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ORIGINAL ARTICLE

Targeting *MET* transcription as a therapeutic strategy in multiple myeloma

Cornel J. Phillip · Christine M. Stellrecht · Ramadevi Nimmanapalli · Varsha Gandhi

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Abstract Multiple myeloma (MM) is an incurable indolent malignancy with an average lifespan of 3 years, underscoring the need for new therapies. Studies have shown that the receptor MET and its ligand hepatocyte growth factor play an important role in proliferation, migration, adhesion, and survival of MM cells. Hence, an effective way to decrease *MET* receptor may act as a viable therapeutic option. Since *MET* mRNA and protein have short half-lives, we hypothesized that transcription inhibitor will reduce *MET* transcript and protein levels and this will lead to cell death. Pharmacological (flavopiridol) and molecular (shRNA) transcription inhibitor were used to impede formation of *MET* transcripts. The diminution of global RNA synthesis with flavopiridol was related to phosphorylation status of Ser residues ($r^2 = 0.90$ and 0.92 for Ser2 and Ser5) on the C-terminal-domain of RNA polymerase II. This was accompanied with a time-dependent decrease in *MET* transcript, which reached to less than 30% (1 μM) and 10% (3 μM) by 24 h. This decline in transcript level was directly associated with a reduction in MET protein level ($r^2 = 0.82$) and resulted in cell death. Assessment of MET in MM survival was done by using shRNA targeted towards *MET*. When cells were infected with shRNA viral construct, there was increased cell death with a decline in MET transcript and protein. Taken together, our study demonstrates that MET plays a critical role in the survival and removal or lowering of MET by flavopiridol or shRNA results in the demise of MM cells.

Keywords Apoptosis · Cell survival · Flavopiridol · MET · Multiple myeloma · Transcription

Abbreviations

CTD	C-terminal domain
CLL	Chronic lymphocytic leukemia
CDK	Cyclin-dependent kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HGF	Hepatocyte growth factor
MM	Multiple myeloma
PARP	Poly (ADP-ribose) polymerase
RNA Pol II	RNA polymerase II

Introduction

Multiple myeloma (MM) is an incurable indolent B-cell malignancy that occurs in the bone marrow and in the advanced stage is associated with formation of osteolytic

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C. J. Phillip · C. M. Stellrecht · V. Gandhi (✉)
Department of Experimental Therapeutics, Unit 71,
Univ. Texas M. D. Anderson Cancer Center,
1515 Holcombe Blvd., Houston, TX, 77030, USA
e-mail: vgandhi@mdanderson.org

C. J. Phillip
Graduate School of Biomedical Sciences,
University of Texas M. D. Anderson Cancer Center,
Houston, TX 77030, USA

V. Gandhi
Department of Leukemia,
University of Texas M. D. Anderson Cancer Center,
Houston, TX 77030, USA

R. Nimmanapalli
Department of Pathobiology, CVMNAH,
Tuskegee University, Tuskegee, AL 36088, USA

lesions [20]. Even with current and newly approved therapies [32], the median survival for an individual is approximately 3–5 years [12, 32]. Therefore, it is imperative that therapies that are targeted at the survival factors in MM [3, 34] be explored.

Previous studies have shown that MET, a receptor tyrosine kinase and its ligand hepatocyte growth factor (HGF) play an important role in migration, proliferation, adhesion [14, 16, 21, 48], and may play a role in survival of MM cells [14, 45, 48]. MET induced signaling initiates when its ligand HGF binds to the receptor and results in the activation of several downstream targets such as the MAPK and PI3K pathways [13, 14, 27, 46]. Investigations regarding biology of the disease have demonstrated that there is an increased level of circulating HGF in plasma of patients with MM [37, 38]. There seems to be a direct relationship between levels of plasma HGF and aggressiveness of the disease. Furthermore, prognosis appears to be worst in patients with higher levels of HGF [22, 39]. Finally, the HGF levels also impact event-free survival of patients [1] with cancer and serve as a significant prognostic factor [39, 47]. Taken together these lines of evidence suggest that MET/HGF are important for survival and maintenance of MM tumor tissue. Therefore, determining an effective way to decrease MET receptor, inhibition of its tyrosine kinase activity, or downstream effector molecules may play a crucial role in the demise of MM cells.

Several different strategies may be used to target HGF/MET in MM therapeutically. One strategy may be to target HGF directly. However, because HGF is present at a very high level in patients with MM, competitive therapeutic antibodies may not be feasible or may be inefficient. Small molecule inhibitors that bind to the ATP binding site on the kinase domain could inhibit MET receptor tyrosine kinase may be considered another viable option [21, 26]. In addition to therapeutic antibodies and small molecule inhibitors, another approach would be to use transcriptional inhibitors that would lower MET receptor expression levels.

Flavopiridol is a general cyclin-dependent kinase inhibitor affecting CDK 4, 6, 7, and 9 [2, 7, 36]. Recent investigations have demonstrated that it functions by impeding transcription initiation and elongation by inhibiting the phosphorylation of the heptamer repeats at serine residues 2 and 5 in the C-terminal domain (CTD) of RNA polymerase II (Pol II). P-TEFb (positive transcription elongation factor b)/ CDK 9/cyclin T have been known to phosphorylate the Ser2 residue of RNA Pol II for transcriptional elongation [31, 43]. CDK 7/cyclin H is known to phosphorylate the Ser5 residue, which plays a role in transcription initiation [43]. Flavopiridol competes with ATP for binding at the active site of several CDKs including CDK 7 and CDK 9 [9]. Overall these actions result in global transcription inhibition.

