
From Structural Plasticity to the Development of Next-Generation Inhibitors to Overcome Drug-Resistant GIST

DISSERTATION

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“Use biology to guide chemistry.

Use chemistry to understand biology.”

- Jun Qi, 12th ICBS 2023 annual conference

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dedicated to my family

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List of Abbreviations

3D	three-dimensional
ABP	ATP-binding pocket
AI	artificial intelligence
AL	activation loop
aq.	aqueous
ATP	adenosine triphosphate
BBB	blood-brain barrier
Boc	<i>tert</i> - butyloxycarbonyl
BTK	BRUTONS tyrosine kinase
Cas9	CRISPR-associated protein 9
CHES	2-(cyclohexylamino) ethanesulfonic acid
CML	chronic myeloid leukemia
CNS-MPO	central nervous system multiparameter optimization
CRISPR	clustered regularly interspaced short palindromic repeats
cryo-EM	cryo-electron microscopy
CTG	CellTiter-Glo
D	day
Da	DALTON
DCM	dichlormethane
DIBAL-H	diisobutylaluminium hydride
DIPEA	diisopropylethylamine
DMF	dimethylformamide
DMP	DESS–MARTIN periodinane
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
del	deletion
dup	duplication
EGFR	epidermal growth factor receptor
X	

ED	e xtracellular d omain
FDA	f ood and d rug a dministration
FRET	F ÖRSTER r esonance e nergy t ransfer
GK	g ate k eeper
GI	g astrointestinal
GIST	g astrointestinal s tral t umors
GR ₅₀	half maximal g rowth r ate inhibition
HATU	h exafluorophosphate a zabenzotriazole t etramethyl u ronium
HBP	h ydrophobic b ack p ocket
H-bonds	h ydrogen b onding
HPLC	h igh p erformance liquid c hromatography
HTRF	h omogeneous t ime- r esolved f luorescence
HSP90	h eat s hock p rotein 90
HTS	h igh t hroughput s creening
IC ₅₀	half maximal inhibitory c oncentration
IG	i mmunoglobulin
ins	i n s ertion
JMD	j uxta m embrane d omain
KD	k inase d omain
k_i	i nactivation rate
K_M	M ICHAELIS constant
KRAS	k irsten r at s arcoma
NSCLC	n on- s mall c ell lung c ancer
MD	m olecular d ynamics
min	m inutes
MMFF94	M erck m olecular f orce f ield
MS	m ass s pectrometry
NMR	n uclear m agnetic r esonance
OS	o verall s urvival

ovn.	overnight
PCR	polymerase chain reaction
POI	protein of interest
PDB	protein data bank
PDGFRA	platelet-derived growth factor receptor alpha
PFS	progression-free survival
quant.	quantitative
PROTACs	proteolysis-targeting chimeras
PTM	post-translational modification
rt	room temperature
RTK	receptor tyrosine kinases
SAR	structure-activity-relationship
SBDD	structure-based drug design
SCFR/KIT	stem cell growth factor receptor
SDH	succinate dehydrogenase
TBAF	<i>tetra-n</i>-butylammonium fluoride
TBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate
TEA	triethylamine
THF	tetrahydrofuran
TKIs	tyrosine kinase inhibitors
TLC	thin-layer chromatography
TMSA	trimethylsilyl acetylene
VEGFR	vascular endothelial growth factor receptor
wt	wild-type

Amino acids are abbreviated with one- and three-letter codes according to the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry and Molecular Biology (IUB).

Abstract

Given the central role of protein kinases in regulating intracellular signaling, it is well-established that mutation-induced dysregulation can lead to aberrant signaling and, consequently, to the development of various diseases, particularly cancer. Kinases are amongst the most frequently mutated proteins responsible for this aberrant signaling and have emerged as valuable targets. Among the various types of cancer, gastrointestinal stromal tumors (GIST) are a prime example where targeted therapy has significantly improved patient outcomes. GIST represent the most common mesenchymal tumors of the gastrointestinal tract, predominantly driven by activating mutations in the receptor tyrosine kinases KIT and PDGFRA. Despite the availability of approved tyrosine kinase inhibitors (TKIs) for the treatment of GIST, these drugs encounter limitations due to the occurrence of acquired resistance mutations and the presence of side effects. Therefore, there is an urgent medical need necessitating the development of next-generation inhibitors. One particular TKI that has transformed the treatment of PDGFRA-driven GIST is avapritinib. However, patients eventually progress, either as a consequence of the emergence of acquired resistance mutations or as a result of treatment discontinuation due to neuro-cognitive side effects. These patients have a poor prognosis and no therapeutic alternatives. At the beginning of this work, the binding mode of this drug was unknown and there was limited knowledge of its medicinal chemistry development - a critical gap in the development of next-generation inhibitors to address these challenges. An in-depth structural analysis and structure-activity relationship (SAR) revealed the existence of a previously undescribed targetable sub-pocket in KIT and PDGFRA. Reflecting its location within the N-lobe of the kinase domain, which is surrounded by amino acids of key regulatory elements such as the Gly-rich loop and the α C-helix it was named G α -pocket. These structural insights fostered the design and synthesis of novel avapritinib derivatives for the determination of key pharmacophoric features to overcome drug resistance and limit potential blood-brain barrier penetration. A comprehensive understanding of the kinase's ability to adopt different conformations and the impact of (resistance) mutations are essential for the development of small molecules that can interfere with and inhibit the activity of the kinase of interest. This work explored the structural basis of drug resistance in GIST, with particular emphasis on mutations within the ATP-binding pocket and activation loop of the kinase domain. Structural analyses and *in silico* modeling approaches revealed diverse mechanisms by which mutations mediate resistance to currently approved drugs. These findings highlight the conformational changes and alterations in the binding affinity that underlie resistance mutations and provide critical insights into the dynamics of KIT and PDGFRA. These structural insights will thus contribute to and facilitate the rational design of next-generation inhibitors to overcome drug resistance and improve outcomes for GIST patients.

To overcome the limitations of currently approved drugs regarding selectivity and resistance, a covalent targeting approach has been performed. Covalent kinase inhibitors such as osimertinib and ibrutinib have shown superiority in the treatment of otherwise drug-resistant cancers, but their development in GIST remains largely unmapped. Against this background, this work explores the potential of covalent KIT and PDGFRA inhibitors designed to overcome drug resistance and offer improved potency and selectivity. Structure-based design, synthesis, and evaluation of derivatives with a covalent mode of action resulted in extensive SAR studies to improve the understanding of protein-ligand interactions in mutant KIT and PDGFRA. The synthesized compounds show encouraging biochemical inhibitory activity in the low nanomolar range, on-target engagement in patient-derived cells, as well as excellent and improved selectivity. It is also noteworthy that this endeavor afforded the first co-crystal structure of PDGFRA in complex with a covalent inhibitor.

In conclusion, this thesis provides critical structural insights into the mechanisms of drug resistance in GIST, emphasizing the conformational dynamics and mutations that underlie therapeutic failure. The rational design, development, and biological evaluation of covalent inhibitors have deepened our understanding of key protein-ligand interactions and demonstrated the potential of covalent targeting of KIT and PDGFRA to overcome resistance while improving selectivity. Moreover, the development of improved avapritinib derivatives as well as the identification of the novel $G\alpha$ -pocket, represent significant advances. According to our studies, targeting the $G\alpha$ -pocket offers great potential to positively impact both potency and selectivity and should be considered for future kinase inhibitor development. Collectively, these findings establish a foundation for the development of next-generation kinase inhibitors, not only to address the current landscape of resistance mutations in GIST, but also in other cancers where mutated receptor tyrosine kinases are oncogenic drivers.

Kurzfassung

Aufgrund der zentralen Rolle von Proteinkinasen in intrazellulären Signaltransduktionsnetzwerken stellen mutationsbedingte Fehlregulationen eine maßgebliche Ursache für die Entstehung zahlreicher Krankheiten wie zum Beispiel Krebs dar. Entsprechend haben sich Kinasen als wertvolle Zielstrukturen für die Behandlung von Tumorerkrankungen durch zielgerichtete Therapieansätze erwiesen. Unter den verschiedenen Krebsarten sind gastrointestinale Stromatumore (GIST) ein Paradebeispiel für erfolgreiche Ansätze der Präzisionsmedizin, die zu einer erheblichen Verbesserung der Lebenserwartung und -qualität von Patienten geführt haben. GIST sind die häufigsten mesenchymalen Tumoren des Gastrointestinaltrakts und werden überwiegend durch aktivierende Mutationen in den Rezeptor-Tyrosinkinasen KIT und PDGFRA verursacht. Obwohl zugelassene Tyrosinkinase-Inhibitoren (TKI) für die Behandlung von GIST zur Verfügung stehen, ist ihre Anwendbarkeit aufgrund des Auftretens polyklonaler Resistenzmutationen und unerwünschter Nebenwirkungen häufig limitiert, wodurch ein dringender medizinischer Bedarf an neuen, potenten und selektiven Inhibitoren besteht. Avapritinib ist der bisher einzige wirksame Arzneistoff, der für die Behandlung von PDGFRA-D842V-getriebenen GIST zugelassen ist. Dennoch manifestiert sich bei den Patienten letztlich eine weitere Progression, die entweder auf das Auftreten erworbener Resistenzmutationen oder auf einen Behandlungsabbruch aufgrund neurokognitiver Nebenwirkungen zurückzuführen ist. Diese Patienten haben schlechte Prognosen und keine verfügbaren therapeutischen Alternativen. Darüber hinaus ist das Wissen über den Bindungsmodus dieses Wirkstoffs sowie über dessen medizinal-chemische Entwicklung unzureichend; dieses Verständnis ist jedoch für die Entwicklung von Inhibitoren, die diese Hindernisse überwinden können, von entscheidender Bedeutung ist. Durch die Durchführung umfassender strukturbiochemischer Analysen, sowie SAR konnte eine bisher unbeschriebene Subtasche identifiziert werden. Aufgrund ihrer Lage, umgeben von Aminosäuren wichtiger regulatorischer Elemente wie der Gly-reichen Schleife und der α C-Helix, wurde sie α C-Tasche genannt. Diese strukturellen Erkenntnisse dienen als Grundlage für das Design und die Synthese neuartiger Avapritinib-Derivate zur Bestimmung pharmakophorer Schlüsseleigenschaften zur Überwindung von Arzneimittelresistenzen und zur Begrenzung einer möglichen Penetration der Blut-Hirn-Schranke. Ein umfassendes Verständnis der Struktur und Funktion von Proteinkinasen ist für die rationale Entwicklung selektiver und wirksamer Inhibitoren von grundlegender Bedeutung. Dazu zählen detaillierte Kenntnisse der verschiedenen Aktivitäts-korrelierten Konformationen von Kinasen, der zentralen Wechselwirkungen, die Liganden innerhalb der Kinase eingehen, sowie der Auswirkungen von (Resistenz-)Mutationen auf die Bindung solcher Substanzen.

Daher wurden im Rahmen dieser Arbeit die strukturellen Grundlagen der Arzneimittelresistenz bei GIST untersucht. Dabei wurde ein besonderes Augenmerk auf Mutationen innerhalb der ATP-Bindungstasche und der Aktivierungsschleife der Kinasedomäne gelegt. Durch Strukturanalysen und *in silico*-Modellierungsansätze konnten verschiedene Mechanismen postuliert werden, durch welche Mutationen die Resistenz gegen derzeit zugelassene Medikamente vermitteln. Diese Studien deuteten auf Mutations-induzierte Konformationsänderungen sowie dadurch bedingte Veränderungen in der Bindungsaffinität und gaben entscheidende Einblicke in die Dynamik von KIT und PDGFRA. Diese strukturellen Erkenntnisse bildeten die Grundlage für die nachfolgenden Design- und Synthesebemühungen und werden dazu beitragen, die rationale Entwicklung von Inhibitoren der nächsten Generation zur Überwindung von Arzneimittelresistenzen zu erleichtern. Während sich kovalente Kinaseinhibitoren wie Osimertinib oder Ibrutinib bei der Behandlung von arzneimittelresistenten soliden Tumoren und hämatologischen Malignomen bewiesen haben, ist ihre Entwicklung bei GIST noch weitgehend unerforscht. Vor diesem Hintergrund wurden in dieser Arbeit kovalente KIT- und PDGFRA-Inhibitoren entwickelt und evaluiert, die darauf ausgelegt sind, Arzneimittelresistenzen zu überwinden und eine verbesserte Potenz und Selektivität zu bieten. Strukturbasiertes Design, Synthese und Evaluierung von neuartigen Inhibitoren mit kovalentem Wirkmechanismus führten zu umfangreichen Struktur-Aktivitäts-Wirkungsbeziehungen (SAR), die das Verständnis der Protein-Ligand-Wechselwirkungen in mutiertem-KIT und -PDGFRA verbesserten. Die synthetisierten Verbindungen zeigten eine vielversprechende biochemische Potenz im niedrigen nanomolaren-Bereich, einen *On-Target*-Effekt in patienten-abgeleiteten Zellen und eine ausgezeichnete und verbesserte Selektivität gegenüber einer Kontrollzelllinie. Darüber hinaus, konnte im Rahmen dieser Arbeit die erste Ko-Kristallstruktur von PDGFRA im Komplex mit einem kovalenten Inhibitor generiert werden.

Zusammenfassend lässt sich sagen, dass diese Arbeit die zugrunde liegende Konformationsdynamik der (Resistenz)Mutationen hervorhebt und wesentliche strukturelle Erkenntnisse über die Mechanismen der Arzneimittelresistenz bei GIST liefert. Des Weiteren hat die Entwicklung und biologische Evaluierung von kovalenten Inhibitoren dazu beigetragen das Verständnis wichtiger Protein-Ligand-Interaktionen aufzudecken und darüber hinaus das Potenzial der kovalenten Adressierung zur Überwindung von Resistenzen bei gleichzeitiger Verbesserung der Selektivität aufzuzeigen. Die Entdeckung der bislang unbeschriebenen α -Tasche eröffnet neue Möglichkeiten für das rationale Design von Inhibitoren und bietet ein vielversprechendes Potenzial, sowohl die Wirksamkeit als auch die Selektivität weiter zu steigern. Insgesamt bilden diese Ergebnisse die Grundlage für die Entwicklung von TKIs der nächsten Generation, die insbesondere auf das derzeitige Spektrum an Resistenzmutationen bei GIST abzielen, aber auch bei anderen Krebsarten, bei denen mutierte Rezeptor-Tyrosinkinasen Onkogene Treiber sind, inspirieren können.

Chapter: 1

INTRODUCTION

1. Introduction

For centuries, drug discovery was largely a journey guided by serendipity. The earliest medicines, like penicillin for bacterial infections, were born from chance observations, often without a comprehensive understanding of their underlying biological mechanisms. However, the advent of technologies and methodologies enabled the systematic search of large substance libraries (high throughput screening, HTS) and iterative optimization processes that made use of a better understanding of molecular and structural biology. This transformation brought about a paradigm shift in drug discovery, moving it from an art of chance to a science of precision.¹⁻³ In the postgenomic era¹ many diseases were not only understood a systemic level but also at the genetic and molecular levels, allowing for the development of therapies tailored to specific targets. This rational approach, grounded in biochemical pathways and structural insights, has redefined therapeutic strategies for addressing complex diseases, such as cancer.^{1, 4-5}

1.1 Precision Oncology and Targeted Approaches

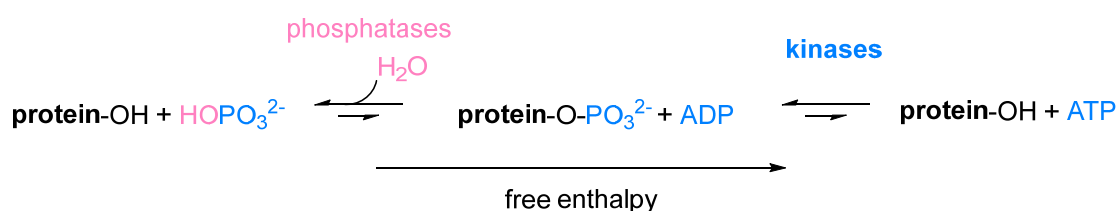
In contrast to the traditional one-size-fits-all approach to cancer therapy, precision oncology seeks to tailor therapeutic strategies based on the genetic, molecular, and cellular characteristics of an individual patient.⁶⁻⁸ These targeted therapies are designed to interact with specific molecular abnormalities that drive cancer progression, taking advantage of a phenomenon first described by BERNARD WEINSTEIN as “oncogene addiction”.⁹⁻¹⁰ This concept refers to the surprising dependency, despite the inherent genetic complexity, of tumor cells on a single oncogene for survival.¹⁰⁻¹¹ By inhibiting these oncogenes, which are responsible for the respective survival and proliferation of the cancer cells, apoptosis is specifically initiated only for the aforementioned cancer cells (oncogenic shock).¹² Thus, unlike conventional chemotherapies that indiscriminately attack rapidly dividing cells, targeted therapies focus on specific targets critical to tumor growth and survival. Ultimately, this specificity reduces collateral damage to normal cells and enhances treatment efficacy, resulting in reduced adverse effects and improved outcomes for patients.¹³⁻¹⁴ The FDA approval of imatinib (Gleevec®, Novartis AG) in 2001 was a milestone, setting the stage for other targeted approaches and marking the emergence of kinase inhibitors as a central drug class.¹⁵⁻¹⁶ Among the various targets identified, kinases have emerged as a particularly valuable target for addressing cancer with, approximately 80 kinase inhibitors currently approved for clinical use.^{6, 17}

¹ Time period following the successful sequencing of the human genome (HUMAN GENOME PROJECT); characterized by research focused on understanding gene function, regulation, and interactions at the molecular level to uncover how genes coordinate complex biological processes and contribute to health and disease.

1.2 Protein Kinases as Valuable Cancer Targets

Today, more than 500 members of the protein kinase family are encoded in the human genome, representing approximately 2 % of all human genes.¹⁸ Kinases are enzymes that catalyze the transfer of the γ -phosphate group from adenosine triphosphate (ATP) to the hydroxy group on the amino acids of their substrates, such as signaling molecules, other proteins, or the kinase itself, in the presence of a divalent metal cation e.g. Mg^{2+} (Scheme 1).¹⁹ The substrate specificity is classified according to aliphatic and aromatic hydroxy amino acids into Ser/Thr- and Tyr-kinases.²⁰⁻²¹ Due to the introduction of the high density of negative charge upon phosphorylation, this post-translational modification (PTM) often induces a conformational change, thereby regulating the activity, localization of the modified (macro)molecule and/or modulating its ability to interact with other proteins.²²⁻²³ Phosphorylation as a reversible PTM is observed in up to one third of all proteins, thus rendering it the most common and important PTM.²⁴⁻²⁵ Upon receiving an external stimulus e.g. a growth factor, receptor kinases on the cell surface often undergo homo- or heterodimerization and subsequent activation via autophosphorylation.^{23, 26} This initiates a cascade of downstream signaling, typically involving a series of phosphorylation events where one kinase activates another, amplifying the signal, enabling a robust cellular response and ultimately controlling cell growth and differentiation.²⁷ To counteract this PTM, so-called phosphatases in turn catalyze the hydrolysis of the phosphoric ester, restoring the original hydroxy group (Scheme 1).²⁸⁻²⁹

Scheme 1. Generic representation of the interplay between phosphatase and kinases, regulating protein activity.



Given the central role of kinases in signal transduction and the orchestrated interplay with phosphatases, they serve as “molecular switches” in order to maintain a “healthy” phenotype.³⁰⁻³³ Therefore, dysregulation of kinases is likely to result in dramatic changes in the control of cellular functions and, indeed, has often been shown to be oncogenic, making them important targets in modern drug discovery, particularly for cancer.³⁴⁻³⁶

1.2.1 Structural Insights into Kinase Structure, Dynamic and Function

In 1991, KNIGHTON *et al.* solved the first structure of a protein kinase (protein kinase A, PKA, PDB ID: 2CPK).³⁷ Since then, the molecular understanding of kinase structure and function has steadily increased.³¹

Today, the Protein Data Bank (PDB) contains about 200000 entries with approximately 10000 experimentally determined structures of human kinases^{11, 38}. Kinases universally have a bilobal structure consisting of an N terminal and a C terminal lobe, which are connected to each other via the hinge region (Figure 1).^{31, 39} At the beginning of the hinge region a key amino acid - the gatekeeper (GK) - is localized, which controls the access to the ATP-binding pocket (ABP) and the hydrophobic backpocket (HBP).⁴⁰⁻⁴¹ The C-lobe consists mainly of α -helices and contains the activation loop (AL). The N-lobe consists of five antiparallel β -sheets, the regulatory α C-helix and the phosphate-binding loop (equivalent to Gly-rich loop, G-loop). The G-loop is formed by a conserved -GXGXYG- motif and represents a highly flexible region. The cofactor ATP binds in the ABP and is sandwich between both lobes. The nucleobase undergoes hydrogen bonding (H-bonds) over the purine-N1 nitrogen and the amino group with the backbone of the hinge and the phosphate moiety interacts with the G-loop (Figure 1B, C). Further, the α - and β -phosphate groups are engaged in coordination of the magnesium ion. Furthermore, involved in the coordination of Mg^{2+} are a conserved glutamate in the α C-helix, the catalytic lysine as well as a conserved tripeptide Asp-Phe-Gly (DFG) motif, inherent to the AL. It can be seen, therefore, that the AL (DFG motif), G-loop, and α C-helix represent crucial structural regulatory elements. These elements engage in a cross-talk which determines the overall conformation and thus the activity state of the kinase. Kinases are not static molecules; they possess inherent flexibility and are subject to a multistate conformational ensemble in equilibrium between inactive and active conformations (conformational plasticity) (Figure 1D).⁴²⁻⁴³ However, for the sake of simplicity, *the* active or *the* inactive state/conformation is the terminology used in the following sections. Structural arrangements and conformations capable of binding ATP and coordinating Mg^{2+} are hallmarks of an active kinase. Such characteristics include the formation of a salt bridge between the catalytic lysine and the glutamate residue located in the α C-helix, as well as the adoption of a so-called DFG-in conformation, in which the aspartate residue is oriented towards the ABP, while the phenylalanine residue is positioned towards the backpocket region (Figure 1C, D).^{31, 42, 44} In addition, and as a consequence the G-loop and α C-helix are lowered down. This is in contrast to the inactive conformation. The G-loop is oriented towards the top, as is the α C-helix, and the DFG motif is in a DFG-out conformation, in which Phe is oriented towards the ABP, while Asp points into the HBP.^{27, 45} Two additional hallmarks of an active state are represented by a network of hydrophobic residues, which can be referred to as a catalytic (C) and regulatory (R) spines.

¹¹ The protein data bank is an open access database for three-dimensional structural data of biological molecules such as proteins and nucleic acids and can be accessed under <https://www.rcsb.org/>. Search was conducted with the following parameters: Methodology: Experimental; Organism: *Homo sapiens*; Polymer entity type: Protein; Enzyme Classification: Transferase; Query: Kinase. Reviewed on 28.10.2024.

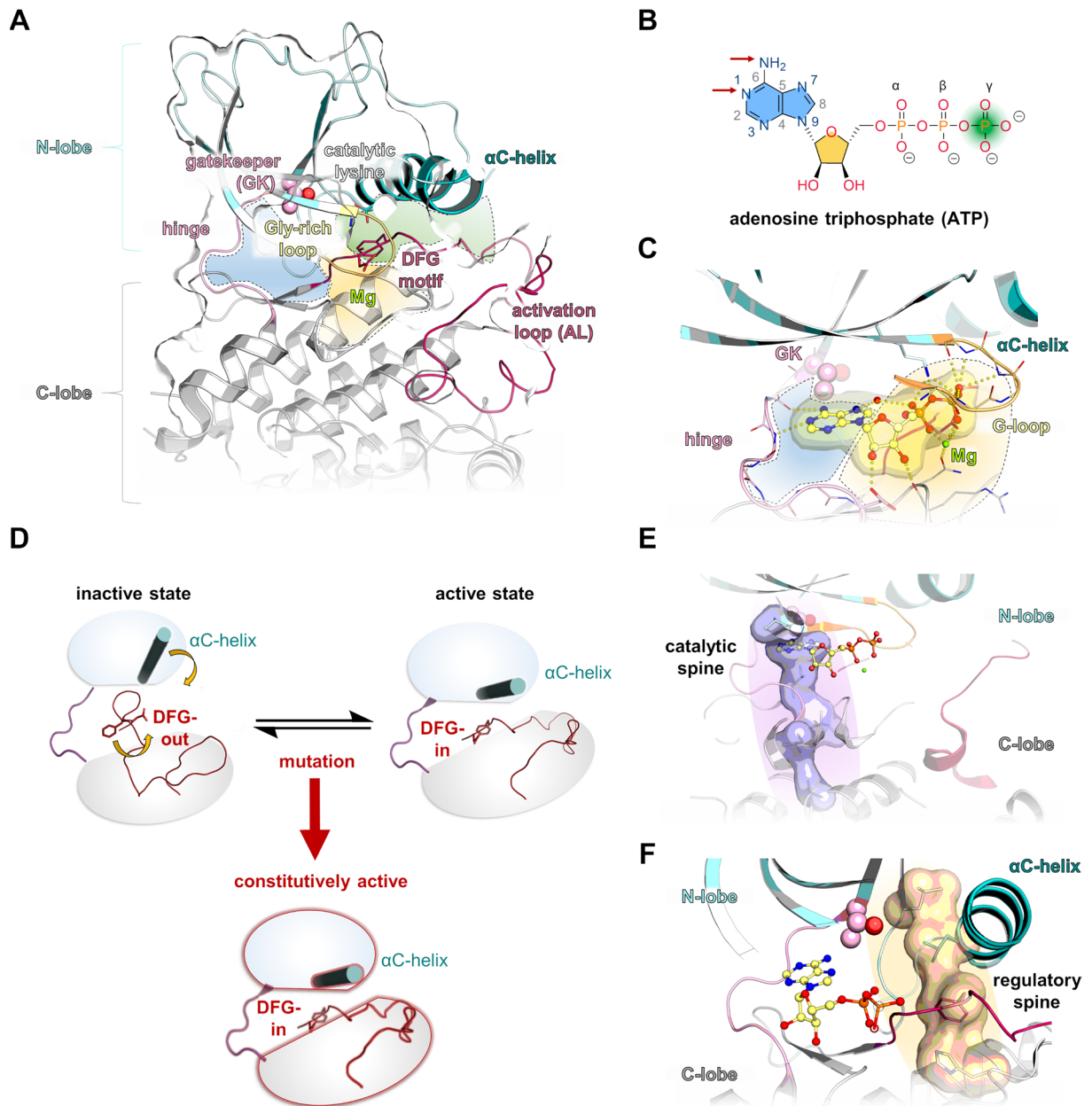


Figure 1. Structural architecture of the catalytic domain of a protein kinase. **(A)** Overview of important structural elements of a protein kinase, including N-lobe (cyan), C-lobe (gray), hinge (pink) and gatekeeper residue (pink spheres), glycine-rich loop (G-loop; yellow), α C-helix (teal), activation loop (red), DFG motif (red sticks), and catalytic lysine (gray sticks). The Mg^{2+} ion is shown as a green sphere. The ATP-binding pocket (blue), the ribose/phosphate binding region (yellow) and the back pocket (green) are indicated by dotted lines. KIT (PDB ID: 1PKG) was used as an example. **(B)** Chemical structure of ATP. The nucleobase is highlighted in blue, the sugar moiety in yellow. Red arrows indicate the interacting parts for hinge binding. The γ -phosphate highlighted in green is transferred during the catalytic process. **(C)** 3D representation of ADP interactions in the active site of a kinase, as exemplified by KIT (PDB ID: 1PKG). **(E)** Assembled regulatory spine in KIT (PDB ID: 1PKG). **(D)** Schematic overview of kinase structure, dynamic equilibrium and conformational activity states (PDB IDs 1PKG and 3G0E). The effect of constitutive activation is schematically implied. **(F)** Assembled catalytic spine in KIT (PDB ID: 1PKG).

These residues can only assemble when the lobes are connected, a structural conformation that is only evident in an active kinase. The C-spine is constituted by hydrophobic residues distributed throughout the kinase linking the N- and C-lobe, as well as the adenine ring of ATP (Figure 1E). This ensures the correct positioning of the ATP. Accordingly, the alignment of the C-spine is of paramount importance for the transfer of a phosphate group.

While the C-spine is directly involved in the catalytic step, the R-spine exerts influence on the activity through structural rearrangement. It also links the N- with the C-lobe, via the formation of an interface through hydrophobic interactions, utilizing residues of the α C-helix, β 4 sheet, DFG motif, and activation segment (α E-helix) (Figure 1F).^{31, 42, 46} It is established that genetic lesions, such as mutations, within this complex network can affect the equilibrium of conformations, thereby increasing the prevalence of active states within the conformational landscape and rendering the kinase constitutively active.⁴⁷⁻⁴⁹ Against this background and given the central role of kinases in various different signaling pathways, there has been a surge in research aimed at devising strategies for the identification and/or development of potent and selective kinase inhibitors.

1.2.2 Molecular Strategies of Kinase Inhibition

However, the development of potent and particularly selective protein kinase inhibitors was long perceived as an insurmountable challenge due to the high sequence and structural similarities, identical catalytic mechanism and substrates, in addition to the fact that ATP is also a substrate of other proteins.^{34, 50-52} Early efforts were further hindered by a lack of structural data, complicating the design of specific inhibitors.⁵³⁻⁵⁴ Around 2000, the group of KEVAN SHOKAT developed a prime example of a chemical genetics approach referred to as the "bump-and-hole" approach. By replacing the bulky gatekeeper residue with, e.g., an Ala, "hole", an orthogonal system is developed to probe a specific protein-ligand pair (a tailored, bulkier small molecule is used to bind the respective "hole-modified" protein, "bump"), excluding interference from naturally related proteins. This work provided fundamental insights into the role of individual kinases in signal transduction and enhanced our understanding of how to achieve selective inhibition of kinases.⁵⁵⁻⁵⁷ This, together with techniques such as protein X-ray crystallography, has paved the way for the development of various types of inhibitors.^{17, 54} Today, there are six principal types of kinase inhibitors (Figure 2, Table 1). Type I inhibitors bind ATP-competitively to the active kinase conformation (DFG-in, α C-in) (Figure 2B).^{17, 39, 58} Prominent examples include two of the earliest inhibitors approved by the FDA that target the epidermal growth factor receptor (EGFR) for the treatment of non-small cell lung cancer (NSCLC), namely gefitinib and erlotinib.⁵⁹ Noteworthy, an unambiguous characterization of the binding type is not always ensured and can be contingent on various factors. Such factors include the availability of complex crystal structures, the target protein, and/or the presence of multiple crystal structures exhibiting disparate binding properties, as exemplified by erlotinib (Supplementary Figure 1, type I in PDB ID: 1M17 or type 1.5 fashion in PDB ID: 4HJO).⁶⁰⁻⁶¹

In contrast, type II inhibitors bind and stabilize the inactive kinase conformation (DFG-out).^{17, 39, 59, 62-65} These inhibitors often engage both the ATP-binding site and the HBP (Figure 2C), which is thought to confer a selective advantage over type I inhibitors.^{59, 65} The first example of this inhibitor type is the first tyrosine kinase inhibitor imatinib (*cf.* chapter 1.1).^{16, 65} Further, the classification of these inhibitors is contingent upon whether they extend beyond the gatekeeper residue either occupying (a) or not occupying (b) the HBP (Table 1).^{17, 59} The same also holds true for type I^{1/2} inhibitors, which are generally defined to bind in and around the ATP binding-pocket to an inactive, yet DFG-in conformation, with other characteristics being variable (Table 1).^{17, 59} One example is lapatinib, approved for the treatment of breast cancer, which binds to epidermal growth factor receptors 1 and 2 (EGFR and HER2) in a DFG-in and α C-helix-out conformation (type 1.5^a).^{59, 66}

Table 1. Summary of hallmarks for the classification of kinase inhibitors, adapted and modified based on R. ROSKOSKI, Jr.⁵⁹ *Approved kinase inhibitor.

property	inhibitor type							
	I	I ^{1/2}	II ^a	II ^b	III	IV	V	VI
ATP competitive	yes	yes	yes	yes	no	no	variable	no
occupies backpocket	no	variable	yes	no	yes	no	variable	variable
DFG-Asp	in	in	out	out	variable	variable	variable	variable
α C-helix	in	variable	variable	variable	out	variable	variable	variable
R-spine	linear	distorted	(usually) distorted	(usually) distorted	distorted	variable	variable	variable
AL	out	variable	variable	variable	variable	variable	variable	variable
reversible	yes	yes	yes	yes	yes	yes	yes	(usually) not
example*	gefitinib	lapatinib	imatinib	nintedanib	cobimetinib	asciminib	-	ibrutinib

Type III inhibitors bind non-ATP-competitively, as they do not interact with the ATP-binding pocket. Instead, they bind to adjacent areas, such as the back pocket (Figure 2D).^{39, 59, 62-63, 65, 67} The assumed type III inhibitor trametinib, which has been approved for different types of cancers with BRAFV600E/V600K mutations, can demonstrate a selectivity advantage through its distinct mode of action.^{17, 63, 68} It is regrettable that no complex structure of trametinib in MEK are available. Nevertheless, an X-ray structure of another type III MEK inhibitor cobimetinib has been solved, which elucidates a DFG-in and α C-helix-out conformation.⁶⁹

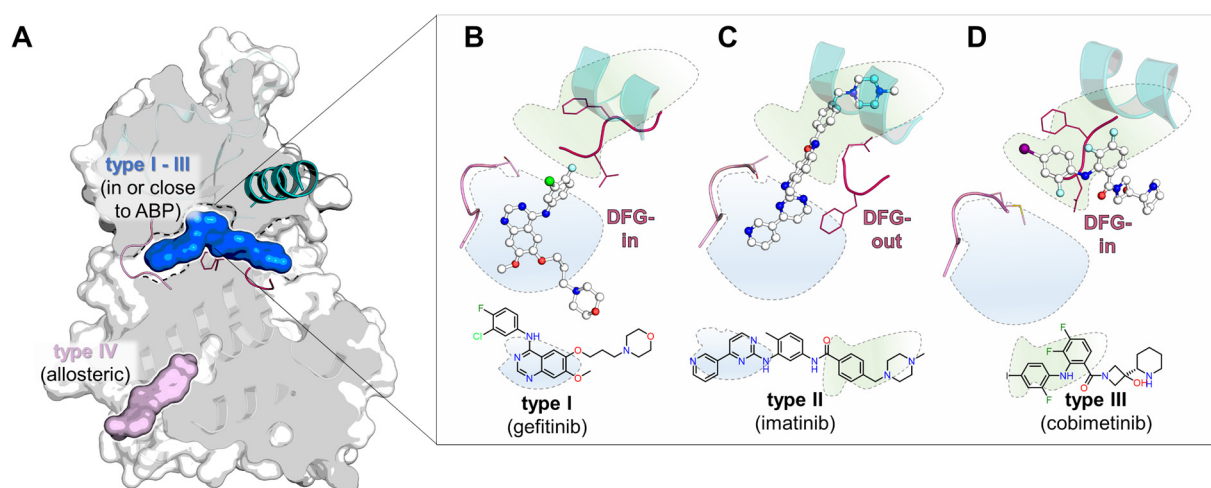


Figure 2. Schematic representation of kinase inhibition modes resp. inhibitor types. **(A)** Overview of the kinase domain and prototypic binders of the ATP (type I-III, indicated in blue (PDB ID: 4U0I) and allosteric (myristate) pocket (type IV, represented in pink, PDB ID: 3K5V). **(B)** Type I inhibitors bind and stabilize an active kinase (DFG-in conformation). Gefitinib (PDB ID: 2ITY) is shown as an example. **(C)** Type II inhibitors bind and stabilize an inactive kinase (DFG-out conformation) and often extend beyond the gatekeeper occupying the backpocket region. Imatinib (PDB ID: 1T46) is shown as an example. **(D)** Type III inhibitors bind non-ATP-competitively to adjacent pocket. Cobimetinib (PDB ID: 7JUS) is shown as an example.

While type III inhibitors already exhibit allosteric character, type IV inhibitors fully bind allosterically (i.e., without binding in or close to the active site) (Figure 2A).^{59, 62-63, 67} It should be noted that additional properties may vary (Table 1).⁵⁹ A key challenge in the development of these inhibitors is the identification of specific allosteric pockets that can achieve robust inhibition. Nevertheless, over a decade ago, e.g., the myristoyl pocket in ABL was identified, paving the way for the development of inhibitors such as GNF-2 or asciminib. In contrast to traditional ATP-competitive inhibitors such as imatinib, these allosteric inhibitors bind outside the catalytic site; inducing a conformational change that effectively inactivates the kinase. This enables the targeting of specific resistance mutations that occur in the vicinity of the active site.⁷⁰⁻⁷¹ Recently, further approaches for the inhibition of kinases have been developed, extending beyond types I-IV. These encompass previously unexplored chemical space and novel chemical modalities, including bivalent (type V) and bifunctional molecules (Figure 3).^{39, 63} Type V inhibitors bind to two different regions (or domains) of the kinase, reflecting their name as bivalent ligands (Figure 3A).^{59, 67} These inhibitors are typically composed of a small molecule, that binds to the ATP-binding pocket, a linker, and a peptide that represents the substrate targeted by the specific kinase, aiming to increase the selectivity.⁶⁰ Early proof of concept studies used Src as a model enzyme and showed considerable promise.⁷² Nevertheless, to date, no inhibitor of this type has been approved.⁷³

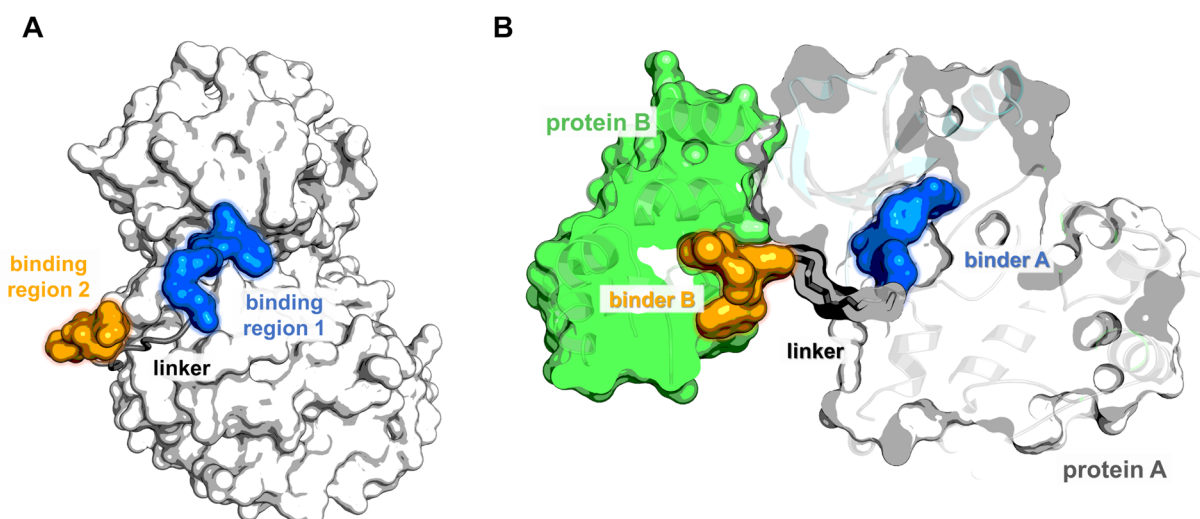


Figure 3. Additional strategies for kinase inhibition. **(A)** Type V (bivalent) inhibitors span two binding regions (or domains) of the kinases simultaneously. PDB ID: 5V61 was used as an example. **(B)** Bifunctional molecules feature a ligand (blue) to bind to the protein of interest (white) and a recruiting moiety (orange) to recognize a second protein (green). PDB ID: 6W8I was used as an example.

Bifunctional molecules generally comprise two distinct functional moieties: one that mediates binding to the protein of interest (POI) and another that recognizes a second protein. These components are connected by a linker. One example of this compound class are proteolysis-targeting chimeras (PROTACs).^{63, 74} In this example, a kinase-binding ligand is linked to a moiety that recruits an E3 ubiquitin ligase. Subsequently, the target kinase will be (poly)ubiquitinated upon achieving proximity to the aforementioned components (ternary complex), which will ultimately result in its degradation via the proteasome (Supplementary Figure 3).⁷⁴⁻⁷⁵ The principal benefit of PROTACs is their catalytic mechanism (event-driven pharmacology), which permits the degradation of target proteins at sub-stoichiometric concentrations.⁷⁴⁻⁷⁷ This may facilitate more sustained therapeutic effects and the targeting of proteins that were previously deemed "undruggable".^{74, 76} Currently, a number of PROTACs are undergoing clinical trials, primarily for the treatment of cancer. However, none have been fully approved yet, but ongoing trials are crucial in determining their future clinical use.^{74, 78}

While these diverse strategies each offer distinct advantages and limitations, competitive inhibition, for instance, must contend with the high intracellular concentration of ATP to effectively occupy the active site. In contrast, covalent targeting (classified as type VI, Table 1) introduces a specialized approach that, when a targetable amino acid is in the vicinity of the ligand, can be effectively integrated with all other types to achieve more profound therapeutic effects. The design and development of covalent inhibitors have gained significant traction in recent years, driven by their distinct mode of action (Supplementary Figure 2A).⁷⁹⁻⁸⁰ In contrast to reversible inhibitors, covalent inhibitors form a permanent bond with their target enzyme, typically with a nucleophilic residue (often a cysteine), leading to irreversible inhibition. The process of forming a covalent bond is comprised of two discrete steps (Figure 4).

The first step entails the reversible binding of the ligand to its biological target. This also results in the proximity of a reactive group (commonly referred to as a 'warhead' and often, but not exclusively, a moderately weak electrophile) on the ligand to the target amino acid on the protein. Subsequently, in the second step, a reaction occurs between the participating functional groups of the ligand and protein, resulting in a covalently modified and inactivated protein.⁸¹

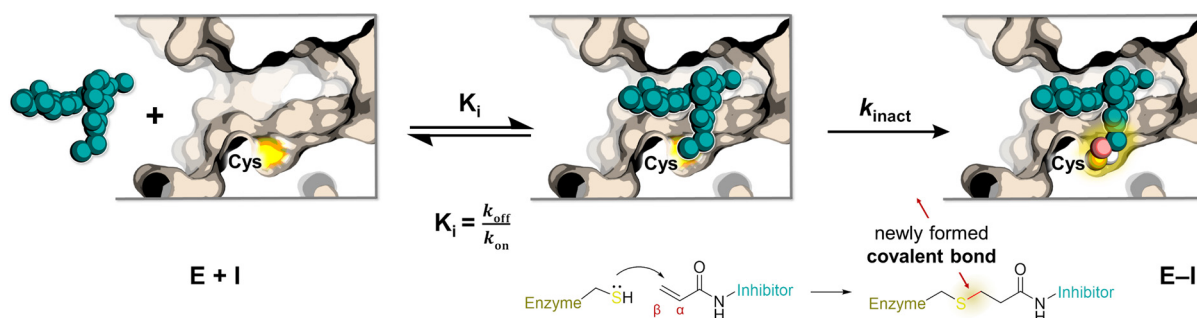


Figure 4. Schematic representation of the formation of a covalent enzyme-inhibitor complex. In the first step, the protein (blue) and ligand (purple) initially form a reversible complex (reversible affinity, K_i , defined as the ratio between off-rate (k_{off}) and on-rate (k_{on})). In the second step, a new covalent bond is formed between the reactive electrophile of the inhibitor and the thiol side chain of the enzyme, described by the maximum inactivation rate (k_{inact}). Figure inspired from Fassunke *et al.*⁸²

The successful implementation of covalent modification is contingent upon the fulfillment of three essential criteria: (i) the presence of a sufficiently nucleophilic and accessible amino acid within the target protein,⁸³ (ii) sufficient reversible affinity to form an enzyme-inhibitor complex, and (iii) the suitability of the warhead for covalent bond formation.⁸⁴ In addition, the reactivity should be carefully balanced to minimize off-target reactions while ensuring effective modification of the desired residue.⁸⁴ Notably, intrinsic reactivity is not the sole determining factor in achieving effective and selective covalent modification. Other considerations, such as the size, orientation, and geometry of the warhead, along with the nucleophilicity and orientation of the targeted amino acid and the surrounding protein environment, are equally important.⁸⁵⁻⁸⁷ Irreversible modification of the target protein confers a multitude of potential pharmacological advantages.^{81, 83, 87-88} Importantly, it allows for maximizing the on-target residence time and thus avoid continuous competition with ATP,⁸⁹⁻⁹⁰ consequently allowing lower dosing.⁹¹ Further advantages include a potentially increased potency and selectivity, which can result in reduced off-target effects.^{81, 90-91} This can, in turn, prevent side effects caused by a high dose and thus, increase the patient's quality of life during therapy. Moreover, it represents a promising avenue to overcome challenges associated with drug resistance and opens the potential to approach previously thought "undruggable" targets, that are difficult to inhibit with reversible agents.^{79, 92} Strategic covalent targeting has become a well-established strategy with a proven track record in cancer treatment. For instance, the clinical success of osimertinib highlights its potent and selective inhibition, effectively overcoming resistance against first-generation EGFR inhibitors by covalently modifying the T790M mutation of EGFR.⁹³⁻⁹⁴

Another notable example is the approval of sotorasib, which targets KRAS-G12C, a GTPase that had evaded direct targeting for more than 25 years. While reversible inhibitors would require competing with the pM affinity of KRAS for its substrates, a disulfide-fragment screening (tethering approach) unveiled a pocket accessible only to covalent ligands, laying the foundation of clinical covalent KRAS-G12C inhibitors.⁹⁵⁻⁹⁶ The exploration of covalent inhibition also extends beyond oncology, with emerging research focusing on inflammatory diseases, viral infections, and neurodegenerative disorders.^{79, 97} In addition, the covalent drug discovery toolbox is expanding with new and advanced warheads, alongside targeting additional amino acids like Lys, Ser, Asp, and Arg⁶⁰ and emerging chemoproteomics platforms to facilitate the identification of novel covalently binding moieties through "electrophile-first" approaches (Supplementary Figure 2B).^{79, 83, 98} As our understanding of kinase biology and covalent binding mechanisms grows, it is anticipated that covalent inhibitors will continue to play a substantial role in the future landscape of targeted therapeutics. However, despite these advantages, the development of covalent inhibitors is not without challenges. As previously stated, the irreversible nature of the bond engenders concerns regarding off-target toxicity via non-selective covalent modification.⁸¹ The application of structure-based drug design (SBDD) and the meticulous selection of reactive warheads have proved to be pivotal in the mitigation of these risks.^{79, 87}

1.3 The Iterative Cycle of Structure-based Drug Design

SBDD has emerged as a powerful approach for the development of innovative therapeutics for a wide range of diseases, including cancer. Over the past two decades, significant advances in techniques like molecular dynamics (MD), nuclear magnetic resonance (NMR) spectroscopy and protein X-ray crystallography have considerably strengthened the role of SBDD as a central strategy in modern medicinal chemistry.⁹⁹⁻¹⁰³ These technologies have facilitated the systematic design of bioactive ligands by assembling atoms or molecular fragments that conform precisely to the target protein binding sites. A particularly notable application of SBDD is in the development of kinase inhibitors.¹⁰⁴⁻¹⁰⁵ Given that kinases are highly conserved enzymes with well-characterized active sites, they are amenable to SBDD, which leverages detailed structural information to inform the design and optimization of potential inhibitors. Central to the SBDD approach is the availability of high-resolution crystallographic data, which provides insights into the three-dimensional (3D) architecture of the kinase.¹⁰⁵⁻¹⁰⁶ The recent advances of artificial intelligence (AI)-driven modeling tools such as AlphaFold have facilitated the prediction of protein structures with remarkable accuracy, thereby expanding the potential for SBDD to previously inaccessible targets.¹⁰⁷⁻¹⁰⁹ Indeed, the 2024 Nobel Prize in Chemistry recognized the impact of AI on protein structure prediction,¹¹⁰ underscoring its transformative role in drug discovery and beyond.

This structural knowledge, whether derived from crystallographic data or AI-based predictions, allows for the precise modeling of protein-ligand interactions, thereby enabling the identification of key binding determinants (pharmacophoric features) that govern affinity and selectivity.¹¹¹⁻¹¹² A salient feature of SBDD is its iterative nature, with each step building upon insights gained from previous cycles of design, synthesis, and biological evaluation (Figure 5).^{102, 113} The process typically commences with the identification of a disease-relevant target, such as a kinase that plays a role in oncogenic signaling. Structural data derived from complex structures, whether existing from previous studies or newly determined through e.g. protein crystallization, serves as the foundation for ligand design. However, if no structure is available, a cheminformatics approach can be used to model the target and predicted binding poses. The aforementioned structural information allows for the rational design of small molecules that exploit key features of the target's binding site.

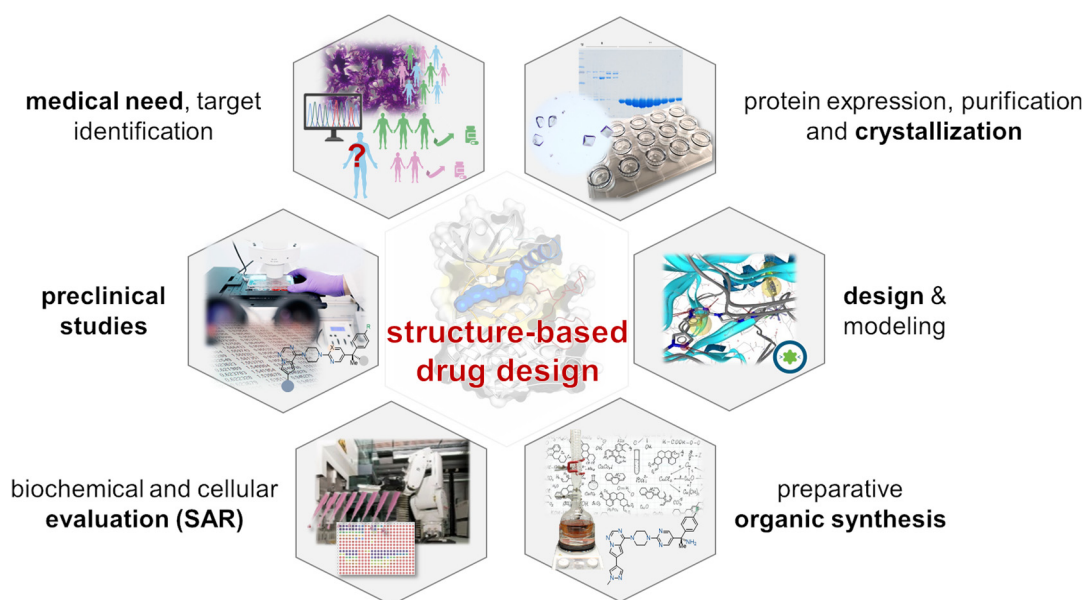


Figure 5. Schematic representation of the iterative structure-based drug design (SBDD) cycle. A disease-relevant target serves as a starting point, followed by either crystallization affords, analysis of already existing complex structures or modeling to rationally design ligands with tailored binding properties. Subsequent synthesis followed by biochemical and cellular evaluation, provides insights into biological properties such as inhibitory potency. Afterwards, in turn, protein expression, purification, and co-crystallization with the best performing modulators are carried out, aiming to obtain a new co-crystal structure, serving as a starting point for a new cycle of SBDD.

These tailored ligands can be designed to overcome specific challenges, such as resistance mutations, that often arise in kinase targets. Subsequent to the initial design phase, the candidate ligands are synthesized and subjected to a comprehensive biological evaluation. This encompasses *in vitro* biochemical assays for the generation of structure-activity relationship (SAR) studies, in addition to cell-based assays for the assessment of permeability and activity in relevant biological contexts. Additionally, preclinical evaluations may be conducted to ascertain the *in vivo* pharmacokinetic properties and potential toxicity of the compounds. The next crucial step is again co-crystallization with the most efficacious ligands.

This high-resolution data then reveals opportunities for further chemical modifications, guiding the next round of optimization. In conclusion, the multi- and interdisciplinary integration of structural biology, structure-based ligand design, organic synthesis, and biochemical and cellular evaluation renders SBDD a highly effective strategy for the identification of potent and selective lead compounds. SBDD has yielded significant breakthroughs in the treatment of kinase-driven diseases. However, notwithstanding the remarkable success of these rational and targeted approaches, the emergence of acquired drug resistance is usually inevitable.¹¹⁴ Gastrointestinal stromal tumors (GIST) can be considered as a prime example of precision medicine, where targeted approaches have transformed treatment. However, unfortunately, they are also prone to drug resistance, representing a persistent challenge.

1.4 Gastrointestinal Stroma Tumors (GIST)

GIST represent a unique and significant subset of mesenchymal tumors found within the gastrointestinal (GI) tract.¹¹⁵⁻¹¹⁶ Although GIST remains a rare subtype of cancer, the estimated annual incidence ranges from 10 to 15 cases per million individuals across the world, equating to thousands of new cases annually. Furthermore, the prevalence of patients with active disease has been observed to increase steadily over time.¹¹⁷⁻¹¹⁸ GIST predominantly affects middle-aged individuals, with a peak incidence observed at approximately 60 years of age, and a similar prevalence among males and females.¹¹⁸⁻¹¹⁹ It is widely accepted that GIST arises from an oncogenic transformation in the pacemaker cells of the gut, the interstitial cells of CAJAL.¹²⁰ GISTs can manifest in any region of the GI tract, with the stomach (approximately 60 %) and the small intestine (approx. 30 %) representing the most prevalent locations. Other sites include the duodenum, rectum, colon, and esophagus.¹²¹ The clinical presentation of these tumors is often nonspecific, exhibiting a wide range of symptoms contingent on their size and localization. The most commonly reported symptoms include GI bleeding and abdominal discomfort. Some patients may present with nausea or anemia, or also nonspecific symptoms as weight loss, fatigue, and early satiety depending on the location and size of the tumor.¹²²⁻¹²⁴ While many GISTs are asymptomatic and discovered fortuitously during imaging or surgical procedures for other conditions,¹²⁵⁻¹²⁶ others can manifest in an aggressive manner, leading to significant morbidity.¹²⁷⁻¹²⁸ The cornerstone of treatment for localized GIST is surgical resection, which aims to achieve complete removal of the tumor.¹²⁹⁻¹³⁰ Incomplete resection is associated with a high risk of recurrence.¹³⁰⁻¹³² In the case of small tumors (less than 2 cm) devoid of high-risk characteristics, a strategy of surveillance may be a viable option.^{130, 133} Nevertheless, surgical intervention is typically recommended for larger tumors or those with high-risk features such as rapid growth or symptomatic presentation.¹²⁹⁻¹³⁰ Unfortunately, in almost half of all cases, there is the potential for metastatic capacity, with the liver and peritoneum being the most common sites of metastasis. In advanced disease progression, metastases can also occur within distant sites, lungs and bone.^{116, 134}

Further, it should be emphasized that conventional radio- and chemotherapy have been demonstrated to offer only limited efficacy and are still associated with serious adverse effects.^{117, 135-136} Nevertheless, despite their relative rarity, the study of GIST has profoundly influenced our understanding of oncogenesis, particularly due to the distinctive molecular characteristics of these tumors. Since 1998, the identification of gain-of-function mutations in KIT as the major oncogenic driver has permitted the deciphering of the molecular mechanisms underlying these cancers.¹³⁷⁻¹³⁸ As a result of these advances, our comprehension of the pathobiology and the specific molecular mechanisms involved has undergone a significant transformation, which has in turn led to a paradigm shift in the successful treatment of these conditions.¹³⁹⁻¹⁴¹ Since effective targeted treatments have been introduced, the overall survival (OS) for patients with advanced GISTs has improved up to 8 years, compared to about 18 month with chemo- or radiotherapy.¹⁴² Nearly 99 % of GISTs have an identifiable driver alteration, guiding targeted therapy treatment strategies.¹⁴³ It is estimated that approximately 70 % of cases are driven by the stem cell growth factor receptor (SCFR, KIT).¹⁴²⁻¹⁴³ Around 15 % are driven by platelet-derived growth factor receptor alpha PDGFRA. The remaining 15 % of GISTs without KIT or PDGFRA mutations may exhibit succinate dehydrogenase (SDH) complex deficiency, or mutations in the GTPase-activating protein neurofibromin 1 (NF1), the kinase BRAF or the small GTPase Ras (Figure 6A).^{139, 143-144} As a result, KIT and PDGFRA have emerged as key targets in GIST. Both KIT and PDGFRA are type III receptor tyrosine kinases (RTK) that possess a conserved structural framework, which is essential for their function in cell signaling (Figure 6G). These RTKs are characterized by an extracellular domain (ED) comprising five immunoglobulin (IG)-like domains (D1-D5), a transmembrane segment, a juxtamembrane domain (JMD), and an intracellular kinase domain (KD), which includes the AL.¹⁴⁵⁻¹⁴⁸ KIT mutations are most frequently observed in the JMD (exon 11) or ED (exon 9) (Figure 6B), with exon 11 mutations occurring in approximately 70 % of GIST cases and thus being the most prevalent alteration.^{138, 149} The JMD is responsible for regulating kinase activity and is located within the back pocket in an inactive kinase state, thereby stabilizing it (*cf.* chapter 1.2.1). The mutations in this region are heterogeneous but appear to have a similar biological consequence (Figure 6D). Certain deletions (del), insertions (ins), or point mutations disrupt the inhibitory function, leading to constitutive receptor activation.¹⁵⁰⁻¹⁵² Approximately 10-15 % of KIT-mutant GISTs are caused by lesions in the ED (exon 9) (Figure 6B). Of these, the most prominent variant is the duplication (dup) A502_Y503dup (Figure 6E).^{150, 152} These mutations are associated with a more aggressive clinical course and are more commonly observed in tumors that originate from the small intestine.¹⁵³⁻¹⁵⁴

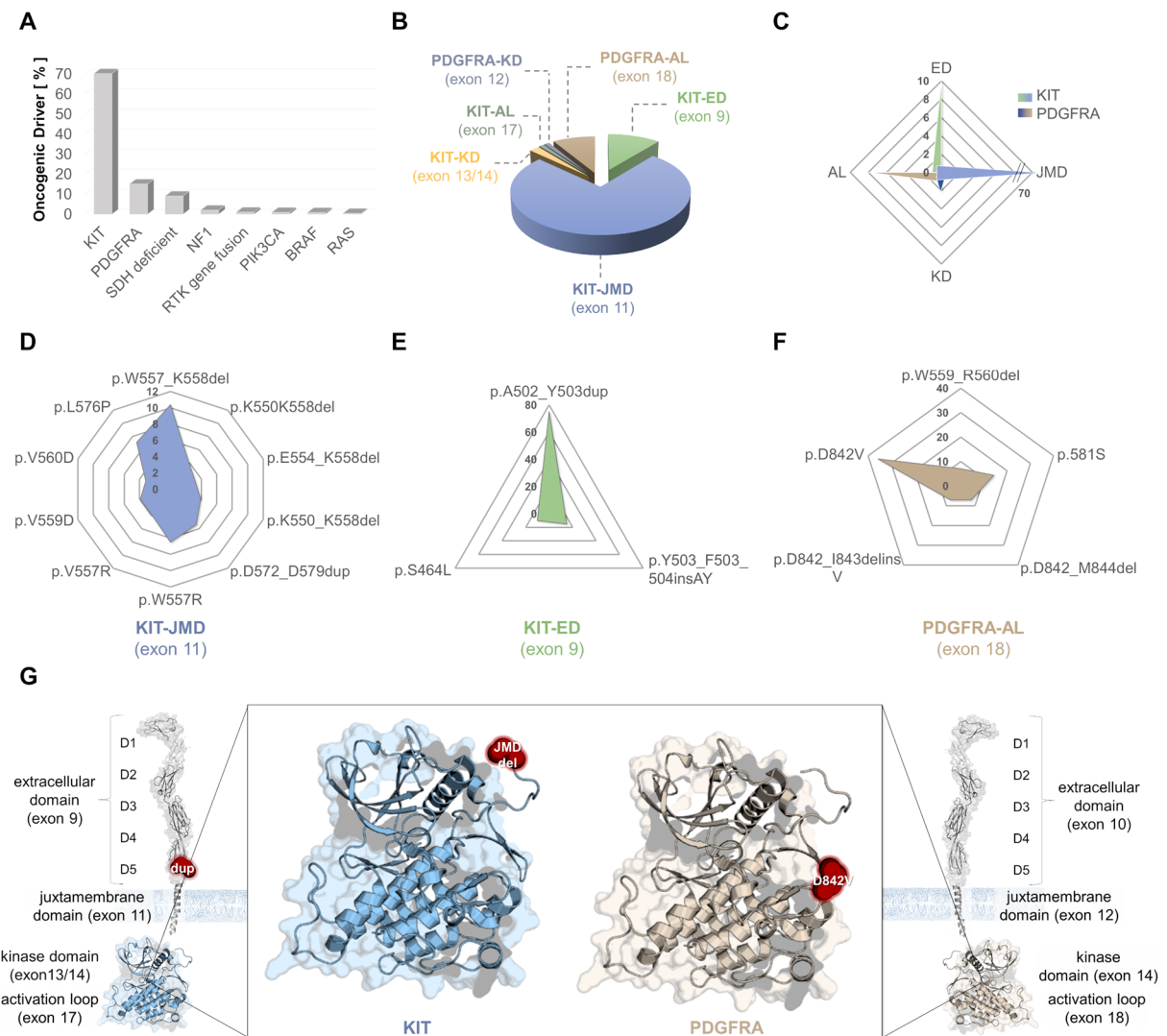


Figure 6. Landscape of driver oncogenes and activating mutations in GIST. **(A)** Bar chart illustrating the proportion of oncogenic drivers in GIST. **(B)** Pie chart indicating the frequency of primary mutations in KIT and PDGFRA by structural domains. **(C-F)** Radar chart displaying the frequency of primary mutations in certain structural domains in KIT and PDGFRA (C), the occurrence of genetic alterations in KIT-JMD (exon 11) (D), KIT-ED (exon 9) (E) and PDGFRA-AL (exon 18) (F) leading to GIST. **(G)** Overview of KIT (blue) and PDGFRA (brown). Localizations of common primary mutations are indicated as red blobs.¹⁵³⁻¹⁵⁸

The occurrence of primary mutations within the APB (exon 13/14) and the AL (exon 17) is relatively rare, with an estimated frequency of approximately 1 % in each case (Figure 6B).^{128, 155} In contrast, PDGFRA mutations are most frequently observed in the AL (exon 18) (Figure 6B), with the D842V point mutation being the most common (Figure 6F).^{150, 156} Therefore, despite their apparent similarity, the mutational transformation of these proto-oncogenes appears to differ (Figure 6C). Typically, an external stimulus, such as the binding of a ligand, causes the receptor to dimerize and undergo autophosphorylation.^{26, 157} Consequently, this phosphorylation event initiates the activation of assorted downstream signaling cascades, including the PI3K-AKT-mTOR, JAK-STAT-JUN, or RAS-RAF-MEK-ERK pathways, which ultimately regulate differentiation, survival, proliferation and apoptosis (Figure 7).^{116, 142, 158}

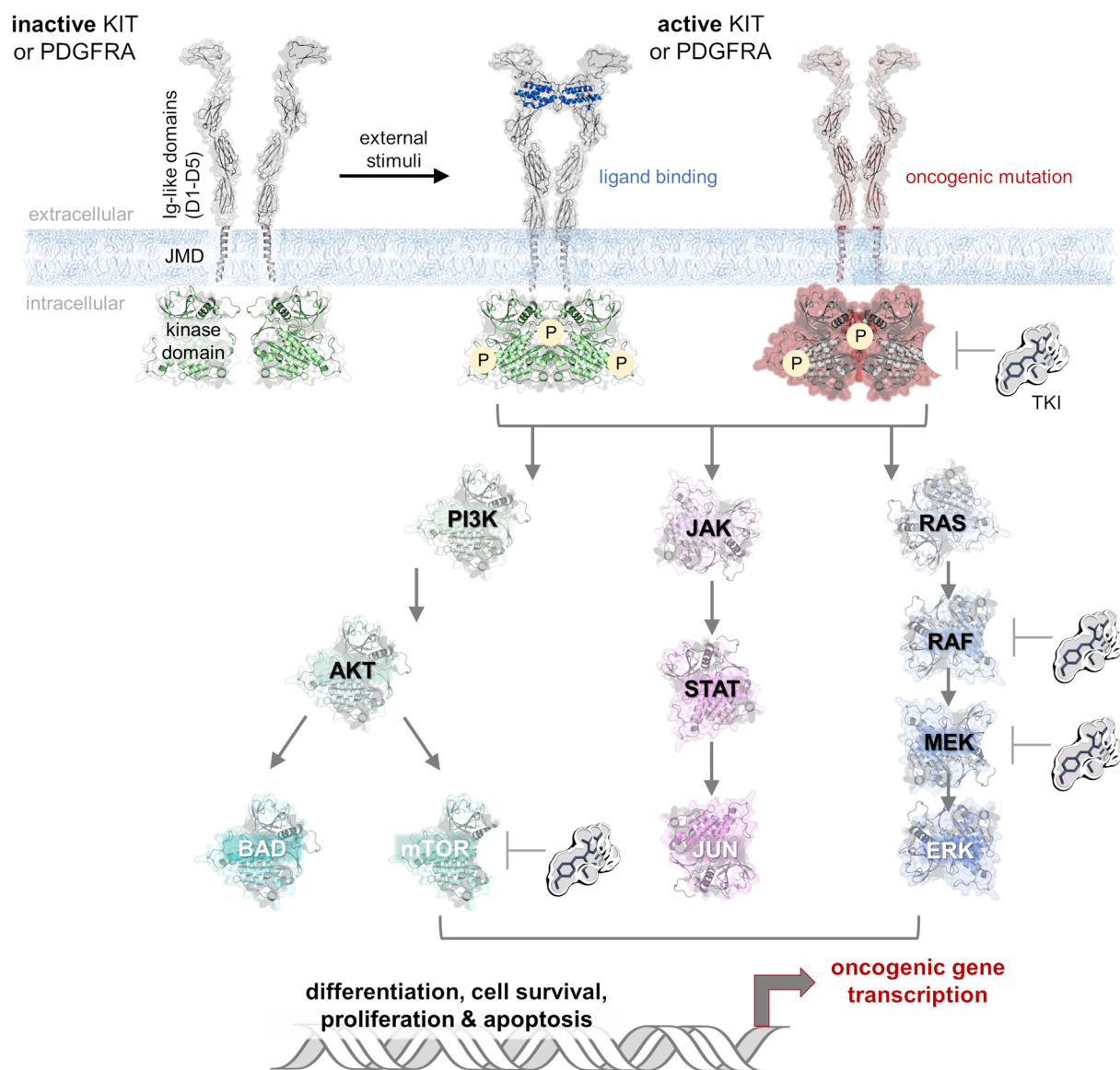


Figure 7. Key signaling pathways in KIT- and PDGFRA-driven GIST. Dimerization via ligand binding or activation by mutations induces phosphorylation and subsequent kinase activation, followed by stimulation of downstream cascades, ultimately controlling the cell fate.

