Aus dem Zentrum für Pathologie der Universität zu Köln
Institut für Allgemeine Pathologie und Pathologische Anatomie
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Mechanistic Insight into RET Kinase Inhibitors Targeting the DFG-out Conformation in *RET*-rearranged Cancer

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Die hier vorgestellten Daten sind in der folgenden Arbeit publiziert worden:

Plenker D*, **Riedel M***, et al.
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* authors contributed equally

Ich habe den Großteil der Daten, die dieser Dissertation zugrunde liegen bzw. die in die Publikation eingeflossen sind, nach Einweisung selbst erhoben und ausgewertet. Dazu zählen vor allem die absolute Mehrzahl der Klonierungs- und Zellkultur- Experimente (die entsprechenden Figures in der Publikation sind in Klammern angegeben). Die Vektoren für die Ba/F3$^{KIF5B\text{-}RET}$ und Ba/F3$^{CCDC6\text{-}RET}$ Zell-Linien inkl. *RET*^{V804M} Mutation wurden vom mir eigenständig kloniert, transfiziert und im Anschluss zusammen mit weiteren Zell-Linien experimentell getestet und ausgewertet (Figure 1A,B,E,F, 3B-D; Supp. Figure 1A-B,F, Supp. Figure 2A-B; Supp. Table 2). Zusätzlich habe ich die Vektoren für die Induktion von *RET*-Fusionen in NIH-3T3 Zellen mittels der CRISPR-Cas9 Gen-Editierungstechnik kloniert und diese transfizierten Zellen anschließend experimentell getestet (Figure 1C-D; Supp. Figure 1C-E). Mittels Mutagenese Screening habe ich *RET*^{I788N} als eine potentielle, sekundär erworbene Resistenz-Mutation unter Therapie mit dem Tyrosinkinase-Inhibitor AD80 gefunden, getestet und diese in selbst etablierten Zell-Modellen validiert. (Figure 4A-D; Supp. Figure 5A-B; Supp. Table 5). Darüber hinaus habe ich resistente TPC-1 bzw. LC-2/AD Zellen vergleichend bzgl. MAPK-Reaktivierung untersucht und die LC-2/AD Zell-Linie mit einem *KRAS*^{G12V} Vektor transfiziert und getestet (Figure 5 C-D; Supp. Figure 5C-F).
Darüber hinaus habe ich alle Daten, an deren Erstellung ich selbst nicht aktiv involviert war, ausgewertet, die Figures der Publikation eigenständig erstellt, das Manuskript mitgeschrieben und aktiv gestaltet, die Beiträge der Mitautoren mitorganisiert, sowie das Review-Verfahren intensiv begleitet.

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Unterschrift:

[1]Bei kumulativen Promotionen stellt nur die eigenständig verfasste Einleitung und Diskussion die Dissertationsschrift im Sinne der Erklärung gemäß dieser Erklärung dar.

Table of Content

I) Abkürzungsverzeichnis

ABL1 - Abelson murine leukemia viral oncogene homolog 1

ALK - anaplastic lymphoma receptor tyrosine kinase

ALT - alternative lengthening of telomeres

ATP - adenosine triphosphate

BCR - breakpoint cluster region

BRAF - v-Raf murine sarcoma viral oncogene homolog B

BRCA1/2 - breast cancer 1/2

CAKUT - congenital abnormalities of the kidney and urinary tract

CCDC6 - coiled-coil domain-containing 6

CCHS - congenital central hypoventilation syndrome

CD74 - cluster of differentiation 74

CDKN2A - cyclin-dependent kinase inhibitor 2A

CML - chronic myeloid leukaemia

CRISPR - clustered regularly interspaced short palindromic repeats

CT - computer tomography

DNA - deoxyribonucleic acid

DOK - docking protein

ECM - extracellular matrix

ED - extensive disease

EGFR - epidermal growth factor receptor

EML4 - echinoderm microtubule associated protein like 4

EMT - epithelial-to-mesenchymal-transition

FGFR1 - fibroblast growth factor receptor 1

FISH - fluorescence in situ hybridziation

FRS2 - fibroblast growth factor receptor substrate 2

GDNF - glial cell line-derived neurotrophic factor

GDP - guanosine diphosphate

GFLs - glial cell line-derived neurotrophic factor (GDNF) family ligands

GI - growth inhibition

GTP - guanosine triphosphate

HPV - human papillomaviruses

HRAS - Harvey rat sarcoma viral oncogene homolog

IHC - Immunohistochemistry

IRS1/2 - insulin receptor substrate 1/2

JAK - janus kinase

KIF5B - kinesin family member 5B

KRAS - V-Ki-ras2 kirsten rat sarcoma viral oncogene homolog

LD - limited disease

LKB1 - liver kinase B1

MAPK - mitogen-activated protein kinase

MEN – multiple endocrine neoplasia

MYC-N - V-Myc Avian myelocytomatosis viral oncogene neuroblastoma derived homolog

NCOA4 - nuclear receptor coactivator 4

NF1 - neurofibromin 1

NRAS - neuroblastoma RAS viral oncogene homolog

NSCLC - non small cell lung cancer

PARP - poly (ADP-ribose) polymerase

PD-1 - programmed death 1

PDL-1 - programmed death ligand 1

PDX - patient derived xenograft

PFS – progression-free-survival

PIK3CA - phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha

PKC - protein kinase C

PR – progressive response

PTEN - phosphatase and tensin homolog

RB1 - retinoblastoma protein 1

RET - rearranged during transfection

ROS1 - ROS proto-oncogene 1

RTK - receptor tyrosine kinase

SCLC - small cell lung cancer

SHC1 - SRC homology 2 domain containing transforming protein 1

SRCC - signet ring cell carcinoma

STAT - signal transducer and activator of transcription

TERT - telomerase reverse transcriptase

TNF - tumor necrosis factor

TP53 - tumor protein p53

TRIM33 - the tripartite motif-containing 33

VEGF - vascular endothelial growth factor

VEGFR - vascular endothelial growth factor receptor

WHO - world health organisation

II) Summary

RET rearrangements have been found among 1-2% of all lung adenocarcinoma patients which form a potentially druggable molecular target for small molecule inhibitors (Pao and Hutchinson 2012). Unfortunately, clinical trials have not provided evidence for a superior treatment regime with small molecule inhibitors in advanced lung cancer in 2018 (Drilon et al. 2018). However, the clinical need for new approaches of targeting *RET*-rearranged lung cancer is high.

To systematically profile AD80 and a panel of other small molecule inhibitors with RET inhibitory effects, I first established *KIF5B-RET* and *CCDC6-RET* viral transduced Ba/F3 cells leading to IL-3 independent, while strongly oncogene-dependent, proliferation. These cell lines together with the *RET*-rearranged lung adenocarcinoma cell line LC-2/AD were the starting point for my project. In addition, to establish and evaluate another endogenously *RET*-rearranged cellular model, I used the recently developed CRISPR/Cas9 genome editing technology. I cloned a pLenti vector with a Cas9 cassette and two promotors for the expression of single-guided RNAs (sgRNAs) and transfected murine fibroblast cells (NIH-3T3) resulting in *KIF5B-RET* translocations in selected cells. By benchmarking potential RET inhibitors against these cell lines in addition to a larger panel of human, patient-derived, lung cancer cell lines, I could not only demonstrate that, at least *in vitro,* currently available anti-RET drugs such as alectinib, cabozantinib or vandetanib may not be potent enough to induce satisfying and lasting responses in RET-fusion driven cancer, but also that AD80 and ponatinib may be able to overcome this shortfall with a 100 to 1000-fold higher cytotoxicity and high RET kinase on-target activity. Next, I tried to relate these differential cytotoxic effects in the dose-response curves to changes on the protein level. The following Western Blots and phosphoproteomic analysis revealed a corresponding strong decrease in phospho-RET and downstream signalling molecules under treatment. Furthermore, these *in vitro* results were supported by *in vivo* data from our PDX mouse models with *CCDC6-RET* rearrangements with strong tumor shrinkage under AD80 treatment.

Parallel to my work, we collaborated with other research groups for this project in order to gain a deeper understanding of the functional mechanisms behind the high on-target efficacy of AD80. We used computational binding mode analysis to provide further evidence that AD80 binds RET in the inactive DFG-out conformation as a type II inhibitor. In addition, thermal shift assays as a surrogate parameter for tighter kinase occupation suggested that the binding of type II inhibitors such as ponatinib and AD80 leads to higher kinase thermal stability as compared to type I inhibitors. That may explain - in part - their strong cytotoxic effects in the cellular experiments.

In the following, the project focused more on the role of resistance mechanisms in *RET*-rearranged cell lines. Using site-directed mutagenesis, I established Ba/F3 *KIF-RET*^{V804M} and *CCDC6-RET*^{V804M} cells with mutations at the strongly conserved gatekeeper position of the RET kinase and tested these cells lines against our panel of RET-inhibitors. Again, AD80 and ponatinib revealed the strongest anti-RET effect with only a minor reduction of cytotoxicity in cell viability assays and RET-dephosphorylation as compared to *RET*^{wt}. Next, I used saturated mutagenesis screening to discover new resistance mutations against AD80. Sequencing of resistant Ba/F3 cells revealed the missense mutation pI788N (c.2363T>A) within the RET kinase domain as a possible resistance mutation against targeted treatment. Cellular viability screening and Western Blot analysis in rearranged Ba/F3 *KIF-RET*^{I788N} and *CCDC6-RET*^{I788N} confirmed the loss of inhibitory effects. Additionally, results from a *RET*-rearranged thyroid cancer cell line (TPC-1) that acquired secondary resistance to RET inhibition and RNA sequencing data from LC-2/AD cells under treatment cells revealed that MAPK signalling reactivation might be a possible resistance mechanism for *RET*-rearranged tumors. To formally assess the role of MAPK activation, I stably transduced LC-2/AD cells with lentiviral *KRAS*^{G12V} leading to an overexpression of KRAS and resistance to AD80 treatment despite RET dephosphorylation.

For ponatinib, clinical phase 3 trials for the treatment of chronic myeloid leukaemia had to be cancelled due to too severe therapy associated complications despite good effects on tumor progression (Lipton et al. 2016). AD80 may be therefore a candidate for future clinical trials. Considering the high clinical need for potent and well tolerated anti-RET drugs for tumor patients, the study provides a broad range of mechanistic insights into optimization of current anti-RET therapy and future drug development

III) Deutsche Zusammenfassung

RET Fusions-Gene können in 1-2% aller Lungen-Adenokarzinom Patienten nachgewiesen werden. Diese genetischen Veränderungen stellen mittels Tyrosin-Kinase Inhibitoren potentiell therapierbare molekulare Zielstrukturen dar (Pao and Hutchinson 2012), jedoch haben klinische Studien im Jahr 2018 für diese Lungenkarzinome bisher noch keine ausreichend erfolgreichen Therapieansätze zeigen können (Drilon et al. 2018).

Um systematisch das therapeutische Profil von AD80 und einer Vielzahl weiterer Tyrosinkinase Inhibitoren gegen *RET*-Fusion getriebene Zellmodelle auswerten zu können, habe ich zunächst Ba/F3 Zellen viral mit *KIF5B-RET* sowie *CCDC6-RET* transduziert. Das Wachstum von Ba/F3 Zellen *in vitro* ist im Normalzustand abhängig von IL-3. Sobald sie mit einem starken Onkogen jedoch transduziert werden, sind sie unabhängig von IL-3 und proliferieren nur noch abhängig von der Aktivität des entsprechenden Onkogens. Durch dieses Modelsystem konnte ich eine große Anzahl verschiedener Inhibitoren gegen RET testen und ihre Potenz untereinander vergleichen. Die selbst etablierten Ba/F3 Zell-Linien zusammen mit der *RET*-Fusion getriebenen Lungen-Adenokarzinom Zelllinie LC-2/AD bildeten den Ausgangspunkt für mein Projekts. Zusätzlich habe ich ein endogen *RET*-mutiertes Zell-Model mittels der Genom-Editierungstechnik CRISPR/Cas9 etabliert. Dafür habe ich einen Vektor mit Cas9 mRNA sowie zwei Promotoren für die Expression von spezifischen „single-guided RNAs“ (sgRNA) kloniert und murine Fibroblast Zellen (NIH-3T3) transfiziert. Mittels sgRNAs gegen die jeweiligen spezifischen Introns von *RET* und *KIF5B* konnte ich somit in selektionierten NIH-3T3 Zellen *KIF5B-RET* Translokationen generieren. Indem ich diese neu etablierten Zelllinien zusammen mit einer größeren Anzahl humaner Lungenkrebs Zell-Linien gegen potentielle RET-Inhibitoren getestet habe, konnte ich die klinische Erfahrung *in vitro* bestätigen, dass die zurzeit gängigen Therapieansätze mit Tyrosinkinase- Inhibitoren wie z.B. Cabozantinib, Alectinib oder Vandetanib wahrscheinlich eine nicht ausreichend hohe therapeutische Potenz besitzen, um eine effektive Wirkung auf RET getriebene Tumore zu entwickeln. Weiterhin deuteten die Daten darauf hin, dass andere Tyrosin-Kinase Inhibitoren, wie z.B. AD80 und Ponatinib, im Vergleich dazu 100 bis 1000-fach potenter sind und spezifisch die RET-Kinase inhibieren.

Als nächstes habe ich die Unterschiede in den Zell-Viabilitäts-Assays mit den Veränderungen auf der Protein-Ebene zu verglichen. Die folgenden Western Blot und Phosphoproteom Analysen haben eine entsprechende Reduktion in phospho-RET und den weiteren nachgeschalteten Signal-Molekülen gezeigt. Zusätzlich haben die *in vivo* Ergebnisse unserer *CCDC6-RET* PDX-Mausmodelle unsere *in vitro* Daten mit gutem Tumoransprechen unter Therapie mit AD80 bestätigt.

Parallel zu meiner Arbeit für das Projekt haben wir mit anderen Arbeitsgruppen zusammengearbeitet, um ein tieferes Verständnis über die funktionellen Mechanismen hinter der hohen Aktivität von AD80 gegen RET zu erhalten. Mittels computerbasierter Modelle haben wir ableiten können, dass AD80 mit hoher Wahrscheinlichkeit als Typ II Inhibitor die RET-Kinase in der inaktiven „DFG-out" Konformation bindet, was mit einer erhöhten Kinase-Thermostabilität im Vergleich zu Typ I Inhibitoren als Bindungspartner einhergeht. Dies wiederum ist ein Surrogat-Parameter für eine engere Kinase-Bindung durch die Inhibitoren und könnte eine mögliche Erklärung für die hohe Cytotoxitität in unseren Experimenten sein.

Im Folgenden hat sich das Projekt mehr auf die Rolle von Resistenz-Mechanismen in RET mutierten Zelllinien konzentriert. Mittels zielgerichteter Mutagenese (site-directed mutagenesis) habe ich an der sogenannten „Gatekeeper Position" mutierte Ba/F3 *KIF-RET*^{V804M} und *CCDC6-RET*^{V804M} etablieren können, um die Wirksamkeit der Inhibitoren dagegen zu testen. Wieder zeigten AD80 und Ponatinib den stärksten inhibitorischen Effekt gegen RET mit nur einer geringen Reduktion der Cytotoxitität in den Zell-Viabilitäts-Assays und RET-Dephosphorylierung im Vergleich zu *RET*^{wt}. Mittels Sättigungsmutagenese (saturated mutagenesis screening) habe ich versucht, Resistenzmutationen zu finden, die neben der bekannten „Gatekeeper Position" pV804M zur Resistenz gegen AD80 führen könnten. Es zeigte sich in der Sequenzierung von resistenten Ba/F3$^{CCDC6\text{-}RET}$ Zellen die missense Mutation pI788N (c.2363T>A) im Bereich der RET-Kinase Domäne als potentielle sekundäre Resistenzmutation unter Therapie mit AD80. Diesen Resistenz-Effekt durch die neue Mutation konnte ich dann in den folgenden zellulären Modellen mittels Zell-Viabilitäts-Assays und Western Blots bestätigen. Zusätzlich haben Ergebnisse einer sekundär gegen AD80 resistent gewordenen TPC-1 Schilddrüsenkarzinom-Zelllinie sowie RNA-Sequenzierungen von LC-2/AD Zelllinien unter Therapie gegen AD80 ergeben, dass MAPK-Reaktivierung potentiell eine Rolle als Resistenz-Mechanismus in *RET*-getriebenen Tumoren besitzen könnte. Um formell die Rolle von MAPK-Reaktivierung in Bezug auf Resistenz-Effekte zu testen, wurden LC-2/AD Zellen von mir lentiviral mit *KRAS*^{G12V} transduziert, was zu einer Überexprimierung von KRAS und zu einer folgenden Resistenz gegen die Behandlung mit AD80 geführt hat.

Für Ponatinib gibt es bereits klinische Phase 3 Studien an Patienten mit Chronisch Myeloischer Leukämie, die jedoch trotz gutem initialen Tumor Ansprechen aufgrund von erhöhten Therapie assoziierten Komplikationen beendet werden musste (Lipton et al. 2016). Der RET-Kinase Inhibitor AD80 wäre daher ein geeigneter Kandidat für zukünftige klinische Studien. Bedenkt man den hohen Bedarf an neuen, wirksamen und verträglichen Therapieansätzen im Rahmen der individualisierten Lungenkarzinom-Therapie, bietet unsere

Studie eine Vielzahl neuer mechanistischer Einsichten in die aktuelle anti-RET Therapie und trägt zur deren zukünftigen Entwicklung bei.

1. Introduction

1.1 Cancer epidemiology

With 8.8 million attributable deaths in 2015 next to cardiovascular disease, lung cancer is the second most frequent cause of death due to non-infectious diseases worldwide (WHO Fact Sheet cancer 2017). The most frequent cancer-related deaths are caused by lung cancer (1.7 million deaths), followed by liver cancer (788,000), colorectal cancer (774,000), breast cancer (571,000) and stomach cancer (754,000) (WHO Fact Sheet cancer 2017). According to the WHO, the number of cancer patients will increase by 70% within the next 20 years and is predicted to rise to 21.4 million in 2030 (WHO 2011). Contributing lifestyle factors are for example tabaco and alcohol abuse, nutrition and lack of physical exercise that are assumed to be responsible for up to one third of all cancer cases (WHO 2014). In developing countries, viral diseases such as chronic liver disease due to Hepatitis B or C and HPV might contribute to up to 25% of all cancer diagnosis (WHO 2014). However, age is the most significant risk factor for cancer, as most patients diagnosed with invasive cancer are over 65 years old (William B. Coleman, Gregory J. Tsongalis 2009).

