Mithramycin Depletes Specificity Protein 1 and Activates p53 to Mediate Senescence and Apoptosis of Malignant Pleural Mesothelioma Cells

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Abstract

Purpose: Specificity protein 1 (SP1) is an oncogenic transcription factor overexpressed in various human malignancies. This study sought to examine SP1 expression in malignant pleural mesotheliomas (MPM) and ascertain the potential efficacy of targeting SP1 in these neoplasms.

Experimental Design: qRT-PCR, immunoblotting, and immunohistochemical techniques were used to evaluate SP1 expression in cultured MPM cells and MPM specimens and normal mesothelial cells/pleura. FTs, chemotaxis, soft agar, β-galactosidase, and Apo-BrdUrd techniques were used to assess proliferation, migration, clonogenicity, senescence, and apoptosis in MPM cells following SP1 knockdown, p53 overexpression, or mithramycin treatment. Murine subcutaneous and intraperitoneal xenograft models were used to examine effects of mithramycin on MPM growth in vivo. Microarray, qRT-PCR, immunoblotting, and chromatin immunoprecipitation techniques were used to examine gene expression profiles mediated by mithramycin and combined SP1 knockdown/p53 overexpression and correlate these changes with SP1 and p53 levels within target gene promoters.

Results: MPM cells and tumors exhibited higher SP1 mRNA and protein levels relative to control cells/tissues. SP1 knockdown significantly inhibited proliferation, migration, and clonogenicity of MPM cells. Mithramycin depleted SP1 and activated p53, dramatically inhibiting proliferation and clonogenicity of MPM cells. Intrapertioneal mithramycin significantly inhibited growth of subcutaneous MPM xenografts and completely eradicated mesothelioma carcinomatosis in 75% of mice. Mithramycin modulated genes mediating oncogene signaling, cell-cycle regulation, senescence, and apoptosis in vitro and in vivo. The growth-inhibitory effects of mithramycin in MPM cells were recapitulated by combined SP1 knockdown/p53 overexpression.

Conclusions: These findings provide preclinical rationale for phase II evaluation of mithramycin in patients with mesothelioma.

Introduction

Malignant pleural mesotheliomas (MPM) are highly lethal neoplasms attributable primarily to occupational or environmental exposures to asbestos and other related mineral fibers such as erionite (1–3). Because of the long latency associated with these neoplasms, the global incidence of MPM continues to increase, despite asbestos being banned in many countries (1, 3). Presently, in the United States, the incidence of MPM is approximately 3,000 cases annually (4). Median overall survival of patients with MPM undergoing aggressive multimodality therapy ranges from 14 to 22 months, depending on tumor stage and histology, extent of surgical resection, and response to chemotherapy (5–7).

Recent investigative efforts have provided new insights regarding the pathogenesis of MPM (11). For example, rare familial MPMs and approximately 65% of sporadic MPM exhibit mutations involving BRCA1-associated protein-1 (BAP1), which encodes a nuclear ubiquitin hydrolase with diverse activities including DNA repair and deubiquitination of the repressive histone mark H2AK119Ub (8–11). In addition, MPMs exhibit recurrent cytogenetic abnormalities including allelic loss of CDKN2A and p14 ARF (12) and amplification of MYC and PVT1 oncogenes (13). Epigenomic perturbations including aberrant activity of DNA methyltransferases and overexpression of polycomb repressor complex 2, silence tumor suppressor genes and noncoding RNAs (14, 15). To date, clinical efforts to specifically target oncogene signaling or reverse epigenomic derangements in MPM have had limited success (16).
Translational Relevance

Pleural mesotheliomas are highly lethal neoplasms for which there are no effective treatments. Experiments described in this article demonstrate that under exposure conditions potentially achievable in clinical settings, mithramycin depletes SP1 and activates p53 signaling to mediate profound growth arrest associated with senescence and subsequent apoptosis of pleural mesothelioma cells in vitro and in vivo. These findings provide the preclinical rationale for evaluation of mithramycin administered systematically or by regional perfusion techniques in patients with mesothelioma.

Specificity protein 1 (SP1) is a zinc-finger transcription factor that binds to GC-rich motifs (17) and mediates diverse physiologic processes such as cell-cycle regulation, apoptosis, and angiogenesis, which are essential for normal embryonic development and tissue differentiation (18, 19). SP1 interacts with a variety of transcription factors, histone acetyltransferases and deacetylases, as well as chromatin remodeling complexes to activate or repress transcription in a context-dependent manner (18, 20). SP1 is overexpressed and contributes to the malignant phenotype of a variety of human cancers by upregulating genes that enhance proliferation, invasion, and metastasis (18, 19), as well as stenosis and chemoresistance (21–24). To date, the role of SP1 in MPM has not been defined. The present study was undertaken to examine SP1 expression in MPM and ascertain the potential clinical efficacy of targeting this transcription factor for mesothelioma therapy.

