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# Cabozantinib and Tivantinib, but Not INC280, Induce Antiproliferative and Antimigratory Effects in Human Neuroendocrine Tumor Cells in vitro: Evidence for 'Off-Target' Effects Not Mediated by c-Met Inhibition

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## Key Words

c-Met · INC280 · Cabozantinib · Tivantinib · Neuroendocrine tumor

## Abstract

**Background/Aims:** The hepatocyte growth factor/transmembrane tyrosine kinase receptor c-Met has been defined as a potential target in antitumoral treatment of various carcinomas. We aimed to investigate the direct effect of c-Met inhibition on neuroendocrine tumor cells in vitro. **Methods:** The effects of the multi-tyrosine kinase inhibitors cabozantinib and tivantinib and of the highly specific c-Met inhibitor INC280 were investigated in human pancreatic neuroendocrine BON1, bronchopulmonary NCI-H727 and midgut GOT1 cells in vitro. **Results:** INC280, cabozantinib and tivantinib inhibited c-Met phosphorylation, respectively. However, while equimolar concentrations (10  $\mu$ M) of cabozantinib and tivantinib inhibited cell viability and cell migration, INC280 had no inhibitory effect. Knockdown experiments with c-Met siRNA also did not demonstrate effects on cell viability. Cabozantinib and tivantinib caused a G2 arrest in neuroendocrine tumor cells. **Conclusions:** Our in vitro data suggest

that c-Met inhibition alone is not sufficient to exert direct antitumoral or antimigratory effects in neuroendocrine tumor cells. The multi-tyrosine kinase inhibitors cabozantinib and tivantinib show promising antitumoral and antimigratory effects in neuroendocrine tumor cells, which are most probably 'off-target' effects, not mediated by c-Met.

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## Introduction

Neuroendocrine tumors (NET) are a heterogeneous group of neoplasms mainly originating from the gastroenteropancreatic system and the lung [1]. Molecular targeted therapy of NETs with the mTOR inhibitor everolimus [2, 3] or the multi-tyrosine kinase inhibitor (multi-TKI) sunitinib (VEGFR, PDGFR, KIT) [4] is currently only approved for pancreatic NETs. There is still an unmet need for further medical therapies including novel targeted therapies [5, 6].

The hepatocyte growth factor (HGF) receptor is encoded by the proto-oncogene c-Met and is a transmembrane tyrosine kinase. The endogenous ligand of c-Met is

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HGF. The HGF/c-Met axis has been characterized as an important target in cancer therapy [7–10] as it mediates tumor cell growth, migration and metastasis. Extracellular anti-HGF antibodies, anti-Met antibodies, as well as ATP-competitive and non-ATP competitive MET inhibitors have been developed and are in clinical trial programs [7, 9]. Cabozantinib (XL-184) is an ATP-competitive multi-TKI (with activity against c-Met, VEGFR2, c-KIT, FLT3, RET and TIE2) that has recently been approved by the FDA and EMEA for the treatment of medullary thyroid carcinoma [11, 12]. Cabozantinib is also currently in phase 3 clinical trials for hepatocellular carcinoma (NCT01908426), prostate cancer (NCT01522443) and renal cell carcinoma (NCT01865747). A phase 2 clinical trial with cabozantinib in patients with NET is currently ongoing (NCT01466036). Tivantinib (ARQ-197) is a non-ATP-competitive c-MET kinase inhibitor [7]. Recently, ‘off-target’ effects of tivantinib have been reported, and antitumoral effects of tivantinib irrespective of c-Met inhibition have been found in vitro [13–16]. Tivantinib has positive phase 2 trial results [17, 18], and current phase 3 clinical trials for hepatocellular carcinoma (NCT02029157) and for NSCLC (NCT01377376) are ongoing. INC280 (c-Met) is an ATP-competitive c-MET kinase inhibitor [7], currently in phase 1/2 clinical trials in various cancer entities.

In preclinical pancreatic neuroendocrine tumor models, various multi-TKIs with combined anti-VEGF and anti-MET efficacy have shown enhanced angiogenesis inhibition, as well as suppression of tumor invasion and metastasis [19–21]. In the Rip-Tag2 mouse model [19], the multi-TKIs foretinib (XL880; with activity against c-Met, VEGFR2, PDGFR, c-KIT, FLT3, RON and TIE2) and cabozantinib (XL184; with activity against c-Met, VEGFR2, c-KIT, FLT3, RET and TIE2), demonstrated superior inhibition of angiogenesis, reduced tumor invasiveness and reduced metastasis in comparison to the multi-TKI XL999 (with activity against VEGFR2, PDGFR, c-KIT and FLT3). This higher antitumoral efficacy of XL880 and XL184 compared to XL999 might have been caused by their different activities against c-Met with IC<sub>50</sub>s of 0.4 and 1.3 versus 463 nM [19]. Inhibition of VEGF signaling by an anti-VEGF antibody or by sunitinib in the RIP-Tag2 mouse model reduced tumor growth but caused an increase in phospho-c-Met expression, paralleled by increased lymphatic metastasis. Invasion and metastasis were reduced by the c-Met inhibitors PF-04217903 (with activity against c-Met, ALK) and crizotinib (PF-02341066; with activity against c-Met and ALK) or cabozantinib (XL184; with activity against c-Met,

VEGFR2, c-KIT, FLT3, RET and TIE2) [20, 21]. Due to these findings, a role of c-Met as a potential target in neuroendocrine tumors has been suggested [19–21]. A phase 2 clinical trial with cabozantinib in patients with neuroendocrine tumors is currently ongoing (NCT01466036).

Despite these promising preclinical data for the multi-TKI cabozantinib in neuroendocrine tumors [19–21], a direct effect of c-MET inhibition on neuroendocrine tumor cell growth and migration seems not yet proven. The multi-TKI tivantinib has recently been reported in various cancer cells to exert its antitumoral efficacy not related to c-Met inhibition but by other ‘off-target’ effects [13–16]. Therefore, we performed a comparative in vitro study using equimolar concentrations of the highly specific c-Met inhibitor INC280 and the multi-TKIs cabozantinib and tivantinib in several human neuroendocrine tumor cell lines. We compared the efficacy of all three compounds on c-Met phosphorylation status, cell viability, cell cycle control, as well as cell migration. In addition, we performed siRNA experiments to knockdown c-MET expression in neuroendocrine tumor cells. Our study demonstrates that c-Met inhibition is not essential for the inhibition of cell growth and cell migration in the investigated neuroendocrine tumor cell models. The observed antiproliferative and antimigratory effects of cabozantinib and tivantinib seem to be mediated by ‘off-target’ effects other than c-Met.

## Materials and Methods

### Materials

Dulbecco’s modified Eagle medium – Nutrient Mixture F-12, 1:1 (DMEM/F12) – and penicillin/streptomycin were purchased from Gibco/Invitrogen (Karlsruhe, Germany), trypsin-EDTA (10×) from PAA Laboratories (Cölbe, Germany), phosphate-buffered saline (PBS) and RPMI medium (with L-glutamine, NaCO<sub>3</sub>) were from Sigma-Aldrich (St. Louis, Mo., USA), and fetal bovine serum (FBS) and amphotericin B were acquired from Biochrom (Berlin, Germany). INC280 was from Novartis (Basel, Switzerland). Cabozantinib and tivantinib were from Selleckchem (Houston, Tex., USA).

### Cell Cultures

All human neuroendocrine cell lines were received and cultured as recently described [22]. The human pancreatic neuroendocrine tumor cell line BON1 [23, 24] (kindly provided by Prof. R. Göke, Marburg, Germany) was grown in DMEM/F12 (1:1) supplemented with 10% FBS, 1% penicillin/streptomycin and 0.4% amphotericin B. The human midgut carcinoid GOT1 cells [25] (kindly provided by Prof. O. Nilsson, Sahlgrenska University Hospital Göteborg, Sweden) and human bronchopulmonary neuroendocrine NCI-H727 tumor cells [26, 27] (purchased from ATCC, Manassas, Va., USA) were cultured in RPMI medium supplement-

ed with 10% FBS, 1% penicillin/streptomycin and 0.4% amphotericin B. The cells were mycoplasma free and incubated at 37°C in 5% CO<sub>2</sub>/95% air.

#### Assessment of Cell Viability

BON1 and NCI-H727 cells were counted by an automated cell counter (Countess™, Invitrogen, Germany), seeded into 96-well plates at densities of 3,000 (BON1) and 4,000 (NCI-H727) cells per well and grown for 24 h in a complete medium containing serum/antibiotic. The next day, the cells were incubated with various concentrations of INC280, cabozantinib and tivantinib (1 nM to 10 μM) in 10% FBS medium (antibiotic free). After 24, 48 and 72 h, the metabolic activity was measured with 'Cell Titer 96 Aqueous One Solution' cell proliferation assay (Promega, Madison, Wis., USA) according to the manufacturer's instructions. The measurement was performed at 492 nm with an ELISA plate reader.

#### siRNA Transfection

The siRNA transfections were performed as described previously [28]. The siRNA oligonucleotide [ON-TARGETplus SMARTpool, Human MET (4233), Cat. No. 003156-00-0005] and the nontargeting siRNA (ON-TARGETplus Non-targeting Pool, Cat. No. D-001810-10-05) were purchased from Thermo Fisher Scientific (Schwerte, Germany). Cells were transfected in an antibiotic- and FBS-free medium using DharmafECT 2 (BON1) and Dharmafect 3 (NCI-H727) according to the manufacturer's instructions (Dharmacon, Lafayette, Colo., USA). Twenty-four hours after transfection, FBS was added for a final concentration of 10%.

#### Cell Cycle Analysis by FACS

Cell cycle distribution was analyzed using propidium iodide staining and flow cytometry (BD Accuri C6 Analysis). Cells were cultured in 6-well plates (4 × 10<sup>5</sup> BON1 cells/well and 5 × 10<sup>5</sup> NCI-H727 cells/well) for 24 h in complete medium. The next day, the medium was replaced with fresh 10% FBS medium and incubated with 10 μM INC280, cabozantinib and tivantinib. After 24, 48 and 72 h, cells were washed with PBS and treated with 300 μl trypsin at 37°C for 4 min. Cells were collected and centrifuged at 2,000 rpm for 5 min. After another wash cycle with PBS, the cells were centrifuged again. The pellets were resuspended in 350 μl propidium iodide. After 2 h, the samples were measured.

#### Cell Migration Assay

BON1 and NCI-H727 cells were seeded at densities of 120,000/140,000 cells/chamber in culture inserts (Ibidi, Munich, Germany). After 24 h, the inserts were removed, and the cells were treated with 100 nM and 10 μM of INC280, cabozantinib and tivantinib. Every 24 h, pictures of the gap between the two cell layers were taken [Zeiss, Axiovert 135 TV (microscope) and Zeiss, AxioCam MRm (camera)]. The assay was stopped after 72 h, and pictures were analyzed.

