Chemical probes for understudied kinases: challenges and opportunities

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ABSTRACT

Over twenty years after the approval of the first-in-class protein kinase inhibitor imatinib, the biological function of a significant fraction of the human kinome remains poorly understood while most research continues to be focused on few well-validated targets. Given the strong genetic evidence for involvement of many kinases in health and disease, the understudied fraction of the kinome holds a large and unexplored potential for future therapies. Specific chemical probes are indispensable tools to interrogate biology enabling proper preclinical validation of novel kinase targets. In this Perspective, we highlight recent case studies illustrating the development of high-quality chemical probes for less-studied kinases and their application in target validation. We spotlight emerging techniques and approaches employed to the generation of chemical probes for protein kinases and beyond and discuss the associated challenges and opportunities.

KEYWORDS

Protein kinase inhibitors; chemical probes; understudied kinome; target validation; hit identification; covalent inhibitors; PROTACs.

INTRODUCTION

The advent of the genomic era raised expectations for the identification of a large number of novel targets to be explored in drug discovery.[1–3] However, these potential new targets did not quickly result in an increase in new medicines, as pharmaceutical companies faced a decrease in the number of drug approvals, especially in the first decade of this century. The attrition rate in the late clinical trial stages were very high, reaching approximately 75% at phase 2 between the years 2005 and 2009.[4] Among several possible reasons for clinical termination (commercial, operational, safety, strategy), the lack of efficacy was the most important factor affecting the success rate in phases 2 and 3. Indeed, lack of efficacy was responsible for approximately half of all phase 2 and 3 clinical terminations in the years 2013-2015.[5] Among the reasons behind failure to achieve clinical efficacy are insufficient preclinical target validation and incomplete understanding of molecular mechanism(s) driving the pathological conditions.[5–7]

Target selection is a critical and committed step made early in drug discovery. Robust preclinical validation of the involvement of the target in health and disease minimizes setbacks at a later stage of drug discovery.[4,8,9] Selection of the highest-quality target possible is a difficult task, but vital to reduce attrition rates and to contain costs.

Protein kinases are one of the most important protein families in human physiology. They are directly or indirectly involved in all cellular biochemical pathways. There are over 500 protein kinases encoded in the human genome (called the "kinome")[10] and currently more than 65 kinase inhibitors are approved by the FDA (for an up-to-date compilation, see Ref.[11]), mainly for targeted cancer therapy although

other indications are successively gaining importance.[12] Given that the first protein kinase inhibitor, imatinib (Gleevec), was approved only 20 years ago[13,14], protein kinase inhibitors represent a remarkable success story in drug discovery.[15] A literature analysis in 2010 showed that most of the kinome was underexplored, with approximately 25% of the protein kinases having no known function and about 50% not being well characterized.[16] Since then there has been significant activity determining protein kinase crystal structures (just over 300 determined to date) and conducting cross-screening of kinase inhibitor libraries[17–19] which has generated much useful information, but the imbalance in understanding protein function has not changed substantially. Notably, poor characterization is not necessarily due to a lack of interest but frequently associated with the unavailability of a suitable high quality pharmacological modulator, a so-called "chemical probe", to study target biology.[20] There is evidence that the understudied fraction of the kinome, now referred to as the “dark kinome”,[21] holds enormous opportunities for discovering new high quality drug targets.

Chemical probes are valuable and powerful tools for target validation. By definition, chemical probes are small-molecules able to selectively modulate functions of a protein (or a defined set of proteins), allowing researchers to address mechanistic questions regarding the respective molecular target *in vitro* (biochemical and/or cell-based) or *in vivo*. The aim is to establish a link between target modulation and specific phenotypes.[22,23] Initial findings on target biology are typically derived from genetic knockdown or knockout models, which have recently become even more powerful with the advent of the CRISPR-Cas system. However, it has frequently been overlooked in the past that hypotheses derived from such genetic models require complementary validation since pharmacological modulation may not reproduce the observed

phenotype, which may only be discovered at an advanced stage of a drug discovery program. Thus, the importance of preclinical target validation using chemical probes is increasingly recognized.[24] Chemical probes are complementary to genetic target validation approaches and offer a variety of advantages including high temporal resolution, reversibility, association of the phenotype with a specific target function rather than with its presence and ultimately, the demonstration of target druggability.[22]

Although chemical probes have long been used to study biology, proper validation of chemical probes has frequently been neglected in the past resulting in piles of corrupted data being generated and published.[25,26] This led chemical biologists and medicinal chemists to define a set of criteria with which chemical probes should comply to be useful to investigate target biology. At the same time, strategic private-public partnerships were established to evaluate and generate chemical probes and make them available via open science portals like the Chemical Probes Portal (https://www.chemicalprobes.org/), the probes portal of the Structural Genomics Consortium (https://www.thesgc.org/chemical-probes), or Boehringer Ingelheim's OpnMe initiative (https://opnme.com/). According to the aforementioned guidelines, high-quality chemical probes for protein kinases should meet the following criteria: biochemical potency against the kinase target below 100 nM (at a K_m of ATP), cellular potency below 1 μM and a ≥30-fold selectivity against other kinases apart from closely-related paralogues.[22] Moreover, a high quality chemical probe should be accompanied by a well-characterized close structural analog devoid of on-target activity to be employed as a negative control.[27] The latter serves to corroborate the relationship between the observed phenotype and the on-target effect, a caveat, however, being that negative controls also tend to lose off-target activity.

Due to the importance of exploring the understudied fraction of the kinome, significant efforts have been directed towards the understanding of such kinases during the last few years. In the first part of this perspective, we highlight a selection of recent studies showcasing the development of chemical probes and the impact they can have for less-studied kinases as well as kinases for which a specific inhibitor was not previously available. In the second part, we discuss the recent advances, novel approaches and strategies in generation of chemical tools and probes for protein kinases.

RECENT ADVANCES IN THE DEVELOPMENT OF CHEMICAL PROBES FOR LESS-STUDIED PROTEIN KINASES

AAK1 and BMP2K/BIKE

The two closely related human kinases AAK1 (adaptor protein 2-associated kinase 1) and BMP2K/BIKE (BMP-2-inducible kinase) share 50% of overall sequence identity and 74% in their kinase domains.[28] AAK1 and BIKE are members of the Numb-associated family of kinases (NAKs) which also includes two other homologs, namely GAK (cyclin G-associated kinase), and MPSK1 (myristoylated and palmitoylated serine/threonine kinase 1, also called STK16).[28] These kinases have been associated with varied diseases such as neuropathic pain, amyotrophic lateral sclerosis and the endocytosis of Rabies viruses in host cells.[29–31] Recently, AAK1 and BMP2K have been of interest for COVID-19 therapy using baricitinib,[32,33] a JAK inhibitor which Sorrell *et al*. identified as also binding AAK1, BMP2K and GAK,[28] and which may therefore hinder viral entry into cells as well as reducing the inflammatory response to infection.

The first crystal structures of AAK1 and BMP2K/BIKE bound to small molecules were released by Sorrell and co-workers in 2016,[28] the same year that chemical starting points for probe development identified by broad screening of the PKIS (Published Kinase Inhibitor Set) were published.[19] The 3-acylaminoindazole scaffold of initial hit compound **1** (Figure 1, IC_{50} = 220 nM against AAK1) was modified to obtain structure-activity relationship (SAR) data, showing the critical importance of the cyclopropyl carboxamide group to retain low nanomolar enzymatic potency.[34] An X-ray crystal structure of the close analog **2** (Figure 1) with BMP2K (PDB code: 5IKW) revealed the key interactions of the compound in the ATP-binding pocket.[35] Modification of the sulfonamide *S*-substituent delivered the AAK1/BMP2K chemical probe SGC-AAK1-1 (**3**, Figure 1) featuring an *N,N*-diethylamino moiety. This compound inhibited AAK1 with an IC_{50} = 270 nM in an enzymatic activity assay and showed a K_i = 9.1 nM and 17 nM for AAK1 and BMP2K, respectively, in a binding-displacement assay. A NanoBRET assay[36] demonstrated cellular target engagement with an $IC_{50} \approx$ 240 nM for **2** and **3** on AAK1 while potencies on BMP2K were slightly lower (IC_{50} = 600 nM and 1.5 µM, respectively).[35] Cross-screening against a panel of 406 kinases (DiscoverX KINOMEscan[37]) confirmed a narrow selectivity profile of **3** having few off-targets at 1 µM (S(10) (1 µM) = 0.02), the selectivity score S(N) being calculated as the number of kinases below N% of control divided by the total number of kinases tested.[38] In a binding assay, only three kinases were bound with a K_D within 30-fold of AAK1 (RIOK1, RIOK3 and PIP5K1C).[34,35] Opening the cyclopropyl carboxamide in **2** yielded 2-methylpropionamide analog **4** (SGC-AAK1-1N, Figure 1), which was devoid of significant AAK1 or BMP2K inhibitory activity and can be used as the negative control.

Dual chemical probe **3** inhibited AAK1-dependent phosphorylation of adaptor related protein complex 2 subunit mu 1 (AP2M1), a known AAK1 substrate, and, for the first time, demonstrated that AAK1 is able to negatively regulate the canonical (β-catenin-dependent) WNT signaling pathway by stimulating clathrin-mediated endocytosis of low-density lipoprotein receptor-related protein 6 (LRP6). These results constitute progress in understanding the role of the WNT pathway in different cancer and neurological disorders. To date, **3** is the only high-quality AAK1/BMP2K chemical probe available.[34,35]

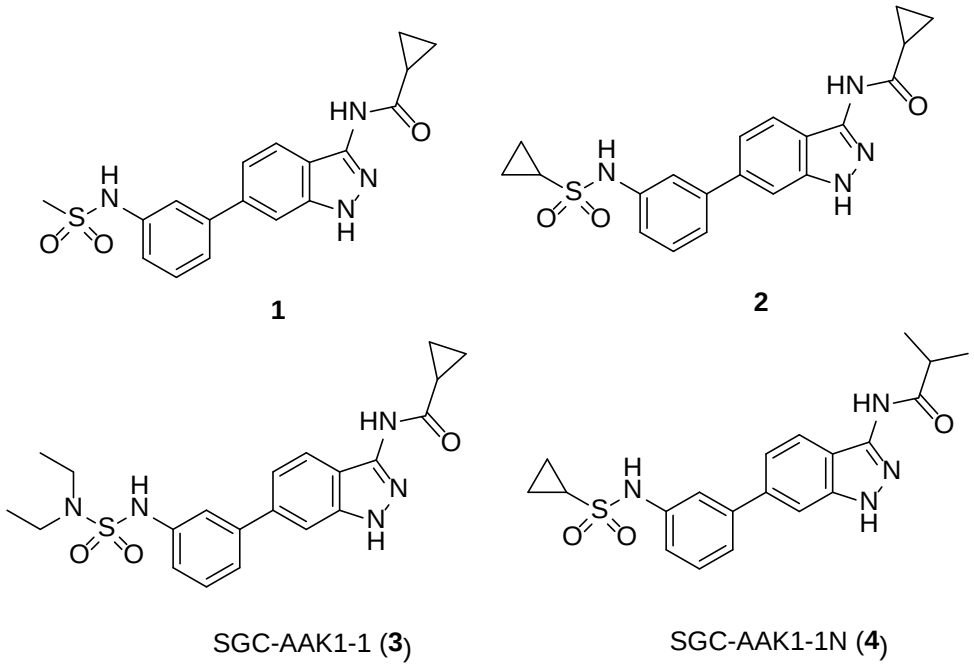


*Figure 1: Molecular structures of AAK1/BMP2K inhibitors: PKIS hit **1** and the close analog **2**, chemical probe **3** and its negative control **4**.*

GAK

Cyclin G-associated kinase (GAK), another member of the NAK subfamily, is a serine/threonine kinase initially found in association with cyclin G.[39] Studies revealed that GAK is present in the cytoplasm and nucleus of the cells[40] and its overexpression is involved in the proliferation and survival of osteosarcoma cells.[41] Interestingly, GAK also

showed an involvement in regulation of hepatitis C virus entrance into host cells.[42] By screening a small library of kinase inhibitor-like compounds followed by structural optimization of screening hit **5** (Figure 2), Kovackova and co-workers were able to identify isothiazolopyridine derivatives exemplified by **6** (Figure 2) as GAK inhibitors with nanomolar binding affinities.[43] Additional optimization efforts yielded a potent compound with broad-spectrum antiviral activity.[44] However, due to the off-target activity on other kinases, such as KIT, PDGFRB, FLT3 and MEK5, these compounds did not meet chemical probe criteria.

A screen of a larger set of protein kinase inhibitors (Published Kinase Inhibitor Set 2, PKIS2)[45] performed by Drewry *et al.* was the source for additional hits, with compounds based on a 4-anilinoquinoline (**7**, Figure 2) or 4-anilinoquinazoline (**8**, Figure 2) scaffold demonstrating inhibitory activities in the nanomolar range. Computational studies including a WaterMap analysis[46] suggested displacement of energetically unfavorable water molecules from the GAK binding site as an important contribution to potency. Initial SAR optimization yielded compound **9** (Figure 2) with high potency (K_i = 0.54 nM) and promising selectivity (>50,000-fold) within the NAK family.[47] By employing a bioisosteric replacement strategy, compound **10** (SGC-GAK-1, Figure 2) carrying a 6-bromo substituent at the quinoline ring was identified with high affinity for GAK (K_d = 4.5 nM by isothermal titration calorimetry, ITC) and remarkable selectivity (>50-fold) over 400 kinases (Discover X), as well >16,000-fold selectivity over the other three NAK family members. As the next most potently inhibited kinase, RIPK2 was further assessed along with GAK in a cellular NanoBRET format. GAK target engagement by **10** was favorable (IC_{50} = 120 nM), however, the compound showed only 3-fold selectivity over RIPK2 (IC_{50} = 360 nM). *N*-Methylation at the secondary amino group and replacement of

the 6-bromide by trifluoromethyl abrogated GAK inhibitory activity, yielding negative control **11** (SGC-GAK-1N, Figure 2). These two compounds were proposed to be used in combination with compound **12** (Figure 2) to study GAK biology, the latter being a potent RIPK2 inhibitor with low activity against GAK in both biochemical and cellular experiments.[48] Subsequent structural modification at the 4-anilinoquinoline scaffold has been performed towards development of an *in vivo* GAK chemical probe.[49]

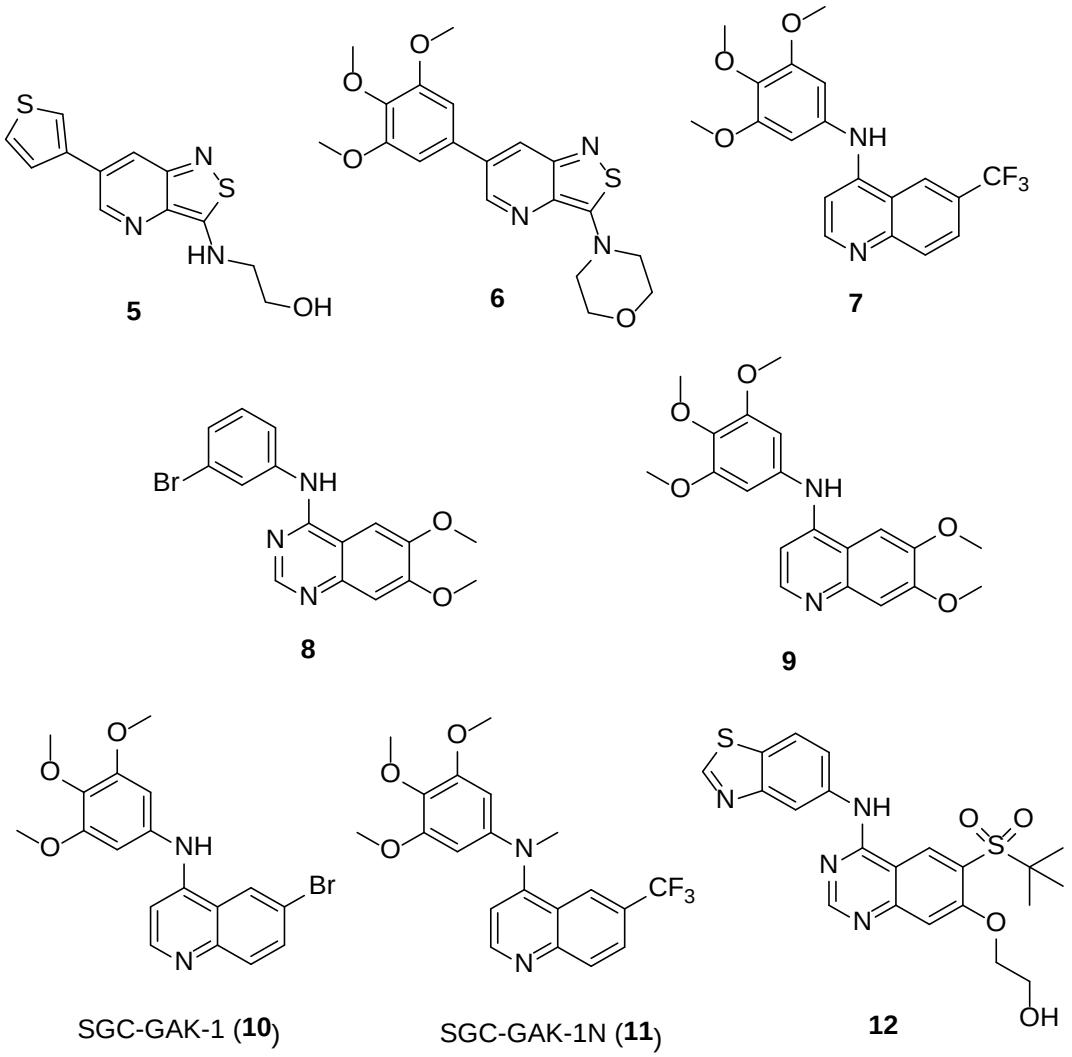


*Figure 2: Molecular structures of GAK inhibitors (**5**, **6**, **7**, **8** and **9**) and the GAK chemical probe package comprising probe **10**, negative control **11** and the RIPK2 inhibitor **12**.*

ALK4, ALK5 and ALK2

The activin receptor-like kinase 5 (ALK5), also known as transforming growth factor beta-receptor (TGFBR1), is able to phosphorylate receptor-regulated Smad proteins (Smad2 and Smad3), which can generate a heterotrimeric complex with Smad4 translocating to the nucleus to regulate gene transcription.[50] Its closest relative is ALK4, which also phosphorylates Smad2 and Smad3, whereas ALK1/2/3/6 phosphorylate Smad1/5/8. Together, all ALKs (ALK1-7; not to be confused with the anaplastic lymphoma kinase ALK) comprise a subfamily of receptor serine-threonine kinases (RSTK), being part of the tyrosine kinase-like branch of the kinome.[51] *In vivo*, ALK5 knockdown reduced plasticity of axonal and dendritic cells with concomitant decrease in neuronal function recovery, suggesting a pivotal role of ALK5 in mediating neuronal plasticity and neurological recovery.[52] Furthermore, ALK5-mediated TGFβ signaling is involved in endometrial homeostasis, tumor suppression, and postpartum endometrial regeneration according to murine ALK5 knockout models.[53]

In 2016, researchers from Takeda identified compound **13** (Figure 3) with suitable ALK5 potency but poor pharmacokinetic properties.[54] After optimization of binding to hydrophobic sub-pockets as well as increasing metabolic stability, compound **14** (Figure 3) was obtained with selectivity over 50 other kinases and an improved pharmacokinetic profile. However, due to cardiovascular toxicity observed in rats, the drug discovery program was discontinued.[54] Nevertheless, **14** may be useful as a chemical tool to explore ALK5 biology.

