Differentiation Inducing Factor-1 Represses c-Myc and VEGF Expression in Myeloma Cells

Rajni KUMARI, Rehan KHAN, Alpana SHARMA

All India Institute of Medical Sciences, Department of Biochemistry, New Delhi, INDIA

ABSTRACT

Cell adhesion mediated-drug resistance (CAM-DR) is designated as the culprit of relapsed and refractory multiple myeloma (MM). c-Myc and VEGF overexpression in MM is correlated with MM progression and CAM-DR in bone marrow microenvironment. c-Myc is responsible for hyperproliferation of myeloma cells whereas VEGF induces angiogenesis to support the hyperproliferation. Hence, c-Myc and VEGF represent the promiscuous targets in MM. In this study, we have examined online databases to verify the severity of c-Myc and VEGF expression in MM. Effect of differentiation inducing factor-1 (DIF-1) was studied by treating RPMI8226 cells with DIF-1 (10 μM) followed by qRT-PCR, western blotting, and cell proliferation by XTT. DIF-1 targets c-Myc and VEGF expression at both transcriptional and translational level in RPMI8226 cells. DIF-1 activates GSK-3β, which may influence c-Myc and VEGF expression. Hence, DIF-1 inhibits cell proliferation of MM cells. Our report is a maiden attempt to reveal that DIF-1 could act as an adjunct drug to overcome chemoresistance in MM in future.

Keywords: c-Myc, VEGF, DIF-1, Multiple myeloma, RPMI8226

ÖZET

Diferansiyasyon İndükleyici Faktör-1, Miyelom Hücrelerinde c-Myc ve VEGF Ekspresyonunu Baskılar

Hücre adezyon aracılı-ilaç direnci (CAM-DR), nükseden ve refrakter multipl miyelom'un (MM) suçlusu olarak belirlenmiştir. MM'de c-Myc ve VEGF aşırı ekspresyonu, kemik iliği mikro çevre MM progresyonu ve CAM-DR ile korelasyon gösterir. C-Myc, miyelom hücrelerinin hiperproliferasyonundan sorumludur, oysa VEGF, hiperproliferasyonu desteklemek için anjiyojenezi indükler. Dolayısıyla, c-Myc ve VEGF MM'deki karışık hedefleri temsil eder. Bu çalışmada, MM'de c-Myc ve VEGF ekspresyonunun şiddetini doğrulamak için online veritabanlarını inceledik. Diferansiyasyon indükleyici faktör-1'in (DIF-1) etkisi RPMI8226 hücrelerini DIF-1 (10 μM) uygulamasını takiben, qRT-PCR, western blot ve XTT ile hücre proliferasyonunu çalışıldı. DIF-1, c-Myc ve VEGF ekspresyonunu etkileyebilecek GSK-3b'yı aktive eder. Dolayısıyla, DIF-1, MM hücrelerinin hücre proliferasyonunu inhibe eder. Raporumuz, gelecekte DIF-1'in MM'de kemorezistansın üstesinden gelmek için yardımcı ilaç olarak işlev görebileceğini ortaya koyan bir ilk çalışmadır.

Anahtar Kelimeler: c-Myc, VEGF, DIF-1, Multipl myelom, RPMI8226

INTRODUCTION

Multiple myeloma (MM) is classified as plasma B-cell neoplasia or plasmacytoma, infiltrating and secreting monoclonal proteins in the bone marrow. As a result, leading to several pathological conditions like hypercalcemia, renal insufficiency, anemia and lytic bone lesions.[1] It is second most common hematological malignancy and develops from asymptomatic monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM).[2] MM cell's interaction in the bone marrow niche with extracellular matrix (ECM) or bone marrow stromal cells (BMSCs) form a protective bone marrow microenvironment for MM progression, survival, angiogenesis, metastasis and chemoresistance. Interaction of MM cells with ECM through integrins or BMSCs results in secretion of various cytokines, chemokines and angiogenic factors. These factors are the drivers of cell adhesion mediated drug resistance in bone marrow microenvironment of MM.[3] Among these secretory factors, secretion of IL-6 induces c-Myc overexpression and serves as an important event for MM cells survival.[4]

Progression from MGUS and SMM to MM involves accumulation of various oncogenic events, among which c-Myc overexpression or translocation to IgH/IgL describes a significant contribution.[5] c-Myc regulates various transcripts in the cell regulating processes like proliferation, metabolism and apoptosis.[6] Role of c-Myc in myeloma cells has been verified with several reports targeting c-Myc with siRNA or chemical inhibitors of Myc-Max heterodimerization.[7-9] These reports enlighten the idea of targeting c-Myc expression in a therapeutic context.