#### Protein Extraction and Western Blotting

For Western blot experiments, 4 × 10<sup>5</sup> cells (BON1) or 5 × 10<sup>5</sup> cells (NCI-H727) were seeded in 6-well plates and grown for 24 h in complete medium. After the medium was replaced by a fresh 10% FBS medium, the cells were incubated with several concentrations of INC280, cabozantinib and tivantinib (1 nM to 10 μM) for 2 and 24 h. The cells were placed on ice, washed twice with cold

PBS and lysed in 200 μl lysis buffer (M-PER® Mammalian Protein Extraction Reagent containing HALT™ protease and phosphatase inhibitor cocktail; Thermo Scientific, Rockford, Ill., USA). Lysates were centrifuged at 13,000 rpm for 10 min. The supernatants were adjusted to the same protein concentration (30–50 μg/50 μl; Roti-quant Universal, Carl Roth, Karlsruhe, Germany). Sodium dodecyl sulfate (SDS) sample buffer (0.25 mM Tris HCL, 40% glycerol, 2% SDS, 1% dithiothreitol, bromophenol blue, pH 8.8) was added, and the samples were boiled for 5 min and separated on an SDS polyacrylamide gel. Proteins were electrotransferred for 60 min onto PVDF membranes (Immobilone; Millipore, Eschborn, Germany) using a semi-dry Western-blot technique. After blocking in 2% nonfat dried milk, the membranes were incubated overnight in appropriate dilutions of antibodies against pMet (Tyr 1234/5; #3077), Met (#3127), pAkt (Ser 473; #4060), Akt (#2920), pERK (Thr202/Tyr204) 1/2 (#4370), pp70S6K (Thr389; #9234), p70S6K (#9202), p4EBP1 (Ser65; #9451), 4EBP1 (#9644), pGSK3 (Ser21/9; #9331), GSK3 (#9315), CDK4 (#12790), CDK6 (#13331), pChk1 (Ser345; #2341), Chk1 (#2360), cyclin B1 (#12231), cyclin D1 (#2978), cyclin D3 (#2936), PARP (#9542), PCNA (#2586), E-cadherin (#3195), N-cadherin (#4061), β-catenin (#8480), src (#2109), vimentin (#5741), ZO-1 (#8193), all from Cell Signaling (Danvers, Mass., USA), twist (sc-15393; Santa Cruz, Dallas, Tex., USA), HGF (701283; Novex-Life, Frankfurt, Germany), actin (A5441; Sigma, St. Louis, Mo., USA), Erk 1/2 (06-182; Merck-Millipore, Darmstadt, Germany). After washing with TBS, the membranes were incubated with a peroxidase-conjugated secondary antibody (1:25,000) for 2 h. The blots were washed and immersed in the chemiluminescent substrate SuperSignal West Dura (Thermo Scientific, Rockford, Ill., USA), and images were taken with an ECL Chemocam Imager (INTAS, Göttingen, Germany).

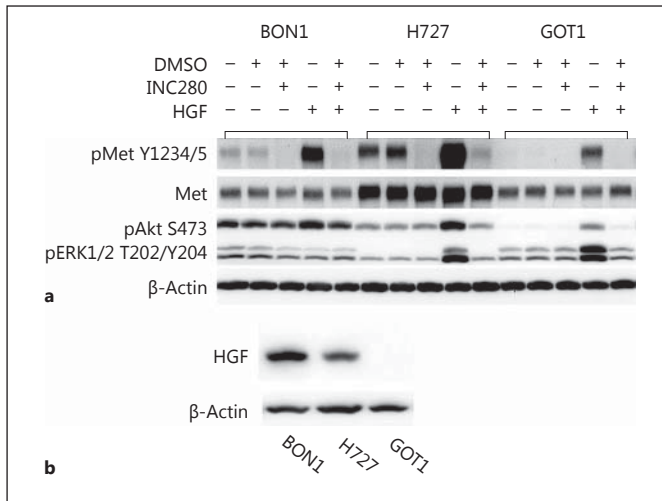
#### Statistical Analysis

For proliferation assays, comparisons were evaluated using two-tailed Student's t test. Results are expressed as mean ± SD of three or four independently performed experiments. Statistical significance was set at p < 0.05.

## Results

### Human Neuroendocrine Tumor Cells Express Functional c-Met and Its Endogenous Ligand HGF

Western blot analysis demonstrated the expression of c-Met in human pancreatic neuroendocrine BON1, bronchopulmonary NCI-H727 and midgut GOT1 tumor cells, respectively (fig. 1a). Expression of the endogenous c-Met ligand HGF was found in BON1 and H727 tumor cells, but not in GOT1 cells (fig. 1b). Incubation of BON1, H727 and GOT1 cells with recombinant human HGF (rhHGF) at a concentration of 1.25 nM for 10 min caused a significant induction of phospho-c-Met Y1234/5, while preincubation with the c-Met-inhibitor INC280 at a concentration of 100 nM for 2 h completely abolished the baseline and HGF-stimulated phospho-c-Met Y1234/5 (fig. 1a). In addition, rhHGF stimulated phosphorylation



**Fig. 1. a** The endogenous c-Met ligand HGF induces c-MET phosphorylation in neuroendocrine tumor cells, which is blocked by the specific c-Met inhibitor INC280. Human pancreatic neuroendocrine BON1, bronchopulmonary H727 and midgut GOT1 cells were incubated with HGF (1.25 nM) for 10 min. A preincubation with INC280 (100 nM) for 2 h was performed in the control group and the HGF treatment group. Subsequently, the expression of phospho-c-Met Y1234/5, c-Met, pAkt S473, pERK1/2 T202/Y204 and  $\beta$ -actin loading control was evaluated by Western blot analysis. A representative blot out of three independently performed experiments is shown. **b** Endogenous HGF expression by neuroendocrine tumor cells. Cell lysates of untreated BON1, NCI-H727 and GOT1 neuroendocrine tumor cells were harvested. Subsequently, the expression of HGF and  $\beta$ -actin loading control was evaluated by Western blot analysis. A representative blot out of three independently performed experiments is shown.

of Akt and ERK1/2, while preincubation with the c-Met-inhibitor INC280 at a concentration of 100 nM for 2 h prevented any stimulation (fig. 1a).

Thus, these data demonstrate the expression of a functional c-Met receptor on all three human neuroendocrine tumor cell lines investigated. However, the expression of the endogenous c-Met ligand HGF and expression of activated c-Met (phospho-c-Met Y1234/5) in untreated cells were only found in BON1 and H727 cells, while it could not be detected in GOT1 cells.

#### *Cabozantinib and Tivantinib Inhibit Cell Viability of Neuroendocrine Tumor Cells, while the Specific c-Met Inhibitor INC280 Has No Effect*

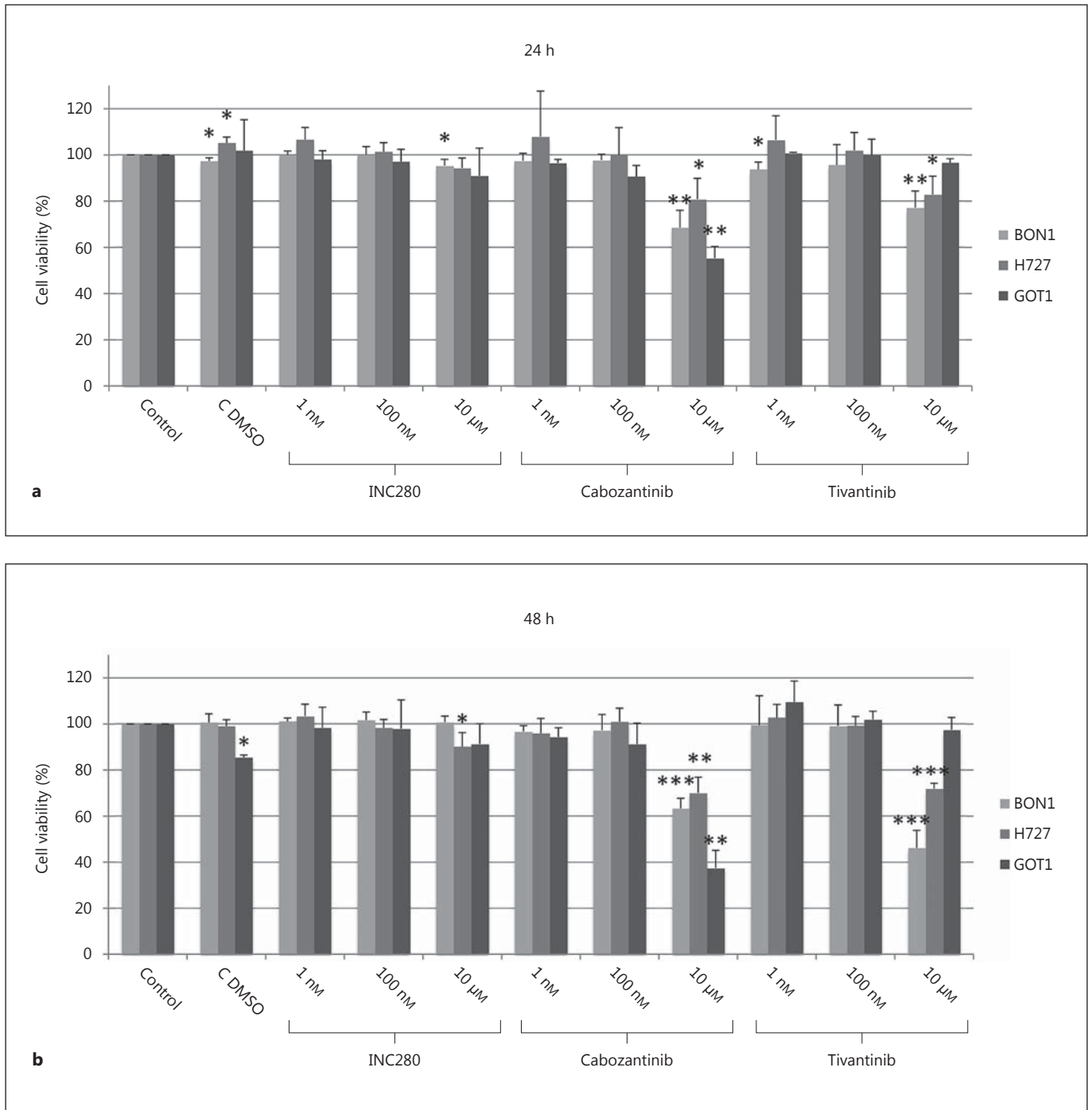
Human pancreatic neuroendocrine BON1, bronchopulmonary NCI-H727 and midgut GOT1 tumor cells were incubated with INC280, cabozantinib and tivantinib at a concentration range of 1 nM to 10  $\mu$ M, for 24 h (fig. 2a),

48 h (fig. 2b) and 72 h (fig. 2c), respectively. Cabozantinib and tivantinib at lower concentrations of 1 and 100 nM demonstrated no constant significant effects on cell viability of the three tumor cell lines. However, cabozantinib 10  $\mu$ M caused a significant decrease in cell viability in BON1, H727 and GOT1 cells, each at 24, 48 and 72 h, respectively (fig. 2). At 72 h, cabozantinib 10  $\mu$ M caused a decrease in cell viability in BON1 cells to  $51.1 \pm 2.3\%$  ( $p < 0.001$ ), H727 cells to  $59.3 \pm 2.1\%$  ( $p < 0.001$ ) and GOT1 cells to  $30.5 \pm 9.3\%$  ( $p < 0.01$ ), respectively. Also tivantinib 10  $\mu$ M caused a significant decrease in cell viability in BON1 and H727 cells at 24, 48 and 72 h, respectively (fig. 2), but had no effect on cell viability of GOT1 cells (fig. 2). At 72 h, tivantinib 10  $\mu$ M caused a decrease in cell viability in BON1 cells to  $28.1 \pm 5.2\%$  ( $p < 0.001$ ) and in H727 cells to  $65.9 \pm 7.4\%$  ( $p < 0.01$ ). In contrast, no significant effect of tivantinib 10  $\mu$ M was observed on cell viability of GOT1 cells [ $98.8 \pm 13.9\%$  (n.s.)]. In contrast, INC280 at all tested concentrations of 1 and 100 nM, and 10  $\mu$ M demonstrated no constant significant effects on cell viability of the three tumor cell lines (fig. 2). At 72 h, INC280 10  $\mu$ M caused only minimal changes in cell viability in BON1 cells to  $105.3 \pm 2.9\%$  ( $p < 0.05$ ), in H727 cells to  $91.0 \pm 4.5\%$  ( $p < 0.05$ ) and in GOT1 cells to  $99.1 \pm 3.9\%$  (n.s.), respectively.