In general, cancer is more frequently observed in developed countries (WHO 2011). Possible reasons are a higher standard of living with different life style factors and a better availability of medical care and infrastructure for a broad share of the population. From an economic point of view, cancer is an increasing burden for society with approximate costs of US$ 1.16 trillion in 2010 (WHO 2014). With increasing life expectancy and rising costs for individual cancer therapies at the beginning of the area of targeted cancer therapy, this amount is very likely to increase and might overstrain health care systems with limited resources (WHO 2014).

1.2 Lung cancer

1.2.1 Epidemiology

Globally, lung cancer is responsible for the majority of cancer related deaths with a total number of 1.7 million deaths in 2015 (WHO Fact Sheet cancer 2017). 80% and 90% of those deaths can be attributed to active or passive tabaco exposure in women and men respectively (American Lung Association 2014). In the developed world, about one third of all cancer deaths are due to lung cancer (Siegel et al. 2014). A difference in prevalence can be seen between the sex. The lifetime risk of developing lung cancer is 8% for men, whereas for women, it is

6% in the USA (Kasper et al. 2015). For men, it causes the highest number of cancer related deaths, for women it is on third place after breast and colorectal cancer (Ferlay et al. 2013). The main reason for this difference is the higher rate of smokers seen in the male population, especially if the past is considered (Hecht 2002). However, the incidence of lung cancer for men has declined from 90/100.000 in 1975 to about 65/100.000 in 2011. During the same period of time, the rate has risen for women from 25 to 48/100.000, with stabilising numbers only recently in the last decade. This can be attributed to equalizing smoking behaviours for both sexes in the last few decades (American Lung Association 2014).

1.2.2 Classification

The term lung cancer is to some extent misleading as it summarises a very heterogeneous group of cancers that differ in terms of aetiology, morphology, histology, clinical presentation, treatment and outcome (Kenfield et al. 2008). The original classification of lung cancer into 'small cell lung cancer' (SCLC), 'non-small cell lung cancer' (NSCLC) and carcinoid has originally been derived from its morphology and histology (Lamb 1984); Plenker 2015).

1.2.2.1 Characteristics of NSCLC

NSCLC can be subdivided into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Other sub-types are very rare. Adenocarcinoma is the most common histological subtype of lung cancer with 40-50% in total. This number is increasing as the absolute number of smokers in the developed world has decreased over the last years (Hanahan and Weinberg 2011). The reason is that the aetiology of lung adenocarcinoma does not seem to be primarily related to tobacco exposure , therefore it is the major type of lung cancer seen among women and never-smokers (Kenfield et al. 2008). The classification of NSCLC has been originally based on certain morphology and immunohistochemical staining properties (Travis 2011). It was established in a time when the genomic and molecular profiling of biopsy samples was not as advanced as it is nowadays. Following the progress of implementing tumor sample genome sequencing in the clinical routine and advancing knowledge about genomic alterations, this rather descriptive categorization is challenged by an approach based on molecular patterns that might be used in the future for an unbiased classification of lung cancers (Bianchi et al. 2008) (Plenker 2015). This is crucial as this has a large impact on individual therapy planning for lung cancer patients in the context of individualised tumor therapy (Travis 2014); (CLCGP and NGM. 2013). A better differentiation between squamous and adenocarcinoma will be possible while the number of large cell cancer diagnosis will likely diminish as it might be considered a merely badly differentiated type of lung cancer (CLCGP and NGM. 2013). About 40% of all adenocarcinomas are driven by *KRAS* and *EGFR* mutations, followed by various oncogenic rearrangements such as *ALK*-, *ROS*-, or *RET* fusions

(Pao and Hutchinson 2012); (Kohno et al. 2015). Additionally, a considerable heterogeneity for Asian and European/US-American populations in lung adenocarcinoma exists (Fig. 1). The second biggest sub-type of NSCLC (30%) is squamous cell carcinoma in which TP53, FGFR1, DDR2 and NFE2L2 are frequently mutated (CLCGP and NGM. 2013; Weiss et al. 2010).

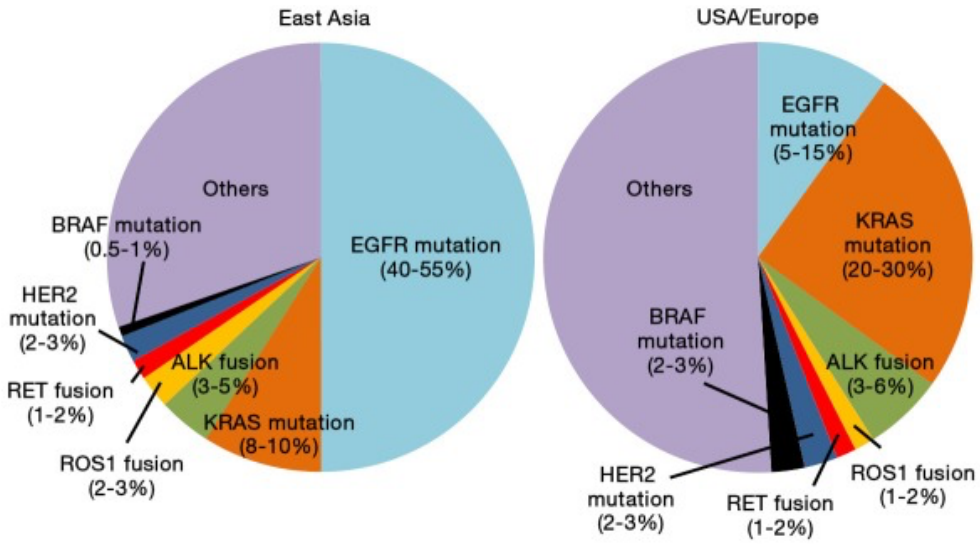

Figure 1: Pie chart showing cumulated data of oncogenic drivers in lung adenocarcinoma for East-Asian (left) and European/US-American populations (Kohno et al. 2015)

1.2.2.2 Clinical background of NSCLC

NSCLC accounts for up to 80% of all lung cancer cases and is not only characteristic in terms of morphology and histology, but also regarding treatment procedures and a better outcome as compared to SCLC. Treatment options depend on the prognosis group as determined by TNM-staging (Table. 1). From stages I-IIIa, NSCLC are potentially treatable in a curative intention with complete surgical resection including a (neo-)adjuvant combined platinum-based radio-chemotherapy. However, over 60% of all NSCLC cases are diagnosed in the later stages IIIB/IV that can only undergo palliative treatment (Brodowicz et al. 2012). Unfortunately, the median survival in this group is below 12 months (Mok 2011).

Stage	T	N	M
Ia	T1a	N0	M0
	T1b	N0	M0
Ib	T2a	N0	M0
IIa	T1a	N1	M0
	T1b	N1	M0
	T2a	N1	M0
	T2b	N0	M0
IIb	T2b	N1	M0
	T3	N0	M0
IIIa	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T3	N1	M0
	T4	N0	M0
	T4	N1	M0
IIIb	T4	N2	M0
	T1	N3	M0
	T2	N3	M0
	T3	N3	M0
	T4	N3	M0
IV	T Any	N Any	M1a or 1b

Table 1: Prognostic groups for NSCLC based on TNM-staging

Especially for patients in a non-curable stage, targeted therapy based on predictive histological, immunohistochemical and genetic markers has become a cornerstone of late NSCLC therapy (Plenker 2015). That has led to the development of therapeutic guidelines for certain genetic alterations for which targeted therapy has been proven beneficial in clinical trials (German NSCLC guidelines in November 2018). Standardised therapeutic guidelines for targeted cancer therapy in late stage NSCLC exist in Germany and Europe for example for *ALK*-rearrangements (Crizotinib, Ceritinib, Alectinib), *ROS*-rearrangements (Crizotinib, Ceritinb, Cabozantinib), *EGFR* mutations (Afatinib, Erlotinib, Gefitinib, Osimertinib) or *PD-L1* overexpression (Pembrolizumab, Nivolumab, Nintendanib, Ramucirumab). Patients with varying oncogenic alterations or non-responders should be, if possible, included in clinical trials to broaden the panel of druggable genetic alterations (German NSCLC guidelines in November 2018). The more oncogenic targets are included in the standard diagnostic sequencing panels, the higher are the chances to detect specific targets for precision cancer medicine (Syn et al. 2016).

A well-documented example is the treatment of patients diagnosed with *EGFR*-mutant end-stage NSCLC with the small molecule inhibitor erlotinib that inhibits the activation of EGFR

and its downstream pathways. EURTAC, a multicentre, open-label, randomised phase 3 trial, showed nearly doubled progression-free-survival (PFS) with 9.7 months (95% CI 8.4-12.3) under erlotinib treatment compared to 5.2 months (4.5–5.8) in the standard chemotherapy subgroup (Rosell et al. 2012). More recently, the third-generation EGFR inhibitor osimertinib received FDA-approval after a positive phase III trial, demonstrating superiority to first-generation EGFR inhibitors like erlotinib (Soria J 2018).

Another recent example is pembrolizumab, a humanized monoclonal antibody against programmed death 1 (PD-1) for the treatment of programmed death ligand 1 (PD-L1) overexpressing NSCLC. The antibody neutralizes PD-L1 on the cancer or ECM cell surface that inhibits activation and chemotaxis of immune cells. In an open-label phase III trial, superiority of immunotherapy compared to standard chemotherapy was demonstrated (Reck et al. 2016) (Fig. 2). PFS was 10.3 months in the pembrolizumab group vs. 6.0 months in the platinum-based chemotherapy group. Accordingly, a higher response rate was with 44.8% vs. 27.8% and a longer duration of response was seen (Reck et al. 2016).

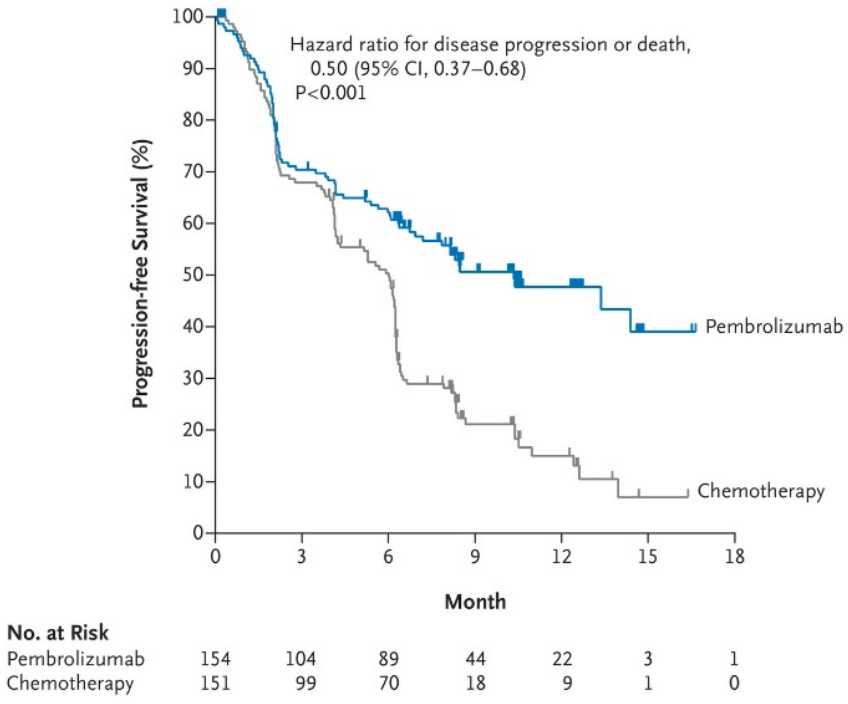

Figure 2: Kaplan Meier curve of progression-free survival according to treatment groups (Reck et al. 2016)

1.3 Tumorigenesis

1.3.1 Aetiology

In general, cancer is caused by a dysregulation of cell growth due to genomic alterations in either tumor suppressive or pro-proliferative (proto-oncogenic) genes and develops in a multi-step process in which different cancer-promoting changes accumulate (WHO 2014). Frequently, this process starts with non-invasive, precancerous lesions followed by malignant transformation as encountered in the concept of an 'adenoma-carcinoma sequence as first suggested for colorectal adenocarcinomas (Vogelstein et al. 1988). This process can be influenced by several environmental factors such as smoking, radiation or by viral and chronic infections. Those factors lead to an altered gene expression that can start the base to cause dysregulated cell growth. Due to somatic predisposition by, for example, germline mutations of *TP53* as in Li-Fraumeni Syndrome or *NF1* alterations in Von-Recklinghausen's Syndrome, the probability to develop cancer can increase up to 100% (Wallace et al. 1990; Brodowicz et al. 2012; Anand et al. 2008; Srivastava et al. 1990). The function of regulatory proto-oncogenes and tumor suppressor genes can be manipulated in various ways, such as point mutations, inversions, deletions or rearrangements, epigenetic modifications, or alternative splicing (Hanahan and Weinberg 2011).

1.3.2 Tumor suppressor genes and proto-oncogenes

Tumor suppressor genes encode for proteins that negatively regulate cell cycle progression, facilitate DNA damage repair and act upon cell differentiation, migration and protein degradation (Hanahan and Weinberg 2011). Therefore, intact tumor suppressor genes are protective against dysregulated cell growth. According to Knudson's two-hit hypothesis, tumor suppressor genes behave in a recessive manner and both alleles of a gene must be altered for loss of function leading to cancer predisposition (Knudson 1971; Plenker 2015). Therefore, patients typically lose both somatic alleles consecutively in a variable period of time or they possess germline mutated heterozygous alleles and gain an additional somatic mutation or they harbour biallelic germline mutations from the beginning in their germline.

A central cell cycle regulatory switch and member of this group of growth suppressing proteins is retinoblastoma protein 1 (RB1) of the "pocket protein family". Its biallelic germline loss-of-function mutation leads to the formation of familial paediatric retinoblastoma. In the non-mutant state, RB1 binds the transcription factor E2F and supresses pro-proliferative signals leading to cell cycle arrest in the G0 or G1 phase (Burkhart and Sage 2008). If the proper function of RB1 is impaired, cells continuously move on to cell division. In contrast to

p53 that regulates the effects of intracellular stress factors, the major role of RB1 is mediating extracellular growth signals (Hanahan and Weinberg 2011). Alterations affecting the function of RB1 are frequently overserved in cancer (Burkhart and Sage 2008). Even though only a minor fraction of lung adenocarcinomas harbour *RB1* mutations, 90-100% of all SCLC show these bi-allelic loss-of-function mutations (George et al. 2015; CLCGP and NGM. 2013).

In lung cancer patients, loss-of-function mutations of the tumor suppressor TP53 negatively affect clinical outcome and survival is further decreased if additional somatic mutations occur (CLCGP and NGM. 2013). Further frequently altered tumor suppressor genes in lung cancer are *PTEN*, *RB1*, *LKB1* and *p16*/*CDKN2A* (Cooper et al. 2013). As it is pharmacologically more difficult to restore activity than to inhibit it, treatment of complete tumor suppressor loss is very complex. Thus, therapeutic targeting of tumor suppressor loss in precision cancer medicine is not feasible yet. An exception form that are approaches of "synthetic lethality" by PARP inhibition in *BRCA* and *BRCA*-like positive malignancies (Lee et al. 2014). The concept of "synthetic lethality" describes the effect that two mutations, if they occur independently from another, are non-lethal, but lead to cell death in combination (Dobzhansky 1946). *BRCA1* and *BRCA2* are tumor suppressor genes that are mutated in more than 80% of all familial breast cancers and are associated with various other cancer types such as ovarian, stomach or pancreatic cancer (Arteaga et al. 2014). Functional loss of *BRCA1/2* leads to improper DNA double-strand break repair. As PARP is essential for single-strand break repair, its therapeutic inhibition in combination with the *BRCA1*/*2* loss leads to a growing accumulation of irreversible DNA damage and eventually (cancer) cell death (Dedes et al. 2011).

In comparison to tumor suppressor genes, oncogenes encode for proteins that enhance cell growth after mutation of one allele in a dominant fashion or gene amplification, leading to dysregulation of potential oncogenic pathways and thus actively induce cancer cell proliferation. Huebner and Todaro proposed this oncogene hypothesis for the first time in 1969 (Huebner and Todaro 1969). Gain-of-function mutations and gene amplifications are the most frequent alterations seen in oncogenes (Hanahan and Weinberg 2011). Genomic rearrangements, translocations and deletions of regulatory elements on the other hand are rarer events (Chin and Gray 2008). The most frequently observed mutated oncogenes in lung cancer are *KRAS*, *PIK3CA*, *EGFR*, *BRAF* and *ERBB2* (CLCGP and NGM. 2013; Plenker 2015)

A well-established model is the *RAS* proto-oncogene that encodes a protein class of small GTPase which act as important molecular signalling mediators (Hanahan and Weinberg 2011). In its active conformation, *RAS* is bound to guanosine triphosphate (GTP) and is

'switched off' when bound to guanosine diphosphate (GDP) leading to specific conformational changes. RAS inactivation is mediated by enzymatic hydrolysis of GTP induced GTPase-activating proteins (GAPs). If GTPase- activity is impaired, for example by the well-described activating mutations at the G12 locus, constitutive GTP-bound *RAS* can drive various malignancies (Eser et al. 2014). With a mutation rate of 16% among all cancer types, *HRAS*, *NRAS* and *KRAS* are the most frequently altered oncogenes (Prior et al. 2012).

1.3.3 Sustaining proliferative signalling in cancer

In normal tissue, production and release of growth factors are tightly regulated by intra- and extracellular (growth) factors and corresponding receptors. This keeps the number of cells and cell architecture in a physiological range and maintains the function of the tissue. Those factors can be produced in a para- or autocrine manner and typically act as ligands on specific membrane proteins (Hanahan and Weinberg 2011). These receptor tyrosine kinases (RTKs) control the downstream cellular signalling that finally leads to gene up- or downregulation. In a broad range of tissues, the phospoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) plays a major role in translating growth signals into a cellular response by inducing cell growth, proliferation and angiogenesis (Hanahan and Weinberg 2011). Hence, dysregulation of this pathway is often recognized in tumor development or in drug resistance (Hanahan and Weinberg 2011).

Cancer cells can acquire different methods to overcome cellular control mechanisms, such as overexpression of the ligand, overexpression of the RTK or via gain-of-function mutations that enhance the kinase activity (Hanahan and Weinberg 2011). One example is the fibroblast growth factor receptor 1 (FGFR1) that is found as a driver of cancer formation in 3.6 % of SCLC and 22% of squamous carcinomas of the lung (Peifer et al. 2012). In those cancers, *FGFR1* is amplified leading to enhanced activation of downstream cellular signalling (Peifer et al. 2012; Weiss et al. 2010). Another example for activating mutations are *BRAF* alterations found in malignant melanoma patients leading to MAPK over-activation (Davies et al. 2002). The clinical development of small molecule inhibitors targeting these alterations has been a great clinical success, as exemplary seen in $BRAF^{V600E}$ positive melanoma under vemurafenib treatment (Bollag et al. 2012).