Angiogenesis is the process which involves regulation and maintenance of sprouting of endothelial cells, hence formation of new blood vessels from the existing ones.[10] Vascular endothelial growth factor (VEGF) serves as a critical factor in this process of angiogenesis. It is secreted in bone marrow microenvironment by myeloma cells as well as neighboring cells and binds to VEGF receptors (VEGFR1 and VEGFR2) to promote the expression of other angiogenic mediators, angiopoietins.[11,12] Bone marrow microenvironment induced angiogenesis of myeloma cells is essential for the chemoresistance of MM and significantly correlates with relapsed and refractory phase of myeloma.[13] Previously, our group has also shown VEGF as a biomarker for MM progression.[14,15] In another study, our group showed the anti-angiogenic effect of cinnamon extract by targeting VEGF and Ang-2.[16] Hence, angiogenesis in MM has always been the promising therapeutic target. Intriguingly, c-Myc also influences vasculogenesis and angiogenesis in MM.[17]

Dictyostelium discoideum differentiation inducing factor-1 (DIF-1) is a chemical morphogen which plays role in stalk cell differentiation in the developmental stages of *Dictyostelium*.[18] Among DIFs, DIF-1 was the first one to be isolated from Dictyostelium and tested for its antitumor properties. DIF-1, being a hydrophobic molecule, does not require any intermembrane transporter or carrier to cross the plasma membrane. Kubohara and colleagues have vastly studied the effect of DIF-1 on various cancer cells proliferation, differentiation and angiogenesis.[19] DIF-1 also shows anti-leukemic effects also on K562 leukemia cells.[20] DIFs have also shown antiproliferative and anti-angiogenic effects on various cancer cell lines previously.[21-23] However, exact mechanism of action of DIF-1 is still under investigation, previous reports suggest DIF-1 alters Wnt/β-catenin signaling through GSK3β activation and β-catenin degradation. β-catenin, if accumulated, translocates to nucleus and binds to DNA after forming heterodimer with TCF/Lef.[24] Derksen *et al*. have shown that levels of β-catenin is also correlated with myeloma progression and survival.[25] Since, β-catenin transactivates c-Myc and VEGF, we sought to determine the effect of DIF-1 on c-Myc and VEGF expression in myeloma cells.

In this report, we have used RPMI8226 to address the effect of DIF-1 in MM as it has c-Myc-IgLλ translocation which mimics the in vivo condition of c-Myc constitutive overexpression in MM.[26] Taken together, we found that DIF-1 repressed c-Myc expression by several fold transcriptionally and translationally. DIF-1 also decreased VEGF expression at both transcript and protein levels. Physiologically, DIF-1 also exerts anti-proliferation on myeloma cells. Hence, this report hints that

DIF-1 has the potential to be a future drug to treat MM either alone or in combination with recent proteasomal or immunomodulatory drugs to rescue cell adhesion mediated drug resistance.

MATERIALS and METHODS

Cell Line and Culture

RPMI8226 (Catalog no. CCL. 155) myeloma cell was purchased from ATCC. RPMI8226 was maintained in suspension culture with RPMI1640culture media (Sigma-Aldrich) supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, 100 μg/ml streptomycin and 5% CO_2 at 37^0C. Cells were routinely monitored for any contaminations.

Reagents and Antibodies

DIF-1 (1-(3,5-Dichloro-2,6-dihydroxy-4-methoxyphenyl) hexan-1-one) was purchased from EnzoLife Sciences, U.K. and 10mM stock solution was prepared in ethanol as instructed. Further dilutions were made in RPMI1640. Equivalent amount of ethanol, present in DIF-1, was used for control experiments. Antibodies for rabbit polyclonal anti-human c-Myc (1:500), rabbit polyclonal anti-human VEGF (0.2 μg/ml), rabbit polyclonal anti-human pSer9GSK3β (1:500), rabbit polyclonal anti-human pTyr216 (1:500) and Anti-rabbit IgG HRP-conjugated (1:25000) was purchased from Abcam, Cambridge, U.K. Housekeeping control GAPDH (1:2000) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Real-time Quantitative PCR

RPMI8226 cells were harvested at 24 hrs after DIF-1 (10 μM) or control ethanol treatment. Total RNA was extracted using TRIzol reagent (Sigma) following manufacturer's instructions. 2 μg of total RNA was used to synthesize cDNA for further reactions. cDNA was diluted 1:100 with nuclease-free water and further used as template for qPCR reactions. Maxima SYBR Green master mix 2X (Fermentas) was used to perform the relative expression analysis of different transcripts using ABI 7500 real-time PCR (Applied Biosystems Inc.). 18S rRNA was used as internal control. Fold change was calculated using ΔΔCt method.

