Inhibition of mTOR's Catalytic Site by PKI-587 Is a Promising Therapeutic Option for Gastroenteropancreatic Neuroendocrine Tumor Disease

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Key Words

PKI-587 · Gedatolisib · mTOR inhibition · Everolimus · Gastroenteropancreatic neuroendocrine tumors · Signaling · 4E-BP1 · LCC-18 cell line

Abstract

Background: The characteristic clinical heterogeneity and mostly slow-growing behavior of gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) cause problems in finding appropriate treatments. Thus, the current therapy options are not satisfactory. PKI-587 is a highly potent, novel dual inhibitor of PI3K and mTORC1/C2. **Aim:** We assessed the effects of PKI-587 in different GEP-NEN tumor models, including the poorly differentiated cell line LCC-18, and compared them with those of the established mTORC1 inhibitor everolimus. **Methods:** We treated BON, QGP-1, KRJ-1, and LCC-18 cell lines with increasing concentrations of the inhibitor PKI-587, and compared the results with those of everolimus and DMSO. We assessed the impact of the

treatments on viability (WST-1 assay), on apoptotic processes (caspase 3/7 assay, JC-1), and on cell cycle regulation (flow cytometry). We determined alterations in signaling mediators by phosphor-specific Western blot analysis and conducted multiplexed gene expression analysis (nCounter[®] technology). **Results:** In all cell lines, PKI-587 dose-dependently inhibited proliferation, whereas everolimus was less effective. Treatment with PKI-587 led to cell cycle arrest and induction of apoptosis and successfully suppressed activity of the direct mTORC1 target 4E-BP1, a crucial factor for tumor genesis only partially inhibited by everolimus. Gene expression analyses revealed relevant changes of RAS, MAPK, STAT, and PI3K pathway genes after treatment. Treatment-dependent and cell line-characteristic effects on AKT/Rb/E2F signaling regarding cell cycle control and apoptosis are extensively discussed in this paper. **Conclusion:** PI3K/mTOR dual targeting is a promising new therapeutic approach in neuroendocrine tumor disease that should be evaluated in further clinical trials.

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Introduction

Current Therapy Options for Gastroenteropancreatic Neuroendocrine Neoplasms and Their Pathogenesis

The rare tumor entity of gastroenteropancreatic neuroendocrine neoplasm (GEP-NEN) shows heterogeneity in its clinical behavior and lacks proper therapeutic options. A small, aggressive subgroup shows high proliferation rates, but most GEP-NENs demonstrate slow-growing characteristics, which makes them inaccessible to classical chemotherapies [1]. Despite their increasing incidence, GEP-NENs are counted among the rare neoplastic diseases. However, regarding their prevalence, they are the second most common malignancy in the gastrointestinal system [2].

Previous developments of molecular targeting substances have led to the approval of two members of this compound class for treatment of GEP-NENs. These are everolimus, an inhibitor of mTOR (mechanistic target of rapamycin) [3–5], and sunitinib, a multikinase inhibitor. Both compounds are approved only for the subgroup of progressive neuroendocrine pancreatic tumors [6–8]. Everolimus showed disease-stabilizing activity in intestinal and bronchial tumors as well, but better responses are rare (RADIANT-4 [9, 10]).

The pathogenesis and regulation of proliferation of GEP-NENs are not well investigated. While causal mutations have rarely been found, several pathways were shown to have a determining influence on extended cell growth [reviewed in 1]. Among these, the PI3K/AKT (phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B) signaling cascade, which regulates mTOR, is regarded as an important switch point for protein translation and therefore inducing proliferation and survival [11, 12] (fig. 1).

Everolimus and Its Limitations

The key mediator mTOR serves as a target for the approved inhibitor everolimus (also known as RAD001) and builds two distinct complexes, mTORC1 and mTORC2 (mTOR complex 1 and mTOR complex 2), with different binding partners and target structures [13]. Everolimus selectively inhibits the function of mTORC1 by interfering with raptor (regulatory-associated protein of mTOR)-dependent complex building and therefore prohibiting phosphorylation of the direct targets p70S6K (ribosomal protein S6 kinase, 70 kDa) and 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein) [14].

However, several factors limit the efficacy of treatment with everolimus. Because of the mTORC1 selectivity, activating feedback occurs via mTORC2. Particularly the activation of upstream PI3K/AKT signaling through p70S6K/mTORC2/AKT but also through p70S6K/IRS-1 (insulin receptor substrate 1)/PI3K are considered to be crucial in this context (fig. 1) [15–18]. Further, it was reported that rapamycin analogs like everolimus only partially inhibit mTORC1, leading to rephosphorylation of 4E-BP1 even though raptor-dependent inhibition of p70S6K is maintained [19, 20]. Comparing p70S6K and 4E-BP1, the former turned out to be dispensable for tumor genesis, while 4E-BP1 and its impact via cap-dependent translation was described as a ‘funnel factor’ for cancer cell growth and progression [21] and was associated with malignancy and adverse prognosis [22]. Use of an inhibitor occupying the catalytic site of mTOR was shown to prevent 4E-BP1 rephosphorylation [21].

PKI-587 and the Aim of the Investigation

A more novel approach to avoid these limitations of feedback and the merely partial inhibition of mTORC1 is the development of dual PI3K/mTOR inhibitors such as PKI-587 by Pfizer (PF-05212384, gedatolisib). These compounds are working ATP competitively [23] and are therefore equally effective in both complexes of mTOR, mTORC1 and mTORC2.

