Tetrathiomolybdate Inhibits Angiogenesis and Metastasis Through Suppression of the NFκB Signaling Cascade

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Abstract
Tetrathiomolybdate (TM), a specific copper chelator, has been shown to be a potent antiangiogenic and antimetastatic compound possibly through suppression of the NFκB signaling cascade. To further delineate the molecular mechanism of the anticancer effect of TM, we investigated whether TM has antineoplastic activity in the setting of genetic NFκB inhibition. In this study, SUM149 inflammatory breast carcinoma cells were transfected with a dominant-negative IκBα (S32AS36A) expression vector. Similar to TM-treated SUM149 cells, SUM149-IκBαMut clones secreted lower amounts of proangiogenic mediators, vascular endothelial growth factor, interleukin-1α, and interleukin-8 and exhibited a less invasive and motile phenotype. The reduction in the angiogenic and metastatic potential of SUM149-IκBαMut clones was not further affected by TM in vitro. SUM149-IκBαMut xenografts grew substantially slower and had less lung metastasis than SUM149 and SUM149-empty vector xenografts. The growth and metastatic potential of SUM149 and SUM149-empty tumors was significantly inhibited with systemic TM treatment, whereas TM had no further antitumor effect on the SUM149-IκBαMut tumors. Additionally, nuclear proteins isolated from TM-treated SUM149 tumors had lower NFκB binding activity, while AP1 and SP1 binding activities were unchanged. Taken together, these results strongly support that suppression of NFκB is the major mechanism used by TM to inhibit angiogenesis and metastasis.

Introduction
Evidence has accumulated in the past decade that establishes an incontrovertible link between uncontrolled NFκB activity and oncogenesis. Numerous reports have characterized various types of solid tumors with deregulated NFκB activity as a result of constitutive activation of the NFκB signaling pathway or inactivating mutations of IκB protein members (1–5). Chromosomal aberrations spanning regions containing RelA, c-Rel, p50, and p52 genes were found in many hematopoietic and solid tumors (1). Amplification of RelA was reported in squamous carcinomas of the head and neck, breast, and gastric cancers (2, 3). Additionally, overexpression of p50 and p52 was observed in colon, prostate, and breast carcinomas (6, 7). As a whole, these reports indicate that constitutive activation of NFκB appears to be an event that frequently occurs during malignant transformation of cells.

NFκB has been shown to regulate a whole cadre of genes important for angiogenesis, invasion, and metastasis (5, 8–12). Blockade of NFκB activity suppressed vascular endothelial growth factor (VEGF) and interleukin (IL)-8 expression, resulting in a decrease in tumor angiogenesis in ovarian and prostate carcinoma cells (13, 14). Additionally, NFκB inhibition was reported to suppress invasion and metastasis of highly metastatic PC-3 prostate carcinoma cells (14). PS-341 (VELCADE), a proteasome inhibitor that blocks NFκB activation through the prevention of IκB degradation, was found to suppress tumor growth that correlated with a decrease in intratumoral microvessel density (15). 2-Methoxyestradiol, an endogenous estrogenic metabolite with antiangiogenic properties, inhibited NFκB activity in DAOY medulloblastoma cells (16). Our laboratory demonstrated that tetrathiomolybdate (TM) inhibits tumor growth and angiogenesis in SUM149 inflammatory breast carcinoma and squamous cell carcinoma V11/SF xenografts (17, 18). In vitro, TM suppressed NFκB activity and decreased the amount of known NFκB-dependent proangiogenic mediators, VEGF, IL-1, IL-6, and IL-8 released by SUM149 cells, indicating that the degree of inhibition of these NFκB-dependent factors was sufficient to drastically affect tumor growth (17). Further, TM was shown to potently block lung metastases in nude mice implanted with DU145 prostate carcinoma cells (19). These results suggest that a major mechanism of the anticancer effect of TM is suppression of NFκB, leading to a global inhibition of NFκB-mediated transcription of proangiogenic and prometastatic genes. However, it is not known whether other mechanisms contribute to the anticancer effect of TM. To discern this question, we set out to independently inhibit NFκB activity in SUM149 inflammatory breast carcinoma cells and assess the effect of adding TM to this intervention.