Thus, these data demonstrate that cabozantinib and tivantinib at a concentration of 10  $\mu$ M potently inhibit cell viability in human neuroendocrine tumor cell lines, while INC280 does not. The antiproliferative effects of INC280, cabozantinib and tivantinib (fig. 2) are not correlated and not congruent with their efficacy as a c-Met inhibitor (see fig. 4a), as demonstrated below. The antiproliferative efficacies of cabozantinib on BON1, H727 and GOT1 cells (fig. 2) do not correlate with the respective expression levels of activated c-Met (phospho-c-Met Y1234/5) in these cell lines (fig. 1a).

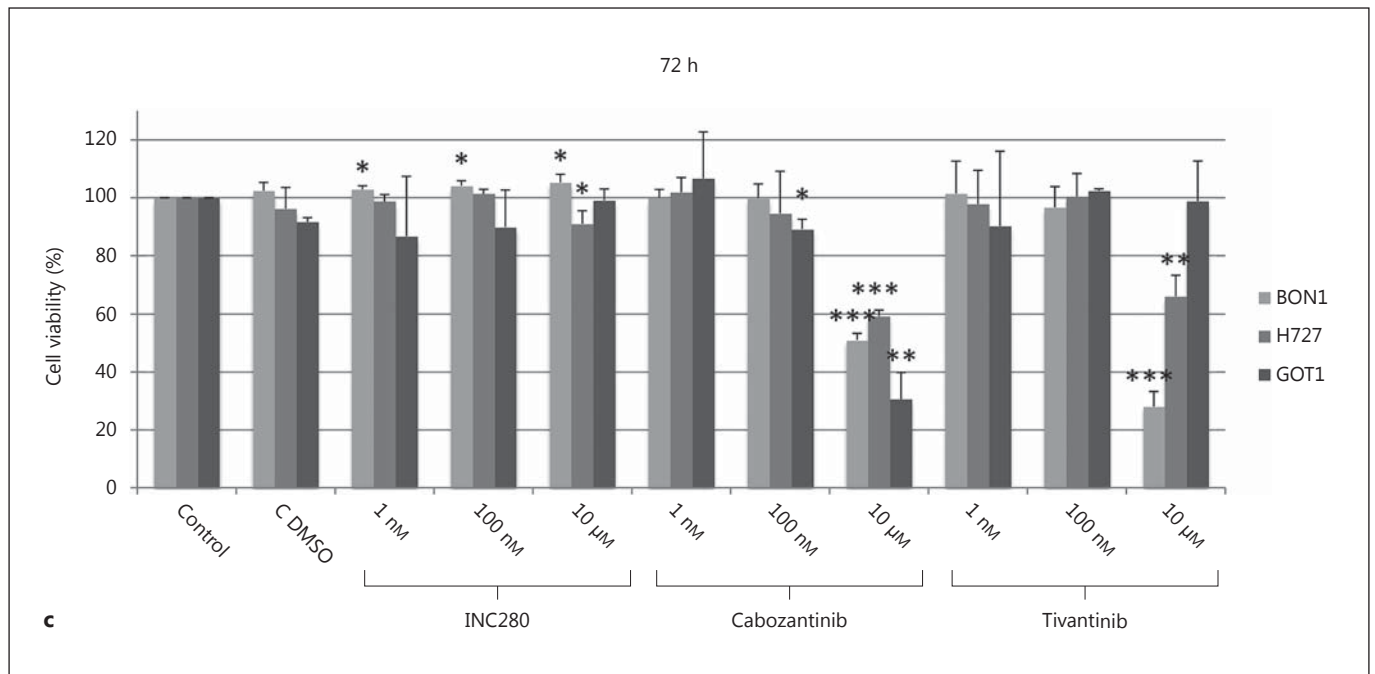
#### *Inhibition of c-Met Expression by c-Met siRNA Does Not Inhibit Cell Viability of Neuroendocrine Tumor Cells*

Human pancreatic neuroendocrine BON1 and bronchopulmonary NCI-H727 cells were transfected with nontargeted  $\beta$ -gal siRNA (50 nM) or siRNA against c-Met (50 nM). The effectiveness of the siRNAs was verified by Western blot analysis 72 h after transfection, and siRNA against c-Met demonstrated a significant decrease in c-Met expression (fig. 3a). Cell viability 72 h after transfection with nontargeted  $\beta$ -gal siRNA versus c-Met siRNA (fig. 3b) was not significantly affected in BON1 cells ( $45.8 \pm 25.7\%$  vs.  $46.6 \pm 25.2\%$ ; n.s.), and only a minimal effect



**Fig. 2.** Differential effects of INC280, cabozantinib and tivantinib on cell viability of neuroendocrine tumor cells. Human pancreatic neuroendocrine BON1, bronchopulmonary NCI-H727 and midgut GOT1 cells were incubated with INC280, cabozantinib and tivantinib at a concentration range of 1 nM to 10 μM for 24 h (**a**), 48 h (**b**) and 72 h (**c**), respectively. Cell viability was measured with Cell Titer 96 kit (Promega). The arithmetic means and standard deviation of four independent experiments are shown. Statistical analysis with t test showed significant results for 1 nM to 10 μM with \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

(For figure 2c see next page.)



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was seen in H727 cells ( $94.7 \pm 8.9\%$  vs.  $89.5 \pm 3.2\%$ ;  $p < 0.05$ ), respectively.

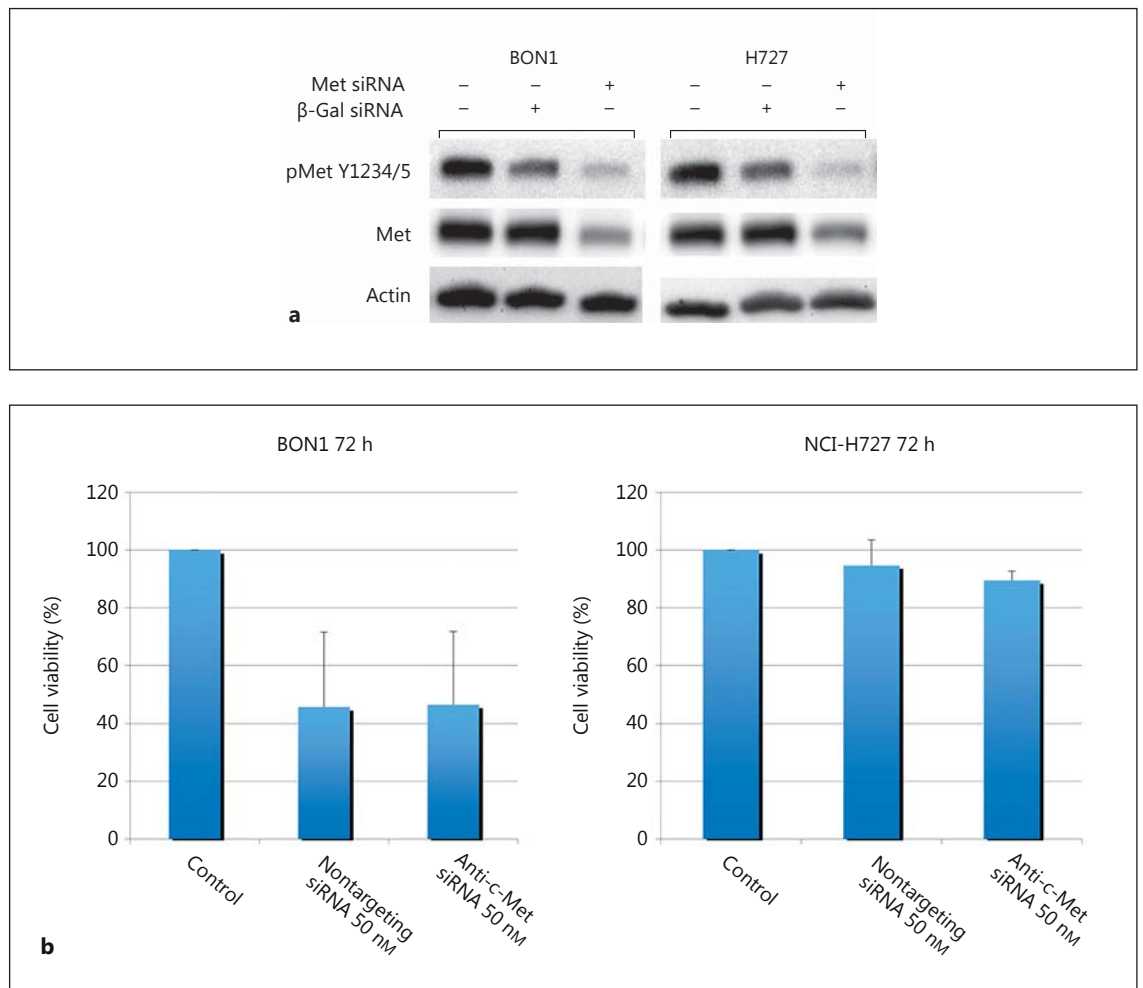
Thus, these data demonstrate that inhibition of c-Met expression does not affect or only minimally affects cell viability of neuroendocrine tumor cells.

#### *Differential Effects of INC280, Cabozantinib and Tivantinib on c-Met Activity and EGFR, Akt and MAPK Signaling*

Human pancreatic BON1 and bronchopulmonary NCI-H727 cells were incubated with INC280, cabozantinib and tivantinib, respectively, in concentrations of 1, 100 and 10,000 nM for 24 h, followed by protein extraction and Western blot analysis (fig. 4a). Expression of activated phospho-c-Met Y1234/5 was already partially inhibited by INC280 and cabozantinib at the lowest concentration of 1 nM, while concentrations of 100 and 10,000 nM completely abolished phospho-c-Met Y1234/5 expression, respectively (fig. 4a). In contrast, tivantinib demonstrated a partial inhibition of phospho-c-Met Y1234/5 expression in BON1 cells only at the highest concentration of 10,000 nM (fig. 4a). Thus, these data demonstrate that the c-Met-inhibitor efficacy of INC280 and cabozantinib in neuroendocrine tumor cells is in a similar low nanomolar range, while tivantinib, even at the highest concentration tested, caused only a partial inhibition of activated phospho-c-Met Y1234/5 expression (fig. 4a).

