

## GSK3 Alpha and Beta Are New Functionally Relevant Targets of Tivantinib in Lung Cancer Cells

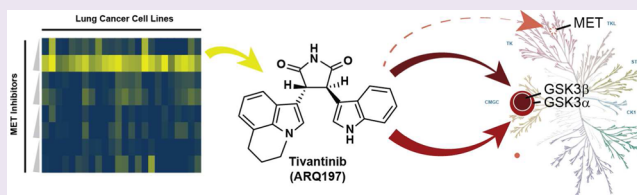
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### S Supporting Information

**ABSTRACT:** Tivantinib has been described as a potent and highly selective inhibitor of the receptor tyrosine kinase c-MET and is currently in advanced clinical development for several cancers including non-small cell lung cancer (NSCLC). However, recent studies suggest that tivantinib's anticancer properties are unrelated to c-MET inhibition. Consistently, in determining tivantinib's activity profile in a broad panel of NSCLC cell lines, we found that, in contrast to several more potent c-MET inhibitors, tivantinib reduces cell viability across most of these cell lines. Applying an unbiased, mass-spectrometry-based, chemical proteomics approach, we identified glycogen synthase kinase 3 (GSK3) alpha and beta as novel tivantinib targets. Subsequent validation showed that tivantinib displayed higher potency for GSK3 $\alpha$  than for GSK3 $\beta$  and that pharmacological inhibition or simultaneous siRNA-mediated loss of GSK3 $\alpha$  and GSK3 $\beta$  caused apoptosis. In summary, GSK3 $\alpha$  and GSK3 $\beta$  are new kinase targets of tivantinib that play an important role in its cellular mechanism-of-action in NSCLC.



The receptor tyrosine kinase c-MET is involved in cell migration and metastasis of various malignancies.<sup>1</sup> c-MET amplification and activity provides an important mechanism by which cancer cells develop resistance to targeted drugs, such as EGFR inhibitors in non-small cell lung cancer (NSCLC).<sup>2</sup> Thus, c-MET is an attractive therapeutic target, and several inhibitors are currently in clinical development. One of the most advanced c-MET inhibitors, tivantinib (ARQ197),<sup>3</sup> has progressed into phase III trials.<sup>4–6</sup> In phase II studies, tivantinib displayed clinical activity in NSCLC patients in combination with the EGFR inhibitor erlotinib, particularly in patients with *KRAS* mutations.<sup>5</sup> This was unexpected as the primary rationale for testing tivantinib in NSCLC was to prevent emergence of resistance to erlotinib due to compensatory c-MET signaling in patients with *EGFR* mutations, which are mutually exclusive with *KRAS* mutations.<sup>7</sup> Moreover, although described to be highly selective for c-MET, reportedly due to its unique ATP-independent binding mode,<sup>3,8</sup> tivantinib showed anticancer activity in various cell lines across diverse tumor types, many of which are not driven by c-MET signaling.<sup>3</sup> We therefore hypothesized that tivantinib inhibits a wider range of targets than appreciated and that some of these are functionally relevant for its activity. Further supporting this hypothesis, two recent studies suggest that tivantinib's anticancer activity in different tumor types may be related to modulation of microtubule dynamics rather than c-MET inhibition.<sup>9–11</sup> Here, we report tivantinib's antiproliferative activity in a large panel of lung cancer cell lines showing that tivantinib action in

