



Concomitant downregulation of proliferation/survival pathways dependent on FGF-R3, JAK2 and BCMA in human multiple myeloma cells by multi-kinase targeting

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ARTICLE INFO

Article history:

Received 25 March 2009

Accepted 16 June 2009

Keywords:

BCMA

FGF-R3

JAK2

Multiple myeloma

RPI-1

ABSTRACT

The identification of proliferation/survival pathways constitutively activated by genetic alterations in multiple myeloma (MM), or sustained by the bone marrow (BM) microenvironment, provides novel opportunities for the development of targeted therapies. The deregulated function of protein tyrosine kinases plays a critical role in driving MM malignant phenotype. We investigated the effects of the multi-target tyrosine kinase inhibitor RPI-1 in a panel of human MM cell lines, including *t*(4;14) positive cell lines expressing the TK receptor FGF-R3. Cells harboring FGF-R3 activating mutations (KMS11 and OPM2) displayed the highest sensitivity to RPI-1 antiproliferative effect. The stimulating effect of the aFGF ligand was abrogated in cells harboring a non-constitutively active receptor. Drug treatment inhibited activation and expression of the FGF-R3^{Y373C} mutant as well as aFGF-dependent signaling involving AKT and ERKs. Inhibition of JAK2, an additional RPI-1 target, resulted in STAT3 inactivation. Blockade of these proliferation/survival pathways was associated with caspase-dependent apoptosis. Moreover, drug treatment abrogated proliferative and pro-invasive stimuli provided by conditioned medium from mesenchymal stromal cells. Gene expression profile of KMS11 cells showed 22 upregulated and 52 downregulated genes upon RPI-1 treatment, with an early modulation of genes implicated in MM pathobiology such as *SAT-1*, *MYC*, *MIP-1α/β*, *FGF-R3*, and the growth factor receptor B-cell maturation antigen (*BCMA*). Thus, concomitant blockade of FGF-R3 and JAK2 results in inhibition of several MM-promoting pathways, including BCMA-regulated signaling, and downregulation of disease-associated proteins. These data may have therapeutic implications in the design of treatment strategies resulting in the concomitant inhibition of FGF-R3 and JAK2 signaling pathways in *t*(4;14) MM.

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1. Introduction

Multiple myeloma (MM), which is regarded as an incurable neoplasia, is characterized by clonal expansion of a monotypic plasma cell population in the bone marrow (BM) and induction of osteolytic bone lesions [1]. MM plasma cells harbor chromosome abnormalities most frequently represented by chromosomal translocations involving the IgH locus at chromosome 14

(14q32), which result in the dysregulation of a variety of oncogenes and are significantly associated with shorter survival [2]. The *t*(4;14) translocation described in 20% of MM patients results in ectopic expression of the fibroblast growth factor receptor 3 (*FGF-R3*) and enhanced expression of *MMSET*, a gene involved in transcription regulation [3–5]. *FGF-R3*, a member of the FGF receptor tyrosine kinase family, has also been found mutated in about 6% of tumors with *t*(4;14) [1,6]. Although the pathogenic role of *FGF-R3* overexpression in MM has not been clearly defined, the acquisition of kinase-activating mutations appears to confirm its role in the multistage disease process [5,7].

In addition to the tumor genetic background, the BM microenvironment is well recognized as a crucial determinant of the biological and clinical behavior of MM [8]. Extracellular matrix components, stromal cells and secreted growth factors support the malignant growth of MM cells in the BM milieu. The best

Abbreviations: aFGF, acidic fibroblast growth factor; BCMA, B-cell maturation antigen; BM, bone marrow; GEP, gene expression profile; HMCL, human multiple myeloma cell line; MM, multiple myeloma; MSC, mesenchymal stromal cell.

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characterized myeloma growth factor is the cytokine interleukin-6 (IL-6) [9]. However, an increasing number of cytokines, chemokines and cell-to-cell contacts provided by the BM have been found to activate a pleiotropic cascade of proliferative/antiapoptotic signaling pathways including JAK/STAT, PI3K/AKT, RAS/ERK and their downstream components [10]. A better understanding of the profile of signaling pathways involved in the pathophysiology of the disease now provides a framework for the identification of novel targets [1,11]. Accordingly, novel agents recently approved in clinical protocols for MM, such as bortezomib, thalidomide and analogs, share the ability of targeting tumor–microenvironment interactions as an essential part of their mechanism of action [8,12].

The complex signaling network activated by oncogenic mutations and by the BM microenvironment sustains growth, survival and migration of MM cells contributing to tumor progression as well as to resistance to conventional chemotherapy [1,8]. Since failure to undergo apoptosis has been suggested to play a main role in MM cell accumulation within the BM as well as in drug resistance [9], blocking both intrinsic and BM microenvironment-driven signaling could be required to induce tumor cell death. Multi-targeting approaches represent thereby an attractive therapeutic strategy for MM.

Simultaneous inhibition by small molecule inhibitors of protein tyrosine kinases oncogenically activated in MM cells, and/or regulated by the BM microenvironment, is an attractive therapeutic approach. The 2-indolinone compound RPI-1 was previously described as a multi-target tyrosine kinase inhibitor showing antitumor and antimetastatic/antiangiogenic activity against human tumor models harboring oncogenic RET or over-expressing c-MET [13–18]. In the present study, the effects of the drug were investigated in a panel of human MM cell lines (HMCLs). FGF-R3 and JAK2 were identified as RPI-1 targets mediating antimyeloma activity in vitro. Moreover, expression analysis of drug-treated cells showed the modulation of several key disease-associated genes and proteins including the growth factor receptor B-cell maturation antigen (BCMA).

2. Materials and methods

2.1. Cell lines and culture conditions

HMCLs (see Table 1) were obtained from DMSZ-German collection of Microorganisms and Cell Culture, Germany (OPM2, JJN3), or kindly provided by Dr. T. Otsuki (Kawasaki Medical School, Okayama, Japan) (KMS-28, KMS-18, KMS-11, and KMS-20). They were maintained routinely in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum. Multipotent human mesenchymal stromal cells (MSCs) from normal BM adherent cells were kindly provided by Dr. M. Inrona (Ospedali

Riuniti, Bergamo, Italy) and cultured in Dulbecco's modified Eagle medium, low glucose, with 10% fetal bovine serum. Conditioned medium from MSCs was harvested after 48 h of culture in complete medium. The murine fibroblast cell lines NIH3T3 and A6-KMS11 (NIH3T3 stably transfected with the FGF-R3 mutant Y373C) [7] were cultivated in Dulbecco's modified Eagle's medium with 10% or 5% fetal calf serum, respectively. Cells were incubated at 37 °C in a 5% or 10% (fibroblasts) CO₂ atmosphere.

