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Development of sulforaphane-encapsulated microspheres for cancer epigenetic therapy

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ABSTRACT

Even though conventional chemotherapeutic management of cancer has reduced morbidity and mortality to a great extent, virtually all chemotherapeutic agents cause damage to healthy cells, necessitating exploration of novel anticancer agents that exert their effects through an alternate mode of action. Objectives of our research were twofold. First, we explored the promising potential of histone deacetylase inhibitor sulforaphane for epigenetic therapy for cancer as this therapeutic approach aims to reverse aberrant epigenetic modifications that affect gene expression. *In vitro* cell culture studies performed using B16 and S91 melanoma cells showed that sulforaphane inhibited growth and proliferation of cancer cells by downregulating deacetylation enzymes. The second part of our research investigated polymeric drug delivery systems to increase therapeutic efficacy and to minimize potential side effects of R,S-sulforaphane. Albumin microspheres encapsulating sulforaphane were developed by spray drying. Microspheres were characterized for their morphology, size and zeta potential. Cell culture studies using melanoma cells and *in vivo* studies in melanoma tumor-bearing C57BL/6 mice demonstrated that albumin based polymeric delivery system was efficacious and has the potential to enhance the therapeutic effect and anticancer activity of sulforaphane.

1. Introduction

Cancer is a complex disease that is characterized by the loss of cellular controls through the accumulation of genetic and epigenetic defects. Genetic alterations are contributed by an array of modifications in DNA including mutations that culminate in aberrant cellular functions such as activation of oncogenes and inactivation of tumor suppressors. Epigenetic changes, on the other hand, affect gene expression without direct alterations in DNA sequences. Although exact mechanisms are largely unknown, of late epigenetic therapy is gaining a lot of attention and is showing great promise as a treatment modality against cancer (Lindemann et al., 2004; Carew et al., 2008; Mariadason, 2008; Paris et al., 2008). Among the various compounds in this category, histone deacetylase inhibitors (HDACIs) represent a new class of molecules for epigenetic therapy. Deacetylation of histones is associated with transcriptional repression (Zhu et al., 2004; Glozak and Seto, 2007). Consistent with this view, histone deacetylases are overexpressed in cancers of varied tissue origin, such as colon, breast and prostate (Zhang et al., 2004; Zhu et al., 2004; Carew et al., 2008).

Histone deacetylase inhibitor sulforaphane has been shown to be an effective anticancer agent in both *in vitro* and *in vivo* studies (Juge et al., 2007; Clarke et al., 2008). It has been proposed that the mode of action of sulforaphane is at least in part due to the induction of Phase 2 enzymes and inhibition of Phase 1 enzymes (Maheo et al., 1997; Myzak and Dashwood, 2006). In addition, sulforaphane can exert its antitumor effect via anti-proliferative mechanisms, such as cell cycle arrest and apoptosis (Singh et al., 2004; Choi and Singh, 2005; Gamet-Payrastre, 2006; Choi et al., 2008). Sulforaphane has been shown to have significant chemopreventive activity in several animal models (Zhang et al., 1994; Chung et al., 2000; Solowiej et al., 2003). This small molecule has also been documented to be effective in blocking initiation and progression of carcinogenesis (Clarke et al., 2008; Yao et al., 2008). Studies have shown that the administration of sulforaphane enhanced natural killer (NK) cell activity and antibody-dependent cellular cytotoxicity in both normal and tumor-bearing animals (Thejass and Kuttan, 2006, 2007; Singh et al., 2009). Although the pure form of sulforaphane has not been tested in humans, broccoli sprouts, which are a rich source of sulforaphane, have been tested in clinical trials. Data from these studies indicate that they are well tolerated with minimal side effects (Kensler et al., 2005; Shapiro et al., 2006).

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Even though sulforaphane shows great promise as an anticancer agent, its potential for clinical use is limited by a number of factors. Sulforaphane has a relatively short half-life of less than 2 h (Petri et al., 2003; Hu et al., 2004). In addition, sulforaphane is not very stable under normal conditions (Van Eylen et al., 2007). Consequently, administration of this agent to attain therapeutically effective doses might be problematic. In addition, to achieve maximal therapeutic response and optimal inhibition of histone deacetylase activity, continuous presence of the HDACI in systemic circulation is required (Drummond et al., 2005). Rapid clearance and metabolism can significantly influence the bioavailability as well as the anticancer activity of HDACIs. Even though sulforaphane rapidly accumulates in cell lines, it is also rapidly metabolized through the mercapturic acid pathway. Sulforaphane reacts with glutathione (GSH), forming a glutathione–sulforaphane conjugate. This conjugate subsequently undergoes several enzymatic modifications to form N-acetylcysteine–sulforaphane conjugate (Fimognari et al., 2008). In rats, within 24 h after administration of a single dose of sulforaphane, about 72% of the drug was recovered in urine as N-acetylcysteine–sulforaphane conjugate (Kassahun et al., 1997; Tang et al., 2006; Zhang et al., 2006). These data further emphasize the need for maintaining therapeutic levels of sulforaphane constantly in systemic circulation to produce effective anticancer effect especially since this compound is rapidly metabolized and excreted. The need for daily infusions of HDACI to maintain continuous drug in circulation in clinical settings can be overcome by designing a good delivery system.