However, in contrast to the ligand-binding-mediated activation, genetic alterations, such as mutations, can lead to a ligand-independent activation (constitutive activity) which results in the development of cancer, as in the case of GIST.^{141, 159} To date, five small-molecule tyrosine kinase inhibitors (TKIs) targeting KIT or PDGFRA have been approved, signifying a breakthrough in the treatment of GIST (Figure 8).

1.4.1 Current Therapeutic Options and Challenges

Imatinib represents the undisputed standard of care as the first-line therapy for unresectable or metastatic GIST, as well as for adjuvant treatment in patients with high-risk primary GISTs following surgical resection.¹⁶⁰ It binds to the inactive (DFG-out) kinase conformation of KIT, acting as an ATP-competitive inhibitor.

Imatinib interacts with the hinge region, specifically the backbone of Cys673 and gatekeeper Thr670, the DFG motif and the back pocket, utilizing both H-bonds and hydrophobic interactions, respectively, thereby stabilizing the inactive conformation (type IIa inhibitor) (Figure 8B).¹⁶¹ Imatinib has been demonstrated to be particularly efficacious against primary activating mutations, including those present in the JMD (exon 11) and, to a lesser extent, the ED (exon 9).¹⁶² Approximately 80 % of patients with GIST respond favorably to treatment with imatinib, which has been shown to provide a progression-free survival (PFS) of approximately two years, with a tolerable side effect profile.^{135, 163-165}

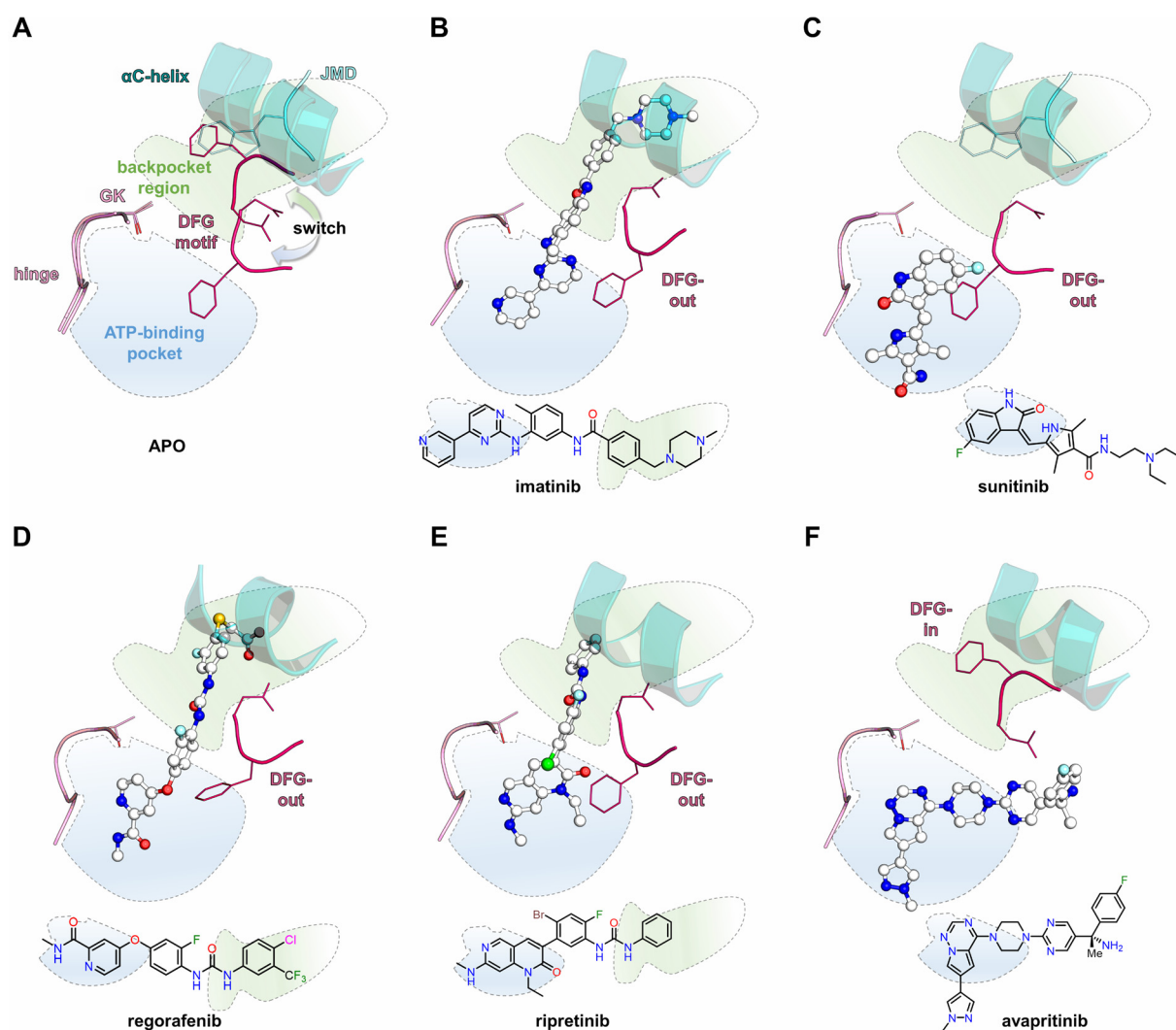


Figure 8. Approved drugs for the treatment of KIT- or PDGFRA-driven GIST and their respective binding modes. (A) Visualized APO structure of KIT-wt in an active (DFG-in, PDB ID: 8PQ9) and inactive (DFG-out, PDB ID: 1T45) conformation, showing important structural features such as the hinge and GK (pink), DFG motif (red), α C-helix (teal) and JMD (pale). The ATP-binding pocket is indicated in blue and the backpocket region in green. (B) Chemical structure and binding mode of imatinib (bound to KIT-wt, PDB ID: 1T46). (C) Chemical structure and binding mode of sunitinib (bound to KIT-wt, PDB ID: 3G0E). (D) Chemical structure and binding mode of a regorafenib derivative (bound to RIPK2, PDB ID 6ES0). (E) Chemical structure and binding mode of a ripretinib derivative (bound to KIT-wt, PDB ID 6MOB). (F) Chemical structure and binding mode of avapritinib (bound to KIT-wt, PDB ID: 8PQ9).

Despite its efficacy, imatinib ultimately fails to treat the majority of patients, with resistance typically developing within two years after treatment initiation.¹⁶⁶⁻¹⁶⁷ Secondary mutations, either localized within the ABP (exons 13/14)^{148, 168-169}, such as V654A or T670I, or within the AL (exon 17/18), like D816A^{III} or A829P, alter the protein structure, thereby rendering imatinib resistance (Figure 9).^{168, 170-171} It is notable that the underlying mechanism of resistance remain poorly understood.^{169, 172} The efforts to overcome these polyclonal resistance mutations resulted in the repurposing of sunitinib (Sutent®, Pfizer Inc.) and regorafenib (Stivarga®, Bayer AG) as second- and third-line therapies, respectively.¹⁶³ Both were developed as vascular endothelial growth factor receptor (VEGFR) inhibitors, yet they were also found to exhibit activity against imatinib-resistant KIT mutants.^{115, 173-174} Both inhibitors bind to the inactive kinase conformation; however, while regorafenib's binding mode also extends the back pocket (type IIa), whereas sunitinib does not (type IIb) (Figure 8C, D). In the context of secondary mutations in KIT, sunitinib has been demonstrated to exhibit therapeutic efficacy against ABP mutations (exon 13 and 14), whereas regorafenib has shown effectiveness in targeting secondary mutations within the AL (exon 17) (Figure 9).¹³⁹ In clinical trials, the inhibitors have exhibited a median PFS rate of 5.6 months for sunitinib and 4.8 months for regorafenib.¹⁷⁵⁻¹⁷⁶ Nevertheless, this relatively moderate clinical activity already indicates that none of the aforementioned agents is capable of addressing the full spectrum of resistance mutations (Figure 9). Furthermore, neither inhibitor was developed with the objective of targeting GIST-relevant oncogenes. Instead, they are multikinase inhibitors with a broader spectrum of kinase inhibition, which results in a less favorable side effect profile.^{115, 135} The most commonly observed adverse effects of sunitinib treatment include anemia, neutropenia, diarrhea, nausea, and hand-foot syndrome.¹⁷⁷ Regorafenib treatment has also been linked to hepatotoxicity, or chemical-induced liver damage.¹⁷⁸ Hence, the development of imatinib-resistance still remains a major challenge in the long-term management of GIST. This has prompted the development of novel inhibitors to circumvent resistance. In 2020, the FDA granted approval for ripretinib (Qinlock®, Deciphera Pharmaceuticals, Inc.), which is now the standard of care for fourth- or later line of treatment.¹⁷⁹ Ripretinib is a next-generation type IIa TKI that effectively inhibits a broad range of primary and secondary mutations, particularly within the AL (exon 17) (Figure 9).¹⁸⁰⁻¹⁸¹ It is one of the first drugs specifically developed for the treatment of GIST.^{180, 182} In the INVICTUS trial, a significant improvement in PFS (around 6 months) and overall survival was observed in GIST patients who had progressed on at least three prior TKIs compared to those who received a placebo.¹⁸³⁻¹⁸⁴ Based on the promising results, a further phase III trial (INTRIGUE) was conducted to assess its use as second-line therapy compared to sunitinib.^{181, 185}

^{III} Hotspot for AL resistance, introducing different amino acids, including Ala or His.

However, ripretinib failed to show an advantage in the PFS endpoint over sunitinib. The trial demonstrated that patients with a secondary AL (exon 17) mutation exhibited significantly improved outcomes with ripretinib compared to sunitinib. In addition, a biomarker substudy utilizing ctDNA revealed that ripretinib was ineffective against primary ED (exon 9) mutations and a range of ABP mutations, including KIT-V654A.^{43, 181} Conversely, it is established that sunitinib has a strong inhibitory potency against this mutation.¹⁷⁵ This provides a potential explanation for the negative outcome of the trial. The findings revealed not only the liabilities of the currently and newly approved drugs, but also highlighted the value of ctDNA biomarker analysis for predicting the response of certain TKIs to imatinib resistance mutations.¹⁸⁶⁻¹⁸⁷

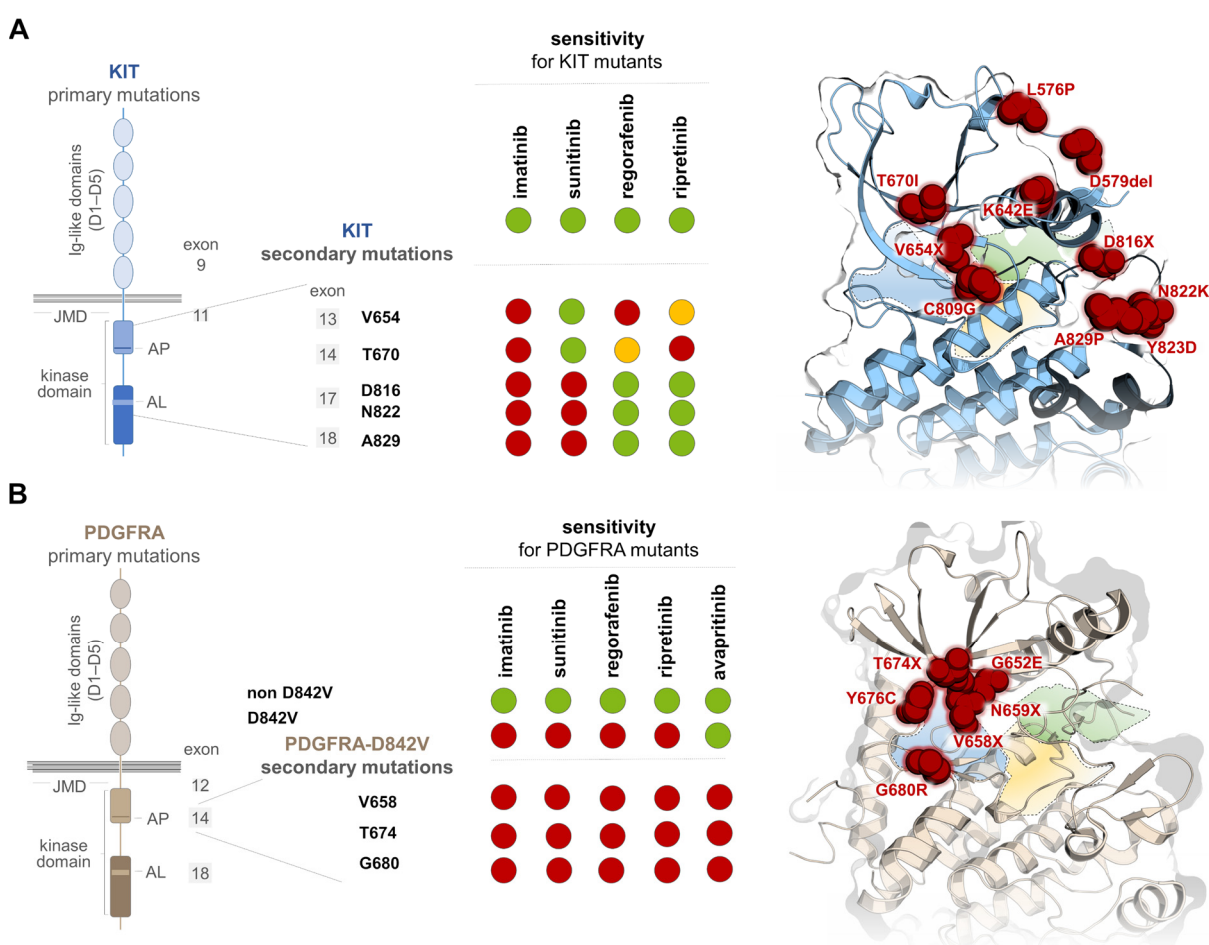


Figure 9. Schematic representation of the sensitivity of the approved drugs for the treatment of GIST on different KIT (**A**) and PDGFRA (**B**) primary and secondary mutations, including the structural localization of certain resistance mutations within the kinase domain. Sensitivity towards specific mutations is summarized and indicated as green (potent), yellow (moderate), and red (resistant) based on data published in S. Grunewald, *et al.* (2021), C. M. Cicala, *et al.*, (2024), and T. Mühlenberg, *et al.*, (2024).^{43, 187-188}

Imatinib resistance in GIST can be classified as either secondary, as described above, or primary (Figure 9).^{139, 189} Primary imatinib resistance has been observed in up to 10 % of cases and is frequently associated with PDGFRA-D842V-driven tumors.¹⁹⁰⁻¹⁹¹ For over a decade, the PDGFRA-D842V mutation has been notoriously unresponsive to any TKI.^{139, 156, 190} It is postulated that this AL mutation induces an active kinase conformation, which renders type II inhibitors such as imatinib ineffective.^{188, 192}

However, the treatment options for patients with this genetic predisposition have recently undergone a significant improvement following the approval of avapritinib (Ayvakit®, Blueprint Medicines Corporation), which demonstrated an overall response rate exceeding 90 % in the NAVIGATOR trial.¹⁹³ Avapritinib was developed with the objective of inhibiting both PDGFRA-D842V and secondary KIT mutations.^{141, 194} It has demonstrated to transform the notorious genotype (D842V) into a chronic disease, in a manner analogous to that observed with imatinib in the case of KIT-driven GIST.^{193, 195} Avapritinib acts as an ATP-competitive inhibitor and is thought to bind in a type I inhibitor fashion (*cf.* Figure 8); yet little has been reported about its exact mode of action and medicinal chemistry development.^{48, 196} Nevertheless, despite its transformational activity in a first line setting, patients eventually progress, mostly due to acquired resistance mutations such as the prominent solvent-front mutation G680R.¹⁸⁸ In addition, the VOYAGER trial showed its liability also against secondary KIT mutations in the ABP.¹⁹⁵ Moreover, avapritinib treatment is associated with neuro-cognitive side effects in a subset of patients which further limits its broad use.^{48, 188, 197} In conclusion, are approved TKIs are reversible ATP-competitive inhibitors, associated with severe side effects, not developed for GIST and are susceptible to resistance mutations in either the ABP or AL. It is therefore imperative that innovative, potent, and selective inhibitors be developed to address this urgent need. The advent of the first tailored inhibitors for GIST has stimulated the development of next-generation inhibitors that seek to overcome the challenges posed by the current landscape of acquired drug resistance (*cf.* Supplementary Figure 4).

1.4.2 Future Directions Targeting Drug-Resistant GIST

Recent, structure-based design efforts afforded the novel type II quinazoline-based inhibitor NB003 (formerly AZD3229).¹⁹⁸ NB003 showed impressive potency against a range of secondary KIT (AP and AL) and PDGFRA-D842V mutant Ba/F3 cell lines.¹⁹⁹ At the moment a phase I study is enrolling patients to assess efficacy and safety as treatment option upon one failed line of treatment.²⁰⁰⁻²⁰¹ IDRX-42 (formerly M4205) is an imidazopyridine-based type II inhibitor that has demonstrated high biochemical potency against a range of secondary KIT mutations in the AP and AL.²⁰² These promising results also translated into efficacy in patient-derived xenograft models.²⁰³ A phase I trial is currently ongoing to investigate its efficacy against patients who progress on imatinib.²⁰⁴ However, in preclinical cell model systems, the gatekeeper mutation (KIT-T670I) and the solvent-front mutation (PDGFR-G680R) were found to be associated with a high level of resistance.⁴³ One candidate that was investigated for repurposing in GIST is ponatinib. Ponatinib has been approved for the treatment of chronic myeloid leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia.²⁰⁵⁻²⁰⁶ This type II inhibitor was developed specifically to target the gatekeeper mutation ABL-T315I, utilizing an ethynyl imidazo-pyridazine scaffold.²⁰⁷⁻²⁰⁸

In the POETIG trial, it demonstrated a distinctive capacity to effectively inhibit both AL mutants, including the D816X variant, as well as the challenging gatekeeper mutation (KIT-T670I).²⁰⁹⁻²¹⁰ However, at a dosage of 30 mg, severe adverse effects, particularly thromboembolic events, including cardiac incidents, were observed.²¹¹⁻²¹² Moreover, combination therapies, which combine TKIs with other agents that e.g. target complementary pathways, or cycling therapies, are being investigated with the aim of enhancing therapeutic efficacy and overcoming resistance.^{187, 213-216} One example is the combination of sunitinib with bezucastinib (CGT9486, previously designated as PLX9486).²¹⁷ Bezucastinib is a novel pyrrolo-pyridine-based type I inhibitor developed through a structure-based approach on a new structure of KIT-D816V.²¹⁸ The rationale for this trial was based on the complementary profile of sunitinib, which has been demonstrated to be effective against secondary AP mutations, and the potency of bezucastinib against AL mutations. Nevertheless, combining two agents with a complementary activity pattern poses a challenge in achieving a clinically effective dose without inducing adverse effects that are too burdensome to the patient.¹⁸⁷ In light of the current challenges associated with KIT/PDGFR inhibitors, alternative strategies are being explored to target drug-resistant GIST.¹⁸⁷ These include other targeted agents, such as heat shock protein 90 (HSP90) inhibitors²¹⁹⁻²²⁰, histone deacetylase inhibitors²²¹⁻²²² or BRAF inhibitors²²³⁻²²⁴, as well as immunotherapy²²⁵⁻²²⁶. Nevertheless, thus far, these agents appear to be of limited clinical value, either due to a lack of efficacy or the occurrence of adverse effects. It is noteworthy that additional studies are still pending, which have yet to elucidate the contribution to clinical efficacy and benefit.^{115, 135} Overall, TKIs have markedly improved clinical outcomes for patients with GIST. Nevertheless, the current drugs are limited by many acquired resistance mutations. Given the prominent role of KIT as the key oncogenic driver and emerging evidence indicating that KIT remains the central driver even in later lines of treatment⁴³, there is a pressing need to develop next-generation inhibitors that can target multiple resistant forms of these kinases and feature improved toxicity profiles.

Chapter: 2

MOTIVATION AND AIMS

2. Motivation and Aims

GIST represent the most common mesenchymal tumor of the GI tract and are predominantly driven by activating mutations in KIT and PDGFRA. Patients harboring mutations in these kinases benefit from first-line treatment with the TKIs imatinib (for primary KIT mutations) and avapritinib (for the primary mutation PDGFRA-D842V). However, despite the transformative impact of these targeted approaches, the emergence of acquired drug resistance - due to secondary mutations in the ATP-binding pocket and/or the activation loop - significantly limits long-term success. Although several TKIs have been approved to target a range of secondary KIT mutations, no single agent effectively inhibits both ABP and AL mutations. Notably, the mutations KIT-D816X (AL mutation, analogous to PDGFRA-D842V) and KIT-T670I (ABP mutation, analogous to PDGFRA-T674I), along with the PDGFRA-G680R solvent-front mutation, remain therapeutically challenging. As a result, the heterogeneity of drug resistance persists a major challenge, underscoring the urgent need for novel, potent, and selective inhibitors. In addition, many of the currently approved drugs have been repurposed from other oncological indications and are often plagued by issues related to selectivity and safety profiles, including serious side effects. While there are therapeutic options for imatinib-resistant KIT-driven GIST, there are currently no effective inhibitors for avapritinib-resistant PDGFRA-driven GIST. In addition, avapritinib treatment is associated with neurocognitive side effects, necessitating the development of next-generation PDGFRA inhibitors. However, there is a lack of detailed knowledge regarding its medicinal chemistry development and binding mode. Nevertheless, understanding these factors is pivotal for developing inhibitors capable of overcoming these challenges. Recently, Dr. ALINA TEUBER, solved the first crystal structures of avapritinib in complex with KIT and PDGFRA. Building on these, one objective was the structural biology analysis to understand critical protein-ligand interactions of avapritinib and to provide insights into resistance mechanisms. Moreover, this structural information should foster the structure-based design, synthesis, and characterization of novel avapritinib derivatives. Uncover key pharmacophoric features that help overcome drug resistance while limiting blood-brain barrier (BBB) penetration to reduce unwanted cognitive side effects. Given the unmet medical need for both highly effective and selective inhibitors, it is imperative to deepen our understanding of the structural mechanisms underlying resistance. These insights are vital to guiding the rational design of next-generation inhibitors. To address these challenges, this work employed a comprehensive approach, integrating in-depth structural biology analysis, *in silico* modeling combined with *in vitro* as well as *in cellulo* evaluation of existing and novel TKIs to investigate how mutations in the ATP-binding pocket and activation loop alter kinase function and affect inhibitor binding. By elucidating key protein-ligand interactions, this research aims to reveal how specific mutations confer drug resistance, ultimately providing the foundation for the rational design of novel inhibitors.

The limitations of currently approved reversible TKIs in terms of selectivity and the lack of efficacy against the plethora of resistance mutations, call for the investigation of innovative approaches. Thus, a key area of exploration was the development of covalent inhibitors, which have demonstrated superior efficacy in overcoming drug resistance in other cancer types. Despite their potential, covalent targeting in GIST has not been extensively pursued. Against this background, this study aims to assess the potential of covalent inhibitors designed specifically to target drug-resistant KIT and PDGFRA variants, with the objective of achieving enhanced potency and selectivity. Therefore, structure-based approaches guided the design and synthesis of inhibitors with a covalent mode of action to establish SAR, offering insights into important protein-ligand interactions to effectively inhibit drug resistance mutations.

AVAPRITINIB-BASED SAR STUDIES UNVEIL THE NOVEL G α -POCKET

Parts of this chapter have been published: Teuber, A.[‡]; **Schulz, T.[‡]**; Fletcher, B. S.; Gontla, R.; Mühlenberg, T.; Zischinsky, M. L.; Niggenaber, J.; Weisner, J.; Kleinbolting, S. B.; Lategahn, J.; Sievers, S.; Müller, M. P.; Bauer, S.; Rauh, D., *Avapritinib-based SAR studies unveil a binding pocket in KIT and PDGFRA*. *Nat Commun* **2024**, 15 (1), 63.

3. Avapritinib-based SAR Studies Unveil the Novel α -pocket

3.1 Avapritinib in PDGFRA-driven GIST: Challenges and Opportunities

Gastrointestinal stromal tumors represent a prime model for precision oncology, showcasing the groundbreaking potential of precision medicine in addressing specific genetic dysfunctions in cancer therapy. The first TKI, imatinib, has been established as an efficacious treatment for primary KIT-mutant GIST.¹³⁹ Nevertheless, for over a decade, the most prevalent PDGFRA primary mutation (D842V) has persistently evaded all known inhibitors.^{156, 160, 193, 227} This resistance is attributed to the preferential promotion of the active kinase conformation, which renders type II inhibitors like imatinib ineffective (Figure 10).^{188, 192} Fortunately, for patients with this mutation, treatment has recently become an option with the approval of avapritinib.¹⁹³

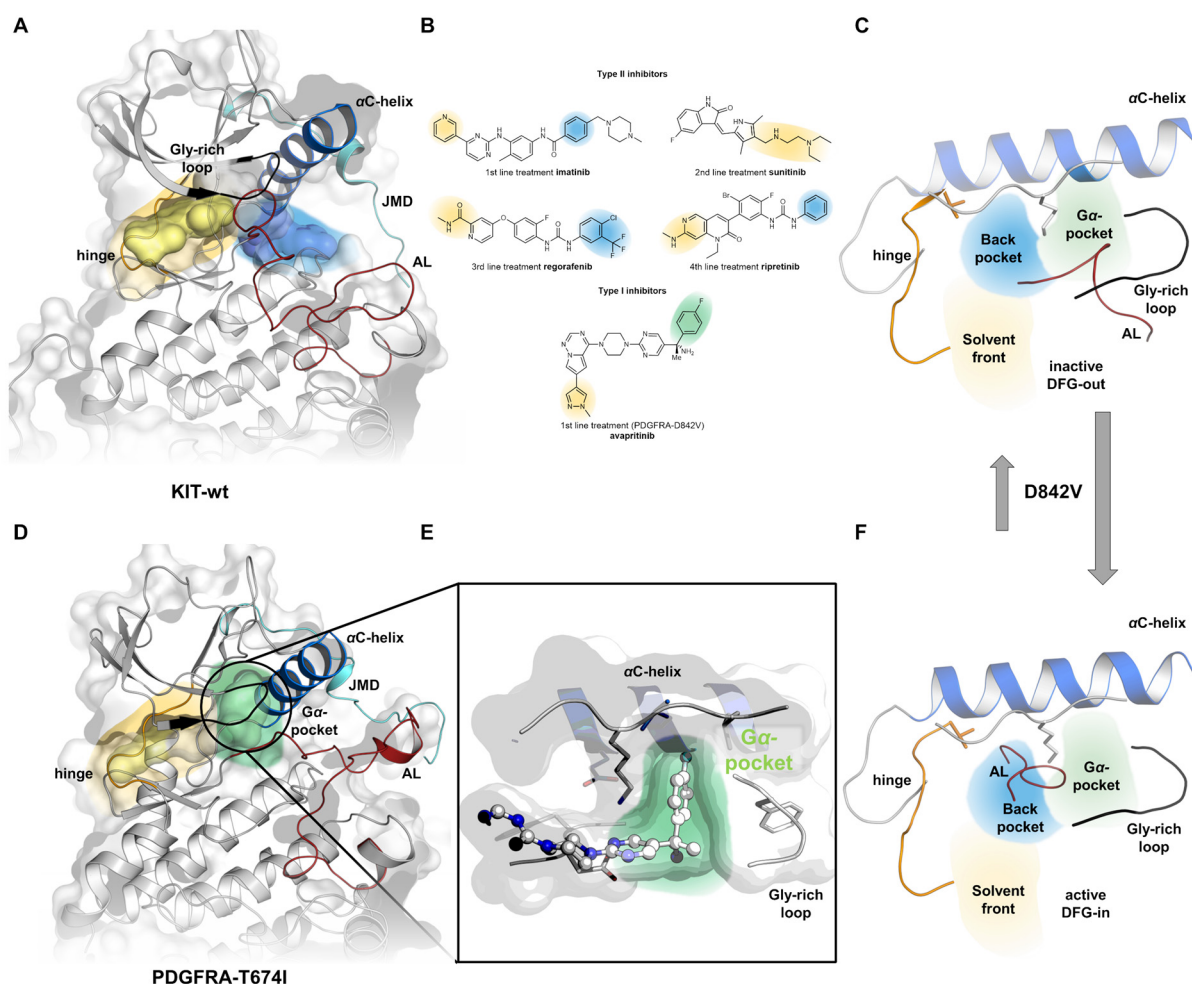


Figure 10. Type I and type II inhibitors targeting KIT and PDGFRA-driven GIST. (A) Inactive kinase conformation engaged by the type II inhibitors: imatinib (PDB-ID: 1T46), sunitinib (PDB-ID: 3G0E) and a ripretinib derivative (PDB-ID: 6MOB). The inhibitors are shown as surfaces. (B) Lewis structure of approved TKIs for GIST treatment. (C, F) Key structural elements of an inactive DFG-out (C) and an active DFG-in (F) conformation, indicating the activating influence of the PDGFRA-D842V mutation. (D) Generated co-crystal structure of avapritinib in PDGFRA-T674I (PDB-ID: 8PQH). (E) Close-up of the identified α -pocket. The figure is adapted from Teuber and Schulz, *et al.* (2024).⁴⁸

Although the clinical efficacy is well documented, its side effects, particularly cognitive issues such as memory impairment and confusion, are a cause for concern for certain patients. It is probable that these (cognitive side effects) are a consequence of the penetration of the blood-brain barrier.^{197, 228} Moreover, acquired resistance further limits the clinical benefit.¹⁸⁸ It is notable that publicly accessible data on medicinal chemistry development is scarce, particularly with regard to structural insights. Nevertheless, this is of considerable importance, as such insights could prove invaluable in understanding the susceptibility to resistance mutations and in guiding derivatization to reduce BBB penetration. Chemoinformatic approaches have been utilized to hypothesize about the binding mode of avapritinib, yet these methods have yielded inconsistent conclusions.^{229, 230} Thus, there is a pressing need for experimental structural data to resolve how avapritinib interacts with KIT and PDGFRA. Against this background, Dr. ALINA TEUBER set out to address this question within her PhD thesis; and successfully crystallized KIT and PDGFRA in complex with avapritinib.^{231IV} The structural data served as the basis for the subsequent structural biology analysis and informed the design and development of novel derivatives for the identification of critical pharmacophoric elements, with the objective of circumventing drug resistance and minimizing BBB penetration.

3.2 Key Protein-Ligand Interactions and the Novel α -pocket

The crystal structure of PDGFRA-T674I complexed with avapritinib (Figure 10D) demonstrated a DFG motif in the "in" conformation, with Asp836, of the DFG motif, oriented towards the ABP, exhibited an extended AL. Consequently, avapritinib binds to an active-like kinase conformation (Figure 10D,F). The binding of avapritinib to the hinge region is facilitated by an H-bond between the pyrrolo triazine scaffold's N2 nitrogen and the backbone of Cys677, as well as hydrophobic interactions with Leu599, Tyr676, Ala625, and Leu825 (Figure 11A,B). Additionally, the solvent-exposed methyl pyrazole engages in a cation- π interaction with Arg597 (Figure 11B). Interestingly, the piperazine moiety did not appear to be involved in crucial protein-ligand interaction. However, contacts were observed with Val607 and Cys835. Additional interactions are facilitated by the pyrimidine ring, which interacts with Val607 and establishes a H-bond with the catalytic lysine (Figure 11E). Further, an ionic interaction was observed between Asp836 (DFG motif) and the amine at the stereogenic center (Figure 11, Supplementary Figure 5).

^{IV} Protein expression, purification, and crystallization carried out by Dr. Alina Teuber, TU Dortmund University (AG Rauh).

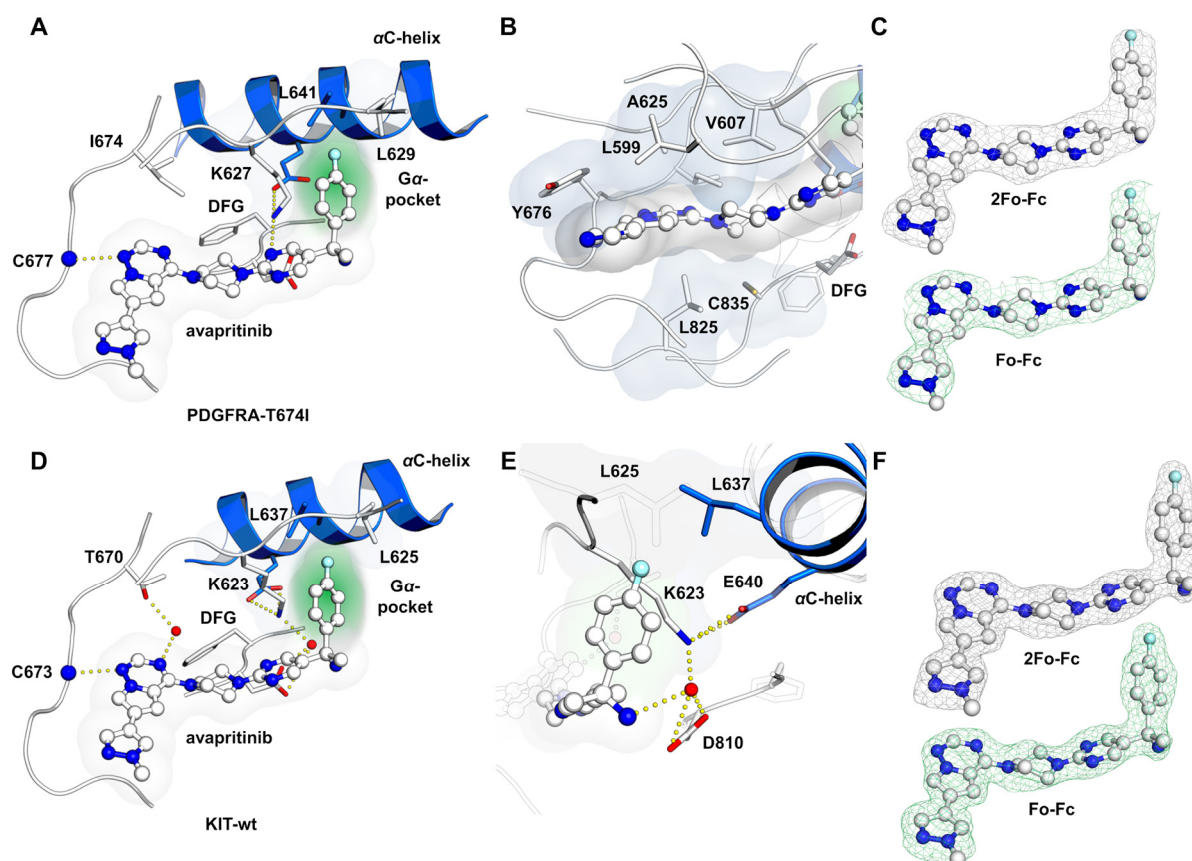


Figure 11 Complex structure of avapritinib bound to mutant PDGFRA (PDB-ID: 8PQH) and KIT-wt (PDB-ID: 8PQ9). (A) Overview of the binding mode of avapritinib in PDGFRA-T674I. (B) Hydrophobic interactions between avapritinib's scaffold and the hinge region of PDGFRA. (C) Respective electron density maps: $|2Fo-Fc|$ - (r.m.s.d. = 1.0) and $|Fo-Fc|$ omit (r.m.s.d. = 2.8) are shown. (D) Overview of the binding mode of avapritinib in KIT-wt. (E) Close-up of the interaction pattern enabled by the primary amine. (F) Respective electron density maps: $|2Fo-Fc|$ - (r.m.s.d. = 1.0) and $|Fo-Fc|$ omit (r.m.s.d. = 2.8) are shown. The figure is modified based on Teuber and Schulz, *et al.* (2024).⁴⁸

Upon avapritinib binding, a specific protein conformation is stabilized (DFG-in, C-helix-out conformation). Within this conformation, residues of the Gly-rich loop (Gly605 and Val607), Leu641 of the α C-helix, Leu629, and the catalytic lysine (Lys627) form a previously unidentified pocket. The pocket is perfectly suited to accommodate the shape of the fluorobenzene moiety, with additional cation- π interactions observed between the fluorobenzene and Lys627. This novel sub-pocket is located distal to the ATP-binding pocket and distinct from the back pocket. Given that this pocket has not been previously studied or observed in KIT/PDGFRA or other kinases, and reflecting its location between the Gly-rich loop and the α C-helix, this novel pocket will henceforth be referred to as the $G\alpha$ -pocket (Figure 10E, 11). It is noteworthy that the $G\alpha$ -pocket is observed to form exclusively upon ligand binding. In both the active (e.g., PDB IDs: 1PKG, 7KHK, 7KHJ) and inactive (e.g., PDB IDs: 1T46, 4U0I, 6MOB) states, this pocket appears to be occluded and inaccessible (Figure 12).

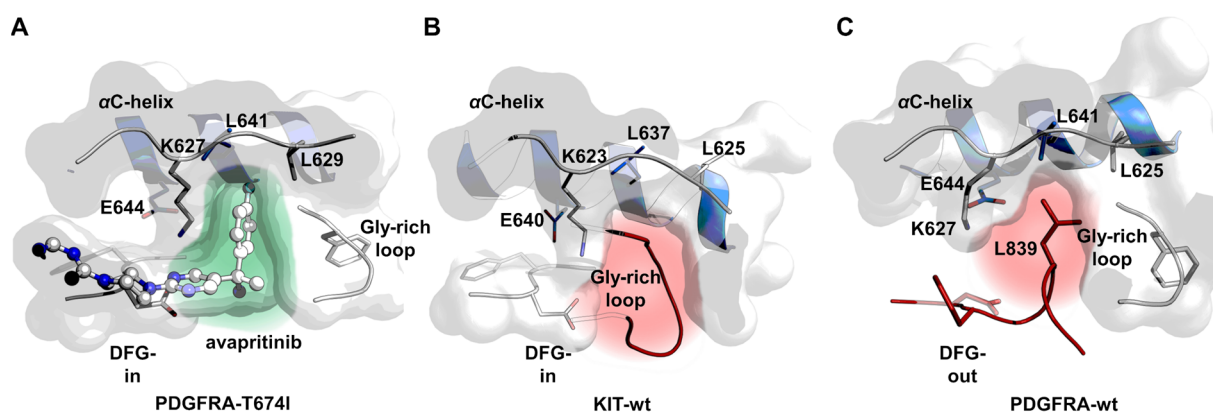


Figure 12. Structural analysis of the α -pocket in different activity states of the kinase. (A) In presence of avapritinib the α -pocket is formed by Phe604, Lys627, Leu629, Glu644, and Leu641 (PDB ID: 8PQH). (B) Active kinase conformation of KIT (PDB ID: 1PKG). The α -pocket is closed by the Gly-rich loop. (C) Inactive kinase conformation of PDGFRA (PDB ID: 8PQJ). The α -pocket is occupied by Leu839 of the activation loop. The figure is modified based on Teuber and Schulz, *et al.* (2024).⁴⁸

In an active kinase conformation, the glycine-rich loop and the α C-helix move toward the ATP-binding site, resulting in the concealment of the α -pocket (Figure 12B). In inactive conformations, the α -pocket is occupied by the AL, particularly amino acid residues from the LARDI motif (Figure 12C). Avapritinib seems to induce this unique pocket by displacing the AL, locking the kinase into a DFG-in yet α C-helix-out conformation. It remains to be established whether this pocket formation occurs as a result of a conformational selection or induced-fit mechanism. Nevertheless, it can be reasonably proposed that this distinctive structural characteristic plays a pivotal role in conferring the high potency and selectivity of avapritinib. The structural analysis suggests that avapritinib exhibits characteristics of a type 1.5 inhibitor, more than the presumed type I mode of action¹⁸⁸, particularly given its binding to a DFG-in but α C-helix-out conformation.²³² In light of these insights, a more detailed examination of diverse conformations of KIT and PDGFRA was conducted with respect to the R-spine assembly, encompassing ligand-bound and apo structures (Figure 13).

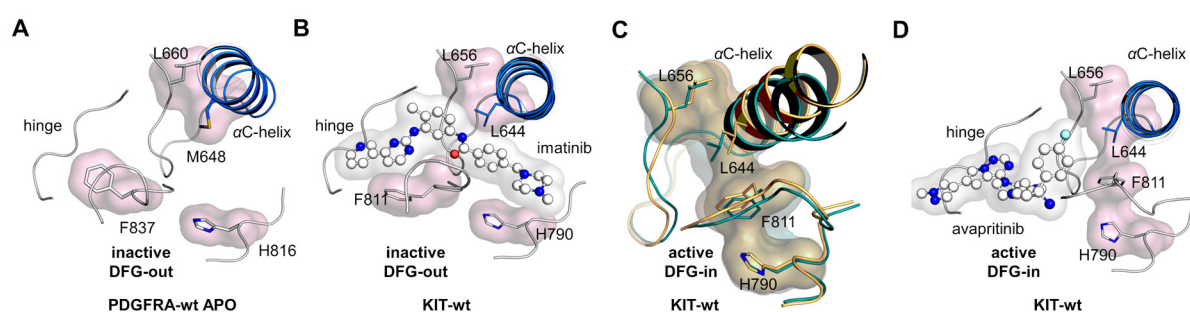


Figure 13. Analysis of the R-spine assembly in KIT and PDGFRA APO and ligand bound states. (A) Inactive PDGFRA-wt structure displaying a DFG-out conformation without formed R-spine (PDB ID: 8PQJ). (B) Inactive co-crystal structure of imatinib bound to KIT-wt, showing a DFG-out conformation without formed R-spine (PDB ID: 1T46). (C) Comparison of an active (teal, PDB-ID: 1PKG) and avapritinib bound KIT-wt structure (TKI not shown, wheat, PDB-ID 8PQ9). (D) Co-crystal structure of avapritinib bound to KIT-wt, displaying R-spine formation (PDB-ID: 8PQ9). The figure is modified based on Teuber and Schulz, *et al.* (2024).⁴⁸

As expected, no assembled R-spine was observed for inactive kinase states in an apo (PDB ID: 8PQJ) and inhibitor-bound (PDB ID: 1T46) scenario (Figure 13A, B), but the R-spine was successfully formed for active conformations (ATP-bound, PDB ID: 1PKG) (Figure 13C). Notably, the complex structures with avapritinib also demonstrated an assembled R-spine (PDB ID: 8PQ9, Figure 13D). The generated crystal structures revealed for the first time the binding mode of avapritinib and led to the identification of a previously identified pocket (α -pocket), providing critical insights into protein-ligand interactions of type I (1.5)-like inhibitors in KIT and PDGFRA. Thus, the findings once again underscore the indispensable role of experimentally determined structures in the drug discovery process.

3.3 Structural Insights into Avapritinib Resistance

In the study by Grunewald *et al.*, clinically relevant secondary gatekeeper mutations (PDGFRA-Thr674) with either an isoleucine (T674I) or an arginine (T674R) were observed to confer resistance to avapritinib.¹⁸⁸ Avapritinib was introduced into the established crystallization pipeline using KIT and PDGFRA wild type and GK mutant variants to elucidate how these mutations mediate resistance.²³³ Thereby, Dr. ALINA TEUBER solved a pair of high-resolution complex structures with avapritinib bound to both KIT-wt and KIT-T670I as well as apo structures of PDGFRA-wt and -T674I. Notably, these structures represent the first structures of KIT to feature the T670I gatekeeper mutation. While a water-mediated interaction between Thr670 and the pyrrolo-triazine scaffold can be observed in the wild type, in the case of the T670I, the isoleucine replaces this. Accordingly, the T670I mutation displaces the water molecule, resulting in a loss of interaction and a reduction in binding affinity (Figure 14A,B). The hydrophobic nature of the substituted isoleucine, which is adjacent to the polar ligand scaffold, is likely to contribute to this observed decrease in binding affinity. Of note, in literature, MD simulations in the literature have proposed that the T670I mutation causes resistance through conformational changes, particularly of the Gly-rich loop.²³⁴ However, our crystallographic data do not support this hypothesis.

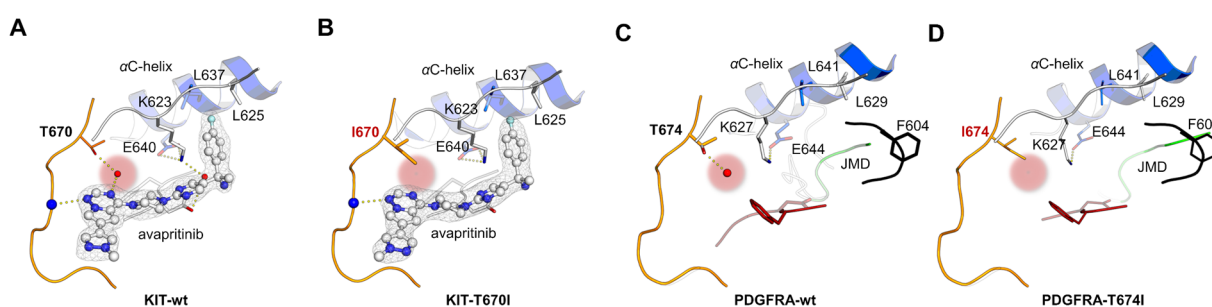


Figure 14. Structural insights into gatekeeper resistance against avapritinib. (A) Complex structure of avapritinib bound to KIT-wt (PDB-ID: 8PQ9) (B) Complex structure of avapritinib bound to KIT-T670I (PDB-ID: 8PQG). (C) Crystal structure of PDGFRA-wt (PDB-ID: 8PQJ). (D) Crystal structure of PDGFRA-T674I (PDB-ID: 8PQK). The $|2F_o - F_c|$ -electron density maps are shown at an r.m.s.d. = 1.0. The figure is modified based on Teuber and Schulz, *et al.* (2024).⁴⁸

Nevertheless, the possibility of other factors, such as a proposed increased binding affinity to ATP²³⁵, cannot be discounted. It is evident that this water molecule is maintained in a fixed position in numerous previously documented structures of KIT and PDGFRA (as exemplified by PDB-IDs: 1T45, 6GQK, 6GQL, and 6GQM). The results of the PDGFRA structure analysis were consistent with these assumptions. In the wild-type structure, the water molecule is present, whereas upon introduction of IleT674, it is displaced (Figure 14C, D). Given the close proximity of avapritinib and the gatekeeper, the structural data suggest that the observed loss of activity in the T674R is likely due to steric repulsion with the sterically demanding and charged Arg674. For the PDGFRA-V658A mutation (homologous to KIT-V654A), which is located adjacent to the α C-helix, this substitution is likely to destabilize interactions within critical regulatory motifs of the kinase, thereby inducing a structural change that is less suitable for efficient binding of avapritinib. Nevertheless, additional research is necessary to gain a deeper understanding of the precise mechanism, including the potential impact on G α -pocket formation and the activity state of the kinase. The structures provide further evidence to support the resistance mechanism proposed by Grunewald *et al.*¹⁸⁸ for the highly resistant G680R mutation. The structures did not show an interaction of avapritinib with Gly680. However, they reveal a close proximity of around 4 Å. This aligns with the assumption, that upon mutation there is a steric conflict between the methyl-pyrazole and Arg680. The results indicate that the observed avapritinib resistance mutations primarily confer resistance through a local conformational change in the vicinity of the binding pocket. This includes the hinge region, as exemplified by T674I and G680R, as well as the α C-helix, as illustrated by V658A. A comparison of avapritinib binding to KIT versus PDGFRA revealed no significant differences. Still, it is noteworthy that additional water-mediated interactions were observed for the primary amine in the KIT structures. Nevertheless, it can thus be postulated that the resistance mutations identified in PDGFRA may also occur in KIT, and *vice versa*.

3.4 Structure-based Design to Identify Important Pharmacophores

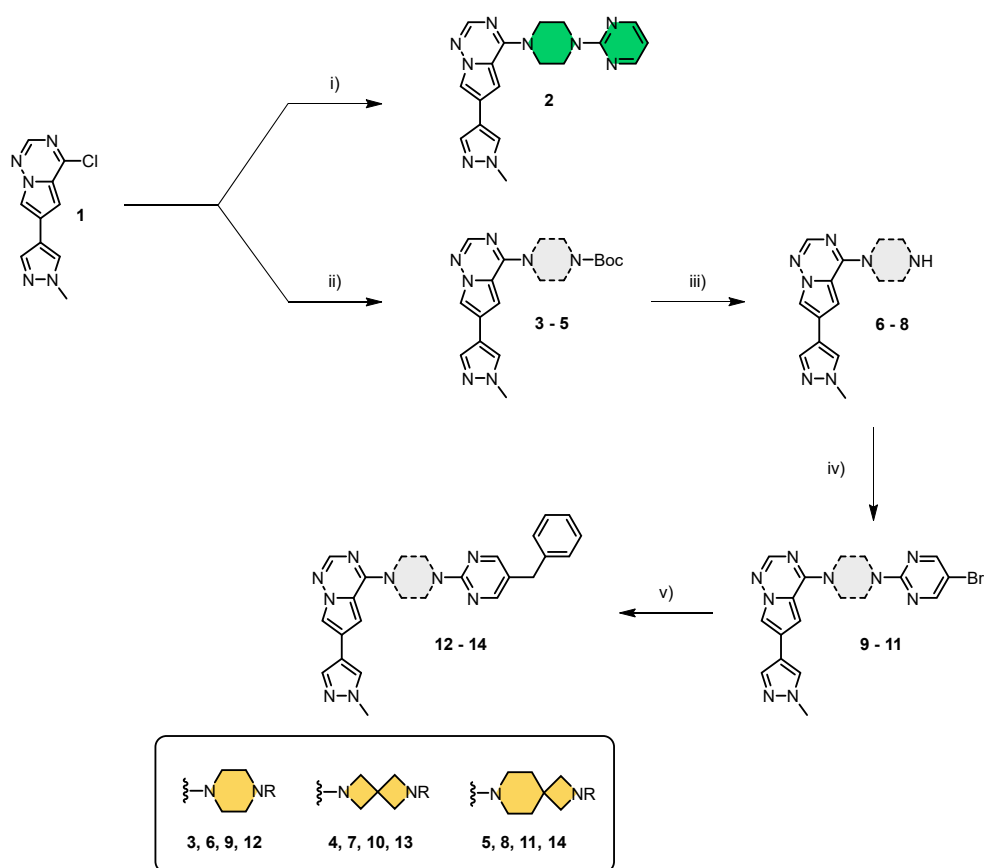
To further elucidate strategies to counteract resistance mutations, key structural elements of avapritinib were modified to evaluate their contribution to potency. Given that the crystal structures revealed and underscored the significance of the G α -pocket, a deconstruction approach was utilized to examine the function of the fluorobenzene group in engaging the G α -pocket (Figure 15A). Additionally, heterocyclic spiro-based linkers as alternatives to piperazine were explored, allowing for variation in linker length between the G α -pocket and the hinge-binding moiety (Figure 15A). The objective was to assess the potential of complex, three-dimensional structures as novel linking motifs. Moreover, the complex structures proposed two solvent-exposed exit vectors for modifying avapritinib, with the objective of limiting its penetration across the BBB while preserving its potency (Figure 15A).

These vectors were identified as i) the free amine (at the stereogenic center) and ii) the methyl-pyrazole. This work examined the derivatization of the primary amine with the objective of refining the physicochemical properties, including hydrophilicity (logP) and molecular weight (MW).

3.5 Preparative Organic Synthesis of Novel Avapritinib Derivatives

In the deconstruction series, derivatives of avapritinib were synthesized, exhibiting a progressive reduction in size and features (Scheme 2). In this context, the smallest compound was represented by the commercially available methyl-pyrazol-pyrrolotriazine **1**. A nucleophilic aromatic substitution was performed with the commercially available (piperazin-1-yl)pyrimidine, based on **1**, affording inhibitor **2** in a good yield of 76 %. Subsequently, **1** was employed in a S_NAr with *tert*-butyl piperazine-1-carboxylate, followed by an acid-mediated carbamate cleavage, resulting in the formation of compound **6**.

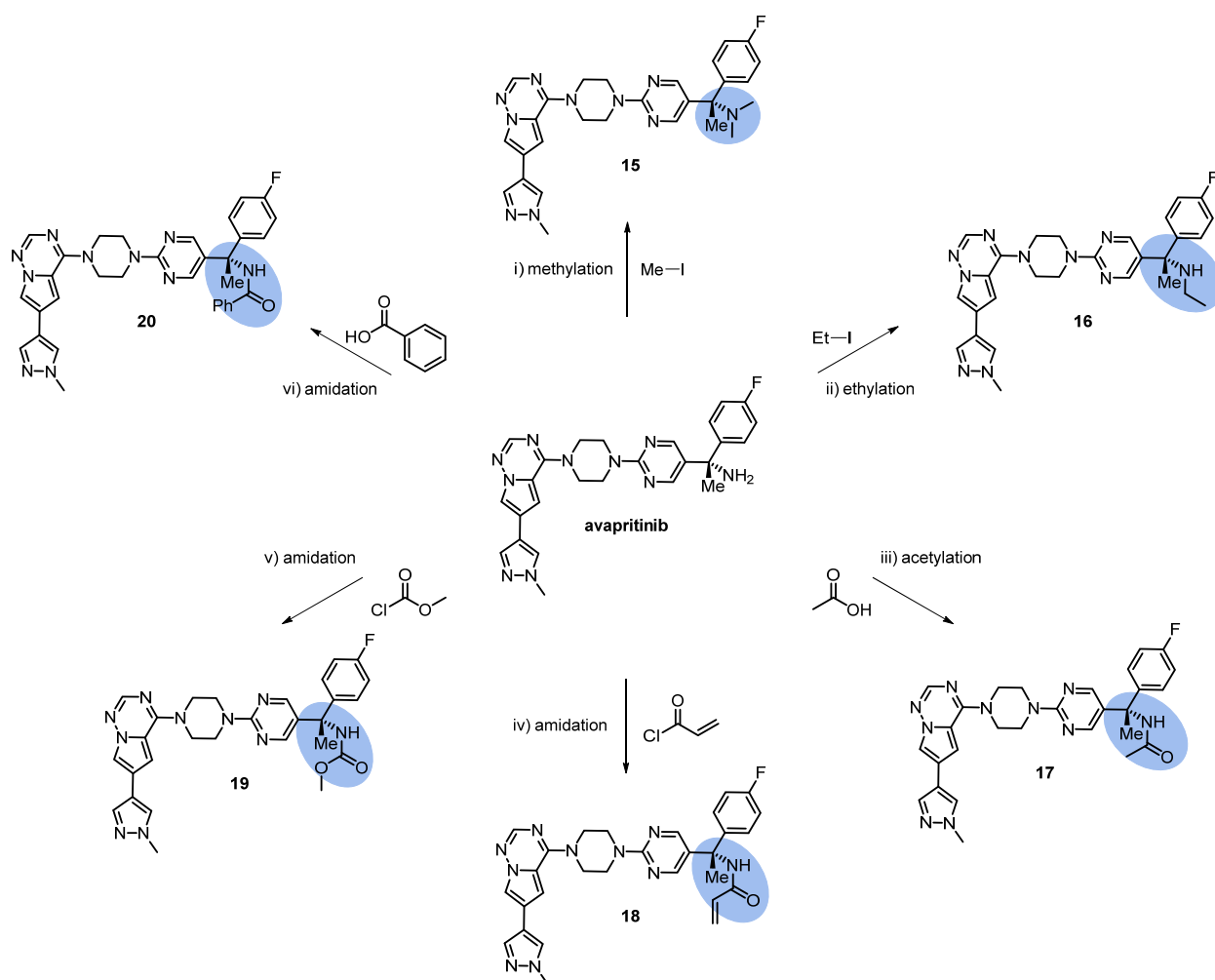
Scheme 2. Synthesis of the deconstruction's series (**1-3**, **12**) and spiro-bearing TKIs (**13-14**)^a



^aReactions and conditions i) 2-(piperazin-1-yl)pyrimidine, TEA, dry THF, rt 6 h, 76 %; ii) Boc protected di-amine, TEA, dry THF, rt, 6 h-ovn, 66-98 %; iii) HCl, DCM, rt, ovn, 63 %-quant.; iv) 5-bromo-2-chloropyrimidine, TEA, EtOH, 60 °C, ovn, 63-85 %; v) benzylboronic acid pinacol ester, Pd(dppf)Cl₂, Cs₂CO₃, Dioxane/water (5:1), 1 h, 120 °C, μ w, 54-72 %.

