Abstract

Purpose: MET, the high-affinity receptor for hepatocyte growth factor, is frequently deregulated in human cancer. Tivantinib (ARQ197; Arqule), a staurosporine derivative that binds to the dephosphorylated MET kinase in vitro, is being tested clinically as a highly selective MET inhibitor. However, the mechanism of action of tivantinib is still unclear.

Experimental Design: The activity of tivantinib was analyzed in multiple cellular models, including: cells displaying c-MET gene amplification, strictly ‘addicted’ to MET signaling; cells with normal c-MET gene copy number, not dependent on MET for growth; cells not expressing MET; somatic knockout cells in which the ATP-binding cleft of MET, where tivantinib binds, was deleted by homologous recombination; and a cell system ‘poisoned’ by MET kinase hyperactivation, where cells die unless cultured in the presence of a specific MET inhibitor.

Results: Tivantinib displayed cytotoxic activity independently of c-MET gene copy number and regardless of the presence or absence of MET. In both wild-type and isogenic knockout cells, tivantinib perturbed microtubule dynamics, induced G2/M arrest, and promoted apoptosis. Tivantinib did not rescue survival of cells ‘poisoned’ by MET kinase hyperactivation, but further incremented cell death. In all cell models analyzed, tivantinib did not inhibit HGF-dependent or -independent MET tyrosine autophosphorylation.

Conclusions: We conclude that tivantinib displays cytotoxic activity via molecular mechanisms that are independent from its ability to bind MET. This notion has a relevant impact on the interpretation of clinical results, on the design of future clinical trials, and on the selection of patients receiving tivantinib treatment.

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Translational Relevance

MET is one of the most frequently activated tyrosine kinases in human cancer. Although a variety of MET inhibitors have been developed, only a few have progressed to the clinic. The recent failure of a phase III trial with tivantinib, the most clinically advanced molecule, could raise concerns about the validity of MET as a therapeutic target. However, the data presented here provide evidence that tivantinib is not a MET inhibitor but an antimitotic agent that kills tumor cells independently of MET. Therefore, the negative results obtained with tivantinib should not be generalized to other MET inhibitors. Moreover, selection of patients undergoing tivantinib treatment should not be based on MET status, and future clinical trials should be designed acknowledging that MET is not the pharmacologic target of tivantinib. Finally, patients who could benefit from MET inhibition should not be subtracted from treatment with an authentic anti-MET drug.

MET signaling in in vitro proliferation assays (7–12) in the conditions used with tivantinib (6).

A second study showed that tivantinib binds to MET only when the kinase is found in the inactive conformation and disclosed the crystal structure of the dephosphorylated MET kinase in complex with the drug (13). In this model, the tricyclic aromatic ring of tivantinib is bound deep inside a hydrophobic niche between the ribose ring sugar and the α-phosphate group of ATP, thus, completely occupying the ATP-binding cleft of MET without directly competing with ATP. The same work also showed that tivantinib is capable of interfering with MET kinase activity only if the latter is found in a totally dephosphorylated state and if the drug is preincubated with the enzyme before addition of ATP (13). In these conditions, tivantinib delays the start of the reaction, but after an initial lag phase, autophosphorylation eventually takes place with a rate that is indistinguishable from that observed in the absence of the drug. Conversely, if ATP is added before tivantinib, the drug has no effect on MET kinase activity. These data suggest that tivantinib may have limited efficacy on tumor cells expressing a constitutively autophosphorylated MET protein. However, in contradiction with this notion, the first study (5) clearly shows that tivantinib effectively inhibits proliferation of MKN-45 human gastric carcinoma cells, which display constitutive MET activation as a result of c-MET gene amplification (9, 14).

A third study analyzed the ability of tivantinib to inhibit metastasis in a mouse model of breast cancer. In this model, both tivantinib and a short hairpin RNA (shRNA) directed against the MET transcript reduced metastatic dissemination of MDA-MB-231 cells to the bones. However, MET shRNA did not affect MDA-MB-231 cell proliferation and survival in vitro, whereas tivantinib displayed potent cytotoxic activity in the same conditions (13). This discrepancy suggests that at least part of the pharmacologic effects of tivantinib is not mediated by MET inhibition in this system.

