

A Selective c-Met Inhibitor Blocks an Autocrine Hepatocyte Growth Factor Growth Loop in ANBL-6 Cells and Prevents Migration and Adhesion of Myeloma Cells

Håkon Hov,¹ Randi Utne Holt,¹
Torstein Baade Rø,¹ Unn-Merete Fagerli,²
Henrik Hjorth-Hansen,³ Vadim Baykov,¹
James G. Christensen,⁴ Anders Waage,³
Anders Sundan,¹ and Magne Børset¹

¹Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway; ²Department of Oncology and ³Section of Hematology, St. Olavs University Hospital, Trondheim, Norway; and ⁴Pfizer Global Research and Development, La Jolla Laboratories, California

ABSTRACT

Purpose: We wanted to examine the role of the hepatocyte growth factor (HGF) receptor c-Met in multiple myeloma by applying a novel selective small molecule tyrosine kinase inhibitor, PHA-665752, directed against the receptor.

Experimental Design: Four biological sequels of HGF related to multiple myeloma were studied: (1) proliferation of myeloma cells, (2) secretion of interleukin-11 from osteogenic cells, (3) migration of myeloma cells, and (4) adhesion of myeloma cells to fibronectin. We also examined effects of the c-Met inhibitor on intracellular signaling pathways in myeloma cells.

Results: PHA-665752 effectively blocked the biological responses to HGF in all assays, with 50% inhibition at 5 to 15 nmol/L concentration and complete inhibition at around 100 nmol/L. PHA-665752 inhibited phosphorylation of several tyrosine residues in c-Met (Tyr¹⁰⁰³, Tyr^{1230/1234/1235}, and Tyr¹³⁴⁹), blocked HGF-mediated activation of Akt and p44/42 mitogen-activated protein kinase, and prevented the adaptor molecule Gab1 from complexing with c-Met. In the HGF-producing myeloma cell line ANBL-6, PHA-665752 revealed an autocrine HGF–c-Met–mediated growth loop. The inhibitor also blocked proliferation of purified primary myeloma cells, suggesting that autocrine HGF–c-Met–

driven growth loops are important for progression of multiple myeloma.

Conclusions: Collectively, these findings support the role of c-Met and HGF in the proliferation, migration, and adhesion of myeloma cells and identify c-Met kinase as a therapeutic target for treatment of patients with multiple myeloma.

INTRODUCTION

Hepatocyte growth factor (HGF) is a pleiotropic cytokine that is mitogenic, motogenic, and/or morphogenic to a variety of cells. The only known high-affinity receptor for HGF is c-Met. This is a tyrosine kinase that is involved in several aspects of malignant cell behavior. Originally identified in 1984 as an oncogene that was responsible for the malignant transformation of a human osteosarcoma cell line (1), c-Met is now known to mediate growth, invasion, motility, and metastasis of cancer cells as well as to promote angiogenesis in tumors (recently reviewed in refs. 2 and 3). An oncogenic variant of c-Met is important for the transformation to malignancy of papillary renal carcinomas and can contribute to hereditary disposition for this cancer (4). HGF and c-Met are reported to be important for cancer cell growth and metastasis in breast cancer (5, 6), lung cancer (7, 8), and several other malignancies (3).

c-Met is produced as a precursor protein (p170) that is cleaved into a heterodimeric molecule consisting of a M_r 50,000 α chain that is disulfide-linked to a M_r 145,000 β chain (p145). The β chain traverses the cell membrane and has both an extracellular and an intracellular domain, whereas the α chain is located only extracellularly. The intracellular part of the receptor can be divided into (a) a juxtamembrane region, followed by (b) a tyrosine kinase catalytic domain, and (c) a COOH-terminal sequence, which is responsible for coupling the receptor to intracellular cell signaling molecules. All of these three domains contain tyrosine residues that are phosphorylated upon ligand binding. The major phosphorylation site of c-Met is in the catalytic domain where three tyrosines (Tyr^{1230/1234/1235}) are located (9). This is the region in which ATP is bound (10). The phosphorylated tyrosines in the COOH-terminal sequence (Tyr¹³⁴⁹ and Tyr¹³⁵⁶) act as docking sites for Src homology 2 (SH2) domains in downstream signaling molecules like Grb2, phospholipase C γ , the p85 subunit of phosphatidylinositol 3'-kinase, and others (11). The juxtamembrane Tyr¹⁰⁰³ of the cytoplasmic part of c-Met is required for ubiquitination and degradation by the proteasome pathway (12), making it a part of a negative feedback loop for c-Met signaling.

After binding to the phosphorylated multisubstrate docking site via its SH2 domain, p85-phosphatidylinositol 3'-kinase leads to phosphorylation of the protein kinase Akt. Grb2 activates the SOS-Ras pathway, leading to activation of MEK1 and

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Requests for reprints: Magne Børset, Department of Cancer Research and Molecular Medicine, Medical Technical Research Center, Norwegian University of Science and Technology, N-7489 Trondheim, Norway. Phone: 47-73866932; Fax: 47-73598801; E-mail: magne.borset@medisin.ntnu.no.

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MEK2, which in turn phosphorylates p44/42 mitogen-activated protein kinase (MAPK; also known as ERK1 and ERK2).

Although c-Met is largely expressed on epithelial cells, certain cells of hematopoietic origin also carry this receptor and are sensitive to HGF. For example, c-Met is found on germinal center B cells (13) and on terminally differentiated plasma cells (14). The receptor also plays a role in some hematologic malignancies, *e.g.*, in primary effusion lymphoma (15) and, notably, in multiple myeloma (16). Malignant plasma cells express c-Met (16–18) and often simultaneously HGF. Hence, autocrine HGF–c-Met loops have been suggested (19). Paracrine growth stimulation of myeloma cells by HGF has been demonstrated (18), and important interactions between the myeloma marker protein syndecan-1 and HGF have been shown in several studies (18, 20, 21). The importance of HGF in multiple myeloma is underscored by the observation that a high concentration of HGF in serum at the time of diagnosis of multiple myeloma imparts an unfavorable prognosis for the patients (22–24).

