Apoptosis induced by the potential chemotherapeutic drug N^1, N^{11}-Diethylnorspermine in a neuroblastoma cell line

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Neuroblastoma is a highly malignant neoplasm found in young children. Although children with high-risk neuroblastoma respond to chemotherapy, relapses are common. On account of poor treatment outcome, new treatment strategies are constantly sought for neuroblastoma. Polyamine analogues are potentially novel substances for treatment of neuroblastoma. In this study, we have treated two neuroblastoma cell lines, SH-SY5Y and LA-N-1, with the spermine analogue N^1, N^{11}-Diethylnorspermine (DENSPM). SH-SY5Y was the most sensitive cell line, in which DENSPM treatment resulted in an inhibition of cell proliferation and an induction of cell death. The cell death induced by DENSPM treatment was apoptotic, as evidenced by cleavage of procaspase 3 and induction of caspase-3 activity. In contrast, DENSPM treatment only resulted in a slight inhibition of cell proliferation in LA-N-1 cells. There were several possible causes for the lower sensitivity to DENSPM treatment in the latter cell line when compared with SH-SY5Y cells. DENSPM-induced polyamine depletion was more extensive in SH-SY5Y cells than in LA-N-1 cells. This was partly because of a higher induction of the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase in the cell line SH-SY5Y. The DENSPM-induced polyamine depletion was also caused by the inhibition of ornithine decarboxylase. LA-N-1 cells contained a higher level of the prosurvival protein survivin, which was further increased after DENSPM treatment. In contrast, DENSPM treatment resulted in a decreased survivin level in SH-SY5Y cells. *Anti-Cancer Drugs* 21:917–926 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2010, **21**:917–926

Keywords: apoptosis, N^1, N^{11}-Diethylnorspermine, neuroblastoma, spermine analogue, spermidine/spermine N^1-acetyltransferase, survivin

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Received 12 October 2009 **Revised form accepted** 11 June 2010

Introduction

Neuroblastoma is a malignant neoplasm found in the chest or abdomen, which originates from the adrenal medulla or the paraspinal sympathetic ganglia [1]. Neuroblastoma comprises 8–20% of all childhood cancers and is the most common solid tumour found in young children. Ninety-six per cent of cases occur before 10 years of age and the long-term survival prognosis is only 15%. If the disease is localized and/or is diagnosed before 1 year of age, the patient can be cured with surgery and little or no adjuvant therapy is needed. When older children are diagnosed with neuroblastoma, they usually have metastases. Despite intense chemotherapy, the majority of children die as the disease progresses. On the basis of the patient's age at diagnosis and biological characteristics, neuroblastoma can be divided into low, medium and high-risk diseases [2–5]. The survival of patients having high-risk neuroblastoma has been improved because of chemotherapy, radiotherapy and bone marrow transplantation, followed by retinoic acid treatment [4]. Although children with high-risk neuroblastoma respond to chemotherapy, relapses are common. When relapsing, the tumour has a multidrug-resistant phenotype, which often includes a mutated or deleted *p53* gene [6,7]. However, mutations of the *p53* gene are very rare in primary neuroblastoma, although *p53* is one of the most common altered tumour suppressor genes in a wide spectrum of human cancers [8]. A negative prognostic marker for neuroblastoma is the *MYCN* proto-oncogene, in which an amplification occurs in 20–25% of all neuroblastoma cases [5]. An amplification of *MYCN* has been shown to be correlated with many advanced-stage diseases and with poor prognosis [2]. On account of poor treatment outcome, new treatment strategies are constantly sought for neuroblastoma.

A new potential chemotherapeutic treatment of neuroblastoma is the polyamine analogues. The natural polyamines – putrescine, spermidine and spermine – have a role in the regulation of cell proliferation, differentiation and cell death in eukaryotes. Cellular levels of polyamines are highly regulated by biosynthesis, catabolism and transport over the cell membrane, thus indicating the importance of polyamines in these processes. Treatment with polyamine analogues almost completely depletes the intracellular polyamine pools; however, the analogues cannot take over the function of the natural polyamines [9–11]. Polyamine pool depletion, induced by polyamine analogues, first results in growth inhibition and thereafter in induction of cell death [12–15]. Polyamine analogues are presently being tested in clinical phase I and phase II trials [16–18].

0959-4973 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32833d1cae

In this study, we have used the spermine analogue N^1, N^{11}-Diethylnorspermine (DENSPM). This analogue induces rapid polyamine pool depletion because of down-regulation of polyamine biosynthetic enzymes and up-regulation of the catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) [13,19]. DENSPM treatment causes growth inhibition in a number of cancer cell lines (pancreas, melanoma, lung, prostate, breast) [20–24], and in some instances, apoptosis is induced as well [13,21,25]. To the best of our knowledge, the effect of DENSPM has not been investigated in neuroblastoma cell lines. However, the outcome of treating neuroblastoma with other compounds that affect polyamine homoeostasis has been reported [26–29]. Treatment of *MYCN*-amplified neuroblastoma with the ornithine decarboxylase (ODC) inhibitor 2-difluoromethylornithine (DFMO) caused growth inhibition in the G_1 phase in the absence of apoptosis [27,29]. Apoptosis was induced in neuroblastoma with wild-type p53 independent of *MYCN* amplification after treatment with an inhibitor of S-adenosylmethionine decarboxylase [28].

