Activation of polyamine catabolism by N^1,N^{11}-diethylnorspermine leads to cell death in glioblastoma

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Abstract. Glioblastoma multiforme (GBM) is one of the most therapeutically refractory human cancers. Elevated cellular polyamine levels are a common feature of cancer cells, including GBM cells, and the polyamine pathway has been explored as a potential therapeutic target to inhibit polyamine biosynthesis or activate polyamine catabolism. In this study, we investigated the effect of N^1,N^{11}-diethylnorspermine (DENSPM), a spermine analog that activates polyamine catabolism, in GBM cells. The *in vitro* cell culture experiments showed that DENSPM increased the sub-G_1 apoptotic cell population in GBM cell lines but caused minimal cytotoxicity in normal astrocytes. Prior to apoptosis induction, DENSPM caused the elevation of spermidine/spermine N^1-acetyltransferase (SSAT) expression accompanied by a decrease in polyamine levels and an increase of acetylated polyamine levels, which temporally coincided with the onset of hydrogen peroxide (H_2O_2) induction in the cells. The cytotoxic effects of DENSPM in the GBM cells could be partially attenuated by either turning down SSAT mRNA with small interference RNA or inhibiting H_2O_2 production with N^1-acetylpolymine oxidase (APAO)/spermine oxidase (SMO) inhibitor. Though mitochondrial damage was induced, neither activation of the caspase cascade nor cytochrome c redistribution between the mitochondria and cytoplasm was observed. Systemic DENSPM treatment of mice with intracerebral GBM led to longer survival. Taken together, our studies indicate that DENSPM kills GBM cells through induction of SSAT coupled with H_2O_2 production, which is a potential target for GBM therapy.

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Abbreviations: DENSPM, N^1,N^{11}-diethylnorspermine; GBM, glioblastoma multiforme; SSAT, spermidine/spermine N^1-acetyltransferase; APAO, N^1-acetylpolyamine oxidase; NHA, normal human astrocyte; SMO, spermine oxidase

Key words: glioblastoma, polyamine, N^1,N^{11}-diethylnorspermine

Introduction

Polyamines are natural factors that are important for cell growth. It has long been recognized that polyamine levels are highly elevated in a broad spectrum of human cancers, including glioblastoma multiforme (GBM) (1-5). GBM is the most advanced and frequently occurring form of glioma. It is also a notoriously refractory cancer, with little improvement in median survival (less than a year) over the past few decades (6,7). The polyamine pathway has been explored as a potential target for cancer therapy through down-regulation of the polyamine biosynthetic pathway or up-regulation of the polyamine catabolic pathway. The initial focus has been on the development of drugs that inhibit polyamine biosynthesis. α-Difluoromethylornithine (DFMO), an inhibitor of the polyamine biosynthesis-limiting enzyme ornithine decarboxylase (ODC), has been tested in various cancer types and shown to exert potent antiproliferative effects as a single agent (8-11). However, DFMO as a single agent was not sufficiently effective in several clinical settings, which has led to the use of combination therapy, with DFMO coupled with methylglyoxal bis-guanylhydrazone or the nitrosourea alkylating agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), procarbazine, *N*-(2-chloroethyl)-*N*'-cyclohexyl-*N*-nitrosurea, and vincristine (PVC) (12-17). Though DFMO combined with BCNU or PVC showed encouraging results in patients with anaplastic gliomas, it had minimal effects on GBM (16,18-20). One possible mechanism for the reduced effectiveness of DFMO alone or in combination therapy in GBM might be compensatory polyamine generation from other sources: increased uptake through the specific polyamine transporter or activation of alternative polyamine biosynthesis

pathways (21,22). In contrast to DFMO, polyamine analogs have been developed to activate the polyamine catabolic pathway. One of these analogs, N^1, N^{11}-diethylnorspermine (DENSPM), can strongly deplete polyamine pools and elevate the expression of spermidine/spermine N^1-acetyltransferase (SSAT) mRNA, resulting in apoptosis with minimal cytotoxicity (23,24). DENSPM has also been shown to be effective in combination therapy. When combined with other chemotherapy drugs, such as 5-fluorouracil and oxaliplatin, DENSPM can markedly elevate SSAT mRNA expression (25,26). The induction of SSAT is accompanied by a synergistic cell kill effect in colon and ovarian cancer cell culture systems (25,27). DENSPM is currently being evaluated as a single agent in several cancers, including lung, breast, and liver cancers (28-31).

The mechanisms of polyamine analog-induced apoptosis have been investigated in a number of studies (23,32-35). SSAT activation is one of the key steps in this process for the majority of polyamine analogs (22). Transfection with SSAT can enhance the apoptosis response to DENSPM (36,37). Reduction of SSAT by small interference RNA (siRNA) attenuates analog-induced apoptosis by inhibiting the depletion of polyamine (23). However, some polyamine analogs deplete polyamine pools in the absence of SSAT activation (38,39), which could lead to apoptosis without SSAT induction (40,41). These findings suggest that polyamine analogs can induce apoptosis in cancer cells via several mechanisms (42).

One key regulator of apoptosis caused by polyamine-analogs that induce SSAT is APAO, which utilizes acetylated spermine/spermidine to produce high levels of H_2O_2. The increased H_2O_2 leads to damage in the mitochondrial membranes, which is believed to be the main cause of the release of cytochrome c. Cytochrome c release is an upstream activator of the cascade of caspases, which in turn initiates the caspase 9-controlled extrinsic cascade of apoptosis (32). However, previous studies have observed inconsistent patterns of caspase involvement in DENSPM-induced cell death (25,34). The application of APAO inhibitor significantly reduced apoptosis induced by polyamine analogs (43). Another key regulator is spermine oxidase (SMO), which converts spermine to spermidine, a reaction that also produces H_2O_2. A small molecule inhibitor of APAO was shown to inhibit SMO as well (44). Thus, it is likely that the balance of these three key enzymes of polyamine catabolism and the composition of polyamine and acetylated polyamines play a critical role in polyamine analog-induced cytotoxicity.