The c-Met-inhibitor efficacy of either drug and the extent of inhibition of activated phospho-c-Met Y1234/5 expression during drug incubation (fig. 4a) do not correlate with the respective antiproliferative efficacies of INC280, cabozantinib and tivantinib on BON1 and H727 cells (fig. 2).

As Akt/mTOR signaling and MAPK signaling is essentially involved in neuroendocrine tumor cell proliferation [28–30], an analysis of various markers of Akt/mTOR signaling and MAPK signaling was performed (fig. 4b, c). Human pancreatic BON1 and bronchopulmonary NCI-H727 cells were incubated with INC280 (10,000 nM), cabozantinib (10,000 nM) and tivantinib (10,000 nM) for 2, 24, 48 and 72 h, respectively, followed by protein extraction and Western blot analysis (fig. 4b, c). Cabozantinib and tivantinib caused late-onset effects at 48 and 72 h with a decrease in phospho-EGFR in BON1 and NCI-H727 cells (fig. 4b, c). Cabozantinib also demonstrated an early negative effect on phospho-IGF-1R at 2 h in BON1 and NCI-H727 cells (fig. 4b, c). Cabozantinib and tivantinib caused inhibition of pAkt and p4EBP1S65 in BON1 cells at 72 h (fig. 4b), but not in H727 cells (fig. 4c). INC280 and cabozantinib caused induction of GSK3 phosphorylation at pGSK3 S21/9 in BON1 cells at 72 h (fig. 4b), while cabozantinib and tivantinib caused induction of pGSK3 S21/9 in H727 cells at 72 h (fig. 4c). A modest compensatory activation of phospho-Erk1/2



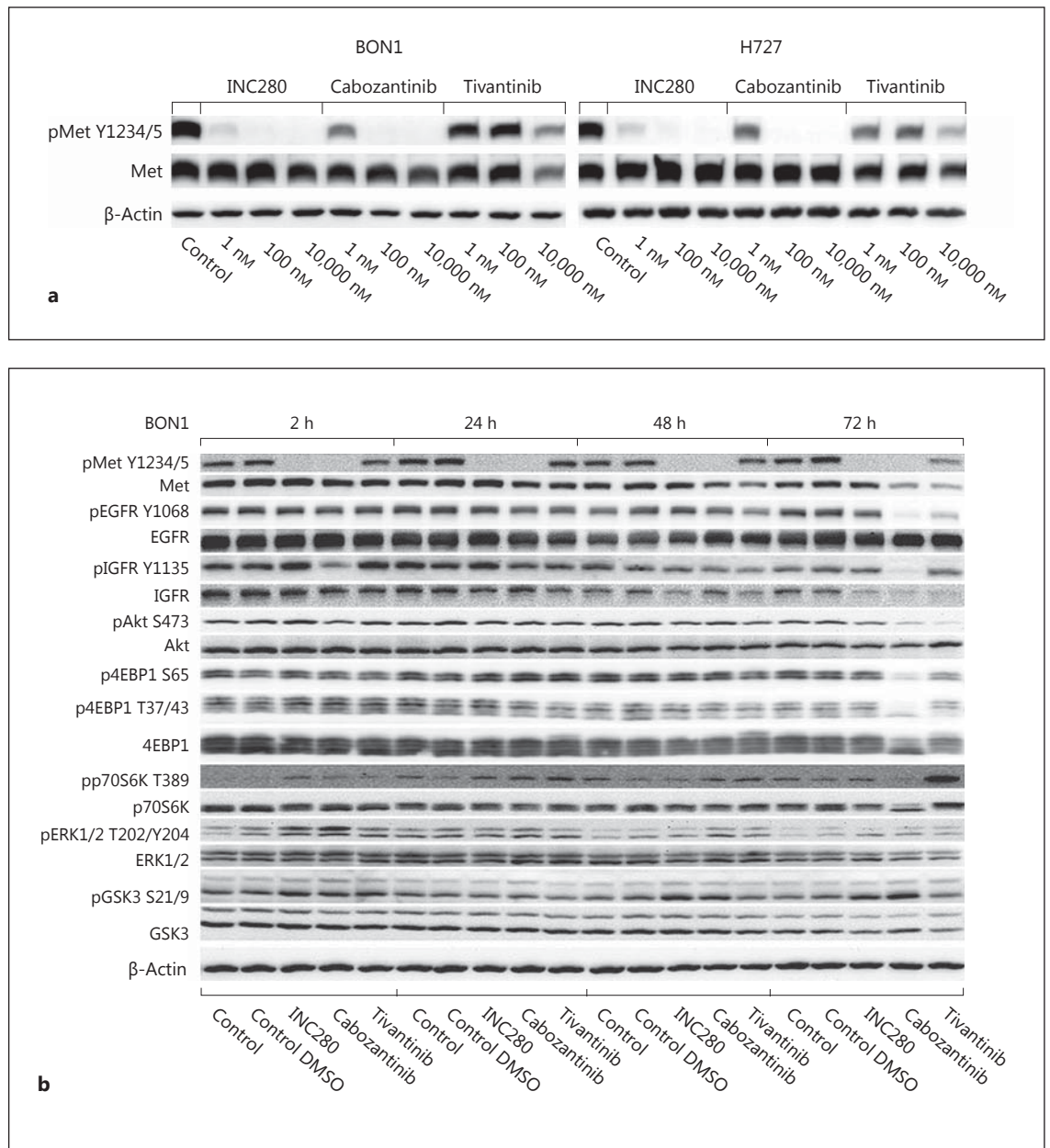
**Fig. 3.** Effects of c-Met siRNA on c-Met expression and cell viability of neuroendocrine tumor cells. Human pancreatic neuroendocrine BON1 and bronchopulmonary NCI-H727 cells were transfected with nontargeted  $\beta$ -gal siRNA (50 nM) or siRNA against c-Met (50 nM). The effectiveness of the siRNAs was verified by Western blot analysis of c-Met expression 72 h after transfection. **a** One representative blot out of three performed experiments is shown. **b** Cell viability in siRNA-treated BON1 and NCI-H727 cells was measured with Cell Titer 96 kit (Promega) 72 h after transfection. The mean values  $\pm$  SD of three independently performed experiments are shown.

T202/Y204 by INC280, cabozantinib and tivantinib could be detected at 2 h in BON1 cells (fig. 4b) and by cabozantinib and tivantinib at 24 h in H727 cells (fig. 4c). In contrast, none of the above described effects were observed in a similar manner with INC280 (fig. 4b, c).

*Cabozantinib and Tivantinib Cause a G2 Arrest of Neuroendocrine Tumor Cells, while the Specific c-Met Inhibitor INC280 Has No Effect*

Human pancreatic neuroendocrine BON1 (fig. 5a, b) and bronchopulmonary NCI-H727 (fig. 5c, d) cells were incubated with equimolar concentrations (10,000 nM) of

INC280, cabozantinib and tivantinib for 24 h, followed by FACS analysis. In BON1 cells, cabozantinib and tivantinib caused a significant accumulation in G2 phase with  $54.9 \pm 13.9\%$  ( $p < 0.05$ ) and  $75.6 \pm 7.9\%$  ( $p < 0.01$ ) versus  $17.5 \pm 3.2\%$  G2 phase in the control group (fig. 5b). In H727 cells, cabozantinib and tivantinib caused a significant accumulation in G2 phase with  $31.9 \pm 4.4\%$  (n.s.,  $p = 0.2$ ) and  $53.7 \pm 4.7\%$  ( $p < 0.05$ ) versus  $26.6 \pm 6.4\%$  G2 phase in the control group (fig. 5d). In contrast, INC280 did not exert a significant effect on the percentage of cells in the G2 phase neither in BON1 cells with  $17.0 \pm 5.3\%$  (n.s.) versus  $17.5 \pm 3.2\%$  G2 phase in the control group

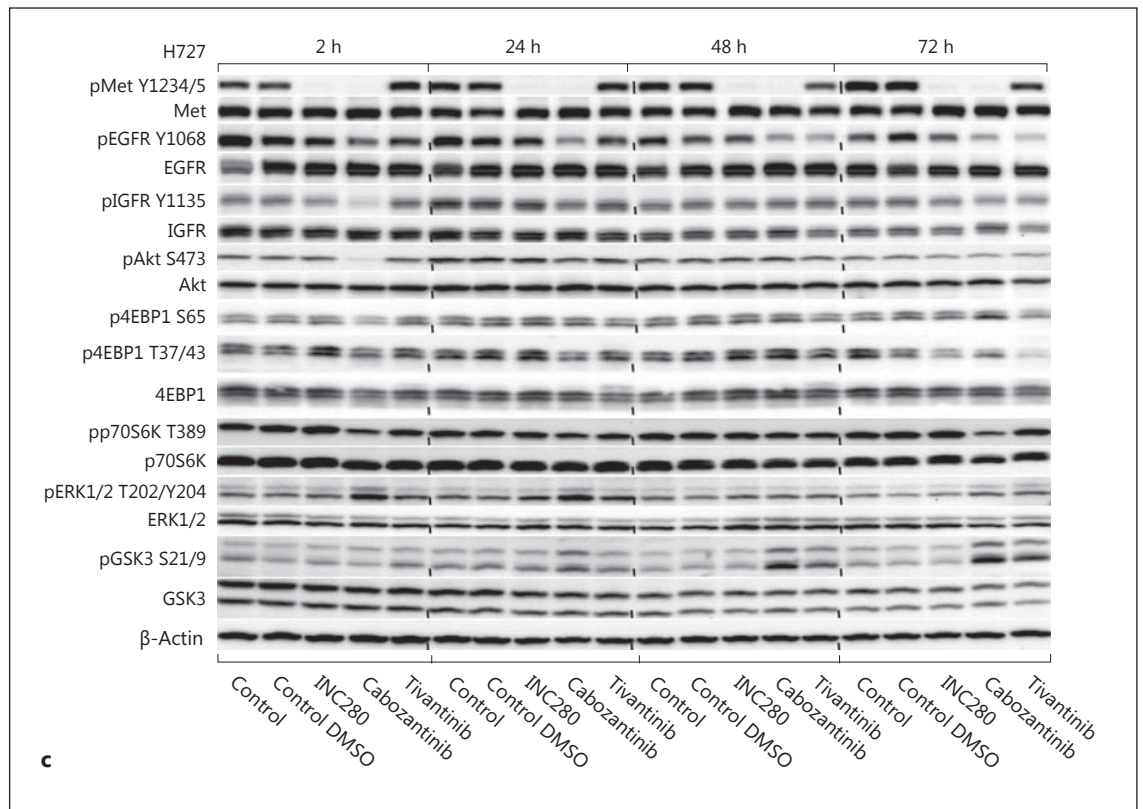


**Fig. 4.** Differential effects of INC280, cabozantinib and tivantinib on c-Met activity and EGFR, Akt and MAPK signaling. **a** Human pancreatic neuroendocrine BON1 and bronchopulmonary NCI-H727 cells were incubated with INC280, cabozantinib and tivantinib, respectively, in increasing concentrations of 1–10,000 nM for 24 h. Subsequently, the expression of phospho-c-Met Y1234/5, c-Met and  $\beta$ -actin loading control was evaluated by Western blot analysis. One representative blot out of three independently performed experiments is shown. Human pancreatic neuroendocrine

BON1 (**b**) and bronchopulmonary NCI-H727 (**c**) cells were incubated with INC280 (10,000 nM), cabozantinib (10,000 nM) and tivantinib (10,000 nM) for 2, 24, 48 and 72 h, respectively. Subsequently, the expression of phospho-c-Met Y1234/5, c-Met, pEGFR Y1068, EGFR, pIGFR Y1135, IGFR, pAkt S473, Akt, p4EBP1 S65, p4EBP1 T37/47, 4EBP1, pp70S6K T389, pERK1/2 T202/Y204, ERK1/2, pGSK3 S21/9, GSK3 and  $\beta$ -actin loading control was evaluated by Western blot analysis. One representative blot out of three independently performed experiments is shown.

