Amino Acids (2012) 42:929–937
DOI 10.1007/s00726-011-1007-y

ORIGINAL ARTICLE

Novel anti-apoptotic effect of the retinoblastoma protein: implications for polyamine analogue toxicity

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Received: 17 March 2011 / Accepted: 28 June 2011 / Published online: 2 August 2011

Abstract The retinoblastoma protein (pRb) pathway is frequently altered in breast cancer cells. pRb is involved in the regulation of cell proliferation and cell death. The breast cancer cell line L56Br-C1 does not express pRb and is extremely sensitive to treatment with the polyamine analogue N^1,N^{11}-diethylnorspermine (DENSPM) which causes apoptosis. Polyamines are essential for the regulation of cell proliferation, cell differentiation and cell death. DENSPM depletes cells of polyamines, e.g., by inducing the activity of the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT). In this study, L56Br-C1 cells were transfected with human pRb–cDNA. Overexpression of pRb inhibited DENSPM-induced cell death and DENSPM-induced SSAT activity. This suggests that the pRb protein level is a promising marker for polyamine depletion sensitivity and that there is a connection between pRb and the regulation of SSAT activity. We also show that SSAT protein levels and SSAT activity do not always correlate, suggesting that there is an unknown regulation of SSAT.

Keywords pRb · Polyamine deficiency · Breast cancer · SSAT · DENSPM

Abbreviations

DENSPM	N^1,N^{11}-Diethylnorspermine
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
FCM	Flow cytometry/flow cytometric
HRP	Horseradish peroxidase
L56Br/pcDNA3	L56Br-C1 cells transfected with the empty vector pcDNA3
L56Br/pRb	L56Br-C1 cells transfected with the pRb-containing pcDNA3 vector
MTT	3(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBST	PBS containing 0.05% Tween 20
PI	Propidium iodide
PPARγ	Peroxisome proliferator-activated receptor gamma
pRb	Retinoblastoma protein
RNase II A	Ribonuclease A type II
SSAT	Spermidine/spermine N^1-acetyltransferase

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Introduction

The retinoblastoma gene is frequently mutated during tumorigenesis, rendering its gene product retinoblastoma protein (pRb) inactive or absent in cancer cells (Hickman et al. 2002). Approximately, 20% of breast carcinomas have inactive pRb, and almost 40% of primary breast cancers have reduced expression of pRb. The expression of proteins that regulate pRb is also often altered in cancer cells, implying that a disrupted pRb pathway in tumours is very common (Sherr 2000; Goodrich 2006). It has been reported that the pRb pathway is inactivated in more than 80% of all sporadic human cancer cases (Chau and Wang 2003). The prognostic and predictive relevance of pRb

status seem to differ between different cancer forms (Scambia et al. 2006). The expression level and/or function of pRb have been suggested to be prognostic factors for the outcome of breast cancer patients, i.e., loss of pRb or low levels of pRb activity is more often found in high-grade breast cancer and breast cancer cell lines (Fung and T'Ang 1992; Pietilainen et al. 1995; Botos et al. 2002).

pRb is a tumour suppressor that acts in the G_1 phase of the cell cycle, and its function is regulated by sequential phosphorylation steps (Adams 2001; Nevins 2001; Poznic 2009). In early G_1 phase, hypophosphorylated pRb binds the transcription factor E2F. During late G_1 phase and early S phase, pRb is increasingly phosphorylated by cyclin–cyclin-dependent kinase complexes. The hyperphosphorylated pRb cannot bind E2F, which is released, and then stimulates the expression of E2F responsive genes that enable cells to progress into and through S phase (Lavia and Jansen-Durr 1999; Nevins 2001; Poznic 2009). Besides the important role in cell cycle control, pRb has also been implicated in the regulation of apoptosis (Sherr 2000; Julian et al. 2007). E2F has been shown to regulate genes involved in apoptosis, e.g., Apaf1 and different caspases. E2F, in turn, is known to be regulated by pRb (Chau and Wang 2003). Recently, pRb has been shown to contain an E2F1-binding domain specific for E2F1-induced apoptosis that has little function for cell cycle control. This suggests the possibility of regulating the cell cycle and apoptosis by the timing of pRb-E2F interactions, as well as by specific physical interactions (Julian et al. 2007). It has also been speculated that pRb inhibits apoptosis by other means than controlling E2F-mediated gene expression. Factors stimulating the release of cytochrome *c* from mitochondria during the apoptotic activation pathway might be sequestered by pRb, thus inhibiting the release of cytochrome *c* (Chau and Wang 2003). It is known that pRb is degraded by caspases in the process of apoptosis (Tan et al. 1997), which could lead to a release of factors stimulating cytochrome *c* release and hence amplification of apoptotic signals (Chau and Wang 2003).