Because of the need for a new therapeutic strategy for GBM, we investigated whether DENSPM induced cytotoxity in two GBM cell lines, U87 and LN229. We explored the underlying apoptotic mechanisms, focusing on the catabolism enzyme SSAT, the mitochondria status/cytochrome c distribution, and the role of H_2O_2 production in the apoptosis process. We further tested whether systemic DENSPM treatment had any therapeutic effect on a GBM xenograft mouse model.

Materials and methods

Reagents. DENSPM was purchased from Tocris (Ellisville, MO) and dissolved in water according to the manufacturer's instructions. CM-H_2DCFDA and rhodamine 123 were purchased from Molecular Probes (Eugene, OR) and dissolved in dimethyl sulfoxide.

Cell lines. The U87 and LN229 GBM cell lines from the American Type Culture Collection (Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% dialyzed serum (Hyclone, Logan, UT) at 37˚C in a 5% CO_2 incubator. Dialyzed serum was used because fetal bovine serum has abundant thymine and polyamine. Normal human astrocytes (NHAs) were purchased from Cambrex Corporation (East Rutherford, NJ) and cultured in AGM™ BulletKit medium according to the manufacturer's instructions.

Cell viability assay. Cell viability was evaluated using the MTS (3-[4,5-dimethythiazol-2-yl]-5-[3-carboxy-methoxyphenyl]-2-[4-sul-fophenyl]-2H-tetrazolium compound) assay (Promega Corporation, Madison, WI). For the MTS assay, we seeded 3,000 GBM cells per well in 100 μl of medium in a 96-well plate. On the second day, various concentrations of DENSPM were added to the wells. For the H_2O_2 effect, GBM cells treated with DENSPM were co-cultured in the presence of the polyamine oxidase inactivator MDL 72527, with a final concentration of 25 μM. After cells were incubated with DENSPM alone or with MDL 72527 for the indicated time, the MTS assay was used to measure cell viability. After 20 μl of MTS solution had been added to each well and mixed, the cells were incubated at 37˚C in the 5% CO_2 incubator according to the product instructions. Absorbance at 490 nm was measured with a microplate reader (MRX, Danatech Laboratory, Houston, TX). All MTS assays were performed at least in triplicate for each treatment condition, and experiments were repeated at least twice.

Cell cycle analysis by flow cytometry. Cells were seeded in a 10-cm^2 dish ($1.5x10^5$ cells/dish) in 10 ml of medium supplemented with 10% dialyzed fetal bovine serum. Twenty-four hours later, 10 μM DENSPM was added. After being incubated with the drug for 48 h (U87 cells) or 72 h (LN229 cells), adherent and non-adherent cells were harvested by trypsinization and centrifugation, washed with ice-cold phosphate-buffered saline (PBS), fixed with ethanol, stained with propidium iodide with RNase A, and analyzed on a FACS-Calibur flow cytometer with Cell Quest Pro software (BD Biosciences, Franklin Lakes, NJ).

Measurement of cellular levels of polyamines. U87 and LN229 cells ($1.0x10^6$ cells/well) were seeded in 25-cm^2 plates and treated with 10 μM DENSPM for 24 h. The cells were collected with trypsin, and the polyamine concentrations were determined by high-pressure liquid chromatography, as described previously (39).

Analysis of mRNA expression by real-time reverse transcription-polymerase chain reaction. The total RNA was extracted from DENSPM treated and untreated U87 and LN229 cells with a Triagent kit (Invitrogen, Carlsbad, CA) and quantified. The mRNA level of SSAT from the cells was quantified using the Applied Biosystems TaqMan method in

conjunction with Assays-On-Demand (ABI PRISM 7900 sequence detection system, Applied Biosystems, Foster City, CA), as described previously (25).

Cellular H_2O_2 staining assay. U87 and LN229 cells ($1.0x10^5$ cells/well) were seeded in 6-well plates and treated with 10 μM DENSPM for 24 and 48 h (U87) and 48 and 72 h (LN229). The cells were treated with CM-H_2DCFDA at a final concentration of 1 μM and incubated for 1 h at 37˚C before being harvested with trypsin and washed twice with ice-cold PBS. Ten thousand cells were analyzed with the FACSCalibur flow cytometer with Cell Quest Pro software. The relative value of H_2O_2 production was calculated on the basis of triplicate experiments.

Mitochondrial transmembrane potential ($\Delta\psi_m$) assay. After $1.0x10^5$ cells/well were seeded in 6-well plates and treated with 10 μM DENSPM for 24 and 48 h (U87 cells) and 48 and 72 h (LN229 cells), cells were treated with rhodamine 123 at a final concentration of 100 nM to detect the disruption of mitochondrial membrane permeability using the flow cytometer. The value of the percent of loss of $\Delta\psi_m$ was calculated based on the triplicate experiments.

Knockdown of SSAT expression by siRNA in GBM cells. Dharmacon SMARTpool® siRNAs (Dharmacon, Lafayette, CO) were used for silencing SSAT with Nucleofector technology, according to the manufacturer's protocol (Amaxa Biosystems, Gaithersburg, MD). For non-specific targeting, negative control siRNA from Ambion was used (Ambion Inc., Austin, TX). Briefly, $2\text{-}3x10^6$ cells were resuspended in 100 μl of Nucleofector solution with 100 nM of siRNAs in the electroporation cuvette. After electroporation, cells were divided into 12-well plates, incubated in the transfection reagent with siRNAs at 37˚C in a humidified incubator with 5% CO_2 for 24 h, and then collected to extract RNA for real-time PCR assay or distributed into 96-well plates to be treated with various concentrations of DENSPM followed by the MTS assay.