(For figure 4c see next page.)





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(fig. 5b), nor in H727 cells with  $27.1 \pm 6.1\%$  (n.s.) versus  $26.6 \pm 6.4\%$  G2 phase in the control group (fig. 5d).

#### *Differential Effects of INC280, Cabozantinib and Tivantinib on c-Met Activity and Various Parameters of Cell Cycle Regulation and Apoptosis*

Human pancreatic BON1 and bronchopulmonary NCI-H727 cells were incubated with INC280 (10,000 nM), cabozantinib (10,000 nM) and tivantinib (10,000 nM) for 2, 24, 48 and 72 h, respectively, followed by protein extraction and Western blot analysis (fig. 6a, b).

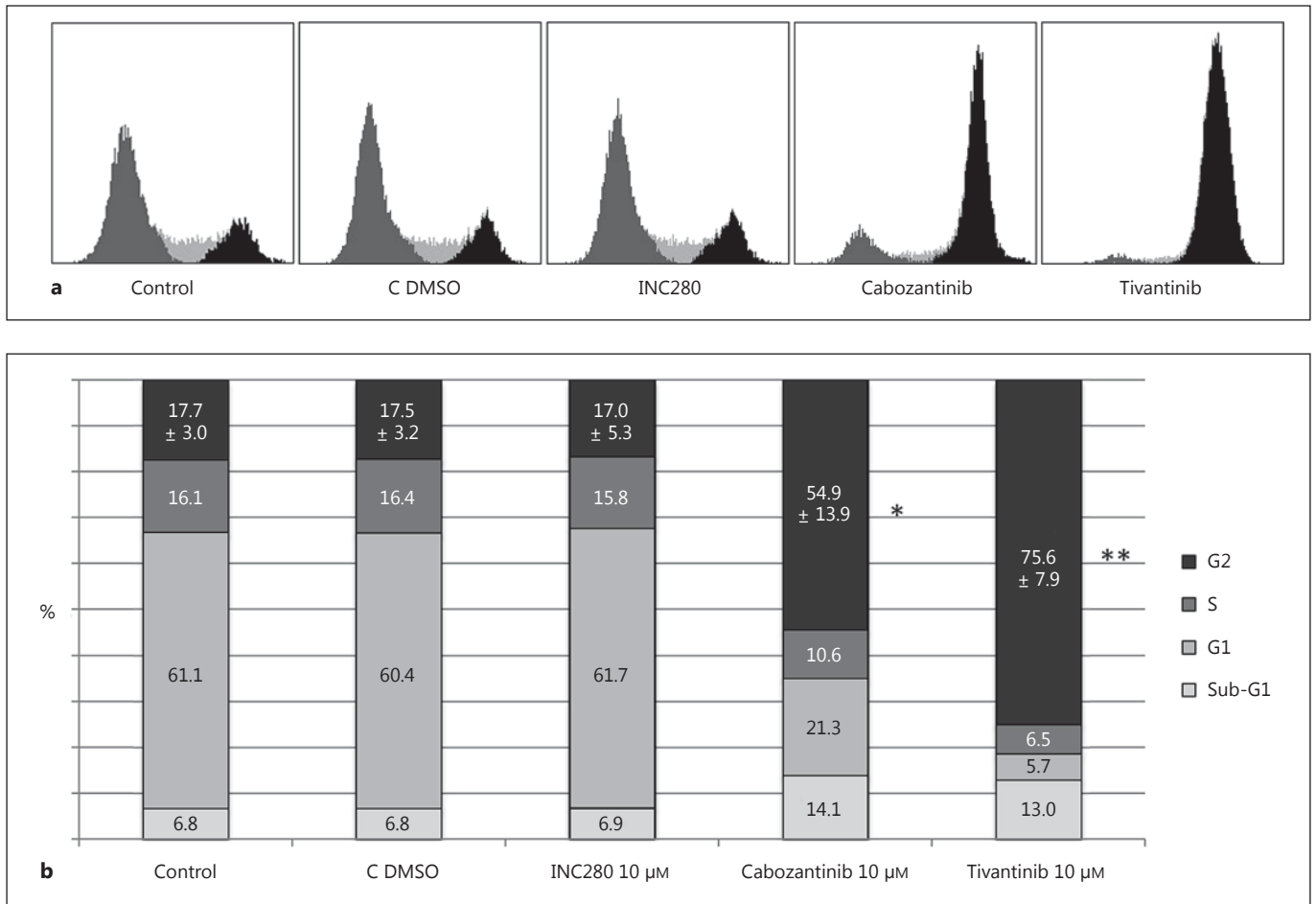
Cabozantinib and tivantinib demonstrated late-onset effects at 72 h with inhibition of the expression of cyclin D1 and proliferation marker PCNA in BON1 cells (fig. 6a) and H727 cells (fig. 6b). Tivantinib upregulated cyclin B1 expression at 24 h in BON1 cells (fig. 6a) and H727 cells (fig. 6b). While these findings indicate that cabozantinib and tivantinib affect cell cycle regulation, cabozantinib or tivantinib had no effect on cleavage of the apoptosis marker PARP (fig. 6a, b).

Cabozantinib and tivantinib cause a G2 arrest in BON1 and H727 cells (fig. 5). Therefore, we also investigated protein expression of the G2 checkpoint regulator Chk1.

The effects of cabozantinib or tivantinib on pChk1S345/Chk1 expression were different in BON1 and H727 cells (fig. 6a, b), indicating a cell type-specific regulation.

#### *Cabozantinib and Tivantinib Inhibit Cell Migration of Neuroendocrine Tumor Cells, while the Specific c-Met Inhibitor INC280 Has No Effect*

Human pancreatic neuroendocrine BON1 (fig. 7a, b) and bronchopulmonary NCI-H727 (fig. 7c, d) cells were incubated with INC280, cabozantinib and tivantinib, respectively, at a concentration of 100 nM and 10  $\mu$ M for 72 h. The gap width at 0 and 72 h, respectively, for each treatment group was analyzed (fig. 7b, d). In BON1 cells, cabozantinib and tivantinib at the highest concentration of 10,000 nM inhibited cell migration into the gap and restored the gap width with  $84.7 \pm 3.4\%$  ( $p < 0.05$ ) and  $82.9 \pm 17.9\%$  (n.s.,  $p = 0.1$ ), respectively (fig. 7b). In contrast, INC280 had no significant inhibitory effect on cell migration into the gap and did not restore the gap width in comparison to control with  $9.3 \pm 11.3\%$  (n.s.,  $p = 0.1$ ) versus  $29.2 \pm 19.2\%$  (fig. 7b). In H727 cells, cabozantinib and tivantinib inhibited cell migration into the gap and restored the gap width with  $102.5 \pm 20.6\%$  ( $p < 0.05$ ) and



**Fig. 5.** Differential effects of INC280, cabozantinib and tivantinib on G2 arrest of neuroendocrine tumor cells. Human pancreatic neuroendocrine BON1 (**a, b**) and bronchopulmonary NCI-H727 (**c, d**) cells were incubated with INC280, cabozantinib and tivantinib, respectively, at a concentration of 10,000 nM for 24 h. FACS analysis analyzed G2, S, G1 and sub-G1 events, respectively. **a, c** A

representative FACS analysis out of three independently performed experiments is shown. **b, d** The mean values ± SD of three independently performed experiments are shown. Statistical analysis with t test showed significant results with \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

(For figures 5c and d see next page.)

$66.1 \pm 2.2\%$  ( $p < 0.05$ ), respectively (fig. 7d). In contrast, INC280 had no significant inhibitory effect on cell migration into the gap and did not restore the gap width in comparison to control with  $33.7 \pm 16.8\%$  (n.s.,  $p = 0.5$ ) versus  $38.9 \pm 7.0\%$  (fig. 7d).

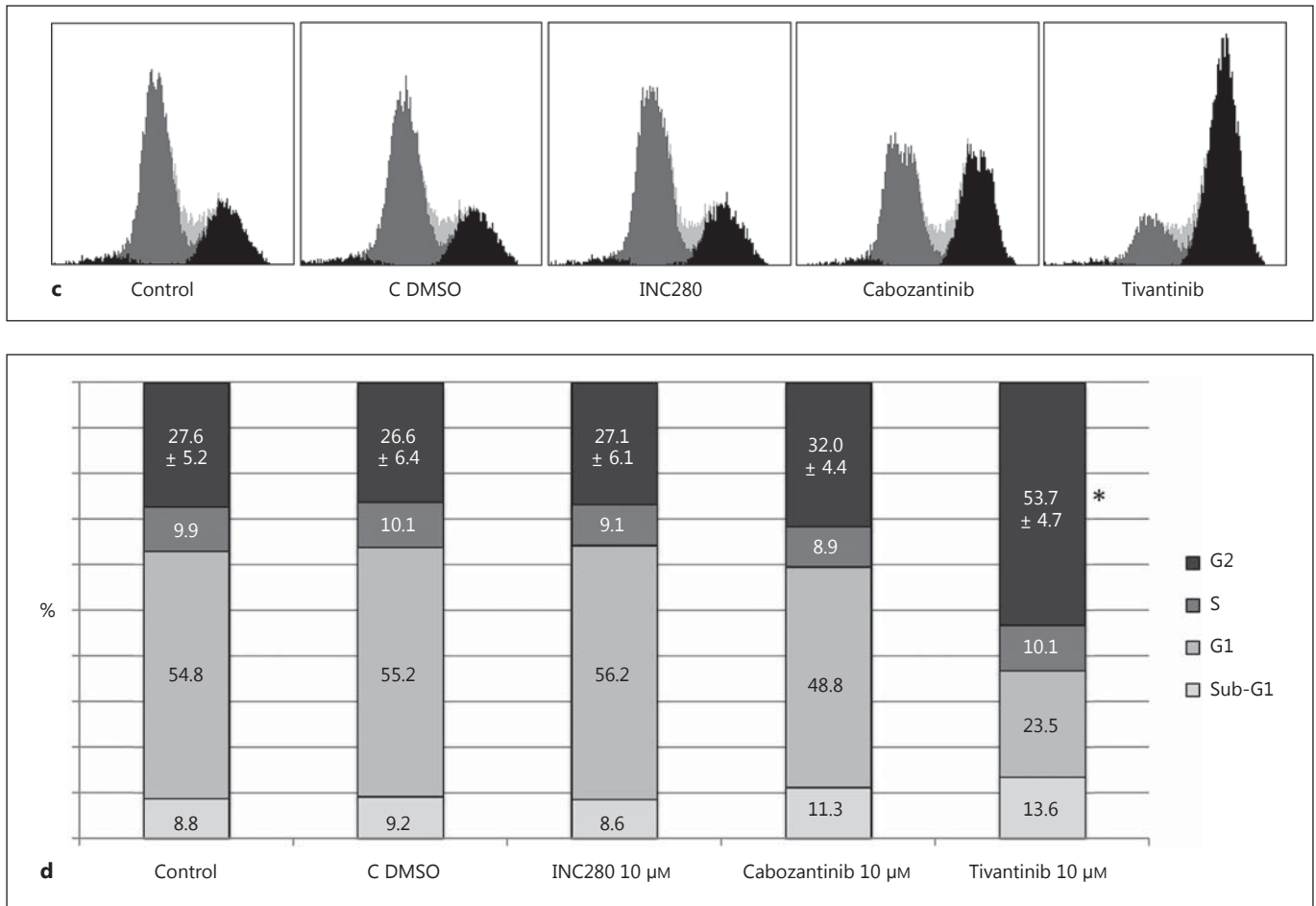
Thus, these data demonstrate that cabozantinib and tivantinib at a concentration of 10 µM inhibit cell migration in human neuroendocrine tumor cell lines, while INC280 does not. The antimigratory effects of INC280, cabozantinib and tivantinib (fig. 7) are not correlated and not congruent with their efficacy as a c-Met inhibitor (fig. 4), as demonstrated before.

