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Irreversible Protein Kinase Inhibitors: Balancing the Benefits and Risks

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INTRODUCTION

In the relatively young but expanding field of irreversible kinase inhibitor drug discovery, there are two main developments that are of central importance. First, the patients have watched the first wave of low molecular weight protein kinase inhibitors becoming available to them over the past decade. These kinase inhibitors have been approved by the U.S. Food and Drug Administration (FDA) for therapeutic use in oncology indications and constitute an important addition to the arsenal of drugs to combat cancer (Table 1). Without exception, these marketed protein kinase inhibitors have been identified and developed using conventional approaches, i.e., reversible inhibitors that (partly) occlude the ATP pocket in the catalytic domain of the kinase. Although protein kinases are regarded as an attractive drug target family, it took (and still takes) huge efforts to master the human kinome, which comprises more than 500 protein kinases. The search for clinically applicable kinase inhibitors that target the highly conserved ATP pocket has been thwarted by a couple of well-known hurdles. Selectivity, cellular potency, and an increasingly crowded intellectual property arena are major points of attention.

The second important development is the renewed interest in covalent binding drugs (reviewed by Potashman and Duggan and by Singh et al).[1,2] This recent revival results from a better understanding of the benefits of the covalent binding principle and the approval of effective and safe covalent drugs. Historically, drug discoverers have been taught to stay away from small molecular entities that harbor reactive electrophilic groups because these used to be equivalent to promiscuity.[3] Promiscuous hits that relied on reactive groups were traditionally hard to optimize toward leads, since these were more than often interfering with the biochemical assay rather than truly modifying the activity of the target of interest.[4] Even if the target modulation was real, indiscriminant reactivity was believed to trigger insurmountable toxic events that may surface in late stage clinical trials when larger patient populations are involved. As a consequence, "suicide inhibitors", "warheads", and covalent irreversible inhibitors developed a negative flavor over time and became almost synonymous with toxicity in some organizations. The skepticism toward irreversible drugs may evaporate as more examples of irreversible drugs progress clinically that demonstrate good efficacy and safety margins.

In a nutshell, the therapeutic applicability or the success of irreversible binding kinase inhibitors is dependent on whether or not the covalent bond can be confined solely to the protein kinase of interest. So this approach is in essence a story about two T's: treatment and toxicity. When relying on the covalent binding principle, it is important to discern adduct-based toxicity and adduct-based treatment, since the adducting molecular entities in question obey overlapping fundamental rules in terms of reactivity. The only key difference is the nature and the function of the proteins that are covalently modified. Covalent kinase inhibitors with well-balanced recognition and reactivity should provide efficacy, selectivity, and ultimately the safety margins that are required for regulatory approval. If we strike the right balance, a third "T" will give enough comfort: therapeutic window.

This Perspective aims to give a comprehensive account from a medicinal chemist's point of view on the progress of irreversible kinase inhibitor drug discovery. The "state of the art" is reviewed by means of reported irreversible kinase inhibitors profiles and their chemical structures. The potential upsides and pitfalls that are associated with this concept are highlighted to provide a general understanding of the differences with respect to conventional drug discovery, as well as the future potential of this approach.

EFFECT OF RESIDENCE TIME ON THE THERAPEUTIC WINDOW

Residence Time and Efficacy. A kinase inhibitor will only be efficacious when it is modulating the action of the physiological kinase and can only do so when the inhibitor is bound. This general paradigm was effectively captured by Copeland and co-workers, who described the potential advantages of long residence time in terms of duration of action and target selectivity.[5] The equilibrium dissociation constant (K_d) for the drug–target binary complex as determined in a closed (in vitro) system is a more accurate measure than the IC_{50} or K_i. Yet the equilibrium setting is different in vivo, since the drug concentrations are no longer constant, and the efficacy is dependent on the on-rate (k_{on}) and even more importantly the off-rate constant (k_{off}). The importance of the related occupancy time or half-life for the protein–drug complex was corroborated in the context of pharmacological agents for whom longer residence times seemed to be beneficial in terms of biological or clinical efficacy.[6,7] In this respect, kinase inhibitors that exert covalent and irreversible binding can achieve the ultimate physiological goal: Efficacy is maintained until the target kinase is physically restored by the body to physiologically relevant levels (Figure 1).[8] In other words, the pharmacodynamics will be a function of the de novo synthesis rate of the target protein rather than the trough level of the compound.

Unlike for kinase inhibitors with a reversible binding mode, we anticipate that not all ADME parameters need to be as efficiently optimized for covalent inhibitors. The general

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Table 1. FDA Approved Protein Kinase Inhibitors (as of March 2012)

generic (brand) name	year of approval	company	indication	target kinase
imatinib (Gleevec)	2001	Novartis	chronic myeloid leukemia (CML)	Abl, c-Kit, PDGFRα/β
gefitinib (Iressa)	2003	AstraZeneca	non-small-cell lung carcinoma (NSCLC)	EGFR
erlotinib (Tarceva)	2004	Genetech, OSI	NSCLC, pancreatic cancer	EGFR
sorafenib (Nexavar)	2005	Bayer, Onyx	hepatocellular carcinoma, renal cell carcinoma (RCC)	Raf, VEGFR2/3, c-Kit, PDGFRβ
sunitinib (Sutent)	2006	Pfizer	gastrointestinal stromal tumor (GIST), RCC	c-Kit, VEGFR, PDGFR, FLT3
dasatinib (Sprycel)	2006	Bristol-Myers Squibb	CML	Abl, c-Kit, PDGFR, Src
nilotinib (Tasigna)	2007	Novartis	CML	Abl, c-Kit, PDGFR, Src, ephrin
lapatinib (Tykerb)	2007	GlaxoSmithKline	breast cancer	EGFR, ErbB2
pazopanib (Votrient)	2009	GlaxoSmithKline	RCC	VEGFR, PDGFRα/β, c-Kit
vandetanib (Caprelsa)	2011	AstraZeneca	thyroid cancer	VEGFR, EGFR, RET
vemurafinib (Zelboraf)	2011	Roche, Plexxicon	CML	Abl, c-Kit, PDGFR, Src, ephrin
crizotinib (Xalkori)	2011	Pfizer	NSCLC (ALK +ve)	ALK, MET
ruxolitinib (Jakafi)	2011	Incyte	myelofibrosis	JAK1/2
axitinib (Inlyta)	2012	Pfizer	RCC	VEGFR, PDGFRβ, c-Kit

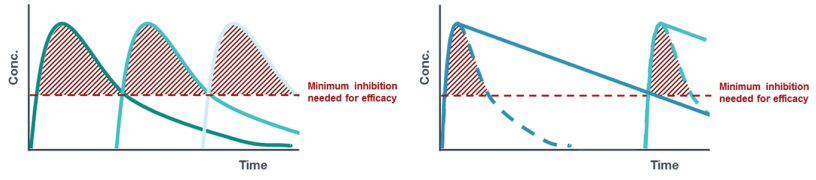

Figure 1. For conventional drugs, the pharmacodynamic effect is driven by the pharmacokinetics of the drug (left). For irreversible covalent drugs, the pharmacodynamic effect is driven by the turnover rate (de novo synthesis) of the protein target (right). The red hatched area is drug exposure not required for efficacy.

requirements for absorption and distribution characteristics remain relatively unchanged with respect to the conventional approach. It is still advantageous to have good oral availability and rapid distribution, but in the case of an irreversible kinase inhibitor it would be favorable to clear the drug from the body once the target kinase is maximally occupied. Thus, the in vitro and in vivo criteria for optimization cycles with respect to metabolism and excretion are very much different and should accentuate a swift clearance of the drug.

Residence Time and Toxicity. Drug-safety-related issues pose a serious problem to the pharmaceutical industry and have been a major contributing factor to attrition rates in the drug development trajectories.[9] As discussed above, since no excessive circulating levels are required to maintain efficacy with irreversible binding drugs, rapid clearance should lead to a lower propensity for off-target related adverse effects. But this can only be achieved when off-target protein−drug interactions are short-lived because of the noncovalent nature of binding.

On the other hand, toxic events can also be associated with covalent binding, and this can be either on-target or off-target related. If the toxicity is a direct consequence of on-target modulation, then irreversible inhibition might exacerbate the adverse effect potential. In this case, the clinical benefit should outweigh the adverse event. Alternatively, prolonged residence times can occur if collateral targets are being adducted by the potential drug. In that case, a high degree of in vivo selectivity can only be accomplished when the off-target turnover rate is (much) faster than the on-target resynthesis rate.

For irreversible kinase inhibitors, the target selectivity thus should not only be discussed in terms of reversible binding to closely related kinases with respect to primary sequence homology. Especially kinases that (also) share the site of covalent modification are highly relevant to include in the selectivity panel. A good level of selectivity for those off-target kinases is suggestive of a high degree of overall selectivity within the human kinome, as well as for other protein classes. Also, potential adverse effects due to covalent inhibition of specific off-target kinases can be designed out early in the optimization cycles to yield a better therapeutic window.

Much of the aversion to irreversible binders is predominantly the result of the negative experiences with reactive metabolites that could bind covalently to a variety of proteins.[10,11] In such a case, the parent drugs are metabolically bioactivated to reactive species that potentially can bind covalently to all sorts of macromolecules. Next to the predictable intrinsic toxicity, this may lead to unpredictable idiosyncratic toxicity (IDT) in late stage clinical testing and beyond. In order to exclude or minimize the propensity of reactive metabolite formation, potential drugs are usually evaluated preclinically for (i) gluthathione (GSH) adduct formation and depletion when incubated with recombinant enzymes or human liver tissue preparations and (ii) magnitude of covalent protein binding in liver microsomal fractions and in rodents. It is obvious that these models are highly relevant for the optimization of irreversible kinase inhibitors as well. The advantage with irreversible kinase inhibitors in this respect is that the parent molecules are the reactive species, which should have a better predictive power for the reactivity assays and body retention models involved.

Recently, the occurrence of IDT or hypersensitivity reaction has been linked by Nakayama et al. to the magnitude of covalent and irreversible binding to off-targets and the daily dose required in patients.[12] A classification system to assess the risk of IDT was proposed based on covalent binding to human hepatocytes as the best predictor, in combination with the daily dose.

Another potential event that has to be considered for covalent and irreversible kinase inhibitors is hapten formation,

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i.e., triggering an immune response to the adducted protein.[13] Experiences with reactive compounds and reactive metabolites indicate that additional danger signals (e.g., mitochondrial damage) are required next to the hapten mechanism to induce an immune response (for a review, see Zhang et al).[14] Except for the β-lactam antibiotics, absence of reports of immune responses for recently marketed irreversible binding drugs does not support this concern as a general problem for covalent drugs.

Reactive Metabolite Considerations. Considerations that have been used historically within the pharmaceutical industry to assess the fate of conventional drugs with suspected reactive metabolites can provide guidance for irreversible binding kinase inhibitors as well.[10] It is obvious that the "avoidance strategy", i.e., complete elimination of reactive groups, cannot be applied on warhead-containing drugs, since the pharmacological effect is largely dependent on the reactive group. Perhaps it would be better to develop a reversible therapy for less severe disorders, especially if one can rationalize that reversible drugs should work equally well. Also, the anticipated human dose (for any drug) has to be considered, as the body burden has to be kept to a minimum (vide infra). In addition, a disease that needs acute dosing rather than chronic treatment might be more appropriate to engage with irreversible binding drugs. These and other questions need to be addressed before a covalent (kinase) inhibitor program can be progressed toward clinical development. Obviously, the key issue here is the benefit/risk ratio, and serious life threatening diseases may be particularly justified in this scenario.

