

## Novel Targeting of Phospho-cMET Overcomes Drug Resistance and Induces Antitumor Activity in Multiple Myeloma

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### Abstract

**Purpose:** The aim of the study was to verify the hypothesis that the *cMet* oncogene is implicated in chemo- and novel drug resistance in multiple myeloma.

**Experimental Design:** We have evaluated the expression levels of cMET/phospho-cMET (p-cMET) and the activity of the novel selective p-cMET inhibitor (SU11274) in multiple myeloma cells, either sensitive (RPMI-8226 and MM.1S) or resistant (R5 and MM.1R) to anti-multiple myeloma drugs, in primary plasma cells and in multiple myeloma xenograft models.

**Results:** We found that resistant R5 and MM.1R cells presented with higher cMET phosphorylation, thus leading to constitutive activation of cMET-dependent signaling pathways. R5 cells exhibited a higher susceptibility to the SU11274 inhibitory effects on viability, proliferation, chemotaxis, adhesion, and to its apoptogenic effects. SU11274 was able to revert drug resistance in R5 cells. R5 but not RPMI-8226 cells displayed cMET-dependent activation of mitogen-activated protein kinase pathway. The cMET and p-cMET expression was higher on plasma cells from patients with multiple myeloma at relapse or on drug resistance than on those from patients at diagnosis, complete/partial remission, or from patients with monoclonal gammopathy of unknown significance. Viability, chemotaxis, adhesion to fibronectin or paired bone marrow stromal cells of plasma cells from relapsed or resistant patients was markedly inhibited by SU11274. Importantly, SU11274 showed higher therapeutic activity in R5- than in RPMI-8226-induced plasmocytomas. In R5 tumors, it caused apoptosis and necrosis and reverted bortezomib resistance.

**Conclusion:** Our findings suggest that the cMET pathway is constitutively activated in relapsed and resistant multiple myeloma where it may also be responsible for induction of drug resistance, thus providing the preclinical rationale for targeting cMET in patients with relapsed/refractory multiple myeloma. *Clin Cancer Res*; 19(16); 4371–82. ©2013 AACR.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Introduction

The hepatocyte growth factor (HGF) receptor cMET is an oncogene that mediates growth, invasion, and metastasis of several tumors, including breast (1), colorectal (2), and lung carcinoma (3), and promotes angiogenesis (4). The cMET is also implicated in resistance to both chemotherapeutics (5) and inhibitors of receptors of VEGF (6) and EGF (7). The cMET and its phosphorylated content as phospho-cMET (p-cMET) are associated with poor survival in colorectal (8) and lung cancer (9), as well as with disease progression in breast cancer (10) and melanoma (11).

Borset and colleagues first described the significance of the cMET in the pathogenesis and progression of multiple myeloma (12), and then observations have been extended to other hematologic tumors (13). Multiple myeloma plasma cells express cMET and often simultaneously HGF (14)

### Translational Relevance

The cMET oncogene is implicated in tumorigenesis and chemoresistance, and it is also implicated in the pathogenesis and progression of multiple myeloma. Here, we studied sensitive and drug-resistant multiple myeloma cell lines and patients, and show that cMET pathway is involved in multiple myeloma relapse and resistance to therapy and that phospho-cMET is a marker of major response to the cMET inhibition. A novel selective c-MET inhibitor SU11274 was able to exert significant therapeutic activity in a multiple myeloma xenograft model. Our results offer a preclinical rationale for targeting this pathway in relapsed and refractory multiple myeloma.

as an autocrine loop, whereas a paracrine loop between cMET-expressing plasma cells and HGF-secreting microenvironment cells has been found (15). HGF also enhances interleukin 6, a plasma cells growth factor (16). HGF and cMET are listed in multiple myeloma-related genes (17).

HGF and the induced p-cMET are crucial in the multiple myeloma progression: HGF levels in blood and bone marrow plasma are substantially increased in newly diagnosed patients compared with healthy controls (18); its serum levels correlate with the Durie & Salmon stage (19), and high levels imply poor prognosis (20); its bone marrow plasma levels decrease with successful treatment response, whereas high pretreatment serum levels mark resistance to high-dose chemotherapy (21) and bortezomib (22).

To date, the role of cMET/p-cMET as a possible pathway mediating multiple myeloma drug resistance remains still unexplored. Also, inhibition of the pathway as a therapeutic approach to several tumors is being developed (23), whereas information on multiple myeloma remains circumstantial (14). Among novel cMET inhibitors, SU11274 is a tyrosine kinase inhibitor (TKI) highly specific for cMET that inhibits HGF-induced p-cMET and its downstream signaling (3).

Here, we studied sensitive and drug-resistant multiple myeloma cell lines and patients' plasma cells, and show that cMET/p-cMET are involved in multiple myeloma relapse and resistance to therapy, and that SU11274 offers in both *in vitro* and *in vivo* assays a preclinical rationale for targeting this pathway in patients with relapsed and resistant multiple myeloma.

### Materials and Methods

#### Patients and cell isolation

Patients fulfilling the International Myeloma Working Group diagnostic criteria (24) for multiple myeloma ( $n = 46$ ) and monoclonal gammopathy of unknown significance (MGUS;  $n = 18$ ) were studied. Patients with newly diagnosed multiple myeloma ( $n = 14$ ), patients at relapse after 1 to 3 therapy lines based on bortezomib and/or thalidomide or lenalidomide in conjunction with melphalan or doxorubicin plus prednisone or dexamethasone, or on resistant phase to these drugs ( $n = 18$ ), or on complete/partial remission ( $n = 14$ ) were studied. They were 27 men and 19 women, ages 47 to 86 years (median 63.5). The patients with MGUS were 11 men and 7 women, ages 41 to 80 years (median 61.5). Eleven patients with anemia due to iron or vitamin B<sub>12</sub> deficiency were studied as controls (25). The study was approved by the Ethics Committee of the University of Bari Medical School (Bari, Italy), and patients gave their informed consent in accordance with the declaration of Helsinki.

Bone marrow mononuclear cells (BMMC) were isolated from heparinized aspirates by the Ficoll gradient: plasma cells were obtained with magnetic anti-CD138 beads (Immunotech), whereas adherent CD138<sup>-</sup> cells were cultured separately as bone marrow stromal cells (BMSC). Fibroblasts were purified from BMSCs of 5 patients with newly diagnosed multiple myeloma through D7-FIB-conjugated (anti-fibroblasts) microbeads (Miltenyi; ref. 26), and cultured in Dulbecco's modified Eagle medium (DMEM) medium with 20% FBS (Sigma). Cells were grown to 80% confluence and incubated in serum-free DMEM medium for 48 hours. Culture supernatants were centrifuged (200 × *g* for 10 minutes), and stored at -80°C as conditioned media.