Next, we evaluated whether the antimigratory effects of cabozantinib and tivantinib are mediated by epithelial

mesenchymal transition (EMT) markers. However, no effects were detected in Western blot analysis of a panel of appropriate EMT markers (fig. 8).

## Discussion

The HGF/HGF receptor c-Met axis has been defined as a potential target in cancer therapy of various tumor entities [7–10]. In this study, we aimed to investigate whether single c-Met inhibition is sufficient to inhibit neuroendocrine tumor cell growth and migration in vitro, and to further characterize the role of the HGF/c-Met axis in neuroendocrine tumors. The effects of the multi-



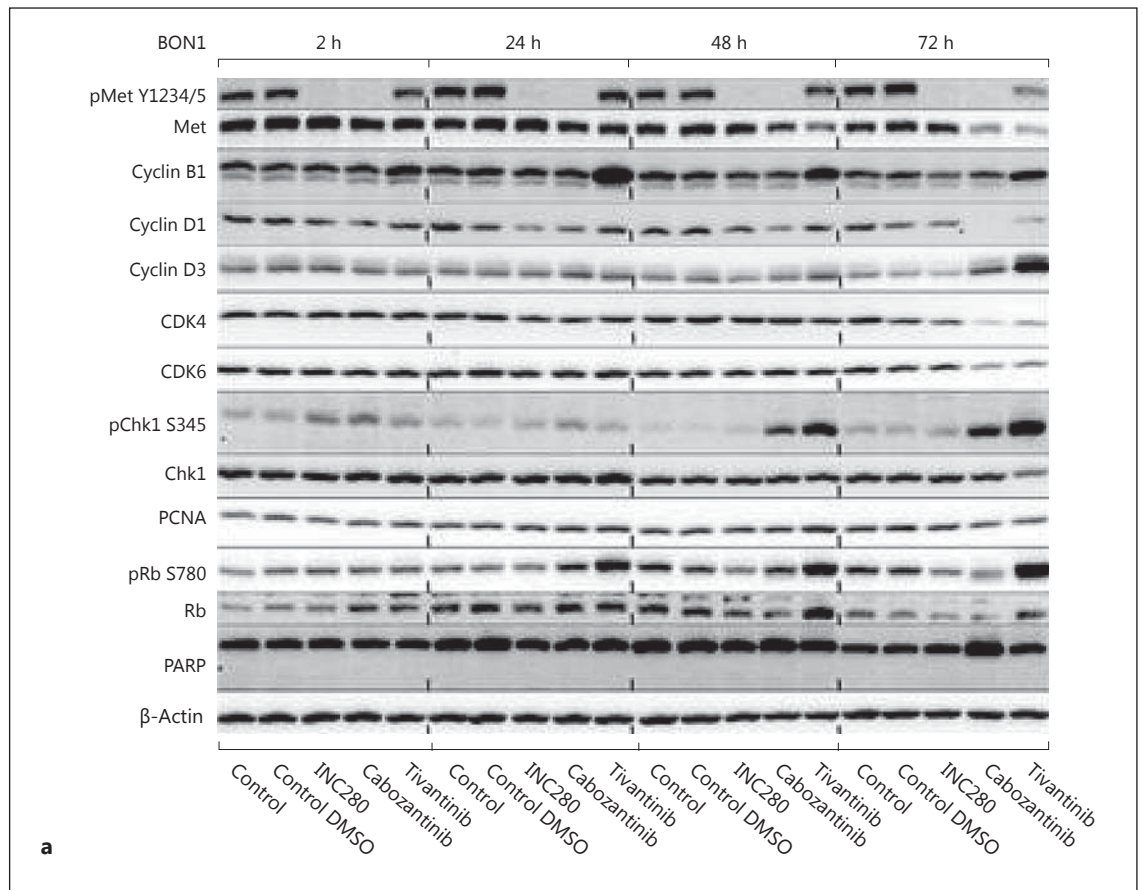
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TKIs cabozantinib and tivantinib and of the highly specific c-Met inhibitor INC280 were investigated in human pancreatic neuroendocrine BON1, bronchopulmonary NCI-H727 and midgut GOT1 cells in vitro.

All three human neuroendocrine tumor cell lines BON1 (pancreatic NET), NCI-H727 (bronchopulmonary carcinoid) and GOT1 (midgut carcinoid) expressed c-Met (fig. 1a). The functionality of c-Met was proven by rhHGF-induced stimulation of phospho-c-Met in all three cell lines. HGF stimulation also induced downstream Akt and ERK signaling. HGF-induced phosphorylation of c-Met and its downstream signals Akt and Erk were inhibited by the specific c-Met inhibitor INC280.

Expression of c-Met has been reported as a putative target in neuroendocrine tumors [31, 32]. In 39 pancreatic neuroendocrine neoplasms, protein expression analysis revealed c-Met overexpression in 17% (4/24) of non-metastasized NET, 33% (5/15) of metastasized NET, 57% (4/7) of lymph node metastases and 56% (5/9) of liver

metastases, respectively [33]. In human pancreatic neuroendocrine BON1 cells, microarray analysis using a small array of 2,503 genes revealed 101 HGF-responsive genes, including genes with a putative function in oncogenesis, cell proliferation, apoptosis or cell adhesion/motility [34]. In 10 gastrinomas, protein expression of HGF receptor c-Met was detectable in 90%, while competitive PCR in 38 gastrinomas revealed c-Met overexpression in 14% compared to normal pancreas [35]. Overexpression of c-Met was a negative prognostic indicator in gastrinomas [35]. In 17 ileal NETs and 28 nonileal NETs, high staining of c-Met immunoreactivity was found in 100% of ileal NETs and 32% of nonileal NETs [36]. The human midgut carcinoid cell line CNDT2 expresses c-Met [37]. In bronchopulmonary neuroendocrine neoplasms, strong c-Met expression was observed in 66% (25/38) of typical carcinoids, 67% (4/6) of atypical carcinoids, 50% (17/34) of SCLC and 55% (6/11) of LCNEC [38]. A strong expression of activated phospho-c-Met was observed in



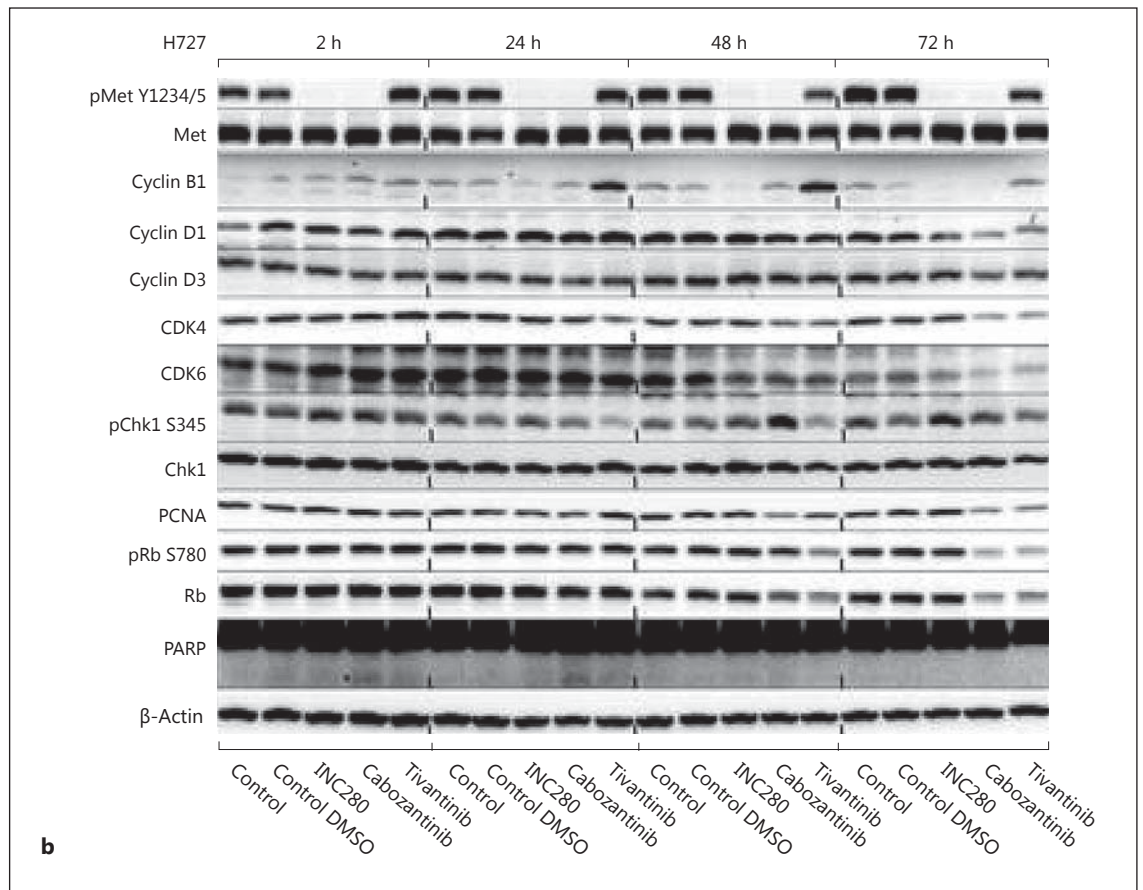
**Fig. 6.** Differential effects of INC280, cabozantinib and tivantinib on c-Met activity and various parameters of cell cycle regulation and apoptosis. Human pancreatic neuroendocrine BON1 (**a**) and bronchopulmonary NCI-H727 (**b**) cells were incubated with INC280 (10,000 nM), cabozantinib (10,000 nM) and tivantinib (10,000 nM) for 2, 24, 48 and 72 h, respectively. Subsequently, the expression of phospho-c-Met Y1234/5, c-Met, cyclin B1, cyclin D1, cyclin D3, CDK4, CDK6, pChk1 S345, Chk1, PCNA, pRb S780, Rb, PARP and  $\beta$ -actin loading control was evaluated by Western blot analysis. One representative blot out of three independently performed experiments is shown.