Materials and Methods

Cell lines, tumor samples, and in vitro mithramycin treatments

H28 and H2452 human MPM lines were obtained from ATCC and maintained in RPMI plus Pen/Strep and 10% FBS (normal media). LP3 and LP9 normal mesothelial cell lines were obtained from the Coriell Institute for Medical Research and cultured per vendor recommendations. MES1-9 cell lines were established in normal media. Validation of these cell lines, which have been described in Supplementary Methods.

RNA isolation, real-time quantitative reverse transcription PCR, and microarray analysis

RNA isolation, quantitative reverse transcription PCR (qRT-PCR) using comparative C_{t} method (25), and microarray analysis are described in Supplementary Methods.

Immunoblotting

Immunoblotting was done as previously described (26) using antibodies listed in Supplementary Methods. Cells for immunoblot analysis were harvested immediately after 24-hour mithramycin treatment in vitro and 3 days following the last intraperitoneal mithramycin injection for xenograft experiments.

Immunohistochemistry of primary tumor samples

See Supplementary Methods.

Morphine xenograft experiments

Athymic nude mice were injected in bilateral flanks with 2 × 10^6 of MES1 or MES7 cells. After 10 to 15 days, mice were sorted by tumor size and randomly assigned to receive either normal saline or mithramycin (1 or 2 mg/kg) intraperitoneally every Monday, Wednesday, and Friday for 3 weeks. Tumor size and mouse weights were measured twice weekly, and mithramycin doses were held for weight loss >20% of initial body weight. After completion of 3 weeks of treatment (day 31, 3 days after last mithramycin injection) or when control tumors reached maximum allowable size, mice were euthanized, tumors were excised and processed for additional studies. For the intraperitoneal tumor model, 3 × 10^6 MES1 cells were injected intraperitoneally into athymic nude mice. Ten days later, mice were randomized to receive saline or mithramycin (1 mg/kg) intraperitoneally every MWF for 3 weeks. Approximately 1 week later, mice were euthanized and laparotomies were performed to assess tumor burden. All animal procedures were approved by the National Cancer Institute Animal Care and Use Committee, and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Generation of stable cells expressing shRNA constructs

MES1 and MES7 were transduced with commercially validated shRNA targeting SP1 or sham sequences (Sigma) according to manufacturer’s instructions (Supplementary Methods). Cell lines were selected with puromycin (Sigma) and expanded after confirmation of knockdown by qRT-PCR and immunoblot techniques.

Chromatin immunoprecipitation experiments

Quantitative chromatin immunoprecipitation (ChIP) assays were conducted as described (26) with minor modifications using either nonspecific immunoglobulin G (IgG) or ChIP-grade antibodies as well as PCR primers listed in Supplementary Methods.

Adenoviral transduction of p53 in SP1-knockdown cells

Both sham control or SP1-knockdown MES1 and MES7 were transduced with high titer Ad-GFP or Ad-GFP-p53 (Vector Labs) for 48 hours. Thereafter, the cells were collected for counting, RNA, and protein analysis. For in vitro studies, 2 × 10^6 cells were injected subcutaneously into the bilateral flanks of athymic nude mice and tumor volume was measured every 4 days until the experiment was completed.

Immunoblotting

Immunoblotting was done as previously described (26) using antibodies listed in Supplementary Methods. Cells for immunoblot analysis were harvested immediately after 24-hour mithramycin treatment in vitro and 3 days following the last intraperitoneal mithramycin injection for xenograft experiments.
Senescence, cell-cycle, and apoptosis analyses
A total of $3 \times 10^4$ MPM cells were seeded in each of four chamber slides overnight. The following day, cells were treated for 24 hours with mithramycin (0–100 nmol/L), and senescence was assessed using the SA-β-gal Staining Kit from Cell BioLabs. Similar techniques were used to detect senescence in sham control or SP1-knockdown MES1 and MES7 cells 48 hours following transduction with high titer Ad-GFP or Ad-GFP-p53. For cell-cycle analysis, knockdown MES1 and MES7 cells 48 hours following transduction were harvested at indicated time points and processed for cell-cycle analysis using flow cytometric techniques (27). Apoptosis was assessed by flow cytometry using reagents and protocols contained in the Apo-BrdU Kit (BD Pharmingen).

Statistical analysis
SD or SEM are indicated by bars on figures and were calculated using GraphPad Prism 6.0. All experiments were conducted with at a minimum of triplicate samples, and all $P$ values were calculated with two-tailed $t$ tests.

