A Drug Resistance Screen Using a Selective MET Inhibitor Reveals a Spectrum of Mutations That Partially Overlap with Activating Mutations Found in Cancer Patients

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Abstract

The emergence of drug resistance is a primary concern in any cancer treatment, including with targeted kinase inhibitors as exemplified by the appearance of Bcr-Abl point mutations in chronic myeloid leukemia (CML) patients treated with imatinib. In vitro approaches to identify resistance mutations in Bcr-Abl have yielded mutation spectra that faithfully recapitulated clinical observations. To predict resistance mutations in the receptor tyrosine kinase MET that could emerge during inhibitor treatment in patients, we conducted a resistance screen in BaF3 TPR-MET cells using the novel selective MET inhibitor NVP-BVU972. The observed spectrum of mutations in resistant cells was dominated by substitutions of tyrosine 1230 but also included other missense mutations and partially overlapped with activating MET mutations that were previously described in cancer patients. Co-crystallization of the MET kinase domain in complex with NVP-BVU972 revealed a key role for Y1230 in binding of NVP-BVU972, as previously reported for multiple other selective MET inhibitors. A second resistance screen in the same format with the MET inhibitor AMG 458 yielded a distinct spectrum of mutations rich in F1200 alterations, which is consistent with a different predicted binding mode. Our findings suggest that amino acid substitutions in the MET kinase domain of cancer patients need to be carefully monitored before and during treatment with MET inhibitors, as resistance may preexist or emerge. Compounds binding in the same manner as NVP-BVU972 might be particularly susceptible to the development of resistance through mutations in Y1230, a condition that may be addressed by MET inhibitors with alternative binding modes. Cancer Res; 71(15); 5255–64. ©2011 AACR.

Introduction

Inhibition of oncogenic tyrosine kinases with small molecules is a commonly pursued approach for cancer therapy. Several kinase inhibitors are approved medications, such as imatinib for the treatment of chronic myeloid leukemia (CML), and numerous others are in preclinical or clinical development. Despite remarkable success in specific cancer indications, where the respective target kinase confers cancer dependence, a recurrent problem encountered in clinical practice is the emergence of resistance. Often resistance results from kinase mutations that interfere with inhibitor binding without compromising kinase function. This is particularly well documented in the case of CML therapy with imatinib, where multiple mutations in the target protein Bcr-Abl conferring various degrees of resistance have been isolated from patients (1, 2). Likewise, resistance mutations have been reported in patients with gastrointestinal stromal tumors that were treated with imatinib (3). In case of Bcr-Abl, the second-generation inhibitors nilotinib and dasatinib are now available. These compounds overcome the majority of imatinib resistance mutations with the notable exception of the cross-resistant gatekeeper mutation T315I (2, 4). The corresponding gatekeeper mutation in epidermal growth factor receptor (EGFR), T790M, has also been found to cause resistance to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib in lung cancer (5).
In vitro identification of resistance mutations in Bcr-Abl has been reported in a number of studies. One successful approach was to generate a library of mutant Bcr-Abl constructs by passaging a retroviral vector containing wild-type Bcr-Abl through the mutagenic Escherichia coli strain XL-1 red (6, 7). Mutated Bcr-Abl constructs were then expressed in BaF3 cells to select inhibitor-resistant clones. One advantage of this method is that one can estimate the complexity of the library of mutants and thus the coverage of the respective kinase. Another example where this methodology was applied successfully is the recent identification of resistance mutations in MET1 by expressing a saturating cDNA library of mutants in a MEK inhibitor–sensitive lung cancer cell line (8). In an alternative protocol, BaF3 cells transformed with an oncogenic kinase are directly mutagenized by exposing them to the alkylating agent N-ethyl-N-nitrosourea (ENU). Subsequently, resistant cells can be selected by incubation with an inhibitor of choice (9, 10). Such a cell-based resistance screen could potentially yield mutations in genes other than the kinase of interest, and an estimation of mutation coverage is more difficult to obtain. However, this simpler method was shown to faithfully reproduce the spectrum of mutations in Bcr-Abl that was observed in imatinib-resistant patients (9). A similar cell-based resistance screen with nilotinib yielded a comparable mutation profile even in the absence of ENU (11), suggesting that at least in Bcr-Abl–driven BaF3 cells, the rate of spontaneous mutations is sufficiently high to screen for resistance.

