Multiple Myeloma Cell Killing by Depletion of the MET Receptor Tyrosine Kinase

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Abstract

Multiple myeloma (MM) is an invariably fatal plasma cell malignancy, primarily due to the therapeutic resistance which ultimately arises. Much of the resistance results from the expression of various survival factors. Despite this, the ribonucleoside analogue, 8-chloro-adenosine (8-Cl-Ado), is cytotoxic to a number of MM cell lines. Previously, we established that the analogue incorporates into the RNA and inhibits mRNA synthesis. Because 8-Cl-Ado is able to overcome survival signals present in MM cells and inhibits mRNA synthesis, it is likely that the drug induces cytotoxicity by depleting the expression of critical MM survival genes. We investigated this question using gene array analysis, real-time reverse transcription-PCR, and immunoblot analysis on 8-Cl-Ado–treated MM.1S cells and found that the mRNAs and protein levels of the receptor tyrosine kinase MET decrease prior to apoptosis. To determine MET’s role in 8-Cl-Ado cytotoxicity, we generated MM.1S clones stably expressing a MET ribozyme. None of the clones expressed <25% of the basal levels of MET mRNA, suggesting that a threshold level of MET is necessary for their survival. Additionally, the ribozyme knockdown lines were more sensitive to the cytotoxic actions of 8-Cl-Ado as caspase-3 activation and the induction of poly-ADP-ribose polymerase (PARP) cleavage were more pronounced and evident 12 h earlier than in the parental cells. We further established MET’s role in MM cell survival by demonstrating that a retroviral MET RNA interference construct induces PARP cleavage in MM.1S cells. These results show that MET provides a survival mechanism for MM cells. 8-Cl-Ado overcomes MM cell survival by a mechanism that involves the depletion of MET.

Introduction

Multiple myeloma (MM) is a progressive, debilitating, and uniformly fatal B cell disorder typified by the accumulation and dissemination of malignant plasma cells in the bone marrow which subsequently induce bone lesions (1). With established therapies, ~50% of the patients with MM show an initial response, but ultimately develop drug resistance (2). Much of the difficulty in effectively treating this disease is due to the indolent nature and longevity of these cells (3); their low proliferative index deters the effectiveness of DNA-directed therapies, and the presence of multiple antiapoptotic signals allows for their inherent as well as escalating resistance (4, 5).

Overcoming the cell survival mechanisms is imperative for effectively treating MM. This can be achieved by inhibiting the expression of key components of the antiapoptotic signaling pathways. Agents directed at RNA synthesis offer an effective approach for abrogating survival signaling (6, 7). Although these agents globally affect RNA synthesis, their activities are more pronounced against transcripts with faster turnover rates. Typically, short-lived transcripts are encoded by oncogenes as well as regulators of other key processes such as cell proliferation, angiogenesis, and cell survival. Therefore, treatments that impede mRNA synthesis would be expected to selectively hinder their expression and be detrimental to neoplastic cells that are dependent on their presence. This may be especially true for indolent cancer cells that are highly dependent on antiapoptotic signals as is evident in studies on chronic lymphocytic leukemia (CLL). Flavopiridol, a cell cycle and transcription inhibitor, as well as the transcription inhibitors, 8-chloro-adenosine (8-Cl-Ado) and 8-aminoo-Ado, all showed potent cytotoxic activity in noncycling CLL cells in vitro and encouraging results were seen in clinical trials for patients with CLL treated with flavopiridol (8–11). In addition to exploiting the tumor cells’ dependency on short-lived transcripts, RNA-directed agents also take advantage of the selective sensitivity that transformed cells have against the inhibition of RNA polymerase II activity that is not seen in nontransformed cells (7, 12), further underscoring the therapeutic effectiveness of these agents.

