RESEARCH

Tropomyosin receptor kinase: a novel target in screened neuroendocrine tumors

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Abstract

Tropomyosin receptor kinase (Trk) inhibitors are investigated as a novel targeted therapy in various cancers. We investigated the in vitro effects of the pan-Trk inhibitor GNF-5837 in human neuroendocrine tumor (NET) cells. The human neuroendocrine pancreatic BON1, bronchopulmonary NCI-H727 and ileal GOT1 cell lines were treated with GNF-5837 alone and in combination with everolimus. Cell viability decreased in a time- and dose-dependent manner in GOT1 cells in response to GNF-5837 treatment, while treatment in BON1 and NCI-H727 cells showed no effect on cellular viability. Trk receptor expression determined GNF-5837 sensitivity. GNF-5837 caused downregulation of PI3K-Akt-mTOR signaling, Ras-Raf-MEK-ERK signaling, the cell cycle and increased apoptotic cell death. The combinational treatment of GNF-5837 with everolimus showed a significant enhancement in inhibition of cell viability vs single substance treatments, due to a cooperative PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathway downregulation, as well as an enhanced cell cycle component downregulation. Immunohistochemical staining for Trk receptors were performed using a tissue microarray containing 107 tumor samples of gastroenteropancreatic NETs. Immunohistochemical staining with TrkA receptor and pan-Trk receptor antibodies revealed a positive staining in pancreatic NETs in 24.2% (8/33) and 33.3% (11/33), respectively. We demonstrated that the pan-Trk inhibitor GNF-5837 has promising anti-tumoral properties in human NET cell lines expressing the TrkA receptor. Immunohistochemical or molecular screening for Trk expression particularly in pancreatic NETs might serve as predictive marker for molecular targeted therapy with Trk inhibitors.

Key Words

- tropomyosin kinase receptor (Trk)
- Trk inhibitor
- neuroendocrine tumor
- everolimus
Introduction

Neuroendocrine tumors (NETs) are highly heterogeneous tumors originating from distinct cell precursors (Schimmack et al. 2011). Current data show an increase in incidence of gastroenteropancreatic neuroendocrine tumors (GEP-NETs) lately (Modlin et al. 2008a, Yao et al. 2008). Despite increasing knowledge on the tumor biology, genetics and epigenetics of GEP-NETs, the overall outcome and survival have gained very little over the last decades (Modlin et al. 2008b, Kidd et al. 2016, Karpathakis et al. 2017). Peptide receptor radionuclide therapy has shown promising results regarding progression-free and overall survival (van Vliet et al. 2013, Bodei et al. 2014), but other systemic therapeutic approaches for GEP-NETs, such as biotherapy, chemotherapy and molecular targeted therapy are limited in their efficiency (Auernhammer & Goke 2011, Pavel & Sers 2016, Cives & Strosberg 2017). Currently, the only approved molecular targeting strategies for NETs are the mTORC1 inhibitor everolimus (Pavel et al. 2011, Yao et al. 2011) and the multi-tyrosin kinase inhibitor sunitinib (Raymond et al. 2011) demonstrating the urgent need for innovative molecular therapeutic targets and strategies.

As recently reviewed by Amatu et al. (2016), the Trk family TrkA, TrkB and TrkC is encoded by the NTRK genes NRTK1, NTRK2 and NTRK3, respectively. Neurotrophins (NTs) are specific ligands to the Trk receptors, especially nerve growth factor (NGF) for TrkA, brain-derived growth factor (BDGF) and NT-4/5 for TrkB and NT-3 for TrkC (Kaplan et al. 1991, Klein et al. 1991, Barbacid 1994, Bibel & Barde 2000, Huang & Reichardt 2003, Amatu et al. 2016). All Trks are transmembrane receptors and ligand binding to the extracellular domain causes receptor oligomerization and autophosphorylation of the intracellular domain containing the kinase region. Subsequently, activated downstream effector proteins of the Trk signaling involve the Ras-Raf-MEK-ERK, the PI3K-Akt-mTOR, the PLCγ-1 and the PKC pathway (Huang & Reichardt 2003, Amatu et al. 2016).

The Trk receptor family has been demonstrated to be implicated in the growth of many different types of cancer, for example, oral squamous cell carcinoma (McGregor et al. 1999), pancreas (McGregor et al. 1999), thyroid (McGregor et al. 1999), lung (Oellman et al. 1995), esophagus (Zhu et al. 2000), prostate (Sortino et al. 2000), breast (Tagliabue et al. 2000) and ovarian cancer (Davidson et al. 2001) and glioblastoma (Oellman et al. 1995). Chromosomal rearrangements with oncogenic gene fusions joining the 3′ region of the NTRK1, NTRK2 or NTRK3 gene to another partner gene resulting in a constitutively activated Trk family kinase have been reported in various cancer types (Amatu et al. 2016).