The receptor tyrosine kinase MET has been linked to cancer by numerous findings, including the presence of activating mutations in cancer patients, hyperactivation due to gene amplification, as well as autocrine growth stimulation by the ligand hepatocyte growth factor (HGF) in certain tumor models (12). Accordingly, several agents targeting the MET–HGF axis are in preclinical and clinical development for cancer treatment (13). Here, we sought to predict resistance mutations in MET that may emerge upon exposure to the highly selective inhibitor NVP-BVU972. The strategy we used was based on the previously described Bcr-Abl ENU mutagenesis screens in BaF3 cells (9, 10), in this case however using BaF3 cells driven by the oncogenic TPR-MET fusion gene, in which the MET kinase domain is constitutively activated. These cells are strictly dependent on MET kinase activity (14) and therefore highly sensitive to MET inhibitors like NVP-BVU972. A screen with various concentrations of NVP-BVU972 resulted in isolation of 85 resistant BaF3 TPR-MET clones containing 10 different mutations in the MET kinase domain. The vast majority of mutations were found in residues Y1230 and D1228. Cocrystallization of NVP-BVU972 with the MET kinase domain provided a clear explanation for the observed mutation profile and revealed a binding mode that is very similar to several other MET inhibitors that are in clinical or preclinical development. A similar screen with the MET inhibitor AMG 458 yielded a distinct spectrum of mutations, which is likely due to its different binding mode. Finally, screens were repeated and the resultant cell populations pooled and sequenced using next generation sequencing (NGS) technologies. The observed mutation spectra largely correlated with the results obtained by traditional sequencing with variants detected at frequencies as low as 0.1%.

Materials and Methods

Compounds

NVP-BVU972 [6-[6-(1-methyl-1H-pyrazol-4-yl)-imidazo[1,2-b]pyridazin-3-ylmethyl]-quinoline] and AMG 458 [1-(2-hydroxy-2-methyl-propyl)-5-methyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazole-4-carboxylic acid [5-(7-methoxy-quinolin-4-yl)-pyridin-2-yl]-amide] were prepared according to the procedures described in the literature (15, 16). XL880 [cyclopropane-1,1-dicarboxylic acid [3-fluoro-4-[6-methoxy-7-(3-morpholin-4-yl-propoxy)-quinolin-4-yl]-phenyl]-amide (4-fluoro-phenyl)-amide; Selleck Chemicals S1111] and PF-02341066 [3–[((R)-1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine; Selleck Chemicals S1018] can be obtained from commercial sources.

Compound profiling assays

Profiling assays as used to generate data in Table 1 are described in detail in the Supplementary Materials and Methods.

Mutagenesis and selection of resistant clones

The protocol was based on procedures published by Bradeen and colleagues (9) and O’Hare and colleagues (10). ENU

| Table 1. Profiling of NVP-BVU972 in biochemical and cellular assays |
|------------------------|-----------------|--------|
| Assay                  | IC\textsubscript{50} ± SD, nmol/L | n     |
| MET kinase assay       | 14 ± 1.7        | 4     |
| 62 Other kinase assays | >1,000/10,000   | 4     |
| Proliferation (GTL-16) | 66 ± 13         | 3     |
| Proliferation (EBC-1)  | 32 ± 16         | 3     |
| Proliferation (MKN-45) | 82 ± 6          | 2     |
| MET phosphorylation (GTL-16) | 7.3 ± 2.2 | 4     |
| MET phosphorylation (A549) | 22            | 1     |
| Proliferation (BaF3 TPR-MET) | 104 ± 42 | 3     |
| Proliferation (13 other BaF3 strains) | >10,000 | 1     |

