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Preclinical *in vitro* investigation to evaluate novel molecular targeted therapy options for neuroendocrine neoplasms

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CONTENTS

1. ABBREVIATIONS

Chk	Checkpoint kinase
CDK	Cyclin-dependent kinase
p21	Cyclin-dependent kinase inhibitor 1
p16	Cyclin dependent kinase inhibitor 2A
Caspase	Cysteinyl-aspartate specific protease
DNA	Deoxyribonucleic acid
MDM2	E3 ubiquitin ligase mouse double minute 2 homolog (MDM2)
EGFR	Epithelial growth factor receptor
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
RAD001	Everolimus, mTORC1 inhibitor (Novartis, Basel)
ERK	Extracellular signal-regulated kinase 1
FACS	Flow cytometric analysis
5-FU	5-fluorouracil
γ-irradiation	Gamma-irradiation
G1 phase	Gap 1 phase
G2 phase	Gap 2 phase
GEP-NET	Gastroenteropancreatic neuroendocrine tumor
GTP	Guanosine triphosphate
MTH1	Human Mut-T homologue 1
IC_{50}	Inhibitory concentration of 50 %
IGFR	Insulin-like growth factor receptor
mTOR	Mechanistic / mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
µM	Micro molar
MEK	Mitogen-activated protein kinase kinase
TH588	MTH1 inhibitor (Thomas Helleday, Karolinska Institutet, Sweden)
nM	Nano molar
NET	Neuroendocrine tumor
%	Percent
PI3K	Phosphoinositid-3-kinase

PARP	Poly (ADP-ribose)-Polymerase
PCNA	Proliferating-Cell-Nuclear-Antigen
Akt	Protein kinase B
Raf	Rapidly accelerated fibrosarcoma protein
Ras	Rat sarcoma protein
ROS	Reactive oxygen species
Rb	Retino-blastoma protein
LEE011	Ribociclib, CDK4/6 inhibitor (Novartis, Basel)
p70S6K	Ribosomal protein S6 kinase beta-1
S phase	Synthesis phase
E2F	Transcription factor E2F1
p53	Tumor suppressor p53

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3. INTRODUCTION

3. 1. General introduction into neuroendocrine tumors

Neuroendocrine tumors (NETs) of the gastroenteropancreatic (GEP) system are highly complex and heterogeneous tumors, regarding their physiologic and genetic make-up and originate from distinct cell precursors [1]. GEP-NETs are frequently metastasized at the time of diagnosis, due to an absence of symptoms and curative resection is often impossible [2-4]. Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) are after colorectal cancer the second most common gastrointestinal malignancy [4] with increasing incidence [5, 6]. Despite an increasing knowledge of the tumor biology, genetics, epigenetics and novel biomarkers, the overall outcome and survival of GEP-NETs has gained very little over the last decades [4, 7, 8]. Furthermore, systemic therapeutic approaches for GEP-NETs, such as biotherapy, chemotherapy, molecular targeted therapy and peptide receptor radionuclide therapy are limited in their efficiency [9-11]. The paucity of successful therapeutic agents in GEP-NET is mostly due to the complexity, the rarity, the intrinsic differences in malignant potential because of their heterogeneity and the dissimilar clinical presentation [1, 4]. Molecularly targeted therapies with the multi-tyrosine kinase inhibitor sunitinib or the mTORC1 inhibitor everolimus are approved for treatment of pancreatic NETs [12-14]. Everolimus has recently also been approved for treatment of lung and gastrointestinal NETs [15]. The promising molecular targeting agent everolimus downregulates the mTOR / p70S6K pathway, one of the most important and oncogenic signaling cascades in GEP-NETs [16-18]. The PI3K-Akt-mTOR axis is frequently over-activated in cancers, including GEP-NETs, causing cellular survival, proliferation and protein synthesis [11, 19]. Unfortunately only a subset of patients respond to everolimus treatment, due to

intrinsic resistance or the development of a *de novo* resistance in response to long term treatment [20-24]. Compensatory feed-back activation of PI3K-Akt signaling in response to mTORC1 inhibition, as well as cross-talk activation of other signaling pathways such as the Ras-Raf-MEK-ERK cascade in response to molecular targeting help to escape cancer cell death and are well described in NET cells [19, 25-27]. Hence, novel therapeutic strategies, including molecularly targeted therapeutics, are urgently needed [9, 14]. Dual-targeting approaches might provide a rationale to overcome acquired or intrinsic resistance, prevent feed-backs within and between signaling pathways and enhance single substance treatment [24, 28, 29].

