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The role of GSK3 and its reversal with GSK3 antagonism in everolimus resistance

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Abstract

Pancreatic neuroendocrine tumors (panNETs) are often inoperable at diagnosis. The mTORC1 inhibitor everolimus has been approved for the treatment of advanced NETs. However, the regular development of resistance to everolimus limits its clinical efficacy. We established two independent everolimus-resistant panNET (BON1) cell lines (BON1 RR1, BON1 RR2) to find potential mechanisms of resistance. After 24 weeks of permanent exposure to 10 nM everolimus, BON1 RR1 and BON1 RR2 showed stable resistance with cellular survival rates of 96.70% (IC_{50} = 5200 nM) and 92.30% (IC_{50} = 2500 nM), respectively. The control cell line showed sensitivity to 10 nM everolimus with cellular survival declining to 54.70% (IC_{50} = 34 nM). Both resistant cell lines did not regain sensitivity over time and showed persistent stable resistance after a drug holiday of 13 weeks. The mechanisms of resistance in our cell line model included morphological adaptations, G1 cell cycle arrest associated with reduced CDK1(cdc2) expression and decreased autophagy. Cellular migration potential was increased and indirectly linked to c-Met activation. GSK3 was over-activated in association with reduced baseline IRS-1 protein levels. Specific GSK3 inhibition strongly decreased BON1 RR1/RR2 cell survival. The combination of everolimus with the PI3K α inhibitor BYL719 re-established everolimus sensitivity through GSK3 inhibition and restoration of autophagy. We suggest that GSK3 over-activation combined with decreased baseline IRS-1 protein levels and decreased autophagy may be a crucial feature of everolimus resistance, and hence, a possible therapeutic target.

Key Words

- ▶ panNETs
- ▶ stable everolimus resistance
- ▶ GSK3
- ▶ combination treatment

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Introduction

Pancreatic neuroendocrine tumors (panNETs) are a type of gastroenteropancreatic neuroendocrine tumor (GEP-NETs) (Yao *et al.* 2008). Due to their slow and frequently asymptomatic growth, panNETs are often advanced when diagnosed, such that curative surgery is no longer an option (Briest & Grabowski 2014). For this reason, the 10-year survival for panNETs is only 44%, in spite of various advances in treatment modalities (Ekeblad *et al.* 2008, Halfdanarson *et al.* 2008, Auernhammer *et al.* 2017, Aristizabal Prada & Auernhammer 2018). Common treatments for advanced disease such as biotherapy, chemotherapy, molecular-targeted therapy and peptide radioreceptor therapy remain limited in their efficacy, and novel treatment options are urgently needed (Frilling *et al.* 2014, Auernhammer *et al.* 2017, Aristizabal Prada & Auernhammer 2018).

One of the most important receptor tyrosine kinase (RTK)-induced oncogenic signaling cascades in GEP-NETs is the mTORC1-p70S6K signaling pathway, which can be inhibited by the mTORC1 inhibitor everolimus (Fig. 1) (Zaytseva *et al.* 2012, Wolin 2013, Shipkova *et al.* 2016).

Figure 1

Proposed mechanism of action of the mTORC1-inhibitor everolimus. Everolimus inhibits the mTORC1 complex and thereby down-regulates the mTORC1/p70S6K signaling cascade, preventing the cell from cell cycle progression, survival and proliferation. Inhibition of mTORC1/p70S6K leads to disinhibition/activation of IRS-1 and thereby activation of PI3K/Akt, which in turn inhibits GSK3 by phosphorylation. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0159>.

The PI3K–Akt–mTORC1 axis is frequently over-activated in cancers, including panNETs, causing increased cellular survival, proliferation and protein synthesis (Briest & Grabowski 2014, Pavel & Sers 2016). Accordingly, the mTORC1 inhibitor everolimus has shown anti-tumor potential in several clinical phase 3 trials in patients with NETs (RADIANT-2, RADIANT-3, RADIANT-4) (Pavel *et al.* 2011, Yao *et al.* 2011, 2016a,b), and has been approved for the treatment of pancreatic, intestinal and pulmonary NETs in many countries (Pavel & Sers 2016, Chan *et al.* 2017). Unfortunately, only around 5% of patients show partial remission in response to everolimus treatment, although many more patients show stable disease. However, most tumors eventually start to progress even if everolimus treatment is continued (Yao *et al.* 2011). Thus, secondary resistance seems to occur in most patients, although primary resistance may also be a problem (Wagle *et al.* 2014, Capozzi *et al.* 2015, He *et al.* 2016, Vandamme *et al.* 2016, Zatelli *et al.* 2016). Advanced panNET patients are treated with a fixed dose of 10mg everolimus daily (NCT00510068) (Yao *et al.* 2013, 2014): the majority develops treatment resistance within 1 year of treatment (Capurso *et al.* 2012, Yao *et al.* 2013). Consequently, reversible adaptive resistance to everolimus in panNET cell lines, and combinatory regimes to overcome acquired resistance, have been the object of previous studies (Zitzmann *et al.* 2010, Passacantilli *et al.* 2014, Vandamme *et al.* 2016). However, long-term resistance to everolimus treatment has only been little studied to date (Capurso *et al.* 2012, Passacantilli *et al.* 2014, Vandamme *et al.* 2016), and thus, there is an unmet need to better understand the mechanisms of resistance and to establish predictive biomarkers for resistance to everolimus, and hence, appropriate treatments. Activation of autophagy by mTORC1 inhibitors has been reported as one potential mechanism for developing mTORC1 inhibitor resistance and for promoting tumor cell growth (Avniel-Polak *et al.* 2016, Avniel *et al.* 2018). Autophagy seems to play a controversial role in cancer: on the one hand, autophagy might offer a protective mechanism in some cancer cells promoting acquired drug resistance (Sui *et al.* 2013, Avniel-Polak *et al.* 2016). On the other hand, autophagy might also induce autophagic cell death, separate from apoptosis (Sui *et al.* 2013).