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#### Cell lines and cMET inhibition

Human multiple myeloma cell lines were RPMI-8226, MM.1S, MM.1R (American Type Culture Collection), and RPMI-8226.R5 (ref. 27; here called R5). R5 cells are resistant to melphalan, doxorubicin, bortezomib, etoposide, tunicamycin, and staurosporin (27); MM.1R cells are resistant to dexamethasone (28). The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (both from Euroclone, Milan, Italy). The selective p-cMET inhibitor SU11274 (Selleck Chemicals) was dissolved in dimethyl sulfoxide at 0.1 to 1 μmol/L for *in vitro* studies (29), whereas it was dissolved in 1% Tween 80 (Sigma-Aldrich Co.) and administered at 100 mg/kg/d for *in vivo* mouse studies (30). In other *in vivo* experiments, bortezomib (Selleck Chemicals) was dissolved in PBS and injected intraperitoneally once a week at the dose of 1 mg/kg. A monoclonal antibody to human HGF (R&D Systems, Inc.) was used at 0.1 to 0.3 μg/mL for the HGF blockade, and an anti-human cMET antibody (R&D Systems) was used at 0.5 to 2 μg/mL for the cMET blockade *in vitro*.

#### Real-time RT-PCR, immunoprecipitation, and Western blot analysis

These were conducted as described (31), and are detailed in supporting information to this manuscript (Supplementary Materials and Methods).

#### Preparation of conditioned media and ELISA

Multiple myeloma cell lines ( $1.5 \times 10^6$  cells/mL) were cultured for 24 hours in serum-free medium (SFM) 1% glutamine, then supernatants centrifuged, concentrated, and stored at -80°C as conditioned media (31). HGF was quantified in conditioned media by an ELISA (R&D Systems, Inc.).

### Fluorescence-activated cell sorting

This was conducted on BMMCs and multiple myeloma cell lines as detailed in supporting information to this manuscript (Supplementary Materials and Methods).

### Functional studies

Viability, chemotaxis, adhesion, proliferation, and apoptosis assays were conducted on both primary plasma cells and multiple myeloma cell lines as described (25, 31) and detailed in supporting information to this manuscript (Supplementary Materials and Methods).

### Microarray hybridization and Proteome Profiler assays

Multiple myeloma cell lines were treated or not (control) with SU11274 1  $\mu\text{mol/L}$  for 6 hours and total RNA extracted and quantified by Experion RNA STN-SENS analysis on EXPERION automated electrophoresis (Bio-Rad). Data were analyzed as described in supporting information to this manuscript (Supplementary Materials and Methods), and are accessible on the Gene Expression Omnibus database (accession number GSE38204; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38204>).

For the Proteome Profiler assay, cells treated as above were lysed, placed (200  $\mu\text{g}$  aliquots) on part A-B membranes (Human Phospho-Kinase array kit, R&D Systems), and spots detected by cocktail A-B of a chemiluminescent kit (LiteAblo), and quantified as for Western blot analysis.

### Multiple myeloma xenografted mice

Six- to 8-week-old nonobese diabetic (NOD) severe combined immunodeficiency (*scid*) NOD.CB17-Prkdcscid/NCrHsd mice (Harlan Laboratories) were housed according to the Institutional Animal Care and Use Committee of the University of Bari Medical School. The R5 and RPMI-8226 cell xenografting, SU11274 treatment, and histology, immunohistochemistry, and cytofluorimetry on R5 plasmocytomas are detailed in supporting information to this manuscript (Supplementary Materials and Methods).

## Results

### Multidrug-resistant multiple myeloma cells present with a constitutive activation of cMET pathway

Expression of cMET and HGF was evaluated in multiple myeloma cell lines either sensitive (RPMI-8226) or multidrug-resistant (R5) to anti-multiple myeloma drugs. R5 cells presented with lower levels of HGF (Fig. 1A) and higher levels of cMET mRNA (Fig. 1B); accordingly, cells showed lower HGF secretion (Fig. 1C) and higher total cMET and p-cMET protein expression (Fig. 1D). Expression of cMET and p-cMET was also analyzed by flow cytometry on the cells cultured in SFM: 90%  $\pm$  8% of R5 cells coexpressed cMET and p-cMET, whereas RPMI-8226 cells showed coexpression in only 52%  $\pm$  5% of cells, while 28  $\pm$  6% expressed cMET alone (Fig. 1E). Furthermore, in SFM a HGF neutralizing antibody was able to reduce markedly p-cMET expression in RPMI-8226 but not in R5 cells (Fig. 1F). Overall findings indicate that R5 cells present with a high

HGF-independent p-cMET content, that is, with a constitutive activation of the cMET receptor.

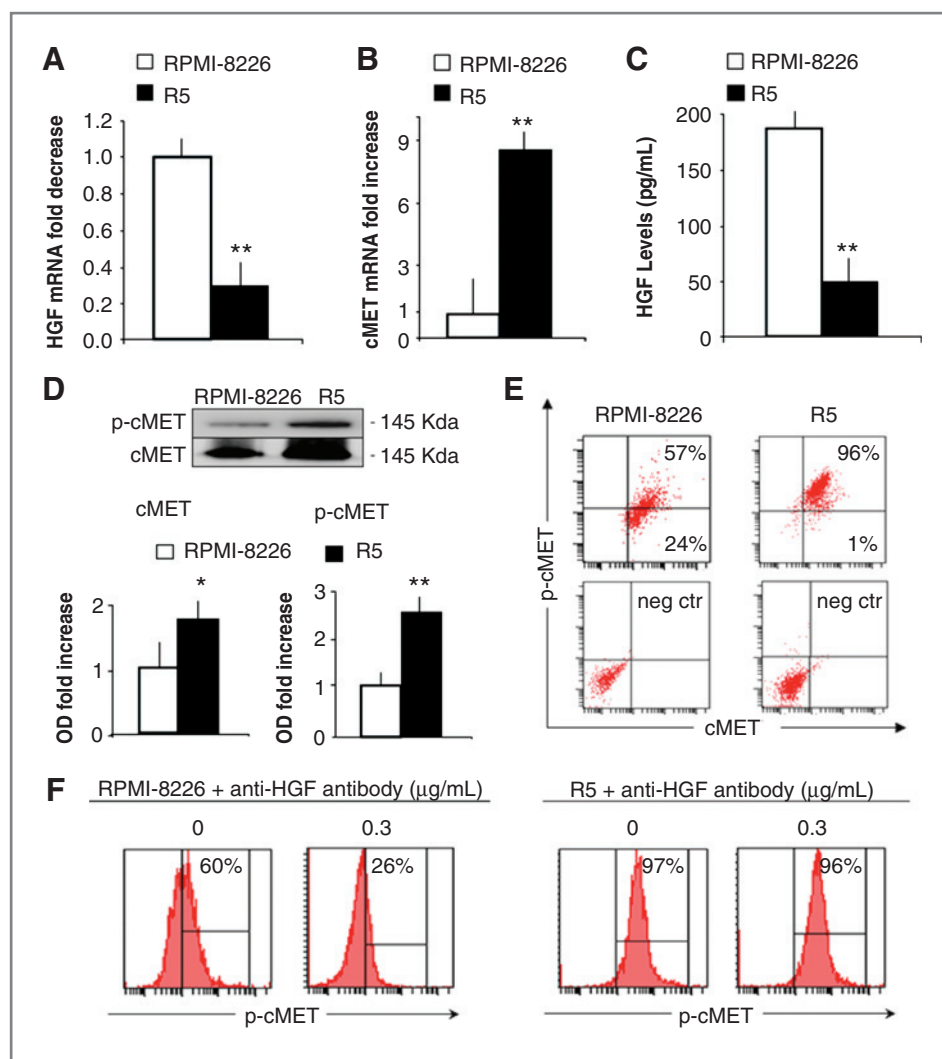
The increase of p-cMET levels on drug-resistant multiple myeloma cells was confirmed in the other pair of isogenic multiple myeloma cell lines: specifically, higher p-cMET expression was found on the dexamethasone-resistant (MM.1R) than on the dexamethasone-sensitive (MM.1S) cells (Supplementary Fig. S1A).