Inhibition of global transcription is an effective strategy in decreasing mRNA with short half-lives because of the rapid turnover [25]. *MET* mRNA has a half-life of 30 min and the protein has a half-life of approximately 5 h [18, 29]. Because of the half-life and rapid turnover of MET at transcript and protein levels, we hypothesized that flavopiridol, a pharmacological transcription inhibitor and *MET* directed siRNA, would serve as strong candidates to target MM cells. To test this hypothesis, we investigated the actions of flavopiridol using micromolar concentration in MM cells with regards to transcript and protein levels of MET and the fate of these cells after flavopiridol treatment. Flavopiridol concentrations used in this current study are attainable clinically during therapy [5]. As an alternate and direct confirmation for the importance of MET for MM cell survival; MET levels were knocked down using shRNA. With these pharmacological and molecular tools, our study shows that MET plays a critical role for the survival of MM and removal or lowering of MET transcript and protein can result in the demise of MM cells.

Materials and methods

Cell lines

The glucocorticoid sensitive MM1.S [24] cell line was obtained from the laboratory of doctors Nancy Krett and Steven Rosen (Northwestern University, Chicago, IL, USA). In addition, U266 cell lines were obtained from the laboratory of Dr. William Dalton (Moffitt Cancer Center, Tampa, FL, USA). These cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum at 37°C in the presence of 5% CO_2. Cells were consistently tested for *Mycoplasma* infection using a manufacturer's kit (Invitrogen, Carlsbad, CA, USA). The doubling time of the cell line is approximately 36–48 h.

Materials

Flavopiridol was acquired from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, USA). It was dissolved in dimethylsulfoxide (DMSO) and stored in aliquots at a concentration of 10 mM at −80°C. [^{3}H]uridine (32 Ci/mmol) was obtained from Moravek Biochemical Inc (Brea, CA, USA).

Flavopiridol treatment

For all experiments, 1 and 3 μM of flavopiridol was used for different times and up to 24 h. These concentrations are

achievable during therapy when flavopiridol was administered using a pharmacologically derived schedule [5]. Additionally, these concentrations of flavopiridol show a biological response in patients with B-cell chronic lymphocytic leukemia during in vitro incubations [9] or during therapy [5].

RNA Synthesis

Global RNA synthesis was measured using [^{3}H]uridine incorporation. MM1.S was first treated with flavopiridol for 2, 4, 6, 8, 10, 12, and 24 h. Cells were labeled for 30 min with [^{3}H]uridine (1 μCi/ml). After uridine incubation, each sample was diluted with 10 ml of cold PBS. Using the Millipore manifold (Fisher Scientific, Pittsburgh, PA, USA) along with Whatman 2.7 cm filters (Whatman Inc, Clifton, NJ, USA), the samples were vacuumed through the filter then washed twice with 5 ml of cold 0.4 N PCA. The filters were rinsed using 70 and 100% ethanol, respectively, dried overnight, and transferred to scintillation vials to count radioactivity.

RNA isolation and quantitation of *MET* transcript levels

MM1.S was treated as indicated. Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA) and was used to quantitate specific mRNA levels. On a 7900HT Sequence Detection System, the relative levels of gene expression were measured using the Taqman One Step RT-PCR master mix reagents (Applied Biosystems, Foster City, CA, USA). The RNA isolation and detection was done according to the manufacturer's instructions. The level of *MET* gene expression was measured in 500 ng of RNA and normalized to thioredoxin 2 (*TXN2*) transcripts levels. *TXN2* was used because its mRNA has a long half-life of 35 h [10, 23]. Primers and probes for *MET* and *TXN2* [45] were designed using PrimerExpress software (Applied Biosystems, Foster City, CA, USA). Experiments were done in triplicate and the results are presented as the percent of control of untreated MM1.S cells.

RNA Pol II and its CTD phosphorylation

Untreated and flavopiridol-treated cells were collected and centrifuged at 1,500 rpm for 5 min, and the pellets were washed twice with PBS. They were lysed using RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail pill) obtained from Roche Diagnostics (Indianapolis, IN, USA). Proteins were separated by electrophoresis with Criterion Bis-Tris gels using the XT MOPS buffer kit (Biorad, Hercules, CA, USA) at approximately 1 h at 200 V and transferred to nitrocellulose membranes at 25 V overnight at 4°C. Using the manufacturer's protocol, membranes were immunoblotted and imaged using the Odyssey imagining system (LI-COR Biosciences, Lincoln, NE, USA). Primary antibodies phosphorylated CTD for Ser2 and Ser5 were H5 and H14, respectively and that for total RNA polymerase II was 8WG16 (Covance Research Products, Inc Berkeley, CA, USA). GAPDH mouse monoclonal antibody (Abcam, Inc Cambridge, MA, USA) was used as loading control.

Cell surface expression of MET

The protein expression of cell surface MET was measured using flow cytometry as indicated. Cells were washed with PBS and resuspended in 100 μl of 5% BSA and were incubated with anti-human c-Met, Rabbit IgG affinity purified primary antibody from Assays Designs (Ann Arbor, Michigan) for 1 h at room temperature. Following incubation of primary antibody, cells were washed with PBS and incubated with Fluorescein (FITC)-conjugated affinipure goat anti-rabbit IgG secondary antibody purchased from Jackson ImmunoResearch (West Grove, PA, USA) for 1 h. After washing, cells were stained with propidium iodide to identify dead cells and the protein expression was measured using the BD FACSCalibur system (BD Biosciences Pharmingen, San Diego, CA, USA). MET protein levels were quantitated from the lower right quadrant representing c-Met FITC positive and PI negative cells. Cells that were c-Met FITC positive and PI positive were excluded from the quantification because the primary antibody may bind nonspecifically to dead cells.

