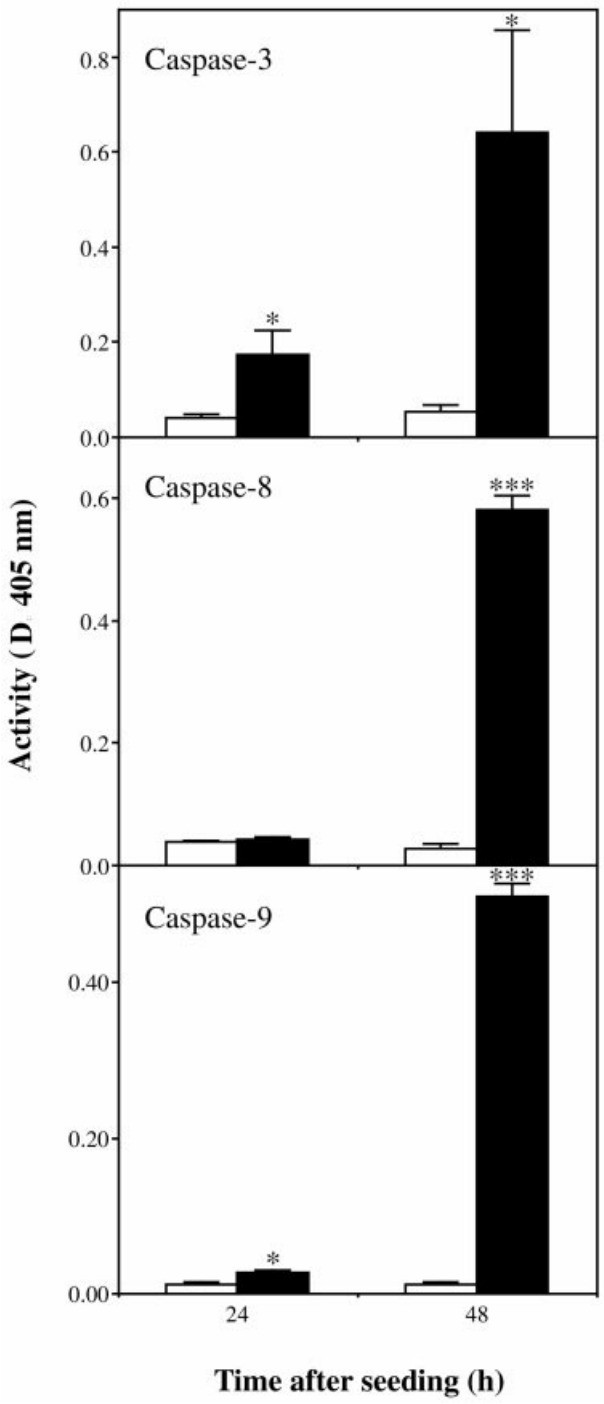

Fig. 6. The effect of DENSPM treatment on the activities of caspase-3, -8 and -9. L56Br-C1 cells were seeded in the absence or presence of 10 μM DENSPM. The results are presented as mean values ($n = 6$) and bars represent ± SEM. White bars, control cells; black bars, DENSPM-treated cells. *, $P < 0.05$; ***, $P < 0.001$.

suppressor gene, the most commonly detected alteration in hereditary breast cancer. The BRCA1 protein is thought to have a role in DNA repair and cell cycle control [23,24]. The cells also have a somatic *p53* mutation. The high sensitivity to DENSPM is interesting in light of the fact that the tumour in the patient was highly refractive to various anticancer treatment regimens including chemotherapy and radiotherapy. DENSPM and other polyamine analogues are presently undergoing Phase I and Phase II clinical evaluations in the US.

In L56Br-C1 cells, DENSPM treatment induced an increase in SSAT activity, which resulted in a decrease in the polyamine pools. The spermine analogue thus activated the catabolism of the natural polyamines. DENSPM presumably also decreased the activities of biosynthetic enzymes. However, we have not measured these activities, as the excessive increase in SSAT is thought to be the primary cause for the decrease in the polyamine pools. We observed a 60- and 240-fold increase in SSAT activity at 24 h and 48 h, respectively, after seeding in the presence of DENSPM. The correlation between the DENSPM-induced increase in SSAT activity and the cellular outcome (inhibition of cell proliferation vs. apoptosis) of DENSPM treatment is not clear. However, a tendency towards higher sensitivity to the drug with massive induction of the catabolic enzyme has been observed when comparing different cell lines [9,12,14]. In the polyamine metabolic pathway, the induction of SSAT results in the acetylation of spermine and spermidine, which are subsequently oxidized by polyamine oxidase to form spermidine and putrescine, respectively. In addition, stoichiometric amounts of acetamidopropanal and H_2O_2 are formed. These latter products have also been suggested to be involved in apoptosis related to analogue induction of SSAT [25]. In MALME-3M and SK-MEL-28 cells the increase in SSAT activity was 650- and 900-fold, respectively, 24 h after seeding [14]. In the former cell line, DENSPM treatment resulted in growth inhibition with a delayed onset of apoptosis and in the latter cell line, apoptosis was found as an early response to DENSPM treatment. In L56Br-C1 cells, the DENSPM-induced increase in SSAT activity was not as extensive as in any of those two cell lines. The depletion of the polyamine pools was however, similar in all three cell lines. The differences in response to DENSPM treatment are presumably reflected in other genetic lesions in the cell lines. One difference between MALME-3M and SK-MEL-28 cells is that the former have the wild-type *p53* gene, while the latter has a mutated *p53* gene resulting in different activation of various cell cycle check point controls [14]. Besides having a mutated *p53* gene, L56Br-C1 cells have a mutated *BRCA1* gene. As polyamines have a role in the stabilization and integrity of DNA [26–28], polyamine depletion is likely to be more deleterious in cells where two genes that are indirectly (*p53*) and directly (*BRCA1*) involved in DNA repair are nonfunctional. *BRCA1* is not mutated in the MCF-7, SK-BR-3 and BT-474 cell lines where we have seen a delayed apoptotic response to DENSPM treatment (S. M. Oredsson, unpublished results). The MCF-7 cell line has a wild-type *p53* gene while the other two have a mutated *p53* gene.

