Tivantinib (ARQ 197) efficacy is independent of MET inhibition in non-small-cell lung cancer cell lines

Antonio Calles\textsuperscript{a, b}, Nicholas Kwiatkowski\textsuperscript{c, d}, Bernard K. Cammarata\textsuperscript{a, b}, Dalia Erkan\textsuperscript{a, b}, Nathanael S. Gray\textsuperscript{c, d}, Pasi A. Jänne\textsuperscript{a, b, e, *}

\textsuperscript{a}Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA
\textsuperscript{b}Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA
\textsuperscript{c}Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02215, USA
\textsuperscript{d}Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02215, USA
\textsuperscript{e}Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115, USA

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\textbf{ABSTRACT}

MET targeted therapies are under clinical evaluation for non-small-cell lung cancer (NSCLC) patients. Tyrosine kinase inhibitors (TKI) against MET have varying degrees of specificity. Tivantinib (ARQ 197) is reported to be a non-ATP competitive selective MET inhibitor. We aimed to compare the activity of tivantinib to established MET TKIs in a panel of NSCLC cell lines characterized by their MET dependency and by different relevant genotypes. A549, H3122, PC9 and HCC827, their respective resistant clones PC9 GR4 and HCC827 GR6 and the MET amplified cell lines H1993 and EBC-1 were treated in vitro with tivantinib, crizotinib or PHA-665752. Crizotinib and PHA-665752 showed growth inhibition restricted to MET dependent cell lines. The pattern of activity was related to MET inhibition and downstream signaling inhibition of AKT and ERK1/2, resulting in G0/G1 cycle arrest and apoptosis. In contrast, tivantinib possessed more potent anti-proliferative activity that was not restricted to only MET dependent cell lines. Tivantinib did not inhibit cellular MET activity or phosphorylation of downstream signaling proteins AKT or ERK1/2, resulting in G0/G1 cycle arrest and apoptosis. In contrast, tivantinib possessed more potent anti-proliferative activity that was not restricted to only MET dependent cell lines. Tivantinib did not inhibit cellular MET activity or phosphorylation of downstream signaling proteins AKT or ERK1/2 in either MET dependent or independent cell lines. Cell cycle analysis demonstrated that tivantinib induced a G2/M arrest in EBC-1. Tivantinib but not crizotinib showed microtubule dynamics, disrupting mitotic spindles by a mechanism consistent with it functioning as a microtubule depolymerizer. Tivantinib activity is independent of MET signaling in NSCLC and suggests alternative mechanisms of action that should be considered when interpreting the results from on-going clinical studies.

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1. **Introduction**

The MET proto-oncogene encodes for the high-affinity cell-surface receptor tyrosine kinase (RTK) for hepatocyte growth factor (HGF), c-MET. MET is commonly expressed in non-small-cell lung cancer (NSCLC) (Ma et al., 2005; Park et al., 2012; Sadiq and Salgia, 2013; Spigel et al., 2013) and its activity may be the result of autocrine or paracrine stimulatory mechanisms, protein overexpression, somatic mutations or gene amplification (Gherardi et al., 2012; Kong-Beltran et al., 2006).

Despite the different mechanisms of MET activation described in NSCLC, only MET gene amplification is to date the most closely associated with dependency on MET signaling both in vitro and in vivo (Lutterbach et al., 2007; Smolen et al., 2006; Tanizaki et al., 2011). MET amplification has been detected in approximately 20% of EGFR mutant NSCLC patients as a mechanism of acquired resistance to EGFR tyrosine kinase inhibitors (TKIs) (Engelman et al., 2007). Moreover, approximately 5% of NSCLC harbor de novo MET amplification including some EGFR-TKI naïve patients with EGFR mutant lung cancer (Cappuzzo et al., 2009; Shibata et al., 2005; Turke et al., 2010; Zhao et al., 2005).