The polyamines, putrescine, spermidine and spermine are ubiquitous positively charged compounds that are essential for the regulation of cell proliferation, cell differentiation and cell death. Cells regulate the level of polyamines meticulously through biosynthesis, catabolism and transport into and out of the cell (Casero and Pegg 1993). Polyamine depletion can be achieved by targeting these mechanisms. The spermine analogue N^1,N^{11}-diethylnorspermine (DENSPM, also known as BENSPM or DE333) depletes cells of polyamines by up-regulating the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) and inhibiting the biosynthetic enzymes ornithine decarboxylase and *S*-adenosylmethionine decarboxylase. DENSPM is also known to suppress the transport of polyamines into cells because it competes with the polyamines for uptake, but also because it induces antizyme (Mitchell et al. 2002). When treating cells with DENSPM, the combined action on polyamine metabolism and uptake adds up to a rapid depletion of all polyamines in the cells (Pegg et al.1989; Fogel-Petrovic et al. 1997). The response to DENSPM-induced depletion of polyamines in cells varies. Some cell lines stop proliferating, while others die by apoptosis (Kramer et al. 1997, 1999; Alm et al. 2000; Hegardt et al. 2002; Holst and Oredsson 2005; Oredsson et al. 2007; Myhre et al. 2008). It has previously been shown that pRb is progressively dephosphorylated in cells where DENSPM causes G_1 arrest (Kramer et al. 1999). The onset of G_1 arrest was found to correlate closely with the appearance of hypophosphorylated pRb, suggesting that the DENSPM-induced growth arrest is due to inhibition of pRb phosphorylation, followed by pRb sequestration of E2F. E2F is thus unable to activate key genes needed for DNA synthesis and S phase progression.

The human breast cancer cell line L56Br-C1 is extremely sensitive to DENSPM treatment and the cells die by apoptosis via the mitochondrial activation pathway (Hegardt et al. 2002; Holst et al. 2008). The L56Br-C1 cells also have a high level of spontaneous apoptosis. We found that this cell line does not express pRb, whereas a cell line that is rather insensitive to DENSPM treatment, MCF-7, does express pRb (Myhre et al. 2008). This observation led us to investigate whether pRb status has a role in determining the sensitivity of cell lines to DENSPM-induced cell death. Our results show that overexpression of pRb inhibited the DENSPM-induced cell death and decreased DENSPM-induced SSAT activation in the breast cancer cell line L56Br-C1.

Materials and methods

Materials

Tissue culture plastics were purchased from Nunc, Roskilde, Denmark, and the cell culture medium components were purchased from Biochrom KG, Berlin, Germany. DENSPM was purchased from Tocris Cookson Ldt., Bristol, UK. The mammalian expression vector pcDNA3, carrying cDNA for pRb, was kindly provided by Professor Urban Gullberg, Department of Hematology, Lund University. DENSPM was made as a 2 mM stock solution in phosphate-buffered saline (PBS, 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l $NaHPO_4$, 0.2 g/l KH_2PO_4, pH 7.3). The stock solution was sterilized by filtration, aliquoted and stored at 20°C. 3(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from ICN Biomedicals Inc., Aurora, OH, USA. Dimethyl sulphoxide (DMSO) was

purchased from Merck KGaA, Darmstadt, Germany. Propidium iodide (PI) was purchased from ICN Biomedicals Inc. Irvine, CA, USA. Ribonuclease A type II (RNase A) was purchased from Sigma Chemicals Co., St. Louis, MO, USA. Hybond™ enhanced chemiluminescence (ECL™) nitrocellulose membrane and ECL™ Advance Western Blotting Detection Kit were purchased from Amersham Biosciences AB, Uppsala, Sweden. The mouse anti-pRb antibody (clone G3245) was purchased from BD Biosciences, and the mouse anti-peroxisome proliferator-activated receptor gamma (PPARγ) antibody (clone A3409A) was purchased from Abcam, Cambridge, UK. The antibody against human SSAT was kindly provided by Professor Anthony E. Pegg, Penn State College of Medicine, Hershey, PA, USA. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibodies were purchased from DAKO, Glostrup, Denmark. Geneticin and all components of the NuPAGE® Novex PreCast Gel System used for Western blot were purchased from Invitrogen Corporation, Carlsbad, CA, USA.