Results

**SP1 expression in MPM**
qRT-PCR and immunoblot experiments were performed to examine SP1 expression in cultured MPM cells and tumors relative to cultured normal mesothelial cells or normal pleura. Seven of nine MPM lines exhibited overexpression of SP1 mRNA relative to normal mesothelial cells (LP3 or LP9; Fig. 1A, top). Immunoblot experiments demonstrated markedly higher SP1 protein levels in MPM cells relative to normal mesothelial cells (Fig. 1A, bottom). Additional qRT-PCR experiments demonstrated overexpression of SP1 in 5 of 7 primary MPM specimens from which the aforementioned cell lines were derived, although the magnitude of SP1 overexpression in cell lines and primary tumors did not exactly coincide (Fig. 1A, middle top). Immunohistochemical experiments demonstrated overexpression of SP1 in 18 of 19 primary MPM specimens compared with normal pleura, including seven from which our cell lines were derived (Supplementary Fig. S1A). To extend these observations, IHC techniques were used to evaluate SP1 expression in commercial tissue arrays containing 59 primary mesotheliomas and 22 normal mesothelial tissues. A spectrum of SP1 expression was detected, with significantly increased SP1 staining in MPMs compared with normal mesothelia (Fig. 1A, middle bottom).

Additional analysis was undertaken to ascertain whether intratumoral SP1 expression detected by Illumina array techniques correlated with survival in 39 patients with locally advanced MPM undergoing potentially curative resections at Brigham and Women’s Hospital. Twenty-four patients had epithelial mesotheliomas, whereas seven patients had biphasic and eight patients had sarcomatoid malignancies. Increased expression of SP1 tended to be associated with shorter survival of patients with MPM, although this was not statistically significant (Fig. 1A, right).

**Effects of SP1 depletion in MPM cells**
Additional experiments were performed to examine whether SP1 expression modulates the malignant phenotype of pleural mesothelioma cells. Briefly, shRNA techniques were used to knockdown SP1 in cultured MPM cells. qRT-PCR and immunoblot experiments demonstrated significant decreases in SP1 expression in MES1 and MES7 cells following transfection with either of two shRNA sequences (shSP1 #1 and #2) relative to controls (Fig. 1B, left and right). Knockdown of SP1 significantly diminished proliferation, migration, and soft agar clonogenicity of MES1 and MES7 cells (Fig. 1C and D).

**Effect of mithramycin in MPM cells**
Seeking potential translation of the aforementioned findings to the clinic, additional experiments were performed to examine whether mithramycin, an antineoplastic agent that inhibits binding of SP1 to DNA (17), could similarly inhibit the malignant phenotype of pleural mesothelioma cells. Briefly, MES1 and MES7 cells were cultured in normal media with or without mithramycin (25–100 nmol/L) for 24 hours. qRT-PCR and immunoblot experiments (Supplementary Fig. S1B) demonstrated dose-dependent decreases in SP1 expression in both cell lines following mithramycin exposure. MTS assays demonstrated that 24-hour mithramycin exposure dramatically inhibited proliferation of MES1 and MES7 cells (Fig. 2A, left and middle). Cytotoxicity was not evident until 24 hours following removal of mithramycin, suggesting delayed effects of drug exposure. Although some recovery of cell growth was observed 3 to 5 days following mithramycin exposures of 25 or 50 nmol/L, treatment with doses of 100 nmol/L or more resulted in progressive decreases in cell viability over the ensuing 4 days. Furthermore, 24-hour exposure of mithramycin significantly inhibited soft agar clonogenicity of MES1 and MES7 cells in a dose-dependent manner (Fig. 2A, right).

Additional experiments were performed to examine whether mithramycin inhibited growth of MPM cells in vivo. As shown in Fig. 2B (left and middle), as well as Supplementary Fig. S1C, mithramycin significantly diminished growth of subcutaneous MES1 and MES7 xenografts in a dose-dependent manner. The growth-inhibitory effects of mithramycin were readily apparent shortly after treatment was initiated, with statistically significant differences in xenograft volumes evident within 7 days of commencing treatment (3 intraperitoneal injections). The 1 mg/kg dose was well-tolerated with no significant weight loss, lethargy, or decreased activity; however, the 2 mg/kg dose group experienced moderate or statistically significant weight loss and some visible toxicities (Fig. 2B, right, and Supplementary Fig. S1C). These latter findings were in contrast to our previously published experiments pertaining to intraperitoneal mithramycin treatment of lung cancer xenografts (21), in which 2 mg/kg mithramycin appeared to be well tolerated, possibly because clinical grade mithramycin was used for the lung cancer studies but not the current experiments.