A small molecule ATP-competitive inhibitor of c-Met tyrosine kinase, PHA-665752, has been identified at SUGEN (San Francisco, CA). PHA-665752 is selective for c-Met with respect to other tyrosine and serine-threonine kinases, including epidermal growth factor receptor, platelet-derived growth factor receptor, fibroblast growth factor receptor 1, vascular endothelial growth factor receptor 2, c-Src, Cdk-2, Akt, and p38 MAPK (25). IC_{50} of PHA-665752 against each of these kinases was more than 300-fold higher than IC_{50} against c-Met (25). Only RON, a tyrosine kinase with close homology to c-Met, was inhibited by a concentration of PHA-665752 within the same order of magnitude as that needed for blockade of c-Met (IC_{50} against RON was 7-fold higher). In the present paper, we examine the effect of this compound on biological responses to HGF, with particular focus on myeloma cells. Collectively, our findings support the role of c-Met and HGF in the molecular regulation of events associated with cancer progression and identify c-Met kinase as a potential therapeutic target for treatment of myeloma patients.

MATERIALS AND METHODS

Compound. PHA-665752 (3Z)-5-[(2,6-dichlorobenzyl)sulfonyl]-3-[(3,5-dimethyl-4-[(2R)-2-(pyrrolidin-1-ylmethyl)pyrrolidin-1-yl]carbonyl]-1H-pyrrol-2-yl)methylene]-1,3-dihydro-2H-indol-2-one was synthesized at SUGEN, Inc., and described by Christensen *et al.* (25).

Cell Lines and Primary Patient Samples. Saos-2 and Mv1Lu cells were obtained from American Type Culture Collection (Manassas, VA). ANBL-6 cells and INA-6 cells were kind gifts from Dr. Diane Jelinek (Mayo Clinic, Rochester, MN) and Dr. Martin Gramazki (University of Erlangen-Nuremberg, Erlangen, Germany), respectively. IH-1, an immunoglobulin A–producing myeloma cell line, was established in our laboratory as described previously (26). Cell lines were grown in RPMI 1640 with 10% fetal calf serum or human serum (IH-1 only), 2 mmol/L L-glutamine, and 40 μ g/mL gentamicin (complete medium). Interleukin (IL)-6 (2 ng/mL) was added to IL-6–dependent cell lines (IH-1, INA-6, and ANBL-6).

After obtaining approval from the regional ethics committee and informed consent from patients, we studied myeloma

cells from six patients admitted to the Section of Hematology, St. Olavs Hospital. CD138-positive cells were purified from bone marrow aspirates by immunomagnetic separation (27). Myeloma cells were purified using Macs MicroBeads (Miltenyi Biotec, Auburn, CA).

Cytokines and Antibodies. Recombinant human IL-1 β , IL-6, insulin-like growth factor-1 (IGF-1), and porcine transforming growth factor- β (TGF β) were from R&D Systems (Abingdon, United Kingdom). Stromal cell-derived factor-1 α (SDF-1 α) was from PeproTech (London, United Kingdom). HGF was purified from medium conditioned by the human myeloma cell line JIN-3 as described previously (19). We used the following anti-c-Met antibodies: sc-161 from Santa Cruz Biotechnology (Santa Cruz, CA; for immunoprecipitation); clone DL-21 from Upstate (Waltham, MA; for detection of total c-Met in immunoblots); rabbit polyclonal antibodies against the phosphotyrosine epitopes phospho-Tyr phospho-Tyr phospho-Tyr^{1230/1234/1235}, phospho-Tyr¹⁰⁰³, and phospho-Tyr¹³⁴⁹ from Biosource (Camarillo, CA; for detection of activated c-Met in immunoblots); clone 95309 from R&D Systems (for neutralization). We used anti-Gab1 from Upstate both for immunoprecipitation and immunoblots. Anti-Akt, anti-phospho-Ser⁴⁷³Akt, and anti-phospho-Tyr²⁰²/phospho-Tyr²⁰⁴-p44/42 MAPK were from Cell Signaling Technology (Beverly, MA). Monoclonal anti-HGF was from R&D Systems, and rabbit anti-HGF serum was raised by us as described previously (23).

Proliferation Assays. For assessment of thymidine incorporation, cells were seeded in 96-well plastic culture plates (Corning Costar, Corning, NY) at 1 to 3 \times 10⁴ cells per well in 200 μ L of complete medium. After 18 hours (Mv1Lu cells) or 48 hours (other cell lines and primary myeloma cells), cells were pulsed with 1 μ Ci of methyl-[³H]thymidine (NEN Life Science Products, Boston, MA) per well and harvested 4 hours (Mv1Lu cells) or 18 hours (other cells) later with a Micromate 96-well harvester (Packard, Meriden, CT). β Radiation was measured with a Matrix 96 β counter (Packard). PHA-665752 was added to Mv1Lu cells 30 minutes before the addition of HGF and TGF β .

Proliferation of Saos-2 cells after an overnight incubation with PHA-665752 was determined with a 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazoliumbromide (MTT) assay. After harvest of supernatants for IL-11 determination (see below), 200 μ L of fresh RPMI 1640 with 2% fetal calf serum and 20 μ L of MTT (5 mg/mL; Sigma-Aldrich, St. Louis, MO) were added to each well. Four hours later, medium was removed, and 100 μ L per well of acid-isopropanol (0.04 N HCl in isopropanol) were added. After shaking on an automated rocker for 1 hour, A_{570} was measured with a UV microplate reader (3550; Bio-Rad, Hercules, CA).

Interleukin-11 Production from Saos-2 Cells. Cells from the osteosarcoma cell line Saos-2 were seeded in 24-well plates at 5 \times 10⁴ cells per well in complete medium. After 24 hours, we replaced the medium with 1 mL of fresh medium with 2% fetal calf serum. Cytokines and PHA-665752 were added as indicated in the legend to Fig. 4. After another 24 hours at 37°C, cell supernatants were collected, centrifuged, and frozen until analysis of IL-11. Detection of IL-11 was done with an enzyme-linked immunosorbent assay (ELISA) from R&D Systems.

Migration Assay. INA-6 cells were washed four times in Hanks' balanced salt solution (HBSS), resuspended in RPMI

1640 supplemented with 0.1% bovine serum albumin and 1 ng/mL IL-6, and seeded (5×10^5 cells per well) in the top compartments of polycarbonate transwells (pore size, 5- μ m; Corning Costar). The total volume of medium was 200 μ L in the top compartments and 700 μ L in the bottom compartments. All samples were performed in duplicates or triplicates. After 18 hours, the number of cells that had migrated through the membrane to the bottom chamber was determined by a Coulter Counter Z1 (Beckman Coulter, Fullerton, CA).

