MK-2461, a Novel Multitargeted Kinase Inhibitor, Preferentially Inhibits the Activated c-Met Receptor


Abstract

The receptor tyrosine kinase c-Met is an attractive target for therapeutic blockade in cancer. Here, we describe MK-2461, a novel ATP-competitive multitargeted inhibitor of activated c-Met. MK-2461 inhibited in vitro phosphorylation of a peptide substrate recognized by wild-type or oncogenic c-Met kinases (N1100Y, Y1230C, Y1230H, Y1235D, and M1250T) with IC50 values of 0.4 to 2.5 nmol/L. In contrast, MK-2461 was several hundredfold less potent as an inhibitor of c-Met autophosphorylation at the kinase activation loop. In tumor cells, MK-2461 effectively suppressed constitutive or ligand-induced phosphorylation of the juxtamembrane domain and COOH-terminal docking site of c-Met, and its downstream signaling to the phosphoinositide 3-kinase–AKT and Ras–extracellular signal-regulated kinase pathways, without inhibiting autophosphorylation of the c-Met activation loop. BIAcore studies indicated 6-fold tighter binding to c-Met when it was phosphorylated, suggesting that MK-2461 binds preferentially to activated c-Met. MK-2461 displayed significant inhibitory activities against fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor, and other receptor tyrosine kinases. In cell culture, MK-2461 inhibited hepatocyte growth factor/c-Met–dependent mitogenesis, migration, cell scatter, and tubulogenesis. Seven of 10 MK-2461–sensitive tumor cell lines identified from a large panel harbored genomic amplification of MET or FGFR2. In a murine xenograft model of c-Met–dependent gastric cancer, a well-tolerated oral regimen of MK-2461 administered at 100 mg/kg twice daily effectively suppressed c-Met signaling and tumor growth. Similarly, MK-2461 inhibited the growth of tumors formed by s.c. injection of mouse NIH-3T3 cells expressing oncogenic c-Met mutants. Taken together, our findings support further preclinical development of MK-2461 for cancer therapy.

Introduction

c-Met, a member of a receptor tyrosine kinase subfamily that also includes Ron, is a transmembrane protein composed of an extracellular α chain disulfide-bonded to a membrane-spanning β chain. The tyrosine kinase resides in the cytosolic portion of the β chain. Binding of hepatocyte growth factor (HGF), the physiologic ligand, to c-Met induces c-Met dimerization and trans-phosphorylation of two tyrosine residues (Y1234 and Y1235) within the activation loop of the c-Met kinase domain, triggering conformational changes that upregulate its catalytic activity (1, 2). Subsequent phosphorylation of Y1349, Y1356, and Y1365 in the c-Met COOH terminus creates a docking site that interacts with scaffold and signaling molecules. The resulting signaling complex activates pathways such as PI3K-PDK1-AKT-mTOR, Ras-Rac-Pak, Ras-Raf-MEK-ERK, and PLC-γ (2–4). In addition to proliferative and antiapoptotic activities, HGF elicits motogenic and morphogenic cellular phenotypes (2, 5). HGF is also known to be an angiogenic factor (6).

c-Met was first identified as oncogenic fusion Tpr-Met (7). Hereditary human papillary renal carcinoma is causally related to germline mutations in the c-Met kinase domain (8, 9). Aberrant c-Met signaling, resulting from MET genomic amplification, c-Met or HGF overexpression, or c-Met mutations, is found in a variety of human cancers and often correlated with poor clinical outcomes (2, 10). Recently, constitutive c-Met activation due to MET amplification was found to be a driver of proliferation and survival of several gastric and lung cancer cell lines (11, 12) and has been linked to acquired resistance of lung cancers to epidermal growth factor receptor (EGFR) inhibitors (13, 14).

c-Met is an attractive cancer drug target. Small-molecule c-Met kinase inhibitors and antibodies targeting c-Met or HGF have exhibited antitumor activities in preclinical models (15–23). Some of these agents are undergoing clinical...
development (22, 23). Here, we describe a novel multitargeted kinase inhibitor that preferentially inhibits the activated c-Met.