Novel insights into the mechanisms of resistance in response to everolimus treatment could provide a rationale for dual-targeting approaches in order to overcome such acquired or intrinsic resistance (Capurso *et al.* 2012, Passacantilli *et al.* 2014, Capozzi *et al.* 2015, Vandamme *et al.* 2016, Avniel *et al.* 2018). Although the study group around Vandamme *et al.* treated BON1 and

QGP1 cell lines over 24 weeks with increasing doses of everolimus up to 1 μ M according to the 'pulse selection model' (McDermott *et al.* 2014), BON1 and QGP1 cells only showed reversible resistance and regained sensitivity to everolimus after a drug holiday of 10–12 weeks (Vandamme *et al.* 2016).

Therefore, our aim was to establish a clinically relevant panNET cell line model from BON1 cells, demonstrating stable, that is, consistent and reliable resistance to everolimus in order to study the mechanisms of long-term resistance and explore novel combinatory treatment options to overcome such resistance.

Besides exploring PI3K-Akt signaling as a potential pathway of resistance (O'Reilly *et al.* 2006, Zitzmann *et al.* 2010, Passacantilli *et al.* 2014, Vandamme *et al.* 2016), we also investigated GSK3 signaling. GSK3 α/β is a direct effector protein of Akt: activated Akt phosphorylates and inhibits GSK3 α/β (Porta *et al.* 2014). GSK3 has been implicated in metabolism since GSK3 is inactivated by insulin leading to disinhibition/activation of glycogen synthase. Moreover, it seems to play a role in diseases such as diabetes, Alzheimer's disease and psychiatric diseases, including bipolar disorder and schizophrenia (Maurer *et al.* 2014). GSK3 α/β has shown controversial effects on cell death and survival, including pro- and anti-apoptotic effects (Maurer *et al.* 2014). Activated GSK β has also been reported to target cell cycle proteins such as Cyclin D for degradation (Maurer *et al.* 2014). On the one hand, treatment with different GSK3 inhibitors (CHIR99021, 6-bromoindirubin-3'-oxime-, 1-azakenpaulone) and siRNA stimulated proliferation of rat insulinoma INS-1E cells (Mussmann *et al.* 2007). Conversely, the non-specific GSK3 α/β inhibitor lithium chloride inhibited neuroendocrine tumor growth *in vitro* and *in vivo* (Greenblatt *et al.* 2010, Lubner *et al.* 2011); however, in a clinical phase 2 study with 15 patients with low-grade NETs (8 carcinoid tumors, 5 islet cell tumors), the non-specific GSK3 α/β inhibitor lithium chloride caused no objective responses, and pre- and post-treatment tumor biopsies showed no consistent GSK3 β inhibitory effects due to insufficient phosphorylation of GSK3 β in patients' serum levels (Lubner *et al.* 2011). To assess the effects of more specific GSK3 α/β inhibition on NETs, we have recently investigated the selective GSK3 α/β inhibitor AR-A014418 in pancreatic, pulmonary and midgut NET cells and reported a significant inhibitory effect on cell viability and strong GSK3 α/β phosphorylation/inhibition (Aristizabal Prada *et al.* 2017). Biochemical studies have focused on GSK3 β inhibition, while other studies have also shown the importance of GSK3 α in drug resistance

(McCubrey *et al.* 2014). Thus, GSK3 appears to play a pleiotropic role in regulating cell proliferation.

We have used our everolimus-resistant cell line model to demonstrate combinatorial treatments that reversed everolimus resistance via GSK3 inhibition *in vitro*. In due course, other cell lines can be studied to explore whether they also develop stable resistance and to compare the intrinsic mechanisms. In a pilot study, we transferred our everolimus-resistant cell line model to an orthotopic intrapancreatic xenograft mouse model providing the basis for translating the *in vitro* approach to an *in vivo* situation.

Materials and methods

Cell culture

The human panNET cell line BON1 (Evers *et al.* 1991) was kindly obtained from Prof. R Göke (Marburg, Germany). The BON1 cell line was analyzed and certified by the German Biological Resource Centre DSMZ (DSMZ, Braunschweig, Germany) using short tandem repeats (STRs). BON1 cells were grown in DMEM/F12 (1:1) (Life Technologies), supplemented with 10% FBS (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Life Technologies) and 0.4% amphotericin B (Biochrom) at 5% CO₂ and 37°C and tested mycoplasma-free for all experiments reported. BON1 is a permanent cell line derived from a lymph node metastasis of a human carcinoid tumor of the pancreas and presents phenotypic NET characteristics (Grozinsky-Glasberg *et al.* 2012).

Experimental design

In order to yield everolimus resistance in panNET cells, BON1 cells (passage 13) were treated continuously with a constant low dose of 10 nM everolimus for 24 weeks. A dose of 10 nM everolimus was chosen since the plasma levels of everolimus in patients on daily therapy with 10 mg everolimus range between around 8 nM ($C_{\min}$ after 2 weeks of daily exposure to 10 mg everolimus) and 59 nM (C_{2h} after 12 weeks of daily exposure to 10 mg everolimus) (Budde *et al.* 2016). Every 3-day medium was exchanged and everolimus treatment was renewed. The concentrations were not increased over time. To determine commonalities in resistance mechanisms in BON1 cells, we have established two independent everolimus-resistant BON1 cell lines (BON1 RR1 and BON1 RR2) in parallel and a control cell line (BON1 Control DMSO) to control for adaptive strategies of long-term *in vitro* culturing and passaging as such. BON1 is the initial parental cell

line, from which the development of resistance started. To obtain a sensitive control cell line (BON1 Control DMSO), BON1 was continuously vehicle treated with a comparable concentration of DMSO (0.00005%) over 24 weeks. We compare our results to the BON1 Control DMSO cell line. In order to assess the stability and reliability of the acquired resistance phenotype, all cells were frozen in liquid nitrogen (minimum for 48h) and thawed, followed by drug withdrawal (McDermott *et al.* 2014). Resistance was defined as at least a doubling of the IC_{50} , compared to the sensitive control cell line (BON1 Control DMSO), after treatment with 10nM everolimus for 144h (McDermott *et al.* 2014).

Inhibitors

The mTORC1 inhibitor everolimus (RAD001), the GSK3 inhibitor AR-A014418 and the PI3K inhibitor BYL719 were acquired from Sigma-Aldrich. All substances were dissolved in dimethyl-sulfoxide (DMSO, Sigma-Aldrich) and stored at -20°C . The stock solutions of everolimus, AR-A014418 and Byl719, were 20mM in DMSO.