MET inhibition is currently being evaluated as a therapeutic strategy in NSCLC and some agents are already in late stage clinical evaluation (Sadiq and Salgia, 2013; Scaglìotti et al., 2012; Spigel et al., 2012). They involve diverse mechanisms of action and are either monoclonal antibodies directed against either HGF or MET or small molecule MET TKIs (Peters and Adjei, 2012; Sadiq and Salgia, 2013; Spigel et al., 2012). TKIs targeting MET include ATP competitive and non-selective small molecules as caboctanizib (XL 184; Exelixis; inhibiting MET, RET, VEGFR2, AXL, Tie-2, KIT and Flt-3), crizotinib (PF2341066; Pfizer; inhibiting MET, ALK and ROS), and foretinib (XL880; Exelixis; inhibiting MET, RON, VEGFR2, PDGFR, Flt-3, Tie-2 and KIT). In contrast, tivantinib (ARQ 197; Daiichi-Sankyo) has been reported to be a non-competitive ATP and highly specific orally bioavailable small molecule for MET. Tivantinib is the most advanced agent under clinical evaluation with a phase III clinical trial in refractory NSCLC in combination with erlotinib (Scaglìotti et al., 2012) (ClinicalTrials.gov identifier: NCT01244191) but recently halted due to futility in an interim analysis (Scaglìotti et al., 2013).

The different and sometimes distinctive mechanisms of action of the different MET inhibitors might have clinical implications on the efficacy observed in different subsets of patients. This could be of particular relevance when these drugs are combined with other therapies currently used in the treatment of NSCLC (Goldman et al., 2012; Scaglìotti et al., 2012; Sequist et al., 2011; Spigel et al., 2013). There are limited preclinical studies analyzing different MET inhibitors in specific genetic subgroups of NSCLC. Furthermore, MET amplification has not been commonly used for the stratification when evaluating the efficacy for the different MET inhibitors.

We aimed to evaluate the efficacy of tivantinib and non-selective MET inhibitors in a panel of NSCLC cell lines selected on the basis of their MET dependency as well as those with EGFR and KRAS mutations or ALK translocation representing the genomic diversity of NSCLC.

2. **Materials and methods**

2.1. **Cell cultures and reagents**

The NSCLC cell lines A549, H3122, the EGFR mutant NSCLC cell lines PC9 (Del E746_A750), PC9 GR4 (Del E746_A750/T790M), HCC827 and HCC827 GR6 and the MET amplified cell lines H1993, EBC-1 were grown in RPMI 1640 supplemented with 10% FBS and 1× Antibiotic-Antimycotic (Invitrogen). Tivantinib (ARQ 197) was obtained from Active Biochem, gefitinib (ZD1839), crizotinib (PF2341066) and PHA-665752 were obtained from Selleck Chemicals and paclitaxel and nocodazole were purchased from Sigma–Aldrich. Stock solutions of all drugs were prepared in dimethyl sulfoxide (DMSO) and stored at –20 °C.

2.2. **Cell proliferation assays**

Cell proliferation and growth assays were performed with the MTS assay as described before (Zhou et al., 2009). All experimental points were a result of six to twelve replications, and all experiments were repeated at least three times. The data was graphically displayed using GraphPad Prism 5 for Windows (GraphPad Software). Each point (mean ( ± standard deviation)) represents growth of treated cells compared to untreated cells. The curves were fitted using a non-linear regression model with a sigmoidal dose response.

2.3. **Antibodies and Western blotting**

Cells grown under the previously specified conditions were lysed in NP-40 buffer. Western blot analyses were conducted after separation by SDS-PAGE electrophoresis and transfer to polyvinylidene difluoride Immobilon-P membranes (Millipore). Immunoblotting was performed according to the antibody manufacturers’ recommendations. Anti-phospho-MET (Tyr1234/1235), total MET, phospho-AKT (Ser473), total-AKT, total-EGFR and anti-α tubulin antibodies were obtained from Cell Signaling Technology. The phospho-EGFR (pY1068), total-ERK1/2, and phospho-ERK1/2 (pT185/pY187) antibodies were purchased from Invitrogen.

2.4. **Cell cycle analysis**

A total of 1 × 10^6 cells treated by triplicate with each of the compound at indicated concentration or DMSO were washed in PBS, fixed in 70% ethanol, incubated with RNAse in PBS and resuspended in 500 μl propidium iodide (PI). The percentage of cells in G1, S-phase and G2/M-phases were analyzed on a FACSCanto machine (Becton Dickinson). Apoptosis was analyzed by flow cytometry using fluorescent conjugates of annexin-V (BioVision). Viable cells are annexin-V and PI negative, and cell death is expressed as a percent of viable cells.

