Dual Inhibition of PI3K and mTORC1/C2 by PKI-587 (PF-05212384) as a Promising Therapeutic Option for Bronchopulmonary Neuroendocrine Tumor Disease

Vanessa Bröker[1,2], Helma Freitag[1], Friederike Christen[1,3], Franziska Briest[1,4,5] , Britta Siegmund[1], Patricia Grabowski[1,5]

[1]Department of Gastroenterology, Infectious Diseases, Rheumatology CC13, Medizinische Klinik 1, CBF, Charité Universitätsmedizin Berlin, Germany

[2]Department Of Natural Sciences, University of Applied Sciences Bonn-Rhein-Sieg, Rheinbach, Germany

[3]Department of Biology, Humboldt-Universität Berlin, Germany

[4]Department of Chemistry and Biochemistry, Freie Universität Berlin, Germany

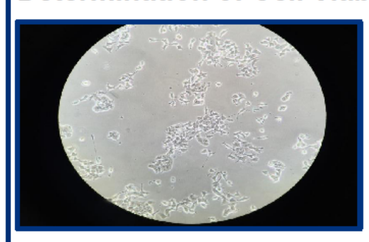
[5]Department of Gastroenterology and Endocrinology, Zentralklinik Bad Berka GmbH, Germany

Background

Bronchopulmonary neuroendocrine tumors (BP-NETs) differ in their clinical behavior, pathology and prognosis from the more common lung cancer populations. A promising therapy is the targeting of the PI3K/AKT/mTOR protein pathway, which plays a key role in cell proliferation, growth and survival. One substance of this group is the mTOR inhibitor Everolimus (RAD001), which has already shown antiproliferative effects *in vitro* on BP-NET cells (1). Clinical Phase III studies like RADIANT 4 (2) have shown beneficial effects *in vivo* and the approval of this substance for well differentiated BP-NETs is awaited. Nevertheless there are some drawbacks, since objective responses are seldom. The dual inhibitory effect on the PI3K/mTOR pathway has already been tested on several gastroenteropancreatic neuroendocrine tumor (GEP-NET) cell lines. Here, treatment with PKI-587 was superior to RAD001 treatment, concluding that the dual inhibition of PI3K and mTOR elicits a stronger antiproliferative effect (3).

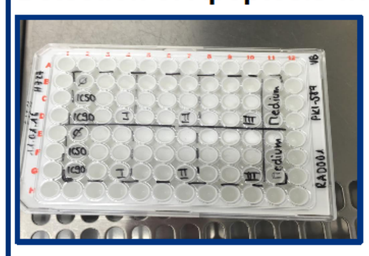
Methods

Determination of Cell Viability with MTS Cell Proliferation Assay (Promega)



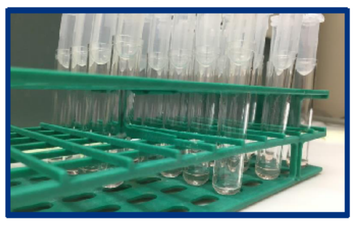
Pulmonary Neuroendocrine Tumor Cell Lines NCI-H727 and NCI-H69 have been treated with different concentrations of RAD001 (0.01 nM – 200 µM) and PKI-587 (0.01 nM – 100 µM) to investigate the cell viability by determination of IC50 for each cell line. The cells were therefore incubated for 48h and 96h.

Detection of Apoptosis with Caspase-Glo® 3/7 Assay (Promega)



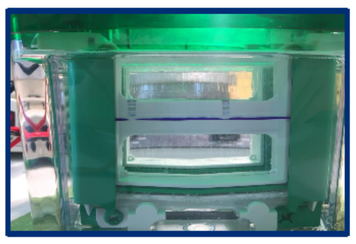
Caspase 3 and 7 play key effector roles in apoptosis of mammalian cells. To investigate the effect of RAD001 and PKI-587 on apoptosis induction in NCI-H727 and NCI-H69, both cell lines were treated with IC50 and IC90 concentrations of each inhibitor and incubated for 24h. Adding Caspase Glo® 3/7 Reagent to the cells led to generation of a luminescent signal proportional to caspase 3/7 activity, which was afterwards detected by a luminometer.

