HelmholtzZentrum münchen
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Norepinephrine transporter (NET) as a predictive marker of response to PI3K/mTOR inhibition in pheochromocytoma

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Abstract:

Pheochromocytomas (PCs) are rare highly vascularized neuroendocrine tumors derived from neural crest-derived chromaffin cells located ether in adrenal medulla or sympathetic ganglia (paragangliomas). Although most cases are benign but about 10% of all PCs can become malignant and resistant to conventional chemotherapy or radiotherapy. Therefore, novel therapeutic approaches are required.

In this study, we verified the antitumor efficacy of the dual PI3K/mTOR inhibitor BEZ235 in a unique model of bilateral PCs, MENX affected rats (Figure 1) and identified molecular read outs of drug treatment. Genome-wide transcriptome profiling of PCs from drug-treated or placebo-treated rats was conducted, and identified the *Slc6a2* gene, encoding the NET protein, as a target of BEZ235, which is inhibited by drug treatment in a dose-dependent manner. Functional analyses confirmed a predictive role for NET expression in the response to PI3K/mTOR inhibition, which can be monitored using NET-selective functional positron emission tomography (PET) imaging with 18F-LMI1195. Moreover, BEZ235 reduced Slc6a2/NET expression also in PC cell line, MPC.

MENX-affected rats develop highly vascularized bilateral pheochromocytomas with 100% penetrance

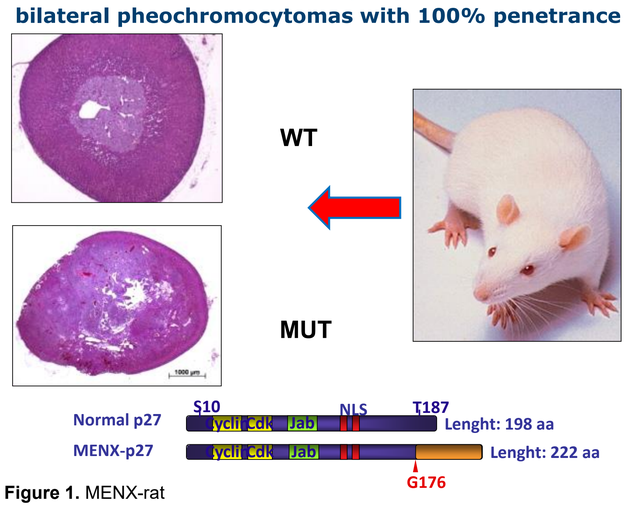


Figure 1. MENX-rat

Material and Methods:

BEZ235:

This compound was kindly supplied by Novartis Pharma and used for *in vitro* and *in vivo* studies.

Rat PC tissue samples:

Sprague-Dawley rats affected by the MENX-syndrome were used for BEZ235 or PEG administration. Rat PCs were used for primary cell culture.

Immunostaining:

Primary antibodies directed against phospho S6 (S6-S240/244; Cell Signaling Technology), p-AKT (Ser473; Cell Signaling Technology), Ki67 (Dako), activated caspase-3 (Cell Signaling Technology), CD31 (Abcam), NuSAP (Proteintech), VEGFA (Santa Cruz), and NET (MAb Technologies) were used.

RT² profiler PCR array, semiquantitative RT-PCR and quantitative TaqMan RT-PCR:

RNA was extracted from the PCs of the BEZ235- treated or untreated rats or MPC cells to perform gene expression array analysis or qRT-PCR.

Cell proliferation and apoptosis assays:

Cell proliferation was measured with WST-1 colorimetric assay (Roche). Apoptosis was measured by assessing the activity of caspase-3/7 using the Caspase-Glo 3/7 Assay Kit (Promega) according to the manufacturer's recommendations.

Magnetic resonance imaging:

MRI was performed using a 3.0 Tesla clinical MRI system. T2-weighted (T2w) turbo spin echo sequence was performed to assess the tumor volume before and after treatment. Diffusion weighted-MRI (DW-MRI) was performed using a multishot spin echo EPI sequence (with a total of 6 diffusion weightings) to assess the median apparent diffusion coefficient (ADC) value before and after treatment.

Results

PI3K/mTOR inhibition shows dose-dependent effects on cell proliferation, cell death and angiogenesis in a model of endogenous PCCs in vivo.