Adhesion Assay. Ninety-six-well plates were coated overnight at 4°C with fibronectin (human plasma fibronectin; 20 μ g/mL in PBS, 80 μ L/well; Becton Dickinson Labware, Bedford, MA) or bovine serum albumin (BSA; 1 mg/mL), blocked with BSA (1 mg/mL, 100 μ L per well) for 1 hour at room temperature, and finally washed three times in HBSS. Cytokines and PHA-665752 were added to the plates 15 minutes before INA-6 cells were seeded. Cells were washed four times in HBSS, resuspended in 5 to 10 mL RPMI 1640 with 0.1% BSA, and incubated for 1 hour at 37°C with 5 μ mol/L acetoxymethyl ester-2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (Sigma-Aldrich). After three washes in HBSS, 10^5 cells were seeded per well in a total volume of 100 μ L and incubated for 1 hour at 37°C in 5% CO₂. The plates were then carefully washed several times in prewarmed (37°C) HBSS, and the remaining cells were solubilized by 50 μ L per well of 1% Triton X-100. Fluorescence level at 538 nm was determined with a Fluoroskan II fluorescence reader (Labsystems, Helsinki, Finland).

Immunoblotting. ANBL-6 cells (but not INA-6 cells) were preincubated with or without anti-HGF overnight before treatment with PHA-665752. First, they were depleted of fetal calf serum and IL-6 by four washes in HBSS and seeded at 1×10^6 cell per milliliter in RPMI 1640 with 0.1% BSA and a 1:750 dilution of rabbit anti-HGF serum or preimmuneserum. INA-6 cells and preincubated ANBL-6 cells were washed four times in HBSS and seeded in 0.5 mL of RPMI 1640 with 0.1% BSA in 24-well plates (4×10^6 cells per well). They were precultured for 15 minutes with or without PHA-665752 before stimulation for 5 minutes with HGF, IGF-1, or medium conditioned by ANBL-6 [medium harvested after conditioning by ANBL-6 cells (10^6 per mL) in RPMI 1640 + 0.1% BSA for 3 days]. After stimulation, cells were pelleted and resuspended in 105 μ L of lysis buffer: 10 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 20 mmol/L Na₄P₂O₇, 2 mmol/L Na₃VO₄, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100, 10% glycerol, and a protease-phosphatase inhibitor mixture (Complete mini tablets; Roche, Basel, Switzerland). After 30 minutes on ice, the nuclei were pelleted by centrifugation at $12,000 \times g$, 4°C for 20 minutes. Supernatants were stored at -80°C until additional processing. Samples were mixed with lithium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) with 10 mmol/L dithiothreitol, heated for 5 minutes at 98°C, and then separated on either 10% NuPAGE Bis-tris gels or 3 to 8% NuPAGE Tris-Acetate gels (Invitrogen), followed by electrophoretic transfer to 0.45- μ m nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% BSA in Tris-buffered saline with 0.05% Tween 20 and incubated with antibodies against phosphorylated proteins overnight at 4°C. Detection was performed with horseradish peroxidase-conjugated antibodies (DAKO Cytomation, Copen-

hagen, Denmark) and chemiluminescence (ECL; Amersham Biosciences, Amersham, United Kingdom). The membranes were stripped at 60°C for 30 minutes with gentle rotation in a buffer containing 62.5 mmol/L Tris-HCl (pH 6.6), 2% SDS, and 10 mmol/L 2-mercaptoethanol, then washed in Tris-buffered saline with 0.05% Tween 20, blocked with 5% nonfat dried milk in Tris-buffered saline with 0.05% Tween 20, and probed with antibodies against non-phospho-epitopes.

Immunoprecipitation. Lysates were made the same way as for immunoblotting but with 10^7 cells and 500 μ L of lysis buffer per sample. The lysates were incubated with protein A Sepharose 4 Fast Flow (Amersham Biosciences) with gentle mixing at 4°C for 1 hour (preclearing). After a short spin, the supernatants were incubated with anti-Gab1 or anti-c-Met for 2 hours at 4°C. The immunocomplexes were precipitated with protein A Sepharose 4 Fast Flow (Amersham Biosciences) for 1 hour at 4°C with gentle mixing. After four washes with cold PBS, 50 μ L of LDS sample buffer with 10 mmol/L dithiothreitol were added to the Sepharose pellet. After incubation at 98°C for 5 minutes, the samples were resolved by gel electrophoresis and examined by Western blotting.