The ribonucleoside analogue, 8-Cl-Ado, exhibits a strong potential for use as an effective therapeutic agent in MM as it mediates cytolysis in a variety of MM cell lines, including lines which are resistant to conventional chemotherapeutic agents (13, 14). In contrast to the dual actions of flavopiridol, which inhibits both RNA synthesis and cell cycle progression, the only known mode of action of 8-Cl-Ado in MM cells is RNA synthesis inhibition. Additionally, we have shown that 8-Cl-Ado cytotoxicity is contingent on its intracellular phosphorylation to 8-Cl-AMP by adenosine kinase (14), which is followed by the formation of 8-Cl-ATP. Due to adenosine kinase’s high specific activity and substantial substrate specificity for 8-Cl-Ado, 8-Cl-ATP accumulates to near millimolar levels in MM cells. Parallel to 8-Cl-ATP accumulation, the endogenous ATP pool decreases, resulting in a high ratio of 8-Cl-ATP to ATP. The net effect of this is a decline in RNA synthesis (14). The RNA synthesis inhibition is caused by the analogue incorporating into RNA and prematurely terminating transcription (15). Of the various types of transcripts, mRNA synthesis is inhibited the most due to the preferential incorporation by RNA polymerase II into the body of the mRNA and the likely detrimental effects on polyadenylate polymerase (16).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).
Although the metabolism of 8-Cl-Ado and its actions on RNA synthesis are well established, little is known about the corollary of this action. Because 8-Cl-Ado is able to overcome strong survival signals present in MM cells and inhibits mRNA synthesis, it is likely to induce cytotoxicity by diminishing the expression of short-lived transcripts critical for MM survival. To decipher possible alterations in survival signaling induced by this analogue, in the present study, we used cDNA arrays for the identification of maximally affected transcripts in MM cells and assessed the role of the depletion of a candidate survival gene, MET, in MM cell survival and 8-Cl-Ado cell killing.

Materials and Methods

Materials and vectors. 8-Cl-Ado was obtained from Dr. V. Rao at the Drug Development Branch of the National Cancer Institute. The MET ribozyme and control plasmids were obtained from John Laterra, Kennedy Krieger Research Institute, The Johns Hopkins University School of Medicine, Baltimore, MD (17). Briefly, the MET560 ribozyme construct containing U1snRNA, MET antisense sequence, and a hammerhead ribozyme that targets MET mRNA at residue 560, was subcloned into a modified pNeo plasmid containing a zeocin-selectable marker. The design of the viral construct, MET-shRNA, was based on the small interfering RNA (siRNA) positioned at MET nt 3310 described by Shinomiya et al. (18). The METmRNA at residue 560, was subcloned into a 5'-GATCCGTGCAGTATCCTCTGACAGTTCAAGAGACTGTCA-3' and 5'-AGCTTTCCTAAAAATGGTCAGTATCTTCTGACAGTTCAGAGTCTTGGT-3' annealed and subcloned into the Retrovector pSilencer 5.1-H1 Retro vector (Ambion) according to the manufacturer's instructions.

Cell culture, transfection, and viral infection. The MM.1S and MM.1R cell lines were obtained from the laboratory of Drs. Nancy Krett and Steven Rosen (Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL) and the RPMI 8226 and the U-266 cell lines were obtained from the American Type Culture Collection maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum in the presence of 5% CO2 at 37°C. Cells were routinely tested for Mycoplasma infection using a commercially available kit (Invitrogen). Doubling times were measured in cultures seeded <1 × 10⁶ cells/mL and duplicate aliquots were each counted twice on a Z2 Coulter Counter (Beckman Coulter, Inc.) daily until reaching a plateau of ~1 × 10⁶ cells/mL. Doubling times were calculated using Prism software (GraphPad Software) and expressed as a mean of triplicate analyses. Cell cycle profiles were determined as described (14). A 0.3% stable transfection rate was estimated for MM.1S cells after nucleofection of 1 × 10⁶ cells with 2 μg of linearized enhanced green fluorescent protein plasmid (BD Bioscience) using Nucleofector Kit V. program T-16 (Amaxa Biosystems) and 45 days of selection with G418. MM.1S cells stably expressing the ribozyme or control constructs were similarly generated by nucleofection with 2 mg linearized ribozyme or control plasmid. The cells were then diluted and ~3,000 cells were plated in 100 μL of culture media containing 200 μg/mL of Zeocin (Invitrogen) for selection per well of a 96-well plate.

