c-Met Inhibitors with Novel Binding Mode Show Activity against Several Hereditary Papillary Renal Cell Carcinoma-related Mutations*

Received for publication, July 13, 2007, and in revised form, November 28, 2007 Published, JBC Papers in Press, November 30, 2007, DOI 10.1074/jbc.M705774200

Steven F. Bellon†, Paula Kaplan-Lefko‡, Yajing Yang‡, Yihong Zhang†, Jodi Moriguchi†, Karen Rex†, Carol W. Johnson§, Paul E. Rose‡, Alexander M. Long‡, Anne B. O’Connor§, Yan Gu§, Angela Coxon§, Tae-Seong Kim¶, Andrew Tasker†, Teresa L. Burgess§, and Isabelle Dussault§†

From the Departments of †Molecular Structure, §Oncology Research, §Pathology, and ¶Medicinal Chemistry, Amgen Inc., Thousand Oaks, California 91320

The c-Met receptor tyrosine kinase is a well established mediator of carcinogenesis. Under physiological conditions such as during embryonic development, c-Met is activated by its ligand, hepatocyte growth factor (HGF),2 to induce an invasive program consisting of cell proliferation, migration, invasion, survival, and branching morphogenesis. However, deregulation of c-Met activity is common in human cancers and is associated with poor outcome (1, 2).

Hereditary papillary renal cell carcinoma is a familial cancer characterized by multifocal and bilateral kidney lesions that becomes symptomatic usually when affected individuals reach their fourth decade of life. Linkage analysis has been used to demonstrate that all patients with this hereditary disorder harbor activating mutations in the c-Met kinase domain (3). Expression of recombinant mutant receptors in cell lines leads to constitutive phosphorylation of the receptor, downstream pathway activation, and cell motility that is consistent with the oncogenic nature of these mutations (4–8). Moreover, these different mutations cause cancer in animal models, proving a causative role for c-Met in carcinogenesis (6).

In addition to activating mutations, c-Met overexpression is frequently observed in tumors, making c-Met an attractive target for cancer therapy. Multiple groups have pursued different approaches to block c-Met activity. Among them are antibodies against HGF (9–11) or c-Met (12), truncated forms of HGF with antagonistic activity on c-Met such as NK4 (13–15), decoy receptors (16), strategies to prevent receptor dimerization such as with a soluble c-Met Sema domain (17), ribozymes targeting either HGF or c-Met (18, 19), and small molecule kinase inhibitors (20–22). Most of these intervention strategies have shown anti-tumor activity in preclinical animal models; however, their clinical utility remains to be determined. Whereas some of these approaches are better suited to block ligand-mediated c-Met activity, small molecule kinase inhibitors offer the most versatile approach by inhibiting HGF-dependent tumors as well as tumors driven by other c-Met-dependent mechanisms, such as receptor amplification and activating mutations.

Clinical experience with small molecule kinase inhibitors has demonstrated that the selectivity of some of these inhibitors for wild-type versus mutant can be a cause of drug resistance (primary drug resistance in the case of pre-existing mutations or acquired resistance due to chronic treatment). For example, imatinib blocks the kinase activity of wild-type Abl and c-Kit but has weaker or no activity against several acquired mutations that arise as a result of imatinib therapy in chronic myelogenous leukemia and gastrointestinal stromal tumor patients (23–26). A well documented mechanism of imatinib-acquired resistance results from the inability of imatinib to bind to the active conformation adopted by the Abl mutants. This type of resistance
can be overcome by other drugs such as dasatinib, an inhibitor of Abl that can bind to both the inactive and the active form of the Abl kinase domain (27–29). The clinical activity of imatinib in gastrointestinal stromal tumor is similarly related to the type of mutations found in these tumors. In this case, imatinib sensitivity is often associated with activating mutations in exon 11 encoding the juxtamembrane domain of c-Kit, whereas patients with mutations in exon 9, which encodes the extracellular domain, are less responsive to the drug (30–32). Exon 9 mutations, thus, appear to confer primary resistance to imatinib. Acquired resistance after chronic imatinib therapy also occurs in gastrointestinal stromal tumor patients and is commonly due to secondary mutations in c-Kit (30, 33).