In this study, the SH-SY5Y and LA-N-1 human neuroblastoma cell lines were treated with DENSPM. The cell lines were chosen on the basis of their different genetic background: SH-SY5Y has wild-type *p53* [30], whereas LA-N-1 has mutated *p53* and a *MYCN* amplification [3,31]. The results show that the two cell lines react differently to DENSPM treatment. SH-SY5Y cells were more sensitive, showing both growth inhibition and apoptosis, whereas LA-N-1 cells were only growth inhibited.

Materials and methods

Materials

Cell culture medium components were purchased from Biochrom (Berlin, Germany). Tissue culture plastics were purchased from Nunc (Roskilde, Denmark). DENSPM was purchased from Tocris Cookson Ltd. (Bristol, UK). Before addition, DENSPM was dissolved in phosphate-buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, pH 7.3) to yield a stock solution of 2 mmol/l. The stock solution was sterile-filtered and stored at −20°C. PBS was purchased from Oxoid Ltd. (Hampshire, UK) and propidium iodide (PI) was purchased from Sigma Chemical Co. (St Louis, Missouri, USA). [^{14}C]Acetyl-coenzyme A (60 mCi/mmol) was purchased from New England Nuclear, DuPont, Scandinavia AB (Stockholm, Sweden). The Caspase 3 Colorimetric Protease Assay Kit was purchased from Medical and Biological Laboratories (Nagoya, Japan). Monoclonal antibodies against Bcl-2 (556354) and Smac/DIABLO (612245) and polyclonal antibodies against Bax (554114) and apoptosis inducing factor (AIF) (551429) were purchased from BD Bioscience PharMingen (San Diego, California, USA). The polyclonal antibody against caspase 3 (AHZ0052) was purchased from Biosource International (Camarillo, California, USA), whereas the monoclonal antibody against survivin (sc-17779) was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Horseradish peroxidase (HRP)-conjugated swine antirabbit immunoglobulins (P0217) and HRP-conjugated goat antimouse immunoglobulins (P0447) were purchased from DAKO A/S (Glostrup, Denmark). NuPAGE 12% Bis–Tris Gels, NuPAGE Transfer Buffer, NuPAGE MOPS, NuPAGE antioxidant and MagicMark XP Western Standard were purchased from Invitrogen Corporation (Carlsbad, California, USA). The enhanced chemiluminescence (ECL) Advanced Western Blotting Detection kit and Hybond ECL Nitrocellulose membranes were purchased from GE Healthcare (Buckinghamshire, UK). L-[1-^{14}C]Ornithine (52 mCi/mmol) was purchased from New England Nuclear Du Pont, Scandinavia AB, Stockholm, Sweden. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from ICN Biomedicals Inc. (Aurora, Ohio, USA).

Cell culture

Two different human neuroblastoma cell lines were used, namely, SH-SY5Y and LA-N-1. These cell lines were purchased from ECACC (Salisbury, UK). The cell lines were chosen on the basis of their different genetic background: SH-SY5Y has wild-type *p53* [30], whereas LA-N-1 has mutated *p53* and a *MYCN* amplification [3,31]. SH-SY5Y cells were cultured in RPMI 1640 medium containing 10% heat-inactivated foetal calf serum, 1 mmol/l nonessential amino acids, 1 mmol/l sodium pyruvate, 10 μg/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin. LA-N-1 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated foetal calf serum, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 1 mmol/l nonessential amino acids, 10 μg/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were incubated at 37°C in a humidified incubator with 5% CO_2 in air, and were subcultured twice a week. Cell proliferation was monitored at each passage by counting in a haemocytometer. For the experiments, cells were seeded (10×10^6 cells in 175 cm^2 in 30 ml of medium) in the absence or presence of 10 μmol/l DENSPM. All cells (floating and attached) were harvested. Only viable cells (opalescent cells excluding trypan blue) were counted in the haemocytometer after 24, 48 and 72 h of treatment. Cells were pelleted at 800*g* for 10 min at 4°C and handled for further analysis as described below.

Flow cytometry and data analysis

Pelleted cells were resuspended in ice-cold 70% ethanol and stored at −20°C until analysis. Nuclear DNA was stained with PI-nuclear isolation medium (PBS containing 100 μg/ml PI, 0.60% Nonidet P-40 and 100 μg/ml RNase A) [32]. Flow cytometric analysis was performed in a Cytoron Absolute flow cytometer (Ortho Raritan, New Jersey, USA) as described earlier [33]. For data analysis of cell cycle phase distribution and the sub-G_1

region, the MultiCycle software program (Phoenix Flow Systems, California, USA) was used.

Polyamine analysis

Pelleted cells were stored at −20°C until analysis. Chromatographic separation and quantification of polyamine levels in cell extracts in 0.2 mol/l perchloric acid were carried out using high-performance liquid chromatography (HPLC) (Hewlett Packard 1100, Scientific Equipment Source, Pickering, Ontario, Canada), with *O*-phthaldialdehyde as a reagent [34].

Spermidine/spermine N^1-acetyltransferase activity assay

Pelleted cells were stored at −80°C until analysis. Samples were sonicated in 50 mmol/l Tris–HCl (pH 7.5) containing 0.25 mol/l sucrose. The SSAT activity of the sonicates was determined by measuring the synthesis of [^{14}C]acetylspermidine after incubation of the cell extracts with [^{14}C]acetyl-coenzyme A and spermidine [35].

