Histone Deacetylase Inhibition Overcomes Drug Resistance through a miRNA-Dependent Mechanism

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Abstract

The treatment of specific tumor cell lines with poly- and oligoamine analogs results in a superinduction of polyamine catabolism that is associated with cytotoxicity; however, other tumor cells show resistance to analog treatment. Recent data indicate that some of these analogs also have direct epigenetic effects. We, therefore, sought to determine the effects of combining specific analogs with an epigenetic targeting agent in phenotypically resistant human lung cancer cell lines. We show that the histone deacetylase inhibitor MS-275, when combined with \((N^1, N^{12})\)-bisethylspermine (BENSpm) or \((N^1, N^{12})\)-bis(ethyl)-cis-6,7-dehydrospermine tetrahydrochloride (PG-11047), synergistically induces the polyamine catabolic enzyme spermidine/spermine \(N^1\)-acyltransferase (SSAT), a major determinant of sensitivity to the antitumor analogs. Evidence indicates that the mechanism of this synergy includes reactivation of miR-200a, which targets and destabilizes \(kelch-like ECH-associated protein 1\) (KEAP1) mRNA, resulting in the translocation and binding of nuclear factor (erythroid-derived 2)-like 2 (NRF2) to the polyamine-responsive element of the SSAT promoter. This transcriptional stimulation, combined with positive regulation of SSAT mRNA and protein by the analogs, results in decreased intracellular concentrations of natural polyamines and growth inhibition. The finding that an epigenetic targeting agent is capable of inducing a rate-limiting step in polyamine catabolism to overcome resistance to the antitumor analogs represents a completely novel chemotherapeutic approach. In addition, this is the first demonstration of miRNA-mediated regulation of the polyamine catabolic pathway. Furthermore, the individual agents used in this study have been investigated clinically; therefore, translation of these combinations into the clinical setting holds promise.

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Introduction

The naturally occurring polyamines, spermine, spermidine, and putrescine, are essential for cellular growth and division (1) and, as polycations, they influence cellular processes such as nucleosome formation, DNA replication, and gene transcription (2–4). Polyamines are typically observed at elevated intracellular concentrations in proliferating cells, particularly in tumor cells, which readily accumulate polyamine analogs such as \((N^1, N^{12})\)-bis(ethyl)-cis-6,7-dehydrospermine tetrahydrochloride (PG-11047) and \((N^1, N^{12})\)-bisethylspermine (BENSpm) used in current studies (Supplementary Fig. S1). PG-11047 is a conformationally restricted version of the anti-tumor, polyamine mimetic \((N^1, N^{12})\)-bisethylspermine (BESpm; ref. 5) and has been safely administered in phases I and II of clinical trials (6). In sensitive cell lines, this class of analogs rapidly and significantly induces polyamine catabolism, depletes the natural polyamine pools, increases reactive oxygen species production, and inhibits growth (7–9). In both \textit{in vitro} and in human tumor xenograft mouse models of various human cancers, PG-11047 treatment causes significant growth inhibition, resulting from a dramatic upregulation of polyamine catabolism and subsequent depletion of the natural polyamines. However, other cell lines, particularly those derived from clinically aggressive small-cell lung cancers, show resistance to this induction of polyamine catabolism and consequently display less growth inhibition following treatment (5, 7, 10–15).

The mechanism for this superinduction of polyamine catabolism by the polyamine analogs occurs mainly through activation of a rate-limiting enzyme, spermidine/spermine \(N^1\)-acyltransferase (SSAT). SSAT mRNA levels are typically expressed at very low levels in the cell, but can accumulate in the presence of natural polyamines...
thereby activating transcription of polyamine-modulating factor 1 (PMF1) binds to NRF2, excess polyamines or their analogs, the NRF2 cofactor in multiple clinical trials. We sought to determine if, in oral bioavailability, long half-life, and safe administration the polyamine analogs. Known clinically as entinostat, (NSCLC) and small-cell lung cancer (SCLC) cell lines that of SSAT expression observed in these cells either before or after analog treatment (7, 20). NRF2 function is primarily regulated by kelch-like-ECH-associated protein 1 (KEAP1), which binds to and sequesters NRF2 in the cytoplasm (21). Inactivation of the KEAP1 protein releases NRF2, allowing its translocation to the nucleus where it binds to specific response elements, including the PRE, and drives gene transcription. Mutations in the KEAP1 gene that disrupt the KEAP1–NRF2 interaction are frequent in lung cancers, resulting in the constitutive nuclear localization of NRF2 that is observed in the polyamine analog-sensitive cell lines (22).