Cytochrome c release assay. Cells were seeded in DMEM/F12 medium that was supplemented with 10% dialyzed fetal bovine serum. Twenty-four hours later, 10 μM DENSPM was added, and the treatment continued for 24 and 48 h. Drug-treated cells were harvested for separation of mitochondria and cytosol using the ApoAlert™ cell fractionation kit (BD Biosciences). Antibodies for cytochrome c and cox4 included in the kit were used in Western blot analysis.

Western blot analysis. Total cellular protein extract was isolated from the harvested cells, the protein concentration determined, and Western blotting carried out as described previously (45). Antibodies for cleaved caspase 9 (c-caspase 9) and cleaved caspase 8 (c-caspase 8) were obtained from Cell Signaling (Danvers, MA).

Measurement of DENSPM's cytotoxicity to NHAs. To compare the cytotoxity of DENSPM in NHA and GBM cells, both cell lines were treated with 10 μM DENSPM and then the MTS assay was performed. The medium (AGM Bulletkit) of NHAs contains various growth factors and may contain high levels of polyamine, which might attenuate the DENSPM effect on NHAs. Thus, we replaced the medium (DMEM/F12) of GBM cells with the medium (AGM Bulletkit) of NHA when DENSPM was applied.

In vivo treatment in mouse models. All animal studies were conducted according to a protocol approved by the Institutional Animal Care and Use Committee. Male 6- to 8-week-old athmic mice (The Jackson Laboratory, Bar Harbor, Maine) were housed within an approved specific pathogen-free barrier facility maintained at the M.D. Anderson Isolation Facility in accordance with Laboratory Animal Resources Commission standards. Appropriate measures were taken to minimize animal discomfort, and appropriate sterile surgical techniques were utilized in tumor implantation and drug administration. Animals that became moribund or had necrotic tumors were euthanized. To induce the intracerebral tumors, logarithmically growing U87 cells were engrafted into the caudate nucleus of athymic mice as previously described (46) at $0.5x10^6$ cells in 3 μl Ca^{++}/Mg^{++}-free PBS. Treatment was performed on animals with evident neurological symptoms, such as a single limb limp, weariness, or other mild clinical manifestations, resulting from the intracranial hypertension caused by engrafted GBM. Two weeks after the implantation of the cells and growth of tumors, DENSPM was administered intraperitoneally at a dose of 0.15 g/kg/day, and 1X PBS was similarly administered into the control mice. The treatment continued for 6 days, and survival was measured for up to 52 days.

Statistical analysis. The effect of DENSPM treatment on H_2O_2 production and mitochondrial membrane status was analyzed using SPSS version 12.0 software for Windows (SPSS, Chicago, IL). Differences in means were evaluated by the two-tailed t-test, assuming unequal variances. The difference in the survival expectancy of mouse models treated with DENSPM or control was tested with χ^2 analysis. In both statistical evaluations, a $P<0.05$ was recognized as a significant change.

Results

DENSPM led to marked cell death in GBM cells. Two GBM cell lines, U87 and LN229, were treated with DENSPM, and cellular viability was measured with the MTS assay. A marked decrease in cell viability after DENSPM treatment was observed in U87 cells after 2 days and in LN229 cells after 3 days (Fig. 1A). The cytotoxic effect of DENSPM was time-dependent: less decrease in viability was seen at earlier time-points for both cell lines (data not shown). A flow cytometric analysis indicated that DENSPM treatment resulted in a substantial sub-G_1 population in U87 cells after 48 h and in LN229 cells after 72 h (Fig. 1B).

DENSPM treatment resulted in polyamine depletion, acetylation of polyamine, and up-regulation of SSAT in GBM cells. DENSPM-treated cells were collected at the indicated time-points and analyzed for changes in polyamines, acetylated polyamines, and SSAT mRNA. The levels of spermidine, spermine, and putrescine decreased, whereas the levels of