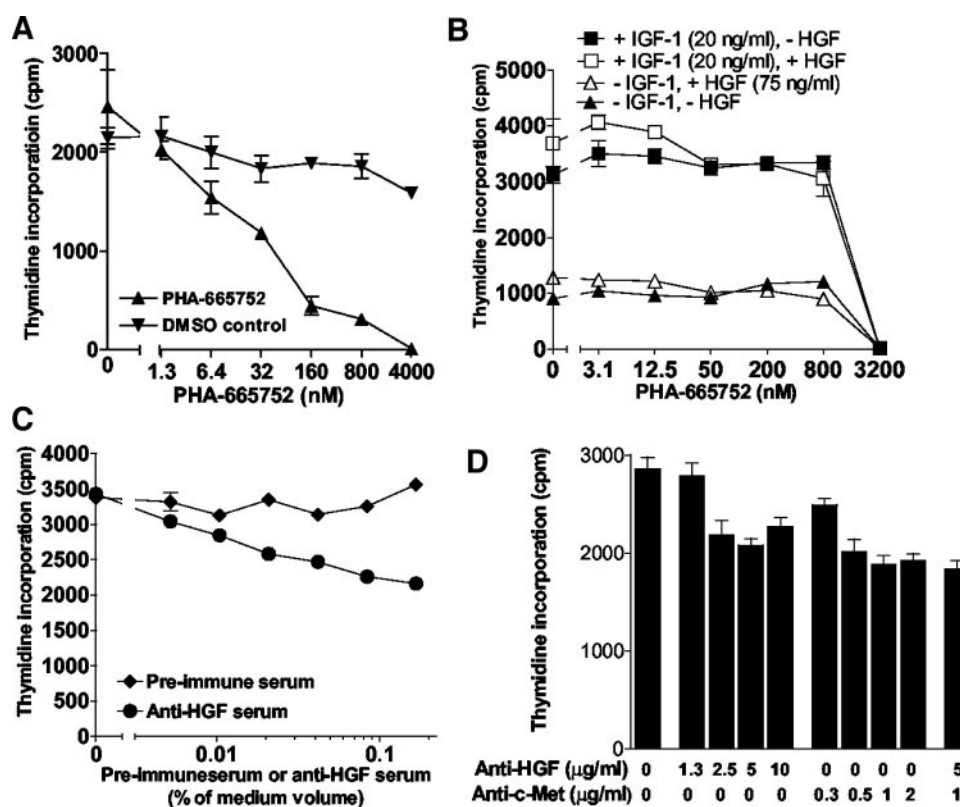
RESULTS

PHA-665752 Revealed an Autocrine Growth Loop in ANBL-6 Cells and in Primary Myeloma Cells. In addition to expressing c-Met, myeloma cell lines often produce HGF. Autocrine phosphorylation of c-Met has been documented in the myeloma cell line JIN-3 (19), but autocrine stimulation of growth has been difficult to demonstrate. ANBL-6 cells secrete more than 20 ng of HGF per 10^6 cells per 24 hours (data not shown). PHA-665752 inhibited the basic proliferation of this cell line, with an IC₅₀ of 15 nmol/L (Fig. 1A), a dose that is more than 1 order of magnitude lower than the threshold for unspecific effects of the compound (compare Fig. 1A with Fig. 1B and Fig. 3). IH-1 myeloma cells do not produce HGF (data not shown), and HGF causes only a moderate stimulation of growth in this cell line (Fig. 1B). However, 50 nmol/L PHA-665752 totally inhibited the contribution of HGF to growth stimulation, whereas inhibition of basal or IGF-1-mediated growth was seen only at doses above 800 nmol/L. These data are in line with the previously reported inability of PHA-665752 to block the IGF-1 receptor, even at doses up to 10 μ mol/L (25).

To more strongly prove the existence of the autocrine growth loop, we treated ANBL-6 cells with serial dilutions of an anti-HGF serum or with preimmuneserum from the same rabbit. There was a dose-dependent inhibition of thymidine incorporation in the presence of antiserum, but not with pre-serum (Fig. 1C). This result was confirmed by experiments with neutralizing monoclonal antibodies against HGF and/or c-Met (Fig. 1D).

We also examined the effect of PHA-665752 on proliferation of six samples of primary myeloma cells purified from bone marrow aspirates with anti-CD138-coated magnetic beads. The majority of such cultures of primary myeloma cells produce HGF, and the presence of autocrine loops is likely (16). One of the examined samples did not incorporate thymidine. Four of the five proliferating samples (80%) responded significantly to PHA-665752 (three responders are shown in Fig. 2). Three of the responders had a very low proliferation rate, a

Fig. 1 Inhibition of c-Met or HGF revealed an autocrine growth loop in ANBL-6 myeloma cells. **A.** ANBL-6 cells were grown with varying doses of PHA-665752. Growth inhibition by PHA-665752 indicates that cells were stimulated by HGF in an autocrine manner. **B.** IH-1 myeloma cells were grown with or without HGF and IGF-1 with varying doses of c-Met inhibitor. PHA-665752 in appropriate doses inhibited only HGF-mediated growth, not basal growth or proliferation induced by IGF-1. **C** and **D.** ANBL-6 cells grown in the presence of varying doses of HGF antiserum (**C**) or neutralizing monoclonal antibodies against HGF or c-Met (**D**). Growth inhibition in the presence of specific antibodies confirmed the existence of an autocrine HGF–c-Met growth loop. *DMSO*, dimethyl sulfoxide.



common finding in primary cultures of myeloma cells. In the presence of PHA-665752, counts per minute in these samples were close to background level. In two other samples, cells proliferated more strongly. In one of these, there was an 88% reduction of thymidine incorporation in the presence of 100 nmol/L PHA-665752, and IL-6 could not rescue the cells (Fig. 2, *bottom left*). Even 50 nmol/L, the lowest dose tested, gave 75% reduction of proliferation, indicating strongly that this was a c-Met-specific effect of the compound. In the nonresponding culture, even 400 nmol/L did not influence a strong proliferative effect of IL-6 (Fig. 2, *bottom right*).

A Wide Range of PHA-665752 Concentrations Gave Only c-Met-Specific Effects. To more clearly explore the difference in concentrations needed to achieve c-Met-specific and c-Met-unrelated effects of PHA-665752, we used Mv1Lu cells (alias CCL-64), a mink lung cell line. These cells are growth-arrested in the presence of TGF β (ref. 19; Fig. 3, ● on Y axis). The growth inhibition by TGF β is totally abolished when HGF is added together with TGF β (ref. 19; Fig. 3, ○ on Y axis). Cells were treated with serial dilutions of PHA-665752, either in the absence of cytokines (Fig. 3, squares) or in the presence of both TGF β and HGF (Fig. 3, ○). A dose of 250 nmol/L PHA-665752 given in the presence of HGF and TGF β almost completely unmasked the TGF β -mediated growth arrest and gave no apparent toxicity in the absence of HGF and TGF β . Only doses of 1000 nmol/L or higher reduced proliferation in the absence of HGF and TGF β . IC₅₀ of PHA-665752 was approximately 10 nmol/L.

These results indicate that PHA-665752 also inhibits cell

proliferation by (a) c-Met-independent mechanism(s) at concentrations above those that give specific c-Met-dependent effects. However, there was about a 100-fold difference between the concentrations needed to achieve c-Met-specific and c-Met-unrelated inhibition. Therefore, the interpretation of specific c-Met-dependent mechanisms is relevant at concentrations below approximately 1000 nmol/L.

PHA-665752 Inhibited Hepatocyte Growth Factor-Mediated Interleukin-11 Production in Saos-2 Cells. HGF stimulation of the human osteosarcoma cell line Saos-2 leads to release of IL-11 into the cell supernatant (28). IL-11 is a stimulator of bone resorption, and it has been argued that HGF-induced IL-11 secretion from osteoblastic cells may contribute to the osteolysis seen in a majority of patients with multiple myeloma (28). HGF-induced IL-11 production was totally abolished at 160 nmol/L PHA-665752, and IC₅₀ was 9 nmol/L (Fig. 4A). IL-11 is released to the medium also when Saos-2 cells are stimulated with IL-1 or TGF β . IL-1- or TGF β -mediated IL-11 production was unaffected even in the presence of 800 nmol/L PHA-665752, showing the specificity of c-Met inhibition by this tyrosine kinase inhibitor. In an MTT assay with Saos-2 cells growing in the absence of HGF, there was no significant toxicity of PHA-665752 at doses up to 800 nmol/L (Fig. 4B).