### SU11274 exerts major antitumor activity in multidrug-resistant multiple myeloma cells

The cMET pathway supports viability of multiple myeloma plasma cells (32) as well as their chemotaxis and adhesion by enhancing, respectively, metalloproteinase 9 (33) and VLA-4 expression (34). Therefore we tested the effects of p-cMET inhibitor SU11274 on the p-cMET expression and key cell functions in the multidrug-resistant R5 cells compared with the sensitive RPMI-8226 cells. The 6-hour SU11274 treatment (range: 0–1  $\mu\text{mol/L}$ ) inhibited the p-cMET expression more intensely in R5 cells, as assessed by flow cytometry (–63% vs. –15% as average at 1  $\mu\text{mol/L}$ ;  $P < 0.001$ ; Wilcoxon signed rank test; Fig. 2A), and Western blot analysis (Supporting Information and Supplementary Fig. S2A). The 12-hour and 24-hour treatments confirmed these results (Supplementary Fig. S3A). Similarly to R5 cells, the dexamethasone-resistant MM.1R cells reduced more intensely the p-cMET expression than the dexamethasone-sensitive MM.1S cells upon the 6-hour SU11274 treatment (–77% vs. –36% as average at 1  $\mu\text{mol/L}$ ;  $P < 0.001$ ; Wilcoxon signed rank test; Supplementary Fig. S1B). In R5 cells, SU11274 inhibited more potently: (i) chemotaxis toward both HGF (Fig. 2B) and conditioned media of bone marrow fibroblasts derived from newly diagnosed patients with multiple myeloma (Supplementary Fig. S3B) and (ii) adhesion to fibronectin (Fig. 2C) and to relapsed/resistant multiple myeloma patients' BMSCs (Fig. 2D). All inhibitory effects were dose dependent.

### The p-cMET inhibition produces apoptosis and prolonged antiproliferative effects in R5 cells, and reverts their bortezomib, melphalan, and doxorubicin resistance

Next, we evaluated the effects of SU11274 on cell apoptosis and proliferation by using propidium iodide (PI) and carboxyfluorescein succinimidyl ester (CFSE) staining, respectively. R5 and RPMI-8226 cells were cultured in SFM with SU11274 (range: 0–1  $\mu\text{mol/L}$ ) for 6, 12, and 24 hours. Multidrug-resistant R5 cells gave irrelevant percentages of spontaneous apoptosis at each time point (9%, 7%, and 8%, respectively; Fig. 3A, X, XIII, and XVI) compared with RPMI-8226 cells (19%, 30%, and 41%; Fig. 3A, I, IV, and VII) that may be related to the higher p-cMET expression (Fig. 1D and E). The SU11274 treatment induced potent apoptosis only in R5 cells: approximately 7-fold higher apoptosis levels with 1  $\mu\text{mol/L}$  with respect to 0  $\mu\text{mol/L}$  at 24 hours versus only approximately 2-fold higher in RPMI-8226 cells (Fig. 3A, XVIII and XVI vs. IX and VII). Worth of note is that the 6-hour SU11274 treatment did not induce apoptosis in both



**Figure 1.** HGF and cMET/p-cMET expression in R5 versus RPMI-8226 cells. Real-time reverse transcriptase PCR of (A) HGF and (B) cMET as mean  $\pm$  SD of 5 determinations per line. C, ELISA for HGF levels in conditioned media of the cell lines as mean  $\pm$  SD of 5 determinations per line. D, Western blot analysis of cMET and p-cMET: fold increase as optical density (OD) in R5 versus RPMI-8226 cells expressed as mean  $\pm$  SD of 5 determinations per line. E, fluorescence-activated cell sorting (FACS) analysis of cMET and p-cMET expression in R5 versus RPMI-8226 cells. The cell rate as coexpression or monoexpression is given. F, FACS analysis of cMET and p-cMET expression in R5 versus RPMI-8226 cells upon treatment with a neutralizing antibody to HGF: contrary to RPMI-8226 cells R5 cells did not reduce the p-cMET expression. A representative experiment out of 5 is shown. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; Wilcoxon signed rank test.

R5 and RPMI-8226 cells (Fig. 3A, I–III and X–XII), implying that the inhibition of p-cMET expression (Fig. 2A) and activities of R5 cells (Fig. 2B–D) that were studied within 6 hours were independent of the drug's apoptogenic effect.

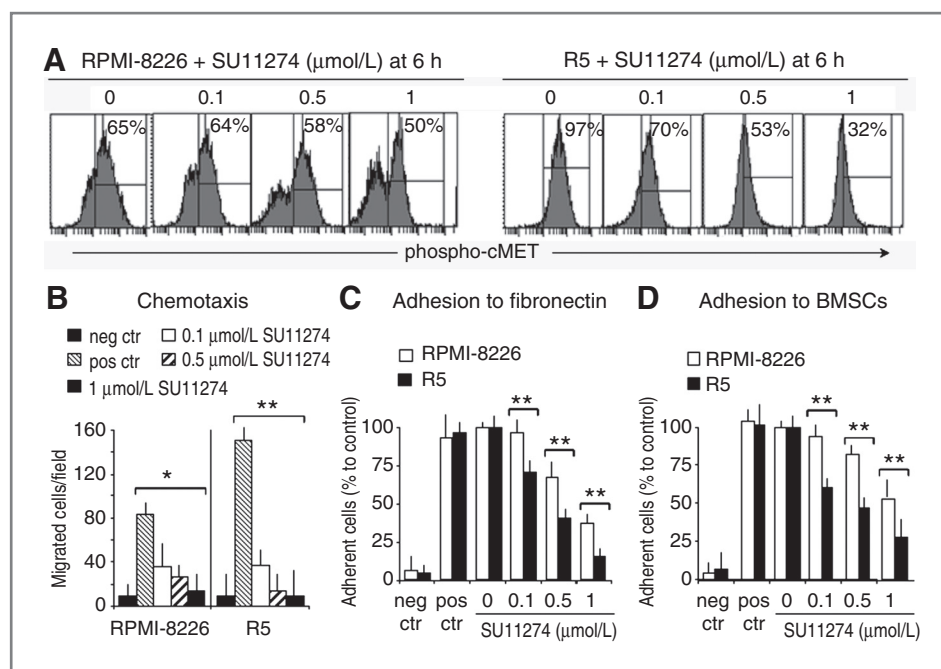
SU11247 inhibited cell proliferation at each time point more strongly in R5 cells (Fig. 3B, green squares). As an example, at 24-hour proliferating R5 cells (blue peaks) were 26% and 17% at 0.5 and 1  $\mu\text{mol/L}$ , whereas 100% at 0  $\mu\text{mol/L}$  (Fig. 3B, XVI–XVIII) versus 98%, 96% and 99% of RPMI-8226 cells (VII–IX). These findings suggest that p-cMET inhibition is able to exert a potent apoptogenic and antiproliferative effect only on the highly expressing p-cMET R5 cells.