Recently, Trk inhibitors have emerged as promising novel targets in molecular targeted cancer therapy (Amatu et al. 2016, Demir et al. 2016, Khotkaya et al. 2017). In a large-cell neuroendocrine carcinoma (LCNEC) cell line the pan-Trk inhibitor AZD7451 showed promising pharmacologic potential, particularly in cells with a NTRK gene fusion with constitutive Trk expression (Tatematsu et al. 2014). Furthermore, BDNF/TrkB expression was significantly upregulated in clinical LCNEC specimens and has shown to be implicated in tumorigenicity and invasiveness in vitro and in vivo (Odate et al. 2013). TrkB signaling has been associated with cellular survival and migration in neuroendocrine lung carcinomas (Osborne et al. 2013).

Several Trk inhibitors, such as LOXO-101 and entrectinib (RXDX-101), are currently investigated in clinical phase 1/2 trials and have shown encouraging activity including patients with molecularly defined cancers with rearrangements of Trk family kinases (Ardini et al. 2016, Passiglia et al. 2016, Drilon et al. 2017).

So far, no data on the putative role of Trk inhibitors in the treatment of GEP-NETs have been available. Here, we demonstrate anti-proliferative effects of the highly specific pan-Trk inhibitor GNF-5837 (Albaugh et al. 2012) in the human neuroendocrine tumor cell line GOT1 in vitro and examine TrkA downstream signal transduction. In addition, we investigated the expression profile of Trk in GEP-NET tumor samples from patients.

Materials and methods

Materials

GNF-5837 was purchased from R&D Systems and recombinant human NGF was purchased from Life Technologies. Human BDNF was obtained from Sigma-Aldrich and everolimus (07741FLUKA) was purchased from Sigma. These substances were diluted in dimethylsulfoxide (DMSO, 10 mM stock solution; Sigma, D8418). Dulbecco’s Modified Eagle medium–Nutrient Mixture F-12, 1:1 (DMEM/F12) as well as penicillin/streptomycin were obtained from Gibco/Invitrogen. RPMI medium (with l-Glutamine, NaCO₃) and phosphate-buffered saline (PBS) were purchased from Sigma, whereas Trypsin-EDTA (10×) was acquired from PAA Laboratories (Cölte, Deutschland). Fetal bovine serum (FBS) and amphotericin B were acquired from Biochrom (Berlin, Germany). Human adult normal brain tissue Lysate (ab29466) was obtained from Abcam and stored at −80°C.
Cell culture

The human pancreatic neuroendocrine BON1 tumor cell line (Evers et al. 1991) (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 cells were grown in RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin and 0.4% amphotericin B. The exact passage status of GOT1 cells is not known, as there is no adequate information from the original institute available.