In the second series of experiments, mice with intraperitoneal mesothelioma carcinomatosis were randomly allocated to receive saline or mithramycin (1 mg/kg) intraperitoneally every Monday, Wednesday, and Friday for 3 weeks, followed by euthanasia 3 days later. Preliminary experiments were performed to confirm the reproducibility and growth kinetics of the carcinomatosis model and timing of intervention. Results of three independent experiments totaling 60 mice are summarized in Fig. 2C. Whereas 29 of 30 control mice (97%) had extensive carcinomatosis when euthanized, 23 of 30 mithramycin-treated mice (77%) had no evidence of disease.
Figure 1.

SP1 is overexpressed in MPM cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001. A, qRT-PCR (left, top) and immunoblot (left, bottom) showing that SP1 mRNA and protein expression levels are higher in cultured MPM cells relative to normal mesothelia; qRT-PCR (middle, top) showing that SP1 mRNA is overexpressed in primary MPM compared with normal pleura; TMA analysis (middle, bottom) of SP1 expression in normal mesothelial tissues (NMT, n = 22) and malignant mesothelial tissues (MMT, n = 59). SP1 staining was scored from 0 to 100 on the basis of percentage of positive cells. The majority of the MMT had significantly increased SP1 staining compared with NMT. Correlation of increased intratumoral SP1 expression with overall survival in 39 patients with locally advanced MPM undergoing potentially curative resections (right). B, confirmation of stable knockdown of SP1 by qRT-PCR and immunoblot analysis in MES1 and MES7 cells (left and right, respectively). C and D, time-dependent inhibition of cell proliferation, migration (after 72 hours) and clonogenicity (after 21 days) of SP1 knockdown in MES1 and MES7 cells, respectively. Both shRNA sequences significantly inhibit soft agar clonogenicity of MPM cells.
Figure 2.
Effects of mithramycin in vitro and in vivo in MPM cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. A, effects of mithramycin on proliferation and soft agar clonogenicity of MES1 and MES7 cells. B, effects of intraperitoneal mithramycin on volume and mass of established subcutaneous MES1 xenografts (left and middle, respectively) and body weight in tumor-bearing mice (right). C, effects of intraperitoneal administration of mithramycin (1 mg/kg intraperitoneal) in nude mice with MES1 induced carcinomatosis. Numbers of mice with residual tumors following mithramycin treatment versus saline injections (left). Representative results depicting carcinomatosis in control mice compared with no residual tumor or minimal residual tumors in mice following intraperitoneal mithramycin (right). D, heatmap (left) and Ingenuity Pathway analysis (right) of genes modulated in MES1 and MES7 xenografts in mice receiving mithramycin (1 mg/kg).
Effects of mithramycin on global gene expression in MPM cells

Affymetrix microarray experiments were performed to examine global gene expression profiles in cultured MES1 and MES7 cells immediately after 24-hour exposure to normal media with or without mithramycin (25 or 100 nmol/L) and their corresponding xenografts harvested three days after the last intraperitoneal saline or mithramycin injection. Mithramycin-mediated dramatic dose-dependent alterations in gene expression in cultured MPM cells. Using criteria of fold change greater than 2 for drug treatment versus control, 986 genes were commonly modulated in MES1 and MES7 cells following 25 and 100 nmol/L mithramycin exposures, respectively (Supplementary Fig. S2A); significant overlap was observed at both drug concentrations. Approximately 65% of genes modulated by mithramycin in cultured MPM cells at either drug concentration were repressed by mithramycin. Top canonical pathways affected by mithramycin exposure in vitro are depicted in Supplementary Fig. S2B. Within each cell line, more overlap was observed with cells treated with 100 nmol/L rather than 25 nmol/L in vitro and tumor xenografts (Supplementary Fig. S2C). Using criteria of fold change equal to or greater than 2 to 100 nmol/L mithramycin as well as xenografts from mice treated with 1 mg/kg mithramycin (Supplementary Table S1). Mithramycin-mediated dramatic in vitro and tumor xenografts (Supplementary Fig. S2C). Similar analysis could not be performed for MES7 because of the limited quantity and poor quality of the RNA from xenografts from 2 mg/kg treated mice. Fifty-six genes were commonly regulated in MES1 and MES7 xenografts from treated mice with 1 mg/kg mithramycin intraperitoneally (heatmap is depicted in Fig. 2D, left); 55% of these genes were downregulated (Table 1). Notably, KIAA1199, which recently has been implicated in modulating metabolism, EGFR and Wnt signaling in cancer cells (28, 29) was markedly repressed in MES1 as well as MES7 xenografts from mithramycin-treated mice. In addition, the oncofetal receptor tyrosine kinase ROR1, which is overexpressed in a variety of human malignancies but to date has not been studied in pleural mesotheliomas, was significantly downregulated in MPM xenografts from mithramycin-treated mice. Top canonical pathways included G-alpha 12/13 signaling, which mediates cytoplasmic β-catenin levels (30), Wnt/Ca²⁺ signaling, axonal guidance signaling, which has recently been implicated recently in pluripotency and metastatic potential of cancer cells (31), p33 signaling, as well as pathways associated with immunologic destruction of cancer cells.