(For figure 6b see next page.)

50% (19/38) of typical carcinoids, 67% (4/6) of atypical carcinoids, 68% (23/34) of SCLC and 36% (4/11) of LCNEC [38]. C-Met mutations have been reported to be relatively rare in bronchopulmonary neoplasias with 6.5% in 46 SCLC and 8.3% in 36 NETs [39]. These mutations were not functionally relevant in regard of c-Met phosphorylation status [39]. Serum levels of HGF were significantly higher in patients with bronchopulmonary carcinoid tumors than in healthy controls [40].

INC 280, cabozantinib and tivantinib inhibited c-Met phosphorylation in neuroendocrine tumor cells (fig. 4). Comparing equimolar concentrations, INC280 was the most potent c-Met inhibitor compared to cabozantinib and tivantinib (fig. 4a). This finding is in accordance with

the literature. For the ATP-competitive c-Met inhibitor INC280 (synonyms: INCB28060, capmatinib) an IC<sub>50</sub> of 0.13 nM towards c-Met in a kinase assay and an IC<sub>50</sub> for c-Met phosphorylation in cells in vitro of 0.3–1.1 nM have been reported [41]. For the ATP-competitive c-Met inhibitor cabozantinib (synonym: XL184), an IC<sub>50</sub> of 1.3 nM towards c-Met in a kinase assay has been demonstrated [42]. The non-ATP-competitive c-Met inhibitor tivantinib (synonym: ARQ197) has been demonstrated as a calculated inhibitory constant K<sub>i</sub> of 355 nM towards c-Met in a kinase assay and an IC<sub>50</sub> for c-Met phosphorylation in cells in vitro of 100–300 nM [43]. Thus, according to the literature [41–43], INC280 inhibits c-Met phosphorylation in vitro with an approximately 10-fold high-



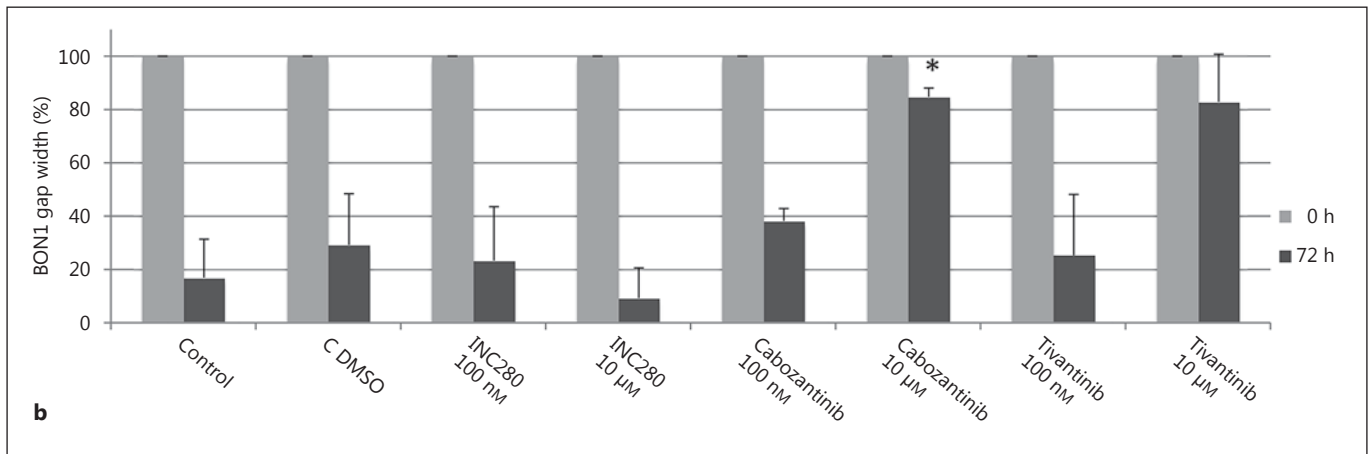
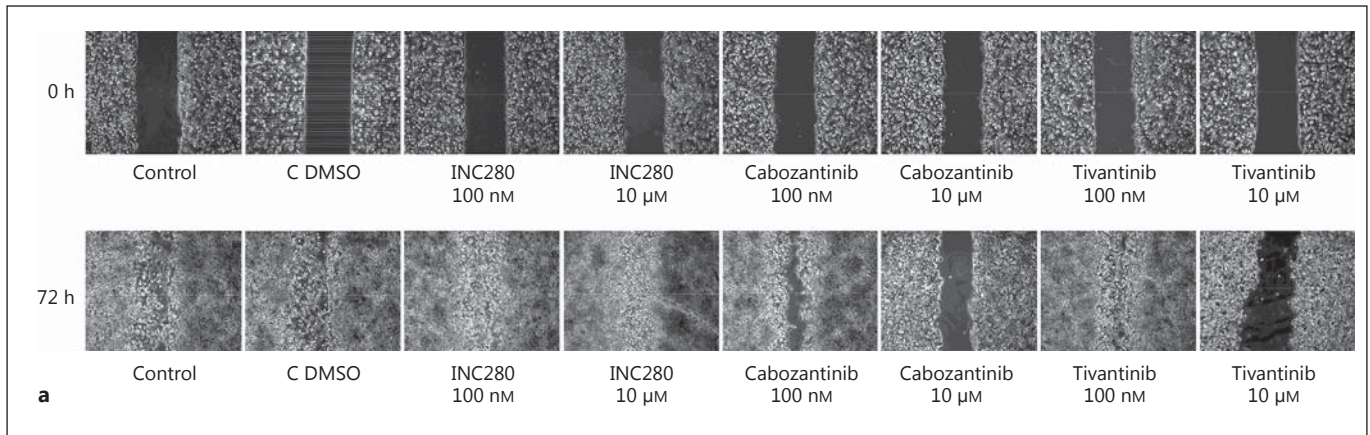
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er efficacy compared to cabozantinib and with an approximately 100- to 1,000-fold higher efficacy compared to tivantinib. This different potency in inhibition of c-Met phosphorylation was also found in our experiments comparing INC280, cabozantinib and tivantinib (fig. 4a). Similar to our data, also in epithelioid sarcoma cell lines has the highly selective c-Met inhibitor INC280 been reported to inhibit phospho-c-Met at concentrations of 1 nM [44].

Although in our study equimolar concentrations (10  $\mu$ M) of cabozantinib and tivantinib potently inhibited cell viability (fig. 2) and cell migration (fig. 7), the highly specific c-Met inhibitor INC280 had no effect on cell viability (fig. 2) or cell migration (fig. 7). Similarly, equimolar concentrations (10  $\mu$ M) of cabozantinib and tivantinib caused a potent G2 arrest in neuroendocrine tumor cells, while INC 280 had no effect (fig. 5). Knockdown experiments with c-Met siRNA also demonstrated no effect or only minor effects on neuroendocrine tumor cell viability (fig. 3). Our in vitro data suggest that c-Met inhibition

alone is not sufficient to exert direct antitumoral or antimigratory effects in neuroendocrine tumor cells in vitro. In contrast, the multi-TKIs cabozantinib and tivantinib show promising antitumoral and antimigratory effects in neuroendocrine tumor cells, which are most probably 'off-target' effects, not mediated by c-Met.

In the Rip-Tag2 mouse model of pancreatic neuroendocrine tumors, the multi-TKI cabozantinib (with activity against c-Met, VEGFR2, c-KIT, FLT3, RET and TIE2) has been demonstrated to be superior in comparison to compounds with anti-VEGF activity only in inhibition of tumor angiogenesis, tumor invasiveness and metastasis [19, 21]. Further studies in the Rip-Tag2 mouse model with an anti-VEGF antibody or sunitinib in combination with the c-Met inhibitor PF-04217903 [19, 21] also showed additive antitumoral effects. The antitumoral effects of cabozantinib in the Rip-Tag2 mouse model have been discussed to be due to simultaneous inhibition of VEGF and c-Met signaling by cabozantinib [19, 21]. However, due to the multi-TKI function of cabozantinib (with known ac-



**Fig. 7.** Differential effects of INC280, cabozantinib and tivantinib on cell migration of neuroendocrine tumor cells. Human pancreatic neuroendocrine BON1 (**a, b**) and bronchopulmonary NCI-H727 (**c, d**) cells were incubated with INC280, cabozantinib and tivantinib, respectively, at a concentration of 100 nM and 10 μM for 72 h. The gap width between the two monolayers was analyzed at

0 and 72 h of the incubation period, respectively. A representative experiment out of three independently performed experiments is shown (**a, c**). **b, d** The gap width at 0 and 72 h, respectively, for each treatment group was analyzed. The mean values  $\pm$  SD of three independently performed experiments are shown. Statistical analysis with t test showed significant results with \*  $p < 0.05$  and \*\*  $p < 0.01$ .

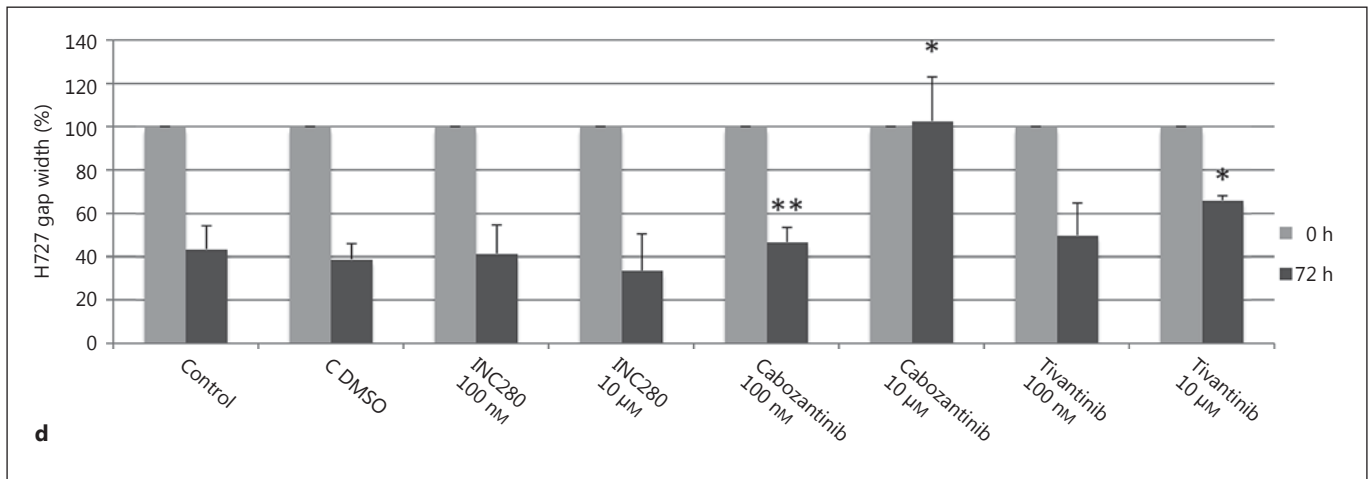
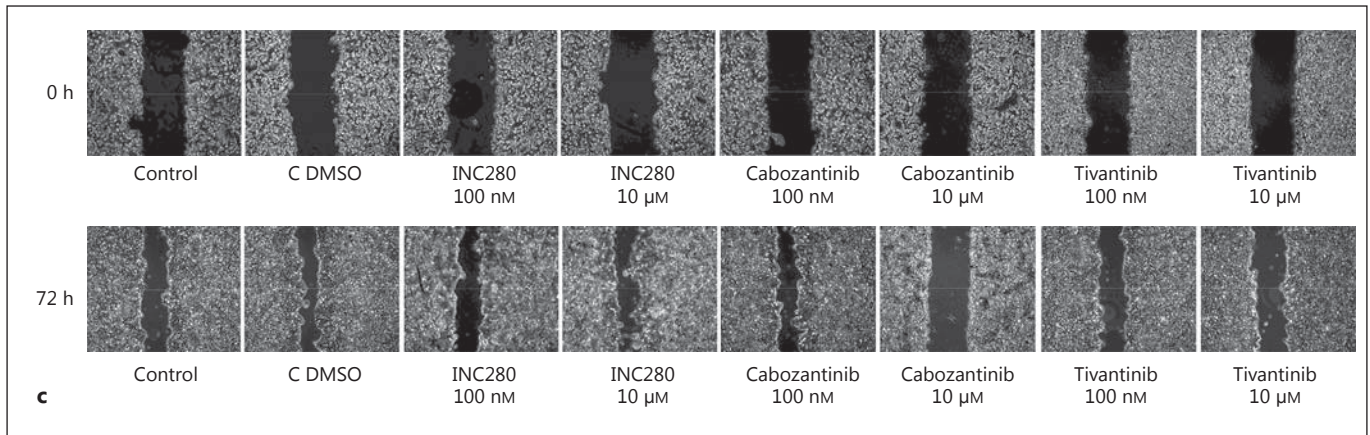
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tivity against c-Met, VEGFR2, c-KIT, FLT3, RET and TIE2), these studies cannot prove the efficacy of c-Met inhibition for the antitumoral effects of cabozantinib [19, 21]. Our in vitro data suggest that c-Met inhibition alone is not sufficient to exert direct antitumoral or antimigratory effects in unstimulated neuroendocrine tumor cells.