Inspired by the promising profile of **14**, Hanke *et al.* performed further characterization to validate **14** (TP-008) as a chemical probe.[55] Screened at 1 µM against a panel of 469 wild-type and mutant kinases (DiscoverX) no off-targets except the closely

related ALK4 were identified. ALK4 and ALK5 potencies in a kinase assay at 1 μM of ATP were very similar (IC_{50} = 113 nM for ALK4 and 343 nM for ALK5) which is not surprising considering the high sequence similarity (~70% overall and >90% in the ATP binding site) of those two gene products. Since previous modeling studies suggested the pyridine nitrogen to be essential for hinge interaction, replacement of this atom by carbon gave negative control **15** (Figure 3) devoid of inhibitory activity. Cellular target engagement was investigated in HEK293 cells using a SMAD2/3-responsive luciferase-based reporter assay, where cells were stimulated with either TGF-β1 for ALK5 or activin A for ALK4, respectively. Compound **14** showed triple-digit nanomolar activities (IC_{50} = 245 nM for ALK5 and 524 nM for ALK4, respectively) while the negative control (**15**) remained inactive (up to 10 μM concentration). Furthermore, and in contrast to compound **15**, probe **14** at 1 μM suppressed SMAD2/3 phosphorylation as shown by immunoblotting. Together, these results validated compounds **14** and **15** as a chemical probe package with dual activity to interrogate ALK4 and ALK5 biology.[55]

Diffuse intrinsic pontine glioma (DIPG), a rare pediatric cancer, and fibrodysplasia ossificans progressiva (FOP), a very rare connective tissue disease, are among the abnormalities linked with mutations and malfunction in the ALK2 signaling pathway.[56,57] In the framework of an industry-academic open science initiative,[58] promising advances have been made towards the generation of chemical probes for this enzyme. Compound **16** (Figure 3) was a good starting point in a pursuit of expanding the SAR of the series, improving potency, selectivity, and pharmacokinetic properties. 2-fluoro-6-methoxybenzamide derivatives (such as **17**, Figure 3) demonstrated suitable biochemical (IC_{50} = 5 nM) and cellular potency (NanoBRET IC_{50} = 52 nM) as well as remarkable selectivity between ALK2 and ALK5 (429-fold), besides promising brain

penetrance properties. Additionally, those derivatives had comparable potencies against ALK2 mutants (R206H, G328V and R258G), which are clinically important in certain patient populations.[59]

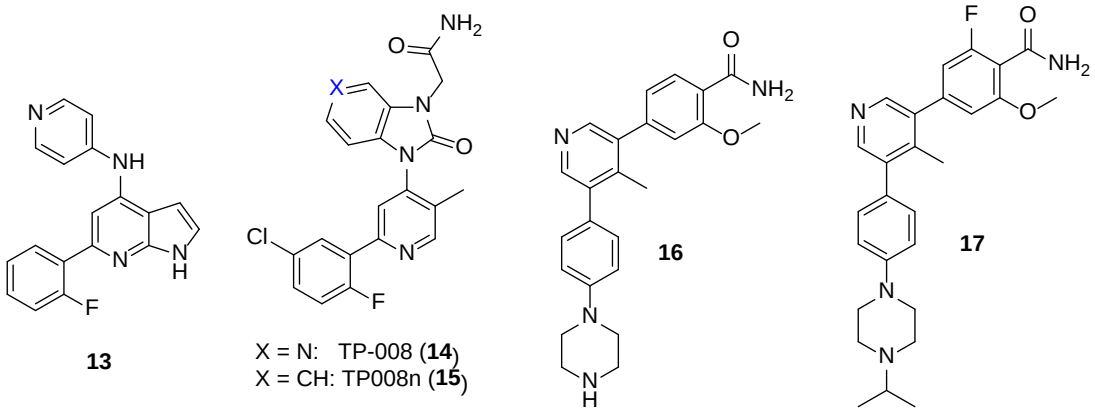


*Figure 3: Molecular structure of ALK4/ALK5 inhibitors: initial hit **13**, chemical probe **14** and its negative control **15** and the ALK2 inhibitors **16** and **17**.*

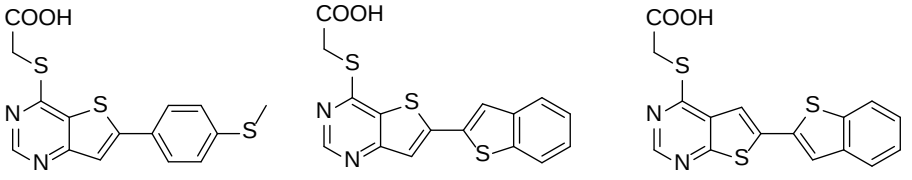
STK17B

The death-associated protein kinase (DAPK) family comprises five proteins: DAPK1-3 and the less-related DRAK1 and DRAK2 (DAPK-related apoptosis-inducing protein kinase 1 and 2, also named STK17A and STK17B, respectively). The DAPK family resides within the calcium/calmodulin-dependent kinase group (CaMK).[60] A correlation between STK17B overexpression and different kinds of tumors has been demonstrated. Its downregulation in MEC-1 cells of chronic lymphocytic leukemia increased cell viability and proliferation, suggesting that STK17B may play an important regulatory role in the progression of the disease.[60,61]

In a search for selective and cell-permeable STK17B inhibitors, the PKIS2[45] was a source for identification of the thieno[3,2-*d*]pyrimidine derivative **18** (Figure 4), showing 60-fold selectivity between STK17B and STK17A (K_d of 3.8 nM and 230 nM, respectively) and a narrow overall kinome selectivity.[62] The co-crystal structure (PDB code: 6Y6F)

confirmed the pyrimidine N1 atom of **18** as the hinge binder and salt-bridges formed between the carboxylate and the catalytic lysine (Lys62) as well as Arg41 bordering the P-loop, the latter interaction inducing a significant conformational change when compared to the apo structure. However, the compound showed poor stability in mouse liver microsomes ($t_{1/2}$ < 5min), which was attributed to the methyl thioether moiety, necessitating further optimization. Three different regions of the molecule, i.e. the carboxylic acid group, heterocyclic core and 6-aryl substituent, were subject to modification to define SAR. Initial profiling was performed using the KinaseSeeker split luciferase binding assay[63] and NanoBRET. The observed SAR was relatively narrow and confirmed the requirement for the α-thioacetic acid side chain, with no sulfur replacements being tolerated. Analog **19** (Figure 4) in which the phenyl ring had been replaced by a 2-benzothiophene moiety, demonstrated high potency (IC_{50} = 34 nM; K_d = 5.6 nM) and cellular target engagement (NanoBRET IC_{50} = 190 nM) while maintaining high selectivity against STK17A. At the same time, the compound featured improved microsomal stability (>50% remaining after 30 min). A co-crystal structure (PDB code: 6Y6H) confirmed similar binding interactions as observed for the parent inhibitor. In comparison with non-selective inhibitors, binding to compound **19** allowed for the mentioned atypically folded P-loop conformation. Although the latter may explain the clean kinome profile, selectivity against STK17A with a virtually identical ATP binding pocket is difficult to rationalize. Therefore, molecular modeling studies including molecular dynamic simulations in microsecond scale were performed. On this basis, the authors proposed that residues outside the ATP site favor a higher flexibility of STK17B compared to STK17A to adopt the P-loop conformation enabling the Arg41 salt bridge, thereby promoting selectivity. In a panel of over 400 human kinases (DiscoverX), **19** had

<10-fold selectivity against only two off-targets (CAMKK1 and CAMKK2). However, high selectivity against these kinases could be shown in an enzymatic and a NanoBRET assay. The thieno[3,2-*d*]pyrimidine regioisomer **20** (Figure 4) proved to be >100-fold less active while having a clean kinome profile suggesting it as a suitable negative control. The steep drop in activity was rationalized by means of density-functional theory (DFT) calculations showing a significantly reduced basicity of the pyrimidine N1 of **20**, which is critical for hinge interaction. Hence, probe **19** (SGC-STK17B-1) together with its negative control **20** (SGC-STK17B-1N) were selected as a probe set which will help to explore STK17B biology.[62]



18 SGC-STK17B-1 (**19**) SGC-STK17B-1N (**20**)

*Figure 4: Molecular structures of STK17B inhibitors: initial hit **18**, chemical probe **19** and its negative control **20**.*

NIK

NF-κB inducing kinase (NIK or MAP3K14) is an understudied non-RD (non-arginine-aspartate) kinase lacking the arginine residue preceding the catalytic aspartate (Asp517) in the conserved HRD motif (HGD in NIK). As a key regulator of the noncanonical NF-κB pathway, NIK is involved in controlling the expression of various proteins including inflammatory cytokines and chemokines.[64] Genetic studies indicated that NIK has an important role in the immune system regulating T cell metabolism and effector function in antitumor immunity.[64,65] Importantly, NIK is thought to promote the survival of autoreactive B cells via the noncanonical NF-κB pathway in response to BAFF

receptor (B cell activating factor receptor) activation suggesting a key role in autoimmune disorders. Therefore, further interrogation of NIK function is important to decipher the molecular basis of immune and inflammatory diseases and certain types of cancer.[66]

In initial efforts toward NIK-selective inhibitors, researchers from Genentech discovered benzoxepine derivatives exemplified by **21** (Figure 5, K_i = 1.5 nM) by merging fragment hit **22** with known NIK inhibitor **23** (Figure 5)[67] targeting the small pocket beyond the methionine gatekeeper residue via the tertiary propargyl alcohol moiety.[68] As observed in the co-crystal structure of **21** and murine NIK (PDB code: 6G4Y), the compound adopts a type I ½ binding mode with the primary amide forming the key hydrogen bond to the hinge region. Despite the high potency and selectivity of several analogs from this earlier series, *in vivo* stability was not ideal for pharmacological studies. Since SAR analyses and crystal structures suggested that replacement of the oxepine ring from the prototype scaffold would be tolerated, a scaffold hopping approach was pursued to design analogs with better synthetic accessibility and opportunities to explore chemical space.[69] The first compound set with a central pyridine ring suffered from glutathione (GSH) adduction upon incubation with human hepatocytes (HH). Replacement by a 1,3-disubstituted benzene ring (exemplified by compound **24**, Figure 5) to reduce alkyne electrophilicity minimized susceptibility to GSH conjugation while maintaining good potency in biochemical and cell-based experiments (K_i = 0.23 nM and IC_{50} = 29 nM in a NF-κB reporter gene assay). However, enzymatic hydrolysis of the essential primary amide remained a concern. To overcome this issue, the steric and electronic environment of the amide was modulated by varying the heteroaryl scaffold and its substituents. This led to optimized 4-methoxy-2-

amidopyridine derivative **25** (NIK SMI1, Figure 5), which maintained potent biochemical and cellular activity (K_i = 0.23 nM and IC_{50} = 34 nM in the same assays) at improved clearance in human liver microsomes and hepatocytes (6.5 and 5.8 mL/min/kg, respectively). Consistent with selective blockage of the non-canonical NF-κB pathway, the compound inhibited the nuclear translocation of p52 (IC_{50} = 70 nM) without influencing the canonical NF-κB nuclear translocation marker RELA up to 20 µM concentration. The compound further showed a suitable *in vitro* and *in vivo* PK profile including moderate to high oral bioavailability in several species and it inhibited BAFF-induced B cell survival *in vitro* and *in vivo*.[69]

The selectivity profile in a panel of 222 kinases (ThermoFisher Scientific SelectScreen) was subsequently reported revealing only KHS1, LRRK2 and PKD1 as off-targets at 1 µM concentration.[70] However, a favorable selectivity window against these kinases (216-, 1077- and 326-fold against KHS1, LRRK2 and PKD1, respectively) was confirmed by K_i determination. The researches further used **25** as a chemical tool and revealed that selective NIK inhibition affects various pathways involved in systemic lupus erythematosus (SLE), improving disease biomarkers, kidney function and survival.[70] Together, those data suggest NIK inhibition as an encouraging strategy for SLE treatment.

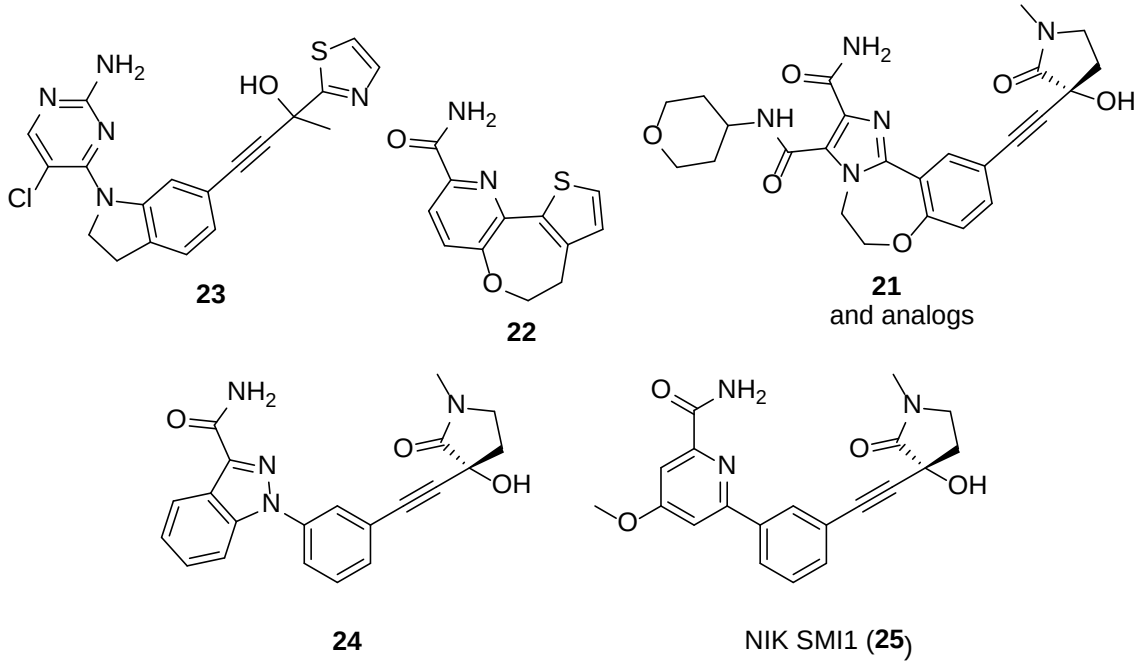


*Figure 5: Molecular structure of prototype NIK inhibitor **23** and fragment hit **22** merged to obtain NIK inhibitors exemplified by **21**, and optimized follow-up compounds **24** and **25**.*

DCLK1

The serine/threonine kinase DCLK1 (doublecortin-like kinase 1) is a microtubule-associated protein. Initially described as a brain-specific protein with an important role in neurogenesis, its functions beyond the CNS especially in cancer are increasingly becoming apparent.[71] DCLK1 is now used as a marker for tuft cells in the gastrointestinal tract and has further been suggested as a marker for certain cancer stem cells and as an anticancer target, e.g. in pancreatic, colon, and renal cancers. Moreover, targeting DCLK1 has been proposed as a strategy to increase anti-tumor immunity.[72,73] Since details on the functional role of DCLK1 remain obscure, chemical tools are fundamental to the validation of DCLK1 as a potential therapeutic target. In 2016, Patel and collaborators published the first X-ray structures of the DCLK1 kinase domain bound to

small molecules (PDB codes: 5JZN and 5JZJ), paving the way for structure-guided medicinal chemistry efforts.[71]

Recently Ferguson *et al.* described the discovery of a highly potent and selective DCLK1 probe based on a 5,11-dihydro-6*H*-benzo(*e*)pyrimido(5,4-*b*)(1,4)diazepin-6-one scaffold. Development started from structurally related compounds like the LRRK2 inhibitor LRRK2-IN-1 (**26**, Figure 6) and the ERK5 inhibitor XMD8-85 (**27**, Figure 6), which show potent off-target activity on DCLK1.[74] Notably, multi-targeted inhibitors **26** and XMD8-92 (not shown), a close analog of **27**, have previously been used to probe DCLK1 function in different cancers. Optimization was pursued by modification the core-scaffold's *N*-substituents. Replacement of the amide *N*-methyl moiety by a [2,2,2]-trifluoroethyl moiety was key to achieving selectivity against LRRK2, ERK5 and the non-kinase off-target BRD4, giving rise to probe DCLK1-IN-1 (**28**, Figure 6). Investigation of SAR further revealed that small substituents at the scaffold's phenyl ring are not tolerated by DCLK1 leading to negative control DCLK1-NEG (**29**, Figure 6) with an additional methyl group in position 8. Notably, **29** did not show significant binding at 1 µM concentration to any of the kinases in a large KINOMEscan panel.

Chemical probe **28** was fully characterized and used to explore DCLK1 biology.[75] In ITC experiments, this compound bound to recombinant DCLK1 with a K_d = 109 nM and showed IC_{50} = 9.5 nM and 57 nM in a complementary KINOMEscan competition binding assay and a radiometric kinase activity assay (50 µM ATP concentration), respectively. Inhibition of close homolog DCLK2 was also confirmed in the two last-mentioned assay formats (IC_{50} = 31 nM and 103 nM, respectively). Cellular DCLK1 engagement was shown by NanoBRET assays in HCT116 cells (IC_{50} = 279 nM). **28** at 1 µM concentration showed a clean profile in a KINOMEscan panel (489 kinases) as well as in a chemoproteomic

KiNativ profiling panel[76] (*vide infra*) against 250 kinases. Docking studies suggested a type I binding mode, with the aminopyrimidine moiety forming two key hydrogen bonds with the hinge region. The trifluoroethyl group directs toward the hydrophobic back pocket, with a proposed interaction between a fluorine atom and the sulfur of gatekeeper residue Met465, which may explain the selectivity against ERK5 which has a leucine gatekeeper. Pharmacokinetic (PK) profiling in mice revealed favorable properties ($t_{1/2} \approx 2$ h, F = 81%) and good tolerability encouraging the use of **28** as a chemical tool for an *in vitro* and *in vivo* studies.[75]

Experiments with **28** in pancreas adenocarcinoma PATU-8988T and PATU-8902 human cell lines showed that aberrant ERK signaling controls DCLK1 expression and that DCLK1 kinase activity is not critical for viability or transformation of those cells. In addition, after treatment with **28**, transcriptome and proteomics analyses indicated only minor changes. Meanwhile, in culture of DCLK1-expressing patient-derived pancreatic ductal adenocarcinoma (PDAC) organoids, proteins related to cell motility (such as HN1, ZIP8 and FYN) were significantly downregulated in response to **28** and reduced phosphorylation of the actin binding proteins CAP1 and DBN1 was observed.[75]

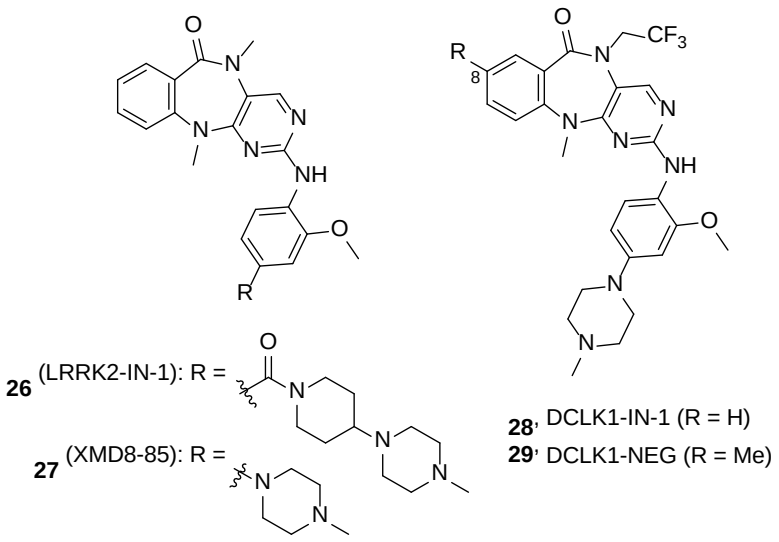


*Figure 6: Molecular structures of DCLK1 inhibitors: LRRK2-inhibtor **26** and ERK5 inhibitor **27** used as starting points to develop high quality DCKL1 chemical probe **28** and negative control **29**.*

TBK1 and IKKε

The two closely related inhibitors of the nuclear factor κB (NF-κB) kinases (IKKs), TBK1 (TANK-binding kinase 1) and IKKε, represent non-canonical IKKs. They play an important and partially overlapping role in regulating innate immune response and a variety of other biological pathways.[77] TBK1/IKKε dysregulation has been linked to cancer, autoimmune disease and inflammation in diabetes and obesity. Several studies have investigated the functional role of TBK1 but the data obtained may be misleading since nonselective inhibitors have been employed. In a search for improved inhibitors Lefranc *et al.* identified the 2-arylamino substituted benzimidazole **30** (Figure 7) as a potent ATP-competitive TBK1/IKKε inhibitor via high-throughput screening of over three million compounds.[78] Aiming to explore the pocket behind the methionine gatekeeper residue, a comprehensive and structurally guided SAR analysis generated compound **31** (Figure 7). In the latter, the benzimidazole's nitrile substituent was replaced by a bulkier *N*-cyclopropylmethyl amide group, which abolished the off-target activity on CDKs

(IC_{50}[CDK9] > 20,000 nM) harboring a larger phenylalanine gatekeeper. Subsequent bioisosteric replacement of the amide by a 1,2,4-oxadiazole ring (**32**, Figure 7) improved cellular permeability. Further optimization included the replacement of the oxadiazole by a 6-dimethylaminopyrimidine and introduction of a methyl group at the methylene bridge connecting the piperazine side chain. This modification created a chiral center, where the (*R*)-enantiomer BAY-985 (**33**, Figure 7) proved to be the eutomer. Compound **33** showed high TBK1/IKKε inhibitory potency (IC_{50} = 2 nM on both enzymes at 10 µM ATP) and a moderate pharmacokinetic profile. Screened at 100 nM against 400 kinases (DiscoverX), inhibitor **33** showed high selectivity against all kinases except YSK4. In contrast, BAY440 (**34**, Figure 7), devoid of the aforementioned methyl group and with an additional chloro-substituent at the pyrimidine C5-position, showed low activity making it a suitable negative control. Despite the high on-target potency, inhibitor **33** manifested only weak antitumor activity in a SK-MEL-2 human melanoma xenograft model. Furthermore, lack of correlation of on-target potency with antiproliferative activity in ACHN cells suggested that effects observed in earlier studies may have resulted from off-target inhibition highlighting the importance of a high-quality chemical probe for further exploration of TBK1/IKKε biology.

In another attempt at developing TBK1 inhibitors, Thomson *et al.* optimized a series of 2,4-diaminopyrimidines from a proprietary library giving rise to GSK8612 (**35**, Figure 7).[79] In a chemoproteomic kinobead selectivity profiling experiment[80] using a mixture of cell lines and tissue extracts to maximize coverage, affinity was determined for 285 kinases. Compound **35** displayed an average pK_d value of 8.0 against TBK1 without off-targets in a 10-fold selectivity window. Only two kinases were identified with an average pK_d ≥ 6.0 including STK17B (average pK_d = 6.2) and closest homolog IKKε

(average pK_d = 6.0) making this molecule a potential tool to study TBK1-specific biology. Affinity and selectivity for TBK1 were rationalized by docking and low micromolar cellular activity was confirmed in Ramos cells by Western blot. Compound **35** also showed similar potency for inhibiting IFN-α release from human PBMCs and IFN-β secretion from dsDNA virus and cGAMP stimulated THP-1 cells.

