Novel targeting of phospho-cMET overcomes drug resistance and induces anti-tumor activity in multiple myeloma

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Author contributions

MM planned and performed experiments and wrote the manuscript; ABo; MAF, AF, LR, RR, AGS, ABa; FF and SR performed experiments; NG and BR contributed material; MC, BN, EM, and DR contributed material and commented on the manuscript; AMR commented on the manuscript; and AV supervised the experiments, provided financing and wrote the manuscript.

List of abbreviations

HGF, hepatocyte growth factor; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; BM, bone marrow; BMSCs, bone marrow stromal cells; BMMCs, bone marrow mononuclear cells; PCs, plasma cells; ECs, endothelial cell; CM, conditioned medium; SFM, serum-free medium; CFSE, carboxyfluorescein N-succinimidyl ester; 7-AAD, 7-aminoactinomycin; MAPK, mitogen-activated protein kinase.
List of online Supporting information

- Supplementary Materials and Methods
- Figure S1: cMET/phospho(p)-cMET expression in MM.1R vs. MM.1S cells.
- Figure S2: Effect of SU11274 treatment on cMET/phospho(p)-cMET expression in (A) R5 vs. RPMI8226 cells; and (B) plasma cells (PCs) from relapsed/resistant vs. newly diagnosed MM patients.
- Figure S3: SU11274 inhibits p-cMET expression and chemotaxis toward fibroblasts’ conditioned medium more intensely in R5 cells.
- Figure S4: An anti-cMET neutralizing antibody leads to modulation of phospho (p)-proteins in MM cell lines.
- Figure S5: Effect of SU11274 treatment on PCs of newly diagnosed MM patients.
- Figure S6: SU11274 impacts marginally on in vivo growth of RPMI8226 plasmocytomas. It reverts bortezomib-resistance in R5 plasmocytomas.

STATEMENT OF TRANSLATIONAL RELEVANCE

The cMET oncogene is implicated in tumorigenesis and chemoresistance and it is also implicated in the pathogenesis and progression of multiple myeloma (MM). Here we studied sensitive and drug-resistant MM cell lines and patients, and show that cMET pathway is involved in MM relapse and resistance to therapy and that p-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 MM xenograft model. Our results offer preclinical rationale for targeting this pathway in relapsed and refractory MM.
ABSTRACT

Purpose: aim of the study was to verify the hypothesis that the cMET oncogene is implicated in chemio- and novel drug- resistance in MM.

Experimental design: we have evaluated the expression levels of cMET/p-cMET and the activity of the novel selective p-cMET inhibitor (SU11274) in MM cells, either sensitive (RPMI8226 and MM.1S) or resistant (R5 and MM.1R) to anti-MM drugs, in primary PCs and in MM xenograft models.

Results: we found that resistant R5 and MM1R 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 RPMI8226 cells displayed cMET-dependent activation of mitogen-activated protein kinase pathway. The cMET and p-cMET expression was higher on PCs from MM patients at relapse or on drug-resistance than on those from patients at diagnosis, at complete/partial remission, or from patients with MGUS. Viability, chemotaxis, adhesion to fibronectin or paired BMSCs of PCs from relapsed or resistant patients was markedly inhibited by SU11274. Importantly SU11274 showed higher therapeutic activity in R5- than in RPMI8226-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 MM, where it may also be responsible for induction of drug resistance, thus providing the preclinical rationale for targeting cMET in relapsed/refractory MM patients.
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 vascular endothelial growth factor (6) and epidermal growth factor (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).

Børset et al. first described the significance of the cMET in the pathogenesis and progression of multiple myeloma (MM) (12), then observations have been extended to other hematological tumors (13). MM plasma cells (PCs) express cMET and often simultaneously HGF (14) as an autocrine loop, while a paracrine loop between cMET-expressing PCs and HGF-secreting microenvironment cells has been found (15). HGF also enhances IL-6, a PCs growth factor (16). HGF and cMET are listed in MM-related genes (17).

HGF and the induced p-cMET are crucial in the MM progression: HGF levels in blood and bone marrow plasma are substantially increased in newly diagnosed patients compared to 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, while high pre-treatment 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 MM drug resistance remains still unexplored. Also, inhibition of the pathway as a therapeutic approach to several tumors is being developed (23), while information on MM 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 MM cell lines and patients’ PCs, and show that cMET/p-cMET are involved in MM relapse and resistance to therapy, and that SU11274 offers in both \textit{in vitro} and \textit{in vivo} assays a preclinical rationale for targeting this pathway in relapsed and resistant MM patients.