The MET-shRNA and the empty vector viral constructs were packaged in the BD RetroPack PT67 cells (Clonetech) and viral stocks were obtained from culture supernatants filtered through a 0.22-μm filter. Infections were done by inoculating MM.1S cells with the viral stocks for 12 h in the presence of 4 μg/mL of Sequa-brene (Sigma-Aldrich Corp.) followed by the addition of a ~10-fold volume of fresh media.

cDNA array analysis. 32P-labeled cDNA generated from the mRNA, isolated with the FastTrack 2.0 mRNA Kit (Invitrogen), was used to screen the Atlas Human Cancer cDNA Expression Arrays (BD Bioscience) according to the instructions of the manufacturer and quantitated with a Storm 840 PhosphorImager (GE Healthcare). Changes in gene expression were calculated using the AtlasNavigator software (BD Bioscience).

Quantitative real-time reverse transcription-PCR. Exponentially growing MM.1S cells were harvested after treatment with 10 μmol/L of 8-Cl-Ado for various amounts of time, and the RNA was isolated with the RNeasy Mini kit (Qiagen S.A.) with three to six replicates for each time point. The gene expression levels were measured on an ABI prism 7900 Sequence Detection System (Applied Biosystems) using one-step real-time TaqMan RT-PCR. The MET, TXN2, and 12 S mitochondrial RNA TaqMan primers and probes (Sigma GenoSys) were designed with PrimerExpress software (Applied Biosystems) and their sequences are presented in Supplementary Table S1. The glycolaldehyde-3-phosphate dehydrogenase (GAPDH) primers and probe mix was purchased from Applied Biosystems "Predeveloped Assay Reagents." The relative gene expression levels were quantitated using standard curves generated from known dilutions of untreated MM.1S cell RNA or the MET-expressing colon cancer cell line, KM-20, and normalized with GAPDH levels in studies not involving drug treatment. Because of their relatively long half-lives, mitochondrial 12 S and TXN2 (19, 20) were used to normalize MET expression in experiments involving 8-Cl-Ado treatment. Each RNA sample was assayed in triplicate and the results are presented as a percentage of the MET expression level in untreated MM.1S cells.

Immunoprecipitation and Western blot analysis. Cells were lysed, electrophoresed, and transferred as described (9) or electrophoresed on Criterion Bis-Tris gels using the XT MOPS buffer kit (Bio-Rad). For detection of MET, 1 to 2 μg of lysate was immunoprecipitated with a 1:50 dilution of the rabbit polyclonal MET antiserum, C12 (Santa Cruz Biotechnology), and survivin (R&D Systems Inc.), and mouse monoclonal antibodies to p-ubulin (Sigma-Aldrich Corp.), poly-ADP-ribose polymerase (PARP; C2-10), BCL-XL, XIAP, (BD Bioscience), and to GAPDH, anti-MET mouse monoclonal antibody (Cell Signaling Technology) or anti-MET rabbit polyclonal antibody (Assay Designs). Other primary antibodies used were rabbit polyclonal antibodies to BCL-2, MCL-1 (Santa Cruz Biotechnology), and survivin (R&D Systems Inc.), and mouse monoclonal antibodies to p-ubulin (Sigma-Aldrich Corp.), poly-ADP-ribose polymerase (PARP; C2-10), BCL-XL, XIAP, (BD Bioscience), and to GAPDH, 6C6 (Abcam, Inc.). The blots were visualized by chemiluminescence (Pierce) or with the Odyssey Infrared Imaging System (LI-COR Biosciences). MET levels were quantitated using Kodak 1D Image Analysis software (Eastman Kodak Company), normalized to controls, and presented relative to intensity.

Caspase-3 activity. Exponentially growing MM.1S cells were harvested after treatment with 10 μmol/L of 8-Cl-Ado for various amounts of time, lysed, and assessed using a caspase-3 fluorometric assay (R&D Systems Inc.) according to the instructions of the manufacturer.

Results

8-Cl-Ado inhibits gene expression. Previously, we showed that treatment of MM.1S cells with 5 to 10 μmol/L of 8-Cl-Ado rapidly inhibits mRNA synthesis within 4 h (15) and initiates apoptosis by 24 h (13). Because these cells are known for their multiple anti-apoptotic signals and 8-Cl-Ado is able to overcome these strong survival signals to induce apoptosis, it is likely that the analogue induces cytotoxicity by diminishing the expression of critical MM survival genes. To determine if the mRNA synthesis inhibition affects the expression of genes involved in cell survival, cDNA arrays were screened to assess changes in gene expression after treatment of MM.1S cells with 8-Cl-Ado. Of the genes probed on the array, eight showed a decrease in expression levels ≥3-fold after an 8-h treatment with 10 μmol/L of 8-Cl-Ado (Fig. L4; Supplemental Table S2).