The major focus of the current investigation was to develop an efficient delivery system to administer sulforaphane in order to overcome the aforementioned drawbacks so that effective inhibition of cancer cells could be achieved. The present study explored the utility of encapsulating sulforaphane in albumin microspheres to overcome the drawbacks mentioned above and as a potential delivery system to attain effective therapeutic effect. Such a delivery system providing sustained drug release would also minimize generalized toxicity. Our results indicated that sulforphane microspheres showed marked inhibition in a panel of tumor cells in culture, and exhibited *in vivo* efficacy against B16 melanoma in C57BL/6 mice. In addition, sulforaphane significantly reduced histone deacetylase levels both *in vitro* and *in vivo* systems. Data obtained from our studies in melanoma cancer cells can be used to design treatment strategies for other types of cancers. The disruption in the equilibrium of histone acetylation and deacetylation is a fundamental process in cancer cells (Carew et al., 2008).

2. Materials and methods

2.1. Formulation and characterization of sulforaphane microspheres

To formulate microspheres, bovine serum albumin (BSA; fraction V) was dissolved in deionized water at a final concentration of 1% and crosslinked chemically using glutaraldehyde for 24 h. R,S-sulforaphane (LKT Laboratories, Inc., St. Paul, MN, USA) was added to the crosslinked solution at concentrations required to achieve the desired drug loading. Blank microspheres were obtained by using similar procedure as above but without the addition of sulforaphane. The solution containing the various constituents was spray dried using a mini spray dryer (Buchi 191; Buchi Corporation, New Castle, DE, USA). The parameters for spray drying were optimized with inlet temperature and outlet temperature settings of 110 and 80 °C, respectively. The air flow rate was maintained at 800 N l/h, and the pump rate was set at 1%. Percent recovery was calculated by dividing the weight of microspheres by the total initial weight of all components in the solution.

2.2. Surface morphology of microsphere formulations

Scanning electron microscopy was performed to determine the size of the microspheres, the uniformity of the microspheres in the preparation as well as to evaluate surface morphology. Microsphere formulations were coated with gold and the images were obtained using a scanning electron microscope (JEOL JSM 5800LV, Tokyo, Japan).

2.3. Particle size distribution

Microspheres were measured using laser diffraction particle sizer (Malvern Zetasizer ZEN1600). Microspheres were suspended in distilled water at a final concentration of 2 mg/ml, and the volume median diameter of microspheres was measured.

2.4. Zeta potential measurement

Microspheres were suspended in distilled water at a final concentration of 2 mg/ml. The suspension was loaded into an optical well and zeta potential was determined (Malvern Zetasizer ZEN1600).

2.5. Dissolution studies

For dissolution studies, approximately 25 mg of microspheres (1 mg of sulforaphane assuming 100% encapsulation) containing sulforaphane were placed in a dialysis bag (MWCO 14 kDa) with 3 ml of PBS, pH 7.4. MWCO 14 kDa effectively block BSA, which is approximately 66 kDa. Dissolution studies were also performed for blank microspheres (no sulforaphane) using similar procedure as above to ensure that the dialysis bag does not cause any interferences to the reading. Dissolution studies were performed using the modified USP type 1 dissolution apparatus Distek at 37 °C and 100 rpm of basket revolution in 15 ml PBS, pH 7.4. At predetermined time intervals, 2 ml samples were withdrawn, and 2 ml of fresh PBS was replaced at each sampling time interval. Sulforaphane concentrations at various time intervals were measured using a UV–vis spectrophotometer at 235 nm.

2.6. Cell culture

Murine S91 and B16 melanoma cells (ATCC) were grown in DMEM and RMPI-1640 media, respectively. The media were supplemented with 10% (v/v) fetal bovine serum, 50 μg/ml penicillin, 50 U/ml streptomycin and 2 mM L-glutamine. The cell cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was renewed every other day. Cells were detached for transfer with 0.1% trypsin and 10 μM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline.

2.7. Uptake studies in cultured cells

Murine S91 and B16 melanoma cells were plated in T25 tissue culture flasks and allowed to adhere. The confluent cells were then treated with fluorescamine-labeled microspheres at a final concentration of 1 mg/ml. Fluorescamine-labeled microspheres were prepared according to previously established procedures (Nettey et al., 2007). At predetermined time points, cells were washed twice with PBS and lysed in lysis buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100). The cell lysate was then analyzed for fluorescamine at excitation 390 nm and emission 465 nm using a fluorescent plate reader (Tecan).

2.8. Cytotoxicity studies with sulforaphane microspheres

To determine the *in vitro* efficacy of R,S-sulforaphane-encapsulated in microspheres, the cytotoxicity of R,S-sulforaphane microspheres were tested in cell cultures. Cell viability was determined by MTS assay (Promega). S91 and B16 melanoma cells were seeded in 96-well plates at a concentration of 1×10^4 cells/well. After incubating for 24 h, cells were treated with various concentrations of R,S-sulforaphane microspheres containing 0.1% dimethyl sulfoxide (DMSO) in culture medium for desired times. The control cultures were treated with 0.1% DMSO alone in culture medium. After the treatment period, dye solution (20 μl), specific for the MTS assay, was added to each well followed by an additional 4 h incubation at 37 °C. After the incubation period, the absorbance at 490 nm was recorded using a microplate reader (BioTek Instruments, Inc.). The percent viability of the treated cells was calculated as follows: $(A_{490\,nm})_{sample}/(A_{490\,nm})_{control} \times 100$.