1.3.4 Fusion genes and cancer development

In the last decades, more and more gene fusions have been identified as oncogenic drivers in a broad range of different tumors. The 'Philadelphia chromosome' *Bcr-ABL1* was the first one described in a patient with chronic myeloid leukaemia (CML) (Nowell P. and Hungerford D.;

Tefferi 2016). A DNA double-strand break results in a translocation of *ABL1* (Abelson murine leukemia viral oncogene homolog 1) gene on chromosome 9 (region q34) to *Bcr* (breakpoint cluster region) gene on chromosome 22 (region q11) (Melo 1996). This gene fusion leads to the expression of a chimeric protein with its promoter within the *Bcr* domain and massive ABL1 tyrosine kinase (over-)expression. Since ABL1 holds a central role in cell cycle control, unrestricted cellular proliferation and eventually tumor formation occurs (Kurzrock et al. 2003). *Bcr-ABL1* is not only a well investigated role model for the oncogenic effect of gene fusions, but with the development of the tyrosine kinase inhibitor imatinib (Gleevec) and others afterwards, it represents a milestone in the development of molecular-based, individualized tumor therapy (Schindler 2000; Kurzrock et al. 2003)

Similar mechanisms of dysregulated kinase domains in a fusion protein also apply to the most common oncogenic rearrangements found in lung cancer (Du and Lovly 2018). These are *CD74-ROS*, *KIF5B-RET* and *EML4-ALK* gene fusions (Cooper et al. 2013).

1.4 Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are polypetides that form transmembrane receptors for many signalling molecules such as cytokines, growth factors or hormones. They do not only regulate physiological cell cycle development and survival but also serve as key components of oncogenic transformation and various other pathologies such as metabolic, immunological, endocrine or neurological diseases (Hubbard 1999; Blume-Jensen and Hunter 2001). Interestingly, from an evolutionary point of view, the core features of the structure, activation and signal processing are highly conserved from the nematode Caenorhabditis *elegans* up to humans highlighting the central role of RTKs for human development (Lemmon and Schlessinger 2010).

1.4.1 Structure, activation and signal transduction of RTKs

All RTKs share a similar molecular structure with an extracellular (N-terminal) ligand binding domain, a hydrophobic transmembrane domain responsible for dimer stabilisation and an intracellular (C-terminal) region that provides catalytically active tyrosine, threonine or serine residues with additional regulatory domains (Du and Lovly 2018). Biochemically, a kinase is an enzyme that transfers phosphate groups from highly energetic donor proteins such as ATP onto a hydroxyl group of a tyrosine, serine or a threonine of lower energetic substrate molecules. In a highly conserved part of the kinase, 12 subdomains form two distinct lobes with a catalytically active central cleft for ATP binding. The adenine ring of ATP binds

through hydrogen bonds in the so called “hinge region” between the N and C-terminal kinase domains while the ribose and triphosphate occupy a hydrophobic pocket (Zhang et al. 2009) (Fig. 3).

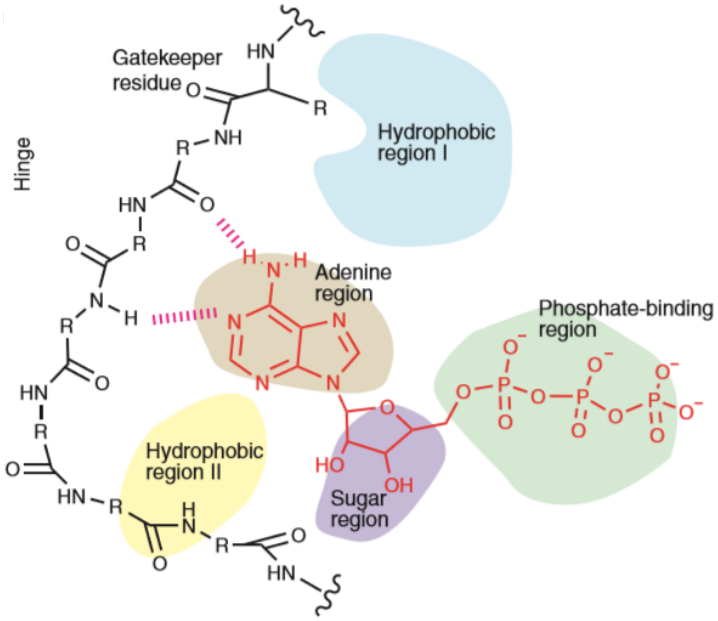

Figure 3: Schematic representation of ATP binding into kinase domain sub pocket (Liu and Gray 2006)

90 genes have been identified that encode for 58 receptor tyrosine kinases that can be subdivided into 20 family groups with distinct molecular characteristics, in particular of the extracellular domain (Blume-Jensen and Hunter 2001). Upon ligand binding of the extracellular domain of the RTK, the most frequent mechanism for signal transduction is by dimerization of two single RTKs leading to trans-phosphorylation of specific cytoplasmatic tyrosine residues. This in turn causes changes in the RTK conformation creating phosphorylation binding sites for additional molecules such as Src homology 2 (SH2) domain- and phosphotyrosine binding (PTB) domain-containing proteins or other adaptor proteins (Pawson 1995). The phosphorylation cascade continues and leads to the activation or deactivation of various transcription factors that are, generally speaking, involved in cell life and death regulation (Ségaliny et al. 2015).

1.4.2 Binding properties of kinase inhibitors

All kinases share the common feature of a conserved “activation loop” that is crucial for the regulation of kinase activity. This loop features the distinct amino acid group “Asp-Phe-Gly” at its base N-terminus, the so called DFG-motif (Treiber and Shah 2013). The activation loop is

not static, but (depending on its phosphorylation state) flips inwards (DFG-in) in the catalytically active kinase state or rotates outward at least 10 Å from its position in the kinase inactive conformation (DFG-out) and blocks phosphate transfer (Dar and Shokat 2011). This leads to an inactive kinase state (Zhang et al. 2009).

The vast majority of small molecule inhibitors developed in the past target the ATP binding site of the kinase in the enzymatically active, phosphate-transferring, DFG-in state (Liu and Gray 2006; Müller et al. 2015). Most of those ATP-competitive type-1 inhibitors consist of heterocycles which core forms hydrogen bonds with the kinase hinge region as well as side groups occupying the neighbouring hydrophobic regions within the kinase (Zhang et al. 2009) (Fig. 4A). Type II inhibitors on the other hand bind the inactive kinase by stabilising the DFG-out formation. The inhibitor utilises additional hydrophobic subgroups (allosteric site) for binding that are only exposed if the DFG-out conformation of the kinase is present and extends into the hinge region as well (Zhang et al. 2009) (Fig. 4B). Type II inhibitors are considered more selective as compared to type I inhibitors, even though the precise mechanisms behind it and the clinical relevance have not been fully understood and exceptions from this assumption exist as well (Davis et al. 2011). One possible explanation could be the effect of the additional binding partners provided by the allosteric site in the DFG-out conformation that are less well conserved among the different kinases (Treiber and Shah 2013).

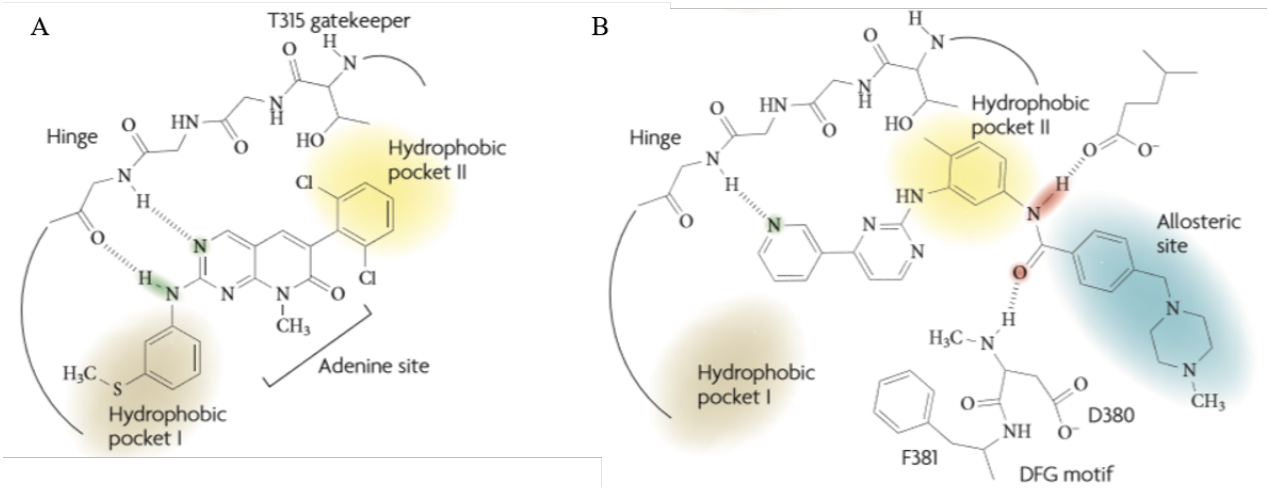

Figure 4: Chemical structures depicting binding properties of the type-1 inhibitor PD166326 (A) and the type-2 inhibitor imatinib (B). The hydrogen bonds to the hinge region (green) and the allosteric site (red) are shown (Zhang et al. 2009).

1.5 The RET proto-oncogene

1.5.1 RET structure and ligands

The RET proto-oncogene is located in the pericentromeric region of chromosome 10q11.2 and encodes for a receptor tyrosine kinase (Andrew et al. 2002). Like other RTKs, RET consists of an extracellular region with cadherin-like repeats for protein stabilisation, a cysteine-rich transmembrane sequence for protein formation and an intracellular kinase domain for autophosphorylation and signal transduction (Takahashi et al. 1988; Anders et al. 2001). Unlike other RTKs, extracellular activation of RET signalling is regulated by glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) which require co-factors (GFRα family members) for the preformation of the heterodimeric complexes (Fig. 5). These ligand-co-factor complexes in turn induce RET conformational changes that lead to facilitated dimerization of RET molecules and eventually trans-autophosphorylation of the intracellular kinase (Arighi et al. 2005).

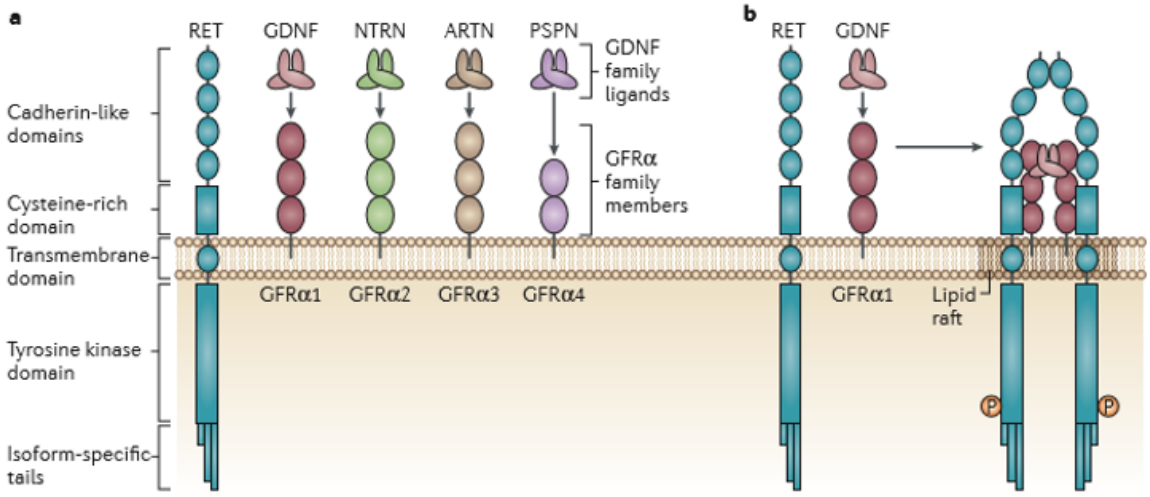

Figure 5: RET receptor tyrosine kinase and its ligand-cofactor complexes (a), ligand activation leads to homo-dimerization (b) (Mulligan 2014)

1.5.2 RET function

1.5.2.1 RET cell signalling

Upon dimerization, autophosphorylation of various intracellular tyrosine sites leads to the upregulation of different downstream pathways such as RAS-MAPK, PI3K-AKT, JAK-STAT or PKC signalling (Ibanez 2013) (Fig.6). In general, these pathways are involved in cell

proliferation, survival and differentiation (Mulligan 2014). The three human RET protein isoforms (RET9, RET43 and RET51) differ in terms of their carboxy-terminal amino acids with distinctive protein interactions and intracellular trafficking due to alternative splicing (Lian et al. 2017). With respect to medullary thyroid carcinoma (MTC) and papillary thyroid carcinoma (PTC), the RET51 isoform had higher oncogenic potential and was more frequently found in human thyroid cancer samples than the other variants (Lian et al. 2017). The most common and best described phospho-tyrosine site of all three isoforms is Y1062 that is recognized by multiple adaptor and signalling molecules such as 'SRC homology 2 domain containing transforming protein 1' (SHC1), 'fibroblast growth factor receptor substrate 2' (FRS2), 'insulin receptor substrate 1' (IRS1), IRS2 and docking protein 2 (DOK2) or DOK4–6 (Mulligan 2014) (Fig. 6).

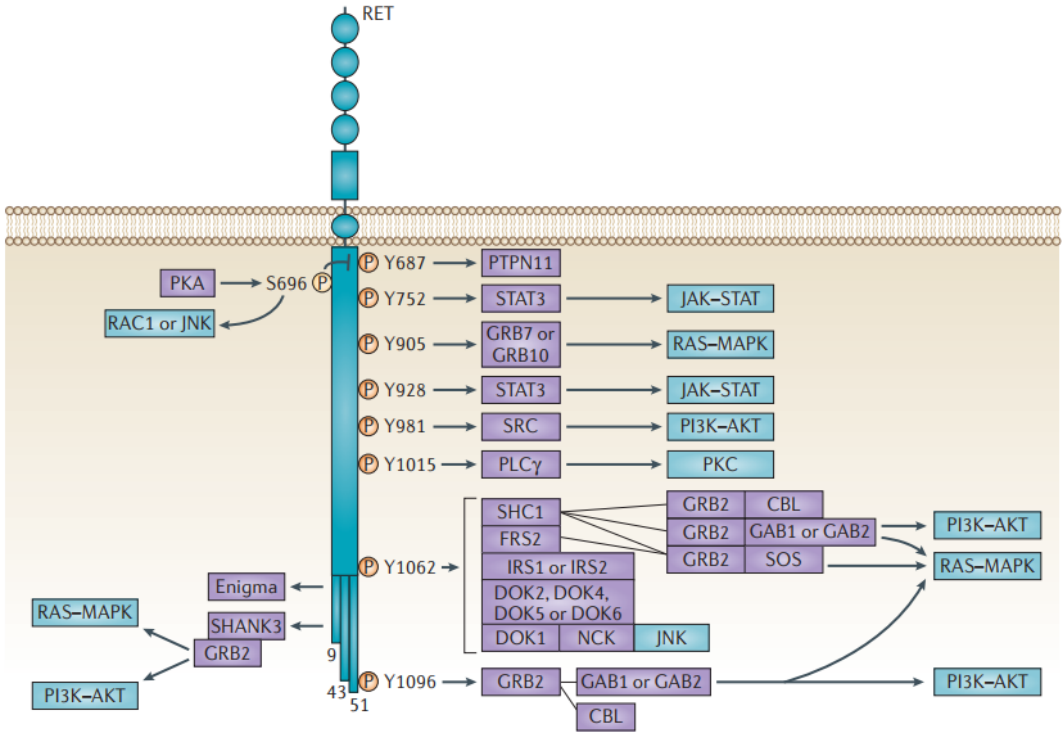

Figure 6: RET downstream signalling (Mulligan 2014)

1.5.2.2 Physiologic role of RET

Physiologic RET function is particularly important for embryonic tissue development that is reflected in a high *RET* conservation among various lower and higher species and a strong expression in early embryogenesis with weak levels in adulthood (Schuchardt et al. 1994; Tsuzuki et al. 1995; Mulligan 2014). RET plays a major role for the growth and morphogenesis of kidneys and the urinary tract system, for the survival of spermatogonial stem cells and for the formation and maintenance of neuroendocrine cells, especially in thyroid c-cells, adrenal chromaffin cells and the enteric nervous system (Mulligan 2014; Arighi et al. 2005). This fact

is also highlighted in the aetiology of Hirschsprung disease (HSCR) due to germline loss-of-function mutations in RET. The lack of sufficient RET signalling leads to an inherent form of megacolon caused by failed migration of neuroblasts in the developing gut resulting in undeveloped submucosal and myenteric plexus (Amiel and Lyonnet 2001). In contrast to specific and very well-described mutations in thyroid or lung cancer, there is a large variety of genetic alterations found in HSCR or other *RET* germline mutation associated disorders, such as 'congenital abnormalities of the kidney and urinary tract (CAKUT) or 'congenital central hypoventilation syndrome' (CCHS) (Drilon et al. 2018).

1.5.3 RET and oncogenesis

Various gain-of-function mutations have been described in different tumor entities with distinct phenotypes leading to an unregulated RET dimerization and activation of its downstream signalling. The alterations mainly consist of activating point mutations in the extracellular or kinase domain of RET (e.g. M918T in MEN2B) or RET rearrangements leading to the formation of fusion proteins (e.g. KIF5B-RET in NSCLC) (Drilon et al. 2018).

RET germline alterations are highly associated with multiple endocrine neoplasia type 2 (MEN2) and its two sub-categories MEN2A and MEN2B. They are characterized by a varying degree of the formation of familial medullary thyroid carcinoma, pheochromocytoma, parathyroid hyperplasia or adenoma and further disorders. (Donis-Keller et al. 1993; Moore et al. 2006; Hofstra et al. 1994). Depending on the specific activating point mutation, ligand independent dimerization or monomer activation leads to aberrant and dysregulated cell signalling (Mulligan 2014). In sporadic medullary thyroid carcinoma, RET fusion genes are found in 20-40% of all patients as oncogenic drivers (Romei and Elisei 2012). Detection of those alterations have a strong impact on early diagnosis, therapy management and improved outcome of thyroid cancer patients (Mulligan 2018).