Primers	Sequence
c-Myc	Forward- 5'-GAGATCCGGAGCGAATAGG-3'
	Reverse- 5'-GCTGCTATGGGCAAAGTTTC-3'
VEGF	Forward- 5'-ACCATGAACTTTCTGCTGTCTTG-3'
	Reverse-5'-ATGGCTTGAAGATGTACTC GATCTC-3'
18S rRNA	Forward- 5'-GTAACCCGTTGAACCCCATT-3'
	Reverse- 5'-CCATCCAATCGGTAGTAGCG-3'

All the primers were synthesized from IDT.

Western Blotting

RPMI8226 cells were harvested after DIF-1 (10 μM) or ethanol control treatment. Cells were lysed in RIPA lysis buffer and Bradford assay was performed for total protein measurement. Equal amount of protein lysates were run on SDS-PAGE gel and transferred on nitrocellulose membrane (MDI). Blocking was done using 5% BSA in TBST. Primary antibodies were incubated overnight after dilution in 5% BSA in TBST. Further, membrane was washed and incubated with secondary antibody for 2 hrs at room temperature. Development was done using enhanced chemiluminicent lightning reagent (ECL) as substrate for HRP after washing. Image was acquired in ChemiDoc machine.

XTT Proliferation Assay

RPMI8226 cell proliferation was monitored after indicated concentrations of DIF-1 for 24 hrs using XTT tetrazolium (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) salt. Briefly, cells were incubated with 20 μl of XTT solution (5mg/ml) in 100 ul cell culture medium for 2-4 hrs after treatment. Absorbance was read at 405 nm and reference at 630 nm.