One has to keep in mind though that most reactive metabolites are by no means tuned, and they can be uncontrollable like "unguided missiles". In contrast, deliberately designed irreversible kinase inhibitors can be subjected to safety assays during optimization cycles to ensure selection of compounds that meet stringent safety prediction criteria. In some other instances, bioactivation is even required to generate the active drug, such as in the case of proton pump inhibitors like omeprazole and $P2Y_{12}$ receptor antagonist clopidogrel.[1] No matter the type of covalent approach involved, a decision tree that incorporates several safety assessment parameters will be required to mitigate the risks. In addition, the level of acceptance that has been set for the magnitude of covalent binding of reactive metabolites in vitro and in vivo also applies for covalent binding parent drugs.

THE CONCEPT APPLIED TO KINASES

The question is whether the members belonging to the superfamily of protein kinases are suitable targets at all for irreversible inhibition. First of all, the replenishment of the target kinase has to be sluggish enough to allow drug dosing once or twice a day. This has to be checked on a kinase-by-kinase basis. Second, unlike for instance the enzyme families of (cysteine) proteases or phosphatases, kinases lack a catalytically active amino acid residue that is key to the function of a particular enzyme by virtue of its overt nucleophilicity. The relatively unreactive cysteines, serines, threonines, and lysines in kinases may have to be exposed to excessive reactivity to ensure covalent bond formation. There are plenty of examples of overly reactive electrophiles that despite the best of intentions, in retrospect never had a chance to make it into a safe drug. Nevertheless, these prototypical molecules definitely marked the beginning of this emerging platform.

Non-ATP Competitiveness. There is one major upside that can specifically be exploited in the kinase field. Non-ATP competitive inhibitors offer distinct advantages to conventional ATP competitive binders, especially in terms of efficacy and selectivity.[15,16] The highly conserved nature of the ATP binding pocket poses a challenge in the identification of selective kinase inhibitors, but more important is that high intracellular ATP concentrations affect the cellular potency of ATP competitive kinase inhibitors under physiological conditions.[17] Besides allosteric binders of protein kinases, blockade of the ATP pocket with an irreversible inhibitor has emerged as an attractive and alternative strategy to achieve non-ATP competitive inhibition of kinase-mediated signaling.[18] As a consequence, caution should be taken with the interpretation and/or comparison of the biochemical and functional IC_{50} values, since irreversible inhibitors usually become more potent over time. The selection of the length of preincubation time will thus influence the observed value, unless maximal inhibition is achieved instantly. The inactivation of the kinase involves two steps: formation of a reversible complex based on affinity (K_i) followed by the covalent bond formation between the inhibitor and the kinase (k_{inact}).[19] The first step depends on the ATP level used in the various assays reported. The second step is primarily influenced by the inherent reactivity of the electrophilic group, as well as the distance to the nucleophilic trap. The better the juxtaposition of the reacting partners, the better the inactivation step. As the kinase is effectively removed from the equilibrium with ATP and substrate, a shift in IC_{50} is expected and, as demonstrated in Figure 2, the inhibitor shows an increasingly non-ATP competitive character as time progresses. Consequently, this would be beneficial for protein kinases with a low $K_{m,ATP}$ in particular.

Many research groups have already embarked intentionally, or unintentionally, on the search for irreversible kinase inhibitors (partly reviewed by Garuti et al).[20] In some instances, these inhibitors have become invaluable assets in the target

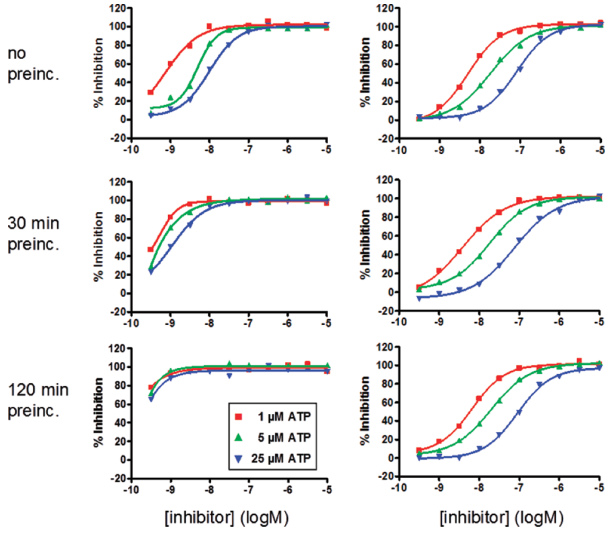

Figure 2. IC_{50} determination depends on the ATP concentration and preincubation time for an irreversible kinase inhibitor (left), whereas an ATP competitive kinase inhibitor is only affected by ATP concentration (right). Data are from an unpublished kinase inhibitor study included for illustration purposes only.

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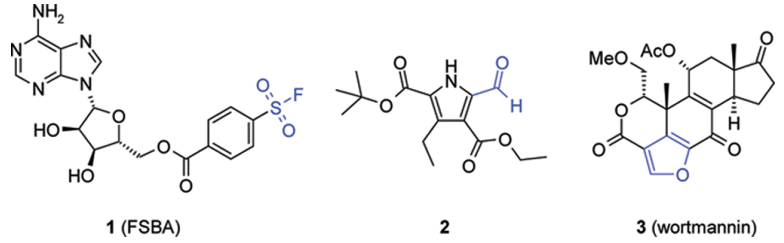

Figure 3. Examples of lysine trapping kinase inhibitors.

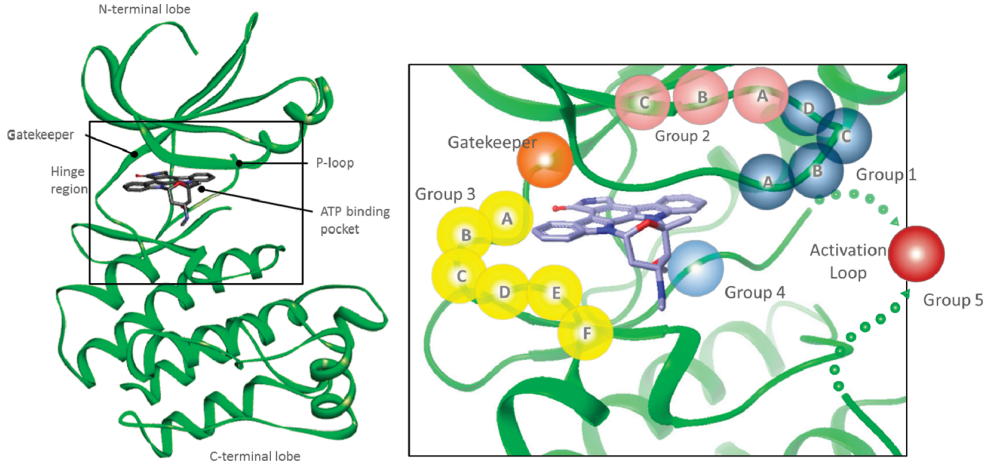

Figure 4. Schematic representation of available cysteines in or near the ATP binding pocket of kinases in the active conformation (based on the crystal structure of interleukin 2 tyrosine kinase, PDB code 1SM2). The spheres indicate the locations of cysteines that are in principle accessible for covalent modification: group 1, glycine-rich- or P-loop (dark blue); group 2, the roof of the ATP binding pocket (pink); group 3, hinge region and front pocket (yellow); group 4, DFG-motif neighboring cysteine (light blue). Group 5 (in dark red) denotes the cluster of cysteines that resides on the activation loop. The gatekeeper group is shown in orange. Adapted by permission from Macmillan Publishers Ltd.: *Nature Reviews Cancer* (http://www.nature.com/nrc/index.html), Copyright 2009.[38]

validation stage. Others were deliberately designed to be the drugs of the future. The selection of examples that is reviewed in this Perspective covers both and in addition is primarily based on the diversity of cysteine locations within the known druggable binding regions of kinases. The electrophilic portions of these inhibitors (highlighted in blue in this Perspective) are either being trapped by the highly conserved lysine or cysteines at various positions of the kinases. To our knowledge, there are no reports to date that describe covalent kinase inhibitors that adduct other amino acid residues, such as serine or threonine.

Lysine-Trapping Covalent Kinase Inhibitors. Because of the well-conserved nature and its abundant presence, the lysine involved in the phosphate transfer machinery is not the most ideal candidate amino acid for trapping electrophilic inhibitors.[21] This particular lysine resides in virtually every ATP-binding pocket of the known human kinases. Nevertheless, a couple of lysine trapping covalent inhibitors have appeared in literature. One of the first reported is ATP analogue 5′-fluorosulfonylbenzoyl adenosine (FSBA, **1**), a tool frequently used as an affinity label for kinases like EGFR,[22] p38γ,[23] and brain PI4K (Figure 3).[24] As per example, Fox and co-workers used **1** for analysis of kinetic mechanisms and ATP-binding site reactivity toward p38γ and found it to bind irreversibly to the unphosphorylated and active form.[23] Digest maps showed that Lys56 of p38γ was selectively and covalently modified by **1**. The use of **1** has been reported in more than 200 publications, underscoring the usefulness of this particular chemical probe.

Bell and co-workers discovered a pyrrole-5-carboxaldehyde class of type 1 insulin-like growth factor receptor (IGF-1R) inhibitors with modest activity but good selectivity over a small panel of other kinases.[25] These aldehyde-based inhibitors were shown to bind in the ATP-binding pocket of IGF-1R in a covalent but reversible manner via imine formation between Lys1003 and the aldehyde functionality. Reduction of the imine adduct with sodium borohydride irreversibly inactivated the kinase, enabling a tryptic digest and X-ray crystallography examination. A representative example is inhibitor **2**, which demonstrated an IC_{50} of 0.49 μM for IGF-1R.

A frequently studied lipid kinase inhibitor is represented by antiproliferative agent wortmannin (**3**, Figure 3). This PI3K inhibitor modifies the ATP pocket covalently and irreversibly via trapping of conserved Lys802 (p110α numbering).[26] Analogues of **3** inhibit PI3K (reported IC_{50} values in the low nanomolar range) by virtue of an activated furan ring as a fairly unusual electrophile, which opens upon nucleophilic attack of the side chain of Lys802.[27] SAR studies on analogues of **3** demonstrated that omission (or modification) of this furan ring results in significant reduction in inhibitory activity. An X-ray crystallographic structure revealed the exact nature of the binding mode of **3**, and clear density was observed for the primary amine linking to the opened furane ring.[28] Later on, **3** was also shown to inhibit the protein kinase Plk1 (IC_{50} = 24 nM), shedding some doubt on the pharmacology that was previously attributed to PI3K inhibition.[29] Subsequently, it was

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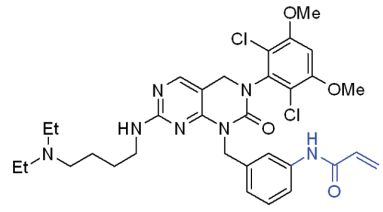
established that **3** also inhibits Plk3 by covalent modification of the conserved lysine in the ATP binding pocket.[30] In general, deliberately designed covalent drugs that target the invariant lysine in kinases are not to be expected. However, uniquely positioned lysines elsewhere in or near the ATP binding pocket may well be suitable for a covalent inhibitor approach.

Cysteine-Trapping Covalent Kinase Inhibitors. Unlike cysteines in kinases, cysteines involved in the catalytic machinery in, for instance, protein tyrosine phosphatases[31] and cysteine proteases[32] are part of very specific environments made up of neighboring amino acids and ternary architecture of these particular proteins. Part of the active site architecture is the position of these cysteines, residing frequently at the "bottom" of one or more α-helixes. Backbone NH groups point downward generating a net positive charge that stabilizes the sulfhydryl anion and augments nucleophilicity.[33] The pK_a of a thiol in a typical cysteine residue is ~8.5, whereas the presence of a polar or positively charged environment can decrease the pK_a up to 5 log units. In many protein families, cysteines contribute to maintaining ternary protein structures via disulfide bond formation. In the ATP-binding pocket of kinases, reduced cysteines generally do not seem overly nucleophilic and the side chain functionality may have a rudimentary role at most. There are, however, several recent reports that suggest that reaction of a cysteine thiol can regulate and control even protein kinase function, for instance, by means of redox signaling[34,35] or S-alkylation with endogenous electrophiles.[36,37] For this very same reason, reducing agents such as dithiothreitol (DTT) or dithioerythritol (DTE) are indispensable ingredients in biochemical assays for kinases, in order to keep the enzyme in an active and sensitive state. This also supports the notion that these cysteines are sensitive enough for electrophilic modification by designer drugs.