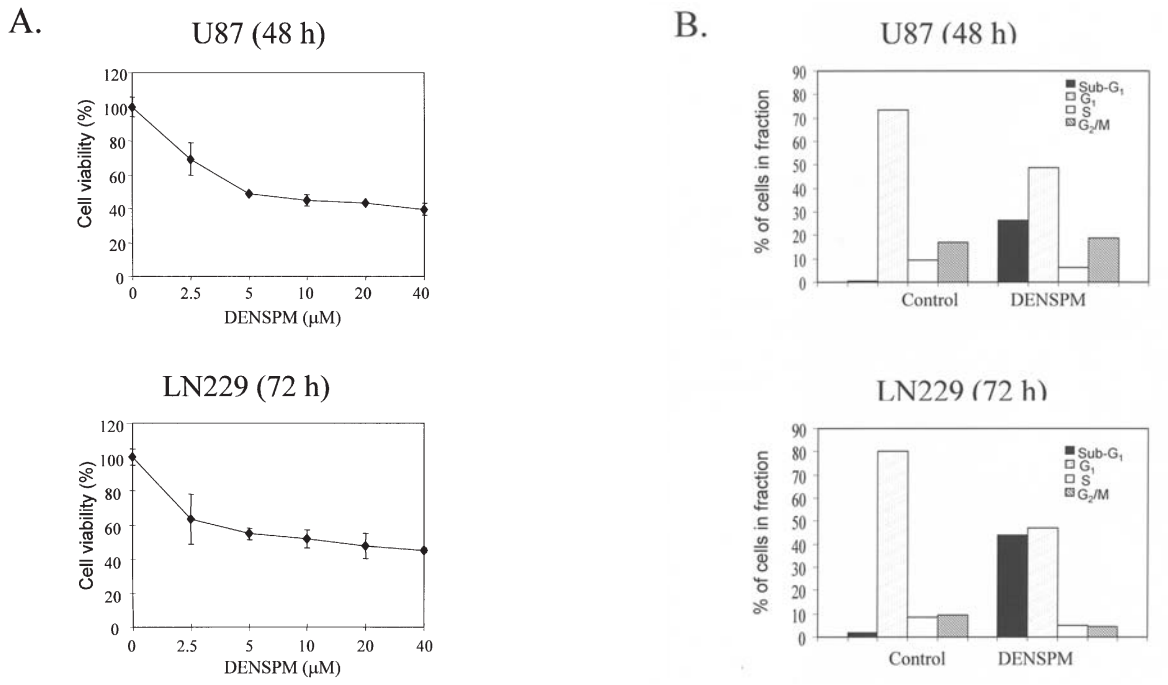

Figure 1. Effects of DENSPM on glioblastoma cell lines. U87 and LN229 cell lines were treated with increasing doses of DENSPM. Cell viability at 48 h for U87 cells and 72 h for LN229 cells was measured with the MTS assay. DENSPM resulted in a dose-dependent decrease in cell viability in both cell lines, and the effect was stronger in U87 cells (A). U87 and LN229 cells were treated with DENSPM (10 μM) and then the cells were harvested for flow cytometry analysis. DENSPM resulted in sub-G_1 peak aggregations in the U87 cell line at the 48-h time-point and in the LN229 cell line at the 72-h time-point (B).

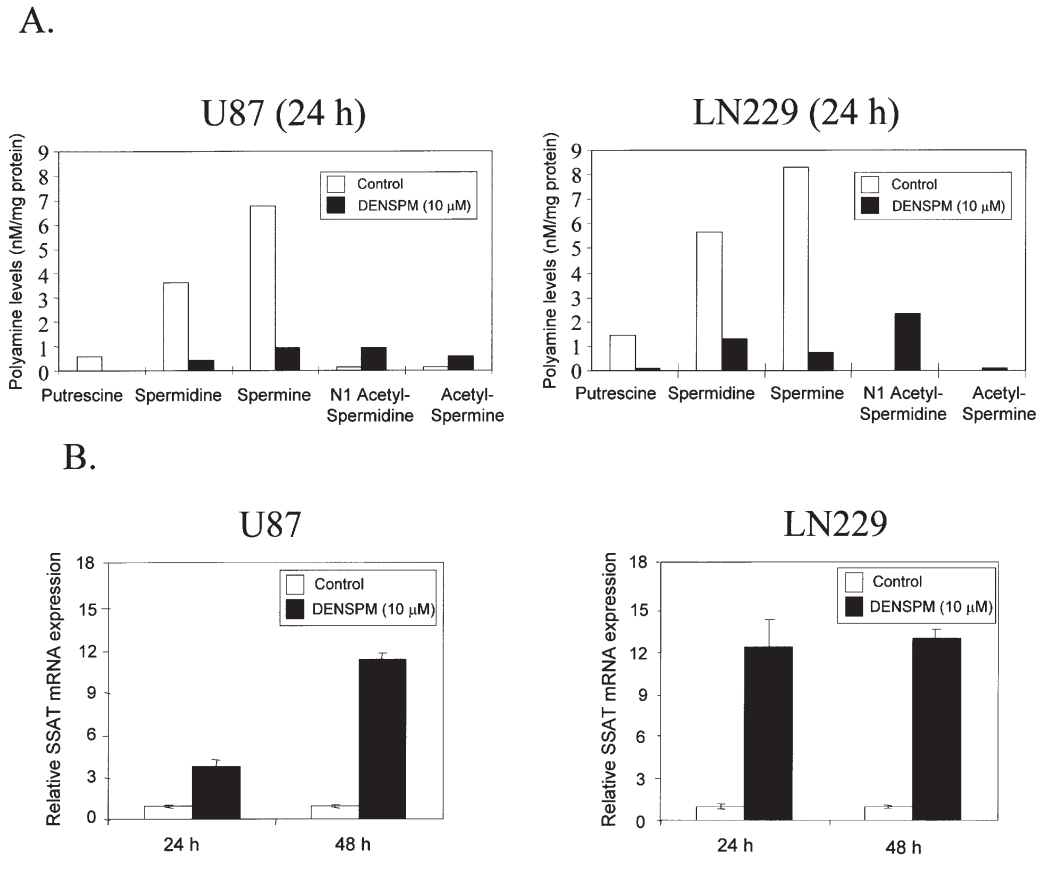

Figure 2. DENSPM led to a decrease of intracellular polyamines and an increase in acetylated spermidine and spermine. A marked elevation in SSAT mRNA was also observed. U87 and LN229 cells were treated with DENSPM (10 μM) for 24 h. The cells were collected, and the levels of intracellular putrescine, spermidine, spermine, N^1-acetyl-spermidine, and N^1-acetyl-spermine were measured by high-pressure liquid chromatography. A marked reduction in putrescine, spermidine, and spermine and an induction in N^1-acetyl-spermidine and N^1-acetyl-spermine were observed (A). Both U87 and LN229 cells were treated with DENSPM (10 μM) and collected to extract the total RNA at 24 and 48 h. A one-step real-time RT-PCR analysis was performed to measure the levels of SSAT mRNA. DENSPM resulted in a marked induction of SSAT in both U87 and LN229 cells (B).