The clinical development of tivantinib is highly advanced. Its safety and efficacy is under investigation in a variety of clinical trials in solid tumors (16). Although the preclinical data do not support a clear role of MET in mediating its pharmacologic activity, tivantinib is being tested in the clinic as a highly selective MET tyrosine kinase inhibitor. In a phase II trial (NCT00777309), the progression-free survival of non–small cell lung carcinoma (NSCLC) patients receiving tivantinib plus erlotinib (Tarceva; Genentech) was compared with that of patients receiving placebo plus erlotinib (17). While the primary end point was not met, the tivantinib–erlotinib association showed a statistically significant benefit in a subset of patients carrying ras mutations, a condition known to promote resistance to MET inhibitors (9, 18). On the other hand, it also showed a significant delay in the development of new metastases among patients with nonsquamous histology, a sub-population displaying higher levels of MET expression. On the basis of these results, a phase III study was designed (NCT01244191, MARQUEE) that tested the same drug scheme in nonsquamous NSCLC patients. Unfortunately, this study was interrupted for futility (19). Several other phase II trials and two phase III trials (NCT01377376, ATTENTION; NCT01755767, METIV) are currently active, and the correlation between MET expression in tumors and the therapeutic response to tivantinib is being investigated.

In order to cast light onto the mechanism of action of this promising drug, we decided to analyze the ability of tivantinib to inhibit MET using several cellular models. These models include: (i) human tumor cell lines displaying c-MET gene amplification, strictly ‘addicted’ to MET; (ii) human tumor cell lines bearing normal c-MET gene copy number, insensitive to MET inhibition in proliferation assays; (iii) a genetically engineered human cell line in which the exon encoding the ATP-binding cleft of the MET kinase—where tivantinib binds—was knocked out by homologous recombination; (iv) two human tumor cell lines not expressing MET at all; and (v) an engineered cell system ‘poisoned’ by MET kinase hyperactivation, where cells survive and grow only in the presence of a specific MET inhibitor.

Materials and Methods

Cell culture
HepG2 hepatocarcinoma cells, MKN-45, Hs746T, and SNU-5 gastric carcinoma cells, H1993, NCI-H441, A549 lung carcinoma cells, TOV-112D and A2780 ovarian carcinoma cells, U87-MG glioblastoma cells, and HCT-116 colon carcinoma cells were obtained from the American Type Culture Collection (Salisbury, Wiltshire, United Kingdom) and cultured as suggested by the supplier. EBC-1 lung carcinoma cells were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). GTL-16 bones. However, MET shRNA did not affect MDA-MB-231 cell proliferation and survival in vitro, whereas tivantinib displayed potent cytotoxic activity in the same conditions (15). This discrepancy suggests that at least part of the pharmacologic effects of tivantinib is not mediated by MET inhibition in this system.
gastric carcinoma cells were derived from MKN-45 cells as described previously (14). EBC-1 and GTL-16 cells were maintained in RPMI medium (Sigma Life Sciences) supplemented with 10% FBS and 2 mmol/L glutamine (Sigma Life Sciences). Isogenic wild-type (WT) and MET exon 16 knock-out (KO) DLD-1 colon carcinoma cells have been described previously (20).

**Drugs and chemicals**

Tivantinib (ARQ197), INJ-38877605, and DN30 Fab were supplied by Arqule, Janssen Pharmaceutical Co. of Johnson & Johnson, and Metheresis (Lugano, Switzerland), respectively. PHA-665752 (Pfizer) was purchased from Tocris Bioscience. Paclitaxel (Taxol; Bristol-Myers-Squibb) was obtained from our hospital pharmacy. Imatinib (Gleevec; Novartis), SU-5416 (Semaxinib; Pfizer), and Iressa (Gefitinib; AstraZeneca) were obtained from Selleck Chemicals. Stauroporine and puromycin were purchased from Sigma-Aldrich.

**In vitro proliferation and apoptosis assays**

For proliferation assays, cells were seeded in 96-well plates (1,000 cells/well) with the appropriate culture medium in the presence of FBS. The day after, cells were treated with increasing concentrations of tivantinib or a control MET inhibitor (INJ-38877605, PHA-665752, DN30 Fab). After 3 days of treatment, cell number was determined using Cell Titer Glo (Promega) with a Victor X4 multilabel plate reader (Perkin Elmer). For apoptosis assays, cells were seeded in 96-well plates as described above. The following day, cells were exposed to increasing concentrations (0–1,200 nmol/L) of tivantinib or INJ-38877605, and apoptosis was determined after 48 hours by the free nucleosome method using a Cell Death Detection ELISAPLUS kit (Roche Diagnostics). Data were analyzed and fit using Prism software (GraphPad).

