

# Methyl Sulfone Manifests Anticancer Activity in a Metastatic Murine Breast Cancer Cell Line and in Human Breast Cancer Tissue – Part I: Murine 4T1 (66cl-4) Cell Line

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## Key Words

Methyl sulfone · Breast cancer · Metastatic phenotypes · Reversal of metastasis · E-cadherin · Differentiation

## Abstract

**Background:** The spread of cancer (metastasis) is usually associated with death. We have identified a new approach that may be useful for treating metastatic cancer. **Methods:** Here we studied the murine breast cancer cell line 66cl-4, because these cells are highly aggressive, potent inducers of metastasis and estrogen receptor negative. **Results:** We found that 200 mM methyl sulfone did not induce apoptosis in cancerous cells but instead decreased cell proliferation and DNA synthesis, inhibited migration of cells through an extracellular matrix and induced contact inhibition and anchorage-dependent growth. Methyl sulfone promoted proper wound healing, reversed the epithelial to mesenchymal transition associated with metastatic disease and increased expression of  $\alpha$ -smooth muscle actin, a differentiation protein of breast myoepithelial cells. **Conclusion:** Methyl sulfone did not kill the cancer cells but instead decreased metastatic phenotypes and increased normal differentiated phenotypes.

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## Introduction

The goal of present-day chemotherapy is to kill cancer cells. While these chemotherapeutic drugs are often effective against primary cells from early-stage tumors, they are less effective against late-stage or metastatic cancer cells. In fact, metastatic cancer accounts for approximately 90% of all cancer deaths [1, 2]. What makes metastatic cancer cells so difficult to kill is not clear. While all cancers have somatic mutations [3, 4], there do not appear to be significant genetic differences between cancer cells found in a primary tumor and metastatic cells found at distal sites in a patient [5, 6]. However, neither broad-based chemotherapy nor more specific drug targeting has proved uniformly successful against metastatic disease.

Methyl sulfone is a small water-soluble molecule that is found in grasses and other vegetation including broccoli, cauliflower, garlic, onions and tomatoes. Mammals, including humans, must acquire the compound through diet. Due to its chemical structure, we identified methyl sulfone as a molecule with potential anticancer activity [7]. We then tested methyl sulfone for effects on the metastatic Cloudman S-91 murine melanoma cell line (subclone M3) [7]. We demonstrated that 200–400 mM methyl sulfone does not kill the malignant melanoma cells but

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instead causes these cells to lose their metastatic hallmarks and to acquire the morphological phenotype of normal melanocytes.

In these studies, we sought to determine whether the antimetastatic activity of methyl sulfone is limited to malignant melanoma or whether this compound is effective against a second type of metastatic cancer. We chose to study breast cancer because this cancer is common and associated with much morbidity and mortality. In the USA during the year 2011, there were approximately 300,000 new diagnoses of breast cancer and approximately 40,000 deaths from the disease [8]. In addition, breast cancer is the leading cause of cancer death among Hispanic women and the second among white, black, Asian/Pacific Islander and American Indian/Alaska Native women. In this paper, we chose to study the murine breast cancer cell line 66cl-4, because this particular cell line is aggressive, with significant metastatic potential [9]. The 66cl-4 cells have a high mitotic index, with cells doubling every 12 h, and *in vivo* studies show that these cells have a significant tumor potential when injected into mice [10]. Histological studies of tumors generated from 66cl-4 cells also demonstrate poorly differentiated adenocarcinomas [11].

Our interest in the 66cl-4 cell line was heightened by the fact that 66cl-4 cells are estrogen-receptor (ER) negative. Women with ER-negative breast cancer have a significantly higher risk of recurrence than women with ER-positive breast cancer [12].

We show here that methyl sulfone does not kill the metastatic breast cancer cells. Instead, the aggressive metastatic phenotypes of 66cl-4 breast cancer cells are reversed by methyl sulfone, and these cells take on attributes of normal breast cells. These data suggest that methyl sulfone may offer a new strategy in the chemotherapeutic treatment of metastatic cancer, in which the goal is not to kill metastatic cancer cells but instead to replace their metastatic phenotypes with properties of normal cells.

## Materials and Methods

### Materials

Methyl sulfone was purchased from Fluka/Sigma Co. (St. Louis, Mo., USA).

### Cell Culture

The murine 66cl-4 cell line was obtained from Kevin Claffey, Department of Cell Biology, University of Connecticut Health Center (Farmington, Conn., USA). This cell line is a subclone of the metastatic breast cancer cell line 4T1 [9–11]. The 66cl-4 cells were grown in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 7% fetal bovine serum (Invitrogen Inc.). Cells were passaged twice per week by treating with trypsin followed by sieving (0.22  $\mu\text{m}$ ) before plating. BD Falcon polystyrene tissue culture plates were purchased from Becton Dickinson and Co. (Franklin Lakes, N.J., USA). Cells were incubated at 37°C with 5% CO<sub>2</sub>.

### Light Microscopy

Light microscopy and photography were performed with a Zeiss Axio Observer A1 microscope using an N-ACROPLAN 10 $\times$ /0.25 objective and an LD A-Plan 20 $\times$ /0.30 objective.

