Cytotoxic Activity of Tivantinib (ARQ 197) Is Not Due Solely to c-MET Inhibition

Ryohei Katayama1,2,3, Aki Aoyama3,5, Takao Yamori4,6, Jie Qi1, Tomoko Oh-hara3, Youngchul Song1, Jeffrey A. Engelman1,2, and Naoya Fujita1,3

Abstract

The receptor tyrosine kinase c-MET is the high-affinity receptor for the hepatocyte growth factor (HGF). The HGF/c-MET axis is often dysregulated in tumors. c-MET activation can be caused by MET gene amplification, activating mutations, and auto- or paracrine mechanisms. Thus, c-MET inhibitors are under development as anticancer drugs. Tivantinib (ARQ 197) was reported as a small-molecule c-MET inhibitor and early clinical studies suggest antitumor activity. To assess whether the antitumor activity of tivantinib was due to inhibition of c-MET, we compared the activity of tivantinib with other c-MET inhibitors in both c-MET–addicted and nonaddicted cancer cells. As expected, other c-MET inhibitors, crizotinib and PHA-665752, suppressed the growth of c-MET–addicted cancers, but not the growth of cancers that are not addicted to c-MET. In contrast, tivantinib inhibited cell viability with similar potency in both c-MET–addicted and nonaddicted cells. These results suggest that tivantinib inhibits its antitumor activity in a manner independent of c-MET status. Tivantinib treatment induced a G2–M cell-cycle arrest in EBC1 cells similarly to vincristine treatment, whereas PHA-665752 or crizotinib treatment markedly induced G0–G1 cell-cycle arrest. To identify the additional molecular target of tivantinib, we conducted COMPARE analysis, an in silico screening of a database of drug sensitivities across 39 cancer cell lines (JFCR39), and identified microtubule as a target of tivantinib. Tivantinib-treated cells showed typical microtubule disruption similar to vincristine and inhibited microtubule assembly in vitro. These results suggest that tivantinib inhibits microtubule polymerization in addition to inhibiting c-MET. Cancer Res; 1–10.

Introduction

The met proto-oncogene (c-MET) was originally identified from N-methyl-N'-nitro-N-nitrosoguanidine-treated human osteosarcoma cell lines. MET is an activated oncogene encoding a receptor tyrosine kinase (RTK) for hepatocyte growth factor (HGF), also called scatter factor (1). The HGF/c-MET–signaling pathway is frequently dysregulated in human cancer (2). Aberrant activation of c-MET can be due to gene amplification, transcriptional upregulation, activating mutations, or HGF-mediated auto- or paracrine stimulation. Activation of c-MET pathway by coexpression of HGF and c-MET was shown to drive tumorigenesis and metastasis in xenograft models and in transgenic mouse models (3). Although HGF/c-MET axis has been associated with metastasis and migration of cancer cells (3, 4), recent studies have shown that some cancers are addicted to the pathway for their growth and survival. In particular, cancers with amplification of c-MET have been shown to be highly sensitive to c-MET kinase inhibitors in cell lines and in the clinic (5–7). In addition, HGF/c-MET pathway was associated with the acquired resistance to inhibitors to EGFR receptor (EGFR) in EGFR mutant non–small cell lung cancers (NSCLC; refs. 8–11). Thus, inhibitors of c-MET have been pursued as therapeutic interventions in oncology. Many low-molecular inhibitors of c-MET and monoclonal antibodies against c-MET and HGF are now entering clinical trials.