**Cell-cycle analysis**

WT and MET exon 16 KO DLD-1 cells were seeded with 600 nmol/L tivantinib or INJ-38877605. After 48 hours, cells were detached with trypsin, washed with PBS, fixed in ice-cold 70% ethanol, and stored at -20°C. The day after, cells were washed with PBS, re-suspended in 0.2 mL of staining solution (50 μg/mL propidium iodide, 100 μg/mL RNase A in PBS), and incubated for 3 hours at 4°C. Samples were analyzed by flow cytometry using a CyANADP apparatus (Dako, Glostrup, Denmark). Cell-cycle distribution was determined using Flowjo software (Tree Star).

**Gene copy number analysis**

Genomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer’s instructions. DNA concentration was determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Quantitative PCR (qPCR) on genomic DNA was carried out using the Go Taq qPCR Master Mix (Promega) and a 7900 HT Abiprism Real-Time System (Applied Biosystems) with the appropriate primers as described previously (21). The ribosomal protein S6 kinase (RPS6K1) gene, located on chromosome 1, was used for gene dosage normalization.

**Confocal microscopy**

WT and MET exon 16 KO DLD-1 cells were seeded in 24-well plates (12,500 cells/well) on glass coverslips coated with human plasma fibronectin (3 μg/mL; R&D Systems). One day after plating, cells were incubated for 36 hours with the appropriate concentrations of tivantinib, INJ-38877605, or paclitaxel and then fixed in 4% formaldehyde for 20 minutes at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 2 minutes on ice, and blocked with 1% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. Fixed cells were stained with rabies anti-α tubulin polyclonal antibody (Abcam) and revealed with Alexa Fluor 555-conjugated secondary antibody (Molecular Probes, Life Technologies). Confocal analysis was carried out on a Leica TCS SP2 AOBS microscope (Leica Microsystems) by maintaining the same laser intensity, gain, and offset settings for all samples including controls. Each image was acquired at a 200-Hz line scanning rate, each line being integrated 8 times. Maximum projection images were created from 2 stacks of 30 to 40 sections (step interval = 0.1–0.2 μm) spanning the whole cell using Leica Confocal Software (LCS). Mean fluorescence intensity (MFI) was determined by analyzing 20 different cells per sample and expressed as 8-bit greyscale values.

**Lentiviral vector engineering**

The amino acid sequence of Tpr-MET and of HGF corresponds to GenBank #U19348 and #M73239, respectively. The cDNAs encoding the above proteins were subcloned into the lentiviral vector backbone pRRL.sin.CPPT.CMV. Wpre (22) downstream to the cytomegalovirus (CMV) promoter. Lentiviral vectors particles were packaged by transient transfection of 293T cells as described previously (23). Vector particle concentration was determined by HIV-1 p24 core profile ELISA (Perkin Elmer).

**HepG2 cell growth rescue assays**

Exponentially growing HepG2 cells were seeded in 96-well plates at a density of 2,000 cells per well. One day later, cells were transduced with purified lentiviral vector particles encoding HGF, Tpr-MET, or with empty vector as control (100 ng/mL HIV-1 p24). One day after transduction and every 2 days thereafter, the medium was replaced with a fresh medium containing increasing concentrations of the various drugs as indicated (0–1,000 nmol/L). Cell number was determined after 10 days using Cell Titer Glo (Promega) as described above. Data were analyzed and fit using Prism software (GraphPad).

**MET expression and activation**

For analysis of MET autophosphorylation, GTL-16 and EBC-1 cells were incubated for 24 hours with increasing concentrations of tivantinib (0–3,000 nmol/L) or INJ-38877605 (0–1,000 nmol/L) and then lysed as previously...
Tivantinib does not inhibit MET autophosphorylation in tumor cells