The aim of this thesis was to evaluate different novel molecular targeted therapy options for neuroendocrine neoplasms based on an *in vitro* model:

3.2. The novel cyclin-dependent kinase 4/6 inhibitor ribociclib (LEE011) alone and in dual-targeting approaches demonstrates antitumoral efficacy in neuroendocrine tumors in vitro

Cyclin dependent kinases (CDKs) are the key regulators of the cell cycle and aberrant expression of these CDKs, due to gene mutation, amplification or overexpression often lead to the formation of cancer [30]. A very promising molecular targeting approach for GEP-NETs is the selective CDK4/6 inhibitor LEE011 (Novartis, Basel) by downregulating the proliferative CyclinD-CDK4/6-Rb axis (Fig. 1) [29]. Currently a clinical phase 2 trial with LEE011 is recruiting patients with advanced NETs of foregut

origin (NCT02420691) and another clinical phase 2 trial is recruiting patients with CDK4/6 pathway activated tumors (NCT02187783), substantiating the probable clinical relevance of targeting the CyclinD-CDK4/6-Rb axis.

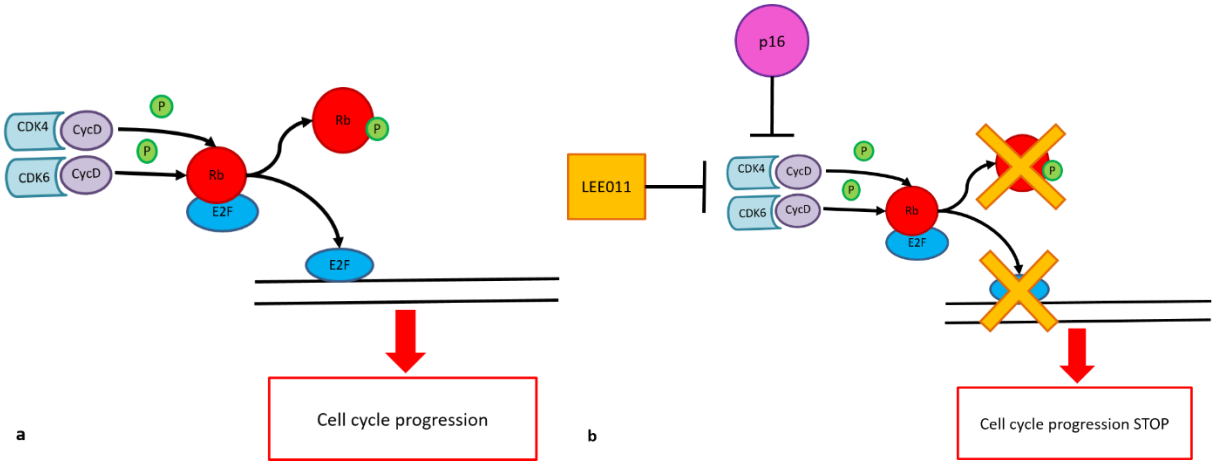

Fig. 1. Proposed and simplified mode of action of the CDK4/6 inhibitor LEE011 on the cell cycle. **a** Activated CyclinD-CDK4/6-Rb axis leads to G1/S cell cycle progression via the phosphorylation of Rb and subsequent activation of the transcription factor E2F. **b** Blocking the CyclinD-CDK4/6-Rb axis leads to G1 phase cell cycle arrest through either the endogenous CDK4/6 inhibitor p16 or the small molecule CDK4/6 inhibitor LEE011

Using human neuroendocrine pancreatic BON1, pancreatic islet QGP1, bronchopulmonary NCI-H727 and ileal GOT1 cell lines we demonstrated the antitumoral efficacy of the novel cyclin-dependent kinase 4/6 inhibitor ribociclib (LEE011) alone and in dual-targeting approaches *in vitro* [29]. In this study we applied cellular survival assays, Western blot analysis, flow cytometric analysis (FACS) and Caspase-3/7 activity assays. The cell viability decreased in a time- and dose-dependent manner in BON1, QGP1 and NCI-H727 cells upon LEE011 treatment, while GOT1 cells were treatment resistant. High expression levels of endogenous Rb and Cyclin D1 were associated with treatment sensitivity towards CDK4/6 inhibition in NETs. LEE011 caused dephosphorylation of Rb and a subsequent G1 phase cell cycle

arrest. The combinational treatment of LEE011 with 5-fluorouracil (5-FU) or everolimus showed a significant enhancement in inhibition of cell viability when compared to the respective single substance treatments, due to PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathway downregulation and cooperative cell cycle component downregulation. Conversely, LEE011 also demonstrated antagonizing effects in combination with 5-FU, by seemingly protecting NET cells from the DNA damaging effects of the chemotherapeutic agent through blocking PARP cleavage and caspase 3/7 activity. Taken together our results, CDK4/6 inhibition with LEE011 might be an efficient new therapeutic rationale for NETs either alone or in dual-targeting approaches, especially in combination with everolimus. We recommend clinical studies to ratify the efficacy of LEE011 and everolimus as dual-targeted therapy in neuroendocrine tumors.