Recently, Zhang et al. have suggested a very useful classification system that originally binned the cysteines into four groups according to the relative positions in the ATP binding pocket (Figure 4).[38] It is justified to add at least two additional groups to this initial classification, involving the cysteines located at the gatekeeper position and a fifth group. Group 5 covers cysteines residing at various locations in the activation loop. Some cysteines are more unique than others, and there are at least 200 kinases that seem tractable for a covalent approach. Given the known conformational flexibility of kinases, this number may be even higher. Structural analyses of the different conformations, including the active kinase state and inactive "DFG-out" and "C-helix-out" conformations, unveil additional cysteines that could be targeted.[39] These cumulative analyses generate a fingerprint of the kinome active site toward cysteines, and the resulting "cysteinome" is of great value for the design of covalent kinase inhibitors. There are now several examples of covalent kinase inhibitors that target this region, and in the following sections the several groups are reviewed. The order of appearance is dictated by the group numbering as indicated in Figure 4.

Group 1 Cysteines. So far, there is only one report that describes the identification of covalent kinase inhibitors targeting a cysteine on the lip of the glycine-rich loop (Figure 4). An irreversible group 1B inhibitor is represented by fibroblast growth factor receptor (FGFR) inhibitor **4** (FIIN-1, Figure 5), which was identified via a structure-based approach using available reversible FGFR inhibitors.[40] The FGFR family consists of four members, FGFR1−4 and plays an important role in tumor formation and progression as well as wound



4 (FIIN-1)

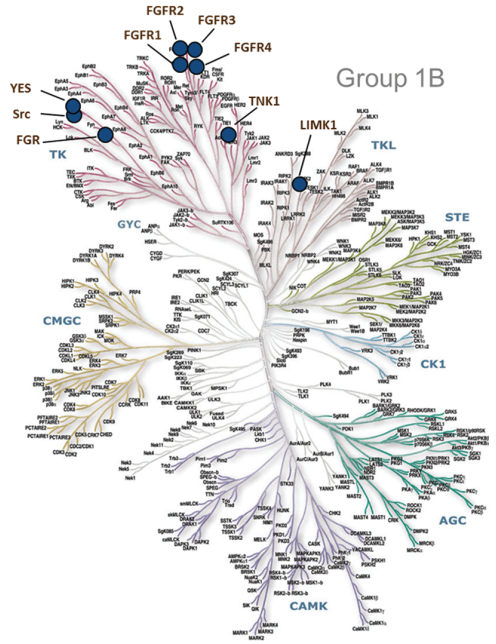


Figure 5. (Top) Chemical structure of FGFR inhibitor **4**. (Bottom) The human kinome and the group 1B cysteines. Adapted illustration reproduced with permission from AAAS (Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, *298*, 1912−1934) and courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).[42]

healing.[41] Compound **4** is a biochemically potent acrylamide-based inhibitor of FGFR1, FGFR2, and FGFR3 (IC_{50} values of 9.2, 6.2, and 11.9 nM, respectively) and is somewhat less potent on FGFR4 (IC_{50} = 189 nM), with decent selectivity over the other group 1B cysteine kinases, such as Src, YES, and TNK1 (Figure 5). The reversible propionyl analogue of **4** was 24- to 100-fold less potent in FGFR1 and FGFR3 harboring cells, respectively. With the aid of a biotinylated version of **4** (Figure 17), specific covalent wild type (WT) FGFR1 labeling was demonstrated in transfected HEK293 cells whereas the C486S mutant was barely modified. This is in line with the binding model that strongly suggests that **4** forms a covalent bond with FGFR1 via Cys486.

Group 2 Cysteines. Within this cluster, subgroup 2B comprises only 11 kinase members and has been the sole subject of proven covalent kinase targeting within the group 2 cysteines. Cohen et al. were the first to exploit this particular cysteine as a selectivity filter in addition to the threonine gatekeeper residue in p90 ribosomal protein S6 kinase (RSK).[43] The four closely related RSKs (RSK1−4) all have the P-loop cysteine, whereas RSK3 has the sterically demanding

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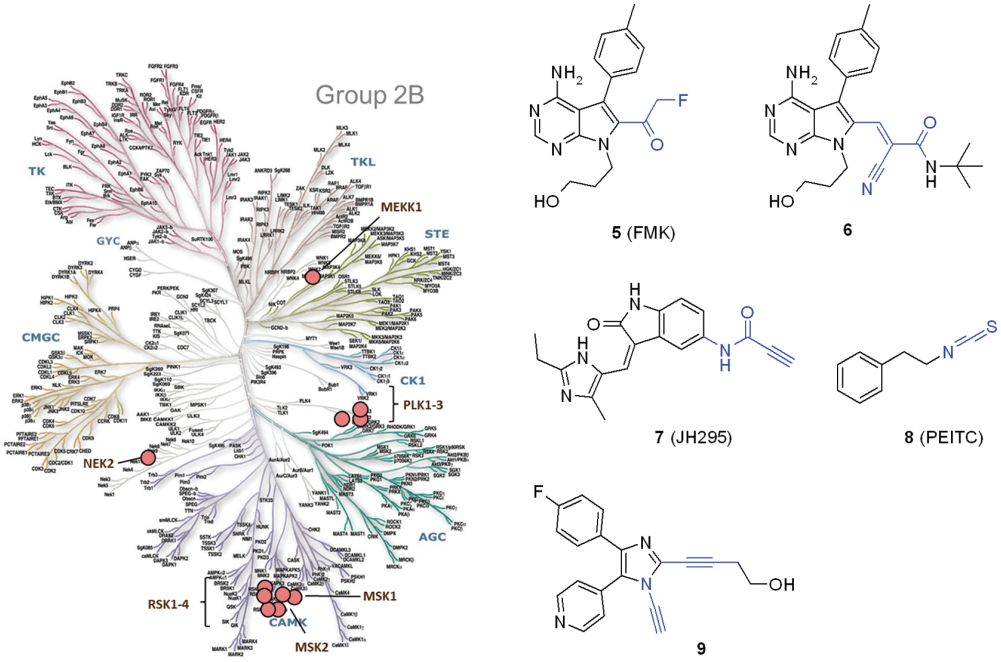

Figure 6. (Left) Human kinome and the group 2B cysteines. Adapted illustration reproduced with permission from AAAS (Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, *298*, 1912−1934) and courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).[42] (Right) Chemical structures of group 2B inhibitor **5**−**8** and group 2C kinase inhibitor **9**.

methionine as a gatekeeper as opposed to threonine in the other three RSK kinase members. This was sufficient to develop a fluoromethylketone (FMK) derivative **5** as a highly potent and selective RSK1 and RSK2 inhibitor, with an IC_{50} value against RSK2 of 15 nM (Figure 6). Selective covalent labeling of WT (but not the C436V mutant) RSK2 was demonstrated by FMK-biotin in transfected HEK293 cells. Also, Michael acceptors such as an acrylate or acrylonitrile were appended from the same pyrrolopyrimidine scaffold, yielding irreversible RSK2 inhibitors with modest biochemical IC_{50} values.[44] By contrast, the doubly activated electrophiles, as in cyanoacrylamide **6**, were 200-fold more potent (IC_{50} = 4 nM) in enzymatic assays than the monoactivated vinyl groups, yet in a fully reversible covalent binding mode. Owing to its greater reactivity, **6** demonstrated faster kinetics than **5** in cellular settings, but the duration of occupancy seemed to rely on protein resynthesis and was indistinguishable between the two inhibitors. Intriguingly, all RSK kinases also accommodate another cysteine which is located in the back of the ATP binding pocket, equivalent to Cys560 in RSK2 (group 4).[45] Potential trapping of derivatives of **5** by this cysteine was not investigated.

NEK2 is a centrosomal kinase that interferes with the spindle assembly checkpoint, affecting mitosis. It harbors a cysteine (Cys22) in an identical position as the RSK family of kinases.[46] Henise and Taunton developed a reasonably potent (IC_{50} = 770 nM) and cell permeable irreversible NEK2 inhibitor (**7**) based on an oxindole scaffold using a propynamide as the warhead. Selective covalent NEK2 inhibitors, such as **7**, are important validation assets to investigate the consequences of intracellular inactivation of NEK2 on regulating the proliferation of tumor cells overexpressing this kinase.

The same specific cysteine on the P-loop of MEKK1 was previously identified as a glutathionylation site following oxidative stress.[47] Bioactive nutrients harbor electrophilic isothiocyanates that intervene with stress-signaling kinase pathways. Reported activities include cell cycle effects and protection of experimental carcinogenesis in animal models. The SAPK/JNK signaling pathway was investigated by means of phenylethyl isothiocyanate (PEITC) **8**, which was shown to inhibit WT MEKK1 (group 2B) but failed to intervene with the C1238V mutant in both biochemical and cellular assays.[48] It was the second covalent kinase inhibitor reported for this subgroup, and although far from being druglike, it demonstrates the potential for covalent kinase inhibitors as research tools in the exploratory biology of kinase signaling pathways.

One group 2C suspect has been reported, which is a p38α inhibitor with moderate biochemical potency (IC_{50} = 200 nM). Dialkynylimidazole derivative **9** did covalently modify p38α following prolonged incubation and as determined by ESI-MS.[49] The authors propose trapping via an intricate cyclization and rearrangement mechanism involving both ethynyl groups of **9**, but the exact mode of action and site of covalent binding were not confirmed. Cys39 could be adducted given its position in the roof of the ATP pocket of p38α and the known binding mode of the imidazole class of p38 inhibitors.[50] However, the cysteine sulfhydryl group is directed away from the pocket itself, and as a consequence, this cysteine is unlikely to be involved in adduct formation.

Group 3 Cysteines. The 3F cluster of the group 3 cysteine-containing kinases has received by far the most attention because of the clinical potential of kinase inhibitors belonging to this subgroup.[18] In addition, the relatively high nucleophilicity with respect to the other cysteines facilitates the reaction with all sorts of electrophilic agents. The pK_a of the sulfhydryl group of this particular cysteine is most likely lower than average because of the position at the end of a C-lobe α-helix (Figure 4). The net positive charge through the backbone NH

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groups stabilizes the thiolate anion, thus making it more amenable for electrophile trapping. Depending on the sequence alignment used, there are 10 kinases with a cysteine on this position (Figure 7). The TEC family (Bmx, Btk, Itk, TEC, and Txk) and ErbB family (EGFR, ErbB2, and ErbB4) constitute the majority of this subgroup and in addition comprise Blk and JAK3.