Cell death assay

After indicated treatments, MM1.S cells were washed with PBS, resuspended in 1 X annexin V binding buffer, and incubated with annexin V-FITC (Pharmingen Biosciences (San Diego, CA, USA) for 15 min at room temperature. Then 400 μl of annexin binding buffer was added along with propidium iodide (PI). The total cell death was measured using the BD FACSCalibur system (BD Biosciences Pharmingen, San Diego, CA, USA). Immunoblots of untreated and flavopiridol-treated cells were prepared and analyzed for PARP protein cleavage. PARP mouse monoclonal antibody, C2-10 was obtained from BD Biosciences Pharmingen (San Diego, CA, USA). The anti-mouse IgG and IgM horseradish peroxidase conjugated antibodies were purchased from Amersham Biosciences (Piscataway, NJ, USA).

MET knockdown

MET knockdown was done using infection of MM1.S cells with a retrovirus that involves a stable shRNA targeting *MET*. The 19-mer target sequence, GTGCAGTATCCTCT GACAG, has been described before [42]. Ambion insert design tool was used to design shRNA sequence to be inserted into the pSilencer 5.1-H1 Retro vector (Ambion, Austin, TX, USA). The oligonucleotide containing shRNA sequence obtained were 5′-GATCCGTGCAGTATCCT CTGACAGTTCAAGACTGTCAGAGGATACTGCAC TTTTTTGGAAA-3′ (forward primer) and 5′-AGCTTTT CCAAAAAAGTGCAGTATCCTCT GACAGTCTCTTG AACTGTCAGAGGATACTGCACG-3′ (reverse primer) (Invitrogen, Carlsbad, CA, USA). The oligonucleotide was ligated into the RetroVector pSilencer 5.1-H1 Retro vector (Ambion, Austin, TX) according to manufacturer's instructions. When the ligation process was complete, for amplification, subcloning efficiency DH5α competent cells (Invitrogen, Carlsbad, CA) were transformed with ligation product and vector only. To ensure the presence of shRNA and its orientation in cloned pSilencer 5.1-H1 Retro vector, plasmid DNA was isolated using GenElute Endotoxin-free maxiprep plasmid purification kit (Sigma-Aldrich, St Louis, MO, USA) and was sequenced at the DNA core analysis facility at M.D. Anderson Cancer Center, Houston, TX. BD RetroPack PT67 (Clonetech, Mountain View, CA, USA) cells were transfected with pSilencer 5.1-H1 Retro vector as control or with pSilencer 5.1 Retro vector with an shRNA (Met-shRNA) using siPORT XP-1 (Ambion, Austin, TX, USA) according to the manufacturer's instruction. Puromycin was used as selection antibiotic for isolation of stable transfected PT67 cells. The BD RetroPack PT67 cell line contains *pol, gag*, and *env* genes from the Moloney murine leukemia virus. Upon completion of transfection and puromycin selection, virus stock was prepared according to pSilencer 5.1 Retro Vector manufacturer's instructions. MM1.S cells were inoculated with this virus stock for 12 h with 4 μg/mL sequa-brene (Sigma-Aldrich, St Louis, MO, USA) and grown for an additional 3 days in fresh media. The infected cells were used for *MET* mRNA, MET protein level and cell death using annexin V-FITC assays.

Statistical analysis

The correlation studies between serine phosphorylation on the CTD of RNA pol II and inhibition of RNA synthesis or decrease in *MET* mRNA and relationship analysis between MET protein and mRNA levels were carried out by the linear regression analyses using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). A P value less than 0.05 indicated a statistically significant relationship.

Results

Inhibition of global RNA synthesis by flavopiridol

To determine if flavopiridol inhibits global RNA synthesis and to identify the concentration and time effect, incorporation of uridine in total RNA was measured. MM.1S cells were incubated with 1 and 3 μM flavopiridol that is clinically achievable [5] for the indicated times (Fig. 1), then 30 min prior to harvesting the cells, [^{3}H]uridine was added to the cell culture. Compared to untreated (control) cells, there was a dose and time dependent decrease in global RNA synthesis. The inhibition reached to 50% at 8 h for 1 μM flavopiridol and at approximately 3 h with 3 μM flavopiridol. At 24 h for both 1 and 3 μM, total RNA synthesis decreased greater than 80% of control.

Effect of flavopiridol on RNA pol II

The decrease in RNA synthesis should be due to loss of function of RNA pol II. The CTD domain of the RNA pol II contains tandem repeats of a heptamer sequence YSPTSPS. Previous studies using cell-free assays with flavopiridol has demonstrated inhibition of CDK 9/cyclin T (P-TEFb) and CDK 7/cyclin H activity. The P-TEFb phosphorylates the Ser2 in the heptamer repeat sequence for transcriptional elongation while CDK 7/cyclin H phosphorylates Ser5 of the heptamer repeat for transcriptional initiation. To determine this effect in MM1.S whole cells, we examined the effects of flavopiridol on phosphorylation of Ser2 and Ser5 residues on the CTD of RNA pol II. Cells were incubated with 1 and 3 μM of flavopiridol for the indicated times, and the phosphorylation status was measured