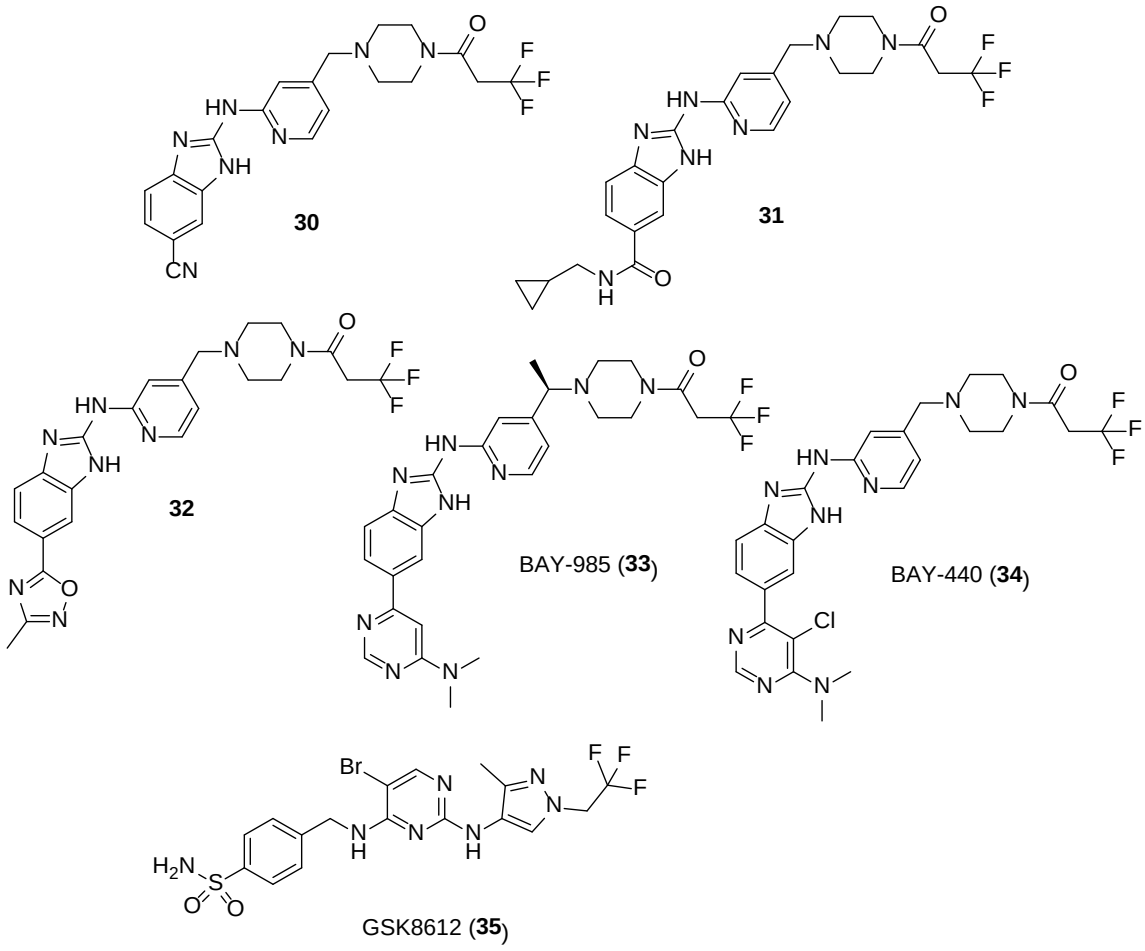


*Figure 7: Molecular structure of TBK1/IKKε inhibitors (**30**, **31**, **32** and **35**) and the TBK1 chemical probe **33** with its negative control **34**.*

CDK12/CDK13

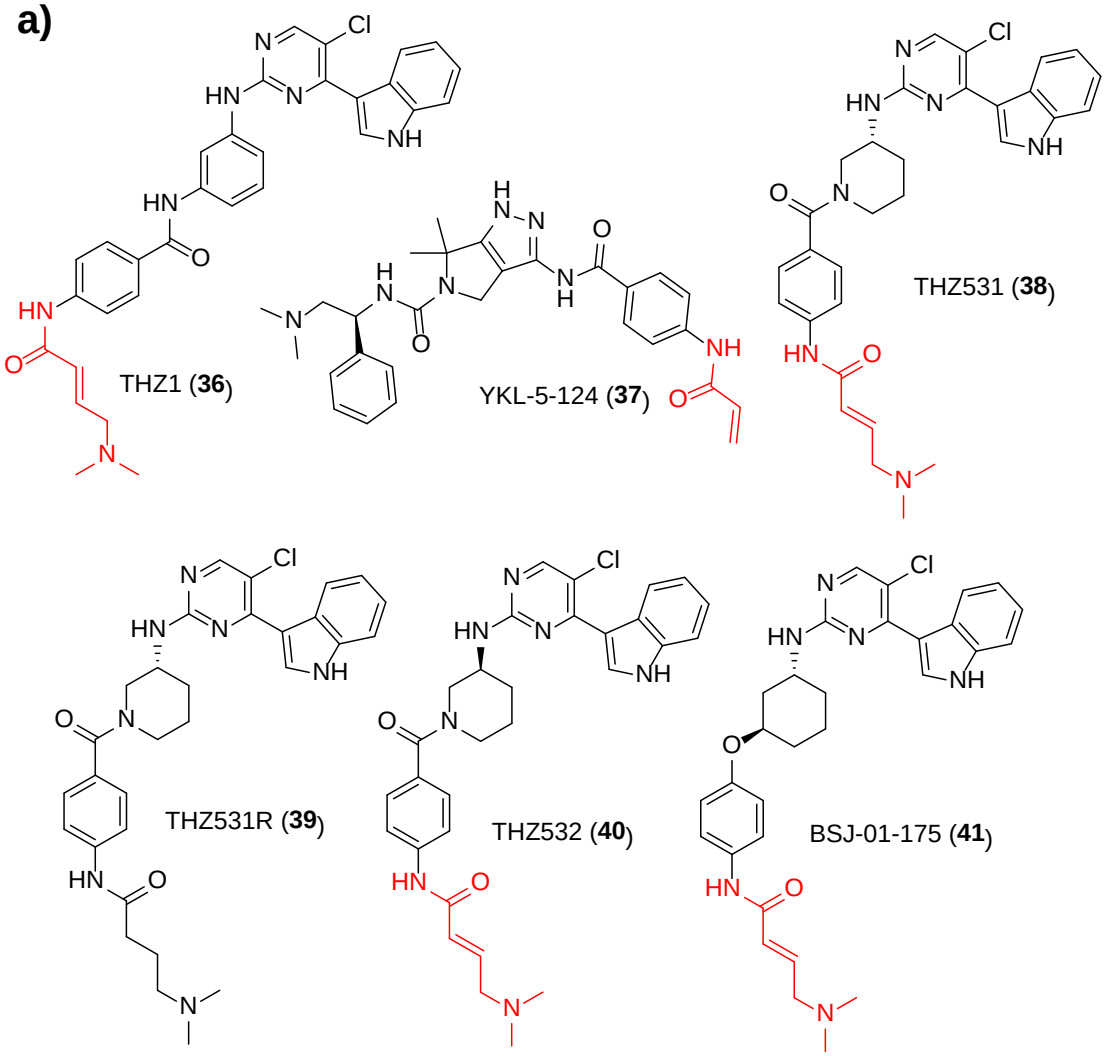
Progression of cellular division, gene transcription and many other biology aspects are driven by cyclin-dependent kinases (CDKs), which are considered as master-regulators of the cell cycle.[81] CDK12 and CDK13 were identified for the first time around

two decades ago from DNA screens for cell cycle regulators related to the CDC2 kinase (CDK1), and are examples of kinases that were little studied for many years before a recent explosion of interest (e.g. CDK12 with ~200 papers published in the last 5 years out of 300 in total) due to the recognition of their importance for various cancers. Both of those enzymes become activated upon binding to their partner cyclin K.[82–84] CDK12 and CDK13 are large serine/threonine kinases (~180 kDa) able to interact with RNA processing factors, and studies have shown the key role of CDK12 in the transcriptional process of the RNA-polymerase II enzyme.[85] Genetic silencing experiments have suggested the involvement of these kinases in different types of cancers, thus making the availability of high quality chemical probes an important requirement to deepen the understanding of their biological functions and to justify further drug discovery efforts.[83,84]

Inspection of the published co-crystal structures of the complexes CDK12-cyclin K and CDK13-cyclin K revealed that both contain a cysteine residue (Cys1039 and Cys1017) outside the canonical kinase domain on a C-terminal extension traversing the ATP pocket.[86] A cysteine (Cys312) with a similar spatial location in CDK7 has previously been identified as the site of modification of inhibitor THZ1 (**36**, Figure 8a) and more optimized follow-up-compounds such as YKL-5-124 (**37**, Figure 8a).[87] Based on this structural information, the first-in-class selective covalent CDK12/CDK13 inhibitor, THZ531 (**38**, Figure 8a), was developed using CDK7 inhibitor **36** as a template.[86] According to the co-crystal structure (PDB code: 5ACB, Figure 8b), the piperidine moiety in **38** orients the acrylamide warhead properly toward Cys1039 of CDK12. Notably, the crystal structure revealed two distinct conformations adopted by both **38** and the loop containing Cys1039. Mass-spectrometry and pull-down from cell lysate with

biotinylated analog bioTHZ531 (not shown) confirmed the covalent attachment to CDK12-cyclin K and CDK13-cyclin K complexes. In a radiometric enzymatic activity assay, **38** showed an apparent IC_{50} = 158 nM and 69 nM against CDK12 and CDK13, respectively, revealing a good selectivity window over CDK7 and CDK9. The saturated analogue THZ531R (**39**, Figure 8a) and mutated CDK12 (Cys1039 to Val) further validated the importance of covalent interaction for the inhibitory activity. Besides non-reactive analog **39**, enantiomer THZ532 (**40**, Figure 8a) was also significantly (>50-fold) less active on CDK12 and CDK13, suggesting the use of these compounds (ideally in combination) as negative controls. Selectivity in cells was shown by pulldown and by chemoprotemic KiNativ profiling capturing more than 200 kinases, none of which was labeled to more than 55% under the tested conditions. Once compound **38** decreased genomic instability and transcription factor genes, experiments to evaluate its anti-cancer activity were carried out in Jurkat cells, demonstrating a potent antiproliferative effect (IC_{50} = 50 nM) and apoptosis induction in a dose- and time-dependent manner.[86]

a)


THZ1 (36)
YKL-5-124 (37)
THZ531 (38)
THZ531R (39)
THZ532 (40)
BSJ-01-175 (41)

b)

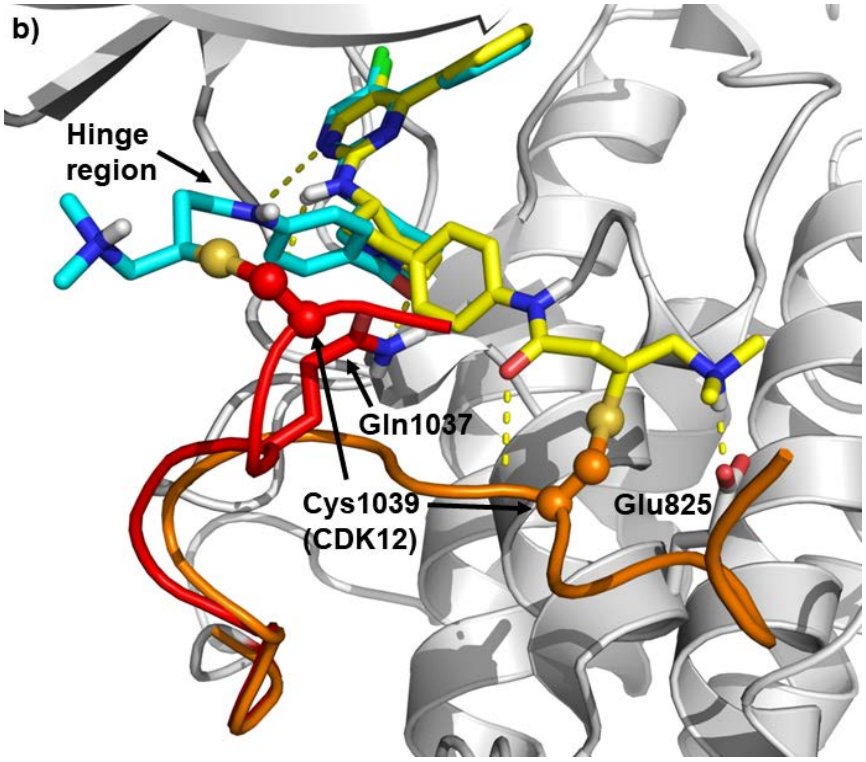

Hinge region
Gln1037
Cys1039 (CDK12)
Glu825

*Figure 8: a) Molecular structure of covalent CDK7 inhibitors (**36**, **37**), CDK12/CDK13 inhibitors (**38**, **41**) and potential negative control compounds (**39**, **40**). Reactive warheads are highlighted in red. b) Inhibitor **38** covalently bound to remote Cys1039 in CDK12 (PDB code: 5ACB). The two distinct binding conformations (inhibitor: cyan/loop: red and inhibitor: yellow/loop: orange) are shown.*

Further research highlighted inhibitor **38** as active against Ewing sarcoma in combination with poly(ADP-ribose) polymerase (PARP) inhibitors,[88] and recent experiments revealed that **38** caused loss of elongation by RNA polymerase II and suppressed gene expression in anaplastic thyroid carcinoma (ATC), one of the most aggressive types of thyroid cancer, as well as showing the potential of this inhibitor to overcome chemoresistance in ATC.[89] Further SAR evaluation yielded the CDK12/13 inhibitor **41** (BSJ-01-175, Figure 8a) with a similar kinase inhibitory and selectivity profile. Due to promising pharmacokinetic data, the compound was tested in preclinical *in vivo* models showing that **41** suppressed tumor growth potential throughout three weeks treatment compared to vehicle control.[90]

Later, several research groups designed and optimized reversible and irreversible inhibitors using different chemical scaffolds (not shown).[91–94] Together, these chemical tools have been used to corroborate CDK12/13 inhibition as a promising anticancer therapeutic strategy.

MKK7

The mitogen-activated protein kinase kinase 7 (MKK7, also known as MAP2K7) is part of the c-Jun N-terminal kinase (JNK) signaling pathway. Excessive MKK7 expression has been linked to many irregular cellular process, for example, promoting progression in T-cell acute lymphoblastic leukemia and colon cancer liver metastasis.[95] Likewise, MKK7-JNK plays a fundamental role regulating gene expression for promoting

mammalian social behavior and staving off depression.[96] However, a deeper understanding of MKK7 biology and the complementarity of its role in relation to MKK4, the other JNK-activating enzyme but which also activates p38 MAP kinases and which has been addressed to promote liver regeneration,[97] is still missing.

In the entire kinome, MKK7 is one of the eleven kinases that have a cysteine residue at the N-terminal end of the αD helix in the solvent exposed front of the ATP binding pocket.[98] A cysteine located at the same position has already given rise to the development of FDA-approved covalent protein kinase inhibitors targeting the epidermal growth factor receptor EGFR (afatinib, dacomitinib, osimertinib), HER2 (neratinib), and BTK (ibrutinib, acalabrutinib and zanubrutinib).[99] Notably, MKK7 features three other cysteines in the ATP binding pocket that may potentially be addressed. Due to the high similarity between the common EGFR T790M gatekeeper mutant and the MKK7 ATP pocket, Wolle *et al.* performed a structure-based optimization of their previously reported EGFR inhibitor (**42**, Figure 9a), a compound structurally related to FDA-approved Bruton's tyrosine kinase (BTK) inhibitor ibrutinib (**43**, Figure 9a) and which showed off-target activity towards MKK7.[100] A small series of analogs was synthesized through copper-catalyzed alkyne-azide click chemistry (CuAAC) aiming to address the back pocket of MKK7 between the Met212 gatekeeper and the catalytic Lys165. The most favorable MKK7 potency was observed for compound **44** (Figure 9a, apparent IC_{50} = 10 nM) carrying a *p*-acetophenone substituent, which also showed a marked selectivity over EGFR wild-type (IC_{50} ≈ 8,000 nM) and the L858R/T790M double mutant (IC_{50} ≈ 10,000 nM). Mass spectrometry and an X-ray crystal structure (PDB code: 6IB2) confirmed covalent engagement of the front-of-pocket Cys218 by the acrylamide warhead, while replacement by propionamide was

detrimental to MKK7 inhibitory activity (IC_{50} > 10,000 nM) highlighting the reliance on covalent bond formation. In a panel of 320 wild-type kinases (ProQinase), compound **44** at 1 µM showed >50% inhibition only for five other kinases that share the targeted cysteine (BLK, BMX, BTK, ITK and JAK3) plus two further kinases (mTOR and S6K). *In vitro* experiments suggested a decent overall pharmacokinetic profile and cellular experiments confirmed submicromolar potency.[100] In a follow-up publication showing further SAR, the authors also obtained apo structures of wild-type MKK7 one of which showed a unique DFG-in/"Leu-in" conformation precluding hinge binding.[101]

Further covalent MKK7 inhibitors based on an indazole scaffold exemplified by compounds **45** and **46** (Figure 9a) were identified by Shraga *et al.* via virtual screening using DOCKovalent followed by minor optimization.[102] Those compounds were profiled in conjunction with negative controls and a clickable proteomics probe (not shown) and showed a remarkable profile with respect to their relatively low molecular complexity. However, all MKK7 inhibitors discussed above were not perfectly selective and showed a rather pronounced loss in potency in cells. The latter might be related to the relatively low reversible binding affinities exacerbating competition with high cellular ATP concentrations while depletion by glutathione may also play a role.

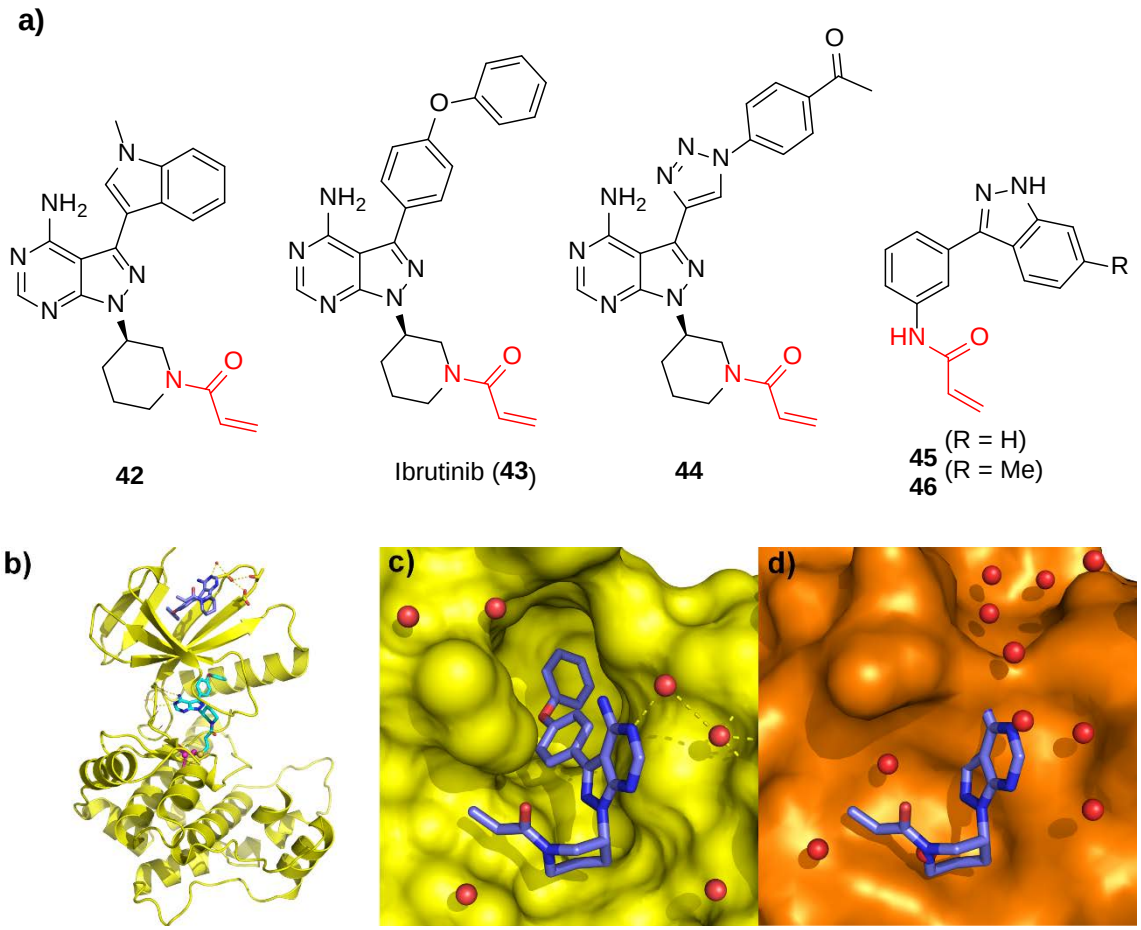


*Figure 9: Molecular structure of covalent EGFR inhibitor **42**, FDA-approved covalent BTK inhibitor **43** and covalent MKK7 inhibitors **44**-**46**. Reactive warheads are highlighted in red. b) MKK7 with two molecules of ibrutinib (**43**) bound (PDB code: 6YG2). The ibrutinib molecule binding the active site (cyan) shows the expected interaction pattern and forms a covalent bond with Cys218. The second ibrutinib molecule (purple blue) is non-covalently bound to an induced allosteric pocket formed by the β-sheets of the N-terminal lobe without forming any direct hydrogen bond to the enzyme. Notably, an alternative crystal structure shows MKK7 with **43** exclusively bound to the allosteric site (PDB code: 6YZ4). c) Surface view of the allosteric binding site. Water molecules are shown as red spheres. d) Overlay of the surface of apo-MKK7 (PDB code: 6YFZ; orange) with the binding pose of **43** (purple blue sticks) highlighting the absence of the allosteric pocket in the unligated structure.*

Recently, Schröder *et al.* reported a wealth of structural information providing insights into MKK7 catalytic domain plasticity and a model of MAP2K allosteric activation.[103] The authors also screened a variety of covalent and non-covalent type I and type II MKK7 inhibitors providing comprehensive selectivity data and X-ray structures, which may be used to generate highly potent and specific MKK7 probes.

Notably, the study also revealed an unprecedented allosteric pocket on the top of the *N*-terminal lobe hosting ibrutinib (**43**) without a covalent bond being formed (Figure 9b-d). This finding could provide a basis for the design of novel allosteric MKK7 ligands or PROTACs (proteolysis-targeting chimera; *vide infra*).