**Materials and Methods**

**Recombinant kinases.** The cytosolic domains of human c-Met, c-Met mutants, Ron, TrkA, insulin-like growth factor I receptor (IGF-IR), and Mer were expressed as NH2-terminal glutathione S-transferase (GST) fusions using Baculogold Baculovirus Expression System (BD Bioscience). GST-tagged KDR and EGFR kinase were from Keith Rickert of Merck Research Labs. His6-tagged Jak2, Flt1, Flt3, Flt4, fibroblast growth factor receptor 1 (FGFR1), FGFR2, FGFR3, and TrkB kinases were from Upstate Biotechnology (Millipore). His6-tagged platelet-derived growth factor receptor β (PDGFRβ) and the c-Met cytosolic domain were from Invitrogen.

**Cell lines.** The GTL-16 gastric cancer cell line was from S. Giordano and P.M. Comoglio (University of Torino Medical School, Turin, Italy); OCUM-1 gastric cancer and EBC-1 lung carcinoma cell lines were from Health Science Research Resources Bank (Japan); Sum-52 breast cancer line was from Asterand, Inc.; NIH 3T3 cell lines harboring c-Met mutations T3936C and T3997C were from L. Schmidt (National Cancer Institute, Bethesda, MD); and Madin-Darby canine kidney (MDCK) type II cell line was from Clontech. All other cell lines were from the American Type Culture Collection.

**Marine myeloid 32D cells harboring Tpr-Met fusions.** Tpr-Met and Tpr-Met (Y362) mutant were cloned into pLenti6/V5-DEST (Invitrogen) and transfected into 293FT packaging cells (Invitrogen) to produce lentiviruses. The lentiviruses were used to infect 32D cells, which were then passaged in interleukin 3 (IL-3)–free medium under blasticidin selection to achieve stable expression of the fusion proteins.

**Antibodies.** The antibodies used included anti-phospho-c-Met (Y1003), anti-phospho-c-Met (Y1003), and anti-phospho-c-Met (Y1003), from Biosource; anti-phospho-c-Met (Y1003), and anti-phospho-c-Met (Y1003), from Biosource; and anti-phospho-c-Met (Y1003), and anti-phospho-c-Met (Y1003), from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource; and anti-phospho-c-Met (Y1234/Y1235) from Biosource.
(Y1234/Y1235), anti-phospho-c-Met (Y1349), anti-phospho-FGFR (Y653/Y654), anti-phospho-PDGFR (Y857), anti-phospho-AKT (S473), anti-phospho–extracellular signal-regulated kinase 1/2 (ERK1/2) (T202/Y204), anti-c-Met, anti-PDGFR, anti-AKT, and anti-ERK1/2 from Cell Signaling Technology; anti-FGFR2 and anti-EGFR from R&D Systems; and anti-phospho-tyrosine antibody 4G10 from Millipore.

In vitro kinase assays. c-Met–catalyzed phosphorylation of N-biotinylated peptide (EQEDEPEGDYFEWLE-CONH₂) was measured using a time-resolved fluorescence resonance energy transfer assay adapted from Park and colleagues (24). To evaluate kinase selectivity, a single concentration (1 μmol/L) of MK-2461 was tested using 216 kinases by Upstate Biotechnology, Inc. (Millipore), which also determined the MK-2461 IC₅₀ for DRAK1, DYRK2, IRAK1, IRAK4, MELK, and MLK1. The MK-2461 IC₅₀ for Ron, Mer, Flt1, Flt3, Flt4, KDR, PDGFRβ, FGFR1, FGFR2, FGFR3, TrkA, and TrkB were determined using time-resolved fluorescence resonance energy transfer assays similar to the c-Met kinase assay.

c-Met autophosphorylation assay. The c-Met cytosolic domain was preincubated with MK-2461. The autophosphorylation was initiated by addition of 50 μmol/L ATP and run for 30 min at 25°C. The reactions were stopped by a denaturing buffer and by boiling, and then the samples were subjected to Western blotting with antibodies targeting various phosphotyrosine residues of c-Met.