2.2. Drug treatment and biological assays

The synthesis and chemical structure of RPI-1 (1,3-dihydro-5,6-dimethoxy-3-[(4-hydrophenyl)methylene]-1-H-indol-2-one) were previously reported [13]. Stock solutions were prepared in DMSO and diluted in culture medium for use (final concentration of DMSO 0.25%, even in controls). Cells were treated with the drug the day after splitting.

Cell viability was assessed by the Trypan blue dye exclusion assay or, alternatively, by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, measuring dye absorbance at 550 nm. IC₅₀ values (drug concentrations producing 50% inhibition) were calculated from dose–response curves. Where indicated, aFGF (50 ng/ml, Sigma Chemical Company, St. Louis, MO) and heparin (100 µg/ml, Serva, Heidelberg, Germany) were added to the cell culture, 24 h after splitting.

Apoptosis was detected by the terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay using the In Situ Cell Detection Kit Fluorescein (Roche, Mannheim, Germany) according to the manufacturer's instructions. Samples were analyzed by flow cytometry using the Cell Quest software (Becton Dickinson, Mountain View, CA).

Invasion assay was performed as previously described [17]. Briefly, cells exposed to vehicle or RPI-1 for 24 h, were transferred on a Matrigel-coated (BD Biosciences, San Jose CA) upper chamber of a Transwell system (Costar, Corning Inc., Corning, NY) in serum-free medium. Complete MM medium or conditioned MSCs medium was placed in the lower chamber. The drug was added in both upper and lower chambers at the same concentration used for cell pretreatment. Cells that invaded Matrigel were counted after 24 h of incubation at 37 °C. Statistical analyses were performed using Student's 2-tailed *t*-test. *P* value <0.05 was considered statistically significant.

2.3. Immunoprecipitation and Western blot analysis

Cells were processed for immunoprecipitation (FGF-R3) or total protein extraction followed by Western blotting as previously described [15]. For immunoprecipitation of FGF-R3, cell lysates were incubated with pre-swelled protein A-agarose resin (Sigma) and anti-FGF-R3 antibody (6 µg for each milligram protein of cell extract), for 2 h at 4 °C. Immunoprecipitates were then washed and eluted as described [15]. For biochemical analysis of FGF-induced effects, cells were previously serum starved for 24 h, treated with RPI-1 for the indicated times, and then stimulated with recombinant human aFGF (50 ng/ml) and heparin (10 µg/ml), in the last 5 min.

The polyclonal antibodies used were: FGF-R3 (C-15), STAT3 (K-15), JAK2 (M-126), caspase-9 (H-83) and Mcl-1 (S-19) from Santa Cruz Biotechnology (Santa Cruz, CA); p44/42 ERKs, phospho-JAK2 (Y1007, Y1008) and PARP from Upstate Biotechnology (Lake Placid, NY); phospho-FGF-R (Y653/Y654), phospho-p44/42 ERKs (T202, Y204), phospho-AKT (S473), phospho-STAT3 (Y705) and cleaved caspase-3 from Cell Signaling Technology (Beverly, MA); caspase-8 and Bcl-X from BD Pharmingen (Franklin Lakes, NJ); AKT from BD Transduction Laboratories (Lexington, KY); survivin and BCMA from Abcam (Cambridge, United Kingdom); actin from Sigma.

Table 1
Antiproliferative effect of RPI-1 on human MM cell lines (HMCLs).

HMCL ^a	t(4;14)	FGF-R3 status	RPI-1 (IC ₅₀ , µM)
OPM2	+	K650E (kinase domain, activating)	4.9 ± 0.1 (<i>n</i> = 2)
KMS11	+	Y373C (transmembrane, activating)	10.8 ± 0.8 (<i>n</i> = 3)
KMS18	+	G384D (transmembrane, not transforming)	36.3 ± 9.9 (<i>n</i> = 5)
KMS28	+	wt	27.6 ± 8.6 (<i>n</i> = 4)
KMS20	–	–	>45 (<i>n</i> = 4)
JJN3	–	–	32.4 ± 6.8 (<i>n</i> = 3)

^a KMS11 and JJN3 cell lines harbor the t(14;16) translocation. OPM2 has a translocation involving MAFB gene at chromosome 20 together with an unidentified partner chromosome. KMS20 is negative for any known chromosome translocation [23].

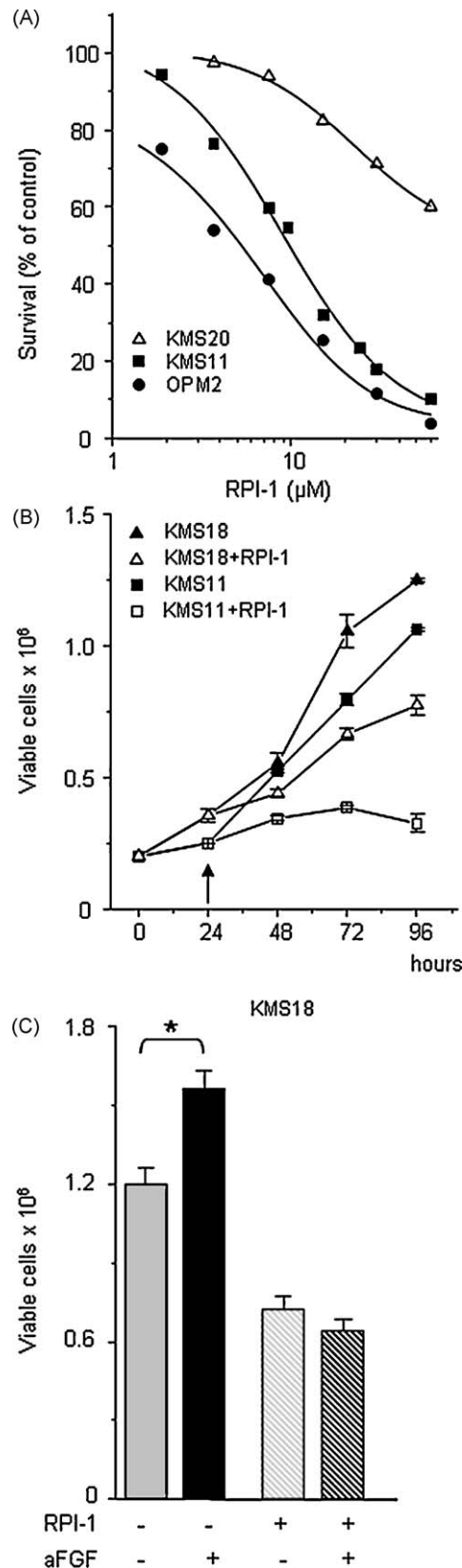


Fig. 1. Effect of RPI-1 treatment on cell viability of representative HMCLs detected by Trypan blue dye exclusion assay. (A) Mean dose–response curves obtained in at least two independent experiments (performed in duplicate) with KMS20, KMS11 and OPM2 cells incubated with the drug for 72 h. Each point represents the mean percentage of the control viable cell number value. (B) Time–course of growth inhibitory effect on KMS18 and KMS11 cell lines. Solvent or RPI-1 (25 μM) were added to cell cultures the day after splitting (arrow). Viable cells were counted every day. Data (mean ± SD) from one experiment representative of three are shown.

