# Long-term acquired everolimus resistance in pancreatic neuroendocrine tumours can be overcome with novel PI3K-AKT-mTOR inhibitors

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**Background:** The mTOR-inhibitor everolimus improves progression-free survival in advanced pancreatic neuroendocrine tumours (PNETs). However, adaptive resistance to mTOR inhibition is described.

**Methods:** QGP-1 and BON-1, two human PNET cell lines, were cultured with increasing concentrations of everolimus up to 22 weeks to reach a dose of 1 $\mu$M everolimus, respectively, 1000-fold and 250-fold initial $IC_{50}$. Using total DNA content as a measure of cell number, growth inhibitory dose–response curves of everolimus were determined at the end of resistance induction and over time after everolimus withdrawal. Response to ATP-competitive mTOR inhibitors OSI-027 and AZD2014, and PI3K-mTOR inhibitor NVP-BEZ235 was studied. Gene expression of 10 PI3K-Akt-mTOR pathway-related genes was evaluated using quantitative real-time PCR (RT–qPCR).

**Results:** Long-term everolimus-treated BON-1/R and QGP-1/R showed a significant reduction in everolimus sensitivity. During a drug holiday, gradual return of everolimus sensitivity in BON-1/R and QGP-1/R led to complete reversal of resistance after 10–12 weeks. Treatment with AZD2014, OSI-027 and NVP-BEZ235 had an inhibitory effect on cell proliferation in both sensitive and resistant cell lines. Gene expression in BON-1/R revealed downregulation of *MTOR*, *RICTOR*, *RAPTOR*, *AKT* and *HIF1A*, whereas *4EBP1* was upregulated. In QGP-1/R, a downregulation of *HIF1A* and an upregulation of *ERK2* were observed.

**Conclusions:** Long-term everolimus resistance was induced in two human PNET cell lines. Novel PI3K-AKT-mTOR pathway-targeting drugs can overcome everolimus resistance. Differential gene expression profiles suggest different mechanisms of everolimus resistance in BON-1 and QGP-1.

Neuroendocrine tumours (NETs) are a diverse group of neoplasms, mainly found in the gastrointestinal tract, lung and pancreas.

Pancreas NETs (PNETs) are relatively rare, with an incidence of 0.43 per 100 000 according to the Surveillance, Epidemiology and End

Results (SEER) registry (Yao *et al*, 2008). However, this rate has doubled during the last 20 years (Lawrence *et al*, 2011; Fraenkel *et al*, 2012). Primary therapy for localised PNET remains surgical excision. However, up to 60% of all patients present with unresectable disease (Halfdanarson *et al*, 2008). In these patients, systemic treatment has an essential role in controlling the disease (Falconi *et al*, 2012). The role of traditional cytotoxic therapies in PNET remains a matter of debate, with only small series showing response to streptozocin-based chemotherapy (Calender, 1997; Hansel *et al*, 2004; Schonhoff *et al*, 2004). Newer chemotherapy regimens with temozolomide alone (Ekeblad *et al*, 2007), or in combination with capecitabine (Strosberg *et al*, 2011), show promise. The low response rate for streptozocin-based chemotherapy and the associated side effects underscore the need for targeted drugs.

The phosphoinositide-3-kinase/Akt/mammalian target of rapamycin (PI3K-Akt-mTOR) signalling pathway has a major role in NET by regulating cell growth, proliferation, survival and protein synthesis (Figure 1A). Furthermore, elevated mTOR expression and activity is associated with a higher proliferative capacity and worse prognosis (Missiaglia *et al*, 2010). Recently, exome sequencing of primary PNET tumour samples revealed mTOR pathway genes to be mutated in 16% of all PNETs, in addition to highlighting mutations in other genes, including *MEN1* (44% of all patients), *DAXX* (25%) and *ATRX* (18%) (Jiao *et al*, 2011). mTOR acts as the catalytic subunit of two functionally distinct complexes, named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Capdevila *et al*, 2011). mTOR proves to be an interesting target for therapy of PNET with mTOR-inhibiting rapamycin and analogues (rapalogues) such as everolimus (RAD001). Rapamycin, everolimus and other rapalogues form a complex with the 12 kD FK506-binding protein FKBP12 (Lach *et al*, 1999; Helpap and Kollermann, 2001). This rapalogue-FKBP12 complex allosterically inhibits mTOR when it is part of mTORC1. However, rapalogues only have limited effect on mTOR when mTOR is part of mTORC2 because of steric hindrance by the Rictor mTORC2-subunit (Goto *et al*, 2001). A phase III trial with everolimus was conducted in 410 patients with well- and moderately differentiated PNETs and showed an improvement in median progression-free survival (PFS) in the everolimus-treated group compared with the placebo group (Yao *et al*, 2011). Similar results were seen in the phase III trial with sunitinib, a pan-tyrosine kinase inhibitor (Raymond *et al*, 2011). On the basis of these results everolimus and sunitinib became the first FDA and EMA approved drugs in 30 years for the treatment of locally advanced, unresectable or metastatic PNETs. However, an objective partial response was only seen in 5% of the patients receiving everolimus or sunitinib. The significant effect on PFS was thus mainly due to disease stabilisation and minor reductions in tumour growth. As PFS in phase III study with everolimus is still limited to 11 months, adaptive resistance to mTOR inhibition with rapalogues was described (Yao *et al*, 2013). To overcome this resistance, novel PI3K-AKT-mTOR targeting drugs have been developed, such as NVP-BEZ235, OSI-027 and AZD2014. Exploiting the homology between the kinase domain of mTOR and PI3K, NVP-BEZ235 docks in the active pocket of both molecules and reduces kinase activity of PI3K and mTOR by competing with ATP-binding. The selective mTOR inhibitors AZD2014 and OSI-027 target the kinase domain of mTOR, blocking both mTORC1 and mTORC2 in an ATP-competitive manner, without blocking PI3K kinase activity (Maira *et al*, 2008; Yu *et al*, 2009). Although the efficacy of novel drugs in PNET cell line model of short-term adaptive resistance to everolimus has been studied (Passacantilli *et al*, 2014), no data are currently available about long-term adaptive resistance in everolimus-treated PNET. A better understanding of the mechanisms underlying resistance to rapalogues is thus necessary for a predictive biomarker for everolimus resistance to be identified.