CK2

Besides the less studied fraction of the kinome, the development of chemical probes for well-studied kinases for which no highly specific inhibitors were previously available has played an important role in clarifying the biological role of such enzymes. Two such examples, namely the protein kinases CK2 and FAK, shall be discussed in the following paragraphs.

The serine/threonine casein kinase 2 (CK2) was among first kinases described and contains a heterodimeric structure that is formed by two catalytic subunits (~42 kDa CK2α/CSNK2A1 and 38 kDa CK2α'/CSNK2A2) and two regulatory subunits (~28 kDa CK2β/CSNK2B).[104] Many reports have described the involvement of CK2 in functions related to atypical cell growth and proliferation, especially because it is able to phosphorylate a large number of nuclear-associated substrates.[105] The literature also provides evidence supporting the role of CK2 in drug resistance (e.g. via mechanisms involving cellular efflux and DNA repair), as well as in disorders beyond oncology.[106,107]

Although CK2 biology can be considered as well-studied with respect to the amount of literature published, a high-quality chemical tool for biological investigations has long remained unavailable. In a recent study, Wells and colleagues describe the development of a CK2 chemical probe based on a pyrazolo[1,5-*a*]pyrimidine scaffold.[108] Their endeavor started from compound **47** (Figure 10) which had shown promising

cellular potency and kinome selectivity. After synthesis of a small series of analogs, all compounds were found to bind both CK2 subunits in cellular target engagement experiments using NanoBRET. Compound **48** (SGC-CK2-1, Figure 10) was identified as the most suitable candidate for use as a chemical probe due to its favorable balance between cellular potency (IC_{50} = 36 nM and 16 nM for the α and α' subunits, respectively; NanoBRET) and selectivity in a >400 kinase panel (DiscoverX; S_{10}(1 µM) = 0.007). DYRK2 was the only notable off-target detected, but with a >100-fold higher IC_{50} value compared to CK2 (IC_{50} = 4.2 nM and 2.3 nM for CK2α and CK2α', respectively and IC_{50} = 440 nM for DYRK2) in an enzymatic assay. A negative control was also obtained, compound **49** (SGC-CK2-1N, Figure 10), with the diaryl nitrogen atom being methylated preventing the scaffold from interacting with the hinge region. A co-crystal structure of probe **48** with CK2 was acquired to confirm the expected type I binding mode in which the inhibitor exclusively occupied the ATP-pocket (PDB code: 6Z83).

Evaluation of the antiproliferative effect of probe **48** in a panel including 140 cancer cell lines surprisingly showed inhibition of only a single cell-line (human histiocytic lymphoma line U-937) below 500 nM. The results using the best CK2 chemical probe yet available revealed that, unlike many previous studies had suggested, selective CK2 inhibition was not able to generate a broad antiproliferative effect.[108] In another recent study, Krämer and colleagues employed a related chemical scaffold to develop the selective and potent CK2 inhibitor **50** (Figure 10; K_d = 12 nM). Despite cellular activity being limited to the low micromolar range, presumably by the presence of a free carboxylic acid function hampering permeability, this compound may also be used as a tool compound to evaluate CK2 biology.[109]

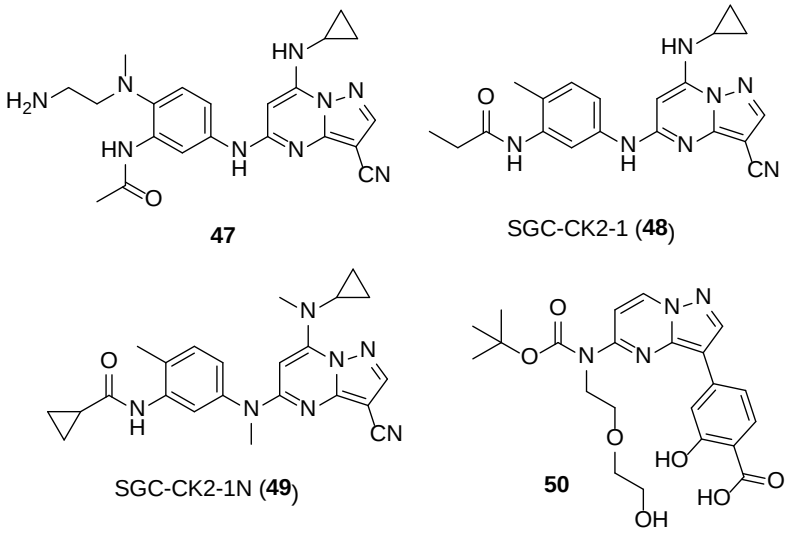


*Figure 10: Molecular structure of CK2 inhibitors: initial hit **47**, chemical probes **48**, **50**, and the negative control **49**.*

FAK

The focal adhesion kinase (FAK), also known as protein tyrosine kinase 2 (PTK2), is a cytosolic tyrosine kinase comprising three different domains with the central kinase domain being located between an N-terminal 4.1 protein, Ezrin, Radixin, Moesin homology domain (FERM) and a C-terminal domain including a focal adhesion-targeting (FAT) domain and two proline-rich regions.[110] It has been shown that FAK is involved in promoting tumor growth and metastasis via various mechanisms.[111] As summarized in a recent perspective,[112] many of the known FAK inhibitors are based on the common 2,4-diaminopyrimidine or 2,4-diaminopyridine scaffold or other related hinge-binding motifs. Moreover, FAK-targeted PROTACs (*vide infra*) as well as allosteric or PPI inhibitors targeting the active site, the FERM domain or the FAT domain have been developed.[112]

In a recent study, Groendyke *et al.* employed a non-canonical pyrimidothiazolodiazepinone scaffold to develop highly FAK-selective chemical

probes.[113] Profiling of compounds from an earlier series identified inhibitor **51** (BJG-01-181, Figure 11) as a starting point with good potency (IC_{50} = 62 nM) and a relatively clean selectivity profile. Interestingly, the structurally related thiazole isomer **52** (BJG-01-085, Figure 11) proved to be inactive (IC_{50} > 10 μM). Simple *N*-methylation at the amide nitrogen gave compound **53** (BJG-03-025, Figure 11) with three-fold increased potency (IC_{50} = 20 nM) while a variety of other modifications did not lead to further improvements. Modeling studies rationalized the weak potency of isomer **52** as due to loss of a hydrogen bond with DFG-Asp564. Compound **53** displayed a clean selectivity profile in a >400 kinase panel (DiscoverX) with 100-300-fold selectivity over minor off-targets being subsequently confirmed. Although showing low oral bioavailability in mice, compound **53** had a low clearance (18.5 mL/min/Kg) and a suitable half-life after iv administration (5.29 h). Investigations using 3D-culture of breast cancer cells (MDA-MB-231 line) showed compound **53** inhibiting cell migratory potential and viability. In addition, the compound inhibited in a dose-dependent manner the proliferation of gastric cancer in organoid models.[113] Taken together, these data showed inhibitor **53** to be a valuable tool to explore FAK biology, with compound **52** being a possible negative control although not further profiled in this study.

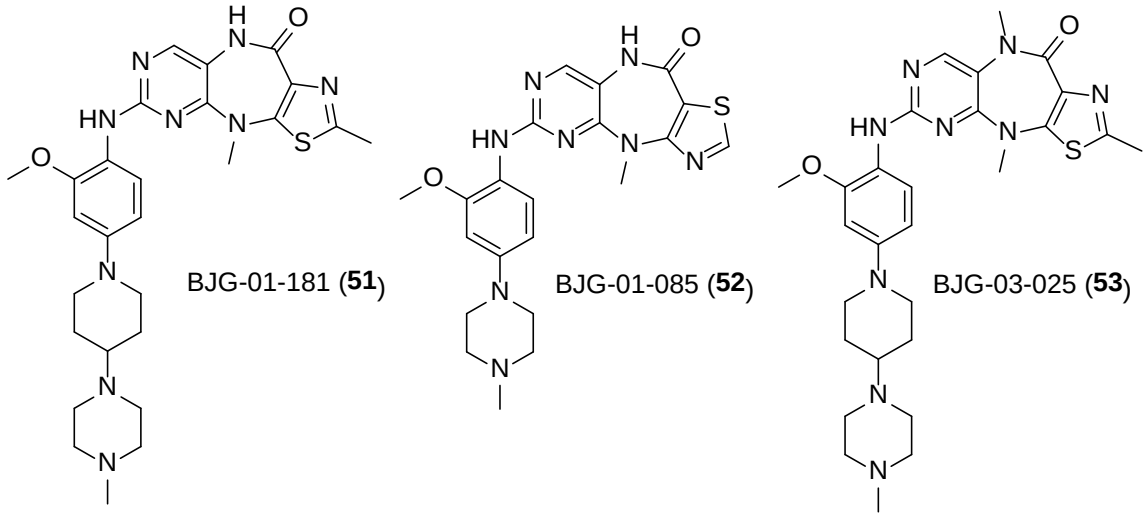


*Figure 11: Molecular structure of FAK inhibitors: initial hit **51**, chemical probe **53** and the possible negative control **52**.*

OPPORTUNITIES AND CHALLENGES

Obtaining Starting Points for Probe Design from DNA-Encoded Chemical Libraries

Generation and screening of DNA-encoded chemical libraries (DEL) represents a powerful and versatile technique to discover new and atypical starting points for drug discovery and probe development programs. This approach recently gained importance in medicinal chemistry projects in both academia and companies, complementing the already well-established screening methods for hit identification.[114–118] Brenner and Lerner,[119] in 1992, envisioned the DEL concept connecting organic chemistry and molecular biology. Typical DELs contain a large number of small molecules (from 10^4 to $>10^9$) each covalently bound to an individual DNA oligonucleotide tag. Part of the DNA acts as a molecular barcode for each single small molecule, providing a robust way to identify minute amounts of compound. Therefore, this technique can be used to generate and screen combinatorial libraries of unprecedented size, allowing for more

extensive sampling of chemical space and increase of screening throughput by several orders of magnitude.[118,120] Significant efforts have been carried out to broaden the scope of amenable building blocks and overcome synthetic challenges of preparing DELs, which is complicated by the presence of the multifunctional and labile oligonucleotide tag and the requirement for reactions to be carried out under mild conditions (pH 4-14, 25-90 °C, no strong oxidants) in an aqueous environment on a very small scale. In this context, photoredox-mediated reactions have proven to be particularly useful.[121–125] In consequence, a growing number of hit compounds with uncommon structures identified from DEL screening are reported in the literature.[117] Notably, the scope of DEL screening has recently been expanded to a physiologically more relevant context with a first proof-of-concept study demonstrating the feasibility of DEL-screening in a living cell.[126]

Showcasing the possibility of expanding the chemical space of protein kinase inhibitors by DEL screening, a highly potent and selective RIPK1 inhibitor based on an unusual benzoxazepinone chemotype was discovered by researchers from GlaxoSmithKline.[127] They screened a DEL collection containing ~7.7 billion compounds resulting in the identification of inhibitor **54** (Figure 12) displaying low nanomolar RIPK1 inhibitory potency and cellular activity. Furthermore, the compound possessed remarkable selectivity for a screening hit with essentially no off-targets at 10 µM in two large kinase assay panels and unusual selectivity for primate vs. nonprimate RIPK1.[127]

The binding mode was initially determined by hydrogen-deuterium exchange mass spectrometry (HDX-MS) and photoaffinity labeling, and later confirmed by co-

crystallisation with RIPK1 (PDB code: 5HX6), revealing the inhibitor to be deeply buried in an allosteric pocket (*vide infra*) pocket behind the ATP binding cleft.[127]

Subsequent SAR exploration revealed the pivotal role of the amide NH and the carbonyl groups for the inhibitory activity. Moreover, the bioisosteric replacement of the isoxazole ring by 1,2,4-triazole (GSK2982772, **55**, Figure 12) improved physicochemical and PK properties while maintaining the favorable biological profile and anti-inflammatory properties. The compound moved to phase 2a clinical trials for treatment of rheumatoid arthritis, psoriasis, and ulcerative colitis.[128] Further efforts towards targeting RIPK1 in oncology led to another clinical candidate, GSK3145095 (**56**, Figure 12).

Seeking to obtain RIPK1 inhibitors for CNS disorders, researchers from Takeda merged HTS hit **57** (Figure 12) binding the same allosteric site with compound **55** to generate inhibitor **58** (Figure 12). This compound showed brain-penetration, oral availability and a promising PK profile. Preclinical efficacy was confirmed in an experimental autoimmune encephalomyelitis model of multiple sclerosis. However, further studies are needed to corroborate the target engagement in brain tissues.[129] Notably, close analogs **59** and **60** (TP-030-1 and TP-030-2, Figure 12) have been further profiled and made available as RIPK1 chemical probes along with negative control **61** (TP-030n, Figure 12).

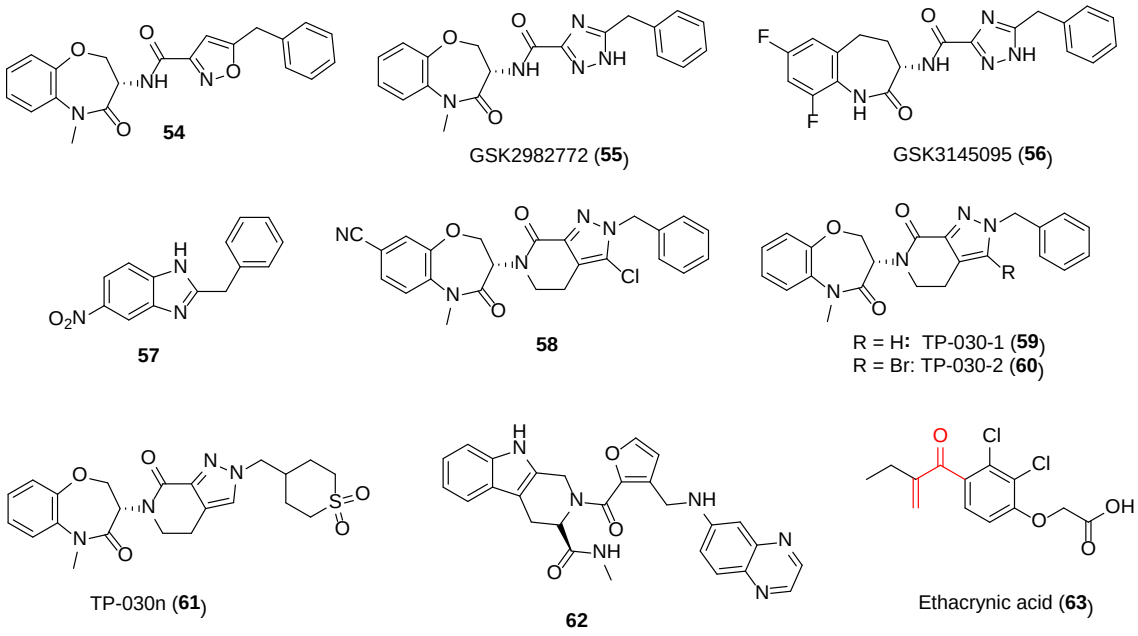


*Figure 12: Examples of inhibitors discovered from DEL screening efforts. The reactive group of compound **63** is highlighted in red.*

Another interesting example of a protein kinase inhibitor with a non-canonical chemotype directly derived from a DEL is BTK inhibitor **62** (Figure 12). Discovered by screening a 110 million compound DEL, the compound displayed subnanomolar BTK affinity. Although this compound bound the ATP pocket and showed a hinge interaction via the quinoxaline, the crystal structure (PDB code: 5U9D) revealed an unprecedented sub-pocket in the back of the ATP binding site usually occupied by the side chain of the catalytic Lys430, which accommodated the tetrahydro-β-carboline moiety. Interaction with this cavity might explain the high affinity of compound **62**.[130] A subsequent study using DELs of reactive compounds yielded potent covalent BTK inhibitors including derivatives bearing epoxides,[131] an electrophile reported for the first time in the context of BTK which had, however, been used before to target an equivalent cysteine in the Janus kinase 3 (JAK3).[132]

DELs can also be employed in combination with other hit identification methods. For instance, application of DELs in conjunction with the IDUP (Interaction Determination using Unpurified Proteins) method enables identification of ligand/target pairs from one-pot mixtures of DNA-linked small-molecules and unpurified protein targets in cell lysates.[133] This approach unexpectedly revealed ethacrynic acid (**63**, Figure 12) to be a weak MAP2K6 (MKK6) kinase inhibitor (IC_{50} = 4.5 μM). According to mass-spectrometry experiments, the mechanism of inhibition relies on covalent binding of the Michael acceptor system to the non-conserved, non-active site Cys38.[134]

Covalent Targeting Approaches and Fragments

Covalent targeting approaches have been very successful in kinase inhibitor discovery.[99,135] They can promote high potency and durable target engagement, potentially decoupling pharmacodynamics from pharmacokinetics. Most covalent kinase inhibitors address non-catalytic cysteines, which are poorly conserved in or around the ATP binding site.[98] Since such cysteines are frequently not even conserved within a certain kinase family, covalent targeting can be used to achieve exquisite selectivity over closely related family members (see for example recent covalent JAK3[136] and FGFR4[137] inhibitors). The field of targeted covalent kinase inhibitors was pioneered by researchers at Parke-Davis in the late 1990s[138,139] and culminated in the FDA-approval of the first two covalent protein kinases inhibitors afatinib (targeting EGFR) and ibrutinib (targeting BTK; see compound **43**, Figure 9a) in 2013. Since then, five other cysteine-targeted covalent protein kinase inhibitors have gained FDA approval, and more are expected to come. Irreversible and reversible covalent targeting has also become an important strategy for the design of chemical probes as highlighted by the irreversible

MKK7 and CDK12/13 inhibitors discussed above, or cyanoacrylamide-based covalent-reversible inhibitors like FM-381 (**64**) and FM-409 (**65**, Figure 13a,b) that were developed as high quality probes for JAK3.[140,141]

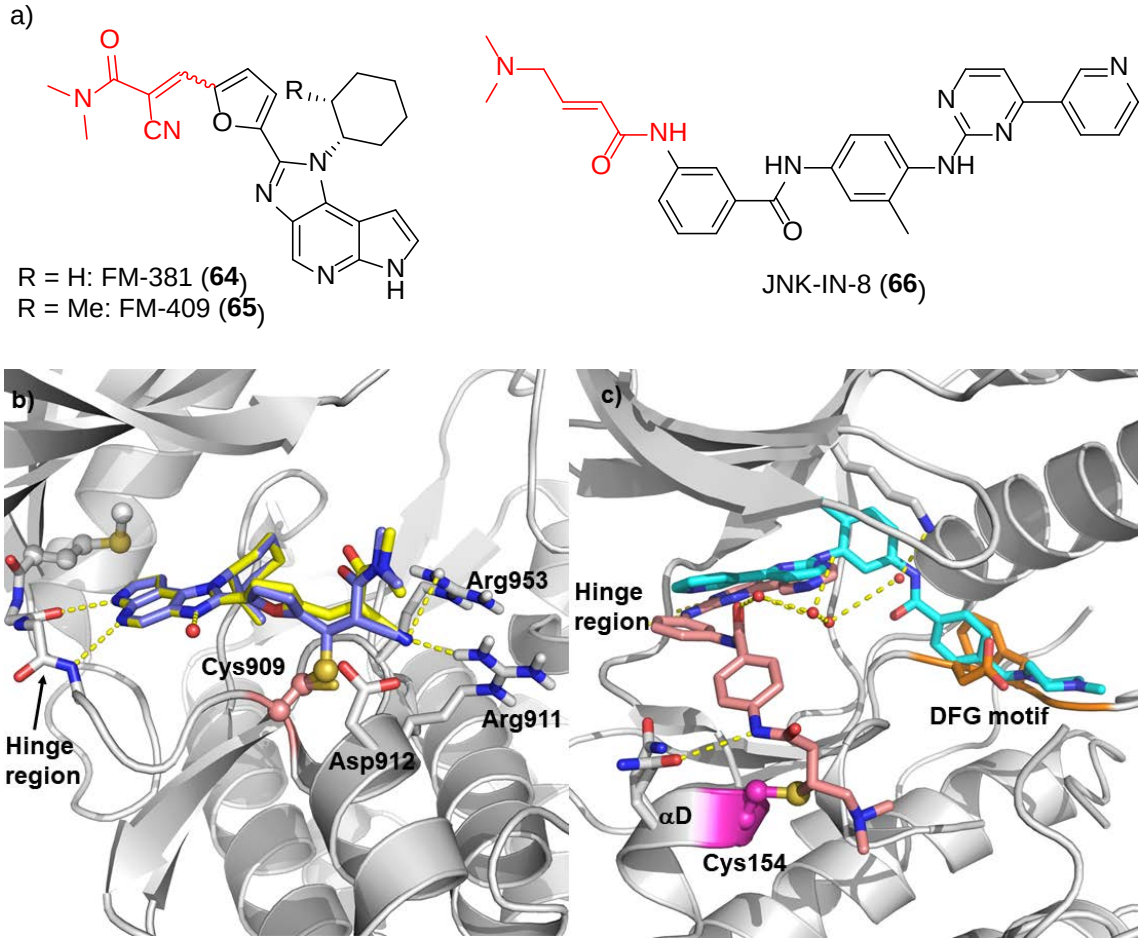


*Figure 13: a) Covalent-reversible JAK3 chemical probes **64** and **65** and covalent JNK inhibitor **66**. Reactive warheads are highlighted in red. b) Covalent-reversible inhibitor **65** bound to the JAK3 ATP pocket (PDB code: 5LWN). The presence of both the covalently bound (purple blue) and the non-covalently bound inhibitor (yellow) is observed. The nitrile group further induces a pocket lined by Arg911, Arg953 and Asp912, which may act as an additional selectivity filter. c) Overlay of the binding mode of an analog of covalent JNK inhibitor **66** (salmon) in JNK3 (PDB code: 3V6R) and imatinib (cyan) bound to p38α MAP kinase (PDB code: 3HEC). Imatinib binds p38α in a DFG-out conformation while **66** covalently binds the related JNK3 in a type I binding mode with a DFG-in conformation (DFG motif highlighted in orange). Only the JNK3 backbone is shown for clarity.*

Covalent inhibitors combine two orthogonal selectivity filters, i.e. reversible recognition between the ligand and the receptor and covalent bond formation, which requires appropriate reactive residues on both the protein and the ligand.[142] The latter

need to be placed in a suitable pre-orientation in the reversibly-bound complex to favor a low energy reaction path increasing reaction rates. As mentioned, covalent binding can be reversible or irreversible depending on the labeling chemistries employed. Irreversible approaches, however, are still much more common in the kinase inhibitor context.[99,135] Irreversible covalent binding is a non-equilibrium process typically involving two steps. The first one is a reversible binding event described by a constant K_I (not to be confused with the equilibrium binding constant K_i) corresponding to the ligand concentration required to achieve the half-maximal rate of covalent inactivation. The maximum achievable rate of covalent inactivation is given by the first-order rate constant k_{inact}, describing the second step.[143] This non-equilibrium behavior results in a time-dependence of potency, with increasing incubation times resulting in higher (apparent) potencies and decreasing competition with ATP.[144] Therefore, covalent kinase inhibitors are best characterized by the (time-independent) quotient k_{inact}/K_I which is, however, more cumbersome to determine with the typical end-point assays used to assess kinase enzymatic activity, and thus is frequently not reported. Nevertheless, the time dependence of IC_{50} values of covalent-irreversible inhibitors should always be kept in mind when interpreting such data[145].