Recent work by Berthou et al. (34) suggests that primary resistance could also be encountered in tumors harboring certain c-Met mutations. They found that the c-Met small molecule inhibitor SU11274 can inhibit the activity of some c-Met mutants previously identified in hereditary papillary renal cell carcinoma patients, including H1094Y3 (H1112Y) and M1250T (M1268T), but that other mutants such as L1195V (L1213V) and Y1230H (Y1248H) were resistant to this inhibitor. As established for imatinib in chronic myelogenous leukemia and gastrointestinal stromal tumor, this information is critical to make decisions on patient treatment. Furthermore, these data imply that different c-Met inhibitors will be needed in the clinic to overcome the predicted resistance.

We have identified novel, selective c-Met small molecule inhibitors that inhibit several c-Met mutations found in hereditary papillary renal cell carcinoma patients that were shown to be resistant to SU11274. We have determined the crystal structure of a representative of this new class of inhibitor bound to unphosphorylated c-Met and compared this structure with the crystal structure of SU11274 also bound to unphosphorylated c-Met. The binding modes of the two inhibitors overlap minimally, which is consistent with their differential abilities to inhibit these c-Met mutations. Our results demonstrate that c-Met inhibitors can bind to the receptor in a drastically different manner that influences their activity on c-Met mutants and that it is possible to identify agents that will inhibit a wide range of c-Met primary mutations.

**EXPERIMENTAL PROCEDURES**

**Cloning and Bacmid Construction**—The wild-type c-Met kinase domain (amino acid 1058–1365) was PCR-amplified from full-length c-Met and inserted into the BamHI-XhoI sites of the Invitrogen pFastBac 1 vector downstream of a GST tag and a thrombin cleavage site previously engineered in pFastBac 1. Site-directed mutagenesis was used to generate the following mutations in the c-Met kinase domain: V1092I, H1094R, D1228H, Y1230H, M1250T. All constructs were confirmed by sequencing. The Invitrogen Bac-to-Bac protocol was followed to obtain recombinant bacmids.

**Preparation of GST Fusion Proteins**—The bacmids were transfected with calcium phosphate into Sf9 cells. Resultant viruses were amplified in SF9 cells. To express GST fusion proteins, Hi5 cells were seeded at a density of 1 × 10^6 cells/ml with a 10% viral addition. Protein expression was allowed to proceed for 48 h at 27 °C. Cells were pelleted, resuspended in 5 volumes of lysis buffer (50 mM HEPES, 0.25 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, pH 8.0) with protease inhibitors and then passed through a Microfluidizer three times. The lysates were centrifuged, and supernatants were mixed with an appropriate amount of glutathione-Sepharose 4B resin at 7 °C overnight. The GST-c-Met fusion proteins were eluted with buffer containing 10 mM glutathione, pooled, and concentrated. Phosphorylation of purified GST-c-Met was performed by mixing the following reactants to the final concentrations indicated: 0.5 mg/ml GST-c-Met, 25 mM ATP, 100 mM MgCl₂, 15 mM Na₃VO₄, 50 mM Tris-HCl at room temperature for 3 h. The identity of all proteins was confirmed by Western blotting.

**In Vitro Kinase Assay**—IC₅₀ measurements of AM7 versus c-Met kinase and its mutants were determined using homogeneous time-resolved fluorescence assays as previously described (35). Molecules were tested in a 10-point serial dilution for each c-Met construct using an ATP concentration of two-thirds the K_m value that was determined for each enzyme preparation and calculated using the Eadie-Hofstee and Lineweaver-Burke methods.

**Cells**—PC3, CT26, A431, and U-87 MG were obtained from American Type Culture Collection (ATCC, Manassas, VA), and MKN-45 human gastric carcinoma cells were obtained from Health Science Research Resources Bank (Japan). Cells were grown as monolayers using standard cell culture conditions.

**Cell-based Autophosphorylation Assay of c-Met**—IC₅₀ measurements of AM7 activity on HGF-mediated c-Met autophosphorylation was determined in serum-starved PC-3 (human) or CT26 (mouse) cells using a quantitative electrochemiluminescent immunoassay. PC3 and CT26 cells were plated at a density of 20,000 cells/well in 96-well plates. 24 h after plating cells were starved in media containing 0.1% bovine serum albumin for 18–20 h. Cells were then treated with a 10-point serial dilution of AM7 for 1 h at 37 °C followed by stimulation with optimal concentrations of recombinant human HGF for 10 min at 37 °C. Cells were washed once with phosphate-buffered saline and lysed (1% Triton X-100, 50 mM Tris, pH 8.0, 100 mM NaCl, 300 μM Na₃VO₄, and protease inhibitors). Cell lysates were incubated with a biotin-labeled goat-anti-c-Met antibody (BAF358, for human c-Met; BAF527, for mouse c-Met; R&D Systems, Minneapolis, MN) for capture followed by a mouse anti-phosphotyrosine antibody 4G10 (Upstate, Charlotteville, VA) and a BV-tag™ labeled anti-mouse IgG (BioVeris Inc., Gaithersburg, MD) as the detection antibody. Levels of c-Met phosphorylation were then measured on a BioVeris M-Series instrument. The IC₅₀ values are calculated using Xlfit4-parameter equation.