RET (over-)expression has also been demonstrated in other tumor entities that could have an implication for patient outcome. 50-65% of pancreatic ductal carcinomas express (excessive) RET that may be associated with a higher rate of metastasis and worse outcome. (Amit et al. 2017; Zeng et al. 2008). Similarly, RET levels are also increased in invasive breast cancer, especially in oestrogen-receptor positive tumors with primary resistance to anti-oestrogen hormone therapy leading to decreased metastasis-free and overall survival (Plaza-Menacho et al. 2010; Morandi et al. 2013). This close interaction between RET and oestrogen receptor signalling is in line with findings that RET transcription is directly affected by oestrogen receptor activity (Boulay et al. 2008). *In vitro* data provide a rational that adjuvant RET inhibitor treatment might be beneficial in terms of response and re-sensitization to endocrine therapy

(Gattelli et al. 2018). Recently Mechera et al. confirmed the association of RET and oestrogen receptor expression in breast cancer patients, however without a significant impact on prognosis or outcome (Mechera et al. 2019). The role of RET signalling for tumor progression in other tissues for example colon, head, prostate or CNS has not been fully understood (Mulligan 2018).

Oncogenic transformation potential of *RET*-fusions and activating mutations *in vitro* were shown for the *CCDC6-RET* harbouring human LC-2 lung adenocarcinoma cell line (LC-2/AD) (Suzuki et al. 2013), IL-3 independently proliferating murine Ba/F3 cells (pro-B lymphocyte) (Lipson et al. 2012) and NIH-3T3 cells (fibroblasts) with anchorage-independent growth and subcutaneous tumor growth in mice (Takeuchi et al. 2012). *In vivo* models of transgenic mice developed adenocarcinomas in *RET*-fusion expressing lung alveolar epithel cells that demonstrate a similar histlogical pattern as seen in human lung adenocarcinoma patient samples (Saito et al. 2014).

1.5.3.1 RET-rearrangements in lung cancer

In *RET* gene fusions, the *RET* 3´ part always contains the kinase domain (exon 12), while the 5´ sequence from a different set of fusion partners encodes a coiled-coil region for protein homo-dimerization. The kinase is preserved and functionally intact despite the genomic breakpoint (Kohno et al. 2012). In contrast to wild type RET, uncontrolled homodimerization leads to hyperactive and ligand-independent RTK signalling (Kohno et al. 2012). This mechanism is similar for *EML4-ALK* gene fusions in NSCLC (Soda et al. 2007), however different as seen in *ROS1* rearrangements without the presence of dimerization domains leading to ligand-independent growth (Takeuchi et al. 2012). The rearrangements likely arise from erroneous DNA double strand breaks and repair mechanisms (Mizukami et al. 2014). Interestingly, it was shown that *CCDC6* and *RET* gene loci are frequently in juxtaposition in the nucleus of non-malignant thyroid cells indicating a possible role of cellular dynamics for gene fusion frequency (Nikiforova 2000)

For lung cancer, especially the ‘in frame’ *RET* rearrangements that induce functionally active fusions are important, particularly in adenocarcinomas where they form a distinct oncogenic group (Pao and Hutchinson 2012). At least 12 recurrent *RET*-fusion partners have been discovered (Ferrera R. 2018). The most frequent and well described fusion partner for *RET* in lung cancer is ‘kinesin family member 5B’ (*KIF5B*) that arises from a pericentric inversion on chromosome 10 (Kohno et al. 2013). Others, such as the ‘coiled-coil domain-containing 6’ (*CCDC6*), the ‘nuclear receptor coactivator 4’ (*NCOA4*) or the ‘tripartite motif-containing 33’ (*TRIM33*) are less frequent (Mulligan 2014) (Fig. 7). In the case of *KIF5B-RET*,

the fusion induces an up to 30-fold increase in RET transcription leading to oncogenic transformation due to the expression of functional active RET kinases (Kohno et al. 2012). Interestingly, it was recently shown that the two RET fusion partners *CCDC6* and *NCOA4* lead to varying degrees of phenotypical changes in a Drosophila transgenic fly model that results in different cellular signalling and sensitivity to kinase inhibition (Levinson and Cagan 2016).

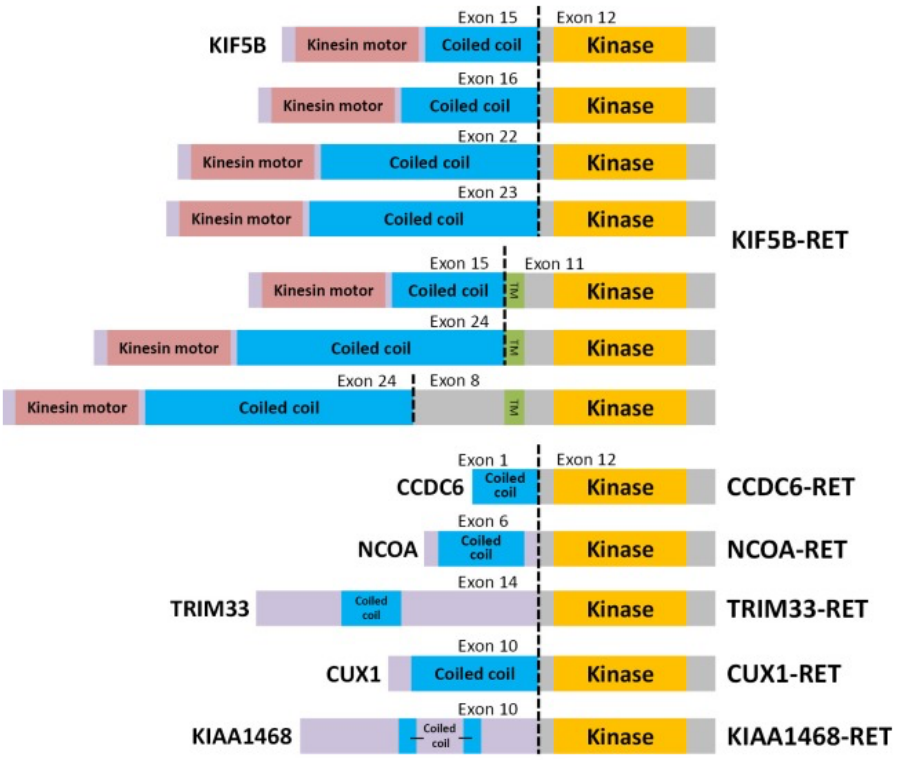

Figure 7: Schematic representation of RET fusions in lung adenocarcinoma (Kohno et al. 2015)

1.5.3.2 Pathological and clinical implications of RET driven lung cancer

The standard method for detecting RET fusions in patient tumor samples in clinical routine is Fluorescence in Situ Hybridziation (FISH). It ptoved good detection quality with 100% sensitivity and 45% - 60% specificity, whereas Immunohistochemistry (IHC) staining proved inferior with 50% sensitivity and variable specificity from 40% - 85% due to a higher false positive rate (Ferrara et al. 2018). Reverse-transcription PCR is specific, but the results can be easily negatively affected by tissue sample quality and it is restricted to already known *RET* fusion partners (Tsuta et al. 2014).

Retrospective studies indicate that *RET*-rearrangements in lung cancer are associated with a more aggressive histological subtype (e.g. presence of signet ring cells), a worse grading, a younger age at diagnosis (<60 years) and non-smkoing history (Tsuta et al. 2014;

Ju et al. 2012). In addition, early metastases seem to be another negative prognostic marker for RET driven NSCLC (Gautschi et al. 2017). Final conclusions about correlations of other genetic alterations parallel to RET rearrangements and their prognostic and therapeutic value are not possible yet due to low incidence rates. At the time of discovery, it was though to be an exclusive oncogenic driver, however more recent studies depict a co-existence of rearranged *RET* with other oncogenic drivers such as mutant *EGFR* in up to 21% of patients (Ferrara et al. 2018). Recently, Piotrowska et al. could demonstrate that CCDC6-RET expressing, EGFR-mutant PC9 and MGH134 cells were resistant to EGFR inhibition and that the RET-inhibitors BLU-667 or cabozantinib resensitized these cells to anti-EGFR targeted therapy. Moreover, combined treatment with osimertinib and BLU-667 in two patients with EGFR-mutant NSCLC and RET-mediated resistance led to lasting tumor response (Piotrowska et al. 2018).

1.5.4 Anti-RET therapy

1.5.4.1 Rationale for targeting RET

For targeted cancer therapy, tailoring a treatment regime for each patient based on the genetic alterations of the tumor is crucial. This “personalisation” is supposed to maximise the individual outcome in end-stage cancer. In the last two decades, cancer drug development has benefited exceptionally from new discoveries of the central role of receptor kinases for oncogenic transformation (Zhang et al. 2009). Taking advantage of this “oncogene addiction” and dependency on therapeutic targets is the key for treatment with small molecule inhibitors (Weinstein and Joe 2006). Clinical trials with improvements in the progression free survival and overall response rate compared to standard chemotherapy or best supportive care have led to the regulatory approval of different drugs as seen, among others, in the treatment of ROS1, ALK or EGFR driven lung cancer (Ferrara et al. 2018)

With RET-rearrangements found in 1-2% of all lung adenocarcinoma, it may be a rather small, but potentially druggable fraction of lung cancer (Pao and Hutchinson 2012). Taking into account that 1.8 million new lung cancer diagnosis are made each year, the clinical need to find treatment options for this relatively rare sub type is high nonetheless (Cheng et al. 2016). In contrast to advanced thyroid cancer, no precision RET therapy in NSCLC has been approved yet (Mulligan 2018). Treatment of MTC or PTC in a palliative stage with the RET-targeting multikinase inhibitors vandetanib, lenvatinib, cabozantinib or sorafenib have significantly improved progression free survival and clinical outcome (Redaelli et al. 2018). That underlines the potential druggability and benefits of targeted anti-RET therapies in RET driven cancers.

1.5.4.2 From bench to bedside – current state of anti-RET therapy in NSCLC

Even though RET is a potential distinct therapeutic target for precision cancer therapy in NSCLC, no standard therapeutic regime of RET driven lung tumors has been approved yet (Mulligan 2018). Based on results from *in vitro* and *in vivo* data as well as promising outcomes of clinical trials in thyroid cancer, multi kinase inhibitors targeting RET in advanced NSCLC have not fulfilled the expectations (Gautschi et al. 2017) (Fig. 8).

Most clinical data exist for vandetanib and cabozantinib (Gautschi et al. 2017). Taking most of the clinical data into account, their median progression free survival (PFS) for treatment of thyroid cancer is 30.5 and 11.2 months respectively. By contrast, those two agents only achieve 3.6- 5.5 months of median PFS in RET driven NSCLC (Drilon et al. 2018) (Fig. 9). This is surprising because these drugs show substantial activity *in vitro* experimental settings with anti-RET half maximal inhibitory concentration (IC_{50}) for vandetanib and cabozantinib around 100nM and 5-20nM respectively, *in vitro* inhibition of downstream cell signalling and *in vivo* anti-cancer activity in genetically-engineered and patient-derived models (Ferrara et al. 2018) (Drilon et al. 2018). One key explanation to this finding is the fact that all agents tested so far are not as specific and potent against RET compared to other targeted drugs (e.g. IC_{50} of 1nM of osimertinib in T790M mutated EGFR), but target a variety of kinases with dose limiting off-target effects (Cross et al. 2014). Comparing the IC_{50} of cabozantinib aginst differnet kinases, a 100-fold stronger inhibition of VEGFR2 than RET (0.035nM vs. 5.2nM) might limit its clincal use (Bentzien et al. 2013). That may contribute to the high dose reduction rate seen for cabozantinib (19/26; 73%) and turned it ineffective for RET inhibition (Drilon et al. 2016). Recently, a phase II trial with lenvatinib resulted in the so far best overall median progression free survival of 7.3 months (Velcheti et al. 2016). Smaller trials with partial response or stable disease were also conducted with alectinib, sunitinib, nintedanib and RXDX-105 while others with ponatinib, apatnib or BLU-667 are still ongoing (Drilon et al. 2018) (Subbiah et al. 2018).

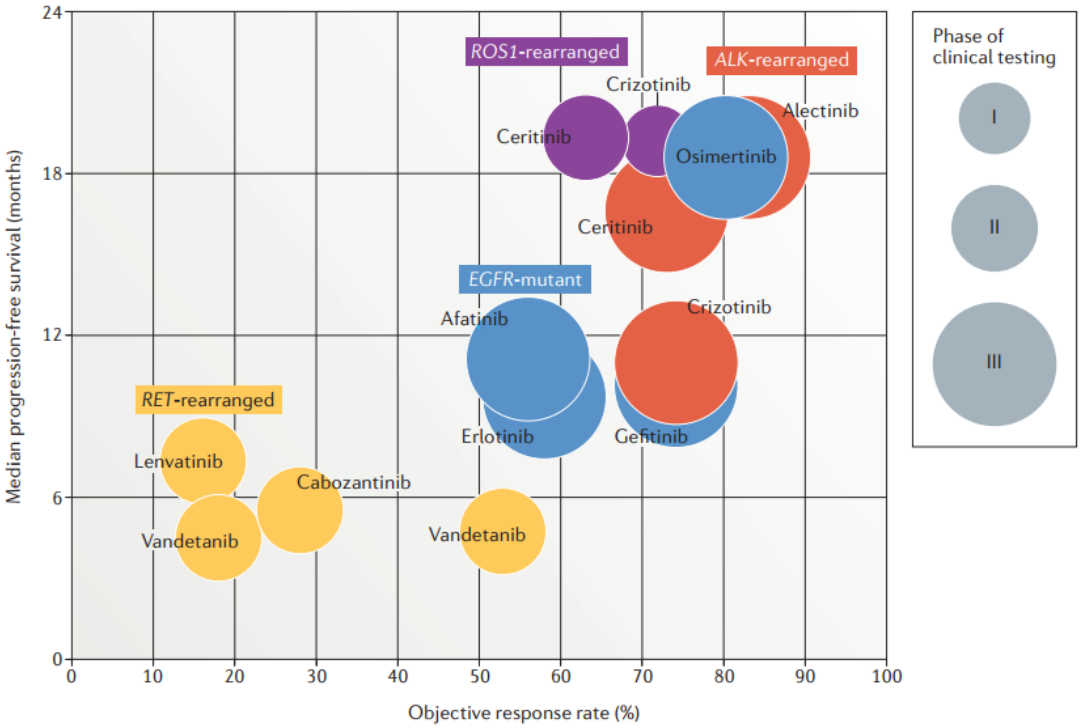

Figure 8: Comparison of clinical results of small molecule inhibition in distinct NSCLC alterations (Drilon et al. 2018)

1.5.4.3 Global multicentre RET registry (GLORY)

In a recent, multicentre, and so far biggest analysis of 165 patients with rearranged *RET* harbouring advanced NSCLC (stage III and IV), 53 of them received therapy with RET targeting TKIs from June 2015 to April 2016 (Gautschi et al. 2017). The spectrum of multikinase inhibitors included cabozantinib, vandetanib, sunitinib, sorafenib, alectinib, lenvatinib, nintedanib, ponatinib, and regorafenib, all of which have known RET inhibitory activity (Gautschi et al. 2017). However, solely for cabozantinib, vandetanib, and sorafenib more than n>10 patients could be included and only patients under cabozantinib therapy tended to benefit from RET targeted treatment (Gautschi et al. 2017) (Table 2).

RET Inhibitor	Complete Response	Partial Response	Stable Disease	Disease Progression
All agents (n = 53)	2 (4%)	11 (22%)	16 (32%)	20 (40%)
Cabozantinib (n = 21)	1 (5%)	6 (32%)	5 (26%)	7 (37%)
Vandetanib (n = 11)	0	2 (18%)	3 (27%)	6 (55%)
Sunitinib (n = 10)	0	2 (22%)	3 (33%)	3 (33%)
Sorafenib (n = 2)	0	0	2	0
Alectinib (n = 2)	0	0	0	2
Lenvatinib (n = 2)	0	1	0	1
Nintedanib (n = 2)	1	0	1	0
Ponatinib (n = 2)	0	0	2	0
Regorafenib (n = 1)	0	0	0	1

Table 2: Number of patients treated and summary of response rates (Gautschi et al. 2017)

Median progression free survival was only 2.3 months (95% CI, 1.6 to 5.0 months) and median overall survival 6.8 months (95% CI, 3.9 to 14.3 months) (Gautschi et al. 2017). Interestingly, no correlation between the binding partner of RET (KIF5B, CCDC6 and others) and clinical outcome was shown (Gautschi et al. 2017). The overall response rate (compete and partial) for cabozantinib, vandetanib and sunitinib was 37%, 18%, and 22%, respectively (Gautschi et al. 2017). This is in line with other published data with overall response rates between 18% - 53% of NSCLC patients with RET alterations (Drilon et al. 2013; Yoh et al. 2017; Falchook et al. 2016) .

In contrast to those rather disappointing results concerning small molecule inhibitor therapy, traditional platinum-based chemotherapy resulted in a complete or partial response rate of 51% (95% CI, 38.1 to 63.4) with a total survival of 24.8 months (95% CI, 13.6 to 32.3 months) (Gautschi et al. 2017). Therefore, a possible sequential treatment regimen proposed by Gautschi et al. could be polychemotherapy followed by RTK inhibitors in case of secondary drug resistance (Gautschi et al. 2017).

2. Aim of the study

With the emergence of more feasible and faster genome sequencing techniques of patients' tumor samples in the last decade, new insights into their oncogenic drivers were gained. Various driver mutations have been discovered in a broad range of cancers that lead to unrestricted growth, but simultaneously provide very promising therapeutic targets in the context of individualised cancer therapy (Hanahan and Weinberg 2011). Mutation-based, precision therapy is especially beneficial and needed for lung cancer patients as they very often present late to the clinic in a palliative setting where curative surgery is not an option (Brodowicz et al. 2012). RET rearrangements represent a distinct oncogenic group in lung adenocarcinomas that lead to constant activation of oncogenic cell signalling causing uncontrolled cellular proliferation and tumor growth (Mulligan 2014).

Small molecule inhibitors that precisely target genomic alterations in specific tumors have proven to be both effective in the experimental setting and in the treatment of patients. Hence, small molecule inhibitors have been increasingly more adopted in the cancer treatment guidelines of lung cancer (Mok 2011; German NSCLC guidelines in November 2018). Unfortunately, compared to the clinical outcomes seen in *ALK*- or *ROS1*-rearranged tumors treated with small molecule inhibitors, the overall response rates in *RET*-fusion driven tumors have been rather disappointing with low overall response rates between 18% - 53% (Gautschi et al. 2017; Drilon et al. 2016; Yoh et al. 2017; Falchook et al. 2016). These studies demonstrate a strong clinical need for the development and therapeutic application of anti-RET drugs.