Histone-modifying enzymes such as histone deacetylases (HDAC) catalyze posttranslational modifications of specific residues on the N-terminal tails of histone proteins, thereby affecting chromatin structure. The combination of these histone marks at a given promoter, together with DNA methylation, ultimately regulates gene transcription (23, 24), and tumor cells have been shown to alter these modifications as a means to evade growth, repair, and death-control mechanisms (25). These observations, together with the fact that epigenetic changes do not alter the primary nucleotide sequence of the gene, suggest the usefulness of strategies reversing these modifications in the treatment of cancer. Several classes of “epi-drugs” have been developed to target specific modifying enzymes with the goal of restoring the natural growth-control pathways of tumor cells. Recent studies have suggested that HDACs play a role in the regulation of KEAP1, thereby influencing nuclear NRF2 translocation and the transcription of antioxidant response genes, although the precise mechanism was not determined (26).

In the current study, we investigate the use of the class I histone deacetylase inhibitor (HDACi) MS-275 (reviewed in ref. 28) in combination with specific antitumor polyamine analogs in human non–small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) cell lines that typically show low sensitivity to the antitumor effects of the polyamine analogs. Known clinically as entinostat, MS-275 was selected for the current studies because of its oral bioavailability, long half-life, and safe administration in multiple clinical trials. We sought to determine if, in phenotypically resistant human lung cancer cell lines, transcription of the SSAT polyamine catabolic enzyme could be enhanced using an HDACi to increase NRF2-mediated transcriptional activation. In addition, we investigated whether the HDACi alleviated epigenetic histone modifications contributing to the low levels of basal SSAT gene expression. Specifically, based on the studies mentioned earlier, we hypothesized and showed that MS-275 could enhance the transcription of SSAT mRNA via activation of a miR-200a–mediated reduction of KEAP1 protein, leading to increased NRF2 translocation and binding to the PRE of the SSAT gene. This transcriptional stimulation, when combined with the induction of transcription provided by the analog and followed by the extensive posttranscriptional effects of the analog on the SSAT protein, sensitized these cells to the antitumor effects of the polyamine analogs.

Materials and Methods

Cell lines, culture conditions, and chemicals

The human anaplastic non–small cell lung carcinoma cell line, Calu-6 [American Tissue Culture Collection (ATCC), Manassas, VA] was maintained in RPMI1640 medium containing 9% FBS, penicillin, and streptomycin at 37°C and 5% CO2. The small-cell lung carcinoma line NCI-H82 (ATCC) was maintained in RPMI1640 containing 9% bovine calf serum. The cell lines were not authenticated after receipt from the ATCC. The polyamine analog PG-11047 was synthesized by Progen Pharmaceuticals, and BENSpm was synthesized as previously reported (29). A stock solution (10 mmol/L) of the HDAC inhibitor MS-275 (Alexis Biochemicals) was prepared in dimethyl sulfoxide (DMSO), with working dilutions in culture medium. Custom primers for PCR were synthesized by Invitrogen, Sigma, and Integrated DNA Technologies.

Treatment conditions for analyses of gene expression and nuclear protein

Calu-6 cells were seeded at 7 × 10^5 cells per 25-cm^2 flask. At the appropriate time, flasks were aspirated and refreshed with medium containing increasing concentrations of PG-11047, BENSpm, and/or MS-275. H82 cells were seeded at 1.67 × 10^6 cells/5 mL medium and treated with the specified combinations. Cells were incubated at 37°C for 24 or 48 hours, as indicated.