NOTE: Biochemical activity of NVP-BVU972 was measured against MET and a panel of 62 other kinases. Inhibition of proliferation was determined in MET-amplified GTL-16, EBC-1, and MKN-45 human cancer cells as well as in transformed mouse BaF3 cells containing a panel of oncogenic kinase fusions including TPR-MET as well as parental interleukin-3–dependent BaF3 cells. Inhibition of MET autophosphorylation and HGF-stimulated MET phosphorylation was measured in GTL-16 and A549 cells, respectively. All IC\textsubscript{50} values are given in nmol/L with SD and number of independent replicates (n).
(Sigma) was dissolved in dimethyl sulfoxide (DMSO) at 100 mg/mL. All the material that came in contact with ENU was decontaminated with 0.2 mol/L NaOH. Per one resistance screen, approximately $1.5 \times 10^8$ BaF3 TPR-MET cells in a total of 160 mL growth media were exposed to a final concentration of 50 μg/mL ENU. After approximately 16 hours, cells were collected by centrifugation, washed, and split into 5 aliquots of $3 \times 10^7$ cells each. Compound (NVP-BVU972 or AMG 458) was added to a final concentration of 50 μg/mL ENU. After approximately 16 hours, cells were collected by centrifugation, washed, and split into 5 aliquots of $3 \times 10^7$ cells each. Compound (NVP-BVU972 or AMG 458) was added to a final concentration of 600, 1,200, 2,400, 4,800, or 9,600 nmol/L, and cells of each aliquot were distributed into three 96-well plates, thereby placing $10^5$ cells in 100 μL media into each well. Plates were incubated over a course of 4 weeks with regular inspection. When clear signs of cell outgrowth were observed in the microscope and by color change of the media, the respective well content was transferred into 3 mL of growth media containing the original concentration of MET inhibitor on a 6-well plate. After approximately 1 week of expansion, the cell number was sufficient for further processing (see below).

**Results**

NVP-BVU972 is a selective MET kinase inhibitor

NVP-BVU972 (Fig. 1A) was synthesized in the context of a medicinal chemistry program that was aimed at identifying selective MET inhibitors. In a panel of biochemical kinase assays, NVP-BVU972 inhibited MET with an IC$_{50}$ of 14 nmol/L but displayed IC$_{50}$ values greater than 1 μmol/L in 62 other

**Identification of MET mutations**

Genomic DNA was prepared from 1 mL of densely grown cells using a Nucleospin Blood kit (Macherey-Nagel), and the remaining 2 mL of expanded cells were cryopreserved for later analysis. For sequence analysis, a DNA fragment covering the entire kinase domain of MET was amplified with a PCR Master kit (Roche) using the primers 5'-AAA-TACTGTCC-ACATTGACCTC-3' and 5'-GCCAGCATTTTAG-CATTACTTC-3', yielding an 818-bp product. PCR products were then purified with a QIAquick gel extraction kit (Qia-gen) and sequenced by standard Sanger sequencing using the reverse PCR primer. Mutations were identified by alignment to the National Center for Biotechnology Information reference sequence NM_000245.2.

**X-ray crystallography**

MET crystals were produced by microseeding at 18°C by the hanging drop method. Purified MET kinase (5 mg/mL) in 20 mmol/L Tris, pH 8.5, 150 mmol/L NaCl, 10% glycerol, and 3 mmol/L Tris (2-carboxy-ethyl) phosphin HCl (TCEP) was incubated with 0.5 mmol/L NVP-BVU972 and was mixed at a ratio of 1:1:0.2 with reservoir solution (100 mmol/L HEPES, pH 7.5, 16% PEG4000, 8% isopropanol, and 3 mmol/L TCEP) and MET microseeds. Crystals appeared overnight, continued to grow for approximately 3 to 5 days, and were transferred to 100 mmol/L HEPES, pH 7.2, 20% PEG4000, and 20% glycerol before flash freezing in liquid nitrogen. Data were collected at beam line 5.0.2 of the Advanced Light Source (Berkeley, California), integrated with X-ray Detector Software (XDS; ref. 17), and scaled with SCALA (18). The structure was solved by molecular replacement with Phaser (19) and refined with Phenix (20) and BUSTER (21). Iterative rounds of model building were done with COOT (22).