It has been previously shown that cMET knockdown by siRNA in U266 multiple myeloma cells increases sensitivity to both bortezomib and doxorubicin (35, 36). We thus wondered whether p-cMET inhibition by SU11274 might revert the resistance to bortezomib, melphalan, and doxorubicin in R5 cells: SU11274 in combination with both bortezomib and doxorubicin successfully targeted these cells in a synergistic way [combination index (CI)  $< 1$ ; isobolo-

gram analysis; Fig. 3C]; and with melphalan gave additive effect (CI = 1; Fig. 3C). SU11274 thus rendered R5 cells as sensitive to the anti-multiple myeloma drugs as RPMI-8226 cells, suggesting that it may overcome drug resistance.

### The cMET inhibition leads to a differential modulation of genes and phosphoproteins in sensitive and multidrug-resistant multiple myeloma cells

Previous studies have analyzed R5 and RPMI-8226 cells at gene level, and shown differentially expressed genes involved in the regulation of cell survival, growth, cytostructure, cell-microenvironment contacts, cholesterol biosynthesis, and protein degradation (27). Here, we wondered whether p-cMET inhibition could lead to a differential modulation of genes in R5 cells versus RPMI-8226 cells, and found that the SU11274 treatment (1  $\mu\text{mol/L}$  for 6 hours) modulated the expression of 2,660 genes (1,336 upregulated and 1,324 downregulated) in R5 cells, whereas of 2,186 genes (1,129 upregulated and 1,057 downregulated) in RPMI-8226 cells, of which 861 were shared by both cells; among these, 83.5% were concordantly up- or



**Figure 2.** SU11274 inhibits p-cMET expression and cell activities more intensely in R5 cells. A, SU11274-treated RPMI-8226 and R5 cells (range 0–1  $\mu\text{mol/L}$ , 6 hours) were assessed by flow cytometry for p-cMET expression: more pronounced lowering of p-cMET expression in R5 cells (–63% vs. –22% as average;  $P < 0.001$ ; Wilcoxon signed rank test). B, chemotaxis assay toward HGF: higher chemotactic activity and major inhibitory effects by SU11274 in R5 cells. Adhesion to (C) fibronectin- and (D) relapsed/resistant multiple myeloma patients' BMSCs-coated plates: more intense inhibition by SU11274 on R5 cells. Chemotaxis assay: negative control (neg ctr) = SFM in lower chamber. Adhesion assay: neg ctr = bovine serum albumin 1%; positive control (pos ctr) = poly-L-lysine. Data are given as mean  $\pm$  SD of 5 independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; Wilcoxon signed rank test.

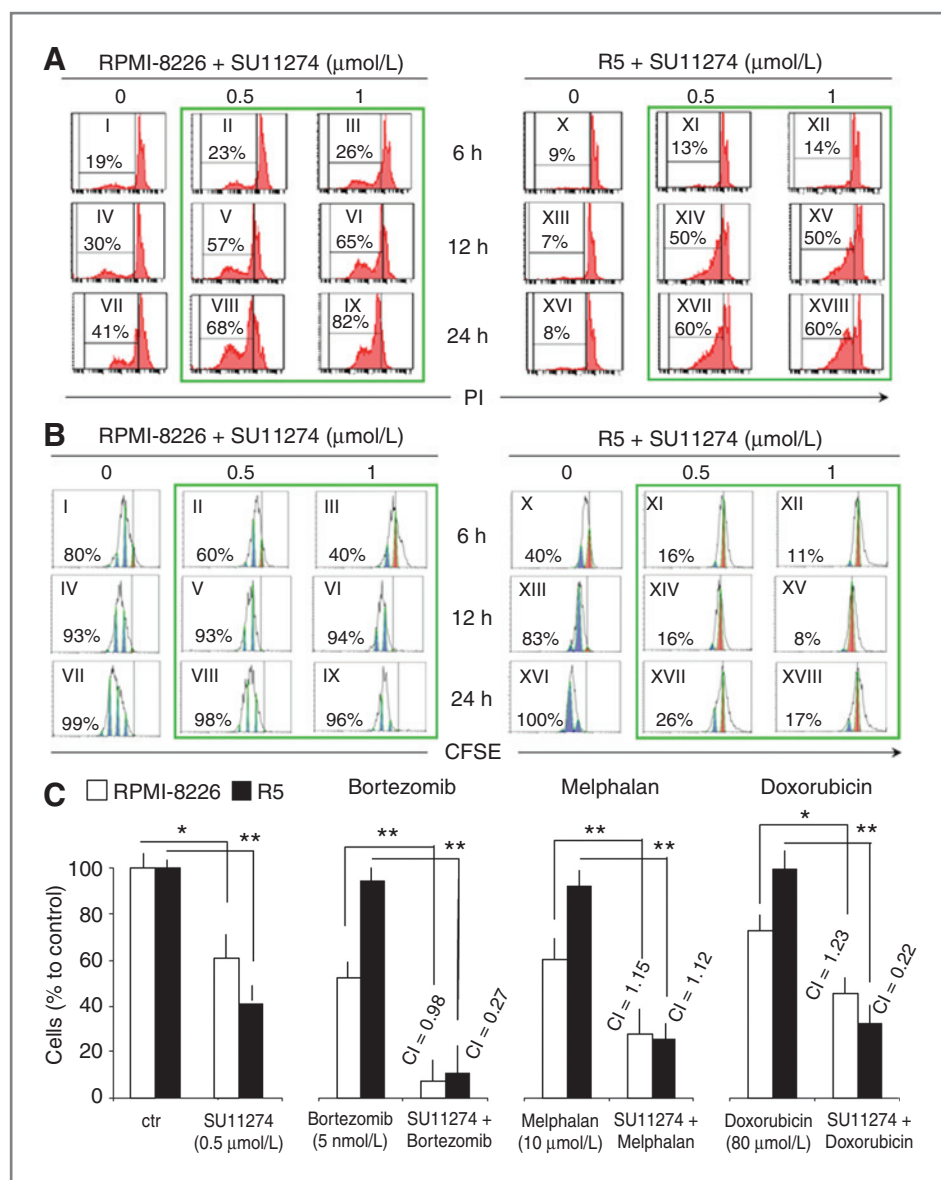
downregulated, whereas 16.5% showed an opposite behavior (detailed results available online at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38204>). Functional analysis of the differentially expressed genes revealed that more pathway maps were modulated in R5 cells by the SU11274 treatment (Supplementary Table S1): genes involved in metabolic pathways (carbohydrate, nucleotide, and amino acid metabolism), signal transduction (ErbB and Wnt signaling); immune and endocrine systems (Fc $\gamma$ R-mediated phagocytosis, T- and B-cell receptor signaling, insulin, adipocytokine and neurotrophin signaling) as well as human cancers. Conversely, RPMI-8226 cells modified the expression of genes involved in nucleotide excision repair, cytokine–cytokine receptor interactions, antigen processing, and axon guidance (Supplementary Table S1).