RNA extraction, gene expression, and miRNA expression studies

For gene re-expression studies using reverse transcription-PCR (RT-PCR), total RNA was extracted using TRIzol reagent (Invitrogen) according to the provided protocol. RNA was quantified by spectrophotometry, and cDNA was synthesized using the SuperScript III First Strand Synthesis System (Invitrogen) with oligo-(dT)_12 as the primer. SYBR green-mediated, real-time PCR was conducted using primer pairs and annealing temperatures as previously reported for SSAT (30) and GAPDH (31). The primers used...
to quantify KEAP1 gene expression were 5′-CAACCGAG-
CAACCAAGACCCC-3′ (sense) and 5′-TCAGTGAGGAG-
GGTGATCATAC-3′ (antisense). NRF2 gene expression
was determined using the primer pair 5′-ACACCGGT-
TCCACGCTCTAC-3′ (sense) and 5′-AAATGGCGCA-
ACCTGGAGTAG-3′ (antisense). The optimum annealing
temperature for each primer pair was determined on
cDNA using temperature gradients followed by melt
curve analyses and visualization on 2% agarose gels with
GelStar staining (Lonza) and KODAK Digital Science
Image Analysis Software (Rochester, NY). Amplification
conditions consisted of a five-minute denaturation step
at 95°C, followed by 40 cycles of denaturation at 95°C for
30 seconds, annealing at the optimized temperature for
30 seconds, and extension at 72°C for 30 seconds. SYBR
green SuperMix for iQ was purchased from Quanta BioSciences. Thermocycling was conducted on BioRad
MyiQ and MyiQ2 real-time PCR detection systems, with
data collection facilitated by the iQ5 optical system software (Hercules, CA). For each of the quantitative
PCR (qPCR) experiments, samples were analyzed in triplicate, normalized to the GAPDH reference gene, and
the fold-change in expression was determined relative to
cDNA from untreated cells using the 2ΔCT algorithm.

For miRNA expression analysis, one microgram of
TRIzol-extracted RNA was converted to cDNA using the
miScript PCR System (SABiosciences). qPCR was con-
ducted using the miR-200a Primer Assay (SABiosciences) according to the manufacturer’s recommendations with amplification of U6 snRNA levels as an internal control.

**Analyses of SSAT enzyme activity and intracellular polyamine concentrations**

Calu-6 cells were seeded at a density of 2.1 × 10^6 cells
per 75-cm² flask and allowed to attach for two nights, at
which time the medium was aspirated and replaced with
that containing 0, 5, or 10 μmol/L PG-11047 or BENS, with
or without 1 μmol/L MS-275. Following 24 hours of
incubation, cells were trypsinized, counted, and quick-
freeze for analysis. H82 cells were seeded and treated at
5 × 10^6 cells/15 mL of medium for 24 or 48 hours.
Measurement of SSAT enzyme activity was done as pre-
viously reported (7, 32). Concentrations of intracellular polyamines were determined by pre-column dansylation followed by reverse-phase, high-pressure liquid chromatography (HPLC), as previously described (33). For each assay, total cellular protein was measured using the method of Bradford (34).

**Cell proliferation assays**

For 96-hour experiments, Calu-6 cells were seeded at
2.8 × 10^5 cells per 25-cm² flask and allowed to attach over-
night. Culture medium was replaced with that containing the
appropriate concentration(s) of PG-11047, BENS, and/or
MS-275. NCI-H82 cells were seeded and treated at
7 × 10^5 cells per 5 mL of medium. Following incubation for
96 hours, cells were collected and counted using a BioRad
TC-10 automated cell counter (Calu-6) or hemacytometer
(H82). Viable cells were determined by their ability to
exclude trypan blue. Cells were quick-frozen and acid-
extracted lysates were used for HPLC analysis of intracel-
lar polyamine pools as described in the previous section.