**Materials and methods**

\textit{Patients and cell isolation}

Patients fulfilling the International Myeloma Working Group diagnostic criteria (24) for MM (n = 46) and MGUS (n = 18) were studied. Newly diagnosed MM patients (n = 14), patients at relapse after one to three 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 MGUS patients were 11 men and 7 women, ages 41 to 80 years (median 61.5). Eleven patients with anemia due to iron or vitamin B\textsubscript{12} deficiency were studied as controls (25). The study was approved by the Ethics Committee of the University of Bari Medical School, and patients gave their informed consent in accordance with the declaration of Helsinki.

Bone marrow mononuclear cells (BMMCs) were isolated from heparinized aspirates by the Ficoll gradient: plasma cells (PCs) were obtained with magnetic anti-CD138 beads (Immunotech, Marseille, France) while adherent CD138- cells were cultured separately as bone marrow stromal cells (BMSCs). Fibroblasts were purified from BMSCs of five newly diagnosed MM patients through D7-FIB-conjugated (anti-fibroblasts) microbeads (Miltenyi) (26), and cultured in DMEM medium with 20% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA). Cells were grown to 80% confluence and incubated in serum-free DMEM medium for 48 h. Culture supernatants were centrifuged (200×g for 10 min), and stored at -80°C as conditioned media (CM).
Cell lines and cMET inhibition

Human MM cell lines were RPMI8226, MM.1S, MM.1R (American Type Culture Collection, ATCC, Manassas, VA) and RPMI8226.R5 (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% FCS (both from Euroclone, Milan, Italy). The selective phospho(p)-cMET inhibitor SU11274 (Selleck Chemicals, Huston, TX, USA) was dissolved in DMSO at 0.1÷1 μM for in vitro studies (29) while it was dissolved in 1% Tween 80 (Sigma-Aldrich Co., St. Louis, MO, USA) and administered at 100 mg/kg/day 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., Minneapolis, MN, USA) was used at 0.1÷0.3 μg/mL for the HGF blockade; an anti-human cMET antibody (R&D Systems) was used at 0.5÷2 μg/mL for the cMET blockade in vitro.

Real-Time RT-PCR, immunoprecipitation and Western blot

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

Preparation of conditioned media (CM) and enzyme-linked immunosorbent assay (ELISA)

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

Fluorescence-activated cell sorting (FACS)

This was performed on BMMCs and MM cell lines as detailed in supporting information to this manuscript (Supplementary Materials and Methods).
**Functional studies**

Viability, chemotaxis, adhesion, proliferation, and apoptosis assays were performed on both primary PCs and MM cell lines as described (25) (31) and as detailed in supporting information to this manuscript (Supplementary Materials and Methods).

**Microarray hybridization and Proteome Profiler™ assays**

MM cell lines were treated or not (control) with SU11274 1 μM for 6 h 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 GEO database (accession number GSE38204; [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=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 μg 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 (LiteAblot®), and quantified as for Western blot.

**MM xenografted mice**

Six to eight week-old non-obese diabetic (NOD) severe combined immunodeficiency (scid) NOD.CB17-Prkdscid/NCrHsd mice (Harlan Laboratories, Udine, Italy) were housed according to the Institutional Animal Care and Use Committee of the University of Bari Medical School. The R5 and RPMI8226 cell xenografting, SU11274 treatment, and histology, immunohistochemistry and cytofluorimetry on R5 plasmacytomas are detailed in supporting information to this manuscript (Supplementary Materials and Methods).

**Results**

*Multidrug resistant MM cells present with a constitutive activation of cMET pathway*
Expression of cMET and HGF was evaluated in MM cell lines either sensitive (RPMI8226) or multiresistant (R5) to anti-MM drugs. R5 cells presented with lower levels of HGF (Figure 1A) and higher levels of cMET mRNA (Figure 1B); accordingly, cells showed lower HGF secretion (Figure 1C) and higher total cMET and phospho(p)-cMET protein expression (Figure 1D). Expression of cMET and p-cMET was also analyzed by flow cytometry on the cells cultured in serum-free medium (SFM): 90 ± 8% of R5 cells co-expressed cMET and p-cMET, whereas RPMI8226 cells showed co-expression in only 52 ± 5% of cells, while 28 ± 6% expressed cMET alone (Figure 1E). Furthermore in SFM a HGF neutralizing antibody was able to reduce markedly p-cMET expression in RPMI8226 but not in R5 cells (Figure 1F). Overall findings indicate that R5 cells present with a high HGF-independent p-cMET content, i.e., with a constitutive activation of the cMET receptor.