## MATERIALS AND METHODS

**Cell lines and culture conditions.** BON-1 and QGP-1, two human PNET cell line models, were used in this study. The BON-1 cell line was a kind gift from Dr Townsend (University of Texas Medical Brachn, Galveston, TX, USA; Townsend *et al*, 1993). The QGP-1 cell line was purchased from the Japanese Collection of Research Bioresources Cell Bank (JRCB, Osaka, Japan; Kaku *et al*, 1980). BON-1 and QGP-1 cell line identity was confirmed using short tandem repeat profiling (Vandamme *et al*, 2015). The BON-1 cell line was cultured in $1:1$ mixture of Dulbecco's modified Eagle medium (DMEM) and F12 medium, supplemented with 10% fetal calf serum (FCS), penicillin ($1 \times 10^5$ units per l), fungizone ($0.5\,mg\,l^{-1}$), and L-glutamine ($2\,mmol\,l^{-1}$). The QGP-1 cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% FCS and penicillin-streptomycin ($1 \times 10^5$ units per l penicillin and $1 \times 10^5$ units per l streptomycin). All cell lines were incubated in an atmosphere of 95% humidity and 5% $CO_2$ at 37 °C. Media and supplements were obtained from Life Technologies Bio-cult Europe (Invitrogen, Breda, The Netherlands).

**Drugs and reagents.** Everolimus (RAD001), AZD2014, OSI-027 and NVP-BEZ235 were purchased from Selleckchem (Selleck Chemicals, Houston, TX, USA). Rapamycin was purchased from LG Laboratories (Woburn, MA, USA). All inhibitors were dissolved in 100% dimethylsulfoxide (DMSO) to a 1 mM concentration and stored in $-20$ °C. All drugs were diluted to working concentrations in 40% DMSO before use. In all the experiments, controls were treated with a vehicle DMSO concentration equivalent to the 0.4% final DMSO concentration in the treatment dilutions.

**Cell proliferation assay using total DNA content.** Cells were plated in 1 ml medium in 24-well plates at the density necessary to obtain a 70–80% cell confluence in the control groups at the end of the experiment. Medium was refreshed and the tested compounds were added to wells in quadruplicate after 24 h for QGP-1 and 72 h for BON-1. Time points were chosen to reduce inter- and intra-experiment variability. The concentrations of compounds tested ranged between 0,1 nM and 1 μM for everolimus, rapamycin and NVP-BEZ235. Given the narrow therapeutic margin of AZD2014 and OSI-027, the used concentrations ranged from 10 nM to 1 μM with an added 250 nM and 500 nM concentration. Every 3 days, the cells were supplied with fresh medium and compounds. After 7 days of treatment, the cells were harvested for DNA measurement. Measurement of total DNA content, as a measure of cell number, was performed with the bisbenzimide DNA-intercalating fluorescent dye (Hoechst 33258; Boehring Diagnostics, La Jolla, CA, USA) as previously described (Barrett *et al*, 1995).