Tivantinib (ARQ 197) was initially reported as a c-MET selective inhibitor in 2010 (12) and entered into clinical trials (13–18). In the initial report, tivantinib inhibited recombinant human c-MET with a calculated inhibitory constant (Ki) of approximately 355 nmol/L and had weak inhibitory effects on p21–activated kinase 3, VEGF receptor-3 (Flt3), calmodulin-dependent kinase II delta, and Pim-1. Tivantinib did not inhibit any of the other 225 human kinases tested, including the Ron kinase, which belongs to the c-MET family of RTKs. The crystal structure of the tivantinib in complex
with the c-MET kinase domain revealed that tivantinib binds to the inactive form of c-MET, suggesting that it inhibits c-MET through a non-ATP–competitive mechanism (19). This suggested that inhibitory mode of action is different from the disclosed c-MET inhibitors under preclinical and clinical development. Recent clinical trial results suggest that tivantinib may be active in KRAS-mutant lung cancers, which is not a cancer type identified in other preclinical studies to be dependent on c-MET signaling (16). In addition, a recent study found that tivantinib was equally potent against MKN-45 cells (with MET amplification) and NCI-H460 cells (KRAS mutation and no MET amplification; ref. 12), although a different study found that another c-MET inhibitor PHA-665752 was effective only in the MKN-45 cells (7). In this study, we aimed to determine whether the toxicity of tivantinib is solely due to inhibition of c-MET, and found that this was not the case. Thus, we sought to determine whether tivantinib inhibits additional target molecules or pathways in the cells. We previously established the COMPARE analysis, which consists of the sensitivity data of a panel of 39 cancer cell lines (termed JFCR39) against numerous drugs (20–24). The COMPARE analysis enables us to putatively identify the molecular target of a test compound by comparing the growth inhibitory patterns (fingerprints) of JFCR39 with those of the known anticancer drug or compounds (24–27). Here, we used COMPARE in silico screening to identify additional molecular targets of tivantinib that may lend insights into its indiscriminatory activity.

Materials and Methods

Cell lines and reagents

EBC1, MKN45, SNU638, A549, NCI-H460, HCC827, SNU-5, BT–77, and SKBR3 were cultured in RPMI-1640 medium with 10% FBS (RPMI growth medium). SNU638 subclones, SR-A1 and SR-C1 cells, were cultured in RPMI growth medium containing 1 μmol/L of a c-MET inhibitor PHA-665752 as described previously (28). SR-A1 and SR-C1 cells were cultured at least 1 week in drug-free RPMI growth medium before experiments. Tivantinib (ARQ 197) and crizotinib were purchased from ChemieTek. PHA-665752 was purchased from Tocris Biosciences. Vincristine and paclitaxel were purchased from Sigma. Compounds were dissolved in dimethyl sulfoxide to a final concentration of 10 mmol/L and stored at −20°C until use.

Survival assays

Assessment of cell viability was conducted as follows. A total of 2,000–3,000 cells were seeded in 96-well plates in sextuplet. On the following day, cells were treated with increasing concentrations of the indicated drugs and incubated for another 72 hours. Cell viability was determined by adding the CellTiter-Glo assay reagent (Promega) for 10 minutes and luminescence was measured using a Centro LB 960 luminometer (Berthold Technologies). The data were graphically displayed using GraphPad Prism version 5.0 (GraphPad Software). IC50 value was determined by a nonlinear regression model with a sigmoidal dose response in GraphPad.

Immunoblot analysis

Lysates were prepared as described previously (9, 29). Equal amounts of lysates were electrophoresed and immunoblotted with the antibodies against phospho-c-MET (Tyr1234/1235) (3D7), c-MET (25H2), phospho-p42/44 extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK; Thr202/Tyr204), p42/44 ERK/MAPK, phospho-AKT (Ser473; D9E), AKT1 (C73H10), β-actin (13E5; Cell Signaling Technology), and α-tubulin (DM1A; Sigma).

Infection of shRNA

Cells were seeded into 96-well plates. After incubation for 24 hours, cells were infected with MET short hairpin RNAs (shRNA; MET KD 345 shRNA and MET KD 4571 shRNA) or scramble shRNA (9, 28). Media was changed the following day. Three days later, cell viability was assessed by CellTiter-Glo assay as described above. Titers of virus were used that gave the same results in the absence and presence of puromycin.