2.5. **Immunofluorescence**

Cells used in immunofluorescence experiments were plated on poly-D-Lysine-coated 12-mm coverslips. Cells were treated at indicated concentration with tivantinib, crizotinib, nocodazole or DMSO for 4 h. Cells were fixed with 4%
paraformaldehyde in PBS at room temperature for 20 min. Following PBS wash, cells were permeabilized with 0.1% Triton-X in PBS (PBST). Cells were then blocked with 5% BSA in 0.1% PBST for 30 min, followed by incubation with Anti-α-tubulin-FITC antibody (Sigma, cat# F2168) for 1 h at room temperature. Coverslips were washed with 0.1% PBST, stained with PBS containing Hoechst 33342 (Life Technologies, cat# H1399), washed, and mounted using ProLong Gold Antifade with PBS containing Hoechst 33342 (Life Technologies, cat# P36935). Images were analyzed on a Nikon Ti motorized inverted microscope with a Perfect Focus System. Widefield images were acquired with a cooled CCD camera (Hamamatsu ORCA-R2) and Metamorph software.

2.6. Microtubule polymerization assay

Microtubule polymerization assays were conducted using the Cytoskeleton Inc. fluorescence-based tubulin polymerization assay kit (Cytoskeleton, Inc., cat# BK011P) according to the manufacturer’s protocol. In brief, 5 μL of 10× concentrations of tivantinib, crizotinib, DMSO or the controls paclitaxel (for tubulin polymerization) and nocodazole (for tubulin depolymerization) were added to a pre-warmed half-area 96-well plate (Corning Costar, cat# 3686). Fifty (50) μL of Tubulin Reaction Mix were added and the plate was read immediately using an Envision plate reader and standard settings. All the experiments were performed in triplicate.

2.7. Statistical analysis

Statistical analyses were performed using Student’s t-test. A p-value < 0.05 was considered statistically significant.

3. Results

We aimed to compare the efficacy of different small molecule TKIs using a small panel of NSCLC cell lines selected on the basis of their MET dependency. We first selected a panel of MET-independent NSCLC cell lines. The EGFR mutant cell lines PC9 and HCC827 harbor the same exon 19 deletion (Del E746_A750) conferring high sensitivity to gefitinib and they have been previously characterized (Ercan et al., 2010; Zhou et al., 2009). PC9 GR4 (Del E746_A750/T790M) is a gefitinib-resistant version of PC9 that contains the EGFR T790M resistance mutation and has been previously described (Zhou et al., 2009). A549 is a KRAS mutant (G12S) cell line, and H3122 harbors an ALK translocation (EML4-ALK variant 1 (E13; A20)). We exposed these cell lines to three different MET inhibitors: tivantinib (ARQ 197; Daiichi-Sankyo) originally described as a highly selective, non-ATP c-Met inhibitor (Adjei et al., 2011; Eathirsaj et al., 2011; Munshi et al., 2010; Yap et al., 2011); PHA-665752 (Pfizer) is a selective ATP competitive small molecule inhibitor of c-Met (Christensen et al., 2003; Smolen et al., 2006); crizotinib (PF2341066; Pfizer) is an ATP competitive, MET and ALK inhibitor already clinically approved for the treatment of ALK translocated tumors (Camidge et al., 2012; Kwak et al., 2010).

The MET-independent cell lines showed resistance to both PHA-665752 and crizotinib (Figure 1A and B). The only exception was cell line H3122 that was sensitive to crizotinib (IC50 of 0.2 μM) but this cell line is known to harbor an ALK translocation that confers sensitivity to crizotinib. Remarkably, all the cell lines tested showed some degree of sensitivity to tivantinib, with IC50s ranging between 0.36 and 0.8 μM (Figure 1B).