Monoclonal antibodies were: c-Myc (9E10) from Santa Cruz, Kip1/p27 from BD Transduction Laboratories; phosphotyrosine (pY, clone 4G10) from Upstate, and β-tubulin from Sigma.

2.4. Microarray data analysis

Total RNA extraction and purification, biotin-labeled cRNA synthesis, fragmentation and hybridization on HG-U133A Probe Arrays and arrays scanning were performed as previously described [19,20]. Samples from three independent replicas for each time point were profiled. The probe level data were converted to expression values using the MAS 5.0 algorithm, and the normalization was performed using the 'global scaling' procedure, which normalizes the signals of different experiments to the same target intensity. Supervised gene expression analyses were performed using the Gene@Work software platform, which is a gene expression analysis tool based on the pattern discovery algorithm Structural Pattern Localization Analysis by Sequential Histograms (SPLASH). Genes@Work discovered global gene expression 'signatures' that were common to an entire set of at least n experiments (the support set), where n was a user-selectable parameter called the minimum support [20,21]. Full support and the value of $\delta = 0.02$ were chosen for the analysis. For each gene, the statistical significance of the differential expression across the phenotype and control sets (gene z-score, Zg) was computed as previously described [20]. The selected probe list was visualized by means of DNAChip Analyzer (dChip) software [22]. The functional analysis on the selected lists was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Tool 2008 (U.S. National Institutes of Health)¹ and NetAffx (Affymetrix)².

3. Results

3.1. Effects of RPI-1 on HMCLs viability

We investigated the effects of the tyrosine kinase inhibitor RPI-1 on a panel of HMCLs, four of which characterized by aberrant expression of FGF-R3 as a consequence of the $t(4;14)$ translocation (Table 1). Among the $t(4;14)$ positive cell lines, OPM2 and KMS11 harbor FGF-R3 activating mutations, KMS18 has a non-transforming mutation, whereas KMS28 expresses a wild type FGF-R3 [7,23]. Representative dose–response and time–course inhibition curves obtained in cell viability assays are reported in Fig. 1A and B. OPM2 and KMS11 cells were the most sensitive to the inhibitory effect of RPI-1. The two $t(4;14)$ negative cell lines, KMS20 and JJN3, as well as KMS18 and KMS28 which harbor an FGF-R3 functionally silent in cell culture in basal conditions [7], showed lesser sensitivity (Table 1). However, cell growth enhancement of KMS18 induced by the specific FGF-R3 ligand, aFGF, was abrogated by RPI-1 treatment (Fig. 1C). The additional $t(14;16)$ chromosome translocation present in KMS11 cells [23] did not appear to influence sensitivity to RPI-1, since the same genetic lesion is present in the less sensitive JJN3 cell line.

3.2. Inhibition of FGF-R3^{Y373C} in NIH3T3 transfectants

To determine whether the high sensitivity of KMS11 and OPM2 cells was related to RPI-1 ability to interfere with FGF-R3 oncogenic activation, we first examined the effects of the drug on NIH3T3 fibroblasts stably transfected with the FGF-R3^{Y373C}

(C) Inhibition of cell growth enhancement induced by aFGF. KMS18 cells were treated with solvent (–) or 25 μM RPI-1 (+) in the absence (–) or presence (+) of aFGF/heparin as indicated. Viable cells were counted after 72 h. Bars represent mean values ± SD. One experiment representative of three is shown. (* $P < 0.0005$).