Immunohistochemistry

EBC1 or A549 cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, followed by 0.5% Triton X-100 for 5 minutes at room temperature. Fixed and permeabilized cells were incubated with 1% bovine serum albumin in PBS for 60 minutes at room temperature. Then, cells were incubated with anti-α-tubulin (1:1,000) as the primary antibody overnight at 4°C and Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (1:1,000) as the secondary antibody with Hoechst 33342 and Alexa Fluor 568 conjugated pharoidine for 1 hour at room temperature. The cells were observed under an Olympus IX71 fluorescence microscopy equipped with a charge-coupled device camera.

COMPARE analysis

On the basis of a series of GI50 values of a panel of 39 human cancer cell lines (termed JFCR39) described previously, fingerprints are presented in the graphic profiles of relative growth inhibition within JFCR39. To analyze the correlation between the fingerprints of drug A and drug B, we exploited the COMPARE computer algorithm as described previously (23, 27, 30). The Pearson correlation coefficient between the fingerprints of drug A and drug B was calculated.

Microtubule polymerization assay

Porcine brain tubulin polymerization assay was conducted in 55 μL volumes at 30°C according to the Tubulin Polymerization Assay Kit (Cytoskeleton) according to the manufacturer’s instructions. Tubulin preparation was incubated at 30°C in the presence of inhibitors (tivantinib, vincristine, paclitaxel, crizotinib, or PHA-665752). Fluorescence emission was read at excitation (355 nm) and emission (460 nm) with a TriStar LB941 plate reader (Berthold Technologies) every minute right after starting incubation.

Flow cytometric analysis

Cells were treated with indicated concentration of tivantinib, vincristine, PHA-665752, or crizotinib for 24 hours. After incubation, the cells were harvested and fixed with 70%
ice-cold ethanol for 30 minutes at 4°C. The cells were washed with PBS and then incubated with RNaseA (0.25 mg/mL, Sigma) for 15 minutes at 37°C, add propidium iodide (final concentration is 50 μg/mL, Sigma) and incubate for 30 minutes at 4°C. Analyses were conducted using a Cytomics 500 flow cytometer (Beckman Coulter) with a Cytomics RXP and FlowJo (Treestar) software.

**Apoptosis assay**

A total of 1 × 10⁵ cells were seeded in 6-well plates. On the following day, cells were treated with the indicated concentration of drugs and incubated for another 48 or 72 hours. After incubation, cells were collected and stained with fluorescein isothiocyanate-labeled Annexin V and 5-fluorouracil for 10 minutes. Cells were then assayed using a Cytomics 500 flow cytometer (Beckman Coulter), and the data were analyzed using FlowJo software (Treestar).

**Results**

**Tivantinib inhibits the growth of both c-MET–dependent and -independent cancer cells**

To examine whether the antiproliferative activity of tivantinib was due to inhibition of c-MET, we examined cancer cell lines previously shown to be addicted to c-MET and sensitive to c-MET inhibitors (NSCLC cell line EBC1 and gastric carcinoma cell lines MKN45 and SNU638) and NSCLC cell lines previously shown to be resistant to c-MET inhibitors, A549, NCI-H460 (H460), and HCC827 (9, 10, 28). As shown in Supplementary Table S1, EBC1 and MKN45 have MET gene amplification (28). The resistant A549 and H460, and HCC827 cells were unaffected (Supplementary Fig. S1B and S1C). These results are consistent with the expected finding that EBC1, MKN45, and SNU638 require c-MET, but A549, H460, and HCC827 do not.