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

**SU11274 exerts major anti-tumor activity in multidrug resistant MM cells**

The cMET pathway supports viability of MM plasma cells (PCs) (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 to the sensitive RPMI8226 cells. The 6-h SU11274 treatment (range: 0÷1 µM) inhibited the p-cMET expression more intensely in R5 cells, as assessed by flow cytometry (-63% vs. -15% as average at 1 µM; P < 0.001; Wilcoxon signed-rank test; Figure 2A), and Western blot (Supporting Information, Supplementary Figure S2A). The 12-h and 24-h treatments confirmed these results (Supplementary Figure 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-h SU11274 treatment (-77% vs. -36% as
average at 1 μM; \( P < 0.001; \) Wilcoxon signed-rank test; Supplementary Figure S1B). In R5 cells, SU11274 inhibited more potently: \( i \) chemotaxis toward both HGF (Figure 2B) and conditioned media of BM fibroblasts derived from newly diagnosed MM patients (Supplementary Figure S3B); \( ii \) adhesion to fibronectin (Figure 2C) and to relapsed/resistant MM patients’ bone marrow stromal cells (BMSCs) (Figure 2D). All inhibitory effects were dose-dependent.

The p-cMET inhibition produces apoptosis and prolonged anti-proliferative 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 PI- and CFSE-staining, respectively. R5 and RPMI8226 cells were cultured in SFM with SU11274 (range: 0±1 μM) for 6, 12, and 24 h. Multidrug resistant R5 cells gave irrelevant percentages of spontaneous apoptosis at each time point (9%, 7% and 8%, respectively, Figure 3A, panels X, XIII and XVI) compared to RPMI8266 cells (19%, 30% and 41%, Figure 3A, panels I, IV and VII) that may be related to the higher p-cMET expression (Figure 1D,E). The SU11274 treatment induced potent apoptosis only in R5 cells: \( \sim 7 \) folds higher apoptosis levels with 1 μM respect to 0 μM at 24 h \( vs. \) only \( \sim 2 \) folds higher in RPMI8226 cells (Figure 3A, panels XVIII and XVI \( vs. \) IX and VII).

Worth of note is that the 6-h SU11274 treatment did not induce apoptosis in both R5 and RPMI8226 cells (Figure 3A, panels I-III and X-XII), implying that the inhibition of p-cMET expression (Figure 2A) and activities of R5 cells (Figure 2B-D) that were studied within 6 h were independent of the drug’s apoptogenic effect.

SU11247 inhibited cell proliferation at each time point more strongly in R5 cells (Figure 3B, green squares). As an example, at 24 h proliferating R5 cells (blue peaks) were 26% and 17% at 0.5 and 1 μM while 100% at 0 μM (Figure 3B, panels XVI-XVIII) \( vs. \) 98%, 96% and 99% of RPMI8266 cells (panels VII-IX). These findings suggest that p-cMET inhibition is able to exert a potent apoptogenic and anti-proliferative effect only on the highly expressing p-cMET R5 cells.
It has been previously shown that cMET knockdown by siRNA in U266 MM 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; isobologram analysis; Figure 3C); and with melphalan gave additive effect (CI=1, Figure 3C). SU11274 thus rendered R5 cells as sensitive to the anti-MM drugs as RPMI8226 cells, suggesting that it may overcome drug resistance.

The cMET inhibition leads to a differential modulation of genes and phospho-proteins in sensitive and multidrug resistant MM cells

Previous studies have analyzed R5 and RPMI8226 cells at gene level, and demonstrated 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 vs. RPMI8226 cells; and found that the SU11274 treatment (1 μM for 6 h) modulated the expression of 2660 genes (1336 up-regulated, 1324 down-regulated) in R5 cells, while of 2186 genes (1129 up-regulated, 1057 down-regulated) in RPMI8226 cells, of which 861 were shared by both cells; among these, 83.5% were concordantly up- or down-regulated, while 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 (Table I): 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, RPMI8226 cells
modified the expression of genes involved in nucleotide excision repair, cytokine-cytokine receptor interactions, antigen processing, and axon guidance (Table I).