Intriguingly, of these eight, one has been shown to be involved in the pathobiology of MM and encodes the MET receptor tyrosine kinase (22–26). MET was first discovered as an oncogene and subsequently found to be the receptor for hepatocyte growth factor/scatter factor (HGF). As an oncogene, MET has been shown to be involved in the growth, survival, and metastasis of numerous forms of cancers (27, 28). In both primary MM cells and cell lines, MET is typically found in an autocrine loop with HGF (25) and HGF/MET signaling has been shown to promote MM cell growth (22, 29). In accord with the concept that short-lived transcripts are
the definitive target of transcription inhibitors, the half-life of the MET transcript has been reported to be <30 min (30). By using the AtlasNavigator software to compare the changes in the hybridization levels on the cDNA arrays, the expression level of MET was determined to be decreased by ~80% (Fig. 1B).

Interestingly, in contrast to reports on the effects of other transcription inhibitors in MM cells (31–34), the expression of the antiapoptotic gene, MCL-1, did not significantly change after 8-Cl-Ado treatment (Fig. 1A), which was confirmed and the analysis was expanded over a 24-h period by real-time reverse transcription-PCR (RT-PCR)4 and Western blot analysis (Fig. 2A). Additionally, we also examined the effects on other short-lived antiapoptotic molecules (BCL-XL, BCL-2, XIAP, and survivin) by real-time RT-PCR5 and/or Western blot analysis and found no significant effects on their expression (Fig. 2A).

8-Cl-Ado inhibits MET expression. Because of the apparent involvement of HGF/MET signaling in MM, we wanted to further explore the effects and possible role of MET in 8-Cl-Ado–mediated cytotoxicity. To examine the effects of 8-Cl-Ado on MET expression and to quantitate the inhibition kinetics, MM.1S cells were treated with 10 μmol/L of 8-Cl-Ado for 24 h and MET mRNA levels were measured by real-time RT-PCR after various times of treatment (Fig. 2B). The results showed that there was an initial decrease in the MET expression levels within 4 h of treatment and continued to decrease up to 60% by 24 h. 8-Cl-Ado has been found to be cytotoxic to all MM cell lines tested including RPMI 8226, U-266, and MM.1S (13). We also examined these three MM cell lines and showed a similar time-dependent depletion of MET mRNA upon treatment with 10 μmol/L of 8-Cl-Ado (Fig. 2C).

Additionally, a block in MET mRNA synthesis would be expected to instigate a decrease in MET protein levels as its also has a short half-life (35). To determine this, MET was immunoprecipitated from cell extracts prepared from MM.1S cells after various times of 8-Cl-Ado treatment and the levels were measured by immunoblot analysis. The analysis revealed that the MET protein levels diminish with the appropriate delayed kinetics as compared with the mRNA inhibition kinetics (Fig. 2D), indicating a temporal relationship between these events. Overall, these results show that 8-Cl-Ado treatment diminishes the expression levels of MET mRNA and protein in MM cells.

Generation of MET knockdown cell lines. Our hypothesis, based on the sequel regarding the 8-Cl-Ado–induced decline in MET transcript and proteins, is that the loss of MET results in the death of MM cells or sensitizes the cells to 8-Cl-Ado killing. To assess the effects of MET expression in MM cells, we used a chimeric U1snRNA/ribozyme construct designed to specifically target and knock down MET levels (17). MM.1S cells were stably transfected with the MET ribozyme or the pU1 control plasmid and subsequently screened for MET mRNA levels by real-time RT-PCR. The levels of MET transcript in 28 of 29 pU1 control-transfected clones were very comparable to mRNA levels in wild-type cells. In contrast, the MET transcript levels in 15 out of 30 MET ribozyme knockdown clones were at least 50% or less of the levels in the parental cells (Fig. 3A) with the lowest expressing clones containing ~25% of the parental cells’ level of MET.