Analysis of phosphorylation status of c-Met, FGFR2, PDGFR, EGFR, AKT, and ERK in cells. Tumor cells were treated for 2 h with MK-2461 or vehicle in RPMI 1640 supplemented with 10% fetal bovine serum and 10 mmol/L HEPES. When called for, the cells were stimulated with HGF or EGF during the last 10 min of the 2-h incubation. The cells were lysed with a denaturing or nondenaturing buffer and then with a denaturing or nondenaturing buffer containing phosphatase and protease inhibitors and subjected to Western blot or immunoprecipitation–Western blot analysis.

Direct binding assay. The His6-tagged c-Met cytosolic domain (Invitrogen) was immobilized on two detection spots of a CM5 sensor chip in a BIAcore 51 instrument through an amino group to both chambers and the plate was incubated for 2 h. HGF was then added to the lower chamber and the plate was incubated for an additional 20 h. Viable cells on the underside of MK-2461 on the abundance of a phosphotyrosine residue(s) of interest was gauged from ion-intensity ratio of the heavy (MK-2461–treated)– and light (vehicle-treated)–labeled tryptic peptide containing the phosphotyrosine(s). The ratio was normalized using the heavy/light ratio of the self-to-self control.

Assessment of motogenic and morphogenic cellular phenotypes. HGF-induced migration of HPAF II pancreatic cancer cells was measured by a modified Boyden chamber method using a Fluoroblock 96-Multiwell Insert System (BD Biosciences). The cells in DMEM plus 10% fetal calf serum were plated into the upper chamber semisubmerged in the lower chamber containing the same medium. MK-2461 was added to both chambers and the plate was incubated for 2 h. HGF was then added to the lower chamber and the plate was incubated for an additional 20 h. Viable cells on the underside of the chamber were counted using a Fluoroblock 96-Multiwell Insert System (BD Biosciences). The surface plasmon resonance (SPR) signals recorded from the spot with unphosphorylated c-Met were subjected to the same procedures. The effect of MK-2461–containing buffer and then with a MK-2461–free buffer. A 2-fold dilution series of MK-2461 was tested. The surface plasmon resonance (SPR) signals recorded from the spot with unphosphorylated c-Met (A) and the spot with phosphorylated c-Met (B) are shown. The rate constants kₘₐₓ and kₜₜₐₐ were obtained from fitting each data set globally with a 1:1 interaction model. The smooth lines represent the best fits. The equilibrium constant Kₐ was calculated as kₜₜₐₐ/kₘₐₓ.
the membrane were stained using Calcein AM (Invitrogen) and quantified using a fluorescence reader. HGF-induced scatter of HPAF II cells was measured as described previously (28). HGF-induced tubulogenesis of MDCK cells was monitored as described by Yamaguchi and colleagues (29).

**Proliferation and mitogenic assays.** Proliferation and viability of tumor cells was measured using the ViaLight PLUS kit (Cambrex). HGF-dependent mitogenic activity of 4MBr monkey lung cells was measured by monitoring bromodeoxyuridine (BrdUrd) incorporation into genomic DNA using the Roche Cell Proliferation ELISA, BrdUrd (chemiluminescence) kit.

**Tumor xenograft models.** GTL-16 cells or c-Met mutant-transformed NIH3T3 cells were inoculated s.c. into the flank of female nude CD-1 nu/nu mice. When mean tumor size reached a predetermined range, the mice were randomized and given vehicle or MK-2461 by p.o. gavage once or twice daily. Tumor volumes were determined using calipers. The percentage increase in the volume of a xenograft tumor on day n versus day 0 (the day when dosing of MK-2461 began) was calculated as (tumor volume on day n – tumor volume on day 0) / tumor volume on day 0 × 100. The mean percentage of tumor growth inhibition in each MK-2461-treated group relative to the vehicle-treated group was calculated as (1 – mean percent increase of tumor volume in the MK-2461-treated group/mean percent increase of the tumor volume in the vehicle-treated group) × 100.