**Analysis of nuclear and total protein expression**

Nuclear protein was harvested from Calu-6 and H82
cells treated with PG-11047, BENS, and/or MS-275 using
NE-PER Nuclear and Cytoplasmatic Extraction Reagents
according to the manufacturer’s protocol (Pierce Biotech-
nology). Total protein was isolated from the same cell
treatments by lysing in buffer containing 25 mmol/L
HEPES, pH 7.9, 150 mmol/L NaCl, 0.5 mmol/L EDTA,
0.1% Triton-X, 10% glycerol, 0.1 mg/mL BSA, 1 mmol/L
DTT, and an EDTA-free protease inhibitor cocktail at 4°C
for 20 minutes. Protein was quantified using the BioRad
DC assay with absorbance measured at 750 nm and
to protein concentration using interolation on a BSA
standard curve.

Nuclear proteins (30 μg per lane) were separated on pre-
cast 10% Bis–Tris NuPAGE gels with 1 × MES running buffer (Invitrogen) and transferred onto Immob-Blot
PVDF membranes (BioRad). Blots were blocked for one
hour at room temperature in Odyssey blocking buffer (LI-
COR), followed by overnight incubation at 4°C with
antibodies specific to NFR2 (H-300, Santa Cruz Biotech-
nology) and β-actin (Sigma). Blots were then incubated
with species-specific, fluorophore-conjugated secondary
antibodies to allow the visualization and quantification of
immunoreactive proteins using the Odyssey infrared
detection system and software (LI-COR).

For total protein Western blots, proteins were separated on
10% Bis–Tris NuPAGE gels in 1 × MOPS running buffer and immunoblotting was conducted as described
earlier. The KEAP1 antibody (1:500 dilution) was pur-
chased from Santa Cruz Biotechnology and β-actin was
used as a loading control and for normalization.

**Quantitative chromatin immunoprecipitation assays**

Calu-6 cells were treated with 0 or 5 μmol/L PG-11047
and/or 1 μmol/L MS-275 for 24 hours. H82 cells were
seeded and treated as indicated for 48 hours. Cells were
cross-linked, resuspended in lysis buffer (6 × 10^6 cells/mL),
and sonication was conducted using a Branson sonifier
with an output of 2.5 and a duty cycle of 40% for
10 seconds with 20-second rests, 10 times per sample.
The amount of chromatin in sheared samples was approx-
imated using UV spectrophotometry and adjusted to a
concentration of 100 μg of chromatin per 400 μL of lysis
buffer for each immunoprecipitation (IP). For histone acety-
ation analysis, an antibody to AcH3K9 (Millipore) was
added to the sheared chromatin and incubated with rota-
tion overnight at 4°C. An antibody to pan histone H3
(Abcam) was used for normalization of the histone mod-
ification, and the negative control rabbit immunoglobulin
G (IgG) was from DAKO. To analyze NRF2 occupancy,
my NFR2 antibody (C-20, Santa Cruz Biotechnology) was
used, and results were compared relative to input DNA.
Protein A and protein G Dynabeads were purchased from Invitrogen.

SYBR green-mediated, quantitative PCR was conducted on the immunoprecipitated DNA to determine the presence and quantity of AcH3K9 occupancy spanning the proximal promoter region of the SSAT gene. Multiple primer sets, the sequences of which are available upon request, were employed that spanned approximately –350 to +310 base pairs relative to the transcriptional start site. A primer pair specific to the PRE, located at –1497 of the transcriptional start site of SSAT, was also used to quantitatively analyze both AcH3K9 and NRF2 chromatin immunoprecipitation (ChIP) products. All primer pairs were optimized using melt-curve and agarose gel analyses of annealing temperature gradients with genomic DNA as the template. Fold enrichment of the modified histone was determined using the \(2^{-\Delta\Delta Ct}\) algorithm, with treated cells relative to untreated cells and normalized to the amount of total H3 protein.