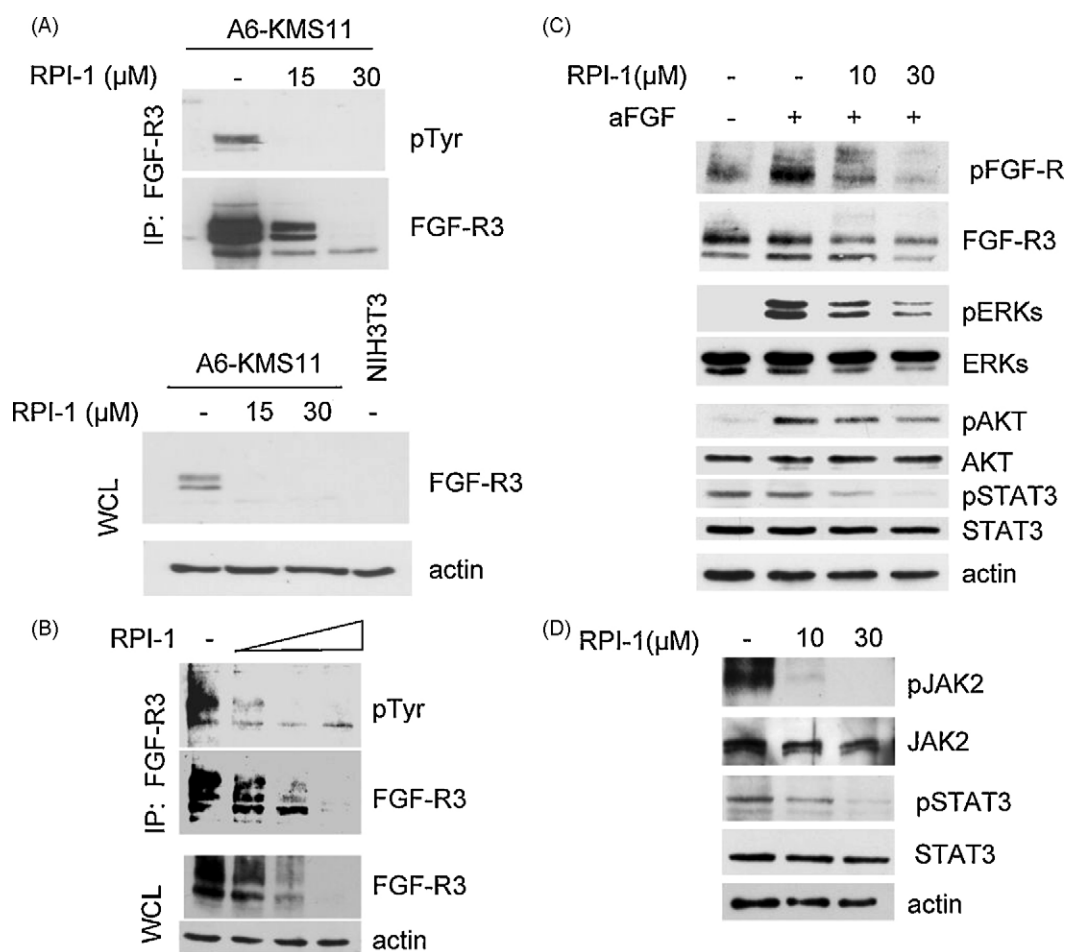


Fig. 2. Inhibition of FGF-R3- and JAK2-mediated signaling by RPI-1. (A) Inhibition of FGF-R3^{Y373C} constitutive activation and expression in NIH3T3 transfectants (A6-KMS11). Cells were treated with the indicated concentrations of the drug for 72 h. Western blot analysis with anti-pan-phosphotyrosine (pTyr) or anti-FGF-R3 antibodies was performed on FGF-R3 immunoprecipitates (IP) or whole cell lysates (WCL) as indicated. In the lower panel, a cell lysate from NIH-3T3 parent cells was run in parallel as a negative control for FGF-R3 expression. (B) Inhibition of endogenous FGF-R3^{Y373C} tyrosine phosphorylation and expression in the HMCL KMS11. Cells were treated with RPI-1 (3, 10 and 30 μM) for 24 h and processed for Western blot analysis as in (A). (C) Inhibition of aFGF-induced signaling in KMS11 cells. Serum starved cells were incubated in the presence of vehicle (–) or RPI-1 (10 or 30 μM) for 6 h. Cells were then left untreated (–) or stimulated with aFGF/heparin (+) for 5 min as indicated. The activated status of receptor and downstream pathways was analyzed in cell lysates by Western blotting with phospho-specific antibodies recognizing activated FGF-R3, ERKs, AKT and STAT3. (D) Inhibition of JAK2/STAT3 activation in KMS11 cells. Serum starved cells were treated with the indicated concentrations of the drug for 24 h. Cell lysates were analyzed by Western blotting with phospho-specific antibodies recognizing activated JAK2 or STAT3. In (C) and (D), each blot was stripped and reprobed with the respective anti-protein antibodies. Protein loading is shown by actin.

mutant present in the KMS11 cell line (A6-KMS11 cells) [7]. Upon treatment with RPI-1, FGF-R3 tyrosine phosphorylation was abolished and its expression was reduced in a dose-dependent manner (Fig. 2A). The drug effect on the receptor levels was confirmed by direct immunoblot detection in whole cell lysates. Consistent with the abrogation of the receptor oncogenic function, RPI-1 induced a reversion of the transformed morphology of A6-KMS11 cells (not shown).

3.3. Inhibition of FGF-R3- and JAK2-dependent signaling in HMCLs

The effect of RPI-1 was then examined in KMS11 cells expressing endogenous FGF-R3^{Y373C}. As observed in A6-KMS11 fibroblasts, drug treatment resulted in a marked dose-dependent inhibition of the receptor tyrosine phosphorylation and expression (Fig. 2B) which was achieved in the range of antiproliferative drug concentrations (3–30 μM corresponding to IC₂₀–IC₈₀, Fig. 1). Since FGF-R3^{Y373C} maintains biochemical responsiveness to the ligand [7], we examined the effects of RPI-1 on the receptor signaling activation in aFGF-stimulated cells. Tyrosine phosphorylation of FGF-R3^{Y373C} under serum starvation confirmed its constitutive activation (Fig. 2C). Stimulation with aFGF enhanced the receptor

signaling as indicated by the increased phosphorylation of the receptor and of the downstream transducers ERKs and AKT. Pretreatment with RPI-1 abolished aFGF-induced activation of FGF-R3^{Y373C}, weakly reduced the receptor expression and inhibited the ligand-induced activation of ERKs and AKT, in a dose-dependent manner (Fig. 2C).

According to previous reports [7,24], the constitutive activation of STAT3 in KMS11 cells was not increased by aFGF stimulation. Nonetheless, STAT3 activation was inhibited by RPI-1 treatment (Fig. 2C) thus suggesting that the drug could affect JAK2, a major receptor-associated tyrosine kinase involved in cytokine intracellular signaling mediated by STAT3 in myeloma cells including KMS11 [24]. Indeed, drug treatment inhibited JAK2 activation which is constitutive in these cells (Fig. 2D). Both JAK2 and STAT3 were completely dephosphorylated in cells exposed to RPI-1 for 24 h, whereas their expression was not affected even with more prolonged treatments (not shown).

3.4. Apoptosis induction

Because activation of signaling pathways dependent on both FGF-R3 and JAK2 is supposed to play a major role in sustaining MM