FACS Analysis of Cell Cycle



To investigate the impact of RAD001 and PKI-587 on the cell cyle, the cells were treated with IC50 and IC90 concentrations of the respective inhibitor and incubated for 48h. DNA was stained with Propidium-Iodide and mitotic cells were detected by tagging them with a fluorescence labeled antibody against phospo-Histon H3.

Analysis of Protein Expression by Western Blot Analysis



IC50 and IC90 treated cells were harvested and lysed after 24h incubation. The intracellular proteins were run through an SDS-Gelelectrophoresis and blotted onto a PVDF membrane for investigation of selected proteins via specific monoclonal antibody binding. Adequate secondary antibodies were used to detect the proteins via a chemoluminescence approach.

Results

The following results show the outcome of the performed methods mentioned above for the bronchopulmonary neuroendocrine cell lines NCI-H727 (well differentiated, carcinoid) and NCI-H69 (poorly differentiated, small cell lung cancer).

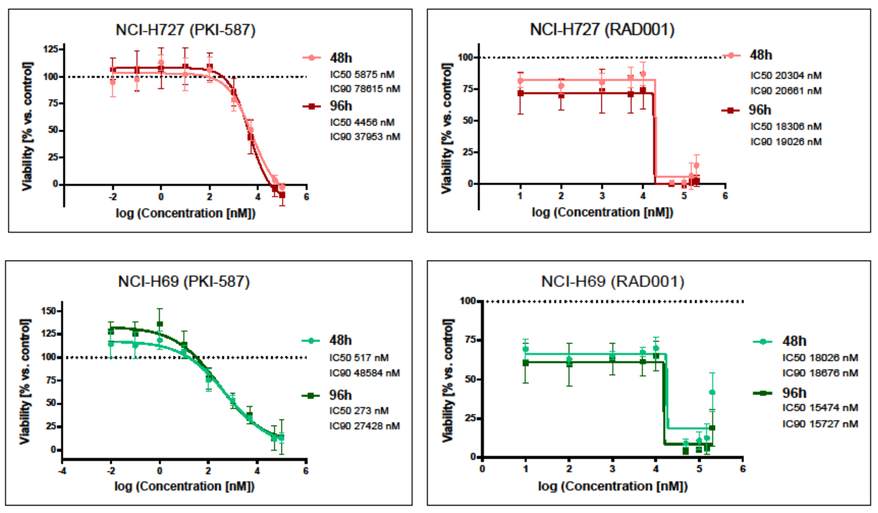


Figure 1: Cell Viability via MTS Cell Proliferation Assay (Promega)

In both cell lines, PKI-587 shows a stronger inhibitory effect than RAD001. However the sheer presence of RAD001 leads to a constant lower cell viabilty (vs. control), which rapidly decreases with increasing concentration. In contrast to that, small amounts of PKI-587 lead to a slight increase of cell viabilty (vs. control) which then decreases at different concentrations.

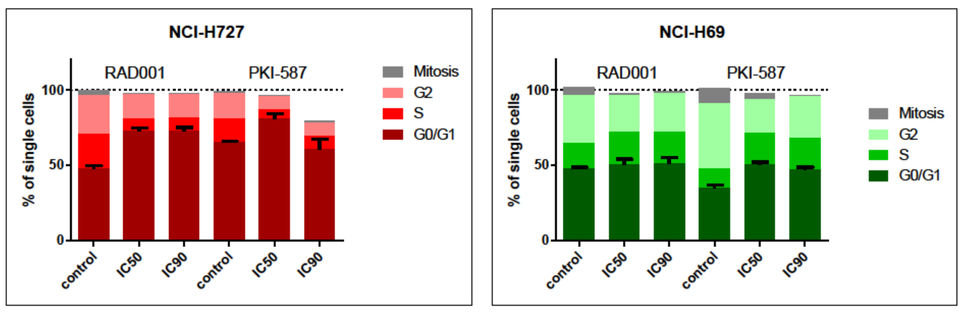


Figure 2: Cell Cycle Analysis with FACS

The antiproliferative effect of PKI-587 can be well observed in the obtained data. In both cell lines, a decrease in mitosis and an increase of G1/G2 can be observed. At an IC90 concentration of PKI-587 in NCI-H727, apoptosis is strongly induced which can be explained by the missing cells, leading to a single cell number below 100% (sub-G1 peak, not shown here). RAD001 affects NCI-H727 cells stronger than NCI-H69 cells.