In a recently published, first-in-human phase I clinical trial, its safety risk was classified as manageable [24]. Other preclinical studies reported an efficacy of PKI-587 in vitro and in vivo [25], but observations of PKI-587 activating other pathways [e.g., MAPK (mitogen-activated protein kinases) signaling] were mentioned as well [26].

Because we consider PKI-587 as a promising compound for treating GEP-NENs, we assessed its in vitro impact on several GEP-NEN cell lines and compared it with everolimus as the reference standard. In our study, we did not restrict ourselves to tumor models of typical slow-growing behavior (BON, KRJ-I, and QGP-1) but also included poorly differentiated, fast-proliferating LCC-18 cells, representing a rare and aggressive tumor subset. We investigated antiproliferative effects, the appearance of apoptosis, and cytotoxic events and performed cell cycle analyses. On the molecular level we conducted phospho-specific Western blot and gene expression analyses in order to gain basic insights into the characteristic behavior of the different tumor subtypes.

Fig. 1. PI3K/Akt/mTOR signaling is highly activated in GEP-NEN [1, 14]. Extracellular signals, bound to RTK (receptor tyrosine kinases), lead to activation of PI3K via IRS-1, and therefore to phosphorylation of PIP₂ [phosphatidylinositol (3,4)-bisphosphate] to PIP₃ [phosphatidylinositol (3,4,5)-trisphosphate] [54–56]. Constitutively active PDK-1 (3-phosphoinositide-dependent protein kinase-1) requires PIP₃ to phosphorylate – and hence activate – Akt, stimulated by prior Akt phosphorylation at Ser473 through mTORC2 [37, 57]. Active Akt phosphorylates a wide range of substrates (e.g., mTORC1), mostly inhibiting their activity, and therefore stimulates protein translation and the cell cycle and inhibits apoptotic processes [1]. mTORC1 is the target complex for both inhibitors of this study. Everolimus (RAD001) is an allosteric raptor-dependent inhibitor of mTORC1, whereas PKI-587 inhibits the catalytic activity of mTOR in both complexes [14, 23]. Crucial targets of mTOR are p70S6K and 4E-BP1. Phosphorylation of p70S6K by mTOR (Thr389) affects ribosomal protein S6, leading to protein translation [58, 59]. 4E-BP1 is a translation suppressor complexed with eIF4E (eukaryotic translation initiation factor 4E) to prevent cap-dependent translational activity. Phosphorylation of 4E-BP1 causes dissociation of the complex, relieving the inhibitory property [60]. mTORC2 is a complex including rictor (rapamycin-insensitive companion of mechanistic target of rapamycin) and is located upstream of Akt, leading to its phosphorylation at Ser473 [61]. As p70S6K is able to disrupt mTORC2, inhibition of p70S6K subsequently leads to Akt activation. This mechanism is regarded as an important feedback loop limiting the efficacy of mTORC1-specific RAD001 treatment [62]. Other important treatment escape mechanisms are activation of Akt via p70S6K/IRS-1/PI3K or activation of connected MAPK signaling [63, 64].

Materials and Methods

Cell Culture

For all in vitro experiments we employed several GEP-NEN cell line models representing different tumor subtypes. In particular, we used pancreatic BON [27, 28] and QGP-1 [29, 30] cells, ileal KRJ-I [31, 32] cells, and colonic LCC-18 [33] cells.

BON and KRJ-I cell lines were grown in DMEM/Ham's F12 (1:1) medium (Biochrom GmbH), QGP-1 in RPMI medium, and LCC-18 in DMEM medium (both PAA Laboratories GmbH). All

media contained 10% FBS Gold (PAA Laboratories GmbH; one single batch for all experiments of this project), 1% penicillin/streptomycin (PAA Laboratories GmbH), and stable glutamine (RPMI and DMEM, also 25 mM HEPES; Biochrom GmbH). The cells received fresh medium twice a week and were held in exponential growth stadium (split once or twice a week). Confluence never exceeded 80% and culture periods were less than 10 weeks. In a regular, recurring procedure, we tested the cell lines for maintenance of specific neuroendocrine marker expression (e.g., chromogranin A, synaptophysin, syntaxin, and synaptobrevin)

by immunofluorescence microscopy. After their receipt in 2012 and 2013, all cell lines were authenticated or verified as unique by the DSMZ (The Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany).

Reagents and Antibodies

The ATP-competitive, dual PI3K/mTOR inhibitor PKI-587 (gedatolisib, PF-05212384) was kindly provided by Pfizer Inc. (Milwaukee, Wis., USA). RAD001 (everolimus) was purchased from Cell Signaling Technology Inc.

Primary antibody against GAPDH was purchased from Gene-Tex Inc.; all other primary antibodies were purchased from Cell Signaling Technology Inc. The secondary antibodies swine anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were purchased from DAKO Deutschland GmbH.

Cell Viability Assay

Cells were seeded into 96-well plates, at 7,000–20,000 cells per well, and were left to adhere overnight. For the viability assay, cells were incubated with PKI-587 or RAD001 at eight different concentrations (0.01 nM to 5 μ M, assessed for each cell line separately in preliminary testing) or with DMSO as a control (in quintuplicate) for 24, 48, and 96 h. The cell proliferation reagent WST-1 (Roche Diagnostics GmbH) was added according to the manufacturer's instructions. Measurement of absorption was followed by use of a Tecan Sunrise microplate reader (Tecan Trading AG). Each experiment was replicated 3 times independently.