More than 1,200 genes and nearly 640 genes were significantly modulated by mithramycin treatment in subcutaneous MES1 and MES7 xenografts, respectively. More than 700 genes were commonly regulated in MES1 cells exposed to mithramycin (100 nmol/L) in vitro and MES1 xenografts from 1 and 2 mg/kg treated mice (Supplementary Fig. S2C). Similar analysis could not be performed for MES7 because of the limited quantity and poor quality of the RNA from xenografts from 2 mg/kg treated mice. Fifty-six genes were commonly regulated in MES1 and MES7 xenografts from treated mice with 1 mg/kg mithramycin intraperitoneally (heatmap is depicted in Fig. 2D, left); 55% of these genes were downregulated (Table 1). Notably, KIAA1199, which recently has been implicated in modulating metabolism, EGFR and Wnt signaling in cancer cells (28, 29) was markedly repressed in MES1 as well as MES7 xenografts from mithramycin-treated mice. In addition, the oncofetal receptor tyrosine kinase ROR1, which is overexpressed in a variety of human malignancies but to date has not been studied in pleural mesotheliomas, was significantly downregulated in MPM xenografts from mithramycin-treated mice. Top canonical pathways included G-alpha 12/13 signaling, which mediates cytoplasmic β-catenin levels (30), Wnt/Ca²⁺ signaling, axonal guidance signaling, which has recently been implicated recently in pluripotency and metastatic potential of cancer cells (31), p33 signaling, as well as pathways associated with immunologic destruction of cancer cells.

Table 1. Genes induced or repressed by mithramycin in MES1 as well as MES7 xenografts

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pathways associated with bladder, pancreas, ovarian, and brain cancer (Fig. 2D, right).

Because p53 signaling was observed to be a top pathway affected by mithramycin in vitro and in vivo, genes from Table 1 and Supplementary Table S1 were screened for SP1- and p53-binding sites within their respective promoters using software guided analysis (DECODE: DECipherment Of DNA Elements or UCSC Genome Browser). Approximately 50% of these genes had recognition elements for either SP1, p53, or both within their promoters.

qRT-PCR and immunoblot techniques were used to validate Affymetrix array results using selected genes modulated by mithramycin in vitro and/or in vivo. qRT-PCR analysis (Fig. 3A) confirmed that mithramycin decreased ROR1 expression in vitro and in vivo. Furthermore, although not identified as a commonly regulated gene by microarray analysis, HDAC4, a well-established SP1 target (32), was repressed by mithramycin in vitro and in vivo in MES1 and MES7 cells. mithramycin also decreased expression of SP1 as well as EZH2, which previously has been shown to be an epigenetic driver of malignancy in pleural mesotheliomas (14; Supplementary Fig. S3A); this phenomenon was not seen in corresponding xenografts possibly due to the timing and intermitten nature of the intraperitoneal drug treatments and interval from last drug treatment to euthanasia. In addition, mithramycin mediated dose-dependent increases in p53, p21, PMAIP1, and PRDM1 in vitro and in vivo (Fig. 3A). Subsequent immunoblot experiments (Fig. 3B) confirmed results of qRT-PCR experiments.

Mithramycin activates p53 in MPM cells

Following stress or DNA damage, p53 undergoes various posttranslational modifications such as acetylation or phosphorylation, mechanisms which are known to activate and stabilize this transcription factor (33). To examine this issue, MES1 and MES7 cells, which express wt p53 were treated with mithramycin at various doses for 24 hours. Immunoblot experiments demonstrated dose-dependent increases in γH2AX in MES1 and MES7 cells indicative of DNA damage, which coincided with increases in total p53 as well as acetylated p53-K320, K373, K382, and phosphorylated p53-S15 levels in MES7 cells, and to a lesser extent, MES1 cells following mithramycin exposure (Supplementary Fig. S3B–S3D). Collectively, these data suggest that DNA damage contributes to mithramycin-mediated activation of p53 in MPM cells.