We next evaluated the inhibition of activity on MET and both MAPK and PI3K signaling in the A549 and PC9 cells. The phosphorylated levels of c-MET did not vary in any of the treated cells with any of the tested drugs. No changes in the MET downstream signaling were noted in the phosphorylated levels of extracellular signal-regulated kinases (ERK) 1 and 2 or AKT (Figure 1C and D). Total MET levels remained stable in treated cells in both MET activated or not activated cells. A549 cells have a constitutive active MET but no changes in either total MET or phosphorylated levels were detected when treated with tivantinib, crizotinib or PHA-668752. The phosphorylation status of downstream effectors such as AKT or ERK1/2 remained high despite treatment (Figure 1C). In contrast to A549 cells, PC9 cells showed no constitutive phosphorylation of MET. As in A549 cells, no changes in AKT or ERK1/2 phosphorylation were detected in PC9 cells when treated with tivantinib, crizotinib or PHA-668752 (Figure 1D). These data suggest that the anti-proliferative activity of tivantinib in MET-independent cell lines is not mediated through the inhibition of MET signaling.

We next aimed to examine the effects of the selected MET inhibitors against a panel of MET-dependent NSCLC cell lines. Both H1993 and EBC-1 cell lines harbor MET amplification with MET overexpression and constitutive phosphorylation with a remarkable MET dependency, as previously described (Lutterbach et al., 2007). HCC827 GR6 is a gefitinib-resistant version of HCC827, harboring the same EGFR exon 19 deletion as the parental HCC827 but with MET amplification as a mechanism of acquired resistance to gefitinib (Engelman et al., 2007). Both H1993 and EBC-1 cells were sensitive to tivantinib, crizotinib or PHA-665752. However, the IC50 for tivantinib was 1 log higher than for crizotinib or PHA-665752 (Figure 2A and B). Interestingly, the growth inhibition for tivantinib was in the same range as was observed for the MET-independent cells. We next evaluated the effect of the three drugs on the phosphorylation status of MET, ERK1/2 and AKT in these cells. Crizotinib and PHA-665752 but not tivantinib effectively inhibited MET phosphorylation in EBC-1 cell line (Figure 2C). Moreover, only crizotinib and PHA-665752 led to complete inhibition of both AKT and ERK1/2 phosphorylation whereas no inhibition of any of these pathways was seen with tivantinib. The same results were obtained on H1993 (data not shown). Consistent with the results obtained in the MET-independent cells, tivantinib activity is also not driven by MET inhibition in the MET-dependent cell lines.

In contrast to H1993 and EBC-1 cells, HCC827 GR6 cells showed sensitivity to tivantinib but resistance to crizotinib and PHA-665752 alone (Figure 2A and B). HCC827 GR6 exhibit a co-dependency on both EGFR and MET and concurrent treatments with both an EGFR and MET inhibitor are required to suppress their growth (Engelman et al., 2007; Turke et al., 2010). MET phosphorylation decreased with increasing PHA-665752 concentration (Figure 2D). This inhibition of MET was coupled with partial inhibition of ERK1/2 phosphorylation and no inhibition of AKT phosphorylation. Despite the ability of tivantinib to inhibit the growth of HCC827 GR6 cells, there...
was no inhibition of MET phosphorylation or downstream effectors such as AKT or ERK1/2 (Figure 2D). We wanted to further evaluate the effects of a combination treatment on HCC827 GR6 cells. The cells were resistant to single-agent treatment with gefitinib, PHA-665752 or crizotinib whereas the combination of gefitinib with either PHA-665752 or crizotinib restored the sensitivity (Figure 3A). The sensitivity-shift effect seen with the combination was less apparent in the tivantinib treated cells, where the addition of gefitinib only added slightly enhanced sensitivity to tivantinib (Figure 3A). Gefitinib alone inhibited EGFR phosphorylation but was decoupled from inhibition of downstream signaling. As we previously observed for PHA-665752, single agent crizotinib resulted in partial inhibition of MET phosphorylation and ERK1/2 phosphorylation and no inhibition of AKT phosphorylation (Figure 3B). Only the combination of crizotinib and gefitinib resulted in complete inhibition of EGFR, MET, and ERK1/2 phosphorylation with almost complete inhibition of AKT phosphorylation (Figure 3B). These results were not observed with tivantinib combined to gefitinib, obtaining no substantial changes in MET, EGFR, AKT, and ERK1/2 phosphorylation compared to gefitinib alone. These findings provide further evidence that the anti-proliferative activity of tivantinib is not correlated with MET dependency and collectively suggest