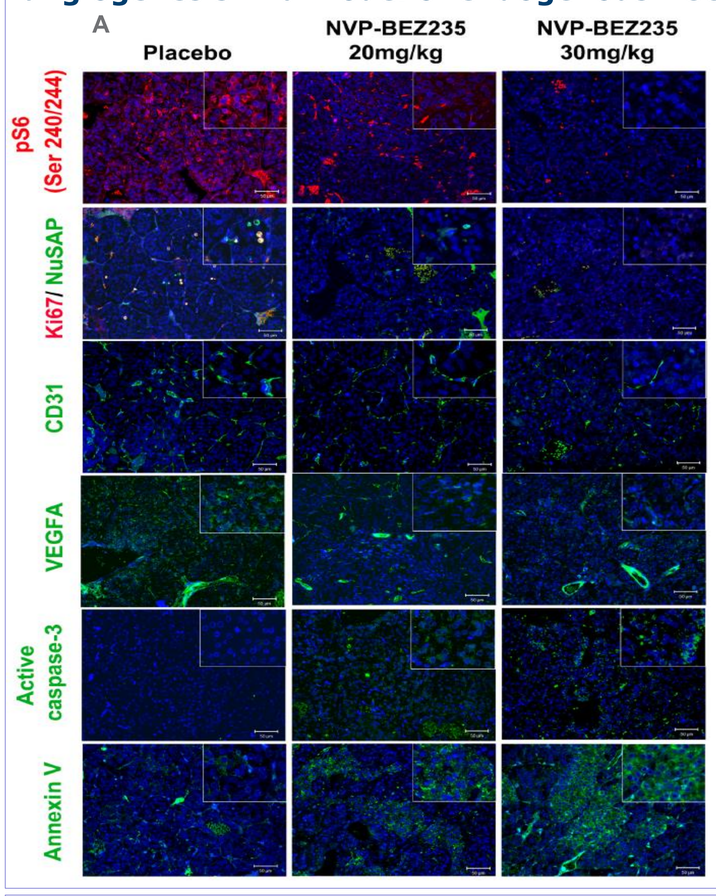
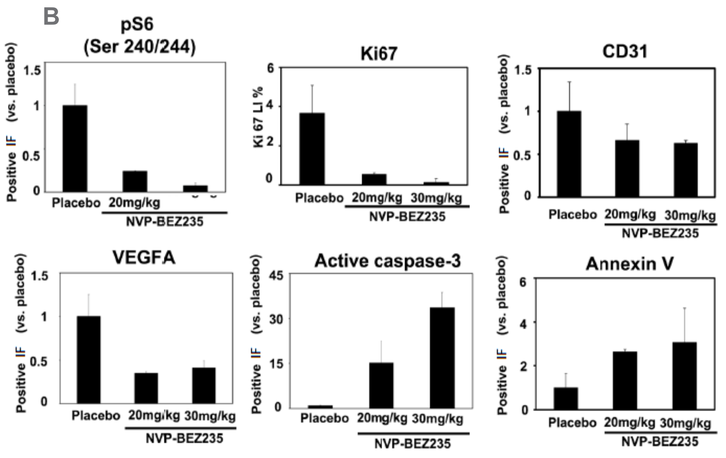


Figure 2. Analysis of PCC tissues from rats treated with placebo or BEZ235.

(A) MENX-affected rats were treated with PEG vehicle (Placebo) or with two doses of BEZ235 by oral gavage for 2 weeks. Tissues were collected, fixed in formalin and embedded in paraffin. Immunofluorescent (IF) staining was performed using specific antibodies against P-S6 (Ser240/244), Ki67, NuSAP, CD31, VEGFA, active caspase-3 or Annexin V). Cell nuclei were counterstained with DAPI. Scale bars: 50µm.

(B) Quantification of the IF signal with Image J software for the antibodies against P-S6, CD31, VEGFA, active caspase-3, and Annexin V. For Ki67, the percentage of the positive nuclei were counted.



Diffusion weighted MRI shows a reduction in cellularity in BEZ235–treated rat PCs

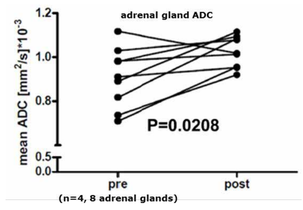


Figure 3. DW-MRI to assess the response of rat PCs BEZ235.

Apparent diffusion coefficient (ADC) values were obtained by performing DW-MRI before (pre) and after (post) treatment of mutant rats with NVP-BEZ235 for 14 days .

Slc6a2/NET (norepinephrine transporter) reduction after BEZ235 treatment in rat PCs and primary cells

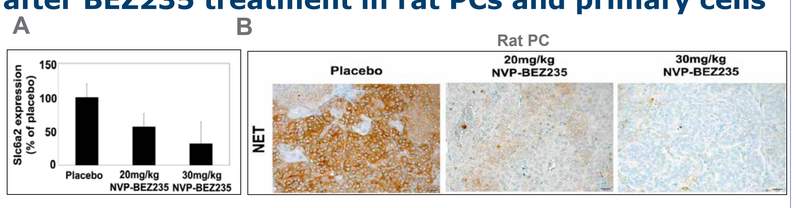
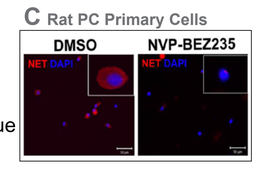


Figure 4. NVP-BEZ235 reduces Slc6a2/NET expression in rat PCs in vivo and in vitro.