**Measurement of c-Met (Y1349) phosphorylation in xenograft tumors.** Mice bearing GTL-16 tumors were euthanized 1 h after p.o. administration of MK-2461. The tumors were excised, snap-frozen, and dispersed using a Qiagen Tissue-Lyser in a non-denaturing lysis buffer containing protease and phosphatase inhibitors. The homogenate was lysed at 4°C for 1 h, clarified by centrifugation, and then analyzed by quantitative Western blotting for phospho-c-Met (Y1349) and total c-Met. The pMet (Y1349) signal of each c-Met band was normalized with its total c-Met signal. To combine or compare data from several gels, the pY1349/total Met ratio for each c-Met band was further normalized to the average pY1349/total c-Met ratio of the vehicle-treated tumor samples on the same gel.

**Results**

**MK-2461 is a potent ATP-competitive kinase inhibitor that preferentially binds the activated c-Met.** MK-2461 (Supplementary Fig. S1) inhibited the kinase activity of human c-Met with a mean IC_{50} of 2.5 nmol/L in the presence of 50 μmol/L ATP. Steady-state kinetics experiments (Supplementary Fig. S2) showed that MK-2461 is competitive with ATP and noncompetitive with respect to the peptide substrate.

The effect of MK-2461 on ATP-induced autophosphorylation of recombinant c-Met cytosolic domain was determined by Western blotting. As shown in Fig. 1A, MK-2461 was substantially more potent against the autophosphorylation of Y1349 (IC_{50} = 100 nmol/L) and Y1365 (IC_{50} = 26 nmol/L) in

![Figure 3. MK-2461 effectively inhibited phosphorylation of the activation loop of FGFR2 and PDGFR in cells. A, Western blots showing that MK-2461 inhibited phosphorylation of the activation loop (Y635/Y654) of FGFR2 in Kato III gastric cancer cells. B, Western blots showing that MK-2461 inhibited phosphorylation of the activation loop (Y849) of PDGFR in H1703 lung cancer cells. C, MK-2461 does not inhibit EGF-induced phosphorylation of EGFR in A549 lung cancer cells. The cells were incubated for 2 h with 2 μmol/L MK-2461, 2 μmol/L gefitinib or vehicle, stimulated with EGF for 10 min, and then lysed. EGFR was immunoprecipitated and subjected to Western blotting with an anti-phosphotyrosine antibody (4G10) and anti-total EGFR antibody. Note that 2 μmol/L gefitinib abrogated EGFR phosphorylation.](image-url)
of a c-Met COOH-terminal peptide dually phosphorylated at Y1349 and Y1356, and 83% decrease in the abundance of the same peptide singly phosphorylated at either Y1349 or Y1356. This was accompanied by a 64% decrease in the abundance of a peptide containing phosphorylated Y1003 of the juxtamembrane domain and a 47% reduction in the abundance of an activation loop–derived peptide singly phosphorylated at Y1234. In contrast MK-2461 caused a moderate increase (25%) in the abundance of the activation loop peptide dually phosphorylated at Y1234 and Y1235.