2.9. Preparation of nuclear extract from in vitro cell cultures

After incubating with various concentrations of sulforaphane microspheres for 24 h, cells were harvested by trypsin–EDTA treatment as previously described. Nuclear extract was obtained from cell cultures according to Akhavein et al. (2009). Approximately 600 μl of ice-cold hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2% Triton X-100, 1 mM PMSF) was added to the trypsinized cells. Cells were lysed on ice for 20 min and centrifuged at 1000 × *g* for 10 min at 4 °C. The pellet was resuspended in 600 μl of lysis buffer, and the cells were lysed using a mechanical homogenizer. The suspension was centrifuged at 8000 × *g* for 20 min at 4 °C. The cell pellet was resuspended in 350 μl of buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM DTT, 1.0% Igepal CA-630, 25% (v/v) glycerol, 1 mM PMSF) and homogenized. The suspension was centrifuged for 10 min at 4 °C at 14,000 × *g*. The supernatant containing the nuclear extract was recovered for subsequent analysis. Protein content was determined using a DC Protein Kit (Biorad). This nuclear fraction was stored at −80 °C until further processing.

2.10. In vitro histone deacetylase (HDAC) activity levels

In vitro HDAC activity was determined using the colorimetric HDAC activity assay kit (BioMol, St. Paul, USA) according to the manufacturer's instructions. Briefly, approximately 10 μg of nuclear extract was incubated with the HDAC assay buffer and the HDAC colorimetric substrate for 30 min at 37 °C. To the above assay solution, lysine developer was added, and the samples were incubated at 37 °C for an additional 30 min. At the end of the incubation period, readings were taken at 405 nm using an ELISA plate reader. HDAC activity levels were measured from S91 and B16 melanoma cell lines. Extracts from cells not treated with sulforaphane served as controls to make conclusions regarding the effect of treatment.

2.11. Global histone changes in cancer cells

After incubating with various concentrations of sulforaphane for 24 h, cells were recovered by trypsin–EDTA as previously described. Nuclei were purified from cancer cells as described by Pai et al. (1998). Histone proteins were extracted from nuclei as described (Green and Do, 2009). Global histone changes induced by sulforaphane in cancer cells were analyzed using two-dimensional gel electrophoresis system containing acetic acid–urea–Triton X-100 in the first dimension and acetic acid–urea in the second dimension (Green and Do, 2009).

2.12. Induction of B16 melanoma tumor in mice

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Animal care and experimentation were conducted according to the protocol approved by Mercer University Institutional Animal Care and Use Committee (IACUC). To induce solid tumor in C57BL/6 mice, 2×10^6 B16 melanoma cells were suspended in RPMI-1640 media and implanted subcutaneously between the ears of mice. Briefly, the skin was gently lifted and the melanoma cell suspension in a 1.0 ml syringe was administered via a 22-gauge needle by making an insertion below the skin layer. Cells were delivered in a 0.1 ml volume. Cells were allowed to grow to form tumors.

2.13. In vivo efficacy of sulforaphane

The study consisted of four groups, with each group consisting of six B16 melanoma bearing mice. *Group A* (controls) did not receive any treatment. *Group B*, mice were treated with blank microspheres (no drug). *Group C*, mice received sulforaphane solution whereas *group D*, mice were treated with microsphere formulation containing sulforaphane. Animals were randomly assigned to receive vehicle control (PBS), blank microspheres, free sulforaphane (500 μM/kg) or sulforaphane microspheres (500 μM/kg) via intraperitoneal injections three times a week for a total period of 4 weeks. Sulforaphane concentrations from microspheres were determined using UV–vis spectrophotometer at 235 nm. For determination of antitumor activity, tumor volumes were measured using a vernier caliper. Tumor volumes were calculated by using the following formula: 0.5 × length (mm) × width (mm) × width (mm). Mice were also weighed twice weekly. Comparison of efficacy of the treatment between sulforaphane microsphere formulation and the sulforaphane solution groups were made.

2.14. Statistical analysis

Data from all *in vivo* studies were analyzed using one-way ANOVA to assess the statistical significance of the observations. A probability of $p < 0.05$ was considered statistically significant.

2.15. Nuclear extract from tumors

All the steps for the preparation of the nuclear extract were performed on ice. Tumor samples from the various animal experimental groups were collected and were rinsed twice with PBS. Samples were homogenized in buffer A (15 mM HEPES, pH 7.5, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 340 mM sucrose, 0.15 mM β-mercaptoethanol, 1 mM NaF, 10 mM sodium butyrate, 1 mM PMSF) on ice using a mechanical homogenizer. The suspension was filtered through three layers of cheesecloth, and the homogenate was layered onto 5 ml of 1:1 buffer A/buffer B (buffer B contains 15 mM HEPES, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 2.1 M sucrose, 0.15 mM β-mercaptoethanol, 1 mM NaF, 10 mM sodium butyrate and 1 mM PMSF). The suspension was centrifuged for 20 min at 14,000 × *g* at 4 °C. The supernatant was aspirated and discarded. The pellet was resuspended in 10 ml of buffer B. The suspension was layered onto 15 ml of buffer B and centrifuged at 20,000 × *g* for 30 min at 4 °C. This nuclear pellet was resuspended in 1 ml of buffer C (20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM DTT, 1.0% Igepal CA-630, 25% glycerol, 1 mM PMSF) and homogenized. The suspension was centrifuged for 10 min at 4 °C at 14,000 × *g*. The supernatant containing the nuclear extract was