**Immunoblot Analysis**—MKN-45 cells were serum-starved for 18–20 h before treatment with increasing concentrations of AM7 for 1 h at 37 °C. U-87 MG cells were similarly treated or received human HGF for 10 min after treatment with AM7.
Monoclonal antibody 2.4.4 (9) was used at a final concentration of 200 nM for 1 h to sequester endogenous HGF from U-87 MG cells. Western blots were done using standard procedures and probed with the following antibodies: anti-c-Met (C12, Santa Cruz Biotechnology), anti-phospho-c-Met (Tyr-1234/1235), anti-phospho-Gab-1 (Tyr-627), anti-phospho-ERK1/2 (Thr-202/Tyr-204), anti-phospho-p70S6K (Thr-421/Ser-424), anti-phospho-S6 ribosomal protein (Ser-240/244) and anti-ERK1/2 (Cell Signaling Technology Inc.), anti-phospho-AKT (Ser-473), anti-c-Met (C12, Santa Cruz Biotechnology), and Anti-Phospho-PRAS40 (Thr-246) (BIOSOURCE Inc., Camarillo, CA). Sections were blocked with CAS-BLOCK (Zymed Laboratories Inc., San Francisco, CA) and incubated with rabbit polyclonal antibody Ki67 antigen (catalog #VP-K451, lot number 301113; Vector Laboratories, Burlington, CA). The antibody was detected with a biotinylated goat anti-rabbit antibody (catalog #BA-1000, lot #R0207; Vector Laboratories). Tissue sections were quenched with Peroxidase Blocking Solution (Dako Corp., Carpinteria, CA) and detected with Vectastain Elite ABC kit (Vector Laboratories). Reaction sites were visualized with liquid DAB+ Substrate Chromagen System (Dako Corp.) and counterstained with hematoxylin.

Crystallography—Crystals of c-Met (residues 1048–1350 with an His6 tag at the C terminus) were grown at room temperature by vapor diffusion. SU11274 was obtained from Calbiochem (#448101). Compounds were dissolved in Me2SO to a final concentration of 20 mM and then mixed with c-Met protein to a final Me2SO concentration of 4% before crystallization. Crystals of AM7 were grown from 12% polyethylene glycol 6000, 1.0 M LiCl2, and 0.1 M sodium citrate, pH 5.0. Large crystals suitable for data collection were obtained by microseeding. Crystals of SU11274 were grown from 0.1 M HEPES, pH 7.8, 15% polyethylene glycol 4000, 6% 2-propanol, 40 mM β-mercaptoethanol, and 3% ethanol. Data were collected on a FRE equipped with an X-8012 and scaled by using Denzo/Scalepack to 2.4 Å (37). The structures were solved by molecular replacement using the published c-Met structure 1R1W as a search model and the program AMORE. The structures were built and refined by using COOT and REFMAC (Table 1). All figures were prepared using PyMOL. Coordinates and experimental data have been deposited with accession codes 2RFN and 2RFS for the structures of AM7 and SU11274, respectively. The compound identification name AM7 was assigned randomly by the Protein Data Bank.