A linear, four-step synthetic route was established for the synthesis of the spiro-bearing inhibitors **12-14** (Scheme 2). The first step was the nucleophilic substitution of **1** with different Boc-protected diazaspino moieties. Subsequently, the protection groups were removed using HCl, resulting in the formation of compounds **7** and **8** in very good yields. Afterwards, **9-11** were obtained through a nucleophilic substitution of **6-8** with 5-bromo-2-chloropyrimidine, respectively. Lastly, SUZUKI-MIYaura coupling of **9-11** with 2-benzyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, afforded the final inhibitors **12-14**.

Scheme 3. Late-stage functionalization's of avapritinib to synthesize **15-20**^a



^aReactions and conditions: i) MeI, DIPEA, dry THF, 3 h, rt, 77 %; ii) EtI, NaH, dry THF, 8 h, rt, 80 %; iii) acetyl chloride, dry THF, 3 h, rt, 89 %; iv) acryloyl chloride, dry THF, 3 h, rt, 90 %; v) methyl chloroformate, K₂CO₃, dry DCM, 5 h, rt, 84 %; vi) benzoic acid, HATU, TEA, dry DCM, 14 h, rt, 85 %.

The selected exit vector reuses a functional group that is capable of facilitating the derivatization of avapritinib in late-stage functionalizations (Scheme 3). Consequently, direct alkylation with iodomethane and ethyl iodide permitted the synthesis of compounds **15** and **16**, respectively.

Additionally, amide formations were conducted using either the corresponding acid chlorides or HATU as a coupling reagent in the case of benzoic acid, resulting in the acetylated **17**, the acrylamide and methylcarbamate-decorated **18** and **19**, as well as the methylbenzamide **20**, respectively, in yields of 85-90 %.^V In summary, a rapid linear stepwise synthesis approach was developed, and concurrently, late-stage modifications of avapritinib facilitated the synthesis of twelve novel inhibitors. The successfully synthesized novel inhibitors were then subjected to a series of biochemical and cellular assays against mutant KIT and PDGFRA.

3.6 Biochemical, Cellular and Structural Evaluation

In order to ascertain the inhibitory potency, a homogeneous time-resolved fluorescence (HTRF) assay was conducted with the objective of establishing the structure-activity relationships (Table 2).^{VI} The impact of the aforementioned modifications on PDGFRA-D842V and KIT-D816H was investigated. Furthermore, a CellTiter-Glo (CTG) assay was performed on primary (GIST-T1 and -T1-a-D842V), secondary (KIT: T1-D816E, T1-T670I; PDGFRA: T1-a-G680R, and T1-a-T674I/R) mutations bearing cell lines as well as -48B as control (Table 2, Supplementary Table 1). In addition, two non-KIT/PDGFRA driven breast cancer cell lines (MDA-MB-175VII and ZR-75-1) and a kinase-independent cancer cell line (SK-LMS 1) were further analyzed for potential off-target toxicity (Supplementary Table 2).^{VII}

3.6.1 SAR Elucidating the Significance of the α -Pocket

Compound **1**, the sole hinge-binding moiety, exhibited a high IC_{50} value of over 7000 nM for PDGFRA-D842V, with minimal potency enhancement upon piperazine addition (**6**) (Table 2). However, additional decoration with pyrimidine moiety (**2**) increased the potency by 160-fold (IC_{50} PDGFRA-D842V=35 nM). Importantly, a comparison with avapritinib highlighted that including the fluorobenzene moiety, which engages the α -pocket, conferred up to a 350-fold increased potency relative to **2** for PDGFRA-D842V ($IC_{50} < 0.1$ nM). The deconstruction of avapritinib revealed an enhanced potency accompanied by augmented molecular complexity. The greatest increase in potency was observed upon the incorporation of the α -pocket-binding element. This observation was consistent across both mutant PDGFRA and KIT. This trend was pronounced in cell-based assays, wherein only inhibitor **12** and avapritinib demonstrated sustained inhibitory potency ($GR_{50} \leq 100$ nM).

^V The synthesis of compound **15**, **16**, **18** was carried out by Dr. Rajesh Gontla, TU Dortmund University (AG Rauh).

^{VI} HTRF measurements were also conducted by Andreas Arndt and Dr. Alina Teuber, TU Dortmund University (AG Rauh).

^{VII} The Cellular experiments were performed by Benjamin S. Fletcher or Dr. Thomas Mühlenberg University Duisburg-Essen, DKTK (KIT-driven GIST cell lines); Dr. Sonja Sievers, COMAS (PDGFRA-driven GIST cell lines); Dr. Jörn Weisner, TU Dortmund University (AG Rauh) (non KIT/PDGFRA dependent control cell lines.)

It seems reasonable to conclude that this is due to the absence of crucial protein-ligand interactions in the simplistic compounds (**1**, **2**, and **6**), most notably those involving the stereogenic center and G α -pocket binding. Therefore, these findings emphasize the significance of ligand binding to the G α -pocket as a crucial structural necessity for achieving high affinity. In line a complex crystal structure of **16** bound to KIT-wt demonstrated that, in the absence of fluorobenzene, the G α -pocket is instead occluded by Phe600 from the Gly-rich loop (Figure 15B). In conclusion, the collective findings of biochemical, cellular, and structural investigations demonstrate the importance of targeting the G α -pocket to achieve efficacious inhibition of mutant PDGFRA/KIT. Given that the majority of secondary mutations are clustered in or around the hinge, focusing on the more distal G α -pocket may offer promising strategies to circumvent the challenges posed by these drug resistance mutations.

3.6.2 Linking Moiety Locking the Bioactive Ligand Conformation

With regard to structural modifications, compound **12** differs from avapritinib in that it is devoid of the methyl and amino group at the stereogenic center. This provided an opportunity to assess the effect of the stereocenter on the orientation of the G α -pocket moiety for effective protein binding. Compound **12** showed IC₅₀ values of 0.6 nM against PDGFRA-D842V and 1.5 nM against KIT-D816H (Table 2), reflecting a 3 to 6-fold reduction in potency relative to avapritinib. In a cellular assay, this decrease was even more pronounced, e.g. 10-fold for PDGFRA-D842V, while no efficacy against secondary mutations was achieved (Table 2). These findings suggest that, while the modified moiety does not appear to be directly involved in critical interactions that would affect the binding affinity, it may play a key role in cellular activity, probably through its effects on solubility or membrane permeability. **92** also serves as a useful reference for **13** and **14**, as these inhibitors vary only in their heterocyclic linker. Instead of piperazine, inhibitors **13** and **14** feature distinct diazaspiro linkers (*cf.* Scheme 2). Interestingly, although there were no obvious protein-ligand interactions involving the piperazine, both derivatives showed markedly reduced potency compared to **12** in both biochemical and cellular assays (Table 2). Notably, the reduction in potency was more pronounced for diazaspiro heptane **13** than for diazaspiro nonane **14**. For instance, **13** was approximately 17-fold less potent on the GIST-T1 cell line, whereas in the case of T1-a-D842V, the reduction exceeded 500-fold. The SAR of this compound series suggests that the piperazine moiety locks the bioactive ligand conformation, facilitating both efficient hinge and G α -pocket binding. In addition, this observation further reinforces the importance of binding to the G α -pocket for potent ligand binding.

Table 2. Determined IC₅₀ and GR₅₀ values on primary and secondary PDGFRA- and KIT-mutants and a control cell line. Data presented as mean values $\pm$ s.d; $n \geq 3$, where n represents the number of independent experiments.

cpd id	HTRF IC ₅₀ [nM]		CTG GR ₅₀ [nM] (KIT)		CTG GR ₅₀ [nM] (PDGFRA) (control)			
	PDGFRA-D842V	KIT-D816H	GIST-T1	T1-D816E	T1-a-D842V	T1-a-G680R	GIST-48B	ratio
avapritinib	< 0.1	0.5 $\pm$ 0.1	36 $\pm$ 10	67 $\pm$ 18	15 $\pm$ 8	1563 $\pm$ 241	1340 $\pm$ 173	89
1	7324 $\pm$ 2078	9303 $\pm$ 1208	6731 $\pm$ 2073	9481 $\pm$ 1038	2733 $\pm$ 517	2000 $\pm$ 403	7967 $\pm$ 2792	3
6	5588 $\pm$ 2557	1541 $\pm$ 852	≥ 10000	≥ 10000	≥ 10000	≥ 10000	≥ 10000	1
2	35 $\pm$ 13	76 $\pm$ 45	5271 $\pm$ 509	8739 $\pm$ 1321	1887 $\pm$ 545	≥ 10000	≥ 10000	5
12	0.6 $\pm$ 0.1	1.5 $\pm$ 1.0	1484 $\pm$ 1203	≥ 10000	163 $\pm$ 47	≥ 10000	≥ 10000	61
13	7351 $\pm$ 884	2158 $\pm$ 167	≥ 10000	≥ 10000	≥ 10000	≥ 10000	≥ 10000	1
14	749 $\pm$ 142	204 $\pm$ 84	709 $\pm$ 196	1962 $\pm$ 443	8216 $\pm$ 1475	≥ 10000	≥ 10000	1
15	0.4 $\pm$ 0.1	2.3 $\pm$ 1.7	254 $\pm$ 74	515 $\pm$ 94	58 $\pm$ 13	2600 $\pm$ 384	1933 $\pm$ 472	33
16	< 0.1	0.4 $\pm$ 0.2	92 $\pm$ 16	235 $\pm$ 51	26 $\pm$ 5	2767 $\pm$ 459	4033 $\pm$ 653	155
17	< 0.1	0.5 $\pm$ 0.1	39 $\pm$ 6	86 $\pm$ 19	26 $\pm$ 21	3311 $\pm$ 534	4833 $\pm$ 2564	186
18	< 0.1	0.5 $\pm$ 0.2	39 $\pm$ 7	89 $\pm$ 23	24 $\pm$ 6	4033 $\pm$ 876	3467 $\pm$ 866	144
19	0.2 $\pm$ 0.1	0.6 $\pm$ 0.4	60 $\pm$ 12	131 $\pm$ 37	19 $\pm$ 6	3167 $\pm$ 58	4867 $\pm$ 1415	256
20	0.2 $\pm$ 0.1	2.0 $\pm$ 0.1	287 $\pm$ 176	637 $\pm$ 111	51 $\pm$ 7	5729 $\pm$ 435	≥ 10000	196

3.6.3 Modifications of the Primary Amine as Useful Exit Vector

Overall, inhibitors with decorations at the amine displayed highly inhibitory potencies (Table 2). In order to gain structural insights supporting the SAR trends, crystallization experiments were carried out, based on the initial identified condition.^{viii} Thereby, high-resolution structures of KIT-wt complexed with **15-19** as well as **16** bound to PDGFRA-T674I were obtained (Supplementary Figure 2).

^{viii} Protein expression, purification, and crystallization carried out by Dr. Alina Teuber, TU Dortmund University (AG Rauh).

Although all inhibitors maintained IC₅₀ values in the low nanomolar range, double methylation of the primary amine (**15**), which prevents the H-bond donor pharmacophore, resulted in a 3- to 5-fold reduction in potency (Table 2). With IC₅₀ values in the same range as avapritinib (PDGFRA-D842V: 0.1 nM; KIT-D816H: 0.4 nM), the ethyl decorated inhibitor **16** retained potency. Nonetheless, cellular assays revealed a 1.7- to 3.5-fold reduced potency (Table 2). The X-ray structures showed that the ethyl moiety in compound **16** dislodged a water molecule; without further conformational changes of the protein (KIT-wt and PDGFRA-T674I) or ligand conformation compared to avapritinib (Supplementary Figure 2E-H). The alkylated inhibitors **15** and **16** also showed high biochemical potency against KIT and PDGFRA (IC₅₀ ≤ 1 nM). Interestingly, they demonstrated lower potency against primary KIT compared to PDGFRA cell lines (Table 2). Acetamide **17** displayed potent inhibition with an IC₅₀ value of less than 0.1 nM for PDGFRA-D842V, and approximately 30 and 40 nM on PDGFRA-D842V and GIST-T1 cells, respectively. The complex structure suggested that the acetyl moiety could adopt different orientations. Accordingly, it was modeled in two different conformations, which were evident in the corresponding electron density maps (Figure 15D, Supplementary Figure 2I). For inhibitors **18** and **19**, the introduction of the carbonyl oxygen in the amide linkage enabled an additional hydrogen bond interaction with the Gly-rich loop (main chain NH of Phe600). This also induced a slightly different loop conformation, potentially intriguing for stronger binding to the Gα-pocket (Figure 15E, F). In particular, methyl ester **19** facilitated further stabilization of this conformation, through additional hydrophobic interactions with Phe600 (Figure 15F, Supplementary Figure 2O). To pronounce this interaction, compound **20** featured a phenyl ring to further engage with Phe600. Inhibitor **20** exhibited potent biochemical inhibition with an IC₅₀ of 0.2 nM against PDGFRA-D842V. Although it showed reduced cellular potency (around 50 nM) against T1-a-D842V relative to avapritinib, it showed no discernible activity against control cell lines, suggesting an optimized off-target profile (Table 2, Figure 15C, Supplementary Table 1, 2). Overall, the amidated derivatives (**17-20**) exhibited notable biochemical potency against mutant KIT and PDGFRA in the sub-nanomolar range, as well as potent inhibition of clinically relevant mutant cell line models (KIT: GIST-T1, T1-D816E and PDGFRA-T1-a-D842V). Importantly, these inhibitors demonstrated an optimized off-target profile with reduced effects in all tested control cell lines (Table 2, Figure 15C, Supplementary Table 1, 2). Therefore, derivatizations of the primary amine were well-tolerated in terms of selectivity and potency against mutant variants of PDGFRA and KIT. Encouraged by the biochemical and cellular potency of these inhibitors, Western blot experiments were conducted to investigate their impact on PDGFRA and downstream cascade phosphorylation inhibition.^{IX}

^{IX} The Western blot experiments were performed by Dr. Thomas Mühlenberg, UKE (AG Bauer).

Ultimately, these studies demonstrated on-target activity at the cellular level and potent inhibition of downstream signaling, comparable to the effect of the approved drug avapritinib (Figure 15G).

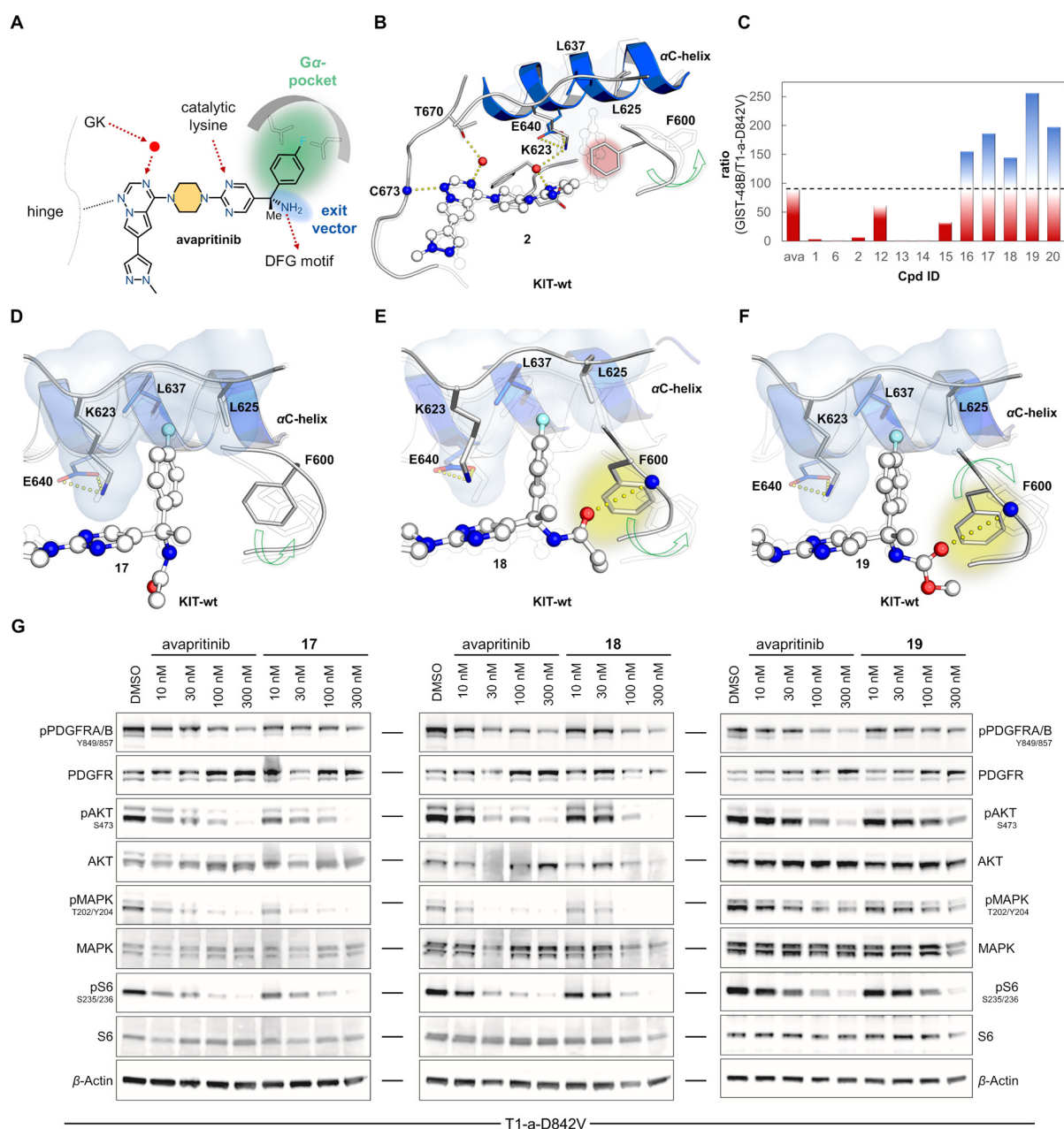


Figure 15. Cellular and Structural investigation of a set of potent inhibitors. **(A)** Schematic depiction of protein-ligand interactions exerted by avapritinib in KIT/PDGFR. **(B)** Aligned crystal structures of **2** (PDB-ID: 8PQA) and avapritinib (shown as outlines, PDB-ID: 8PQ9) in KIT-wt, showing a movement of the Gly-rich loop, resulting in a closed α -pocket. **(C)** Bar chart of the ratios between on-(T1-a-PDGFR-D842V) and off-(GIST-48B) target cell lines, indicating the toxicity profiles of the inhibitors. Threshold (dotted line) is referenced to avapritinib, with red: lower selectivity; white: similar selectivity; blue: increased selectivity. **(D-F)** Complex structure of **17** (**D**, PDB-ID: 8PQD), **18** (**E**, PDB-ID: 8PQE), and **19** (**F**, PDB-ID: 8PQF) each aligned with avapritinib (shown as outlines, PDB-ID: 8PQ9) bound to KIT-wt. **(G)** Western blot analysis PDGFRA and downstream phosphorylation inhibition of **17**, **18**, and **19** compared to avapritinib, at different concentrations, in T1-a-D842V cells. The figure is modified based on Teuber and Schulz, *et al.* (2024).⁴⁸

In light of the promising outcomes and the apparent tolerance of modifications of the amine without a notable reduction in efficacy, this exit vector may provide a strategic avenue to optimize pharmacokinetic parameters with the objective of limiting blood-brain barrier penetration. In order to evaluate the potential for BBB permeability, *in silico* calculations were conducted using central nervous system multiparameter optimization (CNS-MPO)^{236,237} scores (Table 3).

Table 3. Calculated physicochemical parameters, including CNS-MPO scores, and measured MDCKII-MDR1 permeability data for avapritinib and the novel derivatives. ^aCalculated with the MarvinSketch (v. 22.13.0) from Chemaxon. ^bConducted by the Lead Discovery Center (LDC, Dr. Mia-Lisa Zischinsky). n.d. not determined.

cpd id	Physicochemical Parameters ^a						CNS-MPO Score ^a	MDCKII-MDR1 ^b (P _{app} A→B) at 10 μM
	clogP	clogD	MW	TPSA	HBD	pK _a		
avapritinib	3.26	2.12	498.57	106.29	2	8.52	3.52	12.5
1	1.70	1.70	233.66	48.01	0	1.80	6.00	n.d.
6	1.04	-0.31	283.34	63.28	1	8.73	5.38	n.d.
2	2.07	2.07	361.41	80.27	0	3.22	5.96	n.d.
12	4.16	4.16	451.54	80.27	0	2.87	3.77	n.d.
13	3.84	3.84	463.55	80.27	0	2.88	3.92	n.d.
14	4.42	4.42	491.60	80.27	0	2.88	3.35	n.d.
15	4.08	3.59	526.62	83.51	0	7.72	3.67	n.d.
16	4.05	3.25	526.62	91.30	1	8.13	3.46	n.d.
17	3.08	3.08	540.61	109.37	1	2.79	3.52	11.3
18	3.84	3.84	552.62	109.37	1	2.79	2.77	10.1
19	3.70	3.70	556.61	118.60	1	2.79	2.60	12.4
20	4.94	4.94	602.68	109.37	1	2.79	2.14	3.5

The predicted CNS-MPO score of avapritinib is 3.52, indicating the potential for penetration of the BBB. In contrast, the calculated scores suggested that derivatives **18-20** may feature a reduced likelihood of crossing the BBB, with scores ranging between 2.1 and 2.7. To further substantiate these findings, an MDCKII-MDR1 permeability assay²³⁸ was employed to assess the effects of avapritinib and a panel of selected inhibitors **17-20** (Table 3). With regard to avapritinib, a P_{app}(A→B) of 12.5 was observed, which indicates substantial penetration of the blood-brain barrier. However, inhibitor **19** exhibited minimal alteration in BBB permeability, with a P_{app}(A→B) of 12.4, despite its favorable CNS MPO score. Similarly, **17** and **18** displayed only slight variations, with P_{app}(A→B) values of 11.3 and 10.1, respectively.

Analogous results were revealed for **17** and **18**, which demonstrated only modest changes, with Papp(A→B) values of 11.3 and 10.1, respectively. Notably, inhibitor **20** displayed a marked improvement with a Papp(A→B) of 3.5, which signifies a considerable reduction in BBB penetration. In summary, the data illustrates that amide-decorated derivatives emerge as particularly promising inhibitors in this series; maintaining comparable potency to avapritinib in both biochemical and cellular assays, while demonstrating an optimized off-target profile. Moreover, derivatization at this position appears to be a suitable exit vector and strategy to reduce brain permeability, pointing to their potential to mitigate CNS-associated toxicity. However, further studies are needed to refine these inhibitors, specifically *in vivo* experiments will be critical to validate these findings and balance potency and brain permeability.

3.7 Conclusion

Patients with PDGFRA-D842V-driven GIST face limited therapeutic options, with avapritinib being the only approved TKI. So far, avapritinib binding to PDGFRA/KIT has only been inferred by computational models. In this work, an in-depth structural analysis of avapritinib bound to wild-type and mutant variants of PDGFRA and KIT revealed the binding mode for the first time. Moreover, the structural analysis also suggests structural rationales for the effects of clinical-relevant resistance mutations. Most importantly, a previously undescribed sub-pocket in the N-lobe of PDGFRA/KIT, termed the Gα-pocket, was identified, which is bounded by key regulatory structural elements, namely the Gly-rich loop and the αC-helix. The results highlight a link between potent and selective inhibition of PDGFRA and KIT when targeting the Gα-pocket, offering a promising strategy to enhance both potency and selectivity of next-generation inhibitors while potentially counteracting drug resistance mutations. These structural insights inspired the design and led to the development of novel inhibitors, which showed distinct SAR trends and contributed to the understanding of key protein-ligand interactions of mutant PDGFRA/KIT. In particular, modifying the amine of the stereogenic center proved to be a promising starting vector. The novel inhibitors demonstrated high potency, on-target engagement in clinically relevant cell models, and increased selectivity. Moreover, derivative **20** exhibited a notable reduction in the probability of crossing the BBB, thereby addressing significant concerns regarding the use of avapritinib. In conclusion, the insights and results described provide a valuable framework for the development of next-generation inhibitors aimed at overcoming challenges associated with the current landscape of approved TKIs in the treatment of GIST. Moreover, the findings of this research may be extended to the treatment of other cancers where mutated receptor tyrosine kinases drive oncogenesis. This is because it demonstrated the crucial role of X-ray crystallography in conjunction with SAR studies, as well as the potential avenues enabled by analogous pockets to the Gα-pocket present in other kinases.

Chapter: 4

STRUCTURAL INSIGHTS INTO THE MOLECULAR MECHANISMS OF DRUG RESISTANCE IN GIST

4. Structural Insights into the Molecular Mechanism of Drug Resistance in GIST

4.1 Emerging Mechanisms of KIT Inhibitor Escape in GIST

Resistance to imatinib in GIST is predominantly driven by the development of secondary KIT mutations, with clonal heterogeneity posing a significant challenge for treatment.²³⁹ Subsequent KIT inhibitors used after imatinib demonstrate clinical activity, but their efficacy remains limited.^{43, 175-176, 240} Ripretinib has shown clinical benefits in later treatment lines, although these benefits are modest, especially in the fourth-line setting.^{180, 241} The recent INTRIGUE study, which compared ripretinib with sunitinib, highlighted ripretinib's more favorable side effect profile but did not show a significant improvement in median PFS.^{181, 185} Importantly, the molecular mechanisms driving ripretinib resistance remain poorly understood.^{186, 241} To explore the molecular determinants of sensitivity and resistance to ripretinib, a retrospective analysis was conducted. A global collaboration of various research groups contributed to the collection and analysis of patient samples prior to and following treatment with ripretinib.^x Subsequent sequencing yielded insights into the sensitivity profile of the fourth-line inhibitor. In brief, the data demonstrate efficacy on primary and AL mutant KIT and reveal resistance against primary mutations in the ED and secondary AP mutations, in particular KIT-V654A and -T670I. Furthermore, novel tertiary mutations were identified for the first time, bearing an AP and AL mutation in addition to the primary genetic lesion. The combination of AP and AL mutations will be referred to as "AP/AL" mutations. The AP/AL genotype emerged as the most prominent resistance mechanism to ripretinib.

4.2 Cellular Evaluation of Ripretinib and Sunitinib Resistance Profiles

In order to validate the observed resistance to ripretinib in post-progression samples and gain deeper molecular insight, Clustered Regularly Interspaced Short Palindromic Repeats-associated protein 9 (CRISPR-Cas9) technology was employed to generate isogenic GIST cell line models that recapitulate the respective mutation patterns. Sunitinib was employed as a comparator in a manner analogous to that observed in the INTRIGUE study. The cell lines were generated by Dr. THOMAS MÜHLENBERG and BENJAMIN S. FLETCHER (for further details, please refer to MÜHLENBERG *et al.* (2024)⁴³).^{xi} Although ripretinib is known to be a potent KIT-AL inhibitor, it was found to lack efficacy against the second most prominent type of primary KIT mutations, namely lesions in the ED (exon 9). Thus, the initial step involved the generation of an exon 9 (502_503AYdup; AYdup) driven GIST-T1 subline, designated as T1-AY.

^x For further details refer to Mühlenberg and Falkenhorst *et al.*, (2024).⁴³

^{xi} Determination of GR₅₀ values were performed by Dr. Thomas Mühlenberg and Benjamin S. Fletcher, University Duisburg-Essen, DKTK.

Against this cell line, ripretinib displayed GR_{50} values of approximately 80 nM, while sunitinib exhibited a 10-fold greater inhibitory effect, with a GR_{50} of approximately 8 nM (Figure 16). It is noteworthy that the introduction of an additional secondary AL mutation (T1-AY-N822K) resulted in a high level of resistance to sunitinib ($GR_{50} \geq 1000$ nM), while simultaneously enhancing the sensitivity of the cells to ripretinib (20 nM) (Figure 16). In contrast, introducing the V654A (AP) mutation resulted in a slight reduction in potency (Figure 16). Both TKIs exhibited potent inhibitory effects in the low nM range in the GIST-T1 cell line (e11del, JMD) (Figure 16).

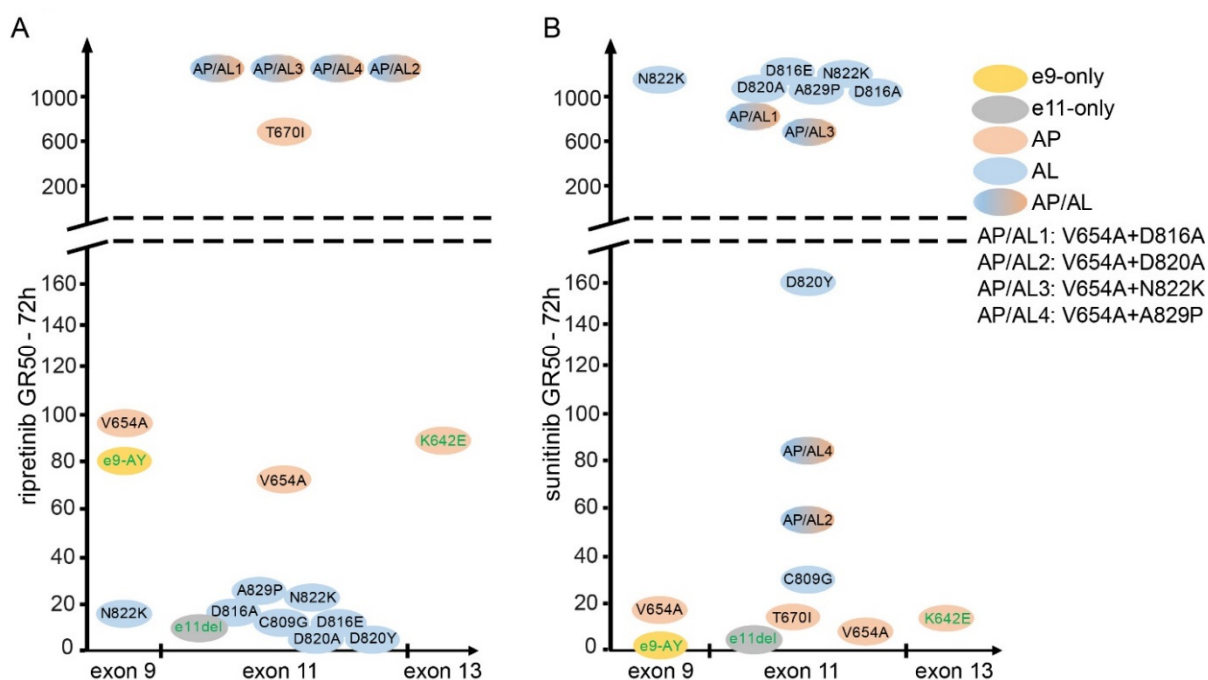


Figure 16. GIST cell line models to recapitulate ripretinib and sunitinib potency against a range of secondary and tertiary KIT mutations. **(A-B)** Overview of GR_{50} values [nM] of ripretinib **(A)** and sunitinib **(B)** on various different GIST cell lines models in ED, JMD, AP and AL. A green font indicates a primary mutation. The figure was adapted and modified based on Mühlenberg and Falkenhorst, *et al.* (2024).⁴³

However, the presence of a secondary mutation within the AL resulted in significantly higher GR_{50} values for sunitinib ($GR_{50} = 350$ to >1000 nM), while ripretinib retained its potency, ranging from 7–26 nM (Figure 16B). In contrast, secondary mutations in the AP resulted in a reduction in potency for ripretinib by approximately eightfold (V654A), whereas sunitinib demonstrated consistent potency (T1-V654A, GR_{50} 15 nM). In particular, the gatekeeper mutation T670I resulted in a significant reduction in the potency of ripretinib (T1-T670I, $GR_{50} = 700$ nM). It is noteworthy that in two instances (AP/AL 2 and 4), the presence of the additional V654A mutations appears to re-sensitize sunitinib potency to a certain extent; yet it remains resistant in the case of D816 and N822 containing AP/ALs. However, all sublines with AP/AL mutations displayed an equivalent level of resistance to ripretinib, with a GR_{50} value exceeding 1000 nM (Figure 16). Notably, the AP/AL cell models exhibit a greater resistance to ripretinib than that resulting from AP mutations alone.

It appears therefore necessary to conduct further research in order to gain a deeper understanding of the clinical challenges posed by AP/AL mutations and the underlying molecular mechanisms. In summary, ripretinib is a highly potent inhibitor of various secondary AL mutations, but mutations in the AP (e.g. V654A, T670I) or AP/AL cause ripretinib resistance. Conversely, resistance to sunitinib is mediated by AL mutations, almost independently from the exact mutation (except C809G), while showing inhibition of AP mutations like V654A or T670I. Moreover, the identified AP/AL mutations appear to constitute a multi-drug resistant genotype.

4.3 Structure-based Modeling of Clinically Relevant KIT Mutants

To gain deeper insights into the underlying molecular mechanisms underlying the observed ripretinib resistance mutations, a combination of structural analysis and *in silico* modelling was employed to assess the structural consequences of ED, JMD, AP and AL mutations. KIT is subject to a conformational equilibrium exhibiting an ensemble of conformations transitioning between inactive and active states (Figure 17A). In brief, three postulated states have been identified in which KIT may exist: an active state, an autoinhibited inactive state, and a non-autoinhibited inactive state (for details regarding specific characteristics, please see Chapter 1.2.1). Structural analysis of a co-crystal structure of ripretinib in KIT-wt evidenced that it binds as a type IIa inhibitor (Figure 17B). Hence, it binds and stabilizes the inactive (α C-helix-out, DFG-out, JMD non-autoinhibitory) conformation. Ripretinib's pharmacophore profiles include the ability to interact via H-bonds with the hinge region, specifically the backbone of Cys673, form contacts with the gatekeeper Thr670 and Val654, and enable hydrophobic interactions with certain side chains within the back pocket (Figure 17C, Supplementary Figure 7). However, most patients show acquired resistance mutations localized within the kinase domain that alter the structural conformation either locally or globally, inducing distinct activation states. Local alterations affect the binding site and include the point mutation T670I (exon 13) concerning the gatekeeper, which exchanges Thr for a sterically more demanding and more lipophilic Ile. This directly interferes with ripretinib binding due to steric repulsion (Figure 17D). Mutations at codon 654 (exon 14), such as Val-to-Ala, introduce smaller and more flexible residues, which accompanies a loss of Van der Waals interactions, resulting in reduced binding affinity. In addition, *in silico* modeling via MD simulations suggested that the V654A potentially perturbs the dynamics of the α C-helix, favoring a kinase conformation, which is less suited for binding of type IIa inhibitors.¹⁶⁸ Notably, inhibitors that do not bind to the back pocket, such as type I or type IIb inhibitors, could likely still bind (*cf.* Figure 17B).

Global structural changes are induced by lesions within the ED, such as Y503_F504insAY (exon 9), which culminates in a sequence duplication (A502_Y503dup). In consideration of the inhibitor profiles of T1-AY (Figure 16), it can be inferred that the protein exhibits a wild-type-like behavior, indicating an auto-inhibited conformation. Considering the 3D structure, the A502_Y503dup mutation is located nearby residues in D5 of the ED.

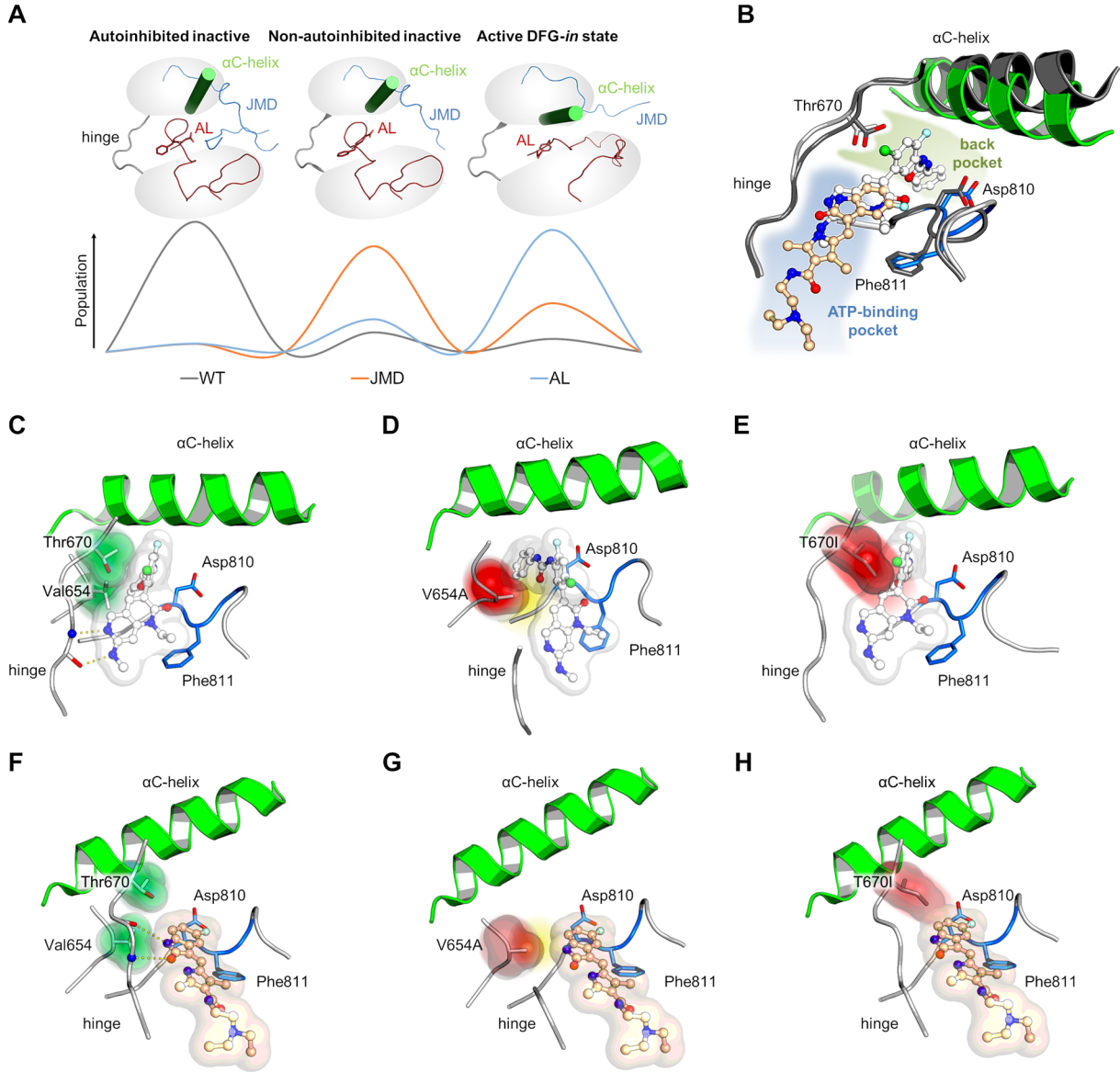


Figure 17. Structural-based modeling of ripretinib resistance mutations in KIT, and their impact on ripretinib and sunitinib binding. (A) Schematic representations depicting three potential conformations of KIT, including the proposed populations that may emerge following specific mutations in various regions of the kinase domains. (B) Alignment of ripretinib and sunitinib, showing their different binding characteristics (PDB ID: 6MOB, 3G0E, and 6JOK). Binding regions are color-coded: ATP-binding pocket (blue) and back pocket (green). (C) Binding mode of ripretinib in KIT-wt (PDB ID: 6MOB). (D) Structural impact of the V654A mutation on the binding of ripretinib. The smaller Ala654 accompanies a reduced binding affinity, attributable to the disruption of van der Waals interactions (yellow). (E) Structural impact of the T670I mutation on the binding of ripretinib. Ile670 sterically interferes with ripretinib. (F) Binding mode of sunitinib (PDB ID: 3G0E, and 6JOK). (H) Structural impact of the V654A mutation on the binding of sunitinib. (G) Structural impact of the T670I mutation on the binding of sunitinib. As a type IIb inhibitor sunitinib does not extend beyond the GK, and thus can better accompany the spatial necessity of Ile670. H-bonds are shown as yellow dotted lines. The figure was adapted from Mühlenberg and Falkenhorst, *et al.* (2024).⁴³

This is thought to facilitate receptor homodimerization through homotypic interactions, resulting in an enhanced binding affinity by decreasing the off-rate or increasing the on-rate.²⁴² This in turn, might influence the intracellular kinase domain to discriminate its non-autoinhibited inactive conformation which is necessary for efficient binding of type IIa inhibitors. Nevertheless, the precise manner by which these effects impact the kinase domain and the rationale behind inhibitor responses remain uncertain. Still, this hypothesis is supported by the observation that resistance is also evident in the case of the IIa inhibitor imatinib, which becomes manageable at higher doses.

Mutations within the flexible AL (exon 17/18), like the D816H, are described to shift the kinase towards a more active state (Figure 17A). The structural analysis of KIT-wt indicates that Asp816 plays a stabilizing role in a helical segment within the AL, thereby contributing to the orientation of the DFG motif (Supplementary Figure 8C). Accordingly, the introduction of a histidine with a different shape and charge is incapable of locking the DFG motif into an inactive conformation, thereby favoring an active state. Similarly, Asn822 forms H-bonds interaction with the surrounding AL side chains of the DFG-out conformation as well as a water network to remain preferably in the (auto)inhibited inactive state (Supplementary Figure 8D). It thus seems reasonable to posit that the positively charged and lengthy lysine residue results in charge repulsion with polar side chains (e.g. Arg796) in close proximity, thereby facilitating a conformational change that favors active states. When the kinase is in an inactive state, Ala829 is capable of forming interactions with Val825, which is also situated within the AL (Supplementary Figure 8C). This stabilizes a sheet-like conformation in this part of the AL. However, Pro829 introduces a ring structure that restricts the degrees of rotation permitted for the ψ and ϕ angles around the peptide bond. Accordingly, the conformational entropy is comparatively low, which would encourage the formation of loops and diminish the stability of α - and β -structures²⁴³, ultimately shifting the conformational equilibrium towards active states. As outlined above, ripretinib binds and stabilizes the inactive conformation. However, AL mutations tend to induce active kinase conformations. The structural analysis indicates that the high potency of ripretinib against AL mutant KIT can be attributed to two key components: i) the back pocket substituent and ii) high binding affinity to the inactive (non-autoinhibited) conformation. Despite being smaller compared to regorafenib or imatinib (phenyl < 4-chloro-3-(trifluoromethyl)phenyl < 4-((4-methylpiperazin-1-yl)methyl)phenyl, respectively, it is most efficiently positioned within this pocket, enabling partial binding to a range of intermediate states and thereby stabilizing the preferred inactive kinase conformation (Figure 18).

Next, a structural analysis of the type IIb inhibitor sunitinib was carried out to see whether the assumptions could also explain the response observed and potentially contribute to better treatment strategies. Based on the assumption that due to lesions in the ED, either the autoinhibited form or the active state is present, the pronounced efficacy of sunitinib in exon 9 patients can be understood. Since sunitinib as a type IIb inhibitor, it can bind more diverse conformations of the KIT kinase domain than ripretinib due to its molecular structure and binding mode not extending beyond the GK towards the back pocket (Figure 17B). So, it could still bind the auto-inhibited inactive conformation or, potentially, the active DFG-in conformation, as well. The latter has yet to be described for KIT; however, sunitinib is described as a type I inhibitor of various kinases.²⁴⁴⁻²⁴⁵ Like ripretinib, sunitinib interacts with Val654 via hydrophobic interactions, yet these are less pronounced (Figure 17G). Moreover, a conformational change induced by a mutation in codon 654 could be compensated by the distinct binding mode, which is less affected by α C-Helix dynamics. This suggests that only a slight loss of affinity is likely. In the case of the gatekeeper mutation T670I, it is notable that sunitinib can accommodate the space requirements of Ile670 more efficiently due to the orientation of its smaller oxindole scaffold (Figure 17H). Therefore, a reduced loss of affinity is anticipated upon mutation of the gatekeeper residue.

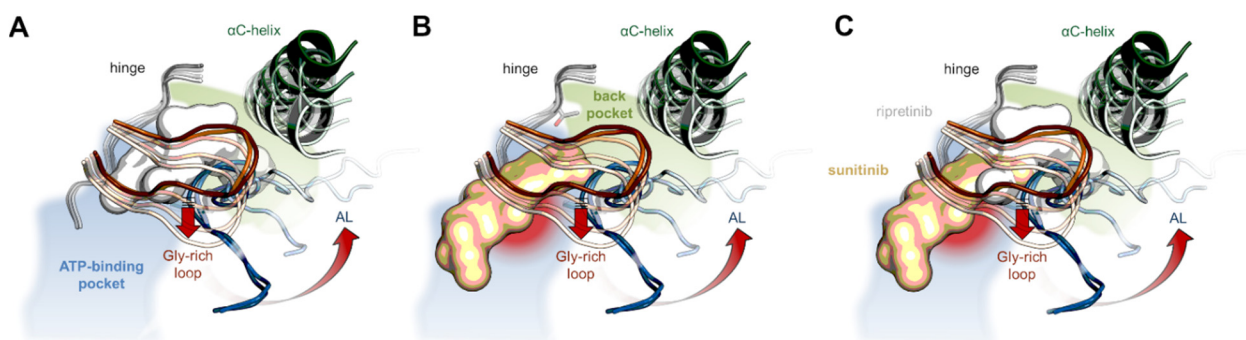


Figure 18. Structural-based modeling of AL mutations in KIT, and their impact on ripretinib and sunitinib binding. (A) Depiction of ripretinib in a multitude of intermediate states transitioning from inactive to active states. Given its interactions within the stable core of the kinase, in particular the back pocket, ripretinib is capable of withstanding a range of intermediate states. (B) Depiction of sunitinib in a multitude of intermediate states transitioning from inactive to active states. The transition towards an active kinase results in a movement of, for example, the AL and Gly-rich loop, which leads to conformations that are unfavorable for sunitinib binding. (C) Alignment of ripretinib (PDB ID: 6MOB) and sunitinib (PDB ID: 3G0E) within the multitude of intermediate states generated. The figure was adapted from Mühlenberg and Falkenhorst, *et al.* (2024).⁴³

However, secondary mutations within the AL result in sunitinib resistance. On the one hand, the observed AL resistance is presumably attributable to the dynamics of pivotal structural components, such as the glycine-rich loop, which impose steric constraints as the protein transitions to its active conformation (Figure 18B). On the other hand, the transition to the active conformation results in rearrangement of the AL distant from the ligand, which in turn impairs the binding affinity. In combination, sunitinib is unable to effectively bind and hence not able to stabilize the favored inactive conformation.

Lastly, in the case of the identified AP/AL genotype, the resistance might be mediated by the interplay of the AP and AL mutants disrupting the binding affinity as well as an overall shift in the equilibrium towards active states. Nevertheless, further research is necessary to elucidate the precise mechanisms by which these tertiary mutations result in multi-drug resistance. Further research may elucidate additional factors contributing to AP/AL resistance, including structural resp. conformational impact, increased ATP affinity, the dimerization process or receptor half-life.

4.4 Conclusion

In-depth structural biology studies revealed insights into the molecular mechanisms of resistance mediated in various regions of the kinase domains. The analysis illustrated that AP mutations primarily mediate resistance through a local influence. The T670I gives rise to a direct steric conflict, whereas for the V654A mutation, it can be postulated that this mutation results in a loss of binding affinity due to reduced protein-ligand interactions. Conversely, it can be proposed that the resistance of ED and AL mutations is mediated via a global effect. The impact of different AL mutations can be attributed to a destabilizing effect on the inactive state, which arises from the introduction of amino acid residues that disrupt or even counteract interaction patterns present in the inactive kinase conformation. This, in turn, promotes a more active conformation. Moreover, the examination of the interactions between ripretinib and KIT, with a particular focus on resistance mutations, pointed to the importance of addressing the back pocket in order to effectively target AL mutations. However, the identified AP/AL genotypes are particularly resistant and represent a significant challenge in treating GIST. These findings provide crucial insights into current resistance mechanisms, emphasizing the necessity for the development of innovative strategies in the creation of next-generation inhibitors that are specifically designed to target the multitude of drug-resistant mutations that are emerging.

Parts of this chapter have contributed to the following publication:

Mühlenberg, T.[‡]; Falkenhorst, J.[‡]; **Schulz, T.**; Fletcher, B. S.; Teuber, A.; Krzeczieska, D.; Klooster, I.; Lundberg, M.; Wilson, L.; Lategahn, J.; von Mehren, M.; Grunewald, S.; Tuns, A. I.; Wardelmann, E.; Sicklick, J. K.; Brahmi, M.; Serrano, C.; Schildhaus, H. U.; Sievers, S.; Treckmann, J.; Heinrich, M. C.; Raut, C. P.; Ou, W. B.; Marino-Enriquez, A.; George, S.; Rauh, D.[#]; Fletcher, J. A.[#]; Bauer, S.[#], *KIT ATP-Binding Pocket/Activation Loop Mutations in GI Stromal Tumor: Emerging Mechanisms of Kinase Inhibitor Escape. J Clin Oncol* **2024**, JCO2301197.

Chapter: 5

STRUCTURAL ANALYSIS TO RATIONALIZE PONATINIB'S INHIBITORY PROFILE

5. Structural Analysis to Rationalize Ponatinib's Inhibitory Profile

5.1 Ponatinib after Resistance to Imatinib in GIST

Despite the advent of more sophisticated therapeutic modalities, KIT remains the key oncogenic driver in GIST, yet is confronted with the challenge of heterogeneous drug resistance.⁴³ It is therefore imperative to develop novel inhibitors and to evaluate the efficacy of existing TKIs, with the objective of improving outcomes for patients with acquired drug resistance. Sunitinib and ripretinib display strikingly disparate inhibitory profiles, with sunitinib exhibiting potent inhibition against AP mutations and ripretinib demonstrating high potency against AL mutations (*cf.* Chapter 4). Nevertheless, to date, no TKI has demonstrated the capacity to effectively target both types of resistance mutations simultaneously. Ponatinib is a multi-kinase inhibitor that demonstrated a strong *in vitro* efficacy against secondary KIT mutations in the AL and the gatekeeper mutation T670I.²⁰⁹ However, patients treated with the standard dose (45 mg) have been observed to experience cardiovascular events.^{210, 246} Subsequent, the phase II open-label POETIG trial (POnatinib after rEsisTance to Imatinib in GIST) was designed to assess the efficacy and safety of ponatinib at a lower dose (30 mg) in patients with KIT-mutant GIST who had previously undergone treatment with TKIs.^{212XII} The study aimed to evaluate the clinical benefit rate in two populations: i) patients in the second line of treatment (post-imatinib resistance), and ii) patients in the last line who had previously received all other approved TKIs. The median OS was 29.7 months in the second-line and 8.7 months in the last-line cohort. This underscores the progressive challenge of sustained benefit as patients advance through multiple lines of therapy. Nevertheless, an OS in the second-line GIST setting of 29.7 months may reflect ponatinib's benefit against certain resistant mutations. Subsequent analysis employed droplet digital polymerase chain reaction (PCR) to identify AP mutations, with a particular focus on KIT-V654A and -T670I mutations.^{XIII} Among patients with pre-treatment detection of the V654A mutation, the median PFS was 2.3 months, which contrasted with the T670I-mutant subset, where an extended median PFS of 13 months was observed. This finding highlighted the association between KIT-T670I and prolonged ponatinib response duration, in contrast to V654A, which typically indicates a more resistant profile. Nevertheless, the safety evaluation showed adverse events similar to those observed in the other ponatinib trials (in GIST²¹⁰ and Leukemia²⁴⁶). Notably, the incidence of vaso-occlusive events was consistent with previous studies. Thus, lowering the ponatinib dose did not appear to reduce the risk of vaso-occlusive events, which further underscores the pressing need to advance the development of TKIs that offer both efficacy and an improved safety profile.

^{XII} Further details can be found in Falkenhorst *et al.*, submitted.

^{XIII} The PCR experiments were conducted by Dr. Johanna Falkenhorst, West German Cancer Center, UKE.

5.2 Cell Line Models to Assess Mutant Specificity

To recapitulate these findings, ponatinib and reference compounds, including approved TKIs and IDRx42, were evaluated in a comprehensive isogenic panel of GIST cell lines with primary and resistance mutations (Table 4).^{XIV} This unique panel allowed a systematic assessment of ponatinib's inhibitory potency across a range of clinically observed mutations, providing insights into its mutation-specific sensitivity. The inhibitory potency of ponatinib was demonstrated against cells with primary KIT mutations, namely the patient-derived GIST-T1 cell line. This result is in accordance with the high sensitivity of this cell line to targeted kinase inhibition. In cell lines with imatinib-resistant AL mutations, ponatinib showed robust high potency regardless of the specific AL mutation present (GR₅₀ 12-20 nM). This potency is within the same range observed for newer TKIs, such as ripretinib and IDRx-42, which are known AL mutant inhibitors. In contrast, regorafenib and sunitinib have been associated with a higher level of resistance, with GR₅₀ values of approximately 200-500 and ≥1000 nM, respectively. However, in the case of KIT-V654A, the resistance landscape shifts. This AP mutation renders the cell lines less sensitive to nearly all TKIs, including ponatinib, with the exception of sunitinib and IDRx-42 (GR₅₀ around 15 nM). Nevertheless, ponatinib achieved inhibition at a concentration slightly above 50 nM.

Table 4. Determined GR₅₀ values for various GIST cell lines bearing primary, secondary AP or AL and tertiary mutations. *published data from Mühlenberg *et al.* for comparison.⁴³ Color-code indicates the type of mutations with primary (gray), secondary AP (orange), AL (blue) and AP/AL (brown). n.r. no response: GR₅₀ ≥ 10000 nM.

mutation	cell line	imatinib	sunitinib	regorafenib	ripretinib	IDRx-42	ponatinib
primary (JMD)	GIST-T1	18	9	34	8	1	2
secondary (AP)	T1-V654A	747	15	314	71	16	53
	T1-T670I	n. r.	28	106	714	681	4
secondary (AL)	T1-D816A	346	n. r.	218	16	20	20
	T1-D820A	642	1634	440	8	16	16
	T1-N822K	2160	3342	366	22	12	12
	T1-A829P	1113	1035	437	26	15	15
tertiary (AP/AL)	T1-816+654	n. r.	1083	n. r.	n. r.	482	218
	T1-820+654	n. r.	59	n. r.	n. r.	295	285
	T1-822+654	n. r.	199	n. r.	n. r.	1622	493
	T1-829+654	n. r.	82	n. r.	2083	276	232

^{XIV} The cellular experiments were performed by Benjamin S. Fletcher and Dr. Thomas Mühlenberg, University Duisburg-Essen, DKTK.

Notably, ponatinib emerged as the most potent inhibitor for the challenging gatekeeper mutation, demonstrating superior potency (T1-T670I: GR₅₀ 4 nM) where most other inhibitors displayed diminished potency. This is particularly interesting in comparison with next-generation inhibitors like ripretinib or IDRx-42, which are regarded as resistant to KIT-T670I. However, efficacy remains constrained across the AP/AL-mutant genotype, where each tested inhibitor exhibited limited potency. In summary, the *in vitro* data are in accordance with the clinical findings, thereby corroborating the mutation-specific nature of ponatinib resistance. Moreover, the assay results underscore ponatinib's distinctive sensitivity profile across different resistance mutations, particularly with regard to the activation loop and the gatekeeper mutation.

5.3 Structural Basis of Potent Ligand Binding in Drug-Resistant Mutants

The conformational shifts that occur in KIT between active and inactive states are determined by key structural regions within the kinase domain. These regions include the Gly-rich loop, the α C-helix, and the DFG motif within the AL. As previously described, mutations or genetic alterations in these critical areas can disrupt the conformational equilibrium, often favoring a more active kinase state, which is associated with drug resistance (*cf.* Chapter 4).^{43, 146} Structural analysis of a ponatinib complex bound to KIT-wt (PDB ID: 4U0I) revealed that the inhibitor binds to and stabilizes an inactive kinase conformation (type IIa), which is marked by a DFG-out conformation and a non-autoinhibitory JMD (Figure 19). Ponatinib establishes H-bonds with the backbone of Cys673 in the hinge region and the side chains of Asp810 of the DFG motif as well as Glu640 in the α C-helix. Moreover, it engages in hydrophobic interactions that extend from the ATP-binding pocket (e.g., Val654) to the back pocket, in particular Ile653, Leu783, and Ile808 (Figure 19B). Although ponatinib shares many structural characteristics with imatinib, it displays two unique pharmacophores that contribute to its potency against mutations in the AL and the gatekeeper mutation (Figure 19A). Firstly, it incorporates a carbon-carbon triple bond linking the hinge and back pocket binding moieties. Secondly, it features a trifluoromethyl group in the eastern part of the ligand. As previously proposed, mutations within the crucial activation loop, such as the D816H mutation, result in a shift towards active kinase conformations (*cf.* Chapter 4).^{43, 170} A comprehensive examination of the crystal structure (PDB ID: 4U0I) elucidated that the trifluoromethyl moiety exhibits shape complementarity within the back pocket (also designated as the switch pocket), specifically interacting with residues Ile653, Leu783, and Ile808, which is -2 of the DFG motif (Figure 19B). Notably, this interaction pattern mirrors that of ripretinib. Analysis of the different conformations that KIT can adopt, including active, inactive, and possible intermediate conformations, highlighted substantial structural rearrangements in the AL and α C-helix.

In contrast, the amino acids associated with CF₃ binding display a relatively rigid arrangement throughout the aforementioned transitions (Figure 19C). Thus, it can be reasonably deduced that these stabilizing interactions facilitate ponatinib's binding across a range of intermediate conformations, thereby promoting and stabilizing the preferred inactive conformation. This, in turn, contributes to the potent inhibition of activation loop mutations.

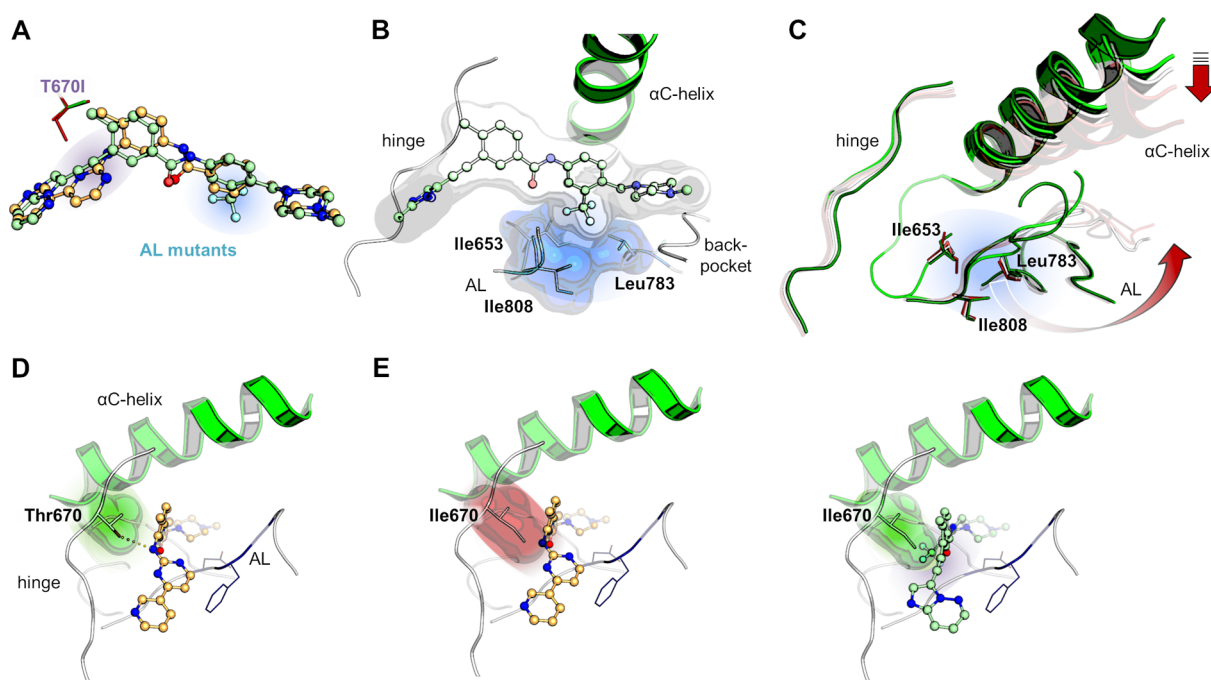


Figure 19. Structural analysis of ponatinib in mutant KIT. (A) Comparison between the structural characteristics of imatinib (orange) and ponatinib (green). Key differences assumed to play a role in potency against AL (blue) and gatekeeper (purple) KIT mutants are indicated. (B) Binding mode of ponatinib to KIT-wt (PDB ID: 4U0I), highlighting the interactions within the back pocket region. (C) Potential conformation that KIT can adapt in the equilibrium between active and inactive states. (D) Binding mode of imatinib to KIT-wt (PDB ID: 4U0I), showing interaction with the gatekeeper residue Thr670 (highlighted in green). (E) Modeled KIT-T670I structure in complex with imatinib (left) and ponatinib (right). Imatinib cannot accommodate the greater steric demand of T670I, while ponatinib can interact with Ile670. Figure adapted from Falkenhorst *et al.*, submitted.