Effects of mithramycin on target gene promoters

ChIP experiments were conducted to further investigate the mechanisms by which mithramycin regulates oncogene and tumor suppressor gene expression in MPM cells. ChIP was performed using primers flanking p53 or SP1 response elements to quantitate occupancy of p53, SP1, or RNA Pol II, as well as levels of H3K4Me3 and H3K27Me3 (euchromatin and heterochromatin histone marks, respectively) within the promoter regions of p21, PMAIP1, HDAC4, ROR1, DACT1, and PRDM1. As shown in Fig. 3C and Supplementary Fig. S4A and S4B, activation of p53 or repression of SP1 by mithramycin increased p53 and decreased SP1 occupancy, respectively, within the p21, PMAIP1, and PRDM1 promoters, which coincided with increased occupancy of RNA Pol II, increased H3K4Me3, and decreased levels of H3K27Me3 in the respective promoters in MES1 and MES7 cells. mithramycin appeared to decrease occupancy of p53 as well as SP1 and RNA Pol II and diminish H3K4Me3 levels, while increasing H3K27Me3 levels within the ROR1 promoter. Furthermore, mithramycin decreased occupancy of SP1 and RNA PolII, coinciding with decreased H3K4Me3 and increased H3K27Me3 within the HDAC4 promoter. Finally, mithramycin increased occupancy of RNASP1 and increased H3K4Me3 levels while decreasing H3K27Me3 within the DACT1 promoter region, which contains no prototypic SP1 or p53 recognition elements (Supplementary Fig. S4A and S4B), suggesting an indirect mechanism of activation of this tumor suppressor gene by mithramycin.

Effects of SP1 knockdown and p53 overexpression in MPM cells

Additional experiments were undertaken to ascertain whether SP1 knockdown (shSP1) and p53 overexpression (p53-OEX) could mimic the effects of mithramycin in MPM cells. MES1 and MES7 cells exhibiting either knockdown of SP1, or respective vector controls, were transduced with adenovirus GFP-p53-WT or control adenovirus. Cell count assays for 48 hours post-adeno viral transduction demonstrated a significant additive effect of shSP1 and p53-OEX in MPM cells (representative results pertaining to MES7 cells are depicted in Fig. 4A, left). Further experiments demonstrated that shSP1 and p53-OEX significantly diminished the growth of subcutaneous MPM xenografts (Fig. 4A, middle and right); the in vivo growth inhibitory effects of combined shSP1/p53-OEX were more pronounced than either shSP1 or p53-OEX alone in MES7 cells. Similar results were observed in MES1 cells (Supplementary Fig. S5A).

Gene expression profiles were obtained from additional microarray experiments in MPM cells following combined shRNA mediated knockdown of SP1 and adenoviral-mediated overexpression of p53 (shSP1/p53-OEX). As shown in Fig. 4B (left), 706 and 806 genes were commonly regulated by combined shSP1/p53-OEX in MES1 and MES7 cells, respectively. Additional analysis was performed to compare gene expression profiles in mithramycin-treated and shSP1/p53-OEX MPM cells. The heatmap for this analysis is shown in Fig. 4B, middle. Fifty-three genes were differentially regulated by mithramycin and shSP1/p53-OEX in either MES1 or MES7 (Supplementary Table S2). Subsequent ingenuity pathway analysis demonstrated that these commonly regulated genes mediated p53 signaling. G1/S, G1/S/M and DNA checkpoint regulation, ATM and
Figure 4.
Effects of SP1 knockdown with or without overexpression of p53 in MPM cells. *P < 0.05; **P < 0.01; ***P < 0.001. A, left, SP1 depletion and p53 overexpression significantly inhibits in vitro proliferation (left) and decreases volume and mass of MES7 xenografts (middle and right, respectively). B, Venn diagram (left) and corresponding heatmap (middle) corresponding to genes regulated by mithramycin and overexpression of p53 in SP1-depleted MES1 and MES7 cells. Ingenuity Pathway Analysis of genes commonly regulated in MES1 and/or MES7 cells by mithramycin and SP1 knockdown with p53 overexpression (right). C, qRT-PCR and immunoblot analysis (left and right, respectively) demonstrating the effects of SP1 knockdown or overexpression of p53 on genes targeted by mithramycin in cultured MES1 and MES7 cells.
Figure A: Distribution of cell cycle phases in MES1 and MES7 cells under different treatments.

- Untreated MES1: G0-G1 = 53.9%, S = 24.5%, G2-M = 21.6%
- MM 25 nmol/L MES1: G0-G1 = 56.5%, S = 20.3%, G2-M = 23.2%
- MM 50 nmol/L MES1: G0-G1 = 60.2%, S = 13.7%, G2-M = 26.1%
- MM 100 nmol/L MES1: G0-G1 = 68.0%, S = 16.0%, G2-M = 16.0%

- Untreated MES7: G0-G1 = 44.3%, S = 33.2%, G2-M = 22.5%
- MM 25 nmol/L MES7: G0-G1 = 48.7%, S = 22.0%, G2-M = 29.3%
- MM 50 nmol/L MES7: G0-G1 = 64.5%, S = 11.2%, G2-M = 24.2%
- MM 100 nmol/L MES7: G0-G1 = 71.6%, S = 10.0%, G2-M = 18.4%

Figure B: Senescence assay in MES1 and MES7 cells.