Ornithine decarboxylase activity assay

Pelleted cells were stored at −80°C until analysis. Samples were sonicated in ice-cold 0.1 mol/l Tris–HCl (pH 7.5) containing 0.1 mmol/l EDTA and 2.5 mmol/l dithiothreitol. To determine the ODC activity, the cell sonicates were used for measuring the release of $^{14}CO_2$ from carboxyl-labelled L-ornithine in the presence of saturating levels of pyridoxal 5-phosphate and L-ornithine.

Caspase-3 activity assay

Pelleted cells were resuspended in 50 µl cell lysis buffer (found in the Caspase 3 Colorimetric Protease Assay kit) and were stored at −80°C until analysis. Caspase-3 activity was determined by measuring the cleavage of chromophore *p*-nitroanilide (*p*NA) from a DEVD-*p*NA-labelled substrate (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions. The samples were incubated with 200 µmol/l of *p*NA substrate at 37°C for 2 h before spectrophotometric (SpectraMax M2 Microplate reader, Sunnyvale, California, USA) measurement of emission at 405 nm.

Western blot analysis

Pelleted cells were stored at −80°C until analysis. Samples were diluted in sample buffer (0.5 mol/l Tris–HCl, pH 6.8, 20% glycerol, 10% SDS, 5% β-mercaptoethanol and 0.5% bromophenol blue), sonicated and incubated at 98°C for 5 min, and then immediately placed on ice. Aliquots equivalent to 75 000 cells were separated using SDS–polyacrylamide gel (12%) electrophoresis. The gel electrophoresis was performed according to the manufacturer's instructions at 150 V/400 mA for approximately 80 min. The separated proteins were then electrophoretically transferred to an ECL nitrocellulose membrane at 30 V/400 mA for 1 h. The membranes were blocked with 5% nonfat dry milk and 0.05% Tween 20 in PBS for approximately 2.5 h. After the addition of primary antibody, the membranes were incubated at 4°C overnight. HRP-conjugated secondary antibodies were used to detect the primary antibody bound to a protein and advanced ECL detection reagent was used according to the manufacturer's protocol. All antibodies were diluted in 0.05% Tween 20 in PBS. The ChemiDoc XRS system and the software Quantity One (Bio-Rad Laboratories Inc., Hercules, California, USA) were used for imaging.

Cytotoxicity assay

Cells were counted and resuspended to a final concentration of 0.6×10^5 cells per millilitre. A 150 µl aliquot of the cell suspension was added per well in 96-well plates. DENSPM in 50 µl was added to the medium to yield a final concentration of 0.01, 0.1, 1, 10, 50 or 100 µmol/l. The sterile-filtered MTT solution (5 mg/ml in PBS) was stored protected from light at −20°C until usage. At 72 h of incubation, 20 µl of MTT solution was added to each well and the 96-well plate was returned to the incubator for 1 h. Thereafter, the MTT-containing medium was removed and the blue formazan product was dissolved by the addition of 100 µl of 100% dimethyl sulfoxide per well. The plates were swirled gently for 10 min to dissolve the precipitate. Absorbance was monitored at 540 nm using a Labsystems iEMS Reader MF (Labsystems Oy, Helsinki, Finland) and the Software DeltaSoft II v. 4.14 (Biometallics Inc., Princeton, New Jersey, USA).

Statistical analysis

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

Results

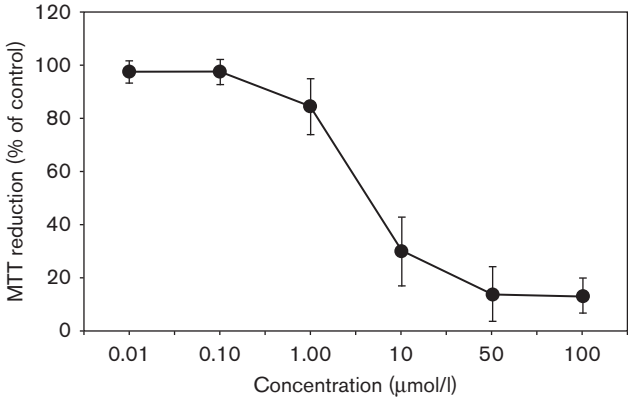
Dose–response curve for SH-SY5Y cells treated with DENSPM

To obtain an overall understanding of the toxicity of DENSPM, a dose–response test was performed. This was only performed with SH-SY5Y cells. The MTT assay could not be performed with the loosely attached LA-N-1 cell line, as the cells detached in the step in which the MTT-containing medium was removed. DENSPM treatment for 72 h at doses above 0.1 µmol/l showed toxicity, as evaluated by measuring the reduction of MTT in mitochondria (Fig. 1). On the basis of these results, published data on DENSPM treatment of cell lines [12–15] and on achievable plasma concentrations in humans (up to 30 µmol/l) [16], we decided to use a 10 µmol/l concentration for further studies.