The baseline phospho-proteome profile of p-proteins closely involved in tumorigenesis and tumor progression (37) showed that R5 cells differentially express 22 out of 46 kinases, as compared to RPMI8226 cells (Figure 4A). Specifically, R5 cells presented with higher phosphorylation rate of proteins belonging to several pro-survival pathways, such as mitogen-activated protein kinases (MAPK), jun-activated kinase (JAK)/STAT, PI3K/Akt, mTOR, Src pathways, and modulation of AMPKα1 and eNOS kinases; together with reduced phosphorylation levels of p53 isoforms and Creb. The differential effects of SU11274 in modulating phospho-proteins in R5 vs. RPMI8226 cells were: inhibition of the MAPK pathway, reinforcement of down-regulation of some p53 isoforms, and of Pyk2, Creb and eNOS, and enhancement of Lyn, Src and mTOR p-proteins (Figure 4B). Similar results were obtained using an anti-cMET neutralizing antibody (Supplementary Figure 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 MM cell growth and survival (38).

cMET and p-cMET expressions reflect the MM disease status

We investigated whether cMET and p-cMET levels could change in MM patients with newly diagnosed disease, relapse, resistance to anti-MM drugs, and remission phase disease. The cMET/p-cMET co-expression on gated CD38^+CD138^+ bone marrow mononuclear cells (BMMCs) showed the highest co-expression on plasma cells (PCs) from both relapsed and resistant patients (95 ± 15% positive cells; Figure 5A) much in the same way as R5 cells. Conversely, newly diagnosed MM patients presented with little co-expression (20 ± 8%; P < 0.01; Wilcoxon signed-rank test), together with a variable expression of cMET alone (28 ± 22%) much in the same way as RPMI8226 cells. MM patients with complete/partial remission and patients with MGUS displayed irrelevant co-expression (0.1-3%). The 6-h SU11274 treatment (range: 0÷1 μM) of PCs from patients with
relapsed/resistant disease reduced more strongly the p-cMET expression than PCs from newly
diagnosed patients (-66% vs. -21% as average at 1 μM, \( P < 0.001 \); Wilcoxon signed-rank test; 
Figure 5B; and Supporting Information, Supplementary Figure S2B), overlapping what was found in R5 vs. RPMI8226 cells (Figure 2A). Also, inhibition by SU11274 of HGF-driven chemotaxis, and cell adhesion to both fibronectin and paired BMSCs was more evident in PCs from relapsed/resistant patients (Figure 5C-E) than in those from newly diagnosed patients (Supporting Information, Supplementary Figure S5A-C). All effects were, again, dose-dependent. Finally, the 24-h drug treatment induced, dose-dependently, cytotoxicity on PCs from relapsed/resistant patients (Figure 5F), but not on BMMCs from control patients (Figure 5G), and reverted the bortezomib resistance of these PCs through a synergistic interaction (Figure 5H). In contrast, minimal cytotoxic effects were observed at higher doses in PCs from newly diagnosed patients (Supporting Information, Supplementary Figure S5D) together with little additive cytotoxicity with bortezomib (Supporting Information, Supplementary Figure S5E). Overall results confirm that highly-expressing p-cMET PCs from relapsed/resistant patients are more prone to be inhibited by SU11274 much in the same way as R5 cells.

**SU11274 targets MM cells in vivo**

The *in vitro* findings were validated using NOD-scid mice, which were xenotransplanted with R5 or 
RPMI8226 cells, and treated with SU11274 (100 mg/kg/day; *per os* (30)) or vehicle. SU11274 was 
able to delay the growth of R5 (Figure 6A) more strongly than that of RPMI8226 plasmocytomas 
(Supplementary Figure S6A), indicating significant anti-tumor activity in multidrug resistant MM 
cells (\( P < 0.05 \) or better; two-way ANOVA and Bonferroni post-test). 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 Figure S6B; \( P < 0.001 \); log-rank test and Bonferroni test).
Moreover, SU11274-treated mice presented with a longer survival, as compared to vehicle-treated mice (Figure 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 plasmacytomas: SU11274-treated mice, as compared to vehicle-treated mice, presented with a significantly higher percentage of mean necrotic area (Figure 6C; $P < 0.01$; Wilcoxon signed-rank test), together with a significantly higher percentage of apoptotic cells (Figure 6D; $P < 0.01$; Wilcoxon signed-rank test). Finally, flow cytometry analysis on tumor cells harvested from plasmacytomas of SU11274- vs. vehicle-treated mice showed very low p-cMET expression in the former (Figure 6E, panels I and II, red squares), together with higher percentages of apoptotic cells (panels 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- vs. vehicle-treated mice (Figure 6F).