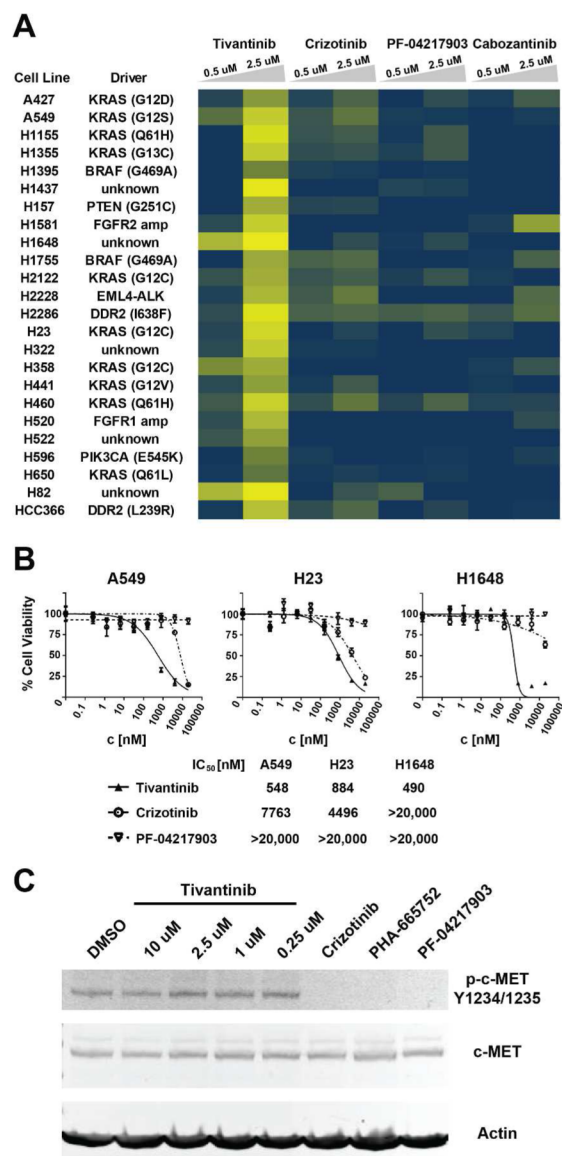
NSCLC is indeed independent of inhibition of c-MET activity, but furthermore also of *KRAS* status. Subsequent cellular target profiling by chemical proteomics identified glycogen synthase kinase (GSK) 3 $\alpha$  and GSK3 $\beta$  as novel tivantinib targets, both being more potently inhibited than c-MET. Loss of function studies suggest that inhibition of these kinases plays an important role for the antiproliferative activity of tivantinib in NSCLC cells.

To obtain a broader view of tivantinib's activity in lung cancer, we screened a panel of 24 *KRAS* mutant and wild-type lung cancer cell lines (Figure 1A). Tivantinib inhibited the viability of the majority of these, whereas crizotinib (PF-02341066), PF-04217903, and cabozantinib (XL-184), which are much more potent c-MET kinase inhibitors than tivantinib, had no significant effects. There was no obvious link between tivantinib sensitivity and *KRAS* mutation status. Determination of the IC<sub>50</sub> values for inhibition of cellular viability confirmed the differential activity of these compounds with tivantinib displaying an IC<sub>50</sub> of about 500 nM for the most sensitive NSCLC cell lines. In comparison, the highly selective c-MET inhibitor PF-04217903 and the less selective crizotinib had no measurable or only weak activity, respectively (Figure 1B). Confirming the functional integrity of these compounds,

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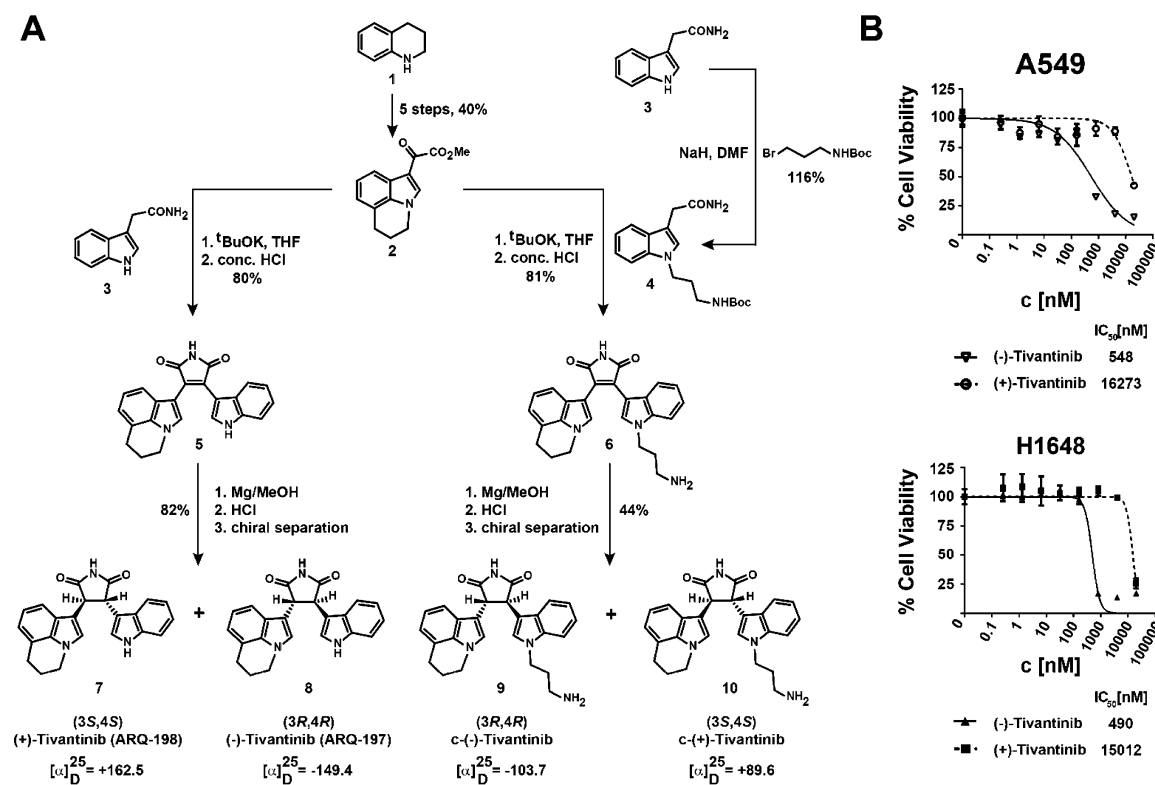