Therefore, the study followed three objectives in order to offer new insights into potential anti-RET drug development:

1. Assessing already established and more recently developed small molecule inhibitors in a broad range of different *in vitro* and in *vivo RET*-rearranged models and benchmarking them in terms of drug on- and off-target efficacy. In 2012, Dar et al. showed potentially strong inhibitory effects of the small molecule inhibitor AD80 against RET using rational synthetic tailoring in a transgenic RET-kinase driven Drosophila model of MEN2 (Dar et al. 2012). Assessing this drug in *RET*-fusion cancer cell models was the starting point for my project. I cloned and transduced the vectors for the Ba/F3$^{KIF5B\text{-}RET}$ and Ba/F3$^{CCDC6\text{-}RET}$ cell lines and the corresponding *RET*^{V804M} gatekeeper mutations. In their normal state, Ba/F3 cells require IL-3 for their growth. However, if they are transduced with a potent oncogene, their proliferation then only depends on the activity of their oncogene. This provides an elegant tool for testing the inhibitory effects of various kinase inhibitors. Additionally, in order to establish another endogenously *RET*-rearranged cell model, I used the recently developed CRISPR/Cas9

genome editing technology. I cloned a pLenti vector including a Cas9 cassette and two single U6 promotors for the expression of single-guided RNAs (sgRNAs) in intron 15 of *KIF5B* and intron 11 of *RET* and transfected murine fibroblast cells (NIH-3T3) resulting in *KIF5B*-*RET* translocations in selected cells. These cell lines along with a large panel of patient-derived lung cancer cell lines representing various oncogenic drivers were then assessed for drug sensitivity and phospho-proteomic changes.

2. Investigating the impact of RET kinase binding modes by small molecule inhibitors that may predict drug vulnerability of AD80 and other type II inhibitors through chemical-genomic and chemical-proteomic analyses. Here, we collaborated with other research groups for a better understanding of the mechanistic background of kinase activity on a structural level.

3. Gaining new insights into rescue mechanisms of *RET*-rearranged tumor cells under treatment through single-nucleotide resistance mutations and signalling pathway alterations. Using saturated mutagenesis screening I tried to discover novel AD80 specific secondary resistance mutations in RET beyond the classical gatekeeper mutation pV804M. By computational binding mode analysis, we also tried to gain a better understanding of structural kinase interactions resulting from the nucleotide changes. Mechanistically, I also assessed cell signalling alterations by comparing secondary resistant *RET*-rearranged TPC-1 thyroid cancer and LC-2/AD lung adenocarcinoma cells in terms of MAPK-reactivation under RET inhibition. This was further evaluated by transfecting LC-2/AD cells with the lentiviral *KRAS*^{G12V} vector and testing it for drug sensitivity and cell signalling changes

3. Publication

The methods and results of this dissertation are included in the following publication:

Plenker D*, **Riedel M***, et al.
Drugging the catalytically inactive state of RET kinase in RET-rearranged tumors.
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CANCER

Drugging the catalytically inactive state of RET kinase in RET-rearranged tumors

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Oncogenic fusion events have been identified in a broad range of tumors. Among them, *RET* rearrangements represent distinct and potentially druggable targets that are recurrently found in lung adenocarcinomas. We provide further evidence that current anti-RET drugs may not be potent enough to induce durable responses in such tumors. We report that potent inhibitors, such as AD80 or ponatinib, that stably bind in the DFG-out conformation of RET may overcome these limitations and selectively kill *RET*-rearranged tumors. Using chemical genomics in conjunction with phosphoproteomic analyses in *RET*-rearranged cells, we identify the CCDC6-RET^{I788N} mutation and drug-induced mitogen-activated protein kinase pathway reactivation as possible mechanisms by which tumors may escape the activity of RET inhibitors. Our data provide mechanistic insight into the druggability of RET kinase fusions that may be of help for the development of effective therapies targeting such tumors.

INTRODUCTION

Targeted inhibition of oncogenic driver mutations with small molecules is a cornerstone of precision cancer medicine. *RET* rearrangements have been identified in a broad range of tumors, including 1 to 2% of lung adenocarcinomas, and their discovery sparked the hope for an effective treatment option in these patients (*1*–*3*). However, when compared to other oncogenic "driver" alterations, such as rearranged anaplastic lymphoma kinase (ALK), rearranged RET seems to be a difficult target, and to date, no drug has been successfully established for the treatment of these tumors (*4*–*6*). Recent clinical data suggest that overall response rates in patients treated with currently available RET-targeted drugs are rather limited and range between 18 and 53% (*7*–*10*). Improved selection of patients based on deep sequencing of individual tumors may help increase these response rates, but still progression-free survival seems to be very limited (*7*, *8*, *10*, *11*). These observations are particularly surprising from a chemical point of view because a broad spectrum of kinase inhibitors is known to bind to RET and to inhibit its kinase activity in vitro (*6*, *12*). On the basis of these observations, we sought to characterize rearranged RET in independent cancer models to identify potent RET inhibitors with high selectivity and optimal biochemical profile to target *RET*-rearranged tumors.

RESULTS

Kinase inhibitor AD80 shows extraordinary activity in *RET*-rearranged cancer models

Because clinical experience with RET-targeted drugs in lung cancer patients is rather disappointing, we sought to test a series of clinically and preclinically available drugs with anti-RET activity in Ba/F3 cells engineered to express either *KIF5B-RET* or *CCDC6-RET* (*1*, *2*, *12*, *13*). In these experiments, AD80 and ponatinib exhibited 100- to 1000-fold higher cytotoxicity compared to all other tested drugs in RET-dependent, but not interleukin-3–supplemented, Ba/F3 cells (Fig. 1A and fig. S1, A and B). In line with these results, AD80, but not cabozantinib or vandetanib, prevented the phosphorylation of RET as well as of extracellular signal–regulated kinase (ERK), AKT, and S6K at low nanomolar concentrations in kinesin family member 5B (KIF5B)–RET–expressing Ba/F3 cells (Fig. 1B and table S1). These data are in line with our own retrospective analysis where out of four patients with *RET*-rearranged tumors, we observed only one partial response in a patient receiving vandetanib (P2) as first-line treatment (fig. S1, C to E, and table S2, A and B) (*9*). Sequencing of rebiopsy samples did not reveal candidate drug resistance mutations, suggesting that the target had been insufficiently inhibited (table S2C).

To validate the efficacy of AD80 and ponatinib in an alternative model, we induced *KIF5B-RET* rearrangements (*KIF5B* exon 15; *RET* exon 12) in NIH-3T3 cells using clustered regularly interspaced short

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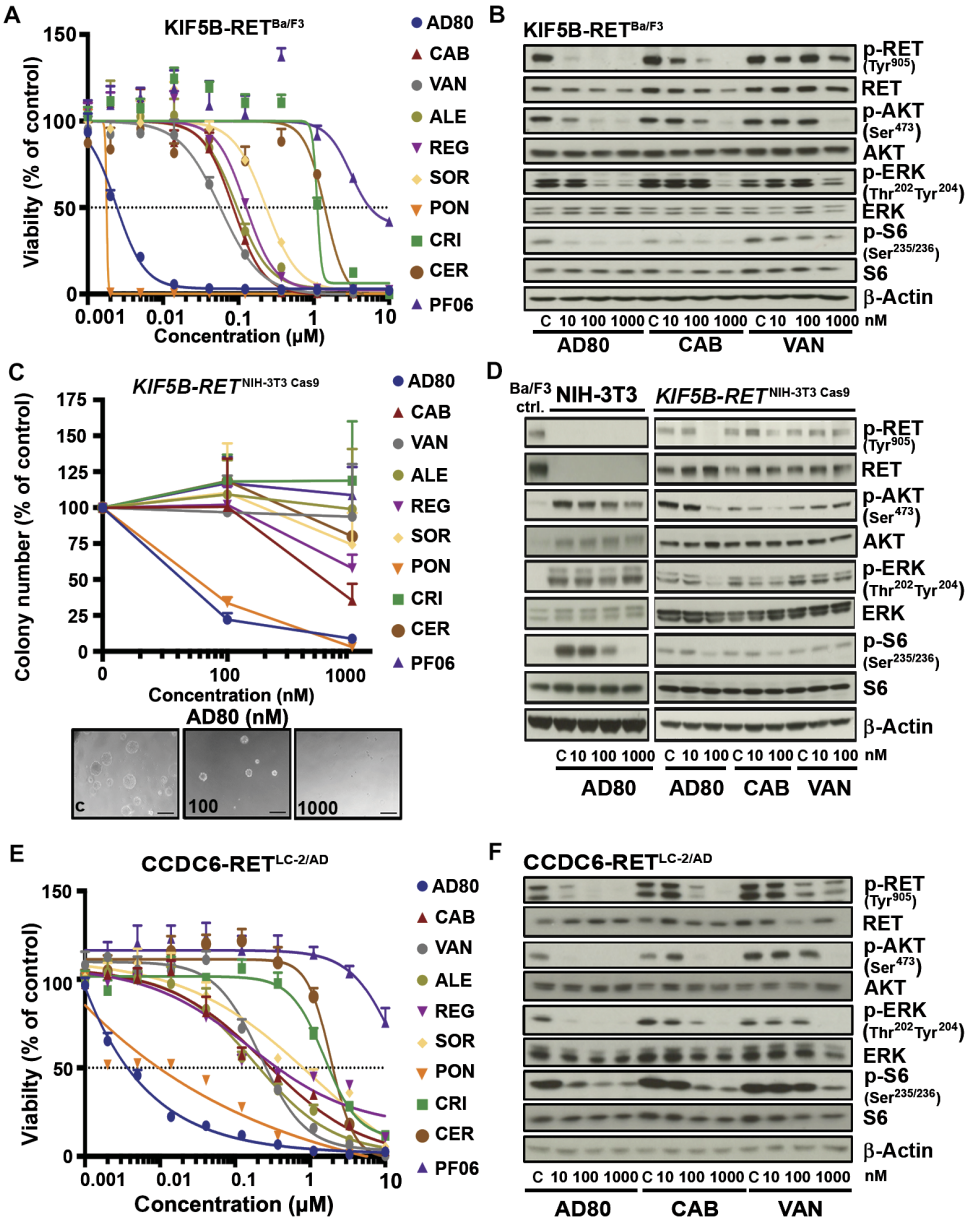

Fig. 1. Cellular profiling of RET inhibitors identifies AD80 and ponatinib as potent compounds. (**A**) Dose-response curves (72 hours) for AD80, cabozantinib (CAB), vandetanib (VAN), alectinib (ALE), regorafenib (REG), sorafenib (SOR), ponatinib (PON), crizotinib (CRI), ceritinib (CER), or PF06463922 (PF06) in KIF5B-RET–expressing Ba/F3 cells ($n = 3$ technical replicates). (**B**) Immunoblotting results of *KIF5B-RET*–rearranged Ba/F3 cells after treatment (4 hours). C, control. (**C**) Relative mean colony number of NIH-3T3 cells engineered with *KIF5B-RET* fusion by CRISPR/Cas9 was assessed in soft agar assays after 7 days under treatment. Representative images of colonies under AD80 treatment are displayed in the lower panel. Scale bars, 100 μm ($n = 3$) (**D**) Immunoblotting of CRISPR/Cas9-engineered, *KIF5B-RET*–rearranged NIH-3T3 cells treated with AD80, cabozantinib, or vandetanib (4 hours). *KIF5B-RET* expressing Ba/F3 cells (Ba/F3 ctrl.) serve as control for RET signaling ($n = 3$) (**E**) Dose-response curves (72 hours) for different inhibitors in LC-2/AD cells. (**F**) Immunoblotting was performed in LC-2/AD cells treated with AD80, cabozantinib, or vandetanib (4 hours).

palindromic repeats (CRISPR)/Cas9–meditated genome editing. We confirmed their anchorage-independent growth, increased proliferation rate, and high sensitivity to AD80 and ponatinib (Fig. 1C and fig. S2, A to C) (*14*). Again, treatment with AD80, but not cabozantinib or vandetanib, led to inhibition of phosphorylated RET (phospho-RET) and of downstream effectors of RET signaling at low nanomolar concentrations (Fig. 1D). AD80 led to dephosphorylation of S6 also in parental NIH-3T3 cells and Ba/F3$^{myr\text{-}AKT}$ control cells, suggesting that S6 may represent an off-target at micromolar concentrations (Fig. 1D and fig. S2D) (*13*).

To further substantiate our results, we next tested our panel of RET inhibitors in the *CCDC6-RET* rearranged lung adenocarcinoma cell line LC-2/AD (*15*). We observed similar activity profiles with AD80 followed by ponatinib as the most potent inhibitors compared to all other tested drugs in terms of cytotoxicity at low nanomolar concentrations (Fig. 1E) and inhibition of phospho-RET and other downstream signaling molecules (Fig. 1F). Overall, our data suggest that in *RET*-rearranged cells, AD80 and ponatinib are 100- to 1000-fold more effective against RET and its downstream signaling than any other clinically tested anti-RET drug.

AD80 and ponatinib effectively inhibit RET kinase in DFG-out conformation

We benchmarked the genotype-specific activity of AD80 and ponatinib against well-described kinase inhibitors, such as erlotinib, BGJ398, vandetanib, cabozantinib, regorafenib, alectinib, and ceritinib, in a panel of 18 cancer cell lines driven by known oncogenic lesions, such as mutant epidermal growth factor receptor (EGFR) or rearranged ALK, including two *RET*-rearranged cell lines (LC-2/AD and TPC-1) (fig. S3A) (*6*, *12*, *16*). Again, we identified AD80 and ponatinib as the most effective drugs and, through the calculation of median on-target versus off-target ratios, also as the most specific drugs in *RET* fusion–positive cells (fig. S3B and table S3).

To further characterize intracellular signaling induced by an RET inhibitor, such as AD80, we performed mass spectrometry–based phosphoproteomic analyses of LC-2/AD cells treated with 10 or 100 nM AD80. In AD80-treated cells, we observed a significant decrease of RET^{Y900} phosphorylation with $\log_2$-fold changes of −1.07 ($P = 0.009$; 10 nM AD80) and −2.11 ($P = 0.0002$; 100 nM AD80), respectively (Fig. 2A). Among all phosphopeptides quantified under control, 10 nM, and 100 nM conditions ($n = 11912$), the abundance of RET^{Y900} was among the most decreased phosphopeptides (control versus 100 nM AD80; $P = 0.00024$) and the most decreased receptor tyrosine kinases (fig. S3C). These results highlight that in these cells, RET is the primary target of AD80.

On the basis of these observations, we speculated that activation of RET-independent signaling pathways should largely abrogate the

cytotoxic effects of AD80. To this end, we supplemented LC-2/AD cells with exogenous receptor ligands and found that the activity of AD80 was significantly reduced ($P \leq 0.05$) through the addition of EGF, hepatocyte growth factor, and neuregulin 1, indicating that RET is the primary cellular target in *RET*-rearranged LC-2/AD cells (fig. S4A).

To further characterize the high potency of AD80 and ponatinib against RET kinase fusions, we expressed and purified different truncated versions of the RET core kinase and juxtamembrane-kinase domain, as well as truncated forms of both coiled-coil domain containing 6 (CCDC6) (ΔCCDC6-KD) and KIF5B (ΔKIF5B-KD) kinase domain fusions (fig. S4, B and C) (*17*). We used these different RET fusion kinase domain constructs to determine the extent to which binding of a given compound has an effect on protein thermal stability, as measured by the melting temperature (T_m). The difference in melting temperature with and without drug (ΔT_m) extrapolates the potency of the individual drugs against the respective constructs (*17*). To our surprise, we found that treatment with the type I inhibitors sunitinib or vandetanib resulted in a ΔT_m of only 1° to 4°C, whereas the type II inhibitors sorafenib, ponatinib, or AD80 increased the ΔT_m of up to 10° to 18°C (Fig. 2B and fig. S4, D to H). We observed the strongest effects in ΔKIF5B-KD and ΔCCDC6-KD constructs treated with AD80 and core KD with ponatinib (Fig. 2B, fig. S4D, and table S4). Such a shift for inhibitors that stabilize the catalytically inactive conformation of RET kinase, in which the DFG motif is flipped out (DFG-out) relative to its conformation in the active state (DFG-in), does not correlate with the differential in vitro kinase activity observed for sorafenib and other RET inhibitors (table S5) (*6*, *18*).

To further characterize the relevance of a DFG-out conformation for the activity of RET inhibitors, we performed structural analyses. We used homology modeling based on a vascular EGFR (VEGFR) kinase [Protein Data Bank (PDB) code 2OH4 (*19*)] in the DFG-out complex similar to a previously published methodology (*20*), followed by extensive molecular dynamics (MD) simulation refinement. We observed that the root mean square deviation (RMSD) values remained largely stable over the time course of the MD simulation (RET^{wt} and RET^{V804M}), thus supporting our proposed model in which AD80 binds in the DFG-out conformation of the kinase (fig. S5A). In this model, AD80 forms a hydrogen bond (H-bond) with the aspartate of the DFG motif that may be involved in the stabilization of the DFG-out conformation (Fig. 3A). A similar H-bond is also observed for cabozantinib, a known type II inhibitor, bound to RET^{wt} (fig. S5B; see the Supplementary Materials and Methods for model generation). This finding corroborates the validity of our binding mode hypothesis, although the pose is biased by construction, being based on the refined RET^{wt}/AD80 structure. Furthermore, we developed a binding pose model for AD57 (derivative of AD80) bound to RET^{wt} (see below), which, upon superimposition, displays considerable similarity with the experimentally determined structure of AD57 bound to cSrc (PDB code 3EL8) in the DFG-out form, again validating our approach (figs. S4H and S5C). Next, we performed free energy MD simulations to transform AD80 into AD57. The calculations yielded a binding free energy difference of $\Delta\Delta G° = -0.21 \pm 0.17$ kcal mol^{-1} at 25°C, which compares well with the values derived from median inhibitory concentration (IC_{50}) in in vitro kinase measurements. These latter concentration-based measurements of binding affinity translate into an experimental estimate of the binding free energy difference of −0.41 kcal mol^{-1} with IC_{50}(AD57) of 2 nM and IC_{50}(AD80) of 4 nM (see the Supplementary Materials and Methods) (*13*). Using an integral equation approximation as an alternative computational approach, we obtained 0.1 kcal mol^{-1}, also in close correspondence with both the MD and experimental results. Thus, these analyses further support the proposed DFG-out conformation as the preferred binding mode because such agreement between the experiment and the theory would not have been expected if the true and predicted binding modes were largely dissimilar.

Overall, our cellular screening, phosphoproteomic, biochemical, and structural data indicate that potent type II inhibitors, such as AD80 or ponatinib, have an optimal RET-specific profile that distinguishes them from currently available anti-RET drugs.