- MES1
  - 0 nmol/L: 25%, 25%, 25%
  - 25 nmol/L: 30%, 30%, 30%
  - 50 nmol/L: 35%, 35%, 35%
  - 100 nmol/L: 40%, 40%, 40%

- MES7
  - 0 nmol/L: 20%, 20%, 20%
  - 25 nmol/L: 25%, 25%, 25%
  - 50 nmol/L: 30%, 30%, 30%
  - 100 nmol/L: 35%, 35%, 35%

Figure C: Analysis of apoptosis in MES1 and MES7 cells.

- MES1
  - Untreated: 1.8%
  - MM 25 nmol/L: 4.3%
  - MM 100 nmol/L: 5.2%
  - Negative control: 0.4%

- MES7
  - Untreated: 0.4%
  - MM 25 nmol/L: 1.5%
  - MM 100 nmol/L: 3.4%
  - Negative control: 0.8%

- Negative control
  - Untreated: 0.3%
  - MM 25 nmol/L: 21.4%
  - MM 100 nmol/L: 34.2%
  - Negative control: 0.3%

Figure D: Analysis of necrosis in MES1 and MES7 cells.

- MES1
  - Untreated: 2.1%
  - MM 25 nmol/L: 47.9%
  - MM 100 nmol/L: 64.5%
  - Negative control: 2.7%

- MES7
  - Untreated: 2.7%
  - MM 25 nmol/L: 29.9%
  - MM 100 nmol/L: 45%
  - Negative control: 3.1%
GADD45 signaling, as well as citrulline, glutamine, and arginine metabolism (Fig. 4B, right). Results of this analysis as well as other aforementioned experiments were further validated by qRT-PCR and immunoblot experiments (Fig. 4C; Supplementary Fig. S5B and S5C). Knockdown of SP1 had no effect on endogenous levels of p53 in MES1 or MES7 cells (Supplementary Fig. 5D) but modestly induced expression of p21 (Fig. 4C). Overexpression of p53 markedly increased p21 levels in these cells. These findings suggest that upregulation of p21 in MPM cells by mithramycin occurs via p53-dependent as well as -independent mechanisms. Modulation of other targets such as ROR1 and EZH2 appeared to be determined by SP1 depletion as well as activation of p53.

Effects of mithramycin on senescence and apoptosis in MPM cells

Because mithramycin modulated a variety of genes including p53, p21, PMAP1, PRDM1 SP1, HDAC4, and EZH2 that either promote or inhibit cell-cycle arrest, senescence, and apoptosis in cancer cells, additional experiments were performed to further characterize the mechanisms by which mithramycin mediated cytotoxicity in MPM cells. Flow cytometric experiments (Fig. 5A) demonstrated dose-dependent accumulation of MPM cells in G0–G1 without a sub-G0 fraction consistent with a G0–G1 arrest immediately following 24-hour mithramycin exposure. Histochemical experiments performed at this time point demonstrated that mithramycin mediated dose-dependent increases in β-galactosidase expression indicative of senescence in MPM cells; similarly, shSP1 and p53-OEX had additive effects on senescent phenotype in MPM cells (Fig. 5B; additional data available upon request). Apo-BrdUrd experiments demonstrated no significant increase in apoptosis in MPM cells immediately following 24-hour mithramycin treatment (Fig. 5C). However, significant dose-dependent apoptosis was observed 48 hours following completion of mithramycin treatment in MES1 as well as MES7 cells (Fig. 5D). These findings were consistent with results of MTT assays depicted in Fig. 2A.

Discussion

Despite being relatively rare, pleural mesotheliomas continue to challenge clinicians due to relentless growth and resistance to conventional treatment modalities as well as novel therapeutics (1). As such, there is an urgent need for innovative treatment regimens targeting specific genetic/epigenetic drivers in pleural mesotheliomas and a more thorough appreciation of drug delivery to these neoplasms in clinical settings.

In the present study, we sought to examine the potential efficacy of targeting SP1 expression in MPM. We observed overexpression of SP1 in the majority of cultured MPM lines and primary pleural mesotheliomas relative to cultured normal mesothelial cells or normal pleura. Knockdown of SP1 inhibited growth, migration, and tumorigenicity of MPM cells, strongly suggesting that SP1 functions as an oncogene in MPM. Whereas SP1 overexpression has been associated with decreased survival of patients with lung and esophageal cancers (34, 35), our microarray experiments did not reveal an association between SP1 mRNA expression and survival in patients with MPM, possibly due to the limited number of samples analyzed, as well as potential discrepancies between mRNA and protein levels detected in MPM specimens using various techniques. Additional experiments demonstrated that mithramycin markedly diminished growth of MPM cells in vitro and in vivo via induction of DNA damage, cell-cycle arrest, and senescence with subsequent apoptosis. To the best of our knowledge, these experiments are the first to demonstrate overexpression of SP1 in MPM and the potential efficacy of mithramycin for mesothelioma therapy.