The baseline phosphoproteome profile of phospho-proteins (p-proteins) closely involved in tumorigenesis and tumor progression (37) showed that R5 cells differentially express 22 out of 46 kinases, as compared with RPMI-8226 cells (Fig. 4A). Specifically, R5 cells presented with higher phosphorylation rate of proteins belonging to several pro-survival pathways, such as mitogen-activated protein kinases (MAPK), Janus-activated kinase (JAK)/STAT, phosphoinositide 3-kinase (PI3K)/Akt, mTOR, Src pathways, and modulation of AMPK $\alpha$ 1 and eNOS kinases, together with reduced phosphorylation levels of p53 isoforms and Creb. The differential effects of SU11274 in modulating p-proteins in R5 versus RPMI-8226 cells were: inhibition of the MAPK pathway, reinforcement of downregulation of some p53

isoforms, Pyk2, Creb and eNOS, and enhancement of Lyn, Src, and mTOR p-proteins (Fig. 4B). Similar results were obtained using an anti-cMET neutralizing antibody (Supplementary Fig. S4). Overall data suggest that multidrug resistant, highly expressing p-cMET R5 cells are dependent on the cMET receptor phosphorylation especially for the downstream modulation of the MAPK pathways that are primarily involved in multiple myeloma cell growth and survival (38).

#### cMET and p-cMET expressions reflect the multiple myeloma disease status

We investigated whether cMET and p-cMET levels could change in patients with multiple myeloma with newly diagnosed disease, relapse, resistance to anti-multiple myeloma drugs, and remission phase disease. The cMET/p-cMET coexpression on gated CD38<sup>+</sup>CD138<sup>+</sup> BMMCs showed the highest coexpression on plasma cells from both relapsed and resistant patients (95%  $\pm$  15% positive cells; Fig. 5A) much in the same way as R5 cells. Conversely, patients with newly diagnosed multiple myeloma presented with little coexpression (20%  $\pm$  8%;  $P < 0.01$ ; Wilcoxon signed rank test), together with a variable expression of cMET alone (28%  $\pm$  22%) much in the same way as RPMI-8226 cells. Patients with multiple myeloma with complete/partial remission and patients with MGUS displayed irrelevant coexpression (0.1%–3%). The 6-hour SU11274 treatment (range: 0–1  $\mu\text{mol/L}$ ) of plasma cells from patients with relapsed/resistant disease reduced more strongly the p-cMET expression



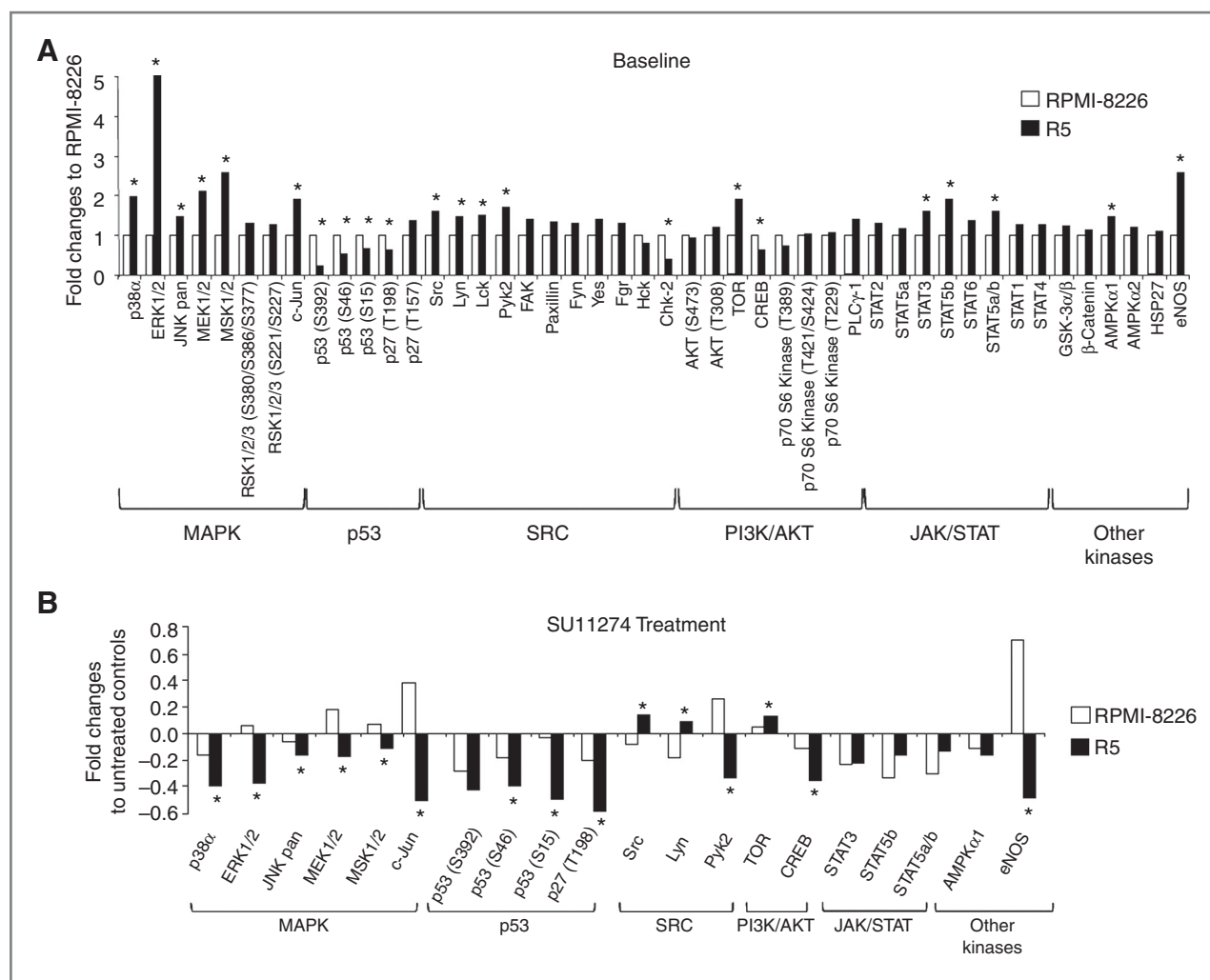
**Figure 3.** SU11274 induces cell apoptosis, inhibits cell proliferation, and overcomes resistance to anti-multiple myeloma drugs in R5 cells. Fluorescence-activated cell sorting (FACS) analysis of (A) cell apoptosis, as PI content, and (B) cell proliferation, as CFSE dilution, upon SU11274 treatment at the indicated doses and time points. A, percentages of apoptotic cells: main apoptogenic effect (green rectangles) versus untreated cells only in R5 cells (~7-fold more apoptotic R5 cells vs. ~2-fold more RPMI-8226 cells at each dose and time point). B, proliferating cells (as blue peaks) in a representative experiment out of 5: more intense and persistent antiproliferative effect (green rectangles) only in R5 cells (~10 and ~5-fold less proliferating R5 cells at 12 and 24 hours with 1 μmol/L SU11274 vs. no effects in RPMI-8226 cells). Red peaks represent not proliferating cells. C, cell cytotoxicity of R5 versus RPMI-8226 cells treated with bortezomib, melphalan, and doxorubicin and/or SU11274 for 24 hours: note the loss of resistance to the above drugs after the SU11274 treatment. The CI <1.0 indicates synergism, = 1 additive effect (isobologram analysis). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; Wilcoxon signed rank test.