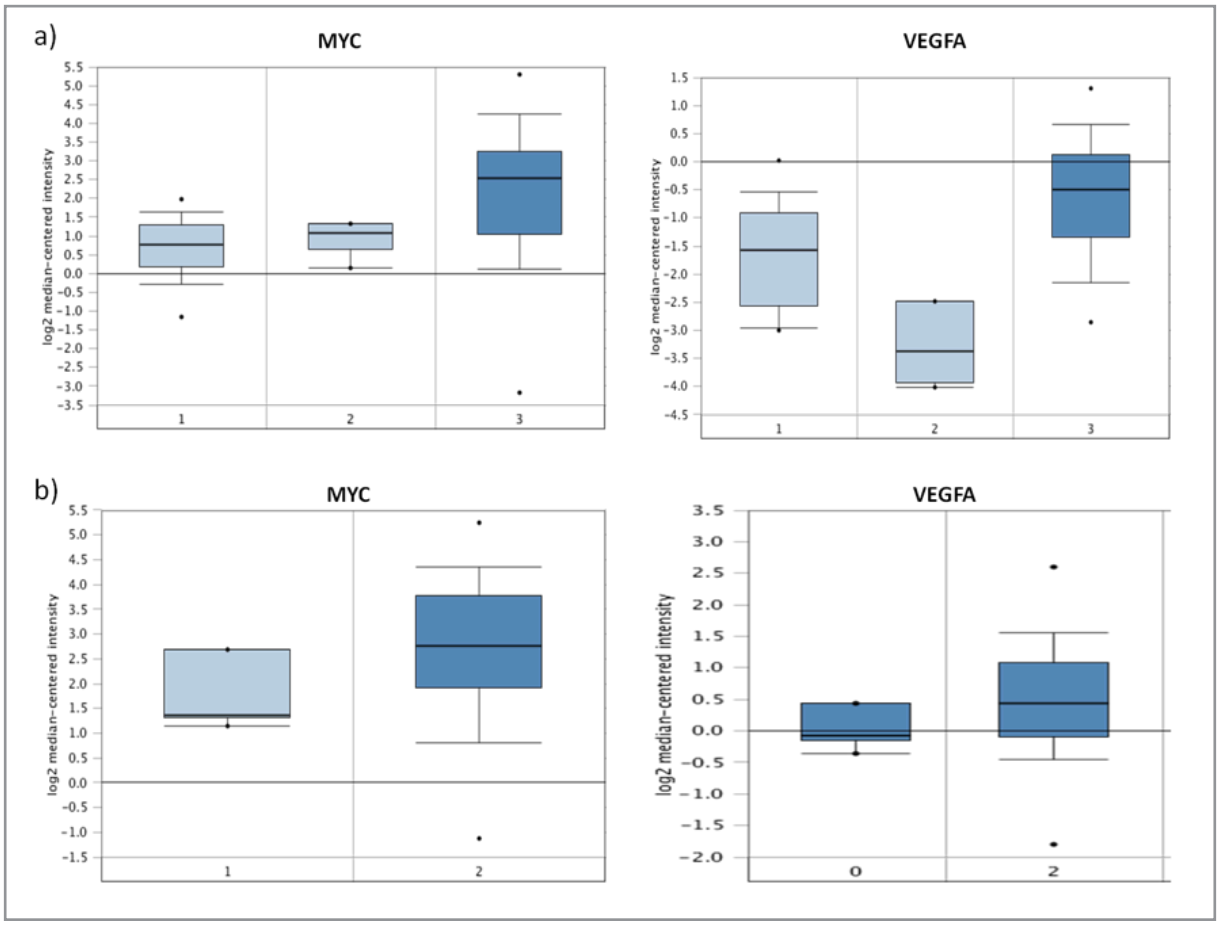

Figure 1. Role of c-Myc and VEGFA in oncogenesis of MM. a) Expression of MYC and VEGF in 1- normal plasma cell, 2- tonsillar lymphoid tissue and 3- multiple myeloma taken from oncomine.com database (Zhan myeloma). b) Expression of MYC and VEGF in 1- MGUS, 2- multiple myeloma taken from oncomine.com database (Agnelli myeloma).

Bioinformatic Analysis

MYC and VEGF expression analysis was done with freely available Oncomine database[27] in myeloma microarray by Agnelli *et al.* (GSE13591)[28] and Zhan *et al.*[29] STRING pathway analysis[30] was done for MYC and VEGF interaction partners with high correlation index.

Statistical Analysis

All the data were analyzed for statistical significance using Student's paired t-test. All analysis were performed using SPSS version 15.0 (SPSS, Chicago, IL, USA) and $p < 0.05$ was considered as statistically significant.

RESULTS

c-Myc and VEGF Levels are Overexpressed in MM Patients

Since MM progression from MGUS relies on enhanced proliferation and angiogenesis, we analyzed available GEO datasets of MM to determine expression levels of c-Myc and VEGF. As a result, we found in both the datasets[28,29] that c-Myc and VEGF expression increases with the progression of MM from MGUS and normal plasma B-cells. Both the studies are completely independent and highlight the significance of c-Myc and VEGF overexpression in MM (Figure 1a and 1b). STRING and KEGG pathway analysis for interaction partners of c-Myc and VEGF reveal their involvement in significant pathways involved in cell proliferation,