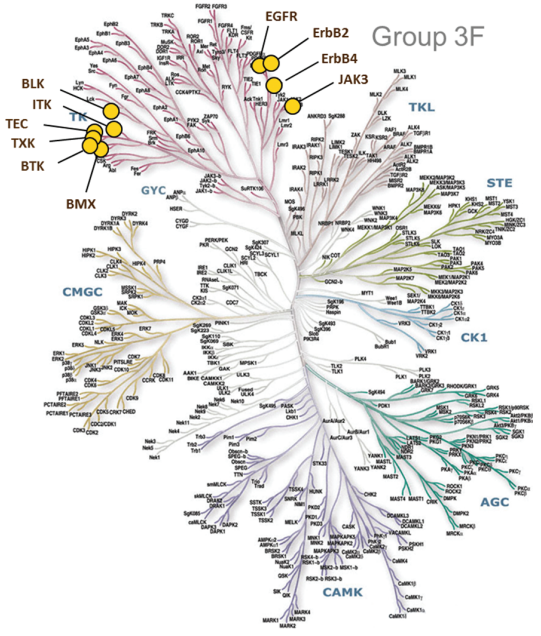

Figure 7. Human kinome and the group 3F kinases. Adapted illustration reproduced with permission from AAAS (Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, *298*, 1912−1934) and courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).[42]

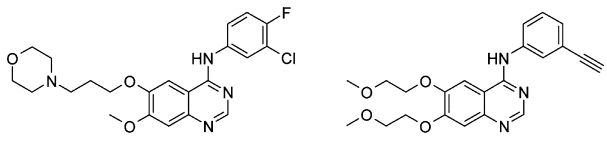

Figure 8. Reversible ATP competitive EGFR inhibitors gefitinib and erlotinib.

The ErbB family kinases were the first ones to be deliberately scrutinized via a targeted irreversible covalent approach. Reversible small molecule kinase inhibitors gefitinib (**10**) and erlotinib (**11**) have shown excellent clinical benefit, particularly in the treatment of non-small-cell lung carcinomas (NSCLC). The chemical structures of these reversible kinase inhibitors are presented in Figure 8. Unfortunately, the ability of these ATP competitive EGFR inhibitors to treat NSCLC patients effectively is short-lived partly because of acquired drug resistant mutations in EGFR which concerns roughly 50% of the treated population.[51,52] One of these mutations involves the threonine gatekeeper, which is converted to a methionine (T790M). This prevents reversible inhibitors from binding at higher ATP concentrations.[53] An additional postulated explanation is the increased internalization of EGFR as observed in drug resistant tumor cell lines.[54] Both resistance mechanisms may efficiently be circumvented by irreversible kinase inhibitors and have spurred the search for irreversible (pan) ErbB inhibiting drug candidates.

In 1997, it was demonstrated by Singh and colleagues of the Parke-Davis group that EGFR and ErbB2 could be covalently modified by an adenosine thiol analogue (**12**, Figure 9).[55] Inactivation of EGFR was shown to be time-dependent, a hallmark for covalent binding, and the binding model suggested the proximity of the 2′-thiol group of **12** to Cys797 in EGFR and to Cys805 in ErbB2. Around the same time, the highly potent and selective **13** (PD-168393) was shown to inactivate EGFR and ErbB2 in an irreversible manner by means of an acrylamide (Figure 9).[56] While the subnanomolar potency of **13** for EGFR (IC_{50} = 0.7 nM) was indistinguishable from the reversible propionyl counterpart, a range of data (MS, site-directed mutagenesis, ^{14}C-labeling) demonstrated unequivocally the alkylation of Cys797. This translated into a significant advantage in a xenograft mouse model, since the duration of action had become a function of the protein turnover rate (de novo synthesis) rather than the pharmacokinetic profile of the inhibitor. Although **13** should be regarded as the prototype 4-anilinoquinazoline-based irreversible EGFR inhibitor, this particular compound did not make it into the clinic. A plethora of newer ErbB inhibitors have been identified that may display advantageous properties. One line of research focused on replacement of the acrylamide function by alternative and less reactive electrophiles, since this warhead was initially considered as too aggressive.

A nearly identical and highly potent analogue discovered by Wyeth researchers is **14** (CL-387,785, IC_{50} = 0.37 nM), which harbors a 2-butynamide as the Michael acceptor (Figure 10).[57] It showed potent inhibition of EGF-induced EGFR autophosphorylation in A431 cells ($IC_{50} \approx 5$ nM) and blocked tumor growth in a xenograft mouse model when given orally (80 mg kg^{-1} day^{-1}, q.d.). This compound, also known as EKI-785, was the first irreversible EGFR inhibitor considered for further clinical development but was abandoned later because of poor bioavailability.[58] A major improvement was observed when introducing a dialkylamino group as in compound **15**. This electrophile serves both as an internal base to accelerate the reaction with the cysteine and as a water-solubilizing group.

Carmi et al. explored a variety of warheads characterized by different reaction mechanisms with respect to nucleophiles.[59] The electrophiles studied included epoxides, phenoxymethylamides, carbamates, nitriles, and (benz)isothiazolinones. Epoxide derivative **16** was the EGFR inhibitor that stood out in terms of biochemical potency (IC_{50} = 1.2 nM) and inhibition of EGFR autophosphorylation in cells with IC_{50} values of 7 nM (immediately following 1 h of incubation with EGF) and 44 nM (8 h after removal of inhibitor from culture medium, following 1 h of incubation with inhibitor), respectively. The hysteresis in efficacy observed is in line with covalent binding, resulting in prolonged inhibition only reversed following de novo synthesis of EGFR.

A less obvious and less reactive electrophile is the acetylene moiety on an electron-deficient thienopyrimidine scaffold as in **17** (Figure 9).[60] Covalent adduct formation of this type of pan-ErbB kinase inhibitors was demonstrated by means of X-ray crystallography and mass spectrometry. Covalent modification of EGFR with **17** was much slower compared to **22** (CI-1033, Figure 11),[61] while the enzymatic IC_{50} values for EGFR, ErbB2,

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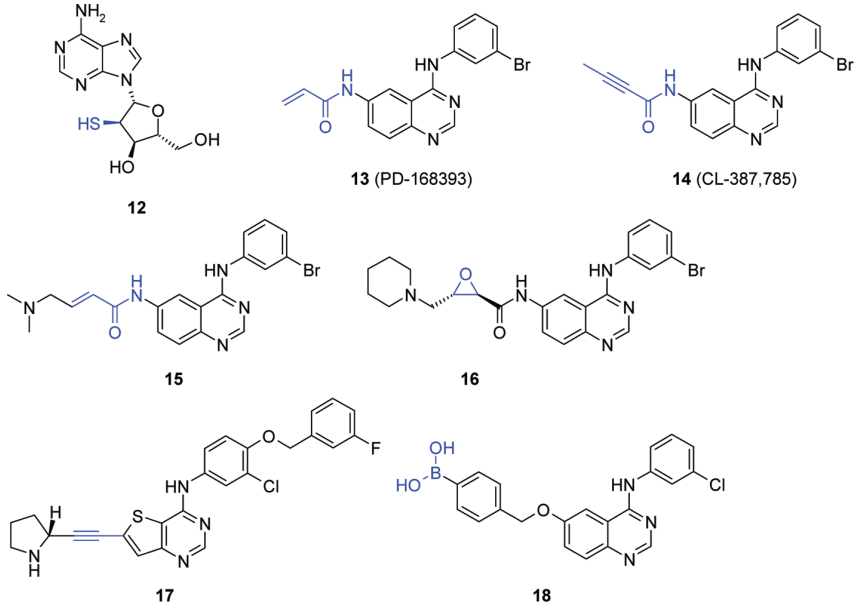

Figure 9. Group 3F irreversible ErbB family kinase inhibitors **12**–**18**.

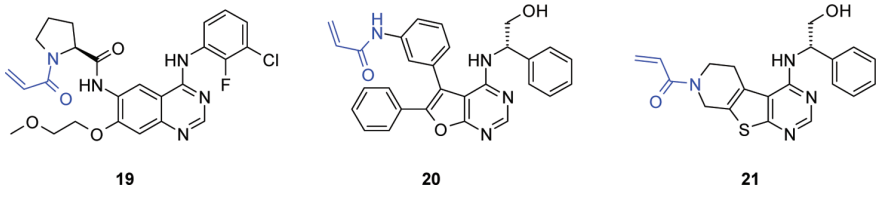

Figure 10. Group 3F irreversible ErbB family kinase inhibitors **19**–**21**.

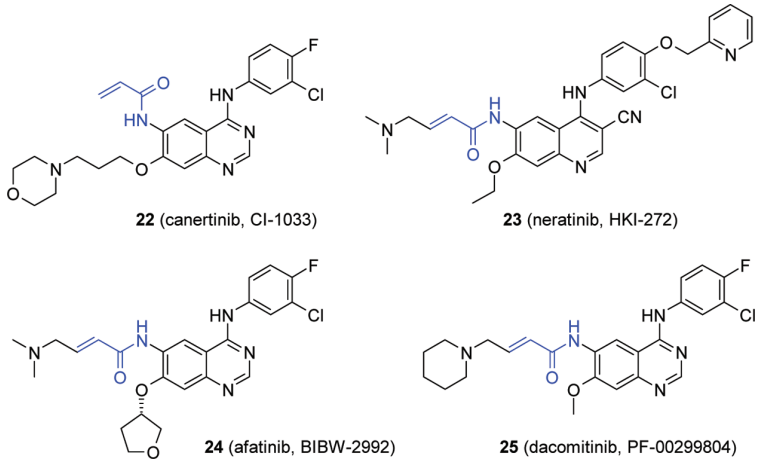

Figure 11. Irreversible pan-ErbB kinase inhibitors in clinical development.

and ErbB4 ranged from 7 to 66 nM. At doses of 10, 30, and 100 mg/kg (b.i.d.), **17** displayed a dose-dependent inhibition of tumor regression in a BT474 xenograft mouse model.

Although not extremely potent, boron-conjugated 4-anilinoquinazolines were also shown to inhibit EGFR with submicromolar IC_{50} values.[62] Unlike the other boronic acid derivatives reported in this paper, compound **18** demonstrated irreversible inhibition, which was attributed to a covalent bond to Asp800 instead of Cys797. Unfortunately, no empirical proof was provided to strengthen this hypothesis. Additional warheads that have been employed in the search for ErbB family kinase inhibitors have recently been extensively reviewed.[63]

Another line of focus is optimization of the nature and size of the linker between the 4-anilinoquinazoline scaffold and the warhead, with the aim to fine-tune warhead orientation. Cha et al. partially locked the orientation of the acrylamide with a conformationally rigid L-proline, arriving at **19** as a potent and

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Table 2. Late Stage Clinical Development Irreversible ErbB Kinase Inhibitors

originator	compd	enzyme IC_{50} (nM) EGFR	ErbB2	ErbB4	stage of development[a]	ref
ParkeDavis[b]	**22**, canertinib	1.5	5	10	Ph 2, discontinued	61
Wyeth[b]	**23**, neratinib	92	59		Ph 3 (breast cancer)	74
Boehringer Ingelheim	**24**, afatinib	0.5	14		Ph 3 (NSCLC, breast neoplasms, squamous cell carcinoma head and neck)	71
Pfizer	**25**, dacomitinib	6	46	74	Ph 3 (NSCLC)	72

[a]Source: Thomson Reuters and ref 67. [b]Now Pfizer.

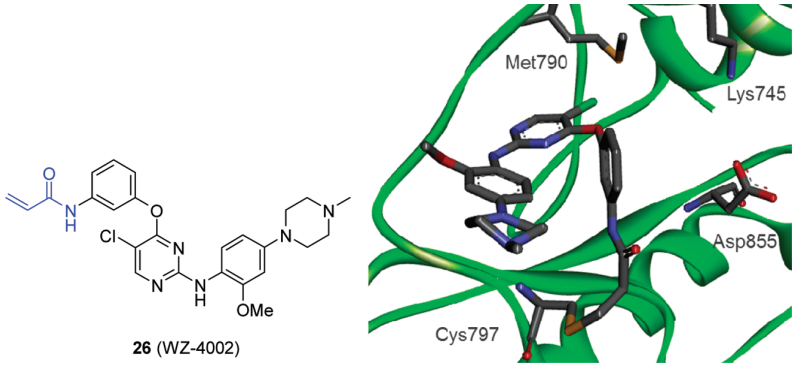

Figure 12. Crystal structure of EGFR T790M covalently bound to **26** (3IKA.pdb; P-loop omitted for clearity).

selective dual inhibitor of EGFR and ErbB2 with IC_{50} values of 13 and 42 nM, respectively (Figure 10).[64] Its favorable cellular potency (IC_{50} = 8 nM) and pharmacokinetic profile translated to robust reductions of tumor size in the A431 xenograft mouse model at 3 mg kg^{-1} day^{-1} (po).