Cell viability assessment

BON1 cells were seeded at ~1500 cells/96-well for 24h in complete medium. Then, the cells were treated with different concentrations of everolimus (from 1nM to 2 μM), BYL719 (from 1 μM to 10 μM) or AR-A014418 (from 5 μM to 20 μM) alone or they were treated with everolimus (from 1nM to 10nM) in combination with BYL719 (from 1 μM to 10 μM) or in combination with AR-A014418 (from 5 μM to 20 μM). The BYL719 concentrations we chose are potentially clinically relevant since in a clinical study assessing population pharmacokinetics and pharmacodynamics of BYL719 in patients with advanced solid malignancies median BYL719 plasma concentrations between 2 μM and 11 μM have been found with 1-month daily oral therapy with therapeutically relevant doses of 300–450mg BYL719 2–8h post dose (De Buck *et al.* 2014). For everolimus alone, the two final DMSO controls were equal to 1 μM everolimus (0.005% DMSO final concentration) and 2 μM everolimus (0.01% DMSO final concentration), respectively, depending on the experiment. For the higher dose BYL719/everolimus combination treatment, the final DMSO control was equal to 1:2000 (Byl719)+1:2,000,000 (everolimus)=0.05005% final DMSO concentration. For the lower dose BYL719/everolimus combination treatment, the final DMSO control was equal to 1:4000 (Byl719)+1:2,000,000

(everolimus)=0.02505% final DMSO concentration. For AR-A014418/everolimus combination treatment, the final DMSO control was equal to 1:1000 (AR-A014418)+1:2,000,000 (everolimus)=0.10005% final DMSO concentration. The final DMSO concentrations were never higher than 0.1% and had no significant effect on cell viability. The metabolic activity of living cells was assessed by the 'Cell Titer Blue' cell viability assay (Promega) after 72h and 144h of incubation with the different agents. Cells were incubated for 4h with 'Cell Titer Blue' solution and fluorescence was measured at 560/590nm using a GLOMAX plate reader (Promega).

Cell cycle analysis by flow cytometric analysis

Cell cycle phase distribution was analyzed (Riccardi & Nicoletti 2006) (BD Accuri C6 Analysis). First, cells were cultured in six-well plates for 24h, before medium was replaced by antibiotic-free medium and incubated with 10nM everolimus. Seventy-two hours later, cells were collected, washed and resuspended in 300 μL propidium iodide (Sigma-Aldrich). After 8h, 20,000 events from each sample were analyzed.

Caspase-3/-7 activity assay

To measure the activity of Caspase-3/-7, we used the Apo-One homogeneous Caspase-3/7-Assay kit (Promega): 10,000 cells were seeded per well, incubated for 24h without treatment or with respective doses of BYL719 (5–10 μM) or everolimus (10nM), and Caspase-3/7 activity was assessed according to the manufacturer's instructions. We compared all results of the BYL719- or everolimus-treated cell lines to the vehicle-treated cell lines. For each experiment, the vehicle represented the highest DMSO concentration present in the respective experiment. The exact DMSO concentrations are detailed under 'Cell viability assessment' section.

Protein extraction and Western blotting

The cells were seeded into 10 cm plates and grown for 24h in complete medium for Western blot analysis. Following medium exchange, cells were incubated with 10nM everolimus, either alone or in combination with BYL719 or AR-A014418. We compared all results to the vehicle-treated cells. For each experiment the vehicle represented the highest DMSO concentration present in the respective experiment. The exact DMSO concentrations are detailed under 'Cell viability assessment' section. The incubation

times were up to 72 h. Western blotting was conducted as described previously (Reuther *et al.* 2016). A cell line screening to determine commonalities between both resistant cell lines (BON1 RR1 and BON1 RR2) in protein expression was performed using the following primary antibodies: pIRS-1 Ser612 (#3203) (1:1000), IRS-1 (#3407) (1:1000), pAkt (Ser473) (#4060) (1:5000), Akt (#2920) (1:5000), pTORC1 (S151) (#3359) (1:1000), TORC1 (#2587) (1:1000), pp70S6K (T387/389) (#9205) (1:2000), p70S6K (#9234) (1:5000), pS6 (S240/244) (#5354) (1:50000), S6 (#2217) (1:5000), p4EBP1 (Ser65) (#9451) (1:1000), 4EBP1 (#9644) (1:10000), pCDK1 (Tyr15) (#4539) (1:1000), CDK1 (#9116) (1:2000), pEGFR (Y1068) (#3777) (1:1000), EGFR (#4267) (1:5000), pMet (Y1234/5) (#3077) (1:2000), Met (#3127) (1:5000), pGSK3 (S21/9) (#9331) (1:2000), GSK3 (#5676) (1:10000), LC3-A (#4599) (1:1000), Caspase 3 (#9662) (1:1000), cleaved Caspase 3 Asp175 (#9661) (Cell Signaling) (1:1000), Actin (A5441) (Sigma) (1:50000), Bcl2 (610539) (BD Transduction Laboratories, Franklin Lakes, NJ, USA) (1:1000), CgA (ab68271) (Abcam) (1:100,000).

Cell migration assay

Cells were seeded at densities of 120,000 (BON1) cells per chamber in culture inserts (Ibidi, Munich, Germany). After 24 h, the inserts were removed, and the cells were treated with 10 nM everolimus for 48 h. We compared the four cell lines BON1, BON1 Control DMSO, BON1 RR1 and BON1 RR2 to each other after treatment with 10 nM everolimus. In this case the BON1 Control DMSO cell line functioned as control and was compared to the resistant cell lines. After 0 h and 48 h of incubation with everolimus, photographs of the gap between the two cell layers were taken (Zeiss, Axiovert 135 TV (microscope) and Zeiss, AxioCam MRm (camera)) and analyzed.

Statistical analysis

The results are displayed as mean $\pm$ 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. *A priori* tests considering the normal distribution and homogeneity of variances were performed applying the Kolmogorov–Smirnov test and Levene's test of the SPSS statistical package SPSS (version 13.0 for Windows, SPSS Inc. (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. When the one group was normalized to 100%, comparisons were performed as one-sample tests.