For Western blot experiments, 1 × 10^6 (GOT1) were seeded in 6-well plates and grown for 24 h in complete medium. Then, the medium was replaced by fresh 10% FBS medium and cells were incubated with different concentrations of GNF-5837 (GNF), NGF and everolimus (RAD001). The incubation times were from 10 min up to 72 h. Short times of incubation (10 min) were needed to see the effects on Trk receptor stimulation (NGF), whereas longer times of incubation (>2 h) were needed to see the effects on Trk receptor inhibition (GNF-5837). After incubation, the cells were washed twice in cold PBS on ice and lysed in 200 μL Lysis buffer (M-PER 1 Mammalian Protein Extraction Reagent containing HALT protease & phosphatase inhibitor cocktail, Thermo Scientific). Lysates were centrifuged at 16,060 × g for 10 min. Supernatants were adjusted to the same protein concentration (30–50 μg/50 μL) (Rotiquant Universal, Carl Roth, Karlsruhe, Germany) and denatured in sodium dodecyl sulfate (SDS) sample buffer (0.25% Tris–HCl, 40% glycerol, 2% SDS, 1% dithiothreitol, bromophenol blue, pH 8.8). Equal amounts of protein were separated on a SDS polyacrylamide gel and electrotransferred for 60 min onto PVDF membranes (Immobilon; Millipore) using a semi-dry Western blot technique. After blocking in 2% skimmed milk powder, the membranes were incubated overnight at 4°C in appropriate dilutions of primary antibodies against pTrkA (Tyr 674/S) 1:1000, pTrkB (Tyr 706/7) (#4621) 1:1000, TrkA (#2508) 1:1000, Trkpan (#4609) 1:1000, pAKT (Ser473) (#4060) 1:20,000, Akt(#2920) 1:10,000, pERK (Thr202/Tyr204) (#4370) 1:10,000, p4EBP1 (Ser65) (#9451) 1:2000, 4EBP1 (#9644) 1:5000; pGSK3 (Ser21/9) (#9331) 1:1000, GSK3 (#5676) 1:5000, pp70S6K (Thr389) (#2448) 1:1000, p70S6K (#9234) 1:1000, p70S6K (#9202) 1:1000, pERK (Thr202/Tyr204) 1:1000, pEGFR (Tyr1068) (#4285) 1:1000, EGFR (#2317) 1:2000, cyclin D3 (#2936) 1:2000 (all from Cell Signaling), Actin (A5441) 1:20,000, TrkA (Tyr 674/5) 1:1000, pTrkB (Tyr 706/7) (#4621) 1:1000, TrkA (#2508) 1:1000, Trkpan (#4609) 1:1000, pAKT (Ser473) (#4060) 1:20,000, Akt(#2920) 1:10,000, pERK (Thr202/Tyr204) 1:2000, p4EBP1 (Ser65) (#9451) 1:2000, 4EBP1 (#9644) 1:5000; pGSK3 (Ser21/9) (#9331) 1:1000, GSK3 (#5676) 1:5000, pp70S6K (Thr389) (#2448) 1:1000, p70S6K (#9234) 1:1000, p70S6K (#9202) 1:1000, pS6 (Ser235/236) 1:25000, S6 (#2317) 1:2000, pEGFR (Tyr1068) (#4285) 1:1000, EGFR (#2317) 1:2000, cyclin D3 (#2936) 1:2000 (all from Cell Signaling), Actin (A5441) 1:20,000, Sigma, ERK1/2 (06–182) (Merck-Millipore) 1:20,000, Bcl2 (ab694) 1:1000, TrkA (ab134155) 1:1000, TrkK (ab11796) 1:500 (all from Abcam), p21 Waf1/Cip1 (BD610233) 1:2000 (BD Biosciences, Heidelberg, Germany), p53 (sc-126) 1:20000 (Santa Cruz) Anti-NGF (EP1320Y) (ab52918) 1:2000 (Abcam). After washing in TBS, the membranes were incubated with a peroxidase conjugated secondary antibody (Anti-Rabbit-IgG, HRP-linked Antibody, Cell Signaling, #7074 or Anti-Mouse-IgG, HRP-linked Antibody, Cell Signaling, #7076) 1:25,000 for 2 h. The blots were washed and immersed in the chemiluminescent substrate Super Signal West Dura (Thermo Scientific), and images were taken with an ECL ChemoCam Imager (INTAS, Göttingen, Germany).
Cell cycle analysis by flow cytometric analysis (FACS)

Cell cycle phase distribution was analyzed using propidium iodide staining and flow cytometry (BD Accuri C6 Analysis, BD Biosciences). Cells were cultured in 6-well plates for 24 h. Subsequently, medium was replaced by fresh medium and cells were incubated with different concentrations of GNF-5837. After 72 h, cells were washed with PBS and treated with 300 μL trypsin for 5 min at 37°C. Cells were collected, washed and resuspended in 300 μL propidium iodide solution (Sigma-Aldrich).

Patient cohort and generation of a tissue microarray (TMA)

Patients with NET of the gastroenteropancreatic system treated at the Klinikum der Universität München were enrolled in the NeoExNET (Network of Excellence for Neuroendocrine Tumors in Munich) program. The NeoExNET program was approved by the Local Ethics Committee of the University of Munich and by the data safety manager (Datenschutzbeauftragter) of the University of Munich, and all patients had given written informed consent. Paraffin-embedded tissue samples from pathology specimens of patients enrolled in the NeoExNET program were used to generate a tissue microarray (TMA) of GEP-NETs. The TMA encompassed tumor samples from 107 patients. Histology was reviewed in all tumors to confirm the diagnosis. The specimens were classified to the new WHO 2010 classification scheme and tumor grading (G) was assessed (‘G1’ = 48 patients, ‘G2’ = 51 patients, ‘G3’ = 3 patients and ‘no information’ = 5 patients). A tissue microarray was assembled using 0.6 mm punch biopsies from all samples according to standard procedures (Knosel et al. 2005, 2006).

Immunohistochemical staining of the TMA

Immunohistochemical staining was performed using TrkA (#2508) and Trkpan (#4609) 1:100 from Cell Signaling. Slides were pre-treated by cooking, and then incubated with the antibodies, followed by antibody detection using a biotinylated anti-mouse secondary antibody and a multilink biotin-streptavidin-amplified detection system (Biogenex, San Ramon, CA, USA). A Fastred chromogen was used to visualize staining. A 4-tier scale was used for a semi-quantitative score (0 = negative, 1 = weak expression, 2 = moderate expression, 3 = strong expression).

Statistical analysis

The results are displayed as mean ± standard deviation of the mean (S.D.) of at least three independently performed experiments. Each cell viability experiment consisted of at least 6 samples per substance concentration and incubation period. Some a priori tests considering the normal distribution and homogeneity of variances were performed applying the Kolmogorov–Smirnov test and the Levene's test of the SPSS statistical package SPSS (version 13.0 for Windows, SPSS (2005)). When parametric criteria were met an ANOVA comparison of means with a post hoc Tukey test or a two-tailed t-test was performed; when non-parametric criteria were met the Kruskal–Wallis followed by the Mann–Whitney test was performed. Statistical significance was assessed at P<0.05. For the immunohistochemistry a Pearson chi-square test was performed.