The ability of MK-2461 to inhibit autophosphorylation of the juxtamembrane domain and COOH-terminal docking site of c-Met while remaining essentially inactive against dual tyrosine (Y1234 and Y1235) phosphorylation of the c-Met activation loop is consistent with MK-2461 binding preferentially to the activation loop–phosphorylated c-Met. To test this

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**Figure 4.** MK-2461 inhibited HGF/c-Met–dependent cellular phenotypes.
A, MK-2461 inhibited HGF-induced scattering of HPAF II pancreatic cancer cells in a two-dimensional culture. The cells were stained with Hoechst 33342 and imaged on an INCell1000. B, MK-2461 inhibited HGF-induced tubulogenesis of MDCK cells in three-dimensional culture. Shown are the images of randomly selected MDCK cysts. C, the effects of MK-2461 on IL-3–independent proliferation of 32D cells transformed with Tpr-Met (○) or Tpr-Met (Y362C) mutant (▵), and on IL-3–dependent growth of parental 32D cells (▼). Each data point represents the average of duplicate determinations.
model, the binding of MK-2461 to phosphorylated and unphosphorylated c-Met cytosolic domain was directly measured using BLAcore (Fig. 2). Two independent experiments showed that the average equilibrium dissociation constant ($K_d$) of MK-2461 for phosphorylated c-Met (4.0 nmol/L) is 6-fold lower than that for unphosphorylated c-Met (25.1 nmol/L). The higher affinity of MK-2461 for phosphorylated c-Met resulted from faster association rate constant ($k_{on}$) and slower dissociation rate constant ($k_{off}$). These findings provided direct evidence that MK-2461 preferentially binds the activated conformations of c-Met.

**In vitro activity of MK-2461 against c-Met mutants.** Several oncogenic c-Met mutations reportedly confer resistance to certain c-Met kinase inhibitors (15, 16, 30, 31). We thus compared *in vitro* potencies of MK-2461 against wild-type c-Met and five c-Met mutants, including three activation loop mutants (Y1230C, Y1230H, and Y1235D), a P + 1 loop mutant M1250T, and N1100Y, which is near the ATP binding site. In the presence of 50 μmol/L ATP, MK-2461 is equally or more potent against the five mutants (mean IC$_{50}$ ranging from 0.4 to 1.5 nmol/L) compared with the wild-type c-Met (mean IC$_{50}$ = 2.5 nmol/L; Supplementary Table S1).

**Kinase selectivity of MK-2461.** *In vitro* kinase assays revealed that Ron (IC$_{50}$ = 7 nmol/L) and Flt1 (IC$_{50}$ = 10 nmol/L) were inhibited by MK-2461 with similar potencies to c-Met (IC$_{50}$ = 2.5 nmol/L), whereas nine other kinases, including FGFFR1, FGFFR2, FGFFR3, PDGFRβ, KDR, Flt3, Flt4, TrkA, and TrkB, were found to be 8- to 30-fold less sensitive to MK-2461 than c-Met (Supplementary Table S1). We further evaluated the effect of MK-2461 on autophosphorylation of FGFFR2 and PDGFRα, respectively, in KATO III cells, which overexpress constitutively activated FGFFR2 (32), and H1703 cells, which overexpress constitutively activated PDGFR-α (33). MK-2461 potently inhibited phosphorylation of the activation loop of FGFFR2 (Y653/Y654) and PDGFR-α (Y849) in the cells with IC$_{50}$ <300 nmol/L (Fig. 3A and B). These observations contrast with the inability of MK-2461 to inhibit activation loop phosphorylation in c-Met. Consistent with its lack of inhibitory activity against recombinant EGFR kinase, MK-2461 did not inhibit ligand-induced phosphorylation of EGFR in A549 lung cancer cells (Fig. 3C).

**Inhibition by MK-2461 of HGF/c-Met–dependent cellular phenotypes.** MK-2461 inhibited HGF-induced mitogenesis (measured as BrdU incorporation) of 4MBr-5 monkey lung epithelial cells (34) with an IC$_{50}$ of 204 ± 47 nmol/L (SE, $n = 5$). (The result of a representative experiment is shown in Supplementary Fig. S3.) The compound inhibited HGF-induced scattering of HPAF II human pancreatic adenocarcinoma cells (35) in a dose-dependent manner (Fig. 4A), with a mean IC$_{50}$ of 416 ± 26 nmol/L (SE, $n = 23$) as determined by an automated imaging assay (28). Using a modified multwell Boyden chamber system, we found that MK-2461 inhibited HGF-induced migration of HPAF II cells with a mean IC$_{50}$ of 404 ± 47 nmol/L (SE, $n = 11$). (A representative experiment is shown in Supplementary Fig. S4.) MK-2461 inhibited, in a concentration-dependent manner, the HGF-induced branching tubulogenesis of MDCK cells in a three-dimensional collagen gel matrix (Fig. 4B).