Nevertheless, in neuroendocrine tumor cells with compensatory upregulation of phospho-c-Met expression, inhibition of c-Met might be a specific target for antitumoral and antimigratory effects [21]. In human pancreatic cancer cells in vitro, INC280 inhibited HGF-induced cell growth and migration, while no effect of INC280 was observed on constitutive cell growth and migration in cells that were not stimulated by HGF [45]. In

hepatocellular tumor cell models, cabozantinib caused a G1 arrest in the phospho-c-Met-overexpressing tumor cell lines MHCC97L and MHCC97H (cabozantinib IC 50 values for inhibition of cell growth 9–13 nM), while in the non-phospho-c-Met-expressing tumor cell lines SK-HEP1 and HepG2 (cabozantinib IC 50 values for inhibition of cell growth 4,300–5,000 nM), a G2 arrest was observed [46]. These data suggest different c-Met-dependent and c-Met-independent mechanisms of cabozantinib on cell cycle in tumor cells [46].

In accordance with our results in neuroendocrine tumor cells, tivantinib has recently been reported to exert antitumoral effects in various tumor entities, which are an off-target of c-Met inhibition [13–16]. In thyroid cancer cells,

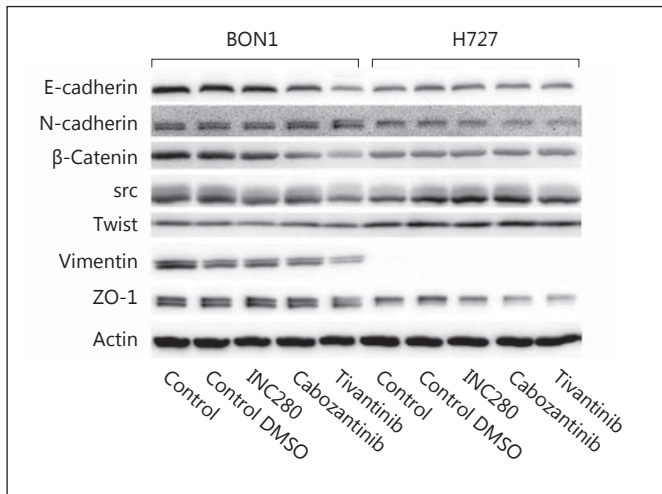


siRNA-mediated downregulation of c-Met did not induce cell cycle arrest or apoptosis [14]. There was no correlation between the c-Met inhibitory potency of crizotinib and tivantinib and their respective antitumoral efficacy in thyroid tumor cells [14]. In various tumor cell entities, tivantinib inhibited c-Met addicted and nonaddicted tumor cells in a similar fashion [15, 16], suggesting a c-Met-independent mechanism of action. Tivantinib has been suggested to cause antitumoral effects by alternative mechanisms as microtubule disruption [15, 16, 43] or inhibition of GSK3 $\alpha$  and GSK3 $\beta$  [44]. Tivantinib caused a significant G2 arrest in various tumor cells [13–16], while the c-Met inhibitors crizotinib and PHA-665752 caused a G1 arrest [15].

In our neuroendocrine tumor cell model in vitro, cabozantinib and tivantinib demonstrated late-onset 72-hour effects with inhibition of the expression of pEGFR (fig. 4), pAktS473 (fig. 4), p4EBP1S65 (fig. 4) and cyclin D1 (fig. 6). In addition, cabozantinib constantly induced GSK3 phosphorylation at pGSK3 S21/9 in both cell lines

at 72 h (fig. 4). Cabozantinib and tivantinib modestly inhibited the proliferation marker PCNA at 72 h (fig. 6) and caused a potent G2 cycle arrest (fig. 5) in BON1 and H727 cells. These data indicate that cabozantinib and tivantinib decrease tumor cell viability, most probably due to inhibition of cell proliferation mediated by inhibition of pAkt and its downstream signals and by upregulation of pGSK3. In contrast, no PARP cleavage as a marker of apoptosis was detected (fig. 6). Accordingly, c-Met inhibitors have been reported to inhibit downstream Akt and MAPK signaling cascades in various tumors [7, 9, 46]. Inhibition of Akt/mTOR signaling is a proven, important target in neuroendocrine tumor cells [28, 30]. The phosphorylation of GSK3 causes its inactivation [47].

Cabozantinib and tivantinib cause a G2 cycle arrest in BON1 and H727 cells (fig. 5). Accordingly to our data, a G2 arrest has also been reported in other in vitro cancer models with cabozantinib [42] and tivantinib [13–16]. Tivantinib has been reported to cause G2 cell cycle arrest in



**Fig. 8.** Lack of effect of INC280, cabozantinib and tivantinib on EMT markers. Human pancreatic neuroendocrine BON1 and bronchopulmonary NCI-H727 cells were incubated with equimolar concentrations (10,000 nM) of INC280, cabozantinib and tivantinib, respectively, for 24 h. Subsequently, the expression of E-cadherin, N-cadherin,  $\beta$ -catenin, src, twist, vimentin, ZO-1 and  $\beta$ -actin loading control was evaluated by Western blot analysis. One representative blot out of three independently performed experiments is shown.

various c-Met addicted and nonaddicted cancer cell lines and to cause microtubule disruption [15]. Phosphorylation and activation of the cell cycle G2 checkpoint regulator Chk1 is known to cause subsequent phosphorylation and inactivation of the phosphatase CDC25. Inactivation of CDC25 phosphatase prevents dephosphorylation and activation of CDK1 in the cyclin B/CDK1 complex and finally inhibits progression to mitosis [48]. On the other hand, Chk1 inhibitors have been suggested as a potential target to sensitize cancer cells [48]. The effects of cabozantinib or tivantinib on the cell cycle G2 checkpoint regulator Chk1 were different in BON1 and H727 cells (fig. 6a, b), indicating a cell type-specific regulation. Further studies are necessary to evaluate the mechanisms by which cabozantinib and tivantinib cause G2 cell cycle arrest in our neuroendocrine tumor cell model.

Our study clearly demonstrates that single c-Met inhibition with the highly specific c-Met inhibitor INC280 is not sufficient to exert direct antiproliferative effects or inhibition of tumor cell migration in neuroendocrine tumor cells in vitro under constitutive conditions. Neuroendocrine tumor cells without pretreatment were not dependent on c-Met signaling for growth, survival or migration. Thus, based on our in vitro data, it seems not to

be a promising strategy to target neuroendocrine tumors in monotherapy with highly specific c-Met inhibitors. However, the limitations of an in vitro model of NET to study their tumor biology have to be stated as follows: (a) there is only a limited number of human cell lines available [24, 27], and (b) the cell proliferation rates of many rapidly growing cell culture models do not match the slow proliferation index Ki-67 of typical G1 and G2 NET.

In contrast, we observed potent antiproliferative effects and inhibition of tumor cell migration by the multi-TKIs cabozantinib and tivantinib in neuroendocrine tumor cells in vitro. Our data suggest these in vitro effects of cabozantinib (multi-TKI with known activity against c-Met, VEGFR2, c-KIT, FLT3, RET and TIE2) and tivantinib (c-Met inhibitor with additional ‘off-target’ effects [13–16]) to be most likely mediated by ‘off-target’ effects besides c-Met. These findings may have implications for the rationale of future selection of multi-TKIs for neuroendocrine tumor treatment and for the evaluation of phospho-c-Met expression as a potential biomarker for response prediction in neuroendocrine tumors.

Single c-Met inhibition seems insufficient to exert direct antitumoral effects in unstimulated neuroendocrine tumor cells. Nevertheless, a synergistic action of c-Met inhibition during combination therapy targeting several signaling cascades cannot be excluded by our data. As stated above, in cancers with compensatory upregulation of phospho-c-Met expression, inhibition of c-Met seems to exert antitumoral and antimigratory effects [21, 49]. In other various tumor entities, resistance against EGFR inhibition or resistance against anti-VEGF therapy has been demonstrated to be mediated by compensatory upregulation of phosphorylated c-Met as an alternative signaling pathway. Inhibition of c-Met has been discussed as a potential target in combination therapies in order to overcome a c-Met-induced escape from EGFR inhibition [50–56] or escape from anti-VEGF therapy [31, 57–60], as has been demonstrated in various tumor entities. These mechanisms have also been discussed for neuroendocrine tumors [19–21, 31, 61]. Further preclinical in vivo studies in xenograft models of human NET treated with appropriate combination therapies including the highly specific c-Met inhibitor INC280 should be performed to address this issue.

## Conclusions

Specific inhibition of c-Met in neuroendocrine tumor cell lines under constitutive conditions in vitro did not cause antiproliferative or antimigratory effects. INC280



is a highly selective c-MET inhibitor, and its further evaluation in c-Met-dependent tumor entities, as cancers with constitutive or compensatory upregulation of phospho-c-Met expression, is warranted.

Our *in vitro* data demonstrate that cabozantinib and tivantinib with potent antiproliferative effects in neuroendocrine tumors are most probably mediated by 'off-target' effects not mediated by c-MET inhibition. Further investigation of these compounds as antitumoral agents in neuroendocrine tumors is warranted. Currently, a clinical phase 2 study of cabozantinib in neuroendocrine tumors (NCT01466036) is recruiting patients.

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### Disclosure Statement

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