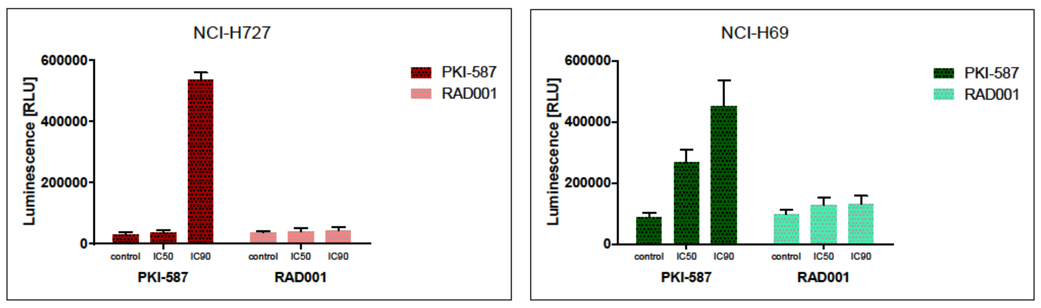


Figure 3: Luminescence measurement with Caspase-Glo ®3/7 Assay (Promega)

The obtained data show that the effect of PKI-587 on Caspase 3 and 7 activity (induction of apoptosis) is superior to the effect of RAD001. While the measured RLU (= relative light units) increases with increasing concentrations of PKI-587 in both cell lines, RAD001 shows no significant effect neither in NCI-H727 nor in NCI-H69 cells.

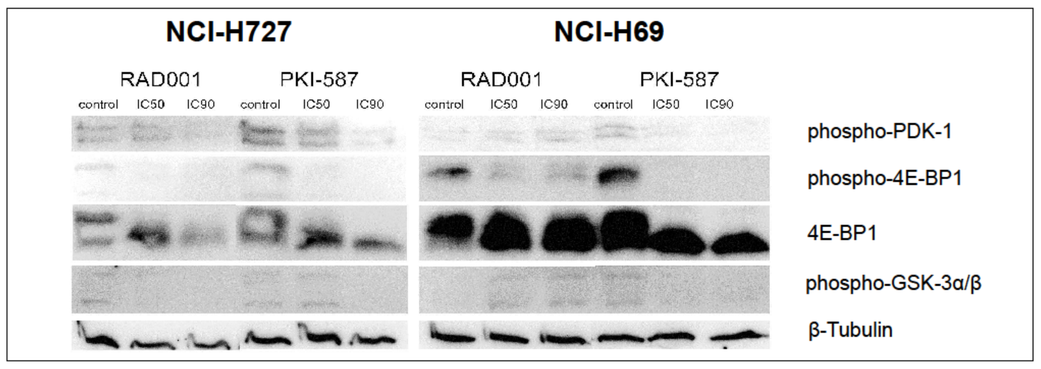


Figure 4: Protein Expression Analysis via Western Blotting

Protein expression analysis with Western Blots shows that both, RAD001 and PKI-587, have an inhibitory effect on phosphorylated PDK-1, which is a member of the PI3K pathway. Also, 4E-BP1 and its phosphorylated form, which are a direct target of mTOR, are affected by both inhibitors whereby PKI-587 seems so have a stronger impact in NCI-H69 cells. This effect is not as strong against phosphorylated GSK-3α/β, a gene inhibited by AKT.

Conclusion

- **In both BP-NET cell lines, the effect of the dual PI3K/mTOR inhibitor PKI-587 was superior to inhibition by Everolimus (RAD001), which solely targets mTOR.**
- **Nevertheless, our preliminary results point to different mechanisms of PKI-587 in the two cell lines: While apoptosis is stronger induced in the well differentiated NCI-H727 cell lines, Western Blot analysis showed that in the SCLC cell line NCI-H69 4E-BP1/p4E-BP1 as direct mTORC1-targets become more inhibited after treatment with PKI-587, pointing to a relevance for proliferation regulation.**
- **PKI-587 is therefore a promising inhibitory substance which should be further investigated in preclinical and clinical trials.**

References

1. Zatelli et al. Endocrine Relat Cancer, 2010
2. Yao et al. The Lancet, 2015
3. Freitag et al. Neuroendocrinology, 2016, in revision

Acknowledgements

- Pfizer
- Theranostics Research Network
- Sonnenfeld-Stiftung

PKI-587 (PF-05212384) was kindly provided by Pfizer.