The use of completely new scaffolds also has led to the discovery of irreversible ErbB family kinase inhibitors, as was demonstrated by Coumar et al., who identified furanopyrimidine derivative **20**.[65] Docking studies of its des-acrylamido hit (IC_{50} = 223 nM on EGFR) suggested the optimal position of the warhead. This led to a 30-fold improvement of the biochemical potency to 7 nM for **20**, along with potent antiproliferative activity in the HCC827 lung cancer cell line. The same group took a hybrid approach with another micromolar tetrahydrobenzothienopyrimidine hit, eventually resulting in **21**.[66] This EGFR inhibitor showed an IC_{50} of 8 nM biochemically, 3 nM toward inhibition of EGFR autophosphorylation in the HCC827 cell line, and again regression of tumor size in vivo in mice following 5−15 mg kg^{-1} day^{-1} iv dosing.

Since the first reports of covalent irreversible ErbB family kinase inhibitors in the late 1990s, a number of these inhibitors have progressed to late stage clinical development.[67] The irreversible inhibitors fall into two related structural categories: 4-anilinoquinazolines and the reminiscent 4-anilino-3-cyanoquinolines. The chemical structures of the most prominent compounds are depicted in Figure 11, and data associated with these compounds are shown in Table 2. So far, these candidates seem to live up to the potential upsides, as prolonged inhibition of both the WT enzymes and primary resistance mutants has been demonstrated preclinically. However, it remains to be established whether the effectiveness observed for all of these agents in vitro against, for instance, the EGFR T790M mutants persists at concentrations realistic for successful clinical use.[68] Dose-limiting toxicity for neratinib (**23**, HKI-272) may have prevented progression toward NSCLC as a primary indication[69] but is currently in phase 3 clinical trials for ErbB2-positive breast cancer.[70] The most advanced clinical trials for afatinib (**24**, BIBW-2992) concern several cancer indications, as a single agent or in combination therapy.[67,71] A phase 3 clinical trial of dacomitinib (**25**, PF-00299804) is ongoing for NSCLC, and the study is expected to be completed in 2013.[72,73] Interestingly, all research groups independently of each other selected candidates that are decorated with a dialkylamino substituted crotonamide warhead. Although ω-alkyl substitution of the acrylamide normally is detrimental to reactivity, introduction of the amine function offsets this loss by deprotonation of the sulfhydryl group (vide infra). In addition, solubility is favorably impacted, and the volume of distribution is high because of the lower intracellular pH, presumably stimulating uptake in the target tissues.

With the observed on-target related adverse effects (skin rash and diarrhea) for EGFR inhibitors, development of EGFR mutant selective inhibitors for relapsed NSCLC due to mutated EGFR could show great promise. These mutant-selective agents may be as efficacious but would be better tolerated when WT EGFR is not affected. As many of the irreversible ErbB family kinase inhibitors reported herein have dual activity toward both the EGFR wild-type and T790M or T790M/L858R mutants, truly effective inhibition of the latter two are usually hampered by a large back-pocket substituent that clashes with the sterically demanding gatekeeper methionine. The first mutant selective EGFR inhibitors that are based on entirely new chemical scaffolds have recently appeared in the literature.[75] To this end, covalent pyrimidine inhibitors were identified by screening, and **26** (WZ-4002) was extracted as the best inhibitor in terms of affinity with a K_d of 0.13 μM for EGFR L858R/T90M and about 100-fold less for WT EGFR (Figure 12). The overall selectivity provides a good starting point for further optimization, but there is clearly room for improvement.[76] Nevertheless, **26** is less potent in vitro and in vivo against WT EGFR than irreversible quin(az)oline-based inhibitors and 30- to 100-fold more potent against EGFR T790M. **26** is efficacious in murine models of T790M-containing cancers, as was shown by reduced tumor volumes

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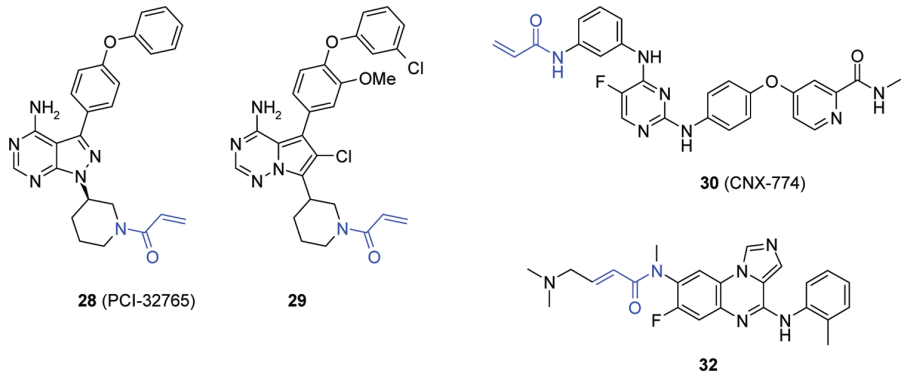

Figure 13. Chemical structures of group 3F irreversible Btk inhibitors **28**–**32**.

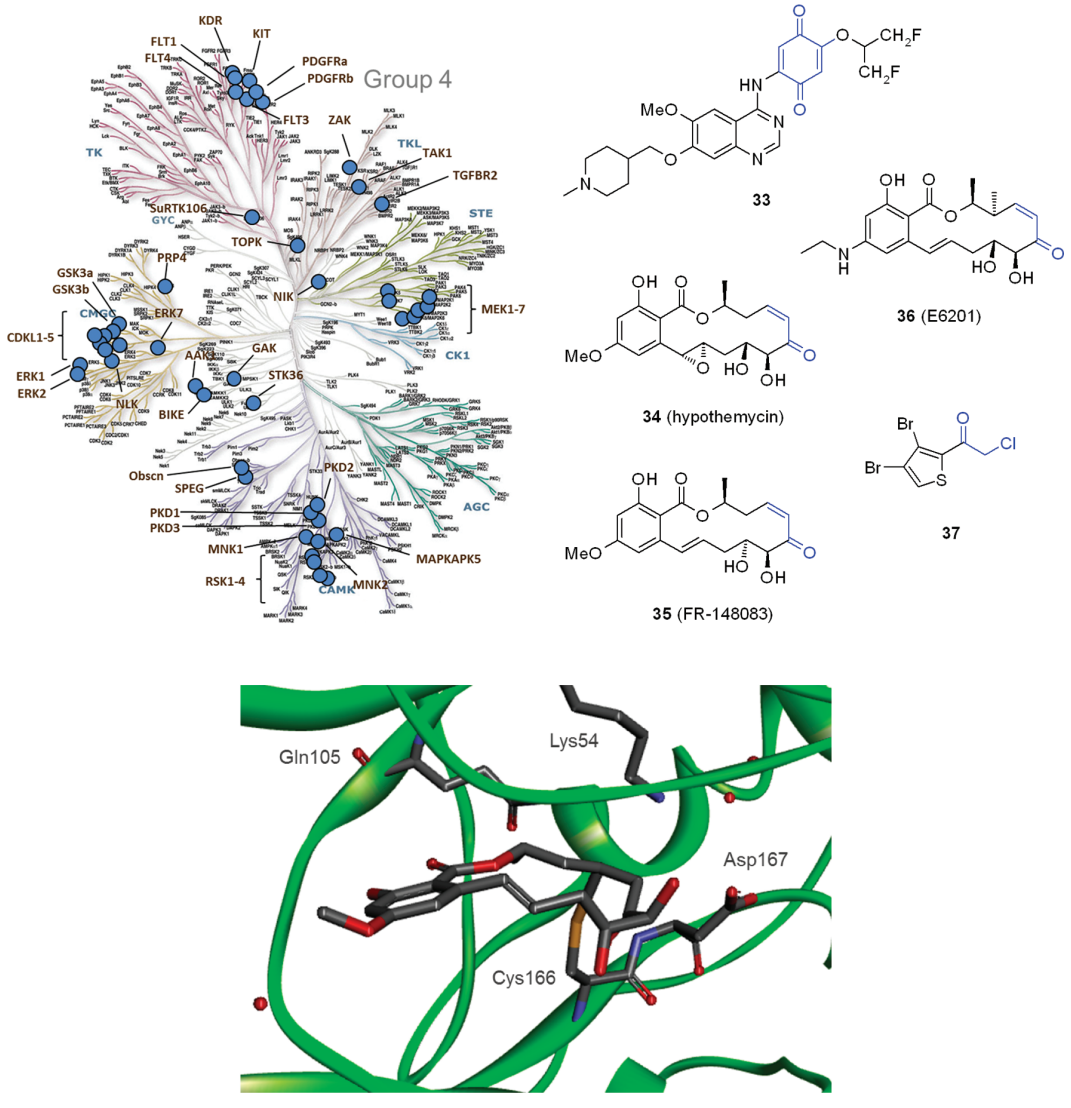

Figure 14. (Top-Left) Human kinome and the group 4 kinases. Adapted illustration reproduced with permission from AAAS (Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, *298*, 1912–1934) and courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).[42] (Top-Right) Chemical structures of selected irreversible group 4 kinase inhibitors **33**–**37**. (Bottom) Compound **35** bound in the active site of Erk2 (2E14.pdb).

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following a daily oral dose of 25 mg/kg.[75] Next to the binding mode, covalent binding to the Cys797 of EGFR T790M was confirmed via X-ray crystallography. Elucidation of the structure also revealed that **26** binds the active conformation of the kinase, as opposed to **23**, which binds the inactive form. One other mutant-selective inhibitor (**27**, CO-1686) has been described in a joint effort by Avila Therapeutics and Clovis Oncology, but this structure has not been disclosed.[77] **27** inhibits cell proliferation of cell lines expressing mutant EGFR T790M and EGFR signaling in, for instance, L858R/T790M expressing H1975 cells with an IC_{50} of 62 nM while sparing WT EGFR. Dose-dependent tumor regression in the H1975 and HCC827 xenograft models was achieved with complete inhibition at the highest dose (100 mg kg^{-1} day^{-1}, po), and **27** has recently been progressed to the clinic for the NSCLC indication. These mutant-selective irreversible inhibitors can be considered the third generation EGFR inhibitors, after the first (reversible, ATP competitive) and second (irreversible) generation WT EGFR inhibitors.

The second kinase of the subgroup 3F that received considerable interest is Bruton's tyrosine kinase (Btk). Btk is a key kinase in the signaling pathway activated via the B-cell receptor (BCR), regulating B-cell proliferation and activation. Inhibition of Btk shows great potential in the treatment of B-cell malignancies and autoimmune diseases. Celera researchers were the first to report on irreversible (Btk) inhibitors, including ibrutinib (**28**, PCI-32765, Figure 13) that was subsequently taken to clinical development by Pharmacyclics.[78] Compound **28** is a potent and fairly selective Btk inhibitor with an IC_{50} of 0.5 nM by virtue of covalent binding to Cys481. It was efficacious in collagen-induced arthritis (CIA) and lupus models in mice and in spontaneous canine B-cell lymphomas.[79] Phase 2 clinical studies in non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL) have been concluded successfully for this Btk inhibitor, showing an excellent response rate in CLL patients and in subtypes of NHL.[80] The reminiscent covalent Btk inhibitor **29** was disclosed recently, displaying an IC_{50} of 2 nM for Btk.[81]

CNX-774 (**30**) from Avila Therapeutics also targets Cys481 and is a potent Btk inhibitor (IC_{50} < 1 nM) of yet another chemotype.[82] Btk activity in Ramos cells was strongly inhibited with an IC_{50} of 1−10 nM, and **30** has demonstrated efficacy in a CIA mouse model at 60 mg kg^{-1} day^{-1}. However, the in vivo target occupancy reached only ∼70% as determined via a chemical probe method. Avila Therapeutics continued activities on a covalent Btk inhibitor, resulting in the development of the more potent compound **31** (AVL-292, structure not disclosed).[83] **31** potently inhibits Btk in biochemical (IC_{50} < 0.5 nM) and cellular assays (EC_{50} = 1−10 nM), including α-IgM stimulation of B-cell proliferation and B-cell activation. The overall favorable efficacy and selectivity profile warranted clinical development, and early observations from a phase 1b clinical study suggest that **31** is safe and well tolerated in NHL and CLL patients.[84]

Pfizer has reported on imidazoquinoxaline **32** and derivatives acting as irreversible Btk inhibitors, emphasizing its interest in this area.[85] Compound **32** combined good biochemical Btk potency (IC_{50} = 1.93 nM; no preincubation) with selectivity over the Src family kinase members albeit not over ErbB family member EGFR. The nanomolar inhibition of B-cell proliferation (IC_{50} = 3.41 nM) in combination with favorable pharmacokinetic properties led to dose-dependent efficacy in the mouse CIA model following 0.3−10 mg/kg via oral route dosing.