Based on the viability data, we calculated the dose-response curve (GraphPad Prism 6) and determined the IC₅₀ (relative IC₅₀, concentration of half-maximal effect [34]), C_{mid} (ca. 80% of the maximal effect, IC₂₀), and C_{max} (concentration of maximal response), considering different incubation times regarding the different requirements of the following experiments.

Multiplexed Viability, Cytotoxicity, and Apoptosis Assay

Cells were seeded into 96-well plates, at 7,000–15,000 cells per well, and were left to adhere overnight. The cells were incubated with PKI-587 or RAD001 at low (IC₅₀) and high (C_{max}) concentrations or with DMSO as a control (in triplicate) for 12 and 36 h. A fluorescence reagent mix for detection of viability and cytotoxicity (different wave lengths) and, later, a chemiluminescence reagent for detection of caspase 3/7 activity were added following the manufacturer's instructions (ApoTox-Glo Triplex Assay; Promega). Measurements were conducted using Tecan Infinite M200 (fluorescence) and Berthold Centro LB 960 plate readers. This experiment was performed 3 times independently.

Cell Cycle Analysis and Determination of Mitotic Index

Cells were seeded into 3.5-cm dishes, at 125,000–500,000 cells per dish, and were left to adhere overnight and incubated with PKI-587 or RAD001 at low (IC₅₀) and high (C_{max}) concentrations or with DMSO as a control for 48 h (all cell lines) and 96 h (just QGP-1 and LCC-18), respectively, in four biological replicates. Fixing and staining of each sample was accomplished as described previously [35]. Mitotic cells were dyed with mitosis-specific Phospho(Ser10)-Histone H3 primary antibody (1:1,600; Cell Signaling Technology Inc., No. 3377) and Alexa Fluor 488 goat anti-rabbit secondary antibody (1:500; Invitrogen, No. A11008); propidium iodide (Invitrogen) was used to quantify the DNA content

of the cells. Fluorescence signals were detected by flow cytometry (BD FACSCalibur and attendant CellQuest Pro software; analyzed by FlowJo 8.7 software).

Assessment of Mitochondrial Membrane Potential $\Delta\psi_m$

Cells were seeded into 3.5-cm dishes, at 375,000 cells per dish, and were left to adhere overnight and incubated with PKI-587 or RAD001 at low (IC₅₀) and high (C_{max}) concentrations or with DMSO as a control for 16 h, respectively, in four biological replicates. The samples were incubated with JC-1 reagent (5 μ M; AAT Bioquest) for 30 min at 37°C; CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone, 50 μ M; Abcam Biochemicals) functioned as a positive control. Fluorescence signals were detected by flow cytometry (BD FACSCalibur and attendant CellQuest Pro software; analyzed by FlowJo 8.7 software).

Western Blot

Following the incubation of cells with PKI-587, RAD001, or DMSO control at intermediate concentrations (C_{mid}) for 24 h, the cells were lysed in NP-40 buffer. The content of total protein was assessed using the Bradford method; 20 μ g protein was used per lane. SDS-PAGE and Western blot were performed using a standard protocol. Membranes were blocked in 5% milk/TBS-T for 60 min and incubated with dilutions of primary antibody overnight. After incubation with secondary HRP-labeled antibody, detection followed using the ECL Prime Western Blotting Reagent (Amersham, GE Healthcare) with a Fujifilm LAS 4000 luminescent image analyzer. For re-probing, antibody binding was removed with acidic glycine buffer (pH 2.3). Always four biological replicates were detected simultaneously. Densitometric analysis was conducted with Fujifilm Multigauge version 3.0 software, and band density was normalized with Coomassie total protein stain ratios for each lane. The densitometric data from the four replicates were averaged and compared with the control.

Analysis of Gene Expression

(NanoString®, nCounter® Technology)

Cells (only KRJ-I, LCC-18, and QGP-1, since p53-mutated BON cells do not provide added information) were seeded in 6-cm dishes (1.5–4 $\times$ 10⁶ cells/dish) and incubated with PKI-587 or RAD001 at intermediate concentrations (C_{mid}) or with control for 60 h, respectively, in two biological replicates.

For extraction of RNA we used the RNeasy Mini Kit (Qiagen), carrying out the instructions of the manufacturer; the total RNA content of each sample was assessed using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). We used 60 ng RNA per sample for processing the nCounter® PanCancer Pathway Panel gene expression analysis (NanoString®). This innovative technology contains 730 different probes binding complementary to corresponding target mRNAs (hybridization at 65°C overnight). The probes were labeled with gene-specific fluorescence bar codes and immobilized on a coated slide. Sample preparation was conducted with nCounter® PrepStation 5s, and microscopy scanning of the bar code signals with nCounter® Digital Analyzer 5s (NanoString®).

The data were analyzed by first principal component analysis using nSolver® 2.5 PanCancer Pathway Analysis Module software (NanoString®). The resulting absolute log fold change data on the single genes were summed up into pathway scores.

Statistical Analyses

For statistical analysis, we used Microsoft Excel and GraphPad Prism 6 analysis software (San Diego, Calif., USA). The data passed a Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lilliefors p value) for a Gaussian distribution, appropriate for data sets below 20 data points. Outliers were identified by Grubbs' test with $\alpha = 0.05$.