Statistical Analysis—Tumor volumes are expressed as the means ± S.E. and plotted as a function of time. The data were statistically analyzed by repeated measures of analysis of variance followed by Bonferroni post hoc analysis for comparison of slopes of volume over time and Dunnett post hoc analysis for comparison of overall means. In addition, univariate analysis of variance and Dunnett post hoc analysis were performed at each
AM7 Is a c-Met Inhibitor

The structure of AM7 (5-(3-fluoro-4-((6-(methyloxy)-7-((3-(4 morpholinyl)propyl)oxy)-4-quinolinyloxy)phenyl)-3-methyl-2-(phenylmethyl)-4(3H)-pyrimidinone) is shown in Fig. 1A. AM7 inhibits c-Met kinase activity with an IC$_{50}$ of 17 nM and shows good selectivity against a panel of kinases (Fig. 1B and data not shown). Despite showing significant inhibitory effect on Lck and BTK kinase activity, AM7 had weak activity in an Lck- and a BTK-dependent cell assay with IC$_{50}$s in the $\mu$M range (data not shown). The only significant activity of AM7 in a cell-based assay identified to date is against c-Met. As shown in Fig. 1B, AM7 inhibits c-Met autophosphorylation mediated by HGF in mouse CT26 and human PC3 cells with IC$_{50}$ values of 130 and 94 nM, respectively.

FIGURE 1. AM7 is a selective c-Met inhibitor. A, structure of AM7. B, effect of AM7 on the enzymatic activity of several kinases and on c-Met phosphorylation in a cell-based HGF-mediated autophosphorylation assay. AM7 has comparable activities in mouse (CT26) and human (PC3) cells ($n=3$). EGFR, epidermal growth factor receptor; VEGFR, vascular endothelial growth factor receptor; IGF, insulin-like growth factor receptor; BTK, Bruton's tyrosine kinase.

RESULTS

AM7 Is a c-Met Inhibitor—The structure of AM7 (5-(3-fluoro-4-((6-(methyloxy)-7-((3-(4 morpholinyl)propyl)oxy)-4-quinolinyloxy)phenyl)-3-methyl-2-(phenylmethyl)-4(3H)-pyrimidinone) is shown in Fig. 1A. AM7 inhibits c-Met kinase activity with an IC$_{50}$ of 17 nm and shows good selectivity against a panel of kinases (Fig. 1B and data not shown). Despite showing significant inhibitory effect on Lck and BTK kinase activity, AM7 had weak activity in an Lck- and a BTK-dependent cell assay with IC$_{50}$s in the $\mu$M range (data not shown). The only significant activity of AM7 in a cell-based assay identified to date is against c-Met. As shown in Fig. 1B, AM7 inhibits c-Met autophosphorylation mediated by HGF in mouse CT26 and human PC3 cells with IC$_{50}$ values of 130 and 94 nm, respectively.

To confirm the inhibitory activity of AM7 on c-Met, we tested its effect on signaling in two c-Met-dependent models. MKN-45 cells are derived from a poorly differentiated gastric adenocarcinoma and have constitutive c-Met activity due to amplification of the receptor (38). c-Met is known to use multiple signaling pathways, including the PI3K and the mitogen-activated protein kinase pathways, to exert its functions (1). This point is well illustrated in cells with constitutive c-Met activity such as the MKN-45 cells. Fig. 2A shows that c-Met as well as several downstream signaling molecules are activated as shown by their phosphorylation status in serum-starved MKN-45 cells. AM7 inhibited c-Met constitutive phosphorylation in a dose-dependent manner. Inhibition of the receptor activity also resulted in the inhibition of signaling pathways downstream of c-Met. Phosphorylation of the adaptor protein Gab1, a major substrate for c-Met (39), was inhibited as a consequence of c-Met inhibition. AM7 also led to inhibition of both the PI3K and the mitogen-activated protein kinase pathways as evidenced by the reduction in AKT, ERK1/2, P70S6K, and S6 ribosomal protein phosphorylation (Fig. 2A). The inhibition of signaling molecules downstream of c-Met is not due to nonspecific activity since AM7 could not inhibit phospho-ERK1/2 in A431 cells stimulated with epidermal growth factor (data not shown). These data suggest that AM7 inhibits c-Met activity and c-Met-mediated signaling.