**Figure 1.** Cellular activity of tivantinib and various c-MET inhibitors in lung cancer cell lines. (A) Effects of tivantinib, crizotinib, PF-04217903, and cabozantinib at 0.5 and 2.5  $\mu$ M on cellular viability across the indicated panel of KRAS WT and mutant lung cancer cell lines. Relative cellular viability is displayed as a gradient from 0% (yellow) to 100% (blue) compared to DMSO control. (B) Dose–response curves and IC<sub>50</sub> values for inhibition of viability by tivantinib, crizotinib, and PF-04217903 in the A549 and H23 (both KRAS mutant) and the H1648 (KRAS-wild-type) NSCLC cell lines. (C) Effect on c-MET autophosphorylation in A549 cells following 30-min treatment at the indicated concentrations of tivantinib and 250 nM each of the c-MET inhibitors crizotinib, PHA-665752, and PF-04217903.

though, c-MET autophosphorylation in A549 cells was effectively inhibited by crizotinib, PF-04217903, and another widely used c-MET inhibitor, PHA-665752, whereas tivantinib showed essentially no effect (Figure 1C). Considering the reported maximum plasma concentration of 5–7  $\mu$ M from phase I clinical trials,<sup>12,13</sup> tivantinib's activity against several of these cell lines was well within physiologically relevant concentrations. In summary, tivantinib displayed potent activity against a broad panel of lung cancer cell lines, which is unrelated to inhibition of c-MET kinase activity and KRAS mutation status.