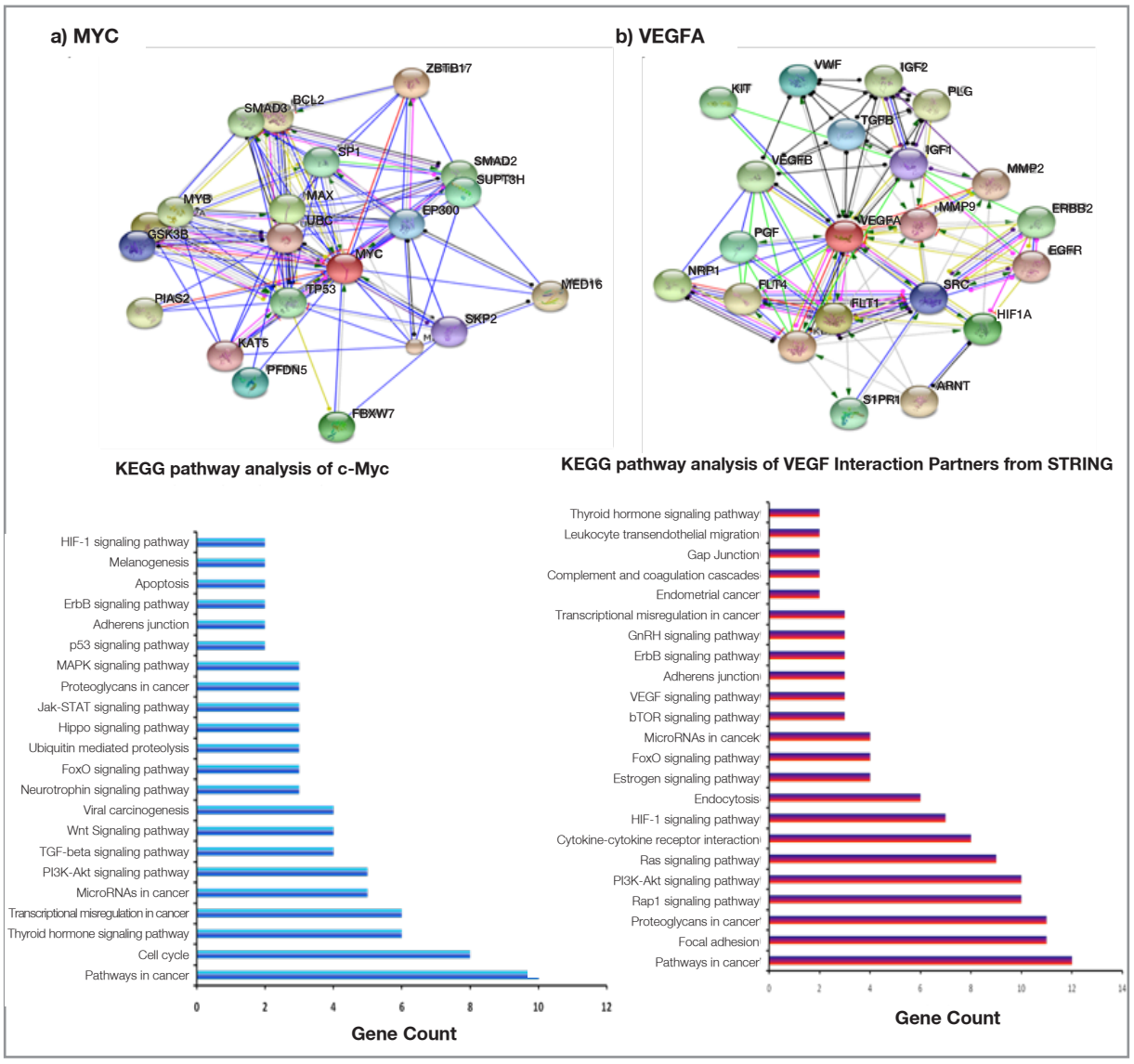

Figure 2. Role of c-Myc and VEGFA in oncogenesis of MM. a) STRING and KEGG pathway analysis for interacting partners of MYC. d) STRING and KEGG pathway analysis for interacting partners of VEGFA

migration and angiogenesis (Figure 2a and 2b). Altogether, we concluded that VEGF and c-Myc overexpression result in MM progression and targeting them at mRNA or protein level could lead to the inhibition and regression of MM in patients.

DIF-1 Inhibits c-Myc and VEGF Expression in MM Cells

As DIF-1 (Figure 3a) has shown anti-angiogenic effects on several leukemic and carcinoma cells *in vitro* and *in vivo*, we wanted to explore its effect on expression levels of c-Myc and VEGF in MM cells. To address this, we performed quantitative real time PCR to analyze change in transcript levels of c-Myc and VEGF upon DIF-1 treatment. We found that c-Myc mRNA expression was profoundly suppressed ($p< 0.0005$) upon treatment of DIF-1 to RPMI8226 myeloma cells (Figure 3b). However, VEGF mRNA expression was also significantly downregulated ($p< 0.0005$) due to DIF-1 (Figure 3c). Furthermore, to check translation of these downregulations at the level of proteins, we performed western blotting for VEGF and c-Myc