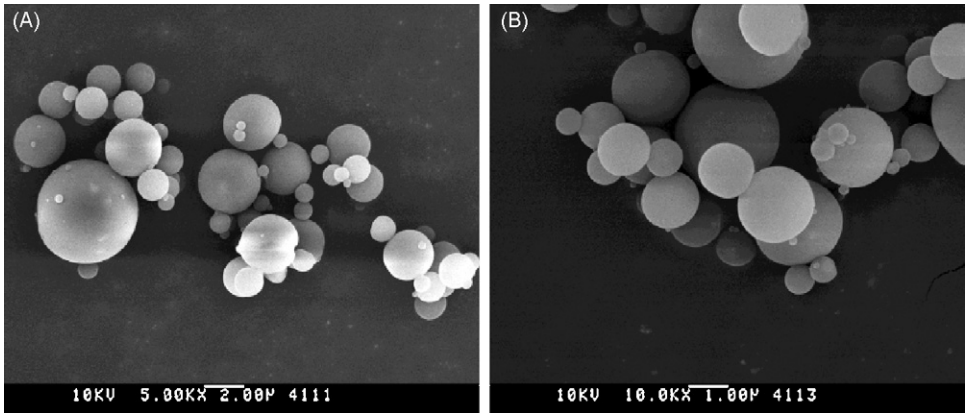

Fig. 1. Scanning electron microscopic images of sulforaphane microspheres. Scanning electron microscopy was done as described in Section 2. A: 5000× magnification, B: 10,000× magnification.

recovered for subsequent analysis. Protein content was determined using DC assay (Biorad).

3. Results and discussion

3.1. Size and surface morphology characterization of sulforaphane microspheres

Microspheres were prepared using spray drying and were characterized for various parameters, including zeta potential, average particle size, and percent recovery (Table 1). Microparticles were spherical in shape, when analyzed by scanning electron microscopy (Fig. 1). This property is important for protecting the integrity of the encapsulated drug. Majority of the microspheres had a uniform surface morphology, with a small fraction exhibiting doughnut shape. Fig. 2 shows a representative analysis for size distribution of microsphere formulations. In the formulation analyzed, a large fraction of microspheres were about 2 μM in diameter. Earlier studies have indicated that microspheres between 1 and 10 μM are phagocytosed efficiently by professional antigen presenting cells, such as macrophages (Ahsan et al., 2002; Makino et al., 2004; Champion et al., 2008). Hence, the size range of sulforaphane microspheres generated by our methodology should facilitate optimal uptake by macrophages.

Table 1
Physicochemical characterization of microsphere formulations.

Microsphere formulations	Zeta potential (mV)	Average particle size (μM)	Recovery (%)
Blank	−32.6 ± 1.5	2.0 ± 0.2	80
Sulforaphane	−32.4 ± 2.1	2.4 ± 0.5	75

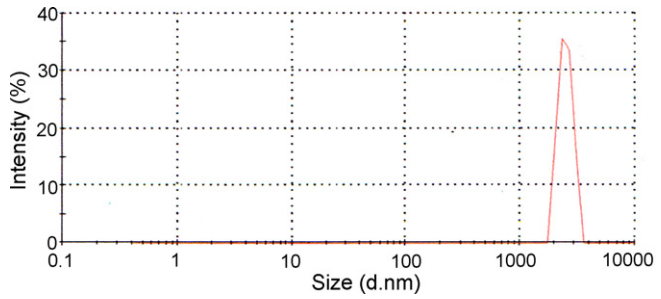

Fig. 2. A representative size distribution of sulforaphane-encapsulated albumin microspheres. Particles suspended in water were analyzed using a laser diffraction particle sizer. Three measurements were recorded, and a representative size distribution is shown.

3.2. Zeta potential measurements

When particles are in suspension, they have the propensity to adhere to one another, forming aggregates. This behavior often poses a major problem since aggregates are larger in size and have the tendency to settle down. Zeta potential provides valuable information on the physical properties of particles in suspension including their stability. This property has been used to optimize drug formulations. Generally, particles that have positive zeta potential greater than +30 mV or negative charge greater than −30 mV are considered stable. Previous studies on the surface charge, as indicated by zeta potential, suggested that the surface charge of microparticles has a significant effect on their uptake by cells. The further the potential is from zero, the more effective is the uptake (Ahsan et al., 2002; Thiele et al., 2003). Our data indicated that the zeta potentials for blank and sulforaphane microspheres were −32 mV. These zeta potential values suggested that these particles are stable.

3.3. Dissolution studies

In this study, the release profile of the drug from microspheres was evaluated. The release profile indicated a release that is characterized by a small burst release effect followed by a phase of slow release (Fig. 3). Our data indicated that 50% of the drug was released from microspheres in about 16–18 h.