To assess the cellular activity of MK-2461 against oncogenic c-Met activation loop mutants, we tested the effect of MK-2461 on proliferation of 32D murine myeloid leukemia cells transformed by Tpr-Met or Tpr-Met (Y362C) mutant, which is equivalent to the c-Met activation loop mutant Y1230C. The 32D/Tpr-Met and 32D/Tpr-Met (Y362C) cells acquired the ability to proliferate in the absence of IL-3, which was needed to support proliferation and survival of the parental 32D cells. As shown in Fig. 4C, MK-2461 potently inhibited IL-3–independent growth of 32D/Tpr-Met and 32D/Tpr-Met (Y362C) cells (IC$_{50}$ = 100 nmol/L). Importantly, MK-2461 was ~100-fold less potent in inhibiting IL-3–dependent proliferation of the parental 32D cells, indicating that the potent antiproliferative effects of MK-2461 on the Tpr-Met–expressing cells resulted from specific inhibition of Tpr-Met signaling.

**Genomic amplification and constitutive activation of c-Met, FGFFR2, or PDGFR are predictors of in vitro tumor response to MK-2461.** The potency of MK-2461 for inhibiting *in vitro* proliferation and viability of tumor cells was evaluated using a tumor cell line panel. The observed antiproliferative potency of MK-2461 varied widely with mean IC$_{50}$ ranging from 0.4 to 1.5 nmol/L compared with the wild-type c-Met (mean IC$_{50}$ = 2.5 nmol/L; Supplementary Table S1).
IC$_{50}$ values ranging from 0.1 to >30 μmol/L (Fig. 5). The cell lines were classified into three groups, described as being sensitive (IC$_{50}$ ≤ 1 μmol/L), moderately sensitive (1 μmol/L < IC$_{50}$ ≤ 5 μmol/L), and insensitive (IC$_{50}$ > 5 μmol/L) to MK-2461. Among the 10 MK-2461–sensitive cell lines, GTL-16 and SNU5 gastric cancer cell lines and EBC-1 and H1993 non–small cell lung carcinoma cell lines were previously reported to overexpress constitutively activated c-Met protein due to MET genomic amplification (11, 12, 36). In addition, KATO III, SNU16 gastric cancer cell lines, and SUM-52 breast cancer cell line were previously shown to overexpress constitutively activated FGFR2 protein due to FGFR2 genomic amplification (32, 37). The H1703 non–small cell lung carcinoma cell line was confirmed, by Western blotting analysis (Fig. 3B), to harbor a high level of constitutively activated PDGFR as previously reported (33). Neither overexpression nor constitutive activation of MET, FGFR, or PDGFR was detected (data not shown) in the two remaining MK-2461–sensitive cell lines, KM12 colon cancer cell line and H1048 small-cell lung carcinoma cell line. With the exception of the MKN-45 gastric cancer line (IC$_{50}$ = 1.2 μmol/L), known to harbor MET genomic amplification (36), all tumor cell lines classified as moderately sensitive or insensitive to MK-2461 did not show MET or FGFR2 amplification and express only low levels of functional c-Met protein, which was not phosphorylated under the condition of the proliferation assay (data not shown). These results show that c-Met or FGFR2 genomic amplification and constitutive activation of PDGFR are predictive biomarkers of in vitro tumor response to MK-2461.