PHA-665752 Inhibited Hepatocyte Growth Factor-Mediated Adhesion of Human Myeloma Cells to Fibronectin. The human myeloma cell line INA-6 adhered to the extracellular matrix protein fibronectin upon stimulation with the cytokines HGF, IGF-1, or SDF-1 α (Fig. 4C). At a dose of 67 nmol/L, PHA-665752 reduced HGF-mediated adhesion by more

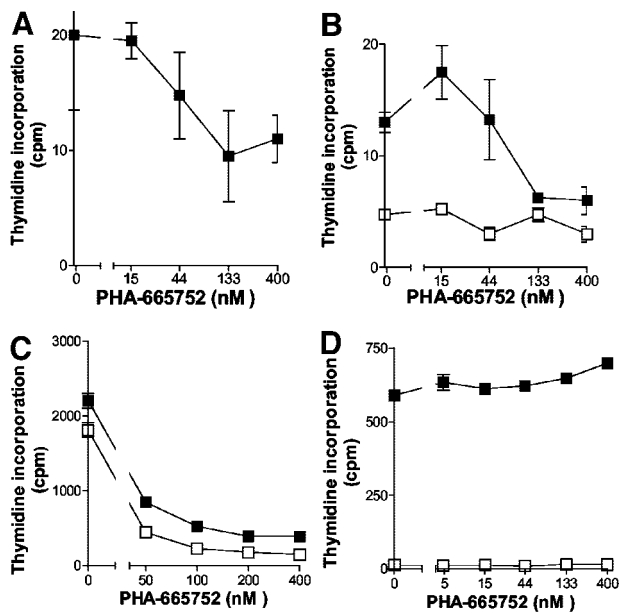


Fig. 2 PHA-665752 prevented growth in a majority of primary myeloma cell samples. Myeloma cells purified from bone marrow samples from different patients were cultured with (□) or without (■) 2 ng/mL IL-6 and with PHA-665752 in concentrations as indicated. Reduced proliferation in the presence of PHA-665752 indicates that the cells were stimulated by autocrine HGF. Error bars represent SEM of quadruplicate measurements.

than 95%, whereas concentrations up to 600 nmol/L did not affect SDF-1 α - and IGF-1-induced adhesion (Fig. 4C). Adhesion induced by these cytokines was reduced more than 50% only at a PHA-665752 dose of 1800 nmol/L or higher (data not shown).

PHA-665752 Inhibited Hepatocyte Growth Factor-Mediated Migration of Human Myeloma Cells. HGF is a chemoattractant for the myeloma cell lines INA-6 (Fig. 4D) and ANBL-6 (not shown). When HGF was added to the bottom compartment of a transwell chamber, there was a 2- to 4-fold increase of the number of cells that traversed the 5- μ m pores of the membrane between the top and bottom compartments. When HGF was added to both the top and the bottom wells, the number of migrating cells was not different from that in controls without HGF, proving that the increased number of cells in the bottom compartment in the presence of an HGF gradient was not caused by increased cell division but by migration. PHA-665752 gave a dose-dependent decrease in number of cells migrating toward HGF, with total inhibition of the HGF effect at 50 nmol/L. A dose of 200 nmol/L did not reduce the number of migrating cells below the level in controls without HGF (Fig. 4D), consistent with experiments showing that doses up to this level did not significantly influence proliferation of INA-6 cells (thymidine incorporation data not shown).

PHA-665752 Prevented Tyrosine Phosphorylation of c-Met in INA-6 Cells and Blocked c-Met-Dependent Signaling Events. The ability of PHA-665752 ability to inhibit activation of c-Met was examined by Western blots probed with monoclonal antibodies directed against three different phosphotyrosine epitopes in the intracellular part of the receptor.

PHA-665752 inhibited the phosphorylation of all examined tyrosines in p145 of c-Met in a dose-dependent way, with 100 nmol/L completely abolishing the effect of 200 ng/mL HGF (Fig. 5A).

After immunoprecipitation of Gab1 from INA-6 myeloma cells, we detected c-Met in the precipitate from cells stimulated by HGF (Fig. 5B), showing for the first time that Gab1 works as an adaptor protein in HGF-mediated signaling in myeloma cells. As expected, PHA-665752 abolished the coupling of Gab1 to c-Met. Conversely, when immunoprecipitation was done with an anti-c-Met antibody, Gab1 appeared in the blot from HGF-stimulated cells but disappeared again when cells were preincubated with PHA-665752 (Fig. 5B).

HGF binding to c-Met in INA-6 cells activated the protein kinases Akt and p44/42 MAPK (Fig. 5C). At 200 nmol/L, PHA-665752 completely inhibited the HGF-induced phosphorylation of Akt and p44/42 MAPK. The same dose of PHA-665752 had no effect on either IGF-1-induced Akt phosphorylation (Fig. 5D) or the background phosphorylation of both Akt and p44/42 MAPK (Fig. 5C).

c-Met and Downstream Signaling Mediators Were Phosphorylated by Autocrine Hepatocyte Growth Factor in ANBL-6 Cells. To demonstrate intracellular effects of autocrine HGF, we chose to block the autocrine loop with anti-HGF serum during an overnight preincubation period. In this period, cells were also serum-starved and grown without IL-6. Blocking the loop this way substantially increased the level of p145 c-Met, the β chain of the active form of the receptor, as shown in Fig. 6A. Thus, autocrine HGF evidently caused degradation of c-Met. HGF-induced degradation was visible after only a 5-minute incubation with exogenous HGF or with conditioned medium from the same cell line (Fig. 6B, total c-Met bands, *third and fifth lanes from the left*). In cells preincubated with anti-HGF serum, autocrine phosphorylation of Tyr¹³⁴⁹ could be clearly demonstrated 20 minutes after removal of the antiserum, whereas phospho-Tyr¹⁰⁰³ was almost invisible (Fig. 6B, *first*

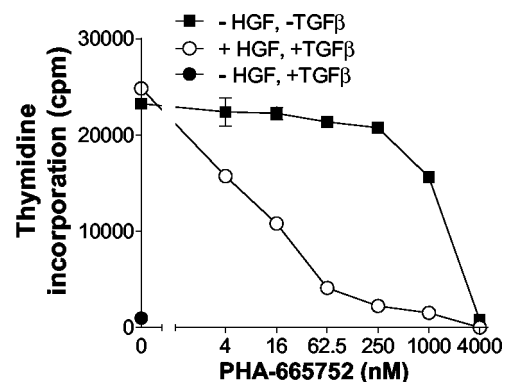


Fig. 3 A wide span of PHA-665752 concentrations gave only c-Met-specific effects on growth of Mv1Lu cells. Mv1Lu cells were grown with (open symbols) or without (filled symbols) HGF (100 ng/mL), with (circles) or without (squares) TGF β (300 pg/mL), and with varying doses of PHA-665752. Cells were growth-arrested when treated with TGF β in the absence of HGF (●). Growth was estimated by incorporation of tritiated thymidine after an overnight incubation. Error bars show SEM of triplicate measurements.