Effects of DENSPM treatment on cell proliferation

We investigated the effect of DENSPM treatment on cell proliferation by seeding cells in the absence or presence of 10 µmol/l DENSPM. In control SH-SY5Y cultures, the cell number increased over the experimental period with a population doubling time of approximately 38 h during exponential growth (Fig. 2). In contrast, when SH-SY5Y cells were seeded in the presence of 10 µmol/l DENSPM, there was no change in cell number during the 72 h

Fig. 1



Effect of different concentrations of N^1, N^{11}-Diethylnorspermine on SH-SY5Y cells evaluated by the MTT assay. The cells were treated for 72 h. Symbols represent mean of 10–12 independent samples from two independent experiments. Bars represent ± standard deviation.

experimental period. In LA-N-1 cells, DENSPM treatment prolonged the lag phase; however, after entering the log phase, the growth rate was similar to that in control cells (Fig. 2). The population doubling time of LA-N-1 cells was approximately 34 h.

Effects of DENSPM treatment on polyamine levels, SSAT and ODC activities

To confirm the expected effect of DENSPM treatment on polyamine pools and the role of SSAT and ODC activities, we determined the size of the cellular polyamine pools by HPLC, and the SSAT and ODC activities by a radiometric assay. Treatment with DENSPM resulted in significantly decreased polyamine pools in both cell lines, compared with control (Fig. 2). However, the polyamine pools were not decreased to the same extent in LA-N-1 cells as in SH-SY5Y cells.

Treatment with DENSPM significantly induced SSAT activity in both cell lines (Fig. 3). In SH-SY5Y cells, induction of SSAT activity increased with time of treatment and was higher than in LA-N-1 cells. In LA-N-1 cells, the increase in SSAT activity induced by DENSPM treatment was constant compared with that in control cells.

Treatment with DENSPM significantly decreased ODC activity in both cell lines (Fig. 4). However, compared with control, DENSPM treatment reduced ODC activity to a greater extent in SH-SY5Y cells than in LA-N-1 cells. The ODC activity was similar in SH-SY5Y and LA-N-1 control cells.

Effects of DENSPM treatment on the sub-G_1 region and the cell cycle phase distribution

The results on cell proliferation led us to investigate whether DENSPM treatment affected the DNA distribution. We investigated the cell cycle phase distribution, and the appearance of cells in a sub-G_1 peak, as a measurement of cell death.

In SH-SY5Y cells, DENSPM treatment significantly increased the percentage of cells in the sub-G_1 region with increased incubation time (Fig. 5). After 72 h of DENSPM treatment, approximately 60% of the SH-SY5Y cells were found in the sub-G_1 region. DENSPM treatment did not result in an increased sub-G_1 region in LA-N-1 cells.

The most evident DENSPM-induced change in cell cycle phase distribution was found in SH-SY5Y cells, in which the G_1 phase increased, whereas the S phase decreased, in DENSPM-treated cultures compared with control (Fig. 5). In LA-N-1 cells, the most pronounced DENSPM-induced change in cell cycle phase distribution was an increase in the G_2 phase population after 72 h of treatment compared with control (Fig. 5).

Effects of DENSPM treatment on caspase-3 activity

To investigate whether apoptosis was involved in the appearance of the sub-G_1 peak in DENSPM-treated SH-SY5Y cells, caspase-3 activity was determined. In SH-SY5Y cells, the caspase-3 activity increased significantly with incubation time, compared with control (Fig. 6). No caspase-3 activity was detected in control or DENSPM-treated LA-N-1 cells (not shown).

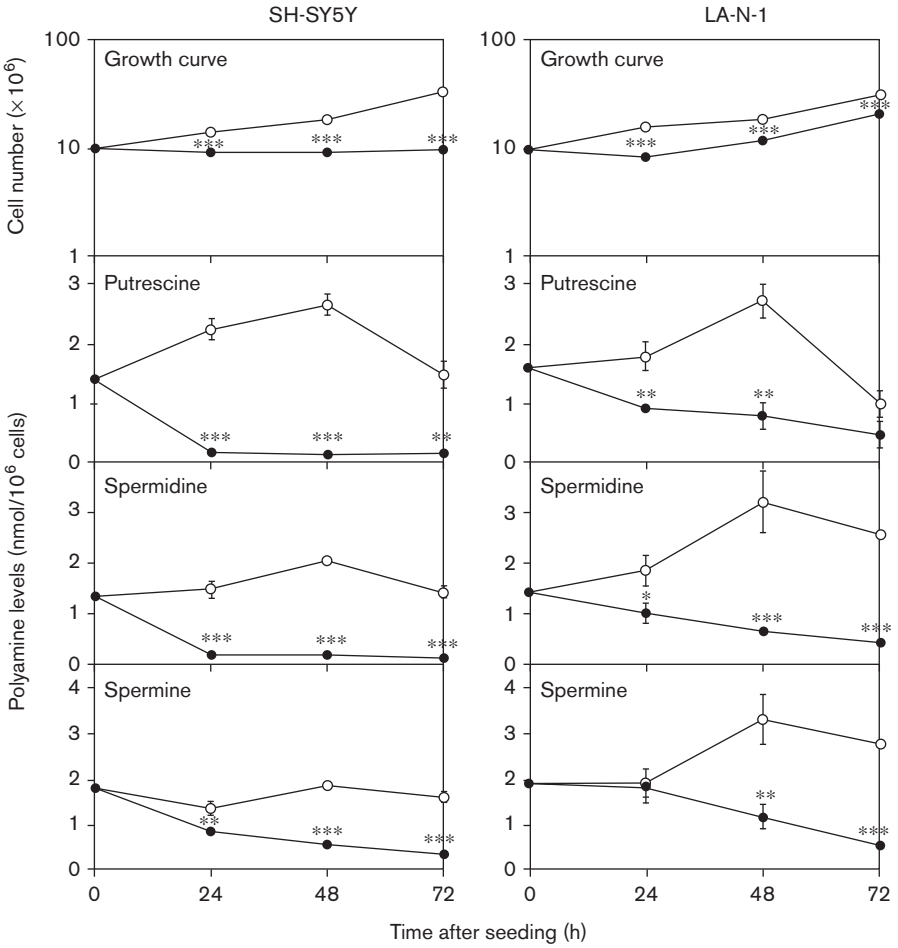
Effects of DENSPM treatment on antiapoptotic and proapoptotic proteins using western blot