3.4. Uptake studies

Blank microspheres (no sulforaphane) were labeled with fluorescamine. S91 and B16 melanoma cells were used for uptake studies. Our data indicated that there was a time dependent increase in uptake of albumin microspheres both in S91 and B16 melanoma cells (Fig. 4). Previous data have indicated that microspheres with this size (less than 10 μM) efficiently enter the cells (Sharma et al., 2004).

3.5. Cytotoxicity profile of S91 and B16 cells treated with sulforaphane-encapsulated microspheres

Studies so far on sulforaphane have shown that it holds great promise for cancer treatment. However, this therapeutic agent has a short half-life of less than 2 h, and is not very stable under normal conditions. Previous studies from Van Eylen et al. (2007) showed that, in general, isothiocyanates (e.g. sulforaphane) are thermolabile. Thus, our primary objective was to develop a delivery system using a biodegradable matrix and which is nontoxic, and that has

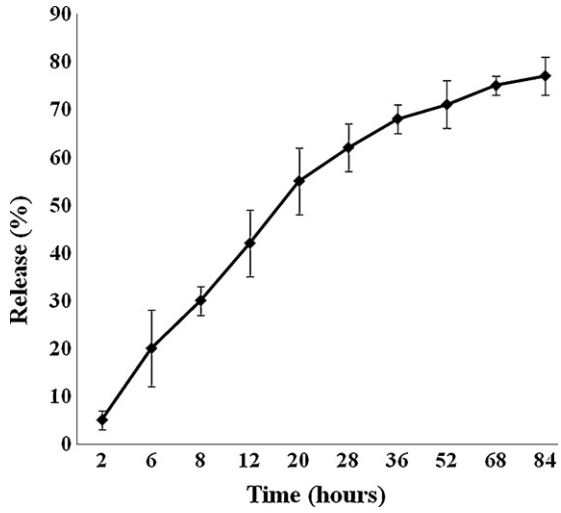

Fig. 3. Dissolution studies of sulforaphane albumin microspheres. Sulforaphane-encapsulated albumin microspheres were suspended in PBS and dissolution studies were performed at 37 °C in a Distek 1 dissolution apparatus. Sampling was done at various time points and sulforaphane concentration was measured using a UV–vis spectrometer at 235 nm. Data represent mean ± SD (n = 3). The experiments were repeated twice.

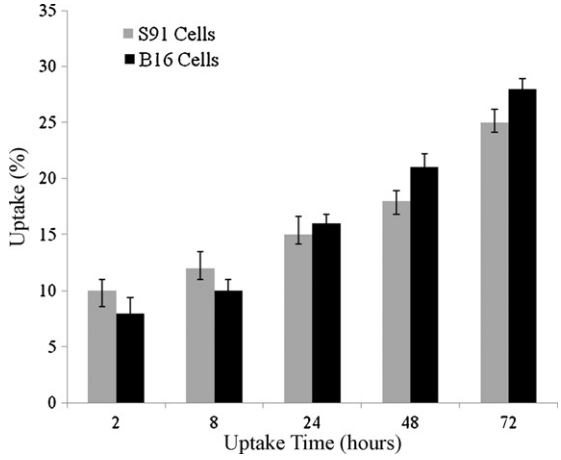

Fig. 4. Uptake of albumin microspheres in S91 and B16 melanoma cells. Determination of the ability of cultured cells to take up the microspheres was made by adding fluorescamine-labeled microspheres to the cultures. Cell lysates were prepared and analyzed for the presence of fluorescamine as described in Section 2. Data represent mean ± SD (n = 4). The experiments were repeated twice.

the ability to deliver sulforaphane to target sites in the body. In addition, although chemotherapeutic agents are effective in killing cancer cells, they can exert adverse reactions on normal cells, especially on proliferating cells. Consequently, R,S-sulforaphane was encapsulated in an albumin polymer matrix. Cytotoxicity studies were done to compare the effectiveness between sulforaphane solution and sulforaphane microspheres. Our data showed that when S91 and B16 melanoma cells were treated with sulforaphane for 24 h, the percent cell viability was higher in group treated with microspheres. Similarly, at 24 h, sulforaphane solution inhibited cell growth more efficiently than sulforaphane-encapsulated in microspheres (Fig. 5A and B). However, at 72 h, 30 or 50 μM concentrations of sulforaphane were used; cell viability was similar in cells treated with sulforaphane solution or sulforaphane-encapsulated in microspheres. Previously, we have shown that approximately 50% of the drug was released from microspheres within 24 h. This release pattern could account for the decreased cell viability seen at 24 and 48 h. Based on the release pattern by 72 h, most of the drug might have been released and taken up by the cells at this point. These data showed that microspheres containing sulforaphane were as efficacious as sulforaphane solution. Microspheres did not improve cell inhibition as compared to solution group because in the *in vitro* cell culture system, sulforaphane in solution is readily available and absorbed by the cells, thus exerting its anticancer activity. For a microparticulate system, the drug release is slower as a result of entrapment in a polymer matrix. However, previous studies have indicated that uptake of a microparticulate system is more efficient than free drug (Sharma et al., 2004). Thus the particulate delivery system offers advantages over free drug in an *in vivo* scenario due to the slow release especially for those drugs that are not stable and are metabolized as well as cleared at a faster rate. In addition, the presence of macrophages in high numbers near tumor site could further enhance the uptake of microspheres as compared to the free drug.