Results

GNF-5837 effectively inhibits GOT1 cell growth

Cell viability assay determined a clear decrease of GOT1 cell viability in vitro in response to the pan-TRK inhibitor GNF-5837 in a time- and dose-dependent manner (Fig. 1 and Table 1). At all three tested incubation periods (48, 96 and 144 h), significant differences when compared to untreated control cells (control DMSO) were detected (P<0.05) (Fig. 1). At a GNF-5837 concentration of 50 nM, a plateau of dose efficacy was reached, where higher doses did not yield a better effect in cell survival decrease (Fig. 1). IC<sub>20</sub> and IC<sub>50</sub> inhibitory concentrations of GNF-5837 in GOT1 cells were calculated (Table 1).

Anti-proliferative effect of the Trk inhibitor GNF-5837 is associated with functional Trk expression in human neuroendocrine tumor cells

GNF-5837 was also tested on the pulmonary carcinoid cell line NCI-H727 and the pancreatic neuroendocrine tumor cell line BON1, however, without any effects on cellular growth inhibition (Supplementary Fig. 1, see section on supplementary data given at the end of this article). In relation with this observed primary GNF-5837 resistance, Western blot analysis failed to detect any expression of TrkA, TrkB or TrkC receptor in BON1 and NCI-H727 cell protein extracts (Fig. 2A). In contrast, protein extracts from human midgut carcinoid GOT1 cells showed significant levels of TrkA expression (145 kDa), faint TrkC
expression (145 kDa) and no TrkB expression (Fig. 2A). A strong signal was also found in protein extracts derived from GOT1 cells, using a pan-Trk antibody detecting TrkA, TrkB and TrkC (Fig. 2A).

A human adult normal brain tissue lysate (ab29466 from Abcam) served as positive control for all three specific Trk receptor antibodies (Numakawa et al. 2010) (Fig. 2A). The different weights of Trk in the human brain tissue (90 kDa) is explained by either alternative splicing forms of Trk (Clary & Reichardt 1994) or an immature form of the receptor (Martin-Zanca et al. 1989) (Fig. 2A).

Next, we investigated the expression levels of the TrkA ligand NGF in all three cell lines. While NGF expression was detectable in human brain tissue (positive control), BON1 and NCI-H727 cells, in GOT1 cells only a low intensity band of NGF could be detected by Western blot analysis (Fig. 2B).

**Demonstration of a functional TrkA receptor in GOT1 cells by NGF-mediated TrkA activation and GNF-5837-mediated TrkA inhibition**

Increasing concentrations of NGF caused a dose-dependent phosphorylation of TrkA in GOT1 cells (Fig. 3A), whereas different concentrations of BDNF failed to stimulate TrkA phosphorylation (Fig. 3A), demonstrating the well-known specificity of the ligand NGF to its receptor TrkA (Amatu et al. 2016). Incubation of GOT1 cells with NGF caused a rapid phosphorylation of TrkA receptor after 10 min, which was partially inhibited by co-incubation with the Trk inhibitor GNF-5837 (Fig. 3B). The phosphorylation status of TrkA decreased continuously with increasing concentration of the pan-TRK inhibitor GNF-5837 (5, 50 and 500 nM) (Fig. 3C). Most prominent dephosphorylation effects were reached at the highest dose of the inhibitor (Fig. 3C). All time periods of incubation showed the same dose-dependent dephosphorylation pattern of TrkA (Fig. 3C). Interestingly, after long periods of incubation (24, 48 and 72 h) with the inhibitor GNF-5837 the unphosphorylated TrkA receptor shows a compensatory upregulation (Fig. 3C).

**Opposite effects of TrkA inhibitor GNF-5837 and TrkA ligand NGF on PI3K-Akt-mTOR signaling, Ras-Raf-MEK-ERK signaling, cell cycle components and anti-apoptotic Bcl2 in GOT1 cells**

Recently, GNF-5837 was reported to effectively inhibit in Trk-driven cancer-specific cellular pathway signaling (Albaugh et al. 2012, Shargh et al. 2016). Here, we aimed to examine which downstream signaling pathways are being affected by the Trk inhibitory function of GNF-5837. We addressed this question using the three Trk expressing NET cell lines (Fig. 2 and 3).

**Table 1**  
IC$_{20}$ and IC$_{50}$ concentration values (in nM) of GNF-5837-mediated inhibition of GOT1 cell proliferation after 48, 96 and 144 h of incubation.