We also decided to investigate the levels of various proteins involved in apoptosis by western blot, aiming to understand whether this could explain the difference in response to DENSPM treatment in SH-SY5Y and LA-N-1 cells. Procaspase 3 was detected in both SH-SY5Y and LA-N-1 cells (Fig. 7). A caspase-3 band only appeared in SH-SY5Y cells after 48 and 72 h of DENSPM treatment (Fig. 7).

In both cell lines, the level of the antiapoptotic protein Bcl-2 increased slightly in DENSPM-treated cells compared with control (Fig. 7). The level of the proapoptotic protein Bax increased in DENSPM-treated SH-SY5Y cells at 48 and 72 h of treatment, compared with control (Fig. 7). In DENSPM-treated LA-N-1 cells, the Bax level increased at 24 and 48 h of treatment compared with control; however, after 72 h of DENSPM treatment, the level was the same in control and treated cells.

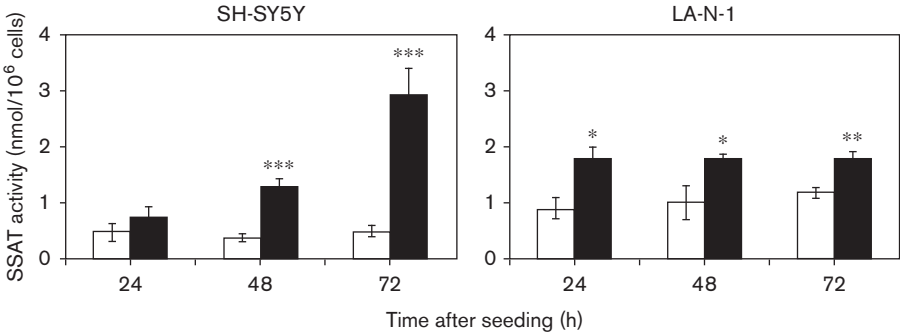
There was no major effect of DENSPM treatment on the AIF level in either of the cell lines (Fig. 8). DENSPM treatment resulted in a lowering of the Smac/DIABLO level in SH-SY5Y cells, whereas there was an increase in LA-N-1 cells at 72 h after treatment. The level of Smac/DIABLO was higher in SH-SY5Y cells than in LA-N-1

Fig. 2



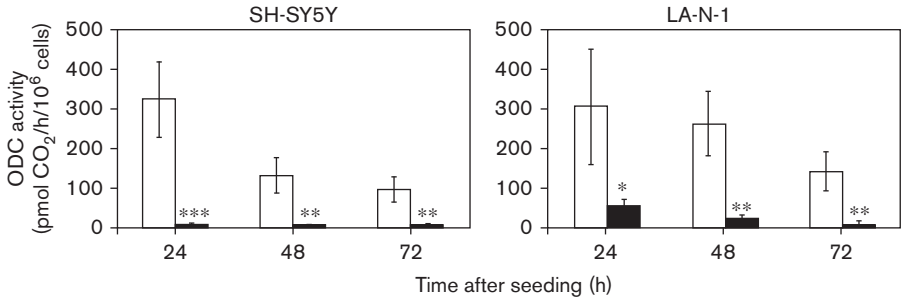
N^1, N^{11}-Diethylnorspermine (DENSPM) treatment resulted in a reduced polyamine content and inhibition of proliferation in SH-SY5Y cells, whereas LA-N-1 cells were less affected. The cells were seeded in the absence or presence of 10 μmol/l DENSPM. The results are presented as mean values ($n=6$ independent samples from two independent experiments). Bars represent ±standard deviation. When not visible, the bars are covered by symbols. ○, control; ●, DENSPM. *P less than 0.05 compared with control; **P less than 0.01 compared with control; ***P less than 0.001 compared with control.