**Quantitative real-time PCR of PI3K-AKT-mTOR pathway genes.** The tested cell line conditions were plated in 3 ml medium in six-well plates at the density required to obtain 70–80% cell confluence at the end of the experiment. Twenty-four hours later for QGP-1 cell line conditions and 72 h later for BON-1 cell line conditions, medium was replaced and cells were incubated for 72 h with vehicle. One-step reverse transcription quantitative PCR (RT–qPCR) was performed on total RNA from six biological replicates in a single reaction using the Power SYBR Green RNA-To-CT 1-Step kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) on a LightCycler 480 instrument (Roche Applied Science, Penzberg, Germany). Primers were designed using QuantPrime software (Arvidsson *et al*, 2008) and RTPrimerDB (http://www.rtprimerdb.org) and have been obtained from Integrated DNA Technologies (Leuven, Belgium) (Supplementary Table 1). All reactions have been performed in triplicates in 384-well plates with 2 μl total RNA (prediluted to 15 ng μl$^{-1}$) as input in a total reaction volume of 10 μl, further comprising 5 μl Power SYBR
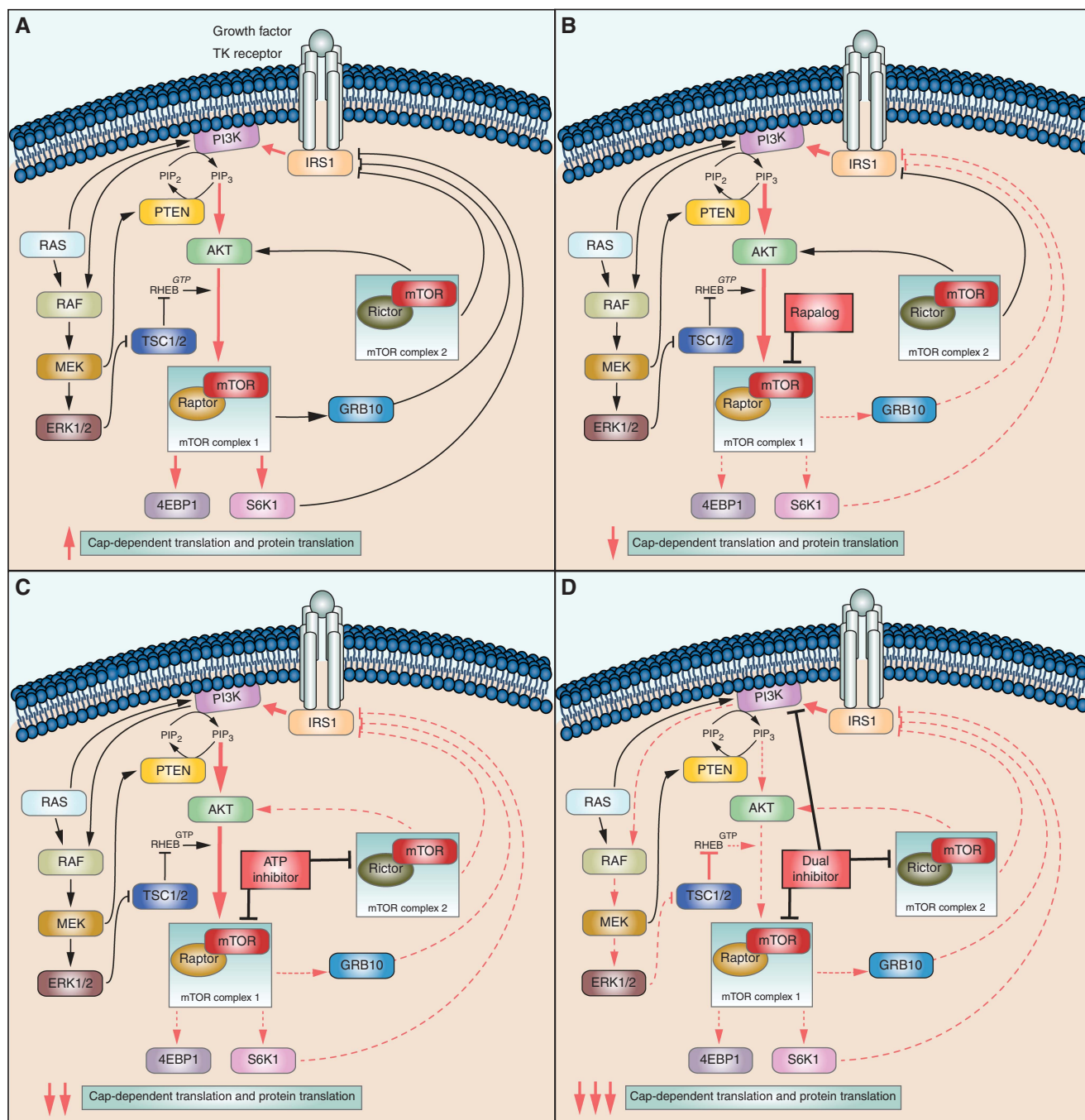
Figure 1. Simplified representation of the PI3K-AKT-mTOR pathway in pancreatic neuroendocrine tumours. Constitutional activation of the pathway (A) and its response to rapalogues (B), ATP-competitive inhibitors (C) and dual PI3K-mTOR inhibitors (D) is shown. Red full lines describe an increase and red dashed lines show a decrease of feedback under different conditions. A detailed description can be found in the text.

Green RT-PCR Mix (2×; Life Technologies, Thermo Fisher Scientific), 0.08 μl RT Enzyme Mix (125×; Life Technologies, Thermo Fisher Scientific) and 200 nM of each primer (final concentration).

**Statistical analysis.** All cell proliferation assays were performed at least twice at different times. The repeated experiments gave comparable results. The comparative statistical evaluations between the different cell line conditions were performed by two-way ANOVA with treatment concentration and cell line condition as variables. For *post hoc* testing, a multiple comparative test with Dunn–Šidák correction was used. For RT–qPCR experiments, normalised relative gene expression values were calculated using qBasePLUS software version 1.5 (Biogazelle,

Zwijnaarde, Belgium). Messenger RNA expression was normalised to household gene expression (*GAPDH* and *RPL13A* for BON-1; *HPRT* and *YWAZ* for QGP-1) according to the geNorm algorithm (Mestdagh *et al*, 2009). Comparison between gene expression levels was done by Student's *t*-test and adjusted for multiple testing using Holm–Bonferroni correction. All statistical analyses were done using GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, CA, USA).