These cell lines were examined for sensitivity to 3 c-MET inhibitors, tivantinib, PHA-665752, or crizotinib. Unexpectedly, tivantinib inhibited cell viability in all of the cell lines examined (Fig. 1A). In contrast, the other 2 c-MET inhibitors, PHA-665752 and crizotinib, inhibited cell viability specifically in the cancer cell lines that were shown to be c-MET dependent in the shRNA experiments (Fig. 1B and C). Similar analyses were conducted on the MET-amplified gastric carcinoma cell line SNU-5 and the HER2-amplified BT-474 and SKBR3 breast cancer cell lines (Supplementary Table S1 and Supplementary Fig. S2). As shown in Fig. 1D, tivantinib is equally potent at diminishing cell viability in cancers cell lines regardless of their dependence on c-MET, whereas the other 2 c-MET inhibitors show substantially greater potency in the cancers driven by c-MET (Fig. 1D and Supplementary Table S2). These data suggest that the toxicity of tivantinib against cancer cell lines may be independent of inhibition of c-MET. Importantly, the IC₅₀ values obtained with tivantinib in these cell lines are equivalent to those reported in earlier publications (12).

In previous studies, we had developed clones of SNU638 with acquired resistance to PHA-665752 (28). Both of these clones
showed resistance to both PHA-665752 and crizotinib (Fig. 2A and B). One of these clones, SR-A1, developed a mutation, Y1230H that was the cause of resistance. Another clone, SR-C1, was resistant due to increased activation of EGFR resulting from increased expression of TGFα (28). In these cells, c-MET was still inhibited by PHA-665752, but EGFR signaling maintained cell viability. Furthermore, the viability of these cells were unaffected by MET knockdown (28). We observed that both of the c-MET inhibitor-resistant clones showed the same sensitivity to tivantinib (Fig. 2C and D and Supplementary Table S2). This was surprising because viability of the SR-C1 clone is not dependent on c-MET (28). These data are consistent with studies in Fig. 1 suggesting that sensitivity to tivantinib is independent of c-MET’s dependence on c-MET.

**Tivantinib does not suppress c-MET phosphorylation and downstream signaling**

To examine the effect of tivantinib on c-MET signaling, MKN45, EBC1, and A549 cells were treated with the increasing concentrations of tivantinib or crizotinib (Fig. 3A). The first 2 cell lines are addicted to c-MET signaling, whereas the A549 cells are not (Fig. 1 and Supplementary Fig. S1). After exposure to 1 μmol/L of crizotinib for 6 hours, there was a marked downregulation of phosphorylation of c-MET and downstream AKT and ERK in MKN45 and EBC1 cells (Fig. 3A). Previous studies have shown that the PI3K-AKT and MEK-ERK signaling are downstream of c-MET in these sensitive cells (7, 9). However, phospho-c-MET was undetectable in the A549 cells and crizotinib did not suppress ERK or AKT phosphorylation. In contrast, tivantinib, used at doses up to 10 μmol/L, failed to impair c-MET, AKT, or ERK phosphorylation in the EBC1 or MKN45 cells. Of note, the IC₅₀ of tivantinib was 300 to 400 nmol/L in these cells (Supplementary Table S2), despite the lack of effect on c-MET phosphorylation or downstream signaling.

When the cells were treated with tivantinib or crizotinib for 24 hours, 1 μmol/L crizotinib potently suppressed c-MET, AKT, and ERK phosphorylation in the MKN45, EBC1, and SNU638 cells. In contrast, tivantinib moderately decreased the phospho-c-MET level in MKN45 and EBC1 cells but not in SNU638 cells (Supplementary Fig. S3). Not surprisingly, there was no suppression of AKT in these cell lines and a modest suppression of ERK in the EBC1 cells treated with tivantinib (up to 10 μmol/L). However, all of these cell lines, including the A549 cells, had submicromolar sensitivity to the tivantinib (Supplementary Table S2). Similar to the results in Figs. 1 and 2, these results are most consistent with the notion that the effect of tivantinib on cell viability is not solely due to inhibition of c-MET.