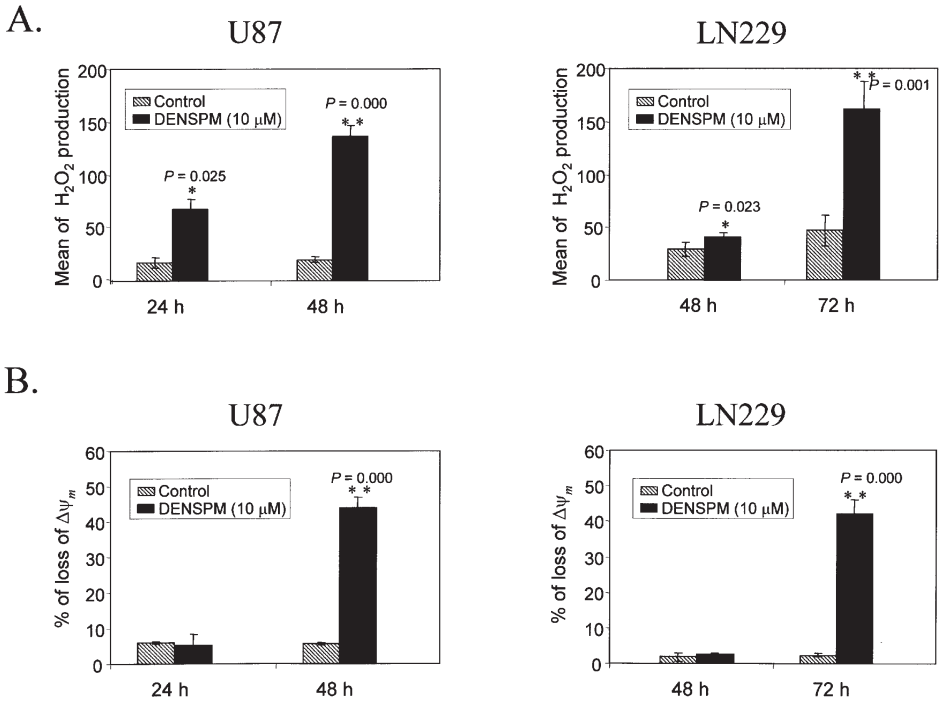

Figure 3. DENSPM induced generation of H_2O_2 and loss of mitochondrial transmembrane potential in GBM cells. After treatment with DENSPM (10 μM) for 24-72 h, both U87 and LN229 cells were divided into 2 groups: the first group was incubated with CM-H_2DCFDA for 1 h at 37˚C and then harvested for analysis by flow cytometry. The relative mean levels of H_2O_2-positive cells were plotted. Each value is an average of the triplicate experiments. H_2O_2 was induced in U87 cells by DENSPM after as early as 24 h and reached a peak at 48 h. In LN229 cells, DENSPM caused initial H_2O_2 production at 48 h and a huge amount at 72 h (A). The second group of cells were incubated with rhodamine 123 for 1 h and then harvested for analysis by flow cytometry. Each value is an average from the triplicate experiments. DENSPM resulted in a significant decrease in mitochondrial membrane potential changes ($\Delta\psi_m$) in both U87 and LN229 cell lines (B). The P values of <0.05 (*) and <0.01 (**) are indicated.

acetylated spermidine and spermine increased (Fig. 2A). The decrease in polyamines and increase in acetylated polyamines coincided with marked induction of SSAT, as measured by real-time RT-PCR analysis (Fig. 2B). The induction of SSAT and changes in polyamine levels in the cells were early events of DENSPM treatment, occurring before marked cytotoxicity was observed.

H_2O_2 was induced and mitochondrial membrane permeability was damaged by DENSPM. H_2O_2 induction and mitochondria damage are two commonly observed features in cancer cells after DENSPM treatment. Similarly, measurements of H_2O_2 using CM-H_2DCFDA showed that incubation of cells with DENSPM led to increased H_2O_2 production in U87 and LN229 cells, beginning after 24 and 48 h of treatment and continuing to increase at 48 and 72 h in these two cell lines, respectively (Fig. 3A). Our measurements of mitochondrial membrane potential changes ($\Delta\psi_m$) showed that DENSPM treatment caused mitochondrial damage in both U87 and LN229 cells (Fig. 3B). The temporal pattern of mitochondria damage was parallel to that of H_2O_2 production and later than SSAT induction and polyamine level changes, suggesting that polyamine alteration occurs upstream of H_2O_2 change and mitochondria damage.

Two enzymatic reactions in polyamine catabolism may be key processes in H_2O_2 production. Acetylated polyamines provide the substrate to an APAO-catalyzed reaction that produces H_2O_2. Pledgie *et al* (44) recently showed that SMO induction by DENSPM treatment is responsible for H_2O_2 production in some cells. To determine whether APAO/SMO plays an important role in DENSPM-induced cell kill and H_2O_2 production, a small molecule inhibitor for APAO/SMO, MDL 72527, was added to both cell lines at a final concentration of 25 μM and incubated in the presence of DENSPM. Cell viability assays showed that MDL 72527 partially attenuated the cytotoxic effect of DENSPM (Fig. 4A) and also blocked H_2O_2 production (Fig. 4B).

The knockdown of SSAT mRNA reduced DENSPM's cytotoxicity in GBM cells. To test the functional significance of SSAT in DENSPM-induced cytotoxicity, we used siRNA to knockdown the expression of SSAT in U87 and LN229 cells. Real-time PCR analysis confirmed that the expression of SSAT mRNA in both U87 and LN229 cells transfected with siRNA was about 70-90% less than that in cells transfected with non-targeting siRNA (Fig. 5A). The knockdown of SSAT mRNA reduced the sensitivity of both U87 and LN229 cells to DENSPM by at least 20% (Fig. 5B).