<table>
<thead>
<tr>
<th>Timing of incubation</th>
<th>IC$_{20}$</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 h</td>
<td>4.30 nM</td>
<td>4.23 nM</td>
</tr>
<tr>
<td>96 h</td>
<td>2.45 nM</td>
<td></td>
</tr>
<tr>
<td>144 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>144 h</td>
<td></td>
<td>660 nM</td>
</tr>
</tbody>
</table>
GNF-5837 (5, 50 and 500 nM) after 24 h of incubation (Fig. 4A and Supplementary Fig. 2). One of the most important cellular signaling pathways, which is often activated during carcinogenesis, tumor proliferation and cellular survival in NETs is the PI3K-Akt-mTOR pathway (Cantley 2002, Briest & Grabowski 2014). Here, we show that key components of that pathway, such as pAkt, pp70S6k, pS6 and p4EBP1 were also clearly downregulated after treatment with the pan-Trk inhibitor GNF-5837 (Fig. 4A and Supplementary Fig. 2). Also another main pathway involved in NET tumorigenesis, the Ras-Raf-MEK-ERK pathway, was downregulated by treatment with the pan-TRK inhibitor GNF-5837, as the expression level of pErk decreased (Fig. 4A and Supplementary Fig. 2). Also the cell cycle component Cyclin D3 and cell cycle regulator p21 were downregulated (Fig. 4A and Supplementary Fig. 2). Interestingly, the expression level of the anti-apoptotic Bcl2 also decreased, suggesting an augmentation in apoptotic cell death mechanisms (Fig. 4A and Supplementary Fig. 2).

In contrast to the effects observed during incubation with the pan-Trk inhibitor GNF-5837 (Fig. 4A and Supplementary Fig. 2), TrkA receptor stimulation with NGF lead to opposite effects regarding PI3K-Akt-mTOR signaling, Ras-Raf-MEK-ERK signaling and cell cycle regulation (Fig. 4B and Supplementary Fig. 3). Only anti-apoptotic Bcl2 expression showed no major change in expression level upon NGF stimulation (Fig. 4B and Supplementary Fig. 3).

**GNF-5837 induces apoptosis and G1 cell cycle arrest**

Cell cycle analysis confirmed the suggested downregulation of the cell cycle and induction of apoptosis in response to GNF-5837 treatment, by significantly increasing the number of cells in G1 and sub-G1 phases, respectively (Fig. 7).

**Combined treatment with GNF-5837 and everolimus causes additive inhibitory effects on GOT1 cell growth mediated by PI3K-Akt-mTOR pathway and Cyclin D3 downregulation**

GOT1 cell viability was also analyzed combining the pan-Trk inhibitor GNF-5837 with the mTOR inhibitor everolimus (Fig. 5A). The combined treatment of GNF-5837 (500 nM) with everolimus (10 nM) showed a modest but significant enhancement over single substance...
Figure 4
(A) Effects of different concentrations of GNF-5837 (5, 50 and 500) on the expression level of different components from the cell cycle, the apoptotic response and the PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathway after 24 h of incubation in GOT1 cells. Basal protein expression level of proteins was evaluated by Western blot analysis. A representative blot out of three independently performed experiments is shown.

Figure 5
(A) Effects of different concentrations of NGF (0.76 nM and 7.6 nM) on the expression level of different components from the cell cycle, the apoptotic response and the PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathway after 24 h of incubation in GOT1 cells. Basal protein expression level of proteins was evaluated by Western blot analysis. A representative blot out of three independently performed experiments is shown.

Immunohistochemistry showed TrkA-positive staining only in pancreatic neuroendocrine tumor specimens

In order to assess the prevalence of Trk receptor expression in actual GEP-NET tumor samples, we immunohistochemically stained a tissue microarray (TMA) with TrkA and pan-Trk antibodies (Fig. 6). We scaled the TMA probes for a semi-quantitative score (0 = negative, 1 = weak expression, 2 = moderate expression, 3 = strong expression).

In a total of 107 (100%) GEP-NET tumor specimens on the TMA, the primary tumor localization was small intestinal (SI)-NET in 65 cases (60.7%), pancreas (p)NET in 33 (30.8%), gastric NET in 1 (0.9%), colon NET in 3 (2.8%), appendix NET in 3 (2.8%) and cancer of unknown primary (CUP) in 2 (1.9%) cases (Table 2).

The antibody against TrkA stained positive (weak and moderate) exclusively in tumor samples of pancreatic primary localization as 24% (8/33) of all pNET samples stained positive for TrkA (Table 2). A statistically significant relation between primary tumor localization and TrkA/Trkpan expression level was proved.

Using a pan-Trk antibody (detecting endogenous levels of total Trk protein encompassing TrkA, TrkB and TrkC), positive IHC expression was again found in all 8 previously TrkA-positive pNET samples (Table 2), as expected. In addition, the pan-Trk antibody caused positive staining in another 3 pNET samples which had shown negative IHC expression in the previous staining with the TrkA antibody. In total, using the pan-Trk antibody 33.3% (11/33) of all pNETs stained positive for...
Trk family receptor expression (Table 2). In addition, 1/65 (=1.5%) SI-NETs also stained positive with the pan-Trk antibody and 1/3 appendix NETs (=33.3%) (Table 2). Taken together, our results demonstrate that a total of 12.2% (13/107) of NET samples stained positive for receptors of the Trk family (Table 2).