Group 4 Cysteines. Although this cluster is confined to a single cysteine adjacent to the DFG-motif and near the bottom of the ATP binding pocket, nearly 10% of all known kinases (48) have this particular cysteine at this very position (Figure 14). Intuitively one would guess that in this case selectivity is a major issue to resolve. However, the fact that the kinases involved are clustered in eight or nine evolutionary families may clearly present options for additional selectivity filters.

After the success of bringing the irreversible EGFR/ERbB2 inhibitors to the clinic, Wissner et al. also explored the 4-anilinoquinazoline scaffold for covalent binding with Cys1045 in KDR (VEGFR-2).[86] In their binding model, the aniline portion was located near the cysteine, and for this reason it was replaced by a reactive quinone group. Inhibitors were screened for KDR inhibition with and without GSH to ascertain a sulfhydryl reaction confined to the kinase of interest and at high and low ATP concentrations to confirm the non-ATP competitive nature. Compound **33** emerged as an attractive irreversible inhibitor with an IC_{50} of 5 nM along with good selectivity and in vivo efficacy in a mouse xenograft model (Figure 14). Although only a small panel of deselection kinases was used, other group 4 member kinases, such as GSK and MEK1, were reportedly not inhibited. Even though caution should be taken (many more group 4 kinases were not included), this suggests that it is feasible to induce selectivity even within this large cluster of kinases, as long as the chemical scaffold is appropriately chosen.

Resorcylic acid lactones (RAL) that contain a *cis*-enone Michael acceptor were shown to be irreversible inhibitors of most of the group 4 kinases. Hypothemycin (**34**) turned out to be rather unselective within this cluster, inhibiting 18 out of 19 tested kinases containing this conserved cysteine.[87] A tryptic digest confirmed covalent binding to Erk2 but did not yet dismiss the other electrophilic functionality, the epoxide, as the reacting warhead. To this end, the crystal structure of the Erk2−hypothemycin complex provided proof that a covalent bond with Cys166 of Erk2 was formed with the *cis*-enone moiety.[88] Around the same time, another group reported on a small set of hypothemycin analogues, exemplified by **35** (FR-148083).[89] In analogy with **34**, X-ray crystallography showed the covalent interaction of **35** with Erk2 (Figure 14). Next to Erk2 (IC_{50} = 80 nM), the MAP2K kinases MEK1 (IC_{50} = 6 nM) and MKK7 (MEK7; IC_{50} = 300 nM) were substantially inhibited, whereas MKK4 (MEK4) showed IC_{50} > 3 μM. No other kinases of this cluster were included in the selectivity profiling. The related analogue **36** (E6201) was evaluated for its anti-inflammatory and antihyperproliferative potential, reportedly owing to the inhibition of MEK1 (IC_{50} = 5.2 nM) as well as several other MAP2K kinases and the upstream group 2B kinase MEKK1 (IC_{50} = 31 nM).[90] Reduction of the *cis*-enone double bond was detrimental to cellular activity, and introduction of the γ-methyl was necessary to enhance metabolic stability. In animal models of dermatitis, compound **36** was active following topical administration[91] and is currently in clinical trials as a topical formulation for psoriasis.[67] *cis*-Enone harboring RAL derivatives remain an intriguing class of irreversible kinase inhibitors that in some instances have shown very potent in vivo effects in preclinical oncology models,[92] but highly selective inhibitors are usually not obtained. Other very similar RAL analogues appeared almost a decade earlier as fairly potent pan-MEK or TAK-1 inhibitors, which is not surprising

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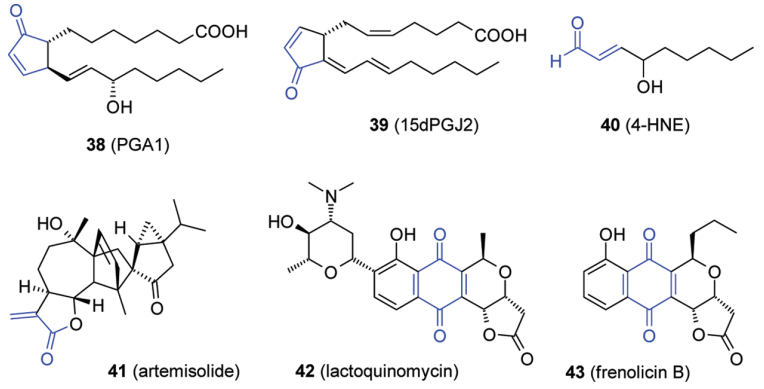

Figure 15. Irreversible group 5 kinase inhibitors **38**−**43**.

given their incorporated α,β-unsaturated ketone moiety and the fact that the kinase involved belongs to group 4.[93] Nevertheless, the potential for irreversible binding was not interrogated at the time.

The latest addition of irreversible kinase inhibitors in this cluster of kinases are thienyl halomethyl ketones.[94] Thiophene derivative **37** displayed a biochemical IC_{50} of 0.5 μM for GSK3β, representing the first reported irreversible inhibitors for this kinase to date. Although moderately potent and far from being druglike, these agents can be considered as fragments that can be optimized for affinity toward GSK3α and/or GSK3β.

Group 5 Cysteines. The location of this cluster is outside the ATP binding pocket and on the activation loop (regulatory or T-loop) of various kinases (Figure 4). It is not within the scope of this Perspective to present a thorough analysis of all potential cysteines via alignment of the kinase sequences. The flexible nature of the activation loops with respect to its length, but also the potential position of the cysteines, hampers a proper analysis. Therefore, it is practical to put all these cysteines in one group. In addition, because of flexibility of the activation loop, it is difficult to target these cysteines deliberately via a structure-based drug design approach, since 3D information for this region is scarce. Density with sufficient resolution is sometimes observed for activation loops but often not visible in crystal structures of kinases. On the other hand, it will probably be only a matter of time before the research community will identify and disclose meaningful ways of visualizing this structural "blind spot" for the majority of the kinases.

Group 5 is an interesting cluster because Mother Nature already seems to incorporate activation loop cysteines in her kinase signaling pathways. For instance, some of the effects of naturally occurring ligands have been tied to allosteric covalent modification of activation loop cysteines. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IKKα/β, and they do so by covalent binding through a cysteine at position 179 within the activation loop.[95] Several lines of evidence were generated. First, the inhibitory effect was completely insensitive to high concentrations (1 mM) of ATP in a biochemical setting, which emphasized the non-ATP competitiveness, albeit not the site of covalent binding per se. Interestingly, both IKKs also have a cysteine located in the ATP binding pocket belonging to group 3B.[38] Second, comparing inhibitory responses of both PGA1 (**38**) and 15dPGJ2 (**39**) on WT IKKβ and the C179A mutant in vitro confirmed the probable site of binding (Figure 15). The WT kinase was fully inhibited, whereas the mutant was not, yet both isoforms were equally responsive to the same stimuli. In line with these findings, the saturated analogous compounds cyclopentanone and cyclopentene derivatives failed to intervene with NF-κB activity.[96] The micromolar concentrations of cyclopentenone derivatives needed for inhibition of IKKβ have been detected at the site of inflammation, and so it can be inferred that inhibition of the NF-κB transcriptional activity is at least partly attributable to this covalent binding mechanism. Another endogenous degradation product, 4-hydroxy-2-nonenal (4-HNE, **40**), was also shown to block the NF-κB pathway.[97] In yet another study, artemisolide (**41**) was presented as an inhibitor of IKKβ by means of Cys179 targeting.[98] The common denominator in all these agents is the α,β-unsaturated ketone functionality, which can act as a Michael acceptor. Interestingly, the kinase LKB1 is a kinase with a nucleophilic Cys210 on the activation loop that aligns nicely with Cys179 in IKKα/β and is also prone to inhibition by some of these reactive lipids, such as **38** and **40**.[99]

The kinase Akt1 accommodates two cysteines on the activation loop (Cys296 and Cys310) and is sensitive to inhibition by pyranonaphthoquinone derivatives, such as lactoquinomycin (**42**, IC_{50} = 149 nM) and frenolicin B (**43**, IC_{50} = 313 nM).[100] Because of the redox sensitive nature of the cysteines involved, the authors speculated righteously that the cysteines could be covalently adducted by these pyranonaphthoquinone-containing inhibitors. First, preincubation with cysteine led to ∼60-fold loss of potency for the inhibitors, and second, both the C296A and C310A mutants were less sensitive to pyranonaphthoquinone derivatives. In contrast, staurosporine inhibited the WT and mutant kinases equally well.

Although the activation loop has not yet been thoroughly addressed, from these collective data it can be inferred that covalent trapping in this region shows potential for the future. Especially when the ATP binding pocket is highly conserved within a kinase family, inactivation via covalent targeting specific activation loop cysteines may be an attractive alternative.

Miscellaneous Cysteines. Beyond the kinase regions discussed so far, a first example of trapping a cysteine located on the catalytic loop has also been reported. While analyzing additional cysteines that are in the vicinity of the ATP binding pocket, taking into account the inactive "DFG-out" and "C-helix-out" conformations of protein kinases, the Winssinger group came across a cysteine located at the beginning of the

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catalytic loop of c-KIT and PDGFR.[39] BCR-Abl inhibitor imatinib (**44**), which also blocks c-KIT and PDGFR via a type II allosteric binding conformation, served as the chemical starting point (Figure 16). The cysteine involved (Cys788 and

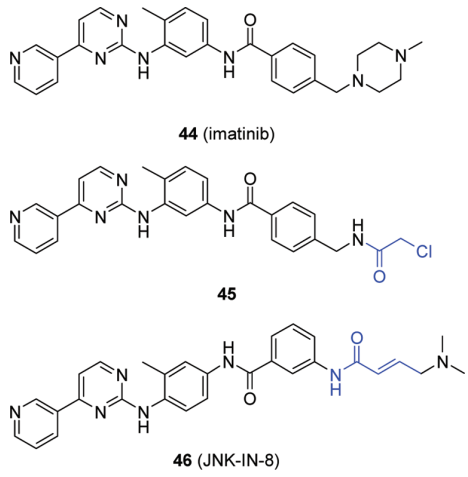

Figure 16. Transformation of imatinib into an irreversible c-KIT/PDGFR inhibitor **45** and JNK inhibitor **46**.