To identify potential synergistic effects between BYL719 and everolimus on cell viability, we used Linear Mixed Effects Models. BYL719, everolimus (each in three different doses) and the interactions between both were considered as fixed effects, the trial as a random effect. If a significant negative interaction between BYL719 and everolimus on cell survival was found, we concluded a synergistic effect. One model was estimated for each cell line. These computations were done with R 3.3.3 (GNU General Public License; R Foundation, Vienna, Austria). For the analysis of the baseline differences in cell viability and Caspase 3/7 assay, we used linear mixed effects models too. While the cell line was considered as fixed, the trial was considered as random.

Statistical significance was assessed at $P < 0.05$, besides for the Western blot analysis, where due to the exploratory character and the sample size, significance was assessed at $P < 0.1$.

Pilot mouse study: establishment of an orthotopic intrapancreatic everolimus-resistant tumor xenograft mouse model

BON1 RR2 (2×10^6 in 20 μ L medium) were implanted into the pancreas of 12-week-old female SCID mice (weight 20 g) ($n = 4$) as described by Soares *et al.* (2014) with minor changes. This technique was previously established by us to generate the orthotopic BON1 xenograft mouse model. Briefly, SCID mice were anesthetized by isoflurane and intraperitoneal injection of ketamine (70 mg/kg). After local shaving and disinfection, the abdominal area was opened by a 1 cm incision along the Linea Alba. The entire pancreatic body was then exposed on sterile gauze and retracted laterally on saline-soaked cotton tips in such way that enough solid pancreatic tissue was available for cell injection, before it was placed back into the abdominal cavity. Tumor growth was monitored once a week by a preclinical 1T PET/MRI (nanoScan, Mediso, Hungary) using a whole-body coil and T1-weighted gradient-echo and T2-weighted spin echo sequences. The metabolic activity of the engrafted tumor was estimated by ^{18}F -FDG PET/MRI, and somatostatin receptor 2 (SSTR2) levels were assessed by ^{68}Ga -DOTATOC PET/CT (15 MBq injected i.v. respectively). When the tumors had reached a size of $\sim 1000 \text{ mm}^3$, the experiment was terminated. Animal experiments were performed in accordance with the national and local guidelines for animal welfare and

approved by the ethics committee of the state Berlin. All *in vivo* experiments were performed at the Berlin Experimental Radionuclide Imaging Center (BERIC), Charité – Universitätsmedizin Berlin, Germany.

Results

Following 24 weeks of permanent exposure to 10nM everolimus, we have, for the first time, been able to develop two independent stable everolimus-resistant panNET cell lines (BON1 RR1 and BON1 RR2). Both resistant cell lines did not regain sensitivity over time, and showed stable resistance after a drug holiday of 13 weeks. Moreover, both cell lines showed a GSK3 over-activation as a crucial commonality.

Stable resistance

After 24 weeks of permanent everolimus exposure, both cell lines (BON1 RR1 and BON1 RR2) showed strong reliable resistance to everolimus, whereas BON1 Control DMSO cells displayed no resistance (Fig. 2). We compared dose–response curves of the resistant cell lines to the dose–response curves of the non-resistant cell lines with 1nM–2µM everolimus and found resistance, i.e. at least a doubling of the IC_{50} in the resistant cell lines, compared to the sensitive controls, after 144-h everolimus treatment. IC_{50} calculations showed a 153-fold increase ($IC_{50}=5200$ nM) in BON1 RR1 and a 74-fold increase ($IC_{50}=2500$ nM) in BON1 RR2 cells when compared to the BON1 Control DMSO cells ($IC_{50}=34$ nM) after 144-h everolimus treatment. BON1 Control DMSO cells demonstrated a significant decrease in cellular survival after re-application of 10nM everolimus for 144 h, whereas

BON1 RR1 and BON1 RR2 cells showed a significant decrease in cellular viability only at 500nM and 60nM, respectively (Fig. 2). The resistance was evaluated after freezing and thawing the cells again to confirm stable resistance. We performed additional dose–response curves after a drug holiday of 13 weeks and again found persistent stable resistance with an $IC_{50}>2000$ nM in BON1 RR1 and BON1 RR2 cells.

Morphologic changes, cell growth and neuroendocrine identity

Everolimus-sensitive BON1 cells showed an oval shape with fibroblastic-like cellular extensions in some cells and regular cluster formation (Fig. 3A). Morphologic characteristics of everolimus-resistant cells displayed an amorphous, flattened cellular shape with lysosome-like bubbles and clumping conglomerates (Fig. 3A). The viable cell number of the resistant cell lines was slightly but significantly increased compared to the sensitive control cell line after 72 h without treatment ($P<0.001$), suggesting more rapid proliferation (Fig. 3B).

We investigated the neuroendocrine identity of the resistant cell lines by assessing chromogranin A (CgA) by Western blot technology (Supplementary Fig. 1, see section on supplementary data given at the end of this article) and found strong expression of CgA in both resistant cell lines.

Heterogeneity of BON1 RR1 and BON1 RR2

We observed inhibition of mTORC1/p70S6K signaling to a differential extent between BON1 RR1 and BON1 RR2 after re-application of everolimus (Supplementary Fig. 1). By using two independently established

Figure 2

Effects of everolimus (1 nM–2 µM) on cell survival of resistant (BON1 RR1 and BON1 RR2) and sensitive (BON1 Control DMSO) cell lines after 144 h of incubation with everolimus. The arithmetic means and standard deviation of at least three independent experiments are shown. Statistically significantly different results in comparison to BON1 Control DMSO are shown, considering $P<0.05$ as significant (*). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0159>.

Figure 3

Everolimus resistant cells show morphologic changes. (A) Everolimus-resistant cells (BON1 RR1 and BON1 RR2) displayed an amorphous, flattened cellular shape with lysosome-like bubbles and clumping conglomerates. (B) The viable cell number of the resistant cell lines was slightly but significantly increased compared to the sensitive control cell line after 72 h without treatment ($P < 0.001$), suggesting more rapid proliferation.

everolimus-resistant cell lines, we have attempted to reduce if not totally eliminate the bias due to tumor heterogeneity in response to long-term everolimus treatment.