3.6. Histone deacetylase activity

High histone deacetylase (HDAC) activity has been linked to chromatin condensation and transcriptional repression. Consequently, elevated expression of this class of enzymes have a negative impact on the expression of many important genes that are needed for several regulatory pathways, including inhibition of cell death, apoptosis and cell cycle arrest. Since sulforaphane is metabolized rapidly as well as cleared from the body at a fast rate, our goal was to design an albumin based polymeric

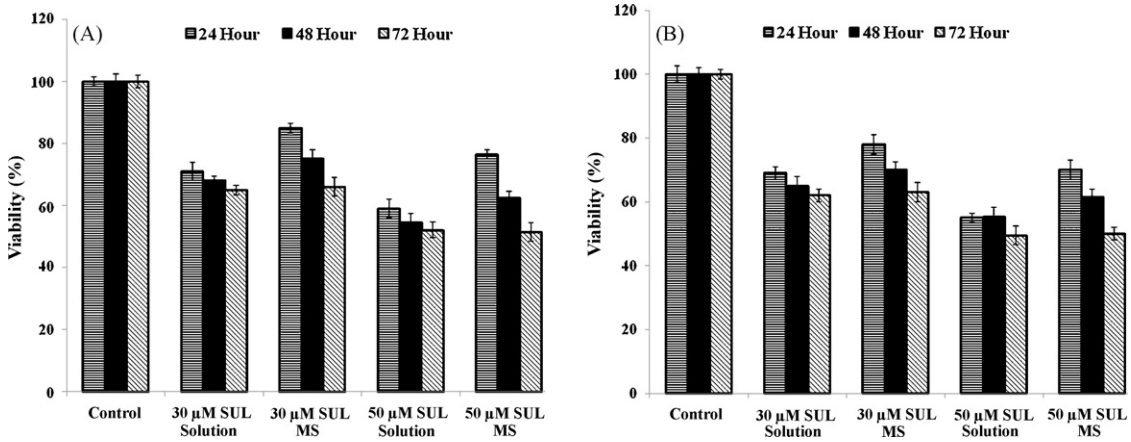

Fig. 5. Evaluation of cytotoxicity of sulforaphane solution and sulforaphane microspheres on melanoma cells. A: S91 cells and B: B16 cells were treated with sulforaphane solution and sulforaphane containing microspheres for 24, 48 and 72 h. Cell viability was determined using the MTS assay. Data represent mean ± SD (n = 3). Each experiment was repeated two times.

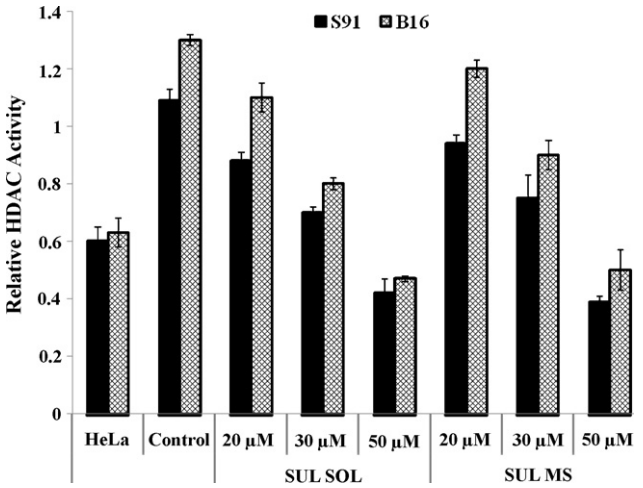

Fig. 6. HDAC activity in S91 and B16 melanoma cells treated with sulforaphane microspheres. Control (untreated) and sulforaphane microsphere treated melanoma cells were lysed and the HDAC activity was determined using the HDAC activity assay kit by recording the product of the reaction at 405 nm. HeLa: Hela nuclear extract (positive control); Control: untreated cells; SOL: solution; SUL: sulforaphane; MS: microspheres. Data represent mean ± SD ($n = 3$). Each experiment was repeated two times.

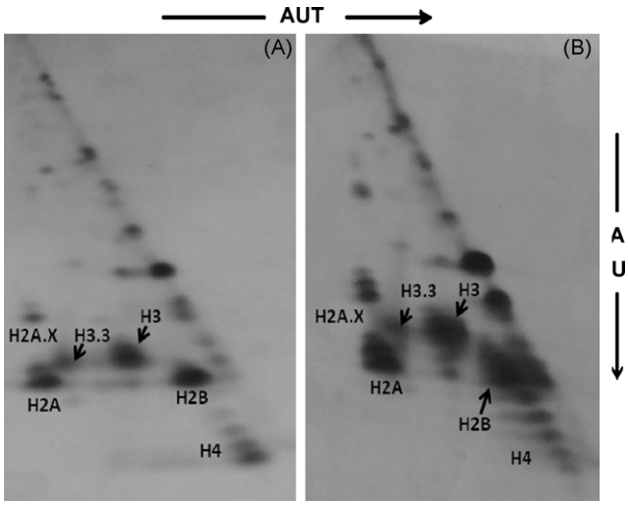

Fig. 7. Analysis of histone acetylation in S91 melanoma cells. Nuclear extracts from the untreated cells and cells treated with sulforaphane were prepared and analyzed by two-dimensional polyacrylamide gel electrophoresis system AUT × AU (acid–urea–Triton × acid–urea) as detailed in Section 2. A: control; B: treated with sulforaphane. Each experiment was repeated three times, and a representative profile is shown.