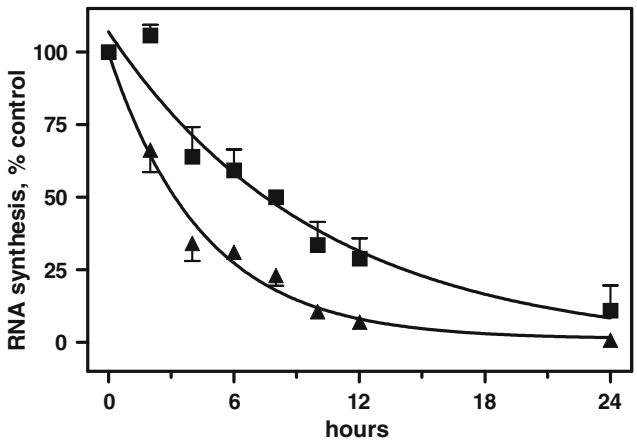

Fig 1 Inhibition of global RNA synthesis by flavopiridol. Cells were treated with 1 (*filled square*) and 3 μM (*filled triangle*) flavopiridol for indicated times and incorporation of [^{3}H] uridine was measured. The data is presented in a time and dose dependent manner as a percentage of time-matched controls (mean ± SE, triplicate samples)

using specific antibodies (Fig. 2a). Both Ser2 and Ser5 phosphorylation was decreased in a time and dose dependent manner; however the effect was more potent on Ser2. Ser2 and Ser5 showed reduction as early as 4 h with flavopiridol (1 and 3 μM) albeit at 3 μM the reduction was more pronounced than at 1 μM. Total protein level of RNA pol II was not affected except for some decrease with 3 μM flavopiridol at 20 and 24 h. This and additional two immunoblots were quantitated, normalized with total pol II, and plotted (Fig. 2b, c). After a 12 h incubation with 3 μM flavopiridol, the Ser2 phosphorylation was less than 25% of controls, while Ser5 phosphorylation was ~35% of controls. At this concentration, 50% of inhibition was achieved at less than 3 h for Ser2 and 6 h for Ser5 phosphorylation. To determine if the Ser2 and 5 dephosphorylation was associated with the global inhibition of RNA synthesis, these two parameters were compared. Inhibition of RNA synthesis for both 1 and 3 μM flavopiridol correlated significantly with the decrease in phosphorylation of Ser2 ($r^2 = 0.90$, $P = 0.0001$, Fig. 3a) and Ser5 ($r^2 = 0.92$, $P = 0.0001$, Fig. 3b).

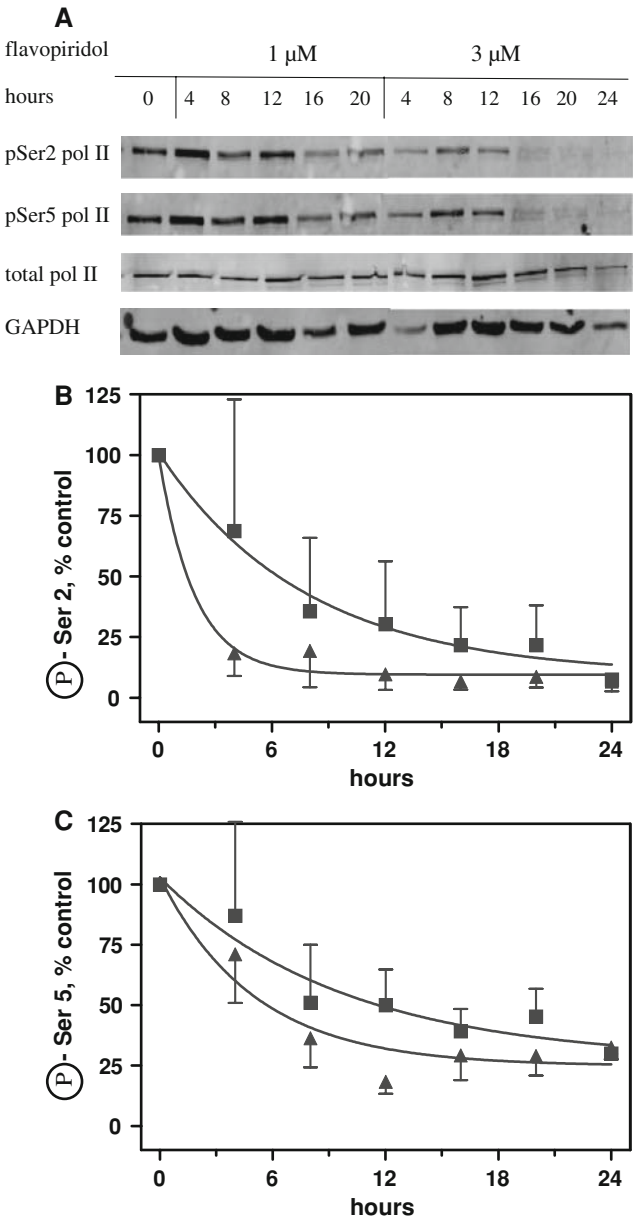

Fig. 2 Effect of flavopiridol on RNA Pol II. MM1.S cells were treated with 1 and 3 μM flavopiridol for indicated times. The phosphorylation of RNA polymerase II was analyzed by immunoblots and antibodies directed at the phosphorylation sites of Ser2 and Ser5 of the CTD domain as well as for total RNA pol II. GAPDH was used to indicate loading control (Fig. 2a). The immunoblots were quantitated using Odyssey Infrared Imaging system application software version 1.2. The phosphorylation levels of Ser2 (**b**) and Ser5 (**c**) in cells treated 1 (*filled square*) and 3 μM (*filled triangle*) flavopiridol were normalized with total RNA pol II. Data are presented as mean ± SE of triplicate immunoblots

Reduction of *MET* transcript levels by flavopiridol

Previous studies have demonstrated that HGF/MET pathway acts as a survival factor in MM [14]. Additionally, because of the rapid turnover rate of *MET* transcripts, inhibition of RNA synthesis by nucleoside analogs lowers *MET* mRNA levels [44, 45]. Thus we investigated whether actions of flavopiridol on RNA synthesis may also reduce *MET* transcripts. Using real-time RT-PCR, the mRNA levels for endogenous *MET* were measured in treated and untreated cells and normalized using *TXN2* transcripts (Fig. 4a). Flavopiridol treatment results in a decrease in the level of *MET* transcripts in MM cells from times of 4–24 h at concentrations of 1 and 3 μM. The number of *MET* transcripts was reduced to 50% at approximately 12 and 8 h with 1 and 3 μM flavopiridol, respectively. The decline in *MET* transcript number was directly related to RNA pol II function measured as dephosphorylation of Ser2 ($r^2 = 0.70$, $P = 0.0002$), and Ser5 ($r^2 = 0.80$, $P = 0.0001$) residues at the CTD of RNA pol II and was also directly related to a decrease in global RNA synthesis ($r^2 = 0.52$, $P = 0.018$, data not shown).