BaF3 proliferation assays, TR-FRET biochemical assay with MET wild type and mutants, and analysis of tyrosine phosphorylation by Western blotting are covered in Supplementary Materials and Methods.
kinase assays, or even greater than 10 μmol/L in 56 of these assays including the most closely related kinase RON (Table 1). Growth inhibition of the MET gene amplified cell lines GTL-16, MKN-45, and EBC-1 (23, 24) as well as suppression of constitutive MET phosphorylation in GTL-16 cells or HGF-stimulated MET phosphorylation in A549 cells was detected in a concentration range that was only slightly above the biochemical activity (Table 1). Furthermore, growth inhibition with low nanomolar potency was also seen in BaF3 cells transformed with a TPR-MET fusion (14) but not in BaF3 wild-type cells grown in the presence of interleukin-3 (IL-3) or in 13 other BaF3 cell models transformed with different activated tyrosine kinases (Table 1). These biochemical and cellular data show that NVP-BVU972 is a selective and potent MET inhibitor.

Selection of BaF3 TPR-MET cells that are resistant to NVP-BVU972

To predict MET mutations that might cause resistance to NVP-BVU972 in MET driven cancers, we conducted a random mutagenesis screen in BaF3 TPR-MET cells. To this end, we exposed mutagenized BaF3 TPR-MET cells in 96-well plates to various concentrations of NVP-BVU972 ranging from 600 nmol/L to 9.6 μmol/L. In this concentration range, the IC50 value for inhibition of proliferation is exceeded by about 6- to 100-fold. Between 2 and 4 weeks after initial plating, resistant cells were isolated from single wells that showed evidence of growth based on media color change and microscopic observations. After expansion in fresh media containing the intact drug concentrations, DNA for sequencing was extracted and an aliquot from each well was cryopreserved for functional characterization. Within a 4-week period, cells grew in 85 of 1,440 wells (5.9%). The relative scarcity of positive wells, together with the microscopic observation that living cells outgrowth of clones in a subset of wells was only seen at 2.4 μmol/L of AMG 458 and to a lesser extent at 4.8 μmol/L, substitutions (with the exception of Y1230F) arising from a single-nucleotide change were detected.

Cocrystal structure of the MET kinase domain and NVP-BVU972 explains frequently observed resistance mutations

The binding mode of NVP-BVU972 to the MET kinase domain was determined by X-ray crystallography (Fig. 2). An important binding interaction is the π-stacking of the core imidazo-pyridazine moiety of NVP-BVU972 with the aromatic side chain of Y1230, explaining why mutations of this residue abrogate binding of the compound and lead to resistance in biochemical and cellular assays. The 3 other residues that were found to be mutated in resistant clones play a role in stabilizing the particular conformation of the activation loop seen in the cocrystal structure: D1228 is engaged in a hydrogen bond with K1110, whereas V1155 and F1200 are involved in hydrophobic interactions with activation loop residues L1225 and F1223, respectively. All 3 interactions appear to be important to keep the activation loop in a conformation allowing for the critical π-stacking interaction between Y1230 and NVP-BVU972.

Mutation spectra obtained with the MET inhibitors NVP-BVU972 and AMG 458 are partially overlapping