Everolimus resistance: G1 phase cell cycle arrest associated with reduced baseline expression of CDK1(cdc2) and of the autophagy marker LC3AII

Both resistant cell lines displayed a significant increase in sub-G1 phase and G1 phase in response to everolimus (Fig. 4A). Associated with the sub G1 increase and G1 phase cell cycle arrest, we found a decreased baseline CDK1(cdc2) expression and reduced baseline expression of the autophagy

Figure 4

(A) Cell cycle analysis via FACS showed a significant increase in sub-G1 population and G1 phase cell cycle arrest in both resistant cell lines (BON1 RR1 and BON1 RR2) after 72 h everolimus treatment. The arithmetic means and standard deviation of at least three independent experiments are shown. Statistically significantly different results in comparison to BON1 Control DMSO are shown, considering * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (B) LC3A consists of two bands: LC3AI (upper band) and LC3AII (lower band). Baseline LC3AII expression level measured by Western blot analysis was decreased in BON1 RR1 and BON1 RR2, compared to BON1 and BON1 Control DMSO. A representative blot out of three independently performed experiments is shown.

marker LC3AII demonstrating reduced autophagy in BON1 RR1 and BON1 RR2 cells (Figs 4B and 5).

Everolimus resistance increased baseline apoptosis

Baseline cleaved caspase 3 as a marker of apoptosis was slightly increased in the resistant cell lines, compared to hardly detectable cleaved caspase 3 in the sensitive controls, indicating slightly stronger baseline apoptosis in the resistant cells (Fig. 6). In order to show these results more clearly, we additionally performed the more sensitive Caspase 3/7 assay and found the same results with significantly increased baseline apoptosis, in both resistant cell lines, compared to the sensitive controls ($P < 0.01$) (Fig. 6). Baseline expression of the anti-apoptotic marker Bcl2 was much lower in the resistant cell lines compared to the sensitive controls and showed a lesser increase in response to BYL719 and everolimus treatment (Fig. 6).

Figure 5

Western blot analysis via densitometry of CDK1(cdc2) showed a baseline decrease in CDK1(cdc2) expression levels in BON1 RR1 and BON1 RR2 cell lines, compared to the sensitive control cells. A representative blot out of three independently performed experiments is shown. For densitometry analysis the arithmetic means and standard deviation of at least three independent experiments are shown. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0159>.

Everolimus resistance increased migration potential and baseline c-Met activation

The migration assay reflected a significant increase in the migration potential of BON1 RR1/RR2 cells, compared to the sensitive control cell line, after treatment with 10nM everolimus (Fig. 7). Both resistant cell lines displayed stronger baseline activation of c-Met when compared to the control cells (Fig. 8).

Everolimus resistance: baseline GSK3 α/β (GSK3) over-activation and strong BON1 RR1/RR2 cell viability reduction by specific GSK3 inhibition

As GSK3 has been shown to be implicated in resistance to radio-, chemo- and molecular-targeted therapy (Shimura 2011, Martelli *et al.* 2014, McCubrey *et al.* 2014),

we examined long-term treatment effects of everolimus on GSK3 (Fig. 9A). GSK3 is inhibited by phosphorylation. BON1 RR1/RR2 cells displayed a pronounced baseline decrease in phosphorylated/inhibited GSK3, and thus, GSK3 over-activation when compared to the sensitive control cell line (Fig. 9A). However, levels of activated and total Akt were similar in resistant and non-resistant cells with and without treatment (Fig. 9A). Akt was phosphorylated/activated in all resistant and sensitive cell lines after 24-h stimulation with everolimus (Fig. 9A).

GSK3 inhibition by the specific GSK3 inhibitor AR-A014418 caused a pronounced decrease in BON1 RR1/RR2 cell viability and – to a much lesser extent – in sensitive control cells (Fig. 9B). In both resistant and in the sensitive control cell line, Western blot analyses showed clear inhibition of GSK3 and an increase in the autophagy marker LC3AII in response to AR-A014418 treatment (Fig. 9C). However, combination treatment of everolimus plus AR-A014418 did not enhance the inhibitory effect of single AR-A014418 treatment on cell viability in resistant cells (Supplementary Fig. 2).

We also performed Western blots on the combination of everolimus plus AR-A014418 and, while single AR-A014418 treatment led to strong GSK3 α inhibition in the resistant cell lines, combination treatment caused no stronger GSK3 α inhibition compared to single AR-A014418 treatment (Supplementary Fig. 2); this may help explain the absence of a synergistic effect.

Everolimus resistance: lower baseline IRS-1 protein levels

Interestingly, baseline IRS-1 protein levels showed a decrease in BON1 RR1 and BON1 RR2 cells compared to parental BON1 and BON1 Control DMSO cells (Fig. 6).

Figure 6

In the resistant cell lines, Western blot analysis showed a baseline decrease in IRS-1 protein levels, an increase in cleaved Caspase 3 and a decrease in anti-apoptotic Bcl-2 as a sign of increased apoptosis, as well as a decrease in LC3AII as a sign of decreased autophagy. LC3A consists of two bands: LC3AI (upper band) and LC3AII (lower band). Caspase 3/7 assay showed significantly increased apoptosis in both resistant cell lines, compared to the sensitive control cell line ($P < 0.01$).

For pIRS-1, the differences between the different cell lines were minor according to the Western blot quantification. Western blot quantifications for IRS-1 and pIRS-1 are shown in [Supplementary Fig. 3](#). The effects of BYL719 and everolimus treatment on IRS-1 are described below.

Total mTORC1 showed no clear differences between resistant cells and non-resistant cells ([Supplementary Fig. 4](#)). No activated pmTORC1 could be detected in our cell lines.

Dual-targeting approach with the PI3K α inhibitor BYL719 overcame everolimus resistance and restored sensitivity through GSK3 inhibition, increased autophagy and decreased apoptosis

Combination treatment with the PI3K α inhibitor BYL719 plus everolimus overcame everolimus resistance and restored treatment sensitivity ([Fig. 10A](#)). A significantly stronger decrease in cellular survival after combination treatment compared to single substance treatment could be observed in both resistant cell lines ([Fig. 10A](#)). We moreover studied additional dose–response relationships combining low doses of 1–10 nM everolimus with 1–5 μ M BYL719 and found significant synergistic effects at almost all drug doses tested in all cell lines, including both

resistant cell lines ($*P < 0.05$) ([Supplementary Figs 5, 6, 7 and 8](#)). This synergistic inhibitory effect on cell survival after the BYL719/everolimus combination treatment was associated with inhibition of GSK3, EGFR, p70S6K and 4EBP1 signaling, as well as showing an increase in IRS-1 protein levels in BON1 RR1/RR2 cells ([Figs 6 and 10B](#)). In both sensitive cell lines, everolimus alone already led to strong GSK3 phosphorylation/inhibition. In BON1 RR1/RR2 cells, single application of everolimus led to lower or no phosphorylation/inhibition of GSK3, but the combination treatment induced stronger inhibition of GSK3, compared to each drug separately. A different response of IRS-1 protein levels to everolimus and BYL719 treatment between resistant and non-resistant cell lines was observed: while sensitive control cells showed a slight increase in IRS-1 protein levels after everolimus treatment, the resistant cells showed a lesser or no IRS-1 increase ([Fig. 6 and Supplementary Fig. 3A](#)). In the non-resistant cells, BYL719 treatment alone was accompanied by increased IRS-1 protein expression, while in the resistant cells a lesser, but nevertheless clear, increase in IRS-1 protein levels was observed in response to BYL719 treatment ([Fig. 6 and Supplementary Fig. 3A](#)).