Overexpression of pRb

Exponentially growing L56Br-C1 cells (8 million) were pelleted and resuspended in 500 µl, 37°C, fresh cell culture medium. Linearized plasmid DNA (15 µg) in 300 µl PBS was added to the cells in a 0.4 cm Gene Pulser® Cyvette (Bio-Rad, CA, USA). The DNA was introduced into the cells by electroporation using a Bio-Rad Gene Pulser® II (BioRad, CA, USA). The cells were pulsed with 300 V at 250 µF. After a 5-min recovery period on ice, the cells were seeded in fresh cell culture medium. Geneticin (0.5 mg/ml) was added after 3 days for selection of stably transfected cells. L56Br-C1 cells transfected with the empty vector pcDNA3 were named L56Br/pcDNA3, whereas the L56Br-C1 cells transfected with the pcDNA3 vector containing cDNA for pRb were named L56Br/pRb. For further maintenance of stably transfected cells, the cell culture medium was continuously supplemented with 25 µg/ml geneticin.

Cell lines and cell culture

The L56Br-C1 cell line was established at the Department of Oncology, Clinical Sciences, Lund University (Johannsson et al. 2003), and MCF-7, L56Br-C1, SK-BR-3 and HCC1937 cell lines were obtained from American Tissue Type Culture Collection (Manassas, VA, USA). The cell lines were cultured as monolayers at 37°C in a water-saturated atmosphere with 5% CO_2 in air. The L56Br/pcDNA3 and L56Br/pRb cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, non-essential amino acids (1 mM), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml insulin and 25 µg/ml geneticin. The cells were subcultured once a week, and the growth culture medium was changed twice a week. The MCF-7, L56Br-C1, SK-BR-3 and HCC1937 cell lines were cultured as described by Myhre et al. (2008).

MTT assay

Cells were trypsinized, counted in a hemocytometer, pelleted and resuspended in cell culture medium. Aliquots of 180 µl cell suspensions containing 8,000 cells were seeded in the wells of 96-well plates. DENSPM was added to the medium to a final concentration of 100, 10, 1, 0.1, 0.01 or 0.001 µM. The sterile MTT solution (5 mg/ml in PBS) was stored at 20°C until usage. At 24 or 48 h of DENSPM treatment, 20 µl of MTT solution was added to each well and the 96-well plates were incubated in a CO_2 incubator for 1 h. The MTT-containing medium was removed and each well was washed gently with 100 µl of PBS. The blue formazan product was dissolved by adding 100 µl of 100% DMSO per well. The plates were incubated at room temperature during gentle agitation for 10 min to dissolve the precipitate. Absorbance was measured at 540 nm using a Labsystems iEMS Reader MF (Labsystems Oy, Helsinki, Finland) and the software DeltaSoft II v. 4.14 (Biometallics Inc., Princeton, NJ, USA). The results are presented as percentage of control.

Analysis of polyamine content

Plateau phase cells were seeded in the absence or presence of 10 µM DENSPM. The cells were trypsinized 24 or 48 h after seeding, counted in a hemocytometer and pelleted. The pellets were stored at 20°C until analysis. The polyamines were separated and quantified in cell extracts in 0.2 M perchloric acid using high-performance liquid chromatography (HPLC) (Hewelett Packard 1100) with *O*-phthaldialdehyde as the reagent (Seiler and Knödgen 1985).

SSAT activity measurements

Plateau phase cells were seeded in the absence or presence of 10 µM DENSPM. The cells were trypsinized 24 or 48 h after seeding, counted in a hemocytometer and pelleted. The pellets 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 amount of [^{14}C]acetylspermidine produced after incubation with [^{14}C]acetyl coenzyme A and spermidine (Matsui et al. 1981).

Flow cytometry

Plateau phase cells were seeded in the absence or presence of 10 μM DENSPM. The cells were trypsinized 24 or 48 h after seeding, pelleted and fixed in 70% ethanol. The samples were stored at 20°C until analysis. At the time of analysis, cells were washed with PBS, pelleted and then incubated with PI nuclear isolation media (PBS containing 100 μg/ml PI, 0.60% Nonidet P40 and 100 μg/ml RNase A) for at least 30 min at 4°C. Immediately prior to flow cytometric (FCM) analysis, the cell suspension was suctioned three times through a syringe (0.7 mm diameter) and filtered through a 50-μm nylon mesh. The cells were analysed on an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ, USA), equipped with a 15 mW air-cooled argon-ion laser. The data were analysed using Multi2D® and MultiCycle® software programs from Phoenix Flow Systems, CA, USA.