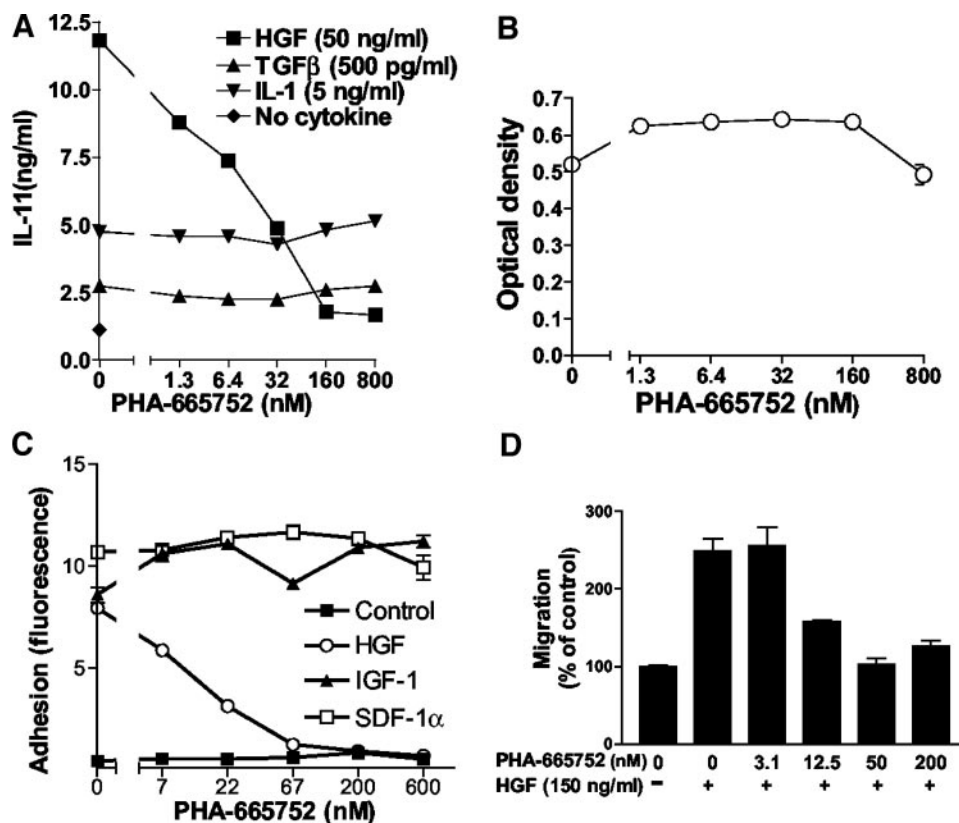


Fig. 4 PHA-665752 prevented HGF-induced adhesion, migration, and cytokine release. **A.** Saos-2 cells were treated with HGF (50 ng/mL), TGF β (500 pg/mL), or IL-1 (5 ng/mL) and varying doses of PHA-665752 for 24 hours. IL-11 concentration in the supernatant was determined by ELISA. **B.** Growth of Saos-2 cells was determined by an MTT assay after a 24-hour culture with the same dose interval of PHA-665752 as in **A.** Error bars represent SEM of triplicate wells. **C.** Adhesion of INA-6 myeloma cells to fibronectin was estimated in an assay in which cells preincubated with a fluorescent dye were seeded in microwells, incubated for 1 hour, washed, and lysed before fluorescence reading. INA-6 cells adhered to fibronectin when stimulated with HGF, SDF-1 α , or IGF-1. Only HGF-induced adhesion was inhibited by PHA-665752. Error bars represent SEM of quadruplicate measurements. **D.** INA-6 cells (5×10^5 per well) were seeded in the top wells of transwell migration chambers. HGF was added to the bottom wells and PHA-665752 to both top and bottom wells. After 18 hours, migration was determined as described in Materials and Methods. HGF-induced migration was inhibited by PHA-665752. Error bars represent SEM of duplicate measurements.

lane). Weak autocrine phosphorylation of p44/42 MAPK (Fig. 6B, fourth panel from bottom, first lane) was also evident after 20 minutes, whereas Akt was still nonphosphorylated. Exogenous HGF or conditioned medium from ANBL-6 cells caused phosphorylation of c-Met in both positions and of both Akt and p44/42 MAPK. In all instances, PHA-665752 counteracted phosphorylation by conditioned medium as efficiently as it eliminated phosphorylation caused by exogenous HGF, indicating that autophosphorylation of Akt and p44/42 MAPK in ANBL-6 cells is caused by HGF and not by other secreted cell products (Fig. 6B, fourth and sixth lanes from the left). We also did experiments in which IL-6 (2 ng/mL) was added during preincubation with anti-HGF serum as well as during the 20-minute stimulation period (Fig. 6B, bottom two panels). As expected, p44/42 MAPK was more strongly phosphorylated than in the absence of IL-6, and PHA-665752 was unable to totally prevent the phosphorylation. Nevertheless, there was a substantial reduction in signal strength by PHA-665752 even in the absence of exogenous HGF, suggesting cooperation between IL-6 and autocrine HGF in MAPK signaling.

DISCUSSION

In this paper, we show that HGF and its receptor c-Met elicited multiple responses in myeloma cells, including proliferation, migration, and adhesion, and that all of these responses could be inhibited by appropriate concentrations of PHA-665752, a novel pharmacologic inhibitor of the c-Met tyrosine kinase. Importantly, PHA-665257 revealed an autocrine HGF-c-Met-driven growth loop in ANBL-6 myeloma cells and in primary myeloma cells.