Dose-response curves were fitted to the measured data using the method of least squares with variable slope; goodness of fit was quantified with R^2 and sums of squares. For detection of significant differences between two or more data groups, we used ANOVA with or without repeated measures (depending on the experimental design) with a p value of 0.05.

Results

Reduction of Cell Viability by PKI-587 in Neuroendocrine Cells (WST-1 Assay)

Incubation of the four cell lines with everolimus (RAD001) or PKI-587 shows a time- and concentration-dependent decrease in cell viability for both treatments. In comparison, this effect of the dual PI3K/mTOR inhibitor PKI-587 was more distinct than that of RAD001 as an mTORC1-selective inhibitor. PKI-587 reduced the amount of living cells by 61–94%, whereas RAD001 reduced it only by 47–52% (fig. 2a).

IC₅₀ of PKI-587 in Neuroendocrine Cell Lines

The concentration of half-maximal effect (relative IC₅₀ [34]) was determined for both substances under the same experimental conditions based on WST-1 data. PKI-587 showed higher IC₅₀ values (25–250 nM) than RAD001 (1–2.5 nM). For C_{max}, the concentration of maximal effect, we chose a low concentration of the lower plateau of the dose-response curve. The intermediate concentration, C_{mid}, was determined relative to IC₂₀, the concentration of 80% of the maximal effect (fig. 2b). The dose-response curves are shown in the online supplementary material (for all online suppl. material, see www.karger.com/doi/10.1159/000448843).

Apoptosis Induction through PKI-587 Treatment (JC-1, Flow Cytometry)

To investigate the occurrence of early apoptotic processes, we assessed the mitochondrial membrane integrity (JC-1, flow cytometry) after treatment of cell lines with RAD001 or PKI-587 for 16 h. Significant changes in the mitochondrial membrane potential ($\Delta\psi_m$) emerged through PKI-587 treatment at high concentrations (C_{max}) in BON and LCC-18 cells. An increase in apoptotic cells was also detectable in KRJ-I cells, but it was not significant ($p = 0.09$) (fig. 2c).

Assessment of Viability, Apoptosis, and Cytotoxicity by the Multiplexed ApoTox-Glo Assay

Viability. The viability data obtained with this assay subsequently confirmed the WST-1-derived data (data not shown).

Apoptosis. We detected caspase 3/7 activity after 36 h of treatment of the cell lines with RAD001 or PKI-587. At high concentrations (C_{max}), the dual inhibitor PKI-587 caused significant increases in apoptosis in BON, KRJ-I, and LCC-18 cells. RAD001 induced a significantly higher caspase 3/7 activity only in KRJ-I cells (fig. 2c). These data confirm the findings from the JC-1 assay of membrane potential integrity.

Cytotoxicity. Measurement of cytotoxicity after 12 h of treatment did not show any increase or decrease in dead cell protease versus the control (data not shown).

PKI-587 Causes Stronger Attenuation of Cell Cycle and G₀/G₁ Arrest

The flow cytometry cell cycle studies after PKI-587 treatment of cells revealed dose- and time-dependent alterations in proliferation. G₀/G₁ arrest occurred along with decreasing amounts of cells in the S, G₂, and M phases. Particularly in the pancreatic cell lines BON and QGP-1, this effect showed significance. These effects could be detected after RAD001 treatment as well, but they were notably weaker (fig. 3a, b).

In addition to these findings, flow cytometry analysis revealed apoptosis in terms of sub-G₁ peaks caused by PKI-587 treatment in the two intestinal cell lines KRJ-I and LCC-18 (fig. 3c).

Fig. 2. a, b We treated cell lines with different concentrations of everolimus (RAD001), PKI-587, or vehicle control for 0, 24, 48, and 96 h and assessed cell viability with the WST-1 assay (Roche). **a** Maximally reached effects on viability in percent of control (indicated by the lower plateau of the dose-response curve; mean $\pm$ SD). Left: RAD001 (BON: 50%; KRJ-I: 52%; LCC-18: 50%; QGP-1: 47%). Right: PKI-587 (BON: 94%; KRJ-I: 61%; LCC-18: 90%; QGP-1: 78%). **b** IC₅₀, C_{mid}, and C_{max} were determined by nonlinear fitting of the dose-response curves (also refer to the online suppl. material). **c** The cell lines were treated with RAD001 (RAD; IC₅₀ and C_{max}), PKI-587 (PKI; IC₅₀ and C_{max}), or control for 16 h (JC-1) or 36 h (caspase 3/7 activity). Subsequently, we detected cells with an altered mitochondrial membrane potential via JC-1 staining and flow cytometry analysis or luminescent signals of caspase 3/7 activity, respectively. JC-1: PKI-587 treatment showed significant results for BON (10.6% of cells; control: 5.5%) and LCC-18 cells (4.9% of cells; control: 3.1%); RAD001 significantly increased the apoptosis rate of KRJ-I cells (27.7% of cells; control: 21.6%). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$: significant difference vs. control.

(For figure see next page.)