So far, design of covalent kinase inhibitors is mostly cysteine-focused.[135] The protein kinase's "cysteinome" has been subject to several analyses with cysteines inside the ATP binding site being relatively well assigned, at least for kinases for which crystal structures or high quality computational models are available.[98,146–148] Our knowledge of cysteines in flexible regions or at remote locations, as well as those potentially accessible in inactive or non-canonical conformations is, however, still far from complete. The same is true for cysteines in allosteric pockets, which are often cryptic in

nature. Engineered cysteines have also been used in the framework of chemical genetics approaches (*vide infra*) to generate specific covalent inhibitors for kinases whose wild-type does not possess a suitable cysteine.[99,135] Such strategies are now facilitated by the advent of CRISPR/Cas9 gene editing enabling the endogenous expression of the cysteine mutant kinase in relevant cellular systems.[149] Beside cysteine-targeted inhibitors, some lysine-targeted compounds are known, most of which bind to the catalytic "roof-top" lysine in the β3-sheet in the N-terminal lobe.[99] For example, KiNativ, a chemoproteomic platform for kinase profiling relies on irreversible transfer of a biotinylated acyl group from an ADP or ATP derivative onto conserved lysines.[76] By MS-based quantification of competition with the KiNativ probe, selectivity against a wide array of protein kinases (and some other ATPases) in tissue or cell lysates can be determined. Similarly, a sulfonyl fluoride-derived probe based on a promiscuous inhibitor scaffold has been used for in-cell selectivity profiling.[150] Examples for covalent targeting of other amino acids (e.g. tyrosine)[151] in protein kinases, however, remain sparse. This is mainly due to the fact that these amino acids hold a significantly lower intrinsic reactivity compared to cysteine and chemistries to selectively address such (non-activated) residues remain poorly explored.[142]

Covalent kinase inhibitor design typically follows structure-based approaches where an unreactive parent inhibitor is equipped with a reactive group often referred to as "warhead", to engage the desired cysteine.[99] An appropriate attachment point and spacer is usually derived from structural information or computational models. Such an approach was, for example, key to the generation of the first covalent EGFR[139] and BTK[152] inhibitors and has resulted in clinically approved drugs. Another common strategy is the optimization towards off-targets identified in screening panels following the logic

of the "selective optimization of side activities" (SOSA) approach.[153] Here, the careful re-design of the ligand allows increasing potency and specificity for the new target (i.e. the former off-target) while minimizing activity on the initial target. Such a strategy was successfully employed, for example, in the design of covalent MKK7 and CDK12/13 inhibitors (*vide supra*) or in recent studies towards chemical probes targeting the protein kinases BMX and BTK.[154] One potential pitfall to be considered in such design approaches is that the ligand may adopt alternative binding modes in other kinases, allowing for unexpected engagement of other (potentially distant) cysteine positions. This is beautifully highlighted by the discovery of covalent JNK-targeted probe JNK-IN-8 (**66**, Figure 13a, apparent IC_{50} = 4.67 nM, 18.7 nM and 0.98 nM against JNK1, JNK2 and JNK3, respectively) and analogs thereof.[155] These compounds were initially designed as covalent inhibitors of PDGFR and c-KIT with an expected type II binding mode (*vide infra*) to address a cysteine accessible in the DFG-out conformation. However, strong off-target activity on JNKs was observed, which feature a rare cysteine in the solvent-exposed front region. X-ray structure determination unraveled an unforeseen, U-shaped type I binding mode in JNKs allowing the inhibitors to reach this alternative site of covalent modification (Figure 13c). Structure-based optimization ultimately gave rise to highly potent and selective JNK inhibitors including compound **66**.

Recently, alternative approaches for covalent ligand discovery have gained traction. For example, the incorporation of covalent inhibitors in DELs has proven successful[131,134,156,157] (*vide supra*), opening up new avenues. However, it remains to be seen to what extent this approach will find broader application. Another promising method increasingly gaining importance uses "covalent fragments", which has been summarized in recent reviews.[158,159] Classical fragment-based drug discovery (FBDD)

starts with screening of fragment-like compounds whose chemical space is loosely defined by the "rule of three" (RO3; i.e. molecular weight <300, a ClogP < 3, and a number of hydrogen bond donors, acceptors, and rotatable bonds < 3).[160] Fragment screens hold several advantages including more efficient sampling of chemical space even at moderate library size (usually few thousand fragments or less) and a high likelihood of retrieving readily optimizable hits with good ligand efficiencies. After discovery of a suitable fragment hit, approaches like growing, linking or merging can be used to generate lead-like high-affinity ligands, which is, however, often not a trivial task.[161] FBDD is considered particularly useful to address difficult targets lacking obvious binding sites as fragments are frequently found to occupy cryptic allosteric pockets or to unravel unprecedented binding modes. At the same time, they can also provide new chemotypes to engage known, druggable binding clefts. Fragment screening is typically conducted in a target-centric fashion by employing biophysical methods for detection. One of its key limitations is linked to the generally low affinities of fragments (often high micromolar or even millimolar K_d values), which are difficult to capture. Covalent fragments can overcome this limitation due to their higher apparent potencies driven by the covalent binding event and the possibility to directly quantify covalent target engagement by mass spectrometry. With the recent improvements in MS-based proteomics, covalent fragments can also widen the scope of FBDD from being largely target-centric to a proteome-wide scale.[158] The power of covalent FBDD to address challenging targets is highlighted by the clinical development of the first inhibitors of the oncogenic KRAS G12C mutant, where covalent ligandability has initially been proven by means of fragment-based disulfide tethering.[162] More recently, screening of covalent fragment libraries has resulted in specific inhibitors for difficult targets such as the RBR

E3 ubiquitin ligase HOIP,[163] the peptidyl-prolyl cis-trans isomerase PIN1,[164] the SARS-COV-2 main protease[165] and some other proteins.[166] Despite several studies showcasing the fragment-based discovery of covalent ligands for kinases such as JAK3, MELK and the RSK family[167,168] (C-terminal domains), these approaches have been comparably sparse and mainly limited to the canonical ATP binding site so far. However, apart from targeting the known cysteinome, it can be assumed that this strategy could become particularly useful for the identification of modulators engaging cryptic allosteric sites or non-canonical binding clefts inside or around the ATP pocket. Moreover, they may be employed in conjunction with suitable warhead chemistries to identify covalent ligands addressing amino acids beyond cysteine. A major problem of covalent fragment library design consists in defining the right reactivity window to avoid binding being excessively reactivity-driven, as well as in the requirement for a nucleophilic amino acid in a suitable position at the target site. Such limitations may be overcome by means of photoreactive fragment libraries,[169] which come, however, with other liabilities that have been reviewed elsewhere and shall not be further discussed here.[158]

Chemoproteomic Profiling

Development of chemical probes has benefited from the increasing availability of large *in vitro* screening panels during the last years allowing for a thorough evaluation of off-targets in an isolated setting. Moreover, live cell methods such as NanoBRET enable the quantification of target engagement under equilibrium conditions in a native cellular environment. Importantly, the latter method takes into account the high and compartment-dependent cellular ATP concentrations giving a more accurate picture of cellular potency and selectivity compared to biochemical kinase panels, which may

provide misleading data when used as a standalone approach to assess selectivity.[36] Even more rigorous off-target profiling in cells has also advanced with state-of-the art chemoproteomic profiling methods now being available to many labs for broader assessment of selectivity. At the same time such methods constitute an integral part of target deconvolution endeavors in phenotypic drug or probe discovery (*vide infra*).[170–172] Chemoproteomic methods have benefited from improvements in sensitivity and throughput of tandem mass spectrometry, which is nowadays the most common detection technique.[173] Chemoproteomic selectivity profiling often makes use of stable isotope labeling either during sample preparation or *in situ*, to directly compare peptides originating from treated and untreated samples.[170] State-of-the-art workflows and modern mass spectrometers allow for the quantification of thousands of proteins. For profiling of non-covalent binders, affinity-based methods necessitating a tagged version of the ligand, with the tag being required for enrichment or immobilization, are usually employed. These have the intrinsic disadvantage that the tag may alter the ligand's biological properties in an unpredictable manner. Alternatively, competition with a (tagged) broad-spectrum reactive probe may be used to investigate selectivity against a defined set of targets, typically enzymes from the same family. Such methods (e.g. the aforementioned KiNativ platform[76] or cell-permeable sulfonyl fluoride probes[150]), however, are limited to targets captured by the reactive probe. Chemical proteomic approaches have been particularly useful to define the off-target labeling profiles of covalent ligands. Similarly, they have been used to identify relevant targets from phenotypic covalent fragment screens. Methods like (competitive) activity-based protein profiling (ABPP) pioneered by the Cravatt group[174] or the recent covalent inhibitor target site identification (CITe-Id)[175] approach have been very successfully

employed in this context. By means of an ABPP approach using stable isotope labeling (isoTOP-ABPP),[174] for example, broadly reactive alkyne-iodoacetamide probes have been used to profile thousands of cysteines in cells and tissues and similar methods have been applied to other amino acids. A very recently developed unbiased chemoproteomic workflow exploiting diverse electrophiles to globally quantify nine different amino acids represents an excellent case study broadening the scope of ABPP.[176] Such advances will become very useful to shed light on the full extent of protein labeling by covalent ligands in a physiologically relevant context.

Beyond the aforementioned methods that either require a tagged ligand, or a (re)activity probe to label a defined set of residues or proteins, label-free profiling methods are gaining traction.[170] The most common approaches are known as cellular thermal shift assays (CETSA)[177] and thermal proteome profiling (TPP)[178], both relying on the same principle as standard thermal shift assays i.e. thermal stabilization of proteins by ligand binding.[179] While classical CETSA methods were based on immunoblotting to detect the soluble (non-denatured) protein fraction in a temperature-dependent manner, recent TPP protocols extend the scope by employing multiplexed quantitative mass spectrometry-based proteomics. Thereby, they enable profiling of thousands of proteins in their native context in cells and tissues.[179] Beyond thermal stability, alternative protocols have been developed to quantify, for example, the capability of a ligand to affect protein stability against proteolytic degradation (drug affinity responsive target stability; DARTS[180]) or oxidation (stability of proteins from rate of oxidation; SPROX[181]). Although CETSA and related methods greatly enhance our understanding of ligands' global interactomes, the current techniques are not free from limitations e.g. with regard to quantification of target engagement/affinity and capturing of low

abundance proteins, or discrimination between functional and chemoproteomic selectivity (or binding vs. pharmacological activity). Consequently, detected off-targets should always be validated by complementary methods.

Degrader Probes

Challenging the paradigm of the occupancy-based pharmacology, induced protein degradation has emerged as a strategy to abrogate protein function in an "event-driven" manner.[182] The most common approach to specific protein degradation employs heterobifunctional molecules called PROTACs (proteolysis-targeting chimera) pioneered in 2001 in the labs of Crews and Deshaies.[183] PROTAC degraders consist of three parts: a ligand binding to the protein of interest, a ligand binding to an E3 ubiquitin ligase and a linker connecting both ligands. Such molecules are designed to recruit an E3 ligase to the target protein to form a ternary complex promoting ubiquitination and subsequent protein degradation via the ubiquitin-proteasome system. Degradation of the target protein offers several potential advantages. For example, degraders abrogate the whole array of a protein's functions thereby copying the phenotype of genetic knockout or knockdown but reversibly and in high temporal resolution.[184] This process of chemical protein knockdown is catalytic in nature, i.e. a single (reversibly binding) PROTAC molecule may induce degradation of a higher number target proteins. Complete ablation of the target protein may also help to overcome drug resistance. At the same time, degraders may be used to address "undruggable" proteins due to their less stringent requirements in terms of target occupancy.[182] Similarly to irreversible covalent inhibitors, degraders may cause prolonged effects since protein resynthesis is required to restore target function. Moreover, PROTAC degraders frequently possess

increased selectivity compared to the parent ligand.[182,184] PROTACs are evaluated by different measures compared to classical probes including the DC_{50} value describing the concentration, at which degradation becomes half-maximal and the D_{max} [%] defining the maximal degradation observed. Notably, PROTACs show bell-shaped concentration-response curves with efficacy being reduced at concentrations significantly above the DC_{50} due to the Hook effect arising from nonproductive binary complex formation.[185] Accounting for the distinct properties of PROTACs, a dedicated set of probe criteria has recently been defined for such probes (see https://www.chemicalprobes.org/protacs).

Despite the recent success of PROTAC degraders, many challenges remain including the lack of general design rules, limited number of amenable E3 ligases, increased complexity in evaluating pharmacokinetics and pharmacodynamics, and the physicochemical property space of PROTAC molecules, which is outside the common rule-of-five (RO5) space.[182] The latter issue may be overcome by smaller "molecular glue" degraders inducing a *de novo* interaction between a target and an E3 ubiquitin ligase without relying on the mentioned chimeric structure.[184] Such molecules, however, are currently difficult to identify. The remarkable pace in the development of degraders, in both academia and companies, is reflected by an estimated 15 or more degrader molecules in clinical trials by the end of 2021.[186,187] A variety of kinases has already been subject to PROTAC-mediated degradation.[187] Although full coverage of this topic is far beyond the scope of this perspective, several relevant recent studies showcasing the development of "kinase degrader probes" shall be highlighted below.

After conflicting reports on synthetic lethality of TANK-binding kinase 1 (TBK1, *vide supra*) loss in mutant K-Ras tumors, Crew *et al.* aimed to assess this question by

means of PROTAC technology. To generate chimeric degraders, they selected aminopyrimidine **67** (K_d = 1.3 nM, Figure 14) derived from a known TBK1 inhibitor and the common hydroxyproline derivative **68** (Figure 14) as pharmacophores binding TBK1 and the E3 ligase VHL (Von Hippel-Lindau), respectively.[188] SAR studies revealed key compound **69** (TBK1 PROTAC 3i, Figure 14) with 15 linker atoms to be highly potent and efficient (DC_{50} = 12 nM and D_{max} of 96%, respectively). Abrogation of VHL binding by epimerization at the proline 4-position or pretreatment with proteasome inhibitor carfilzomib reduced the TBK1 degradation, confirming a VHL-mediated recruitment of the proteasome. Unexpectedly, and in contrast to the parent ligands, compound **69** did not degrade the closely related kinase IKKε (at concentration >50-fold the TBK1 DC_{50}) highlighting the distinct selectivity profiles often observed for degraders. Moreover, although PROTAC **69** promoted virtually complete TBK1 degradation, it did not significantly affect the proliferation of K-Ras mutant compared to K-Ras wild type cell lines thereby contradicting the suggested synthetic lethality of TBK1 with mutant KRAS.[188]

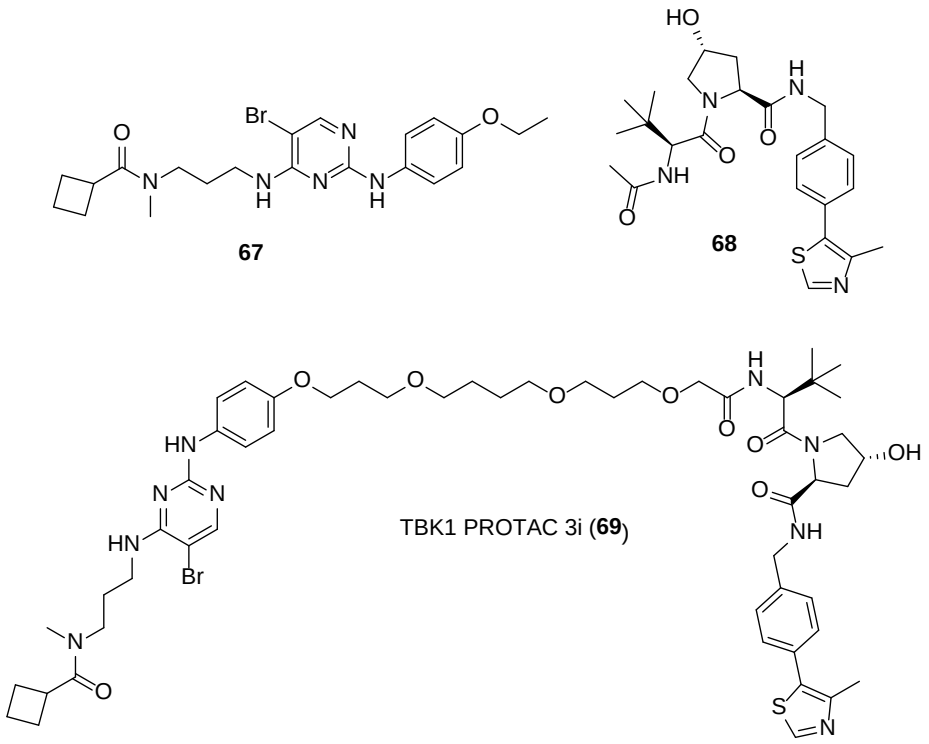


*Figure 14: Molecular structure of TBK1 inhibitor **67**, VHL ligand **68** and TBK1 degrader **69**.*

The first degraders for anaplastic lymphoma kinase (ALK) were reported in 2018 by Powell *et al.*.[189] In a straightforward design approach making use of knowledge from structurally similar multikinase degraders, the parental kinase inhibitors **70** and **71** (ceritinib, Figure 15) were linked to the cereblon ligand pomalidomide to obtain degraders **72** and **73** (Figure 15). The latter showed potent ALK degradation in different cell lines which could be abrogated by treatment with carfilzomib, pomalidomide or by cereblon inhibition. Compounds **74** and **75** (Figure 15) lacking one of the pomalidomide carbonyl groups were suggested as negative controls. However, both degraders were susceptible to ATP-binding cassette subfamily B member 1 (ABCB1)-mediated efflux rendering them ineffective in cells expressing high levels of this transporter. Moreover, proteomic experiments identified several off-targets (PTK2/FAK, FER, RPS6KA1 and AURKA) highlighting the importance of profiling PROTAC's selectivity independently

from their parent ligands. This is a relevant point since proteins may have different susceptibilities to degradation, which does not necessarily correlate with compound potency or affinity.[189] Notably, another study was published just a few months later reporting structurally very similar PROTACs MS4077 and MS4078 (not shown) suitable for *in vivo* studies.[190]

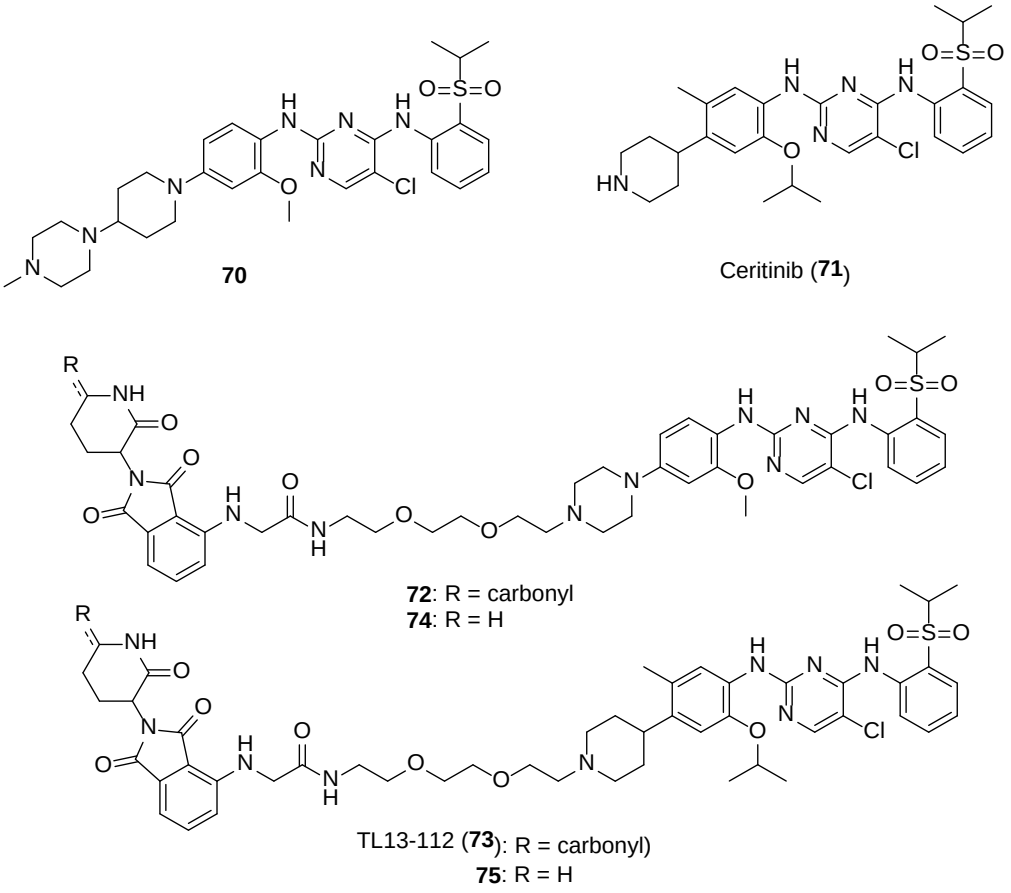


*Figure 15: Molecular structure of ALK degraders (**72** and **73**), their suggested negative controls (**74** and **75**) and the template ALK ligands (**70** and **71**).*