### Fluorescence Microscopy

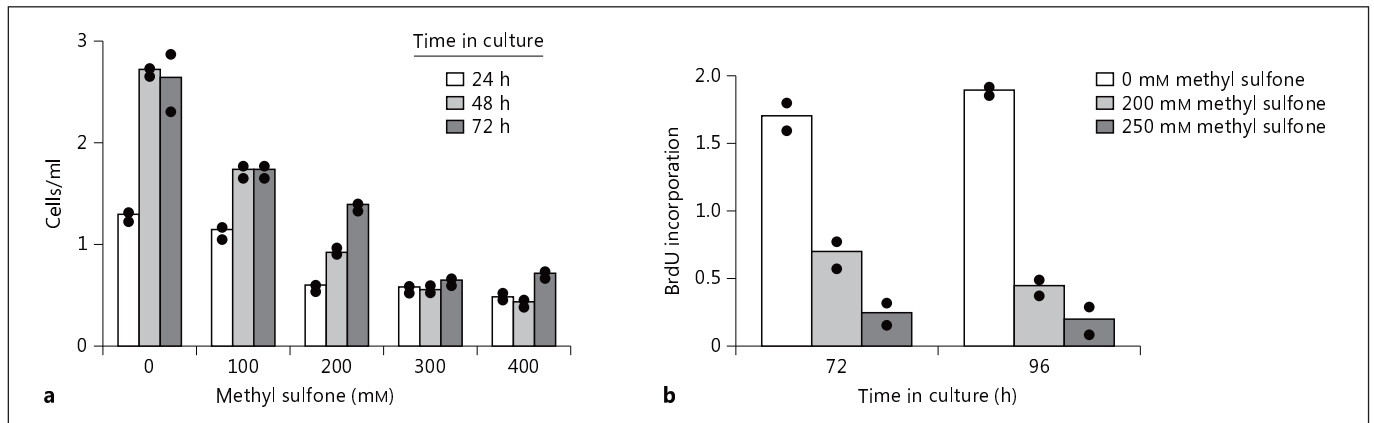
Cells were plated in DMEM in 35-mm tissue culture dishes containing 12-mm glass coverslips at a concentration of 10<sup>5</sup> cells/plate and incubated at 37°C with 5% CO<sub>2</sub>. After 24 h, medium was replaced with control medium (DMEM with no methyl sulfone) or DMEM with 200 mM methyl sulfone. After 24–96 h, cells were fixed in 4% paraformaldehyde, and actin filaments were stained with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Eugene, Oreg., USA). For immunofluorescence staining of E-cadherin and N-cadherin, cells were fixed in 4% paraformaldehyde and prepared for immunofluorescence microscopy using rabbit polyclonal anti-E cadherin antibody and rabbit polyclonal anti-N cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). Secondary antibody for E-cadherin and N-cadherin was goat antirabbit Alexa Fluor 488. Images were obtained at the Richard D. Berlin Center for Cell Analysis and Modeling, University of Connecticut Health Center (Farmington, Conn., USA), with an Axioplan CCD Microscope equipped with a 63 $\times$ 1.4 NA Plan Apo oil immersion objective via Metamorph image acquisition and analysis software (Universal Imaging Corp., Downingtown, Pa., USA). Fluorescent and immunofluorescent microscopy of actin filaments, E-cadherin and N-cadherin were repeated at least 3 times.

### Immunocytochemistry

Immunocytochemistry was performed on cells fixed in 4% paraformaldehyde and incubated with mouse monoclonal anti- $\alpha$ -smooth muscle actin antibody (Santa Cruz Biotechnology), followed by use of an immunocytochemistry staining kit from Santa Cruz Biotechnology (ImmunoCruz Mouse ABC Staining System). Images were visualized and photographed with a Zeiss Axio Observer A1 microscope using an LD A-Plan 20 $\times$ /0.30 objective and no phase ring (phase 0). Immunocytochemistry of  $\alpha$ -smooth muscle actin was repeated at least 4 times.

### Cell Proliferation

Cells (10<sup>5</sup> cells/35-mm plate) were plated in DMEM. After 24 h, medium was replaced with DMEM containing 0–400 mM methyl sulfone. At several time points, plates were washed and cells were released from plates by trypsinization and counted with a hemocytometer. All assays were performed at least 3 times and in triplicate. Apoptosis was assessed with the Annexin V-FITC Apoptosis Kit (PharMingen, Becton-Dickinson, San Diego, Calif., USA). Images were obtained at the Richard D. Berlin Center for Cell Analysis and Modeling, University of Connecticut Health Center, with an Axioplan CCD Microscope equipped with a 40 $\times$ 1.3 NA FL objective lens and a Photometrics PXL-EEV37 high-speed digital cooled CCD camera via Metamorph image acquisition and analysis software (Universal Imaging). All assays were performed at least 3 times and in triplicate.