MET expression promotes cell growth. The MET inhibitor, PHA-665752, inhibits the growth of MM cells (29). To determine the effects of reduced MET expression on cell growth, doubling times were calculated on exponentially growing clones and parental MM.1S cells using Prism software and presented as a fold change over the parental MM.1S cell (Fig. 3B). The results showed that whereas the doubling time for the control clone, pU1-C6, did not significantly vary from the ~40 h MM.1S doubling time, the doubling time for both MET ribozyme clones, MM.D6 and MM.F2, were significantly prolonged to 1.29 (P = 0.003) and 1.66 (P = 0.014) times longer, respectively. These results indicate that MM cells with lower levels of MET grow at a slower rate. To assess the basis of the diminished growth in the cells expressing the MET ribozyme, the cell cycle profile of the ribozyme lines, MM.D6 and MM.F2, were examined and compared with the MM.1S parental cells and the pU1 vector control line (Fig. 3C). The levels of the sub-G1 population of cells, reflecting the level of endogenous cell death, in the parental MM.1S cells and the vector control cells were ~5%. In both MET knockdown cell lines, the levels were more than double this value, ~11%, but this difference was not statistically significant.

8-Cl-Ado further inhibits MET expression in the MET ribozyme clones. To compare the effects of 8-Cl-Ado on MET expression, the parental, control, and MET ribozyme knockdown cells were treated with 10 μmol/L of 8-Cl-Ado for 24 h and the levels of MET mRNA was measured by real-time RT-PCR after various times of treatment (Fig. 4A). In the pU1-C6 control cells, the level of MET expression decreased with a similar kinetics as seen in the MM.1S cells. As expected, both MM.D6 and MM.F2 cells started with a lower level of MET mRNA, which was further reduced with 8-Cl-Ado treatment. In addition, immunoblot analysis of the MET protein levels in MET immunoprecipitates from the cell lysates also showed that the loss of MET mRNA synthesis translated into a decrease in MET protein levels (Fig. 4B) similar to what is seen in the parental MM.1S cells.

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4 C.M. Stellrecht, unpublished data.

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Figure 1. Effect of 8-Cl-Ado on gene expression. A, representative region of the hybridized cDNA arrays, comparing samples from MM.1S cells before and after treatment with 10 μmol/L of 8-Cl-Ado for 8 h. B, normalized array signal intensity of MET.
Depletion of MET accelerates 8-Cl-Ado–induced cytotoxicity. Although we found that 8-Cl-Ado treatment inhibits MET expression prior to the induction of cytotoxicity, it is uncertain if this is merely a casual association or if it is causative. If the decline in MET expression plays a role in the cytotoxic actions of 8-Cl-Ado, then upon treatment, the onset of apoptosis in cells with lower basal levels of MET should be quicker and more pronounced as the minimal threshold level of MET would be attained faster. To address this question, parental, control, and MET ribozyme knockdown cells were treated with 10 μmol/L of 8-Cl-Ado and the degree of PARP cleavage, a marker of apoptosis, was assessed at various times by immunoblot analysis (Fig. 5A). In both the parental MM.1S cells and in the pU1-C6 control cells, the induction of PARP cleavage was not detected until a minimum of 24 h of 8-Cl-Ado treatment. In contrast, 8-Cl-Ado induced PARP cleavage in both of the MET ribozyme lines, MM.D6 and MM.F2, within half this time, ~12 h earlier than in MM.1S cells.

The enhanced induction of apoptosis by 8-Cl-Ado in the MET ribozyme knockdown clones was further confirmed with a fluorometric caspase 3 assay. This apical caspase was found to be activated earlier and to a greater extent in 8-Cl-Ado–treated MET ribozyme line, MM.D6, as compared with MM.1S cells or the pU1-C6 control cells (Fig. 5B). These results show that MM cells with lower basal levels of MET are more sensitive to the cytotoxic activities of 8-Cl-Ado and further implies that MET signaling is necessary for the survival of MM cells. These results also confirm our hypothesis that 8-Cl-Ado induces apoptosis by diminishing the expression of survival genes.

Depletion of MET is cytotoxic for MM cells. The finding that none of the 30 stable MET ribozyme clones expressed MET at levels <25% of the parental cells suggests that MM cells are dependent on MET and lose their survival advantage when MET levels fall below a minimal threshold level. To further address this question, we infected MM.1S cells with MET-shRNA or the vector control retrovirus. Interestingly, cells infected with the control virus showed ~1.5-fold increase in MET transcript levels as compared with untreated MM.1S cells (Fig. 6A). In contrast, as compared with the untreated MM.1S cells, cells infected with the MET siRNA-producing retrovirus depleted the MET transcript levels by ~35%, 80%, and 95% by 24, 48, and 72 h, respectively. Immunoblot analysis of PARP in the infected cells showed that PARP cleavage was readily detected by 48 h in the cells infected with MET-shRNA but not in the untreated or the vector control–infected MM.1S cells (Fig. 6B); thus, conclusively showing that MM.1S cells are dependent on MET for survival.