Tivantinib's *in vitro* inhibition profile was originally determined against a panel of 230 kinases, on the basis of which it was considered a selective c-MET inhibitor.<sup>3</sup> In light of our data, however, we hypothesized that one or more of the remaining almost 300 kinases in the human kinome could be previously unrecognized tivantinib targets responsible for the cellular activity in NSCLC cells. We therefore applied a mass spectrometry-based chemical proteomics strategy to characterize tivantinib's target profile in NSCLC cells in a proteome-wide and unbiased fashion.<sup>14</sup> To this end, we designed the tivantinib analogue c-(–)-tivantinib (**9**, Figure 2A) based on the reported cocrystal structure of tivantinib with c-MET, which suggests that the indole moiety is solvent-accessible.<sup>8</sup> According to our previous experience performing chemical proteomics with various kinase inhibitors, similar structure–activity relationships are likely maintained across the majority of targets.<sup>15,16</sup> c-(–)-tivantinib (**9**) was synthesized by modifying the published synthetic route to tivantinib (**8**) and its enantiomer (+)-3S,4S-tivantinib (**7**), which has been reported to be inactive against c-MET (Figure 2A and Supplementary Figure S1A).<sup>8,17</sup> Interestingly, **7** also showed substantially weaker activity in (–)-tivantinib-sensitive cell lines, making it an excellent control compound (Figure 2B). Separation of the racemic mixture resulting from reduction of the maleimide intermediate **6** by preparative chiral HPLC yielded the optically pure enantiomers c-(–)-tivantinib (**9**) and c-(+)-tivantinib (**10**), which retained differential cellular activity, albeit somewhat reduced possibly due to altered cell permeability (Supplementary Figure S1B). Chemical proteomics with A549 total cell lysates using analogues **9** and **10** (the latter as control) identified several protein kinases not previously implicated as tivantinib targets (Figure 3A, Supplementary Tables S1, S2). Applying stringent filters for reproducibility between replicates and enrichment by **9** over **10**, GSK3 $\alpha$  and GSK3 $\beta$  were found to be the highest confidence target candidates that interacted with tivantinib. Subsequent immunoblotting confirmed the interaction between c-(–)-tivantinib (**9**) and GSK3 $\alpha$  and GSK3 $\beta$  and suggested selectivity of (–)-tivantinib over both (+)-tivantinib and blocked binding to the matrix) for these kinases (Figure 3B). Furthermore, competition with the potent ATP-competitive pan-GSK3 inhibitor BIO suggested that tivantinib interacts directly with GSK3 $\alpha$  and GSK3 $\beta$  by binding to their ATP-binding pockets. In comparison, c-MET was only weakly detectable by LC–MS/MS analysis in A549 cells and did not pass our stringent filtering criteria. Furthermore, while a more sensitive targeted proteomics analysis using multiple reaction monitoring (MRM) with stable isotope-labeled standard (SIS) peptides also detected c-MET as a tivantinib binder, absolute quantification demonstrated that GSK3 $\alpha$  and GSK3 $\beta$  were much more strongly enriched than c-MET (Figure 3C). All three kinases were selectively recovered by c-(–)-tivantinib (**9**) over c-(+)-tivantinib (**10**) (Figure 3D). Chemical proteomics using H1648 cells, which are known to overexpress c-MET, again prominently enriched for GSK3 $\alpha$  and GSK3 $\beta$ , but now also for c-MET (Supplementary Figure S2, Supplementary Tables S3, S4). Consistent with *in vitro* kinase assays (Supplementary Figure S3), these results suggest that c-MET is only a weak tivantinib target. Instead, our data demonstrate that tivantinib is prominently binding to GSK3 $\alpha$  and GSK3 $\beta$ .

To evaluate the functional consequences of tivantinib binding to GSK3 $\alpha$  and GSK3 $\beta$ , enzyme inhibition was determined by

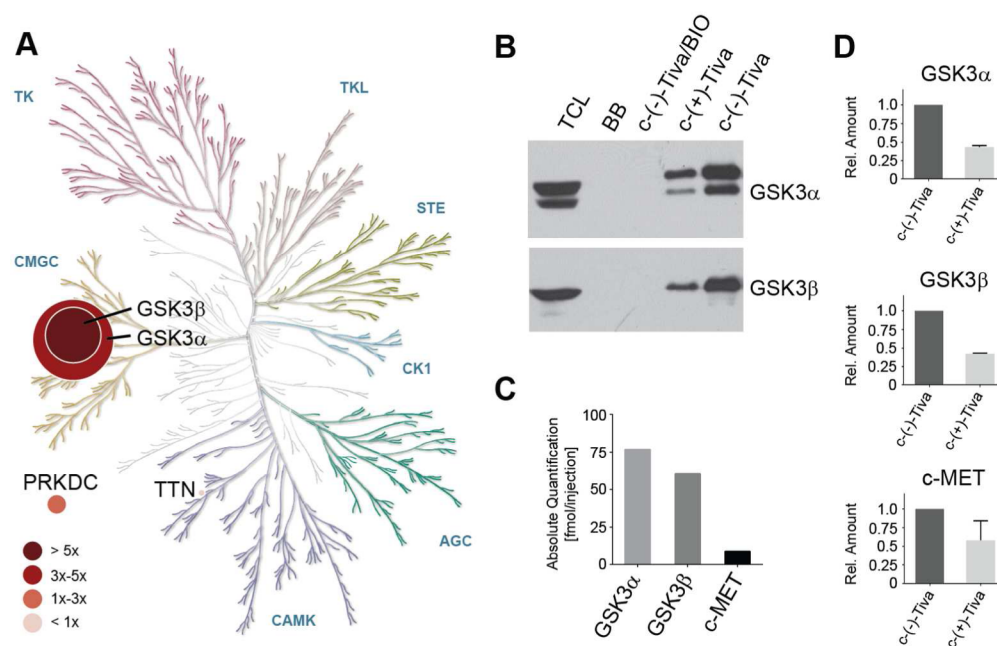


**Figure 2.** Synthesis of tivatinib enantiomers and analogues. (A) Synthetic strategy and chemical structures of (-)-tivatinib (8), its enantiomer (+)-tivatinib (7), and their coupleable analogues 9 and 10, respectively. For the full synthetic route see Supplementary Figure S1A. (B) Differential inhibition of cellular viability of A549 and H1648 cells by (-)-tivatinib and (+)-tivatinib.