Cys814 in c-KIT and PDGFRα, respectively) was targeted by exchanging the *N*-methylpiperazine moiety for electrophilic groups. The resulting chloroacetamide derivative **45** was subsequently shown to adduct c-KIT and PDGFR covalently on the envisaged cysteine as shown in MS/MS experiments. The biochemical activity for the two kinases did not improve compared to **44**, but some advantage in terms of biochemical selectivity was achieved. Also, **45** lost some affinity for BCR-Abl, the prime target for **44**, which has a phenylalanine in the corresponding cysteine position. A serendipitous finding by Gray and co-workers with near identical compounds yielded potent and selective covalent inhibitors of JNK1, JNK2, and JNK3.[101] While working on covalent inhibitors for c-KIT, they observed significant inhibition of JNK. In particular, incorporation of 1,4-dianilino and 1,3-benzamide motifs, such as in **46**, yielded efficient and specific electrophilic inhibitors. The phenylaminopyrimidine analogues trapped Cys116 in JNK1 and JNK2, and equivalent Cys154 in JNK3, via a type I binding conformation, as was demonstrated via cocrystal structures with JNK3. The fairly unique cysteine in JNK1, JNK2, and JNK3 resides just outside the ATP pocket on the C-terminal domain α-helix near the group 3F position (Figure 4). With enzymatic low nanomolar IC_{50} values for JNK1 (4.7 nM), JNK2 (18.7 nM), and JNK3 (0.98 nM) and selective cellular inhibition of c-Jun phosphorylation, **46** is among the JNK inhibitors that are well suited to further study of JNK-mediated intracellular signaling.

■ CHEMICAL REPORTER PROBES FOR PROTEIN KINASES

Highly efficacious and selective kinase inhibitors are extremely useful in in vitro or in vivo validation settings, especially when the kinase involved is blocked irreversibly. First, the inhibition observed is very often time-dependent, which is one of the hallmarks of covalent and irreversible binding. This helps to delineate the pathway involved, and the markers investigated are easier to tie to the kinase under scrutiny. Second and perhaps more importantly, appropriate decoration of a selective inhibitor with a linker-substituted label renders a very powerful chemical probe (also termed activity-based probe) to study intracellular phenomena (comparative application).[102] Also, such a selective chemical probe can aid in the determination of target occupancy of the (pre)clinical candidate irreversible inhibitors (competitive application).

Several compelling examples have been reported for kinases underlining the strength of this platform, and many irreversible kinase inhibitors reviewed herein have been successfully transformed into such useful chemical probes. The irreversible inhibitors can be biotinylated to study in vitro or in vivo selectivity via (strept)avidin enrichment or (once the functional selectivity has been established) appended with a fluorescent tag to support visualization of intracellular processes (Figure 17, see insert).

One of the challenges for designing appropriate probes is the site of attachment of the linker on the core scaffold. Ideally, this position does not interfere with the potency that was obtained with the original parent inhibitor. To this end, Blair and co-workers decided to functionalize the quinazoline C7 position of **13** while searching for a selective EGFR affinity probe.[103] Both the PEG linker and the NBD fluorophore are solvent exposed, and the chance of disrupting the potency is minimal. The resulting fluorescent probe (**47**) was used in this case used to quantitate the relationship between EGFR stimulation and downstream kinases in living cells.

For some of the validation work with respect to covalent FGFR inhibition, FIIN-1-biotin (**48**) was utilized (vide infra).[40] In the design, the authors were allowed to elongate the acrylamide of parent **4** with a dialkylaminomethylene moiety, since the resulting reactivity is normally in the range of the unsubstituted warhead (Figure 17). Also in this case, the cellular EC_{50} was comparable to that of parent inhibitor **4**.

In some instances, the derived chemical probes fail to present the necessary properties like permeability. For this reason, a biotinylated FMK derivative was inappropriate for targeting RSK1 and RSK2 in intact cells, and also FMK-BODIPY (**49**) showed modest cellular activity (Figure 17).[43] This was very elegantly solved by Cohen et al., who incorporated the clickable propargyl linker allowing for postlabeling intracellular conjugation with an azide-labeled biotin or reporter molecule (biotin-azide or TAMRA-azide).[104] On this improved propargyl amine version of FMK (FMK-PA, **50**), the vector of the tethered reporter tag is extending from pyrrolopyridine portion into the ribose pocket and toward the solvent. The application of this highly versatile in situ click conjugation concept contributes significantly to the power of irreversible kinase inhibition with respect to validation of specific target kinases in signaling pathways.

To specifically profile members of the PI3-kinase family, several activity-based probes have been developed based on wortmannin as a scaffold.[105] As per example, a wortmannin-BODIPY probe (structure not shown) turned out to be cell permeable allowing for labeling of proteins in intact living cells.[106] The additional finding by Liu et al. that **3** also targets the conserved lysine of protein kinases Plk1 and Plk3 via covalent binding can in part be attributed to the application of TAMRA-based probe of **3** (**51**, AX-7503) as shown in Figure 17.[29,30]

The same group reported the utility of a selective PKCα/β probe via a slightly unorthodox design angle.[107] It was different, because a cysteine located just outside the ATP pocket of

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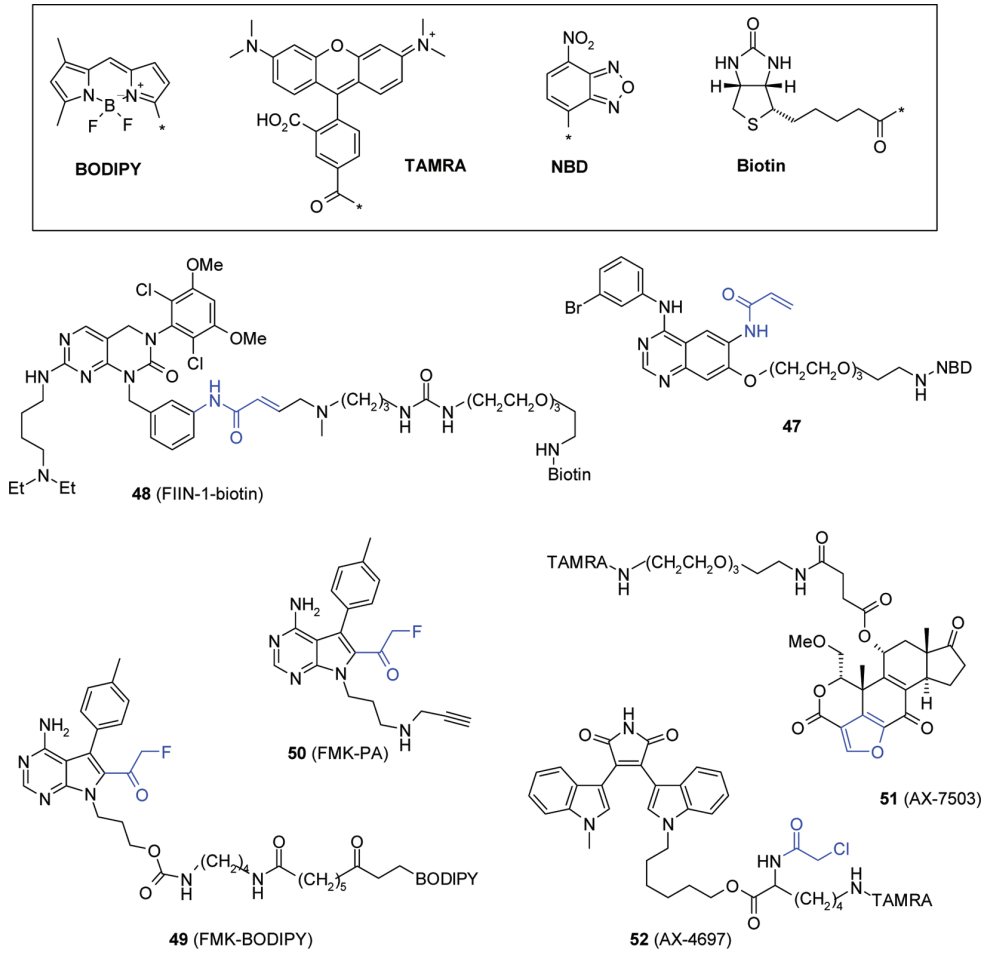

Figure 17. Examples of chemical probes bases on irreversible protein kinase inhibitors. TAMRA = tetramethylrhodamine. NBD = nitrobenzoxadiazole. BODIPY = boron dipyrromethene.

PKCα (C619) and PKCβ (C622) was targeted for covalent trapping. On the well-known PKC inhibitor bisindolylmaleimide core, a chloroacetamide warhead was incorporated on the TAMRA-tagged linker reaching out to the cysteine mentioned. The resulting exo-affinity probe **52** (AX-4697) provided a sensitive and permeable tool for further examination of the consequences of pharmacological intervention in PKC-mediated biology.

The determination of target occupancy has been an integral part of the (pre)clinical development of irreversible Btk inhibitors **31** and **28** (Figure 13). Honigberg et al. created the cell-permeable, fluorescently labeled derivative PCI-33380 (**53**, Figure 18) of their clinical candidate.[79] The level of Btk target occupancy by **28** was determined using **53**, and this occupancy was shown to tightly correlate with the inhibition of BCR signaling and in vivo efficacy in mice and dogs. In the clinic, the same fluorescent probe was used to correlate target occupancy and pharmacodynamics effects in cancer patients.[80] To this end, Avila Therapeutics assessed ex vivo Btk occupancy with a biotinylated covalent probe and quantitated by ELISA.[84]

These probes provide excellent biomarkers, allowing efficacy and target occupancy to be linked in with the analysis of pharmacodynamics. Another upside for the utilization of probes is that it aids the selection of the highest relevant dose in the patient. In oncology clinical trials, patients are treated with

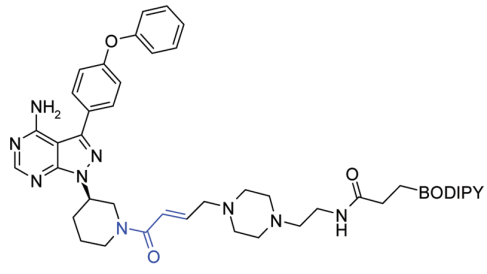

Figure 18. Chemical structure of the BODIPY-derived Btk chemical probe **53**.

increasing doses of the drug until dose limiting toxicity is observed. For a covalent drug, a modest excess of exposure with respect to the dose where full target occupancy is observed should be more than sufficient.

Clearly, one should be able to design and synthesize suitable probes for diagnosis and target occupancy purposes with modest efforts. Covalent kinase inhibitor probes should constitute an integral part of kinase research, especially when the identification and development of irreversible inhibitors come into play.

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CHEMICAL GENETIC ENGINEERING

The physical evidence of irreversible inhibition, and the site of modification on the WT kinase, is frequently corroborated with genetically engineered kinase mutants. To be specific, the naturally occurring nucleophilic amino acid residue is replaced by a nonreactive isoster that is not interfering with the functional activity of the kinase. Hence, the relative contribution of covalent binding to the WT kinase inhibition can be studied. Some research groups have exploited this genetic technique by reversing this concept and introduced a nucleophilic anchor point for irreversible binding that was previously not in the active site. Mutation of strategically positioned key residues into a cysteine creates an engineered pocket that allows unparalleled specificity, especially in combination with additional selectivity filters (often a mutated gatekeeper residue). SAR that has previously been developed for WT kinases, bearing a cysteine in the same position, can now be efficiently exploited for the mutant kinase via selective irreversible inhibition. The groups of Shokat and Rauh have been at the forefront of this so-called chemical genetic engineering platform. In one case, c-Src was subjected to two point mutations in the ATP binding pocket, gatekeeper T338G and S345C at the group 3F position.[103] The latter position is homologous to Cys797 in EGFR, and c-Src-dm has become extremely sensitive to irreversible prototype EGFR inhibitor **13** (Figure 9) and analogues. The mutant IC_{50} values ranged from 1 to 250 nM for these analogues, as opposed to >27 μM for WT c-Src.