The BYL719/everolimus combination treatment showed a strong increase in IRS-1 expression in the non-resistant BON1 and BON1 Control DMSO cells, and a lesser, but still clear, increase in IRS-1 protein levels in the resistant cells ([Fig. 6 and Supplementary Fig. 3A](#)).

We studied the differences concerning cell death mechanisms between resistant and non-resistant cell lines in response to BYL719/everolimus treatment:

Figure 7

Everolimus-resistant cells showed a significantly increased migration potential with a significantly smaller migration gap after 48 h of everolimus treatment. The arithmetic means and standard deviation of at least three independent experiments are shown. Statistically significantly different results in comparison to BON1 Control DMSO are shown, considering $*P < 0.05$. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0159>.

Figure 8

Western blot analysis via densitometry of phosphorylated/activated c-Met and total c-Met in resistant (BON1 RR1 and BON1 RR2) and sensitive control cells showed an increase in phosphorylated/activated c-Met in the resistant cell lines. A representative blot out of three independently performed experiments is shown. For densitometry analysis the arithmetic means and standard deviation of at least three independent experiments are shown. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0159>.

Figure 9

GSK3 over-activation in the resistant cell lines. (A) Both everolimus-resistant cell lines showed significantly enhanced activation of GSK3 α/β (GSK3) compared to the sensitive control cell line (BON1 Control DMSO) and a significantly stronger activation of GSK3 β compared to the parental BON1 cells, but stable phosphorylation/activation of AKT after 24h of incubation with everolimus. A representative blot out of three independently performed experiments is shown. Statistically significant differences (*) in comparison to BON1 Control DMSO are shown. (B) Single substance treatment of AR-A014418 (10–20 μ M) significantly decreased cellular survival in all cell lines tested after 144h of incubation, although much stronger in BON1 RR1 and BON1 RR2, compared to the sensitive control cell line. The arithmetic means and standard deviation of at least three independent experiments are shown. Statistically significant differences in comparison to either single substance treatment are shown, considering *** $P < 0.001$. (C) Western blot analysis showed inhibition of GSK3 and an increase in the autophagy marker LC3AII in response to the selective GSK3 α/β inhibitor AR-A014418. LC3A consists of two bands: LC3AI (upper band) and LC3AII (lower band). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0159>.

We found that in BON1 RR1/RR2 cells, BYL719/everolimus combination treatment was accompanied by an increase in the autophagy marker LC3AII, compared to each drug separately, indicating an increase in autophagy in response to the BYL719/everolimus combination treatment (Fig. 6).

In the sensitive control cells, the Caspase 3/7 assay showed apoptosis induction at 24h with BYL719 treatment, but not in BON1 RR1/RR2; everolimus treatment had no influence on apoptosis induction, while the everolimus/BYL719 combination treatment significantly decreased apoptosis in BON1 RR1/RR2 cells compared to each drug separately, but had no significant influence on apoptosis induction in the sensitive control cells (Supplementary Fig. 9). This was consistent with a decrease in cleaved Caspase 3 in the resistant cell lines after BYL719/everolimus combination treatment, compared to treatment with each drug separately.

Transferability of the resistant cell line model to an orthotopic intrapancreatic xenograft mouse model

In an *in vivo* pilot study, we were able to transfer our resistant cell line model to an orthotopic xenograft SCID mouse model with intrapancreatic tumor growth ($n=4$). Supplementary Figure 10A, B and C show an example of the growth of an intrapancreatic BON1 RR2 tumor in a SCID mouse: 14 days after tumor cell injection into the

pancreas, a detectable tumor developed and reached a size of $\sim 1000 \text{ mm}^3$ around 5 weeks after first detection by MRI (Supplementary Fig. 10A, B and C). Tumor size and tissue morphology were determined by T2w imaging. The resistant tumors showed a similar growth rate and tumor morphology as the native BON1 tumors. When the tumors grew bigger, necrotic areas in the tumor core increased (Supplementary Fig. 10A, B and C) towards the end of the experiment. The glucose uptake (standard uptake value (SUV)) calculated from ^{18}F -FDG PET confirmed that the tumors were metabolically active (Supplementary Fig. 10E). As expected, ^{68}Ga -DOTATOC PET scanning showed no uptake in the resistant tumors, confirming the absence of somatostatin receptor-2 (Supplementary Fig. 10F).

Discussion

Stable resistance and morphologic changes

In our BON1 *in vitro* model, permanent long-term treatment with a constant low dose of 10nM everolimus over 24 weeks caused stable resistance to everolimus. In contrast to the study of Vandamme *et al.*, who found a complete reversal of resistance after a drug holiday of 10–12 weeks (Vandamme *et al.* 2016), we demonstrated stable persistent resistance after a drug holiday of 13 weeks.

Figure 10

Dual-targeting approach with the PI3K α inhibitor BYL719 overcame acquired everolimus resistance and restored sensitivity through inhibition of GSK3, EGFR, p70S6K and 4EBP1 signaling. (A) Combination treatment of everolimus plus BYL719 showed a significantly enhanced inhibitory effect on cell viability compared to single substance treatment in all tested cell lines. Cells were incubated with BYL719 (5 μ M and 10 μ M) and everolimus (10 nM) alone and in combination for 72 h. The arithmetic means and standard deviation of at least three independent experiments are shown. Statistically significantly different results in comparison to either single substance treatment are shown, considering $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (B) Protein levels of components from the EGFR-Akt (pEGFR, EGFR, pAkt, Akt), mTORC1-p70S6K (pp70S6K, p70S6K, pS6, S6, p4EBP1, 4EBP1) and GSK3 (pGSK3, GSK3) signaling pathway are displayed. Inhibition of GSK3, EGFR, p70S6K and 4EBP1 signaling was observed after combination treatment. A representative blot out of three independently performed experiments is shown. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0159>.