To further explore the utility of the BaF3 resistance screening system, we conducted a second resistance screen with AMG 458, a MET inhibitor that is structurally distinct from NVP-BVU972 and predicted to bind MET in a different fashion. The same experimental setup and concentration range as for NVP-BVU972 were used. The previously observed outgrowth of clones in a subset of wells was only seen at 2.4 μmol/L of AMG 458 and to a lesser extent at 4.8 μmol/L, additional residues that were found to be mutated in resistant clones or that interact with such residues to stabilize the activation loop conformation are indicated. Coordinates and structure factors have been deposited to the PDB with the accession no. 3QTI as a marker.
showed signs of growth at 2.4 μmol/L. AMG 458 did not allow growth of any clones (Supplementary Fig. S1). Between 2 and 4 weeks after plating, more than 50% of wells showed signs of growth at 2.4 μmol/L and below. A random selection of 99 clones, mostly outgrowing at 2.4 μmol/L. AMG 458, was further analyzed and only 57 (58%) contained a kinase domain mutation. This is in contrast to the NVP-BVU972 screen where a kinase domain mutation could be detected in every clone obtained (Supplementary Fig. S1). The fact that AMG 458 is less potent and selective than NVP-BVU972 might reduce the selective pressure to acquire resistance through MET alterations, but alternative resistance mechanisms were not investigated in this study.

Within the subset of clones that contained kinase domain mutations, a total of 15 different mutations on the DNA level were observed, resulting in 13 amino acid changes in 10 different residues (Fig. 3A and Supplementary Table S2). Three mutations, V1155L, F1200I, and D1228A, had been found in the NVP-BVU972 screen as well, whereas Y1230 mutations were not detected in the presence of AMG 458. The most frequently altered residue in the AMG 458 resistance screen, accounting for 60% of all kinase domain mutations, was F1200 (Fig. 3A). The chemical structure of AMG 458 suggests that it belongs to the so-called “type II” class of kinase inhibitors that bind to the “DFG out” type of kinase conformation. This is why, in the absence of an available cocrystal structure of AMG 458 and MET, we modeled the binding mode of the inhibitor (Fig. 3B) using the reported crystal structure of MET in complex with a type II pyrrolo-pyridine-pyridone inhibitor of similar chemical structure (25).

This model provides a plausible and simple explanation to the appearance of mutations at residues Y1159, G1163, M1211, V1155, L1195, and F1200 in the resistance screen. All these residues form direct interactions with AMG 458 in the model, the first 3 with the quinoline ring and the last 3 with the dihydropyrazolone moiety. The other more distant mutations may influence the conformation of the kinase and render it unfavorable for type II inhibitors to bind.

**NGS can detect low-frequency variants in pools of resistant BaF3 cells**

Next, we tested whether NGS could be used to determine mutation profiles in resistant BaF3 cell pools. To this end, such pools were generated by repeating resistance screens with either NVP-BVU972 or AMG 458 in a slightly different format. As before, the MET kinase domain was PCR amplified and the single amplicon originating from each pool subjected to NGS (Supplementary Materials and Methods). A high level of sequence coverage was achieved across the 818 bps with a dip in coverage on the ends due to incomplete shearing of the PCR amplicon (Supplementary Fig. S2). Low frequency variants could be detected, and the NGS-derived variants identified in the pooled screens correlated well with those identified previously by single-clone sequencing (Supplementary Table S3). However, several mutations were not identified in the pooled products, notably D1228 mutations in the NVP-BVU972–treated BaF3 cells. This is probably not due to the sensitivity of the NGS platform but could be a consequence of the different selection process (Supplementary Materials and Methods). There was also a strand-specific artifact that manifested in a markedly higher variant frequency in one strand versus the complementary strand. This artifact was largely removed by the analysis pipeline described in Supplementary Materials and Methods.

**Extent of cellular resistance correlates with frequency of mutations in resistance screens**

To functionally validate the resistance mutations, we first compared the ability of NVP-BVU972 and AMG 458 to inhibit proliferation of BaF3 cells containing wild-type or selected mutant versions of TPR-MET by making use of the clones obtained in the 2 resistance screens. In these experiments, we included 2 additional previously described MET inhibitors to cover a more diverse set of chemical structures. The dual MET/ALK inhibitor PF-02341066 is not chemically related to NVP-BVU972 but binds to a similar conformation of MET, also
making a direct contact with Y1230 (26). XL880 has been shown to bind to a DFG out conformation of MET that is significantly different from the one observed with NVP-BVU972 (27). This type II MET inhibitor bears chemical similarity to AMG 458, suggesting a comparable binding mode.