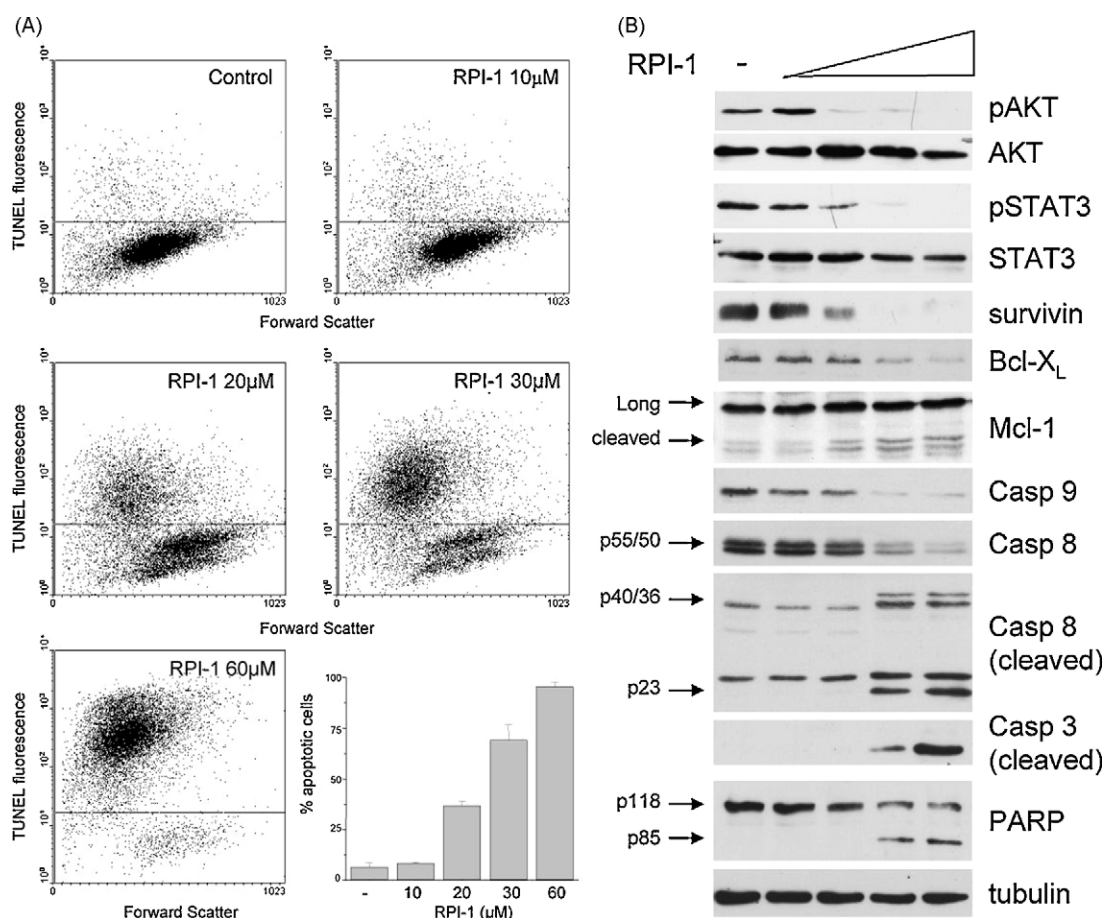


Fig. 3. Apoptosis induction by RPI-1 treatment in KMS11 cells. Cells were incubated in the presence of solvent (control) or RPI-1 (10, 20, 30 and 60 μM), for 72 h. (A) TUNEL assay analyzed by FACS. A representative dose–response experiment is shown together with quantitative data obtained in three independent experiments (panel right bottom). (B) Modulation of anti-apoptotic factors and caspase activation. Cell lysates were analyzed by Western blotting to detect activating phosphorylations of AKT and STAT3, expression of Bcl-X_L, Mcl-1 and survivin, and cleavage of caspases (9, 8 and 3) and PARP. Blots anti-pAKT and anti-pSTAT3 were stripped and reprobed with the respective anti-protein antibodies. Anti-tubulin blot shows protein loading. Quantitative data reported in (A) (panel right bottom) represent mean values of cell death induced by drug treatment both in (A) and (B). Histograms represent mean percentages of apoptotic cells ± SD.

cell survival [9], we examined whether RPI-1 treatment resulted in KMS11 cell death. As determined by TUNEL assay, drug treatment increased the number of apoptotic cells in the KMS11 cell population in a dose-dependent manner (Fig. 3A). Apoptosis was associated with the inhibition of AKT and STAT3 activation and with the downregulation of survivin and the Bcl-2 family member Bcl-X_L (Fig. 3B), known as survival factors in myeloma cells [9,25]. Expression levels of Bcl-2 were not affected by drug treatment (not shown). Likewise, the global expression of Mcl-1, another key regulator of MM survival belonging to the Bcl-2 family [9], was not reduced although an increased detection of two low molecular weight peptides by the anti-Mcl-1 antibody in treated cells (Fig. 3B) suggested the occurrence of truncated pro-apoptotic forms of Mcl-1 [26]. The involvement of both intrinsic and extrinsic apoptotic pathways in cell response to RPI-1 was confirmed by the cleavage of caspases 8, 9, and 3, and of the caspase substrate PARP. These effects were already detectable at 24 h (not shown) and still evident at 72 h of cell exposure to the drug (Fig. 3B).

3.5. Gene expression profiling

To gain further insights into the mechanisms of RPI-1 activity in HCMs, we compared the gene expression profile (GEP) of KMS11 cells prior to and after treatment. The supervised analysis of GEP data revealed, after 24 h of exposure to RPI-1, a total of 92 probe sets differentially expressed, corresponding to 22 upregulated and

52 downregulated genes (Supplementary Data, Tables 3a and 3b). Functional stratification (Fig. 4) evidenced the downregulation of genes involved in sucrose (*ALDOA*, *SORD*, *ME2*, *GPI*, *PYGB*), fatty acid (*FABP5*, *SCD*, *MECR*), protein (*IARS*), and nucleotide (*PAICS*, *NME1*, and *UCK2*) metabolism, in agreement with a decreased cellular growth. Accordingly, genes devoted to mRNA splicing (*SFRS1-2-3-7*, *SNRPF*), rRNA processing (*RRP9*, *EXOSC4*), and translation initiation (*EIF1*, *EIF3S8*, *EIF4a1*, *BZW2*) were also significantly downregulated in KMS11-treated cells. In addition, we evidenced the downregulation of genes encoding receptors (*BCMA/TNFRSF17*, *FGFR3*), chaperone proteins (*HSPA8*, *HSP90B1*, *HSPD1*, *HSPE1*), and the transcription factor *MYC*. Conversely, among the overrepresented genes we identified other transcription factors (*JUN*, *JUN-D*, *ATF3* and *KLF4*), and histones. Notably, spermidine/spermine N(1)-acetyltransferase gene (*SAT-1*) and spermidine synthase (*SRM*), two genes involved in the polyamines metabolism, were respectively up- and downregulated.

With the aim of identifying genes and/or pathways early deregulated by RPI-1, we performed the supervised analysis of GEP data from KMS11 samples upon 6 and 15 h of treatment (Supplementary Data, Tables 1 and 2). Remarkably, analysis at 6 h pointed out the downregulation of *MIP-1α* and *MIP-1β* (macrophage inflammatory protein 1-α and -β, chemokine c-c motif ligands 3 and 4) which encode crucial chemokines involved in the development of osteolytic bone lesions as well as in growth, survival and migration in MM [27,28] (Fig. S1 and Table 1b