Discussion

We show that 8-Cl-Ado induces the depletion of MET expression and that this attenuation promotes the analogue’s cytotoxic activities. These results show that 8-Cl-Ado induces apoptosis in MM cells by diminishing the expression of genes consequential for their survival.

In recent years, it has become apparent that transcription is a potential target for cancer therapeutics as RNA-directed agents exploit the tumor cells’ dependency or addiction (36, 37) on short-lived oncogenes, growth regulators, and survival factors (6, 7). These short-lived transcripts contain specific sequence elements that signal their rapid degradation. The most common determinant of RNA stability in mammalian cells are adenylic/uridylic-rich
elements commonly termed AREs (38). There are multiple potential ARE-like motifs present in the MET mRNA sequence. The possibility that these regions might be functional AREs is consistent with the short half-life (<30 min) of the MET mRNA (30).

Previously, we found that mRNA synthesis was inhibited by ~50% in MM.1S cells treated with 10 μmol/L of 8-Cl-Ado (15).

Because the analogue does not completely stop RNA synthesis, the levels of MET mRNA present during treatment would reflect its decay rate plus the limited amount of its production. Additionally, because 8-Cl-Ado prematurely terminates transcription, total RNA would contain full-length as well as partial transcripts. Using real-time RT-PCR, we determined that the MET transcripts decrease by 60% after 24 h of 8-Cl-Ado treatment (Fig. 2B). In contrast, using cDNA arrays, the levels were measured to diminish by 80% after 8 h (Fig. 1B). One reason for this difference is that for the cDNA arrays, MET mRNA levels were measured in poly(A+)-selected RNA; therefore, only mRNA that were fully transcribed through the adenyate-rich 3'-untranslated region as well as polyadenylated would be detected. Total RNA was used for the real-time RT-PCR and the MET primers, and the probe spanned the boundary of

![Figure 3. Characteristics of ribozyme clones. A, real-time RT-PCR analysis of MET mRNA levels in MM.1S cells stably transfected with the MET hammerhead ribozyme or the ribozyme vector control graphed as a percentage of wild-type MM.1S cells. B, cell doubling time in MM.1S cells stably transfected with the MET hammerhead ribozyme or the ribozyme vector control graphed as a fold change of wild-type MM.1S cells doubling time. C, cell cycle profile of the MET ribozyme lines MM.D6 and MM.F2, the ribozyme vector control line pU1, and the MM.1S parental cells.](image3)

![Figure 4. Effect of 8-Cl-Ado on MET knockdown MM cell lines. A, real-time RT-PCR analysis of MET mRNA levels in MM.1S cells, pU1-C6 vector control cells, MM.D6 MET ribozyme cells, and MM.F2 MET ribozyme cells treated with 10 μmol/L of 8-Cl-Ado for the times indicated and graphed as a percentage of the untreated cells. B, immunoblot analysis of immunoprecipitated MET from lines and treatment as in (A). The normalized signal intensity of MET (bottom).](image4)
exons 20 and 21; thus, any prematurely terminated transcripts that included this sequence as well as full-length transcripts would be detected. Another reason for the discrepancy is the normalization methods used in the two procedures. For the cDNA arrays, the signal value of the entire array was used by the Atlas software to globally normalize gene expression levels. With real-time RT-PCR, the MET gene expression levels were normalized to TXN2 and to MT-RNR1 transcript levels, both of which also would decrease but with a much slower decay rate. Although, real-time RT-PCR is generally a highly accurate way to measure gene expression, in this particular study, it is likely that it underestimated the extent to which 8-Cl-Ado inhibits gene expression.