3.3. The MTH1 inhibitor TH588 demonstrates anti-tumoral effects alone and in combination with everolimus, 5-FU and gamma-irradiation in neuroendocrine tumor cells

Cancer cells often display a dysfunctional redox regulation with a high level of reactive oxygen species damaging the DNA and the free nucleotide pool [31, 32]. The nucleotide pool-sanitizing enzyme MTH1 (human Mut-T homologue 1) was shown to be of pivotal importance for the progression and the survival of cancer cells, preventing the incorporation of damaged nucleotides into the DNA (Fig. 2) [33, 34]. Recently, several new small-molecule inhibitors targeting the nucleotide-sanitizing enzyme MTH1 (TH287, TH588 and S-crizotinib) were described to specifically induce lethality in a broad spectrum of cancer cells without harming untransformed tissues [35, 36].

However the validity of MTH1 as a promising target against cancer has been questioned recently and the striking anti-proliferative effects of MTH1 inhibitors such as TH588 have been attributed to off-target effects [37-39].

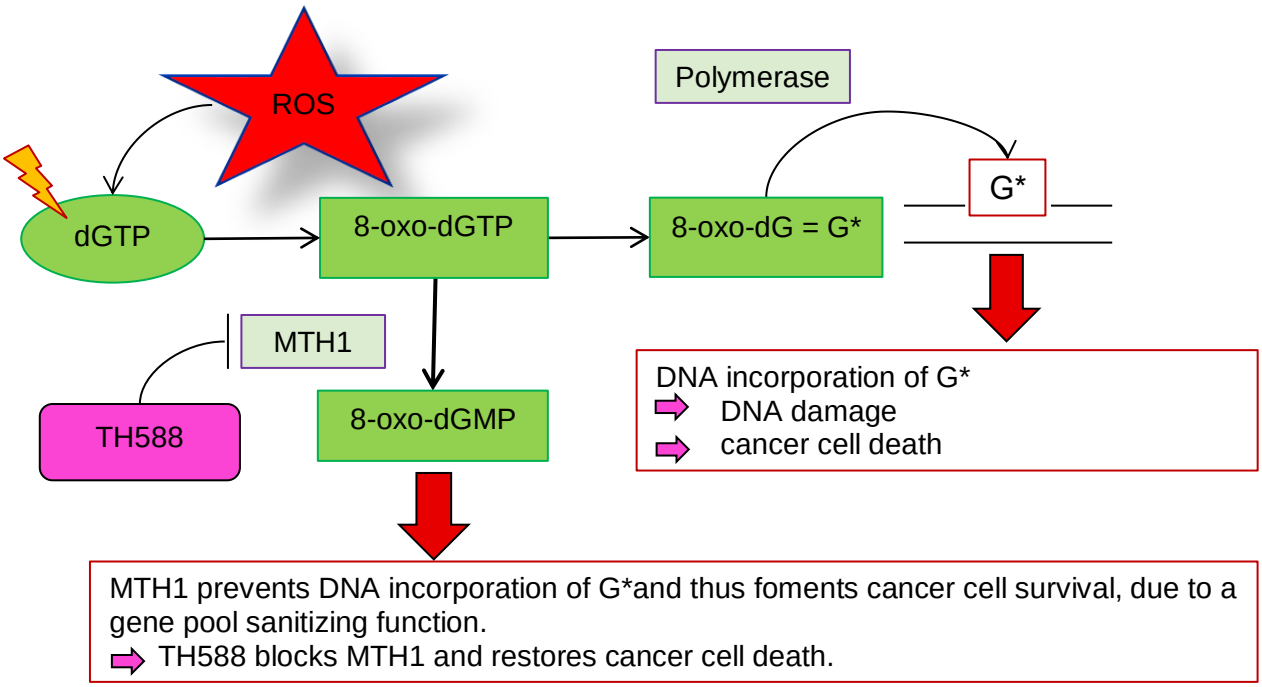

Fig. 2. Proposed mode of action of TH588. MTH1 prevents the incorporation of damaged nucleotides into the DNA and hence protects cancer cells from the lethal effects caused by reactive oxygen species (ROS). By inhibiting MTH1 cancer cell death is restored.