Results and Discussion

**PG-11047 and MS-275 stimulate enhanced catabolism of the natural polyamines in Calu-6 NSCLC cells**

The original rationale for the use of structural polyamine analogs in cancer therapy was based on the self-regulatory nature of polyamine metabolism (35, 36), and PG-11047 has exemplified this ability in tumor cell lines of multiple origins (11, 37, 38). As a polyamine mimetic, PG-11047 uses the polyamine transport system for uptake into dividing cells, stimulating the catabolism and depletion of natural polyamines (38). As the synthetic molecule is incapable of fulfilling the functional requirements necessary to sustain cell growth and division, any remaining natural polyamines are diluted through cell division, and growth is arrested (39, 40). In addition, the superinduction of polyamine catabolic enzymes by polyamine analogs in sensitive cell lines can result in the generation of the reactive oxygen species hydrogen peroxide, resulting in apoptotic cell death (38).

In the current study, we initially examined the effects of PG-11047 exposure on polyamine metabolism in the Calu-6 cells. The Calu-6 NSCLC cell line responds to PG-11047 with a modest induction of polyamine catabolism and growth inhibition; however, compared with the superinduction of SSAT detected in other NSCLC cell lines previously examined (11), Calu-6 cells are relatively resistant. PG-11047 treatment of Calu-6 cells induced small increases in the polyamine catabolic enzyme SSAT at the levels of mRNA (~2-fold) and enzyme activity (~10-fold; Fig. 1A and B). This small induction of catabolism was not sufficient to completely deplete intracellular polyamines; however, it was accompanied by a modest decrease in the concentrations of the higher natural polyamines spermine and spermidine, with accumulation of the analog (Fig. 1B).

Overall, these results are more consistent with those observed in the phenotypically resistant SCLC lines, where the antitumor polyamine analogs, including PG-11047 and BENS, are incapable of superinducing polyamine catabolism and cause only modest growth inhibition (11, 41).

Relative to the superinduction of catabolism often observed in cells of NSCLC origin, the low level of SSAT induction achieved with the polyamine analog alone in Calu-6 cells suggested this would be a good model in which to investigate the polyamine catabolic effects of...
supplementing PG-11047 treatment with MS-275. Single-agent treatment with the HDAC inhibitor MS-275 affected polyamine catabolism in Calu-6 cells, as detected by an induction of SSAT at the level of transcription. After 24 hours, MS-275 induced the expression of SSAT mRNA by approximately 5-fold that of untreated cells, and adding PG-11047 enhanced this expression to approximately 7-fold (Fig. 1A). Because of the substantial post-translational regulation of the SSAT protein by this class of polyamine analogs (42–44), the combination of PG-11047 and MS-275 produced a synergistic increase in SSAT activity (~80-fold) that exceeded the sum of the activities determined with either agent alone (Fig. 1B). This synergy was reflected in the corresponding decreases in intracellular polyamine pools, where MS-275 alone had a minor effect and adding it to the PG-11047 treatment enhanced spermine and spermidine depletion beyond that seen with either agent alone. PG-11047 competes with the natural polyamines for transport into the cell and was accumulated in equal intracellular amounts in all treatment groups. That the SSAT gene can be transcriptionally regulated by a HDAC inhibitor has not been previously reported and provides a completely novel strategy for therapeutic exploitation of polyamine catabolism.

Synergistic induction of SSAT activity in SCLC cells

To confirm this sensitization by MS-275 to the effects of polyamine analogs, we used a cell line of small-cell lung cancer origin, NCI-H82. Well characterized as phenotypically resistant to the induction of SSAT activity, NCI-H82 SCLC cells have extremely low basal levels of SSAT message and activity and have historically displayed little response to the antitumor polyamine analogs (7, 45). In fact, induction of SSAT to a level sufficient to deplete polyamine pools to growth inhibitory levels has never been obtained from the endogenous gene in these cells (45). We examined the effects of combining MS-275 with PG-11047 in this cell line, as well as the combination of MS-275 and BENSpm—an extensively studied polyamine analog known to be one of the most potent inducers of polyamine catabolism—and found that both combinations were capable of synergistically inducing SSAT activity. Treatment with MS-275 alone did not induce transcription of SSAT in H82 cells as it did in the Calu-6 cells, nor did it have an effect on SSAT activity (Fig. 2A). However, an additive induction of SSAT mRNA and a significant synergistic induction of SSAT enzyme activity was detected after 48 hours of cotreatment with PG-11047 (Fig. 2A and B). Increasing the concentration of PG-11047 to 10 µmol/L had no additional effect on either mRNA or...
activity level (data not shown). Most impressively, cotreatment with BENSpm and MS-275 caused an accumulation of SSAT mRNA over 48 hours that resulted in a dramatic, synergistic increase in catalytic activity in a dose-dependent manner (Fig. 2A and C). This is the first demonstration of significant levels of SSAT activity from the endogenous SSAT gene in this cell line. Likewise, similar results were obtained using a second SCLC cell line, NCI-H69 (data not shown).