In line with their high frequency in the NVP-BVU972 screen, Y1230 and D1228 mutations caused dramatic shifts in the measured IC50 values for NVP-BVU972 in cellular proliferation assays (Fig. 4A and Table 2). In agreement with previous data (26), PF-02341066 was also substantially affected by alterations in these 2 residues, whereas no or only mild IC50 shifts were observed with AMG 458 and XL880 (Table 2). F1200I had a clear impact on potency of all tested compounds (Fig. 4A and Table 2), which is expected due to the high frequency of F1200 mutations in the AMG 458 screen and its appearance in the NVP-BVU972 screen. A comparable profile was seen with L1195V, whereas resistance elicited by V1155L was more restricted to NVP-BVU972 and PF-02341066 (Fig. 4A and Table 2).

To rule out the impact of additional undetected mutations in the clones obtained from random mutagenesis screens, selected mutations were produced by site-directed mutagenesis in TPR-MET expression constructs that were then introduced into parental BaF3 cells. Proliferation assays in the resulting strains confirmed the shifts in compound potency that had been measured in the random mutagenesis clones before (Supplementary Table S4). Furthermore, biochemical assays were conducted with recombinant MET proteins, either unmutated or containing Y1230H or F1200I mutations. Data in this assay format were largely in agreement with the cellular resistance pattern (Table 2).

**Resistance correlates with maintenance of tyrosine phosphorylation in the presence of MET inhibitors**

Active recombinant MET protein and TPR-MET fusion protein expressed in BaF3 cells display a high level of tyrosine phosphorylation in the activation loop (14). We compared by Western blot analysis the levels of p-Y1234/Y1235 phosphorylation in recombinant proteins that were used for kinase assays...
as well as in TPR-MET of representative resistance clones covering the most frequent mutations from both screens. Both Y1230H and F1200I were associated with enhanced phosphorylation in purified recombinant proteins (Fig. 4B). Very modest mutation-dependent differences in TPR-MET phosphorylation were also observed in protein lysates from BaF3 cells and detected a large proportion of Y1230 mutations.

Cocrystallization of NVP-BVU972 with the MET kinase domain provided a clear explanation for this resistance hot spot by revealing a π-stacking interaction of the central imidazo-pyridazine ring system of NVP-BVU972 and the activation loop residue Y1230. Therefore, resistance caused by alterations of this residue is likely due to direct disruption of an interaction that is crucial for drug binding. This hypothesis is also supported by data obtained with similar MET inhibitors (see below). Interestingly, we observed with more potent derivatives from the NVP-BVU972 series that the potency shift caused by Y1230H or Y1230F was less pronounced than with Y1230C, suggesting that aromatic side chains as in histidine or phenylalanine can still weakly support an interaction with the central ring system of the compounds (data not shown).

Pronounced resistance was also observed with alterations of the residue D1228, whereas mutations in V1155 and F1200 were infrequent and had milder effects. On the basis of the cocrystal structure, these residues are engaged in interactions that hold Y1230 in place to support the conformation needed for NVP-BVU972 binding. In contrast, a screen with the structurally unrelated MET inhibitor AMG 458 revealed a mutation profile that was clearly distinct and enriched in F1200 mutations. Even though 3 mutations were shared between both resistance profiles, the frequency of these mutations in the respective screens differed significantly. A model of AMG 458 bound to MET suggested a structural rationale for most resistance mutations. The extent of resistance was quantified using cellular and biochemical kinase assay