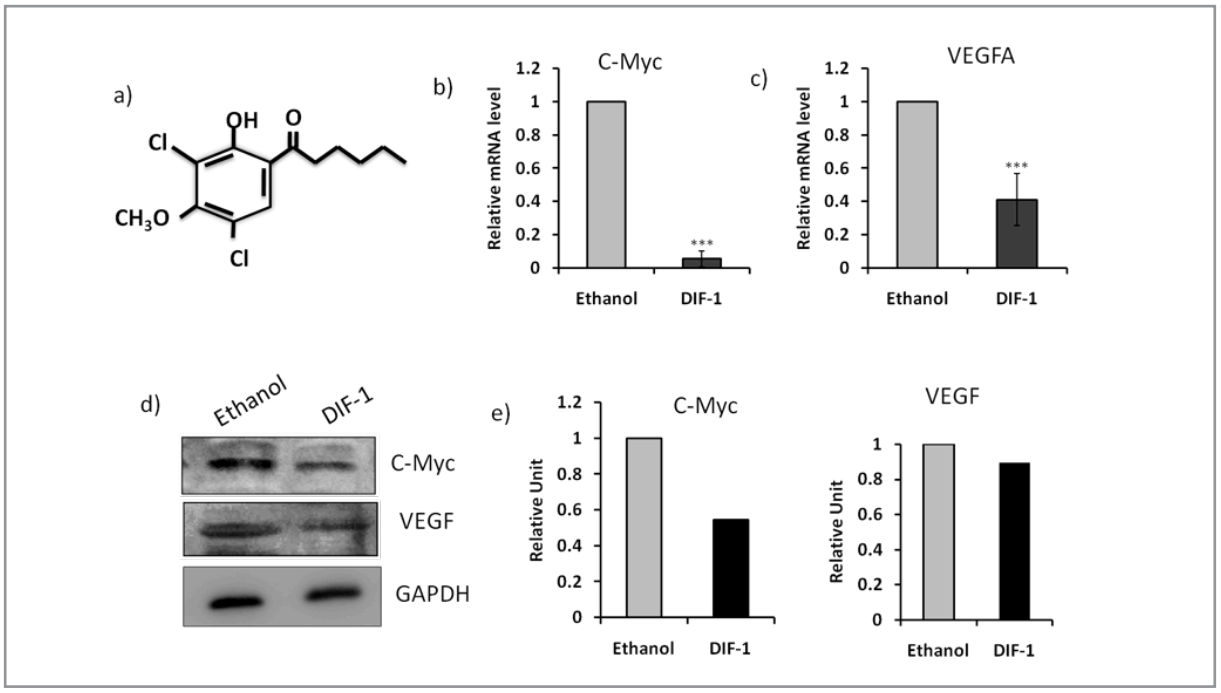

Figure 3. Effect of DIF-1 on c-Myc and VEGFA expression. **a)** Structure of DIF-1. **b)** RPMI8226 cells were treated with ethanol or DIF-1 (10 μM) for 24 hr. Relative mRNA levels were analyzed by RT-qPCR for c-Myc and **c)** VEGFA. Error bars are mean+SD of three independent experiments with triplicate samples. *p< 0.05. **d)** RPMI8226 cells were treated with ethanol or DIF-1 (10 μM) for 24 hr. Cells were then harvested and western blot analysis was done for indicated proteins. **e)** Densitometric analysis of proteins were done with respect to GAPDH and plotted as graph.

after DIF-1 treatment. Protein levels of both c-Myc and VEGF was significantly lowered after the treatment of DIF-1 (Figure 3d). Therefore, DIF-1 inhibited both c-Myc and VEGF expression in myeloma cells.

DIF-1 Activates GSK3β in MM Cells

To explore the mechanism behind c-Myc and VEGF downregulation due to DIF-1, we analyzed GSK3β phosphorylation status in DIF-1 treated myeloma cells. Analysis for phosphorylation of Ser9GSK3β through western blotting revealed its