Discussion

Here, preclinical and clinical evidence showing that the cMET/p-cMET pathway participates to the MM patients’ multidrug resistance is provided. Indeed, both cMET and p-cMET are overexpressed in multiresistant R5 and dexamethasone-resistant MM.1R cells compared to their sensitive counterparts (Figures 1A-E, Supplementary Figure S1). We obtained similar results in plasma cells (PCs) from relapsed/resistant compared to newly diagnosed MM patients, or to patients in complete/partial remission, or with MGUS (Figure 5A). Data are in line with the Børset’s group evidence that cMET expression parallels MM progression (12), and with others who emphasized the role of the cMET pathway in MM PCs proliferation (32), survival (32), adhesion (34) and migration (39).

R5 cells are resistant to several clinically relevant anti-MM agents including bortezomib,
melphalan, and doxorubicin (27), and show a constitutive activation of cMET receptor (Figure 1D-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 (Figure 1C) and differently from RPMI8266 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 (23)) could be involved in constitutive cMET pathway activation in R5 cells. Notably, cMET gene mutations or amplifications have not been reported in MM PCs so far (13).

The novel selective p-cMET tyrosine-kinase inhibitor 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 (Figure 3C). Similar results were observed in PCs from relapsed/resistant MM patients but not in cells from newly diagnosed patients (Figure 5H; Supporting Information, Supplementary Figure 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 to RPMI8226 cells, and on PCs from relapsed/resistant than from newly diagnosed MM patients. These in vitro results point to overactivation of the cMET pathway in terms of p-cMET expression in PCs 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-MM therapy in patients of this type.

Wader et al. showed immunohistochemically that HGF and cMET are co-expressed in MM patients’ PCs, and that cMET does exist in its phosphorylated state in a relevant proportion of patients, implying that the HGF/cMET system is operative in MM patients in vivo (41). The expression of cMET and p-cMET was closely confined to MM plasma cells but absent on PCs of healthy subjects and MGUS, pointing to cMET and p-cMET as one of the factors that distinguishes
malignant from normal PCs (41). Here by using cytofluorimetry on PCs from MM patients at different disease phases and from MGUS patients we observed that the p-cMET amount increases in step with disease progression (Figure 5A), and that SU11274 exerts a marked cytotoxic effect on PCs from relapsed/resistant patients (Figure 5F) but not from newly diagnosed ones (Supporting Information, Supplementary Figure S2). Hence MM patients with PCs displaying high p-cMET expression, e.g., 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 RPMI8226 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 RPMI8226 cells (Table I), suggesting that the former are more addicted to the cMET pathway. Phospho-proteome studies also revealed that R5 differ from RPMI8226 cells in 22 out 46 phospho-proteins, which are involved in the jun-activated kinase (JAK), the mitogen-activated protein kinase (MAPK), the phosphatidyl 3-kinase (PI3K)/Akt pathway together with other phospho-proteins of the Src (Figure 4A). SU11274 inhibitor modulated differently these signal pathways in R5 vs. RPMI8226 cells (Figure 4B), and was able to significantly inhibit phosphorylation of proteins entailed with the MAPK pathway (Figure 4B). These results suggest that multidrug resistant R5 but not RPMI8226 cells become dependent on the cMET receptor for the downstream activation of the MAPK kinase pathway, which is especially involved in MM cell growth and proliferation (38). Accordingly, SU11274 exerts a potent and persistent antiproliferative activity on R5 but not RPMI8226 cells (Figure 3B).

Finally, we evaluated the anti-MM 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-MM activity without determining any toxicity sign. To the best of our knowledge only another pre-clinical study evaluated the anti-MM
efficacy of cMET inhibition in a mouse model by using the HGF competitor NK4 (42). All these results offer both in \textit{in vitro} and \textit{in vivo} a preclinical rationale for targeting cMET pathway in relapsed and resistant MM patients, and point to p-cMET expression on PCs as a potential biomarker of response to novel cMET inhibitors.

References


Legends to figures

Figure 1. HGF and cMET/phospho(p)-cMET expression in R5 vs. RPMI8226 cells. Real-time reverse transcriptase PCR of (A) HGF and (B) cMET as means ± s.d. of five determinations per line. (C) ELISA for HGF levels in conditioned media of the cell lines as means ± s.d. of five determinations per line. (D) Western blot of cMET and p-cMET: fold increase as optical density (OD) in R5 vs. RPMI8226 cells expressed as means ± s.d. of five determinations per line. (E) FACS analysis of cMET and p-cMET expression in R5 vs. RPMI8226 cells. The cell rate as co-expression or mono-expression is given. (F) FACS analysis of cMET and p-cMET expression in R5 vs.
RPMI8226 cells upon treatment with a neutralizing antibody to HGF: contrary to RPMI8226 cells R5 cells did not reduce the p-cMET expression. A representative experiment out of five is shown. *P < 0.05; **P < 0.01; Wilcoxon signed-rank test.