Discussion

Here, we report on the potential role of Trk inhibitors as new molecular targeting approach for NETs of the GEP system. We evaluated the effects of the specific pan-Trk inhibitor GNF-5837 either alone or in a dual-targeting approach on major cellular signaling mechanisms such as the PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathway as well as cell cycle components. Furthermore, we evaluated the prevalence of Trks immunohistochemically in a large patient cohort and could assess possible predictive markers for personalized molecular targeted therapy with Trk inhibitors.

In the first part of this study, effects of the highly selective pan-Trk inhibitor GNF-5837 (Albaugh et al. 2012) were investigated in human neuroendocrine tumor cell lines in vitro.

GNF-5837 significantly decreased GOT1 cell survival (Fig. 1 and Table 1) due to TrkA inhibition (Fig. 2A).
In contrast, lack of Trk expression in BON1 and NCI-727 cell lines (Fig. 2A) and thus obvious independence of cell growth from Trk family receptors caused GNF-5837 treatment resistance in BON1 and NCI-727 cell lines (Supplementary Fig. 1) and confirmed GNF-5837 to be a highly selective pan-Trk inhibitor (Albaugh et al. 2012). The anti-proliferative effects of Trk inhibition in neuroendocrine GOT1 tumor cells are in accordance with findings in other preclinical cancer cell models (Dolle et al. 2003, Albaugh et al. 2012, Seo et al. 2013, Shargh et al. 2016). Accordingly, Trk overexpression showed to enhance tumor growth, metastasis formation and invasiveness in different tumor entities in vitro (Sortino et al. 2000, Lagadec et al. 2009). In the GOT1 cells from a concentration of 50 nM on a plateau effect is observed, where further concentration augmentation did not yield a better cell killing effect (Fig. 1). This might be explained by a maximum TrkA receptor occupation of GNF-5837 at this concentration (Maliartchouk et al. 2000).

Incubation of GOT1 cells with GNF-5837 caused inhibition of TrkA activity as demonstrated by a decrease of TrkA phosphorylation status (Fig. 3A and C), while only specific NGF stimulation caused TrkA phosphorylation (Fig. 3A and B). In contrast, GNF-5837 caused a significant increase of total un-phosphorylated TrkA receptor expression levels in GOT1 cells, possibly due to compensatory feedback mechanisms (Fig. 3C). Similar feedback loops with an upregulated re-expression of TrkA after inhibition have been reported in human airway smooth muscle cells (Freund-Michel & Frossard 2008). In Trk receptor-deficient BON1 and H727 cells, NGF showed a strong expression level (Fig. 2B). Here, we hypothesize that the high expression levels of NGF might be due to the lack of the specific receptor (TrkA), trying to compensate for the missing cellular TrkA-NGF signaling.

**Table 2** TrkA and pan-Trk expression level in different primary tumor localizations from 107 patient probes.

<table>
<thead>
<tr>
<th>TrkA expression level</th>
<th>Small intestine</th>
<th>Pancreas</th>
<th>CUP</th>
<th>Stomach</th>
<th>Colon</th>
<th>Appendix</th>
<th>Total</th>
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<tr>
<td>TrkA negative</td>
<td>n=65</td>
<td>n=25</td>
<td>n=2</td>
<td>n=1</td>
<td>n=3</td>
<td>n=3</td>
<td>n=99</td>
</tr>
<tr>
<td>TrkA weak expression</td>
<td>n=0</td>
<td>n=7</td>
<td>n=0</td>
<td>n=0</td>
<td>n=0</td>
<td>n=0</td>
<td>n=7</td>
</tr>
<tr>
<td>TrkA moderate expression</td>
<td>n=0</td>
<td>n=1</td>
<td>n=0</td>
<td>n=0</td>
<td>n=0</td>
<td>n=0</td>
<td>n=1</td>
</tr>
<tr>
<td>Total TrkA positive</td>
<td>n=0/65 (=0%)</td>
<td>n=8/33 (=24.2%)</td>
<td>n=0/2 (=0%)</td>
<td>n=0/1 (=0%)</td>
<td>n=0/3 (=0%)</td>
<td>n=0/3 (=0%)</td>
<td>n=8/107 (=7.5%)</td>
</tr>
<tr>
<td>pan-Trk expression</td>
<td>n=64</td>
<td>n=22</td>
<td>n=2</td>
<td>n=1</td>
<td>n=3</td>
<td>n=2</td>
<td>n=94</td>
</tr>
<tr>
<td>pan-Trk weak expression</td>
<td>n=0</td>
<td>n=9</td>
<td>n=0</td>
<td>n=0</td>
<td>n=0</td>
<td>n=0</td>
<td>n=9</td>
</tr>
<tr>
<td>pan-Trk Moderate expression</td>
<td>n=0</td>
<td>n=1</td>
<td>n=0</td>
<td>n=0</td>
<td>n=0</td>
<td>n=1</td>
<td>n=2</td>
</tr>
<tr>
<td>pan-Trk strong expression</td>
<td>n=1</td>
<td>n=1</td>
<td>n=0</td>
<td>n=0</td>
<td>n=0</td>
<td>n=0</td>
<td>n=2</td>
</tr>
<tr>
<td>Total pan-Trk positive</td>
<td>n=1/65 (=1.5%)</td>
<td>n=11/33 (=33.3%)</td>
<td>n=0/2 (=0%)</td>
<td>n=0/1 (=0%)</td>
<td>n=0/3 (=0%)</td>
<td>n=1/3 (=33.3%)</td>
<td>n=13/107 (=12.2%)</td>
</tr>
</tbody>
</table>