Fig. 3



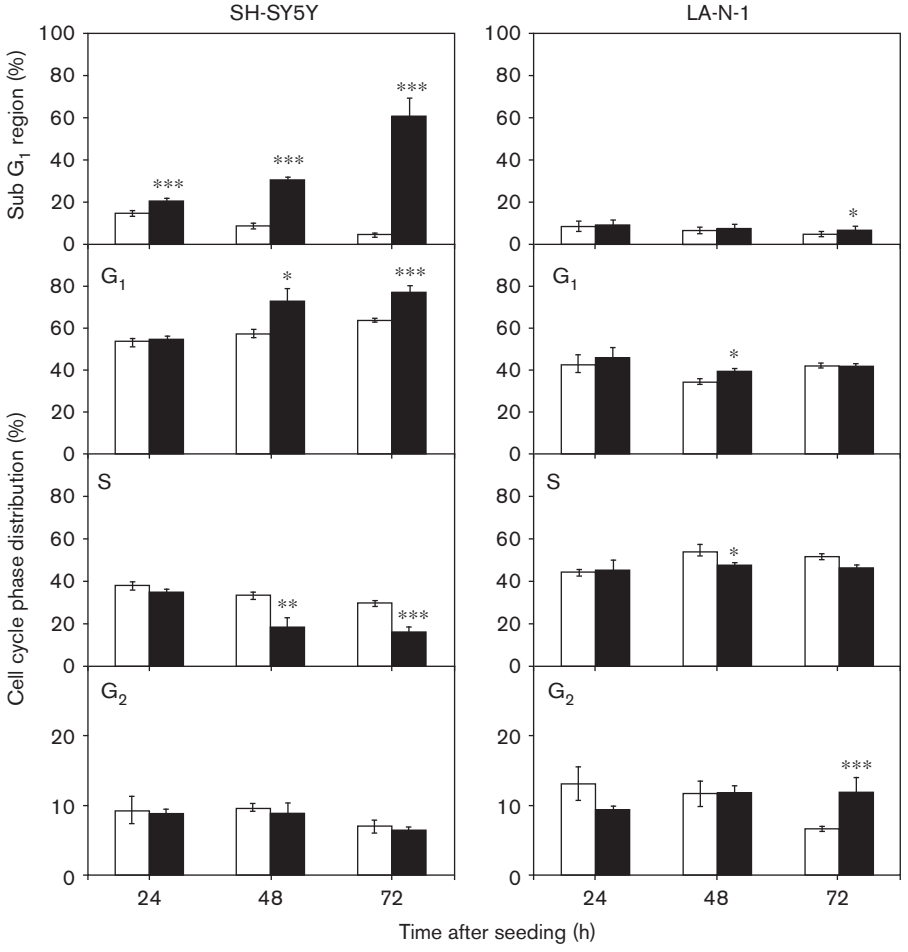
N^1, N^{11}-Diethylnorspermine (DENSPM) treatment resulted in increased spermidine/spermine N^1-acetyltransferase (SSAT) activity in SH-SY5Y and LA-N-1 cells. The cells were seeded in the absence or presence of 10 μmol/l DENSPM. The results are presented as mean values ($n=6$ independent samples from two independent experiments). Bars represent ±standard deviation. White columns, control; black columns, DENSPM. *P less than 0.05 compared with control; **P less than 0.01 compared with control; ***P less than 0.001 compared with control.

Fig. 4



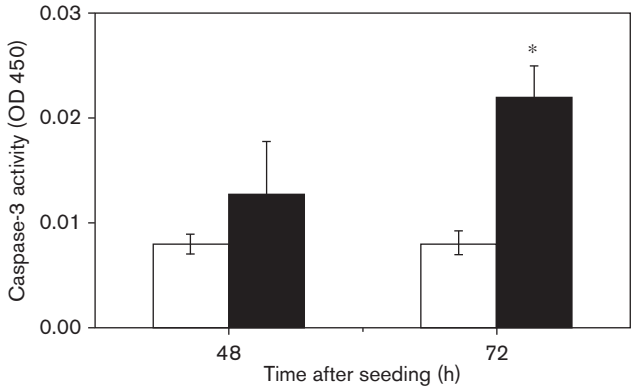
N^1, N^{11}-Diethylnorspermine (DENSPM) treatment reduced ornithine decarboxylase (ODC) activity in SH-SY5Y and LA-N-1 cells. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. The results are presented as mean values ($n=4$ independent samples from two independent experiments). Bars represent ± standard deviation. White columns, control; black columns, DENSPM. *P less than 0.05 compared with control; **P less than 0.01 compared with control; ***P less than 0.001 compared with control.

Fig. 5



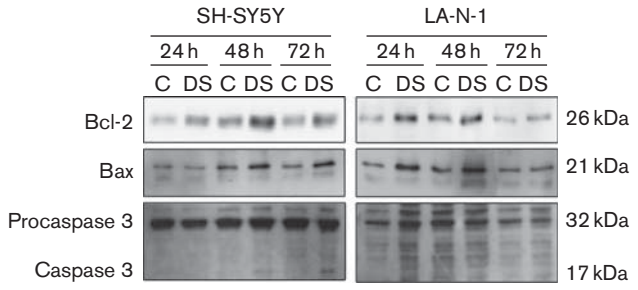
Effects of N^1, N^{11}-Diethylnorspermine (DENSPM) treatment on the sub-G_1 region and cell cycle phase distribution of SH-SY5Y and LA-N-1 cells. Cells were seeded in the absence or presence of 10 μmol/l DENSPM. The results are presented as mean values ($n=6$ independent samples from two independent experiments). Bars represent ± standard deviation. White columns, control; black columns, DENSPM. *P less than 0.05 compared with control; **P less than 0.01 compared with control; ***P less than 0.001 compared with control.

Fig. 6



N^1, N^{11}-Diethylnorspermine (DENSPM) treatment resulted in increased caspase-3 activity in SH-SY5Y cells. The cells were seeded in the absence or presence of 10 μmol/l DENSPM. The results are presented as mean values ($n=4$ independent samples from two independent experiments). Bars represent ± standard deviation. White columns, control; black columns, DENSPM. *P less than 0.05 compared with control. OD, optical density.