U-87 MG glioblastoma cells are known to express c-Met and secrete HGF, thus forming an autocrine loop that activates c-Met-dependent pathways. These cells also exhibit a loss of the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) (40), which results in constitutive PI3K pathway activation. We, therefore, asked what would be the consequence of AM7 on signaling under these circumstances. U-87 MG cells were treated with increasing concentrations of AM7 (Fig. 2B). Although phospho-c-Met (not shown) and phospho-Gab1 were essentially undetectable by direct Western blotting in serum-starved cells, both AKT and ERK1/2 showed constitutive phosphorylation that was inhibited in a dose-dependent manner by AM7, confirming that an HGF/c-Met autocrine loop is active in these cells. Surprisingly, we found that sequestering the endogenous HGF from the U-87 MG cells...
with an HGF-specific antibody partially blocked AKT phosphorylation but had no effect on the low, yet measurable phospho-ERK1/2 levels (Fig. 2C). These results suggest that the HGF/c-Met autocrine loop in U-87 MG cells contributes to AKT activation. To further study the impact of AM7 on the PI3K pathway, we examined signaling downstream of AKT. We found that AM7 was unable to inhibit the strong basal levels of phospho-PRAS40 and phospho-S6 ribosomal protein (Fig. 2B). This is consistent with the remaining AKT activity due to the PTEN mutation in these cells. Finally, we repeated this experiment, but this time c-Met-dependent signaling in the U-87 MG cells was maximally activated with exogenous HGF (Fig. 2D). In this instance c-Met activation was detected in the presence of HGF, and AM7 inhibited the signal in a dose-dependent manner. Gab1 was also clearly activated by HGF and repressed by the c-Met inhibitor. Similar to the situation in the unstimulated U-87 MG cells (Fig. 2B), AKT and ERK1/2 phosphorylation was inhibited in a dose-dependent manner by AM7. However, although AKT phosphorylation was not stimulated by exogenous HGF, ERK1/2 phosphorylation was strongly stimulated by the growth factor. The phosphorylation of PRAS40 and S6 ribosomal protein were unaffected by either the addition of HGF or by the inhibition of c-Met activity by AM7. Together, these results suggest that AM7 inhibits c-Met-dependent signaling specifically and that the inhibitor has no effect when signaling pathways are activated by downstream events such as a mutated PTEN.

**AM7 Inhibits Tumor Growth in Vivo**—The anti-tumor activity of AM7 was evaluated in vivo using the same two c-Met-dependent cell lines grown as xenografts. Mice were administered AM7 at 10, 30, or 100 mg/kg by oral gavage twice daily beginning 1 day after implantation of MKN-45 tumor cells and continuing for 27 days. A dose-dependent response of tumor growth inhibition was observed (Fig. 3A). AM7 demonstrated statistically significant tumor growth inhibition of 42% (p < 0.002) and 62% (p < 0.001) compared with the vehicle control at the 30 and 100 mg/kg doses, respectively. The animals remained healthy, and body weight was maintained in all treatment groups. Efficacy was also examined in the U-87 MG tumor xenograft model. Mice bearing established U-87 MG tumors (~200 mm³) were administered AM7 at 100 mg/kg by oral gavage twice daily for 16 days. AM7 significantly inhibited tumor growth 61% (p < 0.001) compared with the vehicle control (Fig. 3B) and profoundly reduced the number of Ki67-positive cells (Fig. 3C) even though the PI3K pathway was only partially inhibited (Fig. 2, B and D). Taken together, our results demonstrate that AM7 inhibits tumor growth in c-Met-dependent models when given orally and suggest that c-Met inhibitors can have anti-tumor activity even if pathways downstream of the receptor are activated. In the case of U-87 MG, AM7 may act, at least in part, by inhibiting the ERK1/2 pathway.

**AM7 Inhibits the Activity of Multiple c-Met Mutants**—Recently, Berthou et al. (34) demonstrated that cells engineered to overexpress c-Met constructs containing activating mutations found in hereditary papillary renal cell carcinoma patients behaved differently when treated with the c-Met inhibitor SU11274. AM7 was accordingly tested against 5 different c-Met mutations. Because all c-Met-mediated functions are dependent on its kinase activity (41–43), we decided to test the effect on AM7 directly on the purified kinase domain of c-Met (amino acids 1058–1365) that was in vitro phosphorylated to achieve the highest level of intrinsic kinase activity. Kinase assays in the presence of AM7 showed that the tested mutants were all inhibited, some at lower concentrations than the wild-type c-Met (Fig. 4, A and B). These results with AM7 reveal a different pattern of activity against mutations than the findings of Berthou et al. (34) with SU11274 and suggest that AM7 may interact with c-Met differently than SU11274.
AM7 and SU11274 Bind c-Met Differently—To establish the binding mode of AM7 with c-Met, the crystal structure of the complex was determined (Fig. 5). The crystallographic asymmetric unit contained two c-Met complexes, each with identical AM7 binding. The crystal structure reveals a typical kinase fold, quite similar to the existing published c-Met structures 1R1W and 1R0P (44) and 2G15 (45). The protein is organized into two domains; a small N-terminal domain composed mostly of $\beta$ sheet and one helix (termed the C-helix) and a larger C-terminal domain composed mostly of $\alpha$ helices. The C-helix is typically observed to pack tightly against the small domain, but in this structure and all of the published c-Met structures, it is displaced from the small domain by about 3–4 Å. Excellent electron density was observed for the entire inhibitor, which binds at the ATP site located at the interface between the large and small kinase domains (Fig. 5B). Hydrogen bonds are formed between the quinoline of AM7 and the kinase linker that connects the two domains.