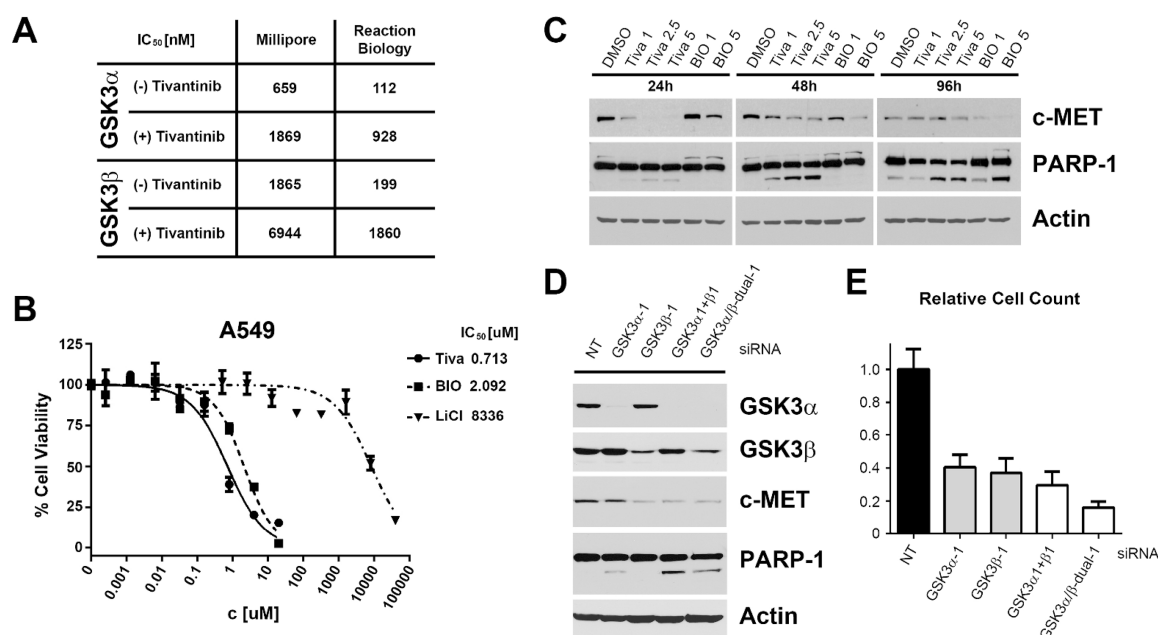
*in vitro* kinase assays. These experiments demonstrated that (-)-tivatinib potently inhibits GSK3 $\alpha$  and GSK3 $\beta$  with IC<sub>50</sub> values in the upper nanomolar range, whereas (+)-tivatinib is a significantly weaker inhibitor of both kinases (Figure 4A). Thus, GSK3 $\alpha$  and GSK3 $\beta$  are more potently inhibited than c-MET by (-)-tivatinib, and the differential activity between the tivatinib enantiomers is supported by the difference observed in their cellular activities. Also consistent with GSK3 inhibition, an increase in total  $\beta$ -catenin levels, which is degraded upon phosphorylation by GSK3,<sup>18</sup> was detected following tivatinib treatment of A549 cells (Supplementary Figure S4). Notably, in contrast to the vast majority of GSK3 inhibitors, which either affect GSK3 $\alpha$  and GSK3 $\beta$  with equal potency or display selectivity for GSK3 $\beta$ ,<sup>19</sup> tivatinib inhibits GSK3 $\alpha$  2- to 3-fold more strongly than GSK3 $\beta$ . Although recently there has been significant progress toward GSK3 $\alpha$ -specific inhibitors,<sup>20,21</sup> tivatinib constitutes to the best of our knowledge the first GSK3 inhibitor in clinical development that has selectivity for GSK3 $\alpha$ . Furthermore, although other targets with different structure–activity relationships cannot be completely ruled out, tivatinib appears to be much more selective for GSK3 $\alpha$  and GSK3 $\beta$  on a kinome-wide level than established GSK3 inhibitors. This relative GSK3 $\alpha$  vs GSK3 $\beta$  specificity, as well as the kinome-wide target selectivity, may harbor novel therapeutic opportunities for tivatinib in malignant diseases, such as acute myeloid leukemia or pancreatic cancer, in which GSK3 $\alpha$  has recently been described to be a promising new target.<sup>22,23</sup> Furthermore, GSK3 inhibitors may have utility in Alzheimer's disease, diabetes, and bipolar disorder.<sup>24,25</sup> Considering the narrow therapeutic index of the FDA-approved GSK3 inhibitor LiCl, the clinical safety and remarkable target selectivity of tivatinib may offer an interesting alternative.