Reduction of MET protein levels by flavopiridol

To determine if decrease in *MET* transcripts resulted in a decline in protein, MET protein levels on the surface of the cell were analyzed using flow cytometry. Cells untreated and treated with 1 μM of flavopiridol were analyzed (Fig. 4b). Isotype control, which was Fluorescein (FITC)—conjugated AffiniPure Goat Anti-Rabbit IgG secondary antibody, was used to determine that there was no nonspecific binding to cells without MET primary antibody (Fig. 4b-I). The majority of cells in untreated population was MET positive (Fig. 4b-II). However, loss of MET positivity followed by cell death (PI positivity) was observed

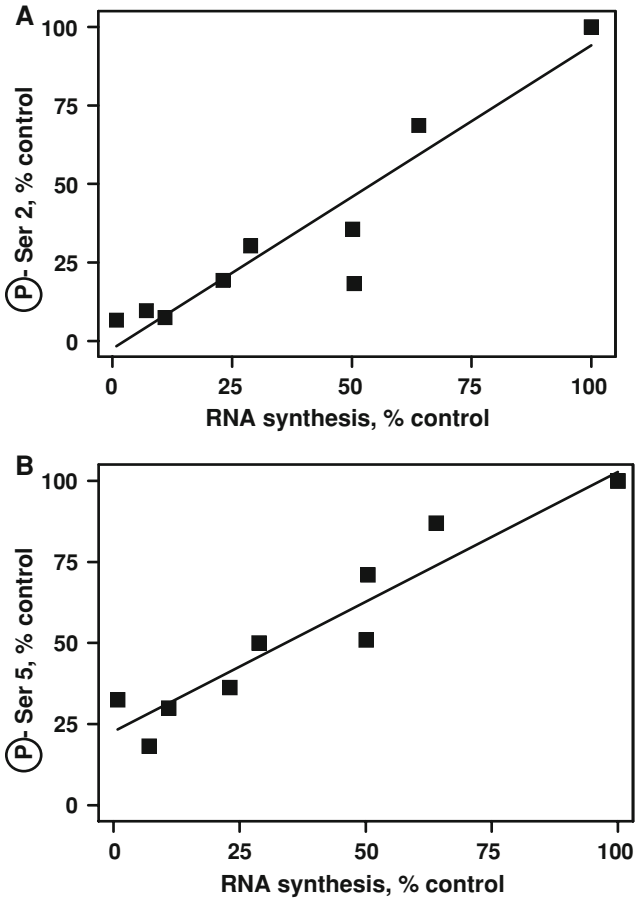

Fig. 3 Relationship between Ser5/Ser2 phosphorylation and RNA synthesis. Data from Fig. 1 regarding inhibition of RNA synthesis at 1 and 3 μM flavopiridol was compared with Ser2 phosphorylation (3A) and Ser5 phosphorylation (3B) in the heptamer repeat of CTD of RNA pol II

when cells were treated with 1 μM flavopiridol for 12 and 24 h (Fig. 4b-III, IV). Similar experiments were performed with 1 and 3 μM of flavopiridol. The protein levels of MET in untreated MM1.S cells for c-Met FITC positive and PI negative were expressed as 100% value. Compared to these untreated controls, at 12 h the decrease of MET protein levels was more than 30% for both 1 and 3 μM, respectively. The protein levels were further declined in a time dependent manner; at 24 h, there was more than 75% reduction of MET protein levels (Fig. 4c). Although the decrease in MET protein level took a longer time, the expression of *MET* transcript was directly related to the cellular level of MET protein ($r^2 = 0.82$, $P = 0.001$, data not shown). MCL-1 and XIAP have been shown to be survival proteins with short half-lives. Both these proteins were reduced after treatment with flavopiridol (Supplementary Fig. 1).

Induction of apoptosis by flavopiridol

To investigate if flavopiridol's effect on MET protein was associated with induction of apoptosis and cell death in multiple myeloma, MM1.S cells were treated with flavopiridol and induction of apoptosis was measured using PARP cleavage, which involves the appearance of 84 kDa PARP cleaved product (Fig. 5a). This cleaved protein was evident as early as 4 h with 1 and 3 μM of flavopiridol albeit with longer time points there was an increased amount of PARP cleavage in a time and dose dependent manner up to 24 h. To further reinforce this observation and to quantitate death in MM1.S cells, cells positive for annexin V PI staining were quantitated using flow cytometry. At 24 h, 1 μM flavopiridol resulted in approximately 40% cell death, whereas, treatment with 3 μM of drug killed 70% of cells (Fig. 5b). Additional MM cell line, U266, was tested for flavopiridol-induced cell death (5C). The data show similar levels of annexin positive cells as observed with MM1.S cell line. Taken together, these apoptosis data in different model systems show that flavopiridol induces cell death in multiple myeloma cells.