Prompted by these data, we analyzed whether tivantinib inhibited MET activity in the same cells where it proved to be active biologically. GTL-16 gastric and EBC-1 lung carcinoma cells, which display HGF-independent, constitutive MET activation due to MET protein overexpression (9), were incubated for 24 hours with increasing concentrations of tivantinib (0–3,000 nmol/L) or JNJ-38877605 (0–1,000 nmol/L), and MET tyrosine autophosphorylation was determined by Western blot analysis using antibodies specific for the two most relevant phosphorylated forms of MET (Tyr1234–Tyr1235 and Tyr1349). While JNJ-38877605 potently inhibited MET autophosphorylation at 100 nmol/L, tivantinib had no significant effect on phospho-MET signal at any concentration (Fig. 2A). As discussed, the failure of tivantinib to inhibit MET kinase activity in these cells could be because of its peculiar binding mode requiring MET dephosphorylation (13). However, tivantinib effectively inhibited GTL-16 and EBC-1 cell proliferation with an EC50 of approximately 300 nmol/L (Fig. 1), thus questioning that the biological activity of this drug is due to MET kinase inhibition in these cells. The ability of tivantinib to inhibit MET autophosphorylation was also analyzed in cells bearing normal c-MET gene copy number (Supplementary Fig. S1). A549 lung carcinoma and HCT-116 colon carcinoma cells were preincubated with increasing concentrations (0–1,000 nmol/L) of tivantinib or JNJ-38877605 and then stimulated with 50 ng/mL recombinant human HGF. MET activation was determined using anti-phospho-MET antibodies as above. Remarkably, MET was found in a completely dephosphorylated state in these cells before HGF stimulation, thus representing an ideal target for tivantinib. In spite of this, tivantinib failed to inhibit HGF-induced MET tyrosine autophosphorylation at either Tyr1234–Tyr1235 or Tyr1349 at any concentration tested (Fig. 2B and 2C). In contrast, JNJ-38877605 effectively reduced phospho-MET levels in both cell lines. A similar analysis was conducted using U87-MG glioblastoma cells and NCI-H441 lung carcinoma cells that also display normal c-MET gene copy number (Supplementary Fig. S1). In these cell models as well, tivantinib did not affect MET protein expression with an EC50 ranging from 60 to 600 nmol/L. These results are surprising for multiple reasons. On one hand, due to its peculiar binding mode requiring MET dephosphorylation (13), tivantinib should not work in cells with high c-MET gene copy number (GTL-16, MKN-45, Hs746T, SNU-5, EBC-1, and HI1993) because these display constitutive MET kinase activation. On the other, if tivantinib were a selective MET inhibitor, it should not inhibit the growth of cells bearing normal c-MET gene copy number (NCI-H441, A549, HCT-116, and U87-MG), as these are not dependent on MET signaling in proliferation assays (7–12). Finally and most importantly, if the biological activity of tivantinib was because of its ability to inhibit MET, this drug should have no effect on cells that do not express the MET protein (A2780 and TOV-112D).

Results

Tivantinib inhibits the growth of tumor cells not expressing MET or not dependent on MET for proliferation

Previous studies suggest that only cells carrying c-MET gene amplification are ‘addicted’ to MET signaling in vitro proliferation assays (7–12). Cell lines with normal c-MET gene copy number, regardless of their MET expression levels, are insensitive to MET blockade in anchorage-dependent conditions and in the absence of HGF (reviewed in ref. 26). To assess the specificity of tivantinib, its ability to inhibit cell proliferation was analyzed on a panel of tumor cells that included six cell lines with high c-MET gene copy number (GTL-16, MKN-45, Hs746T, SNU-5, EBC-1, and HI1993) and six cell lines with normal c-MET gene copy number (NCI-H441, A549, HCT-116, U87-MG, A2780, and TOV-112D; Supplementary Fig. S1A). In addition, the latter group contained two cell lines that do not express MET protein or mRNA (A2780 and TOV-112D; Supplementary Fig. S1B; refs. 27, 28). Using this cell panel, the antiproliferative activity of tivantinib was compared with that of other anti-MET drugs including two ATP-competitive small-molecule inhibitors (JNJ-38877605, Johnson & Johnson; refs. 18, 21, 29; PHA-665752, Pfizer; ref. 30) and a monoclonal antibody Fab fragment causing MET ‘shedding’ (DN30 Fab, Metheresis; refs. 31, 32). This analysis revealed that the response profile of tivantinib clearly segregates it from that of other MET-specific agents (Fig. 1). Indeed, JNJ-38877605, PHA-665752, and DN30 Fab selectively inhibited proliferation of cells displaying c-MET gene amplification in a dose-dependent fashion without affecting the growth of cells with normal c-MET gene copy number. In contrast, tivantinib indiscriminately inhibited proliferation of all cells independently of c-MET gene amplification and MET protein expression with an EC50 ranging from 60 to 600 nmol/L. These results are surprising for multiple reasons. On one hand, due to its peculiar binding mode requiring MET dephosphorylation (13), tivantinib should not work in cells with high c-MET gene copy number (GTL-16, MKN-45, Hs746T, SNU-5, EBC-1, and HI1993) because these display constitutive MET kinase activation. On the other, if tivantinib were a selective MET inhibitor, it should not inhibit the growth of cells bearing normal c-MET gene copy number (NCI-H441, A549, HCT-116, and U87-MG), as these are not dependent on MET signaling in proliferation assays (7–12). Finally and most importantly, if the biological activity of tivantinib was because of its ability to inhibit MET, this drug should have no effect on cells that do not express the MET protein (A2780 and TOV-112D).