Western blot

Plateau phase cells were seeded in the absence or presence of 10 μM DENSPM. The cells were harvested by trypsinization 24 h after seeding. The cells were counted in a hemocytometer, pelleted and then washed once with ice cold PBS. Total cell lysates were made by resuspending the cell pellet in sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.5% bromophenol blue) followed by sonication. The lysates were heat-denaturated at 95°C for 5 min. Cell lysates were loaded according to cell number onto precast NuPAGE© Novex 10% BisTris gels, and the proteins were separated under reducing conditions in a XCell SureLock™ Mini-Cell system at 150 V constant for 1.5 h in NuPAGE© MOPS Running Buffer. The proteins were transferred to a Hybond ECL nitrocellulose membrane during 1 h at 30 V constant using the XCell II™ Blot Module in NuPAGE© Transfer Buffer. The membranes were blocked in 5% non-fat dry milk in PBST (PBS containing 0.05% Tween 20) for 2 h at room temperature. The membranes were incubated with the primary antibodies diluted in PBS (pRb 1:3,000, PPARγ 1:3,000, SSAT 1:2,000) over night at 4°C. After washing the membranes with PBST, they were incubated with HRP-conjugated secondary antibodies in PBS (1:20,000) for 1 h at room temperature.

The bands were detected with the ECL™ Advance Western Blotting Detection Kit from Amersham Biosciences. The ChemiDoc XRS system from BioRad Laboratories Inc., Hercules, CA, USA, was used for detection of the chemiluminescence, and the software Quantity One, also from BioRad Laboratories Inc., was used for imaging.

Results

Protein expression

Western blot analysis showed that the L56Br/pRb cell line had a substantially increased level of pRb compared to the L56Br/pcDNA3 cell line (Fig. 1a). DENSPM treatment did not affect pRb expression in either cell line (unpublished results) (Myhre et al. 2008). The protein expression of SSAT increased in both cell lines with DENSPM treatment, though to a greater extent in L56Br/pRb (Fig. 1b). It has been shown that binding of the transcription factor PPARγ to the SSAT promoter stimulates the expression of SSAT (Ignatenko et al. 2004). It has also been shown that pRb interacts with PPARγ by forming part of a complex that inhibits the activity of PPARγ, suggesting that pRb is an important part of fine-tuning the activity of PPARγ (Fajas et al. 2002). Thus, we decided to investigate whether the level of PPARγ was different in L56Br/pRb and L56Br/pcDNA3 cells and whether the level was affected by DENSPM treatment. The PPARγ level was the same in both cell lines, and DENSPM treatment had no effect on the level (Fig. 1b). Western blot also showed that DENSPM treatment resulted in different SSAT levels in four breast cancer cell lines (Fig. 1c). No SSAT was found in DENSPM-treated MCF-7 cells and a low level in DENSPM-treated SK-BR-3 and L56Br-C1 cells. A very high level of SSAT was found in DENSPM-treated HCC1937 cells.

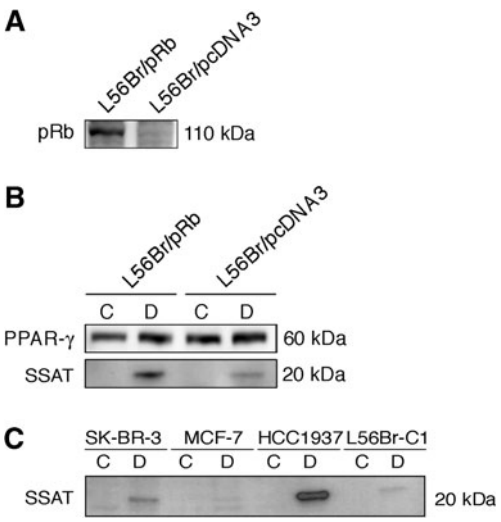

Fig. 1 Protein expression levels detected by Western blot analysis. **a** The protein expression level of pRb in L56Br/pcDNA3 and L56Br/pRb cells. **b** The protein expression levels of SSAT and PPARγ in L56Br/pcDNA3 and L56Br/pRb cells. **c** The protein expression level of SSAT in SK-BR-3, MCF-7, HCC1937 and L56Br-C1 cells. *C* control, *D* treated with 10 μM DENSPM for 24 h. Loading was based on cell number: 50,000 cells per well. The images shown are representative of three independent experiments

MTT reduction

The MTT assay showed that untreated L56Br/pcDNA3 and L56Br/pRb cell populations in 96-well plates reduced MTT similarly during a 96-h assay period (Fig. 2a). For both cell lines 8,000 cells were seeded per well. The increase in absorbance is due to increased MTT reduction due to the increase in cell number. Since the MTT assay is assumed to reflect cell number in untreated cells, the assay shows that L56Br/pcDNA3 and L56Br/pRb proliferate similarly. The MTT assay also showed that the response to DENSPM treatment was significantly different in the two cell lines (Fig. 2b). When treating cells with DENSPM, the MTT reduction decreased with both concentration and time, although the MTT reduction in L56Br/pRb cells did not decrease as rapidly as that in L56Br/pcDNA3. At 48 h of treatment with DENSPM at concentrations above 1 μM, there was no MTT reduction in L56Br/pcDNA3 cells indicating that there were no live cells present. In contrast, L56Br/pRb cells showed MTT reduction although reduced compared to control even at 100 μM DENSPM.