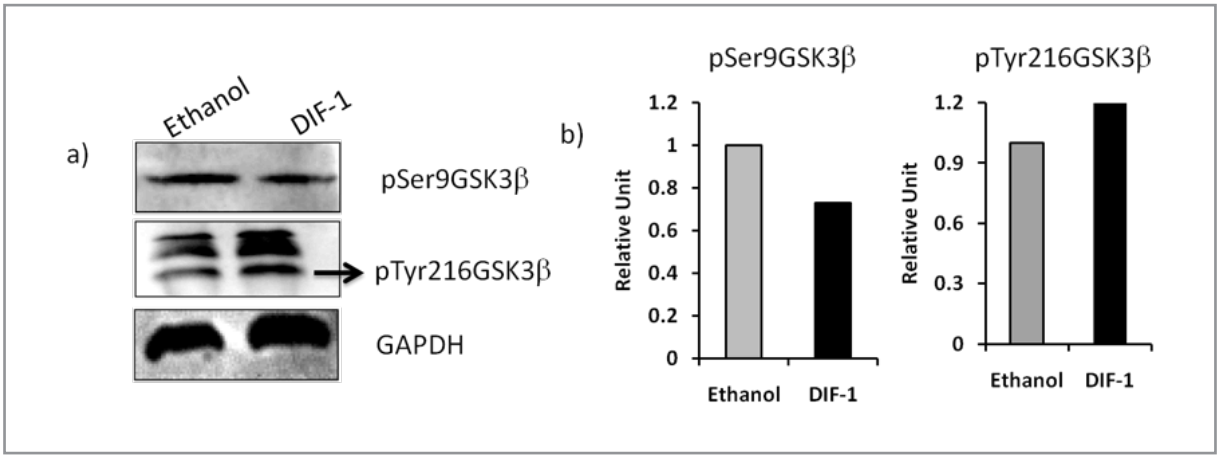

Figure 4. DIF-1 activates GSK3β. a) RPMI8226 cells were treated with ethanol or DIF-1 (10 μM) for 24 hr. Cells were then harvested and western blot analysis was done for indicated proteins. b) Densitometric analysis of proteins were done with respect to GAPDH and plotted as graph.

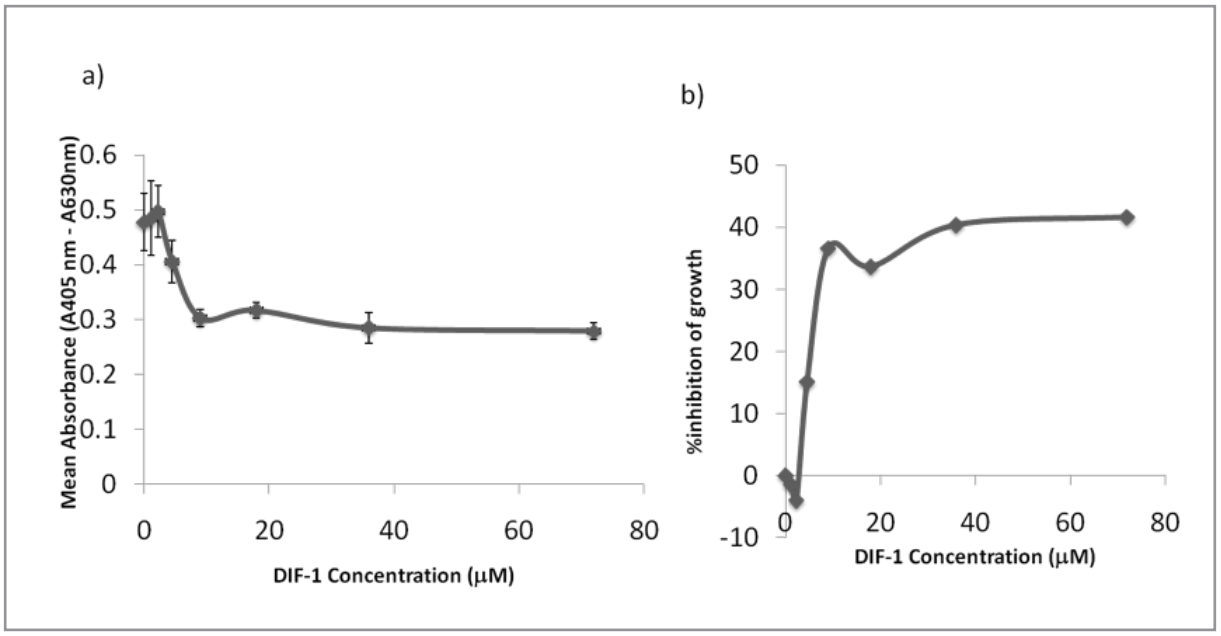

Figure 5. DIF-1 exerts cytostatic effect on RPMI8226 cells. a) RPMI8226 cells were treated with indicated concentrations of DIF-1 for 24 hr and XTT assay was performed. Error bars are means+SD of three independent experiments with triplicate samples. b) Effect of DIF-1 on growth of RPMI8226 cells.

significant absence in DIF-1 treated cells. However, analysis for Tyr216GSK3β phosphorylation reflected its increased presence upon DIF-1 treatment in myeloma cells (Figure 4). These results indicate the activation of GSK3β.

DIF-1 Exerts Cytostatic Effect on MM Cells

Next, we investigated if these biochemical effects of DIF-1 alter RPMI8226 proliferation. We treated RPMI8226 cells with different concentrations of DIF-1 and monitored cell viability through XTT assay. Concentration of DIF-1 as low as 5 μM showed inhibitory effect on RPMI8226 cell proliferation (Figure 5). Intriguingly, at higher concentrations of DIF-1, there was no further decrease in cell viability, which reflects DIF-1 cytostatic effect on myeloma cells.