DENSPM did not induce cytochrome c release. DENSPM has been reported to induce apoptosis in several human cancers, and the accompanying release of cytochrome c from the mitochondria has been observed (32). These observations suggest that cytochrome c redistribution and activation of

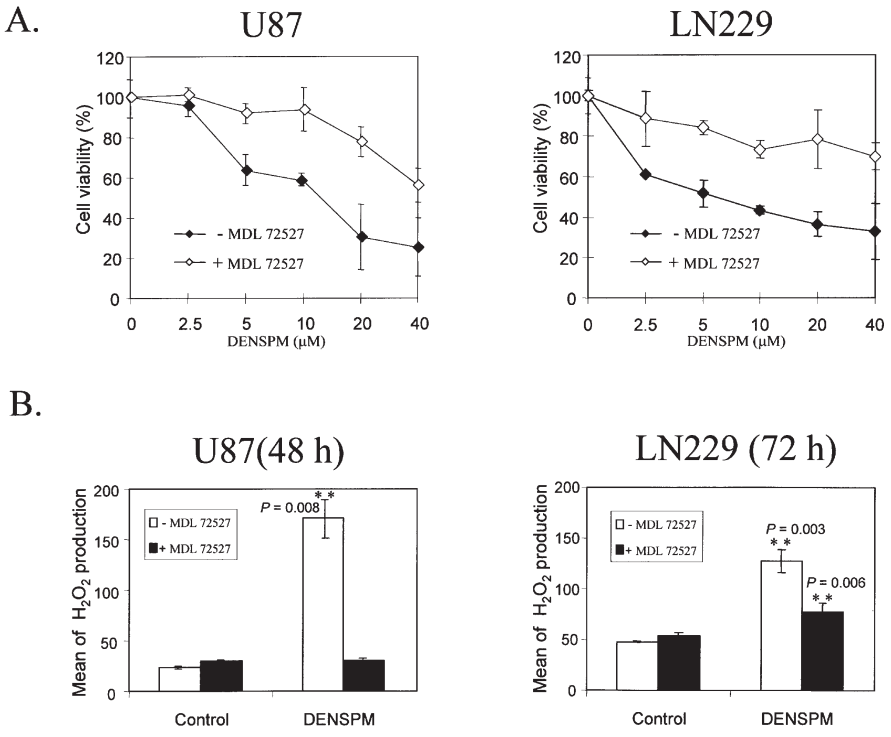

Figure 4. APAO/SMO inhibitor (MDL 72527) attenuated the cytotoxity and blocked the H_2O_2 production induced by DENSPM. Increasing doses of DENSPM were added to U87 and LN229 cells in the presence or absence of a 25-μM final concentration of MDL 72527 for the indicated times (48 h in U87 and 72 h in LN229). This was followed by the MTS assay. The cytotoxic effect of DENSPM was reversed by MDL 72527 both in U87 and LN229 cells (A). In total 4 different media were prepared, including free of drug, containing 25 μM MDL72527 alone, 10 μM DENSPM alone and the medium with 25 μM MDL72527 and 10 μM DENSPM. The cells were selectively collected at the time-point when a large amount of H_2O_2 should be induced by DENSPM (48 h for U87 and 72 h for LN229 respectively). The production of H_2O_2 was significantly attenuated by MDL72527 in both U87 and LN229 cells. The difference in the relative mean value of H_2O_2 between the drug-free control and the treatment group either with DENSPM alone, MDL72527 alone or combination of DENSPM and MDL72527 was calculated (B). The P values of <0.05 (*) and <0.01 (**) are indicated.

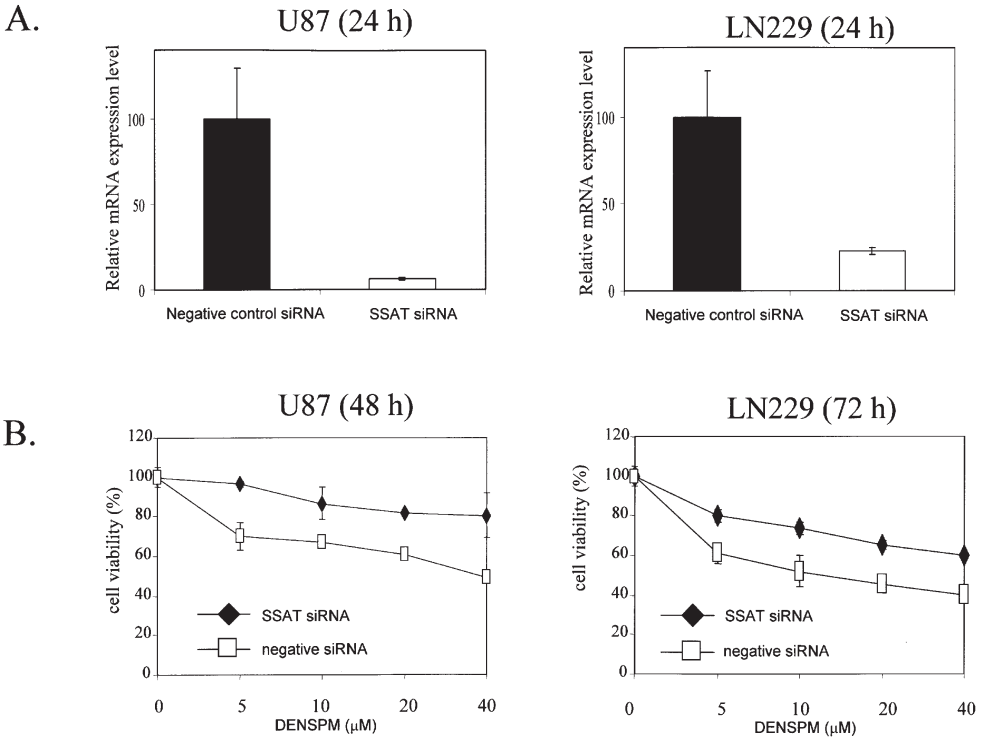

Figure 5. Knockdown of SSAT mRNA decreased the sensitivity of GBM cell lines to DENSPM. U87 and LN229 cells ($2.5x10^6$) were transfected with 100 nM SSAT siRNA and the negative control siRNA. Then the cells were separately seeded into a 12-well plate for RNA extraction and into 96-well plates for the MTS assay. Twenty-four hours later, the real-time PCR results showed that the mRNA expression of SSAT in both U87 and LN229 cells was reduced by about 70-90% compared with negative control siRNA-transfected cells (A). The MTS assay showed that the knockdown of SSAT significantly reduced the sensitivity of both U87 and LN229 cells to DENSPM compared with negative control siRNA-transfected cells (B).