IC₅₀ of PHA-665752 was 5 to 15 nmol/L. Full c-Met blockade was typically achieved at 100 nmol/L. In the biological assays used, the signaling pathways downstream from the receptors for TGF β , IGF-1, SDF-1 α , and IL-1 were not inhibited by much higher doses of PHA-665752 (e.g., 600 nmol/L for IGF-1 and SDF-1 α and 800 nmol/L for IL-1 and TGF β). Likewise, effector functions involving VLA-4-mediated adhesion, production and secretion of IL-11, and cell migration were not affected by doses of PHA-665752 that gave full c-Met blockade. These results show that PHA-665752 does not interfere with a wide spectrum of

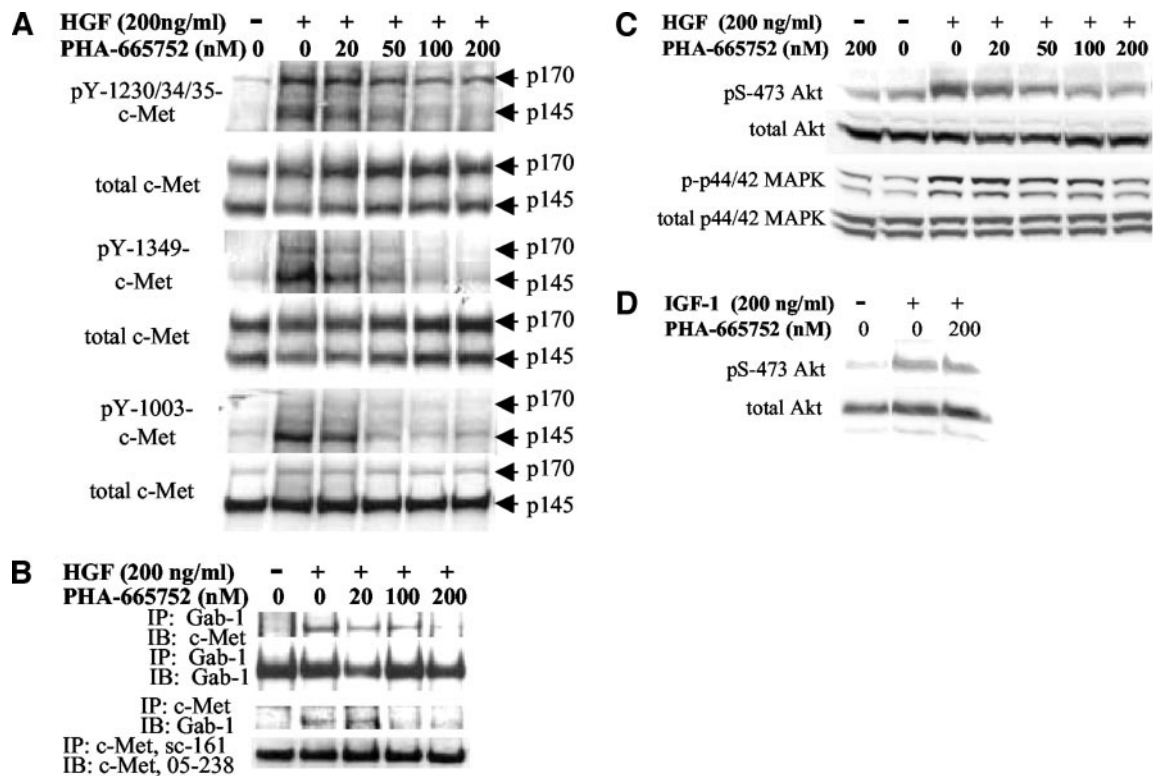


Fig. 5 PHA-665752 blocked tyrosine phosphorylation of c-Met and signaling events downstream of c-Met in myeloma cells. INA-6 cells were treated with HGF (A–C) or IGF-1 (D) after preincubation for 15 minutes with various doses of PHA-665752. Then cells were lysed, and lysates were either processed for protein gel electrophoresis as total lysates (A, C, and D) or subjected to immunoprecipitation with anti-Gab1 or anti-c-Met before gel electrophoresis (B). A, Western blots were probed with antibodies recognizing specific phosphorylated tyrosine residues in the intracellular part of c-Met. Subsequently, the same blots were stripped and probed with an antibody recognizing total c-Met. Arrows point to c-Met precursor protein (p170) and to the β chain of mature c-Met (p145). B, top panel, Gab1 immunoprecipitate, blotted and probed with anti-c-Met; second panel from top, same blot as above, stripped and probed with anti-Gab1; third panel from top, c-Met immunoprecipitate, blotted and probed with anti-Gab1; bottom panel, same blot as above, stripped and probed with anti-c-Met. C and D, Western blots probed with antibodies recognizing phosphorylated Akt (pS-473; C and D) or phosphorylated p44/42 MAPK (C), and the same blots, stripped and probed with antibodies against total Akt or total p44/42 MAPK. pY, phospho-Tyr. IP, immunoprecipitation; IB, immunoblotting.

cell functions, and they confirm recently published data showing that PHA-665752 selectively targets c-Met (25).

The mode of action of PHA-665752 is interference with binding of ATP to the tyrosine kinase domain of c-Met (25). After binding of HGF, ATP is recruited to the receptor and leads to phosphorylation of Tyr^{1230/1234/1235}, which is indispensable for additional catalytic activity (29). We show that PHA-665752 prevented HGF-mediated phosphorylation of Tyr^{1230/1234/1235} in myeloma cells, indicating that PHA-665752 shuts down all signaling due to c-Met phosphorylation. Accordingly, PHA-665752 also prevented phosphorylation of two other tyrosine residues, Tyr¹³⁴⁹ and Tyr¹⁰⁰³.

The c-Met receptor is unique among receptor tyrosine kinases in the sense that a single amino acid sequence (Y¹³⁴⁹VHVNATY¹³⁵⁶VNV) forms a docking region for several SH-2 domain-containing signal transducers (11). It is shown that substitution of Tyr¹³⁴⁹ and Tyr¹³⁵⁶ by phenylalanines blocks HGF-mediated proliferation, motility, invasion, and morphogenesis (30, 31). Phosphorylation of both tyrosines within this multifunctional docking site seems to be critical for binding of most transducers, with the notable exception that Grb2 binds to

Tyr¹³⁵⁶ only (32). Even though the tyrosine kinase domain of the receptor is critical for the actual activation of downstream transducers, binding of ligands to the docking site is probably necessary to bring the transducers in position to be activated. Accordingly, in most studies, prevention of ligand binding to the docking site fully abrogates the c-Met-mediated signal. The prevention of Tyr¹³⁴⁹ phosphorylation suggests that PHA-665752 inhibits binding of transducer molecules downstream of c-Met. This was shown for Gab1, an adaptor molecule that couples to activated c-Met via Grb2. Because Gab1 was prevented from coupling to c-Met, we believe that the bridging molecule Grb2 was also unable to bind to the receptor in the presence of PHA-665752.