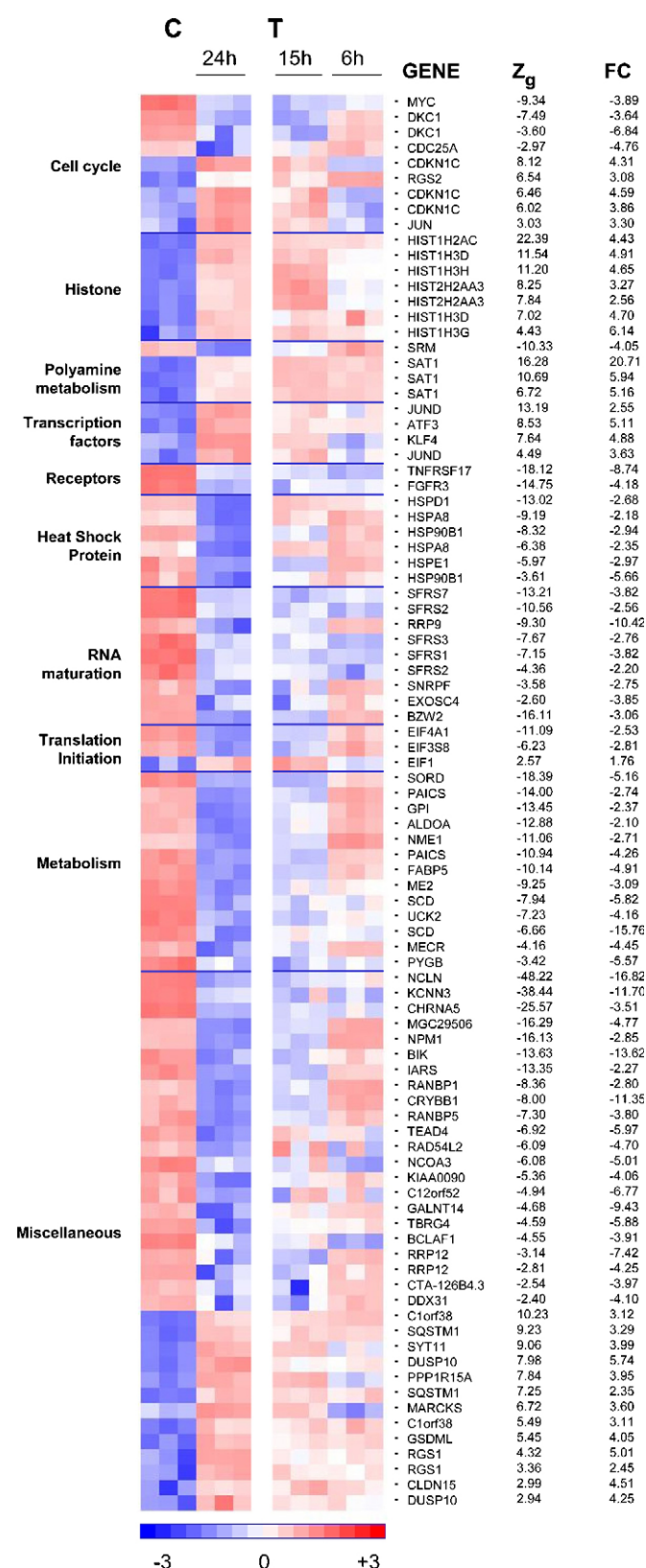


Fig. 4. Supervised gene expression analysis comparing samples from triplicates of KMS11 cells treated with 30 μ M RPI-1 (T) or vehicle (C) for 24 h. The expression levels of the identified genes in cells treated with RPI-1 for 6 h and 15 h are shown on the right side of the figure. Full support and δ value of 0.02 was used in the analysis. Genes differentially expressed in drug-treated vs control cells are grouped according to their functional categories and ranked within each category according to their Z_g score. Z_g scores and fold changes (FC) are expressed using the control group as baseline. The color scale bar represents the relative gene expression changes normalized by the standard deviation.

in Supplementary Data). In addition, we found that several histones, splicing factors (*SFRS1-3*), *SAT1*, *MYC*, and *BCMA*, a B-cell specific TNF receptor family member involved in cell growth and survival regulation of MM [29], were significantly deregulated starting from 6 h of RPI-1 treatment (Fig. 4). *FGF-R3* transcript was significantly downregulated at 15 h, although a negative expression trend was already detectable at 6 h (Supplementary Data, Fig. S1 and Table 2b). Genes involved in metabolic processes were downregulated starting from 15 h, whereas genes encoding heat shock proteins were mostly downregulated upon RPI-1 exposure for 24 h (Fig. 4). Again, the analysis of all samples of RPI-1-treated cells without any temporal segregation, revealed the deregulation of a significant fraction ($P < 0.001$) of genes involved in the MAP kinase signaling pathway, thus confirming the strong impact exerted by the drug on downstream pathways controlling cell proliferation/survival (Tables 1–3 in Supplementary Data).

Validation at the protein level of some of the transcripts modulated upon RPI-1 treatment was performed by Western blot analysis. *FGF-R3* downregulation was already detectable after 6 h of treatment and appeared preceded by a decrease in tyrosine phosphorylation (Fig. 5A). Inhibition of JAK2 phosphorylation followed similar kinetics, although JAK2 transcript/protein expression was not modulated by drug treatment (data not shown and Fig. 5A). A decrease in *BCMA* protein levels was evident starting from 15 h of RPI-1 treatment (Fig. 5B) whereas *Myc* downregulation was already detectable at 6 h (Fig. 5C). Since *Myc* is known to suppress the expression of *p27^{Kip1}* through different mechanisms [30], we also examined the protein levels of this cyclin-dependent kinase inhibitor. RPI-1-induced *Myc* downregulation was in fact reflected in *p27^{Kip1}* upregulation at any time point (Fig. 5C). Such an opposite effect of RPI-1 on *Myc* and *p27^{Kip1}* expression, together with the modulation of other cell cycle regulators (*p21* and *CDC25A*) and histone family members (Tables 1–3 in Supplementary Data), strongly suggested a cell cycle arrest of treated cells at the G1/S boundary [31]. Dose-response experiments (Fig. 5B and C) provided further validation of protein expression modulations in KMS11 cells. Inhibition of JAK2 tyrosine phosphorylation and downregulation of *BCMA* and *Myc*, were also observed in KMS18 cells, thus indicating that these RPI-1 effects are not restricted to the KMS11 myeloma cell line (Supplementary Data, Fig. S2).

3.6. Inhibition of cell growth and Matrigel invasion induced by conditioned medium from mesenchymal stromal cells (MSCs)

The concomitant inhibition of *FGF-R3*, *JAK2/STAT3*, and *BCMA* signaling pathways in RPI-1-treated KMS11 cells suggested a potential ability of the drug to interfere with the tumor cell response to stimuli provided by the BM microenvironment. To explore such issue, we tested the effects of RPI-1 on the MM cells in the presence of culture medium conditioned by BM-derived MSCs (Fig. 6). The conditioned medium significantly increased either cell growth or the cell ability to invade an artificial extracellular matrix such as Matrigel; in both assays, the enhancement was abrogated by RPI-1 treatment (Fig. 6A and B). Similarly, at the biochemical level, increased *STAT3* activation induced by the MSCs' conditioned medium was abrogated (Fig. 6C). Whereas dose-dependent inhibition of KMS11 cell growth was maintained even in the MSC medium, the growth of the MSCs was not significantly affected by the drug (not shown).