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autophosphorylation at either the major phosphorylation site or the docking site (Supplementary Fig. S2). As tivantinib inhibited proliferation of A549, HCT-116, U87-MG, and NCI-H441 cells with an EC50 ranging from 60 (HCT-116) to 600 nmol/L (NCI-H441; Fig. 1), we conclude that the antiproliferative activity of tivantinib is not due to MET kinase inhibition in these cells as well.

Deletion of the ATP-binding cleft of MET does not impair the cytotoxic activity of tivantinib

To further assess whether the pharmacologic activity of tivantinib requires MET, we employed an isogenic pair of human DLD-1 colon carcinoma cells. This pair consists in WT DLD-1 cells—that are diploid and display normal c-MET gene copy number—and an engineered variant in which exon 16 of the c-MET gene, encoding the ATP-binding cleft of the MET kinase, was knocked out on both alleles by homologous recombination (20). The resulting engineered cells (referred to as ‘MET exon 16 KO DLD-1 cells’ in this study) express a deleted MET protein that not only is completely kinase-inactive (20), but also lacks the entire tivantinib-binding domain according to crystal structure data (ref. 13; Fig. 3A). The biochemical and biological response to tivantinib was tested in parallel on both WT and MET exon 16 KO DLD-1 cells, using JNJ-38877605 as a positive control for MET inhibition. In receptor activation experiments, tivantinib failed to inhibit HGF-induced MET autophosphorylation, while JNJ-38877605 completely abrogated it (Supplementary Fig. S3). In cell proliferation assays, tivantinib potently inhibited the growth of both WT and MET exon 16 KO DLD-1 cells with exactly the same efficiency, thus confirming that the antiproliferative activity of tivantinib is not mediated by MET kinase inhibition. In contrast, and consistent with the notion that cells bearing normal c-MET gene copy number are not dependent on MET signaling for proliferation, JNJ-38877605 did not affect the growth of either cell type (Fig. 3B). Flow cytometric analysis of DNA content revealed that tivantinib caused cell-cycle arrest in G2/M independently of the MET status (Fig. 3C). At the same time, tivantinib also reduced...
the fraction of cells in G1 and dramatically increased the sub-G1 population. This suggests that tivantinib promotes mitotic arrest, prevents cells from re-entering G1, and drives them to apoptosis. Analysis of free nucleosomes in the cytoplasm—a hallmark of apoptosis—confirmed that tivantinib induces programmed cell death regardless of the presence or absence of a functional MET kinase (Fig. 3D).