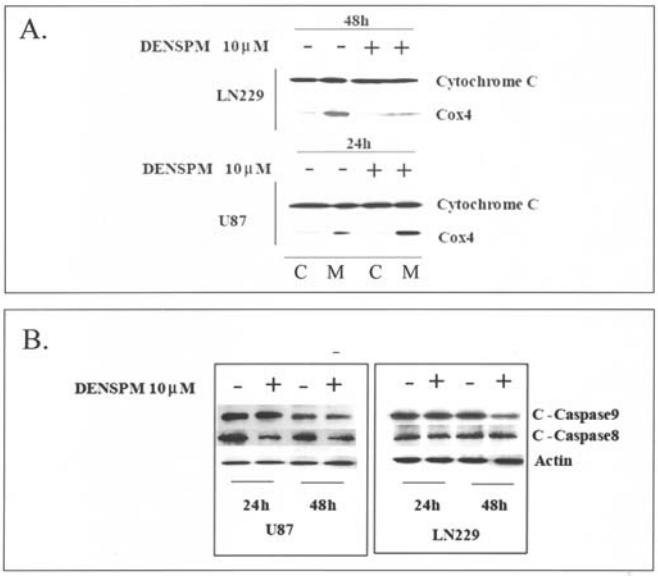

Figure 6. Neither cytochrome c release nor activation of the caspase families occurred after DENSPM treatment. The DENSPM-treated U87 and LN229 cells and their corresponding drug-free controls were collected at the indicated time-points. After their proteins were extracted as the mitochondrial and cytosolic fractionations, Western blotting was performed. The same transferred membrane was probed with Cox4 and cytochrome c antibody successively, which indicated the right protein fractionation and the distribution of cytochrome c, respectively. No marked redistribution of cytochrome c was observed in either U87 or LN229 cells treated with DENSPM, even when apoptosis in these cells was imminent (A). C, cytosol fraction; M, mitochodria fraction. The total proteins were also extracted to measure the caspase families with Western blotting, but no marked change was observed in caspase 8 or 9 (B).

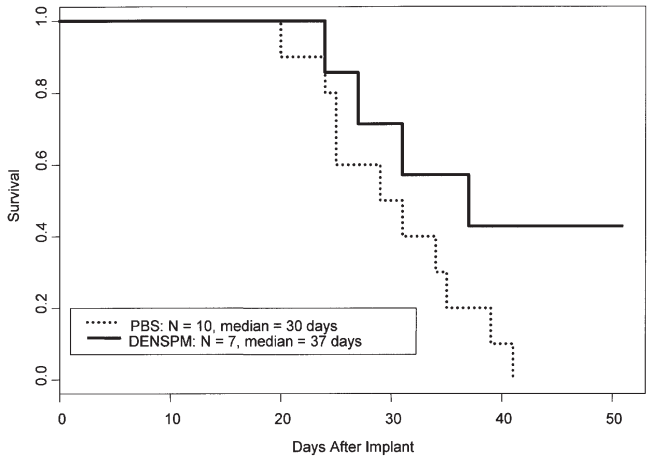

Figure 8. DENSPM improved survival in a GBM mouse model. In the U87 mouse model, all of the sham-treated mice died by the 43rd day, whereas there were still three mice in the DENSPM-treated group who continued to survive without significant neurological signs. The difference in life expectancy between the sham-treated and DENSPM-treated mice was significant (P=0.05).

caspases are downstream mechanisms of DENSPM-induced cell death. We thus examined whether DENSPM induced a release of cytochrome c in GBM cells. However, even after treatment with DENSPM, at which time marked apoptosis had happened (Fig. 1) and a significant disturbance of the mitochondrial membrane was detected in both cell lines (Fig. 3B), no cytochrome c redistribution was observed (Fig. 6A). It is of interest that abundant cytochrome c was also present in the cytoplasm before treatment, in contrast to what we observed in other cell types such as colon cancer (25). Lack of cytochrome c induction was consistent with lack of activation of caspases in the treated GBM cells (Fig. 6B).

DENSPM caused minimal cytotoxicity in NHAs. To determine whether DENSPM's effect is discriminatory between GBM cells and NHAs, which would determine the drug's therapeutic index, we performed drug treatment experiments. We found that the same treatment scheme with DENSPM induced much less cytotoxicity in NHAs than in GBM cells under the same culture conditions (the type of the media and the percentage of serum in the media) (Fig. 7).