MK-2461 inhibited c-Met signaling and tumor growth in tumor xenograft models in mice. To assess the in vivo potency of MK-2461 for inhibiting c-Met, the extent of c-Met Y1349 phosphorylation in GTL-16 tumors was measured 1 hour after the tumor-bearing mice were given an oral bolus of MK-2461 at several dose levels. Plasma samples were analyzed for total concentration of MK-2461. Dose-dependent inhibition of phosphorylation of tumor c-Met (Y1349) was observed and found to be correlated with the concentration of MK-2461 in the plasma. From the relationship between the plasma MK-2461 concentration and tumor phospho–c-Met (Y1349), the in vivo IC$_{50}$ of MK-2461 for c-Met inhibition was estimated to be 1.0 μmol/L (Fig. 6A).

Two independent efficacy studies were conducted to assess the in vivo antitumor activity of MK-2461 in nude mice bearing established subcutaneous GTL-16 tumors. In the first experiment (Fig. 6B), mice were treated with vehicle or one of four 21-day p.o. regimens of MK-2461 [10, 50, and 100 mg/kg twice daily (bid) and 200 mg/kg once daily], which inhibited tumor growth by 62%, 77%, 75%, and 90%, respectively. The mean terminal tumor weights of the four MK-2461 cohorts were 427 ± 91 mg (SE, n = 10), 358 ± 81 mg (n = 12), 340 ± 46 mg (n = 13), and 232 ± 52 mg (n = 13), compared with the mean tumor weight of 1,129 ± 231 mg (n = 9) for the vehicle.
cohort. Statistical analyses showed that each of the four MK-2461 regimens had significant growth-inhibitory effect compared with the vehicle. In the second efficacy study, which evaluated five 21-day p.o. MK-2461 regimens (1, 3, 10, 30, and 100 mg/kg bid), the 100 mg/kg bid regimen caused essentially complete (99%) tumor growth inhibition, whereas the other four MK-2461 regimens did not result in statistically significant tumor growth inhibition (Supplemental Fig. S5). All MK-2461 regimens tested in the two studies were well tolerated, causing neither statistically significant loss in mean body weight nor treatment-related death.

We also tested a MK-2461 p.o. regimen (134 mg/kg bid) in two mouse xenograft models derived from NIH3T3 cells transformed by c-Met single nucleotide mutants T3936C and T3997C, respectively. The mutations are equivalent to Y1230H and M1250T mutations of human c-Met. Based on the tumor growth curves from day 0 to day 16, MK-2461 inhibited growth of T3936C and T3997C tumors by 78% (Fig. 6C) and 62% (Fig. 6D), respectively, and the growth-inhibitory effects of MK-2461 in both models were statistically significant. These results showed the capacity of MK-2461 to inhibit c-Met mutation–driven tumors in vivo.

Discussion

Here, we have identified MK-2461, a novel ATP-competitive multitargeted kinase inhibitor. Several lines of findings strongly suggest that MK-2461 preferentially binds to the activated c-Met with dual-phosphorylated activation loop and inhibits c-Met signaling by suppressing phosphorylation of tyrosine residues in the juxtamembrane domain and COOH-terminal docking site of c-Met. These properties distinguish MK-2461 from other known ATP-competitive tyrosine kinase inhibitors, which either bind to the unactivated and active kinase with similar affinity or preferentially bind the unactivated kinase (38, 39). Additional studies will be needed to decipher the molecular mechanism that allows the activation loop in MK-2461–bound c-Met to remain fully phosphorylated in the face of a cytosolic environment rich in protein phosphatases.