Figure 1 — MET-independent cell lines are resistant to PHA-665752 and crizotinib but sensitive to tivantinib. (A) PC9, PC9 GR4, HCC827, A549 and H3122 cells treated with increasing concentrations of tivantinib (upper left panel), crizotinib (upper right panel) or PHA-665752 (lower left panel). Cell viability relative to untreated controls measured after 72 h. Each data point represents the mean ± SD of 6 wells. (B) Table summarizing the mean IC50 (µM) of each experiment showed in A. (C) A549 cells were treated for 6 h with increasing concentrations of tivantinib, crizotinib or PHA-665752. Cell lysates were immunoblotted to detect indicated proteins. (D) PC9 cells were treated for 6 h with increasing concentrations of tivantinib, crizotinib or PHA-665752. Cell lysates were immunoblotted to detect indicated proteins.
that its main mechanism of action is independent of MET inhibition.

We further investigated of the cellular effects of tivantinib in order to gain insights into possible mechanisms of action other than inhibition of MET. For this purpose we compared the effect on cell cycle and apoptosis induced by crizotinib and tivantinib in A549 and EBC-1 cell lines as an example of MET-independent and dependent cell lines, respectively. Both cell lines were treated with tivantinib or crizotinib for 24 and 48 h and subsequently analyzed by FACS. As expected for crizotinib no significant changes on cell cycle phases or apoptosis were seen in the resistant A549 cells (Figure 4). In contrast, crizotinib induced a G₁ arrest and apoptosis in the sensitive EBC-1 cells as expected for its known mechanism of action (Lutterbach et al., 2007). These results were in contrast to those observed with tivantinib where apoptosis was increased in both MET-dependent and independent cells and was coincident with the increase of G₂/M phase arrest in both cells (Figure 4). We were surprised to observe a G₂/M arrest because typically treatment with a TKI results in a G₁ phase arrest. The profile of G₂/M phase arrest has been described for G₂/M checkpoint modulators like cyclin-dependent kinase (CDK) inhibitors or as secondary
consequence of interfering with microtubule dynamics as is observed for drugs that induce tubulin polymerization (paclitaxel) or that induce depolymerization (vinca alkaloids) (DiPaola, 2002).

To test the effect of tivantinib on microtubule dynamics, A549 and EBC-1 cells were treated with increasing doses of tivantinib, crizotinib, nocodazole or DMSO for 4 h and examined for spindle morphology by immunofluorescence microscopy. In mitotic cells, microtubule disruption following treatment with nocodazole, a known microtubule depolymerizer, results in the formation of monopolar spindles. Similarly, treatment with 1 μM tivantinib increased the frequency of monopolar spindle figures in both EBC-1 and A549 cells. At lower doses of tivantinib (500 nM for A549 and 100 nM for EBC-1) bipolar spindles with unaligned chromosomes were also observed, a finding often seen at lower doses of microtubule polymerizers and depolymerizers (Jordan et al., 1991). In contrast, the percentage of normal bipolar mitotic cells did not change upon crizotinib treatment compared to the DMSO control (Figure 5A and B). We next assessed whether tivantinib could directly affect microtubule stability using a purified microtubule polymerization assay. Microtubules were incubated at 37 °C for 1 h in the presence of experimental compounds in conditions permissive to tubulin polymerization, as monitored by the increased fluorescence of a reporter molecule resulting from its incorporation into the polymerized microtubules. As controls, we used paclitaxel as a polymerization enhancer and nocodazole as a depolymerizer. Tubulin was depolymerized in a dose dependent manner with tivantinib whereas increasing doses of crizotinib had no effect on tubulin stabilization (Figure 5C). These results provide evidence that tivantinib directly affects tubulin polymerization.