It should be noted that PG-11047 and BENSpm display similar abilities to induce SSAT transcription, both alone and in combination with MS-275 over the 48-hour period. It is likely that the well-studied, posttranslational regulatory abilities of BENSpm on the SSAT protein, for example, enzyme stabilization, are more effective than those of PG-11047, thereby accounting for the more dramatic increase in enzyme activity (42, 46). These results also suggest that the main contribution of MS-275 in the observed SSAT induction is at the level of enhanced transcription.

Effects of polyamine analog and MS-275 combination treatments on cell proliferation

The ultimate goal of therapeutic induction of polyamine catabolism is tumor-specific growth inhibition. As the H82 cell line displayed the greatest difference in SSAT activity between the HDACi/BENSpm cotreatment and either of the single-agent treatments, we used this cell line and treatment strategy to evaluate the effects of cotreatment in terms of intracellular polyamine pools and growth inhibition.

Cotreating H82 cells with MS-275 and BENSpm over 96 hours revealed a dose-dependent decrease in growth rate, resulting in complete inhibition of growth ($N_f/N_0 = 1$) in the presence of 0.25 μmol/L MS-275 and 5 μmol/L or more BENSpm (Fig. 3, top). It should be noted that the H82 cells displayed greater sensitivity to the cytotoxic effects of MS-275, and, thus, concentrations were scaled back accordingly. Intracellular polyamine pool analysis correlated with growth inhibition and revealed decreasing concentrations of spermine, spermidine, and putrescine when supplementing BENSpm treatment with increasing concentrations of MS-275 (Table 1). H82 cells maintain much higher basal levels of all three natural polyamines than do the other cell lines examined, reflecting the low endogenous polyamine catabolic enzyme levels. Replacing BENSpm with PG-1047 produced similar dose-dependent results, but did not culminate in cytostatic levels over 96 hours, corresponding to the lower level of SSAT induction and maintenance of higher natural polyamine pools (Fig. 3, bottom).

Alterations in chromatin acetylation following HDACi/polyamine analog cotreatment are not responsible for the observed synergy

As polycations, the polyamines are protonated at physiologic pH and electro-statically interact with negatively charged molecules, including nucleic acids and certain proteins (47). The analogs, therefore, have the potential to displace the natural polyamines from their functional sites, affecting chromatin organization and gene expression (5). Considering the observed transcriptional changes in the SSAT gene induced by the current studies and the fact that MS-275, as well as the natural polyamines and their analogs, are capable of altering chromatin structure, we investigated the changes in an acetylated histone H3 modification known to contribute to an active state of transcription.

Due to the low level of basal SSAT gene expression in the cell lines studied, we sought to determine if the increase in histone acetylation induced by MS-275 correlated with changes in local chromatin architecture at the SSAT promoter following treatment with the HDACi, either alone or in combination with the polyamine analogs. In response to MS-275, quantitative ChIP of H82 cells revealed an increase in acetylated H3K9, a
chromatin mark associated with transcriptionally active chromatin, in the region of the SSAT promoter corresponding to 225–124 nucleotides 5' of the transcriptional start site. However, the addition of PG-11047 or BENSpm to the treatment lessened these increases to a level comparable with that detected in untreated cells (Supplementary Fig. S2), suggesting that the mechanism through which the MS-275-polyamine analog combination is inducing SSAT transcription is not dependent on its ability to increase histone acetylation. Although occupancy by AcH3K9 was also increased at multiple sites spanning the SSAT promoter when Calu-6 cells were treated with MS-275, cotreatment with the analog returned this enrichment to a near-basal level (Supplementary Fig. S2).