Everolimus-resistant B0N1 cells have previously been described as developing an elongated shape with fewer cell-to-cell contacts (Passacantilli *et al.* 2014) or to cause no morphologic changes (Vandamme *et al.* 2016). We, however, found an amorphous, flattened cellular shape with lysosome-like bubbles and clumping conglomerates as a morphologic feature of everolimus-resistant B0N1 cells. The differences in stability of resistance and morphologic appearance between the studies may be explained by experimental design differences: Passacantilli *et al.* treated panNETs cells permanently only for 8 weeks with 10 nM everolimus and did not confirm stable resistance (Passacantilli *et al.* 2014); Vandamme *et al.* followed a pulsed-selection experimental design (McDermott *et al.* 2014) by permanently augmenting everolimus doses up to 1 μ M, which might be less clinically relevant due to the application of high drug doses out of the clinically relevant range (around 8–59 nM) (Budde *et al.* 2016, Vandamme *et al.* 2016).

Since B0N1 RR1 and B0N1 RR2 have both been developed on the same genetic background and B0N1 cells have a higher proliferative index than the typical well differentiated neuroendocrine tumor, we aimed to confirm our model in the novel slow growing human pancreatic NET cell line NT-3 (Benten *et al.* 2018). Unfortunately, due to the slow growing nature of NT-3 cells, these experiments are still ongoing now for more than 12 months. The initial results showed a gradual increase in proliferation over time in the presence of doses up to four times of the IC₅₀ value (4 nM) of the parental cell line, and we observed similar changes in morphology as in resistant B0N1 cells with a switch to a flattened and more pitted appearance under continuous everolimus exposure (unpublished results).

Heterogeneity of B0N1 RR1 and B0N1 RR2

Tumor heterogeneity has been suggested as a possible mechanism of everolimus resistance (Vandamme *et al.* 2016) and could be the driving force of sub-clone selection of the most resistant phenotype in response to treatment (Marusyk *et al.* 2014, Vandamme *et al.* 2016). Our data are compatible with this theory since both resistant cell lines established in parallel do not show a completely identical phenotype with slightly different effects of everolimus on p70S6K phosphorylation in both cell lines. The novelty of our work lies in its ability to elicit *common* features of both independently established everolimus-resistant cell lines in order to reduce the bias due to tumor heterogeneity.

Everolimus resistance: G1 phase cell cycle arrest, reduced baseline autophagy and increased baseline apoptosis

Consistent with an increase in G1 phase cell cycle arrest and sub-G1 population in the resistant cell lines in response to everolimus, we observed a baseline decrease in CDK1(cdc2) expression. CDK1(cdc2) is a key regulator of the mitotic transition (Nurse 1990) and its inactivation causes exit from mitosis and stabilizes G1 phase (Potapova *et al.* 2009). The G1 phase cell cycle arrest/sub-G1 increase in the resistant cell lines might be associated with the observed decrease in baseline autophagy. Inhibition of autophagy has shown to be linked to a G1 phase cell cycle arrest, sub-G1 increase and apoptosis induction in different cancer entities (Choi *et al.* 2013, Liu *et al.* 2014, Almasi *et al.* 2018). Nevertheless, the decrease in autophagy seems unexpected in the context of resistance since mTORC1 inhibitor-induced autophagy has previously been described as a potential mechanism of promoting tumor cell resistance: inhibition of autophagy increased everolimus efficacy in NET cells (Avniel-Polak *et al.* 2016, Avniel *et al.* 2018). The decrease in the autophagy marker LC3AII observed in the resistant cell lines might be a mechanism of adaption to the permanent induction of autophagy in the presence of the mTORC1 inhibitor everolimus. This might indicate a sensitive balance of the dual effects of autophagy as a protective mechanism for the cancer cell on the one hand and as a mediator of cell death on the other (Sui *et al.* 2013).

Since apoptosis negatively correlates with autophagy (Liao *et al.* 2012, Sui *et al.* 2013, Avniel-Polak *et al.* 2016, Mejias-Pena *et al.* 2017), the decrease in autophagy in the resistant cell lines might cause the observed increase in apoptosis. All these effects might be due to GSK3 over-activation. GSK3 over-activation, which is discussed below in detail, has previously been reported to be directly associated with G1 cell cycle arrest, decreased autophagy and increased apoptosis (Li *et al.* 2011, Giancotti 2014, Maurer *et al.* 2014, Weikel *et al.* 2016, Zhang *et al.* 2016, Aristizabal Prada *et al.* 2017) and to be a crucial feature of cancer cell resistance to chemotherapy, radiotherapy and targeted therapies (Domoto *et al.* 2016, McCubrey *et al.* 2016).

Everolimus resistance increased migration potential and c-Met activation

BON1 RR1 and BON1 RR2 cells displayed significantly increased migration potential in response to everolimus

treatment and baseline c-Met over-activation. Temsirolimus-(mTORC1 inhibitor)-resistant renal cell carcinoma cells also showed enhanced migration potential (Juengel *et al.* 2014). Mis-regulation, overexpression or gene amplification of the RTK c-Met have been identified to play a crucial role in tumor invasion, motility and metastasis formation associated with poor prognosis (Di Renzo *et al.* 1992, Camp *et al.* 1999, Chen *et al.* 1999, Takeo *et al.* 2001, Ma *et al.* 2003, Song *et al.* 2010). However, in previous studies, we demonstrated that specific c-Met inhibition by either knock-down experiments with siRNA or the specific c-Met inhibitor INC280 could neither decrease cellular viability nor cellular migration in NET cells *in vitro* (Reuther *et al.* 2016). C-Met activation might be rather indirectly associated with cellular migration potential in NET cells *in vitro* through activation of alternative pathways, such as the PI3K-Akt-mTORC1 pathway (Engelman *et al.* 2007).