We also show that PHA-665752 inhibited the activation of two of the main signaling pathways downstream of c-Met, the phosphatidylinositol 3'-kinase and MAPK signaling pathways. The fact that PHA-665752 did not interfere with IGF-1-induced Akt phosphorylation or with background phosphorylation of Akt and p44/42 MAPK again shows that PHA-665752 works specifically on c-Met.

Multiple myeloma is one of the malignancies in which

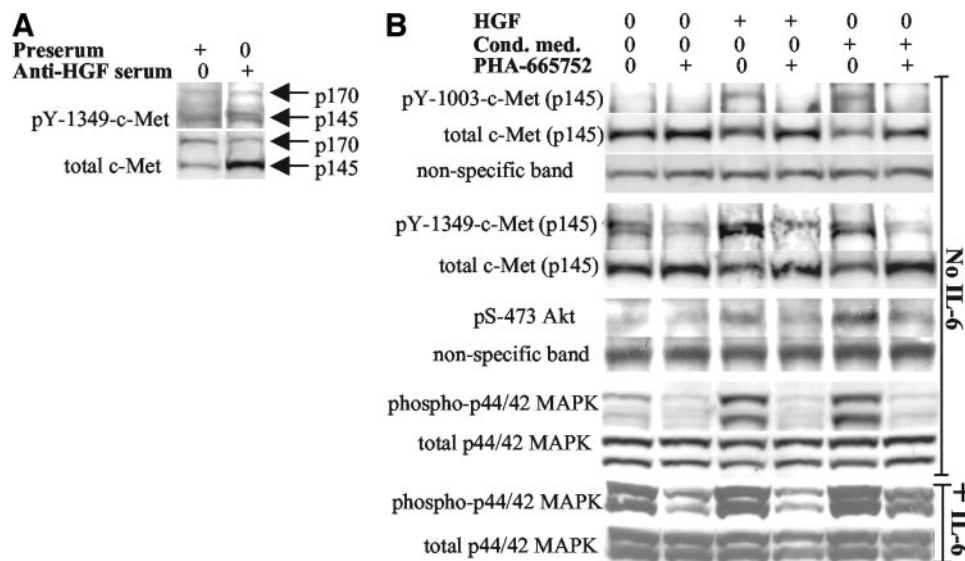


Fig. 6 Autocrine HGF caused phosphorylation of intracellular signaling molecules in ANBL-6 cells. ANBL-6 cells were preincubated overnight with a 1:750 dilution of anti-HGF serum (A and B) or with preimmuneserum (A). Then cells were washed, cultured with or without 200 nmol/L PHA-665752 for 15 minutes, and stimulated for 5 minutes with or without HGF (200 ng/mL) or a 2:3 dilution of conditioned medium from the same cell line. Immunoblots were prepared as described in Materials and Methods. Experiments were either done in the presence of 2 ng/mL IL-6 (two bottom panels of B) or in the absence of IL-6 (all other gels). Arrows in A point to c-Met precursor protein (p170) and to the β chain of mature c-Met (p145). For descriptions of results, refer to the text.

HGF–c-Met seems to be important for progression and spread of the disease. Elevated levels of HGF in serum and in bone marrow fluid from myeloma patients compared with concentrations in body fluids from healthy controls (23, 33, 34), as well as a strong negative prognostic impact of a high serum level of HGF at time of diagnosis (23, 24), support the hypothesis that HGF is indeed of biological significance in this disease. Similarly to normal bone marrow plasma cells (14), malignant plasma cells express the receptor c-Met (16). In addition, myeloma cells, in contrast to normal plasma cells, often express HGF (35). In fact, HGF was the only cytokine on a list of 70 genes (of almost 7000 examined) that were the most significantly up-regulated in myeloma cells compared with normal plasma cells (35). Accordingly, an autocrine HGF–c-Met signal loop was suggested in the myeloma cell line JIN-3, but no biological effect of the putative loop was found downstream from c-Met (19). In the present study, we show that there is an autocrine, HGF–c-Met–mediated growth loop in ANBL-6, another human myeloma cell line, and that the loop can be blocked by PHA-665752. Importantly, four of five samples of proliferating primary myeloma cells responded with reduced proliferation when treated with the c-Met inhibitor, strongly indicating that autocrine HGF-mediated myeloma cell growth is important in patients.

Furthermore, we demonstrate that PHA-665752 was able to completely prevent HGF-induced migration and adhesion of myeloma cells. Multiple myeloma has a disseminated growth pattern, with cancer cells usually spread throughout the skeleton. This dissemination is dependent on migration of cells through endothelial barriers and on adhesion to other cells as well as to matrix components. The importance of myeloma cell adhesion for survival and for resistance to chemotherapeutic

drugs has been documented in several studies (36, 37). Involvement of HGF in transendothelial migration of myeloma cells was recently documented (38). HGF might also be of importance for bone destruction seen in most patients with multiple myeloma. Possible mechanism for this is direct stimulatory effect of HGF on bone-resorbing osteoclasts (39, 40) or HGF-induced release of the potent osteoclast activator IL-11 from osteoblasts (28). In this paper, we show for the first time that a selective inhibitor of c-Met is able to completely block the aforementioned direct effects of HGF on myeloma cells, as well as induction of IL-11 release from an osteoblastic cell line.

HGF and Met are believed to be important contributors to the malignant process in several forms of cancer in addition to multiple myeloma. Treatment directed against the HGF receptor c-Met is therefore an attractive goal. PHA-665752 is a promising substance toward this end. In this study, we found no interference by pharmacologic doses of PHA-665752 with other cell functions than HGF signaling. The study therefore supports the feasibility of c-Met blockade by PHA-665752 or similar agents in cancer patients. In addition, PHA-665752 will become a valuable tool for investigation of the biological roles of HGF and c-Met.

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