Significantly, we found that MK-2461 effectively inhibited autophosphorylation of the activation loop of FGFR2 and PDGFR in cells (Fig. 3). These observations suggest that MK-2461 is capable of preventing activation of FGFR2 and PDGFR, presumably by binding to the unactivated conformation of these kinases. The differences between the binding modes of MK-2461 to c-Met and to FGFR2 may contribute to the observation that MK-2461 exhibits more potent anti-proliferative activity in FGFR2-driven cells than in c-Met–driven cells, despite in vitro kinase assays showing that MK-2461 is more potent against c-Met than against FGFR2. Structural studies of several unactivated tyrosine kinases revealed that parts of the activation loop and/or other structural elements assume configurations that border or block the ATP binding site (40–43). Therefore, the unactivated conformation may have significantly lower affinity for ATP than does the active conformation (38, 39). Compared with inhibitors preferentially binding to the unactivated conformation, inhibitors preferentially binding to the active conformation face stronger competition from cellular ATP and are thus likely to show a larger potency reduction in cell-based assays relative to cell-free assays. Iminatinib, which preferentially binds an unactivated conformation of BCR-Ab1, is known to bind an active, DFG-in conformation of spleen tyrosine kinase, providing a precedent that a tyrosine kinase inhibitor adopts different binding modes in different kinase contexts (44).

In a majority of receptor tyrosine kinases, including c-Met, the phosphotyrosine residues in the juxtamembrane domain and in the region COOH-terminal to the conserved kinase domain seem to be primarily responsible for recruiting adapter proteins and signaling molecules. However, the phosphorylated activation loop of insulin receptor tyrosine kinase has been shown to bind and signal through adaptor proteins APS and Grb10/14 (45, 46). MK-2461 does not block activation loop phosphorylation of c-Met and would be ineffective in inhibiting signaling events that emanate from the signaling or adapter proteins that interact with the activation loop of c-Met. Comparative studies using MK-2461 and c-Met inhibitors capable of inhibiting c-Met activation loop phosphorylation may allow identification of signaling molecules and pathways that are differentially modulated by c-Met inhibitors of different modes of action.

MK-2461 was equally or slightly more potent against five naturally occurring oncogenic human c-Met mutants (N1100Y, Y1230C, Y1230H, Y1235D, and M1250T) compared with wild-type c-Met and inhibited the growth of NIH3T3 tumors harboring murine equivalents of human c-Met Y1230H and M1250T mutants. To date, about 15 c-Met kinase domain missense mutations have been detected in papillary renal carcinomas, with the tyrosine residues Y1230 and Y1235 in the c-Met activation loop being “hotspots” for mutations (2). The missense mutations of Y1230 and Y1235 confer absolute or partial resistance to several known c-Met inhibitors, including PF-04217903, a c-Met inhibitor currently undergoing clinical development (15, 16, 30, 31). A novel class of c-Met inhibitors represented by AM7 (16) exhibits uncompromised inhibitory activities against these c-Met mutants. MK-2461, which is structurally distinct from AM7, represents a new pharmacophore with this desirable biological property. The c-Met inhibitors potent against a broad spectrum of activating c-Met mutants may hold promise for treatment of papillary renal cell carcinoma.

Genomic amplification of c-Met or FGFR2 occurs at higher frequencies in gastric cancers (47, 48) and is believed to be a major driver of malignancy (11, 12, 32). A dual c-Met/FGFR2 inhibitor such as MK-2461 is, potentially, a promising therapeutic for the subset of gastric cancers that display either c-Met or FGFR2 amplification.

In summary, MK-2461 is a novel orally available multitargeted kinase inhibitor, which inhibits c-Met signaling and HGF/c-Met–dependent cellular phenotypes by selectively targeting the active conformation of c-Met. It also exhibits significant activities toward several other oncology kinase targets, including FGFR and PDGFR. It has signifi-
cant in vitro and in vivo antitumor activities against c-Met- and FGFR2-driven tumors. The results described in this report provided justification for advancing MK-2461 to preclinical development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Acknowledgments

We thank Drs. Lex Van der Ploeg, Giulio Draetta, and Mark Goulet for their support.

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Received 7/15/09; revised 10/27/09; accepted 11/17/09; published OnlineFirst 2/9/10.


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Cancer Res 2010;70:1524-1533. Published OnlineFirst February 9, 2010.

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doi:10.1158/0008-5472.CAN-09-2541

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