**Tivantinib promotes microtubule stabilization independently of MET**

The results discussed here suggest that tivantinib is a mitotic inhibitor and a proapoptotic agent that displays its cytotoxic activity independently of MET. Because most antimitotic agents impair cytoskeleton dynamics, we tested whether tivantinib could affect microtubule stability. WT and MET exon 16 KO DLD-1 cells were incubated with increasing concentrations (177–900 nmol/L) of tivantinib or JNJ-38877605 for 36 hours, and the amount of α-tubulin–containing microtubules was determined by confocal immunofluorescence analysis using antibodies directed against the major phosphorylation site (Tyr1234–Tyr1235) or the docking site (Tyr1349). Total MET was determined using anti-MET antibodies. A, GTL-16 human gastric carcinoma cells and EBC-1 human lung carcinoma cells, which display c-MET gene amplification and constitutive MET kinase autophosphorylation, were incubated for 24 hours with increasing concentrations of tivantinib (0–3,000 nmol/L) or JNJ-38877605 (0–1,000 nmol/L), and MET activation was determined by Western blotting using anti-phospho-MET (p-MET) antibodies directed against the major phosphorylation site (Tyr1234–Tyr1235) or the docking site (Tyr1349). Total MET was determined using anti-MET antibodies. B, A549 human lung carcinoma cells, which bear normal c-MET gene copy number and express physiologic MET levels, were preincubated for 24 hours with increasing concentrations (0–1,000 nmol/L) of tivantinib or JNJ-38877605 and then stimulated with 50 ng/mL recombinant HGF. MET activation was determined by Western blotting using anti-phospho-MET antibodies as described. Total MET was determined using anti-MET antibodies. C, HCT-116 human colon carcinoma cells, which bear normal c-MET gene copy number and express physiologic MET levels, were treated, stimulated, and analyzed as described in B. The 170 and 145 kDa bands correspond to the unprocessed and mature form of MET, respectively.
α-tubulin–containing microtubules in a dose-dependent fashion (Fig. 4A). Remarkably, the microtubule-stabilizing activity of tivantinib in MET exon 16 KO cells was indistinguishable from that observed in WT DLD-1 cells. Because the former cells express a deleted form of MET that is kinase inactive (Supplementary Fig. S3; ref. 20) and lacks the tivantinib-binding site (13), these results suggest that tivantinib impairs microtubule dynamics via a molecular mechanism that does not involve MET kinase inhibition or MET binding. Consistent with this idea, MET inhibition by JNJ-38877605 did not have any effect on microtubule stability at any concentration in either cell line (Fig. 4A). Representative confocal microscopy images of cells treated with tivantinib or JNJ-38877605 are shown in Fig. 4B.

Figure 3. Deletion of the ATP-binding cleft of MET does not impair the cytotoxic activity of tivantinib. A, exon 16 of the c-MET gene encodes the ATP-binding cleft of MET (ABC; in red) that contains both the catalytic ATP site and the hydrophobic pouch where tivantinib binds (13). In MET exon 16 knockout (KO) human DLD-1 colon carcinoma cells, exon 16 has been deleted by homologous recombination (22). Therefore, the resulting deleted MET protein lacks both kinase activity and the ability to bind tivantinib. SEMA, 7-bladed β-propeller Semaphorine homology domain; PSI, plexin–semaphorine–integrin homology domain; IPT 1-4, immunoglobulin–plexin–transcription factor homology domains 1–4; KD, kinase domain; CT, C-terminal tail. B, the ability of tivantinib (TVB; red lines) or JNJ-38877605 (JNJ; blue lines) to inhibit cell proliferation was tested in parallel in both wild-type (WT; solid lines) or MET exon 16 KO (dotted lines) DLD-1 cells. Cell number is expressed as percentage relative to untreated control (CTRL). Values are the mean ± SD of 2 sets of experiments conducted in quadruplicate. C, flow cytometric analysis showing cell-cycle distribution of WT and MET exon 16 KO DLD-1 cells treated with no drug, 600 nmol/L tivantinib, or 600 nmol/L JNJ-38877605. Values corresponding to each cell-cycle phase are shown within columns. D, WT or MET exon 16 KO DLD-1 cells were incubated with increasing concentrations (0–1,200 nmol/L) of tivantinib or JNJ-38877605 for 48 hours and then processed for apoptosis analysis. Programmed cell death was measured by the free nucleosome method. Values are the mean ± SD of 2 sets of experiments conducted in quadruplicate and are normalized for cell number. AU, arbitrary units.

An engineered cell system ‘poisoned’ by MET kinase hyperactivation allows identification of MET-specific inhibitors

Previous studies have shown that human HepG2 hepatocarcinoma cells undergo growth arrest and apoptosis—instead of growing—following activation of the MET signaling pathway (33). Exploiting this paradoxical phenomenon, we set up an engineered cell system ‘poisoned’ by MET kinase hyperactivation in which cells die unless they are cultured with a specific MET inhibitor. The principle of this system is illustrated in Fig. 5. HepG2 cells are transduced with an engineered lentiviral vector (LV) expressing HGF or Tpr-MET (34), a constitutively active form of MET found in transformed cells (Fig. 5A). Following transduction, cells are cultured with no drug or with increasing