The results presented suggest that the cell death induced by DENSPM treatment in L56Br-C1 cells indeed was apoptotic. Most 'stress-induced' apoptotic processes proceed via the mitochondrial pathway [16] and we believe that this pathway is activated in DENSPM-treated L56Br-C1 cells. In fact, it has just recently been shown that the mitochondrial apoptotic signalling pathway was activated in DENSPM-treated SK-MEL-28 cells [25], supporting our notion of the mitochondrial pathway being involved in DENSPM-induced cell death in L56Br-C1 cells. The

pathway involves a change in mitochondrial transmembrane potential and in the release of cytochrome *c* from mitochondria. Cytochrome *c* then binds to apoptosis-activating factor 1 and procaspase-9 forming the apoptosome complex that results in activation of caspase-9 by proteolytic cleavage [29]. In this pathway, caspase-3 and -8 are effector caspases activated in turn downstream in the cascade [17]. The higher activation of caspase-3 compared to caspase-9 observed in the DENSPM-treated L56Br-C1 cells at 24 h was probably due to the caspase cascade amplification mechanism. The activated caspase-3 then subsequently activated caspase-8. Other polyamine analogues besides DENSPM have been reported to induce cell death, however, the molecular mechanisms behind the observations have so far not been reported [30].

Apoptotic responses induced by a diverse number of signals are thought to be dependent on p53. Potent DNA-damaging agents are commonly used in cancer chemotherapy and tumour regression after chemotherapy is caused, at least in part, by the ability of DNA damaging agents to activate apoptosis. Mutations in the tumour suppressor *p53* gene are the most frequently reported gene alterations in human cancers. Many cancers seem to be inherently resistant to chemotherapy and apoptosis and this has been attributed to the inactivation of p53 [31]. The successful treatment of p53-deficient tumours is dependent on the development of therapeutic strategies that preferentially induce apoptosis in p53-deficient cells. Apparently, the activation of the mitochondrial apoptotic pathway in DENSPM-treated L56Br-C1 occurs in a p53-independent manner. Thus, DENSPM has the potential to be a drug that can induce apoptosis in tumours with a mutated *p53* gene. However, a deficiency in p53 is not the sole determinant of a rapid apoptotic outcome of DENSPM treatment. There are reports of p53-deficient cells that are inhibited in their growth with no apoptotic response by treatment with DENSPM [32,33]. Other cellular defects are involved and as mentioned above, that may, for example, be important DNA repair genes.

One aim in the treatment of any cancer is to develop treatment strategies that are tailored to individual tumours and patients in order to maximize survival. Treatment strategies should preferably kill the tumour cells rather than just inhibiting their growth, although stable growth inhibition might be an acceptable alternative. Another important property of an anticancer treatment is to minimize the damage to normal cells. DENSPM and other polyamine analogues may have different toxic effects on normal cells and cancer cells. In the present study, we have shown that DENSPM induces mitochondrial dependent apoptosis in L56Br-C1 cells which contain both mutated *BRCA1* and *p53* genes. Our aim is to further clarify the molecular and genetic mechanisms for the sensitivity to DENSPM in the hope of finding a clinically usable marker for sensitivity.

ACKNOWLEDGEMENTS

We wish to thank Ewa Dahlberg for expert technical assistance with the experiments presented in this paper and Lena Thiman for help with the polyamine analysis. We wish to thank Dr Bo Baldetorp for the use of the flow cytometer at the Department of Oncology, The Jubileum Institute, Lund University, Sweden. This work was supported by the Swedish Cancer Foundation, the Crafoord Foundation, the Royal Physiographical Society in Lund, the Mrs Berta Kamprad Foundation, the Gunnar, Arvid and Elisabeth Nilsson Foundation, the IngaBritt and Arne Lundbergs Research Foundation and the Carl Tesdorpfs Foundation.

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