**Fig. 1.** Methyl sulfone inhibited cell proliferation and DNA synthesis. **a** Cell proliferation: metastatic breast cancer cells were incubated with 0, 100, 200, 300 or 400 mM methyl sulfone for up to 72 h and assayed for cell proliferation. **b** DNA synthesis: breast cancer cells were incubated with 0, 200 or 250 mM methyl sulfone for up to 96 h and assayed for DNA synthesis. Data are presented as means  $\pm$  SEM. Dots indicate significant differences between groups ( $p < 0.0001$ ). Data shown in **a** were initially analyzed using a two-way

analysis of variance, which revealed a significant effect of both dose and time of treatment. Subsequently, these data were analyzed by a one-way analysis of variance followed by a Dunnett's post hoc test to detect differences between different doses and times of exposure. Data in **b** were assessed by  $\chi^2$  analysis at both the 72- and 96-hour time points. All the statistical analysis was performed using Prism 5.0 (GraphPad Software, La Jolla, Calif., USA), and differences between groups were considered to be significant if  $p < 0.05$ .

#### DNA Synthesis

Cells ( $10^5$  cells/ml) were plated in DMEM. After 24 h, medium was replaced with DMEM containing 0, 200 or 250 mM methyl sulfone. DNA synthesis was assayed by incubating cells with bromodeoxyuridine (BrdU) followed by fixation and incubation with Alexa Fluor 488-conjugated monoclonal anti-BrdU antibody as described by the manufacturer (Molecular Probes). Images were obtained as described above in the section Cell Proliferation. All assays were performed at least 3 times and in triplicate.

#### Cell Invasion

Invasion assays were performed using Transwell Chambers with 8- $\mu$ m pores (Corning Inc., Lowell, Mass., USA). Membranes were coated with extracellular matrix gel (Engelbreth-Holm-Swarm murine sarcoma, Sigma Co., Mo., USA) that was diluted 1/6 with DMEM containing 0, 200 or 250 mM methyl sulfone. Cells ( $10^5$ ) were seeded into upper chambers in DMEM with and without 200 or 250 mM methyl sulfone. After 48 and 72 h at 37°C, 5% CO<sub>2</sub>, the number of cells that migrated through the extracellular matrix was determined using an Axioplan CCD microscope equipped with a 40 $\times$ 1.3 NA FL objective lens and high-speed digital cooled CCD camera via Metamorph image acquisition and analysis software (Universal Imaging). All assays were performed at least 3 times and in triplicate.

#### Soft Agar Colony Formation

Cells ( $5 \times 10^3$ ) were gently suspended in 37°C DMEM containing 0.66% agar (DNA grade; Difco Bacto Agar, Becton Dickinson and Co., Md. USA) and 0, 200 or 250 mM methyl sulfone. The suspension was placed on solidified 1% agar in DMEM containing 0, 200 or 250 mM methyl sulfone. Cells were incubated at 37°C with 5% CO<sub>2</sub>. After 14 days, cells were stained with crystal blue. Each 35-mm plate was photographed, and colonies were counted with

a dissecting microscope. All visible colonies were counted. All assays were performed at least 3 times and in triplicate.

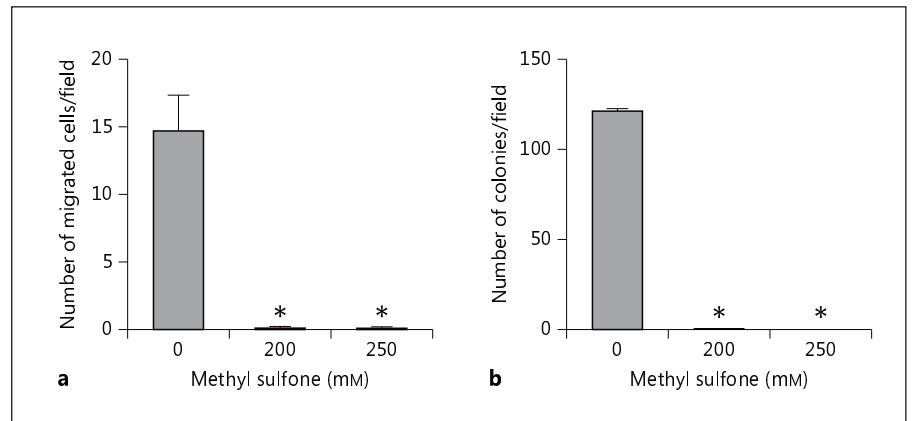
#### Wound Healing

Cells ( $10^5$  cells/ml) were plated in DMEM in 35-mm tissue culture dishes. Once cultures were confluent, medium was replaced with DMEM with and without 200 mM methyl sulfone. After 48 h, cells were wounded with a sterile plastic 1,000- $\mu$ l pipette tip. Cells were washed twice with medium to remove cell debris and incubated at 37°C with 5% CO<sub>2</sub> in DMEM with and without 200 mM methyl sulfone. Wound edges were photographed every 24 h for up to 7 days using a Zeiss Axio Observer A1 microscope with an N-ACROPLAN 10 $\times$ /0.25 objective and an LD A-Plan 20 $\times$ /0.30 objective. Wound healing experiments were repeated at least 3 times.