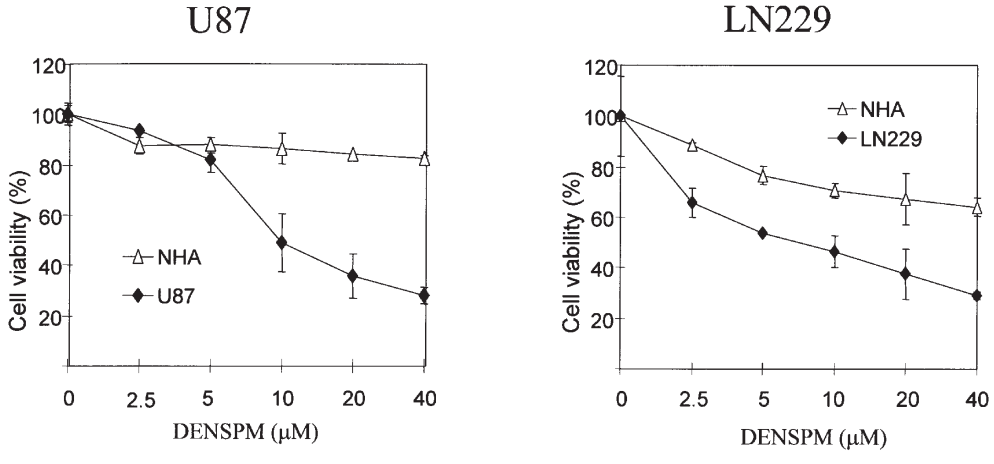

Figure 7. DENSPM caused minimal cytotoxicity to NHAs. U87 and LN229 cell lines were treated with increasing doses of DENSPM for 48 and 72 h, respectively. NHA was included for comparison. Considering that the DENSPM effect on the NHAs might be attenuated by the medium for NHA, the DENSPM-treated GBM cells were also grown in the medium for NHA. DENSPM was much less toxic to NHAs than to GBM cells.

expectancy of GBM tumor-bearing mice. The inhibitory effect of DENSPM on GBM cells *in vitro* prompted us to examine DENSPM treatment in an animal model. A total of 17 mice were randomized to receive DENSPM treatment (n=7) or 1X PBS solution for sham treatment group (n=10). After treatment with DENSPM for 6 days, followed by an additional 46 days of observation, improved survival was observed in the U87 mouse model (Fig. 8). χ^2 analysis showed that the difference in life expectancy between the DENSPM treatment and sham treatment groups was significant (P=0.05).

Discussion

Our results show that activation of polyamine catabolism by DENSPM induced apoptosis in two different GBM cell lines. Whereas the effects of DENSPM on induction of SSAT, polyamine depletion, H_2O_2 production, and mitochondrial damage were similar to that reported in other cancer cells, we observed for the first time that apoptosis of GBM cells by DENSPM may not require the cytochrome c pathway because we did not observe cytochrome c release from the mitochondria after treatment, even in the presence of mitochondria membrane damage. Interestingly, cytochrome c is already abundant in the cytoplasm of GBM cells, without any drug treatment. This is in contrast to other types of cells, such as colon cancer cells, tested in our group (25).

Consistently, we did not observe activation of caspases, which suggests that polyamine activation-mediated apoptosis does not occur via the intrinsic apoptosis pathway mediated by mitochondria. Thus, mitochondria damage could be a secondary event of a key apoptosis inducer, which our data suggest is H_2O_2. First of all, H_2O_2 production occurs temporally parallel with that of mitochondrial damage and apoptosis. Most important, blockage of H_2O_2 production by APAO/SMO inhibitor attenuated apoptosis. However, H_2O_2 induction alone is not sufficient for apoptosis induction. We have also observed H_2O_2 induction in Temozolomide treated GBM cells, which results in G_2/M, rather than sub-G_1, arrest (data not shown). On the other hand, though we confirmed that the knockdown of SSAT reduced the sensitivity of GBM cells to DENSPM, it seems that SSAT alone is also not responsible for DENSPM activity because some polyamine analogs that do not induce large amounts of SSAT mRNA also inhibit cancer cells effectively (42,47). Following this reasoning, we propose that depletion of polyamines and production of H_2O_2 are two key factors in GBM apoptosis induction, which can be fulfilled by activation of polyamine catabolism.

It has been recognized since the 1960s (48) that cancer is a disease of elevated metabolism. The polyamine catabolism pathway is a key pathway for GBM growth and survival. This study shows that this pathway could be an effective target for therapeutic intervention. DENSPM treatment is particularly attractive because it killed fewer normal astrocytes than GBM cells. Our preliminary *in vivo* experiment also shows a promising effect of systemic DENSPM treatment on GBM tumors in an organism.

It is interesting that the polyamine pathway may be the converging pathway in GBM tumors because insulin-like growth factor activation has been shown to lead to an increase in SSAT (49,50), suggesting an elevation of natural polyamine levels in cells. A possible scenario is that oncogenic alterations such as insulin-like growth factor receptor and phosphatidylinositol 3-kinase/akt activation increase the metabolism of cancer cells, leading to a high demand for polyamines. Treatment with polyamine analogs, such as DENSPM deprives the cells of the real polyamines that are required for normal function but triggers the normal feedback mechanism by activating SSAT, thus further removing functional polyamines from the cells through acetylation.

Two known enzymes are candidates for production of H_2O_2: APAO and SMO. The small molecular inhibitor we used in this study has been shown to inhibit both APAO and SMO. Therefore, the inhibitor experiment itself does not seem to differentiate between the two sites of action. However, DENSPM treatment markedly reduced polyamine levels in the U87 cells in the absence of significant SMO induction (data not shown). This reduced substrate level and absence of enzymatic activation potentially excludes SMO as the enzyme responsible for H_2O_2 production, at least in U87 cells and suggests that APAO is the key enzyme in the production of H_2O_2 in treated U87 cells. We did not detect elevated APAO protein or specific enzymatic activity. However, induction of APAO is not necessary to the observed increased activity as it is rate-limited by the availability of its substrates, the acetylated polyamines. Our data indicate that these substrates were markedly induced and should be sufficient to produce the observed increase in H_2O_2. Although further experiments will be required to confirm the source of H_2O_2, the above data are consistent with APAO as the leading candidate.

Taken together, the results of this study reveal that H_2O_2 induction and polyamine depletion by increased polyamine catabolism are targets for therapeutic intervention. Polyamine analogs that modulate these steps may emerge as effective drugs for GBM treatment.

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