Efficacy

Concentrations
of substances

		BON	KRJ-I	LCC-18	QGP-1
PKI-587	IC ₅₀	250 nM	25 nM	250 nM	50 nM
	C _{mid}	1,000 nM	500 nM	1,000 nM	500 nM
	C _{max}	10,000 nM	1,000 nM	10,000 nM	1,000 nM
RAD001	IC ₅₀	1 nM	2.5 nM	2.5 nM	2.5 nM
	C _{mid}	100 nM	100 nM	100 nM	100 nM
	C _{max}	500 nM	500 nM	500 nM	500 nM

Apoptosis

Fig. 3. Cell lines were treated with everolimus (RAD001; IC₅₀ and C_{max}) or PKI-587 (IC₅₀ and C_{max}) for 48 h (BON and KRJ-I) or 96 h (LCC-18 and QGP-1). Cells were stained with PI (DNA content) and mitosis-specific Phospho(Ser10)-Histone H3 immunostain followed by flow cytometry assessment (analysis solely included single cells). According to the stain, cells were divided into groups

of several cell cycle phases (FlowJo software), given in percent of all detected cells (mean ± SD). For the sub-G₁ peak, we detected smaller apoptotic bodies, stated in percent of all detected events (mean ± SD). **a** Cell cycle. **b** Mitotic index. **c** Sub-G₁ events. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$: significant difference vs. control.

Western Blot Analyses

Detailed data are listed in table 1; exemplary bands (one of the four replicates) are shown in figure 4.

AKT Activation. The aim of dual inhibition by PKI-587 is to avoid the limiting feedback activation of AKT. Therefore, we analyzed its direct targets mTORC1, TSC2 (tuberous sclerosis complex 2/tuberin), GSK-3 (glycogen synthase kinase 3), and FOXO1 (forkhead box protein O1), as well as their phosphorylations (fig. 1). In summary, everolimus treatment had almost no detectable effect on AKT target phosphorylation. The only exception was a slight increase in the phosphorylation of GSK-3α

and GSK-3β in KRJ-I and QGP-1 cells (fig. 4a). Further, we detected an elevation of the pan-AKT protein amount after everolimus treatment in the pancreatic cell lines BON and QGP-1 (fig. 4b). In the slow-growing cell lines KRJ-I and QGP-1, the PKI-587-induced AKT activity mainly went along with increased GSK-3 phosphorylation, and additionally, in KRJ-I cells further protein phosphorylations were induced (mTOR, TSC2, and FOXO1). In the two other cell lines, BON and LCC-18, the PKI-587-induced effects focused on the target FOXO1 (fig. 4a).

Proof of Principle. Downstream target proteins of mTOR and PI3K were analyzed. Phospho-4E-BP1 bands

Table 1. Summary of Western blot data clustered according to distinct issues

	BON		KRJ-I		LCC-18		QGP-1	
	RAD001	PKI-587	RAD001	PKI-587	RAD001	PKI-587	RAD001	PKI-587
<i>Proof of principle</i>								
p4E-BP1 (S65)	-	---	---	---	+	-	---	---
4E-BP1	0	---	-	-	+	0	0	---
pp70S6K (T389)	---	---	---	---	---	0	---	---
p70S6K	0	+	0	0	0	0	0	0
pAkt (S473)	-	-	---	---	0	0	0	0
pAkt (T308)	0	0	0	0	0	0	0	0
pan-Akt	+++*	++	0	0	0	0	+	0
<i>Akt activation</i>								
pmTOR (S2448)	0	0	0	++	0	0	-	0
mTOR	+	++	0	-	0	0	0	0
pTSC2 (T1462)	0	0	0	+	0	0	0	0
TSC2	0	0	0	0	+	-	0	---
pFOXO1 (T24)	0	+++*	0	+	0	++	0	0
FOXO1	0	+++*	0	0	0	0	+	+
pGSK-3α (S21)	0	0	+	++	0	0	+	+++*
pGSK-3β (S9)	0	0	+	++	0	0	+	+++*
GSK-3β	0	0	0	0	0	0	0	0
<i>Cell cycle</i>								
pRb (S780)	0	+	---	---	0	0	---	---
Rb	-	-	---	---	---	---	---	---
<i>MAPK signaling</i>								
pERK1/2 (T202/204)	++	+++*	+	++	0	+	0	+++*
ERK1/2	+	0	-	0	-	-	0	0

The cell lines were incubated with everolimus (RAD001; C_{mid}) or PKI-587 (C_{mid}) vs. control for 24 h. Densitometric data on the band intensity of four independent replicates were normalized to Coomassie total protein stain, averaged, and compared with the control. The values describe the impact of treatments on particular proteins regarding the relative data level and p value. - (light blue), -- (middle blue), --- (dark blue) = Decreased band density vs. control, several intensity grades; + (light red), ++ (middle red), +++ (dark red) = increased band density vs. control, several intensity grades; 0 = no difference from control. * Significant difference from control ($p \leq 0.05$).

consistently showed differences between the two treatments (fig. 4b, bands exemplarily shown for BON and QGP-1 cells).

Cell Cycle. A reduced band density of the negative cell cycle regulator Rb (retinoblastoma protein) was detected, and for KRJ-I and QGP-1 cells additionally lowered phospho-Rb levels (fig. 4c).

MAPK Signaling. A greater induction of ERK1/2 (extracellular signal-regulated kinase) phosphorylation was measurable for PKI-587 treatment (fig. 4d).