## RESULTS

**Inducing everolimus resistance.** In untreated human PNET cell lines BON-1 and QGP-1, the everolimus concentration that

reduces growth by 50% (IC$_{50}$) after 7 days of treatment was 1 and 4 nM, respectively (data not shown). Starting from this IC$_{50}$ concentration, QGP-1 and BON-1 were continuously cultured in increasing concentrations of everolimus. The everolimus concentration was progressively doubled every 14 days during 8–10 dose doublings until a final concentration of 1 μM was reached. In parallel, clonal BON-1 and QGP-1 cells were long-term vehicle treated. The established long-term everolimus-treated cell lines (BON-1/R and QGP-1/R) were maintained in the maximally achieved everolimus concentration. No morphological changes were seen between the long-term vehicle-treated cell lines and the long-term everolimus-treated cell lines (Supplementary Figure 2). After the establishment of the long-term everolimus-treated BON-1/R and QGP-1/R, both showed a statistically significant reduced growth inhibitory response to everolimus in comparison with long-term vehicle-treated BON-1 and QGP-1 at everolimus concentrations between 10 nM and 1 μM for BON-1/R and at 1 nM and 1 μM for QGP-1/R, respectively (Figure 2A and B). In addition, BON-1/R and QGP-1/R had a significantly reduced sensitivity to rapamycin in concentrations ranging from 1 nM to 1 μM when compared with their long-term vehicle-treated BON-1 and QGP-1 counterparts (Figure 2C and D).

**Evolution of everolimus-resistance over time.** In order to study reversibility of everolimus-resistance, the BON-1/R and QGP-1/R cell lines were cultured without everolimus maintenance treatment during 10 and 12 weeks, respectively, showing a gradual return of everolimus sensitivity (data not shown). After 10–12 weeks, this resulted in the BON-1/R STOP and QGP-1/R STOP cell line conditions. When comparing these cell line conditions with BON-1/R and QGP-1/R, maintained during 10–12 weeks at maximum 1 μM everolimus concentration, and vehicle-treated BON-1 and QGP-1, a return of BON-1/R STOP and QGP-1/R STOP to the sensitivity levels of BON-1 and QGP-1 was observed (Figure 3A and B).

**Overcoming everolimus-resistance.** A dose–response study in both everolimus-resistant and -sensitive BON-1 and QGP-1 cells to the growth inhibitory effect of AZD2014, OSI-027 and NVP-BEZ235 was executed in parallel. After a 7-day-treatment with AZD2014, cell proliferation was significantly less reduced at the 250 nM and 500 nM concentration of AZD2014 in BON-1/R when compared with long-term vehicle-treated BON-1. When exposing QGP-1/R and QGP-1 to AZD2014 during 7 days, growth reduction was significantly more pronounced in long-term vehicle-treated QGP-1 when compared with QGP-1/R in all tested AZD2014 concentrations above 100 nM. A maximal inhibition of >80% of cell proliferation was obtained at 1 μM of AZD2014 in all cell lines tested (Figure 4A and B). BON-1/R and long-term vehicle-treated BON-1 did not respond significantly different to OSI-027, while QGP-1/R was more resistant to OSI-027 than QGP-1 in all concentrations tested above 100 nM. The maximum inhibition with OSI-027 reached in BON-1/R, BON-1 and QGP-1 cells was a 50% reduction of cell proliferation (Figure 4C and D). No statistically significant difference in the inhibition of cell proliferation was observed after 7 days of treatment with NVP-BEZ235 in BON-1/R when compared with long-term vehicle-treated BON-1 in the 1 nM to 1 μM NVP-BEZ235 concentration range. In both everolimus-sensitive and -resistant BON-1 cells, a maximum inhibition of >92% of control cell proliferation was reached at 100 nM of NVP-BEZ235. When comparing the QGP-1/R and QGP-1, NVP-BEZ235 was less potent at 10 nM, compared with QGP-1/R cells. Maximal cell growth inhibition was achieved a 100 nM in both QGP-1/R and long-term vehicle-treated QGP-1 (Figure 4E and F).
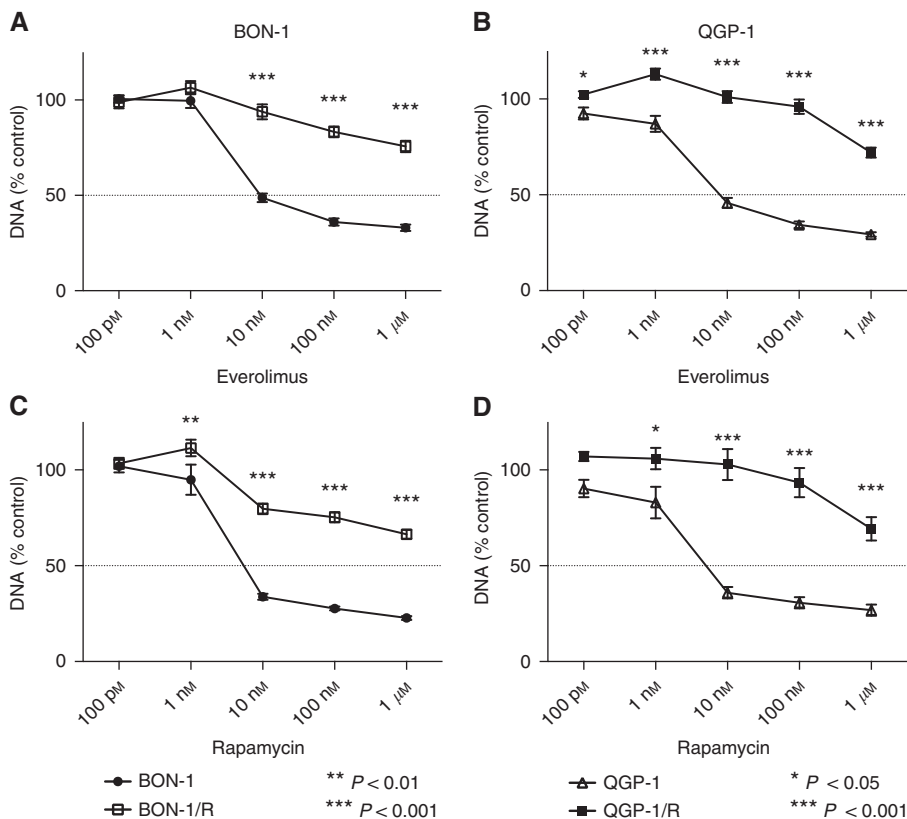