Results and Discussion
Inflammatory breast cancer is highly angiogenic and invasive, giving rise to profusely vascularized tumors at the
primary and distant metastatic sites (20). It is thus an excellent, stringent model to test potential antiangiogenic strategies. Previous reports from our laboratory have demonstrated that SUM149 cells produce high levels of proangiogenic mediators, VEGF, FGF2, IL-6, and IL-8 and exhibit an extremely invasive and motile phenotype (21, 22). Because NFκB activation has been shown to be an inducer of angiogenesis and metastasis and TM, an inhibitor of NFκB, was reported to be antiangiogenic and antimetastatic, we investigated if SUM149 cells are dependent on constitutive NFκB activation for its highly angiogenic and metastatic phenotype. To this end, SUM149 cells were stably transfected with an IκBα mutant expression vector, which encodes a dominant negative IκBα (S32A/S36A). SUM149-IκBαMut-transfected cells were selected in G418-containing medium, and three positive clones (clones 1–3) were expanded and maintained in the selection medium. In Fig. 1A, reverse transcription-PCR analysis showed higher expression of IκBα mRNA in clones 2 and 3 in comparison with untransfected or empty vector-transfected SUM149 (SUM149-empty) cells. Similarly, protein levels of IκBα were significantly increased in clones 2 and 3 (Fig. 1B).

To characterize the effect of NFκB suppression in SUM149 cells, conditioned media from SUM149-IκBαMut clones were collected and measured for VEGF, IL-1α, and IL-8 by ELISA. Consistent with our previous report, secretion of these proangiogenic mediators by SUM149 cells was significantly inhibited following TM treatment (10 nm for 72 h). Similarly, SUM149-IκBαMut clones 2 and 3 secreted lower amounts of these proangiogenic mediators in comparison with untransfected or empty vector-transfected SUM149 cells (P < 0.05) (Fig. 2A). We focused our attention on VEGF, IL-1α, and IL-8 due to their known dependence on NFκB and by no means a complete list of genes regulated by TM. Because induction of tumor angiogenesis is due to a change in the proangiogenic/antiangiogenic balance, it is certainly likely that TM is able to modulate the expression and levels of other proangiogenic and antiangiogenic mediators. Work with cDNA microarray and proteomics is ongoing in our laboratory to address this question in a comprehensive manner. TM (10 nm for 48 h) blocked invasion by 50.8 ± 1.6% (P < 0.04) and motility by 21 ± 2.7% (P < 0.005) in SUM149 cells. Likewise, SUM149-IκBαMut clones are less invasive (40.5 ± 2.2% and 59.5 ± 6.3% inhibition for clones 2 and 3, respectively) and motile (22.8 ± 1.8% and 35.9 ± 4.1% inhibition for clones 2 and 3, respectively) than wild-type SUM149 cells. TM treatment of SUM149-IκBαMut clones resulted in no additional effect on inhibiting invasion, metastasis, and amount of proangiogenic mediators. These results demonstrate that NFκB plays a major role in establishing the angiogenic and metastatic phenotype of inflammatory breast cancer cells. Moreover, because the change in phenotype observed for TM-treated SUM149 cells is similar to untreated SUM149-IκBαMut clones and TM did not change the phenotype of SUM149-IκBαMut clones, there is evidence that inhibition of tumor angiogenesis and metastasis by TM is a direct consequence of the ability of TM to suppress NFκB activation.