## Results

### Methyl Sulfone Inhibited Cell Proliferation and DNA Synthesis in Metastatic Breast Cancer Cells

The 66cl-4 metastatic breast cancer cells were treated with 0, 200, 300 or 400 mM methyl sulfone for 24, 48 and 72 h. At each time point, plates were washed to remove unattached cells. Cells remaining on plates were trypsinized from tissue culture plates, and cells were counted with a hemocytometer. Compared to control cells (no methyl sulfone), cell numbers stopped increasing as the time in culture and dose of methyl sulfone increased (fig. 1a;  $p < 0.0001$ ). For example, at 48 h, when control



**Fig. 2.** Methyl sulfone inhibited migration of breast cancer cells through an extracellular matrix and anchorage-independent growth. **a** Migration through extracellular matrix: metastatic breast cancer cells were incubated in 0, 200 or 250 mM methyl sulfone for up to 72 h and assayed for their ability to migrate through the extracellular matrix. **b** Anchorage-independent growth: breast

cancer cells were incubated in 0, 200 or 250 mM methyl sulfone for 14 days and assayed for their ability to form colonies on soft agar. Data are presented as means  $\pm$  SEM. \*  $p < 0.0001$ . Data were analyzed by a Student's *t* test. All the statistical analyses were performed using Prism 5.0 (GraphPad Software), and differences between groups were considered to be significant if  $p < 0.05$ .

cells were still growing, cell proliferation in methyl sulfone-treated cells was reduced by more than 80%.

We next examined the ability of methyl sulfone to inhibit DNA synthesis. Metastatic breast cancer cells were incubated with 0, 200 or 250 mM methyl sulfone for 72 and 96 h. DNA synthesis was measured by incorporation of BrdU followed by incubation with an anti-BrdU antibody. Treatment of cells with 200 or 250 mM methyl sulfone resulted in approximately 90% inhibition of DNA synthesis (fig. 1b;  $p < 0.0001$ ).

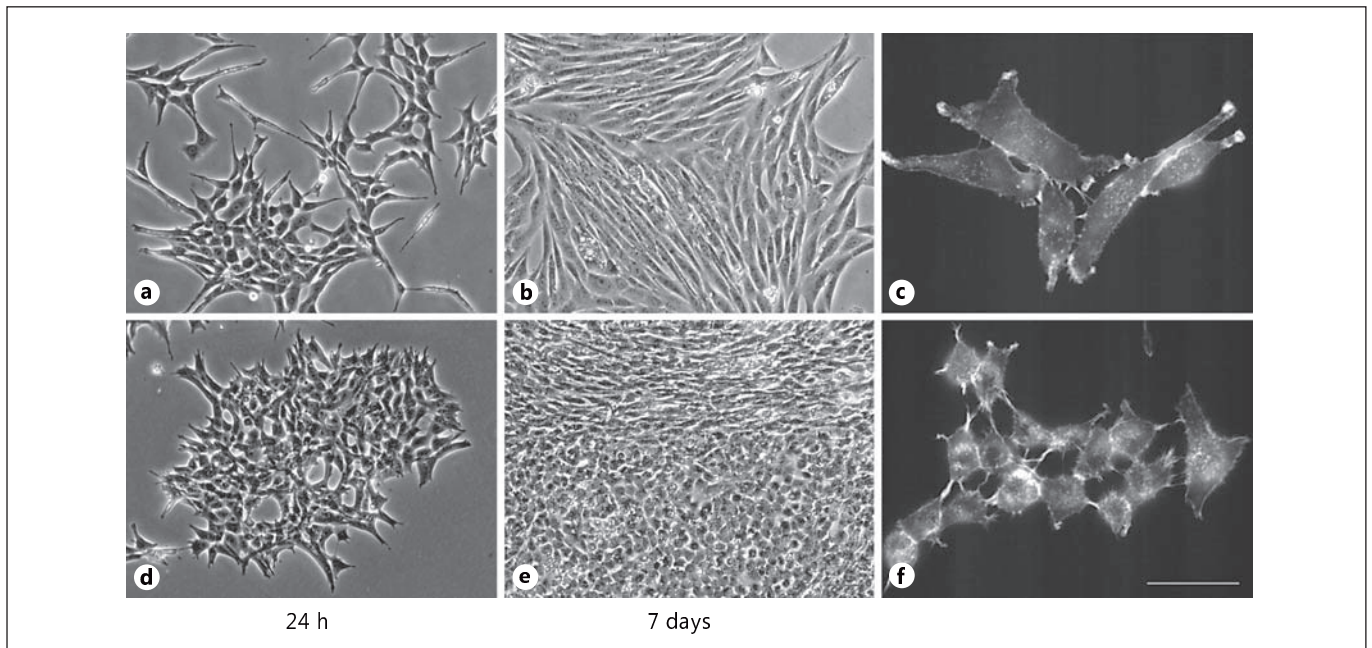
The decrease in cell numbers and DNA synthesis suggested that methyl sulfone was causing growth arrest and/or cell death. To determine whether methyl sulfone was inducing cell death, we measured apoptosis. After treating 66cl-4 cells with 0 or 200 mM methyl sulfone for 20 h, apoptosis was assayed as described in Materials and Methods. Approximately 4% of control cells (no methyl sulfone) and 4% of cells treated with 200 mM methyl sulfone underwent apoptosis ( $p = 1.0000$ ), indicating no significant difference in the percentage of apoptotic 66cl-4 cells cultured in control medium (no methyl sulfone) and 200 mM methyl sulfone. Taken together, our data indicate that methyl sulfone at 200 mM induced significant growth arrest and inhibition of DNA synthesis in 66cl-4 breast cancer cells without inducing cell death.