Upon phosphorylation, the activation loop of kinases (c-Met Asp-1222—Leu-1245) adopts a conserved conformation that properly orients the residues involved in catalysis. The DFG sequence at the N terminus of the activation loop is very highly conserved across the kinome. In the active state the phenylalanine of the DFG motif binds in a hydrophobic pocket termed the DFG pocket, and the aspartate is pointed inward to coordinate Mg$^{2+}$ and ATP. Phosphorylated Lck is a good example of this conserved active kinase conformation (46). In our c-Met structure, perhaps because the kinase is unphosphorylated, only the first residue (Asp-1222) of the activation loop is ordered.

AM7 binds c-Met with an overall extended conformation that stretches from the kinase linker to the C-helix. The most interesting feature of AM7 binding to c-Met is the orientation of the terminal benzyl group. Rather than displacing Phe-1223 and binding in the DFG pocket, our structure reveals that this benzyl group turns upward and binds in a hydrophobic pocket located about 8 Å away from the DFG pocket. This hydrophobic pocket is located behind the C-helix and will be referred to as the C-helix pocket. The C-helix pocket is defined by hydrophobic residues Phe-1124, Val-1155, Leu-1142, Ile-1145, and Gly-1128 and exists because of the movement of the C-helix away from the small domain.

c-Met adopts an inactive conformation in its complex with AM7. Specifically, Asp-1222 is rotated away from the active site where it is unable to coordinate Mg$^{2+}$ and assist in catalysis. In addition, since the C-helix is shifted away from the small domain, Glu-1127 is unable to participate in catalysis. Nevertheless, AM7 potently inhibits all of the activating mutations discussed here (Fig. 4) as well as the unphosphorylated and phosphorylated forms of the wild-type enzyme (Fig. 1B).

To compare the AM7 binding mode with a typical active kinase, we superimposed this AM7 structure onto the structure of phosphorylated Lck in its active conformation (Fig. 6) (46). Interestingly, AM7 docks into the 3LCK structure without any clashes with the Lck activation loop. There are, however, clashes between the terminal benzyl group of AM7 and the C-helix of Lck. This is because AM7 pushes the c-Met C-helix away from the small domain relative to most kinases, including Lck.

The activating mutations that are visible in this structure are colored pink in Fig. 5A. Y1230H, which has been shown to confer resistance to...
SU11274, and D1228H are both located in the activation loop, which is disordered in this structure. Because it is disordered and because the c-Met enzyme used to determine the crystal structure is unphosphorylated, it is difficult to rationalize why AM7 inhibits these mutations equally well as wild-type c-Met. Because it is disordered and because the c-Met enzyme used to determine the crystal structure is unphosphorylated, it is difficult to rationalize why AM7 inhibits these mutations equally well as wild-type c-Met.

To compare the binding modes of AM7 and SU11274 to c-Met, we co-crystallized SU11274 with c-Met and determined the x-ray structure. Excellent electron density was observed for most of the inhibitor (Fig. 7A), but the terminal piperazine was disordered. This is not unexpected considering that this group faces the solvent. The disordered portion of SU11274 was not included in the final structure.

The crystal structures of AM7 and SU11274 are shown superimposed in Fig. 7B. The protein from the SU11274 structure is omitted from Fig. 7B for clarity. It is apparent that the two c-Met inhibitors overlap at the kinase linker but otherwise occupy completely different areas of the c-Met active site. The SU11274 model conformation is roughly C-shaped and wraps around M1211. Whereas AM7 binds c-Met with an extended conformation that stretches from the kinase linker to the C-helix pocket, SU11274 binds 10 Å away from the C-helix pocket. This dramatic difference in binding modes is consistent with the difference in profiles against the activating mutations discussed here.