Direct reduction of *MET* transcript and protein levels by met-shRNA and effect on cell death

To determine if the induction of apoptosis in flavopiridol treated cells was due to decrease in *MET* mRNA and protein levels, a direct assessment of these parameters was done by using shRNA that targets *MET*. To determine if the Met-shRNA was knocking down *MET* mRNA effectively, real-time RT- PCR was done with untreated, vector only and Met-shRNA infected cells (Fig. 6a). At 48 h there was approximately 70% decrease in *MET* mRNA. This observation was consistent at the protein level where there was 50% decline in cell surface expression of MET at 72 h (Fig. 6b). Because the extent of *MET* mRNA and protein decline was similar to that observed with flavopiridol treatment, we measured cell death in shRNA treated cells by annexin V/PI staining. This is illustrated by the increase in number of cells staining positively for annexin V only after infection with shRNA and not with vector control (Fig. 6c). The data from three separate experiments demonstrate that in vector infected cells there is less that 30% cell death, which was consistent with that observed in untreated (normal) MM1.S cells. However, in cells infected with shRNA, there is approximately 60% cell death after 3 days of infection (Fig. 6d). These data suggest that direct targeting MET levels in MM1.S cells result in a decrease in cell survival.

Discussion

Flavopiridol is a general cyclin dependent kinase inhibitor that affects activity of several CDKs [6, 41]. However, it has a more potent effect on CDK 9 with a *Ki* value of 3 nM [8]. CDK 9 partners with cyclin T (P-TEFb), phosphorylates

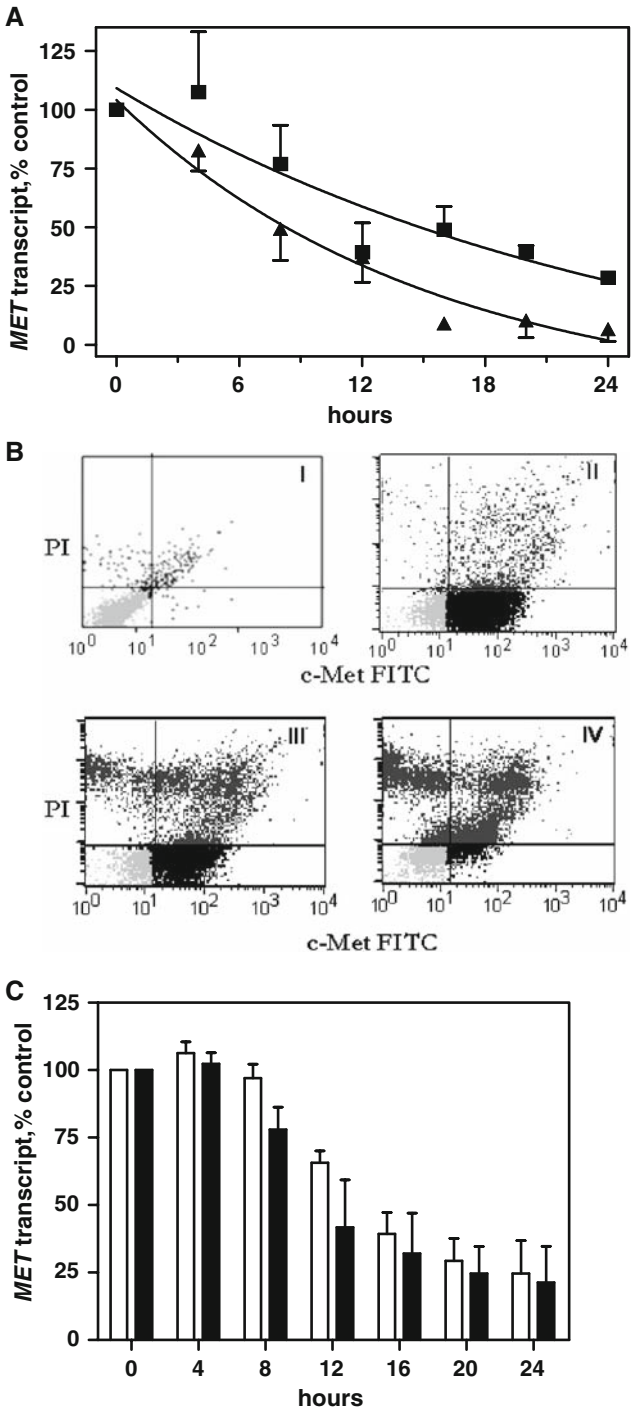

Fig. 4 Reduction of MET transcript and cell surface protein levels by flavopiridol. **a** MM cells were treated with 1 (*filled square*) and 3 μM (*filled triangle*) flavopiridol for indicated times and total RNA was isolated. The expression level of c-Met transcripts was measured using real-time RT-PCR. The *MET* transcripts were normalized using *TXN2* and *MET* mRNA levels were expressed as a percentage of time matched controls. The data is presented in a time and dose dependent manner mean ± SE, triplicate samples. Expression of MET protein levels was measured using flow cytometry analysis after staining of untreated and 1 μM flavopiridol treated cells with c-Met FITC (**b**). Quantitation of Met protein levels after treatment of cells with 1 (*open square*) and 3 μM (*filled square*) flavopiridol at the indicated times (**c**). The data is presented in a time and dose dependent manner as a percentage of time-matched controls (mean ± SE, triplicate samples)

Ser2 on CTD of RNA Pol II, inactivates transcription repressors, and facilitates transcription elongation [43]. Consistent with these actions on CDK 9, flavopiridol had the maximum inhibition of the phosphorylation of Ser2 in the heptamer repeats on the C-terminal domain (CTD) of RNA polymerase II (pol II) (Fig. 2a, b). Although flavopiridol was shown to reduce Ser5 and Ser2 phosphorylation, it did not affect total pol II protein levels (Fig. 2a). Furthermore, dephosphorylation of serine residues on the CTD of RNA pol II was directly related to inhibition of global RNA synthesis (Fig. 3a, b).