Figure 2. Everolimus dose response curves of QGP-1/R and long-term vehicle-treated QGP-1 and BON-1/R and long-term vehicle-treated BON-1. All cells were treated for 7 days with increasing concentrations of everolimus (**A**, **B**) or rapamycin (**C**, **D**), respectively. Growth inhibitory response is expressed as the percentage of vehicle-treated control (± s.e.m.). Control is normalised at 100%.
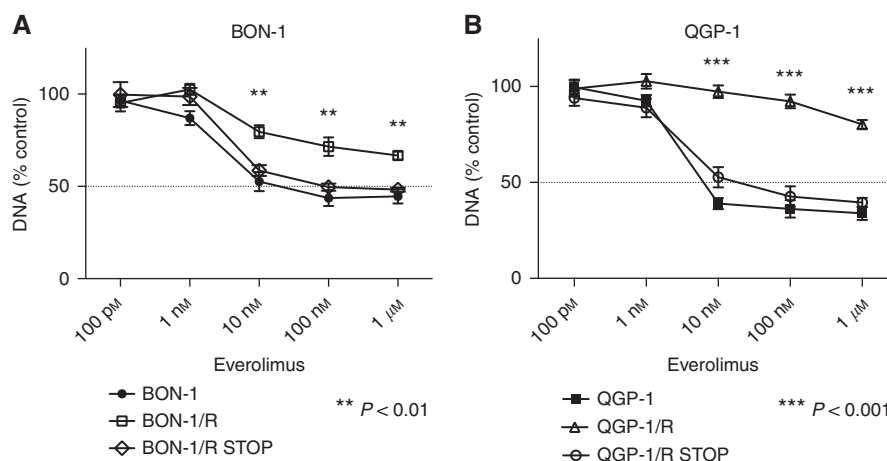
**Figure 3. Everolimus dose response curves after 10 to 12 weeks of wash-out.** Response was evaluated after 7 days of treatment with increasing everolimus concentrations in BON-1/R, long-term vehicle-treated BON-1, and BON-1/R after an everolimus wash-out period of 12 weeks (BON-1/R STOP) (**A**) and QGP-1/R, and in long-term vehicle-treated QGP-1/ and QGP-1/R after an everolimus wash-out period of 10 weeks (QGP-1/R STOP) (**B**). Growth inhibitory response is expressed as the percentage of vehicle-treated control ($\pm$ s.e.m.). Control is normalised at 100%. *P*-values shown are for Dunn Sídák post hoc comparison after two-way ANOVA between BON-1/R and QGP-1/R and BON-1/R STOP and QGP-1/R STOP, respectively.

**Gene expression changes in everolimus resistance.** Differential gene expression of *MTOR, RAPTOR, RICTOR, AKT, S6K1, 4EBP1, ERK1, ERK2, BCL2* and *HIF1A* between BON-1/R and long-term vehicle-treated BON-1 showed a significant downregulation of *MTOR, RICTOR, RAPTOR, AKT* and *HIF1A*, whereas *4EBP1* was significantly upregulated ($P < 0.05$) (Figure 5). When comparing QGP-1/R and QGP-1, a significant downregulation of *HIF1A* and a significant upregulation of *ERK2* were observed ($P < 0.05$).