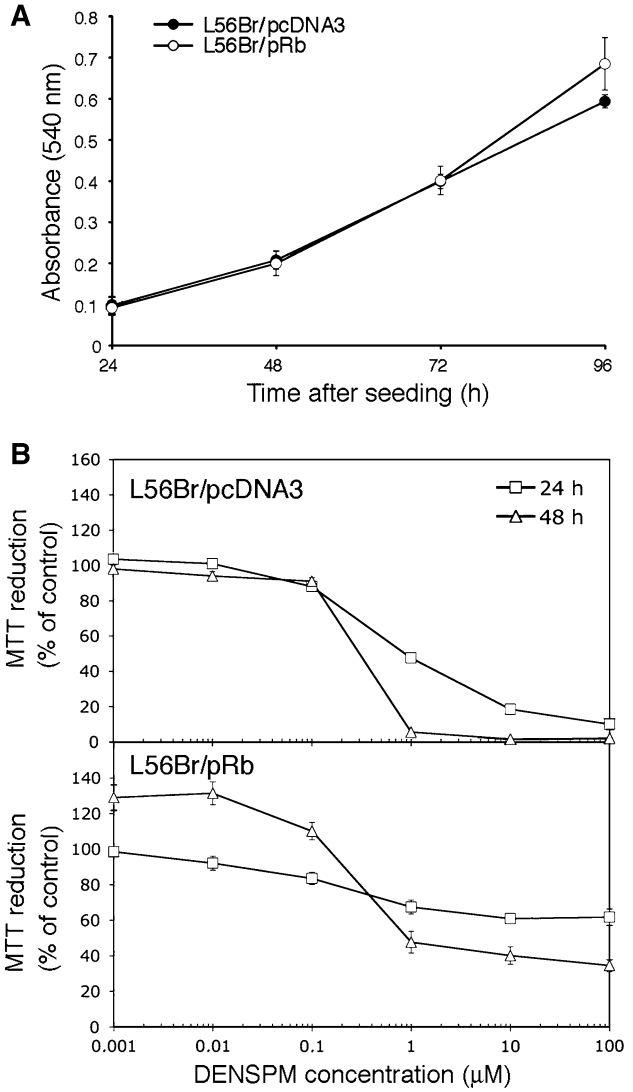

Fig. 2 MTT reduction in L56Br/pcDNA3 and L56Br/pRb cells. **a** The effect of pRb overexpression on MTT reduction during cell proliferation. At time 0, 8,000 plateau phase cells were seeded in 96-well plates. At 24, 48, 72 and 96 h after seeding, MTT reduction was analysed. The results are presented as mean values of $n = 12$–24 from 2 independent experiments with bars representing ± SEM. **b** The effect of DENSPM treatment on MTT reduction in L56Br/pcDNA3 cells and in L56Br/pRb cells. At time 0, plateau phase cells were seeded in the absence or presence of the final concentrations of DENSPM shown in the figure. At time 24 and 48 h, the cellular respiration was analysed by an MTT assay. The results are presented as mean values of $n = 12$–24 from 2 independent experiments with bars representing ± SEM. The data of L56Br/pcDNA3 and L56Br/pRb are significantly different at 24 and 48 h of treatment with 1–100 μM DENSPM ($P < 0.05$)

Cell death

When treating L56Br/pcDNA3 cells with DENSPM, massive cell death was induced after 24 h and even more after 48 h, as indicated by the sub-G_1 region detected by FCM (Fig. 3a) and apoptotic bodies visualized by fluorescence microscopy (Fig. 3b). This is consistent with how the original cell line L56Br-C1 responds to DENSPM treatment (Hegardt et al. 2002; Holst et al. 2008). L56Br/pRb cells treated with DENSPM for 24 or 48 h showed a much lower degree of cell death as investigated by FCM (Fig. 3a), and fluorescence microscopy did not show any apoptotic bodies (Fig. 3b). Also, the spontaneous cell death present in untreated L56Br/pcDNA3 cells was less prominent in L56Br/pRb cells.

Polyamine content

DENSPM treatment depleted spermine and spermidine to the same extent in both L56Br/pcDNA3 and L56Br/pRb cells. Regarding putrescine, the basal level was lower in L56Br/pRb cells than in L56Br/pcDNA3 cells and the effect of DENSPM on putrescine was less obvious (Fig. 4).