Another interesting study highlighting the potential of PROTACs to improve selectivity compared to their parent inhibitors was reported by Olson *et al*..[191] Thalidomide-based chimeric compound **76** (THAL-SNS-032, Figure 16) was designed from non-selective parent scaffold **77** (Figure 16), a known multi-CDK inhibitor. PROTAC

76 proved to be a relatively selective, sub-stoichiometric degrader of CDK9 although it showed a similar inhibitory profile as the parent compound in enzymatic assays and a KiNativ screen. In the same study, the authors also characterized previously known inhibitor **78** (Figure 16)[192] as a highly potent and selective small molecule CDK9 probe to conduct comparative studies. Interestingly, however, **78**-derived degraders (not shown) proved to be rather inefficient. Degrader **76** retained its apoptotic activity even after washout, supporting the notion of degradation promoting superior efficacy compared to inhibition albeit at a slower onset.[191]

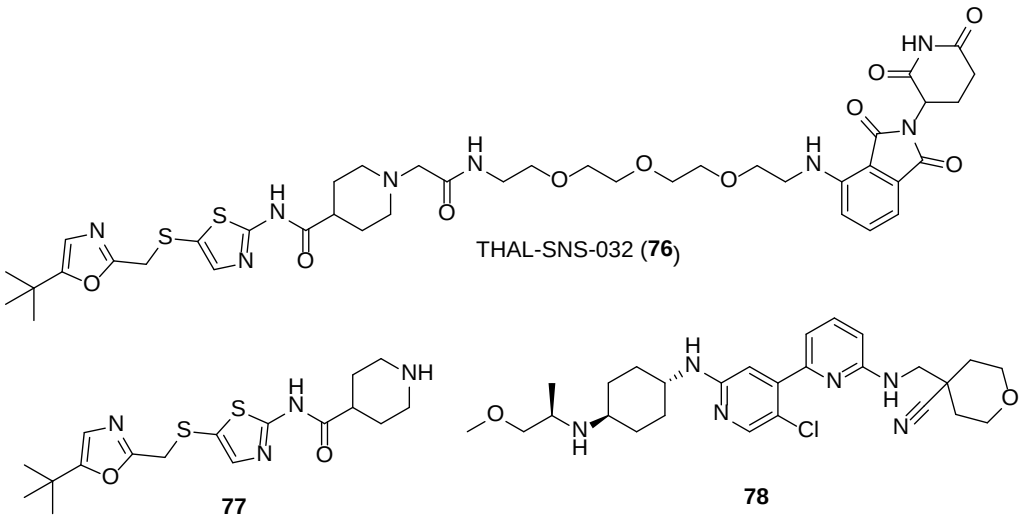
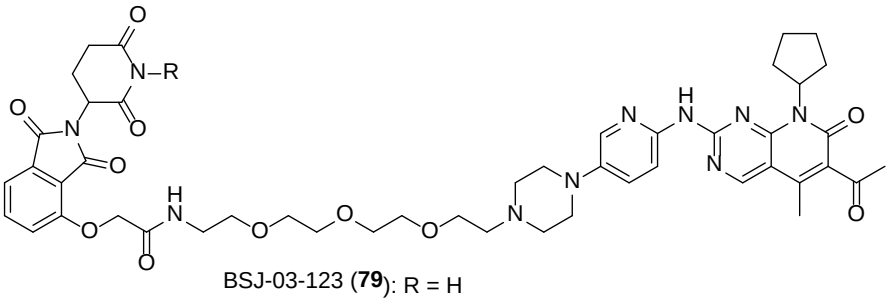


*Figure 16: Molecular structure of the CDK9 degrader **76**, the pan-CDK inhibitor prototype **77** and the highly selective CDK9 inhibitor **78**.*

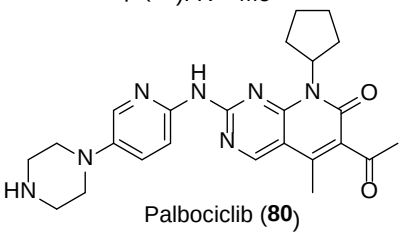
Another study by the same groups reported degrader **79** (BSJ-03-123, Figure 17), representing the first molecule reported to selectively abrogate CDK6 function. Proteome-wide studies revealed CDK6 as the only degraded protein although both the parent compound palbociclib (**80**, Figure 17) and degrader **79** bind the closely related CDK4 with similar affinity. Selectivity was rationalized by differences in ternary complex formation. Compound **79** in conjunction with *N*-methylated negative control **81** (BSJ-

Bump, Figure 17) were used as chemical tools to interrogate CDK6-dependent pathways and gene expression in acute myeloid leukemia (AML). Results suggested that CDK6 involvement in AML is primarily reliant on its kinase function.[193]

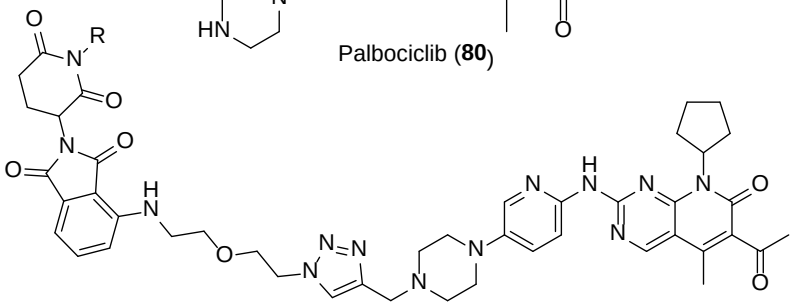
The structurally similar CDK6 PROTAC **82** (CP-10, Figure 17) featuring a clickable triazole spacer was obtained in another study by thorough SAR evaluation using different kinase and E3 ligase ligands and various linkers. Of note, efficient PROTACs could only be generated with cereblon-recruiting ligands. Similar to **79**, degrader **82** showed high efficacy and selectivity against CDK4 while degradation was abrogated by *N*-alkylation at the pomalidomide glutarimide moiety (**83**, Figure 17).[194]



BSJ-03-123 (**79**): R = H
BSJ-Bump (**81**): R = Me



Palbociclib (**80**)



CP-10 (**82**): R = H
CP-23 (**83**): R = Et

*Figure 17: Molecular structures of the CDK6 degraders (**79** and **82**), their negative controls (**81** and **83**) and the CDK6/CDK4 inhibitor **80**.*

Beautiful work recently published by Donovan *et al.* provided a comprehensive assessment of kinase degradability made available as a public resource dataset (see https://proteomics.fischerlab.org/). Making use of MS-based chemoproteomics, they investigated a large set of kinase degraders in different cell lines and assigned degradability scores to create a global map of kinase degradability. The set provides starting points for degraders of over 200 protein kinases along with general design guidelines and broad insights linking degrader characteristics to pharmacologic properties. Importantly, the authors showed that neither cellular target engagement and stability of the ternary complex nor abundance of the target protein represent reliable predictors for degrader efficiency. At the same time, they corroborated the importance of the recruited E3 ligase as a determinant of target space, underlining the need for further E3 ligases to be explored for targeted protein degradation.[195] Recent assay technologies enable real-time characterization and profiling of degraders in live cells improving our mechanistic understanding of targeted protein degradation and assisting PROTAC optimization.[196] Moreover, covalent PROTACs are gaining importance. Despite the fact that irreversible covalent target protein binding is incompatible with the PROTAC's catalytic turnover, such a strategy has proven beneficial in certain contexts. Irreversible binding at the opposite site, i.e. to the E3 enzyme is a promising approach to engage novel ligases. Covalent-reversible approaches, which have recently shown promising results in the kinase context, may further have the potential to improve selectivity, cellular uptake, transport and delivery. For a comprehensive discussion of covalent PROTAC strategies, the reader is referred to excellent recent reviews.[197,198] Furthermore, harvesting alternative protein degradation pathways, like lysosomal degradation[199] is currently expanding the scope of degradable proteins.[187,198]

Kinases Lacking *in vitro* Reagents

Of the around 520 protein kinases in the human genome there are now high resolution structures in the Protein Data Bank for just over 300 (> 95% sequence identity), which illustrates the huge progress in generating purified proteins for this protein family. However, for a significant fraction of the remaining 220 a major challenge hindering the discovery and development of chemical probes is the unavailability of purified protein or assays. While some "easy" kinases can be overexpressed in bacterial systems and purified to homogeneity, others require more elaborate methods and/or may be produced only in low yields. Still other kinases have so far eluded attempts at purification which may in some cases be due to the lack of an obligate protein complex or other cellular components required for protein stability. A rough estimate of the number of these unenabled kinases, based on those kinases with no structure and absent from the catalogues of commercial suppliers of kinase proteins and assays, is 80. Fortunately, the available repertoire of cellular methods for discovering and optimizing small molecule ligands is growing rapidly and many methods now exist that are sufficiently miniaturized and high throughput to be used even for primary compound screening. In addition to traditional methods such as reporter assays, phenotypic screening or measurement of the phosphorylation of a relevant cellular component, some newer methods avoid the difficulties of kinase redundancy and specificity by measuring the direct binding of compounds to protein in cells. Methods such as NanoBRET[36] or competition experiments for binding to immobilized ligands rely upon the ability of the targeted kinase to bind an already known ligand, while others such as SplitLuc CETSA[200] rely on the compound inducing a change in the physical properties of

the target protein. For initial discovery of chemical matter, including for pseudokinases (*vide infra*), cellular fragment screening now offers the hope of identifying ligands for some of the most challenging targets.[169,201]

Targeting Pseudokinases

Kinases share common protein folds and amino acid residues that enable their function as phosphorylation enzymes.[10] A lysine in the VAIK motif in the β3 sheet of the N-terminal lobe and a glycine rich loop (Gly-loop or P-loop) enable kinases to bind ATP (Figure 18). An aspartate from the DFG motif in the activation loop coordinates a magnesium ion while an aspartate from the HRD-loop of the C-terminal lobe acts as a catalytic base in the promotion of phosphoryl transfer. Approximately 10% of protein kinases in the human genome are missing one or more of the residues that support catalytic activity. These kinases are predicted to be enzymatically inactive and are accordingly classified as pseudokinases. Many of these pseudokinases maintain the ability to bind nucleotides or cations or both, serving as the basis for subclassification of pseudokinases.[202] It should be noted, however, that some kinases lacking conserved catalytic residues are nevertheless capable of catalysing phosphorylation. For example, the WNK ("with no lysine") kinases lack the conserved catalytic lysine of the VAIK motif yet remain competent phosphorylation catalysts by compensation from a lysine residue at the terminus of the P-loop.[203]

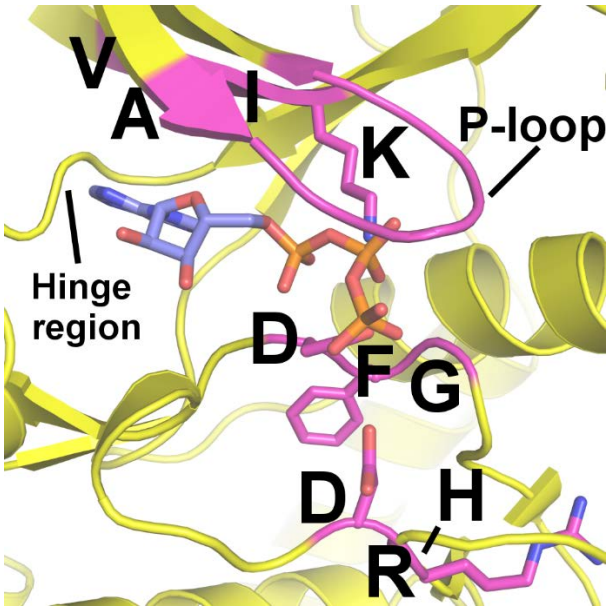


Figure 18: Pseudokinases have the protein kinase fold but lack one or more key motifs essential for nucleotide binding or for phosphoryl transfer. Shown is a schematic representation of an active kinase bound to ATP. Highlighted in magenta are the glycine-rich (P-loop) which is important for nucleotide binding, the VAIK motif whose lysine is important for nucleotide binding and catalysis, the HRD motif whose aspartate is important for catalysis and the DFG motif whose aspartate is important for magnesium binding. Some side chains have been omitted for clarity.

The absence of catalytic activity does not mean that pseudokinases are functionally inert bystanders. Indeed, they retain noncatalytic kinase functions such as allosteric activation, molecular switching, scaffolding, and competitive signaling transduction.[204] Importantly, these noncatalytic functions of pseudokinases underpin their widespread involvement in signaling and in disease. Over two thirds of pseudokinases have been implicated in a range of human pathologies.[205] For example, mutations within the pseudokinase domain of JAK2 underlie human blood cell malignancies[206,207] (for inhibitors targeting the pseudokinase domain of the Janus kinase TYK2, see the allosteric inhibitor section), and TRIB2 plays a role in driving AML via

degradation of the CCAAT-enhancer-binding protein α (C/EBPα) mediated by the pseudokinase.[208,209]

An interesting recent example highlighting the role of pseudokinases was reported by Kahn et al. shedding light on the mechanism of the FDA-approved allosteric MEK inhibitor trametinib (**84**, Figure 19) involving the pseudokinases KSR1 and KSR2 (kinase suppressor of Ras 1 and 2). [210] KSR1/2 are related to the RAF family kinases and known to co-operate with MEK1/2 in regulating the MAPK/ERK pathway.[211] The classification as pseudokinases is due to the replacement of the conserved lysine in the VIAK motif by arginine although there is evidence that KSR1/2 maintain a certain catalytic activity. By elucidating the X-ray structures of different allosteric MEK inhibitors bound to KSR/MEK1 complexes, it was shown that inhibitor **84** gives rise to a cooperative interaction at the KSR/MEK interface remodeling the MEK allosteric binding pocket to exploit molecular glue-like features. In contrast, the compound disrupts the RAF/MEK interaction. This may explain the distinct pharmacological properties of **84** compared to other allosteric MEK inhibitors like cobimetinib (**85**) and selumetinib (**86**, Figure 19), which do not form this interaction, and may help to design a new generation of inhibitors overcoming the limitations of current MEK-targeted drugs in cancer treatment.[210]

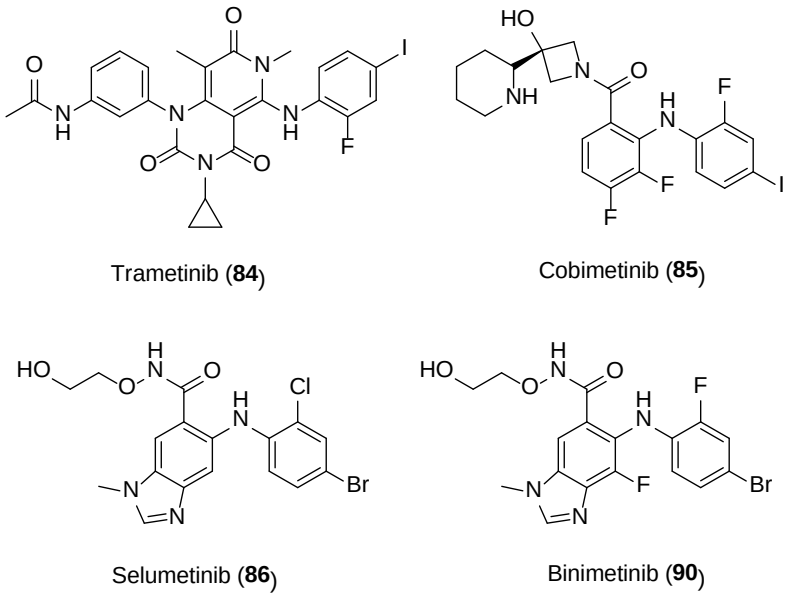


Figure 19: Molecular structures of FDA-approved allosteric MEK inhibitors.

As highlighted by the above examples, the collective evidence strongly suggests opportunities in targeting pseudokinases. A challenge in doing this is that without the ability to measure changes in catalytic activity induced by the developed small molecule modulators, it is necessary to rely upon biophysical or cellular assays. In the case of pseudokinases that retain the ability to bind nucleotides it is common that they also retain the ability to bind ATP-competitive inhibitors. There are also examples of successfully targeting pseudokinases that are unable to bind nucleotides. For example, the covalent EGFR/HER2 inhibitor afatinib forms an adduct with TRIB2 Cys96 in the nucleotide binding site, leading to destabilization *in vitro* and observable degradation in cells.[212] Murphy, Silke, and coworkers identified a kinase inhibitor that bound to the pseudoactive site of mixed lineage kinase domain-like (MLKL) pseudokinase, an effector of necroptosis.[213] Compound binding was shown to inhibit protein translocation and conformational rearrangements and to inhibit necroptosis in cells. For pseudokinases

not amenable to binding ATP-competitive inhibitors the possibilities of targeting other binding pockets or PPI sites using small molecules remain.

In addition to the potential for therapeutic benefit, further study of pseudokinases will help in understanding the contributions from catalytic and noncatalytic functions of all kinases in normal physiology and pathology. The reader is directed to several outstanding pseudokinase reviews.[204,214,215]

Targeting Allosteric Sites

Typically, protein kinase inhibitors bind the conserved ATP binding site. While inhibitors referred to as type I inhibitors target the active kinase with the canonical DFG-motif in an inward-oriented conformation ("DFG-in"), type II inhibitors bind the inactive kinase and extend to a hydrophobic cleft formed in the "DFG-out" conformation (see Figure 20). An intermediate inhibitor type including only parts of the type II pharmacophore is referred to as type I ½.[216,217] Beyond the common type I and type II inhibitors, allosteric protein kinase inhibitors have become more attractive over time, providing distinct opportunities for the development of novel chemical probes and therapeutics.[218–221] Inhibitors targeting allosteric pockets adjacent to the ATP binding site have been classified as type III inhibitors while such targeting more distant allosteric sites are often referred to as type IV inhibitors.[222] It should be noted, however, that these classifications are not used consistently in the literature.

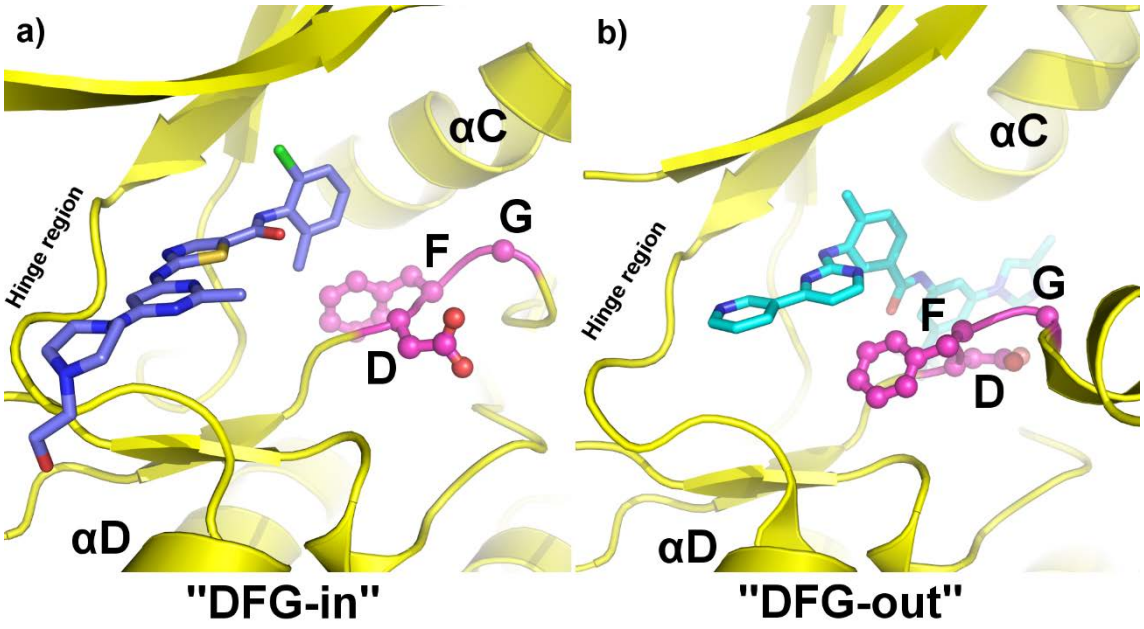


Figure 20: Type I kinase inhibitors target the active conformation of the kinase, in which the aspartate of the DFG motif is directed towards the ATP binding site (DFG-in), while type II kinase inhibitors target an inactive conformation in which the DFG motif is flipped (DFG-out) enabling the inhibitor to access a hydrophobic pocket formerly occupied by the DFG phenylalanine. Type I/DFG-in binding exemplified here by dasatanib binding to ABL1 (PDB code: 2GQG[223], left) and type II/DFG-out binding exemplified here by nilotinib binding to ABL1 (PDB code: 3CS9[224], right).