With this study we contributed to the assessment of cellular mechanisms and molecular signaling pathways implicated in the cellular response to TH588 treatment [40]. Using a panel of heterogeneous NET cell lines (BON1, QGP1, H727 and GOT1), we tested TH588 alone or in combination with the mTOR inhibitor everolimus, 5-FU and γ-irradiation. Here we applied cellular survival assays, Western blot analysis, Caspase-3/7 activity assays, FACS analysis, oxidative stress level assays and colony formation assays to determine the clonogenic cell survival after γ-irradiation. TH588

caused cancer cell death by downregulating the PI3K-Akt-mTOR axis and inducing apoptosis, rather than causing augmentation of the cellular oxidative stress level. These effects were even enhanced when TH588 was combined with everolimus or 5-FU, due to an even stronger downregulation of the PI3K-Akt-mTOR axis and augmentation of apoptosis but not oxidative stress. Furthermore TH588 demonstrated a radiosensitizing potential in combination with γ-irradiation (radiotherapy), one of most important treatment modalities in nowadays cancer treatment [41]. Our data thus not only provided new insights into how TH588 kills cancer cells but also depicted novel perspectives for combinatorial treatment approaches encompassing TH588.

4. ABSTRACT

GEP-NETs are highly complex, rare and heterogeneous tumor entities [1]. Current treatment approaches are unsatisfying, due to their limited efficacy [2-4]. Ergo, novel therapeutic strategies, including molecularly targeted therapeutics, are urgently needed [9, 14]. The aim of this doctoral thesis was to evaluate different novel molecular targeted therapy options for neuroendocrine neoplasms *in vitro*. By targeting different signaling pathways, we obtained novel insights into NET tumor biology and assessed possible novel treatment strategies. Several dual-targeting approaches prevented feed-back loops within a signaling pathway, as well as cross-talk activation between signaling pathways and enhanced single substance treatment. This cumulative doctoral thesis is based on the following original publications:

- The novel cyclin-dependent kinase 4/6 inhibitor ribociclib (LEE011) alone and in dual -targeting approaches demonstrates antitumoral efficacy in neuroendocrine tumors in vitro; Neuroendocrinology. 2017 Feb; doi: 10.1159/000463386. PMID: 28226315. **Aristizabal Prada ET**, Nölting S, Spöttl G, Maurer J, Auernhammer CJ.

- The MTH1 inhibitor TH588 demonstrates anti-tumoral effects alone and in combination with everolimus, 5-FU and gamma-irradiation in neuroendocrine tumor cells. PLoS ONE. 2017 May; doi: 10.1371/journal.pone.0178375. PMID: 28542590. **Aristizabal Prada ET**, Orth M, Nölting S, Spöttl G, Maurer J, Auernhammer CJ.

5. ZUSAMMENFASSUNG

GEP-NETs sind hoch komplexe, seltene und heterogene Tumor-Entitäten [1]. Derzeitige Behandlungsmöglichkeiten sind aufgrund ihrer begrenzten Effektivität nicht zufriedenstellend [2-4]. Daher werden neue Behandlungsstrategien, wie molekular zielgerichtete Therapien, dringend benötigt [9, 14]. Das Ziel dieser Doktorarbeit war es, verschiedene neue molekular zielgerichtete Therapieansätze für neuroendokrine Neoplasien *in vitro* zu evaluieren. Durch das „targeting“ von verschiedenen Signalwegen konnten neue Einblicke in die Tumor-Biologie von NETs erzielt und neue mögliche Behandlungsstrategien erstellt werden. Einige „duale-targeting“ Ansätze konnten „feed-back“ Mechanismen innerhalb eines Signalwegs oder die Aktivierung zwischen unterschiedlichen Signalwegen verhindern und verbesserten zudem die Einzelsubstanzbehandlung. Die kumulative Dissertation basiert auf den folgenden Originalveröffentlichungen:

- The novel cyclin-dependent kinase 4/6 inhibitor ribociclib (LEE011) alone and in dual -targeting approaches demonstrates antitumoral efficacy in neuroendocrine tumors in vitro; Neuroendocrinology. 2017 Feb; doi: 10.1159/000463386. PMID: 28226315. **Aristizabal Prada ET**, Nölting S, Spöttl G, Maurer J, Auernhammer CJ.

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6. PUBLICATION 1

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https://www.ncbi.nlm.nih.gov/pubmed/?term=The+novel+cyclin-dependent+kinase+4%2F6+inhibitor+ribociclib+(LEE011)+alone+and+in+dual+-targeting+approaches+demonstrates+antitumoral+efficacy+in+neuroendocrine+tumors+in+vitro

7. PUBLICATION 2

The MTH1 inhibitor TH588 demonstrates anti-tumoral effects alone and in combination with everolimus, 5-FU and gamma-irradiation in neuroendocrine tumor cells. PLoS ONE. 2017 May; doi: 10.1371/journal.pone.0178375. PMID: 28542590. **Aristizabal Prada ET**, Orth M, Nölting S, Spöttl G, Maurer J, Auernhammer CJ.

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