## DISCUSSION

In this study, to the best of our knowledge, the first two PNET models for long-term acquired everolimus resistance were established. Both QGP-1 and BON-1 were cultured during more than 20 weeks in increasing concentrations of everolimus and continued to grow under a 250 and 1000-fold IC$_{50}$ growth inhibitory concentration of everolimus, respectively. Corresponding *in vivo* concentrations are not reachable in patients (O'Donnell *et al*, 2008). Continued cell growth under these high everolimus concentrations, unreachable in patients, hence indicates an everolimus resistance with possible clinical implications. Both the resulting BON-1/R and QGP-1/R show a significantly decreased response to everolimus in comparison with long-term vehicle-treated BON-1 and QGP-1, even in the highest concentrations tested (1 $\mu$M). Similar results were seen when comparing BON-1/R and QGP-1/R and its vehicle-treated counterparts for response to rapamycin. This indicates that BON-1/R and QGP-1/R are not only everolimus-resistant, but are also resistant to other rapalogues. A previous study looked at everolimus-resistance in BON-1 (Passacantilli *et al*, 2014). However, this study treated BON-1 cells during 8 weeks with a dose of 10 nM, which is a much shorter period and a lower dose than used in this study. In addition, the authors did not perform a resistance induction experiment with QGP-1. Given the long duration of treatment, the resulting BON-1/R and QGP-1/R cell lines in our study could be considered as a representative model for rapalogue resistance seen in PNET patients, where median time to treatment failure and, thus, acquired everolimus resistance is 11 months (Yao *et al*, 2011).

Various mechanisms have been proposed for the limited response to everolimus in PNET (Figure 1B). Not all phosphorylation sites of mTORC1 downstream proteins such as p70 ribosomal S6 kinase 1 (S6K1), growth factor receptor bound protein 10 (GRB10) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) respond to the same extent to allosteric inhibition of mTORC1 by rapalogues(Kang *et al*, 2013), thereby diminishing rapalogue efficacy. Adaptive resistance may also be caused by induction of activated phosphorylation of AKT. This occurs through the lifting of negative feedback of the mTORC1 downstream p70 ribosomal S6 kinase 1 (S6K1) on the PI3K-AKT-mTOR pathway(Ohike *et al*, 2003; O'Reilly *et al*, 2006; Julien *et al*, 2010). S6K1 effects this negative-feedback on insulin receptor substrate-1 (IRS-1), which regulates insulin-like growth factor I (IGF-1; O'Reilly *et al*, 2006). Furthermore, mTORC1 activates GRB10, which negatively regulates IGF-1 signalling. When mTORC1 is inhibited by rapalogues, this negative feedback-loop of IGF-1 is suppressed, synergistically adding to the effect of mTOR inhibition of the S6K1-feedback loop (Emerling and Akcakanat, 2011). As rapalogues effectively block mTORC1 but only have a limited, dose-dependent action on the mTORC2, the effect of rapalogues on mTOR signalling may be circumvented through increased activity of mTORC2 (O'Reilly *et al*, 2006; Julien *et al*, 2010). Furthermore, a direct role of S6K1 on mTORC2-mediated AKT phosphorylation has been described since S6K1 might be instrumental in the inhibitory phosphorylation of Rictor, the rapalogue-insensitive component of mTORC2 (Julien *et al*, 2010). Novel mTOR inhibitors, blocking both mTORC1 and mTORC2 by competitively binding the ATP-binding mTOR kinase pocket, have been developed to overcome these escape mechanisms. AZD2014 is an ATP-competitive mTOR inhibitor, currently undergoing phase II evaluation in different tumour types (Pike *et al*, 2013; Basu *et al*, 2015a) (Figure 1C). In our study, AZD2014 effectively reduces cell proliferation both in the everolimus-sensitive QGP-1 and BON-1, as well as in the everolimus-resistant BON-1/R and QGP-1/R. This is the first time that AZD2014 shows efficacy in PNET models. In addition, these results indicate that AZD2014 overcomes everolimus resistance in PNET in concentrations reachable in patients (Basu *et al*, 2015a). Further development of this drug in PNET could hence benefit PNET patients. In addition, OSI-027, a drug from the same ATP-competitive mTOR inhibiting class was tested. In contrast with the other tested compounds, the growth inhibitory effect of OSI-027 in
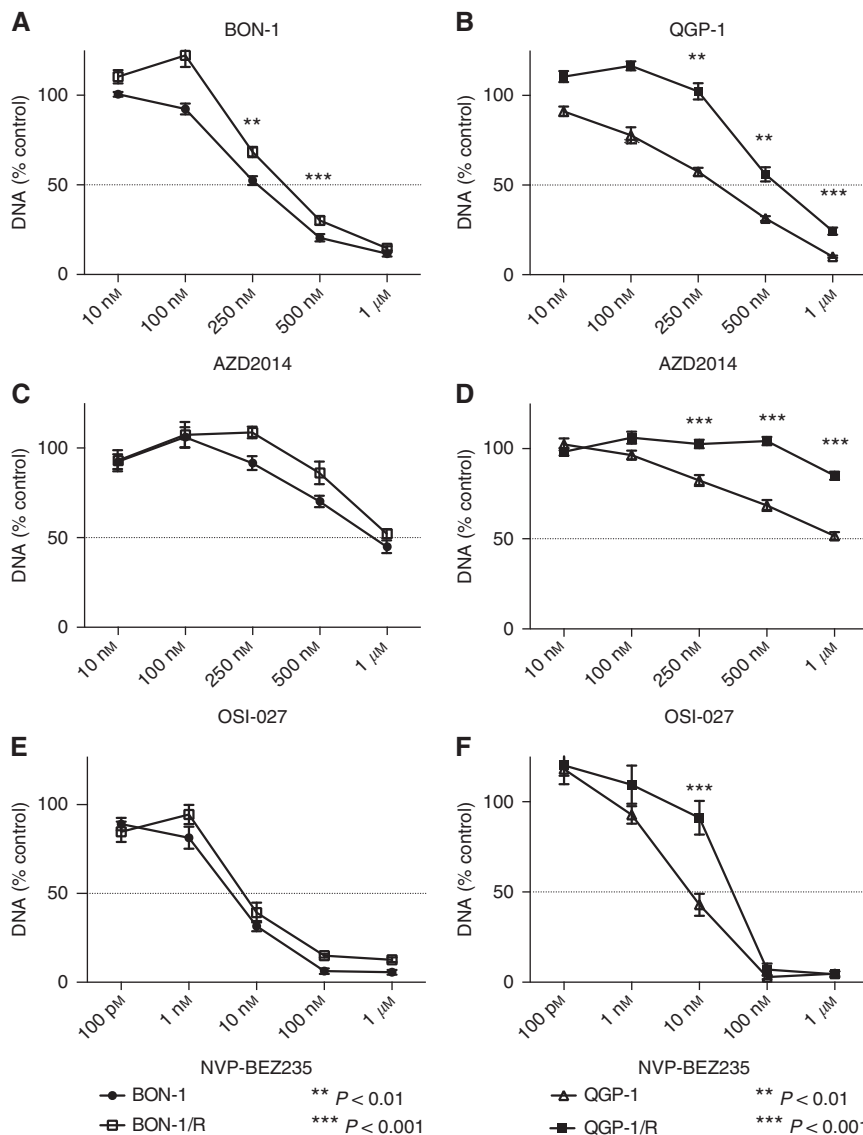
**Figure 4. Dose–response curves with novel PI3K-Akt-mTOR inhibitors in BON-1/R and long-term vehicle-treated BON-1 and QGP-1/R and long-term vehicle-treated QGP-1.** Cells were treated during 7 days with, respectively, AZD2014 (**A**, **B**), OSI-027 (**C**, **D**) and NVP-BEZ235 (**E**, **F**). Response is expressed as the percentage of vehicle-treated control (± s.e.m.). Control is normalised at 100%.