Fig. 7



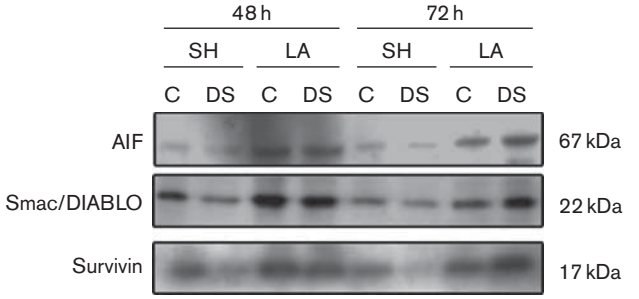
Effects of N^1, N^{11}-Diethylnorspermine (DENSPM) treatment on proapoptotic and antiapoptotic proteins involved in the mitochondrial apoptotic pathway in SH-SY5Y and LA-N-1 cells. The cells were seeded in the absence or presence of 10 μmol/l DENSPM. Western blot was used to detect proapoptotic and antiapoptotic proteins. A total of 75 000 cells were loaded into each well. The shown data are representative of six independent samples from two independent experiments. C, control; DS, DENSPM.

cells, as was the level of survivin. DENSPM treatment resulted in a lowering of the survivin level in SH-SY5Y cells, whereas it increased in LA-N-1 cells at 72 h of treatment.

Discussion

Neuroblastoma is a highly malignant neoplasm found in young children, for which the estimated long-time survival prognosis is approximately 15%. Thus, there is a tremendous need for new chemotherapeutic drugs for the treatment of neuroblastoma. As intracellular polyamines have crucial roles in cell proliferation and cell death pathways, the polyamine metabolic pathway represents a potential target for cancer intervention. In general,

Fig. 8



Comparison of the effect of N^1, N^{11}-Diethylnorspermine (DENSPM) treatment on proapoptotic and antiapoptotic proteins in SH-SY5Y and LA-N-1 cells. The cells were seeded in the absence or presence of 10 μmol/l DENSPM. Western blot was used to detect proapoptotic and antiapoptotic proteins. A total of 75 000 cells were loaded into each well. The shown data are representative of six independent samples from two independent experiments. C, control; DS, DENSPM; LA, LA-N-1; SH, SH-SY5Y.

chemotherapeutic treatment with drugs that manipulate polyamine metabolism has different effects in different cancer cell lines, depending on the genetic aberrations that should be kept in mind when considering their use in the clinic.

In this study, the spermine analogue DENSPM was used to deplete intracellular polyamine pools in neuroblastoma cell lines SH-SY5Y and LA-N-1. SH-SY5Y and LA-N-1 are representative of neuroblastoma at different degrees of malignancy. SH-SY5Y cells containing wild-type p53 are representative of a less-aggressive subtype, whereas LA-N-1 cells containing mutated p53 are representative of a more aggressive form. In addition, the *MYCN* gene is amplified several-fold in LA-N-1 cells, thereby increasing the malignant potential even further.

DENSPM treatment reduced the polyamine levels in both cell lines. Part of the polyamine depletion was because of the activation of SSAT. However, although the activation of SSAT was significant compared with that of control, it was far from the hundred- to thousand-fold activation seen in some cells [13,36,37]. Thus, a major part of the observed DENSPM-induced polyamine depletion in SH-SY5Y and LA-N-1 cells seems to be through DENSPM-mediated inhibition of the polyamine biosynthesis [38]. Indeed, we found that DENSPM treatment resulted in the inhibition of ODC in both cell lines. DENSPM-induced polyamine depletion was more extensive in SH-SY5Y cells than in LA-N-1 cells, which is part of the higher sensitivity in the former cells than in the latter.

As expected, DENSPM-induced polyamine depletion resulted in the inhibition of cell proliferation, which was most prominent in SH-SY5Y cells. The cell cycle phase

distribution is a result of the effect of DENSPM on cell proliferation in each specific cell cycle phase. Similar to what has been found in other cell lines with wild-type *p53* [13,24], DENSPM treatment resulted in an accumulation of cells in the G_1 phase in wild-type *p53*-containing SH-SY5Y cells. In LA-N-1 cells, the G_2 fraction increased with increased incubation time with DENSPM. It has been shown that a high level of survivin may initiate an S-phase accumulation and a G_2/M block [39–41]. In LA-N-1 cells, DENSPM treatment resulted in an increased survivin level, which may explain the increased fraction of cells in the G_2 phase.

The growth curve data of DENSPM-treated SH-SY5Y cells show a constant cell number during the experimental period, which may seem to contrast the increased sub-G_1 region. The increase in SH-SY5Y cells in the sub-G_1 region, despite the fact that there was no decrease in cell number, may imply that cell division was taking place at the same degree as that at which cell death occurred. At 48 h of DENSPM treatment, the labelling index estimated after bromodeoxyuridine labelling was approximately 13%, supporting our notion of cell proliferation and cell death taking place simultaneously (preliminary data not shown). In control SH-SY5Y cells, the labelling index was 26% (not shown). In addition, cells in early stages of apoptosis have an intact cell membrane and may thus have excluded trypan blue and are then counted as live cells. It has been shown that extensive cleavage of DNA and nuclear fragmentation can occur despite an intact cell membrane excluding trypan blue [42,43].