To determine if the antiangiogenic and antimetastatic actions of TM are a result of inhibiting NFκB activation in vivo, SUM149, SUM149-empty, SUM149-IκBαMut clone 2, or SUM149-IκBαMut clone 3 cells (1 × 10⁶) were orthotopically transplanted into the mammary fat pads of 10-week-old female athymic nude mice. Mice were gavaged with water (control) or TM (1.25 mg/day for the first 3 days and 0.7 mg/day for the remainder of the protocol) starting on the day of xenograft transplantation. Ceruloplasmin was followed weekly and used as a surrogate marker for serum copper status. After 1 week of treatment, ceruloplasmin levels of TM-treated mice were maintained at less than 25% of baseline for the remainder of the protocol. Consistent with our previous report, TM significantly inhibited the tumor growth of SUM149 xenografts (74.6 ± 5.0% inhibition; P < 0.001). TM-treated SUM149 tumor-bearing mice had a lower incidence of lung metastasis in comparison with untreated SUM149 tumor-bearing mice (1/6 vs. 6/6 mice). The size of SUM149-empty tumors was smaller by 69.4 ± 1.1% (P < 0.001), and incidence of lung metastasis was lower (0/6 vs. 5/6 mice) as a result of systemic TM therapy (Fig. 3A). SUM149-IκBαMut clones 2 and 3 tumors were significantly less bulky (P < 0.001, n = 6) and less metastatic (only 0/6 and 1/6 mice had lung metastases for clones 2 and 3, respectively) in contrast to the SUM149 and SUM149-empty tumors. Importantly, tumor growth and incidence of lung metastasis of SUM149-IκBαMut clones 2 and 3 bearing mice was unchanged with TM treatment.
To directly determine the effect of TM on NFκB activity of cancer cells in the tumor mass, mammary tumors were resected and nuclear proteins were isolated for electrophoretic mobility shift assay (Fig. 3C). Constitutive intratumoral NFκB binding was observed for untreated SUM149 and SUM149-empty xenografts. Systemic TM therapy resulted in lower NFκB binding in SUM149 and SUM149-empty tumors. As expected, expression of dominant-negative IκBa significantly inhibited NFκB activity of SUM149-IκBaMut tumors. To determine the specificity of nuclear protein binding to NFκB consensus sequence, competition experiments were performed with unlabeled NFκB, AP1, and SP1 (Fig. 3D). Nuclear protein binding to NFκB was specific as excess (100-fold) unlabeled NFκB completely displaced binding, whereas NFκB binding was unchanged with the addition of excess (100-fold) unlabeled AP1 or SP1. Moreover, AP1 and SP1 binding was unaffected with TM therapy, suggesting that TM is specifically targeting NFκB activity of cancer cells within the tumor mass. The observation that these carcinoma cells remained responsive to TM even after 7 weeks of continuous daily therapy suggests that the incidence of resistance to TM may be lower than conventional cytotoxic chemotherapeutic agents. This is exciting from a clinical perspective, as it appears that TM may prove to be more resilient and be efficacious against solid tumors for a prolonged period. These in vivo results are consistent with our in vitro observations and further support the notion that inhibition of NFκB in carcinoma cells is a major component of the antiangiogenic and antimetastatic actions of TM.

Tumor angiogenesis is a dynamic and complex process that involves multiple proangiogenic mediators. Recent reports have demonstrated that a number of proangiogenic mediators are up-regulated in numerous solid tumors, including breast, ovarian, and prostate carcinomas, and sarcomas and a critical event in tumor progression (13, 14, 22, 23). Because several proangiogenic mediators are involved in the stimulation of tumor angiogenesis, it is critical to recognize that blockade of one proangiogenic mediator alone may not be sufficient to inhibit angiogenesis to an extent required for clinical response. Recent failures of late-stage cancer trials for VEGF-KDR antagonists, SU-5416 and Avastin, lend further credence for the need to develop broad-spectrum antiangiogenic agents that can target multiple proangiogenic mediators. In a phase 2 clinical trial for advance renal cell carcinoma, patients rendered copper deficient by TM significantly had lower amounts of proangiogenic mediators, VEGF, FGF2, IL-6, and IL-8 in circulation than prior to therapy (24). It appears that TM is capable of attacking multiple proangiogenic targets and may prove to be more efficacious than single-target therapy for the treatment of solid tumors in future phase 3 clinical trials.
As a whole, these results solidly demonstrate that the major component of the antiangiogenic and antimetastatic actions of TM are due to the direct effect of TM on inhibiting NF-κB activity of cancer cells, thus impairing these malignant cells from producing and releasing mediators required for angiogenesis and metastasis into the tumor microenvironment.

Materials and Methods

Cell Lines

The SUM149 inflammatory breast cancer cell line was developed from a primary inflammatory breast cancer tumor and grown in Ham’s F-12 medium supplemented with 5% fetal bovine serum, 5-μg/ml insulin, and 1-μg/ml hydrocortisone.

Transfection With IκBαMut Expression Vector

SUM149 cells were transfected with pUSEamp-empty vector or pUSEamp-IκBαDN (S32AS36A) (Upstate Biotechnology, Inc., Lake Placid, NY) using FuGene 6 transfection reagent (Roche-Boehringer Mannheim, Mannheim, Germany). Stable transfectants were established by culturing the transfected cells in the described medium supplemented with 100-μg/ml G418 (Life Technologies, Inc., Carlsbad, CA) for 21 days. Expression of the transgene was determined by reverse transcription-PCR and Western blot analysis.