Cancer of unknown primary tumor are designated ‘CUP’.

Figure 7
Cell cycle analysis measured by flow cytometry. The arithmetic means and s.d. of at least three independent experiments are shown. Statistical significant different results in comparison to the Control DMSO are shown, considering *P<0.05; **P<0.01; ***P<0.001. A full color version of this figure is available at [https://doi.org/10.1530/ERC-17-0201](https://doi.org/10.1530/ERC-17-0201).
cascade. Tagliabue et al. found similar results when breast cancer cells with poor TrkA expression showed high NGF expression in order to recruit other receptors to induce cancer cell growth (Tagliabue et al. 2000).

The PI3K-Akt-mTOR and the Ras-Raf-MEK-ERK pathway are often deregulated in human cancers including NETs, and the importance of these pathways comprises cellular oncogenic modulation mechanisms such as cell growth, survival, proliferation, angiogenesis and motility (Cantley 2002, Van Gompel et al. 2005, Kunnimalaiyaan et al. 2007, Zitzmann et al. 2007, 2011, Cook et al. 2010, Iida et al. 2012, Valentino et al. 2014, Briest & Grabowski 2014). TrkA activation and downstream signaling upon NGF stimulation has been shown in multiple cell types to mediate its proliferative and anti-apoptotic characteristics mainly through PI3K-Akt-mTOR and Ras-Raf-MEK-ERK cascade signaling (Sofroniew et al. 2001, Huang & Reichardt 2003, Rahbek et al. 2005, Jang et al. 2007, Lawrence et al. 2008, Julio-Pieper et al. 2009, Amatu et al. 2016, Demir et al. 2016, Pediatitakis et al. 2016). In accordance to these data, in GOT1 cells, NGF stimulation induced the PI3K-Akt-mTOR and Ras-Raf-MEK-ERK signaling cascade (Fig. 4B), while inhibition of TrkA signaling by the pan-Trk inhibitor GNF-5837 caused downregulation of both downstream signaling pathways (Fig. 4A). Accordingly, in a colorectal cancer cell line (KM12) and a LCNEC cell line, pan-Trk inhibition with AZD7451 leads to a cellular survival decrease through pErk or pAkt downregulation (Tatematsu et al. 2014). As a consequence to PI3K-Akt-mTOR and Ras-Raf-MEK-ERK alteration, downstream cell cycle component Cyclin D3 was upregulated upon NGF stimulation or downregulated upon GNF-5837 inhibition, respectively (Fig. 4). Furthermore, a significant population increase in the cell cycle phase G1 confirms a cell cycle downregulation upon GNF-5837 treatment (Fig. 7). GOT1 cells showed not only a strong TrkA expression but also a slight expression of TrkC (Fig. 2A). Besides inhibition of TrkA, also inhibition of TrkC, might contribute to Ras-Raf-MEK-ERK and PI3K-Akt-mTOR pathway inhibition following incubation with the pan-Trk inhibitor GNF-5837. In adenoid cystic carcinoma cells, TrkC signaling was activated and required its ligand NT-3 to stimulate invasive behavior through Ras-Raf-MEK-ERK and PI3K-Akt-mTOR signaling (Ivanov et al. 2013).

Interestingly, the cyclin-dependent kinase inhibitor p21 is upregulated upon NGF stimulation (Fig. 4B and Supplementary Fig. 3) and downregulated upon Trk inhibition by GNF-5837 treatment (Fig. 4A and Supplementary Fig. 2). This fact might be explained by induction of the anti-apoptotic Bcl2 after NGF stimulation and the downregulation of Bcl2 and augmentation of the Sub-G1 population after GNF-5837 inhibition (Figs 4 and 7). Studies have shown that in some systems, the induction of apoptotic mechanisms relate with p21 inhibition, counteracting its tumor-suppressive function (Gartel & Tyner 2002).