A recent report suggests that tivantinib selectively binds to the inactive or unphosphorylated form of c-MET, preventing it from being activated (19). To test whether tivantinib keeps c-MET in inactive form in cells, MKN45 cells were pretreated with 100 nmol/L of crizotinib for 6 hours to suppress c-MET phosphorylation. Pretreatment with crizotinib substantially downregulated the phospho-c-MET level within 6 hours (Fig. 3B). Washout of the crizotinib with fresh medium led to reactivation of c-MET within 15 minutes. Coincubation of tivantinib (up to 10 μmol/L) plus 100 nmol/L of crizotinib for 6 hours also suppressed the phosphorylation of c-MET in MKN45 cells. Surprisingly, washout and continued incubation of the cells with medium containing 10 μmol/L of tivantinib failed to prevent reactivation of c-MET phosphorylation. The reactivation of c-MET was almost the same as observed after wash and additional incubation in the medium containing none (fresh media). When A549 cells, which express c-MET but not phospho-c-MET (Supplementary Fig. S1A), were treated
with HGF ligand, phospho-c-MET level was increased and downstream AKT/ERK signaling was also significantly activated. We did observe that tivantinib pretreatment moderately inhibited HGF-dependent c-MET activation in A549 cells (Supplementary Fig. S4). These results suggest that tivantinib may have the capacity to mitigate c-MET activation following HGF activation. However, the results in Figs. 1–3 show that this activity is not the major mechanism underlying the inhibition of cell viability.

**Tivantinib treatment increases G₂–M phase cells**

To elucidate the mechanism by which tivantinib inhibits cell growth, we conducted cell-cycle analyses after tivantinib treatment. MET-amplified EBC1 cells were treated with 1 μmol/L of tivantinib, PHA-665752, and crizotinib for 24 hours, and the cell cycles were examined by propidium iodide staining. To our surprise, tivantinib markedly increased the number of G₂–M phase cells, whereas the other 2 c-MET inhibitors, crizotinib and PHA-665752, induced G₀–G₁ arrest (Fig. 4A). The effect on cell cycle of tivantinib was more similar to vincristine, a microtubule polymerization inhibitor (31). When MET-amplified EBC1 or MKN45 cells were treated with tivantinib, vincristine, or crizotinib for 48 to 72 hours, induction of apoptosis was observed (Supplementary Fig. S5). The A549 or H460 cells, both of which are resistant to c-MET inhibitors and MET knockdown, were treated with 1 μmol/L of tivantinib, PHA-665752, crizotinib, and vincristine for 24 hours, and the cell cycles were analyzed. Tivantinib treatment markedly increased the number of G₂–M phase cells and sub-G₁ population, whereas the other 2 c-MET inhibitors, crizotinib and PHA-665752 did not affect the cell cycle (Supplementary Fig. S6A and S6B).