DISCUSSION

Despite the development of various chemotherapeutic drugs to target MM at cellular and bone marrow microenvironment level, there is still high rates of relapse cases in MM patients.[13,31] Deciphering molecular events of oncogenesis, researchers have found that dysregulation of c-Myc due to IgH/IgL translocations or overexpression due to epigenetic activation is highly correlated with the occurrence of haematological malignancies and chemoresistance tendency in myeloma and lymphoma.[32-34] Previously, it is reported that malignant myeloma cells acquire oncogenic addiction to c-Myc levels during both *de novo* development from plasma B-cells and progression from asymptomatic MGUS.[7] In this report, our re-examination of online microarray data from Oncomine database also confirms the highly significant contribution of elevated c-Myc levels in myeloma development and progression. Underlying studies on c-Myc molecular functions emerge c-Myc as a master oncogene to function in hyperproliferation of tumor cells by inducing DNA synthesis, suppressing apoptosis and increasing availability of metabolites essential for anabolic needs through angiogenesis.[35,36] Corroborating with this, STRING pathway analysis for c-Myc interaction partners explain its role in oncogenesis.

Since bone marrow microenvironment is a major contributor of CAM-DR in MM, cytokines, chemokines, and growth factors secreted by cells in the bone marrow milieu (BMSCs, endothelial

cells, osteoblasts and osteoclasts) and myeloma cells have been focus of targeted therapy development for the treatment of myeloma.[3] Consequently, angiogenesis in bone marrow microenvironment supports the growth and migration of myeloma cells.[36] This fact is also supported by current treatment regimen of immunomodulatory drugs, thalidomide and lenalidomide, which targets angiogenesis of bone marrow microenvironment.[37] Our group along with others have shown previously that VEGF is a critical factor for maintainence of angiogenesis.[14-15,38] Oncomine database analysis for VEGF expression in myeloma cells also reflected significant contribution of VEGF in myeloma progression, similar to c-Myc levels. Intriguingly, c-Myc in combination with HIF-1α acts as transcription factor for VEGF.[39].So, we determined to target c-Myc and VEGF with a therapeutic molecule under investigation.

Influenced by the studies performed by Kubohara and colleagues on the effects of DIF-1 in cancer cells differentiation, apoptosis and angiogenesis[18-20,23], our search for further literature on molecular mechanisms altered by DIF-1 showed that it activates GSK3β, which targets β-catenin for proteasomal degradation.[24,40] β-catenin acts as a transcription factor for c-Myc and VEGF.[25,41] Therefore, we investigated the potential of DIF-1 in MM.

Interestingly, our report demonstrates that DIF-1 treatment significantly reduces c-Myc and VEGF at mRNA and protein level. Our data is also supported by Jingushi *et al* wherein they have shown downregulation of c-Myc by DIF-1 in colon cancer cell lines.[42] Addressing the issue of CAM-DR, we suggest targeting c-Myc and VEGF level with single drug molecule i.e., DIF-1 may open the door to next generation molecular drugs to treat relapsed and refractory myeloma in future. Concordant with previous studies, our analysis for the activation of GSK3β in myeloma cells after DIF-1 treatment also revealed increased phosphorylation and dephosphorylation at Tyr216 and Ser9 residue respectively. This indicates that activation of GSK3β could be responsible for suppressed levels of c-Myc and VEGF. Additionally, GSK3β is a hub controlling myriad of signaling pathways involved in tumor suppression.[43] Hence, activation of GSK3β by DIF-1 in RPMI8226 enhances its potency as a future myeloma drug.

Significance of our study is also highlighted by the characteristic of myeloma cell line that we have used, RPMI8226. These cells bear c-Myc-IgLλ translocation[26], which further explain the effect of DIF-1 under c-Myc overexpression and hence, the inhibition of myeloma cell proliferation. Anti-proliferative effect of DIF-1 suggests the possibility of its combination with other current therapeutics for relapse-free outcome.

Conclusively, our study is first report to show the efficacy of DIF-1 as a promising drug in MM, after validation in *in vivo* models in future. Furthermore, studies emphasized on synergistic effect of DIF-1 with other myeloma drugs, especially in c-Myc overexpressing MM patients, may also unreveal the utility of DIF-1 as an adjunct drug in precision cancer medicine.

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Correspondence:
Alpana SHARMA
All India Institute of Medical Sciences
Department of Biochemistry
New Delhi / INDIA
e-mail: dralpanasharma@gmail.com
Phone: +911126546665