the concentration ranges tested reaches growth inhibition by only 50% at 1 µM in vehicle-treated BON-1 and QGP-1 cell lines. In a xenograft mouse model, concentrations of up to 2 µM could be reached, but further studies on pharmacokinetics in humans are needed (Bhagwat *et al*, 2011). Although resistant and sensitive BON-1 responded equally to OSI-027 treatment, only limited efficacy in overcoming everolimus resistance with OSI-027 was observed in the QGP-1 cell line. This difference in response to OSI-027 between BON-1/R and QGP-1/R suggests two distinct molecular mechanisms of resistance. NVP-BEZ235 is a dual blocker of mTOR, blocking both mTORC1 and mTORC2, and the upstream PI3K (Doglioni *et al*, 1998) (Figure 1D). NVP-BEZ235 has proven efficacy in *in vitro* and *in vivo* PNET models (Doglioni *et al*, 1998; Paireder *et al*, 2013). Dual inhibition of the PI3K-mTOR pathway could prevent cross-talk activation of the mitogen-activated kinase and extracellular signal-regulated kinase (MAPK-ERK pathway) through PI3K-mediated feedback loop (Helpap and Kollermann, 1999; Carracedo *et al*, 2008; Zitzmann *et al*, 2010; Svejda *et al*, 2011; Paireder *et al*, 2013). This cross-talk could lead to an escape of mTORC1 inhibition and, hence, to rapalogue resistance. Although NVP-BEZ235 has completed phase II studies,

clinical development of this drug might not progress to phase III because of the drug's safety profile (Fazio *et al*, 2016). However, our current study demonstrates that dual blocking of PI3K and mTOR could be an attractive strategy to overcome long-term acquired everolimus resistance. In addition, maximum inhibition in both resistant and sensitive cell lines was reached with NVP-BEZ235 concentrations more than 10-fold lower than dose-limiting plasma concentrations obtained in phase I studies (Bendell *et al*, 2015). As this is in line with previously reported *in vitro* results and an *in vivo* study with lower dose NVP-BEZ235 in a glioblastoma model demonstrated efficacy, it could hence be interesting to evaluate low-dose NVP-BEZ235 in PNET (Maira *et al*, 2008; Passacantilli *et al*, 2014).