Messenger RNA Expression and Protein Levels of IκBαMut Clones

Total RNA was isolated from cells using Trizol reagent (Life Technologies, Inc., Carlsbad, CA).
Technologies) according to the manufacturer’s recommendations. One microgram of total RNA was converted to cDNA using an avian myeloblastosis virus reverse transcription system (Promega, Madison, WI). A 100-μg aliquot of the resulting cDNA was amplified by PCR with 25-ng Lc-Bo or β-actin specific primers. PCR products were separated on a 1.2% agarose gel and imaged on an Alpha Image 950 documentation system (Alpha Innotech, San Leandro, CA). Densitometry of images was performed using NIH Image version 1.62.

Proteins were harvested from SUM149, SUM149-empty vector, and SUM149-Lc-BoMut clones 1–3 cells using radio-immunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1-mg/ml phenylmethyl-sulfonyl fluoride, 1-mM sodium orthovanadate, and 0.3-mg/ml aprotinin; Sigma Chemical Co., St. Louis, MO). Proteins (20 μg) were mixed with Laemmli buffer, heat denatured for 3 min, separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Nonspecific binding was blocked by overnight incubation with 2% BSA in Tris-buffered saline with 0.05% Tween 20 (Sigma). Immobilized proteins were probed using antibodies specific for Lc-Bo and β-actin (Upstate Biotechnology) and visualized by enhanced chemiluminescense (Amersham, Piscataway, NJ).

**Conditioned Medium From SUM149 Cells**

SUM149 and SUM149-Lc-BoMut cells were plated at a density of 2 × 10^4 cells in 100-mm^2 dishes. Cells were treated with vehicle or 10-nM TM for 72 h. Conditioned medium was collected, centrifuged for 5 min at 2500 rpm, and divided into 1-ml aliquots. Quantikine human VEGF ELISA (R&D Systems, Inc., Minneapolis, MN) was used to measure protein levels of the 165 amino acid species of VEGF. ELISAs for IL-1α and IL-8 were performed by the University of Maryland Cytokine Core Laboratory (www.cytokinelab.com).

**Cell Invasion and Motility Assay**

Cell invasion was determined as described from the cell invasion assay kit (Chemicon International, Inc., Temecula, CA). Cells were pretreated with or without 10-nM TM for 48 h, harvested, and resuspended in serum-free medium. An aliquot (1 × 10^5 cells) of the prepared cell suspension was added into the chamber and incubated for 48 h at 37°C in a 10% CO2 tissue culture incubator. Noninvading cells were gently removed from the interior of the inserts with a cotton-tipped swab. Invasive cells were stained and quantified by colorimetric reading at 560 nm. Random cell motility was determined as described from the motility assay kit (Cellomics, Pittsburgh, PA). Cells were pretreated with or without 10-nM TM for 48 h, harvested, suspended in serum-free medium, and plated on top of a field of microscopic fluorescent beads. After a 48-h incubation period, cells were fixed, and areas of clearing in the fluorescent bead field corresponding to phagokinetic cell tracks were quantified using NIH ScionImager.

**Xenograft Model of Breast Cancer**

Ten-week-old athymic nude mice were orthotopically injected with SUM149 cells (1 × 10^6 cells) into the upper left mammary fat pad. Cells were trypsinized, washed, and resuspended in HBSS at a density of 1 × 10^6 cells/200 μl. Mice were anesthetized using 10-mg/ml ketamine, 1-mg/ml xylazine, and 0.01-mg/ml glycopyrrolate, and an incision below the thoracic left mammary fat pad was made. Using a 27-gauge needle, the cell suspension was injected into the exposed mammary fat pad and the wound was closed with a single wound clip.

**Electrophoretic Mobility Shift Assay**

Mammary tumors were resected and nuclear proteins were isolated using NE-PER nuclear protein extraction kit (Pierce Biotechnology, Rockford, IL). Electrophoretic mobility shift assay was performed as described using the LightShift Chemiluminescent EMSA kit (Pierce Biotechnology) with biotin-labeled κB consensus sequence, 5'-AGTTGAGG-GACCTTCCCCAGGC-3', AP1 consensus sequence, 5'-CGCTTGATGATGCAGCGCCGAAA-3', or SP1 consensus sequence, 5'-ATTCCATCGGGGCGGACG-3' in the absence or presence of 100× unlabeled κB, AP1, or SP1 oligonucleotide. Protein-DNA complexes were resolved on a high ionic strength 5% polyacrylamide gel containing 0.5× Tris-borate EDTA buffer.

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**References**


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