**DISCUSSION**

We have identified a novel orally bioavailable c-Met inhibitor that shows activity against a panel of c-Met mutations found in cancer patients. AM7 inhibits c-Met-mediated signaling in a dose-dependent manner in MKN-45 and U-87 MG cells and tumor growth *in vivo*. Interestingly, both the PI3K and mitogen-activated protein kinase pathways are inhibited downstream of c-Met in MKN-45 cells, whereas the PI3K pathway is only partially inhibited in U-87 MG cells in the presence of AM7 due to the PTEN null phenotype of these cells. Nonetheless, U-87 MG xenograft tumor growth is inhibited significantly by AM7. These results are not due to “off target” activity of AM7 for several reasons. First, AM7 has been extensively tested against a large panel of kinases and other receptors and shows good selectivity for c-Met. Second, Western blot experiments in MKN-45 cells demonstrate the ability of AM7 to inhibit c-Met-mediated signaling, whereas epidermal growth factor receptor-mediated signaling was unaffected in A431 cells (data not shown). Furthermore, AM7 inhibits tumor growth in the MKN-45 xenograft model, which is highly dependent on c-Met activity. Finally, specific antibodies against either HGF or c-Met can also inhibit U-87 MG tumor growth (9, 12). Although other groups have shown anti-tumor activity in U-87 MG xenografts with inhibitors of the c-Met pathway, this is the first report to examine the effect of such inhibitors on signaling in these cells. We have shown that the autocrine HGF/c-Met loop that exists in U-87 MG cells contributes partially to the PI3K pathway, whereas the mitogen-activated protein kinase path-
Novel Binding Mode for c-Met Inhibitors

way appears completely dependent on HGF/c-Met. Complete inhibition of c-Met in these cells only partially inhibited phospho-AKT; however, the residual AKT activity was sufficient to maintain downstream activation of PRAS40. Future work will test whether this is the case for other AKT substrates and whether this is also the case in vivo. The fact remains that despite the PTEN null phenotype of these cells, c-Met contribution is critical for the growth of these xenografts. The implication of these results is important, suggesting that tumors possessing activated c-Met may respond to c-Met inhibitors despite harboring downstream molecular defects. Moreover, our results suggest that combinations with inhibitors of the PI3K pathway may offer further anti-tumor benefits in PTEN null tumors.

Several examples now exist in the literature demonstrating that kinase inhibitors show differential ability to interact with, and inhibit the activity of, wild-type and mutant kinases. An understanding of these different abilities to inhibit wild-type and mutant kinases is essential early in the drug development process to design or identify drugs that will be useful for patients harboring these mutations. AM7 is the first c-Met inhibitor to be described that inhibits c-Met mutants that have previously been shown to be resistant to other molecules (34, 48). This finding led us to identify a different binding mode for AM7 and SU11274, which is consistent with their different ability to interact with different c-Met mutants.

Perhaps the most interesting and unexpected feature of the structure of AM7 bound to c-Met is the existence of the C-helix pocket. Before obtaining structural information for this class of c-Met compounds, we had postulated that the benzyl group might bend downward and bind in the DFG pocket typhied by imatinib (49, 50). The AM7 binding mode shown in Fig. 5 leaves the DFG pocket empty and, instead, fills the C-helix pocket located about 8 Å away.

When AM7 is modeled into the activated Lck structure (3LCK), the benzyl group collides with the Lck C-helix. A displaced C-helix could be tolerated in only a subset of kinases, including c-Met, and could potentially be a selectivity determinant of this class of c-Met inhibitor. A displaced C-helix has been observed for several kinases, including Tie-2 (31) and MEK1 (36). In the case of Tie-2, an inhibitor class was postulated to gain part of its selectivity because it binds the kinase with the C-helix shifted away from the small domain in a similar manner to that observed here. The MEK1 inhibitors described in Ohren et al. 36 bind to MEK1 kinase in a pocket created by a large C-helix movement. A portion of the MEK1 inhibitor overlaps quite nicely with the terminal benzyl portion of AM7.

Despite the fact that our c-Met crystal structure and the three published structures referenced herein are of unphosphorylated kinase, the c-Met activation loop likely would assume the conformation seen in phosphorylated Lck (3LCK) upon phosphorylation. This would also be predicted to be the conformation of the activation loop observed for the c-Met activating mutations discussed here. The AM7 binding mode we observe does not clash with the Lck activation loop.