(A) qRT-PCR was performed using TaqMan primer and probe sets specific to rat *Slc6a2*. The obtained relative value was normalized against the average expression of placebo-treated tissues arbitrarily set to 100. (B) PCs were collected after 14 days of daily placebo or BEZ235 administration and analyzed by immunostaining for NET expression. Scale bars: 20µm. (C) In vitro cultures of rat primary PC cells were treated daily with DMSO vehicle or with NVP-BEZ235 (1µM) for 3 days. Then they were fixed and immunofluorescent staining was conducted using the anti-NET antibody. Nuclei were counterstained with DAPI. Scale bars: 50µm.



NET expression is downregulated in MPC cells after BEZ235 treatment

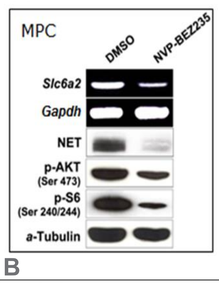
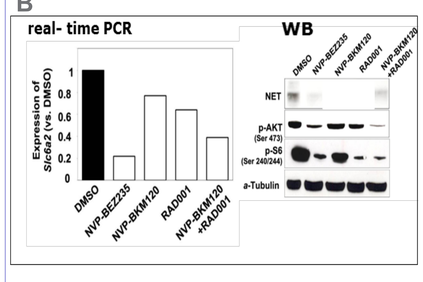


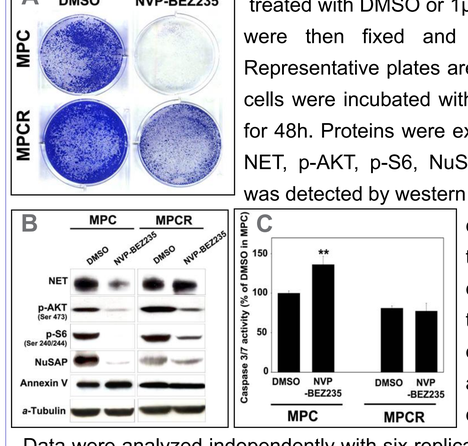
Figure 5. Semiquantitative RT-PCR and western blotting (WB) specific to the *Slc6a2*/NET protein, P-AKT and P-S6 respectively, were performed using MPC cell line. (A) MPC cells were incubated with DMSO or with 1µM BEZ235. RNA and proteins were extracted 48 later. RT-PCR was performed to monitor *Slc6a2* expression. In parallel, NET, P-AKT and P-S6 expression was monitored by WB.



(B) MPC cells were incubated with DMSO, 1µM BEZ235, 1µM NVP-BKM120, 1µM RAD001, or with the combination of NVP-BKM120 and RAD001. RNA was extracted 48h later and *Slc6a2* was amplified using qRT-PCR. Protein was extracted following drug treatment and the expression of NET, P-AKT and P-S6 was monitored by WB.

MPC cells resistant to BEZ235 do not suppress NET expression

Figure 6. The expression of NET in MPC and MPCR cells after BEZ235 treatment. (A) Colony formation assay of MPC and MPCR cells treated with DMSO or 1µM BEZ235 for 2 weeks. Cells were then fixed and stained with crystal violet. Representative plates are shown. (B) MPC and MPCR cells were incubated with DMSO or with 1µM BEZ235 for 48h. Proteins were extracted and the expression of NET, p-AKT, p-S6, NuSAP, Annexin V and α-tubulin was detected by western blotting. (C) MPC and MPCR cells were treated as in B and then were monitored for caspase-3/7 activity. Shown is the average of the relative expression values normalized against the DMSO-treated cells arbitrarily set to 110.

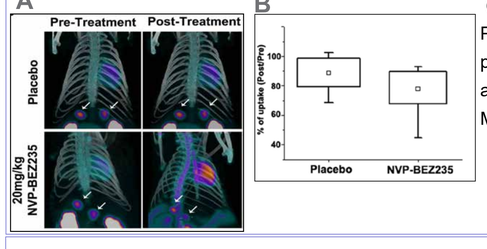


Data were analyzed independently with six replicates each and were expressed as the mean ± SEM. **P < 0.005.

Reduced uptake of 18F-LMI11195 by PCCs after BEZ235 treatment in vivo

Figure 7.Functional imaging with 18F-LMI1195 to monitor the response of rat PCCs to BEZ235 in vivo.

PET imaging was performed before (Pre) and after (Post) treatment with placebo (PEG) or BEZ235 for 14 days. (A) Representative images of the PET scans obtained for mutant rats treated with placebo or BEZ235. Arrows indicate the adrenal glands.



(B) Quantitative analysis of PET images of 18FLMI1195 in placebo-treated (n=5; 10 adrenals) and BEZ235-treated MENX rats (n=8; 16 adrenals).

Conclusions :

Our findings establish PI3K/mTOR inhibition as an effective therapeutic option for PC. NET expression seems to be a predictive biomarker of the response of PC cells to a blockade of PI3K and mTOR signaling, which can be assessed by functional imaging.

References:

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- Majumder PK, Febbo PG, Bikoff R, Berger R, Xue Q, McMahon LM, et al. mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nat Med 2004;10:594–601.

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