than plasma cells from newly diagnosed patients ( $-66\%$  vs.  $-21\%$  as average at  $1 \mu\text{mol/L}$ ;  $P < 0.001$ ; Wilcoxon signed rank test; Fig. 5B; Supporting Information and Supplementary Fig. S2B), overlapping what was found in R5 versus RPMI-8226 cells (Fig. 2A). Also, inhibition by SU11274 of HGF-driven chemotaxis and cell adhesion to both fibronectin and paired BMSCs were more evident in plasma cells from relapsed/resistant patients (Fig. 5C-E) than in those from newly diagnosed patients (Supporting Information and Supplementary Fig. S5A-S5C). All effects were again dose dependent. Finally, the 24-hour drug treatment induced, dose dependently, cytotoxicity on plasma cells from relapsed/resistant patients (Fig. 5F), but not on BMSCs from control patients (Fig. 5G), and reverted the bortezomib resistance of these plasma cells through a synergistic interaction (Fig. 5H). In contrast, minimal cytotoxic effects were

observed at higher doses in plasma cells from newly diagnosed patients (Supporting Information and Supplementary Fig. S5D) together with little additive cytotoxicity with bortezomib (Supporting Information and Supplementary Fig. S5E). Overall results confirm that highly expressing p-cMET plasma cells from relapsed/resistant patients are more prone to be inhibited by SU11274 much in the same way as R5 cells.

#### SU11274 targets multiple myeloma cells *in vivo*

The *in vitro* findings were validated using NOD/scid mice, which were xenotransplanted with R5 or RPMI-8226 cells, and treated with SU11274 ( $100 \text{ mg/kg/d}$ ; *per os*; ref. 30) or vehicle. SU11274 was able to delay the growth of R5 (Fig. 6A) more strongly than that of RPMI-8226 plasmocytomas (Supplementary Fig. S6A), indicating significant antitumor



**Figure 4.** The p-cMET inhibition leads to modulation of p-proteins in multiple myeloma cell lines. A, phospho-proteome profile of 46 p-kinases in R5 versus RPMI-8226 cells showing that the former express differentially 22 of them. B, phospho-proteome profile of the same p-kinases as fold changes in SU11274-treated R5 and RPMI-8226 cells (1  $\mu$ mol/L, 6 hours) versus their untreated controls. Data given as average of 3 independent experiments. \*,  $P < 0.03$  or better; Wilcoxon signed rank test.

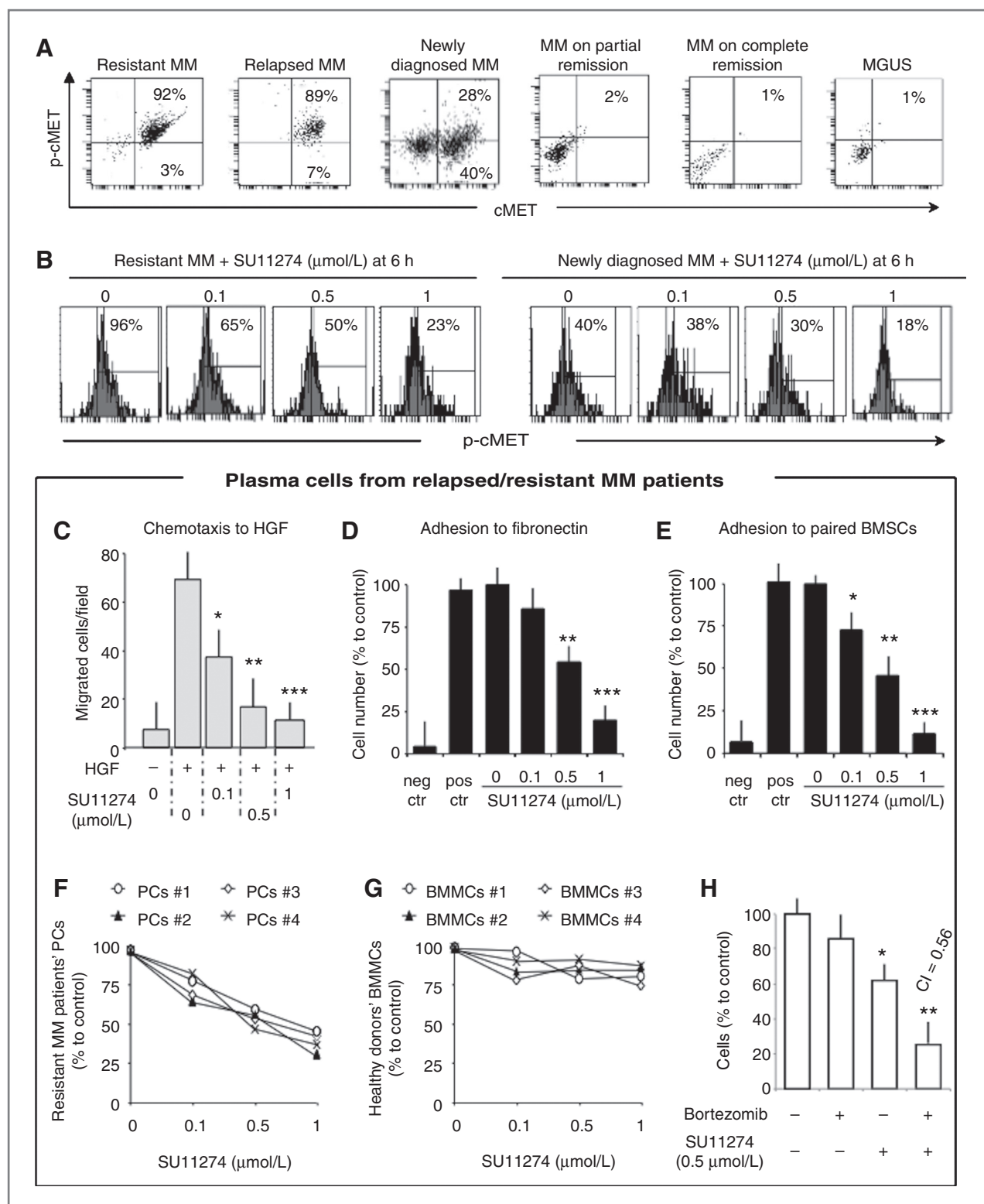
activity in multidrug-resistant multiple myeloma cells ( $P < 0.05$  or better; two-way ANOVA and Bonferroni posttest). Furthermore, SU11274 overcame bortezomib resistance: when R5 plasmocytomas were treated with SU11274 and bortezomib, the tumor growth was significantly slowed down compared with bortezomib alone (Supplementary Fig. S6B;  $P < 0.001$ ; log-rank test and Bonferroni test).