Since allosteric inhibitors do not target the highly conserved ATP site of kinase enzymes, they often achieve higher selectivity, especially against kinases from other families, thus reducing undesired effects. At the same time, addressing allosteric sites is a valuable strategy for targeting specific protein-protein interactions or pseudokinases (*vide supra*). Due to their often limited ATP competitiveness, allosteric kinase inhibitors may have less stringent requirements for potency and may provide improved cellular activity.[219,225] In certain cases, they may also be employed in combination with orthosteric inhibitors to improve efficacy. While ATP-competitive ligands can only serve as inhibitors, allosteric targeting is also a possible strategy to achieve kinase activation, as exemplified by compounds **87** or **88** (Figure 21), both being allosteric c-Abl activators binding to the myristoyl pocket.[226] Such a strategy has not yet received widespread

attention in the protein kinase field but could be useful in a variety of therapeutic contexts. Moreover, allosteric ligands (even silent ones) may be employed to generate highly specific PROTACs, as demonstrated for EGFR and BCR-Abl.[222] Allosteric ligands cover different areas in chemical space compared to ATP pocket binders and may thus hold distinct physicochemical properties and be less likely to cause intellectual property (IP) issues. However, due to lack of generalizable assay technologies, identification of allosteric kinase ligands is not trivial and is typically serendipity-driven. Crystallographic structures of kinase/inhibitor complexes are the most common way to identify and characterize allosteric inhibitors, especially if prior knowledge of binding site and mechanism is unavailable. Despite the advent of high throughput crystallography, this can still represent a substantial bottleneck. In addition, with conformational plasticity being a hallmark of the protein kinases' structures, allosteric sites are often cryptic and thus not observed in the apo-state. For example, and as mentioned before, recent investigation of MKK7 structural biology revealed that ibrutinib (**43**, see Figure 9a) binds in an unexpected 2:1 stoichiometry occupying the ATP binding site as well as an unprecedented induced allosteric pocket on top of the N-lobe (see Figure 9b-d).[103] A recent systematic mapping of kinase/inhibitor X-ray structures has exposed the existence of 10 type IV allosteric sites in kinase domains, showcasing possibilities for hit identification efforts.[220] Beyond the kinase domain, allosteric modulators may also target adjacent domains with regulatory functions such as pseudokinase domains or extracellular domains of receptor tyrosine kinases. For example, addressing the regulatory pseudokinase (JH2) domain of the Janus kinase TYK2 has led to phase III clinical candidate BMS-986165 (**89**, Figure 21), which features exquisite functional selectivity over the other JAK family members, JAK1, JAK2 and JAK3.[227] As stated above,

kinases' structures are very flexible and analyses of protein-inhibitor interactions in a dynamic manner will thus play an increasingly important role. Experimental assays capable of monitoring kinase conformations and/or interactions including and NMR[228] but also fluorescence-based methods such as the (i)FLIK[229] together with molecular dynamics simulations and other modern computational approaches could aid streamlining the identification and structure-based optimization of allosteric inhibitors.[218,222,230,231]

The most common allosteric binding mode is the type III binding mode, where the ligand binds to a pocket in the back of the ATP binding site.[222] Such a binding mode is adopted for example, by the aforementioned RIPK1 inhibitors (see Figure 12) and the approved allosteric MEK1/2 kinase inhibitors trametinib (**84**), cobimetinib (**85**), selumetinib (**86**) and binimetinib (**90**) and (see Figure 19). As is frequently observed for this inhibitor type, the latter engage the active kinase conformation in a manner compatible with simultaneous ATP binding. Other prominent examples of type III inhibitors include EGFR inhibitors like **91** and **92** (Figure 21), which potently inhibit EGFR harboring the gefitinib-resistant T790M mutation. Notably, binding of inhibitor **92** is enhanced by the covalent EGFR inhibitor osimertinib.[232]

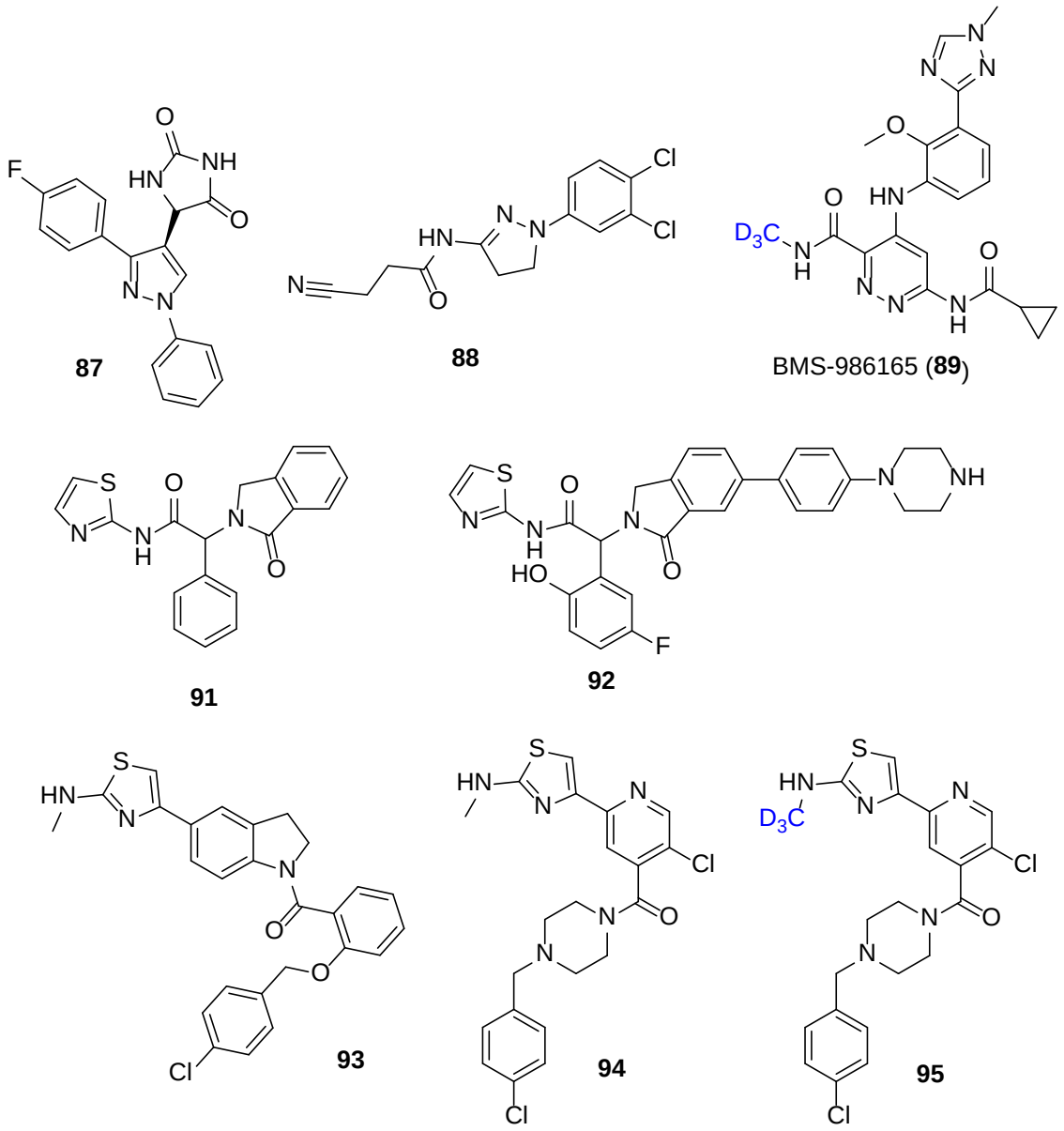


*Figure 21: Selected examples of protein kinase inhibitors targeting allosteric sites. Deuterated groups are highlighted in blue. For allosteric MEK inhibitors **84**-**86** and **90**, see Figure 19.*

A related binding mode is adopted by non-ATP competitive and highly specific inhibitors of WNK1-4 identified by researchers from Novartis in a screen for non-ATP competitive inhibitors. Prioritization of atypical chemotypes and optimization of the most promising hit furnished compound **93** (Figure 21) showing excellent WNK selectivity and a type III binding mode as confirmed by crystal structure (PDB code: 5TF9).[233] A subsequent scaffold morphing approach and induction of torsional strain via

the pyrimidine C5 substituent yielded compounds exemplified by **94** (Figure 21) with improved WNK1 potency (IC_{50} = 4 nM) and selectivity against WNK2 and WNK4, albeit with off-target activity on BTK and FER. Deuteration at the methyl group (**95**, Figure 21) improved PK properties and this compound was used as an *in vivo* probe to investigate the role of WNKs in cardiovascular homeostasis.[234]

Type III inhibitors binding to a DFG-out conformation are also known. For example, highly potent and selective allosteric PAK1 inhibitor **96** (NVS-PAK1-1, Figure 22) and related compounds,[235] which can be used in conjunction with negative control **97** (NVS-PAK1-C, Figure 22) to probe PAK1 biology, adopt such a binding mode. Notably, this results in an indirectly ATP-competitive behavior. Similarly, LIMK1/2 chemical probe **98** (TH-257; negative control: **99**, Figure 22) and analogs show a DFG-out type III binding mode which is assumed to confer the excellent selectivity to these compounds.[236] Covalent allosteric inhibitors with a type III binding mode such as the pan-AKT inhibitor borussertib (**100**, Figure 22) have also been described. Similar to previous reversible allosteric AKT inhibitors, (e.g. **101** and **102**, Figure 22),[222] covalent inhibitor **100** binds the interface of the kinase domain and the N-terminal pleckstrin homology (PH) domain stabilizing an autoinhibited "PH-in" conformation. Additional covalent engagement of activation loop cysteines Cys296 and Cys310 conferred excellent potency (IC_{50} = 0.2 nM, k_{inact}/K_I = 3.29 $\mu M^{-1}s^{-1}$) while maintaining selectivity and the compound showed promising results in preclinical investigations.[237,238]

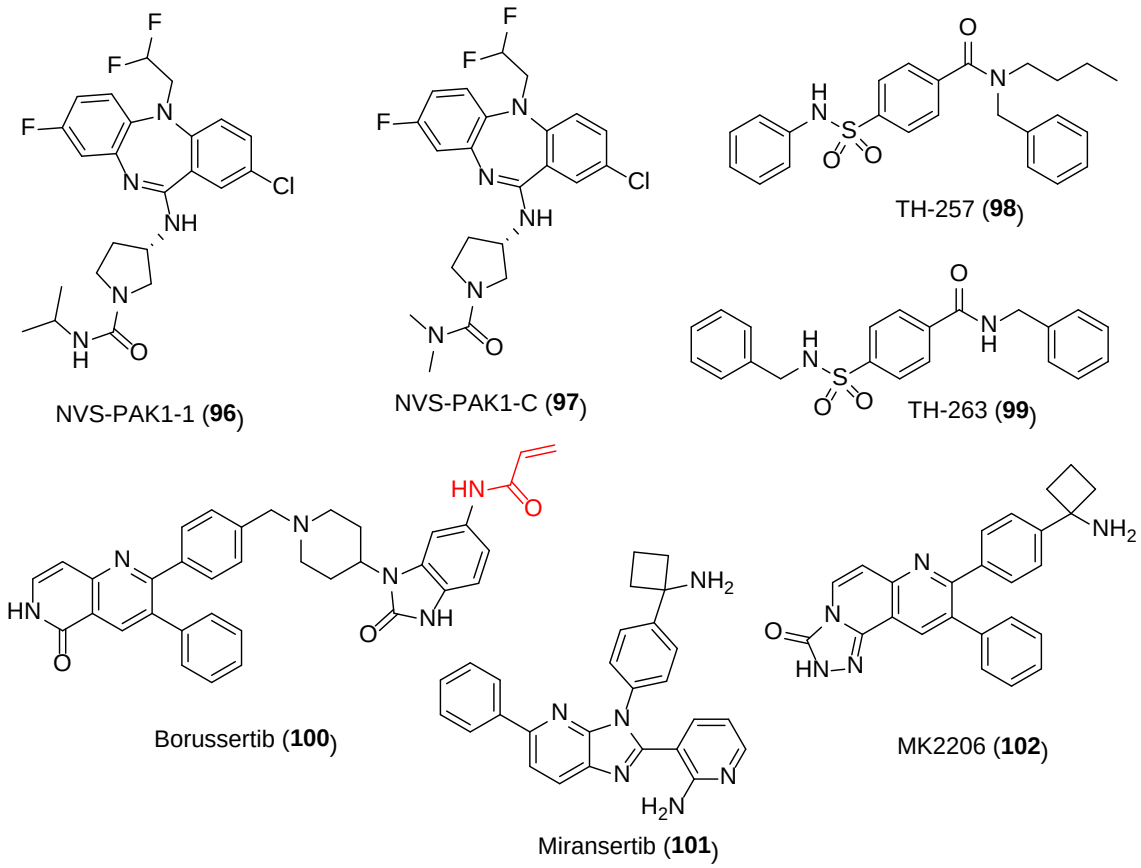


Figure 22: Selected examples of allosteric type III inhibitors targeting kinases in the DFG-out conformation and covalent type III inhibitor borussertib (reactive warhead highlighted in red).

As mentioned above, type IV inhibitors have been reported for a variety of different allosteric sites more distant to the ATP pocket. A thorough discussion of such inhibitors, however, is beyond the scope of this perspective and may be found elsewhere.[221,222,239] It is worth emphasizing here the importance of making use of complementary techniques to confirm an allosteric inhibition mechanism and avoid misinterpreted data. Besides crystallographic structures and biochemical methods such as assays run at varying concentrations of ATP, biophysical experiments such as nuclear magnetic resonance (NMR) or hydrogen–deuterium exchange (HDX) mass spectrometry, photoaffinity labeling in conjunction with mass spectrometry or mutation experiments can give valuable information about binding modes when no co-crystal

structures can be solved. The need for proper characterization is exemplified by aminothiazole derivatives (not shown) that were initially considered as allosteric CK2α inhibitors, but thorough structural and biophysical evaluation revealed that those compounds are in fact ATP-pocket binders.[240]

Targeting Parasite Kinases

Human parasites such as *Plasmodium falciparum* (etiologic agent of malaria) or *Trypanosoma brucei* (etiologic agent of human African trypanosomiasis) have protein kinases that perform crucial functions in the parasite life cycle and targeting these kinases may provide a route to effective anti-parasite drugs, taking advantage of the generally high druggability of protein kinases. Validation of parasitic kinases as potential drug targets has aroused the attention of the scientific community in recent years.[241,242] Considering that many parasitic infections are classified as neglected tropical diseases (NTDs)[243] and affect a significant part of the global population, the need for new drug development is clear. The complexity of parasite life cycles and difficulties of using genetic methodologies to analyze parasite biology inside a host organism or validate parasite protein targets emphasize the potential value of obtaining chemical probes to study parasite biology and to serve as starting points for drug development.[244,245] Often, specific differences between the parasitic and human kinome are important for designing parasite-selective compounds. For instance, differences in one or more amino acid residues lining the ATP-binding pocket often distinguish between human and parasitic orthologs. Furthermore, many human kinases are characterized by the

presence of accessory domains important for protein-protein interactions, which domains are rare, for example, in trypanosomatid kinomes.[246]

Repurposing of human kinase inhibitors on parasites using target-based or phenotypic screening may currently represent the most common and fertile strategy for identifying promising antiparasitic kinase inhibitors.[247,248] Valuable progress has been made recently in obtaining small molecules as chemical tools for understanding certain parasitic kinases. For example, compound **103** (AB1, Figure 23), a covalent human EGFR inhibitor, was identified by phenotypic screening (~2.3 million compounds) and subsequent SAR analysis as a potent antitrypanosomal agent (EC_{50} of 72 nM for *T. brucei*) with high selectivity over human cells.[249] In an experiment involving inducible gain-of-function mutants of individual parasite kinases, only *Tb*CLK1 (also known as KKT10) showed a 6.5-fold increase in resistance against the compound. An enzymatic assay confirmed compound **103** to be a low nanomolar *Tb*CLK1 inhibitor with ~90-fold selectivity against human CLK1. Inactivity of the non-reactive analog **104** (AB2, Figure 23, IC_{50} >20 µM), MS experiments and a co-crystal structure (PDB code: 6Q2A) confirmed covalent interaction with *Tb*CLK1 active site Cys215. The human CLK1 isoform does not contain a cysteine residue at the same region providing a rationale for the observed selectivity. Further experiments demonstrated that *Tb*CLK1 inhibition was detrimental to normal kinetochore function during metaphase and exit from anaphase into cytokinesis, leading to the death of the parasitic cell.[249]

An example of a target-based approach was published in 2019 by Adam *et al.*.[250] Screening of a ~30,000 compound library against the *Plasmodium falciparum* kinase CLK3 (*Pf*CLK3), an enzyme involved in regulation of RNA-splicing, led to the discovery of

pyrrolopyridine derivative **105** (TCMDC-135051, Figure 23) as a potent inhibitor of this plasmodial kinase (IC_{50} = 40 nM). The compound's antiplasmodial activity, which had been reported previously by Gamo *et al*. in 2010,[251] was confirmed (*P. falciparum* 3D7 and Dd2, EC_{50} = 180 nM and 450 nM, respectively). Inhibitor **105** displayed excellent selectivity versus human CLK2 (~100-fold) and the closely related human kinase PRPF4B. It was shown that *Pf*CLK3 inhibition eliminated blood stage and liver-stage parasites and blocked transmission to mosquitos. The compound was also active against other *Plasmodium* species and efficiently cleared parasites from peripheral blood in mice.[250] In a subsequent SAR study, bioisosteric replacement of the carboxylic acid by tetrazole gave compound **106** (Figure 23), which also showed potent *Pf*CLK3 inhibitory activity (IC_{50} = 19 nM) and a similar antiplasmodial profile (EC_{50} = 271 nM, 3D7 strain)[252].

Recently, an unbiased genetic interrogation of protein kinase function in *Leishmania mexicana* identified 44 proteins essential for survival, differentiation or infection success of the parasite.[253] These results may guide prioritization of targets for generation of chemical tools to enable complementary validation.

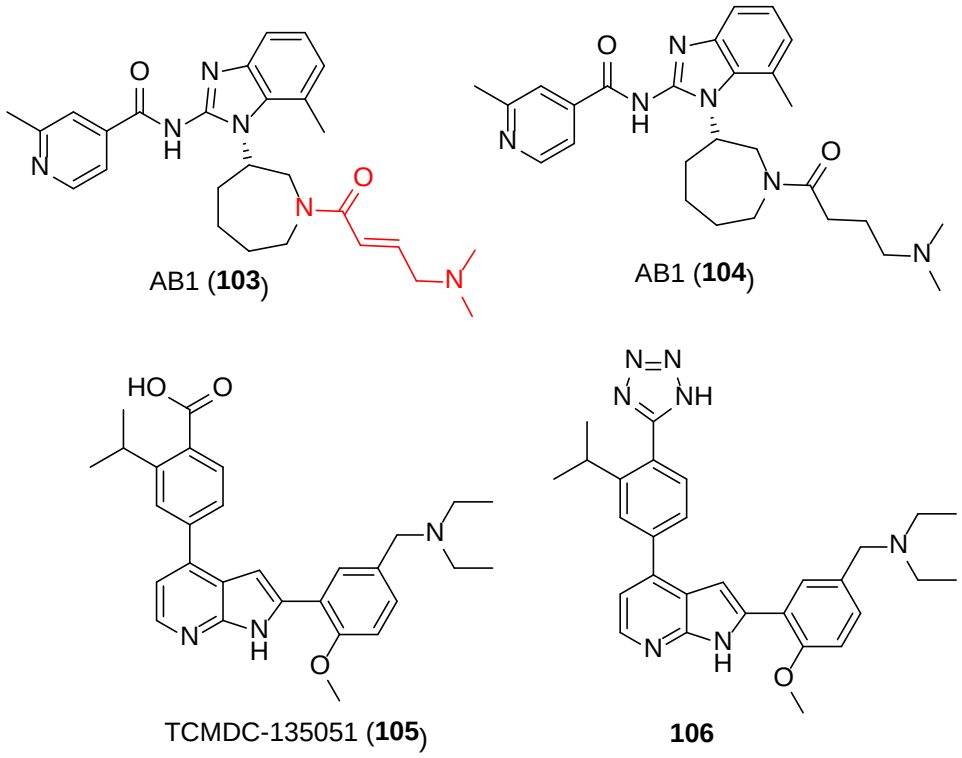


Figure 23: Molecular structure of chemical probes for parasitic kinases: ***103*** *(TbCLK1) and* ***105*** *(PfCLK3) with their negative control (****104****) and bioisoster (****106****), respectively. The reactive warhead of compound* ***103*** *is highlighted in red.*

Chemical-genetic approaches using the bump-and-hole tactic (see Figure 24), which were introduced to the kinase field by the Shokat group approximately twenty years ago,[254] have been widely used for validation of parasitic kinase targets.[255] This strategy makes use of mutant kinases, in which a bulky amino acid (i.e. Phe, Met or Leu) in the gatekeeper position is mutated to a smaller residue (usually Gly or Ala) to create a novel pocket ("hole") that can be exploited for exclusive interaction with complementary "bumped kinase inhibitors" (BKIs).[256] The calcium-dependent protein kinase 1 (CDPK1) of *Cryptosporidium* and *Toxoplasma gondii*, however, naturally contains a glycine gatekeeper residue,[257,258] allowing BKIs to act selectively against such parasitic kinases.[259] On the other hand, bump-and-hole approaches may be flexibly employed either by enlarging the cavity behind the gatekeeper or also by reducing its

size to (in)validate kinase targets. For example, Green *et al.* elegantly employed both strategies to investigate the functional relevance of two related plasmodial kinases.[260] By analogy, protein kinases may also be sensitized to electrophilic inhibitors by introduction of cysteine,[261] as shown for the *Plasmodium falciparum* NEK2 (*Pf*NEK2) kinase.