#### *Methyl Sulfone Inhibited Migration of Metastatic Breast Cancer Cells through an Extracellular Matrix*

One of the most deleterious properties of metastatic cancer is the ability of cells to become mobile and invade surrounding tissue [13]. The 66cl-4 breast cancer cells are aggressive at migrating and invading neighboring epithelial and stromal tissues [10, 11]. Here, we incubated the cells with 0, 200 or 250 mM methyl sulfone and examined the ability of these metastatic breast cancer cells to migrate into an extracellular matrix. As shown in figure 2a, both 200 and 250 mM methyl sulfone blocked migration of 66cl-4 cells into the extracellular matrix by greater than 97% ( $p < 0.0001$ ).

#### *Growth of Metastatic Breast Cancer Cells Became Anchorage Dependent in the Presence of Methyl Sulfone*

Normal cells require anchorage to a substratum for growth [1]. Conversely, growth of metastatic cells, such as the 66cl-4 cells, is anchorage independent. We examined the effect of methyl sulfone on anchorage-dependent growth by incubating the 66cl-4 cells with 0, 200 or 250 mM methyl sulfone in soft agar for 14 days and counting the number of colonies (representing growth of single cells). In the presence of 200 or 250 mM methyl sulfone, no colonies were apparent. In contrast, numerous colonies were visualized in the control sample (no methyl sulfone; fig. 2b;  $p < 0.0001$ ).



**Fig. 3.** Methyl sulfone induced contact inhibition in metastatic breast cancer cells. Breast cancer cells ( $10^5$  cells/ml) were plated in DMEM. After 24 h, medium was replaced with DMEM with and without 200 mM methyl sulfone. **a–c** Breast cancer cells in 200 mM methyl sulfone. **a** Preconfluent cells 24 h after adding methyl sulfone. **b** Confluent cells 7 days after adding methyl sulfone. **c** Fixed

cell microscopy of Alexa Fluor 488-conjugated phalloidin-stained actin filaments in methyl sulfone-treated cells. **d–f** Breast cancer cells without methyl sulfone (control). **d** Preconfluent cells 24 h after plating. **e** Confluent cells 7 days after plating. **f** Fixed cell microscopy of Alexa Fluor 488-conjugated phalloidin-stained actin filaments in control cells. Scale bar = 20  $\mu$ m.

The experiments described above suggested that the lowest optimal concentration of methyl sulfone is 200 mM, which is the concentration we used for the remaining experiments.

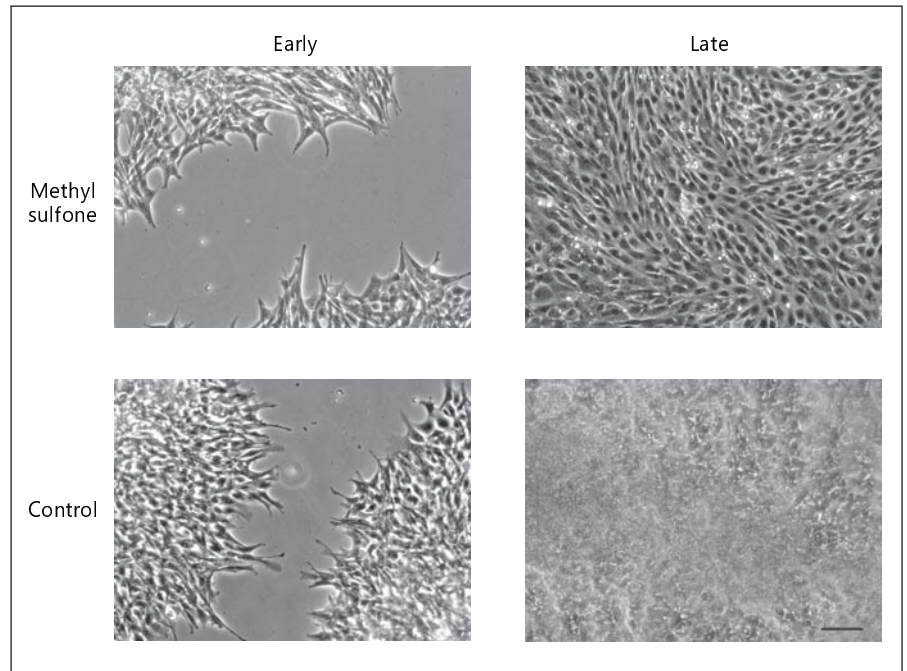
#### *Methyl Sulfone Induced Contact Inhibition in Metastatic Breast Cancer Cells*