Recently, KAITSIOTOU and colleagues examined the potency of new ponatinib-based inhibitors targeting the ATP-binding pocket mutation KIT-V654A using molecular dynamics (MD) simulations to guide the design and support the findings.¹⁶⁸ The simulations pointed to notable structural and physicochemical distinctions in the binding sites of the wild-type and V654A mutant variant of KIT. In particular, the results suggested an increased flexibility among active site residues, thereby impacting the dynamics of the α C-helix and favoring a conformation less suitable for binding type II inhibitors like ponatinib. Moreover, based on the interaction with Val654 and the pronounced shape complementarity (Supplementary Figure 9), it can be postulated that a direct loss of interaction upon mutation will result in a further reduction in binding affinity.

The gatekeeper mutation, KIT-T670I, presents a significant challenge to the effective treatment of GIST, resulting in the reduced efficacy of all approved TKIs (*cf.* Table 4). In-depth structural analysis revealed three pivotal factors that underpin the observed resistance in KIT-T670I: (i) the mutation disrupts crucial interactions with the wild-type Thr670, (ii) replacing the polar threonine with hydrophobic isoleucine, which creates an unfavorable binding interface, and (iii) the bulkier Ile670 introduces steric constraints, prompting the repulsion of inhibitors from the binding site (Figure 19D, E, and Figure 20). Notably, the approved TKIs are not uniformly susceptible to this greater steric demand (Figure 20).

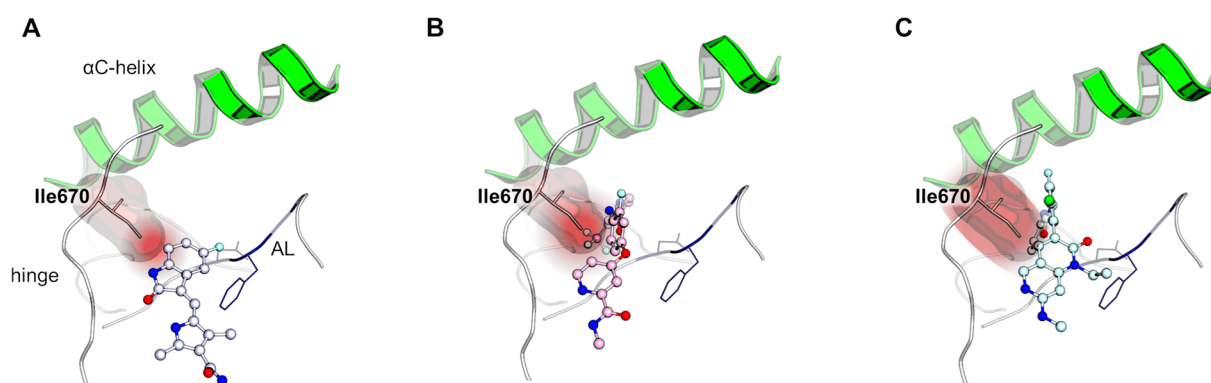


Figure 20. Modeled binding mode of sunitinib (based on PDB ID: 3G0E) (**A**), regorafenib* (**B**) and ripretinib (based on PDB ID: 6MOB) (**C**) in KIT-T670I (based on PDB ID 4U0I). *Shown binding mode was generated via a pharmacophore-based docking in SeeSAR. Figure adapted from Falkenhorst *et al.*, submitted.

Conversely, ponatinib, which was initially developed to target the analogous Abl-T315I mutation, has been demonstrated to exhibit enhanced efficacy against KIT-T670I.²⁰⁸ The structural design, particularly the carbon-carbon triple bond, remains unaltered by the increased steric demand and allows to engage in hydrophobic interactions with Ile670, rationalizing ponatinib's potency against this mutant (Figure 19E).

5.4 Conclusion

In summary, ponatinib showed limited efficacy across the broader study population but provided meaningful clinical benefits for patients with specific resistance mutations, particularly the T670I gatekeeper mutation. Despite the reduced dosage, thromboembolic events persisted, indicating ongoing safety concerns with ponatinib. The structural analysis illustrated that steric and hydrophobic alterations in the binding pocket caused by the T670I mutation significantly hinder the efficacy of most approved TKIs. Ponatinib's alkyne moiety, however, accommodates this increased steric demand and enables hydrophobic interactions, presenting a promising approach for targeting the gatekeeper mutation. Moreover, the observation that a suitable back-pocket binding element enhances the inhibition of AL mutations is supported by the interaction similarities between ripretinib's phenyl group and ponatinib's CF₃ group.

These findings provide valuable insights for the design of future inhibitors, particularly in addressing AP resistance mutations. Overall, this study underscores the pressing therapeutic gap for patients with KIT-mutant GIST who have progressed on ripretinib, a situation in which approved treatment options remain unavailable. The continued occurrence of adverse events, alongside the complex resistance landscape, highlights an urgent need for next-generation inhibitors that offer both efficacy and an enhanced safety profile.

Parts of this chapter have contributed to the following publication:

Falkenhorst, J.; Ivanyi, P.; **Schulz, T.**; Hamacher, R.; Kasper, B.; Hohenberger, P.; Hermes, B.; Pink, D.; Fletcher, B. S.; Haller, V.; Krausmüller, Y.; Rauh, D.; Marino-Enriquez, A.; Mühlenberg, T.; Reichardt, P.; Bauer, S. *Phase 2 trial of ponatinib in patients with metastatic and/or unresectable gastrointestinal stromal tumor (GIST) following failure or intolerance of prior therapy with imatinib (POETIG trial)*. Submitted.

DESIGN, SYNTHESIS AND SAR OF COVALENT KIT AND PDGFRA INHIBITORS

Parts of this chapter have been published in the master thesis of Maria Beerbaum, were part of the master thesis of Tom Schulz, or are recently submitted within **Schulz, T.**; Gontla, R.; Teuber, A.; Beerbaum, M.; Fletcher, B. S; Mühlenberg, T.; Kaitsiotou, H.; Hardick, J.; Jeyakumar, K.; Keul, M.; Müller, M. P.; Sievers, S.; Bauer, S. and Rauh, D., *Design, Synthesis and SAR of Covalent KIT and PDGFRA Inhibitors – Exploring Their Potential in Targeting GIST*. Submitted.

6. Design, Synthesis and SAR of Covalent KIT and PDGFRA Inhibitors

6.1 Need for Innovative Approaches

All current approved TKIs for GIST are reversible ATP-competitive inhibitors, but none can effectively target AP and AL mutations simultaneously. A notable challenge comes from gatekeeper mutations, such as KIT-T670I and PDGFRA-T674I/R (Figure 21). Moreover, many salvage therapies have limited efficacy due to off-target effects, which often lead to undesirable side effects. This highlights the critical need for the development of selective and potent inhibitors that can overcome these limitations. Recently, ponatinib demonstrated the ability to effectively target AL mutants and the challenging KIT-T670I and thereby enabled promising response rates in respective patient populations.²⁰⁹ Unfortunately, cardiac toxic side effects were observed even at a dose of 30 mg.²¹¹⁻²¹²

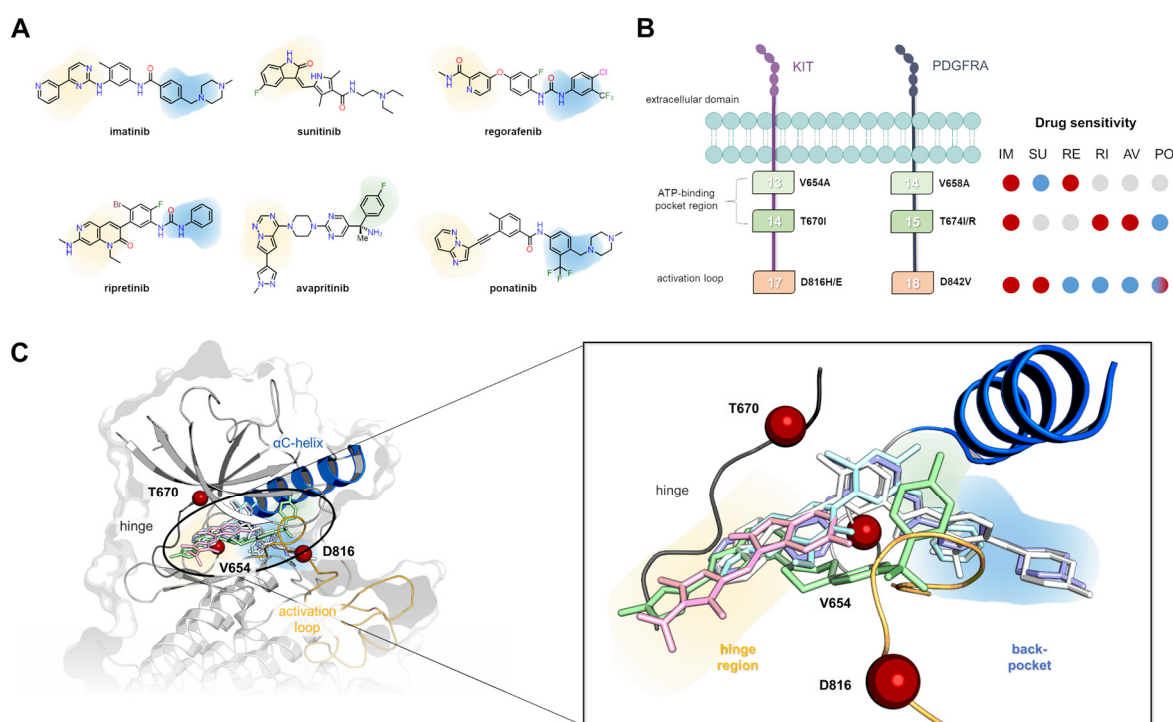


Figure 21. Overview of the current landscape of approved KIT and PDGFRA inhibitors. **(A)** Approved TKI for the treatment of GIST and the clinical investigated inhibitor ponatinib. **(B)** Schematic overview of the sensitivity profiles of selected TKIs against mutant forms of KIT and PDGFRA. Blue = sensitive, gray = moderate activity, red = resistant. **(C)** Localization of AP (Thr670, V654) and AL (Asp816) hotspot mutations are shown in KIT as well as the binding modes of: imatinib, blue, PDB ID: 1T46; sunitinib, pink, PDB ID: 3G0E; ripretinib analog, cyan, PDB ID: 6MOB; avapritinib, green, PDB ID: 8PQ; ponatinib, white, PDB ID: 4U0I. For simplicity the heteroatoms are not color-coded. Hinge binding elements (yellow), back pocket elements (blue) and the Ga-pocket element (green) are highlighted in the respective colors and pockets. Figure adapted from Schulz *et al.*, revised.

Meanwhile, covalent targeting has emerged as a successful strategy and is a well-established approach in the design of small molecule inhibitors, offering several pharmacological benefits.^{83, 87} These include enhanced potency, improved selectivity, and maximized residence time, making it a promising approach to address the limitations of ponatinib.^{83, 87}

Leveraging ponatinib's inherent potency against AL and T670I as well as the proven advantages of covalent TKIs, a structure-based approach inspired the synthesis of novel covalent type II inhibitors with extensive SAR studies targeting mutant KIT and PDGFRA.

6.2 Structure-based Design of Covalent Ponatinib-based Inhibitors

The first step involved identifying amino acids within *KIT* and *PDGFRA* that are amenable to covalent modification. Three cysteine residues are present in their active sites: Cys673 in the hinge region, Cys788 in the back pocket, and Cys809 just in front of the DFG motif (numbering with respect to *KIT*, UniProt ID: P10721; corresponding amino acids in *PDGFRA*, UniProt ID: P16234, are Cys677, Cys814, and Cys835, respectively) (Figure 22).

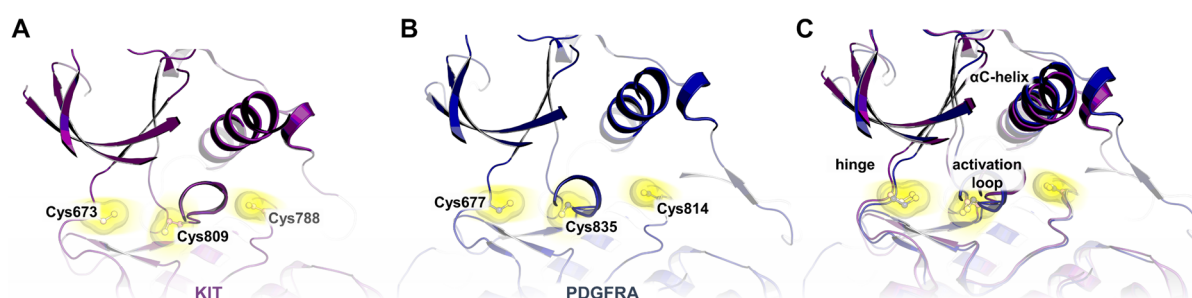


Figure 22. Cysteines present within *KIT* (violet, PDB ID: 4U0I, **A**) and *PDGFRA* (blue, PDB ID: 5K5X, **B**) and an alignment (**C**). Figure adapted from Schulz *et al.*, revised.

Cys788 is the least common, being present in only 18 other kinases (Supplementary Table 3), offering the potential to enhance the selectivity of ponatinib. In 2011, LEPROULT *et al.* utilized an imatinib-based inhibitor decorated with a reactive chloroacetamide warhead to show the general addressability of these cysteine via mass spectrometry (MS);²⁴⁷ no study has yet explored the potential of covalent inhibitors to target drug-resistant GIST, nor has a crystal structure of *PDGFRA* bound to a covalent inhibitor been reported. In this work, the structure-based design and development of novel covalent inhibitors targeting *KIT* and *PDGFRA*, aim to harness the benefits of covalent targeting to address the challenges of side effects and selectivity. Additionally, the key pharmacophoric features necessary for targeting both AP and AL mutants were investigated. Based on the structural analysis (*cf.* Chapter 5), a core structure of ponatinib was defined around which key ligand elements were modified to evaluate their impact on biological activity, particularly in relation to resistance mutations (Figure 21). It was postulated that the alkyne linker, which connects the hinge- and back pocket-binding moieties, represents a pivotal factor in addressing gatekeeper resistance mutations effectively. To test this assumption, inhibitors both with and without this pharmacophore, following a type III inhibitor design approach, were synthesized. Although ponatinib and imatinib share a similar core structure, imatinib features a reversed amide bond (N-C=O) compared to ponatinib (O=C-N). Accordingly, the objective was to gain insight into the functional distinctions between these two linker motifs by synthesizing a series of divergent inhibitors (Figure 23B).

Additionally, benzimidazole derivatives, as a privileged scaffold, were considered as a means of mimicking this pharmacophore. Furthermore, the potential role of the CF_3 group in inhibiting AL mutants was explored through the removal of this moiety and subsequent evaluation of alternative substitution patterns. Furthermore, the range of back pocket-binding elements was expanded by the introduction of various substituents on the phenyl ring, to assess the potential for covalent targeting (Figure 23C). Utilizing the co-crystal structure of ponatinib complexed with KIT (PDB ID: 4U0I), it was feasible to identify a suitable position for integrating a warhead to target Cys788 in KIT (equivalent to Cys814 in PDGFRA). The proximity of approximately 4.0 Å between the benzylic position and the Cys788 suggested that modifying this position with an electrophile would enable successful covalent bond formation.

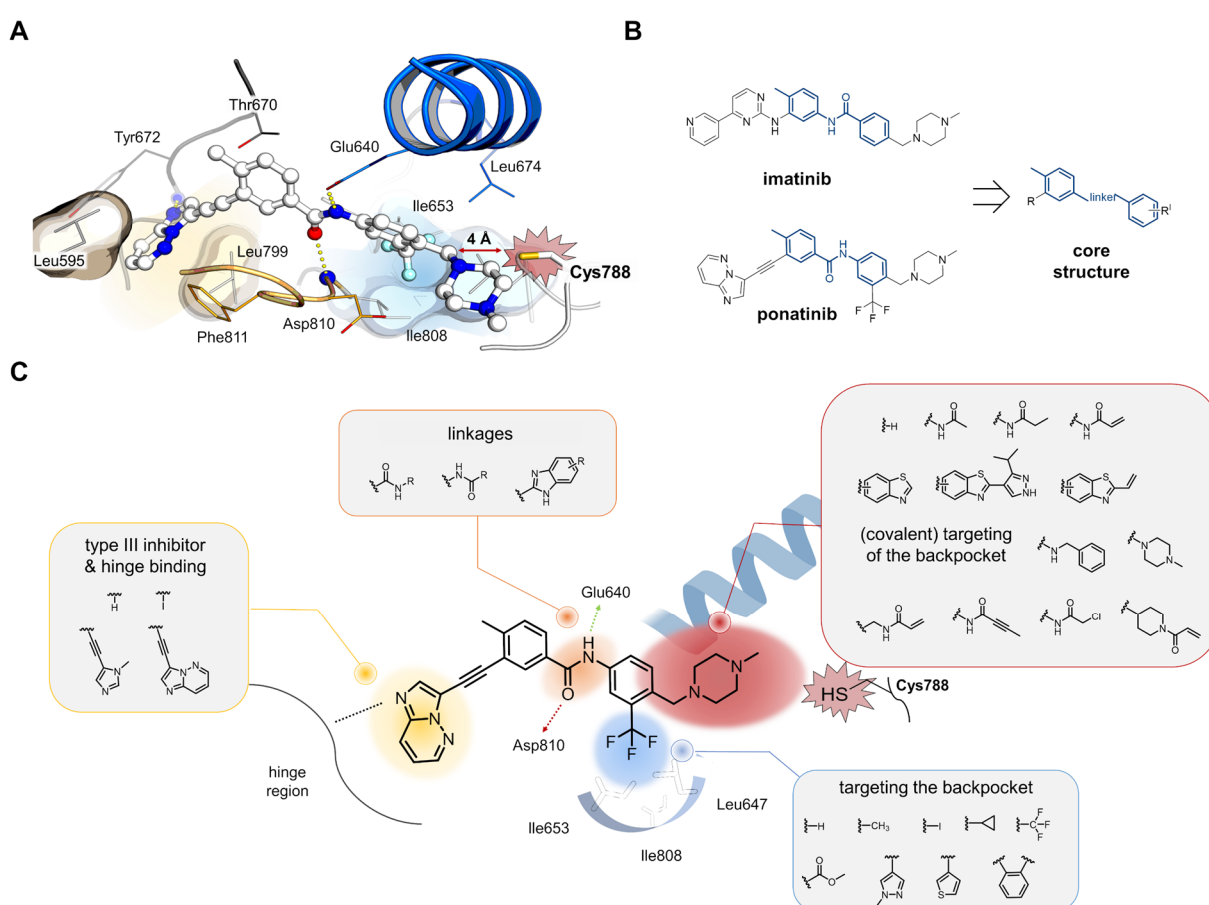


Figure 23. Structure-based design approach to develop covalent type II inhibitors. **(A)** Co-crystal structure of ponatinib bound to KIT-wt (PDB ID: 4U0I). Interactions are highlighted as dotted lines (H-bonds), surfaces (hydrophobic interactions), and the addressed Cys788 is highlighted. **(B)** LEWIS structure of imatinib and ponatinib and the defined core structure for SAR studies. **(C)** Schematic representation of points of derivatization with exemplary substitutions for SAR. Figure adapted from Schulz *et al.*, revised.

Covalent docking studies were conducted to ascertain favorable exit vectors in the vicinity of the benzyl ring to further refine this approach (Figure 24). These studies supported the incorporation of acrylamide at the *ortho* position relative to the CF_3 moiety, which would allow for optimal covalent bond formation while maintaining all key protein-ligand interactions.

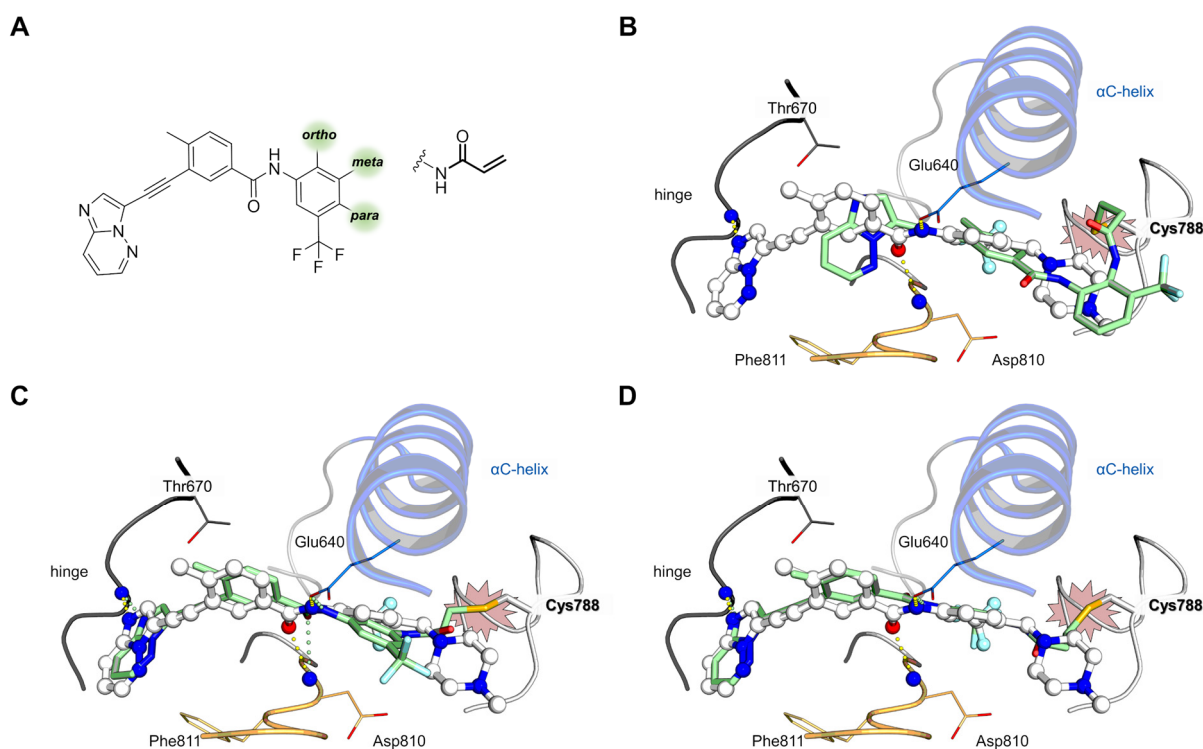
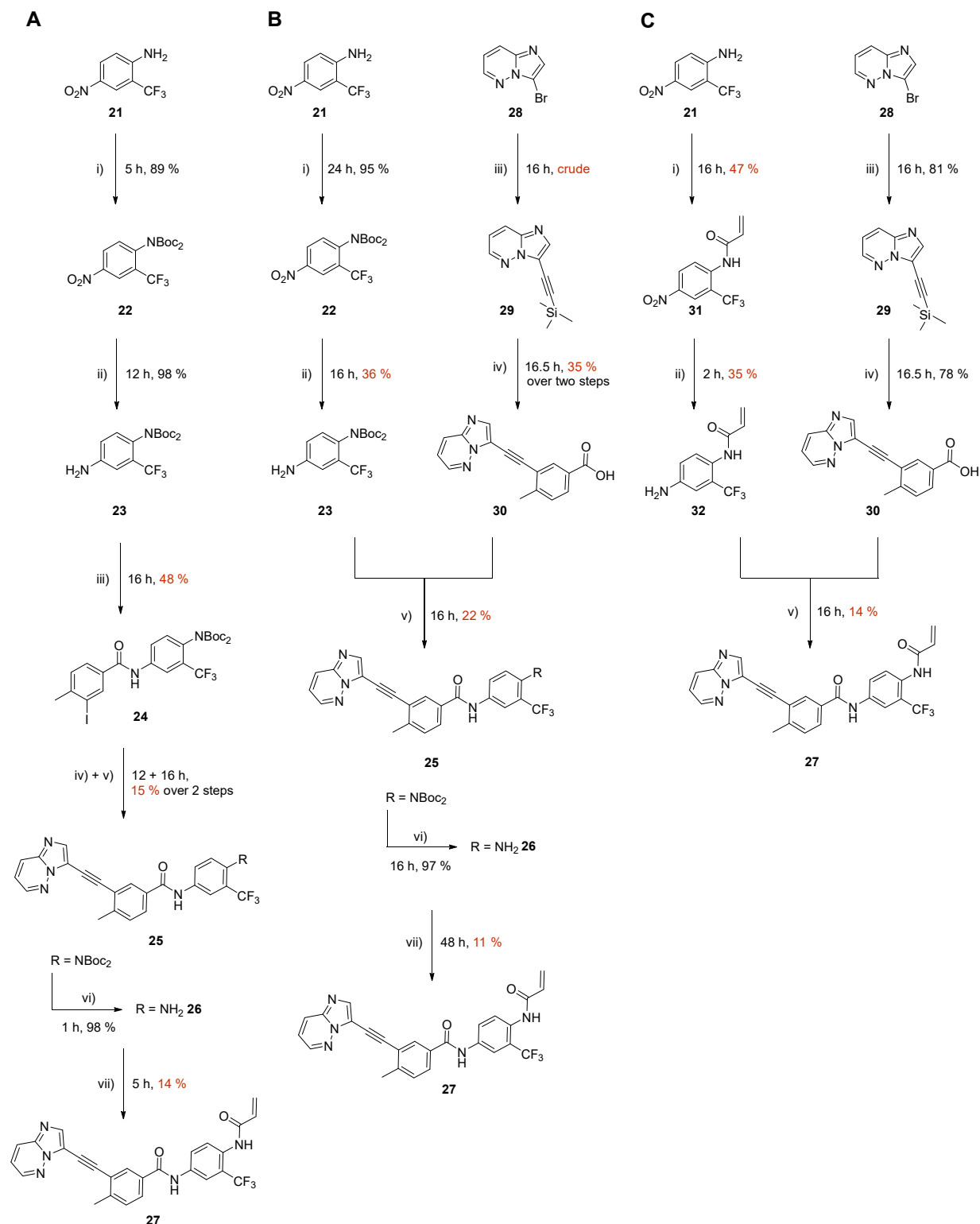


Figure 24. Selected docking poses (green) of ponatinib derivatives decorated with an acrylamide (**A**) in *ortho* (**B**), *meta* (**C**) or *para* (**D**) position in comparison to the parental binding mode of ponatinib (white). Figure adapted from Schulz *et al.*, revised.

6.3 Synthesis of a Structure-based, Focused Covalent Compound Library

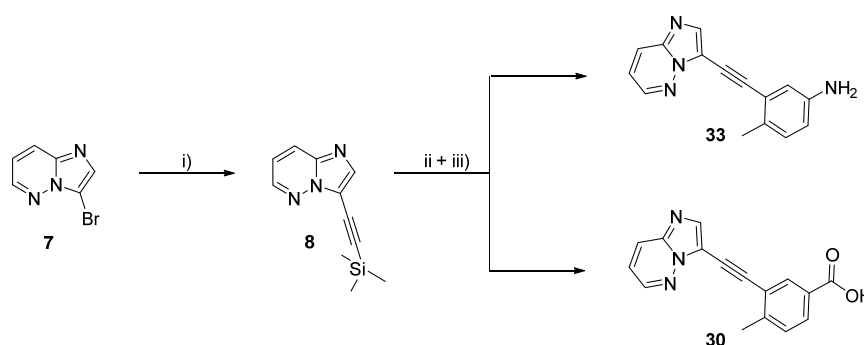
In light of the preliminary work conducted by Dr. HELENA KAITSIOTOU and Dr. JULIA HARDICK, the initial synthesis route was adopted and optimized considering the synthetic accessibility of new derivatives (Scheme 4).²⁴⁸⁻²⁴⁹ The initial linear synthesis comprised seven steps, including protection and deprotection steps to enhance chemoselectivity (Scheme 4A). However, the synthesis route was associated with low yields, particularly in the case of the amidation reactions of 4-nitro-2-(trifluoromethyl)aniline and the SONOGASHIRA coupling. The synthesis was modified to a seven-step convergent route, which did not yield a notable improvement in terms of yield or time efficiency (Scheme 4B). Through screening a variety of reaction conditions (Supplemental Table 4), suitable parameters for directly installing the acrylamide to the 4-nitro-2-(trifluoromethyl)aniline were identified, thereby establishing the basis for a shortened five-step convergent route (Scheme 4C). By modifying the reaction parameters and employing a partial convergent approach to facilitate the synthesis of the hinge binding element, it was possible to achieve higher yields for the SONOGASHIRA reaction. Accordingly, a partially convergent approach was selected to generate a comprehensive array of diverse reversible and covalent compounds, wherein the back pocket binding element was systematically varied. On the one hand, the synthesis of the western parts, featuring the hinge binding elements (Scheme 5); on the other hand, different eastern parts, bearing the warhead and different back pocket elements (Scheme 6-11).

Scheme 4. Development of a synthesis route towards covalent ponatinib derivatives^a

^aReagents and conditions: **A** (i) DMAP, Et₃N, Boc₂O, THF, 5 h, reflux; (ii) Pd/C, H₂, EtOH, 12 h; (iii) HATU, DIPEA, DCM, DMAP, 3-iodo-4-methylbenzoic acid, rt, 16 h; (iv) 29, Pd(PPh₃)₂Cl₂, CuI, (iPr)₂NH, TBAF, ACN, 80 °C, 16 h; (v) 10 % TFA, DCM, 0 °C-rt, 1 h; (vi) acryloyl chloride, DIPEA, THF, 78 °C to rt, 5 h. **B** (i) Et₃N, DMAP, Boc₂O, THF, 80 °C, 1d; (ii) Fe, NH₄Cl, EtOH:H₂O (9:1), 60 °C, 16 h; (iii) TMSA; DIPA, Pd(PPh₃)₂Cl₂, CuI, MeCN, 80 °C, 16 h; (iv) 3-iodo-4-methylbenzoic acid, DIPA, Pd(PPh₃)₂Cl₂, CuI, MeCN, 60 °C, 30 min, then cooling to rt and addition of TBAF, 80 °C, 16 h; (v) HATU; DIPEA, 3-iodo-4-methylbenzoic acid, THF, rt, 16 h; (vi) TFA: DCM (1:3), rt, 16 h; (vii) acryloyl chloride, DIPEA, dry THF, 0 °C to rt, 2d. **C** (i) DIPEA, DCM/DMF, rt to 50 °C, o.v.n.; (ii) Fe, NH₄Cl, EtOH:H₂O (1:1), 60 °C, 2 h; (iii) TMSA, Pd(PPh₃)₂Cl₂, CuI, DIPA, MeCN, 80 °C, o.v.n.; (iv) TBAF, dry ACN, rt, 30 min; 3-iodo-4-methylbenzoic acid, Pd(dppf)₂Cl₂, CuI, DIPA, 80 °C, 16 h. (v) HATU, DIPEA, DCM/DMF, rt to 50 °C, o.v.n.

The first step was a SONOGASHIRA coupling of bromoimidazo-pyridazine **28** with trimethylsilylacetylene (TMSA) to afford trimethylsilyl (TMS) protected imidazo-pyridazine **29**. Subsequently, a second SONOGASHIRA coupling was conducted with either 3-iodo-4-methylbenzoic acid or 3-iodo-4-methylaniline, resulting in the synthesis of the corresponding western parts **30**, **33**, which feature the hinge binding element (Scheme 5). This approach enables the introduction of different constituted amide patterns.

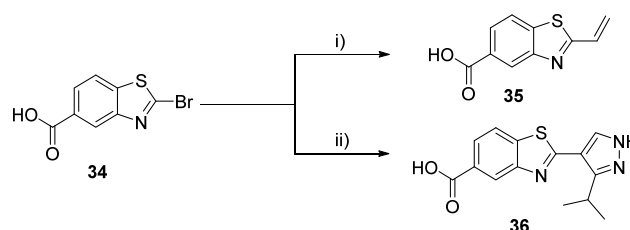
Scheme 5. Synthesis of western parts **30** and **33**^a



^aReagents and conditions: (i) TMSA, Pd(PPh₃)₂Cl₂, CuI, DIPA, dry ACN, 80 °C, 14 h; (ii) TBAF, dry ACN, rt, 30 min; (iii) 3-iodo-4-methylbenzoic acid or 3-iodo-4-methylaniline, Pd(dppf)Cl₂, CuI, DIPA, 80 °C, 16 h.

The corresponding eastern parts were synthesized in different ways (Scheme 6-11). The thiazoles were synthesized in a one-step palladium-catalyzed reaction in either a STILLE or a SUZUKI-MIYAUURA coupling; 2-bromobenzo[d]thiazole-5-carboxylic acid **34** was decorated with vinyl-(tri-*n*-butyl)tin or 3-isopropyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole to yield **34** and **36**, respectively (Scheme 6).

Scheme 6. Synthesis of precursors **35** and **36**^a



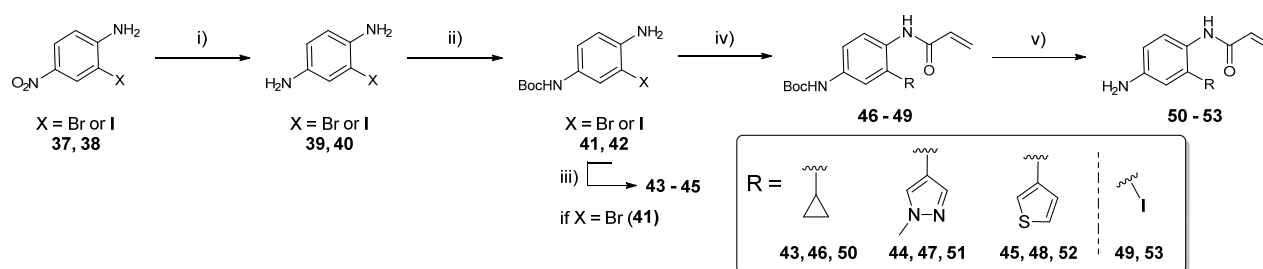
^aReagents and conditions: (i) vinyl-(tri-*n*-butyl)tin, Pd(PPh₃)₄, CuI, dry DMF, 65 °C, 11 min. in a microwave; (ii) 3-isopropyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole, Pd(dppf)Cl₂, KH₂PO₄, dioxane/water, 120 °C, 1 h in a microwave.

Different back pocket elements were introduced by making a small library using SUZUKI-MIYAUURA coupling. Thus, a five-step synthesis route was established (Scheme 7).^{250XV} A BECHAMP reduction of the commercially available bromo- (**37**) or iodo-nitroaniline (**38**) was the first step. Of note, a reduction with H₂, Pd/C led to dehalogenation and no product could be observed.

^{XV} The synthesis of **50-53** was performed by Maria Beerbaum, TU Dortmund University (AG Rauh), 2024.

In the second step, the free amines **39** and **40** were protected with *tert*-butyloxycarbonyl (Boc) group at the 4'-position to ensure chemoselectivity for the upcoming S_N reaction with acryloyl chloride. After that, SUZUKI-MIYAUURA couplings were conducted to introduce different back pocket binding elements on **41**. While coupling with aromatic systems like methyl pyrazole (**44**) or thiophene (**45**) afforded very good yields (81-93 %), coupling with aliphatic groups turned out to be problematic (18 % for cyclopropyl (**43**)). Notably, no product formation was observed for cyclobutyl and cyclopentyl boronic acids. However, dehalogenation was observed at higher temperatures. After derivatization, the warhead was installed, followed by a carbamate cleavage of the BOC group yielding **50-53**.

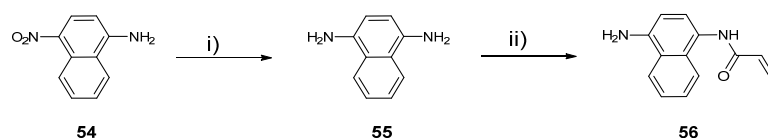
Scheme 7. Synthesis of precursors for compounds **50-53**^a



^aReagents and conditions: (i) Fe, NH₄Cl, aq. MeOH, 65 °C, 24-48 h; (ii) di-*tert*-butyl dicarbonate, TEA, dry MeCN, 0 °C – rt, 24 h; (iii) boronic acid, Pd(PPh₃)₄, Cs₂CO₃, dioxane/water (5:1), 120 °C for 2 h in a microwave; (iv) acryloyl chloride, DIPEA, dry DCM, rt, 1 h; (v) TFA:DCM (1:5), rt, 24 h.

Precursor **56** was prepared in two steps from commercially available 4-nitronaphthalene-1-amine (**54**) (Scheme 8).^{250XVI} Interestingly, direct coupling with acryloyl chloride did not result in any product formation; this may be due to the deactivating M effect, which prevents nucleophilic attack. Thus, the first step was the reduction to form the diamine **55**. It is important to note that **62** was found to be unstable under light and oxygen, which is consistent with the literature on similar substrates.²⁵¹⁻²⁵² In the second step, the warhead was installed. The overall yield was around 5 %, suggesting that optimization of the synthesis to improve stability should be investigated if further derivatives are of interest. One possibility would be to add protecting groups or to couple the key building block directly to the 4-nitronaphthalene-1-amine.

Scheme 8. Synthesis of precursor for compound **56**^a



^aReagents and conditions: (i) H₂, Pd/C, dry MeOH, rt, 24 h; (ii) acryloyl chloride, DIPEA, dry DCM/DMF, rt, 1 h.

^{XVI} The synthesis of **56** was performed by Maria Beerbaum, TU Dortmund University (AG Rauh), 2024.

With these key building blocks in hand, various amide couplings using different acids or amines, including pre-synthesized compounds in-house were performed (Figure 25).

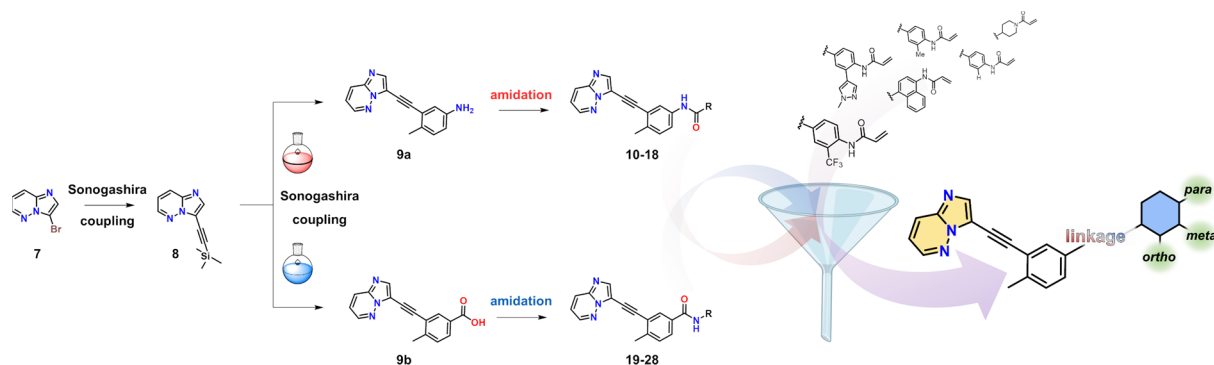
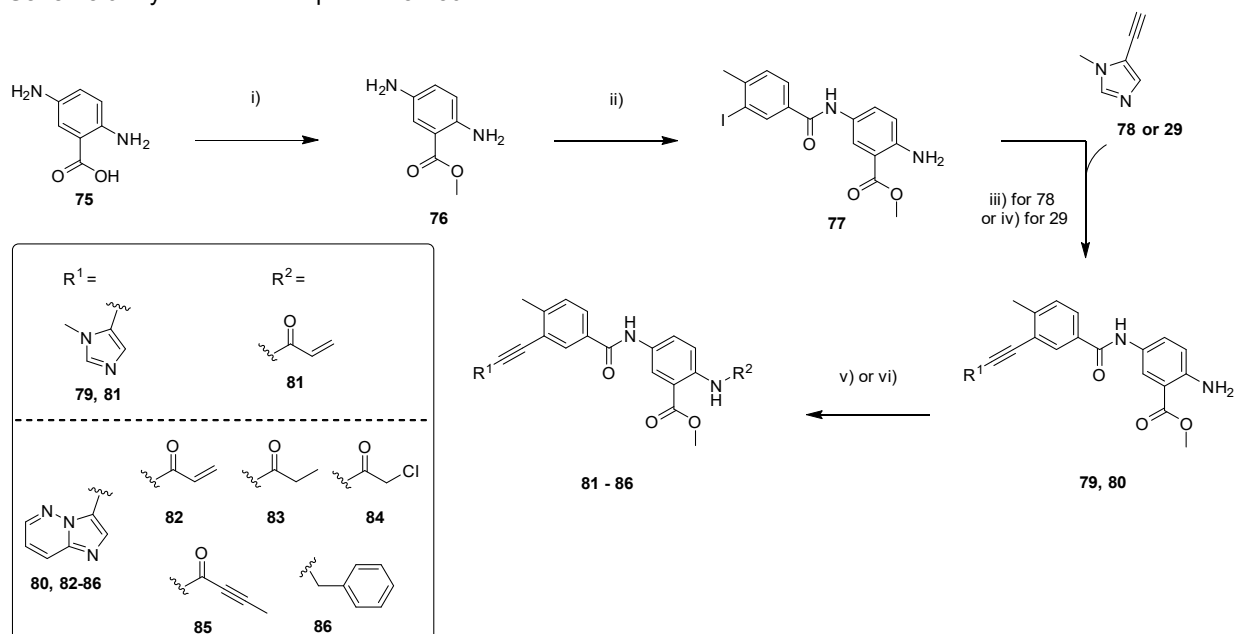


Figure 25. Schematic overview of the synthesis for the focused compound library. Figure adapted from Schulz, *et al.*, revised.

Some of the intended compounds required different synthesis efforts due to better synthetic accessibility of the derivatives or the absence of either the hinge binding element or the amide linkage. Hence, a four-step synthesis was used to generate the ester-containing derivatives **81-86** (Scheme 9).^{xvii} In the first step, the commercially available 2,5-diaminobenzoic acid underwent esterification.

Scheme 9. Synthesis of compounds **81-86**^a

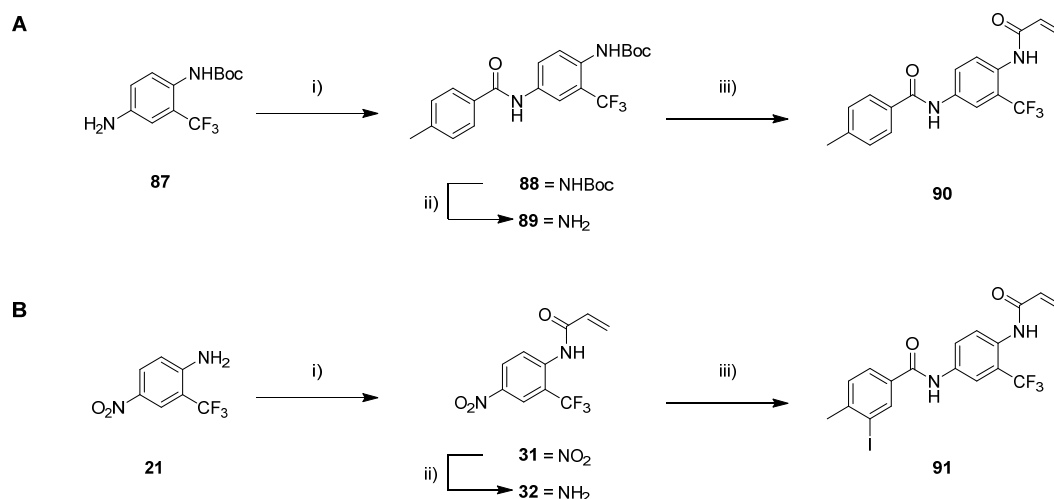


^aReagents and conditions: (i) MeOH, SOCl₂, 90 °C, reflux, ovn.; (ii) 3-iodo-4-methylbenzoic acid, HATU, DIPEA, dry DMF, 95 °C, 3 h; (iii) **78**, Pd(PPh₃)₂Cl₂, CuI, DIPA, dry MeCN, 80 °C, ovn.; (iv) **29**, Pd(PPh₃)₂Cl₂, CuI, DIPA, dry MeCN, TBAF, 80 °C, ovn.; (v) acid chloride, DIPEA, THF, rt, ovn.; (vi) benzyl bromide, K₂CO₃, dry MeOH, 65 °C, ovn.

^{xvii} The synthesis was described in Tom Schulz's master's thesis, TU Dortmund University (AG Rauh), 2021.

Afterwards, an [dimethylamino(triazolo[4,5-b]pyridin-3-yloxy)methylidene]-dimethylazanium; hexafluorophosphate (HATU)-mediated amidation with 3-iodo-4-methylbenzoic acid gave **77**. Notably, the reaction yielded excellent 95 %, although two free amino groups were present. This could be rationalized by the ortho ester (with respect to the non-reactive amine), firstly due to electronic effects (M-effect) and secondly due to steric hindrance. Next, a SONOGASHIRA reaction was performed between the amide **77** and either the previously synthesized alkyne (**29**) or ethynyl-imidazole **78**, yielding **79** and **80**, respectively. **81** was obtained by substitution of **79** with acryloyl chloride. Lastly, three different warheads, a reversible counterpart (propionamide), and a bulky benzyl substituent were attached to the free amine of **80**, resulting in the formation of derivatives **82-86**.

Scheme 10. Synthesis of compounds **90**, **91**^a



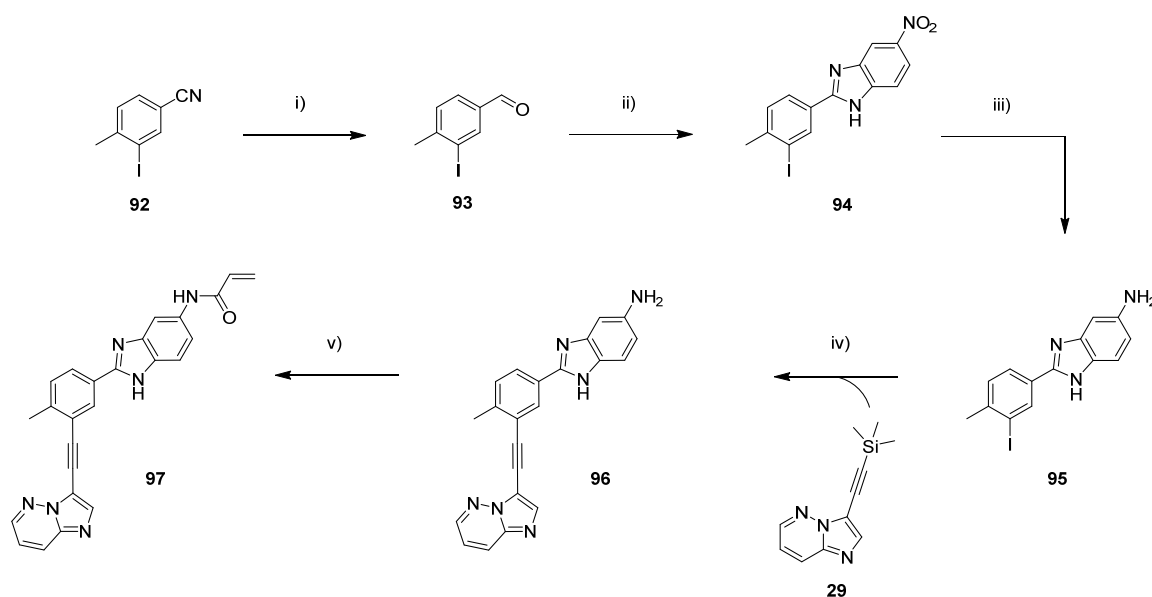
^aReagents and conditions: (A) i) 4-methylbenzoic acid, HATU, DIPEA, dry THF, rt, ovn.; (ii) TFA:DCM (1:3), 0 °C - rt, 5 h; (iii) acryloyl chloride, DIPEA, dry THF, 0 °C - rt, 3 h. B) (i) acryloyl chloride, DIPEA, dry DCM/DMF, rt, ovn.; (ii) Fe, NH₄Cl, EtOH/H₂O (1:1), 60 °C, 2.5 h; (iii) 3-iodo-4-methylbenzoic acid, HATU, DIPEA, dry DCM/DMF, rt, ovn.

90 and **94**, following a type III inhibitor approach, were synthesized in a linear three-step synthesis. Beginning with the amidation of commercially available *tert*-butyl (4-amino-2-(trifluoromethyl)phenyl)(*tert*-butoxycarbonyl)carbamate, which was coupled with 4-methylbenzoic acid. This was followed by BOC deprotection and, finally, the formation of acrylamide to give compound **90** (Scheme 10A).^{249XVIII} Similarly, 4-nitro-2-(trifluoromethyl)aniline was first functionalized with an acrylamide group, then reduced, and subsequently coupled with 3-iodo-4-methylbenzoic acid to yield compound **94** (Scheme 10B).

^{XVIII} The synthesis of **90** was performed by Dr. Julia Hardick, TU Dortmund University (AG Rauh), 2021.

For the benzimidazole derivative **97**, a linear six-step synthetic route was developed (Scheme 11).^{253XIX} The first step was a diisobutylaluminium hydride (DIBAL-H)-mediated reduction of 3-iodo-4-methylbenzonitrile. The benzimidazole moiety was then formed through a kinetically controlled cyclization reaction, followed by a BÉCHAMP-like reduction of the nitro group. A subsequent SONOGASHIRA coupling between benzimidazole **95** and TMS-protected alkyne **29** afforded **96**. Finally, amidation with acryloyl chloride yielded **97**.

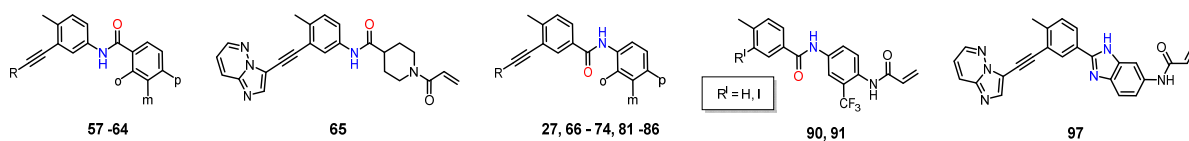
Scheme 11. Synthesis of benzimidazole **97**^a



^aReagents and conditions: (i) DIBAL-H, toluene, 0 °C, 3 h; (ii) 4-nitrobenzene-1,2-diamine, aq. H₂O₂, aq. HCl, MeCN, -5 °C, 4 h; (iii) Fe, CH₃COOH, aq. EtOH, 75 °C, 3 h; (iv) Pd(PPh₃)₂Cl₂, CuI, DIPA, dry MeCN, TBAF, 80 °C, ovn.; (v) acryloyl chloride, TEA, dry THF, rt, ovn.

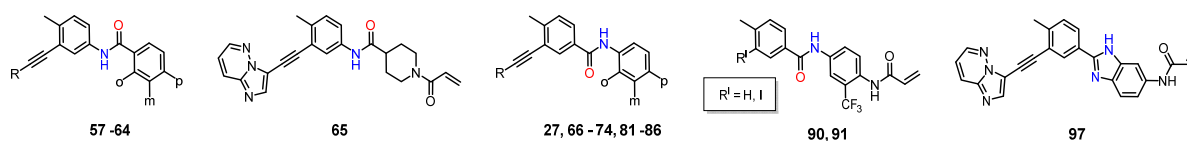
Overall, a variety of synthetic routes were developed, optimized, and pursued in order to synthesize a focused compound library of 28 novel reversible as well as covalent inhibitors (Table 5). The synthesized inhibitors were subsequently evaluated in biochemical and cellular assays for covalent binding, potency towards KIT and PDGFRA, and selectivity.

^{XIX} Details on the development of the synthesis route can be found in Tom Schulz's master's thesis, TU Dortmund University (AG Rauh), 2021.

Table 5. Overview of the synthesized inhibitors for the SAR studies. ^aSynthesized by Dr. Rajesh Gontla, ^binitially synthesized by Dr. Helena Kaitsiotou, ^cSynthesized by Maria Beerbaum.

cpd id	R =	<i>ortho</i>	<i>meta</i>	<i>para</i>
57		H	CF ₃	
58 ^a		H	CF ₃	H
59 ^a		H	CH ₃	
60 ^a		H	H	
61 ^a		H		
62		H		
63		H		
64		H		
27 ^b		H	CF ₃	
66 ^a		H	CF ₃	H
67 ^a			H	H
68 ^a		H	CF ₃	
69 ^a		H	H	
70 ^c		H		
71 ^c		H		
72 ^c		H		
73 ^c		H	I	
74 ^c				

Table 5 continued.



cpd id	R =	<i>ortho</i>	<i>meta</i>	<i>para</i>
81		H		
82		H		
83		H		
84		H		
85		H		
86		H		

6.4 Biochemical and Cellular Evaluation of Covalent KIT/PDGFR α Inhibitors

To determine the half-maximal inhibitory concentration (IC₅₀) and to establish structure-activity relationships, an activity-based HTRF assay was conducted to evaluate the inhibitors against wild-type and mutant forms of KIT (D816H, T670I) and PDGFR α (D842V, T674I, G680R) (Table 6).^{XX} In preliminary work, the parameters for the corresponding wild-types and AL mutants were already established^{231, 248}, but for a more comprehensive profiling of the new inhibitors, assay conditions for the gatekeeper mutant proteins (KIT-T670I and PDGFR α -T674I) had to be identified. In order to accomplish this, it was required that a number of preparatory steps be carried out in accordance with the instructions provided by the manufacturer Cisbio (Codolet, FR).^{254XXI} The initial step was to ascertain the optimal enzyme concentration, which was established at 80 % of the maximum signal for the subsequent reactions (Supplementary Figure 10A). Subsequently, the optimal reaction time was determined through kinetic experiments, in which the linear correlation between signal and incubation time was mapped (Supplementary Figure 10B). Given that enzyme activity is dependent on the concentration of ATP and the substrate, the respective MICHAELIS-MENTEN constants were determined independently (Supplementary Figure 10C, D).

^{XX} HTRF measurements were also conducted by Andreas Arndt, Dr. Alina Teuber, Dr. Marina Keul and Kirujan Jeyakumar, TU Dortmund University (AG Rauh).

^{XXI} The establishment of the PDGFR α -T674I variant was performed together with Maria Beerbaum.²⁵⁰

Subsequently, the determined values were employed in a test run on known inhibitors to further verify the conditions regarding comparable and robust results, thus providing additional evidence to support the identified parameters (Supplementary Table 5). It is noteworthy that, although the majority of compounds analyzed are not ATP-competitive inhibitors, this assay can be used to approximate their potency. However, it should be noted that a determination of the k_{on} and k_{inact} provides a more accurate estimate of the actual potency.^{79, 82} Nevertheless, compounds like **27** and **70** exhibited very potent inhibition against both KIT-wt (2.3 and 1.2 nM) and KIT-D816H (12 and 8.6 nM), surpassing the potency of first to third-line TKIs. Notably, significant inhibition was also observed for PDGFRA-G680R, with compound **27** demonstrating an IC_{50} of approximately 4 nM. The enhanced potency relative to the approved TKIs can be attributed to the imidazo-pyridazine scaffold, which appears to be less impacted by the introduced special requirement of Arg680 (Figure 26A).

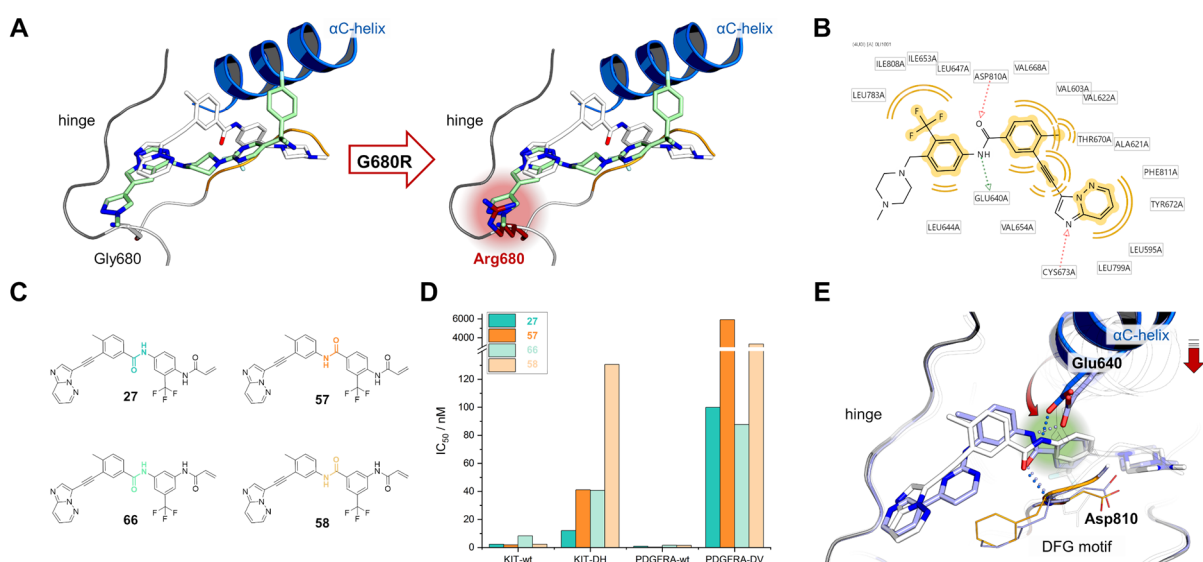


Figure 26. (A) Binding mode of ponatinib (white, PDB ID: 4U0I) and avapritinib (green, PDB ID: 4U0I) in wild-type vs. G680R-mutant PDGFRA. The red sphere indicates a potential steric repulsion with the sterically demanding, basic Arg680 and avapritinib. (B) Pharmacophore model of ponatinib bound to KIT-wt (PDB ID: 4U0I). (C) LEWIS structures of **27**, **57**, **58**, and **66**. Amide linkages are color-coded. (D) Representation IC_{50} values of **27**, **57**, **58**, and **66** for wt and AL mutant KIT and PDGFRA. (E) Structural analysis of ponatinib (white, PDB ID: 4U0I) and imatinib (blue, PDB ID: 1PKG) in an active (shown as outline, PDB ID: 1PKG) and inactive conformation (PDB ID: 4U0I) of KIT-wt. H-bonds are shown as dotted lines. Figure adapted from Schulz *et al.*, revised.

In comparison to a reversible analog (**68**), compound **27** exhibited a notable enhancement in potency, with a two-fold increase observed for PDGFRA-wt and a 14.8-fold increase for PDGFRA-T674I. Both inhibitors demonstrated comparable inhibition at approximately 2 nM against KIT-wt. However, no significant differences in potency were observed between the ester-bearing covalent (**82**) and reversible (**83**) inhibitors. This may be attributed to a reduced propensity for covalent bond formation due to unfavorable (pre)-organization, possibly limited by an intramolecular hydrogen bond between the ester and the warhead.

The use of a rigid butynamide warhead (**85**) resulted in a marked loss of potency against mutant KIT and PDGFR α , likely due to suboptimal geometry and orientation towards the targeted cysteine residue or decreased electrophilicity.