Gene Expression Analyses

Gene expressions of the cell lines QGP-1, KRJ-I, and LCC-18 were screened with innovative NanoString® nCounter® technology after incubation with RAD001 or

PKI-587 to detect treatment-induced alterations in gene regulation. The panel includes 730 relevant genes out of 13 different canonical cancer-associated pathways (nCounter®, PanCancer pathway panel).

Fig. 4. Cell lines were treated with everolimus (RAD001; C_{mid}) or PKI-587 (C_{mid}) versus control for 24 h. We analyzed cell lysates using the Western blot method and conducted immunodetection of several proteins. **a** Analysis of direct target proteins of Akt and their phosphorylations. **b** Analysis of proteins and their phosphorylations downstream from the compound targets mTOR and PI3K. **c** Analysis of the cell cycle-relevant protein Rb. Treatment caused a decrease in Rb and phospho-Rb chemiluminescence. **d** Analysis of ERK1/2, mediator of the MAPK pathway. PKI-587 treatment caused an increase in ERK phosphorylation.

(For figure see next page.)

Fig. 5. Treatment-induced alterations in gene expression. GEP-NEN cell lines were treated (for 60 h) with everolimus (RAD001; C_{mid}), PKI-587 (C_{mid}), or control. Extracted mRNA was analyzed with the NanoString[®] nCounter[®] technology. The differential expression with regard to the impact of treatments vs. control was calculated by nSolver software. **a** For separating the major differentially expressed genes, we excluded genes with a fold change of less than 50% and p value of more than 0.05 and clustered them into 13 different pathways (based on KEGG, Reactome, and GO

annotations; genes participating in several pathways were considered in each of them; total $\neq$ sum). Pathway scores indicate the intensity of the change in expression caused by treatment (sum of absolute log fold change). The column 'genes' shows the number of included genes of this pathway. The intensity of the green color indicates the height of the score/number of genes. **b** log fold change of the relevant signaling genes (mean $\pm$ SD). **c** Genes linked to G₀/G₁ arrest of QGP-1 cells (mean $\pm$ SD).

We selected all genes with a fold change of more than 50% combined with p values lower than 0.05 to discover major differentially expressed genes. We clustered the relevant genes to pathways and calculated a pathway score (fig. 5a). We precisely listed the genes affected by treatments and their log fold change intensity in the on-line supplementary material.

The result is that PKI-587 treatment caused relevant gene alterations more intensely than everolimus, and more genes were affected. For QGP-1 cells, we detected the greatest score for cell cycle-regulating genes. In addition, several genes related to G₀/G₁ arrest in QGP-1 cells were separately analyzed (fig. 5).

Discussion

PKI-587 Is Superior to Everolimus Treatment in Cell Growth Inhibition and Induction of Apoptosis

Comparing the maximal inducible effect of the two substances, cells responded notably better to the ATP-competitive dual PI3K/mTOR inhibitor PKI-587 than to everolimus as an mTORC1-selective inhibitor (fig. 2a), which is consistent with the very recent findings published by Vandamme et al. [36]. However, higher concentrations of PKI-587 are needed to become therapeutically relevant (see the IC₅₀ values in fig. 2b), especially in BON and LCC-18 cells. These assessed dosages are consistent with tolerable drug concentrations used in humans in clinical trials [5, 24].

The measured effect of decreased cell viability is composed of apoptotic processes and of growth inhibition via cell cycle restriction regulated by intracellular signaling networks. Despite reaching a better efficacy with PKI-587 treatment in all four NEN cell lines, the effects on signaling vary characteristically between the different cell line models. Understanding the distinct tumor models might help in developing specific markers to predict the response of a patient to a certain treatment.

KRJ-I cells have the slowest-growing characteristics, and they show the smallest advantage of the dual inhibitor PKI-587 over the selective mTORC1 inhibitor everolimus. The advantage of PKI-587 in BON or LCC-18 cells is greater (fig. 2a). In our study, LCC-18 cells also showed the greatest propensity to induce apoptosis after treatment (fig. 2c, 3c), but they did not display G₀/G₁ arrest like the other cell lines (fig. 3a). Whereas in QGP-1 cells the efficacy of treatment is solely based on restriction of the cell cycle, apoptotic processes were not detectable. The BON and KRJ-I cell lines revealed both cell cycle restriction and apoptotic processes.

In the following, we will discuss this behavior of the different tumor types referring to our protein and gene expression analyses and to known mutations of the cell lines. Because the response of the aggressive cell line LCC-18 was also satisfactory but vastly different in its underlying mechanism from the other cell lines, we analyzed its results in a separate section.

Proof of Principle of PKI-587 in Neuroendocrine Cells: The mTORC1 Target 4E-BP1, a Crucial Factor for Malignancy and Treatment Response

In our study, the mediator 4E-BP1 emerged as an important factor for the antitumoral activity of PKI-587 treatment. The effect on 4E-BP1 protein correlates with

the response to PKI-587 treatment and its advantage over everolimus (regarding cell cycle restriction).

Western blot analyses revealed that everolimus inhibits mTORC1 only partially in GEP-NEN cells in vitro, whereas PKI-587 is able to prevent the rapamycin-typical rephosphorylation of 4E-BP1 (table 1). This advantage of PKI-587 was differentiated depending on the cell type and correlates with the cell cycle-regulating mechanisms leading to G₀/G₁ arrest. In KRJ-I cells, both treatments showed a similar inhibition of 4E-BP1 phosphorylation, and the reduction of viability as well as induction of G₀/G₁ arrest were nearly identical, whereas BON cells responded much better to PKI-587, with strongly reduced phospho-4E-BP1 levels compared with everolimus. These findings confirm the partial inhibition of mTORC1 by everolimus as a crucial limiting factor of this treatment [19, 21].