delivery system that can reduce the frequency of administration of sulforaphane to maintain therapeutic levels of the compound in systemic circulation. HDAC activity levels were measured in S91 and B16 melanoma cells treated with sulforaphane microspheres to determine the efficacy of the microspheres in decreasing deacetylation. Our data showed that HDAC activity levels were marginally higher in S91 and B16 melanoma cells treated with 20 or 30 μM of sulforaphane solution. However, when cancer cells were treated with 50 μM of sulforaphane, microspheres containing sulforaphane decreased HDAC activity to the same extent as that produced with sulforaphane solution (Fig. 6). Interestingly, in the case of S91 melanoma cells, sulforaphane microspheres decreased HDAC activity slightly better than sulforaphane solution.

3.7. Global histone modifications in cancer cells

S91 melanoma cells were treated with 30 μM of sulforaphane. Histone proteins were purified and resolved using two-dimensional gel electrophoresis system AUT × AU. This gel system permits resolution of histones and their variants as well as modified forms due to the mass/charge ratio separation principle on the second dimension. This system is useful for examining charge-altering modifications, such as acetylation, phosphorylation, and ubiquitination. In normal cells, there is an equal expression of histone deacetylases (HDACs) and histone acetyltransferases (HATs) that regulate the dynamics of deacetylation and acetylation, respectively. In cancer cells, HDAC levels increase, thus disrupting the acetylation/deacetylation dynamics that affect the chromatin structure and subsequently cellular functions. Sulforaphane is a histone deacetylase inhibitor, which inhibits the expression of histone deacetylases. Our data showed that when melanoma cells were treated with sulforaphane, there was a significant increase in the level of acetylation in histone proteins, mainly in histone H4 (Fig. 7). Acetylation can be seen as trailing, minor spots on top of the unmodified spots of histones. Acetylation confers a more negative charge on the proteins, thus making them migrate slower on the acid–urea gels. Histone H4 contains tail regions that protrude the nucleosome. Our data suggested that histone H4 acetylation occurred as a consequence of sulforaphane treatment.

B16 and S91 melanoma cells were used as representative cancer cell lines in the current studies. However, changes in HDAC activity have been documented in tumors of varied origins and hence, the results from the current studies have relevance to cancers of other tissue origins.

3.8. In vivo efficacy studies of sulforaphane microspheres

Studies were conducted in C57BL/6 mice to assess the *in vivo* efficacy of sulforaphane microspheres for cancer therapy using the B16 melanoma model. Ability of these cells to form tumors in C57BL/6 mice render them amenable to experimentation on antitumor activity of drugs and drug-encapsulated microparticle formulations. Melanoma tumors were induced using B16 cells. Fig. 8 summarizes the results of the investigation using sulforaphane microspheres. Interestingly, after 1 week of treatment, microspheres containing sulforaphane were less effective in comparison to sulforaphane solution. However, at weeks 2 and 3 post-treatment, sulforaphane microspheres inhibited tumor growth more effectively than sulforaphane solution, suggesting sustained release of sulforaphane from albumin microspheres. At week 4 post-treatment, sulforaphane microspheres inhibited approximately 15% more tumor growth as compared to sulforaphane solution. In this experiment, blank albumin microspheres were used as a negative control. Our data indicated that albumin microspheres had no effect on tumor growth. Mice were weighed regularly to examine adverse effects, if any, of drug treatment. Our data indicated that there were no significant differences in body weight of mice between various treated groups suggesting lack of major toxicities (Fig. 9).

Data from our current studies suggested that microspheres having mean sizes of 2 μM were effective in decreasing the tumor volumes in mice. Previous studies have shown that microspheres less than 10 μM are phagocytosed efficiently by macrophages (Ahsan et al., 2002; Champion et al., 2008). Further studies could explore methodologies for decreasing the wide distribution of microspheres as well as evaluation of the influence of formulations of specific sizes on therapeutic efficiency. This optimization could potentially result in generating drug-encapsulated microparticles with enhanced *in vivo* therapeutic effect.

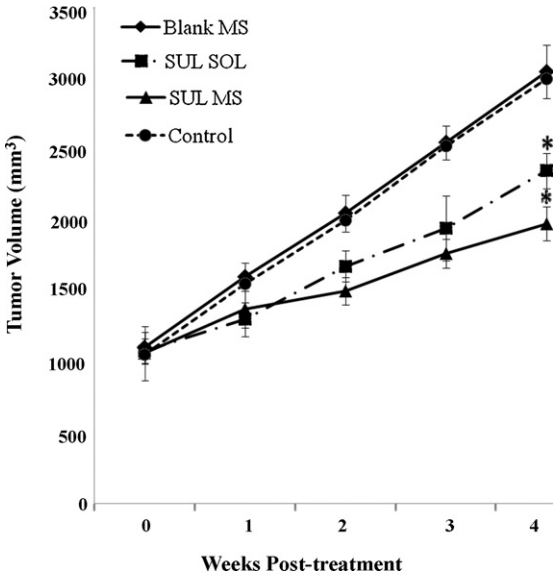

Fig. 8. *In vivo* antitumor efficacy of sulforaphane in C57BL/6 mice bearing B16 melanoma tumor. Tumors were induced in the mice by subcutaneous injection of the melanoma cells. Groups of tumor-bearing mice were treated as described in Section 2. Tumor volumes were determined at weekly intervals until 4 weeks. Data represent mean ± SD derived from six animals treated per experimental group. $^*p < 0.05$ between sulforaphane microspheres and sulforaphane solution.