Introduction of RET kinase gatekeeper mutation reveals differential activity of RET inhibitors

Secondary resistance mutations frequently target a conserved residue, termed gatekeeper, that controls access to a hydrophobic subpocket of

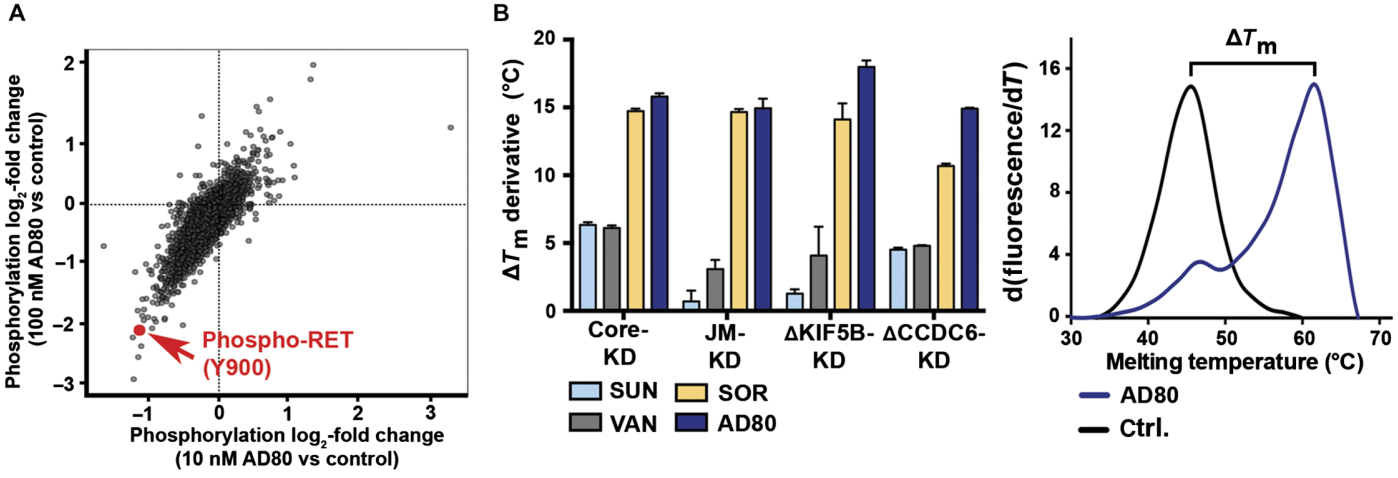

Fig. 2. AD80 specifically targets RET and tightly binds to RET fusion kinase. (**A**) Scatterplot of log_2-fold phosphorylation change for LC-2/AD cells treated (4 hours) with either 10 or 100 nM AD80. Each dot represents a single phosphosite; phospho-RET (Y900) is highlighted in red. (**B**) Difference in melting temperatures after AD80, sorafenib (SOR), vandetanib (VAN), or sunitinib (SUN) addition (ΔT_m) and the respective SEM are shown for each construct. Thermal shift experiments were performed using independent preparations of each protein and were carried out in triplicates (left). Representative thermal melting curves for ΔKIF5B-KD incubated with either AD80 (1 μM) or the equivalent volume of dimethyl sulfoxide (DMSO) (ctrl.) are shown (right).

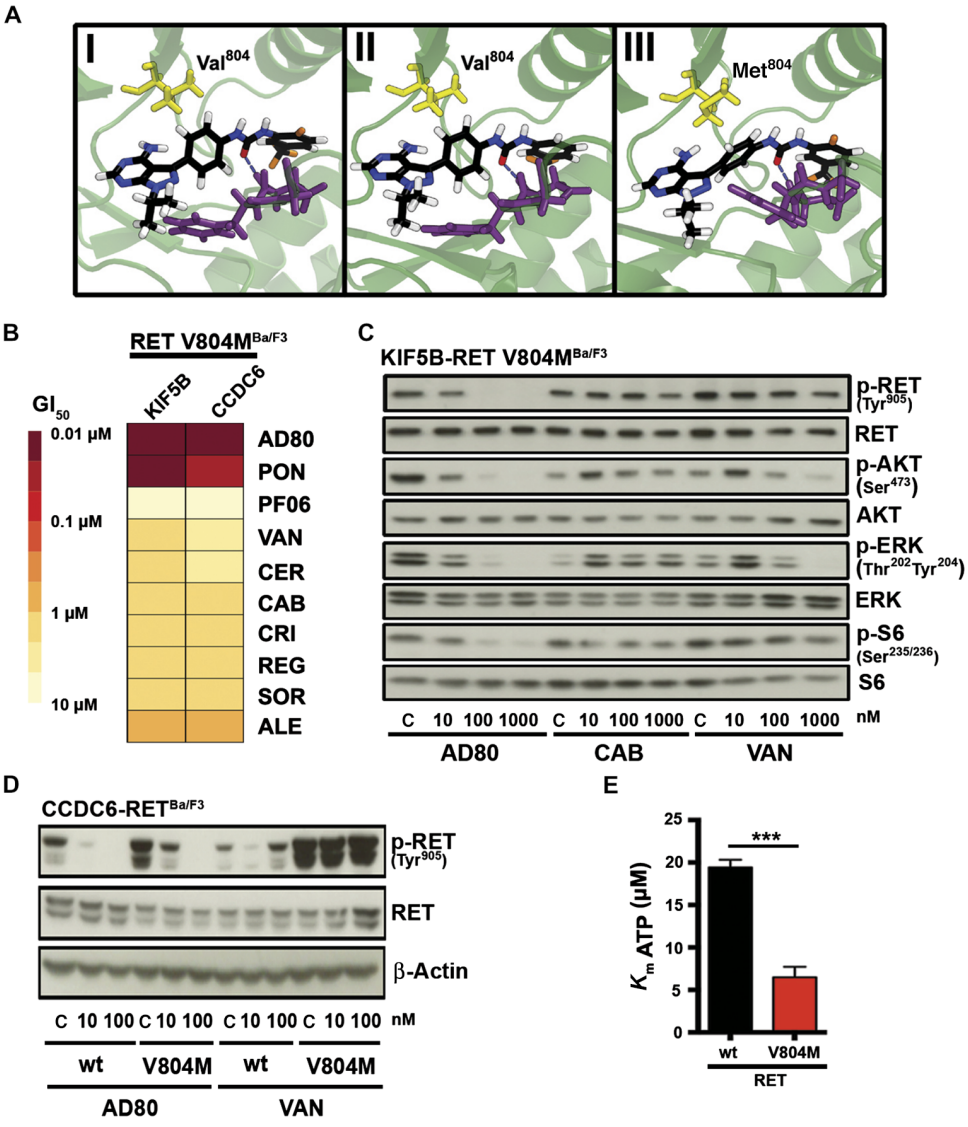

Fig. 3. AD80 is active against gatekeeper mutant RET^{V804M} cells. (**A**) Optimized structures after extensive MD refinement followed by ALPB optimization. (i) RET^{wt}/AD80 after 102 ns, (ii) RET^{wt}/AD57 after 202 ns (92 ns from RET^{wt}/AD80 simulation followed by 110 ns from TI-MD), and (iii) RET^{V804M}/AD80 after 107 ns (side view). The DFG motif is shown in violet. Distances from the center of central phenyl to Val^{804}-C(wt), Ile^{788}-C(wt), and Met^{804}-S(V804M) are 4.77, 3.90, and 4.29 Å, respectively. Dashed lines indicate the H-bond between the bound ligands and aspartate of the DFG motif. (**B**) Heat map of mean 50% growth inhibition (GI_{50}) values ($n \geq 3$) of Ba/F3 cells expressing CCDC6-RET^{V804M} or KIF5B-RET^{V804M} after 72 hours of treatment, as assessed for various inhibitors. (**C**) Immunoblotting of AD80-, cabozantinib-, or vandetanib-treated (4 hours) KIF5B-RET^{V804M} Ba/F3 cells. (**D**) Immunoblotting of Ba/F3 cells expressing CCDC6-RET-RET^{wt} or CCDC6-RET^{V804M} under AD80 or vandetanib treatment (4 hours). wt, wild type. (**E**) Calculated Michaelis constant (K_m) values of ATP binding to RET^{wt} or RET^{V804M} from three independent experiments. ***$P < 0.001$, $n = 3$.

the kinase domain (*21*). To test the impact of the gatekeeper resistance mutations on RET inhibitors, we established Ba/F3 cells expressing *KIF5B-RET^{V804M}* or *CCDC6-RET^{V804M}* and tested them against a panel of different drugs. As expected, only ponatinib and AD80 showed high activity in these gatekeeper mutant cells (Fig. 3B) (*22*). Similar activity was observed when testing the AD80 derivatives AD57 and AD81 for their inhibitory potential on Ba/F3 cells expressing wild-type and V804M-mutated *KIF5B-RET* or *CCDC6-RET* (fig. S6A). This effect was also evident in the ability of AD80 to inhibit phosphorylation of RET as well as of ERK, AKT, and S6K in these cells (Fig. 3C and table S1). Next, we used computational homology modeling coupled with MD refinement of AD80 in RET^{wt} in comparison with RET^{V804M}-mutant kinases. In line with our in vitro results, this analysis revealed high structural similarity and similar binding free energy estimates for both variants (-2.5 kcal mol^{-1} for transforming RET^{wt} to RET^{V804M} bound to AD80 from the integral equation model) (see Fig. 3A and the Supplementary Materials and Methods).

In parallel, we noticed that independent of the individual treatment, RET phosphorylation tended to be higher in gatekeeper mutant cells when compared to wild-type RET (Fig. 3D). To further characterize these differences, we performed in vitro kinase assays and found that the introduction of the *RET^{V804M}* mutation significantly ($P < 0.001$) increases the affinity of the recombinant receptor for adenosine 5′-triphosphate (ATP) when compared to the recombinant wild-type receptor (Fig. 3E). Thus, similar to gatekeeper-induced effects on ATP affinity observed for *EGFR^{T790M}* mutations, our data suggest that these effects may be of relevance for the activity of RET inhibitors in *KIF5B-RET^{V804M}* and *CCDC6-RET^{V804M}* cells (*23*).

Saturated mutagenesis screening identifies CCDC6-RET^{I788N} drug resistance mutation

To identify RET kinase mutations that may be associated with resistance against targeted therapy, we performed accelerated mutagenesis of *RET* fusion plasmids (*24*, *25*). We identified the *CCDC6-RET^{I788N}* mutation by selection of an AD80-resistant cell population (table S6). To validate this finding, we engineered Ba/F3 cells expressing *KIF5B-RET^{I788N}* or *CCDC6-RET^{I788N}* and observed a robust shift in cytotoxicity in response to AD80 treatment (Fig. 4A), as well as the other RET inhibitors, cabozantinib and vandetanib, but not ponatinib (Fig. 4B and fig. S6B). Immunoblotting confirmed that the introduction of the *KIF5B-RET^{I788N}* mutation had a minor effect on the efficacy of ponatinib but a major impact on AD80, as measured by phospho-RET analysis (Fig. 4, C and D). Computational binding mode analysis (Figs. 3A and 4E) suggests that both positions 804 and 788 are adjacent to the location of the central phenyl ring of AD80; characteristic distances between the phenyl center of mass and the nearest adjacent protein nonhydrogen sites to Val^{804}-C(wt), Ile^{788}-C(wt), Met^{804}-S(V804M), and Ile^{788}-C(V804M) are 4.77, 3.90, 4.29, and 4.61 Å, respectively. However, because V804M and I788N mutants responded differently to AD80, a clear conclusion about the molecular origin was

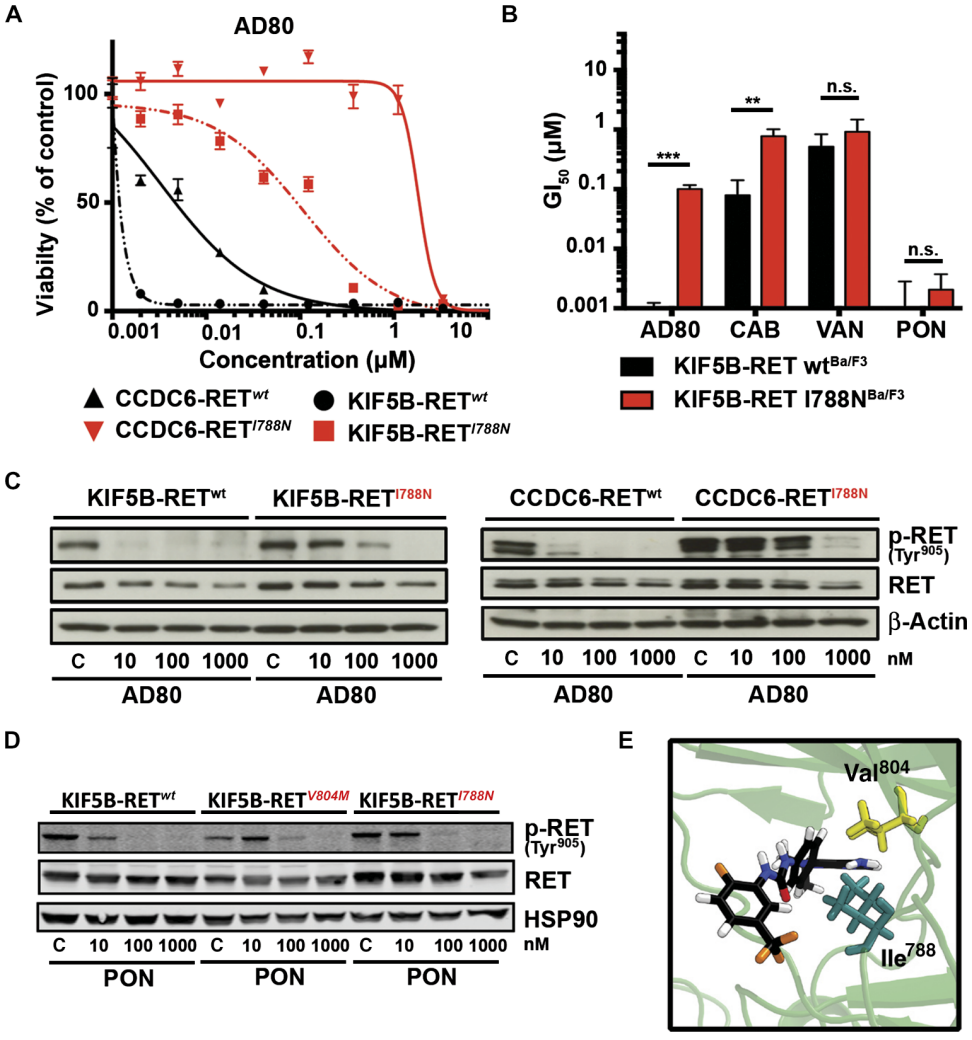

Fig. 4. RET^{I788N} mutations abrogate the activity of AD80 but not ponatinib. (**A**) Dose-response curves for AD80 against Ba/F3 cells expressing KIF5B-RET^{wt} (black) or KIF5B-RET^{I788N} (red) and CCDC6-RET^{wt} (black dashed line) or CCDC6-RET^{I788N} (red dashed line) ($n = 3$). (**B**) Bar graph of mean GI_{50} values + SD (from $n = 3$) for KIF5B-RET^{wt} or KIF5B-RET^{I788N} Ba/F3 cells treated (72 hours) with AD80, cabozantinib (CAB), vandetanib (VAN), or ponatinib (PON). ***$P < 0.001$; **$P < 0.01$; n.s., not significant. (**C**) Immunoblot of Ba/F3 cells expressing KIF5B-RET^{wt} or KIF5B-RET^{I788N} and CCDC6-RET^{wt} or CCDC6-RET^{I788N} treated (4 hours) with AD80. (**D**) Immunoblot of KIF5B-RET^{wt}, KIF5B-RET^{V804M}, or KIF5B-RET^{I788N} expressing Ba/F3 cells treated (4 hours) with ponatinib. HSP90 is used as loading control. (**E**) Optimized structure after extensive MD refinement followed by ALPB optimization. RET^{wt}/AD80 after 102 ns (side view). Distance from the center of central phenyl to Ile^{788}-C(V804M) is 4.61 Å.

not possible based on structural analysis alone, requiring further investigations. Thus, our data uncovered a resistance mutation RET^{I788N} that may arise in *RET*-rearranged tumors under RET inhibitor treatment and that retains sensitivity against ponatinib.

Feedback-induced activation of MAPK signaling modulates activity of RET inhibitors

Beyond the acquisition of secondary mutations, drug treatment of cancer cells may also release feedback loops that override the activity of targeted cancer treatment (*26*, *27*). To systematically characterize these effects, we analyzed altered gene expression by RNA-sequencing (RNA-seq) of LC-2/AD cells under AD80 treatment and performed gene set enrichment analysis (GSEA) (*28*). Our analyses revealed that treatment with AD80 results in up-regulation of genes that are typically repressed by active KRAS (KRAS down; adjusted $P < 0.0001$). On the contrary, genes that are activated by KRAS were down-regulated (KRAS up; adjusted $P = 0.003$) (Fig. 5A). Accordingly, the list of significantly down-regulated genes contained *DUSP6* (adjusted $P < 1 \times 10^{-250}$), *SPRY4* (adjusted $P = 5.75 \times 10^{-89}$), *DUSP5* (adjusted $P = 2.52 \times 10^{-38}$), and other genes that buffer mitogen-activated protein kinase (MAPK) pathway (Fig. 5B) (*29*). This transcriptional deregulation of MAPK signaling was accompanied by residual phospho-ERK staining in immunoblotting analyses of *RET*-rearranged LC-2/AD cells after 24 hours of inhibitor treatment (fig. S6C). Using a Group-based Prediction System (GPS 2.12) to identify kinase-specific phosphosites that are perturbed in AD80-treated LC-2/AD cells assessed in our mass spectrometry–based analysis, we identified a marked enrichment of phosphosites known from different families of noncanonical MAPK kinases (MEKs), such as MAPK8 (66 phosphosites), MAPK13 (21 phosphosites), or MAPK12 (15 phosphosites) (Fig. 5C).

We next tested the relevance of Ras-MAPK pathway reactivation in *RET*-rearranged cells treated with AD80 alone or a combination of AD80 and the MEK inhibitor trametinib. In TPC-1 cells with limited vulnerability to RET inhibition, we observed a pronounced phospho-ERK signal in cells after inhibition with AD80 when compared to LC-2/AD cells (fig. S6D). The combination of AD80 and trametinib fully abrogated MAPK signaling and depleted the outgrowth of resistant cells in clonogenic assays and enhanced the reduction of viability (Fig. 5D and fig. S6, E and F).