Table 6. Determined IC₅₀ values of the synthesized compound library and references against KIT and PDGFR α variants. Values are presented as mean $\pm$ s.d; n.d. not determined; n = number of independent experiments, all with n $\geq$ 3 otherwise marked as ^a n = 2.

cpd id	HTRF IC ₅₀ [nM]						
	KIT-wt	KIT-D816H	KIT-T670I	PDGFR α -wt	PDGFR α -D842V	PDGFR α -T674I	PDGFR α -G680R
57	1.9 $\pm$ 0.9	41.1 $\pm$ 10.9	2237 $\pm$ 529	0.3 $\pm$ 0.2	5905 $\pm$ 1398	227 $\pm$ 58	4.9 $\pm$ 0.3
58	2.3 $\pm$ 0.9	131 $\pm$ 52	5034 $\pm$ 718	1.5 $\pm$ 0.6	3332 $\pm$ 1641	1249 $\pm$ 161	20.7 $\pm$ 5.5
59	9.9 $\pm$ 3.5	$\geq$ 10000	$\geq$ 10000	5.7 $\pm$ 1.8	$\geq$ 10000	$\geq$ 10000	4495 $\pm$ 3976
60	5.9 $\pm$ 3.0	$\geq$ 10000	$\geq$ 10000	217 $\pm$ 38	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000
61	33.2 $\pm$ 14.5	8282 $\pm$ 2943	$\geq$ 10000	44.2 $\pm$ 22.2	2623 $\pm$ 946	$\geq$ 10000	2079 $\pm$ 1005
62	40.2 $\pm$ 12.4	$\geq$ 10000	$\geq$ 10000	43.5 $\pm$ 18.6	7047 $\pm$ 851	$\geq$ 10000	852 $\pm$ 535
63	2.3 $\pm$ 1.0	$\geq$ 10000	$\geq$ 10000	1.4 $\pm$ 0.7	2798 $\pm$ 388	4661 $\pm$ 2751	21.0 $\pm$ 3.3
64	616 $\pm$ 326	$\geq$ 10000	$\geq$ 10000	823 $\pm$ 420	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000
65	3290 $\pm$ 1660	$\geq$ 10000	$\geq$ 10000	73.4 $\pm$ 47.7	$\geq$ 10000	$\geq$ 10000	1941 $\pm$ 262
27	2.3 $\pm$ 0.4	12.0 $\pm$ 4.6	492 $\pm$ 134	0.8 $\pm$ 0.3	99.9 $\pm$ 23.8	42.3 $\pm$ 18.3	4.3 $\pm$ 1.4
66	8.3 $\pm$ 1.6	40.7 $\pm$ 16.9	329 $\pm$ 44	1.6 $\pm$ 0.4	87.7 $\pm$ 39.1	48.5 $\pm$ 4.5	11.4 $\pm$ 2.9
67	44.8 $\pm$ 12.6	$\geq$ 10000	$\geq$ 10000	15.9 $\pm$ 7.2	$\geq$ 10000	$\geq$ 10000	471 $\pm$ 97
68	2.2 $\pm$ 0.3	29.5 $\pm$ 13.5	3858 $\pm$ 1338	1.6 $\pm$ 0.4	380 $\pm$ 106	620 $\pm$ 347	11.0 $\pm$ 2.8
69	7.7 $\pm$ 4.3	$\geq$ 10000	$\geq$ 10000	9.3 $\pm$ 2.9	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000
70	1.2 $\pm$ 0.4	8.6 $\pm$ 4.0	63.1 $\pm$ 25.8	3.2 $\pm$ 1.3	596 $\pm$ 93 ^a	13.8 $\pm$ 1.3	31.1 $\pm$ 16.7
71	11.0 $\pm$ 5.7 ^a	8152 $\pm$ 1490	n.d.	89.2 $\pm$ 49.5	$\geq$ 10000	n.d.	$\geq$ 10000
72	n.d.	63 $\pm$ 14	n.d.	n.d.	2623 $\pm$ 251	n.d.	163 $\pm$ 71
73	1.1 $\pm$ 0.5	20.5 $\pm$ 3.5	212 $\pm$ 97	1.2 $\pm$ 0.5	1169 $\pm$ 479	34.8 $\pm$ 3.4	415 $\pm$ 226
74	0.6 $\pm$ 0.2	198 $\pm$ 30	$\geq$ 10000	1.8 $\pm$ 0.6	305 $\pm$ 134	257 $\pm$ 104	42.1 $\pm$ 22.3
81	127 $\pm$ 14	$\geq$ 10000	n.d.	113 $\pm$ 31	7020 $\pm$ 1256	n.d.	9852 $\pm$ 3168
82	12.8 $\pm$ 5.1	1744 $\pm$ 1000	28.6 $\pm$ 4.6	4.0 $\pm$ 1.6	5801 $\pm$ 1960	$\geq$ 10000	40.9 $\pm$ 10.2
83	8.4 $\pm$ 3.0	1725 $\pm$ 865	$\geq$ 10000	4.3 $\pm$ 1.1	9241 $\pm$ 105	$\geq$ 10000	28.8 $\pm$ 3.5
84	7.2 $\pm$ 1.9	440 $\pm$ 146	$\geq$ 10000	2.4 $\pm$ 0.4	511 $\pm$ 155	9837 $\pm$ 6954	15.1 $\pm$ 3.9
85	15.0 $\pm$ 4.7	$\geq$ 10000	$\geq$ 10000	7.4 $\pm$ 2.6	9975 $\pm$ 4159	$\geq$ 10000	84.6 $\pm$ 18.9
86	167 $\pm$ 62	$\geq$ 10000	$\geq$ 10000	98.4 $\pm$ 38.2	$\geq$ 10000	$\geq$ 10000	1432 $\pm$ 281
90	$\geq$ 10000	$\geq$ 10000	n.d.	n.d.	n.d.	n.d.	n.d.
91	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000
97	3400 $\pm$ 243	$\geq$ 10000	$\geq$ 10000	849 $\pm$ 190	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000

Inhibitor **69** (*para* acrylamide) demonstrated IC₅₀ values of approximately 8 nM and 9 nM, whereas **67** (*ortho* acrylamide) exhibited a lower potency with around 45 nM and 16 nM, against KIT-wt and PDGFR α -wt, respectively. This leads to the conclusion that the *para* position may serve as a more favorable exit vector for covalent modification of the target, which is consistent with the results of the docking studies (Figure 24). In line, a comparison between **27** (*para* acrylamide) and **66** (*meta* acrylamide) revealed a slight advantage for the former, with a 3.6-fold increase in potency against KIT-wt. Subsequently, MS experiments were conducted to elucidate the formation of covalent bonds between KIT-wt and the inhibitors. The protein was incubated with a representative series of inhibitors, which demonstrated successful single labeling by the inhibitors (Figure 27).^{XXII} Of note, not all inhibitors fully modified the protein under the selected conditions. **73** (iodide) displayed a slightly higher labeling efficiency compared to **68** (CF₃) or **82** (ester). Compound **69** (R=H) exhibited no modification, and only slight labeling was observed for aromatic-substituted compounds (e.g., **71**, **72**, **74**). These findings may be attributed to the insufficient engagement of the aromatic substituents with the hydrophobic back pocket, as well as the misalignment of the warhead for effective covalent bond formation with the cysteine residue. Further, the electronic properties of the substituents may also impact the reactivity of the warhead. Moieties that exert an electron-withdrawing effect (-I) featured higher labeling compared to those with electron-donating (+I) or positive mesomeric (+M) effects. However, the observed potency correlated with the labeling efficiency (**67** (*ortho*) < **27/57** (*para*); **81** (ester) < **59** (methyl) < **73** (iodide) $\approx$ **27** (CF₃)).

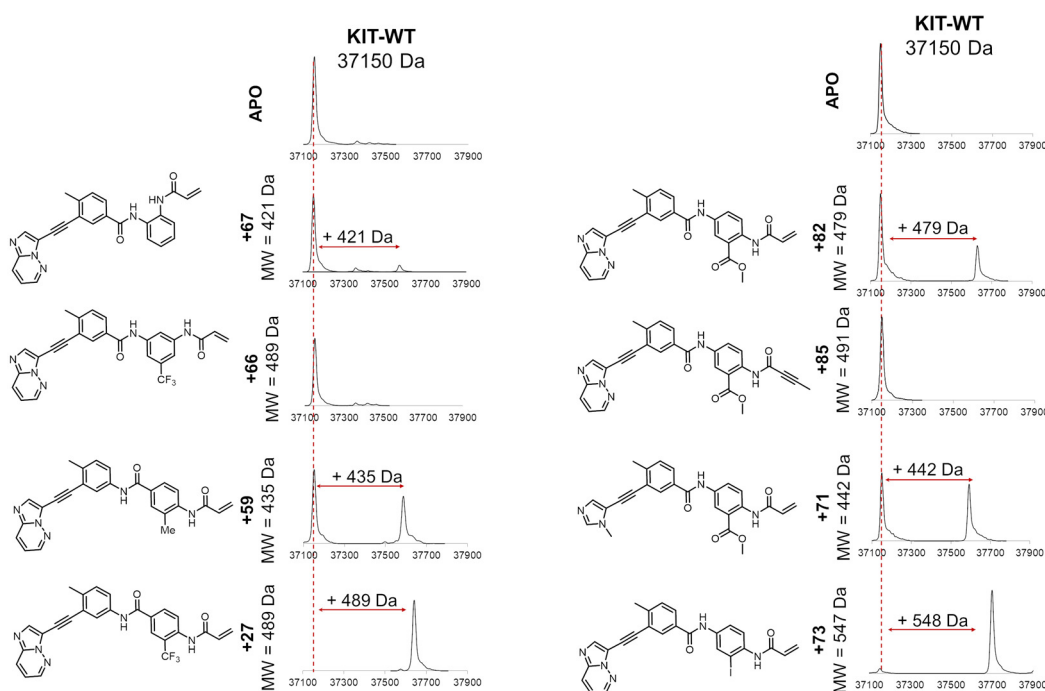


Figure 27. Mass spectrometry-based analysis of covalent bond formation of KIT-wt with selected inhibitors.

^{XXII} The protein was provided by Dr. Alina Teuber. Some of the MS experiments were performed together with Maria Beerbaum as part of her master thesis.²⁵⁰

To further investigate the influence of the substituents additional time-dependent MS experiments were conducted with four inhibitors utilizing saturating conditions in order to determine the inactivation rate k_{inact} (Figure 28, Table 7).^{XXIII} It should be noted that MS is a qualitative method; therefore, the reported k_{inact} values are intended to serve as approximations to permit comparison between the compounds. A comparison of the generated k_{inact} values and half-life ($t_{1/2}$) with existing literature was not applicable, as the inhibitors described herein represent a novel approach to covalent targeting of KIT/PDGFR α .

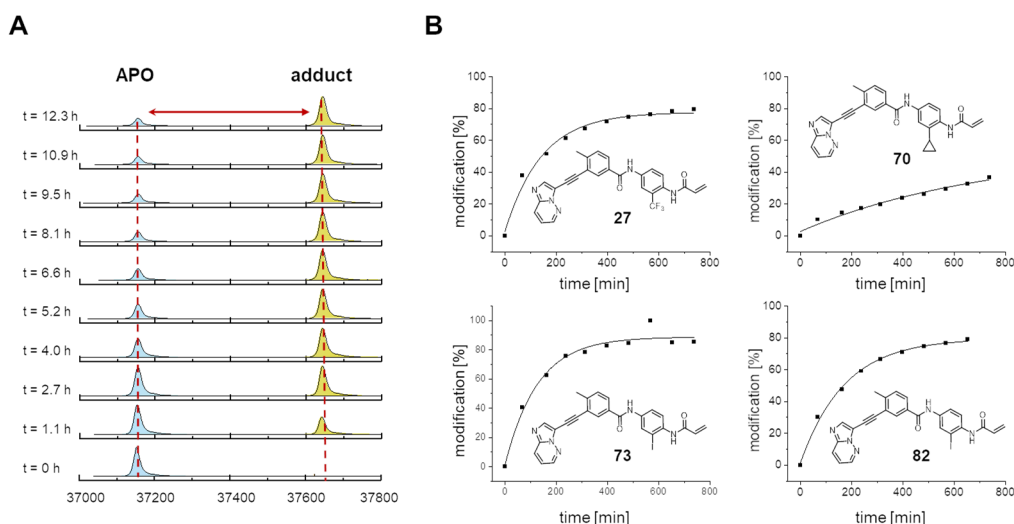


Figure 28. Kinetic evaluation of covalent bond formation in KIT-wt. **(A)** Representative example of deconvoluted MS spectra at different time points for **27**. **(B)** Plot of the adduct formation against the time to determine the k_{inact} values for **27**, **70**, **73**, and **82**.

The modification rates of compounds **68**, **73** and **82** exhibited a rapid initial increase that subsequently declined over time. In contrast, the modification rate of **70** (cyclopropyl) was significantly slower but demonstrated consistent progression, reaching complete modification after 24 hours. This trend is also reflected in the k_{inact} values and $t_{1/2}$ (Table 7). Interestingly, compound **70** had a $t_{1/2}$ five times longer (502 min.) than that of **68**, **73** and **82** (88-120 min.). This further suggests that the orientation of the back pocket moiety in **70** may result in suboptimal positioning of the warhead. Alternatively, the +I effect of the cyclopropyl group may increase the electronic density of the warhead, which could ultimately reduce the electrophilic activity. It is important to note that the data presented were derived from a single measurement and thus require validation through triplicate experiments. Further MS experiments involving PDGFR α -wt and mutant forms of both KIT and PDGFR α could facilitate the acquisition of additional insights. Furthermore, the observed trend indicating that the *ortho*-back pocket moiety appears to influence the reactivity of the warheads should be investigated in additional assays, such as the glutathione assay²⁵⁵⁻²⁵⁶.

^{XXIII} The protein was provided by Dr. Alina Teuber. The MS experiments were performed together with Maria Beerbaum as part of her master thesis.

Table 7. Determined k_{inact} and $t_{1/2}$ for a set of covalent inhibitors against KIT-wt.

cpd id	68	70	73	82
k_{inact} [min ⁻¹]	$6.9 \cdot 10^{-3}$	$1.4 \cdot 10^{-3}$	$7.9 \cdot 10^{-3}$	$5.8 \cdot 10^{-3}$
$t_{1/2}$ [min]	99	502	88	120

Overall, the MS analysis provided confirmation that the inhibitors investigated were able to covalently modify KIT-wt. The data indicated that the substituent in the back pocket affects the capacity for covalent modification, which could be a significant factor to consider in future efforts to develop covalent inhibitors targeting KIT and PDGFRA. To gain structural insights and to ultimately confirm the covalent bond formation, the inhibitors were advanced through our crystallization program.^{233XXIV} This effort resulted in the complex structure of **27** bound to PDGFRA-T674I (Figure 29). Notably, this represents the first reported crystal structure of PDGFRA bound to a covalent inhibitor. The structure revealed that all protein-ligand interactions exerted are consistent with those previously observed for ponatinib. Most importantly, the covalent bond with Cys814 (corresponds to KIT-Cys788) is clearly defined in the electron density (Figure 29D). Moreover, the ethynyl methylbenzene engages in hydrophobic interactions with the mutated gatekeeper residue Ile674 (Figure 29, Supplementary Figure 11). Building on the covalent mode of action, an extensive structure-activity relationship study was pursued with the objective of identifying key pharmacophoric features, with a particular focus on investigating the binding affinity of drug-resistant mutations.

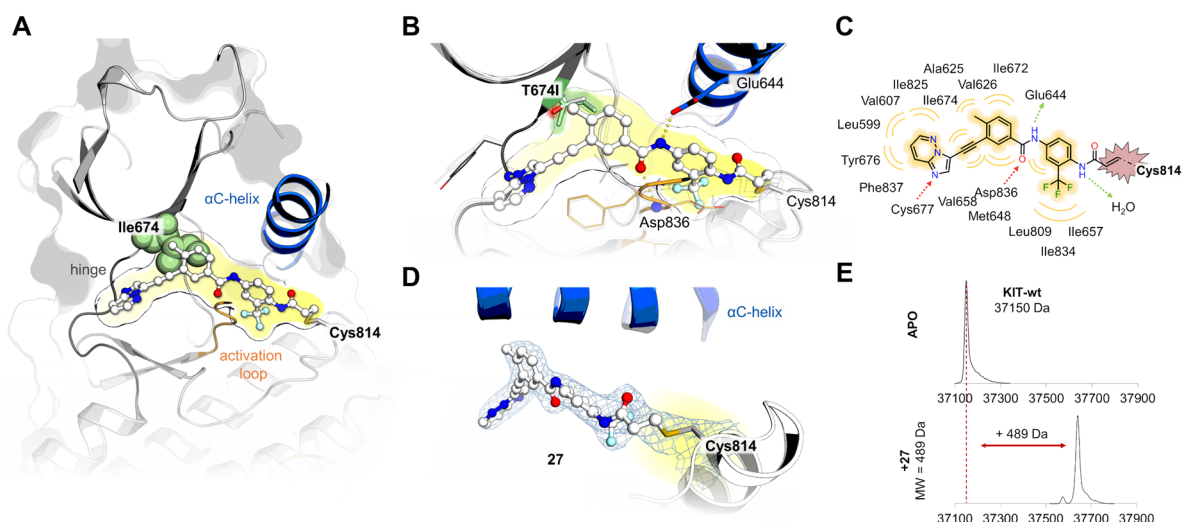


Figure 29. (A) Crystal structure of PDGFRA-T674I in complex with compound **27** (PDB ID: 9GZH). (B) Binding modes of **27** (ball and stick representation) and ponatinib (shown as outline, PDB ID: 40UI) (C) Schematic depiction of the pharmacophoric features of **27** (D) [2Fo-Fc]- electron density map (r.m.s.d. = 1.0) showing covalent bond formation. (E) Deconvoluted MS spectra of KIT-wt before (APO) and after incubation with **27**. Figure adapted from Schulz *et al.*, revised.

XXIV The crystallization experiments were performed by Dr. Alina Teuber.

The type III inhibitors **90** and **91** were found to be inactive ($IC_{50} \geq 10000$ nM) against both wild-type and mutant variants of KIT and PDGFR α , indicating that a hinge-binding element is crucial. Simplifying the hinge-binding element, such as by incorporating a smaller methyl imidazole, resulted in a marked reduction in potency (**81** < **82**). This observation indicates that hydrophobic interactions at the entrance of the kinase (Leu799, Leu595, Tyr672, and Phe811), along with H-bonds to the hinge (Cys673), are vital for strong protein-ligand binding (Figure 23/26). The benzimidazole derivative **97** displayed inhibitory potency in the three- to four-figure nM range against KIT- and PDGFR α -wt, but showed no inhibition of mutant forms ($IC_{50} \geq 10000$ nM). Compound **69** exhibits analogous structural characteristics to **97**, including a Michael acceptor, with the exception of an amide bond instead of the imidazole moiety. Although **69** exhibited no inhibitory effect against mutant KIT or PDGFR α ($IC_{50} \geq 10000$ nM), it demonstrated IC_{50} values of 7.7 and 9.3 nM for the wild-type proteins. This suggests that hydrogen bonding interactions enabled by the amide group, with Glu640 and Asp810 (with regard to KIT), are crucial for potent ligand binding (*cf.* Figure 23). The potency of inhibitors differing only in amide linkage constitution was found to be identical against wild-type KIT and PDGFR α , regardless of whether they were amides (O=C-N, e.g. **27**, **66**) or reverse amides (N-C=O, e.g. **57**, **58**) (Table 6). However, the IC_{50} revealed a striking prevalence of the O=C-N linkage in the case of AL mutants. Inhibitors with the amide linkage of imatinib (N-C=O) demonstrated a reduction in potency of up to 4.6-fold against KIT-D816H and up to 59-fold against PDGFR α -D842V, in comparison to their respective counterparts. The transition to the active conformation is accompanied by a downward movement of the α C-helix, including Glu640 (Glu644 in PDGFR α). These structural alterations might be better tolerated and stabilized by the O=C-N bond due to smaller distances and suitable angles, which are more conducive to the formation of H-bonds (Figure 26). Variations in potency (ranging from 4- to 15-fold against KIT-T670I and 5- to 25-fold against PDGFR α -T674I) were also noted when comparing the inhibitor performance of wt with gatekeeper mutants. These differences may result from altered ligand conformation or potentially suggest an additional effect of the mutation beyond its local impact. Further factors, such as an influence on the ATP affinity or a potential cross-talk between the back pocket and the GK, may also be involved. Vinyl benzothiazole **62** showed an inhibitory potency against the wt's of ca. 40 nM, yet exhibited no inhibition of mutant KIT ($IC_{50} \geq 10,000$ nM) and only modest inhibition of mutant PDGFR α . A simpler benzothiazole (**63**) had an overall increased potency, with a 30-40-fold improvement against PDGFR α -wt and -G680R, and a two-fold increase in potency against PDGFR α -D842V and -T674I. Interestingly, the potency against KIT showed no significant difference. Further modifications, such as adding a piperazine (**61**) or replacing the phenyl group (**65**), resulted in IC_{50} values exceeding 1000 nM for both mutant KIT and PDGFR α . Attempts to introduce an additional hydrophobic moiety (**86**) resulted in high IC_{50} values across all instances.

This may be attributed to the confined spatial limitations of the hydrophobic pocket, which is formed by the amino acids Ile653, Leu783, and Ile808. When comparing the inhibitors with varying in their back pocket-binding element (in the *meta* position), two interesting trends emerged. First, the SAR underscored the significance of interactions within the back pocket, especially involving Ile653, Leu783, and Ile808, in effectively inhibiting AL mutants. Compounds **27** and **70**, which exhibit potency around 1–2 nM for KIT-wt, show similar potent inhibition (around 6–7 nM) compared to **60** and **69**. However, these inhibitors lacking functionalization ($R_2 = H$) are inactive against both AL mutants ($IC_{50} \geq 10,000$ nM). In contrast, **27** and **70**, which feature larger, hydrophobic groups, retain potency at 12 nM and 8.6 nM, respectively. Hydrophilic substituents, like methyl pyrazole **71**, show a reduced potency across all kinase variants studied. It can be concluded that effective inhibition, in particular for AL mutants, necessitates the occupation of the back pocket with substituents of an appropriate size, hydrophobicity, and geometry (Figure 30). The second key finding is that specific substitutions are favored depending on the mutant kinase. For example, the linear iodo-bearing **73** inhibits KIT-D816H at around 20 nM, but shows much lower potency against PDGFRA-D842V ($IC_{50} \geq 1000$ nM). Compound **27**, which carries a bulkier, tetrahedral CF_3 group, inhibits KIT-D816H at 12 nM and, in turn, shows an $IC_{50} \sim 100$ nM on PDGFRA-D842V. Meanwhile, a planar naphthyl group (**74**) displays similar potency for both AL mutant proteins (KIT-D816H: $IC_{50} \sim 200$ nM; PDGFRA-D842V: $IC_{50} \sim 300$ nM). These observations enhance the understanding of the key protein-ligand interactions in targeting AL-mutant KIT and PDGFRA; thereby pointing towards strategies for differentiating between these two structurally related kinases and overcoming drug resistance caused by mutations within the AL (*cf.* Figure 30).

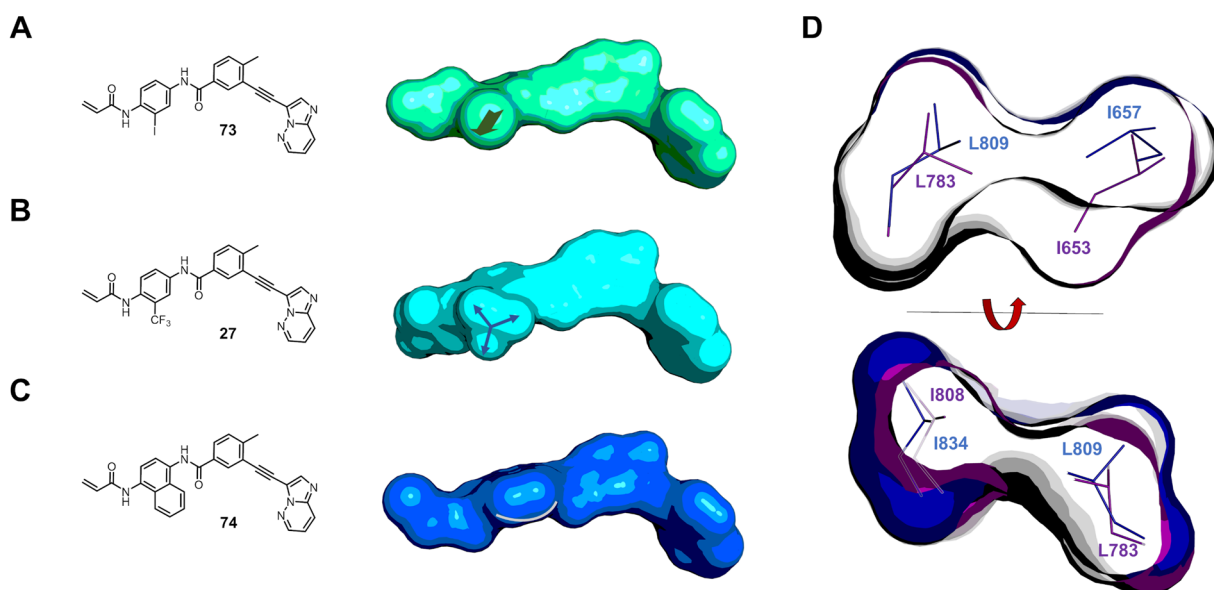


Figure 30. LEWIS structure and 3D surface of **73** (A), **27** (B) and **74** (C) with schematic visualization of the property of the back pocket moiety. (D) Surface representation of the hydrophobic back pocket in KIT (purple, PDB ID: 4U0I) and PDGFRA (blue, PDB ID: (5K5X). Figure adapted from Schulz *et al.*, revised.

In conclusion, the SAR analysis highlighted crucial protein-ligand interaction patterns and provided valuable insights into the effects of resistance mutations and strategies to address them. The results of MS studies demonstrated that the covalent modification of the target protein was achieved. Further, several highly potent covalent inhibitors, with activity in the low nanomolar range, were identified. X-ray crystallography ultimately furnished evidence and provided crucial insights into ligand interactions with drug-resistant PDGFRA-T670I. Encouraged by these promising findings, the SAR studies were continued in a cellular context (Table 8). A CTG assay was conducted on three cell lines: a patient-derived KIT-driven cell line (GIST-T1, primary, p.Val560_Tyr578del), an isogenic PDGFRA-driven cell line (T1-a-D842V, PDGFRA-D842V), and a control line (GIST-48B).^{xxv} The letter is independent of KIT and PDGFRA and thus can indicate potential off-target toxicity. In general, the observed SAR trends were well correlated within cellular systems, thereby supporting the potential of these inhibitors (see Supplementary Table 6 for calculated physicochemical properties). Inhibitor **90**, not featuring a hinge-binding moiety, showed no activity against all cell lines, with a GR₅₀ value exceeding 10000 nM. Also, in cellular experiments benzimidazole **97** was inactive, further emphasizing the critical role of the amide linkage for effective inhibition. In GIST-T1 cells, inhibitors with an amide linkage (O=C-N) display higher potency compared to reverse amides (N-C=O). Notably, this trend was not reflected in the T1-a-D842V, highlighting the nuanced influence of structural elements. The significance of an adequate back pocket substituent was also discernible in the cellular assays. Inhibitors with hydrophobic moieties with distinct geometries, such as **27** and **73**, outperformed more polar group decorated inhibitors, like **71** and **82**. The differences in potency were markedly pronounced in comparison to inhibitors that possessed solely a hydrogen back pocket element, such as **59** and **69**. A comparative analysis of **27**, **66-67** revealed that inhibitors with a *para*-warhead demonstrated the highest potencies, corroborating the assertion that the *para* position is the optimal vector for covalent targeting KIT/PDGFR α . Moreover, the reversible inhibitors **68** and **83** displayed markedly diminished inhibitory effects compared to their covalent counterparts (**27** and **82**), underscoring the advantages of covalent targeting. Overall, the most promising compounds, including **27**, **57**, **73**, and **74**, demonstrated highly potent cellular inhibition in the low nM range (5.4 - 24 nM), proving to be as potent as currently approved TKIs. One of the most noteworthy developments emerging from this study is the markedly enhanced off-target selectivity over the control cell line exhibited by the covalent inhibitors. This was evident in a series of inhibitors, where none affected the GIST-48B control cell line (GR₅₀ $\geq$ 10000 nM).

^{xxv} The CTG assay was performed by Benjamin S. Fletcher or Dr. Thomas Mühlenberg, University Duisburg-Essen, DKTK, for the GIST-T1 cell line and Dr. Sonja Sievers, COMAS for the PDGFRA-T1-a-D842V and the GIST-48B cell lines.

Table 8. GR₅₀ values on KIT- (GIST-T1) and PDGFR-driven (T1-a-D842V) cell lines as well as a control cell line (GIST-48B). GR₅₀ are presented as mean values $\pm$ s.d; n.d. not determined; n represents the number of independent experiments, all with n $\geq$ 3 otherwise marked as ^a n = 2.

cpd id	CTG GR ₅₀ [nM]			ratio (GIST-48B / -T1)
	GIST-T1	T1-a-D842V	GIST-48B	
imatinib	39 $\pm$ 25	1100 $\pm$ 235	$\geq$ 10000	256
sunitinib	30 $\pm$ 18	1004 $\pm$ 159	2714 $\pm$ 216	90
regorafenib	69 $\pm$ 34	370 $\pm$ 123	3966 $\pm$ 1041	57
ripretinib	6 $\pm$ 2	300 $\pm$ 30	2177 $\pm$ 1040	362
avapritinib	22 $\pm$ 4	17 $\pm$ 8	1690 $\pm$ 360	241
ponatinib	23 $\pm$ 9	74 $\pm$ 13	390 $\pm$ 120	17
57	24 $\pm$ 11	3400 $\pm$ 200	7866 $\pm$ 1255	328
59	194 $\pm$ 13	$\geq$ 10000	$\geq$ 10000	52
60	3350 $\pm$ 404	$\geq$ 10000	$\geq$ 10000	3
61	949 $\pm$ 281	3400 $\pm$ 163	2466 $\pm$ 170	3
62	513 $\pm$ 35	$\geq$ 10000	$\geq$ 10000	19
63	54 $\pm$ 11	$\geq$ 10000	$\geq$ 10000	185
65	6047 $\pm$ 252	$\geq$ 10000	$\geq$ 10000	1.6
27	12 $\pm$ 2	8900 $\pm$ 800	$\geq$ 10000	833
66	96 $\pm$ 5	7700 $\pm$ 668	$\geq$ 10000	104
67	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000	1
68	$\geq$ 10000	1367 $\pm$ 235	$\geq$ 10000	1
69	896 $\pm$ 303	$\geq$ 10000	$\geq$ 10000	11
70	27 $\pm$ 4	$\geq$ 10000	$\geq$ 10000	370
71	630 $\pm$ 222	$\geq$ 10000	$\geq$ 10000	16
72	123 $\pm$ 48	2956 $\pm$ 120	3532 $\pm$ 35	81
73	5.4 $\pm$ 3.1	$\geq$ 10000	$\geq$ 10000	1851
74	6.9 $\pm$ 1.3	6720 $\pm$ 1007	6898 $\pm$ 1460	999
82	212 $\pm$ 83	$\geq$ 10000	$\geq$ 10000	47
83	3026 $\pm$ 50	$\geq$ 10000	$\geq$ 10000	3
84	21 $\pm$ 5	$\geq$ 10000	$\geq$ 10000	476
85	46 $\pm$ 17	3400 $\pm$ 300	3776 $\pm$ 124	82
90	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000	1
97	$\geq$ 10000	$\geq$ 10000	$\geq$ 10000	1

Furthermore, the calculation of a selectivity ratio between the target (GIST-T1) and the non-target (GIST-48B) demonstrated that **73** was capable of attaining a selectivity ratio of over 100-fold in comparison to ponatinib while retaining its high potency (Figure 31B).

Ultimately, a subset of inhibitors was examined in western blot studies in patient-derived GIST-T1 cells (Figure 31C).^{xxvi} The western blot analysis indicates that the potency of **27**, **57**, **70**, **73**, and **74** on par with that of ponatinib and is greater than that of the established first-line imatinib. Importantly, this experiment demonstrated on-target engagement and a dose-dependent effect of phospho-KIT inhibition as well as a notable inhibition of downstream signaling. These compelling findings were corroborated by the cell viability data obtained from the CTG assay, thereby providing strong support for the further development of these covalent inhibitors.

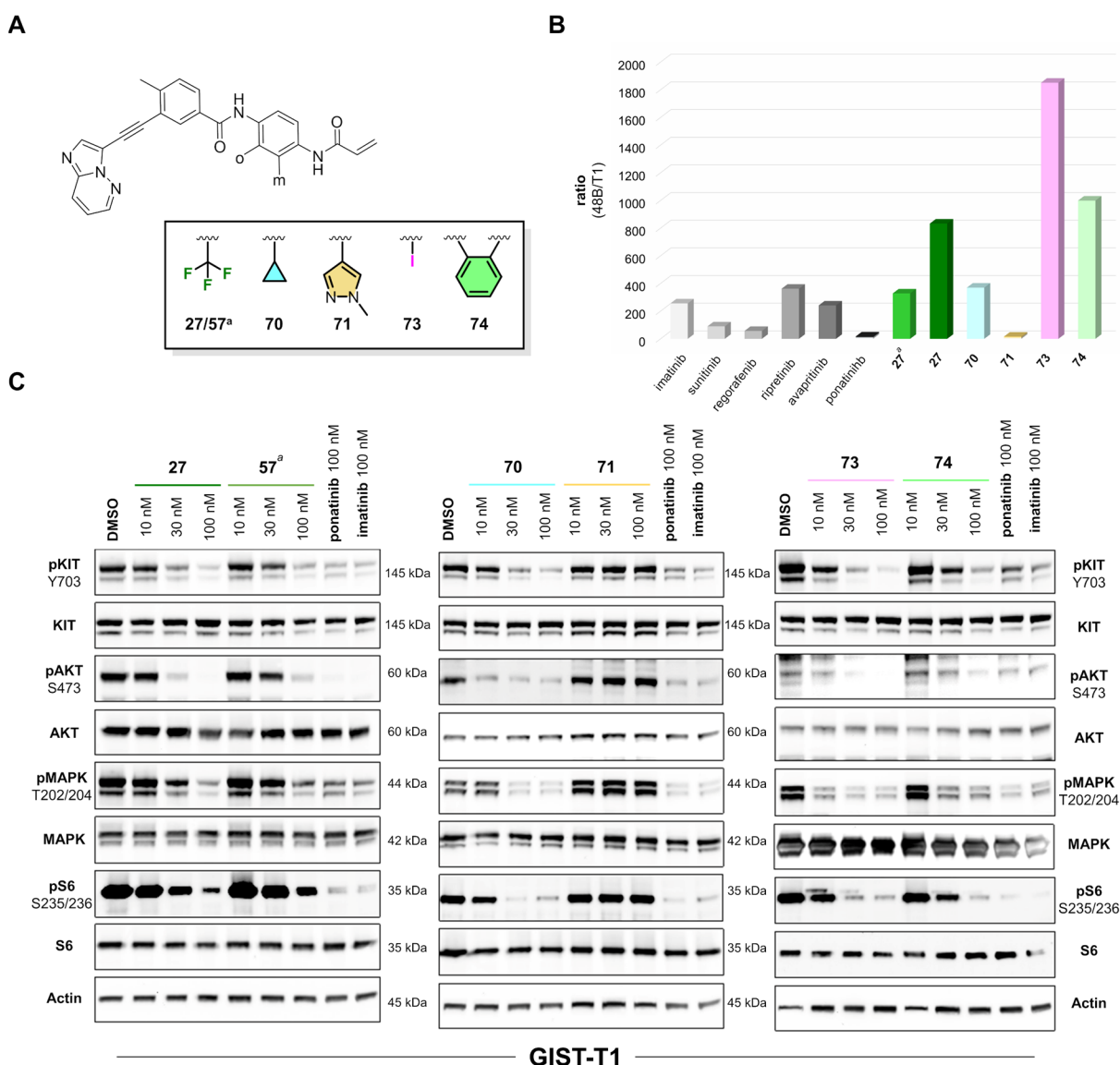


Figure 31. (A) Compounds used for the western blot studies. (B) Bar chart of the off-target selectivity, wherein the ratio between on-target (GIST-T1) and off-target (GIST-48B) cell lines is shown. (C) Western blots of **27**, **57**, **70**, **73**, and **74** as well as imatinib and ponatinib in GIST-T1 cells of KIT and downstream pathway phosphorylation inhibition at different concentrations. ^a reverse amide linkage (N-C=O). Figure adapted from Schulz *et al.*, revised.

^{xxvi} The Western blot experiments were performed by Dr. Thomas Mühlenberg, UKE (AG Bauer).

6.5 Conclusion

Gastrointestinal stromal tumors are primarily driven by genetic lesions in KIT or PDGFRA, and TKIs have been transformative in their treatment, forming the cornerstone of therapy. However, the clinical efficacy of these therapies remains limited due to the emergence of drug resistance and off-target toxicities, thereby underscoring the medical necessity for next-generation inhibitors. Currently approved TKIs rely on an ATP-competitive mode of action. Inspired by the success of covalent inhibitors like osimertinib and ibrutinib, which have demonstrated exceptional efficacy in targeting otherwise drug-resistant cancers, this work sought to explore their untapped potential for targeting GIST-relevant oncogenes. A structure-based approach guided the development of novel covalent type II inhibitors. Covalent binding was demonstrated via protein MS experiments and X-ray crystallography. Further, the first co-crystal structure of a PDGFRA in complex with a covalent inhibitor provided valuable insights into ligand interactions with drug-resistant GK-mutant forms of KIT and PDGFRA. A comprehensive SAR analysis further illuminated key protein-ligand interactions critical for overcoming resistance mutations. The synthesized inhibitors demonstrated highly potent biochemical and cellular inhibition in the low nanomolar range against mutant KIT and PDGFRA variants, with favorable selectivity profiles over currently approved TKIs. Overall, these findings provide substantial advances for developing next-generation inhibitors that circumvent drug resistance in GIST, addressing crucial deficiencies in the current treatment landscape.

Chapter: 7

SUMMARY AND OUTLOOK

7. Summary and Outlook

The treatment of gastrointestinal stromal tumors has revolutionized with the identification of the main oncogenic drivers KIT and PDGFRA. Currently, five reversible, ATP-competitive TKIs that target these mutant receptor tyrosine kinases have been approved, prompting a paradigm shift in the successful treatment of these tumors. However, the emergence of acquired drug resistance, particularly secondary/tertiary mutations in the activation loop and/or the ATP-binding pocket, hampers the long-term success of current therapies. Furthermore, many of the approved drugs have been repurposed and are associated with issues related to selectivity and safety profiles, which give rise to severe adverse effects.

Against this background, the objective of this study was to elucidate structural and mechanistic insights into resistance mutations and to facilitate the rational design, synthesis, and evaluation of next-generation inhibitors tailored to overcome these mutations by combining structural biology and organic chemistry. The structural analysis of avapritinib in wild-type and mutant variants of KIT and PDGFRA elucidated the unique binding mode and provided critical insights into key protein-ligand interactions. Most importantly, it led to the discovery of a previously unidentified α -pocket within the kinase domain. This novel pocket, formed by residues of important regulatory elements (specifically, the Glycine-rich loop and the α C-helix), showed the potential to enhance the potency and selectivity, as implied by the structural and biological evaluation. Leveraging this insight, a series of novel inhibitors was developed, showing specific SAR trends that prioritized efficacy against both PDGFRA and KIT mutants. In particular, derivatization at the amine on the stereogenic center of these inhibitors was identified as a promising approach, yielding compounds with high potency and optimized off-target selectivity. These inhibitors demonstrated on-target engagement and significant inhibition of downstream phosphorylation in a patient-derived cell line. Notably, compound **20** showed comparable inhibitory potency to avapritinib, while featuring a reduced propensity to traverse the blood-brain barrier, addressing a critical limitation of avapritinib related to neurocognitive side effects. Moreover, comprehensive structural biology investigations have elucidated the underlying molecular mechanisms of KIT resistance mutations occurring in diverse regions of the kinase domains. The analysis demonstrated that AP mutations primarily exert their resistance through a local effect. This is exemplified by the KIT-T670I mutation (analogous to the PDGFRA-T674I mutation) inducing a steric repulsion with type IIa inhibitors, such as imatinib or ripretinib. With regard to the type 1.5 inhibitor avapritinib, it has been demonstrated that this mutation causes the loss of a water-mediated interaction, thereby reducing the binding affinity.

In contrast, AL mutations likely have a more global effect, resulting from a destabilization of the inactive state and a shift of equilibrium towards the active state of the kinase, which may be caused by disruption of intermolecular interactions or the introduction of residues that force a conformational change. In this regard, a comprehensive analysis of protein-ligand interactions between AL mutant KIT and TKIs, such as ripretinib and ponatinib, has revealed the importance of targeting the back pocket region for potent inhibition. At the same time, for the recently described tertiary AP/AL mutations, an interplay between AP and AL-mediated resistance mechanisms may be postulated. The question of whether this can be resolved via, for example, X-ray crystallography is of great interest, particularly given the occurrence and impact of resistances observed against all approved and investigational inhibitors. Furthermore, to complement ATP-competitive inhibition strategies, a key component of this research was to delve into the previously unexplored potential of covalent inhibitors, drawing inspiration from the established success of covalent inhibitors in overcoming otherwise drug-resistant cancer. A structure-based design approach was employed to develop novel covalent type II inhibitors following an in-depth structural analysis of ponatinib, which explained the potential for targeting both AL mutations and the gatekeeper mutation. Following extensive synthetic efforts, a comprehensive library of covalent inhibitors was developed, specifically designed to target resistant forms of KIT and PDGFRA. Mass spectrometry and X-ray crystallography demonstrated the covalent mode of action for these inhibitors. Comprehensive SAR analysis further illuminated critical protein-ligand interactions essential for targeting resistance mutations in KIT and PDGFRA. This analysis further emphasized the significance of targeting the back pocket to potentially inhibit AL-resistant forms of KIT/PDGFRA. The newly synthesized inhibitors demonstrated high potency in the low nanomolar range, on-target engagement in clinically relevant GIST-T1 cells, and remarkable selectivity compared to currently approved TKIs. Overall, these results demonstrate and highlight the promising potential of covalent inhibitors in targeting drug-resistant GIST.

Looking forward, these findings pave the way for a nuanced approach to inhibitor development that balances therapeutic efficacy and selectivity. In particular, identifying the α -pocket as a structural feature in KIT and PDGFRA opens new avenues for future inhibitor design. SAR exploring this novel motif appears promising and may also inspire previously unsuccessful type III inhibitor approaches. This represents a promising strategy to overcome drug resistance mediated by AP mutations such as the gatekeeper mutation. In addition, the identification and validation of the primary amine as an exit vector opens up the possibility of installing linker moieties that do not interfere with potent ligand binding. Thus, compounds modified i) with a reporter group, serving as probes, will permit further investigation of drug resistance mutations or ii) decorated with E3 ligase recruiters, following a PROTAC approach, will facilitate the establishment of degrader molecules in GIST.

Furthermore, the covalent targeting approach reported here has demonstrated the great potential of covalent KIT/PDGFR α inhibitors. It thus holds great promise for overcoming resistance mutations that remain challenging for reversible inhibitors. Accordingly, this potential can be transferred to other inhibitors, such as avapritinib or IDRx-42. As the treatment landscape continues to evolve with an increasing emphasis on mutation-dependent therapies, the insights gained from this research underscore the importance of integrating structural biology and medicinal chemistry in a translational effort from bedside to bench and back, particularly for rare cancer types. These methodologies not only shed light on resistance mechanisms but have also identified potential ways to overcome them, thereby facilitating the design of next-generation tailored inhibitors capable of potent inhibition across the spectrum of resistance mutations, with the potential to reduce the side effects associated with currently available TKIs. Collectively, this work advances the field of GIST-relevant oncogenes by elucidating structural mechanisms of drug resistance and opens new avenues for developing next-generation KIT/PDGFR α inhibitors, marking a new frontier in structural guidance for drug-resistant GIST inhibitor design.

Chapter: 8

EXPERIMENTAL SECTION

8. Experimental Section

8.1 Chemoinformatic Methods

A summary of chemoinformatic tools used is provided in Table 9.

Table 9. Summary of chemoinformatic tools used, including their respective functions. ^aThe PyMOL Molecular Graphics System, Version 2.5.2, Schrödinger, LLC. ^bIntegrated in the ProteinsPlus web server (<https://proteins.plus/>). ^cBioSolveIT GmbH, Sankt Augustin, Germany, 2024, www.biosolveit.de/SeeSAR.

tool	function
Coot ²⁵⁷ (v0.9.4)	validation of data quality and analysis of electron density for amino acids and ligands
^a PyMol (v2.2.3)	molecular visualization, introduction of point mutations and structural analysis as well as figure generation
^b Protoss ²⁵⁸⁻²⁵⁹	preparation/protonation of protein structures
LigandScout ²⁶⁰ (v4.4)	pharmacophore model generation, preparation of ligands, including ligand energy minimization via the the Merck Molecular Force Field 94 (MMFF94s)
AutoDock ²⁶¹ (v4.2)	molecular docking studies
GOLD ²⁶²	molecular docking studies, including covalent docking
^c SeeSAR (v13.1.1)	molecular docking studies

8.1.1 Docking for Covalent Derivatives using GOLD

The ligands were subjected to energy minimization using the Merck Molecular Force Field 94 (MMFF94s) and prepared in LigandScout (v. 4.4.8). The protein preparation for the structure (PDB ID: 4U0I) was conducted using Protoss within the ProteinsPlus web server. Covalent docking was conducted using the GOLD software with 20 genetic algorithm (GA) runs, with the "early termination" option disabled. Ponatinib (PDB ID: 4U0I) was employed as a reference ligand, and all non-protein residues were removed. The binding site was defined as a 7 Å region surrounding the co-crystallized ligand. In the context of covalent docking, the sulfur atom of Cys788 was designated as the linking atom to the prepared ligand substructure. Internal hydrogen bond detection was enabled, and both amide bond and ring corner flipping were permitted. The remaining settings were maintained at their default values. The resulting binding poses were then subjected to a visual inspection, and the binding mode that displayed the most favorable interactions was selected for further analysis and visualization.

8.1.2 Generation of Pharmacophore Models

To further assess the interactions of the (crystallized) ligands within the target proteins, two- and three-dimensional pharmacophore models were generated for each ligand using the LigandScout software, version 4.4.8.

8.1.3 Pharmacophore-based Docking in SeeSAR

In the absence of a co-crystal structure of regorafenib bound to KIT, pharmacophore-based docking simulations were conducted using SeeSAR (version 13.0.1). The binding site was automatically defined based on the co-crystallized ligand (PDB ID 4U0I), and the pharmacophore was specified as an acceptor interaction with a 2.0 Å radius. Docking was conducted using the standard settings with ponatinib as a reference, which produced poses comparable to the experimentally determined X-ray structure. The generated regorafenib pose with the highest HYDE score was selected for further analysis.

8.1.4 Structure-based Modeling Analysis

Structural alignments were conducted using PyMOL. The crystal structure of the KIT-wt complexed with a ripretinib derivative (PDB ID: 6MOB) was employed as a template for the generation of the KIT mutants investigated. The point mutations T670I, V654A and N822K were created using PyMOL's mutagenesis wizard tool. After that, the *in silico* mutated proteins were subjected to energy minimization via the Merck Molecular Force Field 94 (MMFF94s) implemented in LigandScout (version 4.4.8) under default settings. In order to create a model of a completely resolved sunitinib structure in KIT, the unresolved diethylamine variant (PDB ID: 3G0E) was used as a basis and subjected to a pair fit command in PyMOL to the resolved sunitinib core (PDB ID: 6JOK). Furthermore, a structural alignment of the KIT complexed with the ripretinib analog (PDB ID: 6MOB) and the modeled sunitinib structure was conducted in PyMOL. The conformational intermediate states of KIT were generated by interpolation between the active and inactive X-ray structures of KIT-wt (PDB ID: 3G0E) and KIT-D816H (PDB ID: 7KHK) using an online morphing tool (<http://www.bioinformatics.org/pdbtools/morph2>). In the analysis presented in Chapter 5, the KIT-wt structure in complex with ponatinib (PDB ID: 4U0I) was employed to model the KIT-T670I mutant via PyMOL's mutagenesis wizard. This modified protein was then energy-minimized using MMFF94s in LigandScout with default settings.

8.1.5 In silico Calculation of Physicochemical Properties

The MarvinSketch software (version 22.13.0, Chemaxon; <https://www.chemaxon.com>) was utilized for the purposes of drawing, visualizing, and characterizing the avapritinib derivatives discussed in Chapter 3. This included an assessment of their physicochemical properties and CNS-MPO scores. The web tool SwissADME was employed to predict the physicochemical properties of the ponatinib-based inhibitor.

8.2 Biochemical Compound Evaluation

8.2.1 Activity-based Assay (HTRF)

Reagents and Materials

All reagents utilized in the KIT and PDGFRA HTRF assay kits were procured from CisBio (Bagnols-sur-Cèze, France). White, round-bottom 384-well plates with a 25 μ L fill volume were obtained from Greiner Bio-One GmbH (Solingen, Germany). The enzymes were procured from the following sources:

KIT-wt (0997-0000-1 (010))	ProQinase
KIT-D816H (#1041-0000-1 (002))	ProQinase
PDGFRA-wt (#1057-0000-1)	ProQinase
PDGFRA-D842V (PV4203, #2343231B)	Invitrogen
PDGFRA-G680R (#X3408-18)	SignalChem

In order for this assay to generate comparable and robust data, it is necessary to complete a series of preliminary steps for each protein variant utilized. The aforementioned steps were completed in accordance with the instructions provided by the manufacturer and are discussed in detail in Chapter 6. A summary of the identified condition for the gatekeeper mutant variants of KIT and PDGFRA is presented in Table 10.

Table 10. Summary of identified HTRF conditions for KIT-T670I and PDGFRA-T674I. Details regarding the protein expression and purification can be found in the Dissertation of Dr. Alina Teuber.

	KIT-T670I	PDGFRA-T674I
Kinase [ng/μL]	0.07	0.4
Substrate [nM]	1000	1000
ATP [μM]	50	234
Biotin-XL665	8:1	8:1
SEB [nM]	50	50
Incubation [min]	30	30
Reaction [min]	15	20
Stop [min]	60	60

HTRF Assay

The IC₅₀ values were determined using the TK HTRF KinEASE assay (CisBio), which was conducted in accordance with the manufacturer's instructions. A 5 µL aliquot of the kinase solution and a 2.5 µL aliquot of the inhibitor solution (8 % DMSO in HTRF buffer) were pre-incubated for 30 minutes. The reaction was initiated by the addition of 2.5 µL of a starting solution containing ATP and substrate peptide. The ATP concentrations were adjusted to their respective *K_M* values, as follows: 200 µM for KIT-wt, 50 µM for KIT-T670I, 12 µM for KIT-D816H, 57 µM for PDGFRA-wt, 14 µM for PDGFRA-D842V, 234 µM for PDGFRA-T674I, and 11 µM for PDGFRA-G680R. The substrate concentrations employed were as follows: The concentrations of the substrate were 550 nM for KIT-wt, 1 µM for KIT-T670I, 1 µM for KIT-D816H, 455 µM for PDGFRA-wt, 1 µM for PDGFRA-T674I, 775 nM for PDGFRA-D842V and 886 nM for PDGFRA-G680R. The reactions were allowed to proceed for defined times, as follows: 20 minutes for KIT-wt, 15 minutes for KIT-T670I, 40 minutes for PDGFRA-wt, 20 minutes for PDGFRA-T674I, and 15 minutes for PDGFRA-G680R. Subsequently, 10 µL of stop solution was added to terminate the reaction. A summary of the respective buffers is presented in Table 11.

Table 11. Overview of the composition of the buffer solution utilized in the HTRF assay.

Enzyme Buffer (pH 7.0)	50 mM HEPES; 0.1 mM Na ₃ VO ₄ ; 0.02 % NaN ₃ ; 0.01 % BSA ; 5 mM MgCl ₂ ; 1 mM DTT; 0.01 (v/v) % TritonX-100; in ddH ₂ O
Detection Buffer (pH 7.0)	25 mM HEPES ; 0.05 % BSA; 400 mM KF ; 10 mM EDTA ; diluted antibody (according to the manufacturer's instructions); in ddH ₂ O

The FRET signal was quantified using an EnVision plate reader (PerkinElmer, Waltham, MA, USA) with excitation at 620 nm and emission at 665 nm. The ratio of the emission intensities was recorded across eight inhibitor concentrations and fitted to a four-parameter HILL equation (1) using Quattro software (Quattro Research GmbH, Martinsried, Germany). Each run was performed in duplicate, with at least three independent determinations for each IC₅₀ measurement.

$$y = A_2 + \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^n} \quad (1)$$

*A*₁ median of the control/DMSO; *A*₂ median of the positive control/staurosporine; *X* ligand concentration; *X*₀ ligand concentration corresponding to the IC₅₀ (inflection point); *n* gradient at inflection point (HILL coefficient)

8.2.2 MS Experiments

A solution of KIT-wt at a concentration of 0.5 mg/ml was incubated with a stock solution of the respective inhibitor at a concentration of 10 mM in DMSO. The mixture was then incubated at 4 °C for one and 24 hours in a buffer containing 20 mM CHES, 200 mM NaCl, and 1 mM TCEP at a pH of 9.5. Following incubation, the samples were subjected to centrifugation at 21160 g for 10 minutes at 4 °C, before undergoing analysis by MS. Aliquots were evaluated via ESI-MS using a Thermo Fisher Scientific Dionex UltiMate 3000 HPLC system, which is connected to a Thermo Fisher Scientific Velos Pro (2D ion trap). A 2 µL sample volume was injected onto a Vydac 214TP C4 5 µm column (150 mm × 2.1 mm). The solvent system consisted of solvent A (0.1 % TFA in water) and solvent B (0.1 % TFA in acetonitrile). The separation began with 20 % solvent B for 5 minutes, followed by a gradient increase to 90 % solvent B over 14 minutes, at a flow rate of 210 µL/min. Data were acquired over a range of 700–2000 m/z. The raw data were deconvoluted and analyzed using Xcalibur (v2.8 rev.2, Novatia) and MagTran (v1.02).

Time-resolved MS Experiments^{xxvii}

To assess the covalent binding of a set of inhibitors to KIT-wt, time-dependent MS analyses were carried out using a Thermo Fisher Scientific Dionex UltiMate 3000 HPLC system, which is connected to a Thermo Fisher Scientific Velos Pro (2D ion trap). The protein sample was initially warmed to be in a liquid state and then subjected to centrifugation at 4 °C for a period of 10 minutes at a rate of 21160 g. The resulting supernatant was subsequently removed. Afterwards, the protein sample was diluted in a buffer at a pH of 9.5, containing 20 mM CHES, 200 mM NaCl, and 1 mM TCEP, to achieve a concentration of 0.1 mg/mL. This prepared protein solution was then utilized for further analysis. The highest possible rate of protein modification is achieved when inhibitor concentrations are saturated. Monitoring the time-dependent covalent modification allows for determining the half-time ($t_{1/2}$) required to achieve 50 % modification of the protein. This value represents the inactivation rate k_{inact} (2).²⁶³⁻²⁶⁴

$$k_{inact} = \frac{\ln(2)}{t_{1/2}} \quad (2)$$

Accordingly, a 50-fold excess of the respective inhibitors was employed in order to guarantee a consistent occupation of the binding pocket. Samples were incubated for a time course of 12 hours at 10 °C. Regularly each 1.5 h 10 µl of the sample was injected and subjected to MS analyzed. The solvent system consisted of solvent A (0.1 % TFA in water) and solvent B (0.1 % TFA in acetonitrile). The raw data were deconvoluted and analyzed using Xcalibur (v2.8 rev.2, Novatia), MagTran (v1.02) and Origin (v.2021, OriginLab).

^{xxvii} Performed together with Maria Beerbaum during her master thesis.

8.3 Organic Chemistry

8.3.1 General Chemistry

Reagents and solvents were obtained commercially from suppliers such as Activate Scientific, Alfa Aesar, Apollo Scientific, BLDpharm, Merck, Sigma-Aldrich, TCI Chemicals, and VWR. All chemicals were utilized as received, without further purification. Air and moisture-sensitive reactions were carried out under inert conditions using either argon or nitrogen atmospheres in prepared glassware (heated and purged with inert gas). The microwave-assisted reactions were conducted in a Microwave 300 reactor, manufactured by Anton Paar. Reaction progress was monitored using thin-layer chromatography (TLC), staining solutions and/or liquid chromatography-mass spectrometry (LC-MS). TLC analysis was performed on Merck 60 F254 aluminum-backed silica gel plates and visualized under UV light at 254 nm and 366 nm. LC-MS data were acquired on an Agilent 1200 series HPLC system fitted with an Eclipse XDB-C18 column (5 μ M, 150 $\times$ 1.6 mm, Phenomenex). For compound purification, column chromatography was conducted using VWR silica gel (40-63 μ m particle size), a Biotage Isolera One™ Flash System, or a Büchi Reveleris PREP HPLC equipped with Büchi Reveleris or FlashPure EcoFlex C18 columns. Preparative HPLC was carried out utilizing a Büchi Reveleris Prep System using a VP 250/21 Nucleodur C18 column (Macherey-Nagel), with detection at 210, 254, and 280 nm. High-resolution electrospray ionization (ESI) mass spectrometry data were collected using a Thermo LTQ Orbitrap mass spectrometer, integrated with an Accela HPLC system containing a Hypersil GOLD column (Thermo Electron). Nuclear magnetic resonance (NMR) spectra for ^1H , ^{13}C and ^{19}F were collected on Bruker AVANCE III HD (400, 500, 600, and 700 MHz) and Agilent DD2 500 MHz instruments. ^1H -NMR multiplicities are noted in accordance with a standardized nomenclature as follows: (s) singlet, (d) doublet, (t) triplet, and (m) multiplet. Chemical shifts (δ) are given in parts per million (ppm), relative to solvent signals as CDCl_3 (7.26 or 77.16 ppm) or DMSO-d_6 (2.50 or 39.52 ppm). Safety considerations: No unusual safety risks or unexpected hazards arose during the experimental procedures.

8.3.2 Synthetic Procedure and Analysis for the Synthesis of Avapritinib Derivatives

The methods used, as well as the reported compound validation, have already been published in: Teuber, A.[‡]; **Schulz, T.[‡]**; Fletcher, B. S.; Gontla, R.; Mühlenberg, T.; Zischinsky, M. L.; Niggenaber, J.; Weisner, J.; Kleinbolting, S. B.; Lategahn, J.; Sievers, S.; Müller, M. P.; Bauer, S.; Rauh, D., *Avapritinib-based SAR studies unveil a binding pocket in KIT and PDGFRA*. *Nat Commun* **2024**, 15 (1), 63.⁴⁸

General Procedure I: Nucleophilic Aromatic Substitution with 4-chloro-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine **1**

To a stirred solution of the corresponding amine (1.0 eq.) and 4-chloro-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine **1** (1.0 eq.) in anhydrous tetrahydrofuran (THF), triethylamine (TEA, 3.0 equivalents) was added. The reaction was allowed to proceed with stirring for 6 hours at room temperature (rt) or for 4 hours at 40 °C. Following completion, the reaction mixture was diluted with water and subjected to extraction with dichloromethane (DCM). The combined organic layers were dried with sodium sulfate filtered and concentrated under reduced pressure. The resulting crude product was adsorbed onto silica gel and subsequently purified by flash column chromatography using a dichloromethane/methanol (DCM/MeOH) solvent system to obtain the desired product.

General Procedure II: TFA-Mediated Carbamate Cleavage

BOC-protected piperazine derivatives (1.0 eq.) were dissolved in a mixture of DCM and trifluoroacetic acid (TFA) in a 3:1 ratio. The reaction mixture was stirred at rt for 12 hours. Reaction progress was monitored by TLC and LC-MS. Upon completion, the mixture was basified by adding 10 M sodium hydroxide solution and then extracted with DCM. The combined organic layers were dried over sodium sulfate, filtered and concentrated under reduced pressure. The resulting crude product was loaded onto silica gel and purified using flash column chromatography with a DCM/MeOH solvent system containing 1 % ammonia to obtain target compound.

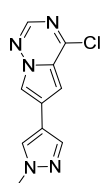
General Procedure III: Nucleophilic aromatic substitution with 5-bromo-2-chloropyrimidine

To a stirred solution of the generated piperazine derivative (1.0 eq.) in EtOH, TEA (3.0 eq.) was added. Subsequently, 5-bromo-2-chloropyrimidine (1.0 eq.) was added. The reaction mixture was then heated to 60 °C and allowed to stir for 16 hours. The completion of the reaction progress was monitored using TLC and LC-MS. Thereafter, the suspension was diluted with water and extracted with DCM. The combined organic phases were dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was adsorbed onto silica gel and purified by flash column chromatography with a DCM/MeOH solvent system to yield the target compound.

General Procedure IV: Suzuki-MIYAUURA cross coupling reactions

A solution of the corresponding bromo pyrimidine (1.0 eq.) and 2-benzyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (1.5 eq.) was prepared in a mixture of 1,4-dioxane and water (0.1 M; 5:1) under an argon atmosphere. Subsequent, the resulting mixture was degassed for 15 minutes, followed by the sequential addition of Cs₂CO₃ (3.0 eq.) and [1,1'-Bis(diphenylphosphino)ferrocene]palladium(II)dichloride (0.15 eq.). The reaction mixture was then heated to 120 °C and stirred for 1 h under microwave irradiation. After cooling, the resulting suspension was filtered through Celite and subjected to five extractions with DCM and water. The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure. After that, the crude was loaded onto silica gel and purified via flash column chromatography using a DCM/MeOH solvent system, yielding the final compound.

4-chloro-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine (**1**) was bought from BLDpharm.

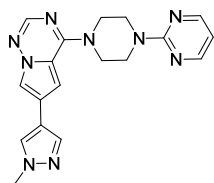


¹H NMR (600 MHz, DMSO-*d*₆) δ ppm 3.88 (s, 3 H) 7.27 (s, 1 H) 7.95 (s, 1 H) 8.20 (s, 1 H) 8.38 (s, 1 H) 8.55 (s, 1 H).

¹³C NMR (151 MHz, DMSO-*d*₆) δ ppm 39.17, 101.24, 115.18, 118.66, 121.95, 123.42, 129.10, 137.49, 145.70, 152.38.

HPLC-MS (ESI): [Rt]: 4.96 min, *m/z* for C₁₀H₈ClN₅ ([M+H⁺]), 233.05 calcd., 234.12, found.

Synthesis of 6-(1-methyl-1H-pyrazol-4-yl)-4-(4-(pyrimidin-2-yl)piperazin-1-yl)pyrrolo[2,1-f][1,2,4]triazine (2**):**



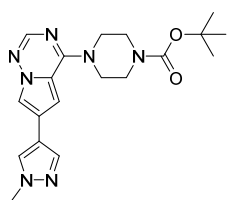
4-chloro-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine (**1**) (10.0 mg, 0.04 mmol, 1.0 eq.), 2-(piperazin-1-yl)pyrimidine (6.1 μL, 0.04 mmol, 1.0 eq.), TEA (6.2 μL, 0.04 mmol, 1.0 eq.) were used following general procedure I (6 h at rt) and yielded the final compound as a white solid (11.8 mg, 76 %).

¹H NMR (600 MHz, DMSO-*d*₆) δ ppm 3.86 (s, 3 H) 3.95 (dd, *J*=6.24, 4.40 Hz, 4 H) 4.12 (dd, *J*=6.24, 4.40 Hz, 4 H) 6.69 (t, *J*=4.77 Hz, 1 H) 7.23 (d, *J*=1.83 Hz, 1 H) 7.82 (d, *J*=0.73 Hz, 1 H) 7.88 (s, 1 H) 7.99 (d, *J*=1.83 Hz, 1 H) 8.42 (d, *J*=4.40 Hz, 2 H).

¹³C NMR (151 MHz, DMSO-*d*₆) δ ppm 39.05, 43.32, 102.16, 110.88, 114.98, 115.94, 116.27, 118.31, 128.03, 136.87, 146.97, 154.18, 158.49, 161.51.

HPLC-MS (ESI): [Rt]: 4.66 min, *m/z* for C₁₈H₁₉N₉ ([M+H⁺]), 361.18 calcd., 362.29, found.