**Figure 2.** SU11274 inhibits p-cMET expression and cell activities more intensely in R5 cells. (A) SU11274-treated RPMI8226 and R5 cells (range 0÷1 µM, 6 h) 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 MM patients’ bone marrow stromal cells (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 = BSA 1%; positive control (pos ctr) = poly-L-lysine. Data are given as means ± s.d. of five independent experiments. *P < 0.05; **P < 0.01; Wilcoxon signed-rank test.

**Figure 3.** SU11274 induces cell apoptosis, inhibits cell proliferation, and overcomes resistance to anti-MM drugs in R5 cells. FACS analysis of (A) cell apoptosis, as propidium iodide content, and (B) cell proliferation, as carboxyfluorescein succinimidyl ester (CFSE) dilution, upon SU11274 treatment at the indicated doses and time points. (A) Percentages of apoptotic cells: main apoptogenic effect (green rectangles) vs. untreated cells only in R5 cells (~7 fold more apoptotic R5 cells vs. ~2 fold more RPMI8226 cells at each dose and time point). (B) Proliferating cells (as blue peaks) in a representative experiment out of five: more intense and persistent anti-proliferative effect (green rectangles) only in R5 cells (~10 and ~5 fold less proliferating R5 cells at 12 and 24 h with 1 µM SU11274 vs. no effects in RPMI8226 cells). Red peaks represent not proliferating cells. (C) Cell cytotoxicity of R5 vs. RPMI8226 cells treated with bortezomib, melphalan and doxorubicin and/or SU11274 for 24 h: note the loss of resistance to the above drugs after the
SU11274 treatment. The combination index (CI) <1.0 indicates synergism, =1 additive effect (isobologram analysis). *P < 0.05; **P < 0.01; Wilcoxon signed-rank test.

Figure 4. The p-cMET inhibition leads to modulation of phospho (p)-proteins in MM cell lines. (A) Phospho-proteome profile of 46 p-kinases in R5 vs. RPMI8226 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 RPMI8226 cells (1 µM, 6 h) vs. their untreated controls. Data given as average of three independent experiments. *P < 0.03 or better; Wilcoxon signed-rank test.

Figure 5. cMET and p-cMET expression in MM patients’ plasma cells (PCs) and effects of the SU11274 treatment. (A) 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 µM) on the p-cMET expression of PCs from a representative resistant and a newly diagnosed MM patient. Note the more intense, dose-dependent, effect in the resistant patient. (C) Chemotaxis and (D) adhesion to fibronectin- or (E) to paired bone marrow stromal cells (BMSCs) of PCs from relapsed and resistant MM patients: note, again, the intense, dose-dependent inhibition by SU11274 on the PCs activities. Chemotaxis assay: negative control (neg ctr) = SFM in lower chamber. Adhesion assay: neg ctr = BSA 1%; positive control (pos ctrl) = poly-L-lysine. Data given as means ± s.d. of the 18 patients. *P < 0.05; **P < 0.01; ***P < 0.001; Wilcoxon signed-rank test. Cytotoxic effect of 24-h SU11274 treatment on (F) PCs from four representative resistant MM patients, but not (G) on bone marrow mononuclear cells (BMMCs) from four representative control patients. (H) SU11274 0.5 µM reverts bortezomib resistance in PCs from five resistant patients. Data are expressed as means ± s.d. The combination index (CI) <1.0 indicates synergism (isobologram analysis). *P<0.05; **P<0.01; Wilcoxon signed-rank test.
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 post-test. (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) Histological 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 100×; inserts 200×. Scale bar = 100 μm at 100×. (D) Tumor apoptosis rate assessed by TUNEL fluorescence staining. Original magnification 400×. Scale bar = 30 μm. **$P<0.01$; Wilcoxon signed-rank test. (E) 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 600×. Scale bar = 10 μm.
Figure 1
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Figure 2
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Figure 3
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Figure 6

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Novel targeting of phospho-cMET overcomes drug resistance and induces anti-tumor activity in multiple myeloma

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