Since flavopiridol is a transcription inhibitor, transcripts with a short half-life will be affected at an early stage. *MET* mRNA has a short half-life of approximately 30 min. Consistent with this fast turnover rate, flavopiridol treatment reduced expression of *MET* transcripts (Fig. 4a). Concurrent to *MET* transcript decrease, there was reduction in MET protein level (Fig. 4b, c). This may be due to several factors. First, the decrease in *MET* transcript will affect formation of new MET protein. Second, endogenous MET protein declines with a half-life of approximately 5 h [18, 29]. So, by reducing the mRNA of *MET*, this will also cause a decrease in the MET protein levels. In agreement with this data there was a direct and statistically significant association between level of MET transcript and protein ($r^2 = 0.8$, $P = 0.001$).

Concurrent with a decline in the receptor tyrosine kinase MET protein and mRNA levels in MM1.S, flavopiridol increased cell death in a time and dose dependent manner (Fig. 5a–c). This finding can also be correlated with the actions of flavopiridol in other cell types such as chronic lymphocytic leukemia (CLL). In CLL there is evidence to show that flavopiridol induces cell death and affects a number of short lived transcripts that act as anti-apoptotic proteins such as Mcl-1 and XIAP [9]. In conjunction with flavopiridol-mediated decrease in these prosurvival factors in CLL, there was apoptosis induction in CLL lymphocytes. It is feasible that in addition to the effect on MET, additional anti-death factors (Mcl-1, XIAP) which have been shown to be important in multiple myeloma cell survival [35, 49] may be involved when MM cells are treated with flavopiridol as decrease in these proteins was observed (Supplementary Fig. 1). Hence with this pharmacological inhibitor of transcription, decline in several survival proteins may contribute to MM cell death.

Since flavopiridol treatment diminished many transcripts with short half-lives including Mcl-1 [19], it was imperative to do a direct assessment to determine if MET is a critical survival factor for multiple myeloma. MET was

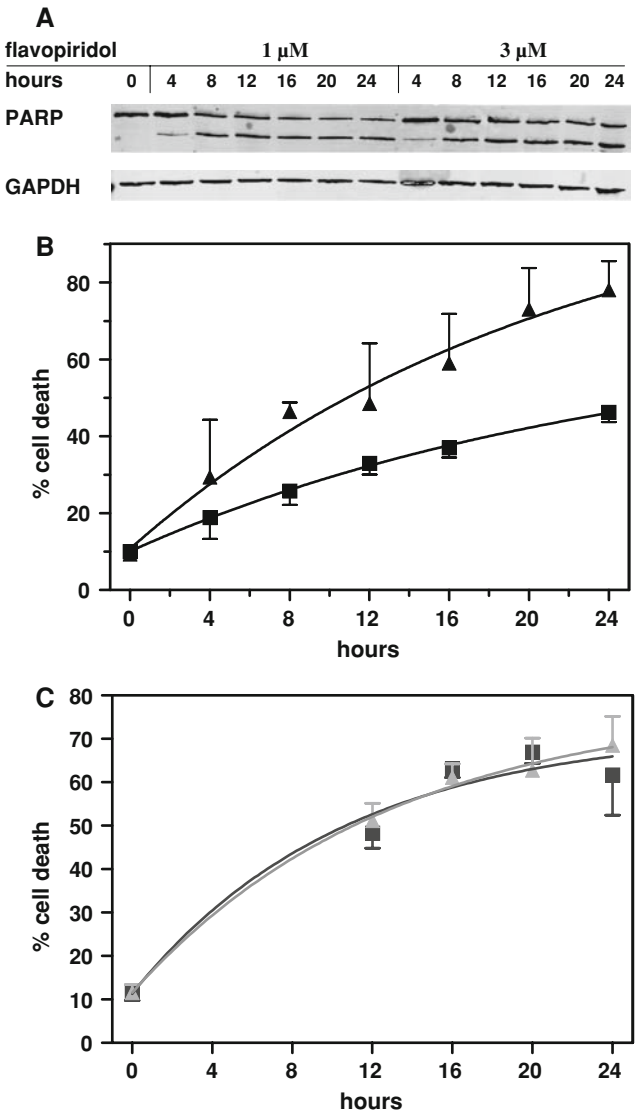

Fig. 5 Induction of apoptosis by flavopiridol. Cells were treated with 1 and 3 μM flavopiridol for indicated times. Protein extracts were analyzed for cleavage of PARP protein (**a**). A second aliquot of cells MM1.S (**b**) and U266 (**c**) was used for flow cytometry to determine the staining of cells with annexin V-FITC and propidium iodide after treatment with 1 (*filled square*) and 3 μM (*filled triangle*) flavopiridol. The data is presented as a percentage of time-matched controls (mean ± SE, triplicate samples)

knockdown using retrovirus infection expressing shRNA targeting MET (Met-shRNA), and there was approximately 50% cell death compared to less than 20% cell death in untreated and shRNA vector treated cells (Fig. 6c, d). Whereas, flavopiridol induces ~40% (1 μM) and 70% (3 μM) cell death in 24 h (Fig. 5b). Hence, this direct assessment shows that MET is one of the important survival protein for multiple myeloma cells. Taken together our results suggest that transcription inhibition by flavopiridol and effect on MET protein levels are associated with MM cell death. However, other effects such as cell cycle mediated growth inhibition of MM cells by flavopiridol, flavopiridol directed effect on mitochondria [11, 30, 40], or flavopiridol mediated downregulation of prosurvival proteins may be additional factors that influence cell death. Based on these results, we can postulate that shRNA vector strategy will be synergistic when combined with flavopiridol. This hypothesis is consistent with our recent data where ribozyme approach was used to decrease *MET* transcript levels followed by an RNA-directed nucleoside analog 8-chloro-adenosine that inhibits transcription [45].