Moreover, SU11274-treated mice presented with a longer survival as compared with vehicle-treated mice (Fig. 6B;  $P < 0.01$ ; log-rank test and Bonferroni test). Importantly, in the treatment period no signs of toxicity were observed, as assessed by closely monitoring mice for clinical condition and body weight (data not shown). These findings were further corroborated by evaluating necrosis and apoptosis in harvested plasmocytomas: SU11274-treated mice, as compared with vehicle-treated mice, presented with a significantly higher percentage of mean necrotic area (Fig. 6C;  $P <$

0.01; Wilcoxon signed rank test), together with a significantly higher percentage of apoptotic cells (Fig. 6D;  $P < 0.01$ ; Wilcoxon signed rank test). Finally, flow cytometric analysis on tumor cells harvested from plasmocytomas of SU11274-versus vehicle-treated mice showed very low p-cMET expression in the former (Fig. 6E, I and II, red squares), together with higher percentages of apoptotic cells (III and IV, green squares), implying that the *in vivo* apoptogenic effect of SU11274 is due to specific inhibition of p-cMET. These findings were corroborated by low immunohistochemical staining with p-cMET of tumor sections from the SU11274-versus vehicle-treated mice (Fig. 6F).

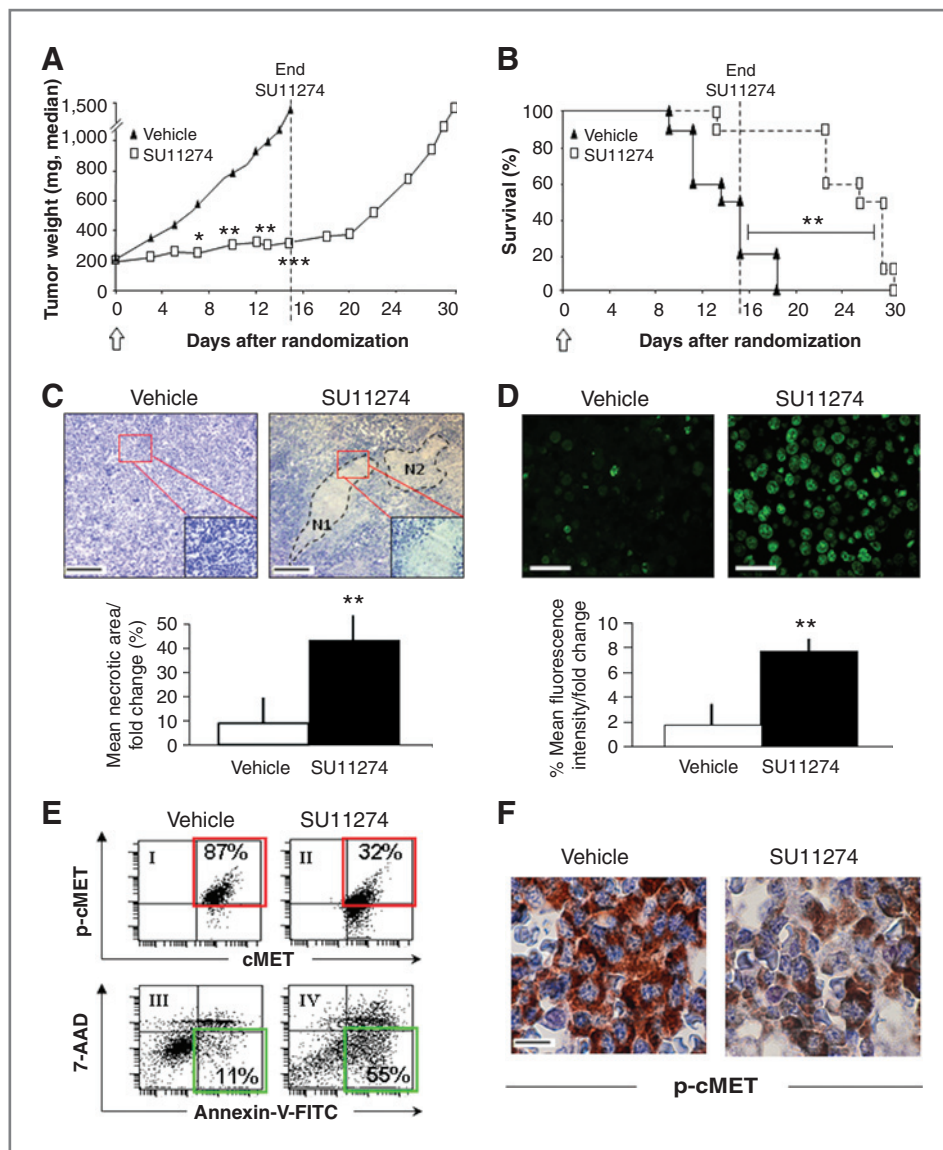
## Discussion

Here, preclinical and clinical evidence showing that the cMET/p-cMET pathway participates to the multiple myeloma patients' multidrug resistance is provided. Indeed, both



**Figure 5.** cMET and p-cMET expression in multiple myeloma (MM) patients' plasma cells (PC) and effects of the SU11274 treatment. A, fluorescence-activated cell sorting (FACS) analysis of representative patients at different disease phases. The cell rate is given as cMET+ positive events (percentages in right bottom), and double-positive events (percentages in right top). B, inhibitory effect of the 6-h SU11274 treatment (range 0–1 μmol/L) on the p-cMET expression of PCs from a representative resistant and a patient with newly diagnosed multiple myeloma. Note the more intense, dose-dependent effect in the resistant patient. C, chemotaxis and (D) adhesion to fibronectin- or (E) to paired BMSCs of PCs from patients with relapsed and resistant multiple myeloma: note, again, the





**Figure 6.** SU11274 delays R5 plasmocytoma growth *in vivo*. NOD/*scid* mice xenografted with R5 cells were treated with vehicle or SU11274 (10 mice per group). **A**, tumor growth curves after randomization as mean tumor weight. Black triangle, vehicle; empty rectangle, SU11274. Empty arrow, start of the SU11274 treatment; dot line, end of the treatment. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; two-way ANOVA test followed by Bonferroni posttest. **B**, overall survival: \*\*,  $P < 0.01$ ; log-rank test and Bonferroni test. Empty arrow, start of the SU11274 treatment; dot line, end of the treatment. **C**, histologic analysis displaying necrotic areas in plasmocytoma sections from the SU11274-treated mice and their lacking in the vehicle-treated ones (particular in the inserts). Original magnification  $\times 100$ ; inserts  $\times 200$ . Scale bar, 100  $\mu\text{m}$  at  $\times 100$ . **D**, tumor apoptosis rate assessed by TUNEL fluorescence staining. Original magnification  $\times 400$ . Scale bar, 30  $\mu\text{m}$ . \*\*,  $P < 0.01$ ; Wilcoxon signed rank test. **E**, fluorescence-activated cell sorting (FACS) analysis of cMET/p-cMET expression and apoptosis as annexin-V/7-AAD staining (red and green squares, respectively) on R5 cells harvested from representative SU11274- and vehicle-treated mice. Note the greater reduction of p-cMET and the higher apoptosis in a representative SU11274-treated mouse. **F**, p-cMET immunohistochemical staining on plasmocytoma sections of representative SU11274- and vehicle-treated mice: note pronounced lowering of p-cMET in the SU11274-treated mouse. Original magnification  $\times 600$ . Scale bar, 10  $\mu\text{m}$ . AAD, 7-amino-actinomycin D; FITC, fluorescein isothiocyanate.