To formally test the relevance of MAPK pathway activation in the context of resistance to RET-targeted therapies in *RET*-rearranged cells, we stably transduced LC-2/AD cells with lentiviral *KRAS*^{G12V}. Introduction of the oncogenic *KRAS* allele into LC-2/AD cells largely eliminated the activity of AD80, as measured in viability assays and by staining of phospho-ERK (Fig. 5, E and F). Overall, our data suggest that drug-induced transcriptional and posttranslational reactivation of Ras-MAPK signaling may modulate the activity of RET-targeted inhibitors in *RET*-rearranged cells.

AD80 potently shrinks *RET*-rearranged tumors in patient-derived xenografts

To compare the in vivo efficacy of AD80 head-to-head with other RET inhibitors, we engrafted NIH-3T3 cells driven by CRISPR/Cas9-induced *KIF5B-RET* rearrangements into NSG (nonobese

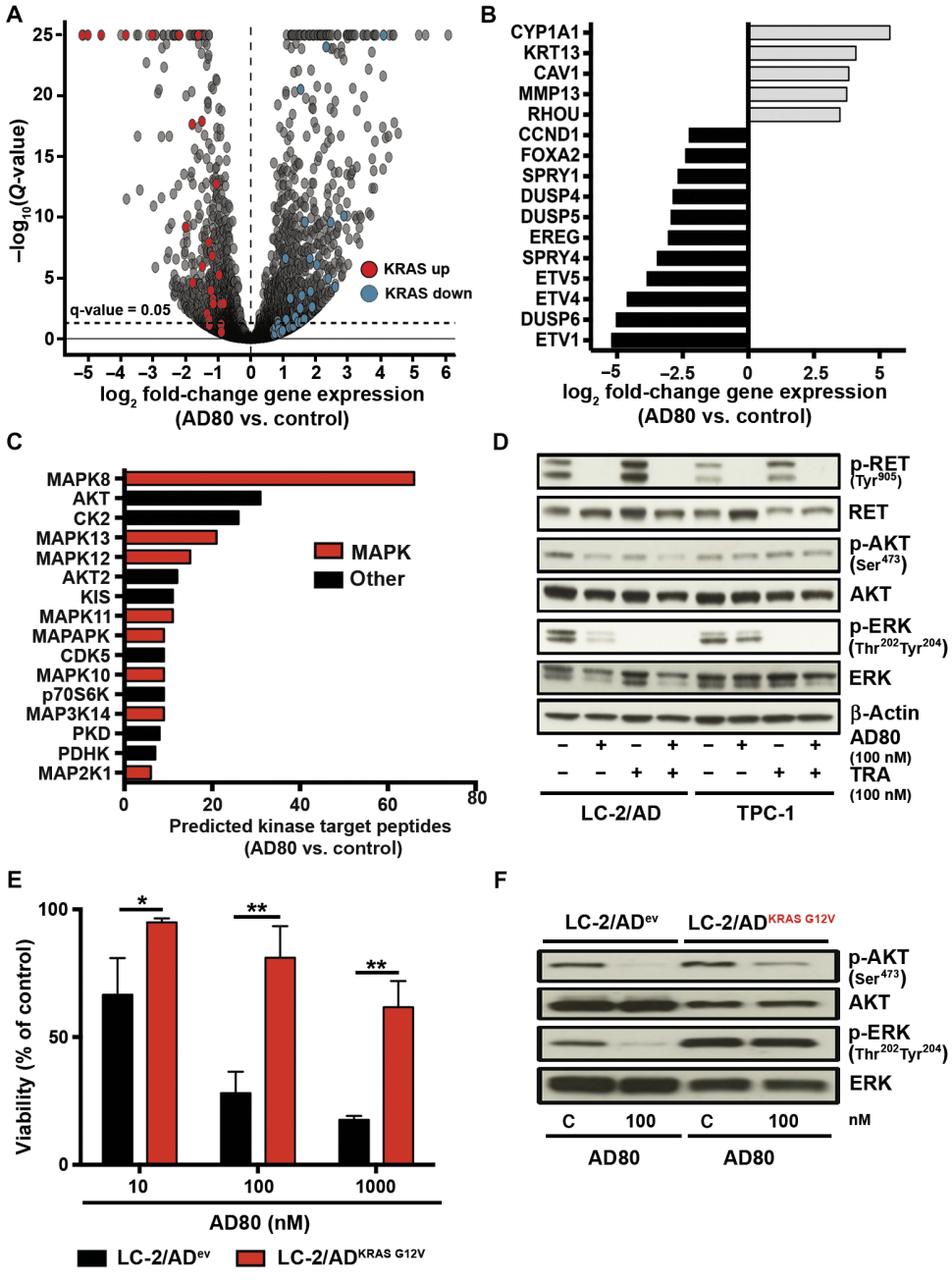

Fig. 5. MAPK pathway activation may be involved in the development of resistance against RET inhibition. (**A**) RNA-seq results of LC-2/AD cells treated (48 hours) with 100 nM AD80. Genes contained within the core enrichments of GSEA against the hallmark gene sets with genes up-regulated (KRAS up) or down-regulated (KRAS down) by active KRAS are highlighted by red and blue, respectively. The dashed line represents false discovery rate–adjusted *Q* value = 0.05. (**B**) Relevant genes from the top 50 genes with the strongest significant changes in RNA-seq after AD80 treatment (100 nM; 48 hours). (**C**) Predicted number of down-regulated phosphorylation sites for each kinase. All kinases with greater than or equal to six down-regulated phosphorylation sites are shown in hierarchical order. Kinases associated with MAPK pathway signaling are highlighted in red. (**D**) In immunoblotting assays, RET signaling was monitored in LC-2/AD and TPC-1 cells treated (48 hours) with AD80 (0.1 μM), trametinib (TRA) (0.1 μM), or a combination of both inhibitors. (**E**) LC-2/AD^{ev} or LC-2/AD$^{KRAS\ G12V}$ cells were treated (72 hours) with AD80. Results are shown as means + SD ($n = 3$). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. (**F**) Immunoblotting of LC-2/AD^{ev} or LC-2/AD$^{KRAS\ G12V}$ cells under AD80 treatment (100 nM; 4 hours).

diabetic/severe combined immunodeficient gamma) mice. After the development of tumors, mice were treated with either vehicle or 12.5 to 25 mg/kg of AD80, cabozantinib, or vandetanib, and tumors were explanted 4 hours later (*30*, *31*). We observed a pronounced reduction in phosphorylation of RET as well as AKT and ERK in tumors treated with AD80 (25 mg/kg) but not in tumors treated with cabozantinib or vandetanib (Fig. 6A). Encouraged by these results, we next treated a cohort ($n = 16$) of patient-derived xenograft (PDX) mice engrafted with tumor tissue from a *CCDC6-RET*–rearranged colorectal cancer (CRC) patient with either vehicle or AD80 (25 mg/kg). Treatment with AD80 induced significant ($P < 0.001$) tumor shrinkage in CCDC6-RET PDX^{wt} (Fig. 6, B and C, and fig. S7A) (*32*). In line with our in vitro data for cells harboring *RET* gatekeeper mutations, tumor shrinkage ($P < 0.01$) was robust but less pronounced when we treated PDX mice ($n = 16$) engrafted with CRC tissue that had developed a *CCDC6-RET*^{V804M} gatekeeper mutation under ponatinib treatment (Fig. 6, B and D, and fig. S7B) (*33*). Furthermore, we observed a robust reduction of cellular proliferation (*CCDC6-RET*^{wt}, $P < 0.001$; *CCDC6-RET*^{V804M}, $P < 0.05$), as measured by KI-67 staining in *CCDC6-RET*^{wt} and *CCDC6-RET*^{V804M} tumors (Fig. 6, E and F). AD80 treatment did not cause body weight loss in either PDX model over the course of the study (fig. S7, C and D). Together, our data indicate that AD80 is a highly potent RET inhibitor with a favorable pharmacokinetic profile in clinically relevant *RET* fusion–driven tumor models.

DISCUSSION

Our chemical-genomic and chemical-proteomic analyses revealed three interesting findings with major implications for the development of effective therapies against *RET*-rearranged tumors: (i) *RET*-rearranged tumors show exquisite vulnerability to a subset of type II inhibitors that target the DFG-out conformation of RET kinase,

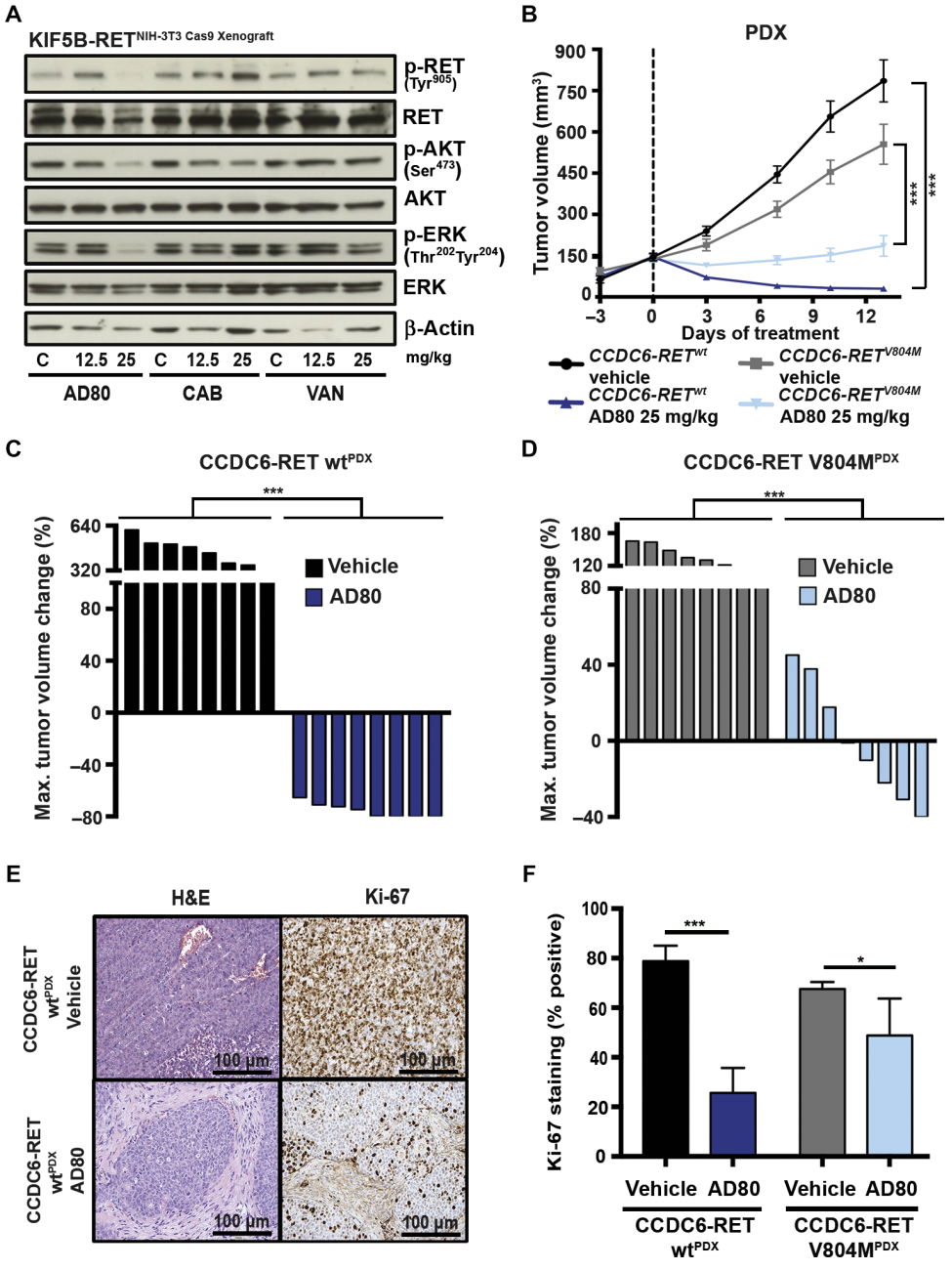

Fig. 6. AD80 treatment effectively shrinks *RET*-rearranged tumors in PDX models. (**A**) Immunoblotting of tumor tissue from CRISPR/Cas9-induced NIH-3T3$^{KIF5B\text{-}RET}$ xenografts was performed. Mice were treated (4 hours) with vehicle control or 12.5 or 25 mg/kg AD80, CAB, or VAN and were sacrificed. (**B**) Median tumor volume was assessed using consecutive measurements of PDX tumors driven by *CCDC6-RET*^{wt} or *CCDC6-RET*^{V804M} rearrangements under treatment with either AD80 (25 mg/kg; 14 days) or vehicle control (14 days). Treatment started at day 0. (**C**) Waterfall plot for each *CCDC6-RET*^{wt} fusion–positive PDX depicting best response (14 days) under AD80 or vehicle control treatment. ***$P < 0.001$. (**D**) Waterfall plot for each *CCDC6-RET*^{V804M}–positive PDX depicting best response (7 days) under AD80 or vehicle control treatment. ***$P < 0.001$. (**E**) Representative immunohistochemistry (IHC) staining for hematoxylin and eosin (H&E) and Ki-67 of AD80- or vehicle control–treated *CCDC6-RET*^{wt} PDX. Scale bars, 100 µm. (**F**) Quantification of Ki-67 IHC staining. ***$P < 0.001$; *$P < 0.05$.

The repurposing of crizotinib for the targeted treatment of *ALK*-rearranged tumors enabled a fast-track introduction of precision cancer medicine for this group of cancer patients and raised hopes that this approach may be a blueprint for the targeted treatment of other driver oncogenes, such as RET (*34*). Although initial clinical response rates were promising in selected patients, a median progression-free survival of less than 6 months and response rates of only about 18% in retrospective studies indicated that *RET* may be a difficult drug target after all (*7*, *9*, *10*, *35*).

Our systematic characterization of anti-RET drugs revealed distinct activity and specificity profiles for the type II kinase inhibitors AD80 and ponatinib in independent in vitro and in vivo models across different lineages of *RET*-rearranged cancer. This finding is noteworthy because the biochemical profiling of these compounds and structurally related compounds would have suggested a broad spectrum of kinase targets (*13*, *36*, *37*). Our data also suggest that an inhibitory profile, including a stable binding in the DFG-out conformation of RET together with a potent in vitro kinase activity, may predict efficacy against *RET*-rearranged cancer cells. At the same time, our study is limited through the lack of insight into drug residence time or structural kinetics that may also contribute to the overall activity of type II inhibitors such as sorafenib and other RET inhibitors (*20*, *38*).

Notably, we identified a *CCDC6-RET*^{I788N} resistance mutation that renders a number of tested RET inhibitors ineffective while retaining vulnerability to ponatinib. These findings resemble the experience with ALK inhibitors in *ALK*-rearranged tumors, where the availability of potent inhibitors allows a mutant-specific selection of inhibitors to overcome drug resistance (*39*). In addition, our results suggest that the reactivation of intracellular networks, including MAPK signaling, may contribute to drug tolerance and, over time, may modulate the efficacy of RET kinase inhibitors in *RET*-rearranged tumors. Given the evident clinical need for effective targeted drugs against RET, our results provide a strong rationale for optimization of current therapeutic strategies and development of RET inhibitors for the effective treatment of *RET*-rearranged cancers.

(ii) compound specificity and compound activity can be faithfully determined in complementary in vitro and in vivo models of rearranged RET, and (iii) resistance mechanisms against targeted inhibition of RET may involve *RET*^{I788N} mutations and the reactivation of MAPK signaling.

MATERIALS AND METHODS

Study design

The goal of our study was to systematically profile a series of kinase inhibitors to identify features that predict high activity against *RET*-rearranged tumors. In particular, we characterized the role of inhibitor binding to RET kinase. Furthermore, we performed chemical genomic analyses and transcriptional profiling to identify mechanisms of resistance against RET inhibitors in *RET*-rearranged cancer cells.

The selection of cell lines was based on availability of *RET*-rearranged cellular models. We used the *RET*-rearranged lung adenocarcinoma cell line LC2/AD and the *KIF5B-RET* and *CCDC6-RET* viral transduced Ba/F3 pro B cell line to benchmark the differential activity of different RET inhibitors. We specifically focused on the characterization of AD80 and ponatinib as the most active drugs. To further profile the intracellular effects of AD80, we used phosphoproteomics to demonstrate that phospho-RET is among the most decreased detected peptides. Because it was not possible for us to obtain crystal structures of AD80 in a complex with RET, we used homology-based modeling of the AD80:RET complex to further substantiate our hypothesis of AD080 binding the DFG-out conformation of RET. To identify resistance mutations against AD80 in *CCDC6-RET*, we performed saturated mutagenesis screening and found a I788N mutation but no mutations at the gatekeeper position V804 of RET. Finally, we used murine PDX models driven by *CCDC6-RET*^{wt} or *CCDC6-RET*^{V804M} showing potent in vivo efficacy of AD80. All experiments were performed at least three times. Screenings were performed in triplicates within each experiment. IHC analyses of PDX tumors were randomly selected and reviewed in a blinded fashion. More details for each individual experiment are indicated in Materials and Methods as well as in the main text and figure legends.

CRISPR/Cas9

CRISPR technology was used via a pLenti vector containing Cas9-IRES-blasticidine and two U6 promoters for expression of individual single-guide RNAs (sgRNAs) [sgRNA1 (intron 15 murine *KIF5B*), GGCACCAAACACTTCACCCC; sgRNA2 (intron 11 murine *RET*), GGGTGTAGCGAAGTGTGCAT) (*14*)]. Twenty-four hours after transfection, the medium was changed to medium supplemented with blasticidin (10 μg/ml) (Life Technologies) for 4 days.

Immunoblot analyses

Immunoblot analyses were performed as previously described (*40*). The individual antibodies are specified in the Supplementary Materials and Methods. Detection of proteins was performed via horseradish peroxidase or via near-infrared fluorescent antibodies using a LI-COR Odyssey CLx imaging system.

Phosphoproteomic analyses

LC-2/AD cells were treated with 0, 10, or 100 nM AD80, lysed, proteolytically digested with trypsin, and labeled with an isobaric mass tag (TMT10plex, Thermo Fisher Scientific). Peptides for global proteome analysis were fractionated by high-pH reversed-phase chromatography. Phosphopeptides were enriched via TiO_2 beads and fractionated using hydrophilic interaction chromatography (*41*). Fractions were analyzed by nano-liquid chromatography–tandem mass spectrometry on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific), and data were analyzed using the Proteome Discoverer 1.4 software (Thermo Fisher Scientific). A detailed description can be found in the Supplementary Materials and Methods.