To identify the genuine target of tivantinib, we conducted COMPARE analysis using the JFCR39 cell line panel. The JFCR39 panel is effective for in silico screening of compounds with specific pharmacologic activities. There is an extensive database assessing the activity of more than 1,000 compounds, including anticancer drugs and known inhibitors of various biologic pathways, across the JFCR39 cell line panel. Using this JFCR39 panel fingerprint, which is created by a series of GI₅₀ value among the JFCR39 cell lines, we can determine potential targets of tests molecules. We have successfully identified the target of new compounds, such as a new telomerase inhibitor (FJ5002), an inhibitor of the Golgi system (AMF-26) and a new phosphatidylinositol 3-kinase (PI3K) inhibitor (ZSTK474; refs. 25–27). From the results of COMPARE analysis, we observed that the fingerprint of tivantinib is similar to that of E7010 and vincristine (Fig. 4B and Supplementary Fig. S7). In contrast, the fingerprints of other 2 c-MET inhibitors PHA-665752 and crizotinib are mutually similar, but different from tivantinib, E7010, or vincristine. The COMPARE analysis of the tivantinib fingerprint identified the tubulin polymerization...
Figure 4. Tivantinib induces a G2–M arrest and exhibits similar activity as tubulin inhibitors across the JFCR39 cell line panel. A, EBC1 cells were treated with 1 μmol/L of tivantinib, vincristine, PHA-665752, or crizotinib for 24 hours. Cells were trypsinized, fixed, and stained with propidium iodide, and the cell cycle was analyzed by flow cytometry. The histogram shows cell distribution versus DNA content. B, growth inhibition against a panel of 39 human cancer cell lines. The mean graph was produced by computer processing of the 50% growth inhibition (GI50) values as described under Materials and Methods. The x-axis represents the logarithm of difference between the mean of GI50 values for 39 cell lines and the GI50 value for each cell line. MG-MID, the mean of log GI50 values for 39 cell lines; Delta, the logarithm of difference between the MG-MID and the log GI50 of the most sensitive cell line; Range, the logarithm of difference between the log GI50 of the most resistant cell line and the log GI50 of the most sensitive cell line. Quantification of the GI50 value is represented as the mean of 4 different experiments. Br, breast; CNS, central nervous system; Co, colon; Lu, lung; Me, melanoma; Ov, ovarian; Re, renal; St, stomach; xPg, prostate. C, the 3 compounds (out of 1,805 compounds), which have high Pearson correlation coefficient (r) with tivantinib, and the results of 2 c-MET inhibitors (crizotinib and PHA-665752) are shown in the table. Each experiment was carried out as described in Materials and Methods.
inhibitor E7010 (32) as the compound with a highest correlation coefficient ($r = 0.74$; 1,805 tests, more than 1,000 compounds). Vincristine and paclitaxel ranked second and third, respectively, also had high-correlation coefficients (Fig. 4C). All of these compounds target microtubule function. These results suggest that tivantinib may disrupt microtubule and suppress growth of the tumor cells.

**Tivantinib disrupts microtubule by inhibiting tubulin polymerization.**

To test whether tivantinib alters microtubule dynamics, c-MET nonaddicted A549 cells and c-MET-addicted EBC1 cells were treated with tivantinib or vincristine for 2 or 16 hours, and the effect on microtubules in those cells was determined by immunofluorescent staining of α-tubulin. Tivantinib treatment led to a loss of microtubules in both A549 and EBC1, similar to the vincristine-treated cells (Fig. 5A and Supplementary Fig. S8 and S9).

To explore whether tivantinib directly affects microtubule formation in vitro, we conducted a tubulin polymerization assay. Highly purified α- and β-tubulin and GTP were mixed with tivantinib, vincristine, crizotinib or paclitaxel, and incubated. Compared with control, paclitaxel enhanced tubulin polymerization, whereas vincristine inhibited tubulin polymerization completely as has been well described previously (Fig. 5B; refs. 31, 33). Similar to vincristine, tivantinib inhibited tubulin polymerization in a dose-dependent manner (Fig. 5B and Supplementary Fig. S10). In contrast, c-MET inhibitors crizotinib and PHA-665752 did not affect tubulin polymerization (Fig. 5B). These results suggest that tivantinib disrupts microtubules in cells by abrogating microtubule assembly.

**Discussion**

The HGF/c-MET pathway is required for the normal development in mammals (34). However, evidence to date suggests that the activity of this pathway is normally low in adults except for some specific physiologic responses including embryogenesis and wound healing (35, 36). However, the HGF/c-MET pathway is frequently dysregulated in many types of human cancers, and the c-MET expression is correlated with the patient poor prognosis (35, 37). c-MET transmits signals for cell proliferation, survival, invasion, and metastasis. In addition, activation of c-MET can lead to resistance to other tyrosine kinase inhibitors, and the HGF/c-MET pathway is believed to be an attractive target for EGFR-resistant cancer therapy.