AM7 does clash with the Lck C-helix, reinforcing that the structure reported here is of an inactive conformation of c-Met. Yet we observe that AM7 inhibits both the unphosphorylated and phosphorylated forms of c-Met with almost equal potency. We assume that the active phosphorylated form of c-Met does not contain the C-helix pocket but, rather, resembles phospho-Lck. It is possible that AM7 inactivates phospho-c-Met by displacing the C-helix in a similar fashion to that observed in our unphosphorylated structure. Structural studies with AM7 and phosphorylated c-Met will be necessary to elucidate this binding mode.

Although the different binding modes of AM7 and SU11274 to c-Met described here probably account for their unique pattern of activity on c-Met mutants, co-crystallography of AM7 and SU11274 in the phosphorylated form of c-Met and in the different mutants will be required to fully understand these differences. Nonetheless, our results indicate that the binding mode of the inhibitor influences the activity on c-Met mutants. This information will be useful to identify c-Met inhibitors with activity against a spectrum of c-Met mutants.

Acknowledgments—We thank the following colleagues for contributions: Tom Graves, Sheila Scully, Barbara Felder, Arisha Ali, and Bob Holzinger. We also thank Matthew Lee for helpful discussions.

REFERENCES

1. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G. F. (2003) Nat. Rev. Mol. Cell Biol. 4, 915–925
2. Corso, S., Comoglio, P. M., and Giordano, S. (2005) Trends Mol. Med. 11, 284–292
3. Dharawandara, P. G., Giubellino, A., and Bottaro, D. P. (2004) Curr. Mol. Med. 4, 855–868
4. Giordano, S., Maffe, A., Williams, T. A., Artigiani, S., Gual, P., Bardelli, A., Basilio, C., Michieli, P., and Comoglio, P. M. (2000) FASEB J. 14, 399–406
5. Jeffers, M., Fiscella, M., Webb, C. P., Anver, M., Koochekpour, S., and Vande Woude, G. F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14417–14422
6. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11445–11450
7. Olivero, M., Valente, G., Bardelli, A., Longati, P., Ferrero, N., Cracco, C., Terrone, C., Rocca-Rossetti, S., Comoglio, P. M., and Di Renzo, M. F. (1999) Int. J. Cancer 80, 640–643
8. Schmidt, L., Junker, K., Nakaigawa, N., Kinijerski, T., Weirich, G., Miller, M., Lubensky, I., Neumann, H. P., Brauch, H., Decker, J., Vocke, C., Brown, J. A., Jenkins, R., Richard, S., Bergerheim, U., Gerrard, B., Dean, M., Linehan, W. M., and Zbar, B. (1999) Oncogene 18, 2343–2350
9. Burgess, T., Coxon, A., Meyer, S., Sun, J., Rex, K., Tsuruda, T., Chen, Q., Ho, S. Y., Li, L., Kaufman, S., McDorman, K., Cattley, R. C., Sun, J., Elliott, G., Zhang, K., Feng, X., Jia, X. C., Green, L., Radinsky, R., and Kendall, R. (2006) Cancer Res. 66, 1721–1729
10. Cao, B., Su, Y., Oskarsson, M., Zhao, P., Kort, E. J., Fisher, R. J., Wang, L. M., and Vande Woude, G. F. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7443–7448
11. Kim, K. J., Wang, L., Su, Y. C., Gillespie, G. Y., Salhotra, A., Lal, B., and Laterra, J. (2006) Clin. Cancer Res. 12, 1292–1298
12. Martens, T., Schmidt, N. O., Eckerich, C., Fillbrandt, R., Merchant, M., Schwall, R., Westphal, M., and Lamszus, K. (2006) Clin Cancer Res. 12, 6144–6152
13. Date, K., Matsumoto, K., Shimura, H., Tanaka, M., and Nakamura, T. (1997) FEBS Lett. 420, 1–6
14. Brockmann, M. A., Papadimitriou, A., Brandt, M., Fillbrandt, R., Westphal, M., and Lamszus, K. (2003) Clin Cancer Res. 9, 4578–4585
15. Tomioka, D., Maehara, N., Kuba, K., Mizumoto, K., Tanaka, M., Matsumoto, K., and Nakamura, T. (2001) Cancer Res. 61, 7518–7524
16. Michieli, P., Mazzone, M., Basilio, C., Cavassa, S., Sottile, A., Naldini, L.,