Synthesis of *tert*-butyl 4-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-*f*][1,2,4]triazin-4-yl)piperazine-1-carboxylate (3):



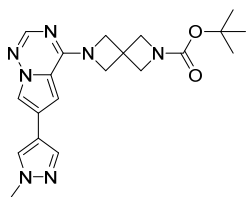
4-chloro-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-*f*][1,2,4]triazine **1** (250.0 mg, 1.10 mmol, 1.0 eq.), *tert*-butyl piperazine-1-carboxylate (219.2 mg, 1.20 mmol, 1.1 eq.), TEA (0.1 mL, 1.1 mmol, 1.0 eq.) were used following general procedure I for 3 h at 40 °C and yielded the title compound as a pale yellow solid (372.1 mg, 91 %).

¹H NMR (600 MHz, DMSO-*d*₆) δ ppm 1.45 (s, 9 H) 3.53 (m, 4 H) 3.86 (s, 3 H) 3.98 - 4.01 (m, 4 H) 7.20 (d, *J*=1.59 Hz, 1 H) 7.81 (s, 1 H) 7.87 (s, 1 H) 7.98 (d, *J*=1.54 Hz, 1 H) 8.03 (s, 1 H).

¹³C NMR (151 MHz, DMSO-*d*₆) ppm 28.55, 39.04, 45.33, 79.66, 102.10, 114.89, 115.93, 116.25, 118.35, 128.01, 136.85, 146.90, 154.12, 154.34.

HPLC-MS (ESI): [Rt]: 5.71 min, *m/z* for C₁₉H₂₅N₇O₂ ([M+H]⁺), 383.21 calcd., 384.03, found.

Synthesis of *tert*-butyl 6-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-*f*][1,2,4]triazin-4-yl)-2,6-diazaspiro[3.3]heptane-2-carboxylate (4).



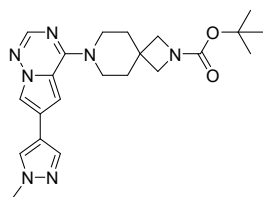
4-chloro-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-*f*][1,2,4]triazine **1** (30.0 mg, 0.1 mmol, 1.0 eq.), *tert*-butyl 2,6-diazaspiro[3.3]heptane-2-carboxylate oxalate (37.0 mg, 0.1 mmol, 1.0 eq.) and TEA (1.8 μL, 0.1 mmol, 1.0 eq.) were used following general procedure I for 6 h at room temperature and yielded the desired product as a white solid (33.4 mg, 66 %).

¹H NMR (600 MHz, CHLOROFORM-*d*) δ ppm 1.46 (s, 9 H) 3.95 (s, 4 H) 4.19 (s, 4 H) 4.69 (s., 3 H) 6.64 (br. s., 1 H) 7.55 (s, 1 H) 7.68 (s, 2 H) 7.86 (s, 1 H).

¹³C NMR (151 MHz, CHLOROFORM-*d*) δ ppm 28.54, 34.25, 39.32, 59.66, 68.67, 80.44, 116.15, 125.03, 127.10, 127.82, 128.38, 137.19, 137.62, 138.16, 151.68, 156.06.

HPLC-MS (ESI): [Rt]: 4.46 min, *m/z* for C₂₀H₂₅N₇O₂ ([M+H]⁺), 395.21 calcd., 396.18 found.

Synthesis of *tert*-butyl 7-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)-2,7-diazaspiro[3.5]nonane-2-carboxylate (5):



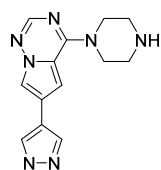
4-chloro-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine **1** (27.9 mg, 0.1 mmol, 0.9 eq.), *tert*-butyl 2,7-diazaspiro[3.5]nonane-2-carboxylate (30.0 mg, 0.1 mmol, 1.0 eq.) and TEA (1.9 μ L, 0.1 mmol, 1.0 eq.) were used following general procedure I for 6 h at rt and yielded the title compound as a white solid (54.9 mg, 98 %).

^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.32 (s, 9 H) 1.73 (t, $J=5.38$ Hz, 4 H) 3.56 (br. s., 4 H) 3.77 (s, 3 H) 3.83 (br. s., 4 H) 7.05 (d, $J=1.47$ Hz, 1 H) 7.74 (s, 1 H) 7.76 (s, 1 H) 7.87 (d, $J=1.47$ Hz, 1 H) 7.96 (s, 1 H).

^{13}C NMR (101 MHz, DMSO- d_6) δ ppm 28.09, 33.47, 34.90, 38.54, 42.83, 78.47, 101.27, 114.41, 115.38, 115.72, 117.71, 127.58, 136.39, 146.49, 153.36, 155.69.

HPLC-MS (ESI): [Rt]: 5.79 min, m/z for $\text{C}_{22}\text{H}_{29}\text{N}_7\text{O}_2$ ($[\text{M}+\text{H}]^+$), 423.24 calcd., 424.24 found.

Synthesis of 6-(1-methyl-1H-pyrazol-4-yl)-4-(piperazin-1-yl)pyrrolo[2,1-f][1,2,4]triazine (6):



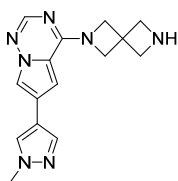
tert-butyl 4-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)piperazine-1-carboxylate **3** (350.0 mg, 0.9 mmol, 1.0 eq.) was used following general procedure II and afforded the target compound as a white solid (166.7 mg, 64 %).

^1H NMR (600 MHz, DMSO- d_6) δ ppm 2.82 - 2.86 (m, 4 H) 3.85 (s, 3 H) 3.89 - 3.93 (m, 4 H) 7.14 (s, 1 H) 7.81 (s, 1 H) 7.84 (s, 1 H) 7.95 (s, 1 H) 8.03 (s, 1 H).

^{13}C NMR (151 MHz, DMSO- d_6) δ ppm 39.02, 46.13, 47.07, 101.92, 114.94, 115.79, 116.26, 118.13, 128.02, 136.85, 146.96, 153.98.

HPLC-MS (ESI): [Rt]: 2.41 min, m/z for $\text{C}_{14}\text{H}_{17}\text{N}_7$ ($[\text{M}+\text{H}]^+$), 283.15 calcd., 284.22 found.

Synthesis of 6-(1-methyl-1H-pyrazol-4-yl)-4-(2,6-diazaspiro[3.3]heptan-2-yl)pyrrolo[2,1-f][1,2,4]triazine (7):



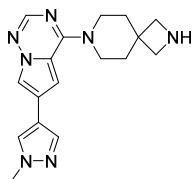
tert-butyl-6-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)-2,6-diazaspiro[3.3]heptane-2-carboxylate **4** (30.0 mg, 0.08 mmol, 1.0 eq.) was used following general procedure III. The target compound was obtained as a white solid (14.2 mg, 63 %).

¹H NMR (600 MHz, DMSO-*d*₆) δ ppm 1.99 (s, 1 H) 3.61 (br. s., 4 H) 3.75 (s., 3 H) 4.32-4.68 (br. s., 4 H) 6.85 (s, 1 H) 7.78 (d, *J*=2.20 Hz, 2 H) 7.90 (s, 1 H) 8.01 (s, 1 H).

¹³C NMR (151 MHz, DMSO-*d*₆) δ ppm 21.24, 39.04, 56.39, 60.24, 99.52, 114.45, 115.24, 116.33, 118.54, 127.93, 136.78, 147.74, 153.70.

HPLC-MS (ESI): [Rt]: 1.89 min, *m/z* for C₁₅H₁₇N₇ ([M+H]⁺), 295,15 calcd., 296.23 found.

Synthesis of 6-(1-methyl-1H-pyrazol-4-yl)-4-(2,7-diazaspiro[3.5]nonan-7-yl)pyrrolo[2,1-f][1,2,4]triazine (8):



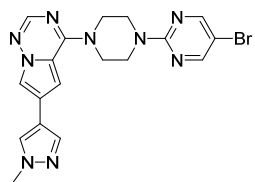
7-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)-2,7-diazaspiro [3.5]nonane-2-carboxylate **5** (45.0 mg, 0.11 mmol, 1.0 eq.) was used according to general procedure III and yielded the target compound as a white solid (34.0 mg, quant.).

¹H NMR (600 MHz, METHANOL-*d*₄) δ ppm 1.90 (br. s., 1 H) 1.96 (br. s., 4 H) 3.62 (s, 4 H) 3.91 (s, 3 H) 3.96 (br. s., 4 H) 7.03 (s, 1 H) 7.76 (d, *J*=1.47 Hz, 1 H) 7.77 (d, *J*=1.83 Hz, 2 H) 7.89 (s, 1 H).

¹³C NMR (151 MHz, METHANOL-*d*₄) δ ppm 36.64, 39.02, 44.40, 46.54, 57.04, 103.08, 116.52, 116.95, 118.08, 119.43, 129.24, 137.89, 147.90, 155.51.

HPLC-MS (ESI): [Rt]: 2.78 min, *m/z* for C₁₇H₂₁N₇ ([M+H]⁺), 323,19 calcd., 324.33 found.

Synthesis of 4-(4-(5-bromopyrimidin-2-yl)piperazin-1-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine (9):



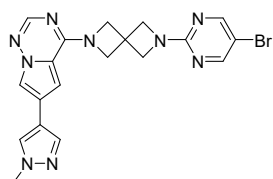
6-(1-methyl-1H-pyrazol-4-yl)-4-(piperazin-1-yl)pyrrolo[2,1-f][1,2,4]triazine **6** (130.0 mg, 0.46 mmol, 1.0 eq.), 5-bromo-2-chloropyrimidine (88.8 mg, 0.46 mmol, 1.0 eq.) and TEA (63.6 μ L, 0.46 mmol, 1.0 eq.) were used according to general procedure III and afforded the target compound as a white solid (127.6 mg, 63 %).

^1H NMR (500 MHz, DMSO- d_6) δ ppm 3.79 (s, 3 H) 3.86 (dd, $J=6.26$, 4.43 Hz, 4 H) 4.06 (dd, $J=6.33$, 4.35 Hz, 4 H) 7.19 (d, $J=1.37$ Hz, 1 H) 7.75 (s, 1 H) 7.83 (s, 1 H) 7.94 (d, $J=1.37$ Hz, 1 H) 7.96 (s, 1 H) 8.46 (s, 2 H).

^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 39.06, 43.46, 106.26, 114.78, 116.15, 116.36, 118.52, 128.06, 130.95, 133.14, 136.87, 153.67, 158.54, 159.83.

HPLC-MS (ESI): [Rt]: 6.25 min, m/z for $\text{C}_{18}\text{H}_{18}\text{BrN}_9$ ($[\text{M}+\text{H}^+]$), 439.09 calcd., 440.32 found.

Synthesis of 4-(6-(5-bromopyrimidin-2-yl)-2,6-diazaspiro[3.3]heptan-2-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine (10):



6-(1-methyl-1H-pyrazol-4-yl)-4-(2,6-diazaspiro[3.3]heptan-2-yl)pyrrolo[2,1-f][1,2,4]triazine **7** (10.0 mg, 0.03 mmol, 1.0 eq.), 5-bromo-2-chloropyrimidine (6.6 mg, 0.03 mmol, 1.0 eq.) and TEA (4.9 μ L, 0.03 mmol, 1.0 eq.) were used following general procedure III. The

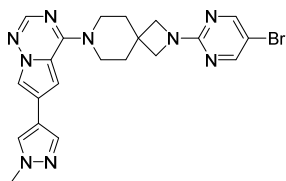
target compound was obtained as a white solid (13.1 mg, 85 %).

^1H NMR (600 MHz, DMSO- d_6) δ ppm 4.29 (s, 4 H) 4.42 (br. s., 2 H) 4.81 (br. s., 2 H) 6.83 (d, $J=1.83$ Hz, 1 H) 7.77 (d, $J=0.73$ Hz, 1 H) 7.81 (s, 1 H) 7.92 (d, $J=1.47$ Hz, 1 H) 8.00 (s, 1 H) 8.48 (s, 2 H).

^{13}C NMR (151 MHz, DMSO- d_6) δ ppm 34.76, 39.04, 60.53, 99.43, 106.96, 114.46, 115.31, 116.30, 118.58, 127.92, 136.76, 147.75, 153.74, 158.48, 161.11.

HPLC-MS (ESI): [Rt]: 4.38 min, m/z for $\text{C}_{19}\text{H}_{18}\text{BrN}_9$ ($[\text{M}+\text{H}^+]$), 451.09 calcd., 452.36 found.

Synthesis of 4-(2-(5-bromopyrimidin-2-yl)-2,7-diazaspiro[3.5]nonan-7-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine (11):



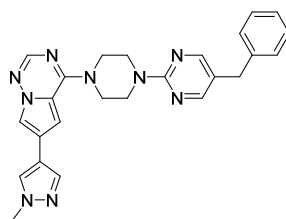
6-(1-methyl-1H-pyrazol-4-yl)-4-(2,7-diazaspiro[3.5]nonan-7-yl)pyrrolo[2,1-f][1,2,4]triazine (**8**, 30.0 mg, 0.09 mmol, 1.0 eq.), 5-bromo-2-chloropyrimidine (17.9 mg, 0.09 mmol, 1.0 eq.) and TEA (19.4 μ L, 0.14 mmol, 1.5 eq.) were used general procedure III and yielded the title compound as a white solid (31.0 mg, 70 %).

^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.79 - 1.84 (m, 4 H) 3.77 (s, 3 H) 3.78 (s, 4 H) 3.86 - 3.90 (m, 4 H) 7.07 (d, $J=1.47$ Hz, 1 H) 7.74 (s, 1 H) 7.77 (s, 1 H) 7.88 (d, $J=1.47$ Hz, 1 H) 7.97 (s, 1 H) 8.38 (s, 2 H).

^{13}C NMR (101 MHz, DMSO- d_6) δ ppm 34.69, 35.53, 39.03, 43.40, 60.24, 100.00, 101.80, 106.51, 114.93, 116.23, 118.21, 128.07, 136.89, 147.00, 153.92, 158.45, 161.32.

HPLC-MS (ESI): [Rt]: 5.70 min, m/z for $\text{C}_{21}\text{H}_{22}\text{BrN}_9$ ($[\text{M}+\text{H}]^+$), 479.12 calcd., 480.33 found.

Synthesis of 4-(4-(5-benzylpyrimidin-2-yl)piperazin-1-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine (12):



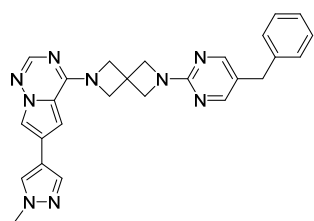
4-(4-(5-benzylpyrimidin-2-yl)piperazin-1-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine (**9** (25.0 mg, 0.1 mmol, 1.0 eq.), 2-benzyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (19.0 μ L, 0.1 mmol, 1.5 eq.), [1,1'-Bis(diphenylphosphino)ferrocene]palladium(II)dichloride (5.9 mg, 8.5 μ mol, 0.15 eq.) and Cs_2CO_3 (55.7 mg, 0.2 mmol, 3.0 eq.) were used according to general procedure IV and yielded **12** as a white solid (18.5 mg, 72 %).

^1H NMR (600 MHz, DMSO- d_6) δ ppm 3.81 (s, 2 H) 3.86 (s, 3 H) 3.89 - 3.92 (m, 4 H) 4.08 - 4.11 (m, 4 H) 7.18 - 7.23 (m, 2 H) 7.25 (d, $J=6.97$ Hz, 2 H) 7.30 (t, $J=7.52$ Hz, 2 H) 7.81 - 7.82 (m, 1 H) 7.87 (s, 1 H) 7.98 (d, $J=1.56$ Hz, 1 H) 8.03 (s, 1 H) 8.34 (s, 2 H).

^{13}C NMR (151 MHz, DMSO- d_6) δ ppm 35.03, 39.04, 43.54, 102.13, 114.97, 115.91, 116.25, 118.29, 123.32, 126.61, 128.03, 128.85, 129.05, 136.86, 141.39, 146.97, 154.17, 158.32, 160.56.

HPLC-MS (ESI): [Rt]: 6.55 min, m/z for $\text{C}_{25}\text{H}_{25}\text{N}_9$ ($[\text{M}+\text{H}]^+$), 451.22 calcd., 452.35 found.

Synthesis of 4-(6-(5-benzylpyrimidin-2-yl)-2,6-diazaspiro[3.3]heptan-2-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine (13):



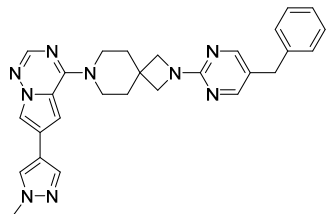
4-(6-(5-bromopyrimidin-2-yl)-2,6-diazaspiro[3.3]heptan-2-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine **10** (13.0 mg, 0.03 mmol, 1.0 eq.), 2-benzyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.01 mL, 0.04 mmol, 1.5 eq.), [1,1'-Bis(diphenylphosphino)ferrocene]palladium(II)dichloride (2.9 mg, 4.3 μ mol, 0.15 eq.) and Cs_2CO_3 (28.2 mg, 0.09 mmol, 3.0 eq.) were used following general procedure IV and afforded the desired compound as a white solid (7.2 mg, 54 %).

^1H NMR (700 MHz, DMSO-d_6) δ ppm 3.33 (s, 8 H) 3.85 (s, 2 H) 4.25 (s, 3 H) 6.84 (d, $J=1.51$ Hz, 1 H) 7.18 - 7.22 (m, 1 H) 7.23 (d, $J=7.10$ Hz, 2 H) 7.30 (t, $J=7.64$ Hz, 2 H) 7.77 (s, 1 H) 7.80 (s, 1 H) 7.92 (d, $J=1.72$ Hz, 1 H) 8.00 (s, 1 H) 8.29 (s, 2 H).

^{13}C NMR (176 MHz, DMSO-d_6) δ ppm 34.36, 34.65, 38.54, 55.78, 60.12, 98.99, 113.98, 114.80, 115.82, 118.09, 123.36, 126.13, 127.43, 128.33, 128.56, 136.27, 140.92, 147.26, 153.26, 157.74, 161.81.

HPLC-MS (ESI): [Rt]: 4.65 min, m/z for $\text{C}_{26}\text{H}_{25}\text{N}_9$ ($[\text{M}+\text{H}]^+$), 463.22 calcd., 464.29 found.

Synthesis of 4-(2-(5-benzylpyrimidin-2-yl)-2,7-diazaspiro[3.5]nonan-7-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine (14):



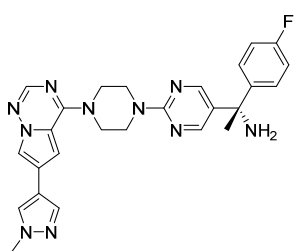
4-(2-(5-bromopyrimidin-2-yl)-2,7-diazaspiro[3.5]nonan-7-yl)-6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazine **11** (28.0 mg, 0.06 mmol, 1.0 eq.), 2-benzyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.02 mL, 0.09 mmol, 1.5 eq.), [1,1'-Bis(diphenylphosphino)ferrocene]palladium(II)dichloride (6.1 mg, 0.01 mmol, 0.15 eq.) and Cs_2CO_3 (57.2 mg, 0.17 mmol, 3.0 eq.) were used following general procedure IV. 14 was obtained as a white solid (15.5 mg, 54 %).

^1H NMR (700 MHz, DMSO-d_6) δ ppm 1.86 - 1.89 (m, 4 H) 3.79 (s, 2 H) 3.82 (s, 4 H) 3.85 (s, 3 H) 3.94 - 3.97 (m, 4 H) 7.14 - 7.15 (m, 1 H) 7.18 - 7.21 (m, 1 H) 7.23 (d, $J=7.10$ Hz, 2 H) 7.28 - 7.31 (m, 2 H) 7.82 (s, 1 H) 7.85 (s, 1 H) 7.96 (d, $J=1.29$ Hz, 1 H) 8.05 (s, 1 H) 8.27 (s, 2 H).

^{13}C NMR (176 MHz, DMSO-d_6) δ ppm 34.16, 34.66, 35.16, 38.51, 40.01, 59.74, 101.28, 114.43, 115.35, 115.73, 117.69, 122.85, 126.08, 127.56, 128.32, 128.53, 136.37, 140.96, 146.49, 153.39, 157.71, 161.88.

HPLC-MS (ESI): [Rt]: 5.77 min, m/z for $\text{C}_{28}\text{H}_{29}\text{N}_9$ ($[\text{M}+\text{H}]^+$), 491.25 calcd., 492.39 found.

Synthesis of (S)-1-(4-fluorophenyl)-1-(2-(4-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)piperazin-1-yl)pyrimidin-5-yl)ethan-1-amine (avapritinib) has been reported.²⁶⁵



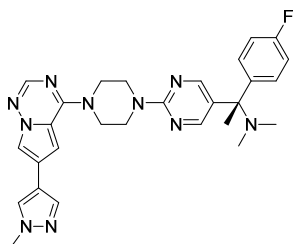
¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.66 (s, 3 H) 3.78 (s, 3 H) 3.81 - 3.85 (m, 4 H) 4.01 (dd, *J*=6.60, 4.16 Hz, 4 H) 7.00 - 7.06 (m, 2 H) 7.15 (d, *J*=1.47 Hz, 1 H) 7.39 (dd, *J*=8.80, 5.38 Hz, 2 H) 7.74 (s, 1 H) 7.79 (s, 1 H) 7.90 (d, *J*=1.47 Hz, 1 H) 7.95 (s, 1 H) 8.33 (s, 2 H).

¹³C NMR (101 MHz, DMSO-*d*₆) δ ppm 31.74, 39.05, 43.53, 55.53, 102.14, 114.84, 114.97, 115.04, 115.92, 116.27, 118.31, 128.03, 128.42, 128.50, 132.14, 136.87, 146.40, 146.97, 154.17, 156.53, 160.19.

¹⁹F NMR (377 MHz, CHLOROFORM-*d*) δ ppm -115.92.

HPLC-MS (ESI): [*R*_t]: 3.30 min, *m/z* for C₂₆H₂₇FN₁₀ ([*M*+*H*⁺]), 498.24 calcd., 498.99 found.

Synthesis of (S)-1-(4-fluorophenyl)-*N,N*-dimethyl-1-(2-(4-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)piperazin-1-yl)pyrimidin-5-yl)ethan-1-amine (15).



A solution of avapritinib (30 mg, 0.06 mmol) in dry THF (3 mL) was treated with 16 μ L of DIPEA (0.09 mmol). Then methyl iodide (11.25 μ L, 0.18 mmol) was dropwise added at 0 °C. The resulting mixture was stirred for or 3 h at room temperature. After the completion of the reaction was monitored via TLC and LC-MS, the reaction was

quenched with sat. NaHCO₃, followed by extraction with EA. The combined organic layers were dried over Na₂SO₄ filtered and concentrated under reduced pressure. The crude was absorbed onto silica gel and purified by flash column chromatography (DCM/MeOH) to yield **15** as off-white solid (24 mg, 77 %).

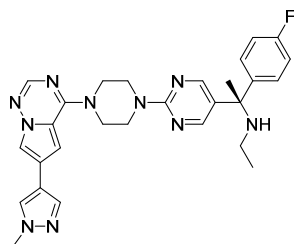
¹H NMR (600 MHz, DMSO-*d*₆) δ ppm 1.69 (s, 3 H) 2.07 (s, 6 H) 3.86 (s, 3 H) 3.92 (dd, *J*=6.12, 4.47 Hz, 4 H) 4.08 - 4.12 (m, 4 H) 7.11 - 7.15 (m, 2 H) 7.22 (d, *J*=1.60 Hz, 1 H) 7.48 - 7.51 (m, 2 H) 7.82 (s, 1 H) 7.87 (s, 1 H) 7.98 (d, *J*=1.56 Hz, 1 H) 8.04 (s, 1 H) 8.42 (s, 2 H).

¹³C NMR (151 MHz, DMSO-*d*₆) δ ppm 18.35, 39.04, 40.53, 43.39, 63.99, 102.13, 114.96, 115.18, 115.32, 115.93, 116.26, 118.30, 126.77, 128.02, 129.35, 136.87, 142.50, 142.52, 146.97, 154.16, 157.50, 160.11, 161.88.

¹⁹F NMR (470 MHz, DMSO-*d*₆) δ ppm -116.83.

HPLC-MS (ESI): [*R*_t]: 5.65 min, *m/z* for C₂₈H₃₁FN₁₀ ([*M*+*H*⁺]), 526.27 calcd., 527.33, found.

Synthesis of (S)-N-ethyl-1-(4-fluorophenyl)-1-(2-(4-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)piperazin-1-yl)pyrimidin-5-yl)ethan-1-amine (16**):**



A stirred solution of avapritinib (40 mg, 0.08 mmol, 1.0 eq.) and freshly washed NaH (60 % in oil, 8 mg, 0.32 mmol, 4.0 eq.) in dry THF (5 mL) was treated with ethyliodide (10 μ L, 0.10 mmol, 1.2 eq.) at 0 °C. The reaction mixture was stirred for 8 h at rt. After the completion of the reaction was monitored via TLC and LC-MS, the reaction was quenched cooled to 0 °C using water and extracted with EA. The combined organic layers were dried over Na₂SO₄ filtered and concentrated under reduced pressure. The resulting crude was absorbed onto silica gel and purified by flash column chromatography. Pure fractions were combined, evaporated to yield the **16** as off-white solid (34 mg, 80 %).

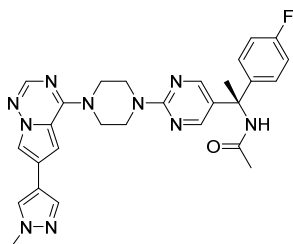
¹H NMR (500 MHz, DMSO-*d*₆) δ ppm 1.02 (t, *J*=7.02 Hz, 3 H) 1.69 (s, 3 H) 2.50 - 2.51 (m, 2 H) 3.86 (s, 3 H) 3.89 - 3.94 (m, 4 H) 4.08 - 4.12 (m, 4 H) 7.12 (t, *J*=8.85 Hz, 2 H) 7.24 (d, *J*=1.37 Hz, 1 H) 7.42 (dd, *J*=8.77, 5.57 Hz, 2 H) 7.83 (s, 1 H) 7.88 (s, 1 H) 7.99 (d, *J*=1.37 Hz, 1 H) 8.04 (s, 1 H) 8.34 (s, 2 H).

¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 15.99, 27.34, 37.24, 39.04, 43.44, 59.53, 102.13, 114.95, 115.15, 115.92, 116.27, 118.30, 128.02, 129.05, 129.12, 129.56, 136.86, 144.30, 146.97, 154.14, 157.20, 160.24, 162.09.

¹⁹F NMR (470 MHz, DMSO-*d*₆) δ ppm -117.05.

HPLC-MS (ESI): [*R*_t]: 3.84 min, *m/z* for C₂₈H₃₁FN₁₀ ([*M*+*H*⁺]), 526.27 calcd., 527.96, found.

Synthesis of (S)-N-(1-(4-fluorophenyl)-1-(2-(4-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)piperazin-1-yl)pyrimidin-5-yl)ethyl)acetamide (**17**):



Acetyl chloride (4.2 μ L, 0.06 mmol, 1.0 eq.) was gradually added to the stirred solution of avapritinib (30 mg, 0.06 mmol, 1.0 eq.) in dry THF (3 mL) at 0 °C. The resulting reaction mixture was allowed to stir for 3 h at room temperature. After the completion of the reaction was monitored via TLC and LC-MS, the reaction was quenched with sat. NaHCO_3 (5 mL) solution. After that two times extracted with ethyl acetate and washed with brine solution. The organic fractions were concentrated, absorbed onto silica and purified by flash chromatography to afford the final product **17** as off-white solid (29 mg, 89 %).

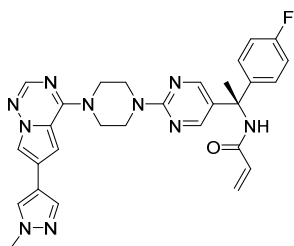
^1H NMR (500 MHz, CDCl_3 -*d*) δ ppm 1.93 (s, 3 H) 2.01 (s, 3 H) 3.87 (s, 3 H) 3.94 (dd, $J=6.26$, 4.27 Hz, 4 H) 4.04 - 4.09 (m, 4 H) 6.06 (s, 1 H) 6.70 (d, $J=1.53$ Hz, 1 H) 6.95 (t, $J=8.54$ Hz, 2 H) 7.15 - 7.20 (m, 2 H) 7.49 (s, 1 H) 7.60 - 7.63 (m, 2 H) 7.82 (s, 1 H) 8.22 (s, 2 H).

^{13}C NMR (126 MHz, CDCl_3 -*d*) δ ppm 24.24, 28.01, 39.07, 43.29, 45.39, 59.03, 101.26, 115.41, 115.58, 115.90, 116.41, 117.78, 126.57, 126.92, 128.03, 136.98, 140.58, 146.84, 154.52, 156.70, 160.28, 160.93, 162.90, 169.22.

^{19}F NMR (470 MHz, $\text{DMSO}-d_6$) δ ppm -117.29.

HPLC-MS (ESI): [R_t]: 5.30 min, m/z for $\text{C}_{28}\text{H}_{29}\text{FN}_{10}\text{O}$ ($[\text{M}+\text{H}^+]$), 540.25 calcd., 541.15 found.

Synthesis of (S)-N-(1-(4-fluorophenyl)-1-(2-(4-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)piperazin-1-yl)pyrimidin-5-yl)ethyl)acrylamide (18**):**



Acryloyl chloride (4.9 μ L, 0.06 mmol, 1.0 eq.) was added to the stirred solution of avapritinib (30 mg, 0.06 mmol, 1.0 eq.) in dry THF (3 mL) at 0 $^{\circ}$ C, by means of a slow and controlled addition. The resulting mixture was stirred at room temperature for 3 h. After the completion of the reaction was monitored via TLC and LC-MS, the reaction was quenched with sat. NaHCO_3 (5 mL) solution. Subsequently, the reaction mixture was extracted with ethyl acetate (EA). The combined organic layers were dried over Na_2SO_4 filtered and concentrated under reduced pressure. The crude was absorbed onto silica gel and purified by flash column chromatography (DCM/MeOH) to yield the target product **18** as off-white solid (29 mg, 90 %).

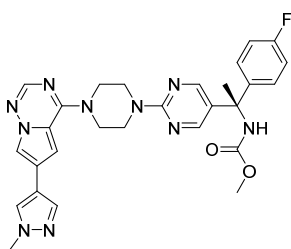
^1H NMR (400 MHz, CDCl_3 -*d*) δ ppm 2.07 (s, 3 H) 3.87 (s, 3 H) 3.94 - 3.98 (m, 4 H) 4.08 (dd, $J=6.36$, 3.91 Hz, 4 H) 5.59 (dd, $J=10.27$, 1.47 Hz, 1 H) 6.01 - 6.11 (m, 1 H) 6.17 - 6.23 (m, 1 H) 6.72 (d, $J=1.47$ Hz, 1 H) 6.93 - 6.99 (m, 2 H) 7.16 - 7.23 (m, 3 H) 7.50 (s, 1 H) 7.62 (s, 2 H) 7.83 (s, 1 H) 8.24 (s, 2 H).

^{13}C NMR (101 MHz, CDCl_3 -*d*) δ ppm 28.11, 39.08, 43.30, 45.55, 59.15, 115.48, 115.70, 116.36, 122.76, 126.47, 126.92, 127.49, 128.02, 128.10, 130.80, 131.09, 137.03, 137.99, 140.40, 144.50, 156.73, 160.20, 163.22, 164.47.

^{19}F NMR (470 MHz, $\text{DMSO}-d_6$) δ ppm -116.53.

HPLC-MS (ESI): [R_t]: 5.66 min, m/z for $\text{C}_{29}\text{H}_{29}\text{FN}_{10}\text{O}$ ($[\text{M}+\text{H}^+]$), 552.25 calcd., 553.14 found.

Synthesis of Methyl (S)-(1-(4-fluorophenyl)-1-(2-(4-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)piperazin-1-yl)pyrimidin-5-yl)ethyl)carbamate (19**):**



Avapritinib (50.0 mg, 0.1 mmol, 1.0 eq.) was dissolved in DCM and K_2CO_3 (13.9 mg, 0.1 mmol, 1.0 eq.) was added. Afterwards, methyl chloroformate (0.01 μ L, 0.1 mmol, 1.0 eq.) was slowly added to the mixture at 0 °C. The reaction was allowed to stir for 5 h at room temperature. After the completion of the reaction was monitored via TLC and LC-MS, the reaction mixture was diluted with water and

subsequently subjected to extraction with DCM. The combined organic layers were dried over Na_2SO_4 filtered and concentrated *in vacuo*. The crude was absorbed onto silica gel and purified by flash column chromatography (DCM/MeOH) to yield the final product **19** as white solid (46.8 mg, 84 %).

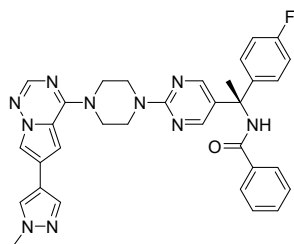
1H NMR (500 MHz, $DMSO-d_6$) δ ppm 1.92 (s, 3 H) 3.48 (s, 3 H) 3.86 (s, 3 H) 3.93 (dd, $J=6.18$, 4.35 Hz, 4 H) 4.09 - 4.13 (m, 4 H) 7.16 (t, $J=8.93$ Hz, 2 H) 7.23 (d, $J=1.53$ Hz, 1 H) 7.30 - 7.34 (m, 2 H) 7.82 (s, 1 H) 7.88 (s, 1 H) 7.92 (s, 1 H) 7.99 (d, $J=1.53$ Hz, 1 H) 8.04 (s, 1 H) 8.28 (s, 2 H).

^{13}C NMR (126 MHz, $DMSO-d_6$) δ ppm 22.96, 39.05, 43.41, 51.56, 58.15, 102.14, 114.98, 115.14, 115.31, 115.94, 116.26, 118.32, 127.75, 128.03, 128.80, 128.86, 136.87, 142.02, 146.97, 154.18, 155.78, 157.17, 160.21.

^{19}F NMR (471 MHz, $DMSO-d_6$) δ ppm -116.58.

HPLC-MS (ESI): [R_t]: 5.92 min, m/z for $C_{28}H_{29}FN_{10}O_2$ ($[M+H]^+$), 556.25 calcd., 557.30 found.

Synthesis of (S)-N-(1-(4-fluorophenyl)-1-(2-(4-(6-(1-methyl-1H-pyrazol-4-yl)pyrrolo[2,1-f][1,2,4]triazin-4-yl)piperazin-1-yl)pyrimidin-5-yl)ethyl)benzamide (20):



In anhydrous DCM, benzoic acid (11.0 μL , 0.1 mmol, 1.1 eq.) was dissolved, and TEA (44 μL , 0.3 mmol, 3.0 eq.) was added. After stirring for 5 minutes, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxide hexafluorophosphate (HATU, 41.9 mg, 0.1 mmol, 1.1 eq.) was introduced into the solution. The mixture was allowed to stir for an additional 15 minutes, followed by the addition of avapritinib (50.0 mg, 0.1 mmol, 1.0 equivalent). The resulting reaction mixture was then allowed to stir for 14 hours at room temperature. Completion of the reaction was monitored by TLC and LC-MS. Upon completion, the mixture was diluted with brine and was extracted with DCM. The combined organic layers were dried over sodium sulfate filtered and concentrated under reduced pressure. The crude product was then adsorbed onto silica gel and purified by flash column chromatography using a DCM/MeOH system as the eluent. Pure fractions were evaporated to dryness, yielding the final compound **20** as a white solid (51.1 mg, 85 %).

^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ ppm 2.08 (s, 3 H) 3.86 (s, 3 H) 3.92 - 3.96 (m, 4 H) 4.10 - 4.13 (m, 4 H) 7.18 (t, $J=8.83$ Hz, 2 H) 7.24 (d, $J=1.38$ Hz, 1 H) 7.39 (dd, $J=8.83$, 5.39 Hz, 2 H) 7.44 - 7.48 (m, 2 H) 7.52 - 7.56 (m, 1 H) 7.82 (s, 1 H) 7.86 (d, $J=7.20$ Hz, 2 H) 7.88 (s, 1 H) 7.99 (d, $J=1.42$ Hz, 1 H) 8.04 (s, 1 H) 8.37 (s, 2 H) 8.76 (s, 1 H).

^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ ppm 38.55, 40.05, 42.93, 58.57, 101.65, 114.48, 114.65, 114.79, 115.44, 115.77, 117.83, 127.19, 127.54, 127.67, 128.09, 128.37, 128.42, 131.23, 135.01, 136.38, 141.26, 146.48, 153.69, 156.83, 159.69, 166.39.

^{19}F NMR (471 MHz, $\text{DMSO}-d_6$) δ ppm -116.73.

HPLC-MS (ESI): [R_t]: 6.54 min, m/z for $\text{C}_{33}\text{H}_{31}\text{FN}_{10}\text{O}$ ($[\text{M}+\text{H}^+]$), 602.27 calcd., 603.13 found.

8.3.3 Synthetic Procedure and Analysis for the Synthesis of Covalent Derivatives

The methods used, as well as the reported compound validation, are part of: **Schulz, T.**; Gontla, R.; Teuber, A.; Beerbaum, M.; Fletcher, B. S; Mühlenberg, T.; Kaitsiotou, H.; Hardick, J.; Jeyakumar, K.; Keul, M.; Müller, M. P.; Sievers, S.; Bauer, S. and Rauh, D., *Design, Synthesis and SAR of Covalent KIT and PDGFRA Inhibitors – Exploring Their Potential in Targeting GIST*. Submitted. And have been published in parts within the master thesis of Tom Schulz and Maria Beerbaum.

General Procedure V: *SONOGASHIRA Coupling with Silyl-Protected Alkynes*

A mixture containing silyl-protected alkynes (1.0 eq.), the appropriate aryl halide (1.0 eq.), and diisopropylamine (3.0 eq.) was dissolved in anhydrous CH₃CN. The solution was degassed for 15 minutes at room temperature under an inert argon atmosphere. *Tetra*-n-butylammonium fluoride (3.0 eq.) was introduced, and stirring continued for an additional 30 minutes. Subsequently, bis(triphenylphosphine)palladium(II)dichloride (10 mol%) and copper(I) iodide (10 mol%) were added. The reaction was allowed to proceed overnight at 80 °C. Progress was monitored using TLC and LC-MS. After completion, the mixture was filtered through celite, and the solvent was evaporated under reduced pressure. The crude was adsorbed onto silica gel and purified using flash column chromatography.

General Procedure VI: *HATU-mediated amide coupling*

The respective carboxylic acid (1.0 eq.) was dissolved in a mixture of anhydrous DCM and DMF. To this, DIPEA (3.0 eq.) and HATU (1.0 eq.) were added, and the solution was stirred for 15 minutes. The corresponding amine (1.0 eq.) was then added gradually to the reaction mixture, which was left to stir at room temperature overnight. Reaction progress was checked using TLC and MS. Upon completion, the mixture was diluted with DCM and washed with saturated brine. The organic layers were combined, dried over Na₂SO₄ filtered and concentrated under reduced pressure. The resulting crude product was adsorbed onto silica gel and purified via flash column chromatography to obtain the desired compound.

General Procedure VII: *Amide formation using acid chlorides*

The corresponding amine (1.0 eq.) was dissolved in dry THF or DCM, and DIPEA or TEA (3.0 eq.) was added. The solution was cooled to -10 °C before the dropwise addition of acryloyl chloride (1.1 eq.) in a pre-diluted form. After confirming reaction completion via TLC and/or MS, the reaction mixture was quenched with aqueous NaHCO₃ and extracted with DCM in three times. The combined organic layers were dried over Na₂SO₄ filtered and concentrated *in vacuo*. The crude product was loaded onto silica gel and purified by flash column chromatography to afford the target product.

General Procedure VIII: *Reduction with hydrogen and Pd/C*

The respective amine (1.0 eq.) was dissolved in dry MeOH, and Pd/C (0.1 eq.) was added under inert conditions. The argon atmosphere was replaced with hydrogen, and the mixture was stirred at room temperature for 6 – 24 hours. After the reaction was completed (monitored via TLC and/or LC-MS), the catalyst was removed by filtration through Celite, and the solvent was evaporated under reduced pressure. The resulting crude was adsorbed onto silica gel and purified using flash column chromatography to yield the target product.

General Procedure IX: *Iron-mediated reduction with ammonium chloride*

The respective amine (1.0 eq.) was dissolved in a 50 – 70 % v/v methanol-water mixture. Iron powder (5.0 eq.) and ammonium chloride (10.0 eq.) were added, and the reaction mixture was stirred at 65 °C under sonication for 2 – 48 hours. The reaction progress was monitored using TLC and/or LC-MS. Upon completion, the mixture was filtered through Celite. The crude product was adsorbed onto silica gel and purified via flash column chromatography to obtain the desired compound.

General Procedure X: *Incorporation of a di-*tert*-butyl dicarbonate protection group*

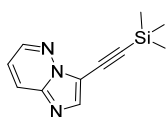
The amine (1.0 eq.) was dissolved in anhydrous CH₃CN under an inert atmosphere, followed by the addition of TEA (1.0 eq.). At 0 °C, a solution of di-*tert*-butyl dicarbonate (1.1 eq.) was slowly added dropwise. The mixture was stirred at room temperature for 24 hours. Upon completion, confirmed by TLC or MS, the reaction was diluted with water and extracted with DCM. The organic layers were combined, dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude product was purified using flash column chromatography.

General Procedure XI: Acidic deprotection of carbamates

The BOC-protected compound (1.0 eq.) was dissolved in a mixture of TFA and DCM (1:5 v/v) and stirred overnight at room temperature. After monitoring completion via TLC and/or LC-MS, the reaction mixture was diluted and neutralized using a saturated aq. solution of NaOH, and extracted three times with DCM. The combined organic fractions were dried over Na₂SO₄, filtered and concentrated under reduced pressure. The resulting crude product was purified by flash column chromatography.

General Procedure XII: SUZUKI-MIYAUURA cross-coupling reactions

The halide (1.0 eq.) and the boronic acid (1.5–3.0 eq.) were dissolved in a 5:1 mixture of dioxane and water under an argon atmosphere. After degassing the solution for 15 minutes, Cs₂CO₃ (3.0 eq.) and Pd(PPh₃)₄ (0.15 eq.) were added. The mixture was heated to 120 °C using microwave irradiation for 1 to 2 hours. Once cooled, the reaction mixture was filtered through Celite and extracted with DCM and water. The combined organic layers were dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was adsorbed onto silica gel and purified via flash column chromatography.

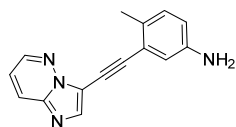
Synthesis of 3-((trimethylsilyl)ethynyl)imidazo[1,2-b]pyridazine (29**):**

A solution was prepared by dissolving 3-bromoimidazo[1,2-b]pyridazine **28** (1.0 eq., 1.0 g, 5.05 mmol), TMSA (5.0 eq., 3.6 mL, 25.3 mmol) and DIPA (3.0 eq., 2.1 mL, 15.2 mmol) in anhydrous CH₃CN. Then, the mixture was degassed for 5 min at room temperature under an argon atmosphere. Copper(I)iodide (10 mol-%) and bis(triphenyl-phosphine)palladium(II)dichloride (10 mol-%) were added to the stirring solution and allowed to stir ovn. at 80 °C. Upon completion of the reaction, as confirmed by TLC and LC-MS analysis, the mixture was filtered, and the solvent was removed under reduced pressure. The crude product was then adsorbed onto silica gel and purified by flash column chromatography. The pure fractions were concentrated to dryness, yielding **29** (883.9 mg, 81 %) as beige crystals

¹H NMR (400 MHz, DMSO-*d*₆) δ 0.00 (s, 9 H) 7.08 (dd, *J*=9.17, 4.40 Hz, 1 H) 7.84 (s, 1 H) 7.93 (dd, *J*=9.17, 1.59 Hz, 1 H) 8.40 (dd, *J*=4.46, 1.53 Hz, 1 H).

¹³C NMR (101 MHz, DMSO-*d*₆) δ -0.24, 91.67, 104.51, 111.88, 118.99, 122.36, 125.98, 138.69, 144.85.

LC-MS (ESI-MS): Calculated: 215.09 for C₁₁H₁₃N₃Si [M+H⁺]. Found: 216.1.

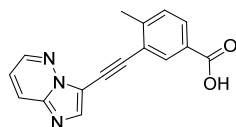
Synthesis of 3-(imidazo[1,2-b]pyridazine-3-ylethynyl)-4-methylaniline (33**):**

29 (1.0 eq., 184.8 mg, 0.86 mmol), 3-iodo-4-methylaniline (1.0 eq., 200.0 mg, 0.86 mmol), DIPA (3.0 eq., 0.36 mL, 2.56 mmol), PdCl₂(PPh₃)₂ (0.15 eq., 90.3 mg, 0.13 mmol), CuI (0.15 eq., 24.5 mg, 0.13 mmol) and TBAF (3.0 eq., 0.74 mL, 2.58 mmol) were used following the general procedure V to yield the product as a pale-yellow solid (189.7 mg, 89 %).

¹H NMR (500 MHz, DMSO-*d*₆) δ ppm 2.34 (s, 3 H) 5.18 (br. s., 2 H) 6.59 (dd, *J*=8.24, 2.44 Hz, 1 H) 6.79 (d, *J*=2.44 Hz, 1 H) 7.00 (d, *J*=8.24 Hz, 1 H) 7.36 (dd, *J*=9.16, 4.58 Hz, 1 H) 8.17 (br. s., 1 H) 8.24 (dd, *J*=9.16, 1.22 Hz, 1 H) 8.69 (dd, *J*=4.27, 1.53 Hz, 1 H).

¹³C NMR (DMSO-*d*₆) δ 19.70, 79.36, 98.49, 116.07, 116.65, 119.18, 121.95, 126.48, 126.84, 130.68, 138.16, 145.38, 146.85.

LC-MS (ESI-MS): Calculated: 248.29 for C₁₅H₁₂N₄ [M+H]⁺. Found: 249.1.

Synthesis of 3-(imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methylbenzoic acid (30**):**

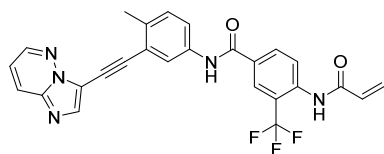
29 (1.0 eq., 400.0 mg, 1.9 mmol), 3-iodo-4-methylbenzoic acid (1.0 eq., 486.8 mg, 1.9 mmol), DIPA (3.0 eq., 0.8 mL, 5.6 mmol), PdCl₂(PPh₃)₂ (0.15 eq., 195.6 mg, 0.3 mmol), CuI (0.15 eq., 53.1 mg, 0.3 mmol) and TBAF (3.0 eq., 1.6 mL, 5.6 mmol) were used following the general procedure V to yield the product as a crystalline light brown solid (404.3 mg, 78 %).

¹H-NMR (600 MHz, DMSO-*d*₆) δ ppm 2.57 (s, 3H), 7.38 (dd, *J* = 9.2, 4.4 Hz, 1H), 7.45 (d, *J* = 8.0 Hz, 1H), 7.87 (dd, *J* = 7.9, 1.8 Hz, 1H), 8.05 (d, *J* = 1.8 Hz, 1H), 8.22 (s, 1H), 8.25 (dd, *J* = 9.2, 1.6 Hz, 1H), 8.72 (dd, *J* = 4.4, 1.6 Hz, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 20.41, 80.73, 96.50, 111.79, 119.00, 121.66, 126.06, 129.68, 129.90, 131.99, 138.16, 139.60, 145.04, 166.71.

LC-MS (ESI): Calculated: 277.09 for C₁₆H₁₁N₃O₂ [M+H]⁺. Found: 278.1.

Synthesis of 4-acrylamido-N-(3-(imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methylphenyl)-3-(trifluoromethyl)benzamide (57):



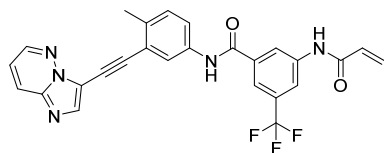
33 (50.0 mg, 0.2 mmol) and 4-acrylamido-3-(trifluoromethyl)benzoic acid (52.0 mg, 0.2 mmol) were used following the general procedure VI to yield the product as a pale-yellow solid (62.1 mg, 62 %).

¹H NMR (400 MHz, DMSO) δ 2.42 (s, 3H), 5.76 (dd, J = 10.2, 1.8 Hz, 1H), 6.23 (dd, J = 17.1, 1.8 Hz, 1H), 6.53 (dd, J = 17.0, 10.2 Hz, 1H), 7.35 – 7.26 (m, 2H), 7.66 (dd, J = 8.3, 2.2 Hz, 1H), 7.72 (d, J = 8.4 Hz, 1H), 7.99 (d, J = 2.1 Hz, 1H), 8.14 (d, J = 5.1 Hz, 1H), 8.22 – 8.16 (m, 2H), 8.25 (d, J = 1.6 Hz, 1H), 8.64 (dd, J = 4.4, 1.5 Hz, 1H), 9.84 (s, 1H), 10.44 (s, 1H).

¹³C NMR (101 MHz, DMSO) δ 20.21, 80.67, 97.47, 112.36, 119.41, 121.70, 122.14, 123.21, 126.30, 126.35, 126.55, 128.46, 130.15, 130.58, 131.37, 132.64, 132.96, 135.33, 137.31, 138.57, 140.03, 145.48, 164.13, 164.71.

LC-MS (ESI-MS): Calculated: 489.46 for C₂₆H₁₈N₅F₃O₂ [M+H⁺]. Found: 490.2.

Synthesis of 3-acrylamido-N-(3-(imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methylphenyl)-5-(trifluoromethyl)benzamide (58):



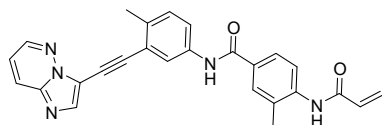
33 (50.0 mg) and 3-acrylamido-5-(trifluoromethyl)benzoic acid (52.0 mg) were used following the general procedure VI to yield the product as a pale yellow solid (77 mg, 78 %).

¹H NMR (400 MHz, DMSO) δ 2.43 (s, 3H), 5.78 (dd, J = 10.0, 1.8 Hz, 1H), 6.27 (dd, J = 17.0, 1.8 Hz, 1H), 6.41 (dd, J = 17.0, 10.0 Hz, 1H), 7.34 – 7.26 (m, 1H), 7.66 (dd, J = 8.3, 2.1 Hz, 1H), 7.98 (d, J = 2.3 Hz, 1H), 8.14 (s, 1H), 8.17 (dd, J = 9.2, 1.5 Hz, 1H), 8.34 (d, J = 10.0 Hz, 1H), 8.64 (dd, J = 4.4, 1.4 Hz, 1H), 10.70 (s, 1H), 10.48 (s, 1H).

¹³C NMR (101 MHz, DMSO) δ 20.22, 80.69, 97.46, 112.37, 118.59, 119.07, 119.39, 121.76, 122.11, 122.89, 123.25, 125.65, 126.53 (s), 128.60 (s), 129.93 (s), 130.28 (s), 130.57 (s), 131.77 (s), 135.41 (s), 136.95 (s), 137.24, 138.52, 140.03, 140.66, 145.47, 164.33.

LC-MS (ESI-MS): Calculated: 489.46 for C₂₆H₁₈F₃N₅O₂ [M+H⁺]. Found: 490.2.

Synthesis of 4-acrylamido-N-(3-(imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methylphenyl)-3-methylbenzamide (59):



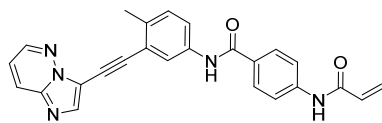
33 (60.0 mg) and 4-acrylamido-3-methylbenzoic acid (50.0 mg) were used following the general procedure VI to yield the product as a pale-yellow solid (89.0 mg, 84 %).

¹H NMR (600 MHz, DMSO) δ 2.34 (s, 3H), 2.51 (s, 3H), 5.80 (d, J = 10.2 Hz, 1H), 6.30 (d, J = 17.0 Hz, 1H), 6.62 (dd, J = 16.9, 10.2 Hz, 1H), 7.34 (d, J = 8.4 Hz, 1H), 7.38 (dd, J = 9.1, 4.4 Hz, 1H), 7.74 (d, J = 8.2 Hz, 1H), 7.80 (q, J = 8.6 Hz, 2H), 7.86 (s, 1H), 8.08 (s, 1H), 8.22 (s, 1H), 8.26 (d, J = 9.1 Hz, 1H), 8.72 (d, J = 4.0 Hz, 1H), 9.61 (s, 1H), 10.23 (s, 1H).

¹³C NMR (151 MHz, DMSO) δ 18.52, 20.19, 80.54, 97.60, 112.41, 119.38, 121.54, 122.03, 122.97, 124.12, 126.09, 126.53, 127.71, 130.34, 130.48, 131.04, 131.31, 132.08, 134.86, 137.72, 138.53, 139.80, 140.02, 145.46, 163.88, 165.56.

LC-MS (ESI-MS): Calculated: 435.49 for C₂₆H₂₁N₅O₂ [M+H⁺]. Found: 436.2.

Synthesis of 4-acrylamido-N-(3-(imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methylphenyl)benzamide (60):

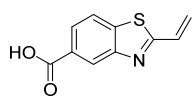


33 (41.0 mg) and 4-acrylamido benzoic acid (35.0 mg) were used following the general procedure VI to yield the product as a pale-yellow solid (54.0 mg, 70 %).

¹H NMR (500 MHz, DMSO) δ 2.50 (s, 3H), 5.82 (dd, J = 10.1, 1.8 Hz, 1H), 6.32 (dd, J = 17.0, 1.8 Hz, 1H), 6.48 (dd, J = 17.0, 10.1 Hz, 1H), 7.34 (d, J = 8.5 Hz, 1H), 7.39 (dd, J = 9.2, 4.4 Hz, 1H), 7.73 (dd, J = 8.3, 2.1 Hz, 1H), 7.83 (d, J = 8.7 Hz, 2H), 7.98 (d, J = 8.7 Hz, 2H), 8.09 (d, J = 2.1 Hz, 1H), 8.24 (dt, J = 28.1, 11.2 Hz, 2H), 8.72 (dd, J = 4.4, 1.2 Hz, 1H), 10.23 (s, 1H), 10.46 (s, 1H).

¹³C NMR (126 MHz, DMSO) δ 20.19, 80.54, 97.61, 119.06, 119.39, 121.55, 122.01, 122.96, 126.54, 128.14, 129.16, 129.73, 130.48, 132.07, 134.83, 137.73, 138.54, 142.53, 145.47, 163.95, 165.35.

LC-MS (ESI-MS): Calculated: 421.46 for C₂₅H₁₉N₅O₂ [M+H⁺]. Found: 422.2.

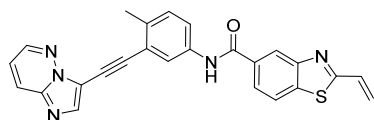
Synthesis of 2-vinylbenzo[d]thiazole-5-carboxylic acid (35):

A microwave vial was loaded with 2-bromobenzo[d]thiazole-6-carboxylic acid **34** (1.0 eq., 200.0 mg, 0.78 mmol), CuI (0.1 eq., 14.8 mg, 0.01 mmol), and tetrakis(triphenylphosphine)palladium(0) (0.05 eq., 44.8 mg, 0.04 mmol). The vial was then sealed, placed under vacuum, backfilled with argon, and filled with DMF (1.0 mL). Vinyl-(tri-n-butyl)tin (1.2 eq., 294.9 mg, 0.9 mmol) was added to the mixture. The reaction was stirred at 65 °C in the microwave for 11 minutes. After cooling the reaction mixture to room temperature, it was extracted with aqueous HCl and ethyl acetate. The combined organic layers were dried over Na₂SO₄, filtered and concentrated under reduced pressure. The resulting crude product was adsorbed onto silica gel and purified by flash column chromatography. After evaporation of the pure fractions, 2-vinylbenzo[d]thiazole-5-carboxylic acid (44.7 mg, 22 %) was obtained as a white solid.

¹H NMR (600 MHz, DMSO-*d*₆) δ 5.94 (d, *J*=11.00 Hz, 1 H) 6.35 (d, *J*=17.24 Hz, 1 H) 7.14 (dd, *J*=17.42, 10.82 Hz, 1 H) 8.05 (s, 2 H) 8.73 (br. s., 1 H) 13.12 (br. s., 1 H).

¹³C NMR (151 MHz, DMSO-*d*₆) δ 123.10, 124.83, 125.93, 127.90, 128.38, 131.35, 134.57, 156.39, 167.35, 170.52.

LC-MS (ESI-MS): Calculated: 205.02 for C₁₀H₇NO₂S [M+H⁺]. Found: 206.1.

Synthesis of N-(3-(imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methylphenyl)-2-vinylbenzo[d]thiazole-5-carboxamide (62):

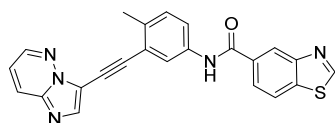
35 (1.1 eq., 22.7 mg, 0.1 mmol), DIPEA (1.2 eq., 13.3 mg, 0.1 mmol), HATU (1.0 eq., 36.8 mg, 0.1 mmol) and **33** (1.0 eq., 25.0 mg, 0.1 mmol) were used following the general procedure VI to yield the product as a pale-yellow solid (2.1 mg, 5 %).

¹H NMR (600 MHz, DMSO-*d*₆) δ 3.86 - 3.87 (m, 1 H) 3.88 - 3.89 (m, 1 H) 5.01 - 5.04 (m, 1 H) 7.36 (d, *J*=8.44 Hz, 1 H) 7.38 (dd, *J*=9.17, 4.40 Hz, 1 H) 7.75 (dd, *J*=8.25, 2.38 Hz, 1 H) 8.07 (s, 1 H) 8.07 (s, 1 H) 8.10 (d, *J*=2.20 Hz, 1 H) 8.23 (s, 1 H) 8.25 (dd, *J*=9.35, 1.65 Hz, 1 H) 8.68 - 8.69 (m, 1 H) 8.72 (dd, *J*=4.40, 1.47 Hz, 1 H) 10.43 (s, 1 H). The DMSO peak covered the protons of the methyl group.

¹³C NMR (151 MHz, DMSO-*d*₆) δ 20.22, 89.58, 90.52, 100.01, 112.40, 115.17, 115.93, 119.42, 122.09, 122.29, 122.48, 123.03, 126.08, 126.58, 130.51, 131.48, 135.08, 135.40, 137.58, 138.49, 139.98, 145.45, 155.06, 165.42, 173.50.

LC-MS (ESI-MS), Calculated: 435.21 for C₂₅H₁₇N₅OS [M+H⁺]. Found: 436.1.

Synthesis of *N*-(3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylphenyl)benzo[*d*]thiazole-5-carboxamide (63**):**



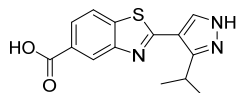
The commercially available benzo[*d*]thiazole-5-carboxylic acid (1.2 eq., 34.6 mg, 0.19 mmol), DIPEA (3.0 eq., 0.08 mL), HATU (1.1 eq., 64.7 mg, 0.18 mmol), and **33** (1.0 eq., 40.0 mg, 0.16 mmol) were used following the general procedure VI to yield the product as a pale-yellow solid (36.6 mg, 55 %).

¹H NMR (700 MHz, DMSO-*d*₆) δ ppm 2.49 (s, 3 H) 7.34 - 7.36 (m, 1 H) 7.36 - 7.38 (m, 1 H) 7.72 (dd, *J*=8.28, 2.26 Hz, 1 H) 8.07 (d, *J*=2.15 Hz, 1 H) 8.11 (dd, *J*=8.50, 1.83 Hz, 1 H) 8.20 (s, 1 H) 8.20 - 8.21 (m, 1 H) 8.21 - 8.22 (m, 1 H) 8.70 (dd, *J*=4.52, 1.51 Hz, 1 H) 8.77 (d, *J*=1.72 Hz, 1 H) 9.54 (s, 1 H) 10.49 (s, 1 H).

¹³C NMR (176 MHz, DMSO-*d*₆) δ 20.18, 80.54, 97.56, 112.40, 119.53, 121.74, 122.06, 123.00, 123.11, 123.28, 126.25, 126.45, 130.59, 132.33, 134.16, 135.32, 137.49, 138.38, 140.01, 145.52, 155.28, 159.72, 165.68.

LC-MS (ESI-MS): Calculated: 409.10 for C₂₃H₁₅N₅OS [M-H⁺]. Found: 408.1.

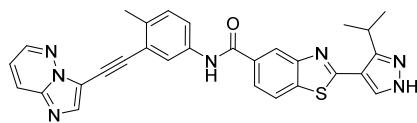
Synthesis of 2-(3-isopropyl-1H-pyrazol-4-yl)benzo[*d*]thiazole-5-carboxylic acid (36**):**



The commercially available 2-bromobenzo[*d*]thiazole-6-carboxylic acid **34** (1.0 eq., 65.0 mg, 0.3 mmol), 3-isopropyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (1.5 eq., 89.2 mg, 0.4 mmol), Pd(dppf)Cl₂ (0.2 eq., 41.1 mg, 0.1 mmol) and potassium phosphate (3.0 eq., 173.8 mg, 0.8 mmol) in dioxane/H₂O (5:1) were used following the general procedure XII for 1 h at 120 °C in the microwave to yield the product as a white solid (25.5 mg, 32 %).

TLC-MS (APCI+): Calculated: 287.07 for C₁₄H₁₃N₃O₂S [M+H⁺]. Found: 288.2.