Due to the PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathway downregulation upon Trk inhibition by GNF-5837 alone (Fig. 4A and Supplementary Fig. 2), we aimed to further investigate the effects of GNF-5837 in a dual-targeting approach with the mTORC1 inhibitor everolimus. The mTOR inhibitor everolimus has proven anti-tumoral efficacy in several clinical phase 3 trials in patients with NET (Pavel et al. 2011, Yao et al. 2016a,b). In NET cell lines, everolimus has been shown to inhibit mTOR downstream signaling and to cause a compensatory upregulation of the Ras-Raf-MEK-ERK signaling cascade (Zitzmann et al. 2010, Wagle et al. 2014, Capozzi et al. 2015, He et al. 2016, Vandamme et al. 2016, Zatelli et al. 2016). Furthermore, development of resistance against everolimus during long-term treatment is often observed in NETs and possibly caused by feedback loops on the PI3K-Akt axis (Zitzmann et al. 2010, Wagle et al. 2014, Capozzi et al. 2015, He et al. 2016, Vandamme et al. 2016, Zatelli et al. 2016). Thus, the potential benefit of a dual-targeting approach is not only to enhance single substance treatment efficacy, but also to prevent and/or counteract resistance mechanisms. We found that the combinational approach of the Trk inhibitor GNF-5837 and the mTOR inhibitor everolimus caused additive anti-proliferative effects (Fig. 5) and agonistically downregulated the PI3K-Akt-mTOR pathway, as well as Cyclin D3 (Fig. 5B and Supplementary Fig. 4). In a Trk-induced leukemia mousemodel, the tumor cells showed a high susceptibility toward the mTORC1 inhibitor rapamycin and RAD001 (everolimus) via PI3K-Akt-mTOR pathway and Ras-Raf-MEK-ERK downregulation (Rhein et al. 2011).

In the second part of this study, we examined the expression of Trk family receptors in a tissue microarray (TMA) encompassing 107 tumor probes of patients with GEP-NETs. Using a specific TrkA antibody, a positive staining in 8/107 tumor probes was exclusively due to the subgroup of pNETs with a positive staining in 8/33 (24%) of all pNETs (Table 2). Using a pan-Trk antibody (detecting endogenous levels of total Trk protein encompassing TrkA, TrkB and TrkC) a positive staining in 13/107 tumor probes
was mainly due to the subset of pNETs with a positive staining in 11/33 (33%) of all pNETs (Table 2). These data demonstrate a high prevalence of Trk receptor expression in pancreatic NETs. Therefore, immunohistochemical or molecular screening for Trk expression in pNETs might be useful as a predictive marker to select patients with pNETs expressing Trks for molecular targeted therapy with Trk inhibitors. Whether Trk overexpression or overactivation caused by chromosomal rearrangements and NTRK gene fusions (Amatu et al. 2016) is also existent in NETs should be investigated in further studies. Interestingly, in the malignant neuroendocrine Merkel cell carcinoma, expression of TrkA was found in all tested tumor specimens (36/36), underlining a possible therapeutic relevance of Trk blockage in NETs not only from the GEP system (Wehkamp et al. 2017).

A limitation of our study is the fact that currently available human neuroendocrine tumor cell lines might not reflect the actual biological expression state of Trk receptors in neuroendocrine tumors in vivo. While the SI carcinoid cell line GOT1 was the only tested cell line expressing a functional Trk receptor (Figs 1, 2A and 3), the TMA analysis revealed Trk expression in SI NETs to be a very rare event as only 1/65 (1.5%) of SI NET tumor probes showed a positive staining with the pan-Trk antibody (Table 2). Further investigation regarding the effects of GNF-5837 in NETs is required in order to validate molecular target credentials and clinical relevance.

Taken together our findings, the highly selective pan-Trk inhibitor GNF-5837 presents significant antiproliferative efficacy in human neuroendocrine GOT1 cells in vitro. Inhibition of TrkA signaling in GOT1 cells caused subsequent downregulation of the PI3K-Akt-mTOR and Ras-Raf-MEK-ERK signaling cascades, as well as the cell cycle and upregulation of apoptosis. A dual-targeting approach using the pan-Trk inhibitor GNF-5837 plus the mTOR inhibitor everolimus showed additive inhibitory effects on PI3K-Akt-mTOR and cyclin D3. Tissue microarray analysis revealed Trk expression in 24% (TrkA)/33% (pan-Trk) of pancreatic NETs. Immunohistochemical or molecular screening for Trk expression in pNETs might be useful as a predictive marker to select patients with pNETs expressing Trks for molecular targeted therapy with Trk inhibitors.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/ERC-17-0201.

Declaration of interest
CJA has received research contracts (Ipsen, Novartis), lecture honorarium (Ipsen, Novartis, Pfizer, Falk) and advisory board honorarium (Novartis). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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