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concentrations of a putative MET inhibitor. Without any treatment, HGF- or Tpr-MET–expressing cells stop growing and die. If the drug analyzed possesses MET inhibitory activity, the survival and growth of HepG2 cells is rescued in a dose-dependent fashion. In contrast, if the drug is not a MET inhibitor, cells die regardless of its concentration. At the same time, cells are also transduced with an empty LV as control and are subjected to the same pharmacologic treatment (Fig. 5B). Without any drug or in the presence of a targeted agent, whether directed against MET or against a different tyrosine kinase, control cells proliferate normally. However, if the drug tested has cytotoxic activity, control cells die. By comparing the drug-response profile observed in cells expressing HGF or Tpr-MET with that observed in control cells, it is possible to determine whether a drug is a MET inhibitor, a targeted agent directed against a different molecule, or a cytotoxic compound.

Tivantinib is a cytotoxic drug and not a MET-targeted agent

Using the HepG2 system, we compared the pharmacologic profile of tivantinib with that of other drugs, including targeted agents and generic cytotoxic compounds (Supplementary Table S1). Targeted agents included JNJ-38877605, PHA-665752, gefitinib (an inhibitor of EGF receptor), imatinib (an inhibitor of platelet-derived growth factor receptor, Bcr-ABL, and KIT) and semaxinib (an inhibitor of VEGF receptor, KIT, and RET). Generic cytotoxic compounds included staurosporine (a protein kinase inhibitor), puromycin (a protein synthesis inhibitor), and paclitaxel (a microtubule inhibitor). This analysis revealed that only JNJ-38877605 and PHA-665752 could rescue survival and growth of cells expressing HGF or Tpr-MET (Fig. 6, blue lines). Gefitinib, imatinib, and semaxinib failed to achieve this rescue, and did not affect control cells either (green lines). In contrast, cytotoxic compounds not only failed to rescue survival and growth of cells ‘poisoned’ by MET activation, but also caused death of control cells (red lines). In this setting, tivantinib clearly behaved as a cytotoxic drug and not as a MET inhibitor (black lines). These data are consistent with our biochemical analysis indicating that tivantinib does not inhibit MET autophosphorylation in tumor cells. In addition, they are consistent with the origin of tivantinib that was discovered as a cytotoxic agent in a cell-based screen (5, 6). Our engineered system confirms that tivantinib displays cytotoxic activity in tumor cells, but does not support the hypothesis that this activity is mediated by MET kinase inhibition.

Discussion

Tivantinib was identified in a cell-based screen for proapoptotic agents (5) as a compound that displayed potent...
Tivantinib Displays MET-Independent Cytotoxic Activity

Figure 5. An engineered cell system 'poisoned' by MET kinase hyperactivation allows identification of MET-specific inhibitors. A, HepG2 human hepatocarcinoma cells, that undergo growth arrest and apoptosis upon activation of the MET signaling pathway (33), are transduced with an engineered lentiviral vector (LV) expressing HGF or Tpr-MET, a constitutively activated form of the MET kinase (red). LV-mediated expression of HGF or Tpr-MET leads to growth arrest and cell death, unless cells are cultured with a MET inhibitor. Any other drug that is not a selective inhibitor of MET fails to rescue cell survival and growth. B, at the same time, HepG2 cells are also transduced with an empty LV as control (green) and are subjected to the same pharmacologic treatment. In this case, targeted agents—whether specific for MET or directed against other tyrosine kinases—do not affect cell proliferation or survival. However, if the drug tested has cytotoxic activity, empty LV-transduced cells die.

cytotoxic activity in a wide variety of tumor cell types (6). The biology of HGF and its receptor MET has been object of intense study in the last three decades. Accurate review of these studies suggests that MET inhibition cannot explain such a widespread cytotoxicity. Inhibition of MET signaling is causally associated to apoptosis—in the conditions used in the screen—only in a very restricted group of tumor cells with a precise genotype (reviewed in ref. 26). Therefore, even considering its unconventional mechanism of binding, the pharmacologic activity of tivantinib can hardly be explained by its ability to interact with MET. The preclinical data also do not show that tivantinib really inhibits MET kinase activity, but rather suggest that tivantinib delays MET kinase activation only if the drug is preincubated with dephosphorylated MET in the absence of ATP (13). The possibility of encountering or reproducing these conditions in tumor cells is uncertain. In any case, the results presented here show that tivantinib does not inhibit HGF-dependent or -independent MET autophosphorylation in any of the tumor cell lines examined, despite the drug concentrations tested were significantly higher than those sufficient for inducing apoptosis in the same cells.