Synthesis of *N*-(3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylphenyl)-2-(3-isopropyl-1*H*-pyrazol-4-yl)benzo[*d*]thiazole-5-carboxamide (64**):**



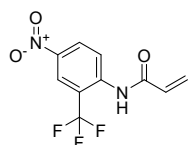
2-(3-isopropyl-1*H*-pyrazol-4-yl)benzo[*d*]thiazole-5-carboxylic acid **36** (1.1 eq., 20.0 mg, 0.07 mmol), DIPEA (3.0 eq., 0.03 mL), HATU (1.3 eq., 31.3 mg, 0.08 mmol) and **33** (1.0 eq., 15.7 mg, 0.06 mmol) were used following the general procedure VI to yield the product as a white solid (23.1 mg, 71 %).

¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.42 (d, *J*=6.36 Hz, 6 H) 4.55 (quin, *J*=6.60 Hz, 1 H) 7.27 - 7.33 (m, 2 H) 7.68 (dd, *J*=8.31, 1.96 Hz, 1 H) 7.95 - 8.04 (m, 3 H) 8.06 (s, 1 H) 8.15 (s, 1 H) 8.18 (dd, *J*=9.29, 1.47 Hz, 1 H) 8.58 (s, 1 H) 8.63 - 8.66 (m, 2 H) 10.38 (s, 1 H). The DMSO peak covered the protons of the methyl group.

¹³C NMR (176 MHz, DMSO-*d*₆) δ 20.21, 22.95, 31.75, 80.58, 97.60, 115.20, 116.69, 116.91, 118.61, 119.41, 121.92, 122.08, 122.55, 123.01, 126.60, 129.17, 130.53, 131.44, 134.20, 135.03, 137.72, 138.06, 145.49, 155.91, 158.48, 158.66, 163.69, 165.49.

LC-MS (ESI-MS): Calculated: 517.17 for C₂₉H₂₃N₇OS [M+H⁺]. Found: 518.2.

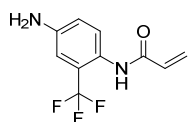
Synthesis of *N*-(4-nitro-2-(trifluoromethyl)phenyl)acrylamide (31**):**



(1.3 eq., 0.16 mL, 1.9 mmol), DIPEA (4.0 eq., 0.9 mL, 6.1 mmol) and *N*-(4-nitro-2-(trifluoromethyl)phenyl)acrylamide (1.0 eq., 316.9 mg, 1.5 mmol) were used following the general procedure VII in DCM/DMF at 50 °C for 48 h to yield the product as a pale-yellow solid (184.4 mg, 47 %).

LC-MS (ESI-MS): Calculated: 260.04 for C₁₀H₇F₃N₂O₃ [M+H⁺]. Found: 261.9.

Synthesis of *N*-(4-amino-2-(trifluoromethyl)phenyl)acrylamide (32**):**

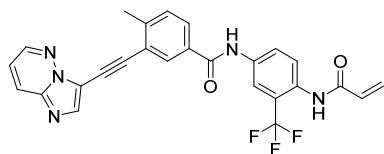


N-(4-nitro-2-(trifluoromethyl)phenyl)acrylamide (1.0 eq., 600.0 mg, 2.3 mmol), NH₄Cl (10.0 eq., 1233.6 mg, 23.6 mmol) and iron (5.0 eq., 643.9 mg, 11.5 mmol) were used following the general procedure XI for 2.5 h at 60 °C in an ultrasonic bath to yield the product as a white crystalline solid (183.0 mg, 35 %).

¹H NMR (500 MHz, DMSO-*d*₆) δ ppm 5.58 (br. s., 2 H) 5.69 (dd, *J*=10.38, 2.14 Hz, 1 H) 6.17 (dd, *J*=17.09, 1.83 Hz, 1 H) 6.43 (dd, *J*=17.09, 10.38 Hz, 1 H) 6.77 (dd, *J*=8.54, 2.44 Hz, 1 H) 6.87 (d, *J*=2.75 Hz, 1 H) 7.03 (d, *J*=8.54 Hz, 1 H) 9.38 (s, 1 H).

¹³C NMR (126 MHz, DMSO-*d*₆) δ 110.6, 117.5, 122.6, 123.1, 125.3, 126.8, 131.9, 148.2, 165.0.

Synthesis of *N*-(4-acrylamido-3-(trifluoromethyl)phenyl)-3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylbenzamide (27):



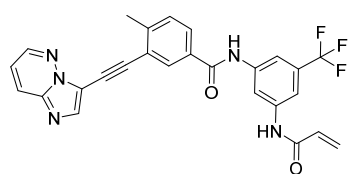
30 (1.0 eq., 30.1 mg, 0.11 mmol), DIPEA (3.0 eq., 0.06 mL, 0.33 mmol), HATU (1.0 eq., 41.3 mg, 0.11 mmol) and **32** (1.0 eq., 25.0 mg, 0.11 mmol) were used following the general procedure XI at 45 °C to yield the product as an off-white solid (18.3 mg, 34 %).

¹H NMR (700 MHz, DMSO-*d*₆) δ ppm 2.62 (s, 3 H) 5.76 - 5.80 (m, 1 H) 6.25 (dd, *J*=17.10, 1.83 Hz, 1 H) 6.53 (dd, *J*=16.56, 10.54 Hz, 1 H) 7.41 (dd, *J*=9.14, 4.41 Hz, 1 H) 7.52 (d, *J*=8.60 Hz, 1 H) 7.57 (d, *J*=8.17 Hz, 1 H) 7.97 (dd, *J*=8.07, 1.83 Hz, 1 H) 8.10 (dd, *J*=8.71, 2.26 Hz, 1 H) 8.24 (d, *J*=1.72 Hz, 1 H) 8.25 (s, 1 H) 8.26 - 8.28 (m, 2 H) 8.74 (dd, *J*=4.52, 1.51 Hz, 1 H) 9.74 (s, 1 H) 10.63 (s, 1 H).

¹³C NMR (176 MHz, DMSO-*d*₆) δ 19.32, 81.65, 96.88, 112.17, 118.12, 119.58, 122.28, 123.09, 124.56, 124.65, 126.60, 127.64, 129.02, 129.27, 130.60, 130.65, 131.36, 132.62, 136.16, 138.15, 138.75, 140.16, 144.06, 145.56, 162.78, 165.11.

LC-MS (ESI-MS): Calculated: 489.14 for C₂₆H₁₈F₃N₅O₂ [M+H⁺]. Found: 490.3.

Synthesis of *N*-(3-acrylamido-5-(trifluoromethyl)phenyl)-3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylbenzamide (66):



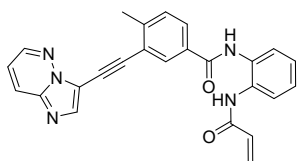
30 (25 mg, 0.09 mmol) and *N*-(3-amino-5-(trifluoromethyl)phenyl)acrylamide (21 mg, 0.09 mmol) were used following the general procedure VI to yield the product as a pale yellow solid (30 mg, 68 %).

¹H NMR (700 MHz, DMSO) δ 2.62 (s, 3H), 5.82 (dd, *J* = 10.2, 1.8 Hz, 1H), 6.32 (dd, *J* = 17.0, 1.8 Hz, 1H), 6.50 (dd, *J* = 17.0, 10.2 Hz, 1H), 7.40 (dd, *J* = 9.2, 4.4 Hz, 1H), 7.56 (d, *J* = 8.1 Hz, 1H), 7.94 (s, 1H), 7.91 (s, 1H), 7.97 (dd, *J* = 8.0, 1.9 Hz, 1H), 8.24 (d, *J* = 1.9 Hz, 1H), 8.25 (s, 1H), 8.27 (dd, *J* = 9.2, 1.5 Hz, 1H), 8.50 (s, 1H), 8.74 (dd, *J* = 4.4, 1.5 Hz, 1H), 10.63 (s, 1H), 10.68 (s, 1H).

¹³C NMR (176 MHz, DMSO) δ 20.88, 81.58, 96.89, 111.10, 112.03, 112.18, 114.54, 119.57, 122.20, 123.69, 125.28 (s), 126.59, 128.10, 129.06, 130.54, 130.74, 132.01, 132.67, 138.74, 140.14, 140.69, 140.88, 144.03, 145.55, 164.10, 165.24.

LC-MS (ESI-MS): Calculated: 489.46 for C₂₆H₁₈N₅F₃O₂ [M-H⁺]. Found: 488.1.

Synthesis of *N*-(2-acrylamidophenyl)-3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylbenzamide (**67**):



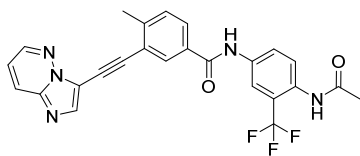
30 (50 mg, 0.18 mmol) and *N*-(2-aminophenyl)acrylamide (35 mg, 0.22 mmol) were used following the general procedure VI to yield the product as an off-white solid (58 mg, 76 %).

¹H NMR (500 MHz, DMSO) δ 2.61 (s, 1H), 5.78 (dd, J = 10.2, 1.6 Hz, 1H), 6.32 (dd, J = 17.0, 1.6 Hz, 1H), 6.55 (dd, J = 17.0, 10.2 Hz, 1H), 7.27 – 7.23 (m, 1H), 7.40 (dd, J = 9.2, 4.4 Hz, 1H), 7.54 (d, J = 8.1 Hz, 1H), 7.64 – 7.60 (m, 1H), 7.72 – 7.67 (m, 1H), 7.92 (dd, J = 8.0, 1.5 Hz, 1H), 8.17 (d, J = 1.3 Hz, 1H), 8.24 (s, 1H), 8.27 (dd, J = 9.2, 1.1 Hz, 1H), 8.73 (dd, J = 4.4, 1.2 Hz, 1H), 9.87 (s, 1H), 10.02 (s, 1H).

¹³C NMR (126 MHz, DMSO) δ 20.85, 81.49, 96.88, 119.55, 122.25, 124.84, 125.54, 126.13, 126.58, 126.97, 127.74, 128.71, 130.49 – 130.39 (m), 131.05 – 130.49 (m), 132.09, 132.78, 138.72, 140.14, 143.71, 145.54, 164.24, 164.67.

LC-MS (ESI-MS): Calculated: 421.46 for C₂₅H₁₉N₅O₂ [M+H⁺]. Found: 421.9.

Synthesis of *N*-(4-acetamido-3-(trifluoromethyl)phenyl)-3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylbenzamide (**68**):



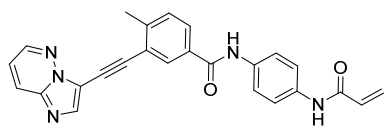
30 (40 mg, 0.14 mmol) and *N*-(4-amino-2-(trifluoromethyl)phenyl)acetamide (34 mg, 0.16 mmol) were used following the general procedure VI to yield the product as a pale-yellow solid (41 mg, 60 %).

¹H NMR (600 MHz, DMSO) δ 2.05 (s, 3H), 2.62 (s, 3H), 7.40 (dd, J = 9.2, 4.4 Hz, 1 H), 7.45 (d, J = 8.7 Hz, 1 H), 7.56 (d, J = 8.1 Hz, 1 H), 7.98 (dd, J = 8.0, 1.9 Hz, 1 H), 8.08 (d, J = 7.3 Hz, 1 H), 8.23-8.26 (m, 3H), 8.27 (dd, J = 9.2, 1.5 Hz, 1 H), 8.74 (dd, J = 4.4, 1.5 Hz, 1 H), 9.57 (s, 1 H), 10.69 (s, 1 H).

¹³C NMR (151 MHz, DMSO) δ 20.88, 23.30, 81.61, 96.89, 112.18, 118.08 (d, J = 5.3 Hz), 119.58, 122.25, 122.99, 124.69 (d, J = 35.6 Hz), 125.03 – 124.84 (m), 126.59, 129.03, 130.62 (d, J = 16.8 Hz), 131.32 (d, J = 42.9 Hz), 131.87 – 131.58 (m), 132.63, 137.96, 138.73, 140.14, 144.00, 145.56, 165.09, 169.78.

LC-MS (ESI-MS): Calculated: 477.44 for C₂₅H₁₈F₃N₅O₂ [M-H⁺]. Found: 476.1.

Synthesis of *N*-(4-acrylamidophenyl)-3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylbenzamide (69):



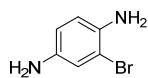
30 (45 mg, 0.2 mmol) and 4-acrylamido aniline (28 mg) were used following the general procedure VI to yield the product as a pale-yellow solid (31 mg, 73 %).

¹H NMR (600 MHz, DMSO) δ 2.61 (s, 3H), 7.40 (dd, J = 9.2, 4.4 Hz, 1H), 7.54 (d, J = 8.1 Hz, 1H), 7.66 (d, J = 9.0 Hz, 2H), 7.78 – 7.73 (m, 2H), 7.94 (dd, J = 8.0, 1.8 Hz, 1H), 8.20 (d, J = 1.8 Hz, 1H), 8.24 (s, 1H), 8.27 (dd, J = 9.2, 1.5 Hz, 1H), 8.73 (dd, J = 4.4, 1.5 Hz, 1H), 10.15 (s, 1H), 10.31 (s, 1H).

¹³C NMR (151 MHz, DMSO) δ 20.84, 81.45, 96.99, 112.22, 119.54, 120.03, 121.34, 122.16, 126.58, 127.09, 128.87, 130.45, 130.60, 132.39, 133.19, 135.18, 135.39, 138.71, 140.12, 143.54, 145.53, 163.38, 164.54.

LC-MS (ESI-MS): Calculated: 421.46 for C₂₅H₁₉N₅O₂ [M+H⁺]. Found: 422.2.

Synthesis of 2-bromobenzene-1,4-diamine (39):

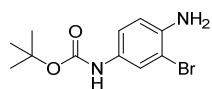


The reaction was performed according to general procedure XI with the commercially available 2-bromo-4-nitroaniline **37** (1.0 eq., 1.0 g, 4.6 mmol), iron powder (5.0 eq., 1.3 g, 23.0 mmol) and NH₄Cl (10 eq., 2.5 g, 46.08 mmol) to yield the product as beige crystals (712 mg, 83 %).

¹H-NMR (600 MHz DMSO-*d*₆) δ ppm 4.42 (d, J = 62.5 Hz, 4H), 6.40 (dd, J = 8.4, 2.5 Hz, 1H), 6.57 (d, J = 8.4 Hz, 1H), 6.66 (d, J = 2.5 Hz, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 108.82, 115.38, 116.85, 117.32, 135.85, 140.59.

LC-MS (ESI): Calculated: 185.98 for C₆H₇BrN₂ [M+H⁺]. Found: 188.9.

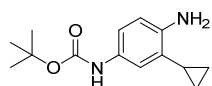
Synthesis of *tert*-butyl (4-amino-3-bromophenyl)carbamate (40):

The reaction was performed according to general procedure X with 2-bromobenzene-1,4-diamine **38** (1.0 eq. 700 mg, 3.7 mmol), TEA (1.1 eq., 0.2 mL, 1.29 mmol) and di-*tert*-butyl dicarbonate (1.0 eq., 0.6 mL, 3.7 mmol) to yield the product as a white solid (704.0 mg, 65 %).

¹H-NMR (500 MHz DMSO-*d*₆) δ ppm 1.44 (s, 9H), 4.94 (s, 2H), 6.70 (d, *J* = 8.7 Hz, 1H), 7.08 (d, *J* = 9.5 Hz, 1H), 7.53 (s, 1H), 9.02 (s, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 28.15, 78.67, 107.09, 115.33, 119.40, 122.13, 129.80, 140.97, 152.88.

LC-MS (ESI): Calculated: 286.03 for C₁₁H₁₅BrN₂O₂ [M+H⁺]. Found: 287.0.

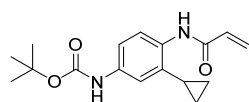
Synthesis of *tert*-butyl (4-amino-3-cyclopropylphenyl)carbamate (43):

The reaction was performed according to general procedure X with *tert*-butyl (4-amino-3-bromophenyl)carbamate **41** (1.0 eq., 20 mg, 0.07 mmol), 2-cyclopropyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (5.0 eq., 83 μ L, 0.35 mmol), CsCO₃ (3.0 eq., 68.1 mg, 0.21 mmol) and Pd(PPh₃)₄ (0.15 eq., 12.1 mg, 0.01 mmol) to yield the product as a brown oil (3.3 mg, 18 %).

¹H-NMR (700 MHz DMSO-*d*₆) δ ppm 0.44 – 0.39 (m, 2H), 0.87 – 0.80 (m, 2H), 1.43 (s, 9H), 1.69 – 1.62 (m, 1H), 4.81 (s, 2H), 6.51 (d, *J* = 8.5 Hz, 1H), 6.93 (d, *J* = 34.5 Hz, 2H), 8.72 (s, 1H).

¹³C-NMR (176 MHz, DMSO-*d*₆) δ 5.83, 11.09, 28.23, 78.11, 113.97, 117.69, 117.83, 126.03, 128.96, 142.51, 153.04.

LC-MS (ESI): Calculated: 248.15 for C₁₄H₂₀N₂O₂ [M+H⁺]. Found: 249.1.

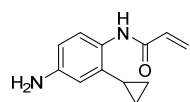
Synthesis of *tert*-butyl (4-acrylamido-3-cyclopropylphenyl)carbamate (46**):**

The reaction was performed according to general procedure VII with *tert*-butyl (4-amino-3-cyclopropylphenyl)carbamate **43** (1.0 eq., 20 mg, 0.08 mmol), DIPEA (3.0 eq., 44 μ L, 0.24 mmol) and acryloyl chloride (1.1 eq., 7.2 μ L, 0.09 mmol) yielding the product as a orange solid (19 mg, 78 %).

$^1\text{H-NMR}$ (600 MHz DMSO- d_6) ppm 0.51 (m, 2H), 0.91 (m, 2H), 1.46 (s, 9H), 1.94 (m, 1H), 5.71 (dd, J = 10.2, 2.0 Hz, 1H), 6.22 (dd, J = 17.1, 2.1 Hz, 1H), 6.53 (dd, J = 17.0, 10.2 Hz, 1H), 7.06 (s, 1H), 7.21 (d, J = 8.9 Hz, 1H), 7.30 (d, J = 8.6 Hz, 1H), 9.21 (s, 1H), 9.46 (s, 1H).

$^{13}\text{C-NMR}$ (151 MHz, DMSO- d_6) δ 7.95, 11.02, 28.13, 78.93, 114.60, 115.46, 125.63, 126.14, 130.84, 131.93, 137.13, 137.41, 152.75, 163.49.

LC-MS (ESI): Calculated: 302.16 for $\text{C}_{17}\text{H}_{22}\text{N}_2\text{O}_2$ $[\text{M}+\text{H}^+]$. Found: 303.2.

Synthesis of *N*-(4-amino-2-cyclopropylphenyl)acrylamide (50**) :**

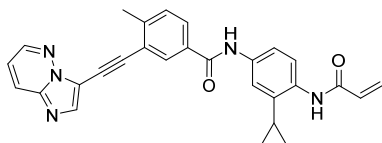
The reaction was performed according to general procedure XI with *tert*-butyl (4-acrylamido-3-cyclopropylphenyl)carbamate **46** (1.0 eq., 50 mg, 0.17 mmol) yielding the product as an orange solid (29.3 mg, 87 %).

$^1\text{H-NMR}$ (600 MHz DMSO- d_6) δ ppm 0.52 – 0.45 (m, 2H), 0.89 – 0.80 (m, 2H), 1.84 (m, 1H), 4.90 (s, 2H), 5.66 (dd, J = 10.2, 2.1 Hz, 1H), 6.12 (d, J = 2.5 Hz, 1H), 6.17 (dd, J = 17.1, 2.1 Hz, 1H), 6.34 (dd, J = 8.4, 2.5 Hz, 1H), 6.48 (dd, J = 17.1, 10.2 Hz, 1H), 6.97 (d, J = 8.3 Hz, 1H), 9.26 (s, 1H).

$^{13}\text{C-NMR}$ (151 MHz, DMSO- d_6) δ 8.33, 11.35, 110.01, 111.61, 125.72, 125.93, 127.21, 132.66, 138.75, 147.29, 164.03.

LC-MS (ESI): Calculated: 202.11 for $\text{C}_{12}\text{H}_{14}\text{N}_2\text{O}$ $[\text{M}+\text{H}^+]$. Found: 203.0.

Synthesis of *N*-(4-acrylamido-3-cyclopropylphenyl)-3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylbenzamide (70**):**



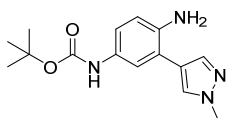
The reaction was performed according to general procedure VI for nine days at rt with **30** (1.5 eq., 55.4 mg, 0.2 mmol), DIPEA (15.0 eq., 369.0 μ L, 2.0 mmol), HATU (6.0 eq., 155.0 mg, 0.8 mmol) and *N*-(4-amino-2-cyclopropylphenyl)acrylamide **50** (1.0 eq., 27.0 mg, 0.13 mmol) yielding the product as a white solid (4 mg, 7 %).

¹H-NMR (700 MHz DMSO-*d*₆) δ ppm 0.62 – 0.57 (m, 2H), 0.98 – 0.94 (m, 2H), 2.04 – 1.99 (m, 1H), 2.61 (s, 3H), 5.77 – 5.72 (m, 1H), 6.25 (dd, *J* = 17.0, 2.0 Hz, 1H), 6.60 – 6.54 (m, 1H), 7.37 (d, *J* = 2.5 Hz, 1H), 7.40 (dd, *J* = 9.2, 4.4 Hz, 1H), 7.46 (d, *J* = 8.7 Hz, 1H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.65 (dd, *J* = 8.6, 2.5 Hz, 1H), 7.94 (dd, *J* = 8.0, 2.0 Hz, 1H), 8.19 (d, *J* = 2.0 Hz, 1H), 8.24 (s, 1H), 8.27 (dd, *J* = 9.2, 1.6 Hz, 1H), 8.73 (dd, *J* = 4.4, 1.6 Hz, 1H), 9.56 (s, 1H), 10.23 (s, 1H).

¹³C-NMR (176 MHz, DMSO-*d*₆) δ 8.56, 11.50, 20.85, 81.52, 96.98, 112.21, 117.44, 118.15, 119.56, 122.16, 125.81, 126.59, 126.82, 128.90, 130.48, 130.54, 132.39, 132.80, 133.12, 137.08, 137.66, 138.71, 140.13, 143.62, 145.55, 164.01, 164.56.

LC-MS (ESI): Calculated: 461.19 for C₂₈H₂₃N₅O₂ [M+H⁺]. Found: 462.2.

Synthesis of *tert*-butyl (4-amino-3-(1-methyl-1H-pyrazol-4-yl)phenyl)carbamate (44**):**



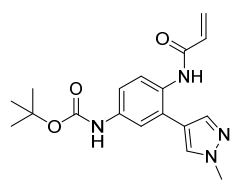
The reaction was performed according to general procedure XII with *tert*-butyl (4-amino-3-bromophenyl)carbamate **41** (1.0 eq., 125 mg, 0.4 mmol), 1-Methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazol

(1.5 eq., 135.6 mg, 0.65 mmol), CsCO₃ (3.0 eq., 425.7 mg, 1.3 mmol) and Pd(PPh₃)₄ (0.15 eq., 75.5 mg, 0.06 mmol) yielding the product as a orange/brown solid (101.1 mg, 81 %).

¹H-NMR (600 MHz DMSO-*d*₆) δ ppm 1.44 (s, 9H), 3.87 (s, 3H), 4.56 (s, 2H), 6.64 (d, *J* = 8.6 Hz, 1H), 7.01 (s, 1H), 7.23 (s, 1H), 7.56 (d, *J* = 0.8 Hz, 1H), 7.88 (s, 1H), 8.84 (s, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 28.22, 38.53, 115.73, 117.56, 119.32, 128.75, 129.53, 137.17, 140.18, 153.05.

LC-MS (ESI): Calculated: 288.16 for C₁₅H₂₀N₄O₂ [M+H⁺]. Found: 289.2.

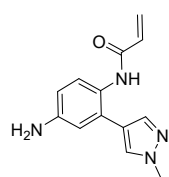
Synthesis of *tert*-butyl (4-acrylamido-3-(1-methyl-1H-pyrazol-4-yl)phenyl)carbamate (47**):**

The reaction was performed according to general procedure VII with *tert*-butyl (4-amino-3-(1-methyl-1H-pyrazol-4-yl)phenyl)carbamate **44** (1.0 eq., 91.1 mg, 0.32 mmol), DIPEA (3.0 eq., 174.9 μ L, 0.95 mmol) and acryloyl chloride (1.1 eq., 28.1 μ L, 0.35 mmol) yielding the product as a yellow solid (19 mg, 78 %).

¹H-NMR (600 MHz DMSO-*d*₆) δ ppm 1.48 (s, 9H), 3.86 (s, 3H), 5.71 (dd, *J* = 10.2, 2.0 Hz, 1H), 6.19 (dd, *J* = 17.1, 2.1 Hz, 1H), 6.45 (dd, *J* = 17.0, 10.2 Hz, 1H), 7.33 – 7.24 (m, 2H), 7.55 – 7.52 (m, 1H), 7.58 (s, 1H), 7.83 (d, *J* = 0.7 Hz, 1H), 9.35 (s, 1H), 9.44 (s, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 28.12, 38.57, 79.07, 116.55, 117.88, 118.87, 126.28, 127.95, 128.01, 128.72, 129.20, 131.80, 137.37, 137.58, 152.79, 163.93.

LC-MS (ESI): Calculated: 342.17 for C₁₈H₂₂N₄O₃ [M+H⁺]. Found: 343.2.

Synthesis of *N*-(4-amino-2-(1-methyl-1H-pyrazol-4-yl)phenyl)acrylamide (51**):**

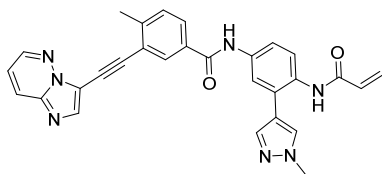
The reaction was performed according to general procedure VII with *tert*-butyl (4-acrylamido-3-(1-methyl-1H-pyrazol-4-yl)phenyl)carbamate **47** (1.0 eq., 75 mg, 0.22 mmol) yielding the product (37.4 mg, 71 %) as a beige solid.

¹H-NMR (600 MHz DMSO-*d*₆) δ ppm 3.84 (s, 3H), 5.07 (s, 2H), 5.67 (dd, *J* = 10.2, 2.1 Hz, 1H), 6.16 (dd, *J* = 17.1, 2.1 Hz, 1H), 6.43 – 6.38 (m, 1H), 6.45 (dd, *J* = 8.3, 2.5 Hz, 1H), 6.64 (d, *J* = 2.6 Hz, 1H), 6.99 (d, *J* = 8.4 Hz, 1H), 7.52 (d, *J* = 0.8 Hz, 1H), 7.76 (d, *J* = 0.8 Hz, 1H), 9.24 (s, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 38.53, 112.34, 112.99, 119.52, 122.36, 125.70, 128.87, 129.40, 132.04, 137.33, 147.12, 164.06.

LC-MS (ESI): Calculated: 242.12 for C₁₃H₁₄N₄O [M+H⁺]. Found: 243.1.

Synthesis of *N*-(4-acrylamido-3-(1-methyl-1*H*-pyrazol-4-yl)phenyl)-3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylbenzamide (71):



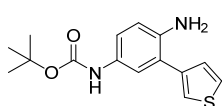
The reaction was performed according to general procedure VI with **30** (1.2 eq., 22.0 mg, 0.08 mmol), DIPEA (8.0 eq., 97.7 μ L, 0.5 mmol), HATU (8.0 eq., 201.1 mg, 0.5 mmol) and *N*-(4-amino-2-(1-methyl-1*H*-pyrazol-4-yl)phenyl)acrylamide **51** (1.0 eq., 16.0 mg, 0.07 mmol) yielding the product as a white solid (20.2 mg, 61 %).

¹H-NMR (700 MHz DMSO-*d*₆) δ ppm 2.61 (s, 3H), 3.88 (s, 3H), 5.74 (dd, *J* = 10.2, 2.0 Hz, 1H), 6.23 (dd, *J* = 17.0, 2.0 Hz, 1H), 6.50 (dd, *J* = 17.1, 10.2 Hz, 1H), 7.40 (dd, *J* = 9.2, 4.4 Hz, 1H), 7.45 (d, *J* = 8.6 Hz, 1H), 7.55 (d, *J* = 8.1 Hz, 1H), 7.62 (s, 1H), 7.68 (dd, *J* = 8.6, 2.5 Hz, 1H), 7.90 (s, 1H), 7.91 (d, *J* = 2.5 Hz, 1H), 7.96 (dd, *J* = 8.0, 2.0 Hz, 1H), 8.22 (d, *J* = 2.0 Hz, 1H), 8.24 (s, 1H), 8.27 (dd, *J* = 9.2, 1.6 Hz, 1H), 8.73 (dd, *J* = 4.4, 1.5 Hz, 1H), 9.55 (s, 1H), 10.37 (s, 1H).

¹³C-NMR (176 MHz, DMSO-*d*₆) δ 19.73, 37.97, 80.41, 95.84, 111.07, 118.07, 118.42, 119.68, 121.06, 125.46, 125.80, 127.05, 127.81, 128.66, 128.82, 129.38, 129.47, 131.15, 131.94, 136.37, 136.78, 137.58, 139.00, 142.56, 144.41, 163.28, 163.58.

LC-MS (ESI): Calculated: 501.19 for C₂₉H₂₃N₇O₂ [M+H⁺]. Found: 502.2.

Synthesis of *tert*-butyl (4-amino-3-(thiophen-3-yl)phenyl)carbamate (45):

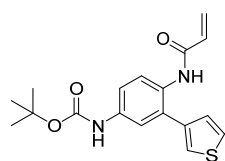


The reaction was performed according to general procedure XII with *tert*-butyl (4-amino-3-bromophenyl)carbamate **41** (1.0 eq., 125 mg, 0.4 mmol), thiophen-3-ylboronic acid (2.0 eq., 111.4 mg, 0.87 mmol), CsCO₃ (3.0 eq., 425.7 mg, 1.3 mmol) and Pd(PPh₃)₄ (0.15 eq., 75.5 mg, 0.06 mmol) yielding the product as a brown solid (117.1 mg, 93 %).

¹H-NMR (600 MHz DMSO-*d*₆) δ ppm 1.46 (s, 9H), 7.13 (d, *J* = 8.7 Hz, 1H), 7.29 (dd, *J* = 4.9, 1.4 Hz, 1H), 7.37 (d, *J* = 7.6 Hz, 1H), 7.49 (s, 1H), 7.67 (dd, *J* = 2.9, 1.3 Hz, 1H), 7.72 (dd, *J* = 5.0, 2.9 Hz, 1H), 9.37 (s, 1H). Primary amine not resolved.

¹³C NMR (126 MHz, DMSO-*d*₆) δ 28.58 79.64 115.81 118.17 118.87 120.66 122.00 124.36 127.61 128.65 138.28 153.31 158.34.

LC-MS (ESI): Calculated: 290.11 for C₁₅H₁₈N₂O₂S [M+H⁺]. Found: 291.1.

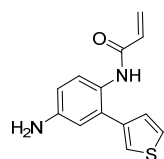
Synthesis of *tert*-butyl (4-acrylamido-3-(thiophen-3-yl)phenyl)carbamate (48**):**

The reaction was performed according to general procedure VII with *tert*-butyl (4-amino-3-(thiophen-3-yl)phenyl)carbamate **45** (1.0 eq., 130 mg, 0.45 mmol), DIPEA (3.0 eq., 247.5 μ L, 1.34 mmol) and acryloyl chloride (1.1 eq., 40.2 μ L, 0.49 mmol) yielding the product as a yellow solid (89.6 mg, 58 %).

$^1\text{H-NMR}$ (600 MHz DMSO- d_6) δ ppm 1.48 (s, 9H), 5.67 (dd, J = 10.3, 2.0 Hz, 1H), 6.16 (dd, J = 17.1, 2.1 Hz, 1H), 6.37 (dd, J = 17.0, 10.2 Hz, 1H), 7.16 (dd, J = 5.0, 1.3 Hz, 1H), 7.40 – 7.31 (m, 2H), 7.52 (dd, J = 2.9, 1.3 Hz, 1H), 7.58 (s, 1H), 7.60 (dd, J = 5.0, 3.0 Hz, 1H), 9.41 (s, 1H), 9.43 (s, 1H).

$^{13}\text{C-NMR}$ (151 MHz, DMSO- d_6) δ 28.11, 79.15, 117.38, 119.15, 123.26, 126.16, 126.25, 127.98, 128.49, 131.70, 132.16, 137.56, 139.15, 152.80, 163.96.

LC-MS (ESI): Calculated: 344.12 for $\text{C}_{18}\text{H}_{20}\text{N}_2\text{O}_3\text{S}$ [$\text{M}+\text{H}^+$]. Found: 345.0.

Synthesis of *N*-(4-amino-2-(thiophen-3-yl)phenyl)acrylamide (52**):**

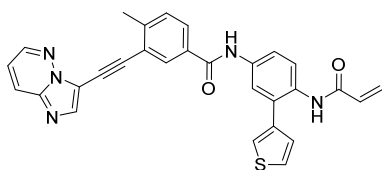
The reaction was performed according to general procedure XI with *tert*-butyl (4-acrylamido-3-(thiophen-3-yl)phenyl)carbamate **48** (1.0 eq., 80.0 mg, 0.23 mmol) yielding the product as a yellow solid (35.0 mg, 63 %).

$^1\text{H-NMR}$ (600 MHz DMSO- d_6) δ ppm 5.12 (s, 2H), 5.62 (dd, J = 10.2, 2.2 Hz, 1H), 6.12 (dd, J = 17.1, 2.1 Hz, 1H), 6.32 (dd, J = 17.1, 10.2 Hz, 1H), 6.52 (dd, J = 8.4, 2.6 Hz, 1H), 6.64 (d, J = 2.6 Hz, 1H), 7.00 (d, J = 8.4 Hz, 1H), 7.15 (dd, J = 5.0, 1.3 Hz, 1H), 7.46 – 7.43 (m, 1H), 7.55 (dd, J = 5.0, 2.9 Hz, 1H), 9.22 (s, 1H).

$^{13}\text{C-NMR}$ (151 MHz, DMSO- d_6) δ 113.09, 114.39, 122.47, 122.73, 125.63, 125.65, 128.10, 128.98, 131.95, 132.99, 139.96, 147.21, 164.15.

LC-MS (ESI): Calculated: 244.07 for $\text{C}_{13}\text{H}_{12}\text{N}_2\text{OS}$ [$\text{M}+\text{H}^+$]. Found: 245.0.

Synthesis of *N*-(4-acrylamido-3-(thiophen-3-yl)phenyl)-3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylbenzamide (72):



30 (1.5 eq., 56.2 mg, 0.20 mmol), DIPEA (9.0 eq., 224.4 μ L, 1.23 mmol), HATU (4.3 eq., 220.8 mg, 0.68 mmol) and *N*-(4-amino-2-(thiophen-3-yl)phenyl)acrylamide (1.0 eq., 33.0 mg, 0.14 mmol)) were used following the general method VI for 6

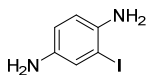
d at rt to yield the product as a yellow solid (17.7 mg, 26 %).

¹H-NMR (600 MHz DMSO-*d*₆) δ ppm 2.61 (s, 3H), 5.70 (dd, *J* = 10.3, 2.0 Hz, 1H), 6.19 (dd, *J* = 17.1, 2.0 Hz, 1H), 6.41 (dd, *J* = 17.1, 10.2 Hz, 1H), 7.24 (dd, *J* = 5.0, 1.3 Hz, 1H), 7.40 (dd, *J* = 9.2, 4.4 Hz, 1H), 7.49 (d, *J* = 8.6 Hz, 1H), 7.55 (d, *J* = 8.1 Hz, 1H), 7.60 (dd, *J* = 2.9, 1.3 Hz, 1H), 7.64 (dd, *J* = 5.0, 3.0 Hz, 1H), 7.92 (d, *J* = 2.5 Hz, 1H), 7.96 (dd, *J* = 8.0, 2.0 Hz, 1H), 8.22 (d, *J* = 1.9 Hz, 1H), 8.24 (s, 1H), 8.26 (dd, *J* = 9.2, 1.6 Hz, 1H), 8.73 (dd, *J* = 4.4, 1.6 Hz, 1H), 10.40 (s, 1H), 9.53 (s, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 20.38, 81.07, 96.48, 111.72, 119.08, 119.52, 121.57, 121.73, 123.41, 126.11, 126.30, 126.43, 127.71, 127.98, 128.46, 129.99, 130.04, 130.12, 131.69, 131.84, 132.54, 137.00, 138.24, 138.97, 139.65, 143.25, 145.07, 163.95, 164.27.

LC-MS (ESI): Calculated: 503.14 for C₂₉H₂₁N₅O₂S [M+H⁺]. Found: 504.2.

Synthesis of 2-iodobenzene-1,4-diamine (40):

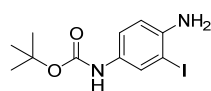


The reaction was performed according to general procedure IX with the commercially available 2-iodo-4-nitroaniline **38** (1.0 eq., 500.0 mg, 1.89 mmol), iron powder (5.0 eq., 528.8 mg, 9.50 mmol) and NH₄Cl (10.0 eq., 1013.0 mg, 18.94 mmol) yielding the product as a yellow solid (296.5 mg, 67 %).

¹H-NMR (500 MHz DMSO-*d*₆) δ ppm 4.37 (d, *J* = 46.1 Hz, 4H), 6.43 (dd, *J* = 8.4, 2.5 Hz, 1H), 6.55 (d, *J* = 8.5 Hz, 1H), 6.90 (d, *J* = 2.5 Hz, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 85.45, 116.18, 116.76, 123.89, 139.25, 141.39.

LC-MS (ESI): Calculated: 233.97 for C₆H₇IN₂ [M+H⁺]. Found: 234.9.

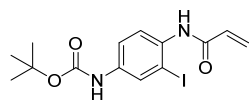
Synthesis of *tert*-butyl (4-amino-3-iodophenyl)carbamate (42**):**

The reaction was performed according to general procedure X with 2-iodobenzene-1,4-diamine **40** (1.0 eq. 275 mg, 1.18 mmol), TEA (1.1 eq., 0.2 mL, 1.29 mmol) and di-*tert*-butyl dicarbonate (1.0 eq., 0.3 mL, 1.18 mmol) yielding the product as light brown crystals (286.5 mg, 73 %).

¹H-NMR (500 MHz DMSO-*d*₆) δ ppm 1.44 (s, 9H), 4.86 (s, 2H), 6.66 (d, *J* = 8.6 Hz, 1H), 7.12 – 7.07 (m, 1H), 7.72 (s, 1H), 8.96 (s, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 28.64, 79.11, 83.28, 114.61, 120.80, 128.78, 130.60, 144.32, 153.36.

LC-MS (ESI): Calculated: 334.02 for C₁₁H₁₅IN₂O₂ [M+H⁺]. Found: 335.0.

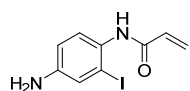
Synthesis of *tert*-butyl (4-acrylamido-3-iodophenyl)carbamate (49**):**

The reaction was performed according to general procedure VII for 90 min at rt with *tert*-butyl (4-amino-3-iodophenyl)carbamate **42** (1.0 eq., 150.0 mg, 0.45 mmol), DIPEA (3.0 eq., 248.7 μ L, 1.35 mmol) and acryloyl chloride (1.1 eq., 39.6 μ L, 0.49 mmol) yielding the product as light brown crystals (145.8 mg, 84 %).

¹H-NMR (600 MHz DMSO-*d*₆) δ ppm 1.47 (s, 9H), 5.74 (dd, *J* = 10.2, 2.0 Hz, 1H), 6.22 (dd, *J* = 17.1, 2.0 Hz, 1H), 6.47 (q, *J* = 10.2 Hz, 1H), 7.27 (d, *J* = 8.7 Hz, 1H), 7.39 (dd, *J* = 8.7, 2.4 Hz, 1H), 9.55 (s, 1H), 9.49 (s, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 28.06, 79.50, 97.02, 118.23, 126.77, 127.39, 127.57, 131.55, 133.42, 138.53, 152.62, 163.46.

LC-MS (ESI): Calculated: 388.03 for C₁₄H₁₇IN₂O₃ [M+H⁺]. Found: 389.0.

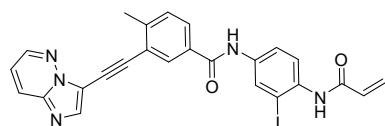
Synthesis of *N*-(4-amino-2-iodophenyl)acrylamide (53):

The reaction was performed according to general procedure XI with (4-acrylamido-3-iodophenyl)carbamate **49** (1.0 eq., 145.8 mg, 0.37 mmol) yielding the product as beige crystals (87.6 mg, 81 %).

¹H-NMR (600 MHz DMSO-*d*₆) δ ppm 5.27 (s, 2H), 5.69 (dd, *J* = 10.2, 2.1 Hz, 1H), 6.18 (dd, *J* = 17.1, 2.1 Hz, 1H), 6.42 (dd, *J* = 17.1, 10.2 Hz, 1H), 6.55 (dd, *J* = 8.5, 2.5 Hz, 1H), 6.95 (d, *J* = 8.5 Hz, 1H), 7.08 (d, *J* = 2.5 Hz, 1H), 9.33 (s, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 98.40, 113.89, 122.61, 126.10, 127.30, 128.32, 131.82, 148.48, 163.60.

LC-MS (ESI): Calculated: 287.98 for C₉H₉IN₂O [M+H⁺]. Found: 289.0.

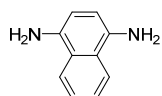
Synthesis of *N*-(4-acrylamido-3-iodophenyl)-3-(imidazo[1,2-*b*]pyridazin-3-ylethynyl)-4-methylbenzamide (73)

The reaction was performed according to general procedure VI with **30** (1.1 eq., 15 mg, 0.05 mmol), DIPEA (6.0 eq., 54.4 μ L, 0.3 mmol), HATU (2.2 eq., 41.2 mg, 0.1 mmol) and *N*-(4-amino-2-iodophenyl)acrylamide (1.0 eq., 14.2 mg, 0.05 mmol) yielding the product as a pale-yellow solid (5.4 mg, 20 %).

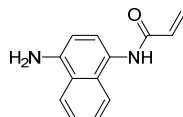
¹H-NMR (600 MHz DMSO-*d*₆) δ ppm 2.61 (s, 3H), 5.77 (dd, *J* = 10.1, 1.9 Hz, 1H), 6.26 (dd, *J* = 17.1, 1.9 Hz, 1H), 6.52 (dd, *J* = 17.1, 10.2 Hz, 1H), 7.44 – 7.37 (m, 2H), 7.55 (d, *J* = 8.1 Hz, 1H), 7.82 (m, 1H), 7.94 (dd, *J* = 8.0, 2.0 Hz, 1H), 8.20 (d, *J* = 1.9 Hz, 1H), 8.24 (s, 1H), 8.27 (dd, *J* = 9.2, 1.6 Hz, 1H), 8.40 (d, *J* = 2.4 Hz, 1H), 8.73 (dd, *J* = 4.4, 1.6 Hz, 1H), 9.63 (s, 1H), 10.41 (s, 1H).

¹³C-NMR (151 MHz, DMSO-*d*₆) δ 20.87, 81.60, 96.91, 112.19, 119.57, 120.86, 122.23, 126.60, 127.44, 127.82, 128.97, 130.40, 130.55, 130.64, 132.02, 132.77, 135.40, 138.40, 138.74, 140.14, 143.89, 145.56, 163.97, 164.83.

LC-MS (ESI): Calculated: 547.05 for C₂₅H₁₈IN₅O₂ [M+H⁺]. Found: 548.1.

Synthesis of naphthalene-1,4-diamine (55):

The reaction was performed according to general procedure XI with the commercially available 4-nitronaphthalen-1-amine (1.0 eq., 1.0 g, 5.3 mmol) and Pd/C (0.1 eq., 56.6 mg, 0.50 mmol). The mixture was filtered over celite and the product was used without further purification.

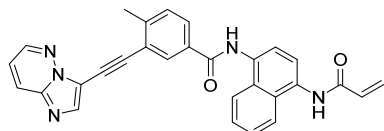
Synthesis of N-(4-aminonaphthalen-1-yl)acrylamide (56):

The reaction was performed according to general procedure VII with naphthalene-1,4-diamine (1.0 eq., 104.0 mg, 0.66 mmol), DIPEA (3.0 eq., 364.1 μ L, 1.97 mmol) and acryloyl chloride (1.1 eq., 584.4 μ L, 0.72 mmol) yielding the product as a white solid. (13.4 mg, 5 %, over two steps).

$^1\text{H-NMR}$ (600 MHz DMSO- d_6) δ ppm 5.76 (d, J = 9.7 Hz, 1H), 6.25 (dd, J = 17.0, 2.0 Hz, 1H), 6.62 (dd, J = 17.0, 10.3 Hz, 1H), 7.43 (d, J = 7.8 Hz, 1H), 6.89 (s, 1H), 7.55 – 7.46 (m, 2H), 7.88 (d, J = 8.3 Hz, 1H), 8.07 (d, J = 7.6 Hz, 1H), 9.86 (s, 1H). Primary amine not resolved.

$^{13}\text{C-NMR}$ (151 MHz, DMSO- d_6) δ 123.00, 123.49, 123.88, 125.11, 126.42, 126.72, 129.54, 132.38, 158.59.

LC-MS (ESI): Calculated: 212.09 for $\text{C}_{13}\text{H}_{12}\text{N}_2\text{O}$ [$\text{M}+\text{H}^+$]. Found: 213.0.

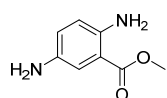
Synthesis of N-(4-acrylamidonaphthalen-1-yl)-3-(imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methylbenzamide (74):

The reaction was performed according to general procedure VI with **30** (1.1 eq., 15 mg, 0.05 mmol), DIPEA (9.0 eq., 54.4 μ L, 0.45 mmol.), HATU (2.5 eq., 46.8 mg, 0.12 mmol) and N-(4-aminonaphthalen-1-yl)acrylamide (1.0 eq., 14.2 mg, 0.05 mmol) yielding the product as a yellow solid (5.1 mg, 22 %).

$^1\text{H-NMR}$ (600 MHz DMSO- d_6) δ ppm 2.64 (s, 3H), 5.83 (dd, J = 10.2, 1.9 Hz, 1H), 6.33 (dd, J = 17.0, 1.9 Hz, 1H), 6.72 (dd, J = 16.9, 10.1 Hz, 1H), 7.39 (dd, J = 9.2, 4.5 Hz, 1H), 7.65 – 7.54 (m, 4H), 7.81 (d, J = 8.1 Hz, 1H), 8.04 (ddd, J = 16.9, 8.3, 1.9 Hz, 2H), 8.11 (dd, J = 8.0, 1.8 Hz, 1H), 8.25 (s, 1H), 8.27 (dd, J = 9.2, 1.6 Hz, 1H), 8.33 (d, J = 1.9 Hz, 1H), 8.73 (dd, J = 4.5, 1.6 Hz, 1H), 10.19 (s, 1H), 10.53 (s, 1H).

$^{13}\text{C-NMR}$ (151 MHz, DMSO- d_6) δ 20.89, 81.50, 97.02, 112.24, 119.54, 121.86, 122.27, 123.42, 124.29, 124.34, 126.50, 126.59, 127.45, 128.68, 128.99, 130.28, 130.52, 130.92, 131.74, 132.23, 132.25, 132.76, 138.73, 140.13, 143.68, 145.55, 164.47, 165.71.

LC-MS (ESI): Calculated: 471.17 for $\text{C}_{29}\text{H}_{21}\text{N}_5\text{O}_2$ [$\text{M}+\text{H}^+$]. Found: 472.2.

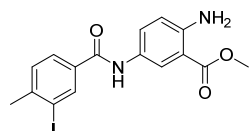
Synthesis of methyl 2,5-diaminobenzoate (76):

A solution of 2,5-diaminobenzoic acid (1.0 eq., 1.0 g, 6.6 mmol) in MeOH (40 mL) was stirred, and SOCl_2 (5.0 eq., 2.4 mL) was added slowly. The mixture was refluxed overnight. Upon confirming reaction completion by TLC and LC-MS, the mixture was cooled to room temperature, and the solvent was evaporated. The crude residue was diluted with ethyl acetate and washed sequentially with saturated NaHCO_3 solution. The combined organic layers were dried over Na_2SO_4 filtered and concentrated under reduced pressure. The resulting crude material was adsorbed onto silica gel and purified by flash column chromatography. After evaporating the pure fractions, the title compound was obtained as a yellow solid (393.0 mg, 60 %).

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 3.67 (s, 3 H) 4.33 (br. s., 2 H) 5.79 (br. s., 2 H) 6.49 (d, $J=8.80$ Hz, 1 H) 6.58 - 6.64 (m, 1 H) 6.92 (d, $J=2.93$ Hz, 1 H).

^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 51.64, 109.80, 114.30, 118.16, 123.83, 138.28, 143.68, 168.47.

LC-MS (ESI-MS): Calculated: 166.07 for $\text{C}_8\text{H}_{10}\text{N}_2\text{O}_2$ $[\text{M}+\text{H}^+]$. Found: 166.9.

Synthesis of methyl 2-amino-5-(3-iodo-4-methylbenzamido)benzoate (77):

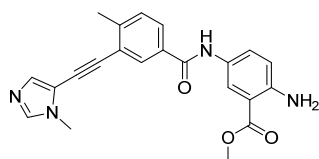
76 (1.2 eq., 235.9 mg, 1.42 mmol), 3-iodo-4-methylbenzoic acid (1.0 eq., 310.0 mg, 1.18 mmol), DIPEA (3.0 eq., 0.6 mL) and HATU (1.1 eq., 475.3 mg, 1.3 mmol) in DMF (10 mL) were used following the general method VI at rt for 3 h obtaining the product as a white solid (460.8 mg, 95 %).

^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 2.44 (s, 3 H) 3.33 (s, 3 H) 6.56 (s, 2 H) 6.78 (d, $J=9.17$ Hz, 1 H) 7.46 (d, $J=8.07$ Hz, 1 H) 7.65 (dd, $J=8.80, 2.57$ Hz, 1 H) 7.89 (dd, $J=7.89, 1.65$ Hz, 1 H) 8.11 (d, $J=2.57$ Hz, 1 H) 8.40 (d, $J=1.47$ Hz, 1 H) 10.05 (s, 1 H).

^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 27.96, 51.92, 101.52, 108.52, 117.02, 122.98, 127.39, 128.01, 128.75, 130.18, 134.52, 137.76, 144.83, 148.69, 163.43, 168.12.

LC-MS (ESI-MS): Calculated: 410.01 for $\text{C}_{16}\text{H}_{15}\text{IN}_2\text{O}_3$ $[\text{M}+\text{H}^+]$. Found: 410.8.

Synthesis of Methyl-2-amino-5-(4-methyl-3-((1-methyl-1H-imidazol-5-yl)-ethynyl)benzamido)benzoate (79):



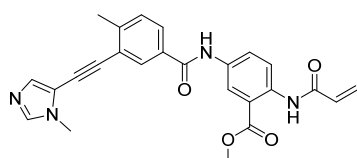
The compound was synthesized following a modified version of general procedure V, omitting the addition of TBAF. A reaction mixture containing methyl 2-amino-5-(3-iodo-4-methylbenzamido)benzoate (1.0 eq., 50.0 mg, 0.12 mmol), 5-ethynyl-1-methyl-1H-imidazole (1.0 eq., 12.9 mg, 0.12 mmol), DIPA (3.0 eq., 0.05 mL, 0.37 mmol), CuI (0.1 eq., 2.3 mg, 0.01 mmol), and Pd(PPh₃)₂Cl₂ (0.1 eq., 8.5 mg, 0.01 mmol) was used. The product was isolated as an off-white solid (59.8 mg, 60 %).

¹H NMR (500 MHz, METHANOL-*d*₄) δ 2.56 (s, 3 H) 3.82 (s, 3 H) 3.85 (s, 3 H) 6.78 (d, *J*=8.85 Hz, 1 H) 7.31 (s, 1 H) 7.42 (d, *J*=8.24 Hz, 1 H) 7.53 (dd, *J*=9.00, 2.59 Hz, 1 H) 7.75 (s, 1 H) 7.83 (dd, *J*=7.93, 2.14 Hz, 1 H) 8.06 (d, *J*=1.83 Hz, 1 H) 8.10 (d, *J*=2.44 Hz, 1 H). Amine protons covered by MeOH peak.

¹³C NMR (126 MHz, METHANOL-*d*₄) δ 11.48, 23.19, 42.49, 72.82, 86.09, 101.19, 108.48, 114.35, 115.77, 118.44, 119.39, 120.31, 120.56, 121.52, 121.91, 122.16, 124.50, 124.65, 135.22, 140.79, 157.98, 160.12.

LC-MS (ESI-MS): Calculated: 388.15 for C₂₂H₂₀N₄O₃ [M+H⁺]. Found: 389.1.

Synthesis of Methyl 2-acrylamido-5-(4-methyl-3-((1-methyl-1H-imidazol-5-yl)ethynyl)benzamido)-benzoate (81):



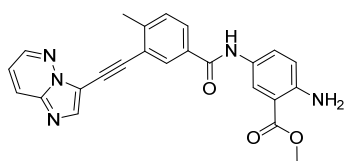
79 (1.0 eq., 15.0 mg, 0.04 mmol), DIPEA (2.0 eq., 0.01 mL, 0.08 mmol) and acryloyl chloride (1.1 eq., 3.8 mg, 0.04 mmol) were used following the general method VII to yield the product as a light brown-white solid (6.1 mg, 35 %).

¹H NMR (700 MHz, DMSO-*d*₆) δ 2.55 (s, 3 H) 3.77 (s, 3 H) 3.89 (s, 3 H) 5.82 - 5.84 (m, 1 H) 6.28 (dd, *J*=17.10, 1.40 Hz, 1 H) 6.45 (dd, *J*=16.99, 10.33 Hz, 1 H) 7.37 (d, *J*=0.86 Hz, 1 H) 7.52 (d, *J*=7.96 Hz, 1 H) 7.82 (s, 1 H) 7.93 (dd, *J*=7.85, 1.83 Hz, 1 H) 8.04 (dd, *J*=9.03, 2.58 Hz, 1 H) 8.16 (d, *J*=1.72 Hz, 1 H) 8.26 (d, *J*=9.03 Hz, 1 H) 8.44 (d, *J*=2.58 Hz, 1 H) 10.42 (s, 1 H) 10.64 (s, 1 H).

¹³C NMR (176 MHz, DMSO-*d*₆) δ 26.57, 32.32, 52.94, 70.21, 82.80, 115.44, 119.29, 120.30, 122.45, 122.61, 126.01, 127.65, 128.58, 130.41, 130.60, 132.72, 132.80, 134.63, 135.26, 135.41, 140.25, 143.48, 163.71, 164.85, 167.75.

LC-MS (ESI-MS): Calculated: 442.16 for C₂₅H₂₂N₄O₄ [M+H⁺]. Found: 443.1.

Synthesis of Methyl 2-amino-5-(3-(imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methylbenzamido)benzoate (80**):**



79 (1.0 eq., 100 mg, 0.2 mmol), **8** (1.0 eq., 104.9 mg, 0.49 mmol), DIPA (3.0 eq., 0.1 mL, 0.7 mmol), TBAF (3.0 eq., 0.2 mL, 0.7 mmol), CuI (0.1 eq., 4.6 mg, 0.02 mmol) and Pd(PPh₃)₃Cl₂ (0.1 eq., 17.1 mg, 0.02 mmol) were used following the general

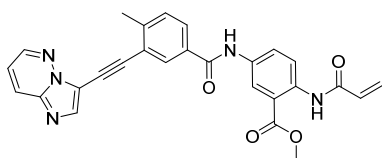
method VII. As complete conversion was not achieved after 12 hours, TBAF (1.5 eq.) was added to the reaction mixture, and stirring was continued for an additional 4 hours. The product was isolated as a beige solid (280.0 mg, 70 %).

¹H NMR (600 MHz, DMSO-*d*₆) δ 2.60 (s, 3 H) 3.82 (s, 3 H) 6.57 (s, 2 H) 6.79 (d, *J*=8.80 Hz, 1 H) 7.40 (dd, *J*=9.17, 4.77 Hz, 1 H) 7.52 (d, *J*=8.07 Hz, 1 H) 7.68 (dd, *J*=9.17, 2.57 Hz, 1 H) 7.94 (d, *J*=8.07 Hz, 1 H) 8.16 (d, *J*=2.57 Hz, 1 H) 8.19 (d, *J*=1.83 Hz, 1 H) 8.24 (s, 1 H) 8.27 (d, *J*=9.17 Hz, 1 H) 8.74 (dd, *J*=4.40, 1.47 Hz, 1 H) 10.14 (s, 1 H).

¹³C NMR (151 MHz, DMSO-*d*₆) δ 20.82, 51.93, 81.39, 97.04, 108.53, 112.24, 117.03, 119.55, 122.11, 122.96, 126.58, 127.52, 128.73, 128.76, 130.42, 130.51, 133.17, 138.68, 140.11, 143.34, 145.55, 148.67, 164.13, 168.15.

LC-MS (ESI-MS): Calculated: 425.15 for C₂₄H₁₉N₅O₃ [M+H⁺]. Found: 426.0.

Synthesis of Methyl 2-acrylamido-5-(3-(imidazo[1,2-b]pyridazin-3-ylethyn-yl)-4-methylbenzamido)benzoate **82:**

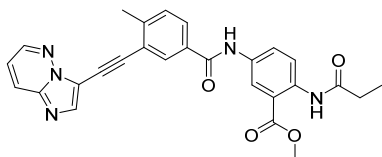


80 (1.0 eq., 15 mg, 0.04 mmol), DIPEA (3.0 eq., 18.8 μL, 0.11 mmol) and acryloyl chloride (1.2 eq., 3.8 mg, 0.04 mmol) were used following the general method VII to yield the product as a white solid (3.6 mg, 24 %).

¹H NMR (500 MHz, DMSO-*d*₆) δ 2.59 (s, 3 H) 5.79 - 5.82 (m, 1 H) 6.23 - 6.28 (m, 1 H) 6.43 (dd, *J*=17.12, 10.27 Hz, 1 H) 7.38 (dd, *J*=9.05, 4.65 Hz, 1 H) 7.53 (d, *J*=8.31 Hz, 1 H) 7.94 (dd, *J*=7.83, 1.96 Hz, 1 H) 8.03 (dd, *J*=8.80, 2.45 Hz, 1 H) 8.21 (s, 1 H) 8.21 (s, 1 H) 8.23 (s, 1 H) 8.25 (dd, *J*=9.29, 1.47 Hz, 1 H) 8.43 (d, *J*=2.45 Hz, 1 H) 8.72 (dd, *J*=4.40, 1.47 Hz, 1 H) 10.48 (s, 1 H) 10.64 (s, 1 H). The CH₃ protons of the methyl ester are covered by a water peak.

LC-MS (ESI-MS): Calculated: 479.16 for C₂₇H₂₁N₅O₄ [M+H⁺]. Found: 479.9.

Synthesis of Methyl 5-(3-(imidazo[1,2-b] pyridazin-3-ylethynyl)-4-methyl-benzamido)-2-propion-amidobenzoate (83):



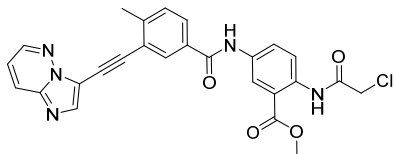
80 (1.0 eq., 17.5 mg, 0.04 mmol), DIPEA (3.0 eq., 21.8 μ L, 0.12 mmol) and propionyl chloride (1.2 eq., 4.3 μ L, 0.05 mmol) were used following the general method VII to yield the product as a white/pastel solid (14.8 mg, 75 %).

^1H NMR (500 MHz, DMSO- d_6) δ 1.19 (t, J =7.48 Hz, 3 H) 2.46 (q, J =7.43 Hz, 2 H) 2.67 (s, 3 H) 3.94 (s, 3 H) 7.45 - 7.48 (m, 1 H) 7.61 (d, J =8.24 Hz, 1 H) 8.02 (dd, J =7.93, 1.83 Hz, 1 H) 8.08 (dd, J =9.00, 2.59 Hz, 1 H) 8.28 - 8.32 (m, 3 H) 8.34 (dd, J =9.31, 1.68 Hz, 1 H) 8.51 (d, J =2.75 Hz, 1 H) 8.80 (dd, J =4.27, 1.53 Hz, 1 H) 10.55 (d, J =3.05 Hz, 2 H).

^{13}C NMR (126 MHz, DMSO- d_6) δ 9.96, 20.87, 30.66, 52.95, 83.45, 93.23, 108.58, 116.06, 118.99, 119.59, 121.28, 122.19, 124.51, 125.55, 126.15, 128.91, 130.54, 130.60, 131.10, 132.78, 135.99, 138.71, 143.81, 145.57, 164.73, 167.88, 172.31.

LC-MS (ESI-MS): Calculated: 481.18 for $\text{C}_{27}\text{H}_{23}\text{N}_5\text{O}_4$ $[\text{M}+\text{H}^+]$. Found: 482.1.