### Table 2. Impact of MET mutations in proliferation and kinase assays

<table>
<thead>
<tr>
<th>Mutation</th>
<th>BVU972 IC50 (SD), nmol/L</th>
<th>PF-02341066 IC50 (SD), nmol/L</th>
<th>AMG 458 IC50 (SD), nmol/L</th>
<th>XL880 IC50 (SD), nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>77 ± 22</td>
<td>88 ± 24</td>
<td>536 ± 63</td>
<td>43 ± 12</td>
</tr>
<tr>
<td>Y1230H</td>
<td>&gt;129</td>
<td>11.6 ± 4.3</td>
<td>1.6 ± 0.5</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>D1228A</td>
<td>&gt;129</td>
<td>13.7 ± 2.2</td>
<td>1.9 ± 0.2</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>V1155L</td>
<td>14.6 ± 0.7</td>
<td>9.5 ± 1.0</td>
<td>2.8 ± 0.6</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>F1200I</td>
<td>14.1 ± 1.0</td>
<td>9.5 ± 1.5</td>
<td>8.0 ± 0.4</td>
<td>19.7 ± 0.7</td>
</tr>
<tr>
<td>L1195V</td>
<td>31.5 ± 10.5</td>
<td>14.3 ± 2.6</td>
<td>&gt;18</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>M1211L</td>
<td>1.2 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>3.8 ± 1.3</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>M1250T</td>
<td>3.6 ± 0.7</td>
<td>2.9 ± 0.9</td>
<td>3.9 ± 1.1</td>
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<tr>
<td>WT</td>
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<td>48</td>
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</table>

**NOTE:** Top section: Antiproliferative activity of 4 structurally distinct MET inhibitors in BaF3 cells containing wild-type (WT) TPR-MET or the indicated mutant TPR-MET versions. Resistant clones from random mutagenesis screens were used. IC50 values for all mutants are expressed as fold shift in comparison to WT (n = 3). Comparable results were obtained with NVP-BVU972 and PF-02341066 in all other clones with Y1230 or D1228 mutations that occurred in the NVP-BVU972 resistance screen (data not shown). Bottom section: Inhibition of MET kinase activity in biochemical assays with WT and mutant recombinant proteins (n = 4 per protein).
assays; however, the interpretation of these data was complicated by the finding that the basal phosphorylation levels of TPR-MET in BaF3 cells and of recombinant MET protein depended to some extent on the respective mutation. In biochemical assays, the $K_m$ value for ATP was found to be about 6 times and about 4 times lower for F1200I and Y1230H compared with wild-type MET. To account for these differences, ATP concentrations in the assay were adjusted accordingly. Even though it is conceivable that differential affinity for ATP and basal phosphorylation levels contribute to the observed shift in IC$_{50}$, we believe that this factor is minor in light of the very pronounced IC$_{50}$ shifts caused by some of the mutations because of the plausible structural explanations for resistance effects.

Activating mutations in the MET kinase domain have been originally reported in patients with hereditary and sporadic papillary renal cell carcinoma (PRCC) and later in several other cancer types (28). Some of these activating mutations are identical to resistance mutations that were discovered in our screens. D1228N, Y1230C, Y1230H, and Y1230D have all been found in hereditary or sporadic PRCC (28, 29), and Y1230C has also been reported in metastases of head and neck cancer (30, 31). In a recently published crystal structure of the autoinhibited, completely dephosphorylated kinase domain of MET, both D1228 and Y1230 were found to be involved in interactions that stabilize the inactive conformation, providing an explanation why mutations of these residues are selected for to enhance kinase activity (32). Because of their activating nature, some of the NVP-BVU972 resistance mutations might thus confer a selective advantage even in the absence of inhibitor treatment.

Several highly selective MET inhibitors including JNJ-38877605 (Johnson & Johnson; phase 1), PF-04217903 (Pfizer; phase 1), SGX523 (SGX Pharmaceuticals; phase 1 discontinued), and probably AMG 208 (Amgen; phase 1) are structurally similar to NVP-BVU972 (26, 33–36). Coocrystal structures of MET and the respective inhibitor have been published for PF-04217903, SGX523, and 2 Amgen triazolo-pyridazines. In all cases, a critical π-stacking interaction with Y1230 was observed. The chemically unrelated dual MET/Alk inhibitor PF-02341066 was also found to bind to a similar MET conformation (26, 33, 34, 37). Because of these concurrent binding modes, the NVP-BVU972 resistance mutation profile might be predictive for many other selective MET inhibitors. Prompted by the literature reports on MET mutations in cancer patients, several MET inhibitors have already been tested against various subsets of these mutations. The Y1230-interacting compounds PF-02341066 and PF-04217903 were both tested in a panel of MET mutations in biochemical as well as cellular assays (26). Y1230C and D1228H were found to profoundly impair the activity of both compounds, and substantial resistance was also seen with Y1235D. Conversely, the related compounds AM7 and AMG 458 retained activity against several mutations including Y1230H and D1228H (16, 38). Our resistance screens confirmed these prior findings.