Our finding that MET depletion plays a role in the cytotoxic activities of 8-Cl-Ado in MM cells complements what is already known about the cellular function of MET and its role in MM. Typically, in both primary MM cells and cell lines, MET is found in an autocrine loop with HGF (39, 40), and MM cells are able to proteolytically convert HGF into its active form (41). HGF/MET signaling promotes the growth of MM cells (22, 29, 42) and boosts MM cell adherence to the bone marrow matrix, which would enhance the survival and drug resistance of MM cells in vivo (24). This correlates with the finding of higher HGF serum and bone marrow levels in advanced stages of MM (23, 26, 43, 44). Moreover, the median survival time in patients with high serum HGF has been shown to be shorter than in patients with low serum HGF levels (45–47), further establishing the significance of HGF/MET signaling in MM.

MET signaling has been shown to be sufficient for rescuing MM cells from serum starvation (22), but it is uncertain if MET is necessary for survival. Of the 30 MET ribozyme knockdown clones that we analyzed, none expressed MET at <25% of the levels found in the parental MM.1S cells. These results might be an indication that there is a minimum level of MET that is required for MM cell survival and any clones that expressed MET at levels lower than this minimum did not survive selection. Similar to our results, Herynk et al. found that the selection of colon cancer cells stably transfected with the same ribozyme only generated clones with a minimal reduction of MET expression (21). Using the pU1-MET ribozyme in an adenovirus construct and measuring cell survival with a colony-forming assay, they were able to show that a greater reduction of MET in colon cancer cells is lethal. In contrast, in cells that are not dependent on MET for survival, this same ribozyme knocks down MET expression ~ 100-fold (17, 27). In these studies, which used either glial, prostate, and breast cancer lines, the cells also lost their tumorigenic phenotype, but unlike the colon cancer cells, they were able to endure selection with very low levels of MET. Additionally, we showed that MET depletion enhances 8-Cl-Ado cytotoxicity, further suggesting that MET signaling is necessary for the survival of MM cells; although it is possible that the depletion of MET in MM cells does not invoke cell death, but increases the sensitivity of the cells to additional activities of the analogue. To conclusively address this question, we generated a MET siRNA–producing retroviral construct, which would enable a greater reduction of MET levels in MM cells without encountering the difficulties imposed by selection. These results showed that this level of MET depletion is cytotoxic to MM.1S even in the absence of 8-Cl-Ado.

Much of the work on MM cell survival has focused on the IL-6–dependent activation of signal transducers and activators of transcription 3, which in turn, induces the expression of MCL-1 (48). It is interesting to note that although IL-6 signaling is able to rescue MM cells from common MM therapeutic agents such as dexamethasone, IL-6 does not rescue MM cells from 8-Cl-Ado (13). Our finding of the lack of involvement of MCL-1 may explain this.
There is increasing evidence that HGF and MET play an instrumental role in the dissemination of MM cells in the bone marrow, which would lead to escalating resistance and the progression of this debilitating bone disease. In general, similar to other cytokine receptors, activation of MET induces proliferation, migration, differentiation, and protection from apoptosis; but uniquely, it also signals a morphogenic invasive-growth program (28). This invasive-growth program allows cells to migrate through the surrounding environment such as in the stimulated release of reticulocytes from erythroid-containing stem cell colonies. HGF/MET signaling stimulates MM cell secretion of matrix metalloproteinase-9 and promotes endothelial cell–stimulated MM cell invasion (49). In addition, MET signaling boosts MM cell adherence to the bone marrow matrix (24) and induces angiogenesis by stimulating the production of vascular endothelial growth factor and inhibiting thrombospondin 1 expression (50). Thus, MET-directed agents are anticipated to alleviate the debilitating bone disease brought on by MM cells (29) as well as being detrimental to their survival.

In conclusion, we used cDNA arrays to analyze the effects of 8-Cl-Ado on gene expression in MM cells. In doing so, we identified eight genes that were down-regulated by 8-Cl-Ado, including the MET oncogene. By stably transfecting MM.1S cells, we showed that this attenuation accelerates the analogue’s cytotoxic activities, demonstrating that 8-Cl-Ado induces apoptosis in MM cells by hindering the expression of genes consequential for their survival. These results provide further evidence for the efficacy of 8-Cl-Ado as a chemotherapeutic agent in MM and suggests that the analogue may also be beneficial for the alleviation of the bone disease. In addition, our results show that 8-Cl-Ado is an effective agent for targeting MET expression. Finally, we showed that MET expression is required for MM cell survival even in the absence of 8-Cl-Ado.

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