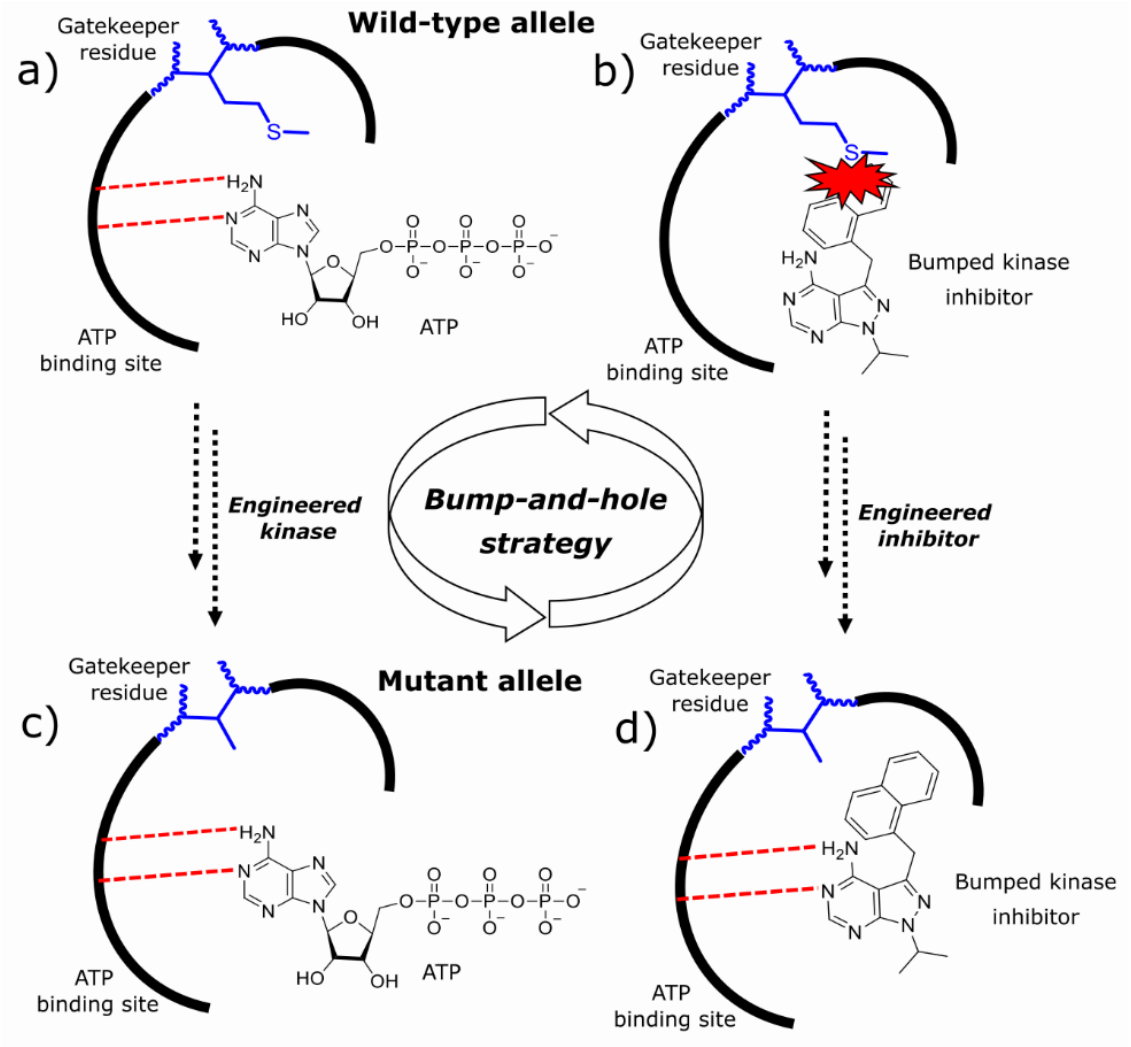


Figure 24: Illustration of the bump-and-hole strategy: a) The gatekeeper in the ATP binding site is usually a large amino acid residue (e.g. methionine). b) Bumped kinase inhibitors are not able to bind to the wild-type ATP binding site, because of the steric hindrance generated by the large gatekeeper amino acid. c) Engineered smaller amino acids (e.g. alanine) in the gatekeeper position do not affect the ATP interaction. d) After gatekeeper mutation, bumped kinase inhibitors are able to access the extended hydrophobic sub-pocket and to inhibit the kinase in an ATP-competitive manner.

Phenotypic Screening and Chemogenomic Profiling

Phenotypic screening, the standard screening approach prior to the target-centric genomic era, has experienced a revival in recent years.[262] This renewed interest stems from the ability of phenotypic screens to directly identify compounds with effects on advanced disease-relevant parameters. Additionally, several technological factors have facilitated phenotypic screening, including the improved ability to develop highly advanced and relevant models of disease in cells, tissues, or even whole organisms coupled with the development of powerful target deconvolution methods.[263]

Kinase inhibitors are commonly identified from phenotypic screens, an unsurprising observation given the ubiquity of kinase involvement in almost all cellular signaling. Kinase target deconvolution from phenotypic screens remains a challenge, particularly with broader spectrum hit compounds. Additional experiments are routinely required for target identification such as affinity selection, proteomic profiling (*vide supra*), and haploinsufficiency profiling.

Chemogenomic profiling has been proposed as a strategy to help bridge the gap between phenotypic and target-based approaches for compound discovery.[264] Chemogenomic compound sets are collections of selective small molecules with well-annotated biological activity. The aspiration is that, by screening highly characterized compound sets, the efficacy target(s) of hit compounds can be identified based on compound activity profile. Kinase inhibitors are well-suited to chemogenomic profiling due to the availability of kinome-wide approaches for compound assessment that can inform the definition of screening sets to exclude broadly active kinase inhibitors.

Drewry and coworkers engaged the research community for the assemblage of an openly-available kinase chemogenomic set (KCGS) that would collectively address the entire human kinome with narrow spectrum inhibitors.[45] The first generation of the KCGS contains 187 inhibitors that address 215 human kinase.[265] Importantly, all compounds in the set have broad kinome profiling data to support their inclusion, and the data across the set was generated in a consistent format (Eurofins/DiscoverX SCANmax panel based on the KINOMEscan technology). Potential limitations of KCGS may arise from biochemical kinase activity profiles not being reflected in cells or organisms or compound interactions with a non-kinase protein. Nonetheless, this publicly accessible resource promises to be useful in identification of kinase roles in advanced cellular models of disease.

PERSPECTIVE AND SUMMARY

Despite the recent increase in the rate of drug approvals by the FDA, which includes a growing number of kinase inhibitors,[266] the problem of attrition in advanced clinical phases due to lack of efficacy remains. To overcome this problem, chemical probes have successively gained importance as tools for preclinical target validation.

Various kinases that would until recently have been considered understudied, such as AAK1, BMP2K/BIKE, GAK, STK17B, NIK, DCLK1, CDK12 and CDK13, now have high-quality chemical tools available for in-depth exploration of their biology (see Table 1 for an overview for the probes discussed in this article). Indeed, use of these chemical probes has already uncovered or validated interesting new insights into human cellular biology such as the link between AAK1 and WNT signaling and the promise of CDK12/CDK13 as anticancer targets. The use of specific kinase chemical probes can also

contradict previous findings made by genetic methods or using non-specific molecules and highlight the need for further studies to understand a protein's function. The need for many more chemical probes is clear and interestingly, in this context, an ambitious private-public open science initiative called "Target 2035" has been recently established with the aim of generating a chemical probe for every protein in the human genome by the year 2035 (see https://www.target2035.net/).[267]

Table 1: Summary of key compounds discussed herein.[a]

Kinase target(s)	Chemical Probe / Tool compound	Negative control	References
AAK1, BIKE	SGC-AAK1-1 (**3**)	SGC-AAK1-1N (**4**)	Wells et al.[34] Agajanian et al.[35]
GAK	SGC-GAK-1 (**10**)	SGC-GAK-1N (**11**)	Asquith et al.[48]
ALK4, ALK5	TP-008 (**14**)	TP-008n (**15**)	Hanke et al.[55]
ALK2	**17**		Ensan et al.[59]
STK17B	SGC-STK17B-1 (**19**)	SGC-STK17B-1N (**20**)	Picado et al.[62]
NIK	NIK-SMI1 (**25**)		Blaquiere et al.[69] Brightbill et al.[70]
DCLK1	DCLK1-IN-1 (**28**)	DCLK-NEG (**29**)	Ferguson et al.[75]
TBK1, IKKε	BAY-985 (**33**) GSK8612 (**35**)	BAY-440 (**34**)	Lefranc et al.[78] Thomson et al.[79]
CDK12, CDK13	THZ531 (**38**), **41**	THZ531R (**39**) THZ532 (**40**)	Zhang et al.[86] Jiang et al.[90]
MKK7	**44**, **45**, **46**		Wolle et al.[100] Shraga et al.[102]
CK2	SGC-CK2-1 (**48**) **50**	SGC-CK2-1N (**49**)	Wells et al.[108] Krämer et al.[109]
FAK	BJG-03-025 (**53**)	BJG-01-085 (**52**)	Groendyke et al.[113]
RIPK1	TP-030-1 (**59**) TP-030-2 (**60**)	TP-030n (**61**)	Yoshikawa et al.[129]
JAK3	FM-381 (**64**) FM-409 (**65**)	FM-479 (not shown)	Forster et al.[140] Forster et al.[141]
PAK1	NVS-PAK1-1 (**96**)	NVS-PAK1-1 (**97**)	Karpov et al.[235]
LIMK1, LIMK2	TH-257 (**98**)	TH-263 (**99**)	Goodwin et al.[236]
*Tb*CLK1	AB1 (**103**)	AB1 (**104**)	Saldivia et al.[249]
*Pf*CLK3	TCMDC-135051 (**105**)		Alam et al.[250]

	Degrader Probes		
TBK1	TBK1 PROTAC 3i (**69**)		Crew et al.[188]
ALK	**72** TL13-112 (**73**)	**74**, **75**	Powell et al.[189]
CDK9	THAL-SNS-032 (**76**)		Olson et al.[191]
CDK6	BSJ-03-123 (**79**) CP-10 (**82**)	BSJ-Bump (**81**) CP-23 (**83**)	Brand et al.[193] Su et al.[194]

[a]Non-exhaustive list of the main chemical probes, tool compounds and negative controls discussed in this perspective. The compounds are listed in order they appear in the text.

Now more than ever, new technologies promise to make such ambitious plans possible. We have highlighted a few such technologies in this perspective (for a selection of relevant techniques discussed here, see Table 2) but cannot do justice to the range of capabilities available today, from AI-driven inhibitor design and synthesis to multiplexed cellular assay systems and ever-increasing ways to measure binding of compounds to proteins with higher sensitivity and greater throughput. New medicinal chemistry technologies such as covalent targeting of amino acids other than cysteine,[142] photoswitchable inhibitors[268] or even degraders,[269–272] or covalent ligand-directed release (CoLDR)[273] where a cargo (e.g. a fluorescent dye, an imaging agents or a toxin) is released from α-substituted methacrylamides upon covalent binding enable new experimental strategies for both inhibitor design and biological discovery.

Table 2: Summary of the major kinase screening and profiling techniques discussed herein.[a]

Technique	Short description	References
NanoBRET	Bioluminescence resonance energy transfer (BRET)-based assay technology using the Nanoluc luciferase and a fluorescent tracer to	Vasta et al.[36]

	quantify target engagement in live cells.	
Kinase panels	Screening platforms available in house or from commercial suppliers (e.g. DiscoverX/Eurofins or Reaction Biology Corp.) typically employing purified enzymes and biochemical or biophysical readouts to evaluate the selectivity of inhibitors within the kinome.	Davis et al.[37] Anastassiadis et al.[18]
KINOMEscan	Screening platform relying on an active site-directed competition binding assay to quantify interactions of inhibitors with a large number of kinases.	Ref[274] Davis et al.[37]
KinaseSeeker	Screening platform relying on an active site-directed competition binding assay exploiting a change in bioluminescence of a split luciferase system.	Jester et al.[63]
KiNativ	Chemoproteomic screening platform relying on an irreversible transfer of a biotinylated group from an ATP analog to conserved lysine moieties. Can be used to quantify interactions of inhibitors with many kinases in cell or tissue lysates.	Patricelli et al.[76]
Kinobead	Bead-based chemoproteomic method using immobilized inhibitors to enrich kinases from cell lysates for LC-MS analyses.	Reinecke et al.[80]
isoTOP-ABPP ("isotopic Tandem Orthogonal Proteolysis – Activity-Based Protein Profiling")	Chemoproteomic method employing stable isotope labeling and residue-specific covalent enrichment probes for global profiling of targets or functional amino acid residues.	Backus et al.[174]
CITe-Id ("Covalent Inhib-itor Target-site Identification")	Chemoproteomic method employing tagged covalent ligands as enrichment reagents for direct and proteome-wide covalent inhibitor target-site identification	Browne et al.[175]
CETSA / TPP ("CEllular Thermal Shift Assay / Thermal Proteome Profiling")	Label-free method exploiting ligand-induced changes in protein thermal stability for small molecule target identification in intact cells and tissue	Molina et al.[177] Savitski et al.[178]

DARTS ("Drug Affinity Responsive Target Stability")	Label-free method exploiting ligand-induced changes in protein stability against proteolysis for small molecule target identification in cell lysates.	Lomenick et al.[180]
SPROX ("Stability of Proteins from Rate of Oxidation")	Label-free method exploiting ligand-induced changes in protein susceptibility towards oxidation for small molecule target identification in cell lysates.	West et al.[181]
SplitLuc CETSA	High-throughput screening method based on the cellular thermal shift assay principle exploiting the luminescence generated by reconstitution of a split Nanoluciferase.	Martinez et al.[200]

[a]The methods and techniques are listed in order they appear in the text.

The recent successes of targeted protein degradation hold promise that PROTAC degraders will become a standard approach that will be tested alongside development of small molecule inhibitors. Here too, the pace of innovation continues to be rapid as exemplified by the application of irreversible and reversible covalent chemistry, at one time avoided in this field, to enable recruitment of novel E3 ligases as well as improving cellular uptake, specificity and degradable target space. Molecular glue degraders inducing a novel interaction between an E3 ligase and the target protein without the requirement for a large, chimeric structure hold the promise of improving the physicochemical properties of degrader molecules while exploitation of the lysosomal degradation system brings extracellular targets within range of targeted degradation.[275]

In conclusion, we see the next ten years as a time of great opportunity for protein kinase research and drug development. With so many kinases for which little knowledge exists and the rapid development of new technologies for discovering and developing

small molecule modulators, the prospects for new discoveries of fundamental importance are enticing.

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establishing the synthetic Medicinal Chemistry lab. While being an SGC-member, he did research stays at the University of North Carolina at Chapel Hill and University of Tübingen, where he is now working as a Teach@Tübingen fellow. His main research interest is on the design and synthesis of reversible and irreversible kinase inhibitors, focused on generation of chemical probes to investigate function and therapeutic potential of understudied kinases.

Jonathan M. Elkins obtained a PhD in chemistry in the laboratory of Prof. Sir Jack E. Baldwin at the University of Oxford. During 16 years at the Structural Genomics Consortium he conducted research on a wide variety of protein families before focussing on chemical probe development for protein kinases and related signalling proteins. He currently leads a research group at the Centre for Medicines Discovery at the University of Oxford.

William J. Zuercher studied chemistry at Grinnell College and obtained his PhD in Organic Chemistry at the California Institute of Technology. He worked for 15 years at GlaxoSmithKline and led many hit-to-candidate programs. He was a founding member and principal investigator of the Structural Genomics Consortium at the University of North Carolina at Chapel Hill and research associate professor in the Division of Chemical Biology and Medicinal Chemistry in the UNC Eshelman School of Pharmacy. His main research interest is in using chemistry to elucidate biology, through the design, synthesis and utilization of chemical probes for understudied protein targets.

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ABBREVIATIONS USED

AAK1; adaptor protein 2-associated Kinase 1; ABCB1, ATP-binding cassette subfamily B member 1; ABPP, activity-based protein profiling; ADP, adenosine diphosphate; AI, artificial intelligence; AKT, RAC-serine threonine kinase; ALK, anaplastic lymphoma kinase; AML, acute myeloid leukemia; AP2M1, adaptor Related Protein Complex 2 Subunit Mu 1; ATC, anaplastic thyroid carcinome; ATP, adenosine triphosphate; BAFF, B-cell activating factor; BCR-Abl, breakpoint cluster region-abelson leukemia; BKI, bumped kinase inhibitor; BLK, B-lymphoid tyrosine *kinase;* BMP2K/BIKE, BMP-2-inducible kinase; BMX, bone marrow tyrosine kinase on chromosome X; BTK, Bruton's tyrosine kinase; CaMK, calcium/calmodulin-dependent kinase; CAMKK, calcium/calmodulin-dependent protein kinase kinase; CAP1, cyclase-associated protein 1*;* Cas9, CRISPR-associated protein 9; CDC2, cell division control protein 2 homolog; CDK, Cyclin-dependent kinase; CDPK, calcium-dependent protein kinase; C/EBPα, CCAAT-enhancer-binding protein α; CETSA, cellular thermal shift assays; cGMP, cyclic guanosine monophosphate; CITe-ID, covalent inhibitor target site identification; CK2, casein kinase 2; CLK, CDC2-like kinase; CNS, central nervous system; CoLDR, covalent ligand-directed release; CRISPR, clustered regularly interspaced short palindromic repeats; CuAAC; copper(I)-catalyzed alkyne-azide cycloaddition; DAPK, death-associated protein kinase; DARTS, drug affinity responsive target stability; DBN1, developmentally regulated brain

protein (also known as drebrin); DC_{50}, half-maximal degradation concentration; DCLK1, doublecortin like kinase 1; DEL, DNA-encoded libraries; DFT, density functional theory; DIPG, diffuse intrinsic pontine glioma; DNA, deoxyribonucleic acid; DRAK, DAPK-related apoptosis-inducing protein kinase;dsDNA, double-stranded deoxyribonucleic acid; DYRK2, dual specificity tyrosine-phosphorylation-regulated kinase 2; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; FAT, focal adhesion-targeting; FBDD, fragment-based drug discovery; FDA, Food and Drug Administration; FERM, 4.1R, ezrin, radixin, moesin; FGFR4, Fibroblast Growth Factor Receptor 4; FLT3, fms-related receptor tyrosine kinase 3; FOP, fibrodysplasia ossificans progressiva; FYN, tyrosine-protein kinase fyn; GAK, cyclin G-associated kinase; HCT116, human colorectal carcinoma 116 cells; HDX, hydrogen-deuterium exchange; HEK293, human embryonic kidney 293 cells; HER2, human epidermal growth factor receptor 2; HH, human hepatocytes; HLM, human liver microsomes; HN1, hematological and neurological expressed 1 protein; HTS, high-throughput screening; IC_{50}, half-maximum inhibitory concentration; IDUP, interaction determination using unpurified proteins; IFN, interferon; IKKs, IkappaB kinases; IP, intellectual property; ITC, isothermal titration calorimetry; ITK, interleukin-2-inducible T-cell kinase; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; KCGS, kinase chemogenomic set; KHS1, mitogen-activated protein kinase kinase kinase kinase 5 (also known as MAP4K5); KIT, tyrosine-protein kinase Kit; KRAS, p21 GTPase (oncogene first found in Kirsten rat sarcoma virus); KSR, kinase suppressor of Ras; LIMK, LIM-kinase; LRRK2, leucine-rich repeat kinase 2; LRP6, low-density lipoprotein receptor-related protein 6; MAPK; mitogen-activated protein kinase; MAP2K, mitogen-activated protein kinase kinase; MDCK, madin-darby canine kidney line model; MEK, Mitogen-activated protein kinase kinase; MEK5, mitogen-activated protein kinase kinase 5; MELK, maternal

embryonic leucine zipper kinase*;* MKK7, mitogen-activated protein kinase 7; MLKL, mixed lineage kinase domain-like; MPSK1, myristoylated and palmitoylated serine/threonine kinase 1; MS, mass-spectrometry; mTOR, mammalian target of rapamycin; NAKs; numb-associated *kinase;* NanoBRET; nanoLuc bioluminescence resonance energy transfer; NEK, NIMA (never in mitosis gene a)-related expressed kinase; NF-κB, nuclear factor kappa B; NIK, nuclear factor kappa B-inducing *kinase;* NTD, neglected tropical disease; PAK1, P21 (RAC1) Activated Kinase 1; PARP, poly (ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cell; PD, pharmacodynamic; PDAC, pancreatic ductal adenocarcinoma; PDB, protein data bank; PDGFR, platelet-derived growth factor receptor A; PDGFRB, platelet derived growth factor receptor beta; PH, pleckstrin homology; PIN1, peptidyl-prolyl cis-trans isomerase; PIP5K1C, phosphatidylinositol-4-Phosphate 5-Kinase Type 1 Gamma; PK, pharmacokinetic; PKD1, polycystic kidney disease 1; PKIS; published kinase inhibitor set; PPI, protein-protein interaction; PROTACS, proteolysis-targeting chimeras; PRPF4B, pre-MRNA processing factor 4B; PTK2, protein tyrosine kinase 2; RIOK, RIO kinase; RIPK, receptor interacting serine/threonine protein kinase; RNA, ribonucleic acid; RO3, rule-of-three; RO5, rule-of-five; RPS6KA1, ribosomal protein S6 kinase A1; RSK, 90 kDa ribosomal S6 kinase; RSTK, receptor serine/threonine kinases; SAR, structure-activity relatioship; SARS-COV-2, severe acute respiratory syndrome coronavirus-2; SK-MEL-2, human melanoma 2 cell line; S6K, ribosomal protein S6 kinase 1; SLE, systemic lupus erythematosus; SOSA, selective optimization of side activities; SPROX, stability of proteins from rate of oxidation; STK17B, serine/threonine-protein kinase 17B; TBK1, TANK binding kinase 1; TGFBR1, transforming growth factor beta receptor 1; TGFβ, transforming growth factor beta; TPP, thermal proteome profiling; TRIB2, tribbles pseudokinase 2; TYK2, non-

receptor tyrosine-protein kinase 2; VHL, von hippel-lindau; WNT, wingless-related integration site; WNK, with-no-lysine kinase; YSK4, mitogen-activated protein kinase kinase kinase 19 (also known as MAP3K19); ZIP8, zinc transporters SLC39A8.

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