3.9. HDAC activity levels in tumors

Histone deacetylase activity level in tumors after treatment with sulforaphane microspheres was tested. At the end of the study (4 weeks), tumors were excised from different experimental groups. Nuclear extract from tumors were obtained and analyzed for HDAC activity levels. Fig. 10 summarizes the HDAC activity in tumors from various treatment groups, significant levels of HDAC activity could be detected in the melanoma tumor. When mice bearing tumors were treated with sulforaphane microspheres or sulforaphane solution, HDAC activity level decreased. In the case of sulforaphane solution treatment, there was approximately 15% reduction in HDAC activity in comparison to untreated controls. When mice were treated with sulforaphane microspheres, there was about 30% reduction in deacetylation activity, suggesting the

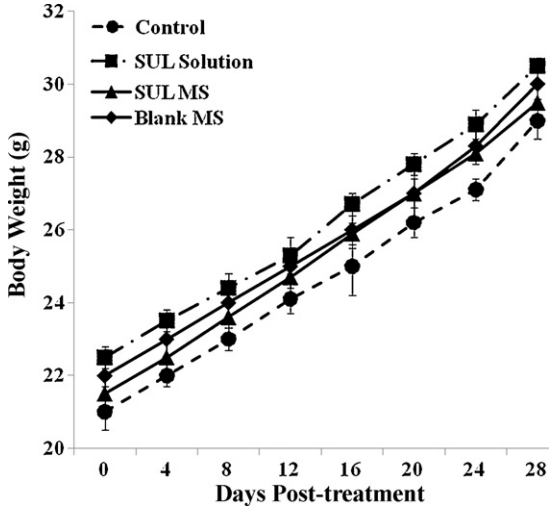

Fig. 9. Body weights of C57BL/6 mice in various experimental groups. Tumor-bearing mice receiving various treatments were routinely weighed. Data represent mean body weight ± SD derived from six animals treated per experimental group in a single experiment.

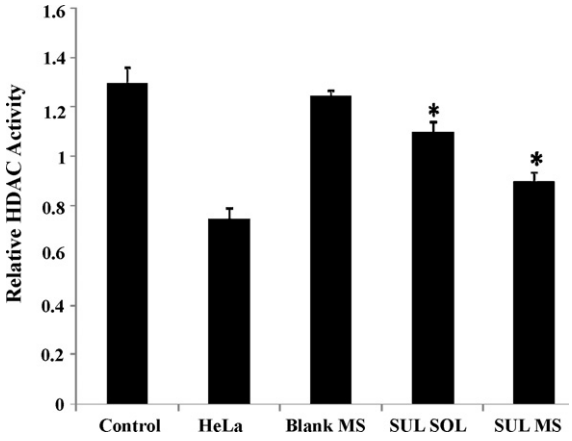

Fig. 10. HDAC activity in mice melanoma tumors after treatment with sulforaphane microspheres. Extracts were prepared from tumors and assayed as described in Section 2. HeLa: Hela nuclear extract (positive control); Control: untreated mice; SOL: solution; SUL: sulforaphane; MS: microspheres. Data represent mean ± SD derived from six animals treated per experimental group in a single experiment. $^*p < 0.05$ between sulforaphane microspheres and sulforaphane solution.

effectiveness of the polymeric delivery system in enhancing anticancer activity of sulforaphane. Blank microspheres did not have any significant impact on HDAC activity levels.

4. Conclusion

Recent advances in understanding of the role of epigenetic changes in the maintenance and progression of the malignant state has opened up avenues for therapeutic intervention (Gronbaek et al., 2008). Specific inhibitors that can reverse some of the aberrant epigenetic changes such as acetylation status of histones have demonstrated potential for inhibition of the cancer cell. In light of the growing body of literature showing the development of mechanisms of drug resistance by tumor cells to conventional modes of chemotherapy, more focus is directed towards development of treatments incorporating epigenetic drugs. This combined modality has shown promise in some malignancies wherein synergistic action has been demonstrated (Bishton et al., 2007). Alternatively, the reversal of epigenetic changes in cancer cells could also facilitate sensitization to chemotherapeutic agents to which the tumor cells were initially non-responsive as well as to alternate treatment regimens (Vogiatzi et al., 2007).

Implementation of epigenetic treatment modalities, however, is not without problems. The efficacy of these class of molecules depend on several factors such as availability of therapeutic levels of the drug in the *in vivo* environment for extended periods of time as well as maintaining the integrity of the drug. Data from our studies suggest that for an epigenetic drug like sulforaphane, enhanced efficacy could be achieved by encapsulation in a biodegradable matrix. These microsphere formulations, in addition to allowing sustained release, also have other desirable properties such as favorable uptake by macrophages. Further development of formulations of drugs and delivery systems analogous to the one reported here should aid in designing therapeutic protocols for efficacious treatment and management of cancer.

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