4. Discussion

We demonstrate the activity of tivantinib, PHA-665752 and crizotinib as examples of specific and non-specific MET TKIs in a panel of NSCLC cells with different genotypes stratified by their MET dependency. Collectively our results demonstrate that tivantinib’s activity is indiscriminate regardless of MET dependency and therefore the mechanism of its activity is unlikely to involve MET inhibition. Furthermore, the complete abrogation of both MAPK and AKT/PI3K/mTOR pathways has been seen as necessary for the complete efficacy of a MET inhibitor in MET dependent cell lines (Lutterbach et al., 2007; Matsubara et al., 2010; Smolen et al., 2006; Tanizaki et al., 2011). Such effects were not observed for tivantinib despite its ability to inhibit the growth of the selected cells. The same pattern of lack of downstream inhibition for tivantinib was observed in the panel of non-MET dependent cell lines that were sensitive to tivantinib including HCC827, A549, H3122, PC9 and PC9 GR4. The indiscriminate efficacy of the drug irrespectively to MET dependency status and the absence of relation with MET inhibition signaling suggest an alternative mechanism of action for tivantinib.

Tivantinib is being currently evaluated in the clinic as a specific MET inhibitor in different tumor types (Fasolo et al., 2013;
Initially described as a specific allosteric MET inhibitor, one of the distinctive features of tivantinib was that it preferentially stabilizes an inactive form of c-MET in a non-ATP competitive way. Tivantinib was described to bind to a hydrophobic pocket in the ATP-binding cleft which prevents MET autophosphorylation, the initial step for the complete activation of the kinase (Eathiraj et al., 2011). The selectivity of the drug was assessed with both in silico and enzymatic assays. The latest included a panel of 230 human kinases where no inhibitory effects were detected against the majority of the kinases tested, including RON, which belongs to the same kinase family as MET (Eathiraj et al., 2011; Munshi et al., 2010). While this approach is routinely used to profile kinase inhibitor selectivity, it is incapable of detecting non-kinase targets. Moreover, initial in vitro experiments validating tivantinib analyzed its efficacy in a panel of 12 human cancer cell lines (Munshi et al., 2010). Although tivantinib’s cytotoxicity was related to apoptosis in the sensitive cells in the original work of Munshi et al. (Munshi et al., 2010) no insights on the cell cycle pattern arrest were performed. We demonstrated that this is due to an unexpected increase in the G2/M phase of the cell cycle and has been recently described by others (Basilico et al., 2013; Katayama et al., 2013). G2/M phase arrest is not usually found with the use of TKIs which instead usually result in a G1 phase arrest. The growth inhibition and apoptosis in sensitive cells treated with crizotinib were related to G1-S cycle arrest showing the same profile as previously reported for shRNA-mediated MET knockdown in these cells (Lutterbach et al., 2007). The G2 checkpoint allows the cell to repair DNA damage before entering mitosis and the profile of G2/M phase arrest has been previously described for G2/M checkpoint modulators like CDK inhibitors (Schwartz and Shah, 2005) and can be also a secondary effect on mitosis by agents affecting the mitotic spindle (Bhalla, 2003; DiPaola, 2002). Agents such as paclitaxel, which stabilize microtubule polymerization, interfere with the normal microtubule dynamics during cell division and induce mitotic arrest beyond the late G2 checkpoint by modulating G2/M checkpoint proteins that are required for entry into mitosis (Bhalla, 2003; DiPaola, 2002).
demonstrated that in contrast to crizotinib, which has no effect on microtubule dynamics, tivantinib was able to disrupt the mitotic spindle at biologically relevant doses leading to unaligned chromosomes and the formation of monopolar spindles in a dose dependent fashion. These are common findings observed for anti-microtubule agents (Jordan et al., 1991). Moreover, we found that tivantinib was acting as a microtubule depolymerizer by a similar mechanism to vinca alkaloids - that include agents such vincristine, vinblastine and vinorelbine (Jordan et al., 1991). These observations provide an alternative explanation for the mechanism of activity of tivantinib off-target of MET inhibition.