cMET and p-cMET are overexpressed in multiresistant R5 and dexamethasone-resistant MM.1R cells compared with their sensitive counterparts (Fig. 1A–E and Supplementary

Fig. S1). We obtained similar results in plasma cells from relapsed/resistant compared with patients with newly diagnosed multiple myeloma, or to patients in complete/partial

intense, dose-dependent inhibition by SU11274 on the PCs activities. Chemotaxis assay: negative control (neg ctr) = SFM in lower chamber. Adhesion assay: neg ctr = bovine serum albumin 1%; positive control (pos ctrl) = poly-L-lysine. Data given as mean  $\pm$  SD of the 18 patients. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; Wilcoxon signed rank test. Cytotoxic effect of 24-hour SU11274 treatment on (F) PCs from 4 representative patients with resistant multiple myeloma, but not (G) on BMMCs from 4 representative control patients. H, SU11274 0.5  $\mu\text{mol/L}$  reverts bortezomib resistance in PCs from 5 resistant patients. Data are expressed as mean  $\pm$  SD. The CI  $< 1.0$  indicates synergism (isobologram analysis). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; Wilcoxon signed rank test.

remission, or with MGUS (Fig. 5A). Data are in line with the Børset's group evidence that cMET expression parallels multiple myeloma progression (12), and with others who emphasized the role of the cMET pathway in multiple myeloma plasma cells proliferation (32), survival (32), adhesion (34), and migration (39).

R5 cells are resistant to several clinically relevant anti-multiple myeloma agents including bortezomib, melphalan, and doxorubicin (27), and show a constitutive activation of cMET receptor (Fig. 1D and E) in terms of the p-cMET content that may entail a therapeutic target (13, 14). However, R5 cells did not show higher HGF secretion (Fig. 1C) and differently from RPMI-8226 did not reduce p-cMET expression upon treatment with a HGF neutralizing antibody: this implies that alternative mechanisms (i.e., receptor activation due to transcriptional upregulation or impaired ligand-receptor internalization; ref. 23) could be involved in constitutive cMET pathway activation in R5 cells. Notably, cMET gene mutations or amplifications have not been reported in multiple myeloma plasma cells so far (13).

The novel selective p-cMETTKI SU11274 was able to exert a marked apoptogenic and antiproliferative effect on R5 cells, and to revert their bortezomib and doxorubicin resistance through a synergistic interaction with these drugs (Fig. 3C). Similar results were observed in plasma cells from patients with relapsed/resistant multiple myeloma but not in cells from newly diagnosed patients (Fig. 5H; Supporting Information and Supplementary Fig. S2). Moreover, the greatest the expression of p-cMET the highest the response to the SU11274: this drug was more apoptogenic and inhibitory on adhesion and chemotaxis of R5 compared with RPMI-8226 cells, and on plasma cells from relapsed/resistant than from patients with newly diagnosed multiple myeloma. These *in vitro* results point to overactivation of the cMET pathway in terms of p-cMET expression in plasma cells as a possible marker of both multidrug resistance and response to the cMET inhibition. Findings agree with those shown in a glioblastoma *in vivo* model in which tumors expressing high p-cMET are more prone to the cMET inhibition (40). Accordingly, results obtained in R5 cells and in relapsed/resistant patients suggest that p-cMET besides being a marker of multidrug resistance offers a strong rationale to apply cMET inhibitors as a plausible new therapeutic tool for anti-multiple myeloma therapy in patients of this type.

Wader and colleagues showed immunohistochemically that HGF and cMET are coexpressed in multiple myeloma patients' plasma cells, and that cMET does exist in its phosphorylated state in a relevant proportion of patients, implying that the HGF/cMET system is operative in patients with multiple myeloma *in vivo* (41). The expression of cMET and p-cMET was closely confined to multiple myeloma plasma cells but absent on plasma cells of healthy subjects and MGUS, pointing to cMET and p-cMET as one of the factors that distinguishes malignant from normal plasma cells (41). Here, by using cytofluorimetry on plasma cells from patients with multiple mye-

loma at different disease phases and from patients with MGUS, we observed that the p-cMET amount increases in step with disease progression (Fig. 5A), and that SU11274 exerts a marked cytotoxic effect on plasma cells from relapsed/resistant patients (Fig. 5F) but not from newly diagnosed ones (Supporting Information and Supplementary Fig. S2). Hence, patients with multiple myeloma with plasma cells displaying high p-cMET expression, for example, those in relapsed and resistant phase are ideal candidates for applying novel cMET inhibitors in clinical trials. In this regard, a phase II trial evaluating therapeutic activity of the cMET inhibitor ARQ-197 (tivantinib) in relapsing patients is ongoing [www.clinicaltrials.gov; id no. NCT01447914].

Gene expression profiles in SU11274-treated RPMI-8226 and R5 cells revealed that these cell lines react differently to the cMET inhibition. A greater number of differentially expressed genes, hence more pathway maps, were enriched in R5 than RPMI8-226 cells (Supplementary Table S1), suggesting that the former are more addicted to the cMET pathway. Phosphoproteome studies also revealed that R5 differ from RPMI-8226 cells in 22 out of 46 p-proteins, which are involved in the JAK, the MAPK, and the PI3K/Akt pathway together with other p-proteins of the Src (Fig. 4A). SU11274 inhibitor modulated differently these signal pathways in R5 versus RPMI-8226 cells (Fig. 4B), and was able to significantly inhibit phosphorylation of proteins entailed with the MAPK pathway (Fig. 4B). These results suggest that multidrug-resistant R5 but not RPMI-8226 cells become dependent on the cMET receptor for the downstream activation of the MAPK kinase pathway, which is especially involved in multiple myeloma cell growth and proliferation (38). Accordingly, SU11274 exerts a potent and persistent antiproliferative activity on R5 but not RPMI-8226 cells (Fig. 3B).

Finally, we evaluated the anti-multiple myeloma activity of SU11274 in a R5 plasmocytoma xenografted mouse model, and showed that by inducing massive necrosis and apoptosis associated to reduction of p-cMET expression, this inhibitor is endowed with potent anti-multiple myeloma activity without determining any toxicity sign. To the best of our knowledge, only another preclinical study evaluated the anti-multiple myeloma efficacy of cMET inhibition in a mouse model by using the HGF competitor NK4 (42). All these results offer both in, *in vitro* and *in vivo*, a preclinical rationale for targeting cMET pathway in patients with relapsed and resistant multiple myeloma, and point to p-cMET expression on plasma cells as a potential biomarker of response to novel cMET inhibitors.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Disclaimer

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# Clinical Cancer Research

## Novel Targeting of Phospho-cMET Overcomes Drug Resistance and Induces Antitumor Activity in Multiple Myeloma

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