In addition, gene expression of 4E-BP1, compared between cell lines, correlates with malignity. Highly proliferating, poorly differentiated LCC-18 cells showed six-fold higher amounts of *EIF4EBP1* mRNA than slowly growing KRJ-1 cells (data not shown). Treatment-induced downregulation of 4E-BP1 mRNA expression (gene: *EIF4EBP1*; fig. 5b) also correlates with treatment response with regard to viability (except LCC-18 cells). These findings provide a reason to investigate 4E-BP1 further as a prognostic marker.

The mTORC1 Target p70S6K

Phosphorylation of the second mTORC1 target p70S6K was equally reduced by both treatments (except in LCC-18 cells; table 1); thus, there is no evidence for p70S6K to play a role in the advantage of PKI-587 treatment in GEP-NEN cells.

Everolimus successfully inhibits the phosphorylation of this target by disrupting the mTORC1 complex, and PKI-587 shows the same effectiveness by active site occupation of the mTOR kinase.

Efficacy of PKI-587 Independent of mTORC2 Inhibition in GEP-NEN Cells

Against our expectations, we saw an equivalent behavior with both treatments: a decrease in AKT phosphorylation at Ser473 in BON and KRJ-I cells, and no effect in LCC-18 and QGP-1 cells. Only the results for KRJ-I cells were clear enough to be significant (Western blot; table 1).

For BON and KRJ-I cells, we assume an everolimus-induced inhibition of mTORC2 suppressing Ser473 phosphorylation of AKT, as it is a rapamycin analog. Similar results caused by rapamycin were published [37, 38], although mTORC2 is generally accepted as being rapalog

insensitive. For LCC-18 and QGP-1 cells, we possibly did not reach the required concentrations of both substances, since there are reports of increased concentrations to be necessary for inhibition of the mTORC2-dependent phosphorylation of AKT at Ser473 [39].

No Experimental Proof of PKI-587 Inhibition of PI3K in GEP-NEN Cells

According to the PI3K-inhibitory feature of PKI-587 proven in a cell-free assay of Venkatesan et al. [23], we expected to find a decrease in AKT phosphorylation at Thr308, but averaging the four biological replicates we were not able to detect any convincing changes (Western blot; table 1). Thus, the effects of PKI-587 on proliferation and survival seem to be independent of PI3K inhibition in our cell lines. Soares et al. [26] recently published similar results concerning dual PI3K/mTOR inhibition. They postulated that growth-inhibitory effects are PI3K independent. Instead, they assumed mTORC2 dependence, which is not consistent with our findings in the GEP-NEN cell lines (see previous subsection).

Induction of ERK Signaling and Minor Relevance of Feedback AKT Activation in GEP-NEN Cells

Because of several cross-activating connections between the PI3K/AKT/mTOR and other mitogen pathways, the dual inhibition of PI3K/mTOR can lead to increased activity in ERK signaling [26, 40]. For all four cell lines, our data ascertained PKI-587-induced activation of ERK signaling and therefore activation of MAPK signaling as an escape mechanism (Western blot; table 1).

The assumption that the everolimus-induced AKT activation due to feedback stimulation poses a considerable limitation to treatment [15–18] cannot be confirmed by our results. The Western blot data indicate that both treatments slightly enhance the phosphorylation of different AKT targets – surprisingly, to a greater extent with PKI-587 than with everolimus. Because we could not detect any activating AKT phosphorylations at Ser473 and Thr308 (Western blot; table 1), the phosphorylations of mTOR, FOXO1, and GSK-3 probably were caused by other kinases than AKT mediated by treatment-induced ERK signaling [41–44].

Treatment-Induced G₀/G₁ Arrest and Decrease in Mitotic Index

The cellular task of the tumor suppressor Rb is complexing and thereby inhibiting the cell cycle-regulating transcription factors E2F1/2/3. Phosphorylation of Rb through cyclin/CDK (cyclin-dependent kinase) com-

plexes, stimulated via AKT, led to disruption of the complex and therefore to cell cycle progression [45]. We detected a downscaled amount of Rb protein for all four cell lines, with hardly any difference between the two treatments (Western blot; table 1), while Rb mRNA expression remained unaffected (fig. 5b). The distinctly decreased phosphorylation status of Rb in the low-proliferating KRJ-I and QGP-1 cells suggests a lower transcriptional activity of E2F1/2/3 and, thus, cell cycle deceleration [45].

The observed differences concerning the intensity of cell cycle restriction (or induction of apoptosis, as discussed later) between the two treatments may arise from distinct conditions such as the composition of interacting proteins, altered posttranslational modifications (e.g., acetylations), or the epigenetic regulation of gene expression [45]. Possibly, the abovementioned PKI-587-induced suppression of cap-dependent protein translation via 4E-BP1 is a further factor influencing the efficacy of treatments.