Similarly, two selectivity filters were applied on fission yeast aurora (Ark1) in order to tailor the kinase for covalent trapping by these quinazoline derivatives.[108] The first mutant filter at the gatekeeper position, L166A, allowed better accommodation of the aniline portion of analogues of **13**, and the second E173C mutation provided the anchor point for covalent modification. The electrophilic inhibitors efficiently inhibited the newly engineered mutant Ark1 in the low nanomolar range, and both selectivity filters were necessary because neither the WT kinase nor the mono E173C mutant was inhibited biochemically (IC_{50} > 20 μM). Subsequently, "analogue-sensitive" Ark1-mediated signaling in recombinant cells was efficiently and irreversibly blocked by the propynamide derivative of **13** while no detectable toxicity was observed.

This covalent complementarity approach was taken one step further by mutating the gatekeeper residue into a unique cysteine.[109] This cysteine has not been previously targeted for covalent binding. Only two human protein kinases (MOK and SgK494) have this endogenous cysteine gatekeeper residue which corresponds with the orange sphere in Figure 4. Thus, cysteine gatekeeper engineering in theory allows electrophilic inhibitor discovery for most kinases, as long as the mutant kinase activity is not impaired. Again, the concept was validated employing c-Src T338C in combination with warhead substituted 3-benzylpyrazolopyrimidines, such as **54**, and 4-anilinoquinazolines (Figure 19). Compound **54** showed a time-dependent inhibition of T338C c-Src and displayed an IC_{50} of 114 nM (versus >5 μM for WT c-Src). MOK inhibition was hardly impacted by **54**, and a few off-target effects were noted when screening against a panel of 307 kinases. A couple of TEC family kinases were hit more than 50% by **54** at 1 μM but not when the fluoromethyl ketone moiety was employed as the electrophile. A crystal structure of **54** in complex with T338C c-Src was solved, which nicely demonstrated the formation of a covalent bond between the vinylsulfonamide warhead and the gatekeeper cysteine residue.

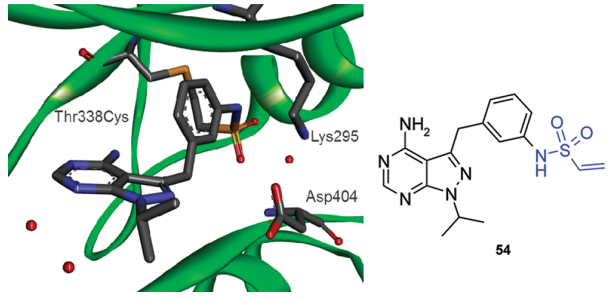

Figure 19. Pyrazolopyrimidine derivative **54** in covalent complex with c-Src T338C (3SVV.pdb; P-loop omitted for clarity).

KINASE KINETICS AND STRUCTURE–*REACTIVITY* RELATIONSHIPS (SRR)

It is easy to get enchanted with extraordinary potencies and superb selectivity. However, embarking on a project with the aim to identify covalent inhibitors that should serve patients warrants an adapted flowchart for selecting candidate compounds with the required druglike properties. It is of paramount importance to incorporate specific off-target assays that are central in the flowchart (vide infra). These can be both computational and empirical of nature. Many of those assays, such as homologue cross-screening and monitoring of glutathione depletion, are already part of the conventional drug discovery toolbox. More importantly, addressing the kinetics of irreversible kinase inhibition and generation of robust structure–reactivity relationships are the real keys to success in the discovery of safe irreversible drugs. The activity of the ultimate irreversible kinase inhibitor should be an optimal mix of reversible binding affinity (SAR) and warhead reactivity (SRR). If the efficacy of the inhibitor depends solely on reactivity, the overall selectivity and consequently the safety profile will undoubtedly be compromised. When the reactivity is too low, the inhibitor will behave like a conventional reversible inhibitor and become more dependent on the clearance of the compound from the circulation again.

A suitable and straightforward assay system for the rate of kinase silencing has been reported by Rauh and co-workers. The kinetics of kinase inhibition were determined by monitoring changes in the intrinsic fluorescence signal upon covalent bond formation between electrophilic inhibitors and the S345C c-Src and the clinically relevant EGFR mutants.[110] Also, electrophilicity calculations by means of enthalpy changes (ΔH) were employed in analogy with previously published procedures for nitrile warhead containing compounds.[111] The potencies of irreversible inhibitors were extrapolated from the initial reaction velocities found in the linear range of the curves. With this technique, the influence of the altered gatekeeper residue size on the initial reactivity rate of the irreversible inhibitors could be directly monitored. A pragmatic way of ranking irreversible kinase inhibitors based on the intrinsic electrophilic strength has been described by Wyeth researchers, who rank-ordered the explored warheads based on simple glutathione adduct formation in a THF–water–methanol solution.[58] Others have incubated irreversible kinase inhibitor leads with glutathione or whole blood and monitored the stability over time.[82]

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OUTLOOK

For many kinases, a crucial regulatory role has been associated with a variety of malignancies, making this protein family an attractive drug target family. The challenge for this target family has been the identification and development of potent inhibitors with sufficient selectivity to generate efficacious drugs with a good safety profile. For a number of reasons, the kinase field in particular seems well equipped for the covalent inhibitor concept with clear potential advantages versus reversible inhibitors. The compelling upsides have been reviewed in this Perspective. First, with high intracellular ATP levels, non-ATP competitiveness is a major upside. Therefore, irreversible protein kinase inhibitors could outperform reversible drugs based on superior efficacy and functional selectivity. Indeed, the loss in cellular potency for biochemically potent reversible inhibitors is in many cases not observed for an irreversible inhibitor. Second, a substantial part of the human kinome harbors a specific cysteine in or near the ATP binding pocket that may be used to achieve both potency and selectivity. Third, the rate of turnover for many kinases, required for return of function of the target that has been effectively knocked down via irreversible kinase inhibition, renders this protein family suitable for less frequent dosing regimens. In addition, lower overall exposure is to be expected, since the pharmacodynamics effects are dependent on de novo synthesis of the target protein, rather than higher dosing required to maintain certain circulating levels for the drug to retain efficacy over time. This lower body burden is beneficial in circumventing off-target related adverse effects. Fourth, protein kinase inhibitors introduced to the market to date have without exception been approved for oncology indications, and it is in this area that irreversible kinase drugs might gain easier acceptance. Drug-induced resistance in many cancers remains a serious problem, and also here, irreversible binding kinase inhibitors could make a difference. It has been proposed that this mutational pressure even might exist for cysteines, which may render the irreversible kinase inhibitors currently in clinical development for oncology indications increasingly ineffective over time.[69] Future follow-up in these patients is required to substantiate the occurrence and prevalence of these inactivating mutations.

Last but not least, irreversible kinase inhibitors as such are invaluable compounds as research tools in the validation of kinase signaling pathways or can with relative ease be appended with reporter tags. This may significantly speed the overall drug discovery process, as the biological validation of the target can be tackled early on and the availability of biomarker target occupancy probes facilitates dose-finding in clinical development.

Monitoring SRR is key in order to progress the most suitable covalent inhibitor candidates through the pipeline, since off-target toxicity related to the electrophile remains a substantial threat. The future will tell whether irreversible binding kinase inhibitors will live up to the expectations that have been generated by the research community; however, the progress that has been made in the past 2 decades has been impressive. The most advanced irreversible kinase inhibitors are in phase 3 clinical trials. Recently, two major deals have been struck within the pharmaceutical industry. Johnson & Johnson purchased phase 2 irreversible Btk inhibitor **28** from Pharmacyclics. Avila Therapeutics, a biotechnology company relying on the covalent binding platform, was recently acquired by Celgene. These developments seem exemplary for the general growing confidence in irreversible kinase inhibitors as effective and safe treatments. If irreversible kinase inhibition is shown to be sufficiently safe in cancer indications, chronic treatment for other disorders may also become accepted. Covalent drugs are already widely applied in indications outside the oncology arena. It is our belief that the covalent binding principle will gain common ground as we get more accustomed to the upsides while still keeping a critical eye on the potential risks. Those covalent drugs that have the appropriate benefit/risk balance are "bound to succeed".

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Notes

The authors declare no competing financial interest.

Biographies

Tjeerd Barf obtained his Ph.D. in Medicinal Chemistry from the University of Groningen, The Netherlands, under the supervision of Prof. Håkan Wikström in 1996. He joined Pharmacia & Upjohn in Uppsala, Sweden, in 1997 and continued his employment in the spin-off company Biovitrum (2000). He has led medicinal chemistry programs concerning metabolic diseases and assumed overall program leadership later on. In 2005, he moved to Legacy Organon (Oss, The Netherlands), and as a Group Leader, his responsibilities encompassed heading immunology lead finding projects. After the acquisition by Schering-Plough and the merger with Merck, he led a global cross-site lead optimization effort for an autoimmune disease. In 2011, he cofounded Covalution Pharma and presently holds the position of Head of Chemistry within that biotechnology company.

Allard Kaptein received his Ph.D. in 1993 in Biopharmaceutical Sciences at Gaubius Institute TNO/University of Leiden, The Netherlands. After 2 years as a Postdoctoral Fellow at Glaxo in Les Ulis, France, he joined the GlaxoWellcome in Stevenage, U.K. In 2000 he joined Organon in Oss, The Netherlands. From 2004 to 2008 he was head of the immunology lead finding program activities, and after 2009 headed a lead optimization program at Schering-Plough and Merck following the takeover of Organon. In July 2011 he founded, together with Tjeerd Barf, the R&D startup Covalution Pharma focusing on covalent kinase inhibitors in oncology and autoimmune diseases. His main areas of expertise include small molecule drug discovery on different target families, translational sciences, and cross-disciplinary knowledge bridging biology and chemistry in research.

ABBREVIATIONS USED

(Bcr)-Abl, (breakpoint cluster region)-Abelson kinase; ALK, anaplastic lymphoma kinase; Akt1 (PKB), Ak mouse strain thymoma; Ark1, Aurora kinase; BCR, B-cell receptor; Blk, B-lymphocyte kinase; Bmx, bone marrow tyrosine kinase gene in chromosome X; Btk, Bruton's tyrosine kinase; CIA, collagen-induced arthritis; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; ErbB, erythroblastosis oncogene B; EGFR (ErbB1), epidermal growth factor receptor; Erk2, extracellular signal-regulated kinase-2; FGFR, fibroblast growth factor receptor; FLT3, fms-like tyrosine kinase-3; GIST, gastrointestinal stromal tumor; GSK, glycogen synthase kinase; IDT, idiosyncratic toxicity; IGF-1R, insulin-like growth factor receptor; IKKα/β, inhibitor of kappa-B kinase subunit α/β; Itk, IL2-inducible T-cell kinase; JAK, Janus kinase; JNK (SAPK), c-Jun N-terminal kinase; c-Kit, steel growth factor receptor;

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LKB1, liver kinase B1; MEK (or MKK or MAP2K), mitogen-activated protein kinase kinase; MEKK1 (MAP3K), mitogen-activated protein kinase kinase kinase 1; MET, hepatocyte growth factor receptor; MOK, MAPK/MAK/MRK overlapping kinase; NEK2 (NIMA), never in mitosis gene a related kinase 2; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung carcinoma; PDGFR, platelet derived growth factor receptor; PI3K, phosphatidylinositol 3-kinase; PI4K, phosphatidylinositol 4-kinase; PKC, protein kinase C; Plk, polo-like kinase; Raf, rapidly accelerated fibrosarcoma; RCC, renal cell carcinoma; RET, rearranged during transfection; RSK, p90 ribosomal protein S6 kinase; SgK494, Sugen kinase 494; c-Src, Rous sarcoma oncogene cellular homologue; SRR, structure–reactivity relationship; TAK, TGF-β activated kinase; TEC, tyrosine kinase expressed in hepatocellular carcinoma; TNK1, tyrosine kinase nonreceptor 1; Txk, T and X cell expressed kinase; VEGFR, vascular endothelial growth factor receptor; WT, wild type; YES, Yamaguchi sarcoma viral oncogene homologue

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