4. Discussion

MM is characterized by complex heterogeneous cytogenetic abnormalities [2]. In cooperation with the driving tumorigenic effects of genetic lesions within MM cells, the BM microenviron-

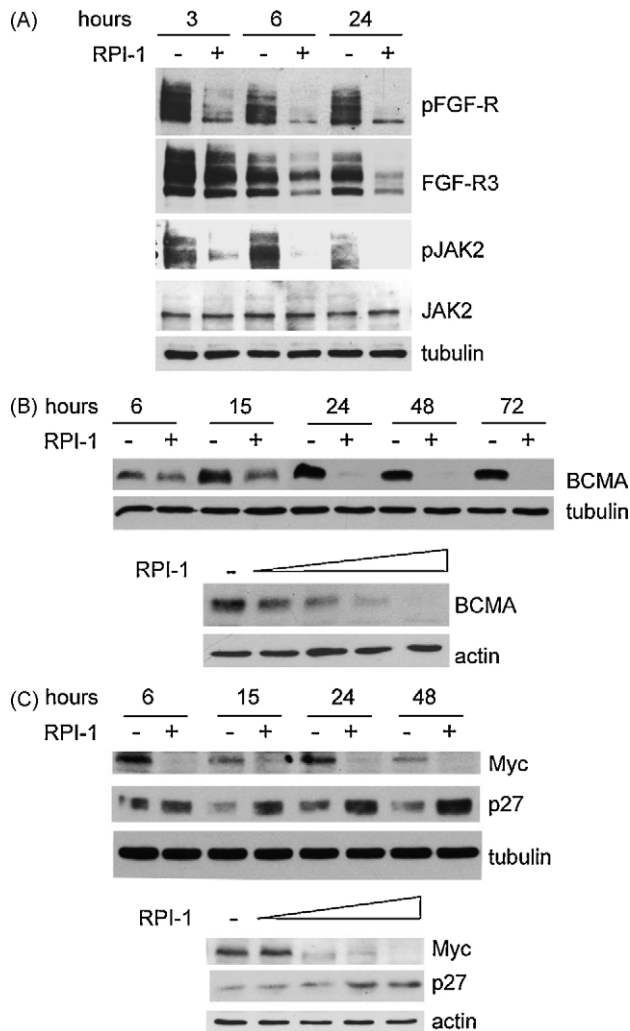


Fig. 5. Modulation of selected gene products in KMS11 cells treated with RPI-1. Whole cell lysates were analyzed by Western blotting. In time-course experiments, cells were treated with solvent (-) or 30 μM RPI-1 (+) for the indicated times. In the dose-response experiments shown in (B) and (C) cells were treated with solvent (-) or increasing drug concentrations (3, 10, 30, 60 μM) for 24 h. (A) Inhibition of FGF-R3 and JAK2 activation evidenced as abrogation of tyrosine phosphorylation (pFGF-R and pJAK2), and downregulation of FGF-R3 protein expression. (B) Downregulation of BCMA expression. (C) Downregulation of Myc and upregulation of p27 expression. Tubulin or actin blots are shown as controls for protein loading.

ment provides essential support to the propagation and expansion of malignant clones and promotes drug resistance [8,26,32]. Based on such evidence, concomitant targeting of intrinsically deregulated and BM microenvironment-sustained proliferation/survival pathways may offer a new exploitable treatment strategy in MM [8,10,26]. The present study indicates that such a goal may be approached by a single multi-target agent. In fact, the 2-indolinone tyrosine kinase inhibitor RPI-1 was shown to inhibit both FGF-R3, aberrantly expressed in *t*(4;14)-harboring MM cells, and JAK2, a major effector of IL-6 signaling. Moreover, analysis of GEP in RPI-1-treated cells revealed the downregulation of BCMA, a B-cell-specific receptor of growth factors that regulate growth and survival of MM. Thereby, three crucial signaling pathways, either dependent on oncogene activation or regulated by the BM microenvironment, were abrogated by drug treatment in MM cells.

Sensitivity to RPI-1 was higher in cells expressing an activated FGF-R3. These findings are consistent with previous reports with other FGF-R3 inhibitors indicating a strong

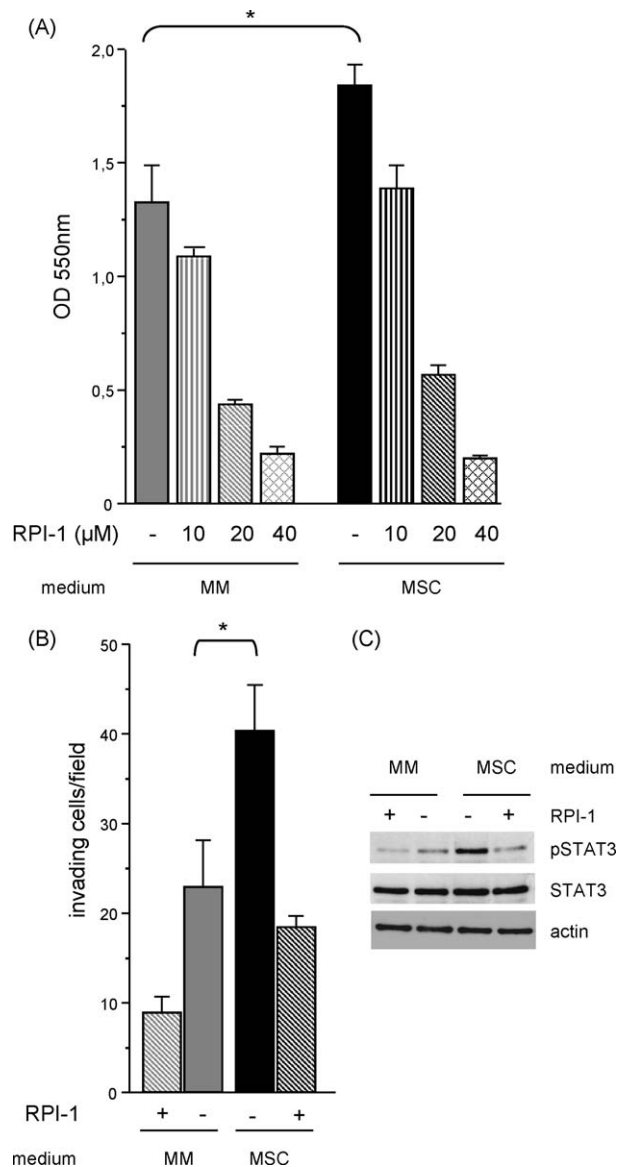


Fig. 6. Abrogation of the stimulating effects of conditioned medium from mesenchymal stromal cells (MSCs) on cell growth and invasive properties of KMS11 cells by RPI-1 treatment. (A) Cell viability assay. KMS11 cells cultured in the proper (MM) or MSCs-derived conditioned medium were treated with solvent (-) or the indicated concentrations of the drug for 72 h. Cell viability was assessed by MTT colorimetric assay and expressed as optical density (OD) at 550 nm. (B) Invasion assay. Cells were exposed to 20 μM RPI-1 for 24 h and then subjected to Matrigel invasion assay in the presence of the proper (MM) or MSCs-derived medium. Invading cells were colored and counted under microscope. Bars represent mean values \pm SD. (C) Inhibition of STAT3 activation. Cell lysates from cells treated as in (B), were subjected to Western blotting and probed with anti-phospho-STAT3 antibody (pSTAT3). The blot was then stripped and reprobed with anti-STAT3 antibody. Anti-actin blot shows protein loading. Data shown are representative of three independent experiments. (* $P < 0.0005$).