DENSPM-induced cell death was studied by monitoring the sub-G_1 region in the DNA histogram. There was a significant increase in the sub-G_1 region in DENSPM-treated SH-SY5Y cells, whereas in LA-N-1 cells there was almost no effect on the sub-G_1 region. In SH-SY5Y cells, DENSPM treatment resulted in an increased G_1 phase fraction pointing to a block in the G_1/S transition. As SH-SY5Y cells contain wild-type p53, both the induction of cell death and the increase in the G_1 phase fraction were presumably because of p53 induction. Similar p53-mediated responses to DENSPM treatment have been found by other investigators [15,21,24]. It has been shown earlier that DENSPM treatment results in cell death in cell lines containing mutated p53 [13,37], which is in contrast to the LA-N-1 cells in this study. LA-N-1 cells have an amplification of the *MYCN* gene and a very high expression of the MYCN protein [44], which presumably protects them from the negative effects of DENSPM treatment. The cell death that took place in SH-SY5Y cells was presumably apoptotic, as evidenced by the procaspase-3 cleavage and increased caspase-3 activity. DENSPM treatment has been shown to induce apoptotic cell death earlier [13,37].

Western blot was used to analyse levels of different apoptosis-related proteins. Two such important proteins with antagonistic properties are Bcl-2 and Bax. Bcl-2 is an antiapoptotic protein, whereas Bax is a proapoptotic protein. One major role of Bcl-2 is to inhibit apoptosis by preventing the translocation of Bax to the outer membrane of the mitochondria, thereby preventing the formation of oligomeric Bax channels through which cytochrome *c* can be released into the cytoplasm. Released cytochrome *c* activates the intrinsic apoptotic pathway [45]. The ratio between Bcl-2 and Bax has been suggested to be a part of the regulation of cell survival versus cell death [46]. In SH-SY5Y and LA-N-1 cells, DENSPM treatment increased the levels of both Bcl-2 and Bax. However, at 72 h of DENSPM treatment in LA-N-1 cells, Bcl-2 increased, compared with control, whereas the Bax level was the same as in control. Thus, in LA-N-1 cells, an increased ratio between Bcl-2 and Bax may have contributed to the insensitivity of cells to DENSPM treatment.

Another contributor to the lower sensitivity of LA-N-1 cells to DENSPM treatment, compared with SH-SY5Y cells, may be the protein survivin. Western blot showed that the level of survivin was higher in the former cells than in the latter. DENSPM treatment of LA-N-1 cells even resulted in an increased survivin level. In contrast, the survivin level decreased in DENSPM-treated SH-SY5Y cells. It has been shown earlier that the survivin level was markedly lowered in DENSPM-treated SK-MEL-28 cells undergoing apoptotic cell death [47]. In the same paper, it was found that DENSPM treatment of SK-MEL-28 cells led to cytosolic release of Smac/DIABLO, a mitochondrial protein known to bind and inhibit the function of the inhibitor of apoptosis proteins. We have only determined the total cellular level of Smac/DIABLO and cannot draw any conclusions from the small changes in the levels observed after DENSPM treatment, nor can we draw any conclusions about DENSPM treatment on AIF. When released from the mitochondria, AIF is known to rapidly translocate to the nucleus, where it induces DNA condensation and fragmentation [48–50]. LA-N-1 cells seem to have higher levels of Smac/DIABLO and AIF than SH-SY5Y cells; however, this does not render them more susceptible to DENSPM-induced apoptosis, as the protein is presumably sequestered in the mitochondria and not released by DENSPM treatment.

In this paper, we show that the MYCN-overexpressing cell line LA-N-1 was less sensitive to DENSPM than the SH-SY5Y cell line that does not overexpress MYCN. When MYCN-overexpressing and nonMYCN-overexpressing cell lines were treated with DFMO, the reverse results were found, that is, the MYCN-overexpressing cell lines were more sensitive than the nonMYCN-overexpressing cell lines [27,29]. The higher sensitivity was shown as a higher degree of DFMO-induced growth inhibition in the former. One of the targets mediating the high malignancy of MYCN tumours may be the polyamine biosynthetic enzyme ODC [27,29], which is inhibited by DFMO [51].

DFMO treatment has also been shown to extend tumour latency and prevent oncogenesis in mouse neuroblastoma models [27,29].

In conclusion, our data show a difference in susceptibility to DENSPM treatment in two neuroblastoma cell lines, in which SH-SY5Y cells were more sensitive than LA-N-1 cells. In SH-SY5Y cells, there was both growth inhibition and cell death. The cell death was apoptotic, as evidenced by procaspase-3 cleavage and increased caspase-3 activity. In LA-N-1 cells, it seems that survivin may have protected the cells from DENSPM-induced negative effects. In LA-N-1 cells, there may also be other protective factors related to the excessive overexpression of MYCN. The presented data support our hypothesis that neuroblastoma with genetic characteristics of SH-SY5Y cells may be successfully treated with DENSPM or other polyamine analogues. This observation, together with the data of others showing efficacy of DFMO in MYCN-overexpressing cell lines, shows that treatment strategies aiming at polyamine depletion may be efficient in neuroblastoma.

Acknowledgements

The authors thank Ewa Dahlberg for expert technical assistance and Lena Thiman for help with the polyamine analysis. They also thank Professor Bo Baldetorp for the use of the flow cytometer at the Department of Oncology, Clinical Sciences, Lund University, Lund, Sweden. This work was supported by grants from the Royal Physiographical Society in Lund, the John and Augusta Perssons Foundation of Scientific Medical Research, the Berta Kamprad Foundation, the Gunnar Nilsson Cancer Foundation and the Research School of Pharmaceutical Science.

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