Treatment of subconfluent metastatic 66cl-4 breast cancer cells with methyl sulfone initially resulted in cell proliferation and migration until cells reached confluency. The extent of cell proliferation and migration was directly proportional to the initial plating concentration of the cells; the lower the plating density, the longer the cells spent proliferating and migrating before reaching confluence. Once confluence was reached, proliferation and migration stopped and cells became contact inhibited, as shown by phase contrast microscopy in figure 3a and b. Contact inhibition was also demonstrated by visualization of actin filaments (fig. 3c). Here, actin filaments could be seen at the cell surface in numerous filopodia, a hallmark of contact inhibition [14–16]. The filopodia stretched across intercellular spaces, touching filopodia

of neighboring cells. In addition to filopodia, several actin filament-based focal adhesions were visible. In contrast, control cells (no methyl sulfone) continued to proliferate and migrate even after reaching confluency. This resulted in multiple layers of cells with no evidence of contact inhibition (fig. 3d–f). This was further demonstrated by visualization of actin filaments. Compared to cells in the presence of methyl sulfone (fig. 3c), actin filaments in control cells were disorganized or appeared as stress fibers. Filopodia were not apparent (fig. 3f).

#### *Methyl Sulfone Reversed Nuclear Pleomorphism Associated with the 66cl-4 Breast Cancer Cell Line*

One important indicator of prognosis for patients with breast cancer is the shape of nuclei in malignant tissue; the greater the pleomorphism, the worse the prognosis [17]. We examined nuclear pleomorphism in cells cultured without methyl sulfone (control) for 2 weeks, cells cultured in 200 mM methyl sulfone for 2 weeks and cells cultured in 200 mM methyl sulfone for 1 week followed by culture in control medium for 1 week. We counted nuclei that were normal in appearance (round/oval) ver-



**Fig. 4.** Methyl sulfone promoted proper wound healing. Breast cancer cells were grown to confluence in the presence or absence of 200 mM methyl sulfone. Scraping a sterile plastic pipette tip through the layer of cells generated a wound. Wounds were monitored by phase contrast microscopy every 24 h. ‘Early’ shows control cells (no methyl sulfone) and 200 mM methyl sulfone-treated cells 24 h after wounding. ‘Late’ shows control cells 5 days after wounding and methyl sulfone-treated cells 6 days after wounding. Scale bar = 20  $\mu$ m.

cells abnormal or irregular. Nuclei were considered abnormal or irregular if there were any apparent deviations from a round or oval shape. As shown in table 1, a high percentage of nuclei in control cells had irregular shapes (76%). This percentage decreased significantly in cells treated with methyl sulfone for 2 weeks (12%) and in cells treated with methyl sulfone for 1 week followed by 1 week in control medium (8%).

#### *Wound Healing Proceeded Normally in the Presence of Methyl Sulfone*

Wound healing is a complicated process that involves commitment to the epithelial to mesenchymal transition (EMT) followed by the reverse process of the mesenchymal to epithelial transition (MET) [18]. In the EMT, epithelial cells take on characteristics of mesenchymal cells, which allow cell proliferation and migration into the wounded area. Once the wound is covered, the reverse process inhibits further cell proliferation and migration, and cells covering the wound become contact inhibited. In early metastatic disease at least, only the EMT occurs, not the MET; cells continue to proliferate and migrate within the area of what becomes a chronic wound [19]. However, we have shown here that proper wound healing occurred when the metastatic 66cl-4 breast cancer cells were incubated with methyl sulfone (fig. 4). Contact inhibition is an integral part of the wound healing pro-

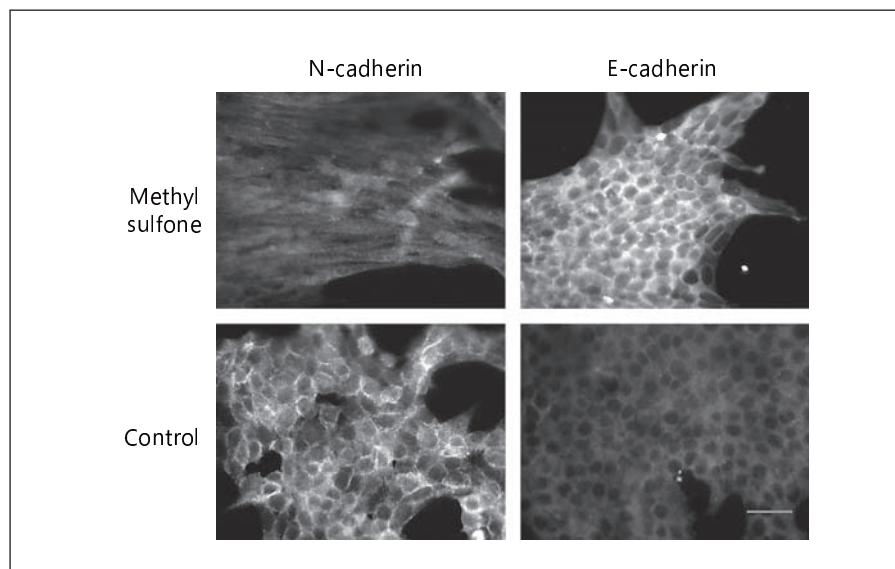
**Table 1.** Effect of methyl sulfone on the shape of nuclei