In the context of cancer, GSK3 is generally considered a tumor suppressor, and thus inhibition of GSK3 by tivatinib as a potential mechanism-of-action in NSCLC is initially counter-intuitive. However, in addition to the aforementioned studies, several recent reports propose GSK3 to be a promising anticancer target, e.g., in glioblastoma multiforme and MLL-rearranged leukemia,<sup>26,27</sup> suggesting that GSK3 function is context-dependent. Consistently, tivatinib also potently inhibited viability of MV-4–11 and RS4;11 MLL-rearranged leukemia cells, stabilized  $\beta$ -catenin, and induced G1 cell cycle arrest, as has been described previously for GSK3 inhibitors in these cells (Supplementary Figure S5).<sup>27</sup> To evaluate the functional relevance of GSK3 inhibition by tivatinib in NSCLC cells, we compared tivatinib activity with the known GSK3 inhibitors BIO and LiCl (Figure 4B). Both compounds showed antiproliferative activity in A549 cells. Although initially it may seem high, the low millimolar activity of LiCl is in excellent agreement with GSK3-dependent growth inhibition in leukemia (Supplementary Figure S5A).<sup>22,27</sup> Like tivatinib, BIO was able to induce apoptosis in A549 cells, albeit slightly delayed, as indicated by PARP-1 cleavage (Figure 4C). Curiously, we also noted decreased c-MET levels upon treatment with either compound. Regulation of c-MET levels may offer an explanation for why tivatinib has clinical activity in tumors driven by mutant or overexpressed c-MET. Interestingly, siRNA-mediated knockdown of GSK3 $\alpha$  and GSK3 $\beta$  suggest that these kinases have nonredundant functions in A549 cells (Figure 4D). Loss of GSK3 $\beta$  was primarily responsible for reducing c-MET levels, whereas GSK3 $\alpha$  knockdown had a stronger effect on PARP-1 cleavage and closely resembled tivatinib's characteristic cell-rounding phenotype, which is possibly due to the observed G2/M cell cycle arrest elicited by





**Figure 3.** Determination of tivantinib's kinase target interaction profile in A549 cells by chemical proteomics. (A) Phylogenetic tree of the human protein kinome displaying targets identified by affinity chromatography experiments with coupled tivantinib analogues in A549 cells. Node size indicates the average ratio of the number of unique spectra to the molecular weight of the proteins identified with the c(-)-tivantinib affinity matrix. Node color corresponds to the ratio of the number of unique spectra identified by c(-)-tivantinib to c(+)-tivantinib affinity chromatography experiments. Displayed are all protein kinases that have been observed in both biological replicates of the (-)-tivantinib affinity purifications with more than two unique spectra across both replicates. (B) Immunoblot analysis of GSK3 $\alpha$  and GSK3 $\beta$  purification by c(-)-tivantinib and c(+)-tivantinib affinity chromatography. Competition experiments were conducted by 2-h treatment of A549 total cell lysate with the pan-GSK3 inhibitor BIO prior to affinity chromatography. (C) Absolute quantification of GSK3 $\alpha$ , GSK3 $\beta$ , and c-MET peptides identified by LC-MS/MS analysis of c(-)-tivantinib affinity purifications using LC-MRM with stable isotope labeled peptides. (D) Relative abundance ratios of GSK3 $\alpha$ , GSK3 $\beta$ , and c-MET peptides between c(-)-tivantinib and c(+)-tivantinib affinity purifications. The Human Kinome Map was adapted with permission from Cell Signaling Technology ([www.cellsignal.com](http://www.cellsignal.com)). TCL: total cell lysate; BB: blocked beads (no immobilized compound); Tiva: tivantinib.