---

**Figure 5.** Inhibition of tubulin polymerization by tivantinib. A, tivantinib treatment disrupted microtubule in A549 cells. Control, tivantinib- (10 µmol/L, 16 hours) or vincristine- (100 nmol/L, 16 hours) treated A549 cells were fixed and stained with Alexa488 labeled anti-α-tubulin antibody, Alexa 568-conjugated phalloidin (F-actin) or Hoechst33342 (nucleus). White scale bars indicate 20 μm. B, tivantinib inhibits tubulin polymerization similar to vincristine in vitro. A tubulin polymerization assay was conducted with or without indicated concentration of tivantinib, vincristine, paclitaxel, crizotinib, or PHA-665752. Experiments were carried out in triplicate. The average values and SEMs are shown.
Tivantinib (ARQ 197) was originally reported as a selective, orally administered, small-molecule, non-ATP competitive inhibitor of c-MET (12). The sensitivity to tivantinib was reported to be dependent on the c-MET status (12). Tivantinib is now entered into clinical trials (13–18) and has shown preliminary activity in combination with erlotinib in KRAS-mutant NSCLCs (16, 38).

We compared the tivantinib sensitivity between c-MET–addicted and c-MET–independent cancer cell lines (Supplementary Fig. S1). To our surprise, we found that the IC50 values of tivantinib in c-MET–independent A549, H460, and HCC827 were similar to those in c-MET–addicted EBC1, MKN45, and SNU638 cells (Fig. 1), suggesting that the toxicity of this compound is unrelated to inhibition of c-MET. In addition, our previously established c-MET inhibitor PHA-665752–resistant SNU638 clones were c-MET–independent and were as sensitive to tivantinib as that in parental SNU638 cells (Fig. 2). Furthermore, tivantinib did not downregulate phospho-c-MET levels after 6 hours treatment (Fig. 3A). When cells were treated with doses of tivantinib exceeding the cytotoxic doses for 24 hours, a modest decrease in phospho-c-MET levels was observed in EBC1 and MKN45 cells (Supplementary Fig. S3). However, this did not lead to marked suppression of downstream PBK/AKT and MAP–ERK kinase (MEK)/ERK pathways, suggesting that this is not the cause of its cytotoxicity. In total, these experiments provide compelling evidence that the cytotoxic activity of this compound is not due to its effects on c-MET.

Tivantinib was reported to stabilize c-MET in inactive form (12, 19). MKN45 cells have MET gene amplification (7) and constitutive c-MET activation. However, when MKN45 cells were treated with 100 nmol/L of crizotinib, phospho-c-MET levels were potently suppressed within 6 hours. Crizotinib binds c-MET in the inactive conformation (39). Even though c-MET was in the inactive conformation, tivantinib was unable to block the reactivation of c-MET (Fig. 3B). In A549 cells, we did observe that tivantinib incubation mitigated activation of c-MET by HGF (Supplementary Fig. S4). Thus, it remains possible that tivantinib has an effect on c-MET activation by ligand. However, the data in this article show that the indiscriminatory toxicity of this compound is not due to its modest effects on c-MET signaling.

Although tivantinib was developed as a c-MET inhibitor and exhibits potent antitumor activity, the data suggested that tivantinib exhibits its antitumor activity by affecting unknown targets in addition to c-MET. Indeed, this was underscored by the effect of tivantinib on the cell cycle, that was quite distinct from other c-MET inhibitors, crizotinib and PHA-665752 (Fig. 4A and Supplementary Fig. S6). To identify a potential target of tivantinib, we used the COMPARE analysis using JFCR39 cell line panel. The fingerprint of tivantinib was most similar to microtubule inhibitors. In contrast, the fingerprints of crizotinib and PHA-665752 were not similar to tivantinib (Fig. 4B and C). Furthermore, tivantinib treatment disrupted microtubules both in c-MET–addicted EBC1 and c-MET nonaddicted A549 cells (Fig. 5A and Supplementary Figs. S8 and S9). These results indicate that tivantinib has an ability to inhibit microtubule function.