Neither Y1235 nor L1195 mutations were observed in our initial NVP-BVU972 screen despite their potential to cause resistance against NVP-BVU972 and related molecules (Table 2 and ref. 26). NGS of a NVP-BVU972–resistant pool revealed a L1195I mutation, but D1228 variants were absent from this pool (Supplementary Materials and Methods). Similar to NVP-BVU972, mutation profiles obtained with AMG 458 were not entirely overlapping when comparing single clones and NGS. Furthermore, all missense mutations of Y1230 that can be obtained with a single bp change were found in the NVP-BVU972 screen with the exception of Y1230F. However, when introducing Y1230F into BaF3 TPR-MET, we observed strong resistance (data not shown). Together these observations indicate that our random mutagenesis screens were not fully comprehensive and a substantial scale-up in the screening format might be needed to reliably cover rare mutations.

High-level amplification of the MET gene has been linked to MET-dependent growth in gastric and lung cancer cell lines (23, 24). In this context, it is important to determine whether resistance mutations in only a fraction of MET copies are sufficient to drive resistance. The T790M mutation in EGFR is frequently found in patients with acquired resistance to the EGFR inhibitors gefitinib and erlotinib. In one report, where the gefitinib-sensitive and EGFR-amplified cell line H3255 was exposed to gefitinib for a prolonged period, a resistant subcloned was obtained that contained T790M in only a small fraction of the amplified alleles (39).

Although the activated TPR-MET fusion used in our cellular screening model may influence protein folding compared with full-length MET, our choice of this construct was motivated by the fact that it readily renders BaF3 cells IL3-independent, that substantial MET inhibitor drug discovery has been carried out across the industry using this fusion protein, and, most importantly, that the structural activity relationship across multiple chemotypes has been found to track between TPR-MET and cell lines expressing native full-length MET. Thus, we can assume that the entire MET kinase domain fused to TPR likely recapitulates the structure of the same domain in the context of full-length MET. We attempted to express full-length MET containing resistance mutations in several cellular systems but failed to obtain sufficient protein expression for functional evaluation. However, while this article was under revision, Qi and colleagues reported MET-Y1230 mutations in resistant derivatives of a gastric cancer cell line expressing full-length MET, which were obtained under treatment with MET inhibitors that rely on π-stacking with Y1230, similar to NVP-BVU972 (40). Hence, confirming that the same predominant mutation arises to confer resistance to π-stacking inhibitors in the context of full-length or TPR-fused MET.

Alternative resistance mechanisms involving the compensatory activation of EGFR or KRAS amplification have also been observed in MET-dependent cells (41–44). Importantly, EGFR-dependent cell lines could be rescued from EGFR inhibition by MET activation, indicating that this mechanism can operate reciprocally (45–47).

To our knowledge, there are no reports of acquired resistance mutations in MET from clinical trials yet. However, the results of our random mutagenesis screens
underline the need to carefully monitor the preexistence or emergence of MET mutations in clinical trials with MET inhibitors. This will not only be crucial to fully understand the observed response patterns but may also guide the choice of the suitable inhibitors, either used sequentially or even concomitantly, in case compounds with different binding modes become clinically available. Furthermore, our resistance mutation profiling setup could be used preclinically to differentiate novel drug candidates from already existing clinical candidates.

Disclosure of Potential Conflicts of Interest
All authors are or have been full-time employees of Novartis.

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A Drug Resistance Screen Using a Selective MET Inhibitor Reveals a Spectrum of Mutations That Partially Overlap with Activating Mutations Found in Cancer Patients

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