Although these data suggest that tivantinib does not inhibit MET kinase activity in tumor cells, the possibility that this drug may modulate some other MET-related function remains open. However, tivantinib elicited a sound cytotoxic effect also in cells expressing a deleted MET protein lacking the tivantinib-binding site and, strikingly, in cells not expressing MET at all. These observations do not support a crucial role of MET in mediating the pharmacologic activity of tivantinib. The virtually universal cytotoxic activity of this drug is more likely to be explained by interference with a more basic cellular function. The observation that tivantinib promotes dose-dependent stabilization of microtubules provides a useful hint for future studies. Microtubules are the target of several antimitotic drugs including taxanes and the more classical colchicine-derived agents. Impairment of microtubule dynamics is causally linked to mitotic blockade and is consistent with the ability of tivantinib to arrest cell cycle in G2/M.

Although these findings do not diminish the therapeutic potential of tivantinib in any way, they do have profound implications in clinical oncology. The first consideration concerns interpretation of clinical results. So far, tivantinib has been tested clinically assuming that it was a highly selective MET inhibitor. This assumption led to testing the tivantinib–erlotinib association in NSCLC, where MET activity is known to mediate resistance to EGF receptor inhibitors (35). A first phase II trial (NCT00777309) did not show any benefit in progression-free survival (17), while the MARQUEE Phase III trial involving almost 1,000 patients was prematurely discontinued due to futility (19). The data presented here...
offer a new interpretation key for these results: these trials did not fail because MET is not a good target in lung cancer as it may be inferred, but because tivantinib is not a MET inhibitor. This notion is also crucial for the design of future clinical trials, as it raises some concern on the rationale for recruiting patients based on their MET status.

A similar case of ‘mistaken identity’ recently involved iniparib (Sanofi-Aventis), a nicotinamide analogue tested clinically as a highly selective, noncompetitive poly-(ADP-ribose) polymerase (PARP) inhibitor. In a phase II trial in triple-negative breast cancer, the iniparib-chemotherapy combination was associated with a significant improvement in overall survival (36). However, these results could not be reproduced in a phase III trial, raising questions about the future of PARP inhibition as a therapeutic strategy. Remarkably, in striking analogy with tivantinib, subsequent studies showed that iniparib is not a PARP inhibitor but a cytotoxic agent that kills tumor cells by an unrelated molecular mechanism (37, 38). In the iniparib as in the tivantinib case, more rigorous preclinical analysis would have addressed these drugs toward a more appropriate clinical testing, preventing failure, and misinterpretation of clinical trials (39).

It is not uncommon that antitumor agents are administered to patients before knowing their mechanism of action. This is fine as long as their safety and efficacy is determined in unbiased clinical trials. However, the assumption that tivantinib is a selective MET inhibitor and not a cytotoxic agent may be misleading in the development of predictive biomarkers. In fact, while targeted agents are effective only in specific subsets of tumors displaying the appropriate genetic features, cytotoxic agents aim at universal mechanisms of cell survival and proliferation and, therefore, exert their activity largely independent of tumor genetics. On the other hand, acquired resistance to a cytotoxic agent is more likely to depend on induction of multidrug resistance and anti-apoptotic mechanisms rather than on activation of alternative oncogenic pathways like in the case of targeted agents. These aspects should be taken into account when searching for markers of response or resistance to tivantinib.

The findings presented in this study offer new perspectives of clinical development for this promising, well-tolerated cytotoxic drug. Continuing to consider tivantinib as a...
selective MET inhibitor may lead, on 1 hand, to the risk of not meeting the clinical end points that authentic MET inhibitors are expected to achieve and, on the other, to the potential subtraction of patients who could benefit from MET inhibition from the proper treatment.

Disclosure of Potential Conflicts of Interest

P. Michieli is a consultant/advisory board member of Metheresis SA and arGEN-X BVBA. No conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Basilico, S. Pennacchietti, E. Vigna, C. Chiriaco, A. Bardelli, D. Valdembri, G. Serini, P. Michieli
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References


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