SSAT activity

DENSPM treatment induced SSAT activity substantially in L56Br/pcDNA3 cells (Fig. 5), as it does in the parental cell line L56Br-C1 (Hegardt et al. 2002). DENSPM treatment also induced SSAT activity in L56Br/pRb cells, but not to the same extent as in L56Br/pcDNA3 cells.

Discussion

In this study, we show that the pRb status is important for the cellular reaction to DENSPM treatment. Several lines

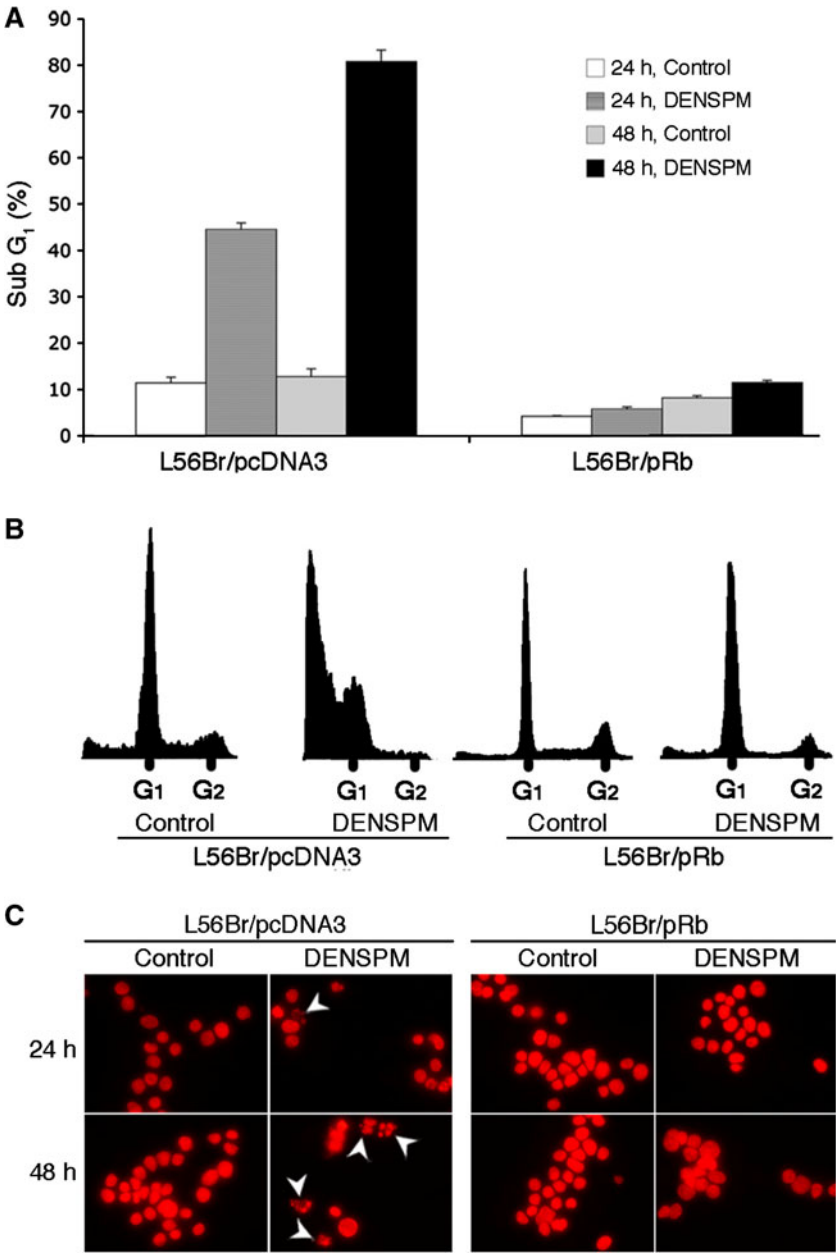

Fig. 3 The effect of DENSPM treatment on apoptosis in L56Br/pcDNA3 cells and L56Br/pRb cells. At time 0, plateau phase cells were seeded in the absence or presence of 10 μM DENSPM. **a** The effect of DENSPM treatment on the sub-G_1 region detected by FCM in L56Br/pcDNA3 and L56Br/pRb cells. The results are presented as mean values of $n = 5$–6 from 2 independent experiments ± SEM. **b** DNA histograms of L56Br/pcDNA3 and L56Br/pRb cells grown in the absence or presence of 10 μM DENSPM for 48 h showing that pRb overexpression reduces the sub-G_1 region. Representative histograms for the data in (**a**). **c** The effect of DENSPM treatment on PI-stained nuclei detected by fluorescence microscopy in L56Br/pcDNA3 and L56Br/pRb cells. The results shown are representative of $n = 5$–6 from 2 independent experiments. *Arrows point* at apoptotic bodies