While our work was in progress, two other groups reported new insights into the cytotoxic mechanism of tivantinib independent of MET inhibition (Basilico et al., 2013; Katayama et al., 2013). Consistent with our results, both studies demonstrated that tivantinib can induce a G2/M cell cycle arrest and also focused on α-tubulin as the possible target for tivantinib for disrupting the microtubule dynamics. However the final mechanism remained unclear and led to seemingly contradictory results. These differences could be the result of the different approaches used. Basilico et al. described tivantinib as a microtubule stabilizer independent of MET, like paclitaxel. They used confocal immunofluorescence to analyze α-tubulin in both wild-type and MET exon 16 KO DLD-1 human colon carcinoma cells after 36 h incubation with tivantinib or controls. In contrast, Katayama et al. described tivantinib as a microtubule disruptor like vincristine by a similar approach but using instead A549 and EBC-1 lung cancer cells and incubating with tivantinib or control for shorter periods of time (2 or 16 h, instead of 36 h for Basilico et al.). In our work we analyzed the effects on microtubule dynamics by two different approaches. First, we examined A459 and EBC-1 cells for spindle morphology after treated for 4 h with selected compounds at biologically relevant doses, showing monopolar spindles upon disruption of microtubule dynamics and bipolar spindles with unaligned chromosomes at lower doses than 1 μM. Second, using the same in vitro polymerization assay than Katayama et al., we also demonstrated that tivantinib inhibited tubulin polymerization in a dose

Figure 5 — Immunofluorescence analyses showing the effect of tivantinib on microtubule dynamics. (A) Normal bipolar spindle formation is disrupted by tivantinib but not crizotinib. A549 cells were treated for 4 h with increasing doses of specified compounds then fixed and stained for tubulin (red) and DNA (blue). Tivantinib led to monopolar spindle formation at higher doses and to bipolar spindles with unaligned chromosomes at lower doses. Crizotinib did not show any effect on mitotic spindles. Nocodazole, a microtubule depolymerizer, and DMSO were used as controls. Similar results were obtained for EBC-1 cells (data not shown). Scale bar represents 10 μm. (B) A549 and EBC-1 cells were examined for spindle morphology and quantified by triplicate for number of normal bipolar mitotic cells. There is a dose dependent effect for tivantinib in both cell lines. At lower doses of tivantinib (500 nM for A549 and 100 nM for EBC-1) bipolar spindles with unaligned chromosomes were also observed. Noc, nocodazole; Crz, crizotinib; DMSO, dimethyl sulfoxide. (C) Microtubule polymerization assay comparing tivantinib versus crizotinib. Paclitaxel, a microtubule polymer stabilizer and nocodazole a microtubule depolymerizer, were used as positive and negative controls, respectively. All experiments were performed in triplicate.
dependent manner. The doses of selected drugs required in this assay were higher than those used for in vitro cellular studies and are a result of the high tubulin concentration required for the assay. The assay is also devoid of other proteins (such as microtubule associated proteins) that serve to stabilize or destabilize microtubules in cells, making microtubules more dynamic. The microtubules in this assay are less dynamic and therefore somewhat artificial and may distort the true intracellular concentration needed to destabilize microtubules. All together these results suggest a potential off-target activity of tivantinib not related to MET and increases the knowledge of the mechanisms of action of this drug. Interestingly, the ability to target microtubules by a kinase inhibitor is not unique to tivantinib and has also been reported for the PI3K inhibitor BKM120 (Brachmann et al., 2012).

Clinical activity of tivantinib has been observed in certain subset of NSCLC patients despite the phase 2 and 3 studies did not met the primary objectives in the overall population (Scaglotti et al., 2013; Sequist et al., 2011). Although limited by the small number of patients and the results came from a sub-group analysis, there was a significant difference in benefit for patients with KRAS mutations when tivantinib was added to erlotinib (Sequist et al., 2011). Given the findings by us and others, these clinical observations may be largely due to the cytotoxic effects of tivantinib and could also possibly be achieved with currently approved cytotoxic chemotherapy agents.

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Conflict of interest

P.A. Jänne is a consultant/advisory board member for Abbott, Astra-Zeneca, Boehringer Ingelheim, Chugai, Clovis, Genentech, Pfizer, and Sanofi. Dr. Jänne receives post-marketing royalties from Dana Farber Cancer Institute licensed intellectual property to Lab Corp. on epidermal growth factor receptors mutations. The rest of authors state there are no conflicts to disclose.

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