Synthesis of Methyl 2-(2-chloroacetamido)-5-(3-(imidazo[1,2-b] pyridazin-3-yl-ethynyl)-4-methylbenz-amido)benzoate (84):



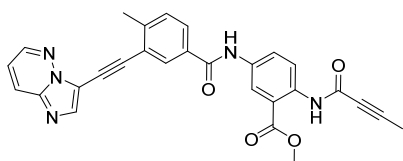
80 (1.0 eq., 34.0 mg, 0.08 mmol), DIPEA (2.0 eq., 0.03 mL, 0.16 mmol) and 2-chloroacetyl chloride (1.0 eq., 9.9 mg, 0.09 mmol) were used following the general method VII to yield the product as a white solid (15.0 mg, 38 %).

^1H NMR (600 MHz, DMSO- d_6) δ 2.62 (s, 3 H) 3.92 (s, 3 H) 4.45 (s, 2 H) 7.38 - 7.42 (m, 1 H) 7.56 (d, J =8.07 Hz, 1 H) 7.97 (dd, J =8.07, 1.83 Hz, 1 H) 8.08 (dd, J =9.17, 2.57 Hz, 1 H) 8.24 (d, J =2.20 Hz, 1 H) 8.25 (s, 1 H) 8.28 (dd, J =9.17, 1.47 Hz, 1 H) 8.36 (d, J =9.17 Hz, 1 H) 8.53 (d, J =2.57 Hz, 1 H) 8.74 (dd, J =4.59, 1.65 Hz, 1 H) 10.54 (s, 1 H) 11.22 (s, 1 H).

^{13}C NMR (151 MHz, DMSO- d_6) δ 23.53, 43.83, 53.16, 81.57, 96.93, 112.19, 118.23, 119.58, 121.83, 122.23, 122.56, 126.31, 126.61, 128.96, 130.56, 130.63, 132.73, 135.16, 135.48, 138.74, 140.14, 143.88, 145.57, 164.82, 165.45, 167.69.

LC-MS (ESI-MS): Calculated: 501.12 for $\text{C}_{26}\text{H}_{20}\text{ClN}_5\text{O}_4$ $[\text{M}+\text{H}^+]$. Found: 502.0.

Synthesis of Methyl 2-(but-2-ynamido)-5-(3-(imidazo[1,2-b] pyridazin-3-yl-ethynyl)-4-methylbenz-amido)benzoate (85**):**



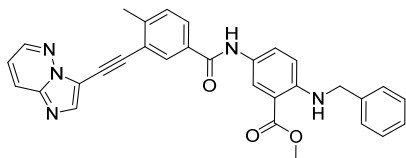
80 (1.0 eq., 40.0 mg, 0.09 mmol), DIPEA (2.0 eq., 0.03 mL, 0.19 mmol) and freshly prepared but-2-ynoyl chloride (1.0 eq., 9.6 mg, 0.09 mmol) were used following the general method VII to yield the product as a white solid (15.0 mg, 38 %).

¹H NMR (600 MHz, DMSO-*d*₆) δ 2.29 (s, 3 H) 2.62 (s, 3 H) 3.89 (s, 3 H) 7.41 (dd, *J*=9.17, 4.40 Hz, 1 H) 7.56 (d, *J*=8.07 Hz, 1 H) 7.97 (dd, *J*=8.07, 1.83 Hz, 1 H) 8.05 (dd, *J*=8.80, 2.57 Hz, 1 H) 8.23 (s, 1 H) 8.25 (s, 1 H) 8.27 (br. s., 1 H) 8.28 - 8.31 (m, 1 H) 8.48 (d, *J*=2.20 Hz, 1 H) 8.74 (d, *J*=3.30 Hz, 1 H) 10.51 (s, 1 H) 10.67 (s, 1 H).

¹³C NMR (151 MHz, DMSO-*d*₆) δ 20.87, 27.82, 53.02, 70.27, 81.55, 96.94, 112.21, 118.53, 119.58, 121.34, 122.19, 122.41, 126.12, 126.60, 128.95, 130.54, 130.62, 132.75, 135.13, 135.50, 138.72, 140.14, 140.94, 143.85, 145.57, 161.95, 164.75, 167.79.

LC-MS (ESI-MS): Calculated: 491.16 for C₂₈H₂₁N₅O₄ [M+H⁺]. Found: 492.2.

Synthesis of Methyl 2-(benzylamino)-5-(3-(imidazo[1,2-b] pyridazin-3-yl-ethynyl)-4-methylbenz-amido)benzoate (86**):**



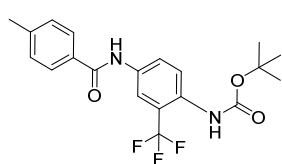
Benzyl bromide (1.0 eq., 10.1 mg, 58.8 μmol) was gradually added to a stirred solution of compound **80** (1.0 eq., 25.0 mg, 58.8 μmol) and K₂CO₃ (2.2 eq., 17.8 mg, 129.2 μmol) in anhydrous MeOH (20 mL). The mixture was stirred overnight

at 65 °C under an argon atmosphere. Upon confirming the reaction's completion via TLC and LC-MS, the mixture was diluted with ethyl acetate and washed with water. The combined organic phases were dried over Na₂SO₄ filtered and concentrated under reduced pressure. The crude product was adsorbed onto silica gel and purified using flash column chromatography. The pure fractions were evaporated to yield compound **34** as a white solid (19.5 mg, 64 %).

¹H NMR (600 MHz, DMSO-*d*₆) δ 2.60 (s, 3 H) 3.85 (s, 3 H) 4.50 (d, *J*=5.87 Hz, 2 H) 6.57 (s, 1 H) 6.75 (d, *J*=9.17 Hz, 1 H) 7.35 - 7.37 (m, 2 H) 7.37 (s, 2 H) 7.40 (dd, *J*=9.17, 4.40 Hz, 1 H) 7.52 (d, *J*=8.44 Hz, 1 H) 7.75 (dd, *J*=8.99, 2.75 Hz, 1 H) 7.92 (dd, *J*=8.07, 1.83 Hz, 1 H) 8.00 (t, *J*=5.87 Hz, 1 H) 8.18 (d, *J*=1.83 Hz, 1 H) 8.24 (s, 1 H) 8.27 (dd, *J*=9.17, 1.47 Hz, 1 H) 8.30 (d, *J*=2.57 Hz, 1 H) 8.73 (dd, *J*=4.40, 1.47 Hz, 1 H) 10.17 (s, 1 H).

LC-MS (ESI-MS): Calculated: 515.20 for C₃₁H₂₅N₅O₃ [M+H⁺]. Found: 516.1.

Synthesis of *tert*-butyl (4-amino-2-(trifluoromethyl)phenyl)carbamate (88**):**

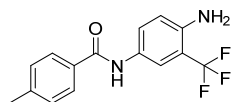


Tert-butyl (4-amino-2-(trifluoromethyl)phenyl)carbamate (1.0 eq., 10 mg, 0.04 mmol) and 4-methylbenzoic acid (1.1 eq., 5.4 mg, 0.04 mmol) were used following the general method VII to yield the title compound (28 mg, 64 %).

¹H NMR (600 MHz, DMSO) δ 10.43 (s, 1H), 8.62 (s, 1H), 8.20 (d, J = 2.4 Hz, 1H), 8.02 (dd, J = 8.7, 2.4 Hz, 1H), 7.90 (d, J = 8.2 Hz, 2H), 7.40 (d, J = 8.7 Hz, 1H), 7.36 (d, J = 7.9 Hz, 2H), 2.39 (s, 3H), 1.43 (s, 9H).

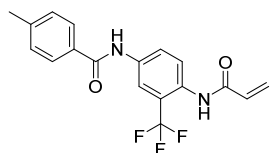
¹³C NMR (151 MHz, DMSO) δ 21.03, 28.04, 79.00, 117.50, 117.54, 122.59, 124.06, 124.40, 125.42, 125.61, 127.73, 128.98, 130.77, 131.44, 137.47, 141.94, 154.16, 165.55.

Synthesis of *N*-(4-amino-3-(trifluoromethyl)phenyl)-4-methylbenzamide (89**):**



The reaction was carried out following the general procedure XI with **88** (1.0 eq., 28 mg, 0.07 mmol). The crude product was utilized without further purification.

Synthesis of *N*-(4-acrylamido-3-(trifluoromethyl)phenyl)-4-methylbenzamide (90**):**

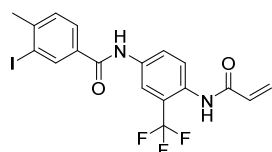


89 (1.0 eq., 20 mg, 0.07 mmol) and acryloyl chloride (1.1 eq., 6.1 μ L, 0.08 mmol) were used following the general method VII to yield the product (6 mg, 25 %).

¹H NMR (700 MHz, DMSO) δ 2.40 (s, 4H), 5.77 (dd, J = 10.3 Hz, 1H), 6.24 (dd, J = 17.1, 1.7 Hz, 1H), 6.52 (dd, J = 16.0, 10.5 Hz, 1H), 7.36 (d, J = 8.0 Hz, 3H), 7.49 (d, J = 8.5 Hz, 1H), 7.90 (d, J = 8.1 Hz, 3H), 8.06 (dd, J = 8.6, 2.2 Hz, 1H), 8.26 (d, J = 2.3 Hz, 1H), 10.47 (s, 1H), 9.71 (s, 1H).

¹³C NMR (176 MHz, DMSO) δ 21.03, 117.46, 122.61, 123.96, 124.17, 127.11, 127.75, 128.97, 129.95, 130.84, 131.12, 131.45, 137.85, 142.03, 164.36, 165.61.

LC-MS (ESI-MS): Calculated: 348.11 for C₁₈H₁₅F₃N₂O₂ [M+H⁺]. Found: 349.1.

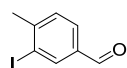
Synthesis of *N*-(4-acrylamido-3-(trifluoromethyl)phenyl)-3-iodo-4-methylbenzamide (91**):**

32 (1.0 eq., 50.0 mg, 0.22 mmol), DIPEA (3.0 eq., 0.12 mL, 0.65 mmol), HATU (1.2 eq., 107.4 mg, 0.28 mmol) and 3-iodo-4-methylbenzoic acid (1.3 eq., 73.9 mg, 0.28 mmol) were used following the general method IX to yield the product as an off-white solid (37.4 mg, 36 %).

¹H NMR (700 MHz, DMSO-*d*₆) δ ppm 2.45 (s, 3 H) 5.74 - 5.79 (m, 1 H) 6.24 (dd, *J*=17.10, 1.83 Hz, 1 H) 6.52 (dd, *J*=16.46, 10.43 Hz, 1 H) 7.51 (dd, *J*=8.39, 3.87 Hz, 2 H) 7.93 (dd, *J*=7.85, 1.83 Hz, 1 H) 8.06 (dd, *J*=8.82, 2.37 Hz, 1 H) 8.22 (d, *J*=2.37 Hz, 1 H) 8.44 (d, *J*=1.94 Hz, 1 H) 9.72 (s, 1 H) 10.54 (s, 1 H).

¹³C NMR (176 MHz, DMSO-*d*₆) δ 28.04, 101.60, 118.09, 123.07, 124.56, 124.63, 127.63, 128.30, 130.31, 130.66, 131.32, 131.56, 133.94, 137.94, 138.05, 145.59, 164.41, 164.85.

LC-MS (ESI-MS): Calculated: 474.01 for C₁₈H₁₄F₃IN₂O₂ [M+H⁺]. Found: 475.1.

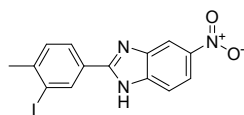
Synthesis of 3-iodo-4-methylbenzaldehyde (93**):**

DiBAL-H (1.5 eq., 1.0 M in THF, 1.2 mL) was gradually added to a stirred solution of benzonitrile **92** (1.0 eq., 200 mg, 0.8 mmol) in toluene (4.0 mL) at 0 °C. The reaction was stirred for three hours while maintaining the same temperature. Upon confirming reaction completion via TLC and LC-MS, the mixture was quenched by adding 1.0 M aqueous HCl (5.0 mL) until a white slurry formed. The mixture was then diluted with DCM and sequentially washed with saturated NaHCO₃ and brine. The combined organic layers were dried over Na₂SO₄, filtered and concentrated under reduced pressure to obtain the crude product. This material was adsorbed onto silica gel and purified by flash column chromatography. After evaporating the pure fractions, 3-iodo-4-methylbenzaldehyde was obtained as a white powder (185.8 mg, 92 %).

¹H NMR (400 MHz, DMSO-*d*₆) δ 2.51 (s, 3 H) 7.61 (d, *J*=7.82 Hz, 1 H) 7.88 (dd, *J*=7.83, 1.47 Hz, 1 H) 8.37 (s, 1 H) 9.96 (s, 1 H).

¹³C NMR (101 MHz, DMSO-*d*₆) δ 28.41, 102.19, 129.31, 131.02, 136.19, 140.24, 148.48, 191.99.

LC-MS (ESI-MS): Calculated: 245.95 for C₈H₇IO [M+H⁺]. Found: 247.7.

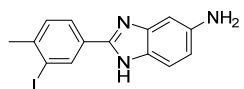
Synthesis of 2-(3-iodo-4-methylphenyl)-5-nitro-1H-benzo[d]imidazole (94**):**

4-Nitrobenzene-1,2-diamine (1.0 eq., 1.0 g, 6.5 mmol) was dissolved in CH₃CN (50.0 mL), and aryl aldehyde **93** (1.0 eq., 1.6 g, 6.5 mmol) was added gradually at -5 °C. To this mixture, aq. 30 % H₂O₂ (5.0 eq., 1.0 mL) and aq. 37 % HCl (2.5 eq., 0.5 mL) were added dropwise from a separatory funnel containing CH₃CN (5.0 mL), while maintaining the temperature at -5 °C. The reaction was stirred under cooling for 4 hours. After confirming completion via TLC and LC-MS, the reaction was quenched by adding water (25.0 mL), and the excess solvent was removed using a rotary evaporator. The reaction mixture was then diluted with ethyl acetate and washed with brine. The organic layers were combined, dried over Na₂SO₄, filtered and concentrated under reduced pressure to obtain the crude product. This material was adsorbed onto silica gel and purified via flash column chromatography. Evaporation of the pure fractions yielded the title compound as an amber-brown solid (1.6 g, 65 %).

¹H NMR (600 MHz, DMSO-*d*₆) δ 2.44 (s, 3 H) 7.52 (d, *J*=8.07 Hz, 1 H) 7.69 (s., 1 H) 8.11 (s., 1 H) 8.12 (s., 1 H) 8.51 (s., 1 H) 8.63 (s, 1 H) 13.58 (br. s., 1 H).

¹³C NMR (151 MHz, DMSO-*d*₆) δ 27.98, 102.25, 112.27, 115.34, 118.81, 127.19, 128.83, 130.88, 136.97, 140.16, 143.26, 144.29, 148.74, 154.08.

LC-MS (ESI-MS): Calculated: 378.98 for C₁₄H₁₀IN₃O₂ [M+H⁺]. Found: 379.9.

Synthesis of 2-(3-iodo-4-methylphenyl)-1H-benzo[d]imidazol-5-amine (95**):**

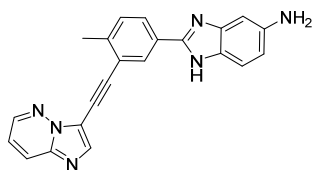
The reaction was carried out following a procedure analogous to the general procedure IX at 75 °C for 3 h with **94** (1.0 eq., 350 mg, 0.9 mmol), iron (4.0 eq., 206.2 mg, 3.7 mmol), in aq. EtOH (20.0 mL, 70 % v/v) containing acetic acid (0.3 mL) yielding the product as a deep brown solid (261.0 mg, 81 %).

¹H NMR (500 MHz, DMSO-*d*₆) δ 2.41 (s, 3 H) 5.02 (s, 2 H) 6.52 (d, *J*=8.54 Hz, 1 H) 6.65 (s, 1 H) 7.28 (d, *J*=8.54 Hz, 1 H) 7.43 (d, *J*=7.93 Hz, 1 H) 8.03 (d, *J*=7.93 Hz, 1 H) 8.54 (s, 1 H) 12.54 (br. s., 1 H).

¹³C NMR (126 MHz, DMSO-*d*₆) δ 27.80, 94.71, 102.14, 112.15, 119.36, 125.98, 126.41, 130.63, 135.80, 136.47, 136.82, 139.42, 145.83, 151.76.

LC-MS (ESI-MS): Calculated: 349.01 for C₁₄H₁₂IN₃ [M+H⁺]. Found: 350.0.

Synthesis of 2-(3-(imidazo[1,2-b]pyridazine-3-ylethynyl)-4-methylphenyl)-1H-benzo[d]imidazole-5-amine (**96**):



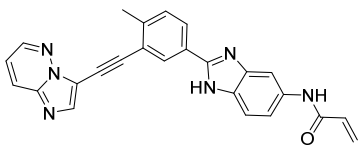
95 (1.0 eq., 250.0 mg, 0.7 mmol), **29** (1.0 eq., 154.2 mg, 0.7 mmol), DIPA (3.0 eq., 0.3 mL, 2.1 mmol), TBAF (3.0 eq., 0.6 mL, 2.1 mmol), Cul (0.1 eq., 13.6 mg, 0.1 mmol) and Pd(PPh₃)₃Cl₂ (0.1 eq., 50.3 mg, 0.1 mmol) were used following the general method V to yield the product as a light brown solid (48 mg, 19 %).

¹H NMR (600 MHz, DMSO-*d*₆) δ 2.58 (s, 3 H) 4.99 (br. m, 2 H) 6.51 - 6.58 (m, 1 H) 6.66 (s., 1 H) 7.30 (s., 1 H) 7.39 - 7.42 (m, 1 H) 7.50 (d, *J*=8.07 Hz, 1 H) 8.05 (d, *J*=7.70 Hz, 1 H) 8.25 (s, 1 H) 8.27 (d, *J*=1.47 Hz, 1 H) 8.28 (d, *J*=1.47 Hz, 1 H) 8.75 (dd, *J*=4.40, 1.47 Hz, 1 H) 12.36 (br. s., 1 H).

¹³C NMR (151 MHz, DMSO-*d*₆) δ 20.67, 81.15, 94.70, 97.25, 112.14, 112.32, 119.50, 122.59, 126.58, 126.74, 128.58, 129.29, 130.86, 136.59, 136.77, 138.65, 140.09, 140.41, 145.53, 145.74, 147.90, 152.77.

LC-MS (ESI-MS): Calculated: 364.14 for C₂₂H₁₆N₆ [M+H⁺]. Found: 365.2.

Synthesis of *N*-(2-(3-(imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methylphenyl)-1H-benzo[d]imidazol-5-yl)acrylamide (**97**):



96 (1.0 eq., 40.0 mg, 0.1 mmol), TEA (1.5 eq., 0.02 mL, 0.2 mmol) and acryloyl chloride (1.0 eq., 9.9 mg, 0.1 mmol) were used following the general method VII to yield the product as an amber solid (32 mg, 70 %).

¹H NMR (600 MHz, METHANOL-*d*₄) δ 2.55 (s, 3 H) 5.69 (dd, *J*=10.09, 1.65 Hz, 1 H) 6.29 (dd, *J*=16.87, 1.83 Hz, 1 H) 6.39 (dd, *J*=16.87, 9.90 Hz, 1 H) 7.25 - 7.26 (m, 1 H) 7.26 - 7.28 (m, 1 H) 7.40 (d, *J*=8.07 Hz, 1 H) 7.47 (d, *J*=8.80 Hz, 1 H) 7.91 (dd, *J*=7.89, 2.02 Hz, 1 H) 7.97 (s, 1 H) 8.00 (dd, *J*=9.17, 1.47 Hz, 1 H) 8.10 (d, *J*=1.83 Hz, 1 H) 8.18 (d, *J*=1.83 Hz, 1 H) 8.53 (dd, *J*=4.40, 1.47 Hz, 1 H).

¹³C NMR (151 MHz, METHANOL-*d*₄) δ 21.02, 81.50, 98.01, 101.56, 109.95, 110.64, 117.86, 120.66, 123.09, 124.09, 124.65, 126.59, 127.74, 128.22, 128.96, 130.76, 131.77, 132.73, 135.69, 138.23, 141.41, 143.77, 146.25, 153.15, 166.27.

LC-MS (ESI-MS): Calculated: 418.15 for C₂₅H₁₈N₆O [M+H⁺]. Found: 419.2.

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Chapter: 10

SUPPLEMENTARY INFORMATION

10. Supplementary Information

10.1 Supplementary Tables

Supplementary Table 1. Determined GR₅₀ values on avapritinib-resistant PDGFRA- and KIT cell lines. Data presented as mean values $\pm$ s.d; $n \geq 3$; n represents the number of independent experiments. ^a $n = 2$; n.d. not determined.

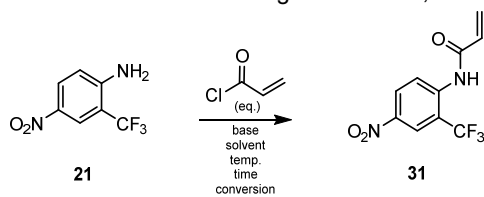
cpd id	PDGFRA CTG GR ₅₀ [nM]			KIT CTG GR ₅₀ [nM]	
	T1-a-T674I	T1-a-T674R	T1-a-5258	T1-T670I	T1-V654A
ava	193 $\pm$ 107	918 $\pm$ 560	540 $\pm$ 30	457 $\pm$ 240	357 $\pm$ 142 ^a
1	4224 $\pm$ 3911	3921 $\pm$ 2783	1340 $\pm$ 70	2867 $\pm$ 510	7556 $\pm$ 991
6	≥ 10000	n.d.	n.d.	n.d.	n.d.
2	8063 $\pm$ 2354	6779 $\pm$ 3777	≥ 10000	≥ 10000	≥ 10000
12	≥ 10000	≥ 10000	8260 $\pm$ 1700	≥ 10000	≥ 10000
13	≥ 10000	≥ 10000	9530 $\pm$ 650	≥ 10000	≥ 10000
14	9030 $\pm$ 1680	≥ 10000	9520 $\pm$ 810	≥ 10000	6730 $\pm$ 439
15	732 $\pm$ 367	880 $\pm$ 298	1660 $\pm$ 250	2560 $\pm$ 258	1171 $\pm$ 264
16	446 $\pm$ 99	1572 $\pm$ 1196	1330 $\pm$ 220	1495 $\pm$ 223	575 $\pm$ 159
17	193 $\pm$ 79	411 $\pm$ 193	2060 $\pm$ 430	386 $\pm$ 39	293 $\pm$ 83
18	256 $\pm$ 115	436 $\pm$ 249	3380 $\pm$ 360	413 $\pm$ 44	347 $\pm$ 108
19	364 $\pm$ 63	597 $\pm$ 235	2220 $\pm$ 350	695 $\pm$ 135	474 $\pm$ 155

Supplementary Table 2. Determined GR₅₀ / EC₅₀ of control cell lines (non KIT- and/or PDGFRA-driven). Data presented as mean values $\pm$ s.d; $n \geq 3$; n represents the number of independent experiments.

cpd id	CTG GR ₅₀ [nM]	CTG EC ₅₀ [nM]	
	SK-LMS-1	ZR-75-1	MDA-MB-175VII
ava	8423 $\pm$ 2731	6558 $\pm$ 705	5778 $\pm$ 88
1	7713 $\pm$ 3929	6484 $\pm$ 1470	9191 $\pm$ 1401
6	≥ 10000	≥ 10000	≥ 10000
2	≥ 10000	≥ 10000	≥ 10000
12	≥ 10000	≥ 10000	≥ 10000
13	≥ 10000	≥ 10000	≥ 10000
14	≥ 10000	≥ 10000	≥ 10000
15	8631 $\pm$ 2371	≥ 10000	≥ 10000
16	9041 $\pm$ 1661	≥ 10000	≥ 10000
17	8787 $\pm$ 2101	≥ 10000	≥ 10000
18	6459 $\pm$ 3067	≥ 10000	≥ 10000
99	7334 $\pm$ 2310	≥ 10000	≥ 10000
20	9498 $\pm$ 869	≥ 10000	≥ 10000

Supplementary Table 3. Summary of kinases, respective Uniprot-IDs and sequence, containing a cysteine equivalent to KIT-Cys788.

protein	Uniprot-ID	sequence alignment
KIT	P10721	QVAKGMAFLAS-KN C IHRDLAARNILLTHGRITKICDFGLARDIKN-D----SNYVVKGN 828
PDGFRA	P16234	QVARGMEFLAS-KN C VHRDLAARNVLLAQGKIVKICDFGLARDIMH-D----SNYVSKGS 854
PDGFRB	P09619	QVANGMEFLAS-KN C VHRDLAARNVLICEGKLVKICDFGLARDIMR-D----SNYISKGS 862
ANPA	P16066	DIVKGMFLHNGAT C SHGNLKSSNCVVDGRFVLKITDYGLESFRDLDPEDQ-----GHT 694
CDKL1	Q00532	QTLQAVNFCHK-HN C IHRDVKPENILITKHSVIKLCDFGFARLLTGPS----DYYT---- 159
CDKL4	Q5MAI5	QTLQALNFCHI-HN C IHRDIKPENILITKQGIKICDFGFAQILI-PG----DAYT---- 158
CSF1R	P07333	QVAQGMAFLAS-KN C IHRDVAARNVLLTNGHVAKIGDFGLARDIMN-D----SNYIVKGN 814
FER	P16591	DAAAGMLYLES-KN C IHRDLAARNCLVGENNVLKISDFGMSRQEDG-G----VYSS-SGL 719
FES	P07332	DAAAGMEYLES-K C IHRDLAARNCLVTEKNVLKISDFGMSREEAD-G----VYAASGGL 719
FGFR1	P11362	QVARGMEYLAS-K C IHRDLAARNVLVTEDNVMKIADFGGLARDIHH-I----DYYKKTN 659
FGFR2	P21802	QLARGMEYLAS-Q C IHRDLAARNVLVTENNVMKIADFGGLARDINN-I----DYYKKTN 662
FGFR3	P22607	QVARGMEYLAS-Q C IHRDLAARNVLVTEDNVMKIADFGGLARDVHN-L----DYYKKTN 653
FGFR4	P22455	QVARGMQYLES-R C IHRDLAARNVLVTEDNVMKIADFGGLARGVHH-I----DYYKKTN 648
FLT1	P17948	QVARGMEFLSS-R C IHRDLAARNILLSENNVVKICDFGLARDIYK-N----PDYVRKGD 1058
FLT3	P36888	QVAKGMEFLEF-K S VHRDLAARNVLVTHGKVVKICDFGLARDIMS-D----SNYVVRGN 847
FLT4	P35916	QVARGMEFLAS-R C IHRDLAARNILLSESDVVKICDFGLARDIYK-D----PDYVRKGS 1073
JAK3	P52333	QICKGMEYLGs-R C VHRDLAARNILVESEAHVKIADFGGLAKLLPLDK----DYYVREP 986
KDR	P35968	QVAKGMEFLAS-R C IHRDLAARNILLSEKNVVKICDFGLARDIYK-D----PDYVRKGD 1064
PYK2	Q14289	QICKAMAYLES-IN C VHRDIAVRNIVASPECVKLGDFGLSRYIED-E----DYYK-ASV 584
STLK6	Q9C0K7	GAVRGLNYLHQ-NG C IHRSIKASHILISGDGLVTLGSLSHLSLVKHGQRHRAVYDFPQF 225

Supplementary Table 4. Optimization of the amidation of 4-nitro-2-(trifluoromethyl)aniline. Conversion determined by LC-MS analysis. ^aSide product formation occurred to a significant level, including di-substitution.

entry	eq. (AcCl)	base (3.0 eq.)	solvent	temp. [°C]	time [h]	conversion [%]
31a	1.5	DIPEA	THF	rt	16	10
31b	5.0	DIPEA	THF	rt	16	≤ 40
31c	8.0	DIPEA	THF	rt	16	≥ 40
31d	1.5	DIPA	THF	rt	16	≤ 5
31e	1.5	TEA	THF	rt	16	~ 45
31f	1.5	DBU	THF	rt	16	≤ 5
31g	1.5	Pyridine	THF	rt	16	≤ 5
31h	1.5	LHMDS	THF	rt	16	~ 45
31i	1.5	-	Pyridine	rt	16	≤ 5
31j	1.5	DIPEA	DCM	rt	16	~ 35
31k	1.5	DIPEA	DMF	rt	16	≤ 5
31l	1.5	DIPEA	DMA	rt	16	≤ 5
31m	1.5	DIPEA	neat	rt	16	≥ 40
31n	5.0	TEA	DCM	40	16	~ 85 ^a
31o	5.0	TEA	DCM	40	4	~ 45 ^a
31p	5.0	TEA	DCM	40	32	100 ^a
31q	5.0	TEA	DCM	70	16	100 ^a

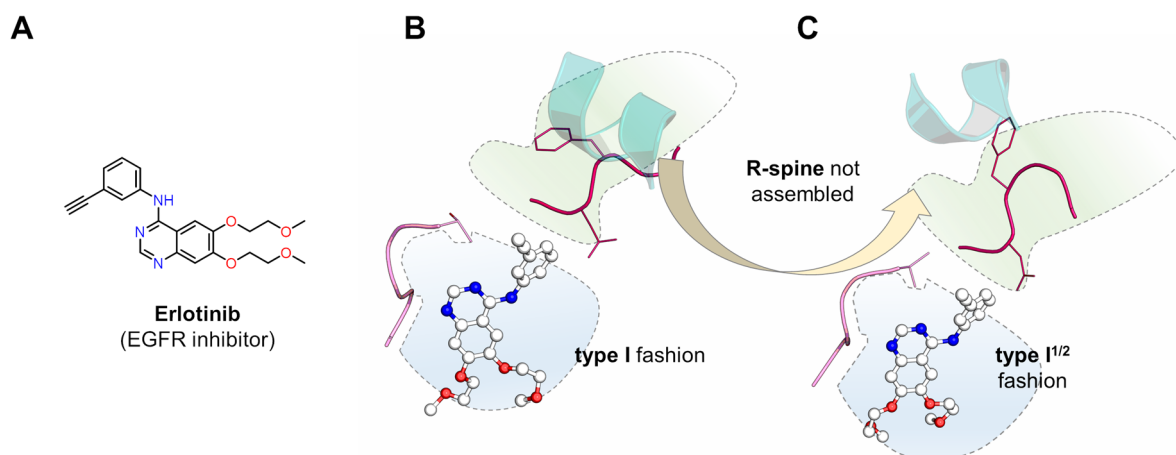
Supplementary Table 5. Determined IC₅₀ values on KIT-T670I and PDGFRA-T674I in comparison to the GIST-T1-T670I cell line. Data are shown from a single measurement. n.d., not determined. The measurement was conducted together with Andreas Arndt (AG Rauh, TU Dortmund University).

cpd id	HTRF IC ₅₀ [nM]		CTG GR ₅₀ [nM]
	PDGFRA-T674I	KIT-T670I	GIST-T1-T670I
imatinib	2407	7617	>10000
sunitinib	4	1	28
regorafenib	300	64	106
ripretinib	255	237	714
avapritinib	1337	192	349
ponatinib	9	21	4
IDRx-42	9649	653	>10000
nintedanib	1	<1	5
osimertinib	1161	7350	n.d.
gefitinib	3843	3562	n.d.

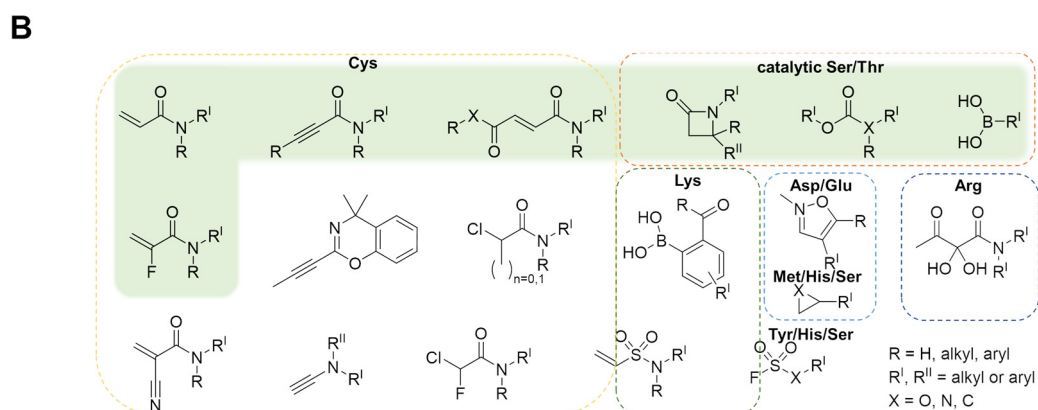
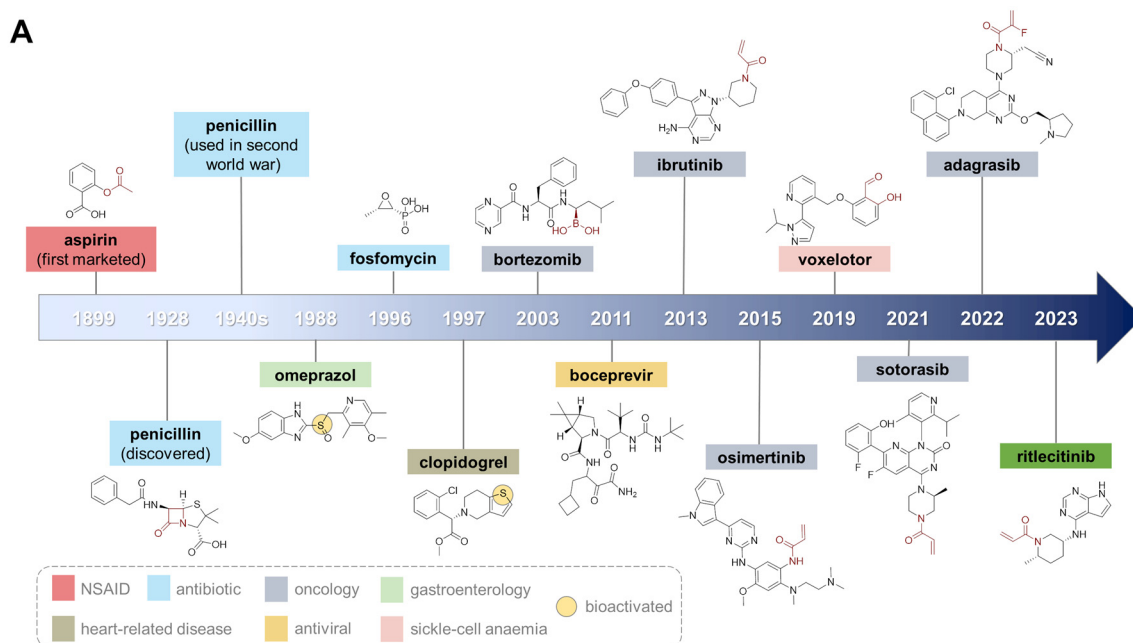
Supplementary Table 6. SwissADME²⁶⁶ calculated physicochemical properties of novel covalent ponatinib-based inhibitors and reference compound, including approved TKIs for GIST.

cpd id	MW [g/mol]	#H-bond donor	#H-bond acceptor	#rotatable bonds	clogP ^a	TPSA [Å ²]
imatinib	493.60	2	6	8	4.04	86.28
sunitinib	398.47	3	4	8	3.55	77.23
regorafenib	482.82	3	8	9	3.51	92.35
ripretinib	510.36	3	4	7	3.10	88.05
avapritinib	498.56	1	7	5	3.54	106.29
ponatinib	532.56	1	8	6	4.42	65.77
57	489.45	2	7	7	3.64	88.39
59	435.48	2	4	6	3.57	88.39
60	421.45	2	4	6	3.17	88.39
61	519.60	2	5	7	4.43	94.87
62	435.50	1	4	4	3.90	100.42
63	409.46	1	4	3	3.14	100.42
65	413.47	1	4	5	3.34	79.60
27	489.45	2	7	7	3.78	88.39
66	489.45	2	7	7	3.50	88.39
67	421.45	2	4	6	3.12	88.39
68	477.44	2	7	6	3.68	88.39
69	421.45	2	4	6	3.12	88.39
70	461.51	2	4	7	3.92	88.39
71	501.54	2	5	7	3.91	106.21
72	503.57	2	4	7	4.25	116.63
73	547.35	2	4	6	3.67	88.39
74	471.51	2	4	6	3.73	88.39
82	479.49	2	6	8	3.69	114.69
83	481.50	2	6	8	3.74	114.69
84	501.92	2	6	8	3.70	114.69
85	491.50	2	6	7	4.25	114.69
90	348.32	2	5	7	2.97	58.20
97	474.22	2	5	7	3.37	58.20
^a iLOGP according to Daina, A. <i>et al.</i> ²⁶⁷						

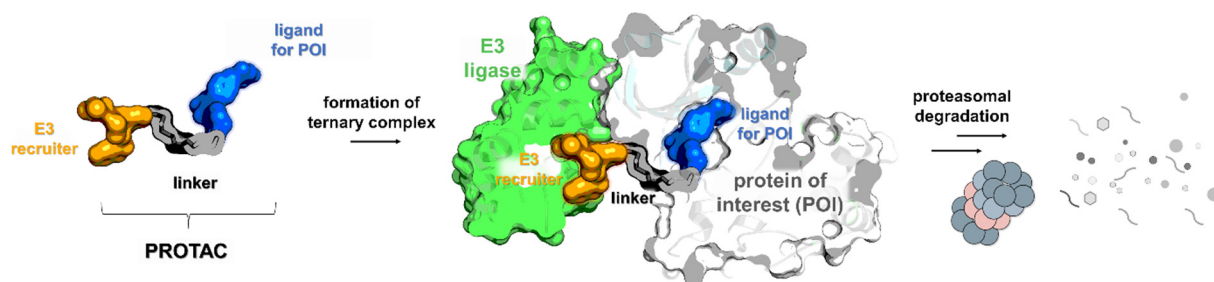
10.2 Supplementary Figures



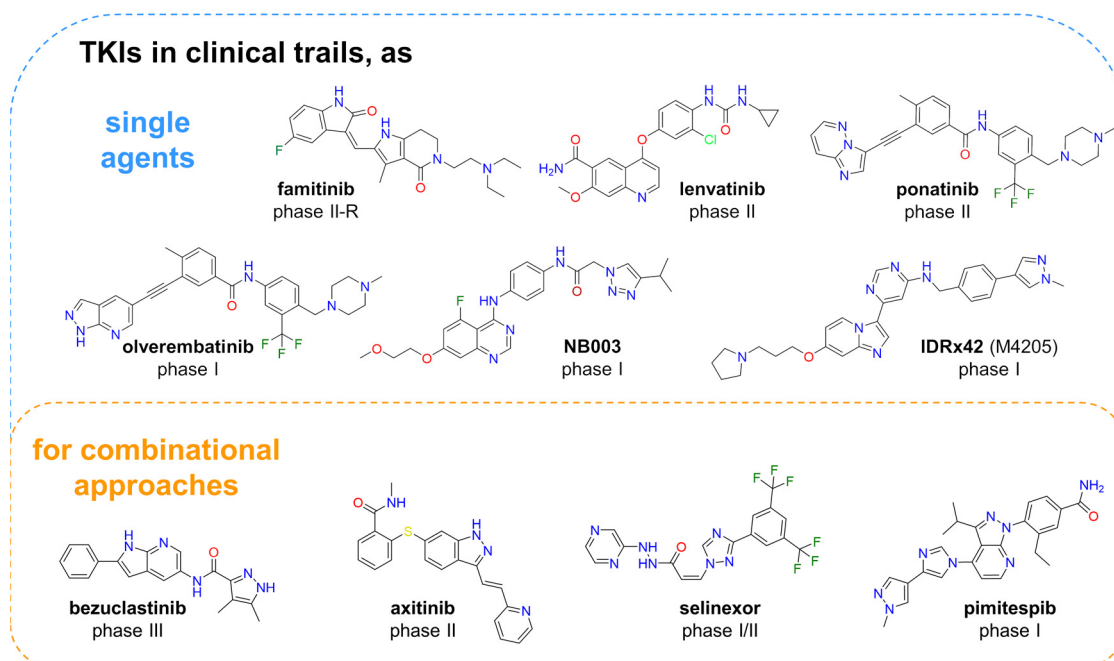
Supplementary Figure 1. Chemical structure of the EGFR inhibitor gefitinib (**A**) and binding modes in a type I inhibitor (**B**, PDB ID: 1M17) and type I^{1/2} inhibitor (**C**, PDB ID: 4HJO) fashion.



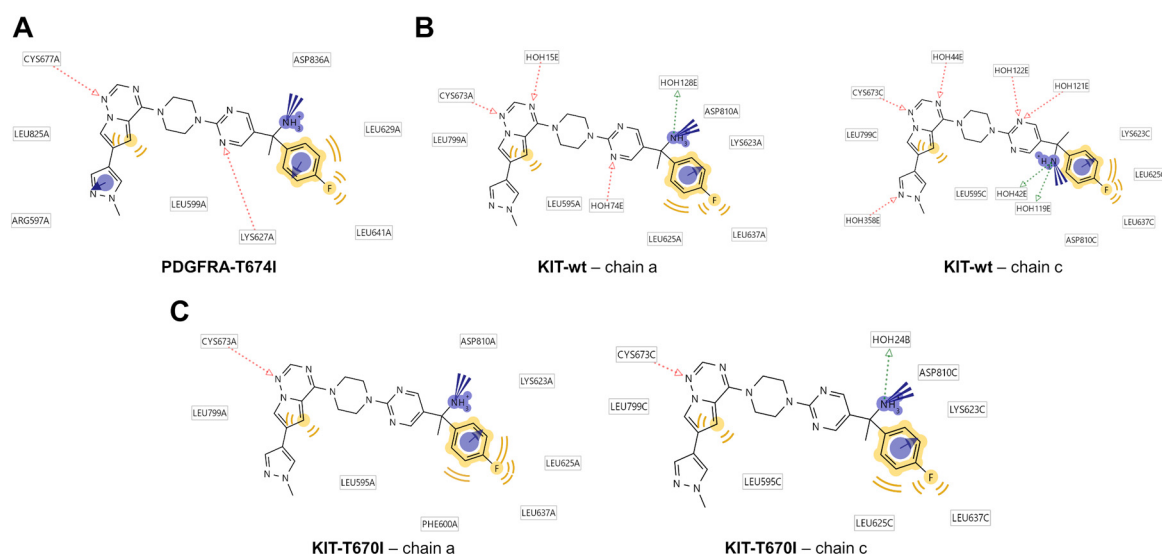
Supplementary Figure 2. (A) Timeline of selected covalent drugs.⁷⁹⁻⁸⁰ NSAID, non-steroidal anti-inflammatory drug.⁷⁹ (B) Selected collection of warheads targeting different amino acids.^{80, 83, 87} Color-coded according to targeted residue; some warheads may target multiple residues. Moieties highlighted in green are used in approved drugs.⁸³ The figure is inspired and based on Boike *et al.* (2022)⁷⁹, and Vita *et al.* (2020)⁸³.



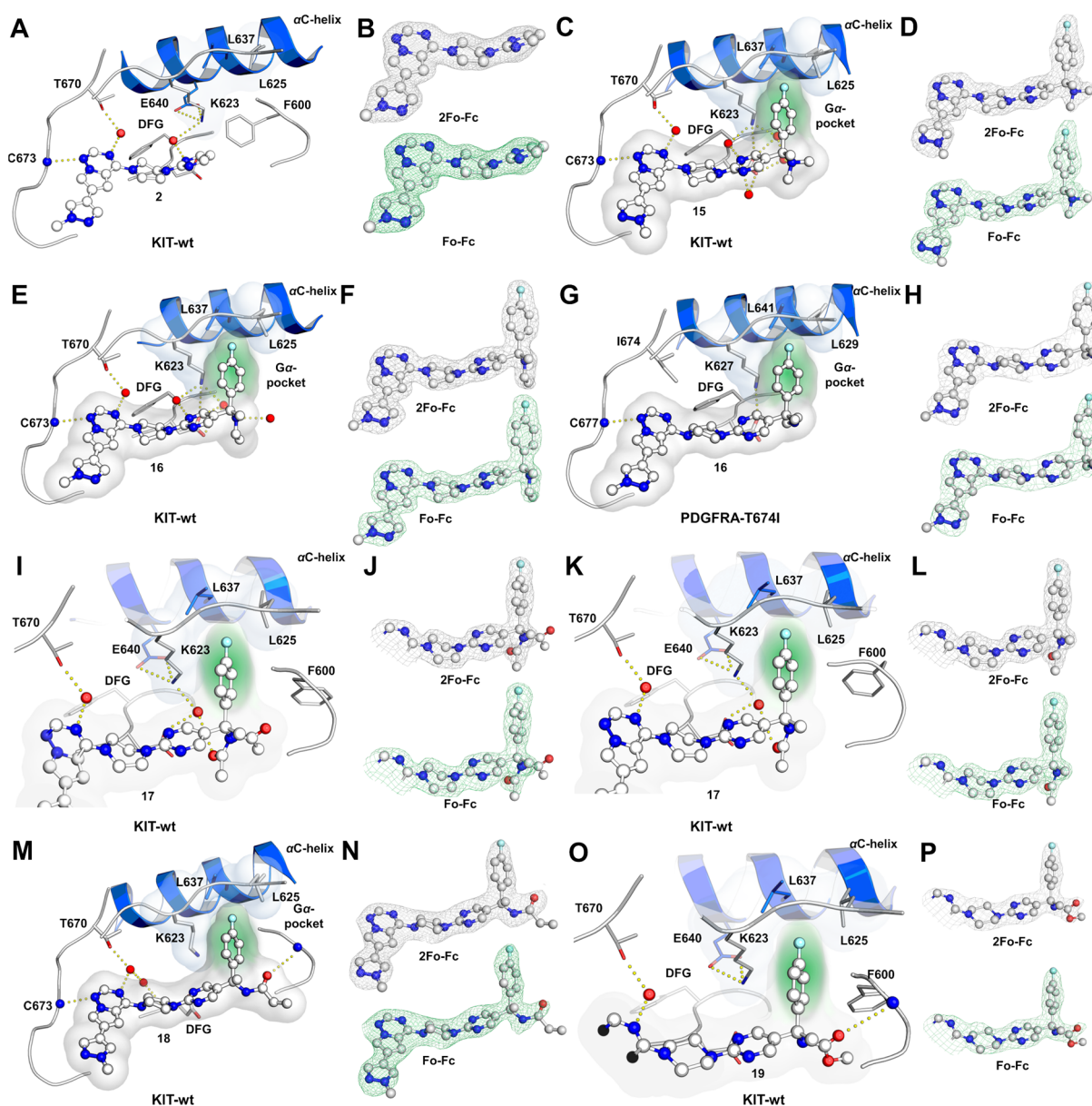
Supplementary Figure 3. Simplified representation of bifunctional degrader exemplified with a proteolysis-targeting chimera (PROTAC) approach. A PROTAC consists of an E3 ligase and a protein of interest (POI) ligand connected by a linker. Upon binding of both the ligase and the POI, a tertiary complex is formed that brings the target into close proximity to the ligase, marking it for ubiquitination. After (poly)ubiquitination, the target is degraded via the proteolytic pathway.⁷⁴



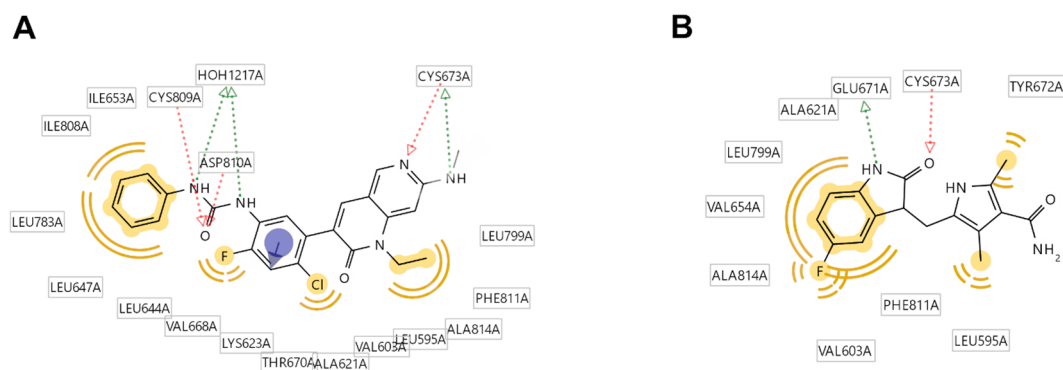
Supplementary Figure 4. Landscape of kinase inhibitors that are or were currently in clinical trials as 2nd – 4th line treatment for imatinib-resistant or advanced GIST. R, randomized.



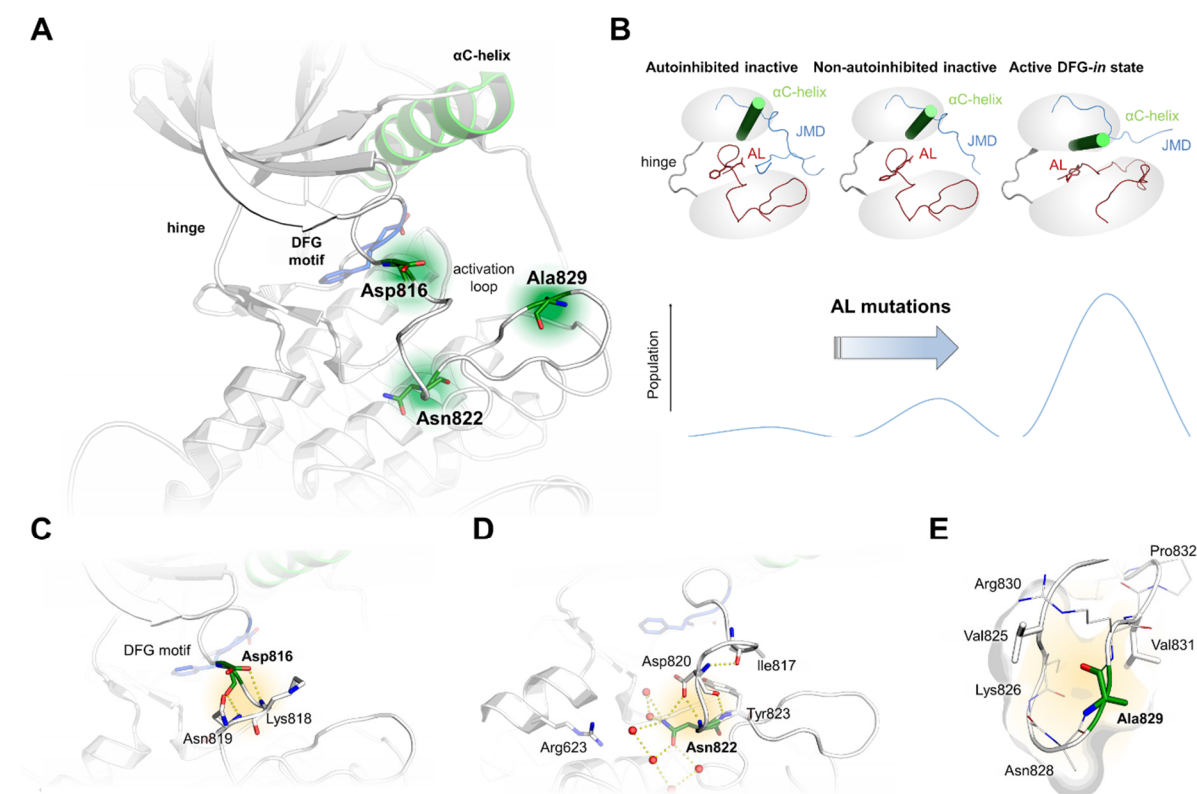
Supplementary Figure 5. Pharmacophore models of avapritinib in PDGFRA-T674I (A), KIT-wt (B) and KIT-T670I (C). The pharmacophore models were generated in LigandScout (v. 4.4.8). The figure is modified based on Teuber & Schulz, *et al.* (2024).⁴⁸



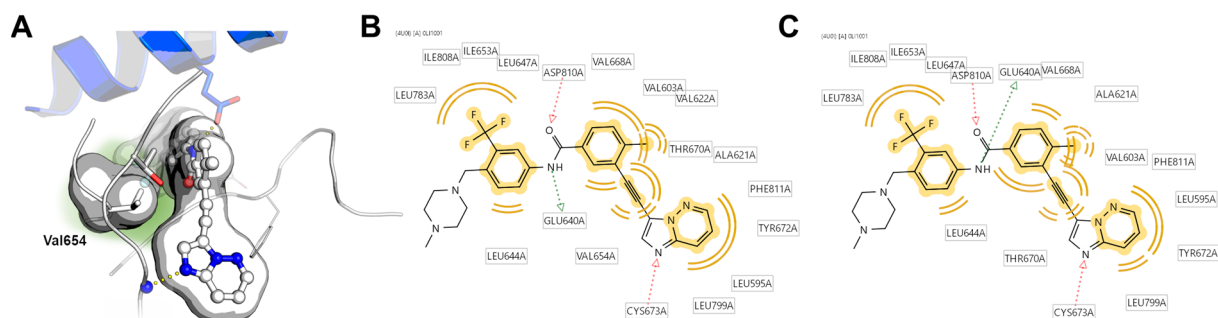
Supplementary Figure 6. Crystal structures of 2, 15-19 bound to KIT and PDGFRA variants. (A-B) Complex structure of **2** bound to KIT-wt (A) with corresponding 2Fo-Fc and Fo-Fc electron density maps (B); PDB-ID: 8PQA. (C-D) Complex structure of **15** bound to KIT-wt (C) with corresponding 2Fo-Fc and Fo-Fc electron density maps (D); PDB-ID: 8PQB. (E-F) Complex structure of **16** bound to KIT-wt (E) with corresponding 2Fo-Fc and Fo-Fc electron density maps (F); PDB-ID: 8PQC. (G-H) Complex structure of **16** bound to PDGFRA-T674I (G) with corresponding 2Fo-Fc and Fo-Fc electron density maps (H); PDB-ID: 8PQI. (I-L) Complex structure of **17** bound to KIT-wt (I, K) with corresponding 2Fo-Fc and Fo-Fc electron density maps (J, L) in different asymmetric units of the crystal structure; PDB-ID: 8PQD. (M-N) Complex structure of **18** bound to KIT-wt (M) with corresponding 2Fo-Fc and Fo-Fc electron density maps (N); PDB-ID: 8PQE. (O-P) Complex structure of **19** bound to KIT-wt (O) with corresponding 2Fo-Fc and Fo-Fc electron density maps (P); PDB-ID: 8PQF. The $|2Fo-Fc|$ - (r.m.s.d. = 1.0) and $|Fo-Fc|$ omit (r.m.s.d. = 2.8) electron density maps were shown at the respective r.m.s.d values. The figure is modified based on Teuber & Schulz, *et al.* (2024).⁴⁸



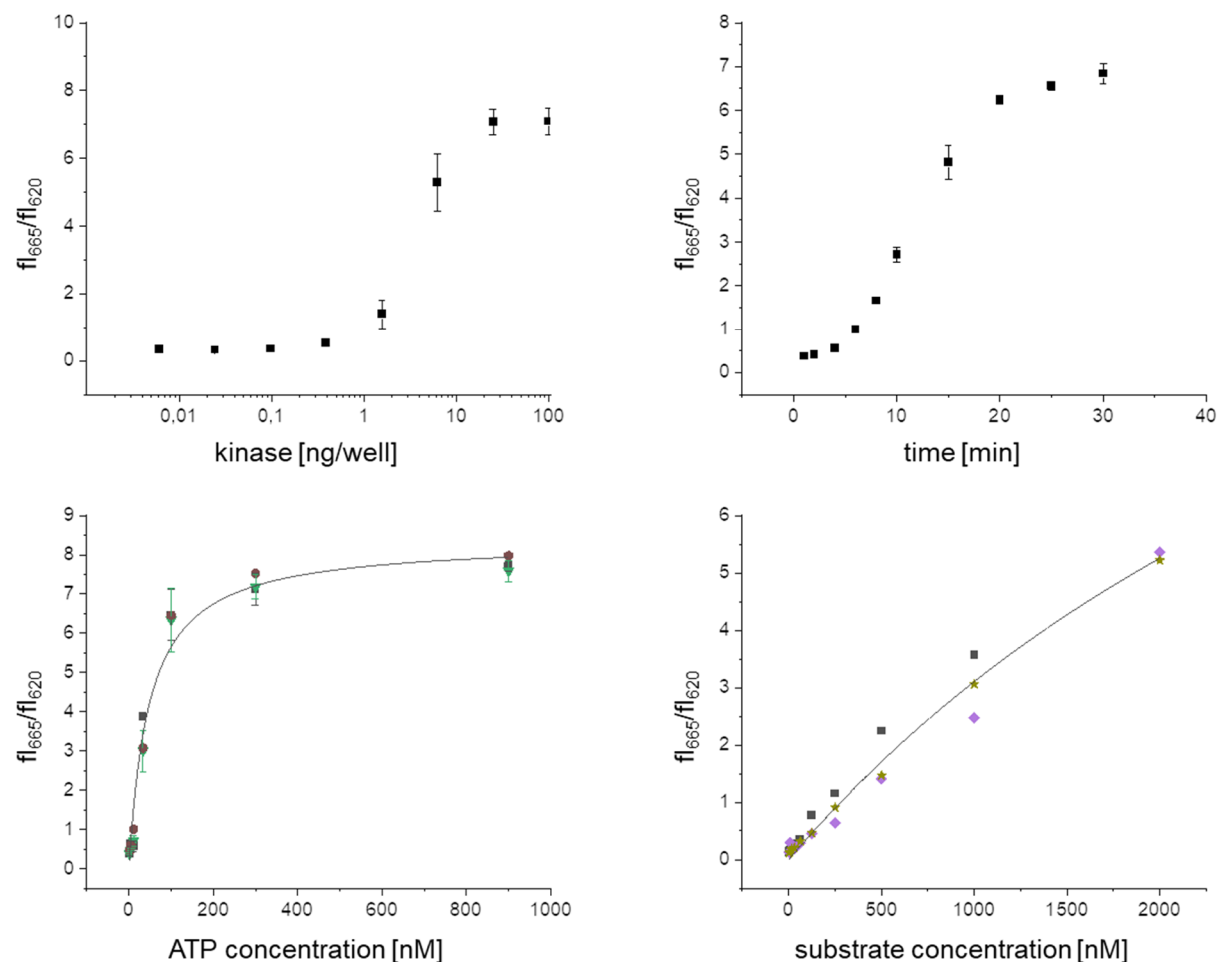
Supplementary Figure 7. Pharmacophore model of ripretinib (**A**) and sunitinib (**B**) in KIT-wt. The models were generated using LigandScout (v.4.4.).



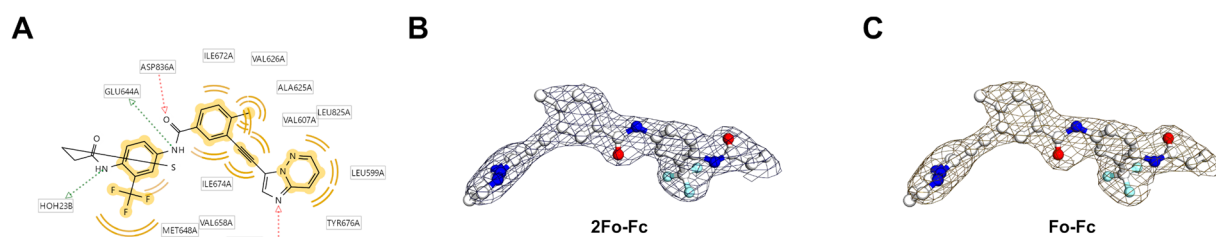
Supplementary Figure 8. Postulated influence upon specific AL mutations in KIT. (**A**) Positions of AL mutations within the KIT kinase domain (highlighted in green). (**B**) Overview of kinase conformations including postulated shift of the conformational equilibrium upon AL mutations. (**C-E**) Interactions of Asp816 (**C**), Asn822 (**D**) and Ala829 (**E**) in a wt-scenario stabilizing the inactive kinase conformation. H-bonds are shown as yellow dotted lines. Analysis was based in particular on PDB ID: 6MOB.



Supplementary Figure 9. Analysis of the influence of KIT-V654A on ponatinib binding. (A) Binding mode of ponatinib in KIT-wt (PDB ID: 4U0I). (B) Pharmacophore model (generated in LigandScout) of ponatinib in KIT-wt (PDB ID: 4U0I). (C) Pharmacophore model (generated in LigandScout) of ponatinib in modeled KIT-V654A (based on PDB ID: 4U0I).



Supplementary Figure 10. Curves for the identification of HTRF assay conditions for KIT-T670I. Graphical representation of the plots for the kinase titration (A), the enzyme kinetic study (B), the determination of the ATP (C) and substrate (D) K_M . (A) Plot of the kinase titration; suitable amount of kinase equals 80 % of the maximum value. (B) Plot for the reaction kinetic; the ideal reaction time is within the linear part featuring an $R^2 > 0.99$. (C,D) Plot of the ATP/substrate concentration. The K_M values were determined in Origin using the MICHAELIS-MENTEN fit. Details regarding the set up for PDGFRA-T674I can be found in the master thesis of Maria Beerbaum.²⁵⁰



Supplementary Figure 11. (A) Pharmacophore model (generated in LigandScout) of **27** in PDGFRA-T674I. |2Fo-Fc|- (B, r.m.s.d. = 1.0) and |Fo-Fc| omit (C, r.m.s.d. = 2.8) electron density maps of **27** bound to PDGFRA-T674I (PDB ID: 9GZH).

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Schulz, Tom

Name, Vorname
(Surname, first name)

Matrikel-Nr.
(Enrolment number)

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From Structural Plasticity to the Development of Next-Generation Inhibitors to

Overcome Drug-Resistant GIST

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