of evidence support the idea that pRb is an inhibitor of apoptosis. Loss of pRb function is connected to an increased incidence of apoptosis (Chau and Wang 2003). We have previously shown that the human L56Br-C1 breast cancer cell line, which reacts with massive apoptosis to DENSPM treatment (Hegardt et al. 2002; Holst et al. 2008), does not express detectable levels of pRb (Myhre et al. 2008). When L56Br-C1 cells were transfected with human cDNA encoding pRb, DENSPM-induced cell death decreased and the spontaneous cell death observed in L56Br-C1 cells was reduced. The lack of functional p53 in L56Br-C1 cells can not alone account for the extreme DENSPM sensitivity of this cell line, as two other breast cancer cell lines with p53 mutations react only by growth inhibition when treated with DENSPM (Hegardt et al. 2002; Holst et al. 2008; Myhre et al. 2008).

We found that pRb overexpression affected the activity of the polyamine catabolic enzyme SSAT. The SSAT activity was lower in DENSPM-treated pRb overexpressing L56Br/pRb cells than in L56Br/pcDNA cells. Thus, pRb may be involved in the regulation of polyamine metabolism and one possible mechanism could involve PPARγ, a transcription factor that binds to the SSAT promoter and stimulates expression (Ignatenko et al. 2004). By binding to PPARγ, pRb has been suggested to be part of the fine-tuning of the activity of PPARγ (Fajas et al. 2002). In L56Br/pRb cells, the increase in SSAT protein levels after DENSPM treatment was more prominent than in L56Br/

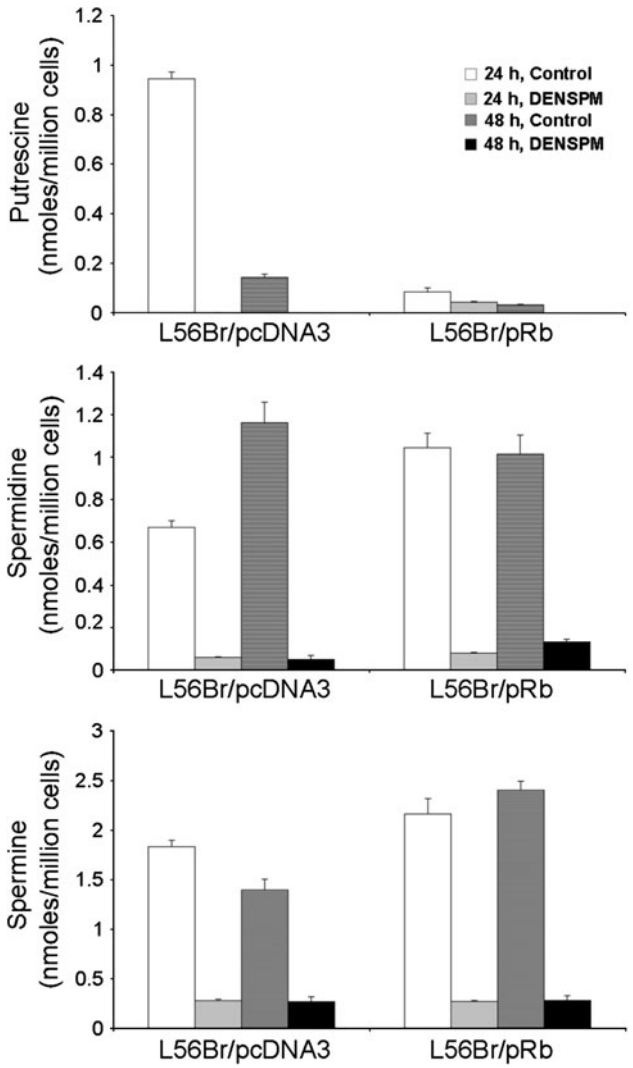

Fig. 4 Effect of DENSPM treatment on putrescine, spermidine and spermine contents in L56Br/pcDNA3 and L56Br/pRb cells. At time 0, plateau phase cells were seeded in the absence or presence of 10 μM DENSPM. The results are presented as mean values of $n = 3$ from independent cultures with *bars* representing ±SD

pcDNA3 cells, although the expression of PPARγ was not affected. It has been shown that pRb is able to interact with over 100 different proteins, leaving the field open to many other possible explanations for our results (Morris and Dyson 2001). The mechanism responsible for the connection between pRb and SSAT remains to be elucidated, and immunoprecipitation of pRb could be useful for further investigations.