Mithramycin is a naturally occurring polyauroleic acid isolated from Streptomyces, which was originally evaluated as a chemotherapeutic agent in patients with a variety of malignancies during the 1960s and 1970s; although complete responses were observed in approximately 10% to 15% of patients with sarcomas and germ cell tumors, the drug was discontinued because of excessive systemic toxicities that were poorly characterized (36, 37). Recently, there has been renewed interest in clinical development of mithramycin and its analogues because of their ability to specifically inhibit binding of SP1 to GC-rich DNA resulting in repression of numerous genes mediating proliferation, invasion, and metastasis of cancer cells (21, 24, 38–40). In an ongoing phase II trial at the NCI using drug of higher purity than previously available, mithramycin has been surprisingly well-tolerated in patients with cancer when administered at the previously recommended dose and schedule (25–30 mg/kg i.v. over 6 hours × 7 days every 4 weeks). Specifically, no nausea, vomiting, bleeding, or myelosuppression have been observed in 12 adult patients with various malignancies; however, nine of these individuals developed dose-limiting transaminitis, which resolved spontaneously following cessation of drug. Affymetrix Drug Metabolizing Elimination and Transport (DMET) microarray experiments demonstrated that mithramycin-induced hepatotoxicity correlated with SNP in several genes encoding transporter proteins regulating bile flow (Schrum and colleagues, manuscript in preparation). On the basis of these findings as well as review of pharmacokinetic data from this trial, the protocol has been amended to enroll only those patients with favorable genotypes while intensifying the treatment regimen to recapitulate drug exposure conditions achieved in our preclinical studies.

Although we initially used mithramycin to target SP1 expression, our microarray and gene manipulation experiments revealed that activation of p53 is a major mechanism by which mithramycin inhibits growth of MPM cells. These findings are consistent with recent studies demonstrating that the

Figure 5.

Effects of mithramycin on cell-cycle progression, senescence, and apoptosis in MPM cells. *, P < 0.05; **, P < 0.01; †††, P < 0.001. A, propidium iodide staining demonstrating that 24-hour mithramycin treatment induces dose-dependent G0–G1 arrest in MPM cells. B, β-galactosidase staining assays demonstrating that 24-hour mithramycin treatment induces dose-dependent senescence in MES1 and MES7 cells. C and D, Apo-BrdUrd analysis demonstrating minimal apoptosis in MPM cells immediately following 24-hour mithramycin exposure (C) but significant dose-dependent apoptosis 48 hours following drug treatment (D).
antiangiogenic effects of mithramycin in myeloma cells in vivo are mediated not by inhibition of SP1 signaling, but rather by activation of p53 (41), and that knockdown of p53 significantly attenuates mithramycin-mediated cytotoxicity in endometrial carcinoma cells (38).

The majority of pleural mesotheliomas retain wt p53 expression and exhibit functional disruption of p53 activity via allelic loss of p14 ARF (1); as such, our experiments did not specifically delineate the effects of mithramycin in MPM cells bearing p53 mutations. However, our recent studies have demonstrated dramatic cytotoxic effects of mithramycin irrespective of p53 mutation status in lung and esophageal cancer cells (21); these latter observations suggest that p53 activation may not be essential for growth arrest induced by mithramycin in MPM cells. Nevertheless, p53 status may affect extent of cell-cycle arrest and propensity for apoptosis in cancer cells following SP1 depletion by mithramycin (42). Recent elegant studies have demonstrated that highly complex interactions between p53, SP1, and mdm2 regulate cell-cycle arrest and apoptosis in cancer cells following exposure to chemotherapeutic agents which activate p53 (43). SP1 is a critical determinant of apoptosis but not cell-cycle arrest mediated by p53. Furthermore, although dispensable for induction of proapoptotic genes, SP1 is required for proapoptotic transcriptional repression by p53 (43). Mdm2, which is upregulated by p53, facilitates proteosomal degradation of SP1, and negatively regulates p53 (44, 45). Conception and design: M. Rao, S.M. Atay, V. Shukla, Y. Hong, R.T. Ripley, W.D. Figg, D.S. Schrump

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Mithramycin Depletes Specificity Protein 1 and Activates p53 to Mediate Senescence and Apoptosis of Malignant Pleural Mesothelioma Cells


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