It is clear that adding either of the polyamine analogs to the HDAC inhibitor treatment affects the histone-modifying abilities of the HDAC inhibitor. One possibility is that the enhanced depletion of natural polyamines following the synergistic induction of SSAT activity by the combination HDACi-analog treatment further facilitates binding of the analog to chromatin, resulting in an altered accessibility of the chromatin to the modifying enzyme. In addition, it is possible that the increased abundance of SSAT protein competes with the histone acetyltransferases for their substrate, acetyl-CoA. Regardless of the mechanism, it does not appear that histone hyperacetylation at the interrogated sites makes a significant contribution to the increase in transcription that is observed when combining MS-275 with the polyamine analogs.

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<tr>
<th>Table 1. Intracellular polyamine concentrations following 96-hour treatments with the indicated combinations</th>
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<tr>
<td><strong>Putrescine (nmol/mg protein)</strong></td>
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<td>BENSpm (μmol/L)</td>
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NOTE: Data is presented as nanomoles of individual polyamine per milligram of total cellular protein and represent dual determinations of two independent experiments, ± range.
Nuclear translocation of NRF2 is enhanced following HDACi and polyamine analog treatment

One of the ways in which the natural polyamines and their analogs exert their transcriptional regulatory effects on polyamine catabolism is through the binding of NRF2 to the PRE of the SSAT gene. Unlike in many of the NSCLC cell lines that are extremely sensitive to the polyamine analogs due to constitutive occupancy of the PRE by NRF2, H82 cells have historically shown little NRF2 presence in the nucleus or at the PRE, even in the presence of polyamine analog treatment (18). We, therefore, analyzed the levels of NRF2 mRNA, total cellular protein, and nuclear protein in these cells. Treatment with MS-275 and/or the polyamine analogs had no effect on NRF2 mRNA expression level or the abundance of total NRF2 protein in the cell (Fig. 4A and B). However, treatment with MS-275 increased the abundance of NRF2 protein that was localized in the nucleus, and this increase was further enhanced by the addition of the polyamine analogs to the treatment, in an MS-275–dose-dependent manner (Fig. 4C).

Changes in NRF2 occupancy at the PRE of SSAT

To confirm that the increased NRF2 in the nucleus of the MS-275-treated cells is indeed playing a role in the enhanced transcription of SSAT, we determined the occupancy of NRF2 at the PRE locus of the SSAT gene by ChIP analysis (Fig. 4D). NRF2 was not detected at the PRE of untreated H82 cells. This is consistent with results from previous studies using electrophoretic mobility shift assay (EMSA) and DNase I protection analyses (18). Treating with either of the polyamine analogs resulted in the detection of low levels of NRF2 bound to the PRE, consistent with the increase in global nuclear NRF2 protein observed. Adding a polyamine analog to the MS-275 treatment had no additional effect. This is not surprising, as the polyamine analog itself is not known to increase NRF2 binding, but increases the binding of the PMF1 cofactor to NRF2, further activating transcription. These results show that treatment with the HDACi MS-275 induces the translocation of NRF2 into the nucleus, where it binds...
to the PRE of the SSAT gene and makes it available for activation by the polyamine analogs in a manner consistent with the constitutive NRF2 binding observed in sensitive cell lines resulting from mutant KEAP1 proteins (18, 22).