Another possible mechanism for everolimus resistance is tumour heterogeneity. Within-patient and within-tumour hetero-geneity in proliferation, genomic alterations and functional imaging characteristics have been demonstrated in neuroendocrine tumour patients (Gebauer *et al*, 2014; Shi *et al*, 2015; Basu *et al*, 2015b). This heterogeneity could be caused by tumoural subclones with different phenotypes and responses to treatement (Marusyk *et al*, 2014). By treating a patient with everolimus, selection of
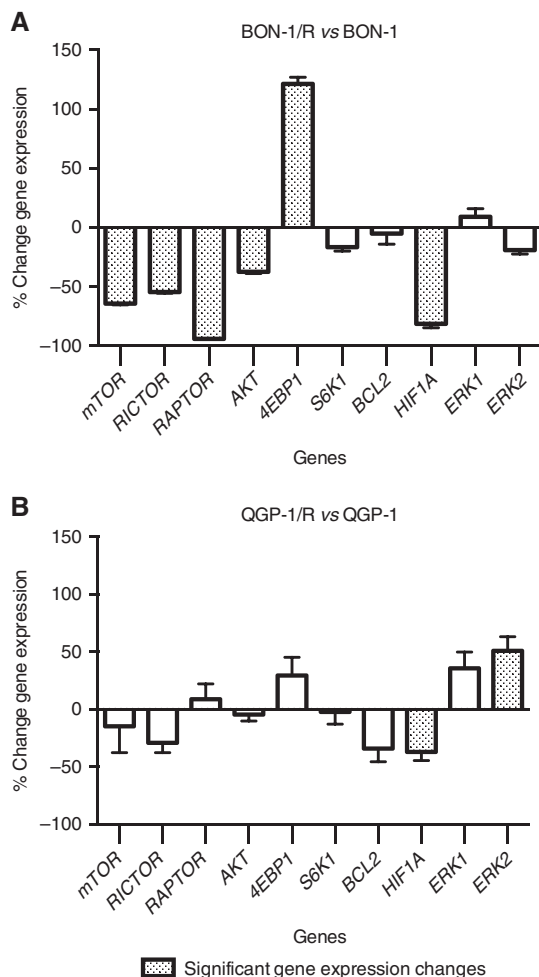
Figure 5. Changes in mRNA expression between resistant and sensitive cell lines. Changes are expressed in mean percentage ± s.e.m between BON-1/R and BON-1 and QGP-1/R (A) and QGP-1 (B). In addition to *MTOR*, mTORC1 component *RAPTOR* and mTORC2 component *RICTOR* expression was studied. Moreover, gene expression of *AKT*, encoding the mTOR upstream AKT, S6K1 and 4EBP1, encoding the mTOR downstream p70 ribosomal kinase 1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), respectively, was determined. In addition, *BCL2*, a gene encoding for anti-apoptotic protein B-cell leukaemia 2 (Bcl-2), and *HIF1A*, encoding the hypoxia-inducible factor 1-alpha protein (HIF-1 alpha) was used to study the overall effect on apoptosis and proliferation in the cell. Finally, expression of *ERK1* and *ERK2*, two components of the MAPK-ERK pathway was determined. Differences were tested using Student's t-test. $P < 0.05$, after Bonferroni correction for multiple testing, was considered significant (hatched bars).

everolimus-resistant subclones could occur, leading to resistance. Although short-term everolimus resistance is mainly driven by phosphorylation changes in PI3K-AKT-mTOR pathway proteins (O'Reilly *et al*, 2006), a shift in clonal population could be a driving force in long-term everolimus resistance. This is illustrated by the gradual reversal of everolimus resistance in BON-1/R and QGP-1/R, with a complete return of everolimus sensitivity only after >2 months of culturing without everolimus. If this resistance would only be caused by phosphorylation and dephosphorylation of proteins, a faster reversal of everolimus resistance could be expected. Long-term treatment with everolimus might hence select or induce subclones with a different genetic, epigenetic or transcriptional make-up that makes them more resistant to everolimus. Future studies using next

generation sequencing would be a good strategy to identify these resistant subclones and yield biomarkers for everolimus resistance. Given the long timeframe of sustained resistance, alterations in gene expression were studied to elucidate possible resistance mechanisms. In BON-1/R, the main components of both mTORC1 and mTORC2 and the important upstream protein AKT were downregulated, whereas effector protein 4EBP1 expression was upregulated, hinting at a compensatory mechanism in which 4EBP1 is less dependent on mTORC1 and mTORC2. On the other hand, the main upregulated gene in QGP-1/R was ERK2, part of the MAPK-ERK pathway, illustrating a possible escape through this pathway. Interestingly, both cell lines seem to have a differential gene expression profile after developing resistance to everolimus. This is corroborated by their different response to novel PI3K-AKT-mTOR pathway targeting drugs. QGP-1/R is more resistant to treatment with the two tested ATP-competitive mTOR inhibitors, AZD2014 and OSI-027, than BON-1/R. Similarly, although full inhibition of proliferation with NVP-BEZ235 could be reached in both BON-1/R and QGP-1/R, a higher concentration of NVP-BEZ235 is needed to overcome resistance in QGP-1/R. Hence, we could conclude that the underlying mechanisms of everolimus resistance in BON-1/R and QGP-1/R might be different. If our data can be extrapolated to PNET patients, our study could provide an insight in the mechanisms determining resistance to mTOR inhibition in the clinic. Ultimately, this might lead to a better selection of patients and true personalised medicine.

In conclusion, the first PNET models for long-term everolimus treatment, resulting in acquired rapalogue-resistance, are presented here. Both the ATP-competitive mTOR blocker, AZD2014, as the dual PI3K-mTOR blocker NVP-BEZ235 are able to overcome this rapalogue resistance. Further evaluation of both drugs in *in vivo* and patient studies, targeted at overcoming everolimus resistance, could hence be challenging. In addition, both models allow the study of the detailed mechanisms of acquired resistance in PNET. Expanding these studies with advanced genetic and genomic techniques, such as next generation sequencing, could lead to the identification of biomarkers for everolimus resistance.

## CONFLICT OF INTEREST

TV, WdH and MP: advisory role and speakers' fees for Ipsen and Novartis. The remaining authors declare no conflict of interest.

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