Flavopiridol may be a desired drug for multiple myeloma although earlier clinical trials for patients with multiple myeloma [15] and CLL [4, 17] were not successful. This lack of early success may be attributed to plasma concentration of flavopiridol being below the effective concentration [4, 17] or the effective concentration was only there for a short duration [15]. In addition, flavopiridol also binds to human serum [9]. However, the current clinical trial has overcome these pharmacokinetic and pharmacodynamic deficiencies by changing the schedule and dose of administration [5]. With this new schedule, flavopiridol has been shown to be efficacious in another B-cell malignancy, CLL. Ongoing clinical trials with associated laboratory biomarkers and endpoints suggest that the effectiveness of flavopiridol is due to transcription inhibition in quiescent CLL lymphocytes [5, 9]. Multiple myeloma and CLL are similar in that they are both B-cell neoplasm and indolent (MM) or quiescent (CLL) malignancies. So, at this pharmacokinetically-guided schedule, flavopiridol may show potential promise as becoming a candidate drug treatment for multiple myeloma.

However, in order to thoroughly evaluate if flavopiridol and targeting MET can be effective in the clinic for patients with MM, additional experiments need to be performed to evaluate the role of microenvironment in MET functions and signaling. The current work used suspension culture of multiple myeloma cell line. This cell line system growing on plastic does not assess role of stroma and growth factors in the survival of multiple myeloma [28]. As a next step to observe the effects of flavopiridol and inhibition of MET in the presence of stromal cells we are pursuing co-combinational cultures. Stromal cells have been shown to release a number of growth factors including hepatocyte growth factor (HGF) [33] which is crucial ligand for MET tyrosine kinase receptor. Upon understanding the role of stromal cells in the survival of multiple myeloma, it would be very informative to observe a direct assessment towards primary tissue cells. Our current efforts are focused on flavopiridol-induced effect on MM cells which are impacted by the stroma.

Taken together, we can conclude that complexity of multiple myeloma is enormous and there seems to be a variety of factors that is important for the survival for multiple myeloma, including MET protooncogene. Because the

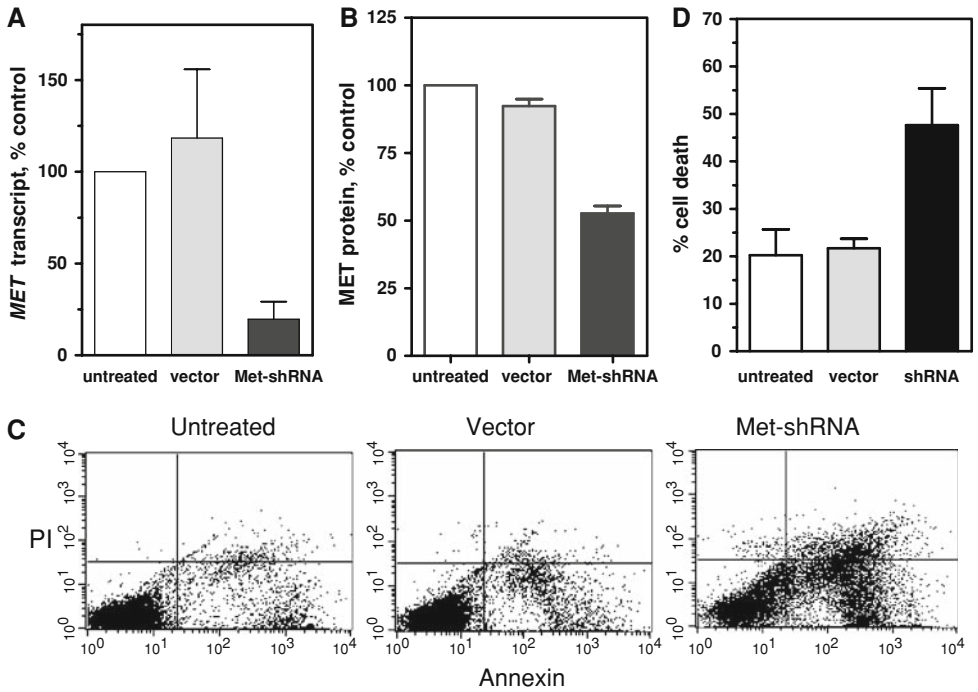

Fig. 6 Reduction of MET transcripts and protein levels by Met-shRNA and effect on cell death. MM1.S cells were infected with PT67 cells with pSilencer 5.1 Retro vector and Met-shRNA. The mRNA levels of *MET* transcripts were measured using real-time RT-PCR (**a**). The *MET* transcripts were normalized using *TXN2* and *MET* mRNA levels were expressed as a percentage control compared to untreated MM1.S cells at 48 h. Protein levels of cell surface MET were measured by flow cytometry and were compared with untreated cells at 72 h (**b**). MM1.S cells were infected with PT67 cells with pSilencer 5.1 Retro vector and with Met-shRNA for 72 h. Cell death was analyzed using annexin PI staining (**c**). The data are quantitated showing the total number of cell death in triplicate samples showing standard error (**d**). Data are presented as mean ± SE of triplicate experiments

MET transcript turn-over rate is fast (t½ 30 min), this could be targeted by molecular (shRNA) and pharmacological (flavopiridol) transcription inhibitors. Since HGF, the ligand for MET tyrosine kinase, levels are high in patients with multiple myeloma, antibody targeted to HGF may not be a viable clinical option, further underscoring the need for targeting MET protein by reducing its level (the current study) or using MET tyrosine kinase inhibitors.

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