Everolimus resistance: baseline GSK3 over-activation and decrease in IRS-1 protein levels

A feedback activation of RTKs such as the insulin receptor due to mTORC1 inhibition has previously been reported (Chandrarapaty *et al.* 2011). The feedback activation of PI3K-Akt signaling by the mTORC1 inhibitor everolimus via disinhibition/activation of IRS-1 has been discussed as a possible cause of resistance in NETs (Fig. 1, red arrows) (O'Reilly *et al.* 2006, Zitzmann *et al.* 2010, Vandamme *et al.* 2016). We have indeed found that pro-survival signaling of the PI3K-Akt pathway through Akt phosphorylation/activation upon everolimus re-application persisted in BON1 RR1 and BON1 RR2, as described previously in long-term everolimus-treated panNET cells (Passacantilli *et al.* 2014). Therefore, we speculate that there was permanent PI3K/Akt activation in the presence of persisting everolimus treatment during the development of resistance. Constitutively active PI3K has previously been reported to induce a reduction in IRS-1/2 protein levels (Pirola *et al.* 2003, Wada 2009). Therefore, IRS-1 downregulation observed in the resistant cell lines might be a compensatory escape mechanism in response to chronic PI3K/Akt over-activation, similar to the mechanism of development of insulin resistance (Pirola *et al.* 2003). Different IRS-1-induced signaling pathways have previously been reported to inhibit GSK3 (Cohen & Frame 2001, Wada 2009, Maurer *et al.* 2014, Vandamme *et al.* 2016). Therefore, lower baseline IRS-1 protein levels in the resistant cells may lead to lower GSK3 inhibition and thus stronger GSK3 activation.

Since baseline Akt activity or expression was not changed in the resistant cells, compared to the sensitive controls, it is unlikely that GSK3 is over-activated through modulation of the Akt pathway, but more likely through an alternative pathway. These mechanisms need to be investigated in future studies in NETs.

Various studies have demonstrated an association between IRS-1/GSK3 α/β deregulation and tumorigenesis (Wada 2009, McCubrey *et al.* 2016). GSK3 over-activation may promote the development of everolimus resistance, as recently reviewed for GSK3 in the context of resistance to chemotherapy, radiotherapy and targeted therapies (Domoto *et al.* 2016, McCubrey *et al.* 2016). Similar effects were observed in transfected MCF-7 breast cancer cells, where a constitutively active GSK3 construct showed resistance to the mTORC1 inhibitor rapamycin (McCubrey *et al.* 2014).

In order to prove the importance of constitutively-active GSK3 in everolimus-resistant panNET cells, we treated BON1 RR1/RR2 cells with the selective GSK3 inhibitor AR-A014418 and found a striking decrease in cellular viability in BON1 RR1/RR2, and to a lesser extent in sensitive control cells, associated with an increase in the autophagy marker LC3AII. However, unexpectedly, combination treatment of AR-A014418 plus everolimus did not enhance the inhibitory effects of single AR-A014418 treatment. The absence of synergism in the AR-A014418/everolimus combination treatment may be explicable by GSK3 α inhibition being similar in response to the combination treatment compared to single treatment with AR-A014418 in the resistant cell lines.

Dual-targeting approach with the PI3K α inhibitor BYL719 overcame acquired everolimus resistance

In a previous study on different NET cell lines, the selective PI3K α inhibitor BYL719 showed synergistic anti-proliferative effects in combination with everolimus through strong GSK3 inhibition (Nölting *et al.* 2017). In contrast to AR-A014418/everolimus combination treatment, the combination of everolimus with BYL719 was synergistic at low therapeutically-relevant doses in both resistant cell lines and restored everolimus sensitivity through more potent GSK3 inhibition, compared to each drug separately; this was associated with IRS-1 up-regulation, increased autophagy associated with decreased apoptosis, and an inhibition of EGFR, 4EBP1 and p70S6K. This is consistent with our previous study showing that selective GSK3 inhibition

led to inhibition of EGFR, p70S6K and 4EBP1 and enhanced the autophagy marker LC3AII in BON1 cells (Aristizabal Prada *et al.* 2017). It has previously been reported that inhibition of GSK3 restored autophagy (Weikel *et al.* 2016, Zhang *et al.* 2016, Aristizabal Prada *et al.* 2017) and re-sensitized different tumor entities to chemotherapy, radiotherapy or targeted therapy (Domoto *et al.* 2016).

Similar combination approaches using PI3K inhibitors (BEZ235 or BKM120), although without taking GSK3 signaling into account, have previously been shown to overcome reversible everolimus resistance (Passacantilli *et al.* 2014, Vandamme *et al.* 2016). BYL719 is currently being investigated in a late phase 3 clinical study (SOLAR-I) for breast cancer (NCT02437318) and will probably enter clinical use. Moreover, a phase 1b clinical study is currently evaluating the safety of BYL719 plus everolimus in advanced breast cancer, renal cell cancer and pancreatic tumors including panNETs (NCT02077933). Therefore, we provide novel data on the potentially clinically relevant PI3K α inhibitor BYL719. GSK3 inhibition by BYL719 might be considered as second-line treatment in everolimus-resistant panNETs if indeed permanent GSK3 over-activation, potentially in combination with decreased IRS-1 protein levels, can be demonstrated as a central mechanism of resistance *in vivo*.

Conclusions and perspectives

In this study, we established two stable everolimus-resistant panNET (BON1) cell lines. The mechanisms of resistance may include G1 cell cycle arrest, decreased CDK1/cdc2 expression, reduced autophagy, increased apoptosis and migration potential potentially due to permanent GSK3 over-activation compatible with downregulation of IRS-1 protein levels. The dual-targeting approach with the PI3K α inhibitor BYL719 restored treatment sensitivity through GSK3 inhibition, IRS-1 upregulation and increased autophagy in the resistant cells. In a pilot study, we have already transferred our resistant cell line model to an orthotopic intrapancreatic tumor xenograft mouse model, and this may represent a valid basis to validate our *in vitro* findings of this first stable everolimus-resistant cell line model and translate it into clinical practice.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-18-0159>.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of the research reported. C J A and A B G have received research contracts (Ipsen, Novartis), lecture honoraria (Ipsen, Novartis) and advisory board honoraria (Novartis). J S has received research contracts (Novartis) and lecture honoraria (Novartis, Ipsen). S N has received a research contract (Novartis) and lecture honoraria (Ipsen).

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