Culture conditions	Round/oval nuclei	Irregular nuclei	Number of cells examined
Control	24%	76%	355
Methyl sulfone	88%	12%	406
Methyl sulfone/control	92%	8%	230

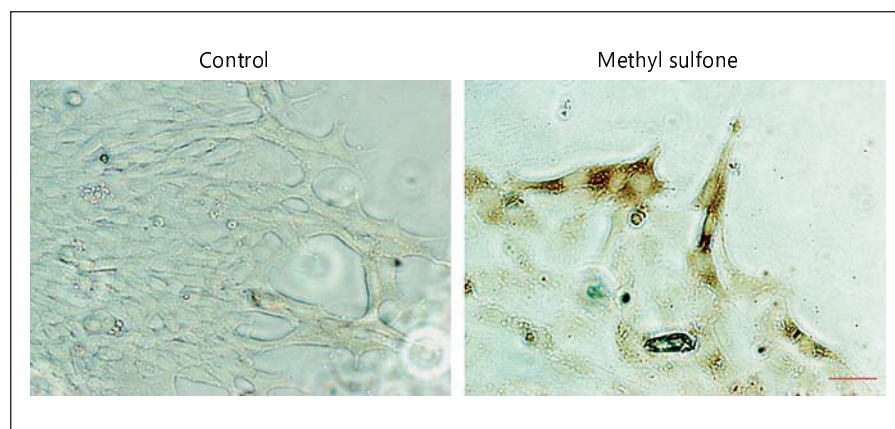
Control: cells were cultured in control medium (DMEM) for 2 weeks. Methyl sulfone: cells were cultured in 200 mM methyl sulfone/DMEM for 2 weeks. Methyl sulfone/control: cells were cultured in 200 mM methyl sulfone/DMEM for 1 week followed by culture in control medium for week. Fisher’s exact test was used to analyze data. p values of <0.05 were considered statistically significant. Control versus methyl sulfone: two-tailed p < 0.0001. Control versus methyl sulfone/control: two-tailed p < 0.0001. Methyl sulfone versus methyl sulfone/control: two-tailed p = 0.1071.

cess [20], and in the presence of methyl sulfone, these cells became contact inhibited once the wound was covered. Conversely, in the absence of methyl sulfone, once the wound was covered, cells continued to proliferate and migrate, displaying no contact inhibition but displaying what appeared to be disorganized tumor masses of cells.

**Fig. 5.** Methyl sulfone decreased expression of N-cadherin and increased expression of E-cadherin. Metastatic breast cancer cells were incubated with and without 200 mM methyl sulfone. After 72 h, cells were processed for immunofluorescence microscopy using antibodies to E-cadherin, N-cadherin and Alexa Fluor 488-conjugated secondary antibody. Scale bar = 20  $\mu$ m.



**Fig. 6.** Methyl sulfone increased expression of  $\alpha$ -smooth muscle actin, a protein specific for myoepithelial cells. Metastatic breast cancer cells ( $10^5$  cells/ml) were plated in DMEM. After 24 h, medium was replaced with DMEM with and without 200 mM methyl sulfone. Two weeks after plating, cells were processed for immu-



nohistochemistry using an antibody to  $\alpha$ -smooth muscle actin. Images were acquired using a Zeiss Axio Observer A1 microscope with an LD A-Plan 20 $\times$ /0.30 objective and no phase ring. Scale bar = 20  $\mu$ m.

#### *Methyl Sulfone Decreased Expression of N-cadherin and Increased Expression of E-cadherin*

Expression of N-cadherin is associated with the EMT described above [21]. However, the appearance of N-cadherin on the cell surface of epithelial cells is also associated with initiation of metastasis. The opposite is true for E-cadherin. Expression of this protein is reduced during the EMT, but it reappears on epithelial cells during the MET [22]. Levels of E-cadherin are also reduced during initiation of metastasis [23]. We found that in the metastatic 66cl-4 cells, N-cadherin was expressed while E-cad-

herin was downregulated (fig. 5). In contrast, incubation of the 66cl-4 breast cancer cells with 200 mM methyl sulfone resulted in a decrease in expression of N-cadherin and an increase in expression of E-cadherin.

#### *Methyl Sulfone Induced Expression of Myoepithelial Cell-Specific $\alpha$ -Smooth Muscle Actin*

Normal breast tissue has 2 major cell types, i.e. ductal epithelial cells and myoepithelial cells [24]. The 66cl-4 cell line contains epithelial cells. While there is no conclusive evidence that this cell line also includes myoepithelial

cells, studies suggest that in the breast there may be a common cellular precursor for both epithelial cells and myoepithelial cells [25]. To examine the possibility that methyl sulfone may induce differentiation of myoepithelial cells, we used immunocytochemistry to identify cells expressing  $\alpha$ -smooth muscle actin. This type of actin is only found in differentiated myoepithelial cells and not in epithelial cells. We identified  $\alpha$ -smooth muscle actin in cells found on the edges of islands of cells (fig. 6). In contrast, we found no immunocytochemical staining of cells in control samples.