Protein thermal shift assay

Different variants of RET kinase domain were designed and ordered from GeneArt (Life Technologies). RET variants were expressed in SF21 cells and harvested 72 hours after transfection. Subsequently, proteins were purified and phosphorylated. To determine the protein thermal shift, protein variants were incubated with DMSO or 1 μM compound. SYPRO Orange dye (Life Technologies) was added to each drug-treated sample, and thermal shift was measured in a 7500 Fast Real-Time PCR machine (Applied Biosystems) in a temperature range of 25° to 90°C. Subsequent analysis was performed using Protein Thermal Shift Software v1.2 (Applied Biosystems). A detailed description can be found in the Supplementary Materials and Methods.

Computational binding mode modeling

Briefly, VEGFR was taken as a template for modeling and filling of sequence gaps, representing the relevant part of the wild-type RET protein. All ligand-bound models were created by superpositioning, followed by extensive MD simulations and energy minimization to relax the structures (RET^{wt}/AD80, RET^{V804M}/AD80, and RET^{wt}/cabozantinib). For comparison with experimentally determined IC_{50} ratios, the binding free energy difference between RET^{wt}/AD80 and RET^{wt}/AD57 was further estimated by MD simulations and integral equation calculations (*42*). The latter approach was also used for approximate determination of the impact of the V804M mutation on the binding affinity of AD80. A detailed description can be found in the Supplementary Materials and Methods.

ATP-binding constant determination

ATP K_m determination for RET^{wt} and RET^{V804M} mutant was performed using the HTRF KinEASE TK assay (Cisbio) according to the manufacturer's instructions. To determine ATP K_m, wild type and V804M mutant were incubated with different ATP concentrations (300 μM to 1.7 nM) for 20 min (RET^{wt}) or 15 min (RET^{V804M}). Phosphorylation of the substrate peptide was determined by Förster resonance energy transfer between europium cryptate and XL665. ATP K_m (app) was calculated using a Michaelis-Menten plot.

Patient-derived xenografts

Tumor fragments from stock mice (BALB/c nude) inoculated with *CCDC6-RET* fusion–positive patient-derived tumor tissues (provided by Crown Bioscience Inc.) were harvested and used for propagation into BALB/c nude mice (*32*). Mice were randomly allocated into vehicle (5% DMSO and 40% PEG400 in saline)– and AD80 (25 mg/kg)–treated groups (oral gavage) when the average tumor volume reached 100 to 200 mm^3. Tumor volume was measured twice weekly in two dimensions using a caliper, and the volume is expressed in cubic millimeters [TV = $0.5(a \times b^2)$, where a and b represent long and short diameter, respectively].

Immunohistochemistry

IHC was performed on Leica BOND automated staining systems using Ki-67 and Mib-1 (Dako) antibodies according to the manufacturer's instructions. Ki-67 labeling index was determined by manually counting 100 tumor cells in the area of the highest proliferation.

Statistical analysis

All statistical analyses were performed using Microsoft Excel 2011 or GraphPad Prism 6.0h for Mac or R (www.r-project.org/). P values were assessed using Student's t test, unless specified otherwise. Significance is marked with $^*P \leq 0.05$, $^{**}P \leq 0.01$, or $^{***}P \leq 0.001$.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Selective inhibition of signaling induced by rearranged RET and clinical activity in vivo.

Fig. S2. Induction of *KIF5B-RET* rearrangements in NIH-3T3 cells via CRISPR/Cas9 and S6 kinase as an off-target of AD80.

Fig. S3. Characterization of the activity profile of AD80.

Fig. S4. Delineation of the cellular targets of AD80 using ligand screens and thermal shift experiments.

Fig. S5. RMSD of RET and AD80 or cabozantinib over time and ALPB-optimized structures.

Fig. S6. Inhibitory potential of AD80 derivatives and resistance mechanisms against RET inhibition.

Fig. S7. Validation of PDX via fluorescent in situ hybridization (FISH) and in vivo effects induced by treatment with AD80.

Table S1. IC_{50} values of AD80, cabozantinib, and vandetanib for phospho-RET in Ba/F3 cells expressing wild type or V804M *KIF5B-RET*.

Table S2. Rates of clinical response to currently available anti-RET drugs and clinical information of patients used in retrospective analysis.

Table S3. GI_{50} values of the panel of patient-derived cell lines.

Table S4. Tabulated derivative melting temperatures (T_m) and differences in melting temperature (ΔT_m) values.

Table S5. In vitro kinase assay of RET^{wt}, RET^{V804M}, and RET^{V804L} mutants with different inhibitors.

Table S6. Experimental setup for saturated mutagenesis screening.

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Drugging the catalytically inactive state of RET kinase in RET-rearranged tumors

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RET-ting out lung tumors

Gene fusions and rearrangements serve as oncogenic drivers in a number of tumor types, and some of these can be targeted with existing drugs. *RET* rearrangements have been identified as drivers in some lung adenocarcinomas, but previous attempts to target RET have not been successful. Plenker *et al*. determined why the drugs previously proposed for inhibiting RET were not sufficiently potent and showed that successful inhibition of RET requires the ability to bind RET in its catalytically inactive conformation, known as the "DFG-out conformation," thus locking it in an inactive state. The authors also identified drugs that bind RET in the desired conformation and demonstrated their efficacy in patient-derived xenograft models.

4. Discussion

Genomic *RET* rearrangements have been shown to play a role as oncogenic drivers in lung adenocarcinoma (Lipson et al. 2012; Takeuchi et al. 2012; Kohno et al. 2012; Plenker et al. 2017). Unfortunately, treatment of those patients with small molecule inhibitors has been rather disappointing without the promising results seen in *EML4-ALK* or *ROS*-fusion driven cancer under crizotinib or ceritinib treatment (Kodama et al. 2014; Kurzrock et al. 2011; Borrello et al. 2013; Plenker et al. 2017). Future trials may show improved response due to a more systematic sequencing of tumor biopsy samples, however progression-free-survival is still rather limited (Plenker et al. 2017). Median progression free survival under small molecule inhibition with anti-RET activity was only 2.3 months (95% CI, 1.6 to 5.0 months) and median overall survival 6.8 months (95% CI, 3.9 to 14.3 months) as seen in the latest multi centre RET registry (Gautschi et al. 2017; Plenker et al. 2017). This is in line with data published before indicating a lack of effective anti-RET cancer treatment (Drilon et al. 2013; Falchook et al. 2016; Lee et al. 2017; Plenker et al. 2017).

Accordingly, the results in our small cohort of four patients with advanced lung cancer revealed only one patient under vandetanib or cabozantinib treatment with progressive response (PR) (Plenker et al. 2017) (Sup. Fig. 1C,D; Sup. Table 6B). This fact contradicts observations that a lot of different kinase inhibitors are known to bind RET and inhibit the kinase effectively *in vitro* (Dar et al. 2012; Plenker et al. 2017). In fact, the GI_{50}-values of currently applied RET inhibitors range between <5 and 100nM (Drilon et al. 2018). The goal of the study has been to use a broad range of both chemical-genomic and chemical-proteomic analyses to give new insights into potential future anti-RET drug development to overcome the limited responses observed in RET-rearranged tumors (Plenker et al. 2017).

Initially, a large panel of drugs with potential anti-RET activity against different *in vitro* models including KIF5B-RET and CCDC6-RET induced Ba/F3 cells, NIH-3T3 cells with KIF5B-RET induced rearrangements using the CRISPR-Cas9 genome editing technique and the patient derived CCC6-RET lung adenocarcinoma cell line LC-2-/AD were tested (Takeuchi et al. 2012; Dar et al. 2012; Song 2015; Choi and Meyerson 2014; Suzuki et al. 2013; Lipson et al. 2012; Plenker et al. 2017) (Fig. 1A,C,E; Sup. Fig. 1A). All three models demonstrated a similar 100- to 1000-times higher cytotoxicity and dephosphorylation of RET and its downstream molecules under AD80 and ponatinib treatment compared to all other drugs with potential RET on-target efficacy (Plenker et al. 2017) (Fig. 1B,D,F). These data illustrate the importance of sufficient on-target activity for a strong decrease of phospho-RET and its downstream signalling molecules activity and indicate the translation into reduced cellular

proliferation (Plenker et al. 2017). Additionally, those *in vitro* data for AD80 were further supported by strong tumor shrinkage in *CCDC6-RET* rearranged patient-derived xenografts (PDX) mice during treatment and followed by resumption of tumor growth as soon as the drug was withdrawn. The mice kept a stable body weight under treatment with good tolerance of the agent (Plenker et al. 2017) (Fig. 6B,C,E; Sup. Fig. 7C). That may be a first indication of a favourable pharmacological side-effect profile under potent AD80 dosage.

The role of different fusion partners of *RET* in terms of tumor phenotype, cell signalling, response to treatment by kinase inhibition or clinical presentation is not clear yet. Levinson and Kagan could demonstrate in a Drosophila transgenic fly model that *NCOA4-RET* rearranged flies developed a more severe oncogenic phenotype with higher rates of RET phosphorylation, cell migration, delamination, and epithelial-mesenchymal transition (EMT) compared to *CCDC6-RET* rearranged Drosophila. Additionally, these different phenotypes were also reflected in a varying response to kinase inhibition (Levinson and Cagan 2016). These results are in line with clinical observations that tumor progression seen in thyroid cancer with *NCOA4-RET* rearrangements is usually more severe if compared to clinical cases with other *RET* fusion partners (Levinson and Cagan 2016). Interestingly, in our experimental setups we also discovered repeatedly stronger *in vitro* cytotoxic effects and RET dephosphorylation in *KIF5B-RET* compared to *CCDC6-RET* rearranged Ba/F3 cells under AD80 treatment (Plenker et al. 2017) (Fig. 4A). Further studies need to explore the precise role of *RET* fusion partners and the mechanistic background in lung cancer as they may have a crucial impact on efficacy of targeted therapy and clinical prognosis.

For drug application, a high ratio between the dose necessary to sufficiently inhibit the primary target and the dose at which unintended therapeutic side- effects occur is crucial (off- vs on-target activity) (Plenker et al. 2017). Benchmarking AD80 against other clinical and pre-clinical available compounds in 18 cancer cell lines with known oncogenic drivers, provided a dataset to calculate the ratio of GI_{50}-values between primary-target and off-target cancer cell lines for each drug (Borrello et al. 2013; Song 2015; Sos et al. 2009; Wilhelm et al. 2011; Guagnano et al. 2011; Karaman et al. 2008) (Sup. Fig. 3A; Sup. Table 2). The median on-target specificity ratios for AD80 and ponatinib were in the range of highly selective kinase inhibitors such as BGJ398, ceritinib, alectinib or erlotinib for their corresponding primary target (Sup. Fig. 3B). This suggests a similar specificity profile and similar rates of genotype-independent off-target effects for AD80 in comparison to these drugs (Plenker et al. 2017). Other drugs currently used as RET-inhibitors such as vandetanib, cabozantinib or regorafenib showed only a low specificity profile (Kodama et al. 2014; Borrello et al. 2013; Song 2015; Wilhelm et al. 2011; Guagnano et al. 2011). Additionally, mass-spectrometry based phosphoproteomic assays revealed highly selective RET dephosphorylation under AD80

treatment as compared to other RTKs (Fig. 2A; Sup. Fig. 3C) (Plenker et al. 2017). This comparative analysis supports our hypothesis that a potent on-target activity as in the case of AD80 and ponatinib may open up a therapeutic window for an effective and genotype-selective inhibition of *RET*-rearranged tumors despite a broad spectrum of potential kinase targets (Plenker et al. 2017).

This is in particular important as so far all RET targeting agents described are not primarily RET specific, but target multiple kinases to a varying degree (Ferrara et al. 2018). Side effects caused by off-target may in part explain the high dose reduction (23%-79%) and treatment discontinuation rates seen under vandetanib, cabozantinib or lenvatinib treatment (Drilon et al. 2018). Rash due to EGFR inhibition or diarrhea due to VEGFR suppression and other side effects could be therapy limiting as necessary target inhibition levels against RET cannot be reached. Regarding ponatinib, the “EPIC” phase 3 trial for the treatment of CML had to be cancelled in 2013 due to higher rates of arterial occlusive diseases and venous thromboembolic complications as compared to the control imatinib group despite good effects seen on tumor progression (Lipton et al. 2016).

Structural analyses based on VEGFR homology modeling and molecular dynamics in the DFG-out conformation with AD80 indicate that the drug is a type II RTK inhibitor (Plenker et al. 2017). That is crucial as other type II inhibitors, e.g. ponatinib and cabozantinib, along with AD80 demonstrated the best results in our *in vitro* experiments (Plenker et al. 2017). In an attempt to gain better mechanistic insights into these drugs, thermal shift assays were used as a surrogate parameter for the potency of AD80 and ponatinib to bind different RET kinase variants (Knowles et al. 2006; Plenker et al. 2017). Our results revealed two observations. First, we saw that the type II inhibitor binding of AD80, ponatinib and sorafenib to RET kinase in the DFG-out conformation resulted in higher thermal stability compared to type I inhibitors that bind the active kinase conformation (Fig. 2B,C; Sup. Table 3) (Plenker et al. 2017). This observation may in part explain the potent cytotoxic effects seen in our cellular viability assays when comparing AD80 and ponatinib to other anti-RET drugs (Plenker et al. 2017). These findings are especially important for the mechanistic understanding of AD80 and the RET kinase function itself, as well as, the development of new anti-RET agents. Second, the strong effects seen for the type II inhibitor sorafenib in the thermal shift assays do not translate into strong cellular toxicity. Moreover, clinical trials with sorafenib in RET-driven lung cancer have been rather disappointing (Horiike et al. 2016; Plenker et al. 2017) (Sup. Fig. 4B-D). Further analyses must explore how other factors such as drug residence time or structural kinetics further explain drug efficacy (Plenker et al. 2017).

Secondary resistance mutations play a major role in drug failure during treatment that have been described in different tumor entities (Carlomagno et al. 2004; Plenker et al. 2017).

Those mutations in the highly conserved gatekeeper residue with replacement of valine against methionine in RET adjacent to its ATP binding-site (pV804M) sterically reduce accessibility to the enzymatic ATP-binding site and, as we could show, increase the < binding affinity of RET for ATP (Fig. 3E) (Drilon et al. 2018). Our cellular experiments revealed that both AD80 and ponatinib remain potent in Ba/F3 cells expressing RET fusions with the most common gatekeeper mutation pV804M with only a small shift in cytotoxicity as compared to other currently used anti-RET drugs (Fig. 3A-D; Sup. Table. 4) (Mologni et al. 2013; Plenker et al. 2017). However, the clinical relevance of this small reduction in drug efficacy in terms of tumor progression under treatment is not clear yet and has to be further investigated (Plenker et al. 2017).

Our PDX mice engrafted with colorectal cancer tissue that had developed a *CCDC6-RET*^{V804M} gatekeeper mutation under ponatinib treatment showed tumor shrinkage during AD80 treatment, however to a lesser degree when compared to PDX mice without a RET gatekeeper mutation (Fig. 6B,D,F) (Plenker et al. 2017). The tumor shrinkage endured only the first few days under treatment and when tumor growth resumed, it was less strong as compared to the control group. The effects of AD80 on other models with RET gatekeeper mutations need to be explored *in vitro* for further evaluation (Plenker et al. 2017).

Using saturated mutagenesis screening we identified the missense mutation pI788N (c.2363T>A) within the RET kinase domain as a potential drug resistance mutation against targeted AD80 treatment (Sup. Tbl. 6) (Heuckmann et al. 2011; Plenker et al. 2017). From a panel of anti-RET drugs only ponatinib remained potent in cellular viability assays with Ba/F3 *KIF-RET*^{I788N} and *CCDC6-RET*^{I788N,}, whereas the cytotoxic effects of AD80 were strongly diminished (Fig. 4A-D) (Plenker et al. 2017). The differential effects seen between AD80 and ponatinib cannot be fully explained with the available structural data and need further investigation (Plenker et al. 2017). Computational modeling revealed a high proximity of the altered amino acid to the inhibitor within the kinase and possible interactions on the molecular level (Fig. 4E) (Plenker et al. 2017). Interestingly, the I788 residue is the orthologous position V654 in *c-KIT* that has been shown to confer resistance to imatinib targeted therapy (Nelkin 2017; Roberts et al. 2007). The clinical relevance of this single RET^{I788N} kinase mutation is unclear, yet it underlines the need for a systematic detection of resistance mutations in patients that relapse under treatment (Plenker et al. 2017).

The fact that we have found a distinct RET single amino acid mutation rendering the complete kinase insensitive to AD80 treatment is another indicator of a high on-target activity of the drug. That is in line with findings in *ALK*-rearranged NSCLC in which acquired resistance mutations can be found more frequently under treatment with the more selective inhibitors alectinib or ceritinib as compared to the first-generation agent crizotinib (Gainor et al. 2016;

Drilon et al. 2018). Accodingly, the fact that until now with only one exception, no clinical data for acquired resistance mutations in RET driven lung cancer exist, emphasizes the current lack of potent anti-RET agents that may change in future trials with a next generation of more potent small molecule inhibitors (Gainor et al. 2016). Only recently it was shown in a patient with metastatic lung adenocarcinoma that the secondary *RET*^{S904F} mutation leads to vandetanib resistance due to higher ATP affinity and autophosphorylation of the mutant RET-fusion kinase (Nakaoku et al. 2018). If secondary resistance mutations will be more frequently observed with the emergence of better anti-RET drugs has to be evaluated in future.

We investigated cell signalling alterations by comparing secondary resistant *RET*-rearranged TPC-1 thyroid cancer and LC-2/AD lung adenocarcinoma cells under AD80 treatment. We found evidence that MAPK upregulation induces insensitivity to anti-RET drugs as a possible resistance mechanism against targeted therapy (Fig. 5D-F; Sup. Fig. 6C-F) (Plenker et al. 2017). Our findings that lentiviral transduction of *KRAS*^{G12V} in LC-2/AD cells leads to an overexpression of KRAS and subsequent resistance to AD80 are supported by a recent study in which MAPK reactivation due to *NRAS* and *KRAS* acquired activating mutations in LC-2/AD cells was shown to lead to reduced cytotoxic effects of various RET-targeting drugs (Nelson-Taylor et al. 2017). This provides a rationale for possible combined RET and MAPK inhibition as seen in *BRAF*^{V600E} mutant melanoma with improved overall response rate under combined vemurafenib and cobimetinib treatment (Larkin et al. 2014).

In summary, our study identifies AD80 and ponatinib as highly potent small molecule inhibitors for targeting RET-rearranged cancers (Plenker et al. 2017). The insights gained in this study for AD80 provide a solid base for the design of future clinical trials that need to further unravel the molecular and biochemical mechanisms of action of RET inhibitors and provide new aspects of potential modes of resistance against targeted therapy in NSCLC.

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6. Anhang

6.1 Abbildungsverzeichnis

6.2 Tabellenverzeichnis

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