Both microtubule polymerization and depolymerization are required for mitosis and normal homeostasis of the cells (40). The in vitro tubulin polymerization assay suggested that tivantinib inhibits tubulin polymerization similar to vincristine (Fig. 5B). Thus, our results suggest that growth inhibition/cytotoxicity of tivantinib may be mainly due to inhibition of tubulin polymerization. In the in vitro tubulin polymerization experiments, we found that 3 μmol/L of tivantinib was required to inhibit polymerization. In this assay, the concentration of purified tubulin is approximately 18 μmol/L (2 mg/mL). Because the concentration of drug required to inhibit tubulin polymerization is likely impacted by the concentration of tubulin, higher inhibitor concentration might be required to inhibit tubulin polymerization in these assays. However, it is important to note 10 μmol/L doses of c-MET inhibitors, crizotinib and PHA-665752, do not inhibit tubulin polymerization (Fig. 5A). Further studies are needed to clarify how tivantinib inhibits tubulin polymerization. Because the in vitro tubulin polymerization assay does not contain microtubule associate proteins, we hypothesize that tivantinib directly binds tubulin.

Currently, many clinical trials are based on the fact that tivantinib is a c-MET inhibitor. The phase II trial of erlotinib with tivantinib or placebo did not reveal a significant difference between 2 groups. Although the study did not meet its primary endpoint, subset analysis suggests that tivantinib showed increased efficacy among patients with KRAS mutation (16). Because our study suggests that tivantinib exhibits antitumor effect via microtubule inhibition, it remains possible that tivantinib may also be effective to other solid cancers. Indeed, the combination with erlotinib may not be the most active because this combination was developed with the notion that tivantinib is a potent c-MET inhibitor. As the mode of action of tivantinib seems to be different from classical antimitotic drugs, further studies are necessary to understand the detailed mechanism how tivantinib inhibits tubulin polymerization and how to use this agent most effectively to treat cancer.

Disclosure of Potential Conflicts of Interest
J.A. Engelman is a co-inventor on a patent combining EGFR and MET inhibitors and is a consultant/advisory board member of Arqule, Genentech, Amgen, and Daiichi. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: R. Katayama, A. Aoyama, T. Yamori, J. Qi, Y. Song, N. Fujita, J.A. Engelman
Development of methodology: R. Katayama, A. Aoyama, T. Yamori, J. Qi, Y. Song, N. Fujita, J.A. Engelman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Katayama, A. Aoyama, T. Yamori, J. Qi, T. Oh-hara, T. Song, N. Fujita
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Yamori, N. Fujita, J.A. Engelman
Writing, review, and/or revision of the manuscript: R. Katayama, N. Fujita, J.A. Engelman
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Katayama, A. Aoyama, N. Fujita, J.A. Engelman
Study supervision: N. Fujita, J.A. Engelman

Acknowledgments
The authors thank Dr. Shingo Dan for valuable discussions and Ms. Sumie Koike for data analysis.
Tivantinib (ARQ 197) Inhibits Tubulin Polymerization

Grant Support

The study was supported in part by R01CA137008, R01CA140594, National Cancer Institute Lung SPORE (J.A. Engelman), and JSPS KAKENHI grant number, 2430044 and 22121008 (N. Fujita).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 26, 2012; revised January 12, 2013; accepted February 6, 2013; published OnlineFirst April 18, 2013.

References


www.aacrjournals.org


## Cytotoxic Activity of Tivantinib (ARQ 197) Is Not Due Solely to c-MET Inhibition

Ryohei Katayama, Aki Aoyama, Takao Yamori, et al.

*Cancer Res* Published OnlineFirst April 18, 2013.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-3256</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2013/09/23/0008-5472.CAN-12-3256.DC1">http://cancerres.aacrjournals.org/content/suppl/2013/09/23/0008-5472.CAN-12-3256.DC1</a></td>
</tr>
</tbody>
</table>

---

**E-mail alerts**  
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.