Our results indicate that SSAT protein levels do not always correlate to SSAT activity. Although the SSAT activity was higher in L56Br/pcDNA3 cells than in L56Br/pRb cells, the protein level investigated by Western blot was lower in the former cell line. DENSPM-induced SSAT protein levels were investigated in four different breast cancer cell lines. The DENSPM-induced SSAT activity has previously been investigated in these four cell lines. It has been shown that DENSPM causes a massive induction of SSAT activity in L56Br-C1 cells, minor activation in HCC1937 cells, slight activation in MCF-7 cells and no activation in SK-BR-3 cells (Holst et al. 2008). Western blot showed that the degree of DENSPM-induced SSAT protein expression differed between the cell lines, and the SSAT activity did not reflect the levels. This is a new observation suggesting that there is a yet unknown regulation of SSAT activity. What is previously known is that SSAT activity is regulated at several levels, including gene transcription, mRNA translation and protein stability (Casero and Pegg 1993; Fogel-Petrovic et al. 1993; Parry et al. 1995; Wang et al. 1998; Coleman and Pegg 2001; Hyvonen et al. 2006; Butcher et al. 2007). To further study the connection between pRb expression and SSAT activity, it would be interesting to study the effect of siRNA-induced knockdown of SSAT activity in L56Br-C1 cells, but also the effect of siRNA-induced knockdown on the pRb expression in p53 expressing breast cancer cell lines.

This study reports that overexpression of pRb inhibits DENSPM-induced cell death and SSAT activity induction.

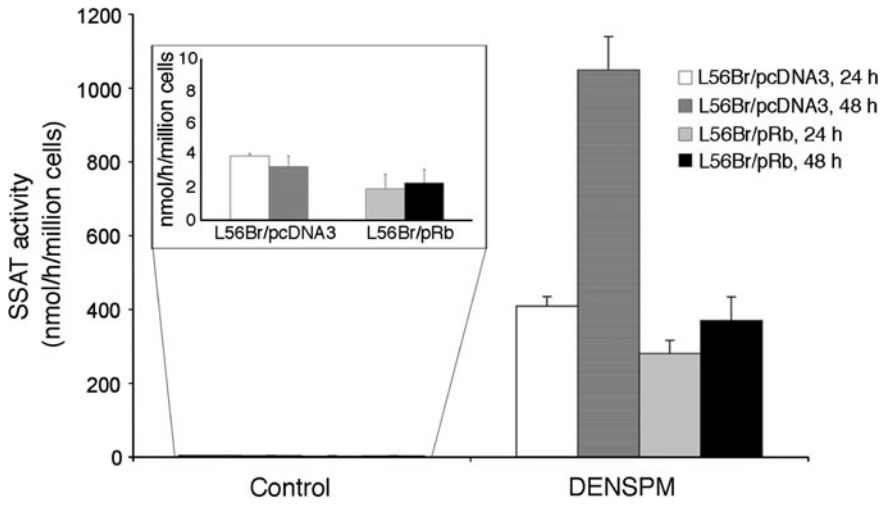

Fig. 5 The effect of DENSPM treatment on the SSAT activity in L56Br/pcDNA3 and L56Br/pRb cells. At time 0, plateau phase cells were seeded in the absence or presence of 10 μM DENSPM. The SSAT activity was determined by using a radiometric assay. The results are presented as mean values from $n = 5$–6 from 2 independent experiments with *bars* representing ±SEM

This suggests that the pRb protein level is a promising marker for polyamine depletion sensitivity and that there is a connection between pRb and the regulation of SSAT activity. We also show that SSAT protein levels and SSAT activity do not always correlate, suggesting that there is an unknown regulation of SSAT.

Acknowledgments We wish to express our gratitude to Prof. Urban Gullberg at the Department of Hematology, Lund University for providing pcDNA3, carrying cDNA for pRb. We wish to thank Ewa Dahlberg for expert technical assistance, and Prof. Bo Baldertorp for the use of the flow cytometer at the Jubileum Institute, Department of Oncology, Lund University Hospital. We also wish to thank Prof. Lo Persson for providing HPLC for the polyamine analysis and Lena Thiman at Department of Physiological Sciences, Lund University for the help with the polyamine analysis. This investigation was supported by grants from the Magnus Bergvall Foundation, the Crafoord Foundation, the Mrs Berta Kamprad Foundation, the Gunnar, Arvid och Elisabeth Nilssons Cancer Foundation, the Per-Eric and Ulla Schybergs Foundation and the Royal Physiographical Society in Lund.

Conflicts of interest The authors declare that they have no conflict of interest.

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