dependence of KMS11 and OPM2 cells on constitutive FGF-R3 signaling for proliferation/survival [33–35]. However, the RPI-1 ability to abrogate KMS18 cell growth enhancement provided by aFGF, suggests a potential benefit extended to *t*(4;14) MM not expressing a constitutively active FGF-R3. Our data indicate that the mechanism of FGF-R3 inhibition by RPI-1 may differ from that of other tyrosine kinase inhibitors. Indeed, RPI-1-induced FGF-R3 inactivation was achieved through both kinase inhibition and expression downregulation as detected at both RNA and protein level. This could be a peculiar aspect of the mechanism of receptor tyrosine kinase inactivation by this drug,

because similar effects were observed on Ret and Met [15–17]. Although elucidating the molecular bases of such RPI-1 mechanism of action was out of the aim of this study, it is worth noting that JAK2 protein expression, similarly to other cytoplasmic tyrosine kinases such as the Ret/ptcs oncoproteins [13,14] was not modulated by drug treatment, thus suggesting a differential target-dependent modality of tyrosine kinase inactivation.

GEP showed that RPI-1 treatment of KMS11 cells modulated genes recently described as specific components of the FGF-R3 activation pathways such as *SAT-1*, *ATF3* and *MIP-1 α /1 β* [36]. In particular, we observed a concordant modulation at every time point of *SAT-1* and *SRM*, both involved in the cellular polyamine metabolism [37]. The concerted up- and downregulation of the catabolic *SAT-1* and the biosynthetic *SRM* enzyme, respectively, is expected to result in a reduction of the cellular polyamine pools, a condition associated with growth inhibition and apoptosis [38]. Indeed, targeting the polyamine pathway may have clinical relevance in anticancer therapy³. The upregulation of *ATF3* induced by drug treatment may be relevant in anti-myeloma therapy as *ATF3* is known to inhibit IL-6 and IL-12B transcription by altering chromatin structure and restricting access to transcription factors [39]. In addition, RPI-1 led to early downregulation of *MIP-1 α* and *MIP-1 β* , two chemokines produced by MM cells and implicated in the pathogenesis of myeloma bone disease [27,28]. Besides its role in the development of osteolytic bone destruction, *MIP-1 α* might play a pivotal role in the pathogenesis of MM directly affecting cell signaling pathways that mediate growth, survival, and migration in MM cells and promoting adhesive interactions between MM and stromal cells [27]. Consistent with the potential implication of DUSP family in FGF-R3 signaling [36], GEP of RPI-1-treated cells also revealed the upregulation of DUSP10, a negative regulator of the MAP kinase cascade. Noteworthy, RPI-1 treatment down-regulated also *SFRS1*, a splicing factor-encoding gene recently described as a proto-oncogene and a potential target for cancer therapy [40].

We show here that, together with FGF-R3, JAK2 is an RPI-1 target in MM cells. The potential therapeutic relevance of JAK2 in the treatment of MM is well recognized and related to its role as an effector of the signaling cascade induced by IL-6, a major growth and anti-apoptotic factor implicated in both autocrine and paracrine MM cell stimulation within the BM milieu [8,12,41]. It has been reported that IL-6 also participate to the regulation of BCMA expression [42], thus the early and stable downregulation of this receptor detected in KMS11 cells after RPI-1 treatment was conceivably an indirect effect of JAK2/STAT3 pathway inhibition. Accordingly, drug treatment inhibited JAK2 and downregulated BCMA also in KMS18 cells. Several lines of evidence point to BCMA as a potential therapeutic target in MM [43]. It represents indeed a major receptor of the two myeloma cell growth/survival factors, B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL), produced by the BM MSCs [29]. A role for BAFF/BCMA signaling has been established in interaction and adhesion of MM cells with BM stromal cells [44]. Moreover, studies in BCMA^{-/-} mice indicate a pivotal role for the receptor in the survival of long-lived BM plasma cells [45] and high levels of BCMA have been found in malignant plasma cells [46].

RPI-1 showed the ability to overcome proliferation and pro-invasive stimuli provided to MM cells by BM-derived MSCs. These findings support that the concomitant inhibition of FGF-R3, JAK2/STAT3 and BCMA signaling pathways may, at least in part, counteract MM cell growth, survival and invasive stimuli sustained by the BM microenvironment. Our in vitro data indicate that the abrogation of growth factor-mediated signaling cascades was reflected in the inhibition of downstream pathways involving ERKs, AKT and STAT3. As a result, a perturbation of cell cycle

progression, as suggested by the modulation of cell cycle regulators including Myc, p27^{Kip1}, p21 and CDC25A, was associated with caspase-dependent apoptosis. Further analysis of the determinants of RPI-1-induced cell death showed that drug treatment affected two additional proteins implicated in the MM pathobiology, Mcl-1 and survivin, both functioning as key regulators of cell survival [25,47]. Apoptosis was in fact characterized by increased detection of low molecular weight forms of Mcl-1 and downregulation of survivin. The generation of pro-apoptotic peptides by caspase-dependent cleavage of Mcl-1 has been described as an effective mechanism contributing to MM cell apoptosis even in the absence of substantial reduction of full-length Mcl-1 levels [26]. Nonetheless, because these peptides could be observed even in untreated cells, the expression of alternatively spliced forms of Mcl-1 cannot be ruled out [48]. The decrease in survivin expression, which was not appreciated at RNA level, was likely a consequence of PI3K/AKT pathway inhibition, as previously shown by specific AKT inhibition in the MM cell context [32]. Notably, modulation of Mcl-1 and survivin, as well as AKT, may have therapeutic relevance in this disease [25,47,49].

A number of tyrosine kinase inhibitors targeting FGF-R3 or JAK2 have shown anti-myeloma activity in preclinical studies [33–35,41,50] and a few of them are currently being evaluated in phase I/II clinical trials in patients with relapsed or refractory MM³. The peculiar FGF-R3/JAK2 targeting profile exhibited by RPI-1 shows the feature to counteract multiple stimuli in MM cells, deriving by either the specific genetic alteration or the BM microenvironment. These findings provide a rationale for the development of treatment strategies resulting in a concomitant inhibition of FGF-R3 and JAK2 kinase signaling. Such therapeutic approaches may have relevance in t(4;14) positive MM which represents the highest risk prognostic variant [3,4] and may potentially contribute to overcoming resistance to conventional therapy.

Disclosure of potential conflicts of interest

The authors reported no potential conflicts of interest.

Acknowledgements

This study was supported in part by Associazione Italiana per la Ricerca sul Cancro to AN and FZ, Ministero della Salute and Alleanza Contro il Cancro. The authors thank the Medicinal Chemistry Department of Cell Therapeutics, Inc., Europe (Bresso, Italy), for the synthesis of RPI-1, Luigia Lombardi for critical review and Laura Zanesi for help in editing the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.06.023.

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