Affirmatively, differences between the two treatments were discernible in the mRNA expression of several proliferation-related genes in QGP-1 cells (fig. 5c). Expression of *E2F1*, physiologically upregulated for G₁/S transition [46], was suppressed, corresponding to PKI-587-induced G₀/G₁ arrest. Further, the genes of cyclin E2 (*CCNE2*) and CDK2 (*CDK2*), also necessary for passing the G₁/S checkpoint, and the genes of the CDK2-activating proteins *cdc25A* (cell division cycle homolog A; gene: *CDC25A*) and *cdc25B* (*CDC25B*) were remarkably downregulated after PKI-587 incubation. Accordingly, expression of the genes of the cyclin D-CDK4/6 complex inhibitors p57 (cyclin-dependent kinase inhibitor 1C; gene: *CDKN1C*) and p15 (cyclin-dependent kinase inhibitor 2B; gene: *CDKN2B*) was upregulated (fig. 5c).

Further, it is likely that FOXO1 also participates in the induction of G₀/G₁ arrest, as the abundance of FOXO1 protein was increased in BON and QGP-1 cells after PKI-587 treatment (table 1). This is one of the major functions of this transcription factor [47] and is associated with elevated p15 mRNA levels [48].

Treatment-Induced Apoptotic Processes

As shown in the cell cycle analyses, the factors Rb/E2F and FOXO1 and their modulations caused by treatments play a crucial role in the cell cycle regulation of GEP-NEN cells. Moreover, these mediators also participate in apoptotic processes [45–47]. Another well-known inducer of apoptosis is the tumor suppressor p53 (tumor protein p53), interacting with both E2F and FOXO1 [46, 47, 49].

FOXO1 and p53 mutually control the induction of apoptosis in reply to cellular stress by transcriptional regulation of apoptotic target genes depending on the cellular context [47]. The aberrantly activated AKT signaling of GEP-NEN might prevent this FOXO-mediated apoptosis. On the other hand, the deregulated activity of E2F1 is able to induce the expression of several proapoptotic genes via p53 or independently of p53 [45, 46].

Inability of QGP-1 Cells to Induce Apoptosis

Although the clinical incidence of p53 mutations is low in GEP-NENs, recent studies have revealed several loss-of-function mutations in cell lines, leading to conformational changes in the DNA-binding domain of p53 (LCC-18) or to truncated (BON) or completely lost expression of the p53 protein (QGP-1) [49–52]. QGP-1 cells in particular seem to be incapable of inducing apoptotic processes. In addition, we observed a lack of caspase 8 and Fas receptor mRNA expression and a comparatively low expression of members of the TNF receptor superfamily in QGP-1 cells (data not shown). All of these findings probably combine in the QGP-1-characteristic prohibition of apoptosis.

Treatment-Induced Regulation of Gene Expression

After a gene expression analysis of the 730 cancer pathway genes in three of the cell lines with and without treatment, we included only differentially expressed genes with significant values and a fold change (treatment vs. control) of 50% or more. Further, we functionally analyzed the intensity of the differential expression using pathway scores.

QGP-1 cells showed the most notably affected gene expression pattern after treatment with regard to both the pathway scores and the number of differentially expressed genes (fig. 5a). Treatment of this cell line particularly interferes with genes participating in cell cycle regulation, confirming our findings of G₀/G₁ arrest (fig. 3, 5c). KRJ-I cells revealed a similar pattern to QGP-1 cells, but it was clearly weaker and without notable results for the cell cycle-related genes. In both cell lines, PKI-587 treatment caused a stronger change in expression of MAPK, PI3K, RAS, and STAT pathway genes than did RAD001 treatment, reflecting the overall stronger effects of PKI-587 compared with everolimus (fig. 5a).

Variational Response of LCC-18 Cells Representing Poorly Differentiated GEP-NENs

Treatment of LCC-18 cells with PKI-587 successfully reduced their viability more than did treatment with

everolimus (fig. 2a). However, there was no association of treatment efficacy with the differing effects concerning 4E-BP1 phosphorylation; surprisingly, everolimus treatment even increased the amount of phospho-4E-BP1 (table 1).

PKI-587 treatment of LCC-18 cells caused apoptosis, but the efficacy of everolimus treatment – not inducing apoptosis, cytotoxicity, or cell cycle arrest but reducing viability – raises the question of how the treatments work. In LCC-18 cells, an unusual form of cell cycle slowdown might occur, without the accumulation of cells in one of the cell cycle phases [53]; however, the knowledge about the possible underlying mechanisms is insufficient.

Conclusion

The dual PI3K/mTOR inhibitor PKI-587 is a promising compound for the treatment of GEP-NENs; it showed good results in our in vitro investigation and displayed a greater efficacy than the approved mTOR inhibitor everolimus. The reduction of cell viability is based on apoptotic processes and cell cycle restriction; whereas BON and KRJ-I cell lines showed both effects, QGP-1 cells were unable to induce apoptosis. PKI-587 treatment of the poorly differentiated cell line LCC-18 successfully decreased cell viability as well, but the underlying mechanisms differed greatly from those in the BON, KRJ-I, and QGP-1 cell lines. The complex processes behind these distinct responses are poorly understood, but phosphorylation of 4E-BP1 and the Rb/E2F axis as well as subsequent modulation of gene expression seem to play crucial roles. Thus, further investigations concerning the different tumor subtypes are required to predict a patient's response to a compound. Because of the convincing efficacy of PKI-587 in GEP-NEN cell models revealed in our study, indicating that PKI-587 is a promising therapy option for GEP-NEN disease, we recommend subsequent in vivo studies of this substance.

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