**Figure 4.** Functional analysis of GSK3 $\alpha$  and GSK3 $\beta$  inhibition. (A)  $IC_{50}$  values for inhibition of GSK3 $\alpha$  and GSK3 $\beta$  *in vitro* kinase activity by (-)-tivantinib and (+)-tivantinib on Millipore and Reaction Biology assay platforms (mean values of independent experimental duplicate analyses). (B) Effect of tivantinib and the known pan-GSK3 inhibitors BIO and LiCl on cell viability of A549 NSCLC cells. (C) Effects of tivantinib and BIO (in  $\mu$ M) on A549 cells regarding PARP-1 cleavage and total c-MET levels. (D) Effects of individual or combined siRNA-mediated knockdowns of GSK3 $\alpha$  and GSK3 $\beta$  in A549 cells on PARP-1 cleavage and c-MET levels 72 h post-transfection. GSK3 $\alpha$ 1+ $\beta$ 1: combination of gene-specific siRNAs GSK3 $\alpha$ -1 and GSK3 $\beta$ -1; GSK3 $\alpha$ / $\beta$ -dual-1: single siRNA targeting both genes. NT: non-targeting siRNA. (E) Effects of individual or combined GSK3 $\alpha$  and GSK3 $\beta$  knockdowns on cell number of A549 cells 96 h post-transfection.

tivantinib (Supplementary Figures S6, S7).<sup>9,10</sup> The combination of GSK3 $\alpha$ - and GSK3 $\beta$ -specific siRNAs or using single siRNAs that cause simultaneous knockdown of both genes, however, was yet more effective in causing apoptosis (Figure 4D, Supplementary Figure S8A). Dual knockdown of GSK3 $\alpha$  and GSK3 $\beta$  furthermore caused a significant reduction of viability in A549 cells compared to non-targeting siRNA (Figure 4E, Supplementary Figure S8B), which is consistent with the essential functions of GSK3 $\alpha$  and GSK3 $\beta$  in many cells.

In summary, applying an unbiased, integrated chemical biology approach, we have identified GSK3 $\alpha$  and GSK3 $\beta$  as novel targets of the clinical kinase inhibitor tivantinib in NSCLC cells. Importantly, tivantinib is a more potent inhibitor of GSK3 $\alpha$  and GSK3 $\beta$  than of its intended target c-MET, which is only weakly inhibited. Furthermore, simultaneous loss of function of GSK3 $\alpha$  and GSK3 $\beta$  causes apoptosis in lung cancer cells suggesting that inhibition of these kinases rather than c-MET plays an important role in tivantinib's mechanism-of-action in NSCLC cells. Considering the large number of substrates and pathways that are modulated by GSK3, many of which also affect microtubules,<sup>18</sup> further global and unbiased studies are required to elucidate the exact downstream mechanism by which tivantinib impairs cell viability. We anticipate that this knowledge will provide the basis for identification of relevant biomarkers that will greatly benefit tivantinib's further clinical development, which is hampered by the erroneous assumption that tivantinib is a potent c-MET inhibitor. In addition, identification of GSK3 $\alpha/\beta$  as tivantinib targets may pave the way for novel clinical studies with tivantinib, for instance, in MLL-rearranged leukemia.

## METHODS

**Cell Culture and Reagents, Flow Cytometry, Immunoblotting, RNA Interference.** For details see Supplementary Methods.

**Viability Assays.** Cell viability assays were conducted using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). For details see Supplementary Methods.

**Chemical Synthesis.** (+)-Tivantinib (7) and (–)-tivantinib (8) were synthesized by the Moffitt Chemistry Core as described in the patent literature<sup>17</sup> followed by an adapted method for chiral separation. For details see Supplementary Methods.

**Kinase Assays.** *In vitro* kinase inhibition assays and IC<sub>50</sub> determinations were performed on the Reaction Biology Kinase Hotspot<sup>SM</sup> and Millipore KinaseProfiler<sup>TM</sup> platforms. For details see Supplementary Methods.

**Affinity Purification and Mass Spectrometry.** Affinity chromatography experiments with c-(–)-tivantinib (9) and c-(+)-tivantinib (10) were performed in duplicate essentially as described previously.<sup>28</sup> For details see Supplementary Methods.

## ASSOCIATED CONTENT

### Supporting Information

Additional data and methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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