## Discussion

In these studies, we have shown that treatment of a highly aggressive and metastatic breast cancer cell line with methyl sulfone resulted in the loss of metastatic properties and the reemergence of normal phenotypes. Interestingly, the optimal concentration of methyl sulfone (200 mM) was the same optimum concentration that we found for metastatic melanoma [7]. Lim et al. [26] recently showed that a similar concentration of methyl sulfone (300–500 mM) decreases growth of cells from breast cancer cell lines by decreasing expression of the transcription factors STAT3 and STAT5b.

### *Methyl Sulfone and the EMT*

The EMT occurs during normal physiological processes [27–30]. One of the most distinguishing characteristics of the EMT is a transient decrease in expression of E-cadherin (an epithelial adherens junction protein) and a simultaneous increase in expression of N-cadherin (a mesenchymal adherens junction protein). This switch in cadherin expression allows cells to detach from the extracellular matrix and neighboring cells and to become mobile. Cells expressing N-cadherin also acquire the ability to synthesize and secrete matrix metalloproteinases, which in turn allows these cells to digest surrounding extracellular matrix and migrate through this material. Each of the activities associated with the EMT is required for normal cell processes. For example, the EMT is required for migration of skin epithelial cells into a wound [31], migration of neuroepithelial cells from the neural crest of the vertebrate embryo [32] and migration of a phalanx of epithelial mammary cells into surrounding stroma during breast development [33]. In all cases, once migration is complete, epithelial cells undergo redifferentiation; N-cadherin expression is downregulated and E-cadherin expression is upregulated. Without this redif-

ferentiation, wounds would not heal, neuroepithelial cells would not be properly positioned in the embryo and mammary epithelial cells would not stop growing into the stroma, all three being characteristics of metastatic cancer.

Breast cancer cells, in their transition to an invasive metastatic state, pirate the EMT [34–40]. Cancer cells of epithelial origin begin to downregulate E-cadherin, a tumor invasion suppressor protein [23, 41], and to express N-cadherin. The decreased expression of E-cadherin is one of the most critical steps for initiation of metastasis [40, 42, 43]. These now metastatic cells acquire the ability to digest the extracellular matrix and to move and invade surrounding tissue. However, we showed that treatment of metastatic breast cancer cells with methyl sulfone ‘re-normalizes’ two physiological functions that utilize the EMT. Firstly, it inhibits the migration of cancer cells through an extracellular matrix, and secondly, it stimulates migration of cancer cells into a wounded area and subsequently inhibits further migration once the wound is covered. Since we also showed that methyl sulfone increased expression of E-cadherin while decreasing the expression of N-cadherin, it is possible that E-cadherin/N-cadherin played a role in the methyl sulfone-induced inhibition of cell migration through an extracellular matrix and proper healing of wounded areas. Similarly, Yi et al. [43] showed that two classes of potential chemotherapeutic agents, histone deacetylase inhibitors and DNA methyltransferase inhibitors, increase the *in vitro* and *in vivo* expression of E-cadherin while at the same time inhibiting cell proliferation in ER-positive endometrial cells. We believe that methyl sulfone will be an important tool for studies of fundamental and complex processes such as migration of metastatic cells and wound healing.

### *Methyl Sulfone and Differentiation*

One might view a differentiated cell as being at the opposite end of the spectrum from a cancer cell. With melanoma cells, differentiation can be deduced from cell shape. For example, we showed previously that methyl sulfone induces melanoma cells to change shape from worm-like cells to cells with functional melanosome-filled arbors as found in normal healthy melanocytes [7]. Similarly, Joung et al. [44] recently showed that methyl sulfone induced the differentiation of primary bone marrow mesenchymal stem cells. In methyl sulfone-treated 66cl-4 breast cancer cells, a similar correlation between structure and function/differentiation is not obvious. Therefore, we sought a different approach to investigate the possibility that methyl sulfone induces differentiation



within the population of metastatic 66cl-4 breast cancer cells. Two major cell types are found in the normal mammary breast, namely ductal epithelial cells that produce milk and myoepithelial cells that line the ductal epithelial cells and push milk through ducts to the nipple. The 66cl-4 breast cancer cells have ductal epithelial phenotypes. Conversely, there is no definitive evidence that the population of 66cl-4 cells also includes myoepithelial cells [11]. However, the studies of Lakhani et al. [25] suggest that epithelial cells and myoepithelial cells derive from a common precursor. Therefore, we examined the possibility that methyl sulfone may induce differentiation of myoepithelial cells. As shown here, treatment of the 66cl-4 metastatic breast cancer cell line with methyl sulfone did indeed induce expression of  $\alpha$ -smooth muscle actin, a myoepithelial-specific and differentiation-associated protein. The significance of methyl sulfone-induced differentiation of myoepithelial cells is further highlighted by the fact that the presence of differentiated myoepithe-

lial cells may be associated with a decrease in the metastatic potential of malignant epithelial mammary cells [45, 46].

To conclude, we have shown here that methyl sulfone induced the loss of metastatic phenotypes with a concomitant gain of normal phenotypes in a highly aggressive metastatic breast cancer cell line. These data demonstrate the possibility that chemotherapeutic agents need not kill cancer cells. Instead, it is possible that metastatic cells may be reprogrammed into normal cells.

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