**HDACi treatment reduces KEAP1 expression in association with the activation of miR-200a**

Under normal, unstressed conditions, the adapter protein KEAP1 sequesters NRF2 in the cytoplasm. A recent study using an *in vivo* cerebral ischemia mouse model reported that various HDAC inhibitors, including MS-275, could reduce KEAP1 mRNA and protein levels, thereby enhancing NRF2 translocation to the nucleus (26). We, therefore, analyzed the mRNA and protein levels of KEAP1 in H82 cells following 48-hour exposures to MS-275 and/or the polyamine analogs. MS-275 treatment significantly decreased *KEAP1* mRNA levels in a dose-dependent manner, and adding either of the polyamine analogs to the treatment had no effect (Fig. 5A). Likewise, the level of KEAP1 protein in the cell decreased with increasing concentrations of MS-275, regardless of the presence of the polyamine analog (Fig. 5B). Therefore, the decrease in KEAP1 protein resulting from MS-275 treatment releases NRF2 from its cytoplasmic sequestration, allowing it to translocate to the nucleus where it can bind to specific gene promoter region response elements and stimulate transcription.

Another recent study provided evidence that KEAP1 can be negatively regulated by the miR-200a miRNA (27). Members of the miR-200 family of miRNAs play a critical role in maintaining the epithelial phenotype and are often downregulated in cancer (48). In addition, these miRNAs are frequently the subject of aberrant epigenetic silencing (49, 50). Eades and colleagues determined that treatment of breast cancer cells with an HDACi restored the expression of miR-200a, which downregulated *KEAP1* mRNA by binding to its 3′-UTR, ultimately activating NRF2-dependent antioxidant response pathways (27). To determine if this same mechanism could be responsible for the activation of the NRF2/SSAT pathway observed in our experiments, we determined the expression levels of miR-200a following treatment with MS-275 and/or the polyamine analogs. PCR amplification of miRNA cDNA using miR-200a-specific primers revealed a very low level of basal miR-200a that was unchanged with analog exposure. Treatment with MS-275 resulted in an obvious dose-dependent increase in miR-200a expression that was not affected by cotreatment with the polyamine analog (Fig. 5C). These results suggest that the epigenetic activation of miR-200a by the HDACi likely contributes to the mechanism responsible for the observed decrease in KEAP1 mRNA and protein that enables NRF2 nuclear translocation.

Overall, the results presented here show that the catabolic enzyme SSAT can be transcriptionally regulated by...
an HDACi; in solid tumor cell lines that are typically resistant to the growth-inhibitory effects of polyamine analogs, cotreating with MS-275 and specific polyamine analogs sensitizes these cells to the antitumor effects of the analogs, as detected by synergistic increases in SSAT activity that further deplete intracellular polyamine concentrations and enhance growth inhibition. We show that these increases in SSAT transcription are not dependent upon histone acetylation changes at the SSAT gene promoter, but rather are the result of an increase in NRF2 nuclear localization resulting from a decrease in KEAP1 protein mediated by the epigenetic activation of miR-200a (Fig. 6). Absent from the PRE of untreated H82 cells, NRF2 binds to the PRE of the SSAT gene in cells treated with MS-275 and poises it for cofactor activation following induction by the polyamine analogs. The resulting increased SSAT mRNA then undergoes posttranscriptional regulation by the polyamine analog as well as SSAT enzyme stabilization, ultimately resulting in enhanced catabolic activity sufficient to deplete intracellular polyamines and arrest growth.

Both of the polyamine analogs used in the current study have been clinically evaluated and were well tolerated by patients (6, 51, 52). The best responders of these studies achieved stable disease; however, current knowledge suggests that the dosing in those trials might not have been optimal and that combining low doses of the analogs with other agents may enhance their therapeutic efficacy. The results provided here represent the first demonstration of synergy between an HDACi and a polyamine analog and provide the first evidence of the effect of miRNA regulation on the polyamine catabolic pathway. The sensitization of tumor cells to the effects of a polyamine analog through the use of an epigenetic therapy targeting polyamine catabolism is a completely novel chemotherapeutic approach. Furthermore, as most epigenetically related clinical studies have focused on hematologic tumors, the current data are derived from solid tumor models, using agents already evaluated as single agents and found to be well tolerated in the clinic. The results of these studies, therefore, hold great potential for rapid and effective translation into the clinic.

Disclosure of Potential Conflicts of Interest
L.J. Marton and R.A. Casero have ownership interest in